ANIMAL WELFARE ASSESSMENT, VOLUME II

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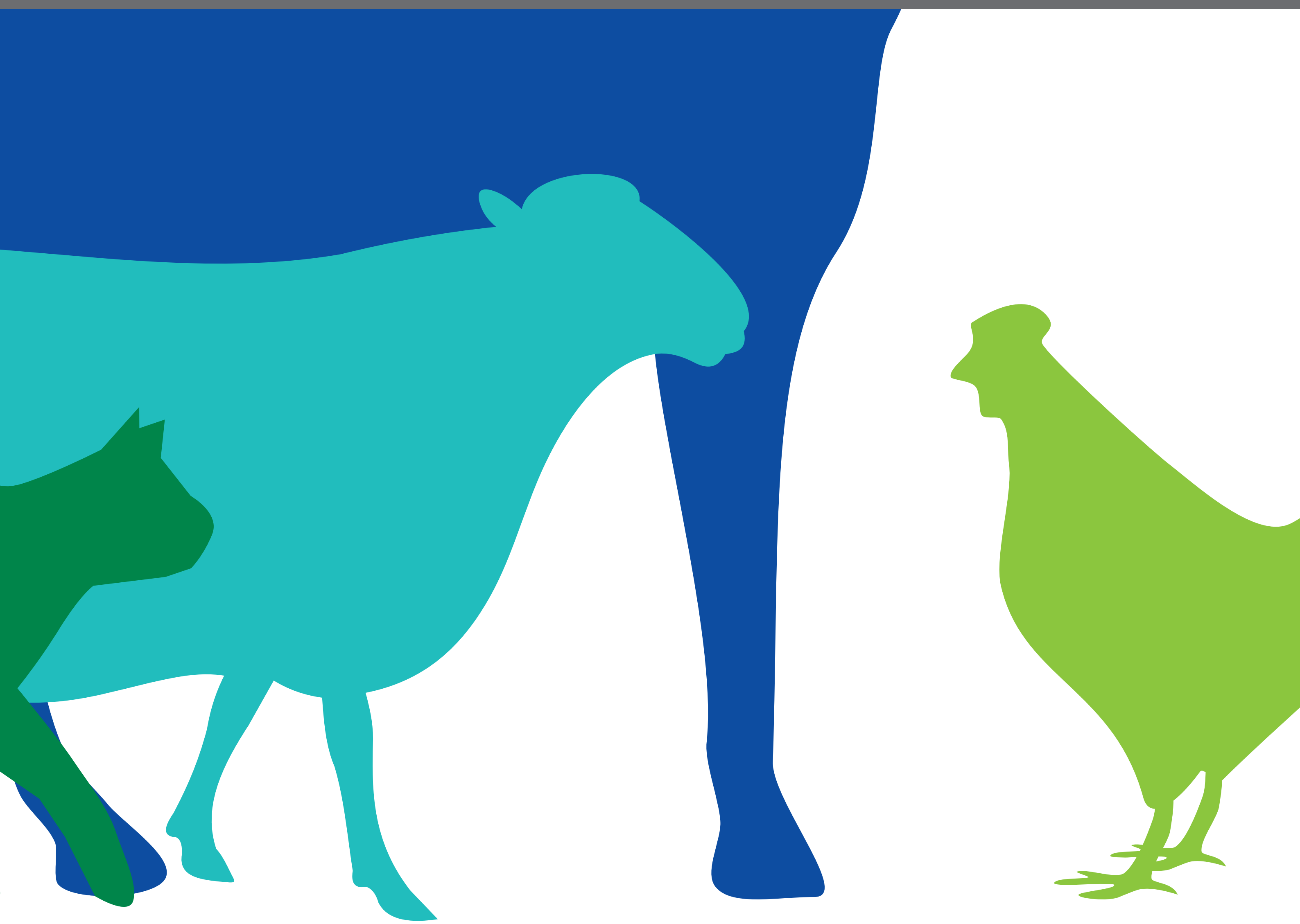

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ANIMAL WELFARE ASSESSMENT, VOLUME II

Topic Editors:
Edward Narayan, The University of Queensland, Australia
Alan G. McElligott, City University of Hong Kong, SAR China

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EDITORIAL
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Editorial: Animal Welfare Assessment: Edition 2

Edward Narayan[1,2]* *and Alan G. McElligott*[3]

[1] School of Agriculture and Food Sciences, Faculty of Science, The University of Queensland, St Lucia, QLD, Australia, [2] Queensland Alliance for Agriculture and Food Innovation, The University of Queensland, St Lucia, QLD, Australia, [3] Jockey Club College of Veterinary Medicine and Life Sciences, City University of Hong Kong, Kowloon, China

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Editorial on the Research Topic

Animal Welfare Assessment: Edition 2

Animal welfare is an important dimension of human-animal interaction in managed settings such as farms and zoos. This field of research can also be a powerful driver to continuously improve the traditional animal production systems to ensure that the animals are able to meet basic requirements of five freedoms (freedom from pain, injury and disease, freedom from fear and distress, freedom from discomfort, freedom to express normal behavior and freedom from hunger and thirst). The recently developed five domains model is also internationally recognized and it attempts to provide an understanding of the emotions of animals (affective state) in response to human interventions. Animal welfare legislation is a complex topic; however, consumer awareness associated with the methods of animal production, health and biosecurity risks increasingly demand stronger investment into research and innovation to continually improve animal welfare standards.

In Edition 2 of the Animal Welfare Assessment *Topic*, we showcased a collection of 13 peer reviewed articles which highlight advancements in animal welfare assessment methods across animal production systems. It includes works of animal welfare experts, veterinarians, animal physiologists and animal managers that will generate a healthy discussion and showcase latest studies working toward finding the harmony between animal performance, health and welfare.

Navarro et al. presented a pharmacological intervention to improving piglet immunity using oral Meloxicam administration to multiparous sows. Early neonatal care of piglets is vital to their survival. The researchers were able to demonstrate that administration of meloxicam orally at the beginning of farrowing in multiparous sows increased immunoglobin and cytokine concentrations in colostrum, improving both humoral and cellular immune response of piglets.

Rodger et al. further studied an app called the health-related quality of life (HRQL) instrument (VetMetrica™) that generates scores in four domains of quality of life in dogs—Energetic and Enthusiastic (E/E), Happy and Content (H/C), Active and Comfortable (A/C), and Calm and Relaxed (C/R). Importantly, the app was able to pick up the disagreement between owner opinion in health status and clinical evidence of chronic disease (40% disagreement), however scores of HRQL were higher in healthy dogs with no clinical information.

Chronic stress can be a significant problem in intensive animal production systems, hence robust quantitative tools are required to measure and evaluate the potential of chronic stress. In their paper, Wiechers et al. studied chronic stress between two different farrowing systems in pigs. Researchers used hair to determine cortisol levels of sows managed either in farrowing crates or in a loose-housing system. They did not find any significant difference in hair cortisol concentrations between the two treatments, however the researchers pointed caution in the potential variation of results due to site of sampling as well as potential modulation of the HPA-axis under exposure to

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Edited and reviewed by:
Severiano Silva,
Universidade de Trás-os-Montes e Alto, Portugal

****Correspondence:***
Edward Narayan
e.narayan@uq.edu.au

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long-term stress.

Hematological methods or blood testing can also boost animal welfare assessment. Seibel et al. discussed the technical developments and opportunities for fish health and welfare monitoring in aquaculture programs. In another study, Ramos et al. discussed important aspects of stress and welfare in fish, highlighting the need for further research based on stress assessment in early life-history stages of fish including focus on egg transport and larval handling.

Emerging animal industries are gaining popularity around the world, such as camel farming. Padalino and Menchetti studied the welfare of camels by applying the principles of Five Freedoms using the Welfare Quality® and AWIN methods adapted to camels. The researchers provided three levels of assessment including (i) Caretaker, (ii) Herd, and (iii) Animal and provided recording sheet for use by Camel producers.

Precision livestock farming (PLF) technologies are gaining popularity as a digitized sensor-based tool to improve the welfare assessment of farm animals. Stygar et al. applied the PRISMA guidelines to evaluate validated and commercially available PLF technologies for welfare assessment of dairy cattle. The study suggests that sensor-based technologies such as accelerometers, milk quality and feeding sensors are useful for assessing welfare status. However, currently available PLF technologies needs to be improved with external validation to boost the assessment of cattle behavior (including calves and heifers) in a reliable way.

In their study, Gómez et al. conducted a literature review on the capability of PLF technologies to contribute to the assessment of pig welfare. Researchers identified 83 PLF technologies commercially available for pigs. However, only 5% were externally validated using a different population than used for system building. Researchers highlighted the need for further validation studies to improve robustness of available technologies as appropriate pig welfare indicators.

Tuyttens et al. discussed the improvements and application specifications of the Welfare Quality® protocol as a user-friendly tool for cost- and time-efficient on-farm monitoring of dairy cattle welfare through application of discrete and continuous animal-based measures feeding into a welfare index (WI). The researchers highlighted that the WI captures most of the welfare key issues dairy cattle, however a list of parameters need to be included as a point of reference to ensure that the data is interpreted correctly using the available anima-based measures.

Brscic et al. evaluated the use of animal-based measures (ABM) in farm animal welfare assessment to standardize terminology that could be applied across sectors. They found that the term ABM was not standardized across sectors and was hardly a common language for different stakeholders. IN order to harmonize the use of ABM in the scientific literature, it was suggested that commonly accepted abbreviations of ABM should be made available in scientific journals.

Lee and Campbell studied virtual fencing technology in cattle to further evaluate the suitability of aversive method such as electrical stimulus. The researchers suggested further research to understand physiological and behavioral responses of animals to see how the virtual fencing technology can be functional animal welfare tool.

In another study, Perea et al. studied the influence of littermate and sex on hormonal and behavioral data from carolic restricted (CR) group housed mice. They showed that grouped male littermates and grouped female male showed less aggressive behavior and physiological stress (measured using serum ACTH levels) during CR, highlighting the welfare benefits of grouping related mice during implementation of CR.

Hempstead et al. studied the welfare assessment of dairy goat farms in the midwestern US, with focus on lactating dairy goats to identify potential welfare issues. Using principal components analysis, the researchers were able to identify physical indicators of welfare issues that will be valuable information to improve goat welfare in dairy industry.

Collectively, the *Topic* further highlights the latest innovations that are helping to boost animal welfare assessment across industries.

AUTHOR CONTRIBUTIONS

EN conceptualized this special issue and collaborated with AM for the editorial role. Both authors contributed to the article and approved the submitted version.

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ORIGINAL RESEARCH
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Analysis of Hair Cortisol as an Indicator of Chronic Stress in Pigs in Two Different Farrowing Systems

*Dierck-Hinrich Wiechers[1], Susanne Brunner[2], Swetlana Herbrandt[2], Nicole Kemper[1] and Michaela Fels[1]**

[1] Institute for Animal Hygiene, Animal Welfare and Farm Animal Behavior, University of Veterinary Medicine Hannover, Foundation, Hanover, Germany, [2] Department of Statistics, TU Dortmund University, Dortmund, Germany

Confinement to farrowing crates is known to prevent sows from performing natural behavior, impairing animal welfare and possibly causing chronic stress. Hair cortisol analyses are increasingly used to detect chronic stress in animals. In the present study, hair samples were collected in the neck of sows kept either in farrowing crates (FC, $n = 31$) or in a loose-housing system (LH, $n = 30$) in six batches. Cortisol was extracted and analyzed using chemiluminescence immunoassay. Mean hair cortisol concentrations (HCC) did not differ significantly between the systems (LH: 1.85 ± 0.82 pg/mg, FC: 2.13 ± 1.53 pg/mg, $P = 0.631$). HCC was also not affected by other factors, such as sows' parity, number of piglets, skin lesion score or sow's weight loss during the farrowing period. However, highly significant differences were found in hair growth rates between different regions within the 20 × 30 cm shaving area. While the hair in both lateral parts of the shaving area grew almost identically (left: 7.48 ± 3.52 mm, right: 7.44 ± 3.24 mm, $P = 1.00$), the hair grew more in the area above the spine (12.27 + 3.95 mm, $P < 0.001$). In both systems, the mean individual lesion score of sows declined from the beginning to the end of the housing period ($P < 0.001$). No difference was found between FC and LH sows at any time ($P > 0.05$). Since neither the amount of skin lesions nor HCC differed between LH and FC sows, it may be concluded that confining sows in farrowing crates did not affect chronic stress levels. However, results may be affected by a downregulation of the hypothalamic-pituitary-adrenal axis during long-term stress, resulting in lower cortisol levels over time. HCC in sows may also be influenced by a dominant stressor, such as farrowing or the presence of suckling piglets. Thus, for a comparison of different farrowing systems regarding chronic stress, the use of hair cortisol measurement seems to be limited. The present results revealed that differences in hair growth rate within the same body region exist. This important finding should be considered when collecting hair samples in pigs, since hair cortisol concentrations may vary depending on hair growth and length.

Keywords: hair cortisol, chronic stress, pig, farrowing pen, hair

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Edited by:
Edward Narayan, The University of Queensland, Australia

Reviewed by:
Sarah Halina Ison, World Animal Protection, United Kingdom
Keelin Katherine Mary O'Driscoll, Teagasc, Ireland

***Correspondence:**
Michaela Fels
michaela.fels@tiho-hannover.de

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INTRODUCTION

The subject of animal welfare in intensive pig farming has become increasingly important for the public in recent years (1). It is already scientifically recognized that farrowing crates restrict sows, not just in their locomotion but also in other natural behaviors (2), causing stress for the confined animals. Loose housing systems without farrowing crates seem to be advantageous in this regard (3), and thus several are currently being researched to improve animal welfare. To evaluate housing systems concerning animal welfare, specific indicators, which refer mostly to physical impacts and the animals' behavior are used (4). However, a housing system should also be evaluated regarding the level of stress which the animals experience there (5). Thus, studies comparing different housing systems often include measurements of stress levels as well. While the term "stress" was only indirectly addressed in earlier definitions of animal welfare, such as the five freedoms (6), today it is often included in the definition of animal welfare itself. From this point of view, the perception of chronic stress is incompatible with good animal welfare (7).

A widely used method to quantify stress is to measure the cortisol level in body fluids or excreta as a biomarker. Cortisol is the main glucocorticoid in most mammals (8) and is produced and released into the blood by the adrenal glands after a stimulus of adrenocorticotropic hormone (ACTH). This hormone is emitted by the activated hypothalamic-pituitary-adrenal axis (HPA axis) (9) after the animal has been confronted with a stressor. The causes of stress in pigs are multifactorial, and can be categorized as social, environmental, metabolic, immunological, or due to handling (10). Acute stress leads to a rapid increase in glucocorticoids, with a peak after about 15–30 min. It is temporarily conducive to adaption to external threats by the redistribution of energy in the organism. In contrast, in case of chronic stress, a long-term elevated glucocorticoid level can be deleterious in many ways (11). Several matrices can be used for cortisol analysis: blood plasma, saliva, urine, feces, milk, and hair (9, 12). Due to the rapid increase after a stimulus occurs and the equally rapid decrease after its removal, cortisol levels in plasma and saliva are highly variable point samples. Even in urine and feces cortisol represents just a time period of 24 h or less of stress experience, so that none of these matrices provide a long-term view of HPA axis activity (13, 14).

Koren et al. (15) carried out one of the earliest studies on the possibility of hair cortisol measurements in animals. Since then, research on this subject has been increasingly intensified in order to be able to measure this hormone as a chronic parameter of stress (14). In addition to providing the advantage of the long-term analysis, collecting hair is a non-invasive method, and the sampling procedure has no influence on the measured values themselves. In contrast, stressful and painful blood sampling can affect cortisol measurements in blood plasma (13). To explain the storage routes of cortisol into the hair shaft, the multicompartment model is often suggested as a basic hypothesis (16). According to this model, the pathway of cortisol release into the hair occurs not only by diffusion from blood into the follicle during the anagen phase of hair formation, but the glucocorticoid can also be incorporated into the hair by an overlaying film of sweat and sebum of hair-associated glands. A further possible way is by incorporating cortisol into the hair via external substances from the environment, after the hair has grown past the outer skin layer. In this case, it would even be conceivable that cortisol is incorporated after hair sampling, thus contaminating the samples. Furthermore, the glucocorticoid can be synthesized and secreted by the hair follicle itself as a functional equivalent of the HPA axis, caused by local stressors on the skin and hair. Since this reaction is independent of the central HPA axis activity, it can be assumed that an additional "peripheral" stress axis exists, with its own local stress response in addition to the systemic reaction (17, 18).

Considering all these possible origins, the question remains to what extent measured hair cortisol concentrations (HCC) are influenced by systemic cortisol levels and whether they actually reflect the HPA axis activity. Some studies have shown that HCC increased in times of higher plasma cortisol levels, or when ACTH was applied to the organism experimentally (19–21). Thus, the HPA axis-dependent cortisol concentration in the hair seems to be sufficiently high to be able to regard the hair as an appropriate medium for chronic stress detection. Further studies showed a correlation between the concentrations of cortisol in hair and feces (22), urine, serum and saliva (23), and underline the possibility of cortisol analyses in hair to detect chronic stress. However, it should be considered that although hair cortisol seems to have a long-term stability of months or years, cortisol can also escape from the hair due to environmental factors (24).

Even if some doubts remain, and there is a need for further research, detection of hair cortisol is increasingly considered a useful marker to determine chronic stress in animals. Therefore, it may be suitable for assessing long-term stress caused by different housing systems for farm animals. Hence, the aim of the present study was to explore the applicability of hair cortisol measurement to detect chronic stress in sows kept in two different farrowing systems. Moreover, factors which affected the sows' stress levels in the farrowing systems should be analyzed as well. Since physical damage in pigs can also influence chronic stress levels (25), the occurrence of skin lesions in sows was investigated using a lesion score and their impact on HCC was also determined.

MATERIALS AND METHODS

Animals, Housing, and Handling

The study was conducted between June 2018 and January 2019 as part of a larger research project at the research farm of the Lower Saxony Chamber of Agriculture in Wehnen, Bad-Zwischenahn, Germany. The animals were kept in accordance with the European Directive 2008/120/EC and the corresponding German national law (Tierschutzgesetz and Tierschutz-Nutztierhaltungsverordnung). The experiments did not include any invasive procedure involving the animals. The study was reviewed and received approval from the Animal Welfare Officer of the University of Veterinary Medicine Hannover, Hannover, Foundation, Germany.

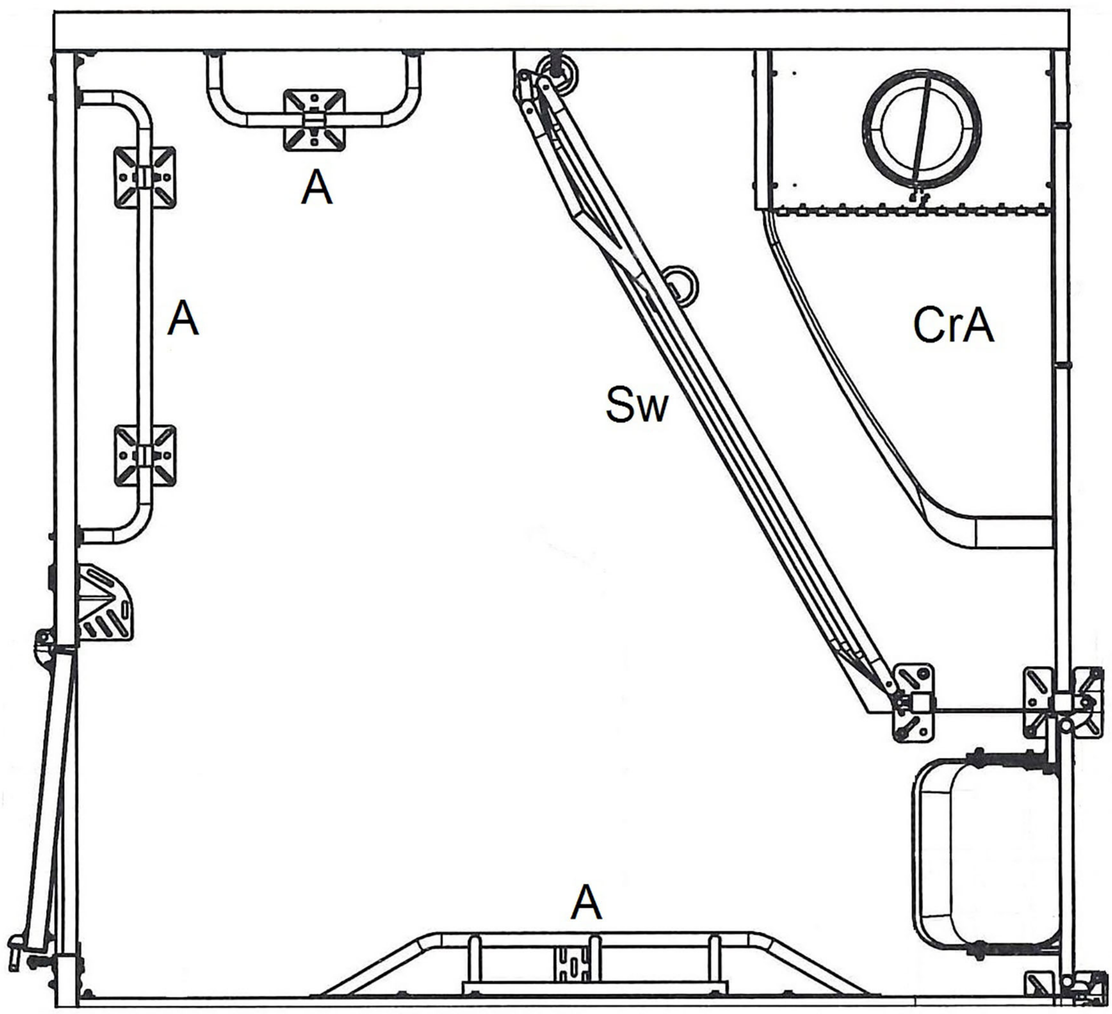

FIGURE 1 | Single loose housing pen (LH). CrA, creep area; Sw, swing gate; A, anti-crushing bars. © Big Dutchman International GmbH, Vechta, Germany.

In the study, two different farrowing systems for sows were compared: conventional pens with farrowing crates (FC) and a loose-housing system without farrowing crates (LH). Both systems were installed in adjacent rooms, and in both systems the sows were single-housed. The LH system had six pens per room and the FC system was equipped with eight pens. Both systems were provided by the same manufacturer (Big Dutchman International GmbH, Vechta, Germany). A single LH pen (**Figure 1**) was 250 cm long and 240 cm wide (6 m^2). A space of 4.01 m^2 was available for the loose-housed sow, separated from the creep area for piglets by a swiveling iron grid, which could be used for confining the sow for different management procedures. The separated two-sided-open creep area (125 × 75 cm) was equipped with a 150 W infrared light heating lamp. The floor of the creep area was covered with a solid rubber mat. To prevent the piglets from being crushed by the sow, anti-crushing bars were installed as a mushroom-shaped protrusion at the long side of the pen. Piglet protection bars were located at the two shorter free sides.

The FC pen (**Figure 2**) measured 260 × 200 cm (5.2 m^2) and included a 190 cm-long and 80 cm-wide (1.52 m^2 usable area for the sow) farrowing crate in the center of the pen. The creep area for piglets was located parallel to the sow's crate and was open on three sides. It was 150 cm long and 60 cm wide and heated by an infrared lamp as well as by a heatable polymer concrete floor. Both housing systems were equipped with the same slatted plastic flooring (10 mm gaps and 11 mm slats), with a non-perforated lying area for the sow, and were subject to the same management procedures. In the LH pen as well as in the FC pen, sows were offered a jute sack as nest-building material in the period before farrowing. As further manipulable material, cotton ropes were offered - one for the sow and a smaller one for the piglets. In addition, a rack with hay was installed in each LH pen. If necessary, all consumed or worn materials were replaced.

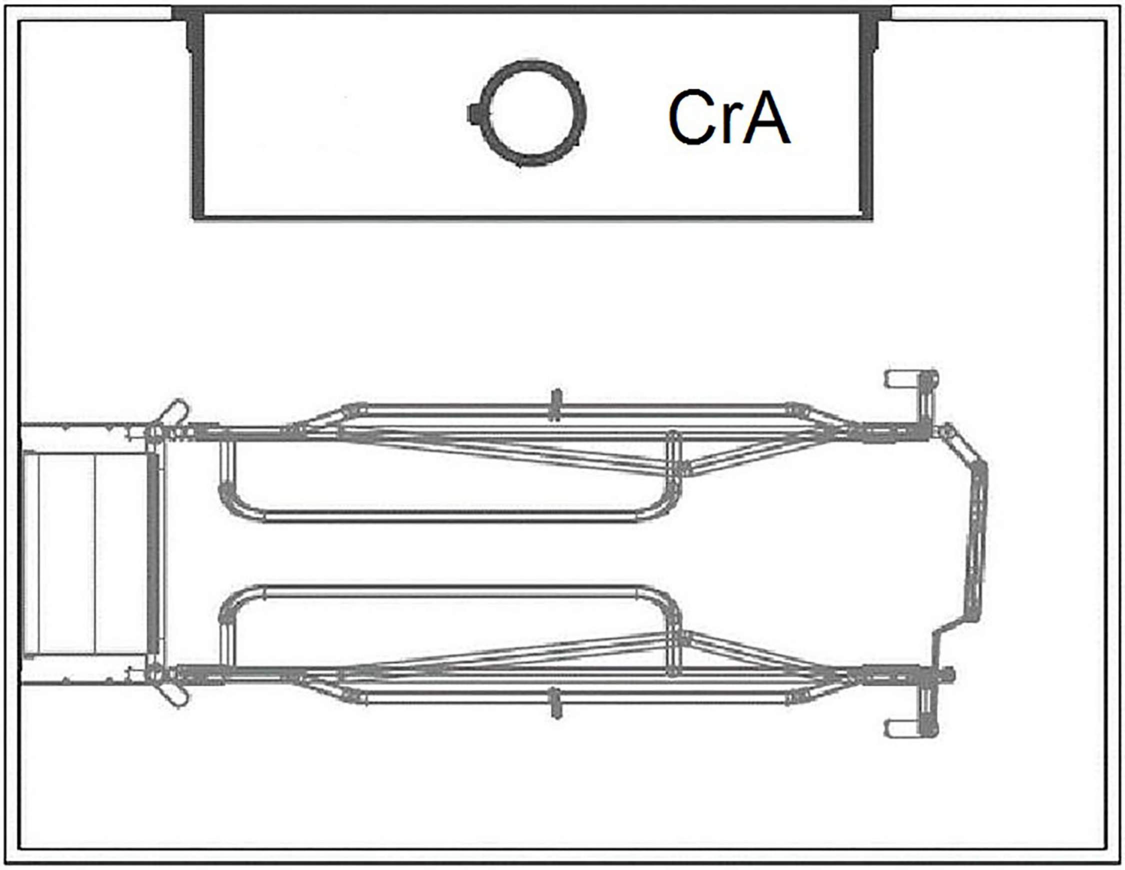

FIGURE 2 | Pen with farrowing crate (FC). CrA, creep area. © Big Dutchman International GmbH, Vechta, Germany.

Before entering the farrowing systems, pregnant sows were housed in groups of three to five animals. Five days before the expected farrowing date, sows were moved to the farrowing pens and thus, were single housed either in FC or in LH pens. Six sows were housed in each farrowing system per batch. At the beginning of the study, the sows were randomly assigned to the two housing systems and thereafter were always allocated to the same housing system. Before entering their pens, the sows were washed and weighed using digital scales (82-b2, RHEWA WAAGENFABRIK, August Freudewald GmbH & Co. KG, Mettmann, Germany). The weight of the piglets was individually recorded within 24 h after birth (scale: SC-A, T.E.L.L. Steuerungssysteme GmbH & Co. KG, Vreden, Germany) and an ear tag was immediately applied to identify the individual animals. The teeth (canines) of the piglets were shortened at the same time. To obtain litter sizes that were as homogeneous as possible, cross-fostering was carried out within the same housing system between three and 72 h after birth. After 28 days, piglets were weaned, reweighed individually and then transferred to the farm's own rearing unit.

While LH sows were never confined during the entire housing period (free farrowing), FC sows were permanently fixed in the crate. Feeding-management in the two systems was the same: sows received a commercial lactation diet twice a day (07:30 and 16:30). The amount of feed was rationed on the days before farrowing (maximum 5 kg per day) and on the day of parturition (maximum 2 kg per day). After parturition, the feed amount was increased by about 0.5 kg per day to reach an ad libitum feeding level after about 14 days (8–9 kg per day).

The sows left the farrowing pens after a period of 33 days, were weighed for the second time and entered the service center for the following reproduction cycle.

In both farrowing systems, temperature and air humidity were measured every 2 min in the respective rooms. The sensors (DOL 114 and DOL 12, dol-sensors A/S, Aarhus, Denmark and 135pro, Big Dutchman International GmbH, Vechta, Germany) were placed at the animals' body height in a farrowing pen in the middle of the room.

In a total of six batches, data of 69 sows (Landrace x Large White, db.Vicoria, BHZP GmbH, Dahlenburg, Germany) from first to seventh parity (LH: 3.8 ± 1.8, FC: 4.3 ± 1.8) were obtained. In five batches, data on all recorded parameters were collected for all sows ($n = 60$). In order to increase the number of hair samples for cortisol analyses, an additional batch was added for this purpose. As some sows were sent for slaughter before hair sampling, and hair length measurement was not possible for

TABLE 1 | Overview of the sows' stereotypies analyzed in the present study.

Stereotypy	Definition
Head waving	Sow moves its head up and down
Bar biting	Sow bites into the bars of the pen
False chewing	Sow chews independently of feed intake, formation of foam at the mouth

TABLE 2 | Scoring scheme for skin injuries (26).

Score	Definition
0	No injuries
1	A small number (<5) of superficial scratches
2	A mean number (5–10) of superficial or a small number of deep scratches (<5)
3	A high number (>10) of superficial or a mean up to a high number of deep scratches (>5)

Superficial injuries were defined as those of the outer skin layers with a minimal reddening or little bleeding at this position. Instead of a blood spot, there could also be a scab. Deep injuries were those that penetrated to the lower skin layers, with reddening, bleeding or scabbing. Furthermore, necrotic or purulent injuries were possible as well.

every animal, the number of sows to be investigated for several parameters was slightly reduced.

Video Analysis

Cameras (Everfocus ez.HD, Everfocus Electronics Corp., New Taipei City, Taiwan) were installed above each pen to record the animals' behavior. They were arranged at the cable duct, directly above the middle of the pen, to observe the entire area from a top view. The cameras were connected to a digital video recorder (Everfocus ECOR FHD 16 × 1, Everfocus Electronics Corp., New Taipei City, Taiwan), which recorded continuously on hard drives over the entire experimental period. The behavior of 60 sows in five consecutive batches was analyzed regarding the occurrence of stereotypies. The associated ethogram is shown in **Table 1**. The animals were observed by the same observer for an 8-h period per Saturday - 4 h in the morning (6:00–10:00) and four in the afternoon (13:00–17:00). There were 5 days of observation: one Saturday before farrowing (mean proximity to farrowing: 3.7 ± 1.5 days), and four Saturdays after farrowing (mean proximity to farrowing: 3.2/10.2/17.2 and 24.2 ± 1.5 days). On those observation days, the occurrence of stereotypies was analyzed for all sows during the time frames using the one-zero sampling method (i.e., Yes-No scale). The general occurrence of stereotypies in a sow (at least one Yes during the 40 h study period) was included in the statistical model. The observed frequencies and types of stereotypical behavior were not part of this analysis.

The total results of the behavioral analyses are planned to be published in a following paper.

Lesion Scoring

In five batches, the sows (n = 60) were scored individually concerning the occurrence of skin lesions by one trained observer at three different time points per batch. Sows were first scored at the day of entering the farrowing systems to record the injuries that resulted from group housing during pregnancy. The next scorings were performed after 13 and 30 days in both farrowing systems. In accordance with the scoring scheme of Nicolaisen et al. (26) (**Table 2**), different body regions were assessed for the two body sides of each sow separately: head, ears, shoulder/neck, forelimbs, lateral side, ham, hind limbs, and the udder. For the loin, the sows received just one scoring grade. For each individual, the scores given for different body regions were added up to a cumulative body lesion score (BLS). Scoring results of the two udder sides were added up analogously to a cumulative udder lesion score (ULS).

Hair Samples

Using electronical clippers, a bilateral symmetric area of 20 × 30 cm was shaved in the transition between neck and shoulder blades (**Figure 3**) as close as possible to the skin. With regard to the multicompartment model (16), this method should rule out, as far as possible, that cortisol in hair originated from outer substances like feces, so as not to falsify the results.

In order to take samples from the newly grown hair, representing the period being in the farrowing systems, the surface of interest was shaved twice. Considering a depth of the hair shaft in the skin of 3–4 mm (27) and an assumed growth rate of 0.7 cm/month (28), it takes about 2 weeks until the lower part of the hair shaft has reached the outermost skin layer to be shaved. Therefore, the sows were shaved 13 days (between 13:00 and 17:00) after entering the farrowing system. Consequently, the newly grown hair in this region should have been formed in the farrowing housing period. To ensure that the hair formed during the experimental period had grown out of the skin, animals were shaved 15 days after leaving the farrowing system again, thus 35 days after their first shaving (**Figure 4**). For measuring the hair length aimed at determining the hair growth rate in the experimental sows (n = 42), the shaved area was divided into three equal sections: a left and a right lateral part and the median subsection over the spine (**Figure 3**). The regrown hairs were first measured in length for the different regions before shaving and then stored in airtight plastic bags under light-protected and dry conditions at room temperature. The hair samples taken from the second shave were sent to the University of Technology, Dresden, Germany for analysis. A total of 61 samples (31 from FC sows and 30 from LH sows) were collected and analyzed. For each section, the length of five hairs per sow was determined with a folding rule after 35 days of growth. Thereafter, the hair growth rate was calculated for a 30-day period in order to compare our own results to those of earlier studies.

Measurement of Hair Cortisol Concentration

Cortisol Extraction

Hair sample washing and the extraction of the hair cortisol based on Davenport's methods (13) were performed. The hair segments were put into a 10 mL glass vessel into which 2.5 mL isopropanol was subsequently added. The tube was then transferred to an overhead rotator, where the prepared samples were mixed for

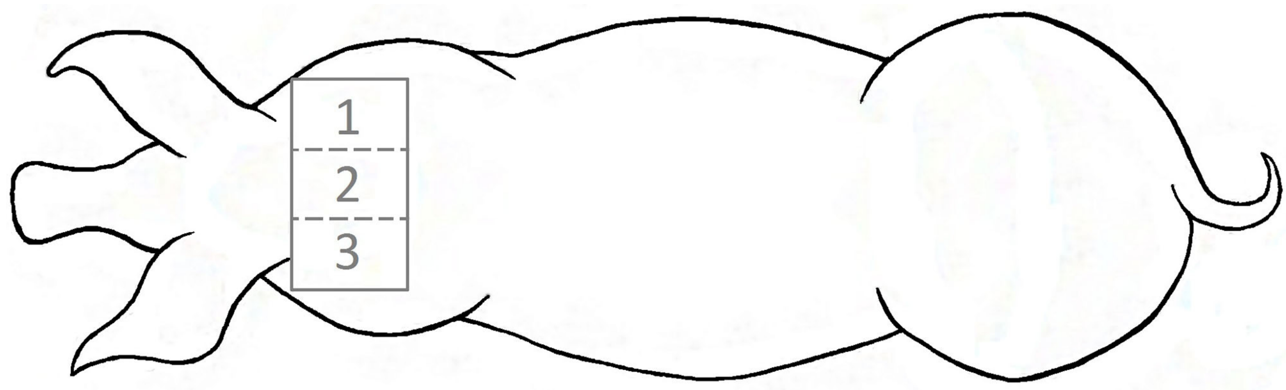

FIGURE 3 | Localization of the shaving area in the transition between neck and shoulder blades with subsections: (1) right lateral part, (2) region over the spine and (3) left lateral part.

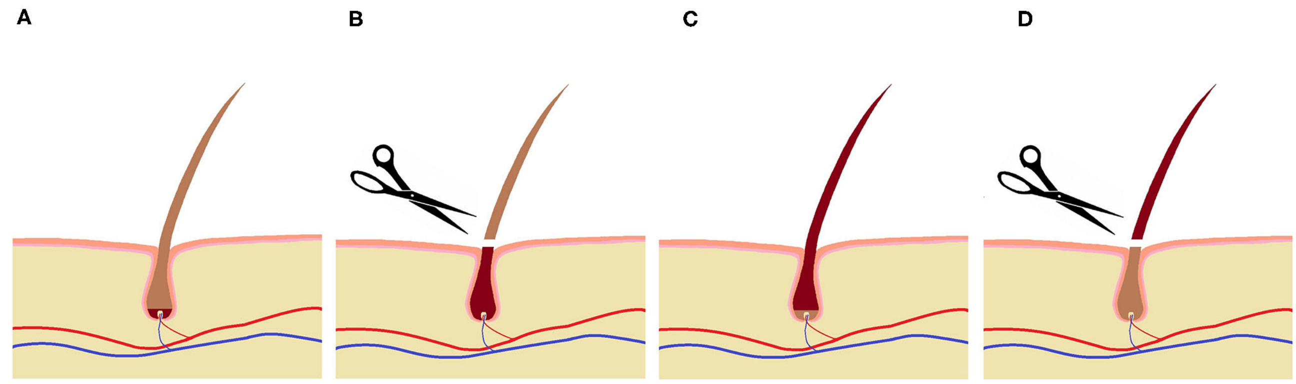

FIGURE 4 | Shaving regime for hair sampling: [dark red] indicates hair formed in the farrowing systems, [light brown] indicates hair formed outside the farrowing systems; **(A)** at the day of entering the systems, **(B)** 13 days after entering (first shaving), **(C)** at the day of leaving the systems, **(D)** 15 days after leaving (second shave for hair sampling).

3 min at room temperature. A short washing time was chosen simply to remove residual traces of externally originating cortisol from the surface of the hair, without extracting steroids from the interior of the hair shaft. The washing procedure was repeated twice and the hair samples were then dried for at least 12 h. A total of 7.5 mg of the washed and dried hair samples was placed in a 2 mL cryo vial for the following 18 h and incubated in 1.5 mL pure methanol for cortisol extraction. The hair samples were then fed into a microcentrifuge and then spun at 10,000 rpm for 2 min. A total of 1 mL of the clear supernatant was transferred to a new 2 mL glass vial. Under a steady stream of nitrogen gas and temperatures of 50 degrees Celsius, alcohol was evaporated and the samples were totally dried. To reconstitute the dried extracts, 0.4 mL of distilled water was added and the tube was vortexed for 15 s.

Cortisol Concentration by Immunoassay

The hair cortisol concentration was finally analyzed using a commercially available immunoassay with chemiluminescence detection (CLIA, IBL International GmbH, Hamburg, Germany). Due to low concentrations of cortisol in the hair, the protocol "RE62019" for ultra-sensitive detection was followed. This included the preparation of an additional standard by diluting standard B 1:3 with standard A. One hundred microliters of each standard, control and sample were pipetted into the respective wells of the microtiter plate. The enzyme conjugate was diluted at 75% and 50 microliters of this was added into each well. The plate was then incubated for 3 h at room temperature on an orbital shaker (400–600 rpm). After washing the plate four times with 250 microliters of diluted wash buffer, 50 microliters of prepared substrate solution mixture were pipetted into each well. The measurement of the relative luminescence units was performed after 10 min.

The assay precision in this study, indicated by the intra- (variation within plates) and interassay (variation between plates) coefficient of variance, was below 10 and 12%, respectively.

Statistical Analysis

Statistical analyses were performed using the R statistics software (29). The level of significance was set at $P < 0.05$.

Data were tested for normal distribution by using histograms.

Hair Growth Rate

A linear mixed effects model was used for hair length analysis in different body regions by implementing the R package *Imer Test* (30), with "hair length" as the dependent variable and "body region" as fixed and "sow" as well as "batch" as random effects. To determine any differences in the hair growth rate between the three different body regions, multiple pairwise comparisons of all body regions were carried out using *t*-tests by implementing the R package emmeans (31). The resulting *p*-values were adjusted using the Bonferroni- Holm method.

In the following, hair lengths are stated as means ± standard deviations (SD).

Hair Cortisol Concentration

First, the measured cortisol values were logarithmized to approach a normal distribution. A linear model was used with hair cortisol concentration as the dependent variable and the following fixed effects: farrowing system, sows' parity, number of piglets born alive, number of weaned piglets, total piglet loss, mean temperature in the experimental period, sows' weight loss in the farrowing system, occurrence of stereotypies, BLS and ULS on the third day of investigation (**Supplementary Table 1**).

Prior to the analysis, the potential effects were prioritized according to their potential influence on HCC. Thereafter, they were included stepwise in the model before the final model was developed.

The effects could potentially affect the stress level of sows and were investigated for the following reasons:

1. The housing system to unveil potential environmental effects on stress level
2. Parity, to show the effects of age and life experience on stress level
3. The number of born, weaned, piglet loss to uncover the effects of litter sizes on the sows' stress level
4. Temperature, to illuminate heat or cold stress
5. Weight loss of sows, to discover possible links between the physical conditions and the sows' stress level
6. Stereotypies, to show any link between behavior and stress
7. Lesions scoring results, to determine if body/udder lesions were stress-related.

The effects of the model were examined for significance using *t*-tests.

The stated mean values and standard deviations were calculated from the measured, not the modeled (logarithmized), HCC values.

Body Lesion Score

A logarithmic mixed-effects regression model was used in conjunction with the R package *Imer Test* (30) to analyze the BLS, considering the following fixed effects: farrowing system, time of investigation, number of weaned piglets, sows' parity, sows' body weight at the day of entering the systems, sows' weight loss in the farrowing system (**Supplementary Table 2**). Sow, pen and batch were considered as random effects. Prior to the analysis, the potential effects were prioritized according to their potential influence on HCC. Thereafter, they were included stepwise in the model before the final model was developed.

The effects could potentially affect the lesion score of sows and were chosen for the following reasons:

1. Farrowing system, in order to reveal environmental effects on the lesion score
2. Time of investigation, in order to reveal the effect of the housing period on the lesion score
3. Number of weaned piglets, in order to reveal the effects of litter sizes on lesions
4. Sows' parity, in order to reveal the effects of age and life experience on the lesion score
5. Sows' body weight at the day of entering the systems, in order to reveal the effects of weight/force on the lesion score
6. Sows' weight loss in the farrowing system to show any link between body weight or nutrition and the lesion score.

Based on the final model, *posthoc*-analysis was conducted using *t*-tests and the R package *emmeans* (31) to examine any differences in BLS between the three times of investigation within each housing system and between the two systems at each time of investigation. Using the Bonferroni-Holm method, the *P*-values were adjusted.

TABLE 3 | Descriptive results of hair cortisol concentrations (pg/mg) in the two farrowing systems (LH, loose housing; FC, farrowing crate).

System	N	Median	Mean	SD	Min	Max
LH	30	1.735	1.853	0.817	0.490	3.730
FC	31	1.630	2.125	1.526	0.940	8.920

RESULTS

Hair Growth Rate

Highly significant differences were found in the hair growth rate between different regions of the shaving area. While the hair in both lateral parts of the shaving area grew almost identically in length within 30 days (left side: 7.48 ± 3.52 mm, right side: 7.44 ± 3.24 mm, $P_{adj} = 1.00$), there was considerably more growth in the 10 cm-wide area above the spine (12.27 ± 3.95 mm, $P_{adj} < 0.0001$) (**Supplementary Tables 3, 4**).

Hair Cortisol Concentration

Overall, HCC were measured from a minimum of 0.49 pg/mg to a maximum of 8.92 pg/mg with a mean of 1.99 ± 1.23 pg/mg for all analyzed samples. Mean HCC did not differ significantly between the farrowing systems (LH: 1.85 + 0.82 pg/mg, FC: 2.13 + 1.53 pg/mg, $P = 0.631$) (**Table 3**). HCC was also not affected by the sows' parity (**Figure 5**), the number of piglets born alive, the number of weaned piglets, the number of total piglet loss, the skin lesion score, the udder lesion score, individual body weight loss during the study period, the occurrence of stereotypies or climatic conditions in the compartment ($P > 0.05$).

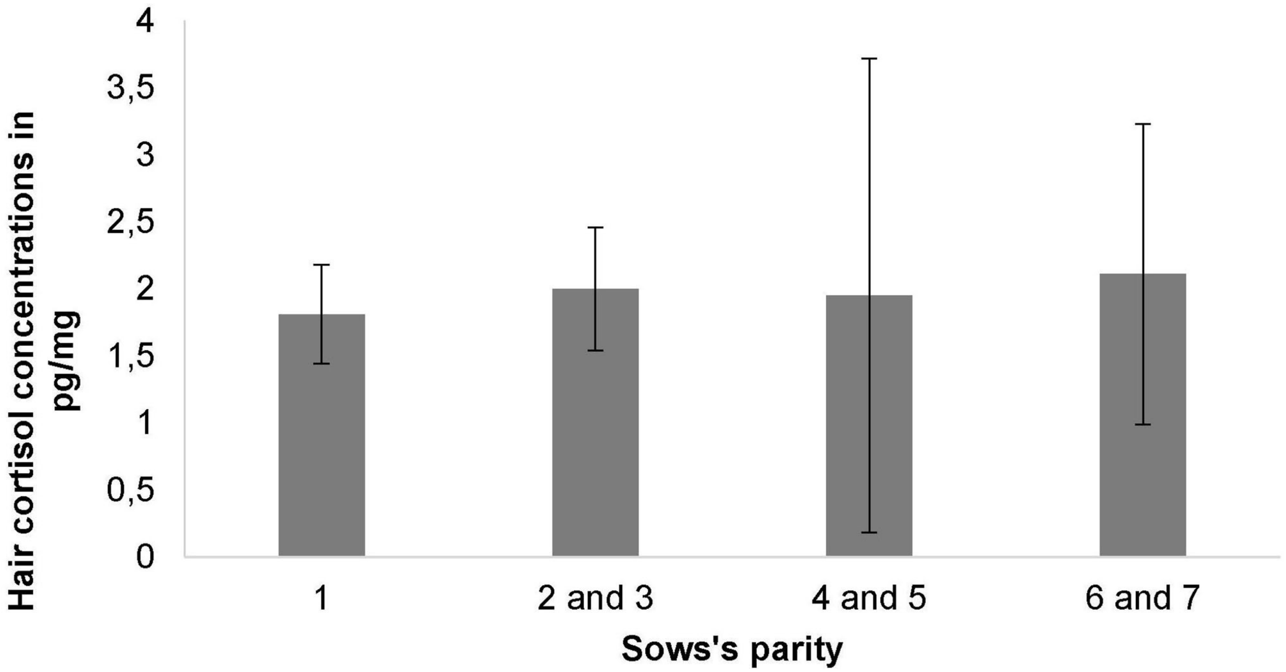

FIGURE 5 | Mean hair cortisol concentrations and standard deviations depending on the sows' parity.

Skin Lesions

In both housing systems, the mean individual BLS declined from the beginning to the end of the housing period ($P < 0.001$) (**Figure 6**). However, no difference in the mean individual BLS was found between FC sows and LH sows, in general ($P = 0.895$). Also, when analyzing each observation time separately (day 0, day 13, day 30), the mean BLS did not differ significantly between FC and LH sows ($P > 0.05$). The number of weaned piglets, sows' parity, body weight at the day of entering the systems, as well as the weight loss of the sows in the farrowing systems had no influence on the BLS (all $P > 0.05$). The results of the BLS at the three examination times are shown in **Figure 6**.

DISCUSSION

Hair Growth Rate

In order to use HCC as a retrospective calendar of stress, knowledge about the speed of hair growth is important. Only then can the period reflected by HCC be narrowed down, as this is how the sampling interval is determined (19). Furthermore, varying hair growth rates between different body regions can cause different results in HCC of these samples within the same examination period (32). Consequently, measurements of the hair growth rate are indispensable when evaluating hair cortisol as an indicator for chronic stress during a specific time frame. However, several studies on HCC in pigs did not consider the hair growth rate in order to adapt the shaving scheme to the period under investigation, or they did not perform a first shave (28, 32). In studies with a non-specific time frame for stress detection (25, 33, 34), prior shaving of the sampling region seems unnecessary. If the stress level should be studied over a certain period of time, shaving should be carried out beforehand.

In human studies, a mean hair growth rate of scalp hair of 1 cm/month is generally accepted. Nonetheless, scalp hair growth varies according to the region, with the posterior vertex region accepted to be the one with the most uniform growth rates, resulting in less intra-individual variation of HCC. Thus, samples are typically obtained from this region (12, 23, 35). However, such a standardized procedure does not yet exist for hair sampling in pigs and it seems useful to identify most suitable body regions for hair sampling in these animals. It was already shown in pigs that the HCC of the neck was lower than that of the lumbar region (32), which in turn was lower than that of the tail (34). Hair growth rates in these regions partly differed significantly, with the lowest one in the neck (34). To the best of our knowledge, there has not yet been any study performed on pigs measuring the growth rate of hair in different subareas within the same or adjacent body regions. In the present study, hair growth rate in the lateral areas of the shaving region was almost identical to those found by Bacci et al. (28) in the rump region of sows and similar to those found by Heimbürge et al. (34) in the neck. However, the growth rate determined in the present study for the subarea above the spine differed from that found in the lateral areas within the same shaving region. Thus, while Heimbürge et al. (34) revealed differences in the hair growth rate between different body regions in pigs, the present study also showed evidence that such differences were present within a single body region in pigs. This important result potentially influencing HCC measurements should be considered in further studies.

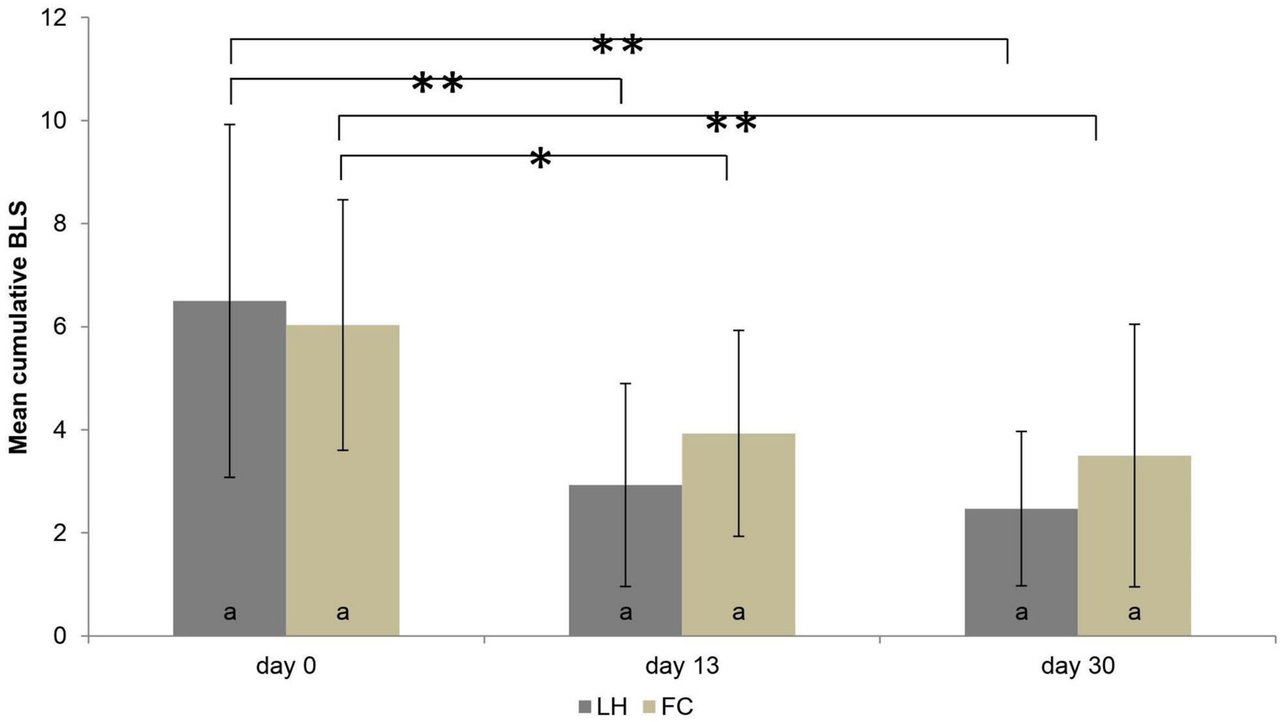

FIGURE 6 | Mean body lesion score (BLS) and standard deviations of the mean in the two systems (pens with farrowing crate = FC, loose housing pens = LH, each $n = 30$ sows) on three examination days. While there were no significant differences within one examination day between the systems (marked by same letters), the significant differences between the examination days are marked by * $P < 0.05$ or ** $P < 0.001$.

Hair Cortisol Concentration

It is assumed that chronic stress can cause both increased and decreased reaction of the HPA axis at different time points during a stressful situation: an HPA activation and elevated cortisol levels at the beginning can be followed by a counter-regulatory response over time, with rebounded glucocorticoid levels below normal ones by feedback mechanisms (36). The adaptation to recurrent or persistent so-called homotypic stressors in terms of a regulation of the HPA-axis, with a reduced physiological response compared to acute stress experiences, has been known for decades and is referred to as "habituation." It depends on characteristics of stress exposure, such as severity, modality and duration (37), and is stressor-specific (38). However, the HPA axis does not get used to particularly threatening stressors (39). Confining sows to crates could be seen as a cause of chronic stress. Even if housing-related hypercortisolemia may be transient (40), hair cortisol is recommended in earlier studies as a good indicator of chronic stress.

In the current paper, we explore if methods adapted from previous successful hair cortisol extraction studies (28, 32, 34) also apply to sows in farrowing systems housed under different farrowing conditions by trying to identify different possible and specific stress factors in the farrowing units.

To the best of our knowledge, this is the first study using hair cortisol analyses for evaluating different farrowing systems for sows.

In addition, it was investigated whether the housing systems had physical impacts on the sows using a lesion score, and whether these were related to measured HCC.

Further data obtained in this study concerning animal behavior and performance will be presented in another paper.

However, the results of the present study do not reveal any effects of either the housing system (farrowing crate or loose housing) or of all other investigated parameters on hair cortisol levels of sows. Thus, the results of the present study seem to be partly contradictory to those obtained by previous studies. Trevisan et al. (41) showed an influence of body weight on HCC, with lighter sows having higher hair cortisol concentrations, which was not confirmed in the present study. However, the cross-breeding sows (local genetic × large white) used in this previous study differed considerably in age, mean body weight and hair growth characteristics from the sows used in our study (1.6 ± 0.2 vs. 0.7 ± 0.3 cm/month in the lateral subareas of the shaving field), thus comparability may be limited. Bacci et al. (28) found an influence of the season on hair cortisol concentration in sows, with the lowest concentrations during the hot season. It was shown by Muns et al. (42) that an increase in temperature from 20 to 25°C can induce heat stress in sows. However, such an influence was neither confirmed by Heimbürge et al. (34) nor by the present study, even if mean room temperatures were closely related to the season (**Supplementary Table 5**) and were quite similar to those measured in the study of Bacci et al. (28) with 20–27°C.

The sampling procedure could also be a cause of conflicting results in different studies. The hair sampling method in the current study was an attempt to improve over previous methods by considering thoroughly the hair growth (which was included for analysis). Due to a standardized procedure in the present study, it is assumed that meaningful results were achieved. In the present study, care was taken to ensure that only hair that was newly formed during the study period was used for hair cortisol measurement. Thus, it was necessary to consider the time delay between incorporating cortisol in the hair and the appearance of this hair section on the skin's surface (24). Therefore, the hair growth rate was determined and the selected body area was shaved when the study started. As this procedure was not performed in all previous studies and in the case of Bacci et al. (28), hair samples were collected alternately from different body sides, the comparability of the results may be limited. Regardless of the selected body site and region for hair sampling, it is important to select the same site for all subjects (14). Since Casal et al. (32) found lower HCC in the dorsal neck of pigs compared to other body regions, suggesting that this lower cortisol concentration may result from less soiling with feces in this body part, this region was also chosen for sampling in the current examination.

Bacci et al. (28) reported that sows had higher hair cortisol values when kept in crates compared to group housing in the gestation area. However, the housing systems changed with the stage of pregnancy, and high cortisol levels in crated sows may also be explained by the systemic rise in cortisol concentration during farrowing (43). In contrast, the present study compared the hair cortisol levels of sows in different housing systems during the same stage of the reproductive cycle. Thus, the effect of the housing system may be better determined. In this case, neither the housing system nor body weight nor other investigated factors affected hair cortisol levels in sows.

Most earlier studies dealing with stress levels of crated and loose-housed sows in farrowing systems used cortisol measurement in saliva or blood and are, therefore, not directly comparable to this study (43–48). Lawrence et al. (43) found higher plasma cortisol values around farrowing independent of the housing system. However, higher cortisol levels were detected in crated sows compared to loose-housed sows during this time. The cortisol levels then decreased rapidly, and were almost identical in sows of both groups only 1 day after farrowing (43).

Hair cortisol is supposed to be an indicator of the previous weeks' or months' adrenocortical activity (14), representing the accumulated hormonal production of the period of hair growth (12). In our study, a fairly long period of investigation after farrowing may have "diluted" higher cortisol levels around farrowing, if followed by lower cortisol values thereafter. Consequently, differences in stress levels between the two housing systems in our study may no longer be obvious when measuring cortisol in hair.

Furthermore, the present study did not reveal any other influencing factors on hair cortisol levels in sows, such as the number of suckling or weaned piglets. This could be explained by the habituation to the constant stressors during the housing period. Suckling was proven to be a stress factor for sows as higher cortisol plasma levels were found the day before weaning compared to the day after weaning. Shortly after weaning, an increase in plasma cortisol was also measured (49). While the weaning procedure is short-lived, the longer suckling period may have a greater impact on cortisol levels. Apart from the possibility that the stimuli were not stressful enough to cause elevated cortisol levels in the sows (50), there is also the possibility of downregulation of the HPA axis in this case. It is also conceivable that dominant stressors with greater influence on cortisol levels mask the impact of other stressors of lesser effect on the HPA axis. An influence of these other stressors (for instance, the housing system) may, therefore, no longer be represented by the measured cortisol values.

Finally, it may also be assumed that measuring hair cortisol in pigs is not the appropriate method to determine stress levels. Heimbürge et al. (51) found no differences in HCC between pigs previously treated with ACTH and control animals, whereas in cattle an effect of ACTH treatment on hair cortisol level was found. The authors concluded that this may be related to a lower systemic cortisol response in pigs, although seasonally lower hair growth or external cross-contamination of hair cannot be ruled out either.

Skin Lesions

As lesion scores are an important animal welfare indicator, they were further investigated.

Although the farrowing crate itself may cause injuries to the sows (52), this was not reflected by the scoring results of the present study, with no indication of any influence of the farrowing system on the BLS. As shown in other studies investigating farrowing housing systems (26, 53), a significant decrease in BLS was observed in both housing systems during the study period. Individually housing sows in the farrowing systems prevented agonistic behavior, as this occurred in the gestation period when sows were group-housed. Thus, over time, skin lesions resulting from previous group housing during pregnancy healed. From this point of view, the time spent in the farrowing systems can be regarded as a recovery phase. Consequently, at no point in time was HCC found to be influenced by BLS. Also, Carrol et al. (25) did not find any influence of skin lesions on HCC in fattening pigs, whereas HCC was affected by tail lesions and lameness.

CONCLUSIONS

The results of the present study revealed that the use of hair cortisol measurements in sows around farrowing seems to be limited. This may be due to the constant stressful conditions in farrowing systems, such as suckling bouts or single housing, to which the sows' HPA axis may adapt over time, resulting in decreased cortisol levels. However, it is also possible that measuring hair cortisol is not the appropriate method for determining stress in pigs. Thus, a meaningful use of HCC in sows for comparing the effect of different farrowing systems on animal welfare remains questionable. Further research on the time course of cortisol levels mapped in the HCC seems necessary to validate the measured values. Also, the hair growth rate should be considered in further studies when measuring HCC. Only if the collected hair grows more or less homogeneously can the time period of hair analysis be defined. Regional differences in hair growth rate within the same shaving area should, therefore, be considered in future studies.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by Animal Welfare Officer of the University of Veterinary Medicine Hannover, Hannover, Foundation, Germany. Written informed consent was obtained from the owners for the participation of their animals in this study.

AUTHOR CONTRIBUTIONS

D-HW and MF designed the experiments. D-HW collected the data. SB and SH analyzed the data and performed the statistical analysis. D-HW, MF, NK, SB, and SH wrote and revised the paper. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2021.605078/full#supplementary-material

REFERENCES

1. Lassen J, Sandøe P, Forkman B. Happy pigs are dirty! – conflicting perspectives on animal welfare. *Livestock Sci.* (2006) 103:221–30. doi: 10.1016/j.livsci.2006.05.008
2. EFSA (European Food Safety Authority). Scientific opinion of the panel on animal health and welfare on a request from the commission on animal health and welfare aspects of different housing and husbandry systems for adult breeding boars, pregnant, farrowing sows and unweaned piglets. *EFSA J.* (2007) 572:1–13. doi: 10.2903/j.efsa.2007.572
3. Pedersen LJ, Malmkvist J, Andersen HML. Housing of sows during farrowing: a review on pen design, welfare and productivity. In: Aland A, Banhazi T, editors. Livestock Housing: *Modern Management to Ensure Optimal Health and Welfare of Farm Animals*. Wageningen Academic Publishers (2013). p. 93–111. doi: 10.3920/978-90-8686-771-4_05
4. EFSA (European Food Safety Authority). EFSA panel on animal health and welfare (AHAW); scientific opinion on the use of animal-based measures to assess welfare in pigs. *EFSA J.* (2012) 10:2512. doi: 10.2903/j.efsa.2012.2512
5. Mkwanazi MV, Ncobela CN, Kanengoni AT, Chimonyo M. Effects of environmental enrichment on behaviour, physiology and performance of pigs - A review. *Asian Austr J Anim Sci.* (2019) 32:1–13. doi: 10.5713/ajas.17.0138
6. FAWC (Farm Animal Welfare Council). *Farm Animal Welfare in Great Britain: Past, Present and Future.* (2009). Available online at: https://www.gov.uk/government/publications/fawc-report-on-farm-animal-welfare-in-great-britain-past-present-and-future (accessed May 26, 2020).
7. Veissier I, Boissy A. Stress and welfare: two complementary concepts that are intrinsically related to the animal's point of view. *Physiol Behav.* (2007) 92:429–33. doi: 10.1016/j.physbeh.2006.11.008
8. Matteri RL, Carroll JA, Dyer CJ. Neuroendocrine responses to stress. In: Moberg GP, Mench JA, editors. *The Biology of Animal Stress: Basic Principles and Implications for Animal Welfare*. Wallingford: CABI Publishing (2000).
9. Mormède P, Andanson S, Aupérin B, Beerda B, Guémené D, Malmkvist J, et al. Exploration of the hypothalamic-pituitary-adrenal function as a tool to evaluate animal welfare. *Physiol Behav.* (2007) 92:317–39. doi: 10.1016/j.physbeh.2006.12.003
10. Martínez-Miró S, Tecles F, Ramón M, Escribano D, Hernández F, Madrid J, et al. Causes, consequences and biomarkers of stress in swine: an update. *BMC Vet Res.* (2016) 12:171. doi: 10.1186/s12917-016-0791-8
11. Herman JP. Neural control of chronic stress adaption. *Front Behav Neurosci.* (2013) 7:61. doi: 10.3389/fnbeh.2013.00061
12. Russell E, Koren G, Rieder M, Van Uum S. Hair cortisol as a biological marker of chronic stress: current status, future directions and unanswered questions. *Psychoneuroendocrinology.* (2012) 37:589–601. doi: 10.1016/j.psyneuen.2011.09.009
13. Davenport MD, Tiefenbacher S, Lutz CK, Novak MA, Meyer JS. Analysis of endogenous cortisol concentrations in the hair of rhesus macaques. *Gen Comp Endocrinol.* (2006) 147:255–61. doi: 10.1016/j.ygcen.2006.01.005
14. Meyer JS, Novak MA. Minireview: hair cortisol: a novel biomarker of hypothalamic-pituitary-adrenocortical activity. *Endocrinology.* (2012) 153:4120–7. doi: 10.1210/en.2012-1226
15. Koren L, Mokady O, Karaskov T, Klein J, Koren G, Geffen E. A novel method using hair for determining hormonal levels in wildlife. *Anim Behav.* (2002) 63:403–6. doi: 10.1006/anbe.2001.1907
16. Henderson GL. Mechanisms of drug incorporation into hair. *Forensic Sci Int.* (1993) 63:19–29. doi: 10.1016/0379-0738(93)90256-A
17. Ito N, Ito T, Kromminga A, Bettermann A, Takigawa M, Kees F, et al. Human hair follicles display a functional equivalent of the hypothalamic-pituitary-adrenal axis and synthesize cortisol. *FASEB J.* (2005) 19:1332–4. doi: 10.1096/fj.04-1968fje
18. Sharpley CF, Kauter KG, McFarlane JR. An initial exploration of *in vivo* hair cortisol responses to a brief pain stressor: latency,

localization and independence effects. *Physiol Res.* (2009) 58: 757–61.
19. Kirschbaum C, Tietze A, Skoluda N, Dettenborn L. Hair as a retrospective calendar of cortisol production-increased cortisol incorporation into hair in the third trimester of pregnancy. *Psychoneuroendocrinology.* (2009) 34:32–7. doi: 10.1016/j.psyneuen.2008.08.024
20. Thomson S, Koren G, Fraser LA, Rieder M, Friedman TC, Van Uum SH. Hair analysis provides a historical record of cortisol levels in Cushing's syndrome. *Exp Clin Endocrinol Diabetes.* (2010) 118:133–8. doi: 10.1055/s-0029-1220771
21. González-de-la-Vara Mdel R, Valdez RA, Lemus-Ramirez V, Vázquez-Chagoyán JC, Villa-Godoy A, Romano MC. Effects of adrenocorticotropic hormone challenge and age on hair cortisol concentrations in dairy cattle. *Can J Vet Res.* (2011) 75:216–21.
22. Accorsi PA, Carloni E, Valsecchi P, Viggiani R, Gamberoni M, Tamanini C, et al. Cortisol determination in hair and faeces from domestic cats and dogs. *Gen Comp Endocrinol.* (2008) 155:398–402. doi: 10.1016/j.ygcen.2007.07.002
23. Sauvé B, Koren G, Walsh G, Tokmakejian S, Van Uum SH. Measurement of cortisol in human hair as a biomarker of systemic exposure. *Clin Invest Med.* (2007) 30:E183–91. doi: 10.25011/cim.v30i5.2894
24. Heimbürge S, Kanitz E, Otten W. The use of hair cortisol for the assessment of stress in animals. *Gen Comp Endocrinol.* (2019) 270:10–7. doi: 10.1016/j.ygcen.2018.09.016
25. Carroll GA, Boyle LA, Hanlon A, Palmer MA, Collins L, Griffin K, et al. Identifying physiological measures of lifetime welfare status in pigs: exploring the usefulness of haptoglobin, C- reactive protein and hair cortisol sampled at the time of slaughter. *Ir Vet J.* (2018) 71:8. doi: 10.1186/s13620-018-0118-0
26. Nicolaisen T, Risch B, Lühken E, van Meegen C, Fels M, Kemper N. Comparison of three different farrowing systems: skin lesions and behaviour of sows with special regard to nursing behaviour in a group housing system for lactating sows. *Animal.* (2019) 13:2612–20. doi: 10.1017/S1751731119000661
27. Mowafy M, Cassens RG. Hair growth in the domestic pig-histological aspects. *J Am Leather Chem Assoc.* (1976) 71:64–70.
28. Bacci ML, Nannoni E, Govoni N, Scorrano F, Zannoni A, Forni M, et al. Hair cortisol determination in sows in two consecutive reproductive cycles. *Reprod Biol.* (2014) 14:218–23. doi: 10.1016/j.repbio.2014.06.001
29. R Core Team. *R: A Language and Environment for Statistical Computing.* R Foundation for Statistical Computing (2019). Available online at: https://www.R-project.org/ (accessed May 30, 2020).
30. Kuznetsova A, Brockhoff PB, Christensen RHB. lmerTest package: tests in linear mixed effects models. *J Stat Softw.* (2017) 82:13. doi: 10.18637/jss.v082.i13
31. Lenth R. *emmeans: Estimated Marginal Means, aka Least-Squares Means. R package version 1.4.3.01.* (2019). Available online at: https://CRAN.R-project.org/package=emmeans (accessed May 30, 2020).
32. Casal N, Manteca X, Peña LR, Bassols A, Fàbrega E. Analysis of cortisol in hair samples as an indicator of stress in pigs. *J Vet Behav.* (2017) 19:1–6. doi: 10.1016/j.jveb.2017.01.002
33. Bergamin C, Comin A, Corazzin M, Faustini M, Peric T, Scollo A, et al. Cortisol, DHEA, and sexual steroid concentrations in fattening pigs' hair. *Animals.* (2019) 9:345. doi: 10.3390/ani9060345
34. Heimbürge S, Kanitz E, Tuchscherer A, Otten W. Within a hair's breadth - factors influencing hair cortisol levels in pigs and cattle. *Gen Comp Endocrinol.* (2020) 288:113359. doi: 10.1016/j.ygcen.2019.113359
35. Pragst F, Balikova MA. State of the art in hair analysis for detection of drug and alcohol abuse. *Clin Chim Acta.* (2006) 370:17–49. doi: 10.1016/j.cca.2006.02.019
36. Miller GE, Chen E, Zhou ES. If it goes up, must it come down? Chronic stress and the hypothalamic-pituitary-adrenocortical axis in humans. *Psychol Bull.* (2007) 133:25–45. doi: 10.1037/0033-2909.133.1.25
37. Grissom N, Bhatnagar S. Habituation to repeated stress: get used to it. *Neurobiol Learn Mem.* (2009) 92:215–24. doi: 10.1016/j.nlm.2008.07.001
38. Jean Kant G, Eggleston T, Landman-Roberts L, Kenion CC, Driver GC, Meyerhoff JL. Habituation to repeated stress is stressor specific. *Pharmacol Biochem Behav.* (1985) 22:631–4. doi: 10.1016/0091-3057(85)90286-2
39. Figueiredo HF, Bodie BL, Tauchi M, Dolgas CM, Herman JP. Stress integration after acute and chronic predator stress: differential activation of central stress circuitry and sensitization of the hypothalamo-pituitary-adrenocortical axis. *Endocrinology.* (2003) 144:5249–58. doi: 10.1210/en.2003-0713
40. Janssens CJ, Helmond FA, Wiegant VM. The effect of chronic stress on plasma cortisol concentrations in cyclic female pigs depends on the time of day. *Domest Anim Endocrinol.* (1995) 12:167–77. doi: 10.1016/0739-7240(94)00018-V
41. Trevisan C, Montillo M, Prandi A, Mkupasi EM, Ngowi HA, Johansen MV. Hair cortisol and dehydroepiandrosterone concentrations in naturally Taenia solium infected pigs in Tanzania. *Gen Comp Endocrinol.* (2017) 246:23–8. doi: 10.1016/j.ygcen.2017.03.007
42. Muns R, Malmkvist J, Larsen MLV, Sørensen D, Pedersen LJ. High environmental temperatures around farrowing induced heat stress in crated sows. *J Anim Sci.* (2016) 94:377–84. doi: 10.2527/jas.2015-9623
43. Lawrence AB, Petherick JC, McLean KA, Deans LA, Chirnside J, Gaughan A, et al. The effect of environment on behaviour, plasma cortisol and prolactin in parturient sows. *Appl Anim Behav Sci.* (1994) 39:313–30. doi: 10.1016/0168-1591(94)90165-1
44. Biensen NJ, von Borell EH, Ford SP. Effects of space allocation and temperature on periparturient maternal behaviors, steroid concentrations, and piglet growth rates. *J Anim Sci.* (1996) 74:2641–8. doi: 10.2527/1996.74112641x
45. Jarvis S, Calvert SK, Stevenson J, van Leeuwen N, Lawrence AB. Pituitary-adrenal activation in pre-parturient pigs (Sus scrofa) is associated with behavioural restriction due to lack of space rather than nesting substrate. *Anim Welfare.* (2002) 11:371–84.
46. Oliviero C, Heinonen M, Valros A, Hälli O, Peltoniemi OA. Effect of the environment on the physiology of the sow during late pregnancy, farrowing and early lactation. *Anim Reprod Sci.* (2008) 105:365–77. doi: 10.1016/j.anireprosci.2007.03.015
47. Hales J, Moustsen VA, Nielsen MBF, Hansen CF. The effect of temporary confinement of hyperprolific sows in Sow Welfare and Piglet protection pens on sow behaviour and salivary cortisol concentrations. *Appl Anim Behav Sci.* (2016) 183:19–27. doi: 10.1016/j.applanim.2016.07.008
48. Goumon S, Leszkowová I, Šimecková M, Illmann G. Sow stress levels and behavior and piglet performances in farrowing crates and farrowing pens with temporary crating. *J Anim Sci.* (2018) 96:4571–8. doi: 10.1093/jas/sky324
49. Tsuma VT, Einarsson S, Madej A, Lundeheim N. Cortisol and β-endorphin levels in peripheral circulation around weaning in primiparous sows. *Anim Reprod Sci.* (1995) 37:175–82. doi: 10.1016/0378-4320(94)01330-O
50. Schmitt O, Baxter EM, Boyle LA, O'Driscoll K. Nurse sow strategies in the domestic pig: I. Consequences for selected measures of sow welfare. *Animal.* (2019) 13:580–9. doi: 10.1017/S175173111800160X
51. Heimbürge S, Kanitz E, Tuchscherer A, Otten W. Is it getting in the hair? - Cortisol concentrations in native, regrown and segmented hairs of cattle and pigs after repeated ACTH administrations. *Gen Comp Endocrinol.* (2020) 295:113534. doi: 10.1016/j.ygcen.2020.113534
52. Anil L, Anil SS, Deen J. Evaluation of the relationship between injuries and size of gestation stalls relative to size of sows. *J Am Vet Med Assoc.* (2002) 221:834–6. doi: 10.2460/javma.2002.221.834
53. Schrey L, Kemper N, Fels M. Behaviour and skin injuries of sows kept in a novel group housing system during lactation. *J Appl Anim Res.* (2018) 46:749–57. doi: 10.1080/09712119.2017.1394308

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The First Protocol for Assessing Welfare of Camels

Barbara Padalino and Laura Menchetti*

Division of Animal Sciences, Department of Agricultural and Food Sciences, University of Bologna, Bologna, Italy

The aim of this study was to develop and describe a protocol for assessing welfare in camels reared in intensive or semi-intensive systems. A literature review was conducted searching for scientific papers on assessment of animal welfare and camel behavior, management, physiology, and pathology. The paradigms of Five Freedoms, the Five Domains Model, and the welfare principles and criteria applied by the Welfare Quality® and AWIN methods were then adapted to camels. A combination of animal-, resource- and management-based indicators were selected and categorized according to three levels of assessment: (i) Caretaker, (ii) Herd, and (iii) Animal. The Caretaker level is an interview of 23 questions exploring the caretaker's background, experience, and routine management practices. The Herd level is a check of the herd and of the place (i.e., box/pen) where camels are kept. The Animal level is a visual inspection aiming at evaluating individual camel behavior and health status. The selected indicators are presented for each welfare principle and level; for instance for the principle of "Appropriate nutrition," feeding management is investigated at Caretaker level; feed availability and quality, the number of feeding points, and camel feeding behavior are recorded at Herd level, while body condition score (BCS) is evaluated at Animal level. In this study recording sheets for the assessment at the three levels are proposed and how to conduct the assessment is described. Limitations of the proposed protocol are also discussed. Further applications of this protocol for assessing camel welfare on a large number of farms is needed to validate the proposed indicators and identify the thresholds for their acceptability as well as to develop overall welfare indices and welfare standards in camels.

Keywords: camel, welfare, behavior, feeding, housing, health

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Annamaria Passantino, University of Messina, Italy

****Correspondence:***
Barbara Padalino
barbara.padalino@unibo.it

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INTRODUCTION

Official FAO statistics report that there are over 35 million camels in the world (last update: 2018). Their number has grown by about 15% in a 10-year period and it is destined to progressively increase in the future (1). Although it is difficult to estimate the economic importance of this species, both present and future (2), some explanations of their growing popularity may be deduced. First of all, there are probably no other animal species as versatile for the human being: the camel is a multipurpose animal, used to produce meat, milk, wool, hides, and skins, with an active role in agricultural, cultural and recreational life of many populations worldwide (2, 3). In recent decades, several studies have confirmed the nutritional quality of camel products, particularly its milk (4–7), suggesting attractive marketing prospects and therapeutic uses (8, 9). Some modernizations in farming techniques, such as machine milking, have been introduced but room for improvement still exists (3, 10, 11). Genetic improvement and rational farm management could enhance the productive efficiency of camels and, therefore, their economic profitability. Finally, climate change and increasing desertification are likely to make camels' adaptive abilities more and more appreciated as they demonstrate peerless productive potential in arid conditions (3, 4).

Despite these promising prospects for camel rearing, there is still very little attention and knowledge about its welfare; these shortcomings concern both the scientific and legislative aspects. Recent bibliometric research (12) pointed out that, although the scientific interest in regards to the camel species has grown, little attention has been paid to camel welfare issues. There are still serious gaps of knowledge in camel physiology and behavior, in the impact of different housing systems on its welfare and relationship with humans. Specific indicators for assessing camel welfare have not been developed yet (12) and camels have been blatantly neglected by international legislation. The World Organization for Animal Health included camels in the document of recommendations for transport by land, but no specific chapters of "Terrestrial Code" addressed the welfare aspects of camels production systems (13). The first European project, named Welfare Quality® project, has focused on other species (14, 15) and the camel did not even appear in the second largest European project, the Animal Welfare Indicators Project (AWIN), which had to cover species not considered in the Welfare Quality® (16). The key idea of both Welfare Quality® and AWIN projects is that animal welfare is a multidimensional concept and multiple aspects of physical and mental health should be stated and evaluated accordingly. The latter protocols organize the welfare dimensions in principles and criteria extending the notions of the Five Freedoms (17) and suggest valid, measurable and reliable indicators for each criterion. The indicators have been further classified according to two generic approaches: (a) animal-based indicators (e.g., behavioral measurements, body conditions, health records); and (b) resource- and management-based indicators (e.g., space allowance, feeding regime, environmental characteristics) (18, 19).

Welfare Quality® and AWIN projects were aimed at developing assessment protocols that provided tools feasible and practical to evaluate animal welfare. Not only animals but also stakeholders would benefit from such a welfare assessment tool. A standardized tool could be used to evaluate individual resources (i.e., diet, housing), compare different husbandry systems, quantify a range for optimal welfare and assess farmers' compliance, develop quality certifications, identify welfare risk factors and give evidence for developing new animal welfare legislation (20).

Hypothesizing that camel welfare could be assessed using animal-based, resource- and management-based indicators and to fill the aforementioned gaps of knowledge, this study was aimed at introducing an innovative protocol for assessing welfare in camels reared in intensive or semintensive farming system conceived by the idea of adapting criteria and principles of Welfare Quality® and AWIN protocols to this peculiar species.

MATERIALS AND METHODS

Selection of Indicators

A group of researchers with experience in camel behavior and animal welfare reviewed the relevant scientific literature to select promising indicators to be included in the protocol. Research databases (PubMed, Web of Science, Google Scholar, and Scopus) were selected and the search was refined limiting the search to recent academic journal articles describing assessment of animal welfare. Since the literature available on camels was very scarce, the researchers mainly referred to the indicators used for horses and ruminants according to the AWIN and Welfare Quality® protocols (15, 18, 21–23), evaluating which ones could be adapted to camels. A combination of animal-, resource- and management-based measures were preferred for inclusion in this protocol as commonly done in the literature (20). The list of indicators was further refined using the experience in the field of the researchers to cover all aspects of camel welfare and consulting articles published on camel physiology, ethology, husbandry, and pathology (24–41). The literature review included only papers written in English.

The selection of the welfare indicators also took into account the principles of validity, reliability, and feasibility as reported in the literature for other species (42, 43). Thus, the indicators that require further laboratory analysis (e.g., metabolic profiling) were excluded to meet the principle of feasibility. All invasive measurements or measurements involving physical contact with animals were also excluded as camels could be untamed making procedures stressful for animals and unsafe for operators. Moreover, although their potential importance in assessing animal welfare is recognized, data such as milk quality and quantity, fertility indexes, mortality or daily body gains were not included in this protocol because they are difficult to be directly verified by an assessor. Finally, only measurable indicators were chosen to comply with the principles of validity and reliability (42). After the aforementioned process, the indicators were organized accordingly with the four principles and 12 criteria developed by Welfare Quality® (14, 15, 18).

Caretaker, Herd, and Animal Levels

Data related to all indicators included in each welfare principle should be collected at three levels depending on their origin: from the caretaker, "Caretaker level," from the direct evaluation of a group of animals and the pen where they are kept, "Herd level," or from the individual camel, "Animal level" (**Figure 1**). A recording sheet was developed for each level of assessment.

The Caretaker level is a face to face interview and comply with the principles of the Terrestrial Code according to which caretakers are responsible for the humane handling and care of the animals, and they should have sufficient skills and knowledge to ensure that animals are treated following animal welfare principles (13). The questions were selected and adapted from a questionnaire previously developed for investigating the knowledge of animal welfare among camel caretakers (44).

Mainly resource- and management-based indicators were chosen for the Caretaker level embracing all principles (**Tables 1**, **2**).

The Herd level is a check of the herd and of the place (i.e., box/pen) where camels are kept. It includes robust and feasible indicators requiring no or minimal handling. Resource- and management-based indicators were chosen for the "Good feeding" and "Good housing" criteria (**Table 1**), while mainly animal-based indicators were chosen for the "Good health" and "Appropriate behavior" criteria (**Table 2**).

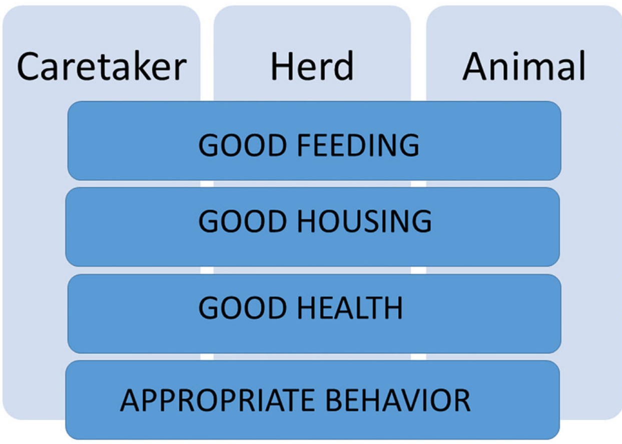


FIGURE 1 | The welfare principles of Good feeding, Good housing, Good health and Appropriate behavior are evaluated at three levels: Caretaker level, Herd level, and Animal level.

The Animal level consists of behavioral observation, behavioral tests, and a visual inspection of individual camels. Mainly animal-based indicators were chosen for all criteria. Among the measures proposed by the AWIN and Welfare Quality® protocols, only the most promising ones in terms of feasibility in the camel field were selected (e.g., BCS) (**Tables 1**, **2**).

RESULTS

Each farm welfare assessment should start with a meeting with the camel farm manager/caretaker, for explaining the protocol. The farm welfare assessment should be carried out at a fixed time, for example, 10:00 a.m., respecting the farm's routine practices. The on-farm welfare assessment would be carried out with some steps taken from outside and other inside the box/pen where the animals are kept (**Supplementary Figure 1**).

Camel Welfare Assessment at Caretaker Level

Table 3 shows the questions of the interview composed of 14 closed-ended and nine open-ended questions. During the interview, general information on the animals and their management is collected. In particular, the questions investigate the following aspects: demographic characteristics of the caretaker and camels, feeding and health management, self-evaluation of their ability to assess pain and distress, and knowledge of animal welfare. This information is aimed at double-checking the reported management with the data collected by the assessor at Herd or Animal level, verifying

TABLE 1 | Camel welfare indicators were selected by researchers for the principles of Good feeding and Good housing.

Principles	Welfare criteria	Welfare indicators		
		Caretaker level	**Herd level**	**Animal level**
Good feeding	Appropriate nutrition	Feeding management	Feed availability Feed quality Feeding points Feeding behavior	BCS
	Absence of prolonged thirst	Watering management	Water availability Water quality Water points Drinking behavior	Bucket test
Good housing	Comfort around resting	Years of experience in working with animals	Bedding Space allowance Rubbish Resting behavior	Resting behavior Insects
	Thermal comfort	Years of experience in working with camels	Temperature Humidity Wind speed Shade Use of the shade	Use of the shade
	Ease of movement	Camel exercise	Pen/box dimension Tethering Fence quality	Tethering Hobbled

The indicators were divided according to welfare criteria and source of information (Caretaker level: indicators collected through an interview of the caretakers; Herd level: indicators collected by a direct evaluation of a group of animals and their pen/box; Animal level: indicators collected by a direct evaluation of individual camels).
BCS, Body Condition Score.

TABLE 2 | Camel welfare indicators were selected by researchers for the principles of good health and appropriate behavior.

Principles	Welfare criteria	Welfare indicators		
		Caretaker level	**Herd level**	**Animal level**
Good health	Absence of injuries	Camel injury observed	Animals injured Type of injury	Injury Scar Swollen Joint Lameness
	Absence of disease	Camel disease observed Camel health check Medical treatments	Sick animals Type of disease	Disease Hair coat conditions Ectoparasites Discharge Diarrhea Abnormal udder Abnormal breathing Coughing
	Absence of pain and pain induced by management procedures	Caretaker's ability to identify pain	Animals in pain Animals with a nose-ring, cauterizations and wounds from halters or similar	Evident pain
Appropriate behavior	Expression of social behavior		Social behavior Aggressive behavior	Social interaction
	Expression of other behavior	Camel behavioral problems observed	Stereotypies Other abnormal behaviors	Stereotypies Other abnormal behaviors
	Good human-animal relationship	Experience in camel handling Caretaker's skills in identifying distress Caretaker's knowledge of animal welfare		Approaching test
	Positive emotional state			Behavior repertoire

The indicators were divided according to welfare criteria and source of information (Caretaker level: indicators collected through an interview of the caretakers; Herd level: indicators collected by a direct evaluation of a group of animals and their pen/box; Animal level: indicators collected by a direct evaluation of individual camels).

the caretaker's knowledge of welfare and at identifying possible hazards. For example, the caretakers' statements relating to the frequency of water distribution would be compared with the Herd level indicators of water quantity and quality. The criteria suggested by the caretaker to evaluate a camel in pain would indicate the ability to early quickly identify a camel that was suffering. Finally, the experience in camel handling and in managing other farm animals would affect farm management, health, and the human-animal relationship.

Camel Welfare Assessment at Herd Level

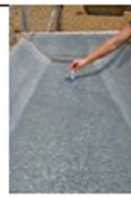
Tables 4–6 show the recording sheets for the assessment at Herd level. They are lists of parameters related to the environment, camel herd and the place where the camels are kept (i.e., pen); their collection should be carried out without disturbing the animals. The first measurements collected from outside the pen are related to animal behavior. After the census of the number of animals, the assessor should observe them and record the behaviors included in the Appropriate behavior section shown by each member of the herd during 3 min (45) (see scan sampling ethogram in **Supplementary Table 1**). Then, the environmental parameters, such as THI, and general characteristics of the pen/box, such as dimension and shape should be recorded. Instruments for detecting environmental parameters should be placed near the fence at the level of the camel's nose. Entrance into the pen is generally required to evaluate indicators of Good health, especially if there are many animals or very large pens, while it is always required to carry out the rest of the measurements and scoring included in the Good housing and Good feeding principles (e.g., dimension of feeding and drinking troughs). In addition to the facilities' dimension, their cleanliness should also be evaluated. The cleanliness of feeding and water points should be scored using a three-point scale: "dirty" if there is an abundant presence of organic or inorganic materials, such feces or debris, "partly dirty," if the facilities are contaminated by a few foreign materials, or "clean" (**Table 4**). Furthermore, the position of the feeding and watering point (i.e., in the sun or in the shade) and the temperature of the drinking water should be noted. Bedding should be similarly evaluated, recording the type of bedding and its cleanliness according to the presence of feces or unsuitable material (**Table 5**). The Herd level assessment also requires a qualitative description of the fences and the rubbish present in the pen. In particular, the condition of the fences should be reported as a binary variable (broken/unbroken) while the rubbish should be scored as "No rubbish," "Small," "Medium," and "Large" size according to its dimension (**Table 5** and **Supplementary Figure 2**). Other indicators such as density and trough space, should be calculated at a later stage. A selection

TABLE 3 | Camel welfare recording sheet at Caretaker level.

Date ___________ **Assessor** ____________	**Farm** ____________	**ID caretaker** ____________
DEMOGRAPHICS	How old are you?	_ _ _ _ _ _ years
	How long have you worked with camels?	_ _ _ _ _ _ years
	What other species have you worked with?	_ _ _ _ _ _ _ _
GOOD FEEDING	How often do you feed camels?	_ _ _ _time(s)/day □ Ad libitum
	How often do you water camels?	_ _ _ _time(s)/day □ Ad libitum
GOOD HOUSING	In your busiest week of the year, approximately how many camels are reared at the farm?	_ _ _ _ _ _ _ _ camels
	Do you keep other animal species at the farm?	□ Yes (specify) _ _ _ _ _ _ □ No
	What is the rearing purpose of your camels?	□ Meat □ Milk □ Other: _ _ _ _
	Are the camels exercised?	□ Yes □ No
	Do you change the management/housing according to season?	□ Yes □ No
GOOD HEALTH	Who assesses the health of the camels?	□ Vet □ Non-vet □ Not conducted
	Who treats the camel when it is sick?	□ Vet □ Non-vet □ Not conducted
	Who administers vaccinations to camels?	□ Vet □ Non-vet □ Not conducted
	Who administers endoparasite treatments to camels?	□ Vet □ Non-vet □ Not conducted
	Who administers ectoparasite treatments to camels?	□ Vet □ Non-vet □ Not conducted
	Which health problems have you observed in camels over the last year?	□ None □ Colic □ Injuries (e.g. cuts, bruises) □ Skin problems □ Muscular problems □ Diarrhoea □ Respiratory problems □ Overheating/sunstroke □ Other _ _ _ _ _
	What criteria do you use to identify a camel in pain or distress?	_ _ _ _ _ _ _ _ _ _ _ _ _ _
APPROPRIATE BEHAVIOR	How many years of experience in camel handling do you have?	_ _ _ _ _ _ years
	Do your camels show behavioral problems?	□ Yes □ No
	If yes, what behavioral problems do camels show?	□ Aggression □ Biting □ Kicking □ Anxiety or escaping from the pen □ Other _ _ _ _ _ _ _ _
	How do you grade your ability in identifying a camel in distress/pain?	□ Low □ Some □ Moderate □ High □ Very high
	What criteria do you use to identify a camel in pain or distress?	_ _ _ _ _ _ _ _ _ _ _ _ _ _
	How do you rank your understanding of animal welfare?	□ Low □ Some □ Moderate □ High □ Very high

Questions to pose during a face to face interview with the farm manager/caretaker divided according to the welfare criteria.

TABLE 4 | Camel welfare recording sheet for indicators of Good feeding collected at Herd level.

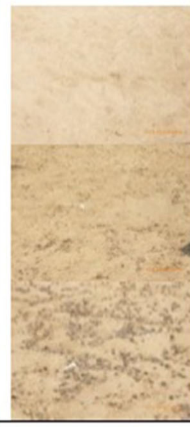
			WATER	FEED
GOOD FEEDING	Trough number		______	______
	Availability		□ Yes □ No	□ Yes □ No
	Trough dimension*	Length	______ meter	______ meter
		Width	______ meter	______ meter
	Trough material*		______	______
	Trough position*		□ In the sun □ In the shade	□ In the sun □ In the shade
	Water temperature		________ °C 	
	Type of food			_____________
	Salt block			□ Yes □ No
	Quality* – Cleanliness	Clean	□ 	□ 
		Partially dirty	□ 	□ 
		Dirty	□ 	□ 
	Number of animals		Drinking: ______ camels	Eating: ______ camels Ruminating: ______ camels

The Herd level consists of a check of the herd and of the place where the camels are kept.
**For each water/feeding point.*

of camel boxes/pens to be assessed may be applied following the rules suggested by the AWIN protocol for goats (section 3.6.1) (46) and stratifying according to the category of animals kept in the pens (young, adults, pregnant, and stage of lactation). The selection of the pen should be randomly conducted excluding the pens used as infirmary, culling, and quarantine. Namely, if <2 pens were present at the farm, all pens would be assessed; if the farm had 3–7 pens, two pens would be assessed; if the farm had

TABLE 5 | Camel welfare recording sheet for indicators of Good housing collected at Herd level.

GOOD HOUSING			
	Camel	Total	_ _ _ _ _ _ _ _ _ camels
		Hobbled/tethered	_ _ _ _ _ _ _ _ _ camels
	Environment	Temperature _ _ _ _ _ Humidity _ _ _ _ _ Wind speed_ _ _ _ _ THI _ _ _ _ _	
	Pen/box	Shape	_ _ _ _ _ _ _ _
		Dimension	Length: _ _ _ _ _ meter Width: _ _ _ _ _ meter
	Shade	Presence	□ Yes □ No
		Dimension of shelter	Length: _ _ _ _ _ meter Width: _ _ _ _ _ meter
		Number of animals in the shade:	_ _ _ _ _ _ _ _ _ camels
	Fence	Material	_ _ _ _ _ _ _ _
		Condition	□ Broken □ Unbroken
	Bedding	Presence	□ Yes □ No
		Type	_ _ _ _ _ _ _ _
		Cleanliness	□ Clean □ Partially dirty □ Dirty
	Rubbish	Dimension	□ No rubbish □ Small size (e.g. ropes, syringes, cans) □ Medium size (e.g. plastic bags, broken troughs) □ Large size (e.g. broken beds, furniture)
		Type	_ _ _ _ _ _ _ _



The Herd level consists of a check of the herd and of the place where the camels are reared.

8–10 pens, three pens would be assessed; finally, if the farm had more than 10 pens, 25% of the pens would be assessed.

Camel Welfare Assessment at Animal Level

Tables 7, **8** show the recording sheets for the assessments at Animal level. The Animal level assessment involves a closer look and contact with the camel without any invasive procedures. The number of animals to be assessed should be chosen following the rules proposed by AWIN for goats' selection assuming a 50% prevalence, a confidence interval of 95%, and an accuracy of 10% (section 3.6.3) (46). However, to minimize the impact on camels, non-restrictive criteria, such as a level of confidence of 90% or less, or rules of thumb could be adopted.

Initially, a behavioral observation of 3 min (direct observation or video taking for further analysis) should be conducted from outside the pen without disturbing the animal. During the behavioral observation, the assessor should record parameters included in Good housing (e.g., position of the camel in the shade or the sun, the presence of insects, and physical restraint) and the other behavior traits included in the recording sheet (see ethogram in **Supplementary Table 1**) using the one-zero (occurrence or non-occurrence) sampling method (45). Then, an approaching test modified by Wulf et al. (48) should be performed (**Supplementary Figure 3**). Briefly, an unfamiliar test person (i.e., tester) enters into the pen where the camel is kept and approaches the camel slowly, one step at a time. The test is stopped if the camel shows avoidance or aggressive behavior (turning the head, running away, biting) or when the tester can approach the camel and put a hand close to the nose of the camel. The tester should be a person with a solid scientific background on animal behavior. The camel behavioral responses should be classified as "Positive," "Neutral," or "Negative" (**Table 9**).

After the approaching test, the assessor should carry out a careful visual inspection of the camel to determine its Body

TABLE 6 | Camel welfare recording sheet for indicators of Good health and Appropriate behavior collected at Herd level.

GOOD HEALTH	Injury	□ Type: _____	_________ camels
	Number of animals	sick	_________ camels
		in pain	_________ camels
		with injuries from halters or tethering	_________ camels
		with cauterizations	_________ camels
		with nose ring	_________ camels
	Disease	□ Type: _____	N°_________ affected camels
		□ Type: _____	N°_________ affected camels
APPROPRIATE BEHAVIOR	Number of animals	resting (i.e. sternal/lateral decubitus)	_________ camels
		standing quietly	_________ camels
		showing social behavior	_________ camels
		showing aggressive behaviors	_________ camels
		showing stereotypies	_________ camels
		showing other abnormal behaviors	_________ camels

The Herd level consists of a check of the herd and of the place where the camels are reared.

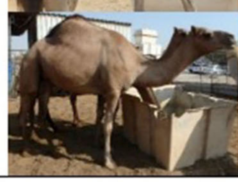
Condition Score (BCS) and the presence of any disease and injuries listed in the Good health section. For the BCS, the scoring (0–5) is based on visual examination of the ribs, the ischial and coxal tuberosities, the hollow of the flank, and the recto-genital zone as suggested by Faye et al. (47) (**Table 7**). If the camel is hobbled or tied up, the type of hobbles, the length of the rope (and whether injuries and scars caused by them were present) should be noted down (**Supplementary Figure 4**). Finally, a bucket-test should be conducted as follows: a bucket is filled with 5 L of fresh and clean water and placed about 1 m far from the camel. The time the camel takes to approach the bucket after it is placed ("latency time," in seconds) is taken using a stop-watch and the volume of water drunk (in liters) is recorded. If the camel does not drink within 60 s, the bucket is removed (**Supplementary Figure 5**). A categorization of these continuous measures is proposed to create a score-based index, called Thirst Index, indicating the animal's thirst (**Table 10**).

DISCUSSION

This study introduced an innovative protocol for assessing welfare in camels reared in intensive or semi-intensive farming systems conceived by the idea of adapting the criteria and principles of Welfare Quality® and AWIN protocols for this species (15, 16, 18). It focused on critical aspects of farming that could negatively impact camel welfare status as indicated by the Five Freedoms paradigm, i.e., thirst, hunger, discomfort, pain, distress, and abnormal behaviors (17). However, based on the current knowledge of the camel species, the proposed tool emphasized positive welfare states and human factors according to the Five Domains Model (49, 50) and proposed indicators *ad hoc* for camels. Among the proposed welfare indicators, some were already validated in camels [e.g., BCS; (47)], others were selected based on their good feasibility, repeatability, and reliability demonstrated in other species (42, 51) and the current knowledge of camel ethology, physiology, and pathology (24–41). The proposed protocol assesses camel welfare applying a multidisciplinary approach (14, 43), suggesting several indicators for each welfare principle assessed at three different levels, namely Caretaker, Herd, and Animal (**Figure 1** and **Table 1**). However, only further applications of the proposed welfare assessment tool on many camel farms will lead to the validation of the proposed indicators and the identification of thresholds for their acceptability as well as to the possible creation of overall welfare indices and welfare standards for camels.

"Appropriate nutrition" and "Absence of prolonged thirst" are the criteria used for the principle of Good feeding (18, 19). Hunger and thirst can occur not only when feed and water are not available, but also when they are not accessible or their quality and quantity do not meet the animals' physiological and behavioral needs (43). Thus, our protocol at the Herd level included structural and technical elements relating to feeding and watering points as well as indicators of effective availability and cleanliness of feed and water in line with the AWIN protocol (18). However, given the high environmental temperatures in which camels are usually reared, the position of the troughs in the shade/sun and the water temperature were added as measures of quality. Herd level was also implemented with some animal-based indicators of positive welfare states, such as feeding and drinking behaviors. Notwithstanding the elevated number of

TABLE 7 | Camel welfare recording sheet for indicators of Good feeding collected at the Animal level.

GOOD FEEDING	BCS*	0		Cachexia; ribs individually visible; ischium, coxal and shoulder very prominent; hollow of the flank visible and very deep; rectogenital zone very deep
		1		Ribs easy visible; ischium, coxal and shoulder very prominent; hollow of the flank visible; rectogenital zone very deep
		2		Ribs visible; ischium, coxal and shoulder prominent; hollow of the flank slightly visible; rectogenital zone deep
		3		Ribs just covered; ischium, coxal and shoulder slightly prominent; hollow of the flank slightly visible; rectogenital zone slightly deep
		4		Ribs well covered; ischium, coxal and shoulder barely visible; hollow of the flank not visible; rectogenital zone not deep
		5		Ribs buried; ischium, coxal and shoulder not visible; hollow of the flank not visible; rectogenital zone full of fat
	Bucket test	Latency time	_ _ _ _ _ _ _ _	sec
		Amount of water drunk	_ _ _ _ _ _ _ _	liters

The Animal level consists of a check of the individual animal and behavioral tests.
**BCS, Body Condition Score, adapted by Faye et al. (47).*

indicators introduced at Herd level, the assessment of welfare at this level has some limitations; firstly, it is only a snapshot of the reality, secondly, camel management and facilities may vary a lot among countries where camels are reared probably more than other livestock. The assessment at Animal level therefore becomes crucial for the evaluation of longer-term welfare conditions of camels. BCS is a robust animal-based measure for evaluating medium to long-term good feeding practices in many species (23, 52) and in camels has been validated by Faye et al. (47) and consequently applied in our protocol. Further studies could identify the welfare implications for each scoring category in camels of different age, physiological states or rearing purposes (i.e., growing camels, lactating she-camels, racing camels). As an indicator of "Absence of prolonged thirst" at Animal level, the protocol proposed a bucket-test, initially designed to evaluate thirst in horses (18). It only requires a graduated plastic container and fresh water as equipment, but biosecurity rules and good hygiene practices have to be respected to avoid the transfer of pathogens while testing animals. During a bucket-test, the latency time and the volume of water drunk can be easily recorded and scored. However, possible confounding factors could arise during the test skewing the results. In particular, different motivation factors could intervene especially if there are other animals in the pen. Furthermore, the latency time could be influenced by the temperament of the camel which could approach out of curiosity the bucket or be reluctant due to shyness or fear. For this reason, a Thrist index was proposed where differential scores were attributed to latency time and volume of water. The motivation

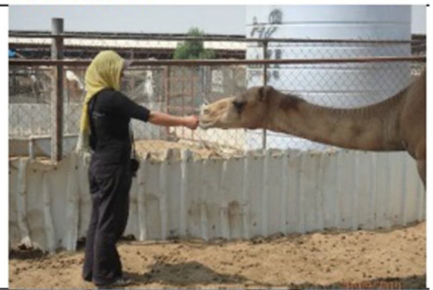
TABLE 8 | Camel welfare recording sheet for indicators of Good housing, Good health, and Appropriate behavior collected at the Animal level.

			Yes	No
GOOD HOUSING	Shade		□	□
	Insects		□ Only a few □ Some on a particular region □ The animal is full	□
	Tethering		□ Length of rope _ _ _ _ _ _ _ cm	□
	Hobbled		□ Material _ _ _ _ _ _ _ _	□
	Resting Behavior		□ Sternal decubitus □ Lateral decubitus	□
GOOD HEALTH			**Yes**	**No**
	Presence of a disease		□ Type _ _ _ _ _ _ _ _	□
	Presence of an injury		□ Type _ _ _ _ _ _ _ _	□
	Swollen joint		□	□
	Lameness		□	□
	Hair/coat	Skin disease	□	□
		Ectoparasites (e.g. tick)	□	□
	Discharge	Nasal discharge	□	□
		Eye discharge	□	□
		Vulva discharge	□	□
	Diarrhea		□	□
	Abnormal udder		□	□
	Breathing	Abnormal breathing	□	□
		Coughing	□	□
	Evident pain		□	□
APPROPRIATE BEHAVIOR			**Yes**	**No**
	Positive social interactions		□	□
	Stereotypies		□	□
	Feeding		□	□
	Ruminating		□	□
	Aggressive behaviours		□	□
	Approaching test		□ Negative response □ Neutral response □ Positive response	

The Animal level consists of a check of the individual animal and behavioral tests.

could also be influenced by the farm system and the type of food: in intensive farm, usually forages are containing more humidity while in extensive areas, where the food is dryer, camels can be more trained to avoid drinking for several days. The results of the bucket-test, thus, should be interpreted with due caution (51) and the results of the test conducted on intensive and extensive

TABLE 9 | Camel approaching test scoring system.

RESPONSE	DEFINITION	EXAMPLE
Positive	The camel moves towards the test person and shows positive signs of interest (i.e. sniffing or looking for a contact, it is possible to pet him/her)	
Neutral	The camel is quiet and relaxed, does not approach the test person or run away	
Negative	The camel is agitated, anxious, moves away or tries to attack/bite the test person	

Definitions of possible camel's responses during an approaching test.

farms should be not compared. Consequently, further studies are necessary to validate both the bucket-test and its scores as well as to develop new indicators to assess the "Absence of prolonged thirst" Criterion in camels.

"Appropriate nutrition" means that physiological and behavioral needs have to be met to ensure a good welfare state. Camels are well-known for their abilities to adapt to resource-poor environments but this could bias their welfare assessment, especially in intensive contexts. The camel, in fact, is well-adapted to the utilization of feed with low nutritional value in its natural habitat where the diet is varied and it can choose the plants by selecting the richest in water and mineral content (53). Under natural conditions, moreover, camels spend most of their time grazing and ruminating (24, 25). In intensive farms, unfortunately, the restricted feed access, as well as a diet usually less varied and poor in low-digestible feeds drastically limit these behaviors. These conditions could also have implications for rumination times, gut microbiota, and, finally, camel health (54). It is interesting to note that, according to Baraka et al. (40), 23% of farmed camels suffer from ruminal acidosis associated with low ruminal pH. Camels, unlike other herbivores, are also predisposed to diabetes mellitus and high-caloric diets can compromise their welfare (32). It is for all of these reasons that several indicators related to the feeding type, feeding strategies, and feeding behavior assume great importance in the welfare evaluation of camels reared in intensive farming systems and they have been included in our protocol. Camels have to digest many mineral salts as they are involved in the homeostatic mechanisms of thermoregulation and water retention. Including mineral supplementation in the diet has shown important effects on their metabolic profile and health (28) as well as on their milk production (33). Thus, the use of salt rocks or other supplements are important, not only for animal welfare, but also for farm productivity. Whilst the proposed protocol only registers the presence-absence of a salt block, quantitative measures of supplements, such as the number of salt blocks, the ease of access, and the physical form (i.e., solid or dissolved in drinking) could be added. Finally, not only animal-based (i.e., BCS), but also resource- and management-based measures indicating "Appropriate nutrition" should be always related to the category of animals present in the pen as nutrient requirements vary according to age, sex, and physiological status (pregnancy and lactation) (55).

"Comfort around resting," "Thermal comfort," and "Ease of movement" are the criteria used for the principle of Good housing (18, 19). In our protocol, the "Comfort around resting" involved measures collected at Herd level describing the space allowance, the type and quality of rubbish and bedding. Since the

TABLE 10 | Parameters and criteria proposed for scoring the results of a bucket-test during welfare assessment in camels.

PARAMETER	CRITERIA	SCORE
Latency time*	≥ 30 s	0
	< 30 s	1
Volume of water drunk[#]	<1 l	0
	1-4 l	1
	>4 l	2
Thirst Index	Latency time score + Volume of water drunk score	0-3

The pre-defined thresholds categorize the latency time and the volume of water drunk into scores ranging from 0 to 2. These scores are added to obtain the Thirst Index, which can range from 0 (not thirsty) to 3 (very thirsty).

Time the camel takes to approach the bucket after it is placed, 1 min maximum.

[#]*5 L maximum.*

latter aspects may depend on managerial decisions, a question about the experience in working with animals was included at the Caretaker level. Even though these aspects may often be neglected by the caretakers on farms, they are very important to respect the natural behavior of camels and to ensure they have a clean and quiet resting site. In extensive contexts, indeed, camels show a strong attachment to sleeping sites and carefully choose the quietest places (35) but intensive farming may affect this natural resting behavior, particularly when the pen is overcrowded. The space allowance could preclude the animal from having an adequate space to rest comfortably leading to various welfare concerns. El Shoukary et al. (36) showed that overstocking resulted not only in reduced lying and rumination time but also in increased serum cortisol concentration, feed competition, aggressive behavior and production losses. Camel's resting time may also be related to the presence and type of rubbish and bedding. Rubbish may be present in camel pens and could limit the space not only for resting but also for walking. Moreover, depending on its size, rubbish could increase the risk of injury and foreign body ingestion. This is the reason why both the presence and the dimension of rubbish are listed as indicators in our protocol. The presence/absence of rubbish inside a pen depends on the caretakers; consequently, their level of general experience in working with animals was included as an indicator and could be a point on which they need to be educated. The cleanliness of bedding is also listed, although dirty assessment at Herd level could be a problem only in case of humidity as the camel feces can dry rapidly. Thus, cleanliness could be also evaluated at Animal level developing a scoring system similar to that proposed for cows (56). Finally, resting behavior is an indicator at both Herd and Animal level, because the behavioral observation becomes crucial to assess whether the camels have, like and use an adequate resting place.

The Criterion of "Thermal comfort" states that "animals should neither be too hot nor too cold" (19). Although the ability of camels to adapt to an arid climate is well-known, the prevention of prolonged heat stress is also a welfare concern for camels. Indeed, physiological adaptive mechanisms may not be adequate to alleviate heat and camels can experience heat stress (29). The primary causes of heat stress are high environmental temperatures and humidity as well as the inadequate facilities to protect the camels from these environmental challenges (29). Thus, the indicators for this Criterion not only concern the environmental parameters, but also the availability and the use of shade as well as the caretaker's experience in working with camels. Although it should be verified, we hypothesized that the knowledge acquired by the caretaker on the thermal needs of camels and the management of adverse climate conditions could optimize the allocation of resources. Heat stress in camelids can cause decreased appetite, reluctance to rise, and lethargy and even result in death of the animals. There are not many statistics on the incidence of heat stress and there is little information on its risk factors (29), but certainly, the effects of heat stress are exacerbated if it is concomitant with water deprivation (41). Some animals could also be predisposed to heat stress by other factors such as parasitism, lameness, weaning, inadequate nutrition, or obesity (29). For this reason, the indicators suggested for the principles of Good health and Good feeding can further contribute to the thermal comfort assessment. A better understanding of the camel's ethology could also be useful to identify indicators of positive experiences related to their "Thermal comfort," as suggested in the Five Domains Model (49, 50). This Model encourages the inclusion of measures that indicate positive experiences for the animal, recognizing that acceptable animal welfare cannot be achieved only by avoiding negative states but agreeable experiences are needed as well. Therefore, minimizing the risk of thermal discomfort would not be enough. It is necessary, at the same time, to offer animals "a life worth living" providing them with opportunities to have positive experiences (50). For example, the number of animals resting or ruminating in the shade might be suggested as a positive welfare indicators although there is still no scientific evidence for this. Preference tests should be conducted to understand whether camels like resting and ruminating in the shade or under the sun.

The "Ease of movement" Criterion responds to the animal's need for an adequate space that guarantees them freedom of movement. In our protocol, a quantitative and qualitative description of the fences was proposed at Herd level as they can be a critical concern of many camel farms. The possibility of exercising was investigated at the Caretaker level, while the numbers of camels hobbled or tethered should be reported at both Herd and Animal level. Health consequences of the tools adopted for restraint are addressed below but, here, their role in the inhibition of movements is emphasized. Camels are usually calm and docile animals that, in feral conditions, live in herds moving over wide areas of land (35). However, in intensive management, it is not uncommon to find them confined in small places or even tied with short ropes and hobbled (37). This condition is a critical welfare concern, both from the point of view of freedom of movement and expressing natural behavior. Indeed, as in other species (57, 58), limited space and social isolation are the cause of chronic stress in camels which can develop stereotypies (38) and show high serum cortisol concentrations (59). Finally, movement control affected metabolism, whereby the increase in locomotory activities favored feed digestion and nutrient absorption (59). Therefore, ensuring the "Ease of movement" Criterion will also enhance camel performance.

"Absence of injuries," "Absence of disease," and "Absence of pain and pain induced by management procedures" are the criteria of the principle of Good health (18, 19). The remarkable resistance and adaptability of the camel can represent serious biases in the evaluation of its health. Several reports testify that camels are susceptible to a lot of diseases and can manifest more severe clinical signs than other animals (30, 31, 60). Some of these diseases mainly occur in certain periods of the year, e.g., breeding season, and could not be noticed on the day of assessment. Thus, caretakers were asked for the pathologies found in their camels during the last year in order to obtain "longitudinal" information on the incidence of the major diseases. The other critical issue is related to their remarkable ability to bear pain. They could continue to work without showing any signs of suffering and therefore medical intervention may be too late (55). In this context, early diagnosis, ability of the handlers to carry out correct evaluations and the frequency of checks assume considerable importance in guaranteeing the principle of Good health. *Ad hoc* indicators were included in our protocol but further considerations are needed. Pastoralists use several strategies to prevent and treat health conditions (61). However, the ineffectiveness of some traditional treatments, the lack of professional surgery as well as the inappropriate use of veterinary drugs and vaccines, not only compromises animal welfare, but contributes to the spread of disease and the development of drug resistance (62). Further epidemiological studies, more training of operators and a constant presence of veterinarians inside the farm would be desirable. In this regard, our protocol proposed a list of indicators at the Caretaker level to investigate the health management of camels and, in particular, to verify if veterinarians are routinely involved. However, further indicators could be added, such as the mortality and morbidity rate, indices to assess udder health, or more questions about the management of hygiene practices considering the growing importance of the camel as a dairy animal.

The measures of "Absence of pain and pain induced by management procedures" selected for the camel protocol are peculiar. Multiple indicators were selected for this Criterion taking into account the practices routinely used in camels for restraint, such as hobbles and nose-ring applications, or curative purposes, such as amputations and cauterization (61–63). Although the procedures for restraining can vary from country to country, halters, nose-rings, and hobbles are commonly used. In general, the nose piercing is a painful procedure which may also cause bacterial infections or lead to mutilation (64, 65). Hobbles, when tied too tight can not only cut the skin, leading to lesions, infection, and swelling but also cause inflammation of the tendons and lameness, and increase the risk of falls. Finally, they can reduce the circulation to the limb causing severe discomfort and pain (65). Cauterization is often practiced by caretakers to treat a wide range of diseases, including traumatic conditions, mastitis, and inflammations (61). Our measures were simplified compared to AWIN method for horses that also includes the Horse Grimace Scale (18) as not validated in camels. Thus, the development of tools for pain assessment in camels is certainly desirable and requires further studies.

"Expression of social behavior," "Expression of other behavior," "Good human-animal relationship," and "Positive emotional state" are the criteria of the principle of Appropriate behavior in AWIN and Welfare Quality (18, 19). The measures of "Expression of social behavior" and "other behavior" include indicators of both negative (i.e., aggressive and other abnormal behaviors) and positive welfare states (i.e., social behaviors), and could be collected both at Herd and Animal level. The present approach could be further implemented including other behavioral tests, such as a Fear test or Avoidance distance, and a Qualitative Behavior Assessment. However, knowledge of camel behavior is still too scarce, and the concept of welfare still seems to be in its infancy, to develop more complex protocols for this species. Social behaviors must surely be considered among indicators as camels were herd animals even before domestication (35). As shown by Padalino et al. (38), social isolation and inappropriate housing increase abnormal behaviors, namely locomotor (head-shaking and pacing in a circle) and oral (self-biting and bar-mouthing) stereotypies in camels. Thus, the presence of stereotypies were selected as indicators in the present protocol. Other behaviors "away from the norm" were defined as "Abnormal Behaviors" (66, 67) and generic examples were reported as there is no literature regarding this so far. The "Good human-animal relationship" Criterion mainly involved the Animal and Caretaker level. A modified version of the approaching test was developed but it is worth noting that the camel's responses could be influenced by the farm's system. Dairy camels, for example, are usually more accustomed to the presence and manipulation by humans than camels used for fattening. Some information on the caretaker's experience in handling camels and knowledge of stress and welfare were also considered important to investigate. According to Mellor (50), several characteristics of the caretaker could affect his relationship with the animals. As shown in other species (68, 69), caretaker's knowledge, training and familiarity with the animals seem to improve empathy, attitudes, and, ultimately, their handling and welfare as well as farm productivity. As regards the Criterion of "Positive emotional state" of camels, the indicators could arise from the evaluation of their behavioral repertoire. It could be possible to suppose that appropriate time spent grazing and rumination could indicate a good welfare state. Free-living camels, indeed, moved frequently from one feeding station to another (24, 25) and their feeding behaviors were characterized by a long eating time (26, 34). However, there is no specific research and further studies still need to be done to consider these behaviors as reliable indicators of positive emotional states. The principle of Appropriate behavior has been linked to several aspects of the camel reproductive sphere (39, 70) and several physiological and pathological consequences in other species (71–73). Consequently, the assessment of indicators included in these criteria could offer possibilities to improve other aspects such as the health and reproductive management of the camel. It is worth highlighting that the assessment of welfare is multidisciplinary and health, production, and welfare are interlinked.

Overall, this study proposes a tool for assessing camel welfare on intensive or semi-intensive systems based on the literature and it is only the first step of a long process. The presented protocol has to be validated by applying it in the field and the proposed measures should also be selected, refined and aggregated to develop overall welfare indices. This protocol, therefore, needs to be implemented by camel scientists, stakeholders, and other members of the various camel industry before suggesting welfare standards for camels.

DATA AVAILABILITY STATEMENT

The original contributions generated in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

BP worked at the conception, designed the study, and edited the manuscript. BP and LM wrote the first draft of the manuscript. Both authors read and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2020.631876/full#supplementary-material

REFERENCES

1. FAO. *Live Animals*. (2020). Available online at: http://www.fao.org/faostat/en/#data/QA/visualize (accessed June 11, 2020).
2. Faye B, Bonnet P. Camel sciences and economy in the world: current situation and perspectives. *3rd ISOCARD Conf Present 29th January—1st February, 2012*. Mascate: Sultanate Oman (2012). p. 2–15.
3. Faye B. The camel today: assets and potentials. *Anthropozoologica*. (2014) 49:167–76. doi: 10.5252/az2014n2a01
4. Zarrin M, Riveros JL, Ahmadpour A, de Almeida AM, Konuspayeva G, Vargas-Bello-Pérez E, et al. Camelids: new players in the international animal production context. *Trop Anim Health Prod*. (2020) 52:903–13. doi: 10.1007/s11250-019-02197-2
5. Konuspayeva G, Faye B, Loiseau G, Levieux D. Lactoferrin and immunoglobulin contents in camel's milk (*Camelus bactrianus*, *Campus dromedarius*, and Hybrids) from Kazakhstan. *J Dairy Sci*. (2007) 90:38–46. doi: 10.3168/jds.S0022-0302(07)72606-1
6. Konuspayeva GS. *Camel Milk Composition and Nutritional Value*. In: Omar A. Alhaj, Bernard Faye Hershey, editors. Pennsylvania: IGI global (2020). doi: 10.4018/978-1-7998-1604-1.ch002
7. Al haj OA, Al Kanhal HA. Compositional, technological and nutritional aspects of dromedary camel milk. *Int Dairy J*. (2010) 20:811–21. doi: 10.1016/j.idairyj.2010.04.003
8. Ayyash M, Olaimat A, Al-Nabulsi A, Liu SQ. Bioactive properties of novel probiotic lactococcus lactis fermented camel sausages: cytotoxicity, angiotensin converting enzyme inhibition, antioxidant capacity, and antidiabetic activity. *Food Sci Anim Resour*. (2020) 40:155–71. doi: 10.5851/kosfa.2020.e1
9. Khatoon H, Ikram R, Anser H, Naeem S, Khan SS, Fatima S, et al. Investigation of anti-inflammatory and analgesic activities of camel milk in animal models. *Pak J Pharm Sci*. (2019) 32:1879–83.
10. Gebremichael B, Girmay S, Gebru M. Camel milk production and marketing: pastoral areas of Afar, Ethiopia. *Pastoralism*. (2019) 9. doi: 10.1186/s13570-019-0147-7
11. Nagy P, Juhasz J. Review of present knowledge on machine milking and intensive milk production in dromedary camels and future challenges. *Trop Anim Health Prod*. (2016) 48:915–26. doi: 10.1007/s11250-016-1036-3
12. Pastrana CI, González FJN, Ciani E, Capote CJB, Bermejo JVD. Effect of research impact on emerging camel husbandry, welfare and social-related awareness. *Animals*. (2020) 10:780. doi: 10.3390/ani10050780
13. OIE. *Terrestrial Animal Health Code*. World Organ Anim Heal (2019). Available online at: https://www.oie.int/en/standard-setting/terrestrial-code/access-online/ (accessed June 15, 2020).
14. Blokhuis HJ, Veissier I, Miele M, Jones B. The welfare quality® project and beyond: safeguarding farm animal well-being. *Acta Agric Scand A Anim Sci*. (2010) 60:129–40. doi: 10.1080/09064702.2010.523480
15. Welfare Quality Network. *Welfare Quality® Project*. (2009). Available online at: http://www.welfarequality.net/en-us/news/assessment-protocols/ (accessed June 15, 2020).
16. European Animal Welfare Indicators Project (AWIN). *Animal Welfare Science Hub*. (2020). Available online at: http://www.animalwelfarehub.com/about-us (accessed June 15, 2020).
17. Farm Animal Welfare Council. *Five Freedoms*. Farm Anim Welf Counc (2009) 5. Available online at: http://www.fawc.org.uk/freedoms.htm (accessed June 15, 2020).
18. AWIN. *AWIN Welfare Assessment Protocol for Horses*. AWIN (2015). p. 1–80. doi: 10.13130/AWIN_HORSES_2015
19. Welfare Quality®. *Welfare Quality® Assessment Protocol for Cattle*. Lelystad: Welfare Quality® Consortium (2009).
20. Main DCJ, Kent JP, Wemelsfelder F, Ofner E, Tuyttens FAM. Applications for methods of on-farm welfare assessment. *Anim Welf*. (2003) 12:523–8. Available online at: http://www.ingentaconnect.com/content/ufaw/aw/2003/00000012/00000004/art00011
21. Battini M, Barbieri S, Vieira A, Can E, Stilwell G, Mattiello S. The use of qualitative behaviour assessment for the on-farm welfare assessment of dairy goats. *Animals*. (2018) 8:123. doi: 10.3390/ani8070123
22. Dalla Costa E, Dai F, Lebelt D, Scholz P, Barbieri S, Canali E, et al. Welfare assessment of horses: the AWIN approach. *Anim Welf*. (2016) 25:481–8. doi: 10.7120/09627286.25.4.481
23. Dunston-Clarke E, Willis RS, Fleming PA, Barnes AL, Miller DW, Collins T. Developing an animal welfare assessment protocol for livestock transported by sea. *Animals*. (2020) 10:705. doi: 10.3390/ani10040705
24. Khan B, Leteef M, Bilal M, Iqbal A, Hassan R. A study on some of the activity patterns of Camelus dromedarius maintained in Thal area of the Punjab Pakistan. *Pak J Agric Sci*. (1998) 33:67–72.
25. Dereje M, Udén P. The browsing dromedary camel: I. Behaviour, plant preference and quality of forage selected. *Anim Feed Sci Technol*. (2005) 121:297–308. doi: 10.1016/j.anifeedsci.2005.01.017
26. Hedi A, Khemais K. Intake, digestion and feeding behaviour of the one-humped camel stall-fed straw-based diets. *Livest Res Rural Dev*. (1990) 2:2.
27. Elmahdi B, Sallmann HP, Fuhrmann H, Von Engelhardt W, Kaske M. Comparative aspects of glucose tolerance in camels, sheep, and ponies. *Comp Biochem Physiol A Physiol*. (1997) 118:147–51. doi: 10.1016/S0300-9629(96)00449-5
28. Faye B, Ratovonanahary M, Chacornac JP, Soubre P. Metabolic profiles and risks of diseases in camels in temperate conditions. *Comp Biochem Physiol Part A Physiol*. (1995) 112:67–73. doi: 10.1016/0300-9629(95)0 0088-O
29. Norton PL, Gold JR, Russell KE, Schulz KL, Porter BF. Camelid heat stress: 15 cases (2003-2011). *Can Vet J*. (2014) 55:992–6.
30. Agab H, Abbas B. Epidemiological studies on camel diseases in eastern Sudan. *World Anim Rev*. (1999) 92:42–51.

31. Sazmand A, Joachim A. Parasitic diseases of camels in Iran (1931-2017)—A literature review. *Parasite*. (2017) 24:21. doi: 10.1051/parasite/2017024
32. Al Haj Ali M, Nyberg F, Chandranath SI, Ponery AS, Adem A, Adeghate E. Effect of high-calorie diet on the prevalence of diabetes mellitus in the one-humped camel (*Camelus dromedarius*). *Ann N Y Acad Sci*. (2006) 1084:402–10. doi: 10.1196/annals.1372.034
33. Onjoro PA, Njoka-Njiru EN, Ottaro JM, Simon A, Schwartz HJ. Effects of mineral supplementation on milk yield of free-ranging camels (*Camelus dromedarius*) in northern Kenya. *Asian-Australasian J Anim Sci*. (2006) 19:1597–602. doi: 10.5713/ajas.2006.1597
34. Aubè L, Fatnassi M, Monaco D, Khorchani T, Lacalandra GM, Hammadi M, et al. Daily rhythms of behavioral and hormonal patterns in male dromedary camels housed in boxes. *PeerJ*. (2017) 5:e3074. doi: 10.7717/peerj.3074
35. Schulte N, Klingel H. Herd structure, leadership, dominance and site attachment of the camel, *Camelus dromedarius*. *Behaviour*. (1991) 118:103–14. doi: 10.1163/156853991X00229
36. El Shoukary RD, Osman A, Mohammed A. Effects of stocking density on some behavioral and some blood biochemical parameters in camel during the rut period. *Egypt J Vet Sci*. (2020) 51:253–62. doi: 10.21608/ejvs.2020.24526.1153
37. Padalino B, Monaco D, Lacalandra M. Male camel behavior and breeding management strategies: how to handle a camel bull during the breeding season? *Emir J Food Agric*. (2015) 27:338–49. doi: 10.9755/ejfa.v27i4.19909
38. Padalino B, Aubé L, Fatnassi M, Monaco D, Khorchani T, Hammadi M, et al. Could dromedary camels develop stereotypy? The first description of stereotypical behaviour in housed male dromedary camels and how it is affected by different management systems. *PLoS ONE*. (2014) 9:e89093. doi: 10.1371/journal.pone.0089093
39. Fatnassi M, Padalino B, Monaco D, Aubé L, Khorchani T, Lacalandra GM, et al. Effect of different management systems on rutting behavior and behavioral repertoire of housed Maghrebi male camels (*Camelus dromedarius*). *Trop Anim Health Prod*. (2014) 46:861–7. doi: 10.1007/s11250-014-0577-6
40. Baraka TA, El-Sherif MT, Kubesy AA, Illek J. Clinical studies of selected ruminal and blood constituents in dromedary camels affected by various diseases. *Acta Vet Brno*. (2000) 69:61–8. doi: 10.2754/avb2000690 10061
41. Bouâouda H, Achâaban MR, Ouassat M, Oukassou M, Piro M, Challet E, et al. Daily regulation of body temperature rhythm in the camel (*Camelus dromedarius*) exposed to experimental desert conditions. *Physiol Rep*. (2014) 2:e12151. doi: 10.14814/phy2.12151
42. Scott EM, Nolan AM, Fitzpatrick JL. Conceptual and methodological issues related to welfare assessment: a framework for measurement. *Acta Agric Scand A Anim Sci*. (2001) 51:5–10. doi: 10.1080/090647001316922983
43. Battini M, Vieira A, Barbieri S, Ajuda I, Stilwell G, Mattiello S. Invited review: animal-based indicators for on-farm welfare assessment for dairy goats. *J Dairy Sci*. (2014) 97:6625–48. doi: 10.3168/jds.2013-7493
44. Menchetti L, Monaco D, Ziani A, Padalino B. Camel welfare: the first survey on camel caretakers' perspective. *J Camelid Sci*. (2020) Accepted paper.
45. Altmann J. Observational study of behavior: sampling methods. *Behaviour*. (1974) 49:227–67. doi: 10.1163/156853974X00534
46. AWIN. *Goats AWIN Welfare Assessment Protocol*. AWIN (2015). doi: 10.13130/AWIN_GOATS_2015
47. Faye B, Bengoumi M, Cleradin A, Tabarani A, Chilliard Y. Body condition score in dromedary camel: a tool for management of reproduction. *Emirates J Food Agric*. (2001) 13:1–6. doi: 10.9755/ejfa.v12i1.5193
48. Wulf M, Aurich J, May AC, Aurich C. Sex differences in the response of yearling horses tohandling by unfamiliar humans. *J Vet Behav Clin Appl Res*. (2013) 8:238–44. doi: 10.1016/j.jveb.2012.09.002
49. Mellor DJ, Beausoleil NJ. Extending the "Five Domains" model for animal welfare assessment to incorporate positive welfare states. *Anim Welf*. (2015) 24:241–53. doi: 10.7120/09627286.24.3.241
50. Mellor DJ. Updating animal welfare thinking: moving beyond the "five freedoms" towards "A life worth living." *Animals*. (2016) 6:21. doi: 10.3390/ani6030021
51. Dalla Costa E, Murray L, Dai F, Canali E, Minero M. Equine on-farm welfare assessment: a review of animal-based indicators. *Anim Welf*. (2014) 23:323–41. doi: 10.7120/09627286.23.3.323
52. Menchetti L, Brecchia G, Cardinali R, Polisca A, Boiti C. Feed restriction during pregnancy: effects on body condition and productive performance of primiparous rabbit does. *World Rabbit Sci*. (2015) 23:1–8. doi: 10.4995/wrs.2015.1703
53. Laudadio V, Tufarelli V, Dario M, Hammadi M, Seddik MM, Lacalandra GM, et al. A survey of chemical and nutritional characteristics of halophytes plants used by camels in Southern Tunisia. *Trop Anim Health Prod*. (2009) 41:209–15. doi: 10.1007/s11250-008-9177-7
54. Samsudin AA, Wright AD, Al Jassim R. The effect of fibre source on the numbers of some fibre-degrading bacteria of Arabian camel's (*Camelus dromedarius*) foregut origin. *Trop Anim Health Prod*. (2014) 46:1161–6. doi: 10.1007/s11250-014-0621-6
55. Previti A, Guercio B, Passantino A. Protection of farmed camels (*Camelus dromedarius*): welfare problems and legislative perspective. *Anim Sci J*. (2016) 87:183–9. doi: 10.1111/asj.12446
56. Faye B, Barnouin J. Objectivation de la propreté des vaches laitières et des stabulations. L'indice de propreté. *Bull Tech Cent Rech Zootech Vétérinaires Theix*. (1985) 59:61–7.
57. Beerda B, Schilder MBH, Van Hooff JARAM, De Vries HW, Mol JA. Chronic stress in dogs subjected to social and spatial restriction. I. Behavioral responses. *Physiol Behav*. (1999) 66:233–42. doi: 10.1016/S0031-9384(98)00289-3
58. Cooper J, McGreevy P. Stereotypic behaviour in the stabled horse: causes, effects and prevention without compromising horse welfare. In: Waran N, editor. *The Welfare of Horses. Animal Welfare, vol 1*. Dordrecht: Springer (2007).
59. El-Shoukary RD, Nasreldin N, Osman AS, Hashem NM, Saadeldin IM, Swelum AA. Housing management of male dromedaries during the rut season: effects of social contact between males and movement control on sexual behavior, blood metabolites and hormonal balance. *Animals*. (2020) 10:1–11. doi: 10.3390/ani10091621
60. Abbas B, Omer OH. Review of infectious diseases of the camel. *Vet Bull*. (2005) 75:1N−16N.
61. Volpato G, Lamin Saleh MS, Nardo A. Ethnoveterinary of Sahrawi pastoralists of Western Sahara: camel diseases and remedies. *J Ethnobiol Ethnomed*. (2015) 11:54. doi: 10.1186/s13002-015-0040-4
62. Basheir BO, ElMalik KH, Abdelgadir AE, Gameel AAR. Traditional and modern practices in the diagnosis, treatment and prevention of animal diseases in South Kordofan State, Sudan. *J Cell Anim Biol*. (2012) 6:213–25. doi: 10.5897/JCAB11.066
63. Ranjan R, Tuteja FC, Kashinath, Patil NV. A survey on traditional practices adopted for restraining camel in Rajasthan. *Indian J Anim Sci*. (2017) 87:118–21. Available online at: http://epubs.icar.org.in/.../66940
64. Stafford KJ, Mellor DJ. Painful husbandry procedures in livestock and poultry. In: Grandin T, editor. *Improving Animal Welfare: A Practical Approach*. London: CABI Publishing. p. 337.
65. Rayner EL, Airikkala-Otter I, Susheelan A, Mellanby RJ, Meunier NV, Gibson A, et al. Prevalence of mutilations and other skin wounds in working donkeys in Tamil Nadu, India. *Vet Rec*. (2018) 183:450. doi: 10.1136/vr.104863
66. Cooper JJ, Mason GJ. The identification of abnormal behaviour and behavioural problems in stabled horses and their relationship to horse welfare: a comparative review. *Equine Vet J Suppl*. (1998) 27:5–9. doi: 10.1111/j.2042-3306.1998.tb05136.x
67. Mason GJ. Stereotypies: a critical review. *Anim Behav*. (1991) 41:1015–37. doi: 10.1016/S0003-3472(05)80640-2
68. Coleman GJ, Hemsworth PH. Training to improve stockperson beliefs and behaviour towards livestock enhances welfare and productivity. *OIE Rev Sci Tech*. (2014) 33:131–7. doi: 10.20506/rst.33.1.2257
69. des Roches A de B, Veissier I, Boivin X, Gilot-Fromont E, Mounier L. A prospective exploration of farm, farmer, and animal characteristics in human-animal relationships: an epidemiological survey. *J Dairy Sci*. (2016) 99:5573–85. doi: 10.3168/jds.2015-10633
70. Bhakat C, Raghavendra S, Sahani MS. Effect of different management conditions on rutting behavior of Indian dromedary camel. *Emirates J Food Agric*. (2005) 17:1–13. doi: 10.9755/ejfa.v12i1.5085
71. Alexander SL, Irvine CHG, Livesey JH, Donald RA. Effect of isolation stress on concentrations of arginine vasopressin, α-melanocyte-stimulating hormone

and ACTH in the pituitary venous effluent of the normal horse. *J Endocrinol*. (1988) 116:325–34. doi: 10.1677/joe.0.1160325

72. Herskin MS, Munksgaard L, Andersen JB. Effects of social isolation and restraint on adrenocortical responses and hypoalgesia in loose-housed dairy cows. *J Anim Sci*. (2007) 85:240–7. doi: 10.2527/jas.2005-346
73. Mumtaz F, Khan MI, Zubair M, Dehpour AR. Neurobiology and consequences of social isolation stress in animal model-A comprehensive review. *Biomed Pharmacother*. (2018) 105:1205–22. doi: 10.1016/j.biopha.2018.05.086

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Effect of Age, Breed, and Sex on the Health-Related Quality of Life of Owner Assessed Healthy Dogs

*Susan Rodger [1,2], E Marian Scott [3], Andrea Nolan [4], Andrea K Wright [5] and Jacqueline Reid [1,2]**

[1] School of Veterinary Medicine, University of Glasgow, Glasgow, United Kingdom, [2] NewMetrica Research, Glasgow, United Kingdom, [3] School of Mathematics and Statistics, University of Glasgow, Glasgow, United Kingdom, [4] Edinburgh Napier University, Edinburgh, United Kingdom, [5] Outcomes Research, International Centre of Excellence, Zoetis, Dublin, Ireland

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***Correspondence:**
Jacqueline Reid
jacky.reid@newmetrica.com

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Using an app, this exploratory study generated information on HRQL in a large cohort of dogs deemed healthy according to the owner. It forms the basis for further studies investigating the natural history of HRQL of dogs to inform the interpretation of interventional studies, but highlights the risks of relying on owner impression of health status. A previously published health-related quality of life (HRQL) instrument (VetMetrica™) that generates scores in four domains of quality of life in dogs - Energetic and Enthusiastic (E/E), Happy and Content (H/C), Active and Comfortable (A/C), and Calm and Relaxed (C/R), generated information on HRQL in 4,217 dogs (3 months—21 years). Dogs were categorized by age; young, 3–47 months, middle-aged, 48–95 months, and old, 96 months and older. Owners considered 2,959 dogs (3–95 months) to be "in perfect health" and these were used to explore the relationship between age, sex, breed and HRQL in apparently healthy dogs. Mean score was significantly greater (better) in young compared to middle-aged dogs in E/E, H/C and A/C and declined with advancing age. In H/C there was a small but significant difference in mean score between female and male dogs (mean greater in females), with a similar rate of decline in each gender with advancing age. In E/E there were very small but statistically significant differences in mean scores between certain breeds. In A/C there was a statistically significant interaction between breed and age and the rate of decline with advancing age differed with breed. Overall, age, breed, and sex predicted very little of the variation seen in HRQL scores. Data from a subset of 152 dogs, for whom clinical information was available, were used to examine the agreement between clinical evidence and owner opinion. According to the clinical records, 89 dogs were healthy and 63 had evidence of chronic disease. There was an approximately 40% disagreement between owner opinion on health status and clinical evidence of chronic disease (35% disagreement in all dogs and 43% in old dogs). HRQL scores were generally higher in dogs for whom there was no evidence of disease in the clinical record.

Keywords: health-related quality of life, dogs, breed, sex, age, owner opinion, health status

INTRODUCTION

Quality of life (QOL) is a general term used in a variety of disciplines in which it is accepted that QOL is, like pain, a multi-dimensional construct that is subjectively experienced by and is uniquely personal to the individual. Health-related quality of life (HRQL) is the subjective evaluation of circumstances that include an altered health state and related interventions.

HRQL instruments can be disease-specific focusing on particular disorders or populations, or they can be generic, designed to be used in a variety of contexts and across a wide range of disease conditions. Generic instruments generate either a single index score or a health profile, which attempts to measure all important aspects of HRQL. Health profiles offer significant advantages – they allow the measurement of the effects of a disease state or its treatment on different dimensions of HRQL e.g., physical or emotional components and they can be applied to any population, sick or healthy. There are several profile measures available to measure HRQL in humans and one of the most popular, the Medical Outcomes Study-Short Form (SF-36) is a generic instrument that generates scores in eight domains of QOL (physical functioning, physical role limitations, bodily pain, general health perceptions, energy/vitality, social functioning, emotional role limitations, and mental health) which can be combined to produce two summary scores in physical and emotional health (1). The authors have previously reported the development of an owner reported 46 item generic profile measure to evaluate HRQL in dogs (VetMetrica™) (2), and subsequently this instrument was shortened to 22 items in order to facilitate its presentation via a smartphone app (3). This instrument generates scores in four domains of QOL - energetic/enthusiastic (E/E), happy/content (H/C), active/comfortable (A/C), and calm/relaxed (C/R) which, like the SF-36, can be combined to create summary scores in physical and emotional health.

The advantages of generic HRQL scales are many, including the comparisons of different disorders, disease severities and treatments. They also can measure the burden of chronic disease in populations as compared with healthy ones (4). However, despite its widespread use among people with chronic conditions, there have been few studies regarding the ways in which the SF-36 performs among healthy populations – see (5) for a useful review. Accordingly it has been difficult to estimate within-person changes that may be the consequence of natural aging. Such norms are important to establish because the effect of any intervention in a sick population may be confounded by changes due to the natural progression of HRQOL over time. Studies using the SF-36 have shown that the SF-36 is reliable and able to detect differences between groups defined by age and gender which are known to impact HRQL scale scores (6). A subsequent study showed that in healthy people emotional health improves with age while physical functioning scores decline, with women scoring consistently lower than men in each age group, with the exception of the general health perception domain (7). To the authors' knowledge there is no information available on HRQL in populations of healthy pet animals in relation to such biological variables, and one aim of this study was to generate such information to expand the body of knowledge in relation to the natural history of HRQL in companion animals.

In human health care, mobile health (m-health) applications are increasing and used in a diverse range of practices (8), but app technology has remained relatively under-exploited in veterinary medicine to date. There are numerous apps available to track HRQL in humans, including some specific to disease states such as cancer and mental health (9–12). The advent of app technology provides a unique opportunity for veterinary surgeons to obtain information about the animals under their care remotely, and although apps have been used in the management of epilepsy (13) and in parasite monitoring in dogs (14), the majority of health and lifestyle apps that are available offer only the ability to record and diarise activity or medical information. One exception is the PetDialog app (Zoetis Inc.) for companion animals which allows owners to record and monitor exercise, nutritional intake and socialization, but also gives them access, through their veterinary surgeon, to the VetMetrica™ HRQL tool for dogs (15). This enables the veterinary surgeon to gather data relevant to animal health and well-being from owners outwith the veterinary consultation, including HRQL data.

The objectives of this work were first to report the effects, if any, of age, sex, and breed on the HRQL of a large cohort of apparently healthy dogs, using app technology to obtain owner responses to a previously validated generic profile measure of HRQL (Study 1), and second to examine the concordance between clinical records, owner opinion and HRQL in dogs (Study 2).

MATERIALS AND METHODS

Data Collection

Health-related quality of life (HRQL) data were collected from dog owners using a validated instrument (Vetmetrica™) (3) via a smartphone app (PetDialog, Zoetis). The HRQL instrument was incorporated as one of several features in the PetDialog app, which also required owners to input their dog's date of birth, breed, sex, and neutering status. Engagement with the HRQL instrument was entirely at the discretion of owners and uptake was not assessed. The PetDialog app was made available to pet owners in the United Kingdom and The Netherlands via 211 veterinary practices and was only accessible using a unique practice code. Due to data protection regulations, the data were anonymized such that the owners' demographic details and geographical location were unknown. These data were screened and where owners had completed multiple assessments for their dog, all except the most recent entry were removed. The Vetmetrica™ HRQL instrument contains 22 items, each of which comprises a descriptor (e.g., "active") with a 7-point Likert rating scale, 0–6 (with 0 meaning "not at all" and 6 meaning "could not be more"), which are used to determine an HRQL score in each of the four domains E/E, H/C, A/C, and C/R (3). An additional question directed at assessing owner opinion was included alongside the 22 items ("Is your dog in perfect health – yes or no?").

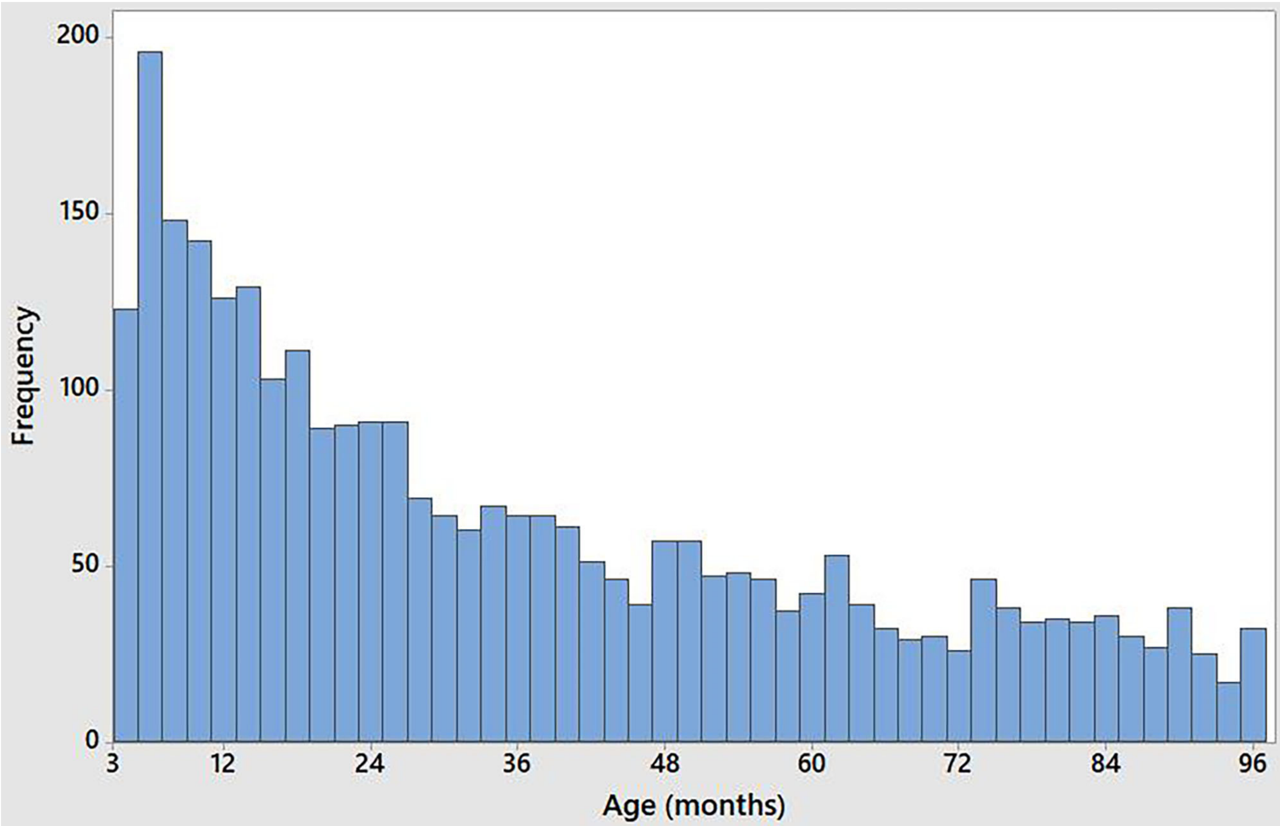

FIGURE 1 | Age distribution of 2,959 dogs aged under 96 months (8 years) of age considered to be in perfect health by their owners.

Statistical Methods

Data were analyzed using Minitab® Statistical Software (2010) (Computer software). State College, PA: Minitab, Inc. (www.minitab.com). ANOVA, ANCOVA and General Linear Modeling (GLM) were used. The level of statistical significance was set at 5% ($p < 0.05$) for all analyses.

Study 1: To assess the effects of age (as a factor) and sex, for each HRQL domain, a GLM was fitted, adding age and sex as main effects and including interactions. Terms which were not statistically significant were removed and the model was re-fitted.

The Tukey HSD test was used as a *post-hoc* test to assess factor level differences. An identical procedure was followed using Age in months (as a continuous variable) using an analysis of covariance (ANCOVA). In order to explore the effect of breed, a subset of data from dogs belonging to those breeds with the greatest representation ($n \geq 30$) were analyzed. For each HRQL domain, a linear model (ANOVA) was fitted, with Age segment, sex, and Breed and their second order interactions. Subsequently, terms which were not statistically significant were removed and the model was re-fitted.

Study 2: Chi-Squared Tests of Association. A Chi-squared test of association was used to assess the association between owner opinion regarding health and clinical record. Follow up confidence intervals for differences in means were used to look at the effect of veterinary assessed health status.

Study 1: HRQL in a Healthy Cohort of Dogs

The aim of these analyses was to examine HRQL in a cohort of healthy dogs. Dogs were categorized as "healthy" or "unhealthy" according to owner opinion and those considered unhealthy (owners answered "No" to the question "Is your dog in perfect health?") were excluded from this study. Due to the fact that the overall prevalence of non-infectious disease increases with age, it was assumed that elderly dogs are more likely to be unhealthy and therefore, in order to reduce the likelihood of including unhealthy dogs in these analyses, data from dogs aged 8 years or 96 months and above were also excluded. The effect of age was considered in two ways, firstly using "Age segment" as a factor whereby dogs aged 3–47 months of age were classified as "young" and dogs aged 48–95 months were classified as "middle-aged" and secondly, as a continuous variable ("Age in months"). The choice of boundaries for age categorization was based on the authors' professional veterinary opinion and clinical experience. It was not intended to define young, middle and old age in dogs but rather as an exploratory analysis of the dataset.

Study 2. Agreement Between Clinical and Owner Opinions on a Subset of Dogs

For most dogs in the database, the basis of health assessment was owner opinion. However, in association with their veterinary practice clinical information was obtained for a small cohort of dogs. These data were accessed and assessed by an independent veterinary surgeon who visited each practice and searched the

TABLE 1 | Dog breeds represented by at least 30 individuals.

Breed	Male	Female	Total
Retriever - Labrador	159	120	279
Spaniel - English Cocker	103	77	180
Jack/Parson Russell Terrier	77	59	136
Border Collie	65	59	124
Spaniel - English Springer	66	56	122
Cockapoo/Cockerpoo/Spoodle (X Spaniel/Poodle)	64	40	104
Bull Terrier - Staffordshire	51	44	95
German Shepherd/Alsatian	39	38	77
Retriever - Golden	45	32	77
Shih Tzu	40	31	71
Pug	48	19	67
Yorkshire Terrier	33	30	63
Border Terrier	27	28	55
Schnauzer (Miniature)	19	29	48
West Highland White Terrier	24	19	43
King Charles Spaniel - Cavalier	18	24	42
Lhasa Apso	17	21	38
Labradoodle (X Labrador/Poodle)	20	14	34
Beagle	21	12	33
Boxer	15	15	30
Total	**951**	**767**	**1718**

clinical records for entries made within 6 months of an owner HRQL assessment. All data were anonymised and apart from the owner's surname which was used to help identify individual dogs, no personal details of the owner were used. These dogs were obtained by random sampling from eight practices with the greatest compliance in data collection and met the following criteria: veterinary practice represented by at least 50 dogs in the dataset; the sample would have a ratio 1:2 of dogs in perfect health and not in perfect health state (owner's opinion); owner and veterinary assessments conducted within 6 months of each other. From these observations, relevant clinical information about chronic disease conditions diagnosed by the examining veterinary surgeon was extracted. Where there was no record of any chronic disease, the dog was classified as being "in perfect health," whereas any evidence of disease in the notes was taken to mean that the dog was "not in perfect health." This study had two objectives, first to assess the concordance between owner and clinical record, second to explore the differences if any between the clinically defined healthy and not healthy dogs.

RESULTS

Study 1. HRQL in a Healthy Cohort of Dogs

Out of a total of 4,217 dogs in the full data set, 3,411 were in "perfect health" according to owner opinion. The dogs considered healthy by owners ranged from 3 to 206 months (17 years) of age and 2,959 were under 96 months (8 years) of age (the majority aged between 3 and 48 months of age) (**Figure 1**). Of the 2,959 considered healthy and aged under 96 months, 1,592 (53.8%) were male, 1,367 (46.2%) were female and of these, 150 (9.42% of) males and 145 (10.60% of) females were neutered.

There were 135 breeds represented by at least one individual dog. There were 20 breeds or specific cross-breeds (i.e., where the dam and sire breeds were known) represented by at least 30 individual dogs (**Table 1**).

The profile of HRQL domain scores amongst young and middle-aged dogs in perfect health according to owner opinion is shown in **Figure 2** with descriptive statistics shown in **Table 2**. These show the differences in mean domain score by age group, with the older group having lower mean scores on average (E/E $p \leq 0.001$; H/C $p = 0.002$; A/C $p \leq 0.001$). There is also clearly variability in the scores, with a large number of potential outliers (identified in **Figure 2** by *). For two of the domains (H/C, A/C) the maximum score of 6 is frequently recorded showing that there are ceiling effects in these domains.

A summary of the results of the statistical analysis using the linear models for each domain is as follows with detail provided in **Table 3**.

There were no statistically significant effects found for CR. For the three other HRQL domains, there was a statistically significant effect of age, both as a factor and as a continuous covariate, with older dogs having lower scores Breed was found to be statistically significant for E/E and A/C, but not for HC. Sex was only found to be statistically significant for HC. There was statistically significant evidence of declining HRQL scores with age, which for AC only included a breed effect (i.e., different breeds declined at different rates). For A/C the annual rate of decline in all breeds ranged from 0.01 to 0.12). **Figure 3** shows the regression model for the domain E/E. It shows the breed differences on average, as well as the same rate of decline with advancing age. For a healthy border collie, over 10 years, its E/E score would be expected to drop from 4 to 3.6 on average. In all domains, the model variation explained was low (R-sq = 6.35%; 4.42% for E/E and A/C, respectively), leaving a large amount of the variation in HRQL domains unexplained.

Study 2: Owner Opinion on Health Status vs. Clinical Data

Of the 152 dogs for whom clinical data were available (3.60% of total dogs), 49 (32.2%) were aged 96 months and older and 103 (67.8%) were aged between 3 and 95 months of age. For each of these 152 dogs, the owner responses and presence or absence of chronic disease in the clinical records are shown in **Table 3**. Overall, the level of agreement between owner opinion on "Is your dog/the dog in perfect health, yes or no?" and the presence or absence of disease according to clinical notes was 65.8% (100/152 dogs), whilst in old dogs it was 57.1% (28/49 dogs). The level of disagreement in older dogs compared to younger dogs was not statistically significant according to Pearson Chi-square test (Chi-square = 2.402, DF = 1, $p = 0.121$). In most cases where there was a disagreement, the owner answered "Yes" whilst the clinical notes indicated that disease was present (20.4% of all dogs and 28.6% of old dogs). However, there were also 21 dogs considered not to be in perfect health by owners for whom there

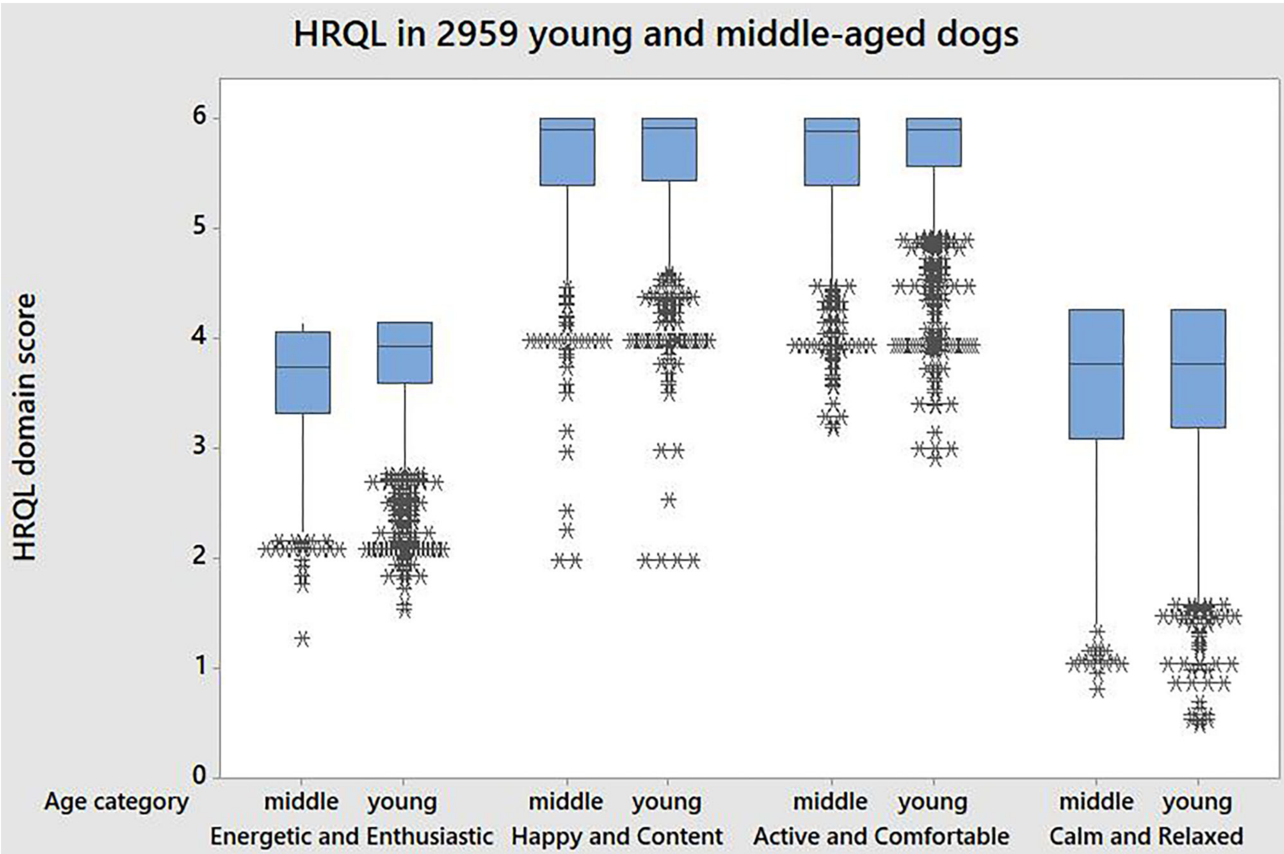

FIGURE 2 | Profile of HRQL domain scores in 2,959 dogs considered in perfect health according to owners. Young refers to dogs aged 3–47 months; middle aged refers to dogs aged 48–95 months.

was no record of disease in the veterinary clinical notes (13.8% of all dogs and 14.3% of old dogs).

HRQL and Clinical Records

From the 152 dogs in the subsample with clinical records, there were 63 dogs with evidence of disease according to the clinical record and 89 without (**Table 4**). The diseases recorded included degenerative joint disease, obesity, cancer, skin disease, cardiac disease, neurological disease, ear disease, dental disease, respiratory disease, eye disease, gastro-intestinal disease, musculoskeletal disease and other medical conditions that did not fit in these categories. For some dogs, a severity was recorded e.g., mild chronic skin disease but for others, the severity was not evident. There were 30 dogs with evidence of degenerative joint disease, ranging in severity from mild to severe and many dogs had evidence of disease in multiple categories e.g., degenerative joint disease with concurrent obesity, skin disease and dental disease etc. Descriptive statistics for HRQL domain scores in dogs with clinical data are shown in **Table 5**. These data suggest that mean HRQL domain scores were generally higher in dogs for whom there was no evidence of disease on the clinical record. There was a statistically significant difference in mean score for "Active and Comfortable" ($p \leq 0.001$). However, there was no statistically significant difference in mean score between these groups for Energetic and Enthusiastic ($p = 0.255$), Happy and Content ($p = 0.163$) or Calm and Relaxed ($p = 0.433$).

DISCUSSION

The objectives of this work were to report the effects, if any, of age, sex, and breed on the HRQL of a large cohort of apparently healthy dogs, using app technology to obtain owner responses to a previously validated generic profile measure of HRQL, and then to examine the concordance between clinical records, owner opinion and HRQL in dogs.

According to the data obtained via the PetDialog app, there is evidence of some variation in three of the HRQL domains (Energetic and Enthusiastic, Happy and Content and Active and Comfortable) according to the explanatory variables studied. In each of these domains, age was a significant factor - there was a general decline in score with advancing age, albeit that the rate of decline was very low. This is understandable given that the prevalence of health problems is higher in older animals and a corresponding decline in HRQL would therefore be expected. It is also in line with similar findings in human studies where older subjects tend to attain slightly lower mean scores for

TABLE 2 | HRQL domain scores across 2959 young and middle-aged dogs considered in perfect health according to owner.

	Mean	SE mean*	Std dev**	Q1***	median	Q3****	IQR*****	*n*
Energetic/enthusiastic								
young	3.78	0.01	0.46	3.60	3.93	4.15	0.55	2,045
middle-aged	3.62	0.02	0.52	3.32	3.75	4.07	0.75	914
Happy/content								
young	5.69	0.01	0.48	5.44	5.92	6.00	0.56	2,054
middle-aged	5.63	0.02	0.53	5.39	5.90	6.00	0.61	914
Active/comfortable								
young	5.72	0.01	0.48	5.57	5.91	6.00	0.43	2,054
middle-aged	5.62	0.02	0.54	5.40	5.89	6.00	0.60	914
Calm/relaxed								
young	3.58	0.02	0.77	3.19	3.77	4.26	1.07	2,054
middle-aged	3.57	0.03	0.77	3.10	3.77	4.26	1.16	914

**Standard error mean, **standard deviation, ***quartile 1, ****quartile 3, *****inter-quartile range.*
Young refers to dogs aged 3–47 months; middle aged refers to dogs aged 48–95 months.

TABLE 3 | Summary of significant effects of Age, Sex and Breed on HRQL domains.

	Energetic and Enthusiastic	Happy and Content	Active and Comfortable	Calm and Relaxed
Age category	Mean score Young > Mean score Middle-aged ($p \leq 0.001$); Average difference between scores in Young and Middle-aged dogs = 0.16	Mean score Young > Mean score Middle-aged ($p \leq 0.002$); Average difference between scores in Young and Middle-aged dogs = 0.06	Mean score Young > Mean score Middle-aged ($p \leq 0.001$); Average difference between scores in Young and Middle-aged dogs = 0.10	No significant difference in mean score; Average difference between scores in Young and Middle-aged dogs = 0.01
Age as continuous covariate	Mean score declined with advancing age at a rate of 0.003582 per month (R-sq adjusted = 3.65%); predicted decline in score over 12 months = 0.04	Mean score declined with advancing age at a rate of 0.001515 per month (R-sq adjusted = 0.71%); predicted decline in score over 12 months = 0.02	Mean score declined with advancing age at a rate of 0.002217 per month (R-sq adjusted 1.3%); predicted decline in score over 12 months = 0.03	Not statistically significant
Sex	No significant difference in mean score	Mean score Female > Mean score Male ($p = 0.039$)	No significant difference in mean score	No significant difference in mean score
Breed	No significant difference in mean score	No significant difference in mean score	Significant difference in rate of decline of mean score with advancing age amongst different breeds; predicted decline in score over 12 months ranged from 0.01 to 0.12 in all breeds;	No significant difference in mean score

HRQL domains such as physical wellbeing, energy/vitality, social wellbeing and pain than younger adults (6). The fact that the fourth factor (Calm and Relaxed) does not show a similar trend is not unexpected because it shows more variability in healthy dogs than was apparent for the other three domains, which is perhaps not surprising given the spectrum of excitability in the healthy dog (3).

In companion animal studies, the effect of sex can be difficult to establish because of the high number of animals that are neutered. However, in this study 91% of males and 89% of females were entire, possibly as a consequence of the young age of many of the dogs. Sex was a statistically significant factor in one domain only (Happy and Content), with females scoring slightly higher on average than males. This is in contrast to studies in human healthcare where women have reported poorer health on all variables of the SF36 than did men ($p < 0.001$) except for general health perception (6). However, in the study reported here it should be noted that the magnitude of difference in mean score was very small and therefore probably of little practical significance.

There was some evidence of significant breed differences in HRQL score in the domains "Energetic and Enthusiastic" and "Active and Comfortable," where small differences in mean score between breeds were found. The rate of decline with advancing age in "Energetic and Enthusiastic" was the same for each breed group. However, for "Active and Comfortable" the rate of decline in HRQL with advancing age was greater in certain breeds. This finding is, perhaps, to be expected, given that there are well

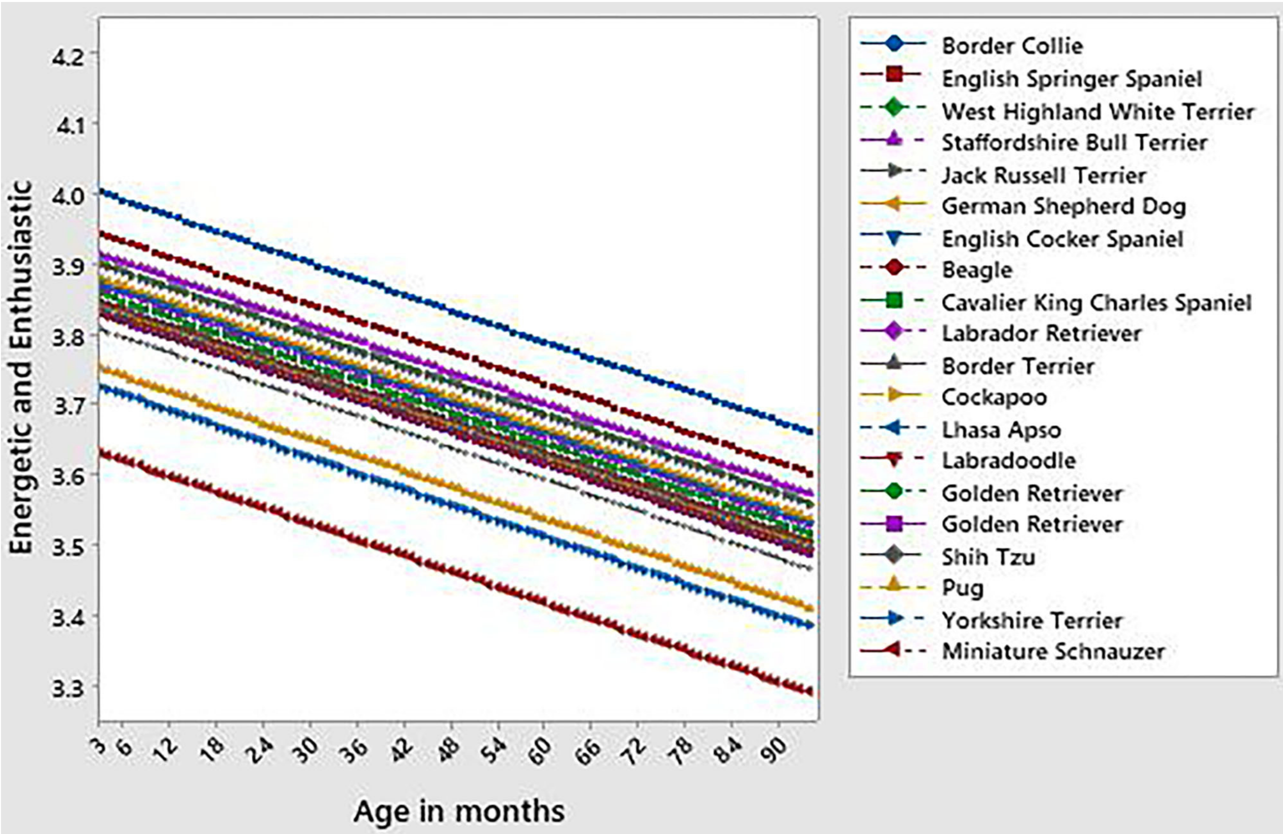

FIGURE 3 | Regression model of the "Energetic and Enthusiastic" HRQL domain, according to age in months and breed for 20 breeds of dog, each represented by at least 30 individuals.

TABLE 4 | Owner Opinion on their Dog's Health Status versus Clinical Evidence.

	Evidence of disease in case record		No evidence of disease in case record	
	All ages	**Old**	**All ages**	**Old**
Owner said dog is not in perfect health	32	18	**21**	**7**
	21.1%	36.7%	**13.8%**	**14.3%**
Owner said dog is in perfect health	**31**	**14**	68	10
	20.4%	**28.6%**	44.7%	20.4%
Total	63	32	89	17
	41.4%	65.3%	58.6%	34.7%

Bold indicates where there was disagreement between owner opinion of health status and clinical evidence.

documented breed differences in longevity (16, 17) and therefore those breeds of dog with shorter average lifespans would perhaps be expected to develop age-related health issues that affect activity at a younger age. Unfortunately there were no Great Danes, whose short lifespan is attributed to faster aging (18), represented. In general, these data suggest that there is a decline in score with advancing age in most breeds and some variability in the rate of decline between breeds. Despite the low breed numbers and restricted breeds studied, the authors consider that these data suggest that further investigation of the effect of breed on HRQL/aging is warranted. For example, in this limited study the mean (confidence intervals) annual rate of decline in healthy dogs for A/C across breeds was 0.03 (−0.05, −0.01) and a notably faster rate of decline could be indicative of an underlying asymptomatic health issue.

There remained considerable variability in HRQL domain scores unexplained by age, sex, and breed in this owner identified "perfect health" sample of dogs, for whom it is likely, given the results of study 2, that as many as 20% may have had underlying health conditions not recognized by the owner. It has been demonstrated that dog owners often underestimate the impact of health issues on the well-being of their pet, even where they are aware of their clinical signs and have knowledge of veterinary interventions. The complexity of the relationship between dog owners and their pets has been cited as one of the reasons for this apparent contradiction, with owners' emotions potentially influencing their response when asked to comment their dog's state of health (19, 20). Further, it has been suggested that owners' ability to recognize signs of ill-health in elderly dogs is particularly poor and that veterinary surgeons cannot rely on owners to report important signs of disease in these animals (21). The observation that owners often reported their dog to be "in perfect health" despite the presence of evidence to the contrary in

TABLE 5 | HRQL domain scores in 152 dogs for whom clinical data was available.

	Mean	SE mean*	Std dev**	Median	IQR***	*n*
Energetic and enthusiastic						
Disease recorded	3.10	0.10	0.76	3.18	1.06	63
No disease recorded	3.24	0.08	0.76	3.45	0.93	89
Difference in mean scores (95% Confidence Interval)	0.143 (−0.105, 0.391) $p = 0.255$					
Happy and content						
Disease recorded	5.15	0.10	0.79	5.30	0.96	63
No disease recorded	5.33	0.08	0.75	5.45	0.94	89
Difference in mean scores (95% Confidence Interval)	0.178 (−0.073, 0.430) $p = 0.163$					
Active and comfortable						
Disease recorded	4.59	0.11	0.90	4.60	1.45	63
No disease recorded	5.26	0.08	0.73	5.41	1.04	89
Difference in mean scores (95% Confidence Interval)	0.675 (0.404, 0.946) $p \leq 0.001$					
Calm and relaxed						
Disease recorded	3.12	0.11	0.88	3.20	1.26	63
No disease recorded	3.23	0.10	0.93	3.43	1.55	89
Difference in mean scores (95% Confidence Interval)	0.117 (−0.177, 0.410) $p = 0.433$					

**Standard error mean, **standard deviation, ***quartile 1, ****quartile 3, *****inter-quartile range.*

the clinical records and further, that this applied more in owners of older dogs, is in concordance with these previous reports. It is interesting to note that in a study of childhood obesity, parents of overweight children invariably underestimated their children's weight, despite being knowledgeable regarding healthy eating patterns and fully conversant with the health risks associated with obesity (22).

In this study, chronic disease was chosen as the focus when clinical records were examined because of the likely associated slow rate of change in health status over time. However, the potential time difference of up to 6 months between owner assessment and clinical examination allows for the possibility that the dog's state of health was genuinely different at these assessments and may explain some of the variation between owner opinion and clinical evidence. It is also possible that despite suffering from a chronic disease, some dogs' clinical signs may have been well controlled such that owners perceived their health to be very good on the day of the assessment. Similarly, some dogs with no evidence of chronic disease according to the case records may have experienced minor acute trauma or self-limiting infectious disease close to the time of the owner assessment which did not necessitate a visit to the veterinary practice and was not therefore recorded. This may in part account for the large numbers of low score outliers shown in **Figure 2**. Nevertheless, it is possible that around 600 dogs in the 2,959 dogs classified as "in perfect health" by their owners may not have been, and this may have accounted for some of the unexplained variability in HRQL domain scores.

A question often asked of the authors, who developed VetMetrica^{TM}, is "Will the scoring be affected by the owner's mood?" and we have no data available to answer that question. Since the scale is developed with the express intention of decreasing respondent bias, it is to be hoped that any such effect is minimized (23). However, there is a growing literature to support the fact that dog behavior can be influenced by human emotion through facial, voice and olfactory cues (24–26) and therefore it is not inconceivable that on any one assessment day the owner's emotional state may have affected their dog's behavior sufficiently to affect the HRQL domain scores. Accordingly, the owner's emotional state could possibly be added to age, gender, breed, and health status as factors accounting for the variability in the HRQL domain scores in this study.

Although not statistically significant in 3 of 4 domains, the general trend toward greater mean HRQL domain score in those dogs with no clinical evidence of disease suggests that the instrument may be discriminatory. This concurs with previous findings where the tool was shown to distinguish healthy from sick dogs (2, 3). It is interesting to note that there was a statistically significant difference between dogs with evidence of disease and those without such evidence in the domain "Active and Comfortable." This may be a reflection of the fact that 30 of the 63 dogs with evidence of disease were suffering from osteoarthritis which would be likely to affect their activity and pain levels.

The work described here was not a prospective study, but rather a retrospective analysis of data and the authors accept that there were clear limitations to their study. Dogs of 8 years and older were excluded from the analysis which is a significant limitation in the context of the investigation of natural aging. However, the fact that more than 1/4 of owners of these older dogs seemed to be incorrect in their interpretation of "perfect health" compared with 1/5 owners of the younger dogs suggests that the decision to restrict this study to dogs under 8 years was appropriate. In the younger cohort studied, the age distribution was heavily skewed to the right with the majority of the 2,959 dogs being between 3 months and 4 years of age. Furthermore, the owner impression of perfect health underpinned Study 1 and Study 2 demonstrated that this might be unreliable in ∼20%

of cases in the age group studied. A veterinary assessment of health status would have resulted in more dependable results, but this was not possible in this study. These limitations contribute to the fact that the results of Study 1 are not generalisable to the healthy dog population *per se*. Nevertheless, some of the findings in Study 1 regarding the effect of age, gender and breed on HRQL, though preliminary, suggest that some variation exists and that further study is warranted. Study 2 provides evidence to support the fact that owners cannot be relied upon to report accurately the health status of their dogs, especially when they are old. In cat studies the disagreement between owner and vet in terms of health status was 29 and 26% which is slightly higher than the 20% reported here for dogs (27). However, the cat study used direct vet clinical assessment compared with case record entries and this may account for the difference.

These limitations notwithstanding, to the authors' knowledge, this is the first study of its kind to generate any detailed information relative to HRQL in a large population of dogs, and additionally to collect data direct from owners by means of digital technology, to provide baseline HRQL data for future studies. The incorporation of the HRQL feature (VetMetrica^{TM}) in the PetDialog app for dogs was first reported in 2015 and since that time data has been gathered for over 9,000 dogs. The results described in this paper suggest that a large number of dog owners were willing to use an app to complete an HRQL questionnaire based on their dog's behavior. However, we have no evidence as to how many owners did not take the opportunity to complete the questionnaire on the app. Nevertheless, the authors are of the opinion that the study has shown the technology to be a useful means of owner engagement and a valid method of obtaining HRQL data remotely. This is significant because owner involvement is a key part of monitoring pet wellness and telehealth is likely to increase as veterinary practices increase their use of digital technologies post Covid19. By providing an app which is easy to use and accessible to owners it should be possible to obtain such information in future in order to track well-being in individual animals as well as for the purpose of surveillance, without reliance on owners and their pets requiring to attend the veterinary surgeon's premises.

In human healthcare it has been reported that in longitudinal studies within person declines (worsening health) with age were greater than estimated by cross sectional data alone (28). Accordingly, determining longitudinal within dog changes with age in a healthy cohort will be important in order to inform the interpretation of interventional studies conducted over time.

In conclusion, this exploratory study has generated valuable information regarding the natural history of HRQL in healthy dogs that lays the foundations for controlled studies to inform the interpretation of population studies as well as treatment effect in longitudinal interventional studies.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

Ethical review and approval was not required for the animal study because the Zoetis Ethical Review Panel deemed ethical permission was not needed on the basis that the ethical review process would not routinely include survey information that clients have provided about their pets. Written informed consent for participation was not obtained from the owners because Owners completed their HRQL assessments (survey relating to their dog's behavior) on a web platform after consenting to their data being used for research purposes.

AUTHOR CONTRIBUTIONS

ES, AN, and JR: conceptualization. AW: data provision. SR: writing. SR and ES: statistical analysis. All authors: review and editing.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2021.603139/full#supplementary-material

REFERENCES

1. Ware J, Kosinski M, Bjorner J, Turner-Bowker D, Gandek B, Maruish M. *User's Manual for the SF-36v2® Health Survey*. Lincoln, RI: QualityMetric Incorporated (2007).
2. Reid J, Wiseman-Orr ML, Scott EM, Nolan AM. Development, validation and reliability of a web-based questionnaire to measure health-related quality of life in dogs. *J Small Anim Pract.* (2013) 54:227–33. doi: 10.1111/jsap.12059
3. Reid J, Wiseman-Orr L, Scott M. Shortening of an existing generic online health-related quality of life instrument for dogs. *J Small Anim Pract.* (2018) 59:334–42. doi: 10.1111/jsap.12772
4. McHorney CA. Methodological and psychometric issues in health status assessment across populations and applications. *Adv Med Soc.* (1994) 5:281–304.
5. Obidoa CA, Reisine SL, Cherniack M. How does the SF−36 perform in healthy populations? A structured review of longitudinal studies. *J Soc Behav Health Sci.* (2010) 4:2.

6. Jenkinson C, Coulter A, Wright L. Short form 36 (SF36) health survey questionnaire: normative data for adults of working age. *BMJ.* (1993) 306:1437–40. doi: 10.1136/bmj.306.6890.1437
7. Hemingway H, Nicholson A, Stafford M, Roberts R, Marmot M. The impact of socioeconomic status on health functioning as assessed by the SF-36 questionnaire: the Whitehall II Study. *Am J Public Health.* (1997) 87:1484–90. doi: 10.2105/AJPH.87.9.1484
8. Boulos MN, Brewer AC, Karimkhani C, Buller DB, Dellavalle RP. Mobile medical and health apps: state of the art, concerns, regulatory control and certification. *Online J Public Health Inform.* (2014) 5:229 doi: 10.5210/ojphi.v5i3.4814
9. Rincon E, Monteiro-Guerra F, Rivera-Romero O, Dorronzoro-Zubiete E, Sanchez-Bocanegra CL, Gabarron E. Mobile phone apps for quality of life and well-being assessment in breast and prostate cancer patients: systematic review. *JMIR mHealth uHealth.* (2017) 5:e187. doi: 10.2196/mhealth.8741
10. Asensio-Cuesta S, Sánchez-García Á, Conejero JA, Saez C, Rivero-Rodriguez A, García-Gómez JM. Smartphone sensors for monitoring cancer-related quality of life: app design, EORTC QLQ-C30 mapping and feasibility study in healthy subjects. *Int J Environ Res Public Health.* (2019) 16:461. doi: 10.3390/ijerph16030461
11. Buitenweg DC, Bongers IL, van de Mheen D, van Oers HA, van Nieuwenhuizen C. Cocreative development of the QoL-ME: a visual and personalized quality of life assessment app for people with severe mental health problems. *JMIR Ment Health.* (2019) 6:e12378. doi: 10.2196/12378
12. Jabrayilov R, van Asselt AD, Vermeulen KM, Volger S, Detzel P, Dainelli L, et al. A descriptive system for the infant health-related quality of life instrument (IQI): measuring health with a mobile app. *PLoS ONE.* (2018) 13:e0203276. doi: 10.1371/journal.pone.0203276
13. App to track epilepsy in dogs. *Vet Rec.* (2015) 177. Available online at: https://apps.apple.com/us/app/rvc-pet-epilepsytracker/id992917809 (accessed January 16, 2021).
14. Jongejan F, de Jong S, Voskuilen T, van den Heuvel L, Bouman R, Heesen H, et al. "Tekenscanner": a novel smartphone application for companion animal owners and veterinarians to engage in tick and tick-borne pathogen surveillance in the Netherlands. *Parasit Vectors.* (2019) 12:1–9. doi: 10.1186/s13071-019-3373-3
15. New quality of life feature for dog app. *Vet Rec.* (2015) 176.
16. Lewis TW, Wiles BM, Llewellyn-Zaidi AM, Evans KM, O'Neill DG. Longevity and mortality in Kennel Club registered dog breeds in the UK in 2014. *Canine Genet Epidemiol.* (2018) 5:10. doi: 10.1186/s40575-018-0066-8
17. O'Neill DG, Church DB, McGreevy PD, Thomson PC, Brodbelt DC. Longevity and mortality of owned dogs on England. *Vet J.* (2013) 198:638–43. doi: 10.1016/j.tvjl.2013.09.020
18. Available online at: https://www.sciencemag.org/news/2017/01/why-large-dogs-live-fast-and-die-young (accessed January 16, 2020).
19. Packer RMA, O'Neill DG, Fletcher F, Farnworth MJ. Great expectations, inconvenient truths, and the paradoxes of the dog-owner relationship for owners of brachycephalic dogs. *PLoS ONE.* (2019) 14:e0219918. doi: 10.1371/journal.pone.0219918
20. Larsen JA, Villaverde C. Scope of the problem and perceptions by owners and veterinarians. *Vet Clin North Am Small Anim Pract.* (2016) 46:761–72. doi: 10.1016/j.cvsm.2016.04.001
21. Davies M. Geriatric screening in first opinion practice – results from 45 dogs. *J Small Anim Pract.* (2012) 53:507–13. doi: 10.1111/j.1748-5827.2012.01247.x
22. Etelson D, Brand DA, Patrick PA, Shirali A. Childhood obesity: do parents recognize this health risk? *Obes Res.* (2003) 11:1362–8. doi: 10.1038/oby.2003.184
23. Choi BC, Pak AW. Peer reviewed: a catalog of biases in questionnaires. *Prev Chronic Dis.* (2005) 2:A13.
24. Müller CA, Schmitt K, Barber AL, Huber L. Dogs can discriminate emotional expressions of human faces. *Curr Biol.* (2015) 25:601–5. doi: 10.1016/j.cub.2014.12.055
25. Yong MH, Ruffman T. Emotional contagion: dogs and humans show a similar physiological response to human infant crying. *Behav Process.* (2014) 108:155–65. doi: 10.1016/j.beproc.2014.10.006
26. D'Aniello B, Semin GR, Alterisio A, Aria M, Scandurra A. Interspecies transmission of emotional information via chemosignals: from humans to dogs (*Canis lupus* familiaris). *Anim Cogn.* (2018) 21:67–78. doi: 10.1007/s10071-017-1139-x
27. Noble CE, Wiseman-Orr LM, Scott ME, Nolan AM, Reid J. Development, initial validation and reliability testing of a web-based, generic feline health-related quality-of-life instrument. *J Feline Med Surg.* (2019) 21:84–94. doi: 10.1177/1098612X18758176
28. Hemingway H, Stafford M, Stansfeld S, Shipley M, Marmot M. Is the SF-36 a valid measure of change in population health? Results from the Whitehall II study. *BMJ.* (1997) 315:1273–9. doi: 10.1136/bmj.315.7118.1273

Conflict of Interest: JR was employed by the company NewMetrica Ltd and AW by Zoetis Inc.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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ORIGINAL RESEARCH
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Oral Meloxicam Administration in Sows at Farrowing and Its Effects on Piglet Immunity Transfer and Growth

Elena Navarro[1]*, Eva Mainau[1], Ricardo de Miguel[2], Déborah Temple[1], Marina Salas[1] and Xavier Manteca[1]

[1] Department of Animal and Food Science, School of Veterinary Science, Universitat Autònoma de Barcelona, Bellaterra, Spain, [2] Department of Animal Pathology, University of Zaragoza, Zaragoza, Spain

Many factors can lead to an inadequate development of piglets during their first days of life, including poor maternal behavior, which can be due to pain caused by farrowing, and reduced colostrum ingestion. This study investigates the action of meloxicam administered orally at farrowing on piglet weight gain and immunity transfer. Thirty-five multiparous sows were divided into two groups and treated with 0.4 mg/kg of oral meloxicam (oral meloxicam group; $n = 18$) or with a mock administration (control group; $n = 17$). A total of 382 piglets were individually weighed on the farrowing day (day 0), as well as on days +9 and +20. Immunoglobulin G (IgG) and A (IgA) concentrations in piglet serum and in sow's saliva, colostrum and milk were measured. Additionally, Interleukin-2 (IL-2), Interleukin-4 (IL-4) and Interferon gamma (IFN-ϒ) in serum of piglets and in sow's milk or colostrum were studied. All samples were obtained on days +1, +9, and +20. Piglets from sows in the oral meloxicam group tended to grow faster from day +9 to day +20 than did piglets from control sows ($p = 0.059$), and this difference was also observed in piglets with low body weight (BW) at birth ($p = 0.056$). The oral meloxicam group sows tended to increase the colostrum levels of IgA and IgG, as compared with control sows on day +1 ($p = 0.068$ and $p = 0.072$, respectively). IgA levels in piglet serum from the oral meloxicam group were significantly higher than in the control group on day +1 and +9 ($p = 0.019$ and $p = 0.011$ respectively). Furthermore, IL-2 and IL-4 levels in the serum of piglets from sows in the oral meloxicam group tended to be higher than that in the control group on day +9 ($p = 0.078$ and 0.056, respectively). The administration of meloxicam orally at the beginning of farrowing in multiparous sows increased immunoglobin and cytokine concentrations in colostrum, improving both humoral and cellular immune response of piglets. Pre-weaning growth of piglets born with a low BW improved in the meloxicam-treated group.

Keywords: pain, farrowing sow, piglet, immunity transfer, weight gain, meloxicam, immunoglobulin

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***Correspondence:**
Elena Navarro
elena.navarro@uab.cat

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INTRODUCTION

Piglets are born agammaglobulinemic because of the epitheliochorial placentation of swine (1, 2).

An early and sufficient intake of colostrum is crucial for piglet growth and survival (3, 4), as it is the source of energy as well as active and passive immunity. Colostrum is a complex mammary secretion released from the time of farrowing (early-colostrum) to 12 h (mid-colostrum) and up to 36 h post-farrowing (late-colostrum) (5). Early-colostrum is mostly produced before farrowing and contains up to 75% of Immunoglobulin G (IgG) and 20% of Immunoglobulin A (IgA), which are central elements of humoral immune responses. After farrowing, IgG concentration drastically drops, whereas IgA reduction during lactation is more gradual due to its role in the regulation of piglet intestinal microbiota, which is critical for the prevention of digestive problems (5).

Colostrum-associated cellular immunity has been overlooked for a long time. It contains around 10^6 cells/mL, up to 25% of them being lymphocytes (6, 7). Immune responses are orchestrated via complex signaling pathways within cells mediated by cytokines, which are small proteins with a plethora of effects. IL-2, IL-4 and IFN-ϒ are important cytokine mediators of the adaptive immune response, thus their quantification allows for partial characterization of the immune response. IL-2 is mainly produced by T lymphocytes and induces the proliferation of T and B lymphocytes and the activation of Natural Killer cells (NK) (8–10). IL-4 triggers differentiation of T helper lymphocytes toward the Th2 subset, which is related to humoral and anthelmintic responses (8, 10), while IFN-ϒ activates macrophages and elicits the differentiation of T helper lymphocytes toward the Th1 subset, thus favoring cellular responses and boosting protection against intracellular microbes (10).

Non-steroidal anti-inflammatory drugs (NSAIDs) have analgesic, anti-inflammatory, anti-endotoxic and anti-pyretic effects. It has been proven that NSAIDs administered to sows help them recover from a painful situation such as lameness (11) or post-partum dysgalactia syndrome (12, 13). NSAIDs also decrease the mortality rate at weaning in litters from healthy sows (14) and in sows with dysgalactia syndrome (13). However, studies on the effect of NSAIDs, on sow welfare, piglet growth and immunity transfer in healthy sows show discrepancies. Meloxicam administered to healthy sows around farrowing improves post-farrowing sow recovery (15, 16) and enhances piglet growth, especially at weaning (17, 18) and in piglets with low body weight (BW) at birth (18, 19). Nevertheless, other studies administering NSAIDs to healthy sows around farrowing did not find enhanced sow welfare and recovery post-farrowing (20) or improve piglet growth (15, 21).

To our knowledge, only two studies have looked into the effects of NSAIDs administered to sows around farrowing and have assessed passive immunity transfer via colostrum and immune system development in piglets (18, 20). Both studies recorded IgG transfer measured in piglet serum without exploring immunoglobulins in sow colostrum or milk. These studies did not measure other relevant immune factors for piglet growth and survival, such as IgA or cytokines. Mainau et al. (18) demonstrated that the administration of meloxicam orally at the beginning of farrowing in multiparous sows increased the concentration of IgG in piglet serum and enhanced their pre-weaning growth.

The present study aims to evaluate the effect of meloxicam administered orally to healthy sows at the beginning of farrowing on piglet growth, also including the effect of sex and immune transfer via colostrum of immunoglobulins (A and G) and cytokines (Interleukins IL-2 and IL-4, and Interferon Gamma IFN-ϒ), taking into account the sow parity effect.

MATERIALS AND METHODS

The experimental protocol described in this experiment was approved by the Institutional Animal Care and Use Committee of the Universitat Autònoma de Barcelona (CEEAH-1591) and the Generalitat de Catalunya (DMAH-6720). Written informed consent was obtained from the owners for the participation of their animals in this study.

Animals, Housing and General Management

Sample size was calculated by means of ENE 3.0. The sow was the experimental unit. Based on two previous studies carried out by Mainau et al. (18, 19), a reference mean average daily gain (ADG) of 0.2 kg/day was established at sow level for the control group and an expected mean ADG of 0.225 kg/day was considered for the treatment group. An overall standard deviation of 0.025 kg/day (at sow level) was assumed with a power of 80% and confidence level of 95%. A prevision of 17 sows per group was predicted.

The experimental procedure was carried out on a commercial farm (Heura S.L.; Santa Perpètua de Mogoda, Barcelona, Spain), with 9 farrowing barns equipped with an evaporative cooling system each. From December 2017 to March 2018, a total of 35 hybrid (Landrace x Duroc) multiparous sows from 2nd to 7th parturition were randomly selected the day of farrowing. At least 5 replicates with 5 to 10 sows per replicate were studied.

On day 109 of gestation, sows were moved to the farrowing barn and were housed in individual farrowing crates (1.95 × 0.60 m) built with steel bars. Farrowing crates were centrally located in farrowing pens (2.40 × 1.80 m) with fully metal-slatted floors for sows and plastic-slatted floors for piglets. A metal pad ensured 36°C of heat for the piglets during the first week of life, and heat lamps were placed over the metal pad the first day of life. The temperature in the farrowing barn was kept constant at ∼20°C, and the light was on from 07:00 to 17:00 h every day. Sows were offered 2.6 kg of feed per day, divided into two meals (07:00 and 15:00 h) and water was available *ad libitum* from drinkers.

Thirty days before farrowing, all sows were vaccinated with Clostridium novyi (2 mLSuiseng®, Hipra SA; Girona, Spain). Sixteen days after farrowing, sows that were not expected to be culled ($n = 31$ sows) were vaccinated with Parvovirus and Erysipelothrix rhusiopathiae (2 mL Eryseng®parvo, Hipra SA; Girona, Spain) and with Leptospira spp (2 mL Autovacuna®syva, Syva SAU; León, Spain). On day 113 of gestation, farrowing was hormonally induced with 2 mL of Planate® (Cloprostenol 0,092 mg/mL, MSD Animal Health; Friesoythe, Germany) divided into two injections of 1 mL (07:00 h and 11:00 h). Only hormonally induced farrowings that started on the morning of day 114 of gestation were included in the study. Lame sows or those with any kind of visible disease symptoms such as mastitis, diarrhea, fever, or respiratory problems were not included in the study.

Treatments and manual interventions during farrowing followed the usual routine of the farm and were performed by the same person. The following treatments were allowed during farrowing and were administered intramuscularly (IM) in the neck. When the time interval between the birth of two piglets exceeded 1 h and the cervical canal was dilated, 1 mL of Oxytocin (Hormonipra®, HipraSA, Girona, Spain) was injected. When the cervical canal was not sufficiently dilated, sows were treated with 200 mg of Vetrabutine hydrochloride (Monzal®, Boehringer Ingelheim España; SA, Barcelona, Spain). When sows were very nervous around farrowing Carazolol (Suacron®, Divasa Farmavic SA; Barcelona, Spain) or Azaperone (Stressnil®, Janssen Animal Health, Elanco; Brussels, Belgium) were administered.

A total of 382 piglets, identified individually by a numeric ear tag, were included in the study. Piglets were weaned at 21 days of age, according to veterinary recommendations, and moved to another barn of the farm equipped with conditioned infrastructures for very young piglets.

Experimental Procedure

In each replicate, sows were randomly allocated into two homogeneous groups, regarding parity, and treated with either 0.4 mg/kg body weight of meloxicam (Metacam® 15 mg/mL Oral Suspension, Boehringer Ingelheim Vetmedica GmbH; Ingelheim, Rhein, Germany) or a mock administration with an empty syringe. Treatments were administered at the beginning of the farrowing, between the first and the third piglet. If any further anti-inflammatory treatment was required, the sow was excluded from the study.

Litter size was standardized at 11–12 piglets by cross-fostering within 6–8 h post-farrowing. Cross-fostering was carried out within each treatment. Each treatment was identified with two different colored cards in order to make the treatment blind to farm and laboratory staff.

Data Collection

For each sow, the following parameters were registered during farrowing by direct observations: the duration of farrowing (defined as the period of time between the first and the last piglet born), the condition of each piglet at birth (born alive, stillborn or mummified fetus), the piglet's sex (male or female), the number of treatments and manual interventions during farrowing, and the number of piglets cross-fostered and weaned. The presence of placenta retention was also recorded. During lactation, piglet mortality was registered. The number of sows culled after weaning and the interval between farrowing and the following fertile insemination were recorded.

One and 9 days after farrowing (day +1 and day +9) and the day before weaning (day +20), saliva samples were collected from all sows using Salivette® tubes (Sarstedt; Nümbrecht, Germany). Each tube contained a cotton swab, which was clipped with a Kocher clip, and sows were allowed to chew it for around 1 min. Then, the cotton swab was placed in the tube and centrifuged at 6,048 × g for 13 min. Saliva samples (∼1–2 mL per cotton swab) were stored in Eppendorf tubes and frozen at −80°C until analysis. Colostrum and milk samples were collected from all sows on day +1 (colostrum) and on days +9 and +20 (milk). Sows were injected with 0.7 mLof Oxytocin IM (Hormonipra®, Hipra SA; Girona, Spain), and 30 s later, 2 mL of colostrum and milk were collected into sterile tubes. Colostrum and milk samples were immediately frozen at −20°C until analysis.

Each pig was individually weighed at farrowing (day 0), and on days +9 and +20. One day after farrowing, during one suckling event, 3–4 piglets of each litter were selected for blood sampling. Piglets were chosen so that at least one of them was suckling from the sow's craneal teats, another one from middle teats and yet another one from caudal teats. The same piglets from each litter were blood sampled on days +1, +9, and +20. Blood samples (1–2 mL) were collected into heparinized tubes from the anterior vena cava. Samples were centrifuged for 6 min at 2,058 × g and plasma was stored in Eppendorf tubes at −80°C until analysis.

All samples were analyzed at the Murcia University Veterinary Hospital Laboratory. Immunoglobulin G (IgG) and A (IgA) concentrations in piglet serum and sow saliva, colostrum and milk were quantified by using two commercially available sandwich ELISAs (IgA and IgG ELISA Quantitation Kit; Bethyl Laboratories; Montgomery, TX, USA). Interleukin-2 (IL-2), Interleukin-4 (IL-4) and Interferon gamma (IFN-ϒ) in piglet serum, sow saliva, milk or colostrum were analyzed using MILLIPEX® MAP Porcine Cytokine/Chemokine Panel Kit (EMD Millipore; Darmstadt, Germany).

Statistical Analysis

Data were analyzed using the SAS software (SAS Institute Inc.; Cary, NC, USA). The experimental unit for data analysis was the individual sow. All descriptive values in the Results section are shown as the mean ± standard error (SE). Significance was set at $p < 0.05$, and tendency at $p < 0.1$, in all cases.

The Mann-Whitney Wilcoxon test was used to test whether the performance values (other than piglet weight and average daily gain) obtained at the individual sow level were significantly different between treatments.

Normality tests of residuals were performed for each dependent variable. Weight of piglets and ADG (From birth to day+9, from day+9 to weaning and from birth to weaning) were normally distributed without data transformation. A general linear mixed model (proc MIXED) for repeated measures was used. Model included the fixed effects of treatment (control vs. oral meloxicam), day (at birth, day +9 and at weaning), sex (males vs. females) and their pair interactions. Day and piglet within sow were introduced as repeated effects. Weight at birth was introduced as a covariate for the analysis of weight at day +9 and at weaning, and litter size was introduced as a covariate in all the models. The residual maximum likelihood was used as a method of estimation. Differences in least-square means were investigated after using a Tukey adjustment for multiple comparisons. The same models were used to study the performance of piglets categorized by quintiles according to their weight at birth: very light (from 0.670 to 1.294 Kg), light (from >1.294 to 1.492 Kg), mid (from >1.492 to 1.625 Kg), heavy (from >1.625 to 1.878 Kg) and very heavy (from >1.878 to 2.427 Kg).

IgG and IgA concentrations in piglet serum, sow saliva and colostrum or milk, IL-2, IL-4 and IFN-ϒ in colostrum or milk,

TABLE 1 | Mean, standard error (SE), median (MED) and 95% confidence intervals for median (95% CI) of performance parameters and treatment records studied in the control and oral meloxicam groups during the whole trial period (from farrowing to weaning at 21 days).

Items	Control (*n* = 17 sows)				Oral meloxicam (*n* = 18 sows)				*P*-value
	Mean	SE[a]	MED	95%CI	Mean	SE	MED	95%CI	
Parity	4.06	0.441	3	2–7	4.28	0.394	4	2–7	0.582
Piglets born at the moment of the treatment	1.70	0.143	2	1–3	1.78	0.117	2	1–3	0.856
Total duration of farrowing (h)	3.27	0.328	3.47	1.12–5.47	3.31	0.387	3.06	1.38–7.28	0.817
Total piglets born per litter	13.47	0.912	13	6–21	12.73	0.576	13	9–17	0.621
Live born per litter	11.88	0.624	12	6–18	11.36	0.584	11	8–16	0.489
Stillborn per litter	0.94	0.358	1	0–6	0.83	0.259	0.5	0–4	0.986
Mummified fetus per litter	0.65	0.226	0	0–2	0.55	0.217	0	0–3	0.815
Cross-fostered piglets per litter	11.18	0.231	11	10–13	10.67	0.256	10.5	8–12	0.209
Crushing deaths per litter	0.47	0.229	0	0–3	0.17	0.121	0	0–2	0.322
Total liveborn mortality	0.76	0.304	0	0–4	0.61	0.282	0	0–4	0.662
Total weaned piglets	10.41	0.193	10	9–12	10.06	0.338	10	6–12	0.569
Manual intervention per sow	0.47	0.174	0	0–2	0.38	0.230	0	0–4	0.422
Oxytocin treatment per sow	0.24	0.106	0	0–1	0.17	0.900	0	0–1	0.637
Total treatments per sow	0.24	0.106	0	0–1	0.33	0.140	0	0–2	0.731

A total of 35 sows and 354 piglets were included in the study. P-value from Mann-Whitney Wilcoxon test is shown.
[a]SE, standard error.

and IFN-ϒ in serum of piglets were normally distributed after a log transformation. IL-2 and IL-4 in piglet serum followed a normal distribution without data transformation. Additionally, extreme outliers detected by proc UNIVARIATE box plot procedures were deleted.

Immunity measurements in piglets and sows were analyzed using general linear mixed models (proc MIXED) for repeated measures. Models for immunity sow measurements included the fixed effects of treatment (oral meloxicam vs. control), day (day +1, +9, and +20), parity (from 2nd to 7th) and their pair interactions. Day was introduced as a repeated effect. Models for immunity piglet measurements included the fixed effects of treatment (oral meloxicam vs. control), day (day +1, +9, and +20), sex (male vs. female), the position at the udder (anterior, middle and posterior teats) and their pair interactions. Day and piglet within sow were introduced as repeated effects. The residual maximum likelihood was used as a method of estimation. Differences in least-square means were investigated after using a Tukey adjustment for multiple comparisons. All general linear mixed models included replicate (from 1 to 5) and farrowing barn (from 1 to 9) as random effects.

RESULTS

Performance Parameters and Treatment Records

Results on performance and treatment records at the individual sow level are summarized in **Table 1**. Both treatment groups (oral meloxicam vs. control) were similar when the experimental procedure started in terms of performance data recorded during farrowing.

TABLE 2 | Mean and standard error (SE) of the piglet weight at birth, 9 days after farrowing (day + 9) and at weaning (day + 20) in Kilograms and the Average Daily Gain (ADG) of piglets from birth to day + 9 after farrowing, from birth to weaning and from day + 9 to weaning in Kilograms per day for 354 piglets regarding treatment (control vs. oral meloxicam).

	Control		Oral meloxicam		
	Mean	SE	Mean	SE	*P*-value
Weight at birth (Kg)	1.510	0.065	1.600	0.063	0.996
Weight at day +9 (Kg)	3.556	0.071	3.499	6.538	0.909
Weight at weaning (Kg)	6.479	0.109	6.538	0.103	0.644
ADG from birth to day +9 (Kg/day)	0.218	0.010	0.217	0.010	0.981
ADG from birth to weaning (Kg/day)	0.243	0.006	0.251	0.006	0.275
ADG from day +9 to weaning (Kg/day)	0.261[b]	0.007	0.275[a]	0.007	0.059

Different superscripts (a, b) in the same row indicate significant differences within each effect ($p < 0.05$). Tendency has been shown at $p < 0.1$.

Treatment did not have an effect on the time interval between weaning and the following fertile insemination (4.769 ± 0.121 days in oral meloxicam group vs. 7.071 ± 1.811 days in the control group; $p = 0.893$), or on the number of sows culled after weaning (0.111 ± 0.076 in the oral meloxicam group vs. 0.176 ± 0.095 in the control group; $p = 0.608$).

Twenty-four piglets died during lactation, which represents 6.28% of mortality. Oral meloxicam treatment of sows did not significantly affect piglet mortality (6.84% from the control group and 5.73% from oral meloxicam group; $p = 0.661$).

The mean and standard error (SE) of weight at birth, weight on day +9 and weight at weaning are summarized in

Table 2. Piglet weight at birth, on day +9 and at weaning was not different between the control and the oral meloxicam group. Piglet sex had a significant effect on the weight of the piglets, males being heavier than females at weaning (**Table 1**, **Supplementary Material**).

Average daily gain (ADG) data are also summarized in **Table 2**. Oral meloxicam treatment of sows tended to increase piglet ADG from day +9 to weaning. Piglet sex had a significant effect on the ADG, males growing faster than females from birth to weaning and from day +9 to weaning.

Piglet weights (at birth, on day +9 and at weaning) and ADG (from birth to day +9, from day+9 to weaning and from birth to weaning) were not affected by treatment in piglets born with light, mid, heavy or very heavy weight at birth. Piglets with very light weight at birth tended to have a higher ADG from day+9 to weaning in the oral meloxicam group (267.93 ± 7.793 gr) than in the control group (240.11 ± 9.207 gr) ($p = 0.056$).

Immunoglobulins G and A Concentrations in Saliva and Colostrum or Milk of Sows and in Piglet Serum

Immunoglobulin G and A (IgG and IgA) concentrations in saliva, colostrum or milk of sows and in piglet serum by treatment effect on days +1, +9, and +20 after farrowing are shown in **Figure 1**.

IgG levels in sow saliva (μg/mL) were affected by day after farrowing (day +1: 44.89 ± 4.486; day +9: 9.08 ± 1.810 and day +20: 3.28 ± 0.475; $p < 0.001$ in all pair comparisons), but were not affected by treatment ($p = 0.547$) irrespectively of the day studied.

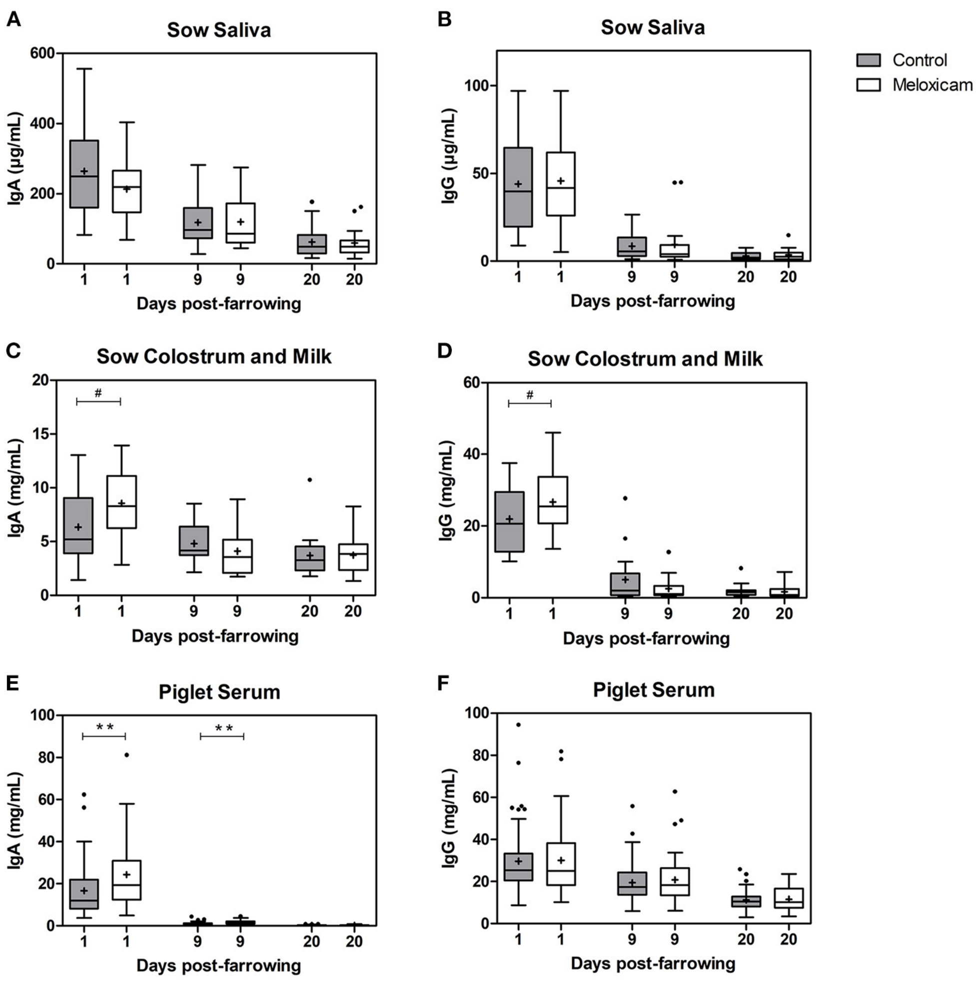

FIGURE 1 | IgA and IgG at days +1, +9, and +20 after farrowing regarding treatment received by sows (control vs. oral meloxicam) in sow saliva **(A,B)**, sow colostrum and milk **(C,D)** and piglet serum **(E,F)**. Significant differences were established at $p < 0.01$(**) and tendency was set at $p < 0.1$(#). Boxes represent the interquartile range (IQ = Q3-Q1), horizontal lines inside the boxes represent the median and the cross (+) represents the mean values of the data. Whisker bars were calculated from the IQ (Upper: Q3 + 1.5 × IQ; lower: Q1 – 1.5 × IQ), and reflect the variability of the data outside Q1 and Q3. Points outside the box-and-whiskers plot represent extreme values of the population.

IgA levels in sow saliva (μg/mL) were affected by day after farrowing (day +1: 239.64 ± 21.202, day +9: 118.477 ± 12.267 and day +20: 60.81 ± 7.295; $p < 0.001$ in all pair comparisons), but were not significantly affected by treatment; ($p = 0.704$) irrespective of the day studied.

IgG levels in colostrum or milk of sows (mg/mL) were affected by day after farrowing and were higher on day +1 (24.48 ± 1.484) than on days +9 (3.75 ± 0.953) and +20 (1.77 ± 0.298) ($p < 0.001$ in both cases). IgG levels in colostrum from the sows treated with oral meloxicam tended to be higher than in the control group on day +1 ($p = 0.072$). However, on days +9 and +20, IgG concentration in sow milk was not affected by treatment.

IgA levels in colostrum or milk of sows (mg/mL) were affected by day after farrowing and were higher on day +1 (7.48 ± 0.577) than on days +9 (4.41 ± 0.347; $p < 0.001$) and +20 (3.72 ± 0.315; $p < 0.001$). IgA concentration on day +9 and +20 were similar ($p = 0.246$). Furthermore, IgA levels in colostrum of sows treated with oral meloxicam tended to be higher than in the control group on day +1 ($p = 0.068$), but on day +9 and +20, IgA levels in sow milk were not affected by treatment.

IgA and IgG concentrations in saliva and in colostrum or milk were not affected by parity (saliva: $p = 0.290$ and $p = 0.192$, respectively, and colostrum or milk: $p = 0.127$ and $p = 0.232$). The interaction between treatment and parity was not significant (saliva IgA $p = 0.113$; IgG $p = 0.925$ and colostrum or milk IgA $p = 0.239$; IgG $p = 0.112$).

IgG levels in piglet serum (mg/mL) were affected by day after farrowing (day +1: 29.93 ± 1.377; day +9: 20.26 ± 0.935 and day +20: 11.48 ± 0.466; $p < 0.001$ in all pair comparisons). IgG levels in piglet serum were not significantly affected by treatment ($p = 0.963$), sex ($p = 0.189$) or piglet position at the udder ($p = 0.811$) irrespective of the day studied.

IgA levels in piglet serum (mg/mL) were affected by day after farrowing (day +1: 20.63 ± 1.314, day +9: 1.36 ± 0.080 and day +20: 0.27 ± 0.018 $p < 0.001$ in all pair comparisons), and there was an interaction between treatment and sampling day ($p = 0.020$). IgA levels in piglet serum from sows treated with oral meloxicam were significantly higher than in piglets from the control group on day +1 ($p = 0.019$) and day +9 ($p = 0.011$). However, on day +20, IgA level in piglet serum was not significantly affected by treatment ($p = 0.943$). IgA levels in piglet serum were not significantly affected by sex (7.79 ± 0.944 in females vs. 7.89 ± 1.001 in males; $p = 0.633$) or by piglet position at the udder (anterior teats: 7.67 ± 1.048, middle teats: 7.17 ± 1.328 and posterior teats: 9.07 ± 1.448, $p = 0.725$) irrespective of the day studied.

Concentration of Cytokines (IL-2, IL-4 and IFN-ϒ) in Colostrum or Milk of Sows and in Piglet Serum

Concentration of interleukins (IL-2 and IL-4) and interferon gamma (IFN-ϒ) in colostrum or milk of sows and in piglet serum by treatment effect on days +1, +9, and +20 after farrowing are shown in **Figure 2**.

IL-2, IL-4 and IFN-ϒ concentration in colostrum or milk of sows (ng/mL) were affected by day after farrowing ($p < 0.001$ in all cases). IL-2, IL-4 and IFN-ϒ in sow colostrum on day +1 (IL-2: 1.51 ± 0.166; IL-4: 10.12 ± 1.289; IFN-ϒ: 62.99 ± 5.505) showed higher concentrations than in sow milk on day +9 (IL-2: 0.50 ± 0.079; IL-4: 2.60 ± 0.540; IFN-ϒ: 24.31 ± 3.827) and on day +20 (IL-2: 0.73 ± 0.089; IL-4: 4.02 ± 0.641; IFN-ϒ: 41.86 ± 4.585), whereas concentrations on day +20 were higher than on day +9.

IL-2, IL-4, and IFN-ϒ concentrations in colostrum or milk of sows were not significantly affected by treatment ($p = 0.206$, 0.142, and 0.322 respectively).

IL-2, IL-4 and IFN-ϒ concentrations in colostrum or milk of sows were affected by parity ($p = 0.010$, $p < 0.001$ and $p = 0.008$ respectively). The general pattern was that sows in their second parity showed lower levels of cytokines than did older sows (three parturitions or more). Specifically, IL-2 levels in colostrum or milk of sows in their second parity were lower than those in sows in their fourth ($p = 0.016$) and fifth parity ($p = 0.023$) and tended to be lower than in sows in their seventh parity ($p = 0.098$). IL-4 levels in colostrum or milk of sows in their second parity were lower than those in sows in their third ($p = 0.005$), fourth ($p = 0.001$), fifth ($p = 0.001$), sixth ($p = 0.035$) and seventh parity ($p = 0.002$). IFN-ϒ levels in colostrum or milk of sows in their second parity were lower than those in sows in their fourth parity ($p = 0.008$) and tended to be lower than in sows in their sixth parity ($p = 0.070$).

IL-2 and IL-4 levels in piglet serum (ng/mL) were affected by day after farrowing, and were higher on day +9 (IL-2: 2.40 ± 0.173; IL-4: 17.15 ± 1.442) than those on days +1 (IL-2: 1.33 ± 0.070; IL-4: 8.53 ± 0.526; $p < 0.001$ in both cases) and +20 (IL-2: 1.55 ± 0.145; IL-4: 10.67 ± 1.156; $p < 0.001$ and $p = 0.001$, respectively).

IL-2 and IL-4 levels in piglet serum were affected by treatment, and tended to be higher in the oral meloxicam group than those in the control group on day +9 ($p = 0.078$ and $p = 0.056$, respectively). IL-2 and IL-4 levels in piglet serum were not significantly affected by sex of piglets ($p = 0.596$ and $p = 0.868$, respectively) or by piglet position at the udder ($p = 0.888$ and $p = 0.715$, respectively) irrespective of the day studied.

IFN-ϒ levels in piglet serum (ng/mL) were affected by day after farrowing, and were lower on day +1 (6.00 ± 0.334) than those on day +9 (10.74 ± 0.654) and day +20 (12.07 ± 0.758) ($p < 0.001$ in all cases).

IFN-ϒ levels in piglet serum were not significantly affected by treatment ($p = 0.409$), sex of piglets ($p = 0.858$), or piglet position at the udder ($p = 0.320$), irrespective of the day studied.

DISCUSSION

In the present study, both treatment groups (oral meloxicam and control) were well-matched in terms of performance variables recorded during farrowing. Early administration of oral meloxicam treatment did not negatively affect total piglets born alive per litter, total duration of farrowing, treatments administered during farrowing (such as oxytocin) or the number

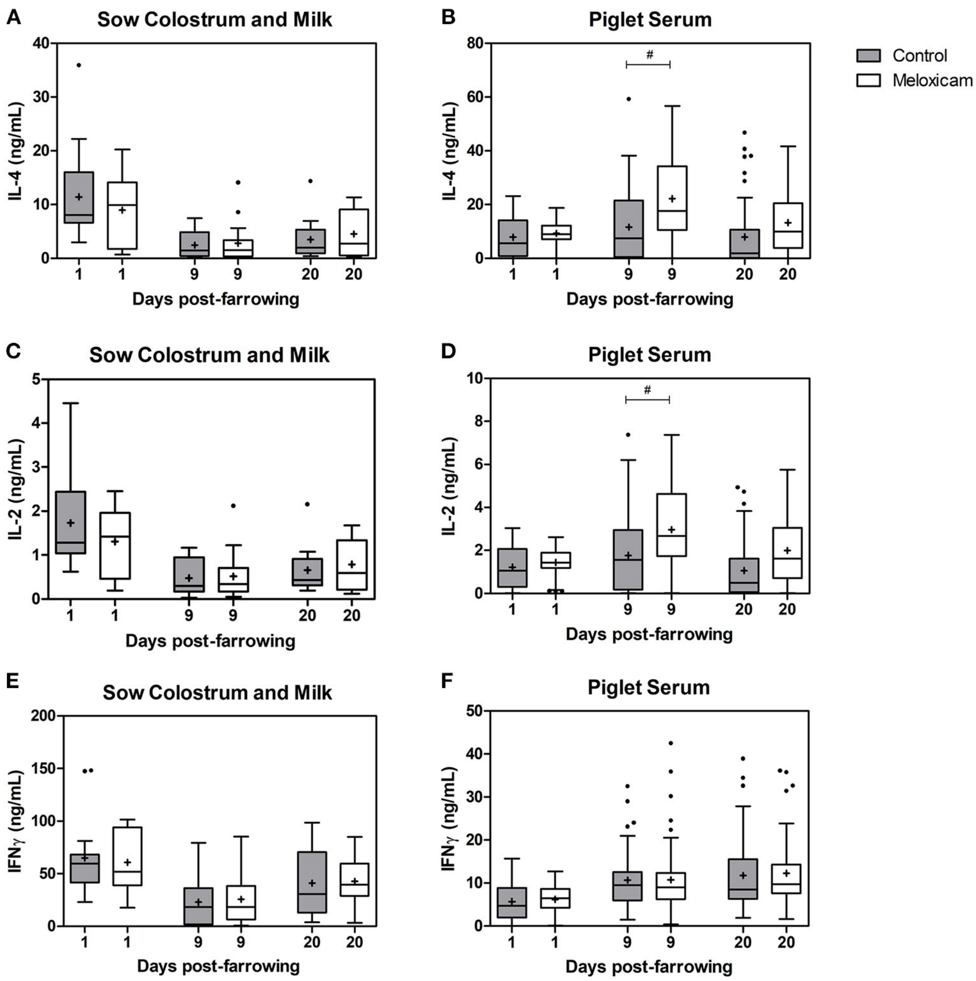

FIGURE 2 | Concentration of IL-4, IL-2, and IFN-ϒ at days +1, +9, and +20 after farrowing regarding treatment received by sows (control vs. oral meloxicam) in sow colostrum and milk (a, c, e) and piglet serum (b, d, f). Tendency differences were established at $p < 0.1$(#). Boxes represent the interquartile range (IQ = Q3 – Q1), horizontal lines inside the boxes represent the median and the cross (+) represents the mean values of the data. Whisker bars were calculated from the IQ (Upper: Q3 + 1.5 × IQ; lower: Q1 - 1.5 × IQ) and reflect the variability of the data outside Q1 and Q3. Points outside the box-and-whiskers plot represent extreme values of the population.

of manual interventions during farrowing. Hence, it appears that the use of oral meloxicam during parturition (more specifically between the first and the third piglet born) did not interfere with the progression of the birth process.

Piglet Mortality and Growth

In agreement with other authors that studied the effect of NSAIDs around farrowing (17, 18, 20, 21), oral meloxicam administered to healthy sows did not show an effect on pre-weaning piglet mortality. On the contrary, Homedes et al. (14), in a large-scale study on commercial farms with a high incidence of pre-weaning mortality (±10%), showed lower pre-weaning piglet mortality after ketoprofen administration to sows within 12 h after farrowing. Homedes et al. (14) explained such an effect due to higher milk production by the sow ketoprofen-treatment group. We assume that a larger sample size enrolling different farms with high pre-weaning mortality would be needed to observe differences in piglet mortality (piglet mortality in our study was 6.8%).

Piglet weights at birth were similar (16, 17) or slightly higher (3, 22, 23) than values reported in other studies. The administration of oral meloxicam at the beginning of farrowing tended to enhance the ADG of piglets from day +9 to weaning, and particularly for the lightest piglets. A similar effect was described by Mainau et al., in two studies (18, 19), treating the sows around farrowing with injectable and with oral meloxicam. Tenbergen et al. (17), injected meloxicam intra-muscularly within 12 h of farrowing and found that piglet ADG tended to be higher for piglets from the meloxicam group sows than for control piglets in medium-sized litters (11–13 piglets). Ketoprofen is another AINE used in pig production, but Viitasaari et al. (21) and Ison et al. (15), who both injected sows with ketoprofen during farrowing, did not find that it had any effect on piglet average daily gain to weaning. These discrepancies in the effects of NSAIDs administered to healthy sows around farrowing on piglet growth could be due to different factors such as the active principle administered. Meloxicam is a selective COX-2 inhibitor and may be a more specific treatment for inflammation caused by farrowing than a non-selective COX inhibitor, like ketoprofen (24). The time of administration is another important factor to take into consideration. Studies administering meloxicam at the beginning of farrowing (17–19) show the effect on weaning weights of piglets and ADG. Thus, the active ingredient administered (preferably a selective COX-2 inhibitor) and the administration time (as soon as possible after farrowing starts) are presumably important factors to improve piglet growth and weight at weaning.

Transfer of Passive and Active Immunity

Colostrum intake is crucial for development of piglet immunity. In this study, and in accordance with normal colostrum and milk immunoglobulin kinetics (5), sow colostrum and milk IgG and IgA showed an abrupt and steady decrease respectively (**Figures 1C,D**). Interestingly, colostrum immunoglobulin content on day +1 was higher in the oral meloxicam group then in the control group. The difference between groups was more pronounced in IgA than in IgG, which could be explained by the switch between the IgG/IgA ratio after farrowing (5). One-hundred percent of colostrum IgG and 40% of colostrum IgA come from sow blood via an Ig receptor, whereas up to 60% of IgA is directly synthesized in the mammary gland (1). Our data support the local role of oral meloxicam in the mammary gland, which likely decreases local inflammation, thus favoring both immunoglobulin recruitment from plasma and local production of IgA in plasma cells (1). Indeed, *in vitro* studies developed in cattle have shown the anti-inflammatory effect of meloxicam in mammary epithelial cells (25). Furthermore, mastitis in cows has been associated with reduced pre-weaning immunity, growth, and health of the offspring (26, 27), so the anti-inflammatory effect elicited by meloxicam treatment is presumed to have the opposite effect.

In comparison with blood sampling, saliva sampling is generally considered to be a non-invasive and stress-free methodology (28). IgG levels in sow saliva are directly proportional to the levels in sow serum, whereas IgA in saliva is mostly produced locally, so IgA levels are highly variable in response to environmental factors such as stress and oral infections (29). In our study, saliva IgG levels, a marker of plasma IgG levels, showed no differences between groups, which probably rules out a systemic effect of oral meloxicam administration on the Ig increase in piglets from treated sows.

IgA and IgG concentration in piglet serum during lactation is the result of intake of immunoglobulins from colostrum. The quick drop of IgA and the slow drop of IgG in piglet serum is likely explained by the different half-lives of these immunoglobulins in serum, being 6 days for IgA and 21 days for IgG (30). IgA concentration was higher in piglet serum in the oral meloxicam group on days +1 and +9. Interestingly, diarrhea of newborn piglets is one of the biggest health issues in pig production, and increased IgA levels could play a major role in preventing these problems by their protective effect on the intestinal mucosa (1). Mainau et al. (18) found that the administration of meloxicam orally at the beginning of farrowing (on average, when 2.6 piglets had already been born) increased the concentration of IgG in the serum of piglets. In the present study, sows were treated early at the beginning of farrowing, when early colostrum (with the highest IgG levels) has already been produced and thus the influence of treatment on the IgG serum levels of piglets fed with this colostrum was lower. Nevertheless, weaker piglets and those born later during parturition are known to suffer from delayed and reduced colostrum intake (31). These animals have lower survival and growth rates, which may be improved by treatment with meloxicam, as shown by our results with piglets born with a very light weight at birth, as well as by other studies (31). These differences could be explained by a higher IgG and IgA immunity transfer in the treatment group in these weaker animals, which are likely to consume a smaller quantity of early-colostrum and a larger proportion of mid- and late-colostrum. Unfortunately, in this study a low percentage of piglets with low BW at birth were blood sampled, thus hampering a proper analysis of their serum IgG and IgA levels.

Regarding colostrum and milk cytokines, higher levels of IL-2, IL-4, and IFN-Y were found on day +1, which is likely to be related to pain and to contamination of the reproductive tract induced by farrowing. Milk cytokine levels moderately decreased between day +1 and +9 and increased again between day +9 and +20, likely in response to the vaccination given to sows on day +16. Cytokines and lymphoid cells have been demonstrated to cross the intestinal barrier of newborn piglets (32–34). In piglet serum, cytokine levels measured on day +1 are expected to be the result of both colostrum-derived cytokines and cytokines produced by the piglets. In contrast, taking into consideration the short half-life of these cytokines (minutes for IL-2 and IL-4 and a few hours for IFN-ϒ) and the loss of piglet intestinal permeability, cytokine levels on days +9 and +20 reflect only the activity of the piglet immune system. Higher concentrations of all cytokines were found on day +9, likely due to the immune challenge elicited by tail docking (in both sexes) and castration (in males), which were performed on their second day of life. Interestingly, higher IL-4 and IL-2 levels were measured in piglets from the meloxicam treated group on days day +9 (**Figures 2B,D**). Increased secretion of IL-4 and IL-2 in piglets has been related to better Th2 and Th1 immune responses, respectively (8). Moreover, IL-4 induces antibody production and tissue repair, whereas IL-2 plays a major role in the activation of NK-cells and the generation of effector and memory cells (9, 10). This positive influence of colostrum on the immune system development could be related to the transfer of colostrum-associated immune cells, which are absorbed selectively in the newborn gut, although the precise mechanisms remain unclear (1). Therefore, it could be hypothesized that meloxicam treatment around farrowing had an impact on the concentration of immune cells in colostrum, but further studies are needed to investigate this hypothesis.

This study was developed on a commercial farm with really good sanitary and husbandry conditions. Further research is required to determine if these positive results on piglet welfare can be even more pronounced by studing a larger set of commercial farms with higher mortality rates and lower growth rates during lactation. In summary, the results of this study show that early administration of oral meloxicam improves some aspects of piglet performance and welfare. Further research is needed to study whether these effects are also observed in primiparous sows or could be improved by administering meloxicam before the onset of farrowing.

CONCLUSIONS

The administration of meloxicam orally at the beginning of farrowing in multiparous sows increased the concentration of immunoglobins and cytokines in sow colostrum and improved both humoral and cellular immune response in piglets. Pre-weaning growth of piglets, especially in piglets born with low BW, tended to be higher in the meloxicam-treated group than that in the control group.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The experimental protocol described in this experiment was approved by the Institutional Animal Care and Use Committee of the Universitat Autònoma de Barcelona (CEEAH-1591) and the Generalitat de Catalunya (DMAH-6720). Written informed consent was obtained from the owners for the participation of their animals in this study.

AUTHOR CONTRIBUTIONS

EN: data acquisition, structuralization, and interpretation, drafting of the manuscript and final approval of the version to be published. EM: study concept and design, data acquisition, data analysis and interpretation, drafting of the manuscript and final approval of the version to be published. RM: immunology data analysis and interpretation, preparation of figures, and drafting of the manuscript and final approval of the version to be published. DT and MS: data acquisition and final approval of the version to be published. XM: study concept and design, drafting of the manuscript and final approval of the version to be published. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

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REFERENCES

1. Salmon H, Berri M, Gerdts V, Meurens F. Humoral and cellular factors of maternal immunity in swine. *Dev Comp Immunol*. (2009) 33:384–93. doi: 10.1016/j.dci.2008.07007
2. Zanello G, Meurens F, Serreau D, Chevaleyre C, Melo S, Berri M, et al. Effects of dietary yeast strains on immunoglobulin in colostrum and milk of sows. *Vet Immunol Immunopathol*. (2013) 152:20–7. doi: 10.1016/j.vetimm.2012.09023
3. Decaluwé R, Maes D, Wuyts B, Cools A, Piepers S, Janssens GPJ. Piglets' colostrum intake associates with daily weight gain and survival until weaning. *Livest Sci*. (2014) 162:185–92. doi: 10.1016/j.livsci.2014.01024
4. Quesnel H, Farmer C, Devillers N. Colostrum intake: influence on piglet performance and factors of variation. *Livest Sci*. (2012) 146:105–14. doi: 10.1016/j.livsci.2012.03010
5. Theil PK, Lauridsen C, Quesnel H. Neonatal piglet survival: impact of sow nutrition around parturition on fetal glycogen deposition and production and composition of colostrum and transient milk. *Animal*. (2014) 8:1021–30. doi: 10.1017/S1751731114000950
6. Evans PA, Newby TJ, Stokes CR, Bourne FJ. A study of cells in the mammary secretions of sows. *Vet Immunol Immunopathol*. (1982) 3:515–27. doi: 10.1016/0165-2427(82)90017-4
7. Le Jan C. A study by flow cytometry of lymphocytes in sow colostrum. *Res Vet Sci*. (1994) 57:300–4. doi: 10.1016/0034-5288(94)90121-X
8. Junttila IS. Tuning the cytokine responses: an update on interleukin (IL)-4 and IL-13 receptor complexes. *Front Immunol*. (2018) 9:888. doi: 10.3389/fimmu.201800888
9. Abbas AK, Trotta E, Simeonov D, Marson A, Bluestone JA. Revisiting IL-2: biology and therapeutic prospects. *Sci Immunol*. (2018) 25:3;6. doi: 10.1126/sciimmunolaat1482
10. Kumar V, Abbas AK, Aster JC. *Robbins & Cotran Pathologic Basis of Disease, 10th Edn*. Philadelphia, PA: El Sevier (2020).
11. Conte S, Bergeron R, Gonyou H, Brown J., Rioja-Lang FC, Connor ML, et al. Use of an analgesic to identify pain-related indicators of lameness in sows. *Livest Sci*. (2015) 180:203–8. doi: 10.1016/j.livsci.2015.08009
12. Hirsch AC, Philipp H, Kleemann R. Investigation on the efficacy of meloxicam in sows with mastitis-metritis-agalactia syndrome. *J Vet Pharmacol Ther*. (2003) 26:355–60. doi: 10.1046/j.1365-2885.2003.00524x
13. Sabaté D, Salichs M, Bosch J, Ramó P, Homedes J. Efficacy of ketoprofen in the reduction of pre-weaning piglet mortality associated with sub-clinical forms of post-partum dysgalactia syndrome in sows. *Pig J*. (2012) 67:19–23.
14. Homedes J, Salichs M., Sabaté D, Sust M, Fabre R. Effect of ketoprofen on pre-weaning piglet mortality on commercial farms. *Vet J*. (2014) 201:435–7. doi: 10.1016/j.tvjl.2014.05038
15. Ison SH, Jarvis S, Rutherford KMD. The identification of potential behavioural indicators of pain in periparturient sows. *Res Vet Sci*. (2016) 109:114–20. doi: 10.1016/j.rvsc.2016.10002
16. Schinckel AP, Cabrera R, Boyd RD, Jungst S, Booher C, Johnston M, et al. Impact of birth and body weight at twenty days on the post-weaning growth of pigs with different weaning management. *Prof Anim Sci*. (2007) 23:197–210. doi: 10.15232/S1080-7446(15)30965-7
17. Tenbergen R, Friendship R, Cassar G, Amezcua MR, Haley D. Investigation of the use of meloxicam post farrowing for improving sow performance and reducing pain. *J Swine Heal Prod*. (2014) 22:10–5.

18. Mainau E, Temple D, Manteca X. Experimental study on the effect of oral meloxicam administration in sows on pre-weaning mortality and growth and immunoglobulin G transfer to piglets. *Prev Vet Med*. (2016) 126:48–53. doi: 10.1016/j.prevetmed.2016.01032
19. Mainau E, Ruiz-De-La-Torre JL, Dalmau A, Salleras JM, Manteca X. Effects of meloxicam (Metacam®) on post-farrowing sow behaviour and piglet performance. *Animal*. (2012) 6:494–501. doi: 10.1017/S1751731111001790
20. Ison SH, Jarvis S, Ashworth CJ, Rutherford KMD. The effect of post-farrowing ketoprofen on sow feed intake, nursing behaviour and piglet performance. *Livest Sci*. (2017) 202:115–23. doi: 10.1016/j.livsci.2017.06001
21. Viitasaari E, Hänninen L, Heinonen M, Raekallio M, Orro T, Peltoniemi O, et al. Effects of post-partum administration of ketoprofen on sow health and piglet growth. *Vet J*. (2013) 198:153–7. doi: 10.1016/j.tvjl.2013.06013
22. Roelofs S, Godding L, de Haan JR, van der Staay FJ, Nordquist RE. Effects of parity and litter size on cortisol measures in commercially housed sows and their offspring. *Physiol Behav*. (2019) 201:83–90. doi: 10.1016/j.physbeh.2018.12014
23. Udomchanya J, Suwannutsiri A, Sripantabut K, Pruchayakul P, Juthamanee P, Nuntapaitoon M, et al. Association between the incidence of stillbirths and expulsion interval, piglet birth weight, litter size and carbetocin administration in hyper-prolific sows. *Livest Sci*. (2019) 227:128–34. doi: 10.1016/j.livsci.2019.07013
24. Ison SH, Jarvis S, Hall SA, Ashworth CJ, Rutherford KMD. Periparturient behavior and physiology: further insight into the farrowing process for primiparous and multiparous sows. *Front Vet Sci*. (2018) 5:122. doi: 10.3389/fvets.201800122
25. Caldeira MO, Bruckmaier RM, Wellnitz O. Meloxicam affects the inflammatory responses of bovine mammary epithelial cells. *J Dairy Sci*. (2019) 102:10277–90. doi: 10.3168/jds2019-16630
26. Heravi M.oussavi A, Danesh Mesgaran M, Gilbert RO. Effect of mastitis during the first lactation on production and reproduction performance of Holstein cows. *Trop Anim Health Prod*. (2012) 44:1567–73. doi: 10.1007/s11250-012-0107-3
27. Ferdowsi Nia E, Nikkhah A, Rahmani HR, Alikhani M, Mohammad Alipour M, Ghorbani GR. Increased colostral somatic cell counts reduce pre-weaning calf immunity, health and growth. *J Anim Physiol Anim Nutr*. (2010) 94:628–34. doi: 10.1111/j.1439-0396.2009.00948x
28. Escribano D, Gutiérrez AM, Martínez Subiela S, Tecles F, Cerón JJ. Validation of three commercially available immunoassays for quantification of IgA, IgG, and IgM in porcine saliva samples. *Res Vet Sci*. (2012) 93:682–7. doi: 10.1016/j.rvsc.2011.09018
29. Muneta Y, Yoshikawa T, Minagawa Y, Shibahara T, Maeda R, Omata Y. Salivary IgA as a useful non-invasive marker for restraint stress in pigs. *J Vet Med Sci*. (2010) 72:1295–300. doi: 10.1292/jvms10-0009
30. Curtis J, Bourne FJ. Half-lives of immunoglobulins IgG, IgA and IgM in the serum of new-born pigs. *Immunology*. (1973) 24:147–55.
31. Devillers N, Le Dividich J, Prunier A. Influence of colostrum intake on piglet survival and immunity. *Animal*. (2011) 5:1605–12. doi: 10.1017/S175173111100067X
32. Elahi S, Thompson DR, Van Kessel J, Babiuk LA, Gerdts V. Protective role of passively transferred maternal cytokines against *Bordetella pertussis* infection in newborn piglets. *Infect Immun*. (2017) 85:1–16. doi: 10.1128/IAI01063-16
33. Bandrick M, Theis K, Molitor TW. Maternal immunity enhances *Mycoplasma hyopneumoniae* vaccination induced cell-mediated immune responses in piglets. *BMC Vet Res*. (2014) 10:124. doi: 10.1186/1746-6148-10-124
34. Perrine S. 基 因 的 改 变 NIH public access. *Bone*. (2005) 23:1–7. doi: 10.1038/jid.2014.371

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OPINION
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About Welfare and Stress in the Early Stages of Fish

Juan Ramos, Joan Carles Balasch and Lluis Tort*

Department Cell Biology, Physiology and Immunology, Universitat Autonoma de Barcelona, Bellaterra, Spain

Keywords: welfare, early fish stages, zebrafish, anesthesia, stress, fish husbandry

INTRODUCTION

Fish are the most phylogenetically ancient vertebrates and the most varied group in terms of genetic and morphological diversity. Hence, the considerations about fish welfare and the physiological bases for such welfare have been adopted always later than higher vertebrates and it has been more difficult to generalize protocols and methodologies. In recent years there has been a greater social sensitivity in terms of fish welfare, which has been reflected in an increasingly protective (European) legislation of fish, whether they are for aquarium trade (006/88/EU), production (standing committee of the European convention for the protection of animals kept for farming purposes: recommendation concerning farmed fish adopted by the standing committee on 5 December 2005) or research, (2010/63/EU https://eur-lex.europa.eu/eli/dir/2010/63/oj). This social defendant has been associated to a change in the growing scientific perspective and research regarding animal welfare. The development of different indicators for evaluating the status of the fish has resulted in a quantifiable set of parameters, either individually or for a given population. But some questions arise regarding welfare during earlier fish stages: When and how the fish start to experience stress and pain along development? Are stress and pain experiences in mature fish applicable to the earliest stages of development? In this contribution, we review the state of the art regarding the studies dealing with stress and welfare in eggs, larvae and early stages of fish. Provided that zebrafish, *Danio rerio* is, by far, the most used species in biomedical research, we focused this opinion paper in this species, although most of the conclusions can be applied to other species, such as medaka or killifish. By law only welfare of fish with independent feeding, should be considered, as they are protected by European directive, and considered legally as research animals. But the implications on welfare during the early stages (no independent feeding), can affect to the adults and further generations. Early stages welfare is not required by law but will affect the normal development and reliability of the research. Our conclusion is that current protocols of egg transport and larval handling, lack of solid analytical background and therefore there is a need of specific studies (1, 2) (**Table 1**).

Welfare during early stages begins with the paternal welfare. The experiences of the parents (nutritional, social, environmental), especially during the gametogenesis period, are of great importance for the progeny. During the gametogenesis the DNA will be reprogrammed, so this information will be transmitted to the progeny, thus involving transgenerational effects with a direct impact in the quantity, viability, social status, neurogenesis, and adaptation of the further generations (3–5). Welfare has to be understood as continuous and intergenerational, linking progeny adaptation to parents resources.

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Edward Narayan, The University of Queensland, Australia

Reviewed by:
Jose Fernando Lopez-Olmeda, University of Murcia, Spain
Patrick Prunet, Institut National de la Recherche Agronomique (INRA), France

***Correspondence:**
Juan Ramos
juanramoblas@gmail.com

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TABLE 1 | Type of protocol and the variables that should be considered to preserve welfare and normal development.

Protocol	Variables	Author
Environmental parameter	Oxygen	Reed et al., Aleström et al.
	Temperature	Sfakianakis et al., Scott et al., Long et al.
	Light and photoperiod	Villamizar et al., Basili et al.
	Nitrogen compounds	Lin et al.
	Noise and vibrations	Lara et al., Bhandiwad et al.
	pH	Reed et al., Aleström et al.
	Conductivity	Reed et al., Aleström et al.
	Hardness/alkalinity	Reed et al., Aleström et al.
Incubation of eggs	Density	
	Egg and gametes quality	Yilmaz et al., Labbé et al.
	Time to be collected	Lin et al.
	Environmental parameters	
	Transgenerational effects	Wang et al., Labbé et al., Long et al.
	% Renovation water	Water quality
Larval rearing	Environmental enrichment	Lee et al.
	Maternal social status	Best C. et al.
	Social and schooling parameters	Gerlach et al., Biechl et al., Groneber et al.
	Diet and nutrition	Monteiro et al., Martins et al., Chang et al.
	Water flow	Oteiza et al.
	Rearing larval density	Ribas et al.
	Behavior	Tudorache et al.
	Microbiota	Davis et al., Basili et al.
	Environmental parameters	
Transport of embryos	Acclimation period	Dhansari et al.
	Insulation	Barton et al.
	Disinfection method	Barton et al.
	Environmental parameters	Barton et al.
Handling	Analgesics	Lopez-Luna et al.
	Refinement technique	Oteiza et al.
	Environmental parameters	
Anesthesia and euthanasia	Type of anesthesia	Felix et al., Strikowski et al., Collymore et al.
	Refinement technique	
	Environmental parameters	

EGG QUALITY AND DEVELOPMENT: HANDLING AND ENVIRONMENTAL EFFECTS

Fish have been able to colonize many ecological niches, so they have developed multiple adaptive strategies, thanks to their genetic plasticity. Thus, when the environmental parameters are not optimal, they try to adapt to the new conditions. If the adaptation is successful, no welfare problems will arise, but some alterations can often occur. The genetic quality of embryonic eggs is determined by the gametes, which results from the parents plus any own experience. Assessing the protein and genetic profiles of the eggs, may help to predict their quality and the viability of the embryos (6). In this way, altered embryos can be identified and discarded avoiding further welfare implications.

During their development, embryonic eggs are very sensitive to environmental influences (7). To ensure its correct embryonic development and to avoid future alterations in the juvenile or adult stages, the environmental conditions must be adjusted to the optimal ranges. But how can we assure it along all the development period? Adult zebrafish are kept under recirculation but not when they are mating (1, 2). Zebrafish eggs are usually obtained under static conditions, so water has low oxygen and high ammonia values and kept in these conditions 4 h affecting their normal development, but this is not usually taken in account in the protocols (8).

Environmental influences on embryogenesis vary between species and individuals, thus modifying the normal development of fish larvae and, so the animal may not be able to cope with some environmental conditions. Regarding zebrafish, no studies have been done to stablish a proper protocol of incubation. Thus, the influence conditions like the use of fungustatics or disinfectants, density of eggs, % of water removal, type of water or type of incubator (with or without photoperiod control) have not been established. The influence of environmental conditions is not a trivial issue and should be investigated before stablishing the protocols in order to assure the normal development and avoid further welfare problems (1, 2) (**Table 1**).

LARVAL RESPONSES

The alteration of appropriate conditions during development will produce changes in DNA methylation, with the consequent physiological change (7), that may remain for all live stages and the offspring. In zebrafish larvae, an inadequate density induces a stress response and can influence sex determination (9). Also like in previous stages, water quality and light conditions modulates gene expression and development (10, 11).

Zebrafish larvae are able to process external stimuli, thanks to the presence of neural centralized circuits, and not by automatic or ecotaxic processes as it was previously thought (12). For example, zebrafish larvae adapt their swimming depending on water flow (13, 14) thanks to the integration of the stimuli perceived by the lateral line. During the early stages of development, the nociceptive pathways are already active, allowing the larvae to escape from painful stimuli. This is possible thanks the activation of oxytocin neurons, which produce a locomotor reaction, whose activation can be modulated by analgesics (15–17). So analgesic drugs could be used in zebrafish larvae in order to avoid pain and preserve welfare, but also their impact in the larvae, as bioactive molecules, should be studied (18). As fish develop they are able to process more environmental stimuli and elaborate a strategy to cope with them. If the environment during early stages is complex they will have more strategies and less anxiety. So enrichment and complexity of environmental conditions during early stages should be taken in consideration in order to help their adaptation strategies and improve welfare.

Another way for fish larvae to avoid external dangers such as predation is schooling, especially in social fishes such as zebrafish. In order to develop this aggregation mechanism, they need to differentiate their congeners from other fish. Thus, the olfactory cues (19–21) are key signals, and these have been found in zebrafish brain from day 6 of development, allowing them to recognize their siblings and perform the schooling pattern. This process would be impossible without memory, that performs the integration of the stimuli and the identification of the habitual environment (22). The social conditions of early stages should be also considered in protocols, as they are developing social patterns: schooling and social avoidance (23).

Zebrafish larvae can sometimes adopt different coping strategies in front of the same stimulus. The stimuli can be processed as an opportunity, as for proactive fish, or like a risk, as for reactive ones, so they develop anxiety. These differences may be determined by the paternal genetic load, as well as the experiences of the embryo or larva during development (24), The use of substrates and enrichment makes the ambient more complex and reduces anxiety, improving the boldness (proactive) (25). Environmental enrichment is not common in zebrafish tanks because of technical implications but this may result in more anxious fish.

Nutrition has a direct impact in animal welfare especially during the growing o development period. The use of life preys in zebrafish (*Artemia*, paramecium, and rotifers) allows to display the natural behavior as a predator and also use them as vehicle for different nutrients such as polyunsaturated fatty acids. So new protocols have been developed using life preys and special dry foods that help zebrafish larvae to grow and develop faster (26–28). The use of probiotics has been tested in zebrafish larvae as a way to improve welfare by modulating anxiety, immunity, or gut function (29).

WELFARE IN EARLY FISH STAGES AND THE ANTHROPOGENIC IMPACT

Transport is a highly stressful process for adult fish (30) if environmental conditions are not properly controlled. Zebrafish eggs are commonly transported between facilities as they are cheaper and easier to ship than adults. If there is an improper isolation and no heater or chiller inside the box, during the transport, eggs can be exposed to extreme temperatures (higher or lower than their optimal range). Till the eggs arrive to the new facility no water or air is exchanged, as they are in watertight containers, and no light is received. Although the importance of the photoperiod, for the activation of circadian rhythms (11, 31, 32), and the importance of temperature for a normal development (33–35) has been widely studied in zebrafish, none of these factors are usually considered for the shipping protocols of embryos neither the relevance of the acclimation period afterwards. The evaluation and standardization of environmental conditions during transport of the early stages of zebrafish should then be revisited (1, 2, 36), as it should be during the standard incubation period (**Table 1**).

Fish experimental facilities usually involve noises and vibrations produced by water pumps or working routines that will affect the normal development of fish (37, 38). Fish husbandry facilities could be designed in order to minimize noise impact, removing pumps, machines from the facility. The routines have to be also reduced to minimum, especially in the breeding area.

Visual techniques are very common in zebrafish research, due to the translucency of embryos. It is useful for developmental studies, but it usually requires immobilization of the larvae or embryos with an anesthetic. Nevertheless, the use of anesthetics, especially in the early stages, has a broad implication on the present and future welfare of the individual. So, it must be carefully considered when carrying out the experiments even if these early phases are not protected by welfare laws. For instance, the most common anesthetic for zebrafish, Tricaine metanosulphonate (MS-222), is capable of generating oxidative stress, altering the normalized development of cartilage and inducing apoptotic processes (39–41).

In terms of euthanasia, an anesthetic overdose is the usual procedure, but its efficiency in larvae is very limited. Since oxygen is taken by zebrafish through the skin until day 14 for respiration, it makes them resistant to most of the anesthetics, as the muscular contraction is not related to respiration. Furthermore, it should be taken into account that cessation of the heartbeat, usually taken as an indicator of death, could not be considered as such, since heart fibers are capable of beating for more than 20 min after death (42, 43). For these reasons, especial euthanasic protocols and more clear death indicators should be addressed for zebrafish early development stages.

CONCLUSION

In conclusion, the investigations carried out up to now demonstrate that during the early stages fish show high sensitivity to many types of stressors involving an array of responses to overcome alterations that could affect the animal and be transmitted to the progeny. Welfare in eggs and larvae is a continuous process that involve both parental experience and development, so environmental parameters have to be controlled during all the life time, especially during the gamete and organogenesis stages. Standard protocols should be developed including all environmental parameters by studying not just the zoothecnical indexes but also stress and welfare-related indicators such as behavioral traits, stress hormones, or the expression of genes associated to them. In our opinion, the results of the research on these early stages also points out a lack of adequate standards for reliable welfare results in relation with the procedures for maintenance, husbandry or transport (**Table 1**).

AUTHOR CONTRIBUTIONS

JR collected data and wrote the first draft. JCB and LT designed the paper structure. All authors completed, revised and finished the work, and approved it for publication.

REFERENCES

1. Aleström P, D'Angelo L, Midtlyng PJ, Schorderet DF, Schulte-Merker S, Sohm F,et al. Zebrafish: housing and husbandry recommendations. *Lab Anim.* (2020) 54:213–24 doi: 10.1177/0023677219869037
2. Reed B, Jennings M. *Guidance on the Housing and Care of Zebrafish Danio rerio*. Res Anim Dep Sci Group (2011).
3. Best C, Kurrasch DM, Vijayan MM. Maternal cortisol stimulates neurogenesis and affects larval behaviour in zebrafish. *Sci Rep.* (2017) 7:40905. doi: 10.1038/srep40905
4. Wang SY, Lau K, Lai KP, Zhang JW, Tse ACK, Li JW, et al. Hypoxia causes transgenerational impairments in reproduction of fish. *Nat Commun.* (2016) 7:12114. doi: 10.1038/ncomms12114
5. Wang M, Chen J, Lin K, Chen Y, Hu W, Tanguay RL, et al. Chronic zebrafish PFOS exposure alters sex ratio and maternal related effects in F1 offspring. *Environ Toxicol Chem.* (2011) 30:2073–80. doi: 10.1002/etc.594
6. Yilmaz O, Patinote A, Nguyen TV, Com E, Lavigne R, Pineau C, et al. Scrambled eggs: proteomic portraits and novel biomarkers of egg quality in zebrafish (*Danio rerio*). *PLoS ONE.* (2017) 2:e0188084. doi: 10.1371/journal.pone.0188084
7. Labbé C, Robles V, Herraez MP. Epigenetics in fish gametes and early embryo. *Aquaculture.* (2017) 47:96–103. doi: 10.1016/j.aquaculture.2016.07.026
8. Lin LY, Zheng JA, Huang SC, Hung GY, Horng JL. Ammonia exposure impairs lateral-line hair cells and mechanotransduction in zebrafish embryos. *Chemosphere.* (2020) 257:127170. doi: 10.1016/j.chemosphere.2020.127170
9. Ribas L, Valdivieso A, Díaz N, Piferrer F. Appropriate rearing density in domesticated zebrafish to avoid masculinization: links with the stress response. *J Exp Biol.* (2017) 220:1056–64. doi: 10.1242/jeb.144980
10. Long Y, Yan J, Song G, Li X, Li X, Li Q, et al. Transcriptional events co-regulated by hypoxia and cold stresses in Zebrafish larvae. *BMC Genomics.* (2015) 16:385. doi: 10.1186/s12864-015-1560-y
11. Villamizar N, Vera LM, Foulkes NS, Sánchez-Vázquez FJ. Effect of lighting conditions on zebrafish growth and development. *Zebrafish.* (2014) 11:173–81. doi: 10.1089/zeb.2013.0926
12. Bahl A, Engert F. Neural circuits for evidence accumulation and decision making in larval zebrafish. *Nat Neurosci.* (2020) 23:94–102. doi: 10.1038/s41593-019-0534-9
13. Dunn TW, Gebhardt C, Naumann EA, Riegler C, Ahrens MB, Engert F, et al. Neural circuits underlying visually evoked escapes in larval Zebrafish. *Neuron.* (2016) 89:613–28. doi: 10.1016/j.neuron.2015.12.021
14. Oteiza P, Odstrcil I, Lauder G, Portugues R, Engert F. A novel mechanism for mechanosensory-based rheotaxis in larval zebrafish. *Nature.* (2017) 547:445–8. doi: 10.1038/nature23014
15. Lopez-Luna J, Al-Jubouri Q, Al-Nuaimy W, Sneddon LU. Impact of stress, fear and anxiety on the nociceptive responses of larval zebrafish. *PLoS ONE*. (2017) 12:e0181010. doi: 10.1371/journal.pone.0181010
16. Wee CL, Nikitchenko M, Wang WC, Luks-Morgan SJ, Song E, Gagnon JA, et al. Zebrafish oxytocin neurons drive nocifensive behavior via brainstem premotor targets. *Nat Neurosci.* (2019) 22:1477–92. doi: 10.1038/s41593-019-0452-x
17. Lopez-Luna J, Canty MN, Al-Jubouri Q, Al-Nuaimy W, Sneddon LU. Behavioural responses of fish larvae modulated by analgesic drugs after a stress exposure. *Appl Anim Behav Sci.* (2017) 195:115–20. doi: 10.1016/j.applanim.2017.05.021
18. Lopez-Luna J, Al-Jubouri Q, Al-Nuaimy W, Sneddon LU. Impact of analgesic drugs on the behavioural responses of larval zebrafish to potentially noxious temperatures. *Appl Anim Behav Sci.* (2017) 188:97–105. doi: 10.1016/j.applanim.2017.01.002
19. Gerlach G, Hodgins-Davis A, Avolio C, Schunter C. Kin recognition in zebrafish: a 24-hour window for olfactory imprinting. *Proc R Soc B Biol Sci.* (2008) 275:2165–70. doi: 10.1098/rspb.2008.0647
20. Biechl D. *Neuronal basis of olfactory imprinting and kin recognition in the zebrafish Danio rerio* (Dissertation), München: Faculty of Biology, LMU (2017). doi: 10.5282/edoc.21625
21. Romano D, Elayan H, Benelli G, Stefanini C. Together we stand – Analyzing schooling behavior in naive newborn guppies through biorobotic predators. *J Bionic Eng.* (2020) 7:174–84. doi: 10.1007/s42235-020-0014-7
22. Bruzzone M, Gatto E, Xiccato TL, Valle LD, Fontana CM, Meneghetti G, et al. Measuring recognition memory in zebrafish larvae: issues and limitations. *PeerJ.* (2020) 8:e8890. doi: 10.7717/peerj.8890
23. Groneberg AH, Marques JC, Martins AL, Diez del Corral R, de Polavieja GG, Orger MB. Early-life social experience shapes social avoidance reactions in larval zebrafish. *Curr Biol.* (2020) 30:R1275–6. doi: 10.1016/j.cub.2020.07.088
24. Tudorache C, Ter Braake A, Tromp M, Slabbekoorn H, Schaaf MJM. Behavioral and physiological indicators of stress coping styles in larval zebrafish. *Stress.* (2015) 18:1–23. doi: 10.3109/10253890.2014.989205
25. Lee CJ, Paull GC, Tyler CR. Effects of environmental enrichment on survivorship, growth, sex ratio and behaviour in laboratory maintained zebrafish *Danio rerio*. *J Fish Biol.* (2019) 94:86–95. doi: 10.1111/jfb.13865
26. Monteiro JF, Martins S, Farias M, Costa T, Certal AC. The impact of two different cold-extruded feeds and feeding regimens on zebrafish survival, growth and reproductive performance. *J Dev Biol.* (2018) 6:15. doi: 10.3390/jdb6030015
27. Martins G, Diogo P, Pinto W, Gavaia PJ. Early transition to microdiets improves growth, reproductive performance and reduces skeletal anomalies in zebrafish (*Danio rerio*). *Zebrafish.* (2019) 16:300–7. doi: 10.1089/zeb.2018.1691
28. Chang CT, Benedict S, Whipps CM. Transmission of Mycobacterium chelonae and *Mycobacterium marinum* in laboratory zebrafish through live feeds. *J Fish Dis.* (2019) 42:1425–31. doi: 10.1111/jfd.13071
29. Davis DJ, Bryda EC, Gillespie CH, Ericsson AC. Microbial modulation of behavior and stress responses in zebrafish larvae. *Behav Brain Res.* (2016) 311:219–27. doi: 10.1016/j.bbr.2016.05.040
30. Dhanasiri AKS, Fernandes JMO, Kiron V. Acclimation of zebrafish to transport stress. *Zebrafish.* (2013) 10:87–98. doi: 10.1089/zeb.2012.0843
31. Villamizar N, Blanco-Vives B, Oliveira C, Dinis MT, Di Rosa V, Negrini P, et al. Circadian rhythms of embryonic development and hatching in fish: a comparative study of Zebrafish (Diurnal), Senegalese Sole (Nocturnal), and Somalian Cavefish (Blind). *Chronobiol Int.* (2013) 30:889–900. doi: 10.3109/07420528.2013.784772
32. Basili D, Lutfi E, Falcinelli S, Balbuena-Pecino S, Navarro I, Bertolucci C, et al. Photoperiod manipulation affects transcriptional profile of genes related to lipid metabolism and apoptosis in zebrafish (*Danio rerio*) larvae: potential roles of gut microbiota. *Microb Ecol.* (2020) 79:933–46. doi: 10.1007/s00248-019-01468-7
33. Sfakianakis DG, Leris I, Laggis A, Kentouri M. The effect of rearing temperature on body shape and meristic characters in zebrafish (*Danio rerio*) juveniles. *Environ Biol Fish.* (2011) 92:197–205. doi: 10.1007/s10641-011-9833-z
34. Scott GR, Johnston IA. Temperature during embryonic development has persistent effects on thermal acclimation capacity in zebrafish. *Proc Natl Acad Sci USA*. (2012) 109:14247–52. doi: 10.1073/pnas.1205012109
35. Long Y, Li L, Li Q, He X, Cui Z. Transcriptomic characterization of temperature stress responses in larval zebrafish. *PLoS ONE.* (2012) 7:e37209. doi: 10.1371/journal.pone.0037209

36. Barton CL, Baumann DP, Cox JD. Export and transportation of zebrafish. In: *The Zebrafish in Biomedical Research: Biology, Husbandry, Diseases, and Research Applications*. p. 443–50. doi: 10.1016/B978-0-12-812431-4.00037-3
37. Bhandiwad AA, Raible DW, Rubel EW, Sisneros JA. Noise-induced hypersensitization of the acoustic startle response in larval zebrafish. *J Assoc Res Otolaryngol*. (2018) 19:741–52. doi: 10.1007/s10162-018-00685-0
38. Ayala R., Ornelas R. Impact of noise exposure on development, physiological stress and behavioural patterns in larval zebrafish. *Res Square* [Preprint]. (2020). doi: 10.21203/rs.3.rs-126894/v1
39. Félix L, Coimbra AM, Valentim AM, Antunes L. Review on the use of zebrafish embryos to study the effects of anesthetics during early development. *Crit Rev Toxicol*. (2019) 49:357–70. doi: 10.1080/10408444.2019.1617236
40. Félix LM, Luzio A, Themudo M, Antunes L, Matos M, Coimbra AM, et al. MS-222 short exposure induces developmental and behavioural alterations in zebrafish embryos. *Reprod Toxicol*. (2018) 81:122–31. doi: 10.1016/j.reprotox.2018.07.086
41. Félix LM, Luzio A, Santos A, Antunes LM, Coimbra AM, Valentim AM. MS-222 induces biochemical and transcriptional changes related to oxidative stress, cell proliferation and apoptosis in zebrafish embryos. *Comp Biochem Physiol Part C Toxicol Pharmacol*. (2020) 237:108834. doi: 10.1016/j.cbpc.2020.108834
42. Strykowski JL, Schech JM. Effectiveness of recommended euthanasia methods in larval zebrafish (*Danio rerio*). *J Am Assoc Lab Anim Sci*. (2015) 54: 81–4.
43. Collymore C, Banks EK, Turner PV. Lidocaine hydrochloride compared with MS222 for the euthanasia of zebrafish (*Danio rerio*). *J Am Assoc Lab Anim Sci*. (2016) 55:816–20.

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A Multi-Disciplinary Approach to Assess the Welfare Impacts of a New Virtual Fencing Technology

Caroline Lee and Dana L. M. Campbell*

CSIRO, Agriculture and Food, FD McMaster Laboratory, Armidale, NSW, Australia

Virtual fencing involving the application of audio cues and electrical stimuli is being commercially developed for cattle. Virtual fencing has the potential to improve productivity through optimized pasture management and utilization by grazing animals. The application of virtual fencing initiates public concern for the potential welfare impacts on animals due the aversive nature of using an electrical stimulus. It is therefore important to provide welfare assurance of the impacts of virtual fencing on livestock. In this paper, we provide an overview of the welfare assessment and validation stages for virtual fencing which could be applied to other new technologies utilizing novel systems. An understanding of stress measures and their suitability for use in specific contexts is discussed, including the use of glucocorticoids to measure both acute and chronic stress, and behavioral responses and patterns to indicate welfare states. The importance of individual differences in relation to learning and cognition are also highlighted. Together, this multi-disciplinary approach to welfare assessment provides a tool kit that may be applied for welfare assurance of some new technologies and systems for farm animals.

Keywords: animal welfare, behavior, cattle, cortisol, cognition, stress, sheep

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****Correspondence:***
Caroline Lee
caroline.lee@csiro.au

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INTRODUCTION

Utilization of livestock by humans has depended on the capacity of animals to adapt to new farming technologies like herding, milking and harvesting of fiber and eggs. Further advances in husbandry systems and management technologies, such as virtual fencing, intensive housing, and automated milking parlors have increased complexity of the environment farmed animals must learn to engage with. Adaptation to new systems involves cognitive evaluation of environmental stimuli which influences the stress response and subsequent adaptation (1). Assessment of the welfare impacts of implementing new technologies and systems is needed to ensure welfare is acceptable.

Virtual fencing involves the containment of animals without the use of a physical fence by using signals from a device that is attached via a neckband. Using GPS technology to monitor animal movement and behavior, an audio cue signal warns the animal that it is approaching the virtual boundary, and this is followed by an electrical pulse only if the animal does not respond to the audio cue (2–5). The device applies an electrical pulse sequence in the kilovolt range with an intensity that is lower in energy than an electric fence (6). Successful learning occurs when the animal responds to the audio cue to stay within the boundary and avoids receiving the electrical pulse. On some occasions, an animal may cross the virtual fence line and no stimuli are applied if the animal turns and moves toward the inclusion zone to encourage movement back within the boundary (7). In a 44 day study, the virtual fence was 99.8% effective at preventing cattle accessing a sapling regeneration area (8). As the virtual fencing is not 100% effective at containing livestock, fixed fencing should be used for external boundaries and the virtual fence should only be used for internal fencing to reduce the risks of animals accessing roads or public areas. When the virtual fence location is moved, both cattle (9) and sheep (10) enter the new paddock area within hours, demonstrating that they learn to respond to the audio cue and not the location that cues are given, this has important implications for pasture management and strip grazing applications. Virtual fencing has the potential to transform livestock (cattle and sheep) farming (11, 12) by optimizing pasture management, managing weeds in mixed farming systems, maintaining separation to prevent fighting (13) and protecting environmentally sensitive areas (7, 8). Removal of physical fencing also has the potential to benefit wildlife conservation (14). The virtual fencing technology is being commercialized by Agersens (Melbourne, VIC, Australia) and a product for cattle (eShepherd®) will be released imminently. However, the use of an aversive electrical pulse generates concern from the public in relation to animal welfare impacts and science-based evidence to provide welfare assurance is required (15).

Assessing the welfare impacts of virtual fencing in livestock, requires a multi-disciplinary approach to account for the complexity of the animal interacting with and learning about a new technology autonomously, while in a field situation. Consideration of physiological indicators of acute and chronic stress, behavioral responses and patterns, cognition, associative learning, and social learning are all necessary. This review will discuss and highlight the challenges of providing a comprehensive assessment of animal welfare in relation to a new livestock farming technology. The findings from studies investigating the effects of virtual fencing on measures of acute and chronic stress and animal learning will be considered in relation to the welfare implications of this technology and ethical assurance for stakeholders.

STAGES OF LEARNING

We propose that the stress responses of livestock differ in relation to the stages of virtual fence learning. The first stage of virtual fence learning requires the animal to experience both the audio cue and the aversive electrical pulse to enable subsequent associative learning to occur (**Figure 1**). In this initial period, animals cannot avoid receiving the electrical pulse [but see (16) for impacts of social facilitation on behavioral responses], and so the relative aversiveness of the electrical pulse will determine the intensity and duration of the acute stress response (17, 18). Following this, there is a period of adaptation (stage 2) to the virtual fencing system where animals may be in an aroused state until they have learnt to respond to the audio cue and are able to avoid receiving the electrical pulse. Finally, stage 3 is where learning has occurred, and the animals are able to predict and control their interaction with the fence. In this final stage, the fence position is indicated by an audio cue and may shift location. Thus, cattle need to rely on responding correctly to the sound to avoid the electrical pulse without any accompanying visual information which contrasts with being able to see the visual barrier of a standard (electric) fence. For each of these stages, the timelines vary, and different measures are relevant. The acute stress measures are applicable to the initial learning period which typically has a duration of minutes and the chronic stress measures are applicable to later stages of learning. The stage 2 period of adaptation may last for a few hours up to a few days, but stage 3 implementation of a virtual fence could be weeks, months, or potentially years. For welfare to be assured, the effects of virtual fencing on key measures during stage 1 and 2 should be minimal and in stage 3, should not differ from control treatments or normal baseline measures.

PHYSIOLOGICAL INDICATORS OF STRESS

Physiological measures that have been applied to assess acute stress responses to virtual fencing include circulating plasma concentrations of stress hormones, such as cortisol and β-endorphin, measures of heart rate and body temperature increases that may indicate stress induced hyperthermia. To ensure that the stress response measured is due to the exposure to the virtual fencing stimuli themselves and not due to other factors, it is important to have in place a robust experimental design including a control treatment, minimal handling and/or habituation. Controlled studies are necessary where the stimuli are manually applied to account for potential variation in self-exposure to stimuli among individual animals. Other physiological measures that could be applied to the assessment of stress include infrared thermography (19), functional near-infrared spectroscopy (20), and electroencephalography (21).

Concentrations of Stress Hormones

The hypothalamic-pituitary-adrenal (HPA) axis is activated in response to a stressor with a clear relationship between stressor intensity and duration and the HPA axis activation. Stress hormones including glucocorticoids (e.g., cortisol) and opioids (e.g., β-endorphin) are released as part of a cascade when stressors are perceived by the brain (22, 23). As handling itself is stress inducing, blood samples should be collected within 2–3 mins of restraint, before the adrenal cortex has been activated (23). An alternative is to habituate animals to handling prior to the study and include a control treatment to show that cortisol responses are not elevated by handling itself (18). These considerations for the measurement of acute stress hormone concentrations in the context of controlled experimental studies enables comparisons to be made between treatments. To demonstrate this, plasma cortisol and β-endorphin concentrations were assessed in beef cattle receiving an electrical pulse compared with a range of common husbandry procedures and this showed that the stress response to an electrical pulse was not different to being restrained in a crush (17). In a similar comparison study with sheep, a mild cortisol response to an electrical pulse was shown and this was similar to hearing a barking dog (18) and sheep did not differ in their cortisol responses to the audio cue once they had successfully learnt the virtual fence (24). Overall, these results indicate that while the electrical pulse is aversive, it is not more stressful than common handling procedures in both sheep and cattle.

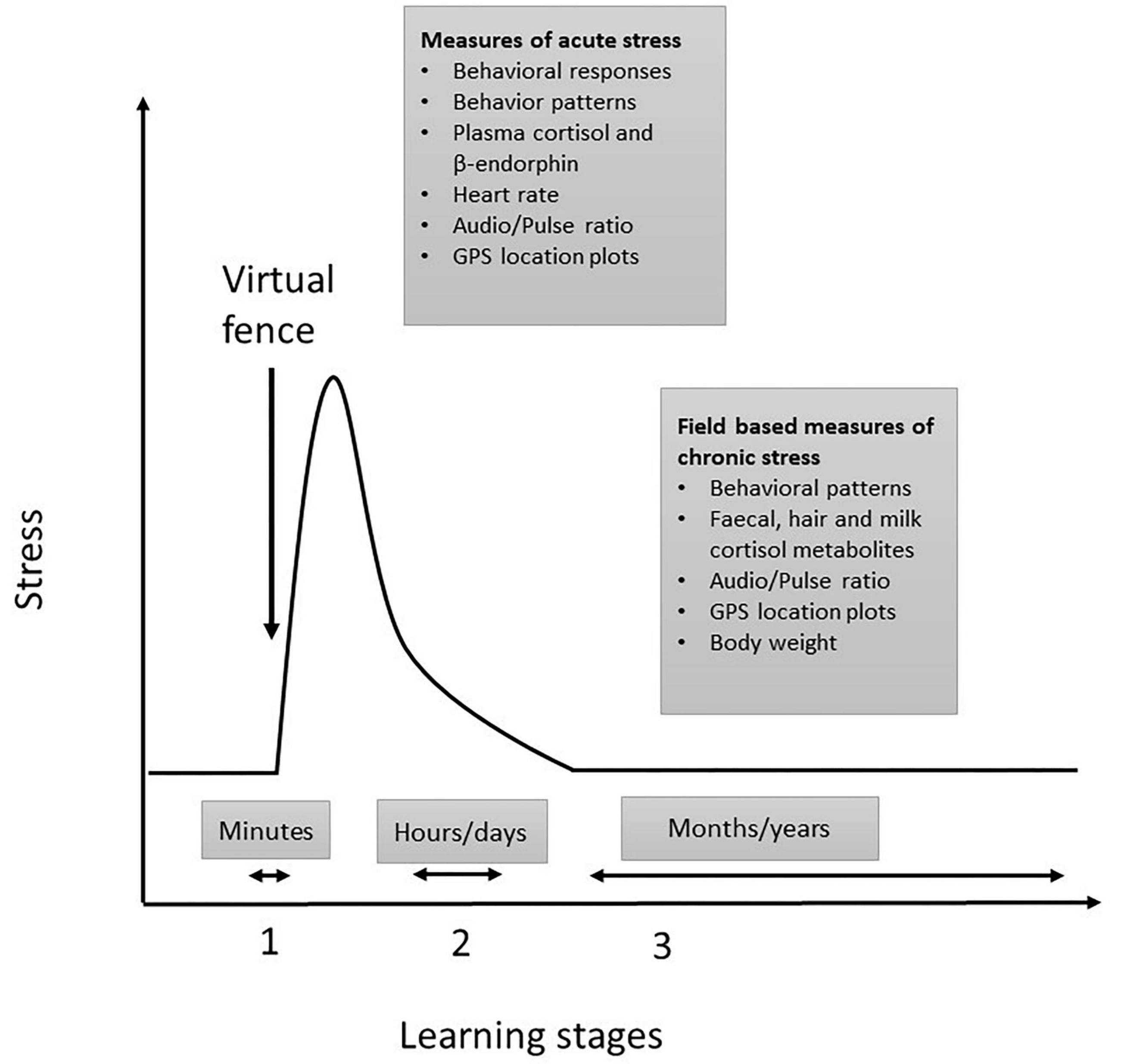

FIGURE 1 | Proposed relationship between learning the virtual fence and stress in livestock. Stage 1 represents the acute phase of learning when animals cannot avoid receiving the electrical pulse and this induces an acute stress response. Stage 2 describes animals adapting to the virtual fence. Stage 3 is where associative learning has occurred, and animals can control their interaction with the fence and avoid receiving the electrical pulse. Relevant measures for acute and chronic stress responses are listed within the text boxes.

While plasma stress hormones are good measures of arousal in short-term controlled experiments, they are less suitable for measurement of chronic stress in field-based studies. Plasma cortisol is affected by the sampling procedure itself and levels usually decline after the acute response so are not very informative for states of chronic stress (23). In addition, chronic stress can modify the responsiveness of the HPA axis, with a range of effects, including both an increase in the responsiveness (25) and a decrease in the sensitivity of the HPA axis following negative events (26, 27). Measurement of cortisol metabolites in feces, hair or milk are more stable and therefore are practical options for assessment of chronic stress in longer-term field studies (28, 29). When virtual fencing was compared with conventional electric fencing, fecal cortisol metabolites did not differ over a 4-week period, indicating that there were no differences in stress responses over that period between fencing groups although the metabolites did reduce across time (6). Similar findings were reported in dairy cows, with no differences between virtual and conventional electric fencing on milk cortisol concentrations for a 5-day period, however longer-term assessment is needed (30).

Heart Rate

Other physiological measures of stress include heart rate and heart rate variability (HRV), which indicate a change in cardiac function and provide an early indicator of stress responses (31, 32). A heart rate device is strapped around the girth area of an animal and the area is shaved to enable close contact of the electrodes with the skin. While heart rate and HRV measures are feasible in controlled experimental contexts, they are not yet practical for longer-term field deployment mainly due to issues with attachment (33). However, progress in developing heart rate measures in cattle with high accuracy for use in the field is occurring (34). In addition, heart rate is affected by locomotion (35) so care should be taken when designing studies using this measure. In the cattle study that measured stress hormone responses to the electrical pulse and common husbandry procedures, a second experiment assessed heart rate responses and found that they did not differ between any treatments which confirmed the stress hormone findings (17).

Stress-Induced Hyperthermia

Stress-induced hyperthermia (SIH), a rapid increase in core body temperature due to exposure to a stressor, can be used to measure acute stress responses (31). Small temperature loggers collect data and are placed in the vagina or rectum of the animal (36, 37). SIH has been demonstrated in sheep during shearing (38), isolation (39) and when anxious (40–42), and in cattle during handling (43) or when anxious (44). However, SIH was not observed in sheep exposed to virtual fencing stimuli either in a controlled experiment (18) or in the field (45). This may have been due to the stimuli intensity or duration not being sufficiently aversive to induce a stress response. Thus, while SIH has been an accurate and practical measure of stress response deployed in both experimental and field contexts, its relevance to welfare assessment of virtual fencing is uncertain. The short-lived duration (<1 s) of electrical pulse exposure and the substantial variation in self-exposure both within and among individuals may limit interpretations of this measure.

Body Weight

A coarser indicator of welfare is changes in body weight over time where a lower body weight gain may be indicative of a welfare issue (46). But this can be influenced by many factors, including feed and water availability, health, climate, and physiological status and thus may be most informative if paired with other simultaneous welfare measures. If used as part of a controlled study, it may be a valuable measure but to date has not provided consistent indications of welfare impacts of virtual fencing (6).

BEHAVIORAL INDICATORS OF STRESS

Behavioral Responses

Immediate behavioral responses specifically to the stimuli provide an indicator of their aversiveness and effectiveness. The audio cue alone should be benign, eliciting no specific reaction beyond ear movement until it has been associated with the electrical pulse. This has been observed when cattle first hear the audio tone (4) although sheep appear more sensitive to the audio signal with first exposure (5, 47). With the electrical pulse, it needs to be aversive enough that it deters the animal, but extreme and extended behavioral responses such as leaping forward, vocalizing, and jumping are undesirable and may reduce an animal's learning ability while in such a heightened state (2). A stimulus that is highly aversive is inappropriate to use (2, 5), and in the case of developing the virtual fencing pre-commercial prototypes in cattle, alternative pulse durations and intensities were tested to optimize the electrical pulse (4). Additionally, poor or inconsistent pulse delivery may result in animals that show a minimal behavioral response (e.g., head tossing or turning in cattle) to both the audio and pulse stimuli whilst continuing to move past the virtual fence (8). Ultimately, this could have welfare consequences if they attract others to follow, thus increasing stimuli delivery for some individuals, potentially causing confusion, frustration and stress. Individual variation in skin sensitivity and pain perception may increase the aversiveness for some animals with some evidence of variation in dairy cows (48, 49) but further investigation into this is required.

Behavioral Patterns

Monitoring of behavioral patterns of the individual and the herd are a practical indicator of welfare to deploy in field studies. Although precise behavioral patterns vary among individuals and herds, and within herds relative to season or across age (50), deviations from what is expected to be "normal" for that species may be indicative of chronic stress. With the availability of increasing numbers of off-the-shelf monitoring products such as IceQubes® and Moomonitors® (51) for cattle (6) and HOBO's for sheep (45) that have relatively long battery life, long-term monitoring of cattle and sheep behavior is now possible. Disturbances in normal behavioral patterns over time may indicate that welfare is not optimal, for example, lying time has been demonstrated to indicate comfort of lying surfaces in cattle (52). In a study using pre-commercial eShepherd® prototype devices for cattle where virtual fences were moved at regular intervals for a 22-day period, behavioral time budget changes were minor (9). Similarly, minimal behavioral pattern changes were reported in a longer 4-week study using the virtual fencing system in beef cattle (6) or for a shorter 5-day period with dairy cows (30). Further assessments of behavioral time budgets over longer periods would be recommended in future research to confirm these findings.

GPS Location Data

GPS location of individual animals can be used to assess if animals show a lack of understanding of where the virtual fence is located as evidenced by thigmotaxis, a tendency to move toward physical contact, such as an increased following of fixed fences. Rodents show thigmotaxis when anxious (53) and it is thought to be a protection against predators (54). No evidence has been reported of thigmotaxis in any of the virtual fencing studies using the Agersens system (eShepherd®) and manual dog collars. All GPS plots to date of sheep and cattle locations in the presence of a virtual fence indicate usage of all paddock areas including those immediately in front of the virtual barrier (6–10). Interpretation of GPS data showing spatial distribution of animals should consider the uniformity of the paddock and position of preferred resources as these will influence the time animals spend in certain areas.

COGNITIVE MEASURES OF WELFARE

Associative Learning

The ability of animals to predict and control their situation in the long term is strongly related to welfare outcomes (55). Consideration of the impact of sudden changes to predictable routines such as feeding times, and regrouping can have negative impacts (56). As proposed in a welfare assessment framework of virtual fencing (57), once animals learn the association between the audio and electrical stimuli, the cues are both predictable (the audio cue always precedes the electrical pulse) and controllable (animals can choose to avoid the electrical pulse by stopping or turning), thus minimizing negative welfare impacts. Indeed, cattle learn rapidly after an average of 2.5 interactions with the virtual fence before responding to the audio cue alone (6). This hypothesis was tested Kearton et al. (24) in a study that assessed the influence of controllability on stress responses to virtual fencing stimuli. Sheep that had learned to predict and control receiving the electrical pulse through their behavioral responses, did not differ in their cortisol, core body temperature and behavioral responses compared with a control treatment that did not receive any cues. This shows that the sheep perceived the audio cue as benign once they had successfully learnt.

Inclusion of a measure that indicates learning of the virtual fence such as the relative proportions of audio and electrical pulse cues is of value for welfare assessment. This could be used to ensure all animals are learning and have reached set thresholds within a certain number of interactions with the virtual fence. Additionally, it would allow confirmation that all animals being managed by the system have successfully learnt to respond to the audio cue so that it is both predictable and controllable. Identification of animals that are not learning (as indicated by an audio cue always being followed by an electrical pulse) may indicate a learning or equipment failure and providing an alert will enable the animal to be checked and if necessary, removed from the virtually fenced paddock.

Social Learning

Livestock are social animals that are typically managed in groups forming dominance relationships and social networks (58–60). Associative learning of the virtual fence occurs more rapidly when applied to a group of cattle (7–9, 61) or sheep (10, 47) than when applied to individuals (3, 4). It is likely that the social attraction to remain with the group provides encouragement to respond by turning and re-joining the herd or flock. Previous experience can also affect learning of the virtual fencing stimuli, with pre-exposure to an electric fence in dairy heifers resulting in more rapid associative learning (62). Recently, social facilitation of virtual fence learning was reported in cattle (16) with animals responding when others interacted with the fence. Social influences on the effectiveness of the virtual fence were also shown in sheep, with collaring two thirds of the group with virtual fence collars being equally as effective at containing sheep as having all animals collared (45). More research is needed to understand social learning aspects of virtual fencing, particularly in larger, commercially relevant group sizes.

OTHER CONSIDERATIONS

With the identification of distinct personalities (63) and coping styles in sheep (64) and temperament in cattle (65), consideration of individual differences is recommended in evaluating welfare impacts of management practices and new technologies such as virtual fencing. In addition, further research to investigate application of virtual fencing to different stock classes such as cows and calves or ewes and lambs and the impact on animal welfare is needed.

CONCLUSIONS

Welfare assessment of a virtual fencing system requires consideration of the nature of the stress response during the different stages of learning and adaptation to the system. A multi-disciplinary approach applied to assess both acute and chronic stress is needed that also accounts for individual differences in cognition, physiology and behavioral responses. Of importance is the assessment of the chronic stress measures as the acute stress response is short lived and animals quickly adapt. Welfare assessment and validation that focusses on the longer-term impacts across different situations is needed for welfare assurance of new technologies and systems. Application of a range of measures over the short and longer term, have confirmed that welfare impacts of virtual fencing on cattle and sheep are minimal. Further studies to assess the impacts over even longer periods are recommended to confirm these findings in a commercial setting.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

CL prepared the first draft and edited the manuscript. DC contributed to the content, wrote parts, and edited the manuscript. All authors contributed to the article and approved the submitted version.

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REFERENCES

1. Ursin H, Eriksen HR. The cognitive activation theory of stress. *Psychoneuroendocrinology*. (2004) 29:567–92. doi: 10.1016/S0306-4530(03)00091-X
2. Lee C, Prayaga K, Reed M, Henshall J. Methods of training cattle to avoid a location using electrical cues. *Appl Anim Behav Sci.* (2007) 108:229–38. doi: 10.1016/j.applanim.2006.12.003
3. Lee C, Henshall JM, Wark TJ, Crossman CC, Reed MT, Brewer HG, et al. Associative learning by cattle to enable effective and ethical virtual fences. *Appl Anim Behav Sci.* (2009) 119:15–22. doi: 10.1016/j.applanim.2009.03.010
4. Campbell DLM, Lea JM, Haynes SJ, Farrer WJ, Leigh-Lancaster CJ, Lee C. Virtual fencing of cattle using an automated collar in a feed attractant trial. *Appl Anim Behav Sci.* (2018) 200:71–7. doi: 10.1016/j.applanim.2017.12.002
5. Marini D, Meuleman MD, Belson S, Rodenburg TB, Llewellyn R, Lee C. Developing an ethically acceptable virtual fencing system for sheep. *Animals.* (2018) 8:33. doi: 10.3390/ani8030033
6. Campbell DLM, Lea JM, Keshavarzi H, Lee C. Virtual fencing is comparable to electric tape fencing for cattle behavior and welfare. *Front Vet Sci.* (2019) 6:445. doi: 10.3389/fvets.2019.00445
7. Campbell DLM, Haynes SJ, Lea JM, Farrer WJ, Lee C. Temporary exclusion of cattle from a riparian zone using virtual fencing technology. *Animals.* (2019) 9:5. doi: 10.3390/ani9010005
8. Campbell DLM, Ouzman J, Mowat D, Lea JM, Lee C, Llewellyn RS. Virtual fencing technology excludes beef cattle from an environmentally sensitive area. *Animals.* (2020) 10:1069. doi: 10.3390/ani10061069
9. Campbell DLM, Lea JM, Farrer WJ, Haynes SJ, Lee C. Tech-savvy beef cattle? How heifers respond to moving virtual fence lines. *Animals.* (2017) 7:72. doi: 10.3390/ani7090072
10. Marini D, Llewellyn R, Belson S, Lee C. Controlling within-field sheep movement using virtual fencing. *Animals.* (2018) 8:31. doi: 10.3390/ani8030031
11. Anderson DM, Estell RE, Holechek JL, Ivey S, Smith GB. Virtual herding for flexible livestock management - a review. *Rangel J.* (2014) 36:205–21. doi: 10.1071/RJ13092
12. Umstatter C. The evolution of virtual fences: a review. *Comput Electron Agric.* (2011) 75:10–22. doi: 10.1016/j.compag.2010.10.005
13. Lee C, Prayaga KC, Fisher AD, Henshall JM. Behavioral aspects of electronic bull separation and mate allocation in multiple-sire mating paddocks. *J Anim Sci.* (2008) 86:1690–6. doi: 10.2527/jas.2007-0647
14. Jachowski DS, Slotow R, Millspaugh JJ. Good virtual fences make good neighbors: opportunities for conservation. *Anim Conserv.* (2014) 17:187–96. doi: 10.1111/acv.12082
15. RSPCA. *What Is Virtual Fencing (or Virtual Herding) and Does it Impact Animal Welfare?* (2020). Available online at: https://kb.rspca.org.au/knowledge-base/what-is-virtual-fencing-or-virtual-herding-and-does-it-impact-animal-welfare/ (accessed February 2, 2021).
16. Keshavarzi H, Lee C, Lea JM, Campbell DLM. Virtual fence responses are socially facilitated in beef cattle. *Front Vet. Sci.* (2020) 7:543158. doi: 10.3389/fvets.2020.543158
17. Lee C, Fisher AD, Reed MT, Henshall JM. The effect of low energy electric shock on cortisol, beta-endorphin, heart rate and behaviour of cattle. *Appl Anim Behav Sci.* (2008) 113:32–42. doi: 10.1016/j.applanim.2007.10.002
18. Kearton T, Marini D, Cowley F, Belson S, Lee C. The effect of virtual fencing stimuli on stress responses and behavior in sheep. *Animals.* (2019) 9:30. doi: 10.3390/ani9010030
19. Stewart M, Webster JR, Schaefer AL, Cook NJ, Scott SL. Infrared thermography as a non-invasive tool to study animal welfare. *Anim Welf.* (2005) 14:319–25.
20. Gygax L, Reefmann N, Wolf M, Langbein J. Prefrontal cortex activity, sympatho-vagal reaction and behaviour distinguish between situations of feed reward and frustration in dwarf goats. *Behav Brain Res.* (2013) 239:104–14. doi: 10.1016/j.bbr.2012.10.052
21. Ong RM, Morris JP, Odwyer JK, Barnett JL, Hemsworth PH, Clarke IJ. Behavioural and EEG changes in sheep in response to painful acute electrical stimuli. *Aust Vet J.* (1997) 75:189–93. doi: 10.1111/j.1751-0813.1997.tb10064.x
22. Moberg GP. Biological response to stress: implications for animal welfare. In: *Biology of Animal Stress.* CAB International. (2000). p. 1–23. doi: 10.1079/9780851993591.0001
23. Mormede P, Andanson S, Auperin B, Beerda B, Guemene D, Malmkvist J, et al. Exploration of the hypothalamic-pituitary-adrenal function as a tool to evaluate animal welfare. *Phys Behav.* (2007) 92:317–39. doi: 10.1016/j.physbeh.2006.12.003
24. Kearton T, Marini, D, Cowley, F, Keshavarzi, Mayes B, Lee C. The influence of predictability and controllability on stress responses to the aversive component of a virtual fence. *Front Vet Sci.* (2020) 7:580523. doi: 10.3389/fvets.2020.580523
25. Andres R, Marti O, Armario A. Direct evidence of acute stress-induced facilitation of ACTH response to subsequent stress in rats. *Am J Physiol.* (1999) 277:R863–8. doi: 10.1152/ajpregu.1999.277.3.R863
26. Janssens C, Helmond FA, Wiegant VM. Increased cortisol response to exogenous adrenocorticotrophic hormone in chronically stressed pigs - influence of housing conditions. *J Anim Sci.* (1994) 72:1771–7. doi: 10.2527/1994.7271771x
27. Janssens C, Helmond FA, Loyens LWS, Schouten WGP, Wiegant VM. Chronic stress increases the opioid-mediated inhibition of the pituitary-adrenocortical response to acute stress in pigs. *Endocrinol.* (1995) 136:1468–73. doi: 10.1210/endo.136.4.7895656
28. Palme R. Non-invasive measurement of glucocorticoids: advances and problems. *Phys Behav.* (2019) 199:229–43. doi: 10.1016/j.physbeh.2018.11.021
29. Mostl E, Maggs JL, Schrotter G, Besenfelder U, Palme R. Measurement of cortisol metabolites in faeces of ruminants. *Vet Res Commun.* (2002) 26:127–39. doi: 10.1023/A:1014095618125
30. Verdon M, Langworthy A, Rawnsley R. Virtual fencing to intensively graze lactating dairy cattle II: effects on cow welfare and behavior. *J Dairy Sci.* (in press).
31. Blache D, Maloney SK. New physiological measures of the biological cost of responding to challenges. In: *Advances in Sheep Welfare*. Woodhead Publishing (2017).
32. von Borell E, Langbein J, Despres G, Hansen S, Leterrier C, Marchant-Forde J, et al. Heart rate variability as a measure of autonomic regulation of cardiac activity for assessing stress and welfare in farm animals - a review. *Phys Behav.* (2007) 92:293–316. doi: 10.1016/j.physbeh.2007.01.007
33. Stucke D, Ruse MG, Lebelt D. Measuring heart rate variability in horses to investigate the autonomic nervous system activity - pros and cons of different methods. *Appl Anim Behav Sci.* (2015) 166:1–10. doi: 10.1016/j.applanim.2015.02.007
34. Wierig M, Mandtler LP, Rottmann P, Stroh V, Mueller U, Buescher W, et al. Recording heart rate variability of dairy cows to the cloud-why smartphones provide smart solutions. *Sensors.* (2018) 18:3541. doi: 10.3390/s18082541
35. Hagen K, Broom DM. Emotional reactions to learning in cattle. *Appl Anim Behav Sci.* (2004) 85:203–13. doi: 10.1016/j.applanim.2003.11.007
36. Lea JM, Niemeyer DDO, Reed MT, Fisher AD, Ferguson DM. Development and validation of a simple technique for logging body temperature in free-ranging cattle. *Aust J Exp Agric.* (2008) 48:741–5. doi: 10.1071/EA07422
37. Lees AM, Lea JM, Salvin HE, Cafe LM, Colditz IG, Lee C. Relationship between rectal temperature and vaginal temperature in grazing *Bos taurus* heifers. *Animals.* (2018) 8:156. doi: 10.3390/ani8090156
38. Sanger ME, Doyle RE, Hinch GN, Lee C. Sheep exhibit a positive judgement bias and stress-induced hyperthermia following shearing. *Appl Anim Behav Sci.* (2011) 131:94–103. doi: 10.1016/j.applanim.2011.02.001
39. Pedernera-Romano C, Ruiz de la Torre JL, Badiella L, Manteca X. Effect of perphenazine enanthate on open-field test behaviour and stress-induced hyperthermia in domestic sheep. *Pharmacol Biochem Behav.* (2010) 94:329–32. doi: 10.1016/j.pbb.2009.09.013
40. Monk JE, Belson S, Colditz IG, Lee C. Attention bias test differentiates anxiety and depression in sheep. *Front Behav Neurosci.* (2018) 12:246. doi: 10.3389/fnbeh.2018.00246
41. Monk JE, Lee C, Belson S, Colditz IG, Campbell DLM. The influence of pharmacologically-induced affective states on attention bias in sheep. *PeerJ.* (2019) 7:e7033. doi: 10.7717/peerj.7033
42. Monk JE, Lee C, Dickson E, Campbell DLM. Attention bias test measures negative but not positive affect in sheep: a replication study. *Animals.* (2020) 10:1314. doi: 10.3390/ani10081314

43. Lees AM, Salvin HE, Colditz IG, Lee C. The influence of temperament on body temperature response to handling in Angus cattle. *Animals.* (2020) 10:172. doi: 10.3390/ani10010172
44. Lee C, Cafe LM, Robinson SL, Doyle RE, Lea JM, Small AH, et al. Anxiety influences attention bias but not flight speed and crush score in beef cattle. *Appl Anim Behav Sci.* (2018) 205:210–15. doi: 10.1016/j.applanim.2017.11.003
45. Marini D, Kearton T, Ouzman J, Llewellyn R, Belson S, Lee C. Social influence on the effectiveness of virtual fencing in sheep. *PeerJ.* (2020) 8:e10066. doi: 10.7717/peerj.10066
46. Fisher AD, Crowe MA, de la Varga MEA, Enright WJ. Effect of castration method and the provision of local anesthesia on plasma cortisol, scrotal circumference, growth, and feed intake of bull calves. *J Anim Sci.* (1996) 74:2336–43. doi: 10.2527/1996.74102336x
47. Marini D, Cowley F, Belson S, Lee C. The importance of an audio cue warning in training sheep to a virtual fence and differences in learning when tested individually or in small groups. *Appl Anim Behav Sci.* (2019) 221:104862. doi: 10.1016/j.applanim.2019.104862
48. Norell RJ, Gustafson RJ, Appleman RD, Overmier JB. Behavioral studies of dairy cattle sensitivity to electrical currents. *Trans ASAE.* (1983) 26:1506–11. doi: 10.13031/2013.34160
49. Reinemann DJ, Stetson LE, Reilly JP, Laughlin NK. Dairy cow sensitivity to short duration electrical currents. *Trans ASAE.* (1999) 42:215–22. doi: 10.13031/2013.13198
50. Gou X, Tsunekawa A, Tsubo M, Peng F, Sun J, Li Y, et al. Seasonal dynamics of cattle grazing behaviors on contrasting landforms of a fenced ranch in northern China. *Sci Total Environ.* (2020) 749:141613. doi: 10.1016/j.scitotenv.2020.141613
51. Verdon M, Rawnsley R, Raedts P, Freeman M. The behaviour and productivity of mid-lactation dairy cows provided daily pasture allowance over 2 or 7 intensively grazed strips. *Animals.* (2018) 8:115. doi: 10.3390/ani8070115
52. Haley DB, Rushen J, de Passille AM. Behavioural indicators of cow comfort: activity and resting behaviour of dairy cows in two types of housing. *Can J Anim Sci.* (2000) 80:257–263. doi: 10.4141/A99-084
53. Treit D, Fundytus M. Thigmotaxis as a test for anxiolytic activity in rats. *Pharmacol Biochem Behav.* (1988) 31:959–62. doi: 10.1016/0091-3057(88)90413-3
54. Grossen NE, Kelley MJ. Species-specific behavior and acquisition of avoidance behavior in rats. *J Comp Physiol Psychol.* (1972) 81:307–10. doi: 10.1037/h0033536
55. Weiss JM. Somatic effects of predictable and unpredictable shock. *Psychosom Med.* (1970) 32:397–408. doi: 10.1097/00006842-197007000-00008
56. Boissy A, Lee C. How assessing relationships between emotions and cognition can improve farm animal welfare. *Rev Sci Tech.* (2014) 33:103–10. doi: 10.20506/rst.33.1.2260
57. Lee C, Colditz IG, Campbell DLM. A framework to assess the impact of new animal management technologies on welfare: a case study of virtual fencing. *Front Vet Sci.* (2018) 5:187. doi: 10.3389/fvets.2018.00187
58. Shackleton DM, Shank CC. A review of the social-behavior of feral and wild sheep and goats. *J Anim Sci.* (1984) 58:500–9. doi: 10.2527/jas1984.582500x
59. Bouissou M-F, Boissy A, Le Neindre P, Veissier I. The social behaviour of cattle. In: *Social Behaviour of Farm Animals*. CAB International (2001). p. 113–45.
60. Foris B, Zebunke M, Langbein J, Melzer N. Comprehensive analysis of affiliative and agonistic social networks in lactating dairy cattle groups. *Appl Anim Behav Sci.* (2019) 210:60–7. doi: 10.1016/j.applanim.2018.10.016
61. Colusso PI, Clark CEF, Lomax S. Should dairy cattle be trained to a virtual fence system as individuals or in groups? *Animals.* (2020) 10:1767. doi: 10.3390/ani10101767
62. Verdon M, Lee C, Marini D, Rawnsley R. Pre-exposure to an electrical stimulus primes associative pairing of audio and electrical stimuli for dairy heifers in a virtual fencing feed attractant trial. *Animals.* (2020) 10:217. doi: 10.3390/ani10020217
63. Reale D, Gallant BY, Leblanc M, Festa-Bianchet M. Consistency of temperament in bighorn ewes and correlates with behaviour and life history. *Anim Behav.* (2000) 60:589–97. doi: 10.1006/anbe.2000.1530
64. Lee TK, Lee C, Bischof R, Lambert GW, Clarke IJ, Henry BA. Stress-induced behavioral and metabolic adaptations lead to an obesity-prone phenotype in ewes with elevated cortisol responses. *Psychoneuroendocrinol.* (2014) 47:166–77. doi: 10.1016/j.psyneuen.2014.05.015
65. Boissy A, Bouissou MF. Assessment of individual differences in behavioural reactions of heifers exposed to various fear-eliciting situations. *Appl Anim Behav Sci.* (1995) 46:17–31. doi: 10.1016/0168-1591(95)00633-8

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A Systematic Review on Commercially Available and Validated Sensor Technologies for Welfare Assessment of Dairy Cattle

Anna H. Stygar[1]*, *Yaneth Gómez*[2], *Greta V. Berteselli*[3], *Emanuela Dalla Costa*[3], *Elisabetta Canali*[3], *Jarkko K. Niemi*[1], *Pol Llonch*[2] *and Matti Pastell*[4]

[1] Bioeconomy and Environment, Natural Resources Institute Finland (Luke), Helsinki, Finland, [2] Department of Animal and Food Science, Universitat Autònoma de Barcelona, Cerdanyola del Vallès, Spain, [3] Dipartimento di Medicina Veterinaria, Università degli Studi di Milano, Milan, Italy, [4] Production Systems, Natural Resources Institute Finland (Luke), Helsinki, Finland

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***Correspondence:**
Anna H. Stygar
anna.stygar@luke.fi

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In order to base welfare assessment of dairy cattle on real-time measurement, integration of valid and reliable precision livestock farming (PLF) technologies is needed. The aim of this study was to provide a systematic overview of externally validated and commercially available PLF technologies, which could be used for sensor-based welfare assessment in dairy cattle. Following Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines, a systematic literature review was conducted to identify externally validated sensor technologies. Out of 1,111 publications initially extracted from databases, only 42 studies describing 30 tools (including prototypes) met requirements for external validation. Moreover, through market search, 129 different retailed technologies with application for animal-based welfare assessment were identified. In total, only 18 currently retailed sensors have been externally validated (14%). The highest validation rate was found for systems based on accelerometers (30% of tools available on the market have validation records), while the lower rates were obtained for cameras (10%), load cells (8%), miscellaneous milk sensors (8%), and boluses (7%). Validated traits concerned animal activity, feeding and drinking behavior, physical condition, and health of animals. The majority of tools were validated on adult cows. Non-active behavior (lying and standing) and rumination were the most often validated for the high performance. Regarding active behavior (e.g., walking), lower performance of tools was reported. Also, tools used for physical condition (e.g., body condition scoring) and health evaluation (e.g., mastitis detection) were classified in lower performance group. The precision and accuracy of feeding and drinking assessment varied depending on measured trait and used sensor. Regarding relevance for animal-based welfare assessment, several validated technologies had application for good health (e.g., milk quality sensors) and good feeding (e.g., load cells, accelerometers). Accelerometers-based systems have also practical relevance to assess good housing. However, currently available PLF technologies have low potential to assess appropriate behavior of dairy cows. To increase actors' trust toward the PLF technology and prompt sensor-based welfare assessment, validation studies, especially in commercial herds, are needed. Future research should concentrate on developing and validating PLF technologies dedicated to the assessment of appropriate behavior and tools dedicated to monitoring the health and welfare in calves and heifers.

Keywords: PLF, accelerometer, camera, milk sensor, scale, bolus, dairy cow, calf

INTRODUCTION

Recently introduced concept of One Welfare recognizes the interconnections among animal welfare, human well-being, and the environment (1). Better understanding of the values of high welfare standards can, among others, support food security, improve productivity, reduce antimicrobial use, and greenhouse gas emission [e.g., (2–4)].

Animal welfare is also a highly interesting topic for European consumers (5, 6). This interest is seen in production statistics and consumer purchases decisions. Consumers are willing to pay a premium price for credence attributes of milk (7, 8), such as organic, environmentally friendly, or high animal welfare (on average 28, 25, and 31% of premium). Moreover, consumers appreciate proactive approach to managing animal health and welfare (9), and there is evidence that the animal-friendly marketing strategies influence the uptake of products (10).

Animal welfare friendly products can be identified through labeling. Most dairy welfare labeling schemes in Europe have requirements concerning resource-based welfare indicators such as space allowance, provision of bedding and enrichments, minimum transportation time, outdoor access, or absence of mutilations (8). Recently, animal-based indicators have gained more attention, especially following the publication of Welfare Quality® (WQ®) protocols and a few labeling schemes highlighting animal-based measures have been introduced during the past years (e.g., AENOR welfare certificate in Spain, Arla one farm milk in Finland, and ClassyFarm in Italy). However, existing animal welfare assessment protocols show some inaccuracies as: (1) they are only applied at group level, (2) are unable to continuously monitor animal welfare, and (3) they rely on human judgments and decisions-making facilitating some degree of subjectivity on the assessment. Moreover, those protocols are not practical for detecting early-warning signals which could result in implementation of preventive measures. Abovementioned limitations could be, at least partially, solved by application of precision livestock farming (PLF) technologies.

Different PLF techniques have been developed for monitoring dairy cattle production. Sudden change in the activity, feeding and drinking, physical condition, and health of animals can be detected by different sensors [e.g., radio-frequency identification (RFID), accelerometers, load cells, and cameras]. Change in behavior or in physical state may indicate problems related to management (e.g., feeding system failure) or disease, as well as can signal specific physiological status such as estrus. PLF technologies can potentially add value to the farm management process by improving data processing, decision making, and implementation of everyday herd management decisions (11). Moreover, PLF technologies could also be applied for monitoring animal welfare [e.g., (12)]. On the other hand, as demonstrated by large-scale studies (13, 14) investment in sensor systems might not necessarily lead to the economic gain for farmers. Therefore, the merits of each sensor system need to be assessed individually and the performance should to be verified before the promise of improved management can be realized.

Research groups and companies around the world has been engaged in developing new PLF sensors, however, not all PLF solutions developed in a lab environment can be successfully implemented as commercial products on dairy farms. The reason can be that some technologies will still be too expensive or will perform better in an experimental setting, where conditions are controlled, and sample size is small, compared to the farming environment. Therefore, for successful assessment of on-farm welfare using PLF technology, it is essential to validate this technology at the commercial level (external validation). Furthermore, applying sensor-based welfare assessment for labeling schemes or welfare support payments should be based on widely available and validated technologies.

The main goal of this review was to assess which welfare aspects of cows', heifers', and calves' husbandry can be addressed by available (and validated) technologies. To reach this goal, commercially available and/or externally validated technologies with potential use for animal-based welfare assessment in dairy herds were first identified. Validated technologies were later grouped according to their performance. Finally, possible gaps between available and validated tools and needs for animal-based welfare assessment were identified based on the principles of the WQ® protocol, including appropriate nutrition, housing, health, and behavior.

MATERIALS AND METHODS

Market Availability Search

A broad market research (using web Google search) on commercially available PLF systems with potential application for animal-based welfare assessment was conducted. This research was done by exploring the assortment of technology providers that cover a wide range of sensors which could provide information on animal base indicators for welfare. The search criteria used included *"dairy cow"* and one of the following terms describing sensors: *(automatic drinker OR automatic waterer), (automatic feeder), (activity sensor OR activity monitor), (RFID), (Global Positioning System OR GPS), (thermal camera), (thermography), (mastitis sensor), (automatic mastitis detection), (somatic cells counter), (milk analyzer), (automatic weigh scale*

OR automatic weigh), (lameness sensor), (automatic lameness detection), (pressure mat OR force sensor), (body condition score sensor OR automatic body condition score), (body condition camera), (rumen bolus), (milking robot), and (accelerometer). Also, search for *calf automatic feeder* was performed. As an example, the following word combinations were used to look for feeding equipment available on the market: *"dairy cow"* plus *"automatic feeder."* The first five pages (50 hits) from Google search were scanned. Additionally, the availability of sensors was scanned using dedicated on-line marketplace for providers (https://www.agriexpo.online/). Search was performed between March and May 2020. Tools with exclusive use for reproduction (for estrus detection or calving alarms) were excluded from the final list. Information on sensor name, provider name, internet link, sensor type (with attachment position to animal when applicable), aim, and country of origin (headquarters) for 129 technologies are provided in **Supplementary Table A1**.

Literature Search and Exclusion Criteria

To explore technology limitation, a systematic literature search based on Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) methodology (15) was conducted. Literature search was conducted through Web of Science and Scopus. Altogether, 147 different search terms were used. Each search included terms describing different phases in the production cycle (*"cow" OR "calves" OR "calf" OR "heifer"*) and validation (*"test" OR "assess*" OR "evaluat*" OR "validat*"*) as well as several exclusion terms: NOT (*"review"* OR *"survey"* OR *"beef"* OR *"sheep"* OR *"goat*"* OR *"hors*"* OR *"buffalo"* OR *"steer*"* OR *"ewe"* OR *"leg calf"* OR *"muscle*"*). Additionally, each search was supplemented with physiological and behavioral term (e.g., feeding behavior), or sensor type (e.g., camera), or the commercial name (e.g., CowView). For physiological and behavioral term as well as sensor types the following terms were used: *("feeding behavior" OR "feeding behavior" AND "monitoring"), ("monitoring feeding"), ("drinking behavior" OR "drinking behavior" AND "monitoring"), ("vocalization"), ("vision"), ("camera"), ("accelerometer*"), ("temperature AND sensor"), ("mastitis AND sensor"), ("image analyses"), ("scale AND body weight"), ("pressure mat"), ("bolus"), ("indoor AND position"), ("in-line"), ("tracking system"), ("RFID"),* and *("microphone")*. The commercial names used in the search are presented in **Supplementary Table A1**, column A.

The example search looked as follow: *("cow" OR "calves" OR "calf" OR "heifer") AND ("test" OR "assess*" OR "evaluat*" OR "validat*") AND ("camera")* NOT *("review"* OR *"survey"* OR *"beef"* OR *"sheep"* OR *"goat*"* OR *"hors*"* OR *"buffalo"* OR *"steer*"* OR *"ewe"* OR *"leg calf"* OR *"muscle*")*.

Only studies in peer-reviewed scientific journals published in English between January 2000 and May 2020 were included to this review. Since this review focuses on dairy production, all validation trials conducted on beef breeds or steers were omitted. Articles were excluded if not dealing with aspects directly related to the welfare of dairy cows (e.g., reproduction related problems such as estrus detection, and environmental aspects such as methane emission, etc.). We further excluded papers with only internal validation, which was defined as validation data set used to assess the performance originating from the same animals or herd/herds as used in the developing of the technology (16).

Study Classification

This review includes papers presenting the higher standards of objective validation, which is external validation. Based on the approach presented by Altman et al. (16) we have defined two levels of external validation:

1. External self-validation was defined for studies where the system was evaluated using a fully independent data set, that means data was collected from different herds not used for system development. Research was conducted by the same scientist (at least one author involved in developing and validation) or had been validated by at least one author representing a company providing a technology.
2. External independent validation was defined for studies where the system was evaluated using a fully independent data set, which means data was collected from different herds not used for system building. Research was conducted by scientists not involved in technology development.

In order to determine the validation level, both origin of the technology and validation location (herd) needed to be known. Technology was identified through commercial name or based on studies describing building phase (for prototypes). Origin of the validation herd was identified through information on location (country), and type (if a herd was commercial or research). We have assumed that criteria of external validation were fulfilled if commercially available technology was validated in a commercial herd or a research herd (different from the company/developer own research herd). For prototypes, the criteria of external validation were fulfilled only if the scientific paper clearly described where technology was validated, and validation place was different from the herd used for technology building (based on information from scientific publication describing building phase). If both country and herd specifications (commercial or research) could not be identified, then the study was excluded from this review (due to not enough information in materials and methods). However, papers stating that herds used for validation were different than those used for technology development (without mentioning location, for example due to privacy concerns) were included into this review.

Performance Measures for Validated Trials

In this review, we distinguished regression and classification measures for performance reporting. Regression measures, reflects the agreement between a continuous trait measured by validated technology (predictor) and the golden standard (outcome). For example, the agreement between body weight measured by a conventional scale and partial scale attached to a milk feeder. Regression measures can be presented using any of the following measures including Pearson correlation coefficient (r), Spearman's rank correlation coefficient (rs), coefficient of determination (R^2), mean bias from the Bland–Altman plots (B–A plots), significance tests for intercept and slope of linear regression (I/S), or concordance correlation coefficient (CCC). Classification measures refers to the ability of a technology to predicting categorical outcomes e.g., locomotion score. Classification performance was usually reported using either area under the receiver operating characteristics curve (AUC) or sensitivity (Se) and specificity (Sp) or Cohen's kappa coefficient (κ).

In this review, we have distinguished tools validated for high performance and lower performance. It was assumed that high performance was reached when all indicators defined/selected by authors of studies fulfilled following criteria: r, rs, CCC, Se, and Sp, or AUC was >0.9, R^2 and κ was >0.81, I/S did not differ significantly from 0 or 1, respectively, and B–A plot included zero with the 95% interval of agreement. Criteria for high performance (precision and accuracy) were accepted similarly to those referred by studies assessing technology performance (17–19).

Assessment of Welfare Relevance

Welfare Quality® is a scientifically rigorous animal welfare assessment protocol (20), which follows four animal welfare principles (good housing, good feeding, good health, and appropriate behavior). WQ® principles were used as a reference to classify indicators measured by technologies. In this review, members of the ClearFarm project with expertise in animal welfare were asked to evaluate the relevance of each indicator measured by the PLF technologies listed in this review for assessing WQ® principles. Possible scores were: "relevant" and "not relevant." For example, the panel was asked to evaluate whether grazing time is relevant for the principles of good feeding, good housing, good health, and appropriate behavior. Experts votes were categorized based on "relevant" votes, so that all traits with more than 80% votes were grouped in "very relevant" category, traits receiving from above 20% up to 80% votes were in "moderately relevant" category, and all traits with 20% or less votes were in "not relevant" category.

RESULTS

Technologies Commercially Available

The full list of commercially available technologies is presented in the **Supplementary Table A1**. In total, 129 technologies were found from 67 different providers located in 21 countries. The United Kingdom, the Netherlands, and the United States, are the leaders for providing technologies with potential use for animal-based welfare assessment. Technologies were grouped according to the used sensor. Accelerometer-based technologies and load cells were the largest group on the list (37 different products for each group) and constituted 57% of all found tools. Commercially available accelerometers were offered with different animal attachment solutions (collar, leg, ear, and halter), and some companies offered products with more than one attachment option. The collar was the most popular solution (65%, $N = 24$), while leg (30%, $N = 10$), ear (14%, $N = 6$), and halter (3%, $N = 1$) were less frequent. We have identified 14 boluses and 10 products using vision-based monitoring. Regarding milk quality, 25 sensor technologies (19% of a market share) for health monitoring were identified (including 13 milking robots). GPS sensors were used in eight different products offering the possibility to locate animal position. Additionally, two systems using microphone, as well as one mobile app for body condition scoring was identified. All products based on accelerometers offered health alerts. Only one accelerometer-based product was dedicated for calves, the remaining products were advertised for cows or heifers. Systems based on load cells in combination with RFID were most often used for managing and tracking the feeding program of individual animals. Also, few systems were used for body weight monitoring. Boluses were advertised as tools to measure body temperature, pH, and rumen activity as well as for animal identification. Among cameras, seven were dedicated for body temperature monitoring (thermal cameras), two were used for body condition scoring (BCS), and one camera for feeding monitoring.

Peer-Reviewed Records on Technology Validation

The literature search resulted in 1,111 titles, but after duplicate removal and exclusion criteria throughout the review process, 1,069 papers were omitted. A modified PRISMA flow diagram provides information on the number of excluded papers and reason for exclusion (**Figure 1**). A total of 42 articles satisfied the selection criteria, and 38 publications validated commercially available technologies. Moreover, we have identified four studies on prototype validation (**Table 1**). Only two papers validated more than one product, however several papers validated more than one indicator measured by the technology. The performance of technologies with accelerometer sensors were the most often assessed (26 technology validation trials). Validation trials for load cells ($N = 6$), bolus, and camera (four trials each), RFID ($N = 3$), microphone and viscosity sensor (two trials each), and conductivity and spectroscopy (1 trial each) were less frequent (**Table 1**). Regarding accelerometers, the precision and accuracy of products offering different attachments to the animal were assessed in 11 sensors [leg ($N = 5$), collar ($N = 3$), ear ($N = 2$), and halter ($N = 1$)]. The most often validated technology originated from Itin+ Hoch GmbH, Liestal, Switzerland (six trials), Afimilk, Kibbutz Afikim, Israel (five trials), and Agis, Harmelen, the Netherlands (five trials). Information on the study design (herd type, size, and location) for all qualified papers is presented in **Supplementary Table A2**. In total, 28 studies presented validation trials conducted on research farms. The remaining studies (33%, $N = 14$) were conducted on commercial herds. The sample size used in validation trials varied substantially. In general, the smallest sample size was selected for experiments concerning cannulated cows [below 10 animals for bolus validation, e.g., (52)], while the highest sample size was selected for experiment testing performance of online somatic cell count (SCC) estimation in automatic milking system [above 4,000 milking cows (43)]. When it comes to the geographical location of the herds, most technologies were validated in the United States (11 studies) and Canada (5 studies). The performance of tools was assessed using regression measures (27 papers), classification measures (7 papers), and

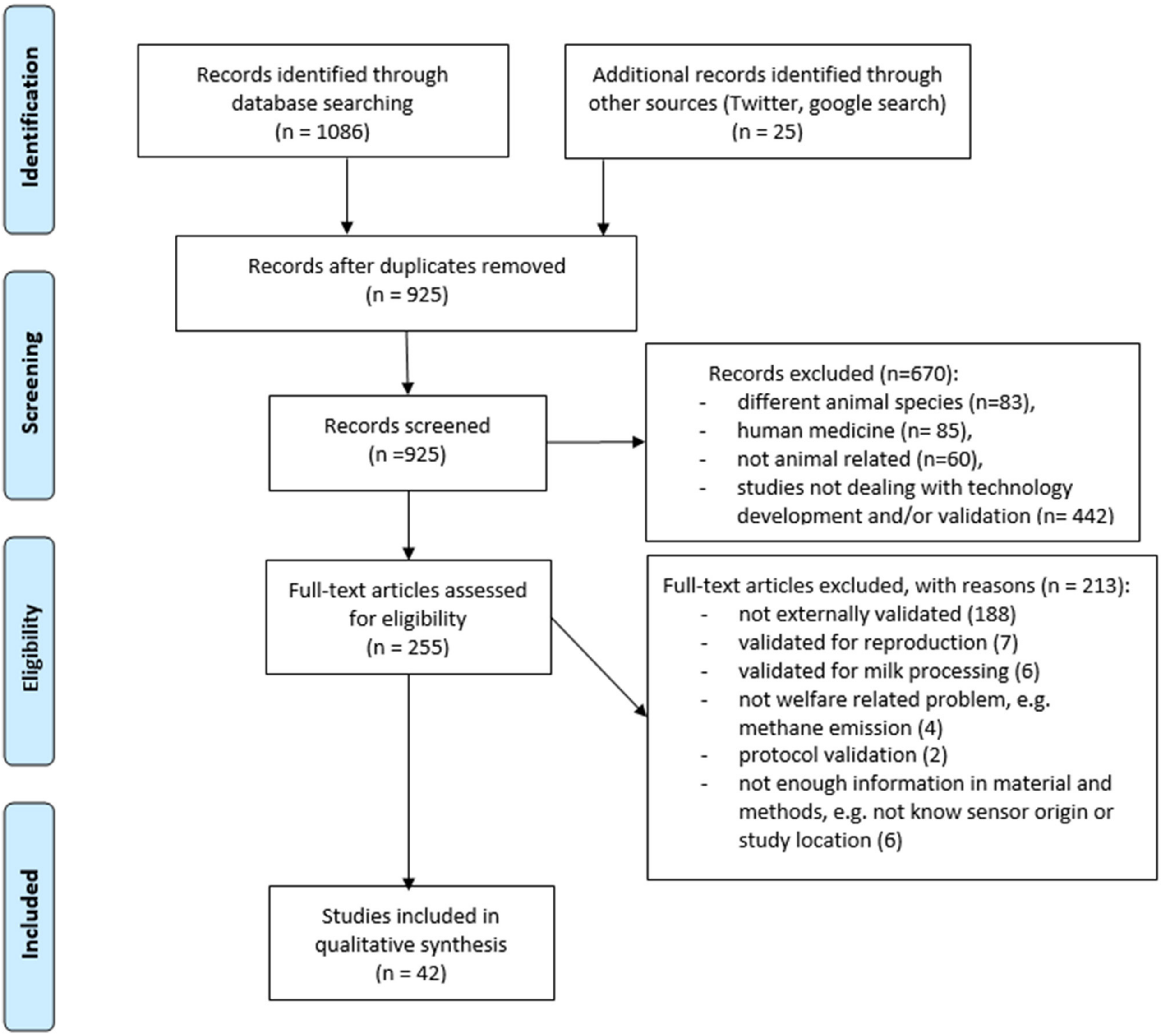

FIGURE 1 | Modified Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) flow diagram (15) with the systematic review search strategy and study selection.

both measures (8 papers). Most of the reviewed papers were classified as full independent validation, and only 33% ($N = 14$) of reviewed papers were self-validated.

Validation Rate

According to the obtained results, only 18 commercially available sensors listed in the **Supplementary Table A1** have been externally validated (14%). The highest validation rate was found for systems based on accelerometers (30% of tools available on the market have validation records), while the lower rates were obtained for cameras (10%), load cells (8%), miscellaneous milk sensors (8%), and boluses (7%).

Performance of Technology Validated for Dairy Cows

Table 2 summarizes tools with available validation trials which could have practical application in welfare assessment for dairy cows. Validated animal-based traits concerned animal activity (walking, number of steps, lying, lying and standing, and standing), feeding and drinking behavior (feeding time, presence at feeder, intake, grazing, rumination, drinking duration, presence at a drinker, and water intake) physical condition, and health (locomotion score, BCS, rumen pH, body temperature, health disorder detection, and milk quality).

Non-active behavior (lying, lying and standing, and standing) as well as rumination and feeding time were the most often

TABLE 1 | Summary of externally validated technologies with potential use for dairy welfare assessment.

Technology name	Technology provider	No. of validation trials for technology provider	Used sensors and attachment position[a]	Independent validation[b]	Self-validation[c]
AfiAct Pedometer Plus, AfiTagII, Pedometer Plus	Afimilk, Kibbutz Afikim, Israel (S.A.E.Afikim, Israel)	5	Accelerometer, leg	(21–24)	
AfiLab real-time milk analyzer			Spectroscope	(25)	
CowAlert IceQube, IceTag	IceRobotics Ltd., Edinburgh, Scotland	4	Accelerometer, leg	(21, 26–28)	
Track A Cow	ENGS, Rosh Pina, Israel	1	Accelerometer, leg	(21)	
MooMonitor+	Dairymaster, Tralee, Ireland	2	Accelerometer, collar	(18, 29)	
HerdInsights	Alanya Ltd., Cork, Ireland	1	Accelerometer, collar	(30)	
Hi-Tag	SCR Engineers Ltd., Netanya, Israel (currently Allflex)	2	Microphone, collar	(31, 32)	
RumiWatch	Itin+ Hoch GmbH, Liestal, Switzerland	7	Accelerometer and pressure sensor, halter (29, 33–37), halter and leg (38)	(34, 35)	(29, 33, 36–38)
CowScout Leg	GEA Farm Technologies, Bonen, Germany	1	Accelerometer, leg		(27)
CowManager SensOor	Agis, Harmelen, Netherlands	5	Accelerometer, ear	(21, 39–41)	(17)
The Smartbow	Smartbow GmbH, Jutogasse, Austria	1	Accelerometer, ear	(21)	
Lely activity	Lely, Maassluis, the Netherlands	2	Accelerometer, collar	(42)	
Lely- on-line California mastitis test			Viscosity meter	(43)	
GrowSafe	GrowSafe Systems Ltd., Airdrie, AB, Canada	1	RFID (neck collar), load cell	(44)	
Insentec	Insentec, Marknesse, the Netherlands (now Hokofarm group)	1	RFID (ear), load cell	(45)	
Intergado	Intergado Ltd., Contagem, Minas Gerais, Brazil	2	RFID (ear), load cell		(46, 47)
Body Condition Scoring	DeLaval International AB, Tumba, Sweden	2	Camera	(48)	
Combi			Load cell	(49)	
eCow Farmer bolus	eCow, Dekon, UK	2	Bolus (reticulum)	(50, 51)	
KB 3/04 bolus	Kahne Limited, New Zealand	1	Bolus (rumen sac)	(52)	
Bella Ag Cattle	Bella Ag LLC, USA	1	Bolus (reticulum)	(53)	
Stepmetrix	BouMatic, Madison, USA	1	Load cell	(54)	
Optris	Optris, Berlin, Germany	1	Camera	(55)	
Prototypes—locomotion score	ns	2	Camera		(19, 56)
IMAG model—prototype mastitis detection	ns	1	Conductivity meter, thermometer (milk temperature)		(57)
Prototype-mastitis detection	detection model (prototype) and online cell counter, DeLaval International AB, Tumba, Sweden	1	Viscosity meter		(58)
Prototype-activity	ns	1	Accelerometer		(59)

[a] *Sensor location is provided only for sensors attached to the animal.*
[b] *Validated using independent data set (different animals and herd than for technology building) and co-authors were not involved in technology development.*
[c] *Validated using independent data set (different animals and herd than for technology building) and was developed and validated by at least one the same co-author (based on the authorship of papers) or have been validated by at least one co-author representing a company providing a technology.*

validated attributes (20, 15, and 11 trials, respectively). There are several different commercially available technologies classified with high performance for non-active behavior (**Table 2**). For active behavior (walking, number of steps), lower performance of tools was reported. Regarding feeding and drinking, the performance of the tool varied depending on measured traits and used sensor. Feeding time, which was monitored using accelerometer-based sensors, was validated for lower

TABLE 2 | Results of validation trials for dairy cows in respect to measured traits.

Category	Measured trait	Validation place (farm)	Technologies validated for high performance[a]	Technologies validated for lower performance[b]
Activity	Non-active behavior (lying, lying and standing)	Research	AfiAct Pedometer Plus (21), AfiTagII (22), CowAlert IceQube (21), CowManager SensOor (17, 39), Track A Cow (21), RumiWatch (38)	CowManager SensOor (40), Lely activity (42), MooMonitor+ (18), Pedometer Plus (23)
		Commercial	CowScout Leg (27), Ice Tag (26, 27), prototype (59)	
	Standing—identified as a separate behavior	Research	RumiWatch (38)	Lely activity (42)
		Commercial	CowScout Leg (27), Ice Tag (26, 27)	
	Active behavior (walking, no. of steps)	Research		CowManager SensOor (17, 39, 40), IceTag (28), Lely activity (42), RumiWatch (38),
		Commercial		CowScout Leg (27), Ice Tag (26, 27)
Feeding and drinking	Feeding time	Research		CowManager SensOor (17, 21, 39, 40), MooMonitor+ (18), RumiWatch [V0.7.0.0 (33) and V0.7.4.5 34], Track A Cow (21)
		Commercial	RumiWatch V0.7.3.2 (36)	prototype (59), RumiWatch V0.7.2.0 (36)
	Presence at the feeder[c]	Research	GrowSafe (44), Insentec (45), Intergado (46)	
	Feed intake (kg)	Research	Insentec (45), Intergado (46)	
	Grazing time	Research	MooMonitor+ (29), RumiWatch (29, 38)	
	Rumination	Research	CowManager SensOor (17) MooMonitor+ (18, 29), Rumi Watch (29, 33–35, 38), Smartbow (21), Hi-Tag (31)	CowManager SensOor (21, 39, 40), Lely activity (42)
		Commercial	Prototype (59), Rumi Watch (36)	
	Drinking time	Research		Rumi Watch [V0.7.0.0 (33) and V0.7.4.5 (34)]
		Commercial		RumiWatch (36)
	Water intake	Research	Insentec (45)	
	Presence at the drinker	Research	Insentec (45)	
Physical condition and health	Locomotion score	Commercial		Prototype (19), prototype (56), Stepmetrix (54),
	Body condition scoring	Commercial		DeLaval Body condition scoring (48)
	Rumen pH	Research		eCow bolus (50, 51), KB 3/04 bolus (52)
	Body temperature	Research		KB 3/04 bolus (52), OPTRIS (55)
	Health disorder[d]	Commercial		HerdInsight (30)
	Milk quality[e]	Research		AfiLab real-time milk analyzer (25)
	Mastitis detection	Commercial		Lely-on-line California mastitis test (43), prototype -IMAG model (57), prototype (58)

[a]*All indicators defined/selected in validation trail (by authors of studies) were above high-performance threshold. High precision threshold was reached when Pearson correlation, Spearman's rank correlation, concordance correlation coefficient, sensitivity, specificity, area under the curve (AUC) was higher than 0.9, regression coefficient and Kappa coefficient is higher than 0.81, significance tests for intercept and slope of linear regression did not differ significantly from 0 or 1, respectively, and Bland–Altman plot included zero with the 95% interval of agreement.*
[b]*Any indicator validated with lower performance (below threshold defined above).*
[c]*Animal identification and time.*
[d]*e.g., mastitis or pneumonia.*
[e]*Fat, lactose, and protein as indicator for mastitis.*

performance. Conversely, presence at the feeder and feed intake (observed in feeding stations) and grazing time (monitored through accelerometer-based sensor) were evaluated for high performance, but only in the research farm conditions. Drinking time was assessed using accelerometer-based tools, and the pressure sensor was evaluated for lower performance. All tools used for physical condition evaluation and health were classified under lower performance. Assessment of locomotion score varied between presented tools [poor (54) or fair classification performance (19, 56)], and in general, none of reviewed technologies was able to outperform the human observer. Regarding BCS, the technology was reliable for dairy cattle with average body condition (scoring between 3.00 and 3.75 on the five-point scale) but did not score accurately for thinner or fatter cows. The only validated study on the accelerometer-based system used for health alarms (30), reported a high number of false positives, but the true health disorders were alerted by the system before the farmer noticed them. Regarding technologies applied for monitoring milk quality and mastitis, real-time milk analyzers agreed moderately with SCC (43), protein, lactose, and fat determined in the laboratory (25), while mastitis detection models have acceptable results for sensitivity, specificity, and error rates (57, 58).

Performance of Technology Validated for Calves and Heifers

Table 3 summarizes the tools with available validation trials which could have practical application in welfare assessment for young cattle (calves and heifers). Validated traits concerned active behavior (walking), non-active behavior (lying), feeding (time, presence at the feeder, and intake), rumination, drinking (presence at the drinker and intake), body weight, and body temperature. For calves and heifers, rumination and body temperature were the most often validated traits (three and two trials, respectively). Tools measuring active and non-active behavior (lying and walking), feeding and drinking behavior (feed and water intake and presence at the drinker or feeder), and body weight were validated for high performance. Feeding time, rumination, and body temperature were validated for lower performance.

Experts' Assessment

Answers from animal welfare experts concerning the relevance of the indicator in assessing good feeding, housing, health, and appropriate behavior are summarized in **Table 4**. For good health, nine traits received "very relevant" evaluation (body temperature, BCS, lameness, mastitis, water consumption, rumination, rumen pH, feed intake, and non-active behavior). Regarding good feeding, seven traits were categorized as "very relevant" (BCS, water consumption, rumination, rumen pH, grazing, feed intake, and feeding time). For good housing evaluation, experts agreed on the usefulness of non-active behavior monitoring. While, for appropriate behavior, only grazing monitoring was evaluated as "very relevant."

DISCUSSION

Retailed and Validated PLF Technologies for Welfare Assessment

The aim of this review was to identify validated and/or commercially available technologies for measuring animal-based welfare indicators in dairy cattle. Currently, farmers can select from at least 129 different sensors to monitor animal-based indicators of health and welfare in dairy production. However, there is still limited information on the performance of these tools. According to our results, only 14% of commercially available sensors have external validation trials available, which may thwart confidence on these technologies.

We identified four potential reasons for such a small number of validation trials: (1) insufficient reporting (2) low scientific interest for validating technology not for research (3) high cost and labor intensity of data collection (4) reluctance to publish negative results.

Regarding reason (1), altogether six studies reporting validation trails were excluded from this review due to insufficient information provided about study design.

Reason (2), there might be lower scientific interest to validate technologies that are not used for research experiments or are not yet integrated as data sources for other systems. For example, for many commercially available sensors based on scales (like individual feed intake measurement), there are no validation trials available. However, the required precision for feeding monitoring tools (as well as the interest in validation) might increase if the data from these tools, as in the example from pig production (60), would be integrated into marketing or health monitoring systems. Furthermore, the validation rate could be increased if technologies, similar as medical industry, receive specific certification [e.g., International Organization for Standardization (ISO) standards]. Currently, devices and systems used for the purposes of official milk recording (e.g., milk meters, samplers, and milk analyzers) need to meet the requirements specified in ISO standards and must be tested to achieve approval from The International Committee for Animal Recording (ICAR) (61). However, the data from the validation process conducted by ICAR are not publicly available. This could also explain a low number of validation records in peer-reviewed literature for milk recording devices and systems.

Reason (3), validation studies can be labor intensive and costly, due to the need to collect the reference data set. For example, accelerometer-based systems are the most widely available and validated among all PLF technologies. But, as demonstrated in this review, the majority of the accelerometer-based validation studies concerned behavioral monitoring and only one validation study for the performance of accelerometer system for health monitoring was found. Validation of health monitoring technology requires obtaining reference data set containing data on veterinary examinations and blood or milk samples to detect among others lameness, mastitis, ketosis, and pneumonia. The substantial costs needed for the reference data set might affect the number of available publications.

Finally, for reason (4), it could be pondered, if the reason behind the relatively small number of validation studies is due to reluctance in publishing negative results. Technology providers are involved in the validation process and altogether, about one-third of all validation studies presented in this review were classified as self-validation. Self-validation could raise the question of conflict of interest in reporting negative results. However, it is impossible to conclude how many of the negative results were never published due to the conflict of interest.

Certainly, technologies which are commercially available may not all have been identified in this study. The search was conducted using internet websites in English, therefore all tools without English marketing material or presented in printed company catalogs were omitted. The biggest producers will have information provided in English, but smaller companies offering products for local markets or startups might not yet have information available for international buyers. Therefore, a constructed list of retailed products is an approximation of the current market. Our goal was not to identify every single technology but to use this list to identify tendencies on the

TABLE 3 | Results of validation trials for calves and heifers in respect to measured traits.

Category	Measured trait	Validation place (farm)	Technologies validated for high performance[a]	Technologies validated for lower performance[b]
Activity	Non-active behavior (lying time, lying bouts)	Research	AfiTag II (24)	
	Active behavior (walking, no. of steps)	Research	AfiTag II (24)	
Feeding and drinking	Feeding time	Research		CowManager SensOor (41)
	Presence at the feeder	Commercial	Intergado (47)	
	Feed intake	Commercial	Intergado (47)	
	Rumination	Research		CowManager SensOor (41), Hi-Tag (32), RumiWatch (37),
	Water intake	Commercial	Intergado (47)	
	Presence at the drinker	Commercial	Intergado (47)	
Physical condition	Body weight	Research	Combi DeLaval (49)	
	Body temperature	Research		Bella Ag Cattle (53), OPTRIS (55)

[a]*All indicators defined/selected in validation trail (by authors of studies) were above high-performance threshold. High precision threshold was reached when Pearson correlation, Spearman's rank correlation, concordance correlation coefficient, sensitivity, specificity, are under the curve, is higher than 0.9, regression coefficient and Kappa coefficient is higher than 0.81, significance tests for intercept and slope of linear regression did not differ significantly from 0 or 1, respectively, and Bland–Altman plot included zero with the 95% interval of agreement.*

[b]*Any indicator validated with lower precision and or accuracy (threshold defined above).*

TABLE 4 | Indicator evaluation for relevance in assessing good feeding, housing, health, and appropriate behavior[a].

Indicator	Good feeding	Good housing	Good health	Appropriate behavior
Body temperature	+−	+−	+	−
Body condition scoring	+	−	+	−
Lameness	−	+−	+	−
Mastitis	−	+−	+	−
Water consumption	+	−	+	+−
Drinking duration	+−	−	+−	+−
Rumination	+	+−	+	+−
Rumen pH	+	−	+	−
Grazing time	+	+−	+−	+
Feeding intake	+	−	+	+−
Feeding time	+	−	+−	+−
Active behavior	−	+−	+−	−
Non-active behavior	−	+	+	+−

[a]*Symbols +, +−, − refer to "very relevant," "moderate," and "not relevant" evaluation, respectively.*

market and set possible market constraints for developing sensor-based welfare assessment. One must also remember that not all validation studies available for a device were reported in this review. We have included only validation studies for attributes related to animal welfare, therefore, some validation studies for performance of estrus detection on accelerometer-based devices [e.g., (62)] or pregnancy detection from in-line analyzer [e.g., (63)] were excluded.

Precision livestock farming uses technology for real-time, continuous monitoring of individual animals and/or groups of animals, which provides an opportunity to improve welfare assessment. Applying sensor-based welfare assessment for labeling scheme or welfare subsidies should be based on widely available technologies. This review shows that reliable technologies for monitoring welfare-related traits exist, however, there are areas concerning sensors and algorithms which require further developments. For example, based on the presented summary, it can be concluded that while recording behavior of farm animals using machine-vision has shown great progress in research (64, 65), it is only entering the commercial market, and external validation will be needed to confirm the performance. Furthermore, according to our results, the performance of existing health and welfare monitoring systems was sporadically tested on young animals (heifers and calves). Validation studies with accelerometers based on collar were rare and only 14% of validated traits for activity monitoring was obtained from the collar devices. On the other hand, this was the most often marketed attachment point for the accelerometer. Therefore, further validation studies for collar-based systems are needed. To successfully assess welfare of young animals, more work on dedicated systems might be required. Further technological and validation gaps regarding assessment of welfare will be discussed according to the principles defined in the WQ® protocol.

Sensor-Based Welfare Assessment for Dairy Cows and Young Cattle—How Far Are We?

The concept of welfare has multidimensional nature, there is no one indicator that can be used to assess the welfare of an animal, but there are some indicators which are linked to several aspects of welfare. Quite often, welfare assessment is performed using a combination of animal and resource-based indicators (as in WQ® protocol, for instance) and the evaluation is performed by a human observer. Some of the aspects which are evaluated using welfare protocols could be addressed by sensor-based technologies. Below, we will discuss the availability of technologies for the assessment of each welfare principle:

Good Feeding

To fulfill the good feeding principle, animals should not suffer from prolonged hunger or thirst. For prolonged hunger, the WQ® protocol adopts animal-based indicator. Regarding thirst criterion, only resource-based indicators are evaluated (20). Therefore, PLF technologies can provide additional single-animal level information for good feeding evaluation.

There were several attributes monitored by PLF technologies (BCS, rumination time, rumen pH, grazing time, feed and water intake, and feeding time), which have high potential for "good feeding" assessment. Some of the attributes (rumination time, rumen pH, grazing time, feed and water intake, and feeding time), when frequently monitored, can be used for designing early warning systems for disease detection and/or feeding system failures [e.g., (66)]. On the other hand, BCS, which assess the proportion of body fat, can have practical application for decision support systems (e.g., predicting the risk of cow developing ketosis or having reproduction problems) (67). The good feeding assessment might be hampered by the commercial availability of technologies. Based on our search, only two providers offered a camera-based sensor used for BCS monitoring. There is also a shortage of tools able to assess grazing time (only two technologies had validation studies for grazing monitoring). Finally, measurements on individual feeding and drinking were performed at feeding stations, mostly used for research (feeding experiments), and due to the high costs of equipment might have little relevance for commercial application. Potentially, systems based on cameras can also have application for feed availability and intake monitoring (68). However, these systems are still in development and only one commercial camera-based system for feed accessibility monitoring was identified. There are several providers of boluses for rumen pH monitoring, but still, relatively little is known on the performance of the detection models (with alarm-based monitoring) for rumen pH monitoring. Additionally, short functional life of the pH boluses [around 40 days due to loss in accuracy of the electrode (69)], does not allow long lasting individual-animal based assessment. Animal presence at the feeding trough or water bin, can be monitored using RFID technologies [e.g., (46)], but available technologies have been tested mostly in experimental farms, and examples with commercial farm validation are rare. Increased competition among cattle at the feed bunk can be currently detected in experimental settings [e.g., (70)] and can indicate shortage of food (decrease feeding time or dry matter intake). However, there is a need for further validation studies on systems based on RFID for detecting food or water shortage at an individual level.

According to our results, good feeding assessment based on animal indicators in commercial settings could be primarily conducted using accelerometer technologies. Accelerometers-based systems are easily available and can assess rumination (with high performance) and feeding time (with lower performance). Moreover, accelerometers together with noseband pressure sensors were used to measure drinking duration [e.g., (36)]. In the future, good feeding assessment could be further improved by integrating information from emerging technologies (such as video-based assessment of BCS).

Good Housing

In order to ensure good housing, animals should have thermal and resting comfort as well as enough space to move freely (20). For assessing comfort around resting, the WQ® protocol uses animal-based (e.g. time to needed lie down, animals colliding with housing equipment during lying down, animals lying partially or completely outside the lying area) and management-based indicators (e.g., presence of tethering and access to outdoor loafing area or pasture). Therefore, measuring the activities of animals and the physical state using PLF technologies can provide a more accurate assessment at an individual level. Regarding the evaluation of experts, non-active behavior (lying or standing still) has the highest potential to be used for the assessment of good housing. Allowing dairy cows adequate space and facilities to lie down is considered an important aspect for production as well as animal welfare (71). As recently reviewed, the lying time will depend on individual cow-based factors (reproductive status, age, and milk production), health status (lameness and mastitis), and the comfort of housing facilities (72). For example, pasture-based cows are characterized by longer, undisrupted lying times compared to cows kept in cubicles (73). Lameness can result in longer lying times while mastitis can reduce it (72). For this reason, to avoid confounding factors between animal health and housing conditions, an integration with other data sources, such as milking or breeding records, presence of lameness or mastitis is necessary. Non-active and active behavior as well as grazing time can be assessed using accelerometers. However, performance of technologies varied in different farm conditions. For example, CowManager sensor was evaluated for high (17, 39) and lower (40) performance in measuring lying behavior of cows. High performance was obtained in tie stall and free stall barn and lower performance for grazing cattle. These somehow varying performance results raise the question, if sensor systems should be adjusted (and also validated) for different environmental/housing conditions. Cleanliness of udder, cleanliness of flank, and cleanliness of upper and lower legs are other animal-based indicators recorded in the WQ® protocol to assess the criterion of comfort around resting and consequently the principle of good housing (20). To the best knowledge of authors, currently there are no available technologies able to assess the cleanliness of animals. However, rapid development in vision-based monitoring for automatic individual identification [e.g., (74)] can prompt the development of algorithms capable of evaluating this welfare aspect. Thermal comfort can be assessed on an individual basis by application of invasive (e.g., boluses) and non-invasive sensors (thermal cameras). Both options are available on the market; however, there is a clear shortage of validation studies for monitoring systems based on those sensors.

Good Health

For good health, animals should be free from physical injuries (like lameness and integument alterations) and disease and should not suffer pain induced by inappropriate management or handling (20). As agreed by experts, several traits measured by PLF technologies have a potential application for assessment of good health (body temperature, BCS, lameness, mastitis, water consumption, rumination, rumen pH, feed intake, and non-active behavior). The listed attributes can be categorized as direct or indirect health indicators. Indirect indicators, such as active and non-active behavior, rumen pH, and feeding quantity, on its own does not indicate health status of an animal, but changes in the behavior of animals possibly in combination with other data sources (e.g., lactation status and reproduction) can be processed to obtain early-warning signals for health problems (e.g., lameness, mastitis, and ketosis) and potentially prevent them. Direct welfare indicators, like the number of cows with increased SCC, brings knowledge on health (if an animal is sick or not), and can be useful for operational decisions (e.g., antimicrobial treatment). Injuries, such as lameness, can be detected measuring animal behavior (accelerometers), gait (load cells), posture (cameras), and increased body temperature (thermal camera). Though, the performance of accelerometer-based systems and thermal cameras for lameness detection is unknown (we have not identified external validation studies for lameness detection using those techniques), while the commercial availability and performance of two remaining methods are still low. According to our knowledge, there are no commercially available systems able to detect skin lesions; however, similar to cleanliness evaluation, development in camera-based monitoring systems could in the future allow identification of animals with such problems.

Assessment of good health (and especially presence of diseases) should be based on integrating and analyzing data from different sources. There are commercially available examples of systems using multiple sensors (e.g., milking robots and activity collars) which provide data on milk production, SCC, and animal behavior. System using both an automatic milking system and an activity collar was presented by Elischer et al. (42). However, there are no external validation studies on the performance of these systems for disease detection. There are already examples of flexible models able to handle different sensor or non-sensor data for disease detection [e.g., for mastitis prediction (75)] but the performance of these tools need still to be tested in commercial settings. Even if a technology is not able to provide highly accurate health data on individual level, it could still be useful to estimate herd level prevalence of health problems. Potential integration could concern milk sensor data, accelerometer data, load cells with RFID, boluses (for body temperature and rumen pH), cameras (for body temperature, gait, BCS), and microphones (cough detection).

Appropriate Behavior

Appropriate behavior concerns expression of social behavior, expression of other behaviors, good human-animal relationship, and positive emotional state (20). Based on the answers of experts, it can be concluded that PLF technologies currently have a low potential to address appropriate behavior. With the exception of grazing behavior, none of the evaluated attributes was evaluated as “very relevant” by all the experts. However, some of the attributes related to activity as well as feeding and drinking monitoring were evaluated as moderately relevant. From all available technologies, only two tools were tested for evaluation of appropriate behavior (namely, for grazing monitoring). There is, however, a substantial scientific interest in developing research tools aiming to address this welfare principle. For example, accelerometer-based tools were already applied to monitor social behaviors, such as discriminating spontaneous locomotor play (76) and licking/suckling (77) in dairy calves. Also, data from feeding and drinking stations were applied for monitoring social competition (70, 78, 79). In recent years, scientists pointed out the importance of positive emotions as key elements to ensuring good animal welfare (80). In experimental conditions, both ear postures (81) and nasal temperature (82) have been proven to be useful measures of a change in emotional state of cows. For example, the drop in nasal temperatures of cows can be a result of the experience of a positive, low arousal experience. However, further research is needed to design systems able to monitor positive emotional state in commercial settings. Also, there is a technological gap concerning monitoring good human-animal interaction with no retailed technologies intended for this purpose. There is also very scarce information on any experimental techniques for measuring avoidance distance (which is used to assess good human-animal interaction) at the individual level (12). Human-animal relationship could be automatically monitored using 3D cameras, which can capture the distance between a target and camera. However, application of vision technologies requires more research effort.

Performance Results—Quality and Quantity of Validation Studies

Validation studies are essential for further use of the tool in scientific experiments as well as for welfare labeling or subsidy payments. Therefore, there should be more emphasis on the quantity and quality of conducting and reporting of validation studies for PLF technologies. The results of validation studies are quite often presented as *technical notes* or *short communications* with rather limited space for detail description; however, this does not absolve authors from presenting information necessary for readers to assess the risk of reporting bias. Based on this review, similar suggestions to those presented by Hendriks et al. (83) on how to improve reporting can be made. The location of the trial (commercial vs. experimental herd), criteria for animal selection (e.g., random or based on a stage of lactation), building and management characteristics (e.g., floor type and grazing), and feeding system should be always reported, since results obtained in the different production settings might not be comparable. Furthermore, the validated tool, especially if not commercially available, should be described in enough detail for correct technology identification. Also, when possible, the software version should be reported.

In this review, we have grouped tools based on reported performance measures. Threshold for high performance was selected based on available literature and represents very good agreement. Here, it should be noted that tools which did not fulfill high performance criteria still have practical relevance. For example, online California Mastitis Test performed by milking robots, agreed only moderately with laboratory measurements on SCC. Even though this data was not very precise, nevertheless can be very useful for on-farm decision making, due to the high sampling frequency (43). Therefore, the practical relevance of tools need to be assessed based on their objectives (84) and judged one by one. The results from a single validation trial are not yet conclusive regarding the tool performance. Ideally, tools should be validated in different production conditions (e.g., different countries and housing). According to our results, some of the traits were validated multiple times, by different research groups around the world. And as expected, some presented performance results were inconsistent (as in the example of the CowManager sensor evaluated in different housing conditions). Also, as seen from the example of **Supplementary Table A2**, authors varied in reported statistics. There seems to be no clear guideline on sufficient level of information regarding performance which should be reported. For example, for classification models, reports should not be based on presenting only sensitivity and specificity of the model without information on selected thresholds for detection. Instead, the performance of the classification model should be preferably presented in receiver operating characteristic curve, which is the overall performance indicator (85). Regarding regression models, the adequate statistical tests are presented for example in Tedeschi (84). Including only Pearson correlation coefficient allows assessing precision of the tool, while nothing is known about its inaccuracy (the systematic deviation from the truth). Testing for tool performance is especially important for technologies from which data will be post-processed and used for building further algorithms. In this review, we have not removed or distinguished in result table studies which provided somehow limited information on the tool performance (for example, only results on Pearson correlation). It could be possible to set additional exclusion criteria for papers selection; however, one must remember that even in the limited form, these studies provide some partial information about the validity.

Application of Sensor-Based Technology for Welfare Assessment on Farms and Beyond

The primary goal of a sensor system is to improve animal management. Sensor systems provide information for decision making which may, among others, influence farm profitability and animal health and welfare as well as have environmental impact (11). However, potential application of sensor systems can go beyond a single farm level. There are studies demonstrating that data routinely recorded from milking robots provide information which can assist in genetic evaluation [e.g., (86)]. Moreover, production data could be utilized for designing health surveillance systems. For example, an attempt was made to use milk yield data to detect outbreaks of Bluetongue and Schmallenberg viruses (87). PLF technologies may provide evidence-based approach to the monitoring and surveillance of animal welfare not only at the farm but also during transport or at slaughter (88). Already now, in some countries, there are suggestions to base the certification system of livestock farming on real-time measurements and using animal behavior as a criterion for quality labeling (89). This kind of policy could increase transparency of the sector and could result in a wider selection of welfare friendly products. As demonstrated in a previous review, data routinely collected on the farm (e.g., on milk yield, culling, and reproduction) and available in national data base, were associated with dairy cow welfare (90). Also, meat inspection data can have practical application for welfare assessment (91). This review demonstrates that data collected during on-farm monitoring has high potential to assess different aspects of dairy cow welfare, and that currently available technologies can provide animal-based welfare information. However, for the data to be fully utilized for this purpose, there is a need to develop new methodologies for data integration and processing. Data collected from various automatic recording technologies need to be processed and integrated into a single outcome of animal welfare (which is easy to understand by the consumer). This challenging task will be considered by the ClearFarm project, which aims to develop a platform to control animal welfare in pig and dairy farming. The integration of technologies for welfare, health surveillance, or breeding evaluation will require access to a vast amount of PLF data from different devices and different users. The utilization of these data requires that data ownership rights, privacy, and confidentiality issues are resolved and agreed between the parties involved. For example, for the EU markets, non-binding guidelines on data sharing from PLF technologies are available (92) and cover, among others, ownership, access, control, and privacy. However, according to the recent review on digital agriculture, the area of data ownership regulations could receive more attention (93). Another challenge concerns data storage capacity and strong computational power. However, there are already efforts to design a set of industrial, large-scale high-performance computing solutions to support the processing of very large PLF data sets from different users (94).

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

MP, JN, AS, EC, and PL conceived the presented idea. AS defined literature search. AS, GB, and EDC carried out literature search. YG defined and performed company search. AS wrote the paper with input from all the co-authors. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

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REFERENCES

1. Pinillos RG, Appleby MC, Manteca X, Scott-Park F, Smith C, Velarde A. One welfare – a platform for improving human and animal welfare. *Vet Rec.* (2016) 179:412–3. doi: 10.1136/vr.i5470
2. Stygar AH, Chantziaras I, Toppari I, Maes D, Niemi JK. High biosecurity and welfare standards in fattening pig farms are associated with reduced antimicrobial use. *Animal.* (2020) 14:1–9. doi: 10.1017/S1751731120000828
3. von Keyserlingk MAG, Rushen J, de Passillé AM, Weary DM. Invited review: the welfare of dairy cattle—Key concepts and the role of science. *J Dairy Sci.* (2009) 92:4101–11. doi: 10.3168/jds.2009-2326
4. Llonch P, Somarriba M, Duthie C-A, Haskell MJ, Rooke JA, Troy S, et al. Association of temperament and acute stress responsiveness with productivity, feed efficiency, and methane emissions in beef cattle: an observational study. *Front Vet Sci.* (2016) 3:43. doi: 10.3389/fvets.2016.00043
5. European Commission. *Attitudes of EU Citizens Towards Animal Welfare, Report; Special Eurobarometer 442*. Brussels: European Commission (2016).
6. European Commission. *Online Consultation on the Future of Europe. Second Interim Report*. (2019). Available online at: https://ec.europa.eu/commission/sites/beta-political/files/online-consultation-report-april-2019_en.pdf (accessed September 18, 2020).
7. Yang W, Renwick A. Consumer willingness to pay price premiums for credence attributes of livestock products – a meta-analysis. *J Agric Econ.* (2019) 70:618–39. doi: 10.1111/1477-9552.12323
8. Heinola K, Latvala T, Raussi S, Kauppinen T, Niemi J. Kuluttajanäkökulmia eläinten hyvinvointimerkin kehittämiseen. *SMST.* (2020) 38:1–12. doi: 10.33354/smst.89454
9. Clark B, Panzone LA, Stewart GB, Kyriazakis I, Niemi JK, Latvala T, et al. Consumer attitudes towards production diseases in intensive production systems. *PLoS ONE.* (2019) 14:e0210432. doi: 10.1371/journal.pone.0210432
10. Riemsdijk L, Ingenbleek PTM, Veen G, Trijp HCM. Positioning strategies for animal-friendly products: a social dilemma approach. *J Consum Aff.* (2020) 54:100–29. doi: 10.1111/joca.12240
11. Rojo-Gimeno C, van der Voort M, Niemi JK, Lauwers L, Kristensen AR, Wauters E. Assessment of the value of information of precision livestock farming: a conceptual framework. *NJAS Wageningen J Life Sci.* (2019) 90–1:100311. doi: 10.1016/j.njas.2019.100311
12. Maroto Molina F, Pérez Marín CC, Molina Moreno L, Agüera Buendía EI, Pérez Marín DC. Welfare Quality ® for dairy cows: towards a sensor-based assessment. *J Dairy Res.* (2020) 87:28–33. doi: 10.1017/S002202992000045X
13. Steeneveld W, Hogeveen H, Oude Lansink AGJM. Economic consequences of investing in sensor systems on dairy farms. *Comput Electron Agric.* (2015) 119:33–9. doi: 10.1016/j.compag.2015.10.006
14. Steeneveld W, Vernooij JCM, Hogeveen H. Effect of sensor systems for cow management on milk production, somatic cell count, and reproduction. *J Dairy Sci.* (2015) 98:3896–05. doi: 10.3168/jds.2014-9101
15. Moher D, Liberati A, Tetzlaff J, Altman DG, The PRISMA Group. Preferred reporting items for systematic reviews and meta-analyses: the PRISMA statement. *PLoS Med.* (2009) 6:e1000097. doi: 10.1371/journal.pmed.1000097
16. Altman DG, Vergouwe Y, Royston P, Moons KGM. Prognosis and prognostic research: validating a prognostic model. *BMJ.* (2009) 338:b605. doi: 10.1136/bmj.b605
17. Bikker JP, van Laar H, Rump P, Doorenbos J, van Meurs K, Griffioen GM, et al. Technical note: evaluation of an ear-attached movement sensor to record cow feeding behavior and activity. *J Dairy Sci.* (2014) 97:2974–9. doi: 10.3168/jds.2013-7560
18. Grinter LN, Campler MR, Costa JHC. Technical note: validation of a behavior-monitoring collar's precision and accuracy to measure rumination, feeding, and resting time of lactating dairy cows. *J Dairy Sci.* (2019) 102:3487–94. doi: 10.3168/jds.2018-15563
19. Van Hertem T, Tello AS, Viazzi S, Steensels M, Bahr C, Romanini CEB, et al. Implementation of an automatic 3D vision monitor for dairy cow locomotion in a commercial farm. *Biosyst Eng.* (2018) 173:166–75. doi: 10.1016/j.biosystemseng.2017.08.011
20. Welfare Quality®. *Welfare Quality® Assessment Protocol for Cattle*. Welfare Quality® Consortium, Lelystad (2009). Available online at: http://www.welfarequalitynetwork.net/media/1088/cattle_protocol_without_veal_calves.pdf (accessed September 18, 2020).
21. Borchers MR, Chang YM, Tsai IC, Wadsworth BA, Bewley JM. A validation of technologies monitoring dairy cow feeding, ruminating, and lying behaviors. *J Dairy Sci.* (2016) 99:7458–66. doi: 10.3168/jds.2015-10843
22. Henriksen JC, Munksgaard L. Validation of AfiTagII, a device for automatic measuring of lying behaviour in Holstein and Jersey cows on two different bedding materials. *Animal.* (2019) 13:617–21. doi: 10.1017/S1751731118001623
23. Mattachini G, Antler A, Riva E, Arbel A, Provolo G. Automated measurement of lying behavior for monitoring the comfort and welfare of lactating dairy cows. *Livestock Sci.* (2013) 158:145–50. doi: 10.1016/j.livsci.2013.10.014
24. Swartz TH, McGilliard ML, Petersson-Wolfe CS. Technical note: the use of an accelerometer for measuring step activity and lying behaviors in dairy calves. *J Dairy Sci.* (2016) 99:9109–13. doi: 10.3168/jds.2016-11297
25. Kaniyamattam K, De Vries A. Agreement between milk fat, protein, and lactose observations collected from the Dairy Herd Improvement Association (DHIA) and a real-time milk analyzer. *J Dairy Sci.* (2014) 97:2896–908. doi: 10.3168/jds.2013-7690
26. Mattachini G, Riva E, Bisaglia C, Pompe JCAM, Provolo G. Methodology for quantifying the behavioral activity of dairy cows in freestall barns. *J Anim Sci.* (2013) 91:4899–907. doi: 10.2527/jas.2012-5554
27. Nielsen PP, Fontana I, Sloth KH, Guarino M, Blokhuis H. Technical note: validation and comparison of 2 commercially available activity loggers. *J Dairy Sci.* (2018) 101:5449–53. doi: 10.3168/jds.2017-13784
28. Shepley E, Berthelot M, Vasseur E. Validation of the ability of a 3D pedometer to accurately determine the number of steps taken by dairy cows when housed in tie-stalls. *Agriculture.* (2017) 7:53. doi: 10.3390/agriculture7070053
29. Werner J, Umstatter C, Leso L, Kennedy E, Geoghegan A, Shalloo L, et al. Evaluation and application potential of an accelerometer-based collar device for measuring grazing behavior of dairy cows. *Animal.* (2019) 13:2070–9. doi: 10.1017/S1751731118003658

30. Brassel J, Rohrssen F, Failing K, Wehrend A. Automated detection of health disorders in lactating dairy cattle on pasture: a preliminary study. *Polish J Vet Sci.* (2019) 22:761−7. doi: 10.24425/pjvs.2019.131406
31. Schirmann K, von Keyserlingk MAG, Weary DM, Veira DM, Heuwieser W. Technical note: validation of a system for monitoring rumination in dairy cows. *J Dairy Sci.* (2009) 92:6052–5. doi: 10.3168/jds.2009-2361
32. Burfeind O, Schirmann K, von Keyserlingk MAG, Veira DM, Weary DM, Heuwieser W. Technical note: evaluation of a system for monitoring rumination in heifers and calves. *J Dairy Sci.* (2011) 94:426–30. doi: 10.3168/jds.2010-3239
33. Ruuska S, Kajava S, Mughal M, Zehner N, Mononen J. Validation of a pressure sensor-based system for measuring eating, rumination and drinking behaviour of dairy cattle. *Appl Anim Behav Sci.* (2016) 174:19–23. doi: 10.1016/j.applanim.2015.11.005
34. Steinmetz M, von Soosten D, Hummel J, Meyer U, Daenicke S. Validation of the RumiWatch Converter V0.7.4.5 classification accuracy for the automatic monitoring of behavioural characteristics in dairy cows. *Arch Anim Nutr.* (2020) 74:164–72. doi: 10.1080/1745039X.2020.1721260
35. Kroeger I, Humer E, Neubauer V, Kraft N, Ertl P, Zebeli Q. Validation of a noseband sensor system for monitoring ruminating activity in cows under different feeding regimens. *Livestock Sci.* (2016) 193:118–22. doi: 10.1016/j.livsci.2016.10.007
36. Zehner N, Umstatter C, Niederhauser JJ, Schick M. System specification and validation of a noseband pressure sensor for measurement of ruminating and eating behavior in stable-fed cows. *Comput Electron Agric.* (2017) 136:31–41. doi: 10.1016/j.compag.2017.02.021
37. Eslamizad M, Tuemmler L-M, Derno M, Hoch M, Kuhla B. Technical note: development of a pressure sensor-based system for measuring rumination time in pre-weaned dairy calves. *J Anim Sci.* (2018) 96:4483–9. doi: 10.1093/jas/sky337
38. Werner J, Leso L, Umstatter C, Niederhauser J, Kennedy E, Geoghegan A, et al. Evaluation of the RumiWatchSystem for measuring grazing behaviour of cows. *J Neurosci Methods.* (2018) 300:138–46. doi: 10.1016/j.jneumeth.2017.08.022
39. Zambelis A, Wolfe T, Vasseur E. Technical note: validation of an ear-tag accelerometer to identify feeding and activity behaviors of tiestall-housed dairy cattle. *J Dairy Sci.* (2019) 102:4536–40. doi: 10.3168/jds.2018-15766
40. Pereira GM, Heins BJ, Endres MI. Technical note: validation of an ear-tag accelerometer sensor to determine rumination, eating, and activity behaviors of grazing dairy cattle. *J Dairy Sci.* (2018) 101:2492–5. doi: 10.3168/jds.2016-12534
41. Reynolds MA, Borchers MR, Davidson JA, Bradley CM, Bewley JM. Technical note: an evaluation of technology-recorded rumination and feeding behaviors in dairy heifers. *J Dairy Sci.* (2019) 102:6555–8. doi: 10.3168/jds.2018-15635
42. Elischer MF, Arceo ME, Karcher EL, Siegford JM. Validating the accuracy of activity and rumination monitor data from dairy cows housed in a pasture-based automatic milking system. *J Dairy Sci.* (2013) 96:6412–22. doi: 10.3168/jds.2013-6790
43. Deng Z, Hogeveen H, Lam TJGM, van der Tol R, Koop G. Performance of online somatic cell count estimation in automatic milking systems. *Front Vet Sci.* (2020) 7:221. doi: 10.3389/fvets.2020.00221
44. DeVries TJ, von Keyserlingk MAG, Weary DM, Beauchemin KA. Technical note: validation of a system for monitoring feeding behavior of dairy cows. *J Dairy Sci.* (2003) 86:3571–4. doi: 10.3168/jds.S0022-0302(03)73962-9
45. Chapinal N, Veira DM, Weary DM, von Keyserlingk MAG. Technical note: validation of a system for monitoring individual feeding and drinking behavior and intake in group-housed cattle. *J Dairy Sci.* (2007) 90:5732–6. doi: 10.3168/jds.2007-0331
46. Chizzotti ML, Machado FS, Valente EEL, Pereira LGR, Campos MM, Tomich TR, et al. Technical note: validation of a system for monitoring individual feeding behavior and individual feed intake in dairy cattle. *J Dairy Sci.* (2015) 98:3438–42. doi: 10.3168/jds.2014-8925
47. Oliveira BR, Ribas MN, Machado FS, Lima JAM, Cavalcanti LFL, Chizzotti ML, et al. Validation of a system for monitoring individual feeding and drinking behaviour and intake in young cattle. *Animal.* (2018) 12:634–9. doi: 10.1017/S1751731117002002
48. Mullins IL, Truman CM, Campler MR, Bewley JM, Costa JHC. Validation of a commercial automated body condition scoring system on a commercial dairy farm. *Animals.* (2019) 9:287. doi: 10.3390/ani9060287
49. Cantor MC, Pertuisel CH, Costa JHC. Technical note: estimating body weight of dairy calves with a partial-weight scale attached to an automated milk feeder. *J Dairy Sci.* (2020) 103:1914–9. doi: 10.3168/jds.2019-16918
50. Neubauer V, Humer E, Kroeger I, Braid T, Wagner M, Zebeli Q. Differences between pH of indwelling sensors and the pH of fluid and solid phase in the rumen of dairy cows fed varying concentrate levels. *J Anim Physiol Anim Nutr.* (2018) 102:343–9. doi: 10.1111/jpn.12675
51. Falk M, Münger A, Dohme-Meier F. Technical note: a comparison of reticular and ruminal pH monitored continuously with 2 measurement systems at different weeks of early lactation. *J Dairy Sci.* (2016) 99:1951–5. doi: 10.3168/jds.2015-9725
52. Lohoelter M, Rehage R, Meyer U, Lebzien P, Rehage J, Daenicke S. Evaluation of a device for continuous measurement of rumen pH and temperature considering localization of measurement and dietary concentrate proportion. *Landbauforschung.* (2013) 63:61–8. doi: 10.3220/LBF_2013_61-68
53. Knauer WA, Godden SM, McDonald N. Technical note: preliminary evaluation of an automated indwelling rumen temperature bolus measurement system to detect pyrexia in preweaned dairy calves. *J Dairy Sci.* (2016) 99:9925–30. doi: 10.3168/jds.2015-10770
54. Bicalho RC, Cheong SH, Cramer G, Guard CL. Association between a visual and an automated locomotion score in lactating Holstein cows. *J Dairy Sci.* (2007) 90:3294–300. doi: 10.3168/jds.2007-0076
55. Hoffmann G, Schmidt M, Ammon C, Rose-Meierhoefer S, Burfeind O, Heuwieser W, et al. Monitoring the body temperature of cows and calves using video recordings from an infrared thermography camera. *Vet Res Commun.* (2013) 37:91–9. doi: 10.1007/s11259-012-9549-3
56. Schlageter-Tello A, Van Hertem T, Bokkers EAM, Viazzi S, Bahr C, Lokhorst K. Performance of human observers and an automatic 3-dimensional computer-vision-based locomotion scoring method to detect lameness and hoof lesions in dairy cows. *J Dairy Sci.* (2018) 101:6322–35. doi: 10.3168/jds.2017-13768
57. de Mol RM, Ouweltjes W, Kroeze GH, Hendriks M. Detection of estrus and mastitis: field performance of a model. *Appl Eng Agric.* (2001) 17:399–407. doi: 10.13031/2013.6201
58. Sorensen LP, Bjerring M, Lovendahl P. Monitoring individual cow udder health in automated milking systems using online somatic cell counts. *J Dairy Sci.* (2016) 99:608–20. doi: 10.3168/jds.2014-8823
59. Tamura T, Okubo Y, Deguchi Y, Koshikawa S, Takahashi M, Chida Y, et al. Dairy cattle behavior classifications based on decision tree learning using 3-axis neck-mounted accelerometers. *Anim Sci J.* (2019) 90:589–96. doi: 10.1111/asj.13184
60. Stygar AH, Kristensen AR. Detecting abnormalities in pigs' growth – a dynamic linear model with diurnal growth pattern for identified and unidentified pigs. *Comput Electron Agric.* (2018) 155:180–9. doi: 10.1016/j.compag.2018.10.004
61. ICAR. *The Global Standard for Livestock Data*. Section *11 -Guidelines for Testing, Approval and Checking of Milk Recording Devices*. (2020). Available online at: https://www.icar.org/Guidelines/11-Milk-Recording-Devices.pdf
62. Schweinzer V, Gusterer E, Kanz P, Krieger S, Suess D, Lidauer L, et al. Evaluation of an ear-attached accelerometer for detecting estrus events in indoor housed dairy cows. *Theriogenology.* (2019) 130:19–25. doi: 10.1016/j.theriogenology.2019.02.038
63. Bruinjé TC, Ambrose DJ. Technical note: validation of an automated in-line milk progesterone analysis system to diagnose pregnancy in dairy cattle. *J Dairy Sci.* (2019) 102:3615–21. doi: 10.3168/jds.2018-15692
64. Guzhva O, Ardo H, Nilsson M, Herlin A, Tufvesson L. Now you see me: convolutional neural network based tracker for dairy cows. *Front Robot Ai.* (2018) 5:107. doi: 10.3389/frobt.2018.00107
65. Wurtz K, Camerlink I, D'Eath RB, Fernández AP, Norton T, Steibel J, et al. Recording behaviour of indoor-housed farm animals automatically using machine vision technology: a systematic review. *PLoS ONE.* (2019) 14:e0226669. doi: 10.1371/journal.pone.0226669
66. Schirmann K, Weary DM, Heuwieser W, Chapinal N, Cerri RLA, von Keyserlingk MAG. Short communication: rumination and feeding behaviors

differ between healthy and sick dairy cows during the transition period. *J Dairy Sci.* (2016) 99:9917–24. doi: 10.3168/jds.2015-10548

67. Roche JR, Friggens NC, Kay JK, Fisher MW, Stafford KJ, Berry DP. Invited review: body condition score and its association with dairy cow productivity, health, and welfare. *J Dairy Sci.* (2009) 92:5769–801. doi: 10.3168/jds.2009-2431
68. Bloch V, Levit H, Halachmi I. Assessing the potential of photogrammetry to monitor feed intake of dairy cows. *J Dairy Res.* (2019) 86:34–9. doi: 10.1017/S0022029918000882
69. Phillips N, Mottram T, Poppi D, Mayer D, McGowan MR. Continuous monitoring of ruminal pH using wireless telemetry. *Anim Prod Sci.* (2010) 50:72. doi: 10.1071/AN09027
70. Huzzey JM, Weary DM, Tiau BYF, von Keyserlingk MAG. Short communication: automatic detection of social competition using an electronic feeding system. *J Dairy Sci.* (2014) 97:2953–8. doi: 10.3168/jds.2013-7434
71. Haley DB, Rushen J, de Passillé AM. Behavioural indicators of cow comfort: activity and resting behaviour of dairy cows in two types of housing. *Can J Anim Sci.* (2000) 80:257–63. doi: 10.4141/A99-084
72. Tucker CB, Jensen MB, de Passillé AM, Hänninen L, Rushen J. Invited review: lying time and the welfare of dairy cows. *J Dairy Sci.* (2020) 104:20–46. doi: 10.3168/jds.2019-18074
73. Olmos G, Boyle L, Hanlon A, Patton J, Murphy JJ, Mee JF. Hoof disorders, locomotion ability and lying times of cubicle-housed compared to pasture-based dairy cows. *Livestock Sci.* (2009) 125:199–207. doi: 10.1016/j.livsci.2009.04.009
74. Li W, Ji Z, Wang L, Sun C, Yang X. Automatic individual identification of Holstein dairy cows using tailhead images. *Comput Electron Agric.* (2017) 142:622–31. doi: 10.1016/j.compag.2017.10.029
75. Jensen DB, Hogeveen H, De Vries A. Bayesian integration of sensor information and a multivariate dynamic linear model for prediction of dairy cow mastitis. *J Dairy Sci.* (2016) 99:7344–61. doi: 10.3168/jds.2015-10060
76. Groessbacher V, Buckova K, Lawrence AB, Spinka M, Winckler C. Discriminating spontaneous locomotor play of dairy calves using accelerometers. *J Dairy Sci.* (2020) 103:1866–73. doi: 10.3168/jds.2019-17005
77. Roland L, Schweinzer V, Kanz P, Sattlecker G, Kickinger F, Lidauer L, et al. Evaluation of a triaxial accelerometer for monitoring selected behaviors in dairy calves. *J Dairy Sci.* (2018) 101:10421–7. doi: 10.3168/jds.2018-14720
78. Foris B, Thompson AJ, von Keyserlingk MAG, Melzer N, Weary DM. Automatic detection of feeding- and drinking-related agonistic behavior and dominance in dairy cows. *J Dairy Sci.* (2019) 102:9176–86. doi: 10.3168/jds.2019-16697
79. McDonald P, von Keyserlingk MAG, Weary DM. Technical note: using an electronic drinker to monitor competition in dairy cows. *J Dairy Sci.* (2019) 102:3495–500. doi: 10.3168/jds.2018-15585
80. Mellor DJ. Animal emotions, behaviour and the promotion of positive welfare states. *N Z Vet J.* (2012) 60:1–8. doi: 10.1080/00480169.2011.619047
81. Proctor HS, Carder G. Can ear postures reliably measure the positive emotional state of cows? *Appl Anim Behav Sci.* (2014) 161:20–7. doi: 10.1016/j.applanim.2014.09.015
82. Proctor HS, Carder G. Nasal temperatures in dairy cows are influenced by positive emotional state. *Physiol Behav.* (2015) 138:340–4. doi: 10.1016/j.physbeh.2014.11.011
83. Hendriks SJ, Phyn CVC, Huzzey JM, Mueller KR, Turner S-A, Donaghy DJ, et al. Graduate student literature review: evaluating the appropriate use of wearable accelerometers in research to monitor lying behaviors of dairy cows. *J Dairy Sci.* (2020) 103:12140–57. doi: 10.3168/jds.2019-17887
84. Tedeschi LO. Assessment of the adequacy of mathematical models. *Agric Syst.* (2006) 89:225–47. doi: 10.1016/j.agsy.2005.11.004
85. Dominiak KN, Kristensen AR. Prioritizing alarms from sensor-based detection models in livestock production - a review on model performance and alarm reducing methods. *Comput Electron Agric.* (2017) 133:46–67. doi: 10.1016/j.compag.2016.12.008
86. Heringstad B, Kjøren Bugten H. Genetic evaluations based on data from automatic milking systems. In: *39th ICAR Session, May 19-23.* Berlin (2014).
87. Veldhuis A, Brouwer-Middelesch H, Marceau A, Madouasse A, Van der Stede Y, Fourichon C, et al. Application of syndromic surveillance on routinely collected cattle reproduction and milk production data for the early detection of outbreaks of Bluetongue and Schmallenberg viruses. *Prev Vet Med.* (2016) 124:15–24. doi: 10.1016/j.prevetmed.2015.12.006
88. Buller H, Blokhuis H, Lokhorst K, Silberberg M, Veissier I. Animal welfare management in a digital world. *Animals.* (2020) 10:1779. doi: 10.3390/ani10101779
89. Council on Animal Affairs (RDA). *Digitisation of the Livestock Farming Sector*. (2019). Available online at: https://english.rda.nl/publications/publications/2020/02/13/digitisation-of-the-livestock-farming-sector (accessed January 4, 2020).
90. de Vries M, Bokkers EAM, Dijkstra T, van Schaik G, de Boer IJM. Invited review: associations between variables of routine herd data and dairy cattle welfare indicators. *J Dairy Sci.* (2011) 94:3213–28. doi: 10.3168/jds.2011-4169
91. Nielsen S, Denwood M, Forkman B, Houe H. Selection of meat inspection data for an animal welfare index in cattle and pigs in Denmark. *Animals.* (2017) 7:94. doi: 10.3390/ani7120094
92. COPA-COGECA, CEMA. *EU Code of Conduct on Agricultural Data Sharing by Contractual Agreement*. (2018). Available online at: https://www.fefac.eu/files/81630.pdf (accessed February 4, 2020).
93. Klerkx L, Jakku E, Labarthe P. A review of social science on digital agriculture, smart farming and agriculture 4.0: new contributions and a future research agenda. *NJAS Wageningen J Life Sci.* (2019) 90–1:100315. doi: 10.1016/j.njas.2019.100315
94. Perakis K, Lampathaki F, Nikas K, Georgiou Y, Marko O, Maselyne J. CYBELE – fostering precision agriculture & livestock farming through secure access to large-scale HPC enabled virtual industrial experimentation environments fostering scalable big data analytics. *Comput Netw.* (2020) 168:107035. doi: 10.1016/j.comnet.2019.107035

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REVIEW
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Blood Will Tell: What Hematological Analyses Can Reveal About Fish Welfare

*Henrike Seibel[1,2], Björn Baßmann[3] and Alexander Rebl[4]**

[1] Institute of Animal Breeding and Husbandry, Christian-Albrechts-University, Kiel, Germany, [2] Gesellschaft für Marine Aquakultur mbH (GMA), Büsum, Germany, [3] Department of Aquaculture and Sea-Ranching, Faculty of Agricultural and Environmental Science, University of Rostock, Rostock, Germany, [4] Institute of Genome Biology, Leibniz Institute for Farm Animal Biology (FBN), Dummerstorf, Germany

Blood analyses provide substantial information about the physiological aspects of animal welfare assessment, including the activation status of the neuroendocrine and immune system, acute and long-term impacts due to adverse husbandry conditions, potential diseases, and genetic predispositions. However, fish blood is still not routinely analyzed in research or aquaculture for the assessment of health and/or welfare. Over the years, the investigative techniques have evolved from antibody-based or PCR-based single-parameter analyses to now include transcriptomic, metabolomic, and proteomic approaches and from hematological observations to fluorescence-activated blood cell sorting in high-throughput modes. The range of testing techniques established for blood is now broader than for any other biogenic test material. Evaluation of the particular characteristics of fish blood, such as its cell composition, the nucleation of distinct blood cells, or the multiple isoforms of certain immune factors, requires adapted protocols and careful attention to the experimental designs and interpretation of the data. Analyses of fish blood can provide an integrated picture of the endocrine, immunological, reproductive, and genetic functions under defined environmental conditions and treatments. Therefore, the scarcity of high-throughput approaches using fish blood as a test material for fish physiology studies is surprising. This review summarizes the wide range of techniques that allow monitoring of informative fish blood parameters that are modulated by different stressors, conditions, and/or treatments. We provide a compact overview of several simple plasma tests and of multiparametric analyses of fish blood, and we discuss their potential use in the assessment of fish welfare and pathologies.

Keywords: erythrocytes, hematology, leukocytes, teleost fishes, well-being, transcriptomics, proteomics, stress

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The University of Queensland, Australia*

****Correspondence:***
*Alexander Rebl
rebl@fbn-dummerstorf.de*

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INTRODUCTION

A significant segment of the constantly growing aquaculture sector is represented by intensive farming practices (1) aimed at meeting the increasing demand of a growing world population. However, at the same time, this emphasis raises many concerns due to the competition for resources and the negative impact of intensive aquaculture on the environment and fish welfare (2). Consequently, the visionary concepts for future fish farming include welfare-friendly systems that can monitor animal-based parameters and quickly regulate disruptive environmental variables when necessary (3, 4). Certified farming procedures should be based on a comprehensive, science-based understanding of how teleost fish respond to anthropogenic environmental disruptions and challenging aquaculture-related conditions. This requires multidisciplinary investigations to define and evaluate optimal species-specific husbandry conditions, especially for newly introduced aquaculture species (5). Consequently, research groups and large scientific consortia are currently studying the influences of a variety of factors, ranging from global climate change, ocean acidification, and eutrophic habitats to introduced pathogens and pollution by environmental toxins or microplastics, on fish breeding and wild fish stocks. The use of more extensive research approaches to screen the effects of environmental conditions can increase the probability of detecting disturbances and even dangerous confounding factors, thereby allowing the discovery of relevant diagnostic biomarkers for fish health.

One favorable option for rapid and non-lethal sampling of large numbers of fish individuals is blood analysis. Blood is a complex mixture of heterogeneous cell populations (6–8) that include erythrocytes (red blood cells), leukocytes (white blood cells), and thrombocytes (analogs of mammalian platelets) (9). These fish blood cells are broadly similar in function to their mammalian counterparts and are found in all other tissues and organs throughout the body. Blood transports an immense variety of substances (gases, water, minerals, nutrients, hormones, immune effectors, toxins, microbial structures, or waste products), so its analysis can provide a wealth of information about fish physiology and health status. Alterations in informative blood-based indicators like metabolite concentrations, hormonal profiles, and transcript abundances can reflect systemic reactions to changes or disturbances in homeostasis that can alert scientists and veterinarians (as well as fish farmers), who monitor the physiological status of an individual fish or an entire population.

Blood tests on fish have been carried out for decades (10) in laboratory and field settings to assess endocrine, reproductive, and immune functions; maturation; nutrition and health status or to perform genetic studies (11). More analysis techniques have been established for blood as a test material (**Figure 1**) than for any other tissue or fluid sample. Nevertheless, interpreting the obtained data to extract meaningful information on the individual condition remains difficult. On the one hand, a few excellent reviews have recently updated the history of selected hematological techniques (12, 13); however, remarkably, blood analyses using systems biological approaches or even PCR-based techniques are still underrepresented in fish physiology (14). On the other hand, the omics-based hypotheses put forward by fish geneticists and molecular biologists are correlated only on a small scale with non-transcript parameters extracted from fish blood.

Abbreviations: ACTH, adrenocorticotropic hormone; CRH, corticotropin-releasing hormone; Hb, hemoglobin; HSP, heat-shock protein; IL, interleukin; KEGG, Kyoto Encyclopedia of Genes and Genomes; MHC, major histocompatibility complex; NOD, nucleotide binding oligomerization domain containing protein; NADH, Nicotinamide Adenine Dinucleotide - Hydrogen; RIA, radioimmunoassay; RNA-seq, RNA sequencing; scRNA-seq, single-cell RNA sequencing; TLR, toll-like receptor.

A growing part of the research on farmed fish focuses on "animal welfare" involving optimization of husbandry conditions, stress avoidance, and improvement of fish quality of life (15, 16). The focus is largely on the ability of domesticated fish populations to cope with environmental and/or anthropogenic challenges (17). In general, animal-welfare programs aim to ensure freedom from (i) thirst, hunger, and malnutrition; (ii) discomfort; (iii) pain, injury, and disease; (iv) restriction of normal behaviors; and (v) fear and suffering (18). These basic aspects also apply to some extent to the welfare of fish in aquaculture. However, fish require different treatment than terrestrial animals in many ways due to their aquatic nature and differences in their anatomy and physiology, as well as in their required husbandry conditions. According to Huntingford et al. (19), fish welfare can be expressed by (i) a feelings-based definition, focusing on a reduction in pain and negative emotions, and/or increased access to positive experiences; (ii) a nature-based definition assuming that every fish species must express its inherent biological nature; or (iii) a function-based definition targeting the ability of fish to adapt to environmental demands.

The well-being of fish in aquaculture is impaired by acute environmental changes, coupled with husbandry practices, such as sorting, grading, and transport that can induce stress responses. Most research studies are based on the third concept (iii), which involves fish health and the adequate functioning of fish biological systems, especially those involved in managing a compromised homeostasis imposed by aquaculture conditions. The physiological state of a fish with disturbed homeostasis is usually captured by observing and recording indicative signs and measurable characteristics of the response to environmental challenges or stressors. Restoration of homeostasis usually requires the invocation of complex behavioral and physiological adaptive responses (20).

Primary stress responses are characterized by the release of catecholamines and corticosteroids (21). The subsequent secondary stress responses have a multitude of actions in many tissues, including blood, and can range from accelerated mobilization of energy *via* glucose, altered hydromineral balance, and increased lactate levels to decreased blood pH, hematocrit, and sodium levels and lower liver glycogen levels (20, 21). Increased cardiovascular activity and breath rate that enhance the uptake and transport capacity for oxygen are accompanied by a redistribution or suppression of immune functions (21, 22). Tertiary responses are reflected by behavioral adaptions, inhibited growth, decreased reproduction, and a compromised capacity to endure any additional stressors. The repeated exposure to a low-intensity stressor triggers the development of an adaptive response over time and attenuates acute stress reactions, whereas the exposure to harmful, persistent stressors is likely to intensify the physiological response (23). When stressors co-occur with a high pathogenic pressure, they provoke serious diseases (24) and strongly impair fish welfare (25).

Many previous and current studies on fish welfare have measured the main components of the primary physiological stress response, largely plasma cortisol and glucose (26–28). Both components are informative stress markers, but

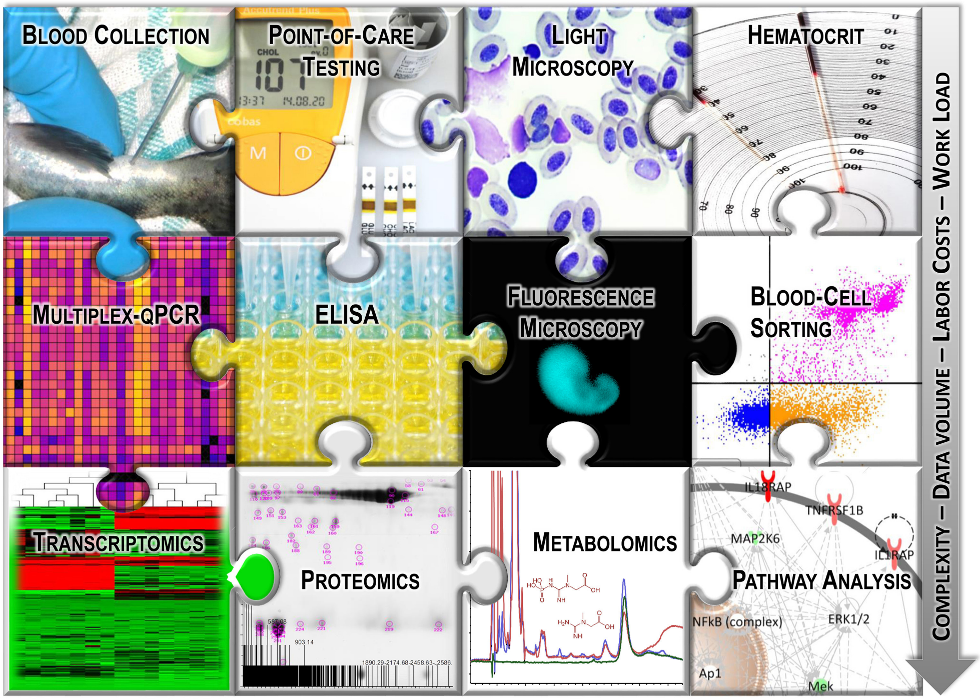

FIGURE 1 | Graphical overview of the selected analysis techniques used with fish blood.

they have limitations (29). The current scientific consensus is that the assessment of fish welfare is a complex task (30), in part because the absence of a physiological stress response does not necessarily imply adequate welfare (31). Each fish species has distinct "ecological and behavioral demands" (15); therefore, the responses to adverse conditions vary across taxa (32, 33). In recent years, the scientific evaluation of fish physiology has shifted from the conventional, limited biomarker approach to comprehensive and rather holistic approaches (34, 35). The spectrum of the parameters now recorded has expanded and is accompanied by an increasingly well-equipped fish-specific toolbox (**Figure 1**). These advances now facilitate the generation of weighted welfare scores (36, 37) that can integrate operational and laboratory-based parameters.

Laboratory data, such as gene expression studies (30, 38, 39), are based on RNA specimens that are mostly obtained from the organs of previously killed fish. By contrast, fish blood collection can be conducted non-lethally (11); therefore, blood represents an alternative and preferable matrix for ethical reasons. Though relatively non-invasive, blood sampling is generally stressful for fish, but repeated blood sampling from the same individual provides the possibility of tracking the time course of various processes after the treatment or determining fish welfare during the developmental stages. Other operational parameters, such as the monitoring of exploratory and swimming behavior (40, 41) or the rate of gill ventilation (42), can readily be recorded on commercial farms, even those that lack the well-equipped wet laboratories.

This review compiles the most commonly reported techniques for obtaining chemico-physical, cellular, metabolic, and transcriptional information from fish blood. It also provides interpretations of the various methods in terms of their importance in assessing the impact of changing environmental conditions or experimental treatments and the intensity and duration of the response of fish.

ANESTHESIA AND BLOOD SAMPLING PROCEDURES

For gentle handling and for safety reasons, fish should be sedated prior to and during blood sampling to minimize pain or discomfort and to prevent defensive or flight reactions and subsequent injuries. An analgesic alone may mask the sensation of pain but will not prevent the perception of subsequent treatments. A loss of consciousness, therefore, ensures the welfare of the fish during blood collection. The most commonly used anesthetic is MS-222 (also known as Tricaine-S or 3-ethoxycarbonylanilinium methanesulfonate) (43), although clove oil (containing eugenol, 4-allyl-2-methoxyphenol), quinaldine (2-methylquinoline), 2-phenoxyethanol, and benzocaine (ethyl 4-aminobenzoate) are also commonly used to stun fish (44). The legal provisions of a given country determine the circumstances that justify the use of particular fish anesthetics. Anesthetics are usually administered as an immersion bath, but the capture of the fish and its transfer to the anesthetic bath, with the brief exposure to air, are likely to evoke a stress reaction (45, 46). Therefore, these

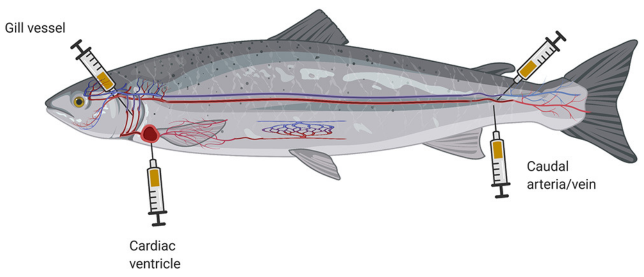

FIGURE 2 | Schematic illustration of different cannula placements for blood collection from fish (created with BioRender.com).

steps should be carried out quickly. The anesthetic *per se* can also act as a stressor on the individual fish being treated.

MS-222 blocks the sodium channels and action potentials of neurons, but concentrations of up to 300 mg MS-222 per liter were shown to have no significant impact on the plasma cortisol levels of South American silver catfish (*Rhamdia quelen*) (47). By contrast, gilthead sea bream (*Sparus aurata*) showed significantly increased cortisol levels following the exposure to even 25 mg/L MS-222 or 0.075 mg/L 2-phenoxyethanol (48). In zebrafish, 15 mg/L of buffered MS-222 altered various hematological characters, including hematocrit, coagulation, and the amount of blood collected (49). The influence of a given anesthetic on the biochemical profile of a sample obviously depends on the dose and on the treated species. These considerations should therefore be taken into account during the evaluation of fish blood parameters. Nevertheless, the anesthesia certainly facilitates the blood collection and prevents a more pronounced stress response, so these advantages may outweigh the potential difficulties related to stress diagnostics.

Optimal blood collection depends on the size and anatomical characteristics of the investigated individual (11). The least traumatic and most widely used blood sampling procedure is to withdraw blood from the caudal vessels—laterally or ventrally—using a cannula and syringe filled with an anticoagulant (sodium citrate, heparin, or potassium ethylenediaminetetraacetic acid) (**Figure 2**). Some analyses require quite large amounts of blood, which creates difficulties when the blood is collected from small teleosts with an estimated whole-blood volume of 3–7 ml per 100 g (50–52). Fish smaller than 8 cm in length are difficult to bleed, but one option is to sever the fin of an anesthetized small fish and then centrifuge the fish at low force (14). Larger teleosts may also be bled by puncture of the cardiac ventricle or other vessels, such as gill-blood vessels (**Figure 2**) (53).

These blood sampling techniques are reserved for studies aimed at answering distinct research questions (54), as they bear a high risk of late complications. In this context, a point worth noting is that the site of blood collection often influences the obtained biomarker profile (55). More detailed information on blood collection techniques has been provided in recent review articles (45, 56). After blood sampling, the fish should be transferred to an aerated recovery tank to allow it to regain perception, awareness, and ability to react. Immediate return of a fish to its usual environment poses a risk that it may be attacked by its tank mates if it remains in a state of non-reactivity.

The liquid part of the blood—the plasma fraction—transports diverse metabolites of interest, including hormones and catabolic products, such as proteins, amino acids, glucose, lipids, and organic acids. For this reason, many researchers separate the plasma *via* centrifugation with an anticoagulant or obtain the serum after blood clotting and subsequent centrifugation (57). Hence, plasma contains natural coagulation factors, whereas serum contains all plasma components except the coagulation factors. Thus, the choice of using plasma or serum depends largely on the research question and its related need for coagulation factors.

IMMUNOASSAYS AND CLINICAL TEST KITS

Many studies that have evaluated fish welfare in aquaculture have revealed that aversive husbandry conditions alter the stress-hormone levels in the farm fish. Stressors and adverse conditions can trigger the chromaffin cells of the head kidney to rapidly release catecholamines, such as noradrenaline/norepinephrine and adrenaline/epinephrine. These reactions may also occur in direct response to the catching process, the subsequent anesthesia, and/or the blood sampling, so they complicate the evaluation of experimentally induced challenges. However, the hypothalamus generates a parallel production of corticotropin-releasing hormone (CRH), which then stimulates the pituitary gland to release adrenocorticotropic hormone (ACTH) (19, 58, 59). This is followed by the synthesis and secretion of cortisol by the interrenal tissue in the head kidney. Many scientists prefer to measure cortisol or ACTH as blood-based indicators of acute stress responses due to the slow increase (within minutes) of these compounds.

Cortisol is probably the most commonly measured molecule for determining the effects of stress in fish (16, 35). Cortisol and ACTH are commonly measured using radioimmunoassays (RIAs). Typically, RIA incorporates a radioactive iodine isotope due to its decay properties. However, the higher technical effort required in an isotope laboratory has led to an increasing preference for ELISAs (60) as an alternative non-radioactive method for the cortisol and ACTH measurements. Both RIAs and ELISAs are antibody-based detection methods, but ELISAs use specific antibodies that bind to the substance (antigen) and rely on an enzymatic color reaction for antigen detection. Electrochemical immunoassays (61), gas or liquid chromatography (62), mass spectrometry (63), and fluorescence-based methods (64) are alternatives to RIA and ELISA for determining blood cortisol levels.

Increased cortisol levels stimulate a number of metabolic processes, such as glucogenolysis and gluconeogenesis, to mobilize and allocate energy reserves (e.g., glucose release into the bloodstream) (21). Increased, as well as decreased, glucose levels are considered signs of stress (**Table 1**) (88), as stress-induced alterations in muscle activity accelerate the conversion

TABLE 1 | Overview of the selected physiological parameters that change in blood, plasma, or serum in response to stressful conditions in various fish species.

Stressor/condition	Species[a]	Duration of challenge	Putative indicator	Direction	References
Handling:					
Transportation	*I. punctatus*	20 min	Circulating granulocytes and T cells	Up	(65)
Transportation	*I. punctatus*	20 min	Circulating B cells	Down	(65)
Catching, sorting, transportation	*R. rutilus, A. brama*	3 h	Sodium-ion contents	Down	(66)
Handling (15 s)	*S. salar*	2 weeks	O-phosphocholine, lactate, carbohydrates, alanine, valine, trimethylamine-N-oxide	Up	(67)
Handling (15 s)	*S. salar*	4 weeks	Di-O-acetylated sialic acids	Up	(68)
Transfer to another tank	*S. trutta*	1 min	Cortisol, glucose, lactate	Up	(69)
Aerial exposure	*H. sabinus*	30 min	Lactate, acidosis, glucose	Up	(9)
Aerial exposure	*S. senegalensis*	3 min	Cortisol, glucose, lactate, osmolality levels	Up	(70)
"Hook and line" stress[b]	*O. mykiss*	2 min	Cell counts, hematocrit, glucose, thrombocytes	Up	(71)
"Hook and line" stress[b]	*O. mykiss*	2 min	Clotting time	Down	(71)
Manual stripping procedure	*O. mykiss*	5 days	Cortisol, *TNF*	Up	(72)
Temperature					
16°C	*G. morhua*	5 days	*B2M*, *MHC-I*, *IGL* mRNA	Up	(73)
20°C	*C. catla*	12 h	*TLR2,−4,−5* and *NOD1,−2* mRNA	Up	(74)
23°C	*D. labrax*	12 h	Cortisol, glucose, superoxide dismutase activity	Up	(75)
30°C	*P. mesopotamicus*	5 days	Glucose	Up	(76)
35°C	*C. catla*	12 h	*TLR2,−4,* and *NOD1,−2* mRNA	Up	(74)
36°C	*R. holubi*	7 days	Cortisol	Up	(28)
37°C	*E. coioides*	1 h	Immunoglobulin M	Up	(77)
Oxygen saturation					
0.3 ppm	*O. niloticus*	3 days	Heat-shock protein 70	Up	(78)
1.0 ppm	*C. catla*	1 h	*HMBG1, TLR4, MYD88, NOD1, RICK, IL6, CXCL8, IL10* mRNA	Up	(79)
1.3 ppm	*S. aurata*	>11 h	Hematocrit, hemoglobin, glucose, lactate	Up	(80)
1.3 ppm	*S. aurata*	>11 h	*UCP2* mRNA	Down	(80)
1.3 ppm	*S. aurata*	4 h	*GST3*, *UCP2*, *ATPAF2*, *SCO1*, *MIRO1a*, *TIM44, TIM10, ACAA2* mRNA	Down	(81)
1.3 ppm	*S. aurata*	4 h	Glucose, lactate, hematocrit, hemoglobin	Up	(81)
2.3 ppm coupled with stocking of 19 kg/m^3	*S. aurata*	22 days	*NDUFAF2* mRNA	Down	(82)
2.3 ppm coupled with stocking of 9.5 kg/m^3	*S. aurata*	22 days	Hematocrit, growth hormone, lactate, erythrocyte number	Up	(82)
Stocking:					
30 kg/m^3	*S. salar*	10 weeks	Alkaline phosphatase	Up	(83)
30 kg/m^3	*S. salar*	10 weeks	Immunoglobulin M	Down	(83)
30 kg/m^3	*S. salar*	10 weeks	Cortisol	Up	(83)
30 kg/m^3	*S. salar*	10 weeks	Maleic dialdehyde	Up	(83)
34 kg/m^3	*S. aurata*	15 weeks	Cortisol, plasma proteins, hematocrit, hemoglobin, erythrocyte number	Up	(84)
40 kg/m^3, 80 kg/m^3	*O. mykiss*	9 months	Cortisol	Down	(85)
45 kg/m^3	*O. mykiss*	1 month	Cholesterol	Down	(86)
45 kg/m^3	*O. mykiss*	1 month	Glucose	Up	(86)
45 kg/m^3	*O. mykiss*	1 month	Triglyceride	Down	(86)
70 kg/m^3	*O. mykiss*	2 days	Lactate	Up	(87)

(Continued)

TABLE 1 | Continued

Stressor/condition	Species[a]	Duration of challenge	Putative indicator	Direction	References
100 kg/m^3	*C. maraena*	9 days	Circulating myeloid cells	Up	(32)
100 kg/m^3	*C. maraena*	9 days	Circulating thrombocytes	Down	(32)
120 kg/m^3	*S. fontinalis*	1 month	Glucose	Down	(88)
Nutrition:					
Food deprivation	*O. mykiss*	28 days	Very-low-density lipoproteins	Up	(89)
Food deprivation	*O. mykiss*	28 days	High-density lipoprotein, choline, β-glucose, lactate	Down	(89)
Plant-based diet with yeast fraction	*O. mykiss*	84 days	Histidine	Down	(90)
Food supplementation with menthol oil	*O. niloticus*	15 days	Hematocrit, erythrocytes, leukocytes, globulin and albumin content, protein concentration, lysozyme and phagocytic activity	Up	(91)
Food supplementation with roselle powder	*O. mykiss*	60 days	Erythrocytes, hematocrit, activities of superoxide dismutase and catalase	Up	(92)
Pollution					
Metallic/organic compounds	*D. labrax*	15 days	Glucose, cortisol, superoxide dismutase activity	Up	(75)
Oxytetracycline	*O. mykiss*	14 days	Sodium dismutase, erythrocyte and leukocyte number	Down	(93)
Nitrite	*P. fulvidraco*	4 days	Sodium- and chloride-ion contents, erythrocyte number, hemoglobin, total antioxidant capacity, activities of superoxide dismutase and catalase and glutathione peroxidase	Down	(94)
Nitrite	*P. fulvidraco*	4 days	Sodium- and chloride-ion contents, leukocyte number, malondialdehyde content	Up	(94)
Polystyrene nanoplastics	*C. idella*	20 days	Erythrocyte nuclear abnormalities, altered erythrocyte morphometry	Up	(95)
Other environmental conditions					
Low-dose ultraviolet B radiation	*C. carpio*	6 weeks	Total protein concentration, oxidative burst activity	Down	(96)
Low-dose ultraviolet B radiation	*O. mykiss*	6 weeks	Oxidative burst activity, cortisol, lymphocyte number	Up	(96)
High CO_2 levels	*H. hippoglossus*	14 weeks	Complement C3, fibrinogen	Up	(97)
Low salinity	*D. labrax*	12 h	Glucose, cortisol, hemoglobin, peroxidase and superoxide-dismutase activity	Up	(75)
Open field (absence of shelter)	*B. episcopi*	2 min	Cortisol	Up	(98)

[a] *Abramis brama (A. brama), Brachyrhaphis episcopi (B. episcopi), Catla catla (C. catla), Coregonus maraena (C. maraena), Ctenopharyngodon idella (C. ide), Cyprinus carpio (C. carpio), Dicentrarchus labrax (D. labrax), Epinephelus coioides (E. coioides), Gadus morhua (G. morhua), Hippoglossus hippoglossus (H. hippoglossus), Hypanus sabinus (H. sabinus), Ictalurus punctatus (I. punctatus) Oncorhynchus mykiss (O. mykiss), Oreochromis niloticus (O. niloticus), Pelteobagrus fulvidraco (P. fulvidraco), Piaractus mesopotamicus (P. mesopotamicus), Rhabdosargus holubi (R. holubi), Rutilus rutilus (R. rutilus), Salvelinus fontinalis (S. fontinalis), Salmo salar (S. salar), Salmo trutta (S. trutta), Solea senegalensis (S. senegalensis), Sparus aurata (S. aurata).*

[b] *Hook insertion into the caudal peduncle forcing fish to swim for 2 min by applying tension to the line.*

of glucose to lactate (or alternatively to pyruvate) by anaerobic glycolysis. The resulting plasma levels of lactate and glucose are often used in conjunction with cortisol levels to establish the physiological state and to assess the severity of a stress response (9, 87, 99–101). Some studies have demonstrated that even the removal of an individual *S. aurata* fish evoked an acute stress reaction in the remaining fish in the tank. The plasma cortisol levels in the tank fish returned to the initial level after 8 h, whereas the plasma glucose and lactate levels and other hematological parameters required 24 h for recovery (102). The plasma triglycerides or cholesterol levels can also reflect the metabolic changes and serve as physiological indices (86), as these lipids can be used as alternate sources of energy by the fish (103–105). Blood glucose, lactate, and triglyceride levels can be determined easily and quickly using appropriate cuvette tests or test strips in portable devices designed for clinical test kits (also known as point-of-care tests) (76). The advantages of point-of-care tests are their time-saving and simple operation, with their reliable and easily readable results.

Changes in cortisol levels indicate the magnitude of a primary stress response, but usually do not provide sufficient information to gauge the ability of an animal to cope with a challenging condition (106). Notably, cortisol levels can vary widely among individuals in response to diurnal or seasonal fluctuations and the ambient temperature (107–109) or according to gender and maturity (58, 106, 110, 111). The cortisol level depends on many factors that can have diverse interactions (29). In optimal cases, the cortisol level rises in correlation with the intensity of a stressor and returns to its baseline level if the stressor does not persist. Multiple stressors occurring simultaneously or persistent chronic stress conditions can complicate the interpretation of a measured cortisol level, as cortisol release might be suppressed by feedback interactions through the activated stress axis. In particular, differences in treatments can complicate comparisons between experiments (112); therefore, we recommend measuring other parameters of the secondary and tertiary stress response to establish the ability of the animal to cope with stressors and to assess its well-being.

HEMATOLOGICAL PROFILING AND BLOOD CELL SORTING

The original technique for obtaining a differential blood count is simple and relatively inexpensive, but time-consuming and tedious (113). Hematological evaluations are usually based on blood smears and require a sound knowledge of blood cell morphology.

The vast majority of blood cells are erythrocytes, which ensure a sufficient supply of oxygen in the various body tissues. Metabolic alterations associated with physical work, excitement, and stress responses increase the tissue oxygen requirements, so large numbers of erythrocytes are additionally recruited and mobilized from depots in the spleen (114). For instance, rainbow trout (*Oncorhynchus mykiss*) responded to a 2-month food supplementation with roselle (*Hibiscus sabdariffa*) meal by significantly increasing the number of erythrocytes in the blood and simultaneously lowering the blood cortisol and glucose levels (92). By contrast, Indian major carp (*Labeo rohita*) exposed to water 6°C warmer than usual showed significantly increased glucose levels and a reduction in erythrocyte counts (115). A recent examination of the erythrocytes from grass carp (*Ctenopharyngodon idella*) did not reveal any significant alteration in numbers after 20 days of exposure to polystyrene nanoplastics, but numerous abnormalities were observed regarding the shape and size of the cells and their nuclei (95).

Adverse environmental conditions can affect the numbers and shapes of circulating erythrocytes, but they can also change the composition of leukocytes in circulation (58, 116, 117). Leukocytes are generally subdivided into monocytes, lymphocytes, and granulocytes (118). Granulocyte staining with Romanowsky/Wright or May–Grünwald–Giemsa stains can differentiate the eosinophilic, basophilic, and neutrophilic types (119, 120) that occur in tetrapods. Some fish species possess heterophilic granulocytes that are characterized by the presence of additional eosinophilic granules (121, 122).

As reported in other vertebrates (123), the ratios of certain leukocyte populations in fish blood can provide insights into the response to defined treatments or environmental variables. Stress hormones inhibit the proliferation of lymphocytes (58), the apoptosis of granulocytes (124), and the emigration of monocytes and neutrophilic granulocytes from the hematopoietic tissue of the head kidney into the peripheral blood (125). A high number of circulating leukocytes (leukocytosis) can therefore reflect an increased number of monocytes (monocytosis) and neutrophilic/heterophilic granulocytes (neutrophilia) and a concomitant decrease in the number of lymphocytes (lymphopenia) (126). The resulting dysregulation of the immune system can lead to a persistent inflammatory state in fish (127).

Although the neutrophil/heterophil-to-lymphocyte ratio is considered as an important indicator of distress across vertebrate species, it does not necessarily correlate with stress-hormone levels (128). The delayed mobilization of immune cells following the cortisol peak has been regarded as a mechanism that enables the immune system to respond once the primary threat has been overcome (129). The response of the neutrophil/heterophil-to-lymphocyte ratio to long-term environmental stressors is detectable over relatively long periods of time, in contrast to the temporary increase in hormone levels (130). Infections also have a decisive influence on the proliferation and trafficking of leukocytes (131), and the coincidence of stress and immune responses can have an antagonistic effect on the ultimate number of peripheral leukocytes (132).

Differential blood counts have been used to assess the effects of acute and chronic stress events, such as heavy metal exposure in common carp *(Cyprinus carpio)* (133), organochloride herbicide exposure in African catfish (*Clarias gairepinus*) (134), or crowding in Atlantic salmon (*Salmo salar*) (135). Nevertheless, conducting a differential blood count in fish is not very common in clinical laboratory diagnostics, often because reference values are missing.

Flow cytometry is an alternative method for studying the blood cell composition and has the advantage of high sample throughput. In the flow cytometer, individual blood cells successively pass by a laser beam, and the light they scatter is characteristic for a specific blood cell population, allowing their separation. The use of specific antibodies facilitates a more precise determination of the proportions of specific immune cell subpopulations in the blood. Fish cell sorting has depended more on cell characteristics, such as size and granularity, because of a general lack of fish-specific antibodies except for a few model fish species (7, 136).

Similar alterations in blood cell composition have been studied in different fish species exposed to various types of stress (137). For instance, in channel catfish (*Ictalurus punctatus*), the number of circulating neutrophil granulocytes increased after transportation stress (138), while the overall number of leukocytes (including lymphocytes) decreased. Another study on *I. punctatus* confirmed the occurrence of the previously observed neutrophilia simultaneously with decreasing numbers of peripheral B-lymphocytes in response to handling and transportation (**Table 1**) (65). In Gulf killifish (*Fundulus grandis*) and sea trout (*Cynoscion nebulosus*), the response to crude-oil pollution was characterized by a significantly decreased number of circulating lymphocytes and an increased number of monocytes and eosinophilic granulocytes, respectively (139). Maraena whitefish (*Coregonus maraena*) exposed to high stocking densities (100 kg/m^3) showed strong increases in the numbers of myeloid cells (granulocytes, monocytes, myeloid dendritic cells, and mast cells), whereas the number of thrombocytes was significantly reduced (**Table 1**) (32). Overall, different types of stress apparently trigger an increased mobilization of myeloid cells and a reduction in the levels of lymphocytes in the circulation of the affected fish.

HEMATOCRIT MEASUREMENTS

As outlined in the previous section, the stress-related secretion of cortisol might provoke contractions of the fish spleen to induce the release of stored erythrocytes into the peripheral blood (114). This would then elevate the volume percentage of erythrocytes, also referred to as the hematocrit value. Along with the hemoglobin (Hb) content and the leukocyte count, the hematocrit is regarded as a key indicator of the secondary stress response. The hematocrit measurement is easy and relatively inexpensive, as the collected whole blood is simply centrifuged in heparinized micro-hematocrit capillaries that are then read off a measuring template (140). A substantially greater effort is required to establish leukocyte profiles; consequently, hematocrit measurements are about 50 times more common in fish studies, as evident from our recent literature search in the Web of Science.

One important aspect for fish physiologists is the association between the hematocrit and the blood viscosity (141, 142). Wells and Weber measured the blood viscosity in *O. mykiss* kept under stressful conditions and found that a 30% hematocrit indicates an optimal relative oxygen transport capacity in the presence of a blood viscosity with a variable hematocrit but a constant Hb concentration (142). The hematocrit in *S. salar* ranges between 44 and 49% (143); however, the levels depend on the temperature (115, 144), fish strain (145), diet (91), and body weight. Large, active fish generally have a high muscular oxygen demand, which can lead to a stimulation of erythropoiesis in the head kidney (146, 147). Accordingly, the physiological hematocrit differs between fast-swimming pelagic fish and fish living sedentarily or in benthic habits (148).

Anesthesia can increase the hematocrit (149), while malnutrition, infection, or environmental toxins can reduce hematocrit and Hb values in fish (150). Non-physiologically low hematocrit values are considered hallmarks of anemia, a specific pathophysiological stress response. Anemia in fish can be easily diagnosed by examining the gills, although more detailed blood analyses help to identify the cause of anemia (53). A significantly reduced hematocrit and an increased erythropoietin production, for instance, can be observed with experimental hemolysis induced by the hemolytic compound phenylhydrazine in *S. salar* (151).

The hematocrit and Hb values associated with erythrocyte count can also depend on the electrolyte–water balance of the fish blood (152). Stressed freshwater fish undergo a drop in their plasma sodium concentrations, and this drop activates counter-transporting ion channels on the erythrocyte membranes (66). The rising ion concentration then induces an inflow of water, causing the erythrocytes to swell and increase their binding capacity for oxygen. These responses are accompanied by the release of additional erythrocytes from splenic stores to compensate for the increased oxygen demands.

MEASUREMENTS OF OSMOLALITY AND ION CONTENTS

The electrolyte–water balance in the body is termed its osmolality. The plasma osmolality and osmotic regulatory capacity are measured with an osmometer, whereas a spectrophotometer can identify the contributing ions; these are predominantly sodium, chloride, calcium, magnesium, potassium, and phosphate (46).

Freshwater fish species are hyperosmotic in relation to their habitat, whereas marine fishes are hypo-osmotic to the surrounding sea. The resulting differential osmotic pressures force teleost fishes to undergo continuous osmoregulation. Water and ions are exchanged primarily *via* the skin, gills, intestines, and kidneys (52, 153, 154), though freshwater and marine fishes have developed different strategies to maintain their internal blood osmolality within narrow limits. Osmoregulation is a persistently energy-intensive process, even in the absence of additional stress. However, since stress hormones control both the hydromineral balance and energy metabolism in fish, variations in the osmolality of the blood plasma, including changes in the ion composition, are part of the secondary stress response (155, 156). In general, aversive conditions decrease the osmolality in freshwater fish and increase osmolality in marine fish (**Table 1**) (59, 69, 70, 145, 157, 158). The exposure to high nitrite concentrations, for example, caused a significant reduction in sodium and chloride ion contents in the blood of the freshwater species yellow catfish (*Pelteobagrus fulvidraco*) (94). Catching and a subsequent 3-h-long transportation of freshwater roach (*Rutilus rutilus*) and common bream (*Abramis brama*) reduced the level of plasma sodium by one-third (66). By contrast, the osmolality significantly increased in the plasma of the marine Japanese flounder (*Paralichthys olivaceus*) after the acute exposure to air (159).

Adverse environmental conditions (e.g., hypoxia, which is often associated with high ambient temperature) require an increased branchial activity to enhance the uptake of oxygen. In (hypotonic) freshwater, this hyperventilation accelerates the loss of osmolytes. The conflicting demands of osmoregulation and respiratory gas exchange cause an increased oxygen uptake and loss of ions from the plasma in freshwater fishes. This concept of the "osmorespiratory compromise" has been well-researched in salmonids (160, 161) and hypoxia-tolerant and euryhaline fishes (162). The salinity level (162, 163), the species-specific cellular gill architecture (164), and various extreme environmental variables

(165) influence the osmoregulatory ability of fishes under stressful conditions and can maintain physiological osmolality (166, 167) and ion levels (168) in response to diverse challenges in freshwater and saltwater species in their native osmotic environments. For example, ion concentrations were unaffected in Pacific hagfish (*Eptatretus stoutii*) exposed to hypoxia (169) or in Gulf toadfish (*Opsanus beta*) stimulated with spill oil (170).

Some fishes compensate relatively quickly for increased ion flux rates within a certain range; therefore, ion concentrations and osmolality can serve as suitable indicators of acute environmental stressors (140). On the other hand, both parameters change less rapidly compared to the dynamics of stress-hormone levels, making this difference advantageous for recording post-stress responses.

ASSESSMENT OF THE HUMORAL IMMUNE CAPACITY

The response to distinct external signals involves the neuroendocrine system and the immune system (171). For decades, researchers have extracted various immune-relevant parameters from fish blood and evaluated their potential as indicators for compromised homeostasis (172). The central question addressed by these studies is the extent that stress and related adaptive responses influence immunocompetence in fish.

An initial test for assessing immunocompetence is to record the bacterial growth rate in blood plasma from treated vs. control fish (173–176). For example, the growth of the bacterium *Aeromonas salmonicida* after a 24-h incubation was significantly enhanced in plasma from *O. mykiss* with impaired immune capacity due to the exposure to high temperature coupled with crowding (177). By contrast, the non-stressed fish clearly showed potent bactericidal mechanisms that depended mainly on the concentration of a range of immunocompetent macromolecules known as humoral factors. These humoral factors consist of antimicrobial peptides, antibodies, and complement components (178) that circulate in the body fluids. Many fish physiologists have therefore examined the activity of these specific humoral factors rather than the general bactericidal activity of the plasma.

The bactericidal enzyme lysozyme and the microbe-clearing complement components are important humoral molecules of the teleostean innate immune defense (179, 180) and are frequently used as non-specific immune markers (181). In fish, lysozyme has a broader activity than its mammalian counterpart, and several complement components are present as multiple isoforms in bony fishes (182). Lysozyme and complement components are mainly synthesized by leukocytes and the liver, respectively (183–185), but they are secreted into the blood to eliminate invasive pathogens and harmful agents. However, the actual concentrations of the multiple immune factors that are synthesized and secreted into the blood of various fishes at different stages of an infection process or during a challenging situation are not known. Therefore, the measured values for supposedly one factor could actually represent multiple variants and should be interpreted with caution.

The activity of lysozyme and the complement system are measured using turbidimetric assays (186), lysoplate assays (187), lysorocket electrophoresis (188), or microplate assays (189). These assays have demonstrated, for example, that acute handling increased the activity of lysozyme in *O. mykiss* (181) and goldfish (*Carassius auratus*) (190). By contrast, lysozyme activity decreased in Siberian sturgeon (*Acipenser baerii*) after heat stress (189), in sheatfish (*Silurus glanis*) after exposure to intense halogen light (191), in Nile tilapia (*Oreochromis niloticus*) after exposure to the pesticide chlorpyrifos (91), and in *O. mykiss* after transport and exposure to chemicals (192, 193) or low stocking densities (85). Subordinate *O. niloticus* individuals had lower lysozyme activity levels than their dominant conspecifics (194). Overall, stress seems to dampen the lysozyme activity in fish (193).

The influence of stress on the complement system is ambiguous. For example, heat-stressed *A. baerii* (189) and acutely stressed *S. aurata* (195) showed increased complement activity, whereas this activity decreased in European bass (*Dicentrarchus labrax*), red porgy (*Pagrus pagrus*), and *S. aurata* exposed to crowding (84, 196–198). The complement gene families are expanded in several fishes (180, 185), and this has often been associated with newly acquired or partitioned functions of the original complement factors. Consequently, complement-involving events are likely to be more complex than indicated by the snapshot provided by a complement test result.

Other parameters have also proven significant for the assessment of fish welfare. These include the coagulation capacity of the blood (71), the antibody titer (83, 96), the phagocytic activity (32, 91), or the oxidative burst (199, 200), including the activities of the myeloperoxidase (201), superoxide dismutase (75, 202), glutathione peroxidase (93, 94), or glutathione reductase (152). Together with the bactericidal activity, these parameters provide valuable downstream information about the effects of stress hormone release on the immune performance under challenging conditions. Hormones are also likely to induce rather subtle changes in the activity of these immune parameters, either daily or seasonally, and between the sexes (203–205). Most of these analyses are carried out *ex vivo* within a limited time after the previous collection. The oxidative burst assay or phagocytic tests can also be performed *in vitro* on cultured cells (206).

IN VITRO TESTS ON PRIMARY BLOOD CELL CULTURES

The development of *in vitro* systems has allowed testing of the effect of certain stressors or challenging conditions delivered in defined doses, intensities, time frames, and/or time points. The dominant model systems for primary fish cell cultures are still derived from the head kidney and spleen (207–209); however, many protocols for blood cell cultures from different fish species have been established (210–213). These models are mainly used to investigate the influence of microbial structures/vaccines and viruses on particular immune cascades (212, 213). In addition, blood cells from fish are useful for investigating the influence of drugs or environmental toxins on the cellular homeostasis. For example, low concentrations of a halogenated hydrocarbon (once used as the insecticide lindane) were shown to increase intracellular calcium levels in peripheral blood leukocytes of *O. mykiss*, whereas high concentrations reduced the synthesis of vital cytokines and induced cell death (214). The polycyclic aromatic hydrocarbon 3-methylcholantrene stimulated the proliferation of blood leukocytes from *C. carpio*, but inhibited the lymphocyte proliferation in response to immunostimulants (215). Similarly, the toxin microcystin-LR (produced by cyanobacteria) and bisphenol A (the monomer component of polycarbonate plastics) modulated the proliferation of lymphocytes isolated from the blood of *O. mykiss* (216) and *C. auratus* (217), respectively. Leukocytes from *C. carpio* subjected to the "alkaline comet assay" have been used to assess the genotoxic potential of organic sediment by determining the DNA damage (218).

Apart from these toxicological studies, blood cell cultures are actually not suitable for modeling aquaculture-relevant problems, such as malnutrition or stocking density stress. For this reason, generalizing the data obtained from *in vitro* systems to an entire organism or a population is controversial, as the response to environmental stress is usually systemic and involves complex cellular networks and tissue systems that communicate with each other *via* stress-inducible mediators, such as steroids and amines. Only a few studies have investigated how stress hormones affect events like the *in vitro* proliferation of blood cells (219). The further development of three-dimensional cultures from fish cells (220) will bring *in vitro* data one step closer to their practical use as fish model systems.

EXPRESSION PROFILING OF SELECTED GENES IN BLOOD CELLS

Quantitative PCRs (qPCRs) detect the smallest alterations in the expression of genes (221) that are subject to modulation by environmental challenges or stressors. Importantly, gene expression profiles rarely allow absolute statements about the functioning of biological systems, for a number of reasons. One is that many genes are not completely switched on or off in response to a specific treatment or under certain environmental conditions. Instead, most treatments induce a stronger (upregulated) or a reduced (downregulated) expression of a distinct set of genes, and these expression changes only become evident when treated cells are compared with an untreated matching control. One case in point is the expression of potential thermal indicator genes that correlate with the well-studied response of many fish to suboptimal water temperatures (189). Heat stress is well-known to induce the expression of certain heat-shock protein (HSP) genes, such as *HSP70* (*HSP1A1*) and *HSP90* (*HSP90AA1*) (189, 222, 223), whereas hypothermia also induces the copy number of *HSP90* in the blood of *C. carpio* (224). The HSP-encoding transcripts have also been proposed as indicators of the potentially destructive effects of environmental toxins and pollutants. For example, the level of *HSP90* copies dropped almost by half in the blood cells of *C. carpio* exposed to cadmium for 24 h (224). By contrast, the abundance of *HSPA8* copies increased, together with the *HSP70* level, in silver sea bream (*Sparus sarba*) exposed to sublethal concentrations of cadmium for only 2 h (225).

Several investigations have also demonstrated that thermal stress modulates the expression of immune genes in the blood cells of different fish species. In particular, cytokine-encoding transcripts appear to mirror the immune status during stress (226). The classic cytokines, such as interleukins (IL), tumor necrosis factor α (TNF), interferon (IFN), and transforming growth factor (TGF), are relevant in this context (72, 226, 227). The head kidney, spleen, and liver are the usual tissue choices for quantifying immune-relevant transcripts in stimulated or stressed fish, whereas the skin, gills, and blood are used to detect impaired homeostasis. For instance, a temperature study exposed Atlantic cod (*Gadus morhua*) to water temperatures rising from 10°C to 16°C or 19°C and reported slightly increased plasma glucose and cortisol levels (73). In parallel, upregulated expression was observed for the genes encoding interleukin-1β (*IL1B*), β2-microglobulin (*B2M*), major histocompatibility complex class I (*MHC-I*), and immunoglobulin M light chain (*IGL*) in leukocytes of the thermally challenged *G. morhua* (**Table 1**). These findings were partly confirmed by a report of increased IgM-transcript levels in blood cells of orange-spotted grouper (*Epinephelus coioides*) after heat shock (77). The Indian major carp (*Catla catla*) exposed to temperatures above and below the optimum temperature of 25°C showed a significant increase in blood expression levels of immune genes coding for toll-like receptors (TLR2,−4,−5) and nucleotide binding oligomerization domain containing proteins (NOD1,−2) (**Table 1**) (74).

An expanded set of immune genes was profiled in *C. catla* exposed to an oxygen saturation below 3 ppm for 1 h (79). The increased expression of the genes coding for the transcriptional regulator high-mobility-group-box-1 protein HMBG1, the receptors TLR4 and NOD1, and their associated adapter proteins myeloid differentiation primary-response protein 88 (MYD88) and receptor interacting serine/threonine kinase 2 (RIPK2), as well as the cytokines IL6, CXCL8, and IL10 suggested an activation of early innate immune mechanisms by hypoxia (**Table 1**). Other hypoxia studies reported that considerably fewer genes were regulated in fish blood cells, and most were downregulated. For example, a 1-h exposure of *S. aurata* to hypoxic conditions with an oxygen saturation of 1.3 ppm increased hematocrit, Hb content, glucose and lactate levels, but the level of uncoupling protein 2 (*UCP2*) transcripts in the blood cells was strongly decreased (**Table 1**) (80). Another research group also investigated *S. aurata* under similarly acute hypoxic conditions but with an extended set of qPCR assays (81). They reported a significant downregulation of *UCP2* along with reduced levels of transcripts coding for antioxidant enzymes (*GST3*), outer and inner membrane translocases (*TIM44* and *TIM10*), respiratory enzyme subunits (*SCO1* and *NDUFAF2*), and also markers of mitochondrial dynamics (*MIRO1a*) and fatty acid β-oxidation (*ACAA2*) (**Table 1**). A subsequent experiment by the same group exposed *S. aurata* to a lowered oxygen saturation of 2.3 ppm combined with crowding (82). The slightly higher oxygen concentration (compared with the previous hypoxia experiment) or an antagonistic effect of hypoxia and density stress was proposed as reasons for the unexpected lack of modulation of either *UCP2* or 42 other profiled genes (**Table 1**). The exception was *NDUFAF2* (**Table 1**), which codes for an assembly factor of the Nicotinamide Adenine Dinucleotide - Hydrogen (NADH): ubiquinone oxidoreductase complex and had been included in the list of differentially regulated genes in their previous hypoxia experiment.

Gene profiling can significantly extend the list of stress response parameters beyond hematological, immunological, and metabolic types by identifying negative biomarkers whose values drop below the control levels (39, 80). A key advantage of gene profiling over the detection of stress hormones is that the stressful events that occur immediately prior to sampling (e.g., due to the capture, stunning, and killing of the animal) are usually not reflected in altered transcript levels, while the levels of ACTH or cortisol increase promptly (see section Immunoassays and Clinical Test Kits). Stress hormones are stored as preformed molecules that can be released within seconds, whereas several minutes are required for activation of the appropriate signaling pathways that culminate in the activation and nuclear transfer of the respective transcription factors that initiate the gene transcription process (228). Nonetheless, stress is mechanistically defined by a hormonal response (229), and this response cannot be adequately demonstrated at the transcript level. Without accompanying data on stress hormone levels, alterations in gene expression might only reflect the adaptive changes in the pathways that reestablish homeostasis.

A shortcoming of transcript-specific assays is that the selection of supposedly suitable parameters is left to the skill and knowledge of the experimenters. Studying the complete set of transcripts facilitates the identification of novel indicators that may not previously have been recognized as relevant. However, in truth, exploratory omics approaches are less effective in elucidating mechanistic insights than they are in generating hypotheses on how subsequent experiments can validate a selection of meaningful biomarkers.

BLOOD TRANSCRIPTOMICS

High-throughput transcriptomic approaches, such as microarray or RNA-sequencing (RNA-seq) analyses, allow monitoring of the transcriptional changes in a comprehensive panel of potential indicators for a defined research setting (230, 231). In this way, transcriptomic approaches help to arrange traditional and novel parameters into virtual pathways and/or gene networks. RNA-seq is certainly the transcriptomic method of choice over microarrays, which have only been available for a few fish species for decades (230, 232, 233). This shortage is unlikely to be overcome in the future since the availability of reference genomes from a constantly increasing number of fish species and the reduced costs of deep sequencing have now made RNA-seq analyses highly attractive. Another benefit is that RNA-seq analysis distinguishes between individual transcript variants and ohnologous genes, whereas standard PCRs and microarrays typically do not.

The blood cells of most fish species are nucleated (234), whereas the mature erythrocytes and thrombocytes of mammals lack nuclei. This fact alone makes teleostean erythrocytes and thrombocytes highly interesting for (comparative) transcriptomic analyses to record the constitutively expressed transcripts in either cell type (8, 231, 235). Moreover, erythrocytes have been proven to actively supplement allostatic reactions by the induced expression of certain genes. Most investigations have focused so far on the immune responses of erythrocytes after stimulation with pathogen-associated microbial patterns (236), bacteria (237), or viruses (238, 239).

Beyond this, the impact of only a few other stressors has been investigated on the transcriptional response of blood cells. Following an acute exposure of *O. mykiss* to a 25°C water temperature, the erythrocytes showed altered (at least 2-fold) expression of 26 genes at 4 h and 33 genes at 24 h (240). The panel of upregulated genes comprised a cluster of molecular chaperones, including the genes coding for HSP70 (constitutive and inducible forms), HSP90, and the heat-shock factor-binding protein HSBP1. The genes *HSP90*, *HSP70,* and zinc-finger AN1-type-containing protein 2B (*ZFAND2B*) were later confirmed by literature-mining approaches to represent robust biomarkers for temperature stress in different tissues of salmonid fish (241, 242). Heat stress also increased the transcription of additional stress-related genes, such as stress-induced-phosphoprotein (*STIP1*) and *JUN*, and also immune-relevant features, including NF-κB inhibitor α (*NFKBIA*) and interferon regulatory factor 1 (*IRF1*) in erythrocytes from *O. mykiss*, whereas immunoglobulin-encoding genes were downregulated. This example once again points to the close interdigitation of immune and stress pathways in teleost fishes (171, 243, 244).

High water temperatures are often associated with oxygen depletion as a co-occurring stressor. The schizothoracine fish (*Gymnocypris eckloni*) is an established model for the study of adaptation (245) and hypoxia tolerance (246). A comparison of two *G. eckloni* cohorts exposed to water containing ∼8 mg oxygen per liter (normoxia) or ∼3 mg oxygen per liter (hypoxia) for 3 days revealed differential expression of about 70 genes in the blood (q-value <0.05) of the stressed fish (246). Insulin-like-growth-factor-binding protein 1 (*IGFBP1*) was among the upregulated genes and had previously been identified in the liver of hypoxia-stressed goby fish (*Gillichthys mirabilis*) (247). The differentially expressed genes were assigned to nine Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways, including hypoxia-inducible factor 1-alpha (HIF1α) signaling and fructose/mannose metabolism. Both pathways are expected to be regulated in the context of oxygen depletion in particular and stress in general.

The transcriptome of blood samples from *C. auratus* was analyzed to identify a suitable anesthesia method for routine use in aquaculture (see section Anesthesia and Blood Sampling Procedures) (248). This study revealed that most genes were differentially regulated after percussive stunning (877 at least 2-fold regulated features, q-value <0.05), compared with two chemical anesthetics, MS-222 (487 genes) and eugenol (208 genes). Handling of *C. auratus* triggered the upregulation of a large cluster of genes involved in general stress responses (including heat and cold shock, oxidative stress, and endoplasmic reticulum stress), whereas the anesthetized groups showed comparably fewer differentially regulated stress genes. In addition, all three anesthetics effectively maintained the serum cortisol at low levels (<100 ng/ml).

Transcriptomic profiles are critical for understanding relevant functional pathways and networks, but they also have limitations. One serious problem regarding transcriptome analyses of blood samples (and in other samples from whole tissues and organs as well) is that blood is generally a very heterogeneous mixture of cells, so the transcripts represent an average over a broad range of populations. Single-cell RNA sequencing (scRNA-seq) is one step beyond whole-transcriptome analysis, as it identifies the entirety of the transcriptional changes at the level of an individual cell. The use of an scRNA-seq approach in zebrafish provided novel insights into the unique expression patterns of rare immune cell subsets in the teleostean spleen (249) and documented that scRNA-seq created multifold possibilities for recording the tailored response of distinct blood cells to a defined stressor.

BLOOD PROTEOMICS

The debate regarding how well-transcript and protein levels correlate (250, 251) is fueled by continuously published confirmatory and contradictory results. Therefore, the safest policy is to consider transcriptome and proteome datasets as complementary. Before qPCR analyses became a standard method in research, antibodies were exploited to record specific biomarkers in blood. The parameters chosen for examination were mostly the already established markers. An additional limitation was that the antibodies, which had generally been produced for mammalian antigens, needed to be cross-reactive (i.e., be able to recognize well-conserved epitope sequences). For instance, the elevated levels of HSP70 in the blood of *O. niloticus* (**Table 1**) were reported to indicate acute hypoxia (78), whereas the elevated levels of ubiquitin in the erythrocytes of blue maomao (*Scorpis violaceus*) suggested confinement stress (252). The protein analysis conducted by 2D- or differential polyacrylamide-gel electrophoresis or by liquid chromatography coupled to tandem mass spectrometry and using matrix-assisted laser desorption/ionization and time-of-flight-mass analysis, now provides more comprehensive insights into the dynamic allostatic events occurring at the protein level (253).

The effects of handling stress in *S. salar* were examined by Liu et al. (68), who profiled the O-acetylation of sialic acids in the serum. They found that the levels of di-O-acetylated sialic acids increased (**Table 1**), whereas the levels of mono-O-acetylated sialic acids decreased significantly in stressed fish (68). The exposure of Atlantic halibut (*Hippoglossus hippoglossus*) to an optimal temperature of 12°C and a suboptimal temperature of 18°C in combination with high-CO_2 water (1,000 matm) for 14 weeks resulted in increased levels of the complement component C3 and fibrinogen γ chain (FGB) in the plasma of both high CO_2-exposed groups (**Table 1**) (97). A synthesis of these two factors is triggered very early after injury and pathogen invasion. The plasma of salinity-stressed Mozambique tilapia (*Oreochromis mossambicus*) also showed high concentrations of C3, together with NADH dehydrogenase, Mg^{2+}-dependent neutral sphingomyelinase, semaphorin, and caspase-3 (254). The phagocytic activity of leukocytes from the head kidney and spleen also decreased in parallel, suggesting that both aspects might be causally connected.

BLOOD PLASMA METABOLOMICS

Metabolome research is increasingly finding its way into aquaculture research, but it still lags behind the metabolomic-based research in mammalian models (46). Most metabolite structures are identical across species, in contrast to gene and protein sequences; therefore, the analytical assays do not need to be customized for a particular investigated species (34). The metabolites found in the blood plasma include various intermediates from a wide range of biochemical pathways. For this reason, metabolomic analyses of blood serum can be used to understand nutritional (89, 90), developmental (255), or pathophysiological (256) aspects of fish physiology and are increasingly being used for disease diagnostics (257). The most commonly used analytical techniques for studying endogenous metabolite profiles are nuclear magnetic resonance spectroscopy in combination with mass spectrometry or vibrational spectroscopy (258). Most metabolomic studies that have been conducted on plasma samples from fish have dealt with toxicological questions (259–262). Application of a five-percent-by-weight concentration of heavy oil has been reported to increase the levels of several plasma metabolites, including amino acids, butyrate derivatives, creatinine, glycerol, and glucose, in *C. carpio* (259). These findings suggested a perturbed tricarboxylic acid cycle of energy metabolism. A similar conclusion was drawn following the analysis of plasma samples from zebrafish exposed to the herbicide acetamiprid (260). The insecticide chlorpyrifos was found to enhance gluconeogenesis (glucose and glycerol), fatty acid metabolism (3-D-hydroxybutyrates and acetoacetate), energy metabolism (creatine), and glutamate generation (glutamine and proline) in *C. carpio* (261). *O. mykiss* exposed to the synthetic contraceptive estrogen ethinylestradiol revealed increased vitellogenin levels, concomitant with significant changes in the plasma lipid profiles that, in turn, were attributed to the high lipid content of vitellogenin (262).

Metabolomic approaches for blood plasma analysis have also been utilized to address aquaculture-related issues. Food deprivation in juvenile *O. mykiss* increased the level of very-low-density lipoproteins while reducing the concentrations of high-density lipoproteins, choline, β-glucose, and lactate, in fasted fish (**Table 1**) (89). The daily netting of juvenile *S. salar* for 2 weeks disturbed the plasma metabolic balance, as reflected by altered levels of lipoproteins, lipids, lactate, carbohydrates, and specific amino acids (**Table 1**) (67).

The concentration of a particular enzyme does not necessarily increase or decrease (coupled to an up- or downregulated gene expression) due to varying environmental conditions, though the efficiency in converting certain metabolites may vary. Therefore, metabolic profiling can provide an alternative list of highly sensitive potential biomarkers (34) that can complement the findings of PCR-based techniques and transcriptomics or antibody-based techniques and proteomics. This type of holistic approach can help to coordinate the differentially regulated features in blood and plasma/serum samples in cases where elevated concentrations of a certain metabolite co-occur with increased levels of the associated catalyzing enzyme and with upregulated expression of the enzyme-encoding gene. However, these holistic high-level approaches (cf. **Figure 1**) remain to be performed in fish.

CONCLUSIONS

Blood contains easily accessible information about the individual physiological state of a fish. Nonetheless, blood is not the appropriate matrix for every research question; for instance, not all aspects of "welfare" can be detected in the blood. Several studies have reported the isolation of steroids from matrices other than blood (i.e., mucus, scales, feces, or water) (263–266); however, the data obtained directly from blood are still far more accurate, as the risk of rapid cortisol degradation and contamination from external cortisol sources are evidently lower (16). Most blood sampling techniques are considered minimally invasive for fish above a given size, though sampling activates primary stress responses within minutes. During the experimental manipulations, the researcher should remain aware that the sampling itself might conceal the hallmarks of a (stress) response to previous treatments, thereby biasing the interpretation of the extracted data. In general, the interpretation of blood-derived parameters requires caution, since particular physiological perturbations do not necessarily depend on a given experimental protocol. The metabolic changes, for instance, might also result from persistent chronic disturbances and/or causally independent events (e.g., circadian rhythms, seasonality, feeding times, conspecific aggressions, water quality, etc.) or substandard sampling and laboratory-specific procedures. The influence of sex and body weight/size of the individual fish should also not be underestimated. Multiple parameters should be recorded simultaneously, preferably from different analysis techniques, to disclose unsuitable husbandry conditions and to identify less obvious or previously unnoticed environmental stressors that exceed the adaptive capacity of fish. This approach supports identification of the comprehensive signature of a distinct stressor, thereby allowing valid conclusions to be drawn regarding fish welfare aspects. Unfortunately, the question of which method(s) should be used to detect the signature of a distinct stressor cannot be answered given the current state of knowledge.

This manuscript reviews different methods for recording welfare-related physiological processes in fish blood. Over the past few decades, a broad repertoire of fish-specific tools and methods has been established that enables the quantification of the concentrations of numerous hormones, metabolites, immune factors, and relevant transcripts that now supplement the panel of traditional biomarkers in blood. In the future, high-throughput -omics technologies (particularly transcriptomics, proteomics, and metabolomics) are expected to provide holistic snapshots of the physiological state of an individual. Assembling the ever-growing number of -omics puzzle pieces will ultimately provide a comprehensive picture of the metabolic, transcriptional, and immunological activities in the blood (and other tissues) of fish. Recent technological innovations, such as scRNA-seq and spheroid cell cultures, will further boost the identification of transcriptional signatures in blood cells of farmed and model fish species.

AUTHOR CONTRIBUTIONS

HS wrote sections anesthesia and blood sampling procedures, hematological profiling and blood cell sorting, hematocrit measurements, measurements of osmolality and ion contents, and assessment of the humoral immune capacity. BB wrote sections anesthesia and blood sampling procedures and immunoassays and clinical test kits. AR wrote sections hematological profiling and blood cell sorting, assessment of the humoral immune capacity, *in vitro* tests on primary blood cell cultures, expression profiling of selected genes in blood cells, blood transcriptomics, blood proteomics, and blood plasma metabolomics. All authors wrote the introduction, conclusion, and edited the entire manuscript.

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REFERENCES

1. Leal JF, Neves MGPMS, Santos EBH, Esteves VI. Use of formalin in intensive aquaculture: properties, application and effects on fish and water quality. *Rev Aquac.* (2018) 10:281–95. doi: 10.1111/raq.12160
2. Leith P, Ogier E, Haward M. Science and social license: defining environmental sustainability of Atlantic Salmon Aquaculture in South-Eastern Tasmania, Australia. *Soc Epistemol.* (2014) 28:277–96. doi: 10.1080/02691728.2014.922641
3. Dupont C, Cousin P, Dupont S. IoT for Aquaculture 4.0 Smart and easyto- deploy real-time water monitoring with IoT. In: *2018*

Global Internet of Things Summit (GIoTS) (Bilbao: IEEE). p. 1–5. doi: 10.1109/GIOTS.2018.8534581

4. Antonucci F, Costa C. Precision aquaculture: a short review on engineering innovations. *Aquac Int.* (2020) 28:41–57. doi: 10.1007/s10499-019-00443-w
5. Mylonas CC, Robles R, Tacken G, Banovic M, Krystallis A, Guerrero L, et al. New species for EU aquaculture. *Food Sci Technol.* (2019) 33:22–6. doi: 10.1002/fsat.3302_6.x
6. Fazio F, Saoca C, Costa G, Zumbo A, Piccione G, Parrino V. Flow cytometry and automatic blood cell analysis in striped bass *Morone saxatilis* (Walbaum, 1792): a new hematological approach. *Aquaculture.* (2019) 513:734398. doi: 10.1016/j.aquaculture.2019.734398
7. Korytár T, Dang Thi H, Takizawa F, Köllner B. A multicolour flow cytometry identifying defined leukocyte subsets ofrainbow trout (*Oncorhynchus mykiss*). *Fish Shellfish Immunol.* (2013) 35:2017–9. doi: 10.1016/j.fsi.2013.09.025
8. Shen Y, Wang D, Zhao J, Chen X. Fish red blood cells express immune genes and responses. *Aquac Fish.* (2018) 3:14–21. doi: 10.1016/j.aaf.2018.01.001
9. Lambert FN, Treberg JR, Anderson WG, Brandt C, Evans AN. The physiological stress response of the Atlantic stingray (*Hypanus sabinus*) to aerial exposure. *Comp Biochem Physiol A Mol Integr Physiol.* (2018) 219–220:38–43. doi: 10.1016/j.cbpa.2018.02.009
10. Field JB, Elvehjem CA, Juday C. A study of the blood constituents of carp and trout. *J Biol Chem.* (1943) 148:261–9. doi: 10.1016/S0021-9258(18)72280-1
11. Cooke SJ, Lawrence MJ, Raby GD, Teffer AK, Jeffries KM, Danylchuk AJ, et al. Comment: practices for drawing blood samples from teleost fish. *N Am J Aquac.* (2019) 81:424–6. doi: 10.1002/naaq.10115
12. Fazio F. Fish hematology analysis as an important tool of aquaculture: a review. *Aquaculture.* (2019) 500:237–42. doi: 10.1016/j.aquaculture.2018.10.030
13. Burgos-Aceves MA, Lionetti L, Faggio C. Multidisciplinary haematology as prognostic device in environmental and xenobiotic stress-induced response in fish. *Sci Total Environ.* (2019) 670:1170–83. doi: 10.1016/j.scitotenv.2019.03.275
14. Babaei F, Ramalingam R, Tavendale A, Liang Y, Yan LSK, Ajuh P, et al. Novel blood collection method allows plasma proteome analysis from Single Zebrafish. *J Proteome Res.* (2013) 12:1580–90. doi: 10.1021/pr3009226
15. Lugert V, Steinhagen D, Reiser S. Lack of knowledge does not justify a lack of action: the case for animal welfare in farmed fish. *J Sustainable Organic Agric Syst.* (2020) 70:31–4. doi: 10.3220/LBF1592499937000
16. Sadoul B, Geffroy B. Measuring cortisol, the major stress hormone in fishes. *J Fish Biol.* (2019) 94:540–55. doi: 10.1111/jfb.13904
17. Broom DM. Indicators of poor welfare. *Br Vet J.* (1986) 142:524–6. doi: 10.1016/0007-1935(86)90109-0
18. Webster J. *Animal Welfare. A Cool Eye Towards Eden*. Blackwell Science (1995). Available online at: https://www.wiley.com/en-us/Animal$+$Welfare%3A$+$A$+$Cool$+$Eye$+$Towards$+$Eden-p-9780632039289 (accessed October 12, 2020).
19. Huntingford FA, Adams C, Braithwaite VA, Kadri S, Pottinger TG, Sandoe P, et al. Current issues in fish welfare. *J Fish Biol.* (2006) 68:332–72. doi: 10.1111/j.0022-1112.2006.001046.x
20. Chrousos GP. Stress and disorders of the stress system. *Nat Rev Endocrinol.* (2009) 5:374–81. doi: 10.1038/nrendo.2009.106
21. Wendelaar Bonga SE. The stress response in fish. *Physiol Rev.* (1997) 77:591–625. doi: 10.1152/physrev.1997.77.3.591
22. Schreck CB, Tort L, Farrell AP, Brauner CB. Biology of stress in fish. In: Schreck CB, Tort L, Farrell AP, Brauner C, editors. *Biology of Stress in Fish*. Cambridge, MA: Academic Press (2016). p. iii.
23. von Borell E. Stress and coping in farm animals. *Arch Fur Tierzucht-Archives Anim Breed.* (2000) 43:144–52. doi: 10.5194/aab-43-441-2000
24. Segner H, Sundh H, Buchmann K, Douxfils J, Sundell KS, Mathieu C, et al. Health of farmed fish: its relation to fish welfare and its utility as welfare indicator. *Fish Physiol Biochem.* (2012) 38:85–105. doi: 10.1007/s10695-011-9517-9
25. Broom DM. Behaviour and welfare in relation to pathology. *Appl Anim Behav Sci.* (2006) 97:73–83. doi: 10.1016/j.applanim.2005.11.019
26. Silbergeld EK. Blood glucose: a sensitive indicator of environmental stress in fish. *Bull Environ Contam Toxicol.* (1974) 11:20–5. doi: 10.1007/BF01685023
27. Sumpter JP, Dye HM, Benfey TJ. The effects of stress on plasma ACTH, α-MSH, and cortisol levels in salmonid fishes. *Gen Comp Endocrinol.* (1986) 62:377–85. doi: 10.1016/0016-6480(86)90047-X
28. van der Vyver JSF, Kaiser H, Potts WM, James N. Using blood plasma cortisol concentration and fish behaviour to determine temperature avoidance in the estuarine-dependent fish species *Rhabdosargus holubi* (Steindachner, 1881) (Sparidae). *J Appl Ichthyol.* (2013) 29:1275–8. doi: 10.1111/jai.12268
29. Martinez-Porchas M, Martinez-Cordova LT, Ramos-Enriquez R. Cortisol and glucose: reliable indicators of fish stress? *J Aquat Sci.* (2009) 4:158–78. Available online at: https://panamjas.org/pdf_artigos/PANAMJAS_4(2)_158-178.pdf
30. Noble C, Gismervik K, Iversen MH, Kolarevic J, Nilsson J, Stien LH, et al. (2018). *Welfare Indicators for farmed Atlantic salmon: tools for assessing fish welfare An FHF-financed project, led by Nofima in partnership with*. Available online at: www.nofima.no/fishwell/english (accessed October 12, 2020).
31. Moberg GP. *Problems in defining stress and distress in animals*. (1987). Available online at: https://www.semanticscholar.org/paper/Problems-in-defining-stress-and-distress-in-Moberg/fd97479bd5536a7842a3faf1135138aa8ff33a3d (accessed October 12, 2020).
32. Korytár T, Nipkow M, Altmann S, Goldammer T, Köllner B, Rebl A. Adverse husbandry of maraena whitefish directs the immune system to increase mobilization of myeloid cells and proinflammatory responses. *Front Immunol.* (2016) 7:631. doi: 10.3389/fimmu.2016.00631
33. Rebl A, Zebunke M, Borchel A, Bochert R, Verleih M, Goldammer T. Microarray-predicted marker genes and molecular pathways indicating crowding stress in rainbow trout (*Oncorhynchus mykiss*). *Aquaculture.* (2017) 473:355–65. doi: 10.1016/j.aquaculture.2017.03.003
34. Alfaro AC, Young T. Showcasing metabolomic applications in aquaculture: a review. *Rev Aquac.* (2018) 10:135–52. doi: 10.1111/raq.12152
35. Aluru N, Vijayan MM. Stress transcriptomics in fish: a role for genomic cortisol signaling. *Gen Comp Endocrinol.* (2009) 164:142–50. doi: 10.1016/j.ygcen.2009.03.020
36. Turnbull J, Bell A, Adams C. Stocking density and welfare of cage farmed Atlantic salmon: application of a multivariate analysis. *Aquaculture*. (2005) 243:121–32. doi: 10.1016/j.aquaculture.2004.09.022
37. Pettersen JM, Bracke MBM, Midtlyng PJ, Folkedal O, Stien LH, Steffenak H, et al. Salmon welfare index model 2.0: an extended model for overall welfare assessment of caged Atlantic salmon, based on a review of selected welfare indicators and intended for fish health professionals. *Rev Aquac.* (2014) 6:162–79. doi: 10.1111/raq.12039
38. Wiseman S, Osachoff H, Bassett E, Malhotra J, Bruno J, Vanaggelen G, et al. Gene expression pattern in the liver during recovery from an acute stressor in rainbow trout. *Comp Biochem Physiol D Genom Proteomics. (*2007) 2:234–44. doi: 10.1016/j.cbd.2007.04.005
39. Krasnov A, Afanasyev S, Nylund S, Rebl A. Multigene expression assay for assessment of the immune status of Atlantic Salmon. *Genes*. (2020) 11:111236. doi: 10.3390/genes11111236
40. DePasquale C, Wagner T, Archard GA, Ferguson B, Braithwaite VA. Learning rate and temperament in a high predation risk environment. *Oecologia.* (2014) 176:661–7. doi: 10.1007/s00442-014-3099-z
41. Baker MR, Goodman AC, Santo JB, Wong RY. Repeatability and reliability of exploratory behavior in proactive and reactive zebrafish, *Danio rerio*. *Sci Rep.* (2018) 8:12114. doi: 10.1038/s41598-018-30630-3
42. van de Nieuwegiessen PG, Olwo J, Khong S, Verreth JAJ, Schrama JW. Effects of age and stocking density on the welfare of African catfish, *Clarias gariepinus* Burchell. *Aquaculture.* (2009) 288:69–75. doi: 10.1016/j.aquaculture.2008.11.009
43. Topic Popovic N, Strunjak-Perovic I, Coz-Rakovac R, Barisic J, Jadan M, Berakovic AP, et al. Review Tricaine methane-sulfonate (MS-222) application in fish anaesthesia. *J Appl Ichthyol.* (2012) 28:553–64. doi: 10.1111/j.1439-0426.2012.01950.x
44. Uehara SA, Andrade DR, Takata R, Gomes Júnior AV, Vidal MV. The effectiveness of tricaine, benzocaine, clove oil, and menthol as anesthetics for lambari-bocarra *Oligosarcus argenteus*. *Aquaculture.* (2019) 502:326–31. doi: 10.1016/j.aquaculture.2018.12.054
45. Lawrence MJ, Raby GD, Teffer AK, Jeffries KM, Danylchuk AJ, Eliason EJ, et al. Best practices for non-lethal blood sampling of fish *via*

the caudal vasculature. *J Fish Biol.* (2020) 97:4–15. doi: 10.1111/jfb.14339
46. Young T, Walker SP, Alfaro AC, Fletcher LM, Murray JS, Lulijwa R, et al. Impact of acute handling stress, anaesthesia, and euthanasia on fish plasma biochemistry: implications for veterinary screening and metabolomic sampling. *Fish Physiol Biochem.* (2019) 45:1485–94. doi: 10.1007/s10695-019-00669-8
47. Teixeira N dos S, Marques LS, Rodrigues RB, Gusso D, Fossati AAN, Streit DP. Effects of anesthetic tricaine on stress and reproductive aspects of South American silver catfish (*Rhamdia quelen*) male. *bioRxiv.* (2019) 759340. doi: 10.1101/759340
48. Molinero A, Gonzalez J. Comparative effects of MS 222 and 2-phenoxyethanol on gilthead sea bream (*Sparus aurata* L.) during confinement. *Comp Biochem Physiol A Physiol.* (1995) 111:405–14. doi: 10.1016/0300-9629(95)00037-8
49. Deebani A, Iyer N, Raman R, Jagadeeswaran P. Effect of MS222 on hemostasis in zebrafish. *J Am Assoc Lab Anim Sci.* (2019) 58:390–6. doi: 10.30802/AALAS-JAALAS-18-000069
50. Smith LS. Blood volumes of three salmonids. *J Fish Res Board Canada.* (1966) 23:1439–46. doi: 10.1139/f66-129
51. Tort L, González-Arch F, Torres P, Hidalgo J. On the blood volume of the Mediterranean dogfish, *Scyliorhinus canicula*. *Fish Physiol Biochem.* (1991) 9:173–7. doi: 10.1007/BF02265133
52. Olson KR. "Blood and extracellular fluid volume regulation: Role of the renin-angiotensin system, kallikrein-kinin system, and atrial natriuretic peptides," in Fish Physiology (Elsevier Inc.), 135–254. doi: 10.1016/S1546-5098(08)60010-2
53. Noga EJ. "Major Cultured Species," in Fish Disease (Ames, Iowa USA: Blackwell Publishing, Inc.), 1–8. doi: 10.1002/9781118786758.ch1
54. Chakraborty S, Rout SK, Anupama RR, Milli K, Sona RR, Behera L. Response of salinity (brine solution) induced stress on cortisol hormone in Indian major carp, *Labeo rohita*. *J Exp Zool India.* (2017) 20:1377–81. Available online at: http://www.connectjournals.com/toc.php?bookmark=CJ-033215&did=Supplement&volume=20&year=2017&issue_id=Supplement&issue_month=October
55. Bando K, Kawahara R, Kunimatsu T, Sakai J, Kimura J, Funabashi H, et al. Influences of biofluid sample collection and handling procedures on GC-MS based metabolomic studies. *J Biosci Bioeng.* (2010) 110:491–9. doi: 10.1016/j.jbiosc.2010.04.010
56. Duman M, Saticioglu IB, Suzer B, Altun S. Practices for drawing blood samples from teleost fish. *N Am J Aquac.* (2019) 81:119–25. doi: 10.1002/naaq.10077
57. Tuck MK, Chan DW, Chia D, Godwin AK, Grizzle WE, Krueger KE, et al. Standard operating procedures for serum and plasma collection: early detection research network consensus statement standard operating procedure integration working group. *J Proteome Res.* (2009) 8:113–7. doi: 10.1021/pr800545q
58. Barton BA, Iwama GK. Physiological changes in fish from stress in aquaculture with emphasis on the response and effects of corticosteroids. *Annu Rev Fish Dis.* (1991) 1:3–26. doi: 10.1016/0959-8030(91)90019-G
59. Barton BA. Stress in fishes: a diversity of responses with particular reference to changes in circulating corticosteroids. *Integr Comp Biol.* (2002) 42:517–25. doi: 10.1093/icb/42.3.517
60. Martos-Sitcha JA, Wunderink YS, Straatjes J, Skrzynska AK, Mancera JM, Martínez-Rodríguez G. Different stressors induce differential responses of the CRH-stress system in the gilthead sea bream (*Sparus aurata*). *Comp Biochem Physiol A Mol Integr Physiol.* (2014) 177:49–61. doi: 10.1016/j.cbpa.2014.07.021
61. Wu H, Ohnuki H, Hibi K, Ren H, Endo H. Development of a label-free immunosensor system for detecting plasma cortisol levels in fish. *Fish Physiol Biochem.* (2016) 42:19–27. doi: 10.1007/s10695-015-0113-2
62. Blahová J, Dobšíková R, Svobodová Z, Kaláb P. Simultaneous determination of plasma cortisol by high pressure liquid chromatography and radioimmunoassay methods in fish. *Acta Vet Brno.* (2007) 76:59–64. doi: 10.2754/avb200776010059
63. Raposo De Magalhães C, Schrama D, Farinha AP, Revets D, Kuehn A, et al. Protein changes as robust signatures of fish chronic stress: a proteomics approach to fish welfare research. *BMC Genom.* (2020) 21:309. doi: 10.1186/s12864-020-6728-4
64. Tschmelak J, Proll G, Gauglitz G. Verification of performance with the automated direct optical TIRF immunosensor (River Analyser) in single and multi-analyte assays with real water samples. *Biosens Bioelectron.* (2004) 20:743–52. doi: 10.1016/j.bios.2004.04.006
65. Ainsworth AJ, Dexiang C, Waterstrat PR. Changes in peripheral blood leukocyte percentages and function of neutrophils in stressed channel catfish. *J Aquat Anim Health.* (1991) 3:41–7. doi: 10.1577/1548-8667(1991)003<0041:CIPBLP>2.3.CO;2
66. Martemyanov VI. Patterns of changes in sodium content in plasma and erythrocytes of freshwater fish at stress. *J Ichthyol.* (2013) 53:220–4. doi: 10.1134/S0032945213020094
67. Karakach TK, Huenupi EC, Soo EC, Walter JA, Afonso LOB. 1H-NMR and mass spectrometric characterization of the metabolic response of juvenile Atlantic salmon (*Salmo salar*) to long-term handling stress. *Metabolomics.* (2009) 5:123–37. doi: 10.1007/s11306-008-0144-0
68. Liu X, Afonso L, Altman E, Johnson S, Brown L, Li J. O-acetylation of sialic acids in N-glycans of Atlantic salmon (*Salmo salar*) serum is altered by handling stress. *Proteomics.* (2008) 8:2849–57. doi: 10.1002/pmic.200701093
69. Pickering AD, Pottinger TG, Christie P. Recovery of the brown trout, *Salmo trutta* L., from acute handling stress: a time-course study. *J Fish Biol.* (1982) 20:229–44. doi: 10.1111/j.1095-8649.1982.tb03923.x
70. Costas B, Conceição LEC, Aragão C, Martos JA, Ruiz-Jarabo I, Mancera JM, et al. Physiological responses of Senegalese sole (*Solea senegalensis* Kaup, 1858) after stress challenge: effects on non-specific immune parameters, plasma free amino acids and energy metabolism. *Aquaculture.* (2011) 316:68–76. doi: 10.1016/j.aquaculture.2011.03.011
71. Casillas E, Smith LS. Effect of stress on blood coagulation and haematology in rainbow trout (*Salmo gairdneri*). *J Fish Biol.* (1977) 10:481–91. doi: 10.1111/j.1095-8649.1977.tb04081.x
72. Stone DAJ, Gaylord TG, Johansen KA, Overturf K, Sealey WM, Hardy RW. Evaluation of the effects of repeated fecal collection by manual stripping on the plasma cortisol levels, TNF-α gene expression, and digestibility and availability of nutrients from hydrolyzed poultry and egg meal by rainbow trout, *Oncorhynchus mykiss* (Walbaum). *Aquaculture.* (2008) 275:250–9. doi: 10.1016/j.aquaculture.2008.01.003
73. Pérez-Casanova JC, Rise ML, Dixon B, Afonso LOB, Hall JR, Johnson SC, et al. The immune and stress responses of Atlantic cod to long-term increases in water temperature. *Fish Shellfish Immunol.* (2008) 24:600–9. doi: 10.1016/j.fsi.2008.01.012
74. Basu M, Paichha M, Swain B, Lenka SS, Singh S, Chakrabarti R, et al. Modulation of TLR2, TLR4, TLR5, NOD1 and NOD2 receptor gene expressions and their downstream signaling molecules following thermal stress in the Indian major carp catla (*Catla catla*). *Biotech.* (2015) 5:1021–30. doi: 10.1007/s13205-015-0306-5
75. Roche H, Bogé G. Fish blood parameters as a potential tool for identification of stress caused by environmental factors and chemical intoxication. *Mar Environ Res.* (1996) 41:27–43. doi: 10.1016/0141-1136(95)00015-1
76. Pinto RD, Nascimento DS, Reis MIR, do Vale A, dos Santos NMS. Molecular characterization, 3D modelling and expression analysis of sea bass (*Dicentrarchus labrax* L.) interleukin-10. *Mol Immunol.* (2007) 44:2056–65. doi: 10.1016/j.molimm.2006.09.014
77. Cui M, Zhang Q, Yao Z, Zhang Z, Zhang H, Wang Y. Immunoglobulin M gene expression analysis of orange-spotted grouper, *Epinephelus coioides*, following heat shock and *Vibrio alginolyticus* challenge. *Fish Shellfish Immunol.* (2010) 29:1060–5. doi: 10.1016/j.fsi.2010.08.018
78. Delaney MA, Klesius PH. Hypoxic conditions induce Hsp70 production in blood, brain and head kidney of juvenile Nile tilapia *Oreochromis niloticus* (L.). *Aquaculture.* (2004) 236:633–44. doi: 10.1016/j.aquaculture.2004.02.025
79. Basu M, Paichha M, Lenka SS, Chakrabarty R, Samanta M. Hypoxic stress: impact on the modulation of TLR2, TLR4, NOD1 and NOD2 receptor and their down-stream signalling genes expression in catla (*Catla catla*). *Mol Biol Rep.* (2016) 43:1–9. doi: 10.1007/s11033-015-3932-4
80. Bermejo-Nogales A, Calduch-Giner JA, Pérez-Sánchez J. Tissue-specific gene expression and functional regulation of uncoupling protein 2 (UCP2) by hypoxia and nutrient availability in gilthead sea bream (*Sparus aurata*):

implications on the physiological significance of UCP1-3 variants. *Fish Physiol Biochem.* (2014) 40:751–62. doi: 10.1007/s10695-013-9882-7

81. Martos-Sitcha JA, Bermejo-Nogales A, Calduch-Giner JA, Pérez-Sánchez J. Gene expression profiling of whole blood cells supports a more efficient mitochondrial respiration in hypoxia-challenged gilthead sea bream (*Sparus aurata*). *Front Zool.* (2017) 14:1–12. doi: 10.1186/s12983-017-0220-2
82. Martos-Sitcha JA, Simó-Mirabet P, de las Heras V, Calduch-Giner JÀ, Pérez-Sánchez J. Tissue- specific orchestration of gilthead sea bream resilience to hypoxia and high stocking density. *Front Physiol.* (2019) 10:1–18. doi: 10.3389/fphys.2019.00840
83. Liu B, Liu Y, Wang X. The effect of stocking density on growth and seven physiological parameters with assessment of their potential as stress response indicators for the Atlantic salmon (*Salmo salar*). *Mar Freshw Behav Physiol.* (2015) 48:177–92. doi: 10.1080/10236244.2015.1034956
84. Montero D, Tort L, Robaina L, Vergara JM, Izquierdo MS. Low vitamin E in diet reduces stress resistance of gilthead seabream (*Sparus aurata*) juveniles. *Fish Shellfish Immunol.* (2001) 11:473–90. doi: 10.1006/fsim.2000.0324
85. North B, Turnbull J, Ellis T. The impact of stocking density on the welfare of rainbow trout (*Oncorhynchus mykiss*). *Aquaculture.* (2006) 255:466–79. doi: 10.1016/j.aquaculture.2006.01.004
86. Yarahmadi P, Miandare HK, Hoseinifar SH, Gheysvandi N, Akbarzadeh A. The effects of stocking density on hemato-immunological and serum biochemical parameters of rainbow trout (*Oncorhynchus mykiss*). *Aquac Int.* (2014) 23:55–63. doi: 10.1007/s10499-014-9797-z
87. Conde-Sieira M, Aguilar AJ, López-Patiño MA, Míguez JM, Soengas JL. Stress alters food intake and glucosensing response in hypothalamus, hindbrain, liver, and Brockmann bodies of rainbow trout. *Physiol Behav.* (2010) 101:483–93. doi: 10.1016/j.physbeh.2010.07.016
88. Vijayan MM, Ballantyne JS, Leatherland JF. High stocking density alters the energy metabolism of brook charr, Salvelinus fontinalis. *Aquaculture.* (1990) 88:371–81. doi: 10.1016/0044-8486(90)90162-G
89. Kullgren A, Samuelsson LM, Larsson DGJ, Björnsson BT, Bergman EJ. A metabolomics approach to elucidate effects of food deprivation in juvenile rainbow trout (*Oncorhynchus mykiss*). *Am J Physiol.* (2010) 299:1440–8. doi: 10.1152/ajpregu.00281.2010
90. Roques S, Deborde C, Richard N, Marchand Y, Larroquet L, Prigent S, et al. Proton-NMR Metabolomics of rainbow trout fed a plant-based diet supplemented with graded levels of a protein-rich yeast fraction reveal several metabolic processes involved in growth. *J Nutr.* (2020) 150:2268–77. doi: 10.1093/jn/nxaa206
91. Dawood MAO, El-Salam Metwally A, Elkomy AH, Gewaily MS, Abdo SE, Abdel-Razek MAS, Soliman AA, Amer AA, Abdel-Razik NI, Abdel-Latif HMR, et al. The impact of menthol essential oil against inflammation, immunosuppression, and histopathological alterations induced by chlorpyrifos in Nile tilapia. Fish Shellfish Immunol (2020) 102:316–25. doi: 10.1016/j.fsi.2020.04.059
92. Hoseini SM, Hoseinifar SH, Van Doan H. Growth performance and hematological and antioxidant characteristics of rainbow trout, *Oncorhynchus mykiss*, fed diets supplemented with Roselle, *Hibiscus sabdariffa*. *Aquaculture.* (2021) 530:735827. doi: 10.1016/j.aquaculture.2020.735827
93. Enis Yonar M, Mişe Yonar S, Silici S. Protective effect of propolis against oxidative stress and immunosuppression induced by oxytetracycline in rainbow trout (*Oncorhynchus mykiss*, W.). *Fish Shellfish Immunol.* (2011) 31:318–25. doi: 10.1016/j.fsi.2011.05.019
94. Zhang M, Yin X, Li M, Wang R, Qian Y, Hong M. Effect of nitrite exposure on haematological status, oxidative stress, immune response and apoptosis in yellow catfish (*Pelteobagrus fulvidraco*). *Comp Biochem Physiol C Toxicol Pharmacol.* (2020) 238:108867. doi: 10.1016/j.cbpc.2020.108867
95. Guimarães ATB, Estrela FN, Pereira PS, de Andrade Vieira JE, de Lima Rodrigues AS, Silva FG, et al. Toxicity of polystyrene nanoplastics in *Ctenopharyngodon idella* juveniles: a genotoxic, mutagenic and cytotoxic perspective. *Sci Total Environ.* (2021) 752:141937. doi: 10.1016/j.scitotenv.2020.141937
96. Markkula E, Salo HM, Rikalainen K, Jokinen IE. Long-term UVB irradiation affects the immune functions of carp (*Cyprinus carpio*) and rainbow trout (*Oncorhynchus mykiss*). *Photochem Photobiol.* (2009) 85:347–52. doi: 10.1111/j.1751-1097.2008.00446.x
97. De Souza KB, Jutfelt F, Kling P, Förlin L, Sturve J. Effects of increased CO_2 on fish gill and plasma proteome. *PLoS ONE.* (2014) 9:102901. doi: 10.1371/journal.pone.0102901
98. Archard GA, Braithwaite VA. Increased exposure to predators increases both exploration and activity level in Brachyrhaphis episcopi. *J Fish Biol.* (2011) 78:593–601. doi: 10.1111/j.1095-8649.2010.02880.x
99. Acerete L, Balasch J., Espinosa E, Josa A, Tort L. Physiological responses in Eurasian perch (*Perca fluviatilis*, L.) subjected to stress by transport and handling. *Aquaculture.* (2004) 237:167–78. doi: 10.1016/j.aquaculture.2004.03.018
100. Ashley PJ. Fish welfare: current issues in aquaculture. *Appl Anim Behav Sci.* (2007) 104:199–235. doi: 10.1016/j.applanim.2006.09.001
101. Arends RJ, Mancera JM, Muñoz JL, Wendelaar Bonga SE, Flik G. The stress response of the gilthead sea bream (*Sparus aurata* L.) to air exposure and confinement. *J Endocrinol.* (1999) 163:149–57. doi: 10.1677/joe.0.1630149
102. Molinero A, Gomez E, Balasch J, Tort L. Stress by fish removal in the Gilthead Sea Bream, *Sparus aurata*: a time course study on the remaining fish in the same tank. *J Appl Aquac.* (1997) 7:1–12. doi: 10.1300/J028v07n02_01
103. Di Marco P, Priori A, Finoia MG, Massari A, Mandich A, Marino G. Physiological responses of European sea bass *Dicentrarchus labrax* to different stocking densities and acute stress challenge. *Aquaculture.* (2008) 275:319–28. doi: 10.1016/j.aquaculture.2007.12.012
104. Sánchez-Muros MJ, Villacreces S, Miranda-de la Lama G, de Haro C, García-Barroso F. Effects of chemical and handling exposure on fatty acids, oxidative stress and morphological welfare indicators in gilt-head sea bream (*Sparus aurata*). *Fish Physiol Biochem.* (2013) 39:581–91. doi: 10.1007/s10695-012-9721-2
105. Assem H, Khalifa A, ELSalhia M. Physiological and microbiological indices as indicators of evaluating dietary fungi degraded date pits as a probiotic for cultured Nile tilapia *Oreochromis niloticus* fingerling and its effect on fish welfare. *Egypt J Aquat Res.* (2014) 40:435–41. doi: 10.1016/j.ejar.2014.10.004
106. Ellis T, Yildiz HY, López-Olmeda J, Spedicato MT, Tort L, Øverli Ø, et al. Cortisol and finfish welfare. *Fish Physiol Biochem.* (2012) 38:163–88. doi: 10.1007/s10695-011-9568-y
107. López-Patiño MA, Gesto M, Conde-Sieira M, Soengas JL, Míguez JM. Stress inhibition of melatonin synthesis in the pineal organ of rainbow trout (*Oncorhynchus mykiss*) is mediated by cortisol. *J Exp Biol.* (2014) 217:1407–16. doi: 10.1242/jeb.087916
108. Manuel R, Boerrigter JGJ, Cloosterman M, Gorissen M, Flik G, van den Bos R, et al. Effects of acute stress on aggression and the cortisol response in the African sharptooth catfish *Clarias gariepinus*: differences between day and night. *J Fish Biol.* (2016) 88:2175–87. doi: 10.1111/jfb.12989
109. Almazan-Rueda P, van Helmond ATM, Verreth JAJ, Schrama JW. Photoperiod affects growth, behaviour and stress variables in *Clarias gariepinus*. *J Fish Biol.* (2005) 67:1029–39. doi: 10.1111/j.0022-1112.2005.00806.x
110. Pottinger TG, Carrick TR. Modification of the plasma cortisol response to stress in rainbow trout by selective breeding. *Gen Comp Endocrinol.* (1999) 116:122–32. doi: 10.1006/gcen.1999.7355
111. Pottinger TG, Moran TA, Morgan JAW. Primary and secondary indices of stress in the progeny of rainbow trout (*Oncorhynchus mykiss*) selected for high and low responsiveness to stress. *J Fish Biol.* (1994) 44:149–63. doi: 10.1111/j.1095-8649.1994.tb01591.x
112. Ellis T, Berrill I, Lines J, Turnbull JF, Knowles TG. Mortality and fish welfare. *Fish Physiol Biochem.* (2012) 38:189–99. doi: 10.1007/s10695-011-9547-3
113. Blaxhall PC, Daisley KW. Routine haematological methods for use with fish blood. *J Fish Biol.* (1973) 5:771–81. doi: 10.1111/j.1095-8649.1973.tb04510.x
114. Pearson MP, Stevens ED. Size and hematological impact of the splenic erythrocyte reservoir in rainbow trout, *Oncorhynchus mykiss*. *Fish Physiol Biochem.* (1991) 9:39–50. doi: 10.1007/BF01987610
115. Ashaf-Ud-Doulah M, Mamun AAl, Rahman ML, Islam SMM, Jannat R, Hossain MAR, et al. High temperature acclimation alters upper thermal limits and growth performance of Indian major carp, rohu, *Labeo rohita* (Hamilton, 1822). *J Therm Biol.* (2020) 93:102738. doi: 10.1016/j.jtherbio.2020.102738

116. Wojtaszek J, Dziewulska-Szwajkowska D, Łozińska-Gabska M, Adamowicz A, Dzugaj A. hematological effects of high dose of cortisol on the carp (*Cyprinus carpio* L.): cortisol effect on the carp blood. *Gen Comp Endocrinol.* (2002) 125:176–83. doi: 10.1006/gcen.2001.7725
117. Pulsford AL, Lemaire-Gony S, Tomlinson M, Collingwood N, Glynn PJ. Effects of acute stress on the immune system of the dab, *Limanda limanda*. *Comp Biochem Physiol C Pharmacol Toxicol Endocrinol.* (1994) 109:129–39. doi: 10.1016/0742-8413(94)00053-D
118. Ainsworth AJ. Fish granulocytes: morphology, distribution, and function. *Annu Rev Fish Dis.* (1992) 2:123–48. doi: 10.1016/0959-8030(92)90060-B
119. Balla KM, Lugo-Villarino G, Spitsbergen JM, Stachura DL, Hu Y, Bañuelos K, et al. Eosinophils in the zebrafish: prospective isolation, characterization, and eosinophilia induction by helminth determinants. *Blood.* (2010) 116:3944–54. doi: 10.1182/blood-2010-03-267419
120. Nakada K, Fujisawa K, Horiuchi H, Furusawa S. Studies on morphology and cytochemistry in blood cells of ayu *Plecoglossus altivelis* altivelis. *J Vet Med Sci.* (2014) 76:693–704. doi: 10.1292/jvms.13-0584
121. Hine PM. The granulocytes of fish. *Fish Shellfish Immunol.* (1992) 2:79–98. doi: 10.1016/S1050-4648(05)80038-5
122. Dikić D, Lisičí D, Matić-Skoko S, Tutman P, Skaramuca D, Franić Z, et al. Comparative hematology of wild Anguilliformes (*Muraena helena*, L. 1758, *Conger conger*, L. 1758 and *Anguilla anguilla* L. 1758). *Anim Biol.* (2013) 63:77–92. doi: 10.1163/15707563-00002395
123. Silva MB da, Fraga RE, Nishiyama PB, Silva ISS da, Costa NLB, de Oliveira LAA, et al. Leukocyte profiles in *Odontophrynus carvalhoi* (Amphibia: Odontophrynidae) tadpoles exposed to organophosphate chlorpyrifos pesticides. *Water Air Soil Pollut.* (2020) 231:372. doi: 10.1007/s11270-020-04726-4
124. Weyts FA, Verburg-van Kemenade BM, Flik G. Characterisation of glucocorticoid receptors in peripheral blood leukocytes of Carp, *Cyprinus carpio* L. *Gen Comp Endocrinol.* (1998) 111:1–8. doi: 10.1006/gcen.1998.7080
125. Ortuño J, Esteban MA, Meseguer J. Effects of short-term crowding stress on the gilthead seabream (*Sparus aurata* L) innate immune response. *Fish Shellfish Immunol.* (2001) 11:187–97. doi: 10.1006/fsim.2000.0304
126. Pickering AD. Cortisol-induced lymphocytopenia in brown trout, *Salmo trutta* L. *Gen Comp Endocrinol.* (1984) 53:252–9. doi: 10.1016/0016-6480(84)90250-8
127. Weyts FAA, Cohen N, Flik G, Verburg-van Kemenade BML. Interactions between the immune system and the hypothalamo-pituitary-interrenal axis in fish. *Fish Shellfish Immunol.* (1999) 9:1–20. doi: 10.1006/fsim.1998.0170
128. Davis AK, Maney DL. The use of glucocorticoid hormones or leucocyte profiles to measure stress in vertebrates: what's the difference? *Methods Ecol Evol.* (2018) 9:1556–68. doi: 10.1111/2041-210X.13020
129. Frank MG, Watkins LR, Maier SF. Stress-induced glucocorticoids as a neuroendocrine alarm signal of danger. *Brain Behav Immun.* (2013) 33:1–6. doi: 10.1016/j.bbi.2013.02.004
130. Goessling JM, Kennedy H, Mendonça MT, Wilson AE. A meta-analysis of plasma corticosterone and heterophil: lymphocyte ratios - is there conservation of physiological stress responses over time? *Funct Ecol.* (2015) 29:1189–96. doi: 10.1111/1365-2435.12442
131. Katakura F, Nishiya K, Wentzel AS, Hino E, Miyamae J, Okano M, et al. Paralogs of common carp granulocyte colony-stimulating factor (G-CSF) have different functions regarding development, trafficking and activation of neutrophils. *Front Immunol.* (2019) 10:255. doi: 10.3389/fimmu.2019.00255
132. Kepka M, Verburg-van Kemenade BML, Chadzinska M. Neuroendocrine modulation of the inflammatory response in common carp: adrenaline regulates leukocyte profile and activity. *Gen Comp Endocrinol.* (2013) 188:102–9. doi: 10.1016/j.ygcen.2012.11.014
133. Vinodhini R, Narayanan M. The impact of toxic heavy metals on the hematological parameters in common carp (*Cyprinus carpio* l.). *Iran J Environ Heal Sci Eng.* (2009) 6:23–8. Available online at: https://www.semanticscholar.org/paper/THE-IMPACT-OF-TOXIC-HEAVY-METALS-ON-THE-PARAMETERS-Vinodhini-Narayanan/5390407c1f96e4ea1c3457c19485d12fe3546449
134. Oluah NS, Aguzie IO, Ekechukwu NE, Madu JC, Ngene CI, Oluah C. Hematological and immunological responses in the African catfish *Clarias gairepinus* exposed to sublethal concentrations of herbicide Ronstar®. *Ecotoxicol Environ Saf.* (2020) 201:110824. doi: 10.1016/j.ecoenv.2020.110824
135. Delfosse C, Pageat P, Lafont-Lecuelle C, Asproni P, Chabaud C, Cozzi A, et al. Effect of handling and crowding on the susceptibility of Atlantic salmon (*Salmo salar* L.) to *Lepeophtheirus salmonis* (Krøyer) copepodids. *J Fish Dis.* (2020) 6:jfd.13286. doi: 10.1111/jfd.13286
136. Gansner JM, Leung AD, Superdock M, Blair MC, Ammerman MB, Durand EM, et al. Sorting zebrafish thrombocyte lineage cells with a Cd41 monoclonal antibody enriches hematopoietic stem cell activity. *Blood.* (2017) 129:1394–7. doi: 10.1182/blood-2016-12-759993
137. Morgan JAW, Pottinger TG, Rippon P. Evaluation of flow cytometry as a method for quantification of circulating blood cell populations in salmonid fish. *J Fish Biol.* (1993) 42:131–41. doi: 10.1111/j.1095-8649.1993.tb00311.x
138. Ellsaesser CF, Clem LW. Haematological and immunological changes in channel catfish stressed by handling and transport. *J Fish Biol.* (1986) 28:511–21. doi: 10.1111/j.1095-8649.1986.tb05187.x
139. Ali AO, Hohn C, Allen PJ, Ford L, Dail MB, Pruett S, et al. The effects of oil exposure on peripheral blood leukocytes and splenic melano-macrophage centers of Gulf of Mexico fishes. *Mar Pollut Bull.* (2014) 79:87–93. doi: 10.1016/j.marpolbul.2013.12.036
140. Sopinka NM, Donaldson MR, O'Connor CM, Suski CD, Cooke SJ. Stress indicators in fish. In: Schreck CB, Tort L, Farrell AP, Brauner CJ, editors. *Biology of Stress in Fish* (Amsterdam: Elsevier Inc.). p. 405–62. doi: 10.1016/B978-0-12-802728-8.00011
141. Rand PW, Lacombe E, Hunt HE, Austin WH. Viscosity of normal human blood under normothermic and hypothermic conditions. *J Appl Physiol.* (1964) 19:117–22. doi: 10.1152/jappl.1964.19.1.117
142. Wells RMG, Weber RE. Is there an optimal haematocrit for rainbow trout, *Oncorhynchus mykiss* (Walbaum)? An interpretation of recent data based on blood viscosity measurements. *J Fish Biol.* (1991) 38:53–65. doi: 10.1111/j.1095-8649.1991.tb03090.x
143. Sandnes K, Lie O, Waagbo R. Normal ranges of some blood chemistry parameters in adult farmed Atlantic salmon, *Salmo salar*. *J Fish Biol.* (1988) 32:129–36. doi: 10.1111/j.1095-8649.1988.tb05341.x
144. Sambraus F, Olsen RE, Remen M, Hansen TJ, Torgersen T, Fjelldal PG. Water temperature and oxygen: the effect of triploidy on performance and metabolism in farmed Atlantic salmon (*Salmo salar* L.) post-smolts. *Aquaculture.* (2017) 473:1–12. doi: 10.1016/j.aquaculture.2017.01.024
145. Iversen M, Finstad B, Nilssen KJ. Recovery from loading and transport stress in Atlantic salmon (*Salmo salar* L.) smolts. *Aquaculture.* (1998) 168:387–94. doi: 10.1016/S0044-8486(98)00364-0
146. Putnam RW, Freel RW. Hematological parameters of five species of marine fishes. Comp Biochem Physiol Part A Physiol (1978) 61:585–8. doi: 10.1016/0300-9629(78)90132-9
147. Jawad LA, Al-Mukhtar MA, Ahmed HK. The relationship between haematocrit and some biological parameters of the Indian shad, *Tenualosa ilisha* (Family Clupeidae). *Anim Biodivers Conserv.* (2004) 27:47–52. Available online at: http://abc.museucienciesjournals.cat/volume-27-2-2004-abc/the-relationship-between-haematocrit-and-some-biological-parameters-of-the-indian-shad-tenualosa-ilisha-family-clupeidae-2/?lang=en
148. Wells RMG, Davie PS. Oxygen binding by the blood and hematological effects of capture stress in two big gamefish: Mako shark and striped marlin. *Comp Biochem Physiol A Physiol.* (1985) 81:643–6. doi: 10.1016/0300-9629(85)91041-2
149. Phuong LM, Damsgaard C, Huong DTT, Ishimatsu A, Wang T, Bayley M. Recovery of blood gases and haematological parameters upon anaesthesia with benzocaine, MS-222 or Aqui-S in the air-breathing catfish *Pangasianodon hypophthalmus*. *Ichthyol Res.* (2017) 64:84–92. doi: 10.1007/s10228-016-0545-4
150. Witeska M. Anemia in teleost fishes. *Bull Eur Assoc Fish Pathol.* (2015) 35:148–60. Available online at: https://eafp.org/download/2015-volume35/issue_4/35-4-148-witeska.pdf
151. Krasnov A, Timmerhaus G, Afanasyev S, Takle H, Jørgensen SM. Induced erythropoiesis during acute anemia in Atlantic salmon: a transcriptomic survey. *Gen Comp Endocrinol.* (2013) 192:181–90. doi: 10.1016/j.ygcen.2013.04.026

152. Islam MJ, Kunzmann A, Thiele R, Slater MJ. Effects of extreme ambient temperature in European seabass, *Dicentrarchus labrax* acclimated at different salinities: growth performance, metabolic and molecular stress responses. *Sci Total Environ.* (2020) 735:139371. doi: 10.1016/j.scitotenv.2020.139371
153. Varsamos S, Flik G, Pepin JF, Bonga SEW, Breuil G. Husbandry stress during early life stages affects the stress response and health status of juvenile sea bass, *Dicentrarchus labrax*. *Fish Shellfish Immunol.* (2006) 20:83–96. doi: 10.1016/j.fsi.2005.04.005
154. Whittamore JM. Osmoregulation and epithelial water transport: lessons from the intestine of marine teleost fish. *J Comp Physiol B.* (2012) 182:1–39. doi: 10.1007/s00360-011-0601-3
155. Veiseth E, Fjaera SO, Bjerkeng B, Skjervold PO. Accelerated recovery of Atlantic salmon (*Salmo salar*) from effects of crowding by swimming. *Comp Biochem Physiol B Biochem Mol Biol.* (2006) 144:351–8. doi: 10.1016/j.cbpb.2006.03.009
156. Robertson L, Thomas P, Arnold CR, Trant JM. Plasma cortisol and secondary stress responses of red drum to handling, transport, rearing density, and a disease outbreak. *Progress Fish-Culturist.* (1987) 49:1–12. doi: 10.1577/1548-8640(1987)49<1:PCASSR>2.0.CO;2
157. Liebert AM, Schreck CB. Effects of acute stress on osmoregulation, feed intake, IGF-1, and cortisol in yearling steelhead trout (*Oncorhynchus mykiss*) during seawater adaptation. *Gen Comp Endocrinol.* (2006) 148:195–202. doi: 10.1016/j.ygcen.2006.03.002
158. Cataldi E, Di Marco P, Mandich A, Cataudella S. Serum parameters of Adriatic sturgeon *Acipenser naccarii* (Pisces: Acipenseriformes): effects of temperature and stress. *Comp Biochem Physiol A Mol Integr Physiol.* (1998) 121:351–4. doi: 10.1016/S1095-6433(98)10134-4
159. Lim HK, Hur JW. Effects of acute and chronic air exposure on growth and stress response of juvenile olive flounder, *Paralichthys olivaceus*. *Turkish J Fish Aquat Sci.* (2018) 18:143–51. doi: 10.4194/1303-2712-v18_1_16
160. Randall DJ, Baumgarten D, Malyusz M. The relationship between gas and ion transfer across the gills of fishes. *Comp Biochem Physiol A Physiol.* (1972) 41:629–37. doi: 10.1016/0300-9629(72)90017-5
161. Onukwufor JO, Wood CM. The osmorespiratory compromise in rainbow trout (*Oncorhynchus mykiss*): the effects of fish size, hypoxia, temperature and strenuous exercise on gill diffusive water fluxes and sodium net loss rates. *Comp Biochem Physiol A Mol Integr Physiol.* (2018) 219–220:10–8. doi: 10.1016/j.cbpa.2018.02.002
162. Giacomin M, Bryant HJ, Val AL, Schulte PM, Wood CM. The osmorespiratory compromise: physiological responses and tolerance to hypoxia are affected by salinity acclimation in the euryhaline Atlantic killifish (*Fundulus heteroclitus*). *J Exp Biol.* (2019) 222:206599. doi: 10.1242/jeb.206599
163. Damsgaard C, McGrath M, Wood CM, Richards JG, Brauner CJ. Ion-regulation, acid/base-balance, kidney function, and effects of hypoxia in coho salmon, *Oncorhynchus kisutch*, after long-term acclimation to different salinities. *Aquaculture.* (2020) 528:735571. doi: 10.1016/j.aquaculture.2020.735571
164. Robertson LM, Val AL, Almeida-Val VF, Wood CM. Ionoregulatory aspects of the osmorespiratory compromise during acute environmental hypoxia in 12 tropical and temperate teleosts. *Physiol Biochem Zool.* (2015) 88:357–70. doi: 10.1086/681265
165. Fiess JC, Kunkel-Patterson A, Mathias L, Riley LG, Yancey PH, Hirano T, et al. Effects of environmental salinity and temperature on osmoregulatory ability, organic osmolytes, and plasma hormone profiles in the *Mozambique tilapia* (*Oreochromis mossambicus*). *Comp Biochem Physiol A Mol Integr Physiol.* (2007) 146:252–64. doi: 10.1016/j.cbpa.2006.10.027
166. Barton BA, Zitzow RE. Physiological responses of juvenile walleyes to handling stress with recovery in saline water. *Progress Fish-Culturist.* (1995) 57:267–76. doi: 10.1577/1548-8640(1995)057<0267:PROJWT>2.3.CO;2
167. Nakamura M, Watanabe S, Kaneko T, Masuda R, Tsukamoto K, Otake T. Limited adaptation to non-natal osmotic environments at high water temperature in euryhaline wanderer fishes. *Environ Biol Fishes.* (2020) 103:137–45. doi: 10.1007/s10641-019-00940-0
168. Barton BA, Ribas L, Acerete L, Tort L. Effects of chronic confinement on physiological responses of juvenile gilthead sea bream, *Sparus aurata* L., to acute handling. *Aquac Res.* (2005) 36:172–9. doi: 10.1111/j.1365-2109.2004.01202.x
169. Giacomin M, Dal Pont G, Eom J, Schulte PM, Wood CM. The effects of salinity and hypoxia exposure on oxygen consumption, ventilation, diffusive water exchange and ionoregulation in the Pacific hagfish (*Eptatretus stoutii*). *Comp Biochem Physiol A Mol Integr Physiol.* (2019) 232:47–59. doi: 10.1016/j.cbpa.2019.03.007
170. Alloy MM, Cartolano MC, Sundaram R, Plotnikova A, McDonald MD. Exposure and recovery of the gulf toadfish (*Opsanus beta*) to weathered Deepwater Horizon slick oil: impacts on liver and blood endpoints. *Environ Toxicol Chem.* (2020) etc.4966. doi: 10.1002/etc.4966. [Epub ahead of print].
171. Tort L. Stress and immune modulation in fish. *Dev Comp Immunol.* (2011) 35:1366–75. doi: 10.1016/j.dci.2011.07.002
172. Nakanishi T. Seasonal changes in the humoral immune response and the lymphoid tissues of the marine teleost, *Sebastiscus marmoratus*. *Vet Immunol Immunopathol.* (1986) 12:213–21. doi: 10.1016/0165-2427(86)90125-X
173. Trust TJ, Courtice ID, Khouri AG, Crosa JH, Schiewe MH. Serum resistance and hemagglutination ability of marine vibrios pathogenic for fish. *Infect Immun.* (1981) 34:702–7. doi: 10.1128/IAI.34.3.702-707.1981
174. Ourth DD, Wilson EA. Bactericidal serum response of the channel catfish against Gram-negative bacteria. *Dev Comp Immunol.* (1982) 6:579–83. doi: 10.1016/S0145-305X(82)80044-X
175. Nagai T, Nakai T. Growth of *Flavobacterium psychrophilum* in fish serum correlates with pathogenicity. *J Fish Dis.* (2011) 34:303–10. doi: 10.1111/j.1365-2761.2011.01245.x
176. Jiang X-F, Liu Z-F, Lin A-F, Xiang L-X, Shao J-Z. Coordination of bactericidal and iron regulatory functions of hepcidin in innate antimicrobial immunity in a zebrafish model. *Sci Rep.* (2017) 7:4265. doi: 10.1038/s41598-017-04069-x
177. Rebl A, Korytár T, Borchel A, Bochert R, Strzelczyk JE, Goldammer T, et al. The synergistic interaction of thermal stress coupled with overstocking strongly modulates the transcriptomic activity and immune capacity of rainbow trout (*Oncorhynchus mykiss*). *Sci Rep.* (2020) 10:1–15. doi: 10.1038/s41598-020-71852-8
178. Magnadottir B, Lange S, Gudmundsdottir S, Bøgwald J, Dalmo RA. Ontogeny of humoral immune parameters in fish. *Fish Shellfish Immunol.* (2005) 19:429–39. doi: 10.1016/j.fsi.2005.03.010
179. Saurabh S, Sahoo PK. Lysozyme: an important defence molecule of fish innate immune system. *Aquac Res.* (2008) 39:223–39. doi: 10.1111/j.1365-2109.2007.01883.x
180. Sunyer JO, Boshra H, Lorenzo G, Parra D, Freedman B, Bosch N. Evolution of complement as an effector system in innate and adaptive immunity. *Immunol Res.* (2003) 27:549–64. doi: 10.1385/IR:27:2–3:549
181. Demers NE, Bayne CJ. The immediate effects of stress on hormones and plasma lysozyme in rainbow trout. *Dev Comp Immunol.* (1997) 21:363–73. doi: 10.1016/S0145-305X(97)00009-8
182. Sunyer JO, Tort L, Lambris JD. Structural C3 diversity in fish: characterization of five forms of C3 in the diploid fish *Sparus aurata*. *J Immunol.* (1997) 158:2813–21.
183. Lie Ø, Evensen Ø, Sørensen A, Frøysadal E. Study on lysozyme activity in some fish species. *Dis Aquat Organ.* (1989) 6:1–5. doi: 10.3354/dao006001
184. Goshima M, Sekiguchi R, Matsushita M, Nonaka M. The complement system of elasmobranches revealed by liver transcriptome analysis of a hammerhead shark, *Sphyrna zygaena*. *Dev Comp Immunol.* (2016) 61:13–24. doi: 10.1016/j.dci.2016.03.009
185. Köbis JM, Rebl A, Kühn C, Korytár T, Köllner B, Goldammer T. Comprehensive and comparative transcription analyses of the complement pathway in rainbow trout. *Fish Shellfish Immunol.* (2015) 42:98–107. doi: 10.1016/j.fsi.2014.10.032
186. Parry RM, Chandan RC, Shahani KM. A rapid and sensitive assay of muramidase. *Proc Soc Exp Biol Med.* (1965) 119:384–6. doi: 10.3181/00379727-119-30188
187. Osserman EF, Lawlor DP. Serum and urinary lysozyme (muramidase) in monocytic and monomyelocytic leukemia. *J Exp Med.* (1966) 124:921–52. doi: 10.1084/jem.124.5.921

188. Virella G. Electrophoresis of lysozyme into Microscoccus-containing agarose gel: quantitative and analytical applications. *Clin Chim Acta.* (1977) 75:107–15. doi: 10.1016/0009-8981(77)90505-8
189. Simide R, Richard S, Prévot-D'Alvise N, Miard T, Gaillard S. Assessment of the accuracy of physiological blood indicators for the evaluation of stress, health status and welfare in *Siberian sturgeon* (*Acipenser baerii*) subject to chronic heat stress and dietary supplementation. *Int Aquat Res.* (2016) 8:121–35. doi: 10.1007/s40071-016-0128-z
190. Eslamloo K, Akhavan SR, Fallah FJ, Henry MA. Variations of physiological and innate immunological responses in goldfish (*Carassius auratus*) subjected to recurrent acute stress. *Fish Shellfish Immunol.* (2014) 37:147–53. doi: 10.1016/j.fsi.2014.01.014
191. Caruso D, Schlumberger O, Dahm C, Proteau J-P. Plasma lysozyme levels in sheatfish *Silurus glanis* (L.) subjected to stress and experimental infection with *Edwardsiella tarda*. *Aquac Res.* (2002) 33:999–1008. doi: 10.1046/j.1365-2109.2002.00716.x
192. Mock A, Peters G. Lysozyme activity in rainbow trout, *Oncorhynchus mykiss* (Walbaum), stressed by handling, transport and water pollution. *J Fish Biol.* (1990) 37:873–85. doi: 10.1111/j.1095-8649.1990.tb03591.x
193. Yildiz H. Plasma lysozyme levels and secondary stress response in rainbow trout, *Oncorhynchus mykiss* (Walbaum) after exposure to leteux-meyer mixture. *Turk J Vet Anim Sci.* (2006) 30:265–9. Available online at: https://dergipark.org.tr/tr/download/article-file/132614
194. Caruso D, Lazard J. Subordination stress in Nile tilapia and its effect on plasma lysozyme activity. *J Fish Biol.* (1999) 55:451–4. doi: 10.1111/j.1095-8649.1999.tb00690.x
195. Sunyer JO, Tort L. Natural hemolytic and bactericidal activities of sea bream *Sparus aurata* serum are effected by the alternative complement pathway. *Vet Immunol Immunopathol.* (1995) 45:333–45. doi: 10.1016/0165-2427(94)05430-Z
196. Mauri I, Romero A, Acerete L, Mackenzie S, Roher N, Callol A, et al. Changes in complement responses in Gilthead seabream (*Sparus aurata*) and European seabass (*Dicentrarchus labrax*) under crowding stress, plus viral and bacterial challenges. *Fish Shellfish Immunol.* (2011) 30:182–8. doi: 10.1016/j.fsi.2010.10.006
197. Montero D, Marrero M, Izquierdo M, Robaina L, Vergara J, Tort L. Effect of vitamin E and C dietary supplementation on some immune parameters of gilthead seabream (*Sparus aurata*) juveniles subjected to crowding stress. *Aquaculture.* (1999) 171:269–78. doi: 10.1016/S0044-8486(98)00387-1
198. Rotllant J, Tort L. Cortisol and glucose responses after acute stress by net handling in the sparid red porgy previously subjected to crowding stress. *J Fish Biol.* (1997) 51:21–8. doi: 10.1111/j.1095-8649.1997.tb02510.x
199. Herron CL, Cogliati KM, Dolan BP, Munakata A, Schreck CB. Stress up-regulates oxidative burst in juvenile Chinook salmon leukocytes. *Fish Shellfish Immunol.* (2018) 80:655–9. doi: 10.1016/j.fsi.2018.06.038
200. Narra MR, Rajender K, Reddy RR, Murty US, Begum G. Insecticides induced stress response and recuperation in fish: biomarkers in blood and tissues related to oxidative damage. *Chemosphere.* (2017) 168:350–7. doi: 10.1016/j.chemosphere.2016.10.066
201. Caipang CMA, Berg I, Brinchmann MF, Kiron V. Short-term crowding stress in Atlantic cod, *Gadus morhua* L. modulates the humoral immune response. *Aquaculture.* (2009) 295:110–5. doi: 10.1016/j.aquaculture.2009.06.036
202. Karadag H, Firat Ö, Firat Ö. Use of oxidative stress biomarkers in *Cyprinus carpio* L. for the evaluation of water pollution in Ataturk Dam Lake (Adiyaman, Turkey). *Bull Environ Contam Toxicol.* (2014) 92:289–93. doi: 10.1007/s00128-013-1187-0
203. Montero R, Strzelczyk JE, Chan JTH, Verleih M, Rebl A, Goldammer T, et al. Dawn to dusk: Diurnal rhythm of the immune response in rainbow trout (*Oncorhynchus mykiss*). *Biology*. (2020) 9:1–13. doi: 10.3390/biology9010008
204. Shahsavani D, Mohri M, Gholipour Kanani H. Determination of normal values of some blood serum enzymes in *Acipenser stellatus* Pallas. *Fish Physiol Biochem.* (2010) 36:39–43. doi: 10.1007/s10695-008-9277-3
205. Svoboda M, Kouril J, Hamáčková J, Kaláb P, Savina L, Svobodová Z, et al. Biochemical profile of blood plasma of tench (*Tinca tinca* L.) during pre- and postspawning period. *Acta Vet Brno.* (2001) 70:259–68. doi: 10.2754/avb200170030259
206. Lulijwa R, Alfaro AC, Merien F, Meyer J, Young T. Advances in salmonid fish immunology: a review of methods and techniques for lymphoid tissue and peripheral blood leucocyte isolation and application. *Fish Shellfish Immunol.* (2019) 95:44–80. doi: 10.1016/j.fsi.2019.10.006
207. Goetz FW, Planas JV, Díaz M, Iliev DB, MacKenzie S. Culture of fish head kidney mononuclear phagocytes and muscle satellite cells: valuable models for aquaculture biotechnology research. In *Aquaculture Biotechnology* (Oxford: Wiley-Blackwell). p. 207–21. doi: 10.1002/9780470963159.ch13
208. Anderson DP, Dixon OW, Lizzio EF. Immunization and culture of rainbow trout organ sections *in vitro*. *Vet Immunol Immunopathol.* (1986) 12:203–11. doi: 10.1016/0165-2427(86)90124-8
209. Martorell Ribera J, Nipkow M, Viergutz T, Brunner RM, Bochert R, Koll R, et al. Early response of salmonid head-kidney cells to stress hormones and toll-like receptor ligands. *Fish Shellfish Immunol*. (2020) 98:950–61. doi: 10.1016/j.fsi.2019.11.058
210. Pierrard M-A, Roland K, Kestemont P, Dieu M, Raes M, Silvestre F. Fish peripheral blood mononuclear cells preparation for future monitoring applications. *Anal Biochem.* (2012) 426:153–65. doi: 10.1016/j.ab.2012.04.009
211. Miller NW, Deuter A, Clem LW. Phylogeny of lymphocyte heterogeneity: the cellular requirements for the mixed leucocyte reaction with channel catfish. *Immunology.* (1986) 59:123–8.
212. Forlenza M, Walker PD, de Vries BJ, Wendelaar Bonga SE, Wiegertjes GF. Transcriptional analysis of the common carp (*Cyprinus carpio* L.) immune response to the fish louse *Argulus japonicus* Thiele (Crustacea: Branchiura). *Fish Shellfish Immunol.* (2008) 25:76–83. doi: 10.1016/j.fsi.2007.12.013
213. Scapigliati G, Buonocore F, Randelli E, Casani D, Meloni S, Zarletti G, et al. Cellular and molecular immune responses of the sea bass (Dicentrarchus labrax) experimentally infected with betanodavirus. *Fish Shellfish Immunol.* (2010) 28:303–11. doi: 10.1016/j.fsi.2009.11.008
214. Duchiron C, Betoulle S, Reynaud S, Deschaux P. Lindane increases macrophage-activating factor production and intracellular calcium in rainbow trout (*Oncorhynchus mykiss*) leukocytes. *Ecotoxicol Environ Saf.* (2002) 53:388–96. doi: 10.1016/S0147-6513(02)00007-6
215. Reynaud S, Deschaux P. The effects of 3-methylcholanthrene on lymphocyte proliferation in the common carp (*Cyprinus carpio* L.). *Toxicology*. (2005) 211:156–64. doi: 10.1016/j.tox.2005.02.015
216. Rymuszka A, Sierosławska A, Bownik A, Skowroński T. *In vitro* effects of pure microcystin-LR on the lymphocyte proliferation in rainbow trout (*Oncorhynchus mykiss*). *Fish Shellfish Immunol.* (2007) 22:289–92. doi: 10.1016/j.fsi.2006.06.002
217. Yin D-q, Hu S-q, Gu Y, Wei L, Liu S-s, Zhang A-q. Immunotoxicity of bisphenol A to *Carassius auratus* lymphocytes and macrophages following *in vitro* exposure. *J Environ Sci.* (2007) 19:232–7. doi: 10.1016/S1001-0742(07)60038-2
218. Kammann U, Riggers JC, Theobald N, Steinhart H. Genotoxic potential of marine sediments from the North Sea. *Mutat Res.* (2000) 467:161–8. doi: 10.1016/S1383-5718(00)00030-9
219. Kemenade BV, Verburg-Van Kemenade BML, Nowak B, Engelsma MY, Weyts FAA. Differential effects of cortisol on apoptosis and proliferation of carp B-lymphocytes from head kidney, spleen and blood. *Fish Shellfish Immunol.* (1999) 9:405–15. doi: 10.1006/fsim.1998.0197
220. Lammel T, Tsoukatou G, Jellinek J, Sturve J. Development of three-dimensional (3D) spheroid cultures of the continuous rainbow trout liver cell line RTL-W1. *Ecotoxicol Environ Saf.* (2019) 167:250–8. doi: 10.1016/j.ecoenv.2018.10.009
221. Kralik P, Ricchi M. A basic guide to real time PCR in microbial diagnostics: definitions, parameters, and everything. *Front Microbiol.* (2017) 8:108. doi: 10.3389/fmicb.2017.00108
222. Urquhart K, Collins C, Monte M, Sokolowska J, Secombes C, Collet B. Individual measurement of gene expression in blood cells from Rainbow trout *Oncorhynchus mykiss* (Walbaum). *J Exp Appl Anim Sci.* (2016) 2:1. doi: 10.20454/jeaas.2016.1077
223. Zhang A, Zhou X, Wang X, Zhou H. Characterization of two heat shock proteins (Hsp70/Hsc70) from grass carp (*Ctenopharyngodon idella*): evidence for their differential gene expression, protein synthesis and secretion in LPS-challenged peripheral blood

lymphocytes. *Comp Biochem Physiol B Biochem Mol Biol.* (2011) 159:109–14. doi: 10.1016/j.cbpb.2011.02.009
224. Ferencz Á, Juhász R, Butnariu M, Deér KA, Varga IS, Nemcsók J. Expression analysis of heat shock genes in the skin, spleen and blood of common carp (*Cyprinus carpio*) after cadmium exposure and hypothermia. *Acta Biol Hung.* (2012) 63:15–25. doi: 10.1556/ABiol.63.2012.1.2
225. Fulladosa E, Deane E, Ng AHY, Woo NYS, Murat JC, Villaescusa I. Stress proteins induced by exposure to sublethal levels of heavy metals in sea bream (*Sparus sarba*) blood cells. *Toxicol Vitr.* (2006) 20:96–100. doi: 10.1016/j.tiv.2005.06.005
226. Irwin MR, Cole SW. Reciprocal regulation of the neural and innate immune systems. *Nat Rev Immunol.* (2011) 11:625–32. doi: 10.1038/nri3042
227. Elenkov E, Chrousos C. Stress Hormones, Th1/Th2 patterns, pro/anti-inflammatory cytokines and susceptibility to disease. *Trends Endocrinol Metab.* (1999) 10:359–68. doi: 10.1016/S1043-2760(99)00188-5
228. Vihervaara A, Duarte FM, Lis JT. Molecular mechanisms driving transcriptional stress responses. *Nat Rev Genet.* (2018) 19:385–97. doi: 10.1038/s41576-018-0001-6
229. Lightman SL. The neuroendocrinology of stress: a never ending story. *J Neuroendocrinol.* (2008) 20:880–4. doi: 10.1111/j.1365-2826.2008.01711.x
230. Denslow ND, Garcia-Reyero N, Barber DS. Fish "n" chips: the use of microarrays for aquatic toxicology. *Mol Biosyst.* (2007) 3:172–7. doi: 10.1039/B612802P
231. Götting M, Nikinmaa MJ. Transcriptomic analysis of young and old erythrocytes of fish. *Front Physiol.* (2017) 8:1–11. doi: 10.3389/fphys.2017.01046
232. Kumar Roy A. Microarray analysis of fish genomic data for enhancing aquaculture productivity of India. *Ann Proteom Bioinform.* (2017) 1:6–17. doi: 10.29328/journal.hpbr.1001002
233. Douglas SE. Microarray studies of gene expression in fish. *Omi A J Integr Biol.* (2006) 10:474–89. doi: 10.1089/omi.2006.10.474
234. Claver JA, Quaglia AIE. Comparative morphology, development, and function of blood cells in non-mammalian vertebrates. *J Exot Pet Med.* (2009) 18:87–97. doi: 10.1053/j.jepm.2009.04.006
235. Puente-Marin S, Nombela I, Ciordia S, Mena MC, Chico V, Coll J, et al. *In silico* functional networks identified in fish nucleated red blood cells by means of transcriptomic and proteomic profiling. *Genes.* (2018) 9:40202. doi: 10.3390/genes9040202
236. Morera D, Roher N, Ribas L, Balasch JC, Doñate C, Callol A, et al. RNA-Seq reveals an integrated immune response in nucleated erythrocytes. *PLoS ONE.* (2011) 6:e26998. doi: 10.1371/journal.pone.0026998
237. Li Z, Liu X, Liu J, Zhang K, Yu H, He Y, et al. Transcriptome profiling based on protein-protein interaction networks provides a core set of genes for understanding blood immune response mechanisms against *Edwardsiella tarda* infection in Japanese flounder (*Paralichthys olivaceus*). *Dev Comp Immunol.* (2018) 78:100–13. doi: 10.1016/j.dci.2017.09.013
238. Puente-Marin S, Nombela I, Chico V, Ciordia S, Mena CM, Perez L, Coll J, Ortega-Villaizan MdM. Potential role of rainbow trout erythrocytes as mediators in the immune response induced by a DNA vaccine in fish. *Vaccines.* (2019) 7:60. doi: 10.3390/vaccines7030060
239. Dahle MK, Wessel Ø, Timmerhaus G, Nyman IB, Jørgensen SM, Rimstad E, et al. Transcriptome analyses of Atlantic salmon (*Salmo salar* L.) erythrocytes infected with piscine orthoreovirus (PRV). *Fish Shellfish Immunol.* (2015) 45:780–90. doi: 10.1016/j.fsi.2015.05.049
240. Lewis JM, Hori TS, Rise ML, Walsh PJ, Currie S. Transcriptome responses to heat stress in the nucleated red blood cells of the rainbow trout (*Oncorhynchus mykiss*). *Physiol Genomics.* (2010) 42:361–73. doi: 10.1152/physiolgenomics.00067.2010
241. Akbarzadeh A, Günther OP, Houde AL, Li S, Ming TJ, Jeffries KM, et al. Developing specific molecular biomarkers for thermal stress in salmonids. *BMC Genom.* (2018) 19:749. doi: 10.1186/s12864-018-5108-9
242. Rebl A, Verleih M, Nipkow M, Altmann S, Bochert R, Goldammer T. Gradual and acute temperature rise induces crossing endocrine, metabolic and immunological pathways in maraena whitefish (*Coregonus maraena*). *Front Genet.* (2018) 9:241. doi: 10.3389/fgene.2018.00241
243. Rebl A, Goldammer T. Under control: the innate immunity of fish from the inhibitors' perspective. *Fish Shellfish Immunol.* (2018) 77:328–49. doi: 10.1016/j.fsi.2018.04.016
244. Harris J, Bird DJ. Modulation of the fish immune system by hormones. *Vet Immunol Immunopathol.* (2000) 77:163–76. doi: 10.1016/S0165-2427(00)00235-X
245. Zhou C, Xiao S, Liu Y, Mou Z, Zhou J, Pan Y, et al. Comprehensive transcriptome data for endemic Schizothoracinae fish in the Tibetan Plateau. *Sci Data.* (2020) 7:28. doi: 10.1038/s41597-020-0361-6
246. Qi D, Chao Y, Wu R, Xia M, Chen Q, Zheng Z, et al. Transcriptome analysis provides insights into the adaptive responses to hypoxia of a schizothoracine fish (*Gymnocypris eckloni*). *Front Physiol.* (2018) 9:1–12. doi: 10.3389/fphys.2018.01326
247. Gracey AY, Troll JV, Somero GN. Hypoxia-induced gene expression profiling in the euryoxic fish *Gillichthys mirabilis*. *Proc Natl Acad Sci USA.* (2001) 98:1993–8. doi: 10.1073/pnas.98.4.1993
248. Le Q, Hu J, Cao X, Kuang S, Zhang M, Yu N, et al. Transcriptomic and cortisol analysis reveals differences in stress alleviation by different methods of anesthesia in Crucian carp (*Carassius auratus*). *Fish Shellfish Immunol.* (2019) 84:1170–9. doi: 10.1016/j.fsi.2018.10.061
249. Carmona SJ, Teichmann SA, Ferreira L, Macaulay C, Stubbington MJT, Cvejic A, et al. Single-cell transcriptome analysis of fish immune cells provides insight into the evolution of vertebrate immune cell types. *Genome Res.* (2017) 27:451–61. doi: 10.1101/gr.207704.116
250. Vogel C, Marcotte EM. Insights into the regulation of protein abundance from proteomic and transcriptomic analyses. *Nat Rev Genet.* (2012) 13:227–32. doi: 10.1038/nrg3185
251. Schwanhäusser B, Busse D, Li N, Dittmar G, Schuchhardt J, Wolf J, et al. Global quantification of mammalian gene expression control. *Nature.* (2011) 473:337–42. doi: 10.1038/nature10098
252. Ryan SN, Pankhurst NW, Wells RM. A possible role for ubiquitin in the stress response of the teleost fish blue mao mao (*Scorpis violaceus*). *Physiol Zool.* (1995) 68:1077–92. doi: 10.1086/physzool.68.6.30163794
253. Karim M, Puiseux-Dao S, Edery M. Toxins and stress in fish: proteomic analyses and response network. *Toxicon.* (2011) 57:959–69. doi: 10.1016/j.toxicon.2011.03.018
254. Kumar VB, Jiang I-F, Yang H-H, Weng C-F. Effects of serum on phagocytic activity and proteomic analysis of tilapia (*Oreochromis mossambicus*) serum after acute osmotic stress. *Fish Shellfish Immunol.* (2009) 26:760–7. doi: 10.1016/j.fsi.2009.03.005
255. Yi S, Liu L-F, Zhou L-F, Zhao B-W, Wang W-M, Gao Z-X. Screening of biomarkers related to ovarian maturation and spawning in blunt snout bream (*Megalobrama amblycephala*) based on metabolomics and transcriptomics. *Mar Biotechnol.* (2020) 22:180–93. doi: 10.1007/s10126-019-09943-5
256. Xiao M, Qian K, Wang Y, Bao F. GC-MS metabolomics reveals metabolic differences of the farmed Mandarin fish *Siniperca chuatsi* in recirculating ponds aquaculture system and pond. *Sci Rep.* (2020) 10:6090. doi: 10.1038/s41598-020-63252-9
257. Sitjà-Bobadilla A, Gil-Solsona R, Estensoro I, Piazzon MC, Martos-Sitcha JA, Picard-Sánchez A, et al. Disruption of gut integrity and permeability contributes to enteritis in a fish-parasite model: a story told from serum metabolomics. *Parasit Vectors.* (2019) 12:486. doi: 10.1186/s13071-019-3746-7
258. Young T, Alfaro AC. Metabolomic strategies for aquaculture research: a primer. *Rev Aquac.* (2018) 10:26–56. doi: 10.1111/raq.12146
259. Kokushi E, Uno S, Harada T, Koyama J. 1H NMR-based metabolomics approach to assess toxicity of bunker a heavy oil to freshwater carp, *Cyprinus carpio*. *Environ Toxicol.* (2012) 27:404–14. doi: 10.1002/tox.20653
260. Zhang H, Zhao L. Influence of sublethal doses of acetamiprid and halosulfuron-methyl on metabolites of zebra fish (*Brachydanio rerio*). *Aquat Toxicol.* (2017) 191:85–94. doi: 10.1016/j.aquatox.2017.08.002
261. Kokushi E, Uno S, Pal S, Koyama J. Effects of chlorpyrifos on the metabolome of the freshwater carp, *Cyprinus carpio*. *Environ Toxicol.* (2015) 30:253–60. doi: 10.1002/tox.21903
262. Samuelsson LM, Förlin L, Karlsson G, Adolfsson-Erici M, Larsson DGJ. Using NMR metabolomics to identify responses of an environmental

estrogen in blood plasma of fish. *Aquat Toxicol*. (2006) 78:341–9. doi: 10.1016/j.aquatox.2006.04.008

263. Carbajal A, Monclús L, Tallo-Parra O, Sabes-Alsina M, Vinyoles D, Lopez-Bejar M. Cortisol detection in fish scales by enzyme immunoassay: biochemical and methodological validation. *J Appl Ichthyol*. (2018) 34:967–70. doi: 10.1111/jai.13674
264. Guardiola FA, Cuesta A, Esteban MÁ. Using skin mucus to evaluate stress in gilthead seabream (*Sparus aurata* L.). *Fish Shellfish Immunol*. (2016) 59:323–30. doi: 10.1016/j.fsi.2016.11.005
265. Félix AS, Faustino AI, Cabral EM, Oliveira RF. Noninvasive measurement of steroid hormones in zebrafish holding-water. *Zebrafish*. (2013) 10:110–5. doi: 10.1089/zeb.2012.0792
266. Cao Y, Tveten AK, Stene A. Establishment of a non-invasive method for stress evaluation in farmed salmon based on direct fecal corticoid metabolites measurement. *Fish Shellfish Immunol*. (2017) 66:317–24. doi: 10.1016/j.fsi.2017.04.012

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Caloric Restriction in Group-Housed Mice: Littermate and Sex Influence on Behavioral and Hormonal Data

Cristina Perea[1†], *Ana Vázquez-Ágredos*[2†], *Leandro Ruiz-Leyva*[3,4†], *Ignacio Morón*[5*], *Jesús Martín Zúñiga*[1] and *Cruz Miguel Cendán*[3,4]

[1] *Center of Scientific Instrumentation, University of Granada, Granada, Spain,* [2] *Department of Psychobiology, Institute of Neurosciences, Center for Biomedical Research (CIBM), University of Granada, Granada, Spain,* [3] *Department of Pharmacology, Faculty of Medicine, Biomedical Research Center, Institute of Neuroscience, University of Granada, Parque Tecnológico de Ciencias de la Salud, Granada, Spain,* [4] *Biosanitary Research Institute ibs.GRANADA, Granada, Spain,* [5] *Department of Psychobiology, Faculty of Psychology, Center of Investigation of Mind, Brain, and Behavior, University of Granada, Granada, Spain*

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Alan G. McElligott,
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Vootele Voikar,
University of Helsinki, Finland

***Correspondence:**
Ignacio Morón
imoron@ugr.es

†*These authors have contributed equally to this work and share first authorship*

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Much of the research done on aging, oxidative stress, anxiety, and cognitive and social behavior in rodents has focused on caloric restriction (CR). This often involves several days of single housing, which can cause numerous logistical problems, as well as cognitive and social dysfunctions. Previous results in our laboratory showed the viability of long-term CR in grouped rats. Our research has studied the possibility of CR in grouped female and male littermates and unrelated CB6F1/J (C57BL/6J × BALBc/J hybrid strain) mice, measuring: (i) possible differences in body mass proportions between mice in *ad libitum* and CR conditions (at 70% of *ad libitum*), (ii) aggressive behavior, using the number of *pushes* and *chasing behavior time* as an indicator and social behavior using the *time under the feeder* as indicator, and (iii) difference in serum adrenocorticotropic hormone (ACTH) concentrations (stress biomarker), under *ad libitum* and CR conditions. Results showed the impossibility of implementing CR in unrelated male mice. In all other groups, CR was possible, with a less aggressive behavior (measured only with the number of *pushes*) observed in the unrelated female mice under CR conditions. In that sense, the ACTH levels measured on the last day of CR showed no difference in stress levels. These results indicate that implementantion of long-term CR in mice can be optimized technically and also related to their well-being by grouping animals, in particular, related mice.

Keywords: caloric restriction, grouped mice, littermate mice, adrenocorticotropic, eating behavior, social behavior

INTRODUCTION

Caloric restriction (CR) has been widely used in experimental research (1, 2). CR has been used in different modalities (moderate, 70–80% and intense restriction, 50–60%), with respect to the maximum *ad libitum* (AL) intake. It is proposed as a maintenance method between 6 and 24 months or more (3). Overfeeding is considered one of the most uncontrolled variables in bioassays in general (4). The most commonly used in chronic (24 months) and/or subchronic (12 months) evaluation studies is the moderate (70–80%) CR procedure (4–7). Traditionally, CR research has focused on how it influences increased longevity (2, 8, 9). CR has also been studied in relation to oxidative stress, where it was found to have an antioxidant effect (10), or in relation to the reduction of inflammatory processes induced by aging and measured in microglia levels (11). Another context in which CR has been studied is anxiety. Thus, CR has proven to have an anxiolytic effect, tested in the open field and in elevated plus maze (12, 13) and it enhances fear extinction learning (14), but has no effect on post-traumatic stress disorder (15). CR research also studies cognitive functions and social behavior. CR has been observed to have negative effects on cognitive functions, probably caused by lower glucose levels (16), and on maternal care, inducing a decline in maternal behavior toward pups (17). However, CR also has positive effects, such as heightened social behavior between mice (18).

Typically, CR experiments require extended periods of time. Body mass control and avoiding potentially aggressive behavior (19–21) can force researchers to use single housing for animals. Especially if the aggressive mice behavior is considered (22). Single housing can cause many logistical issues (it requires more cages and racks, space and maintenance staff, etc.) as well as problems related to the well-being of animals (stress induction) (23). Regarding the logistical problems, current legislation [for a review see (24)] limits research installations and resources, discouraging individual maintenance of animals for extended periods of time. Single housing also impacts negatively on the animal's well-being [for a review see (25)], which could be a potential confounding variable in future protocols applied to animals in single housing. It is important to note that mice display complex social behavior such as empathy [for a review see (26, 27)], and the social deprivation associated with single housing in CR research may therefore have severe effects on the animal's behavior, by denying the animal access–for example–to the various benefits of social interaction. In this sense, social interaction has been shown to improve memory processes, reduce hippocampal damage in aged mice (28), induce brain-derived neurotrophic factor (29), reduce the impact of CR (30), induce higher food consumption (31), reduce anorexic behavior in adolescent mice (32) and facilitate cognitive recovery after a social defeat experience (33). All these aspects show the potential benefits of carrying out abundant research into CR with grouped animals. The benefits of grouping animals are not only logistical and economic, they are also very much related to their well-being, as described above.

Previous research in our laboratory showed the viability of group-housing while sustaining CR for long periods in male rats (34). Our results indicated the effectiveness of CR in different groups, regardless of the relationship between the rats. No extreme body mass changes were observed in CR rats, nor did they display aggressive behavior or show alterations in their corticosterone levels. To our knowledge, no similar data has been reported about the possibility of group-housing mice under CR. We decided to study CR in CB6F1/J mice for two reasons: it is a inbred strain often used in experimental research (35) and has a tendency to show aggressive behavior under grouped conditions (22). Our main objective was to determine how CR at 70% of *ad libitum* affects the body mass, relationships and behavior of littermates and unrelated (male or female) grouped mice, as well as to study as an indicator of stress, in relation to animal welfare (36, 37) by analyzing serum adrenocorticotropic hormone (ACTH) levels. It is expected that against aggressive behavior in C57BL/6 mice (22), a normal interaction with absence of significant aggressive behaviors will be observed in the CR at 70% mice. Likewise, we expected to find no significant differences in serum ACTH levels between the groups.

MATERIALS AND METHODS

Ethical Approval and Other Ethical Considerations

Animals were kept in accordance with EU Directive 2010/63/EU and Spanish Royal Decree 53/2013. The University of Granada's Research Ethics Committee and the Junta de Andalucía, Consejería de Agricultura, Ganadería, Pesca y Desarrollo Sostenible approved the experimental protocol with reference number 09/08/2019/137. All animal procedures carried out for this study were subject to review by Animal-Welfare Officials and a designated veterinarian of the Animal Facility at CIBM/UGR.

Animals

The experiment used fifty-four CB6F1/J (#100007; F1 generation hybrids from breeding of BALB/cJ females and C57BL/6J males from Jackson Laboratories) mice (27 female) from the Biomedical Investigation Center (CIBM) of Granada. Mice were divided into groups of three and kept in transparent methacrylate cages (215 × 465 × 145 mm) in rooms at 22 ± 1°C and with a 12-h light/darkness cycle (lights on at 7:30 AM). Standard Type-II Tecniplast LTD 370 cm^2 cuvettes -allowing a maximum of 5 mice- were used for the maintenance of mice in the experimental phase, with pine wood shavings from Rettenmair Ltd, and enrichment elements (pieces of paper). This is the usual size used in the experimental maintenance of chronic and subchronic mice at the SPEA/IC/CI/CIBM facilities. The experimental subjects came from a 10 monogamous breeding pairs, in which the offspring were separated into groups of three after weaning in separate cages into males and females of the same litter, with *ad libitum* feeding for the littermate groups. For the unrelated groups, males and females were randomly selected from the cages of unrelated individuals. At the beginning of the experiment, the average body mass was 20.9 ± 1.13 g for females and 27.2 ± 1.53 g for males. Throughout the 23 days of the experiment, water was accessible *ad libitum* and a standard laboratory pellet diet (Harlan Teklad Research diet, Madison, WI, USA) was administered as described in the "Method" section below.

Method

Cages with CR and *ad libitum* groups had the same body mass proportions and housekeeping conditions. To control the effectiveness of the restriction process, 18 unrelated mice (Group *ad libitum*; nine females and nine males housed in groups of three mice per cage) were designated as control groups. Since there was an absence of interaction during feeding in these unrelated controls groups, we consider not necessary to include another 18 littermate mice control group. Thirty-six mice (18 females and 18 males) were exposed to 70% food restriction. Each cage held a group of three mice, and they were distributed into four groups. In two groups, 18 unrelated mice (Group *Restricted unrelated; nine females and nine males*) were subjected to 70% food restriction, and 70% food restriction was also introduced in the other two groups of nine littermates mice (Group *Restricted-Related; nine females and nine males*). The tails of mice from each cage were marked in different colors (red, blue or no mark) to identify them.

Recording Body Mass and Observing Behavior

Every day at 1:00 p.m., each mouse was weighed on a scale and food was administered. The *ad libitum* group was given 200 g of food, and groups on food restriction were given 70% of the food eaten each day by the *ad libitum* group of mice (The uneaten food from the *ad libitum* group was weighed). The remaining food (pellets) was removed before the CR groups had access to their food, to ensure there was a 70% food reduction. The order in which food was administered was rotated each day, thus producing parity between groups with regard to the time that mice had to wait for food (and the resulting added stress). After calculating the mean and standard deviation from the recorded body mass the body mass proportion between the three animals in each cage was calculated by considering the weight of the heaviest cage-mate mouse as 100% and applying the following equation:

$$\%\ Body\ Mass = \frac{mouse\ weight\ \ (g)\ *100}{heaviest\ mouse\ weight(g)}$$

The greater differences between mice weight, the lower the average % Body mass per cage.

Animals were recorded in their respective cages for 15 min every day, using a digital JVC camera model Everio HDD GZ-MG680BE, immediately after making the food available to both groups under CR. Of that time, the first 5 min were used to analyze behavior. At the end of the experimental procedure, a global analysis of social behavior was performed with the recorded material by using Behavioral Observation Research Interactive Software (BORIS) (38). Based on data obtained with rats in past research (34), the behavioral analysis focused on the number of times each mouse *pushed* its cage companions while eating. This *push* action can be compared to wrestling behavior observed in other studies (39, 40); a form of defense from other mice, using the front or back paws to indicate fighting behavior, or an attempt to force the mouse who is receiving the *push* to submit (submission response) (41). However, our previous results with rats (34) showed that *pushes*, under CR conditions, can be interpreted as social behavior. Also, potentially aggressive behavior (26) was recorded during the 15 min after food was made available to CR mice. Whenever such behavior was observed, the cage was eliminated from the experiment and CR was suppressed to prevent physical injuries.

In addition to the measurement of pushes, two additional behavioral from the total of 15 min recorded were analyzed. On the one hand, and within the aggressive behaviors, there was the recording of chasing behavior, understood as the time in which at least two mice chased each other to get a piece of food. On the other hand, and as part of the recording of more social behaviors, we measured the time in which the three animals were eating at the same time under the feeder without the presence of pushing and shoving. There were no other significant behavior to analyze.

Hormonal Analysis

On the 23rd day of the experiment, after being deeply anesthetized with isoflurane, fifty microliters of blood were extracted intracardially from the 44 mice (10 samples were not collected due to the elimination of the Unrelated Male CR (9 samples) and the insufficient volume of blood for one female from the Unrelated CR group). To acclimatize mice to the procedure, they were previously exposed to the isoflurane box on two occasions. After the blood draw, animals were sacrificed with a cervical dislocation. Of the 44 mice, 18 were unrelated mice with food *ad libitum* (nine females and nine males), and another eight were unrelated, female mice on 70% food restriction. The remaining 18 were littermates on 70% food restriction (nine females and nine males). Serum was obtained from these blood samples for the hormonal analysis. Hormonal analyses were performed using the Milliplex map pituitary magnetic bead panel kit (MPTMAG-49K-01) for ACTH and the Luminex 200TM HTS, FLEXMAP 3D. Preparation of serum samples was performed as follows: blood was allowed to clot for at least 30 min before centrifugation, for 10 min at 1,000 x g. Serum was removed and assayed immediately. For each serum sample, 150 μl of the antibody-bead and assay buffer were added to the mixing bottle, resulting in a total volume of 2,850 μl. Next, samples were incubated overnight on a shaker at 4°C. Samples were then measured on the Lumina 200TM. Median Fluorescent Intensity (MFI) was recorded using a weighted 5-parametrer logistic or spline curve-fitting method to analyze concentrations in samples. The validation of the measurements made on the hematology counter was performed with a commercial artificial blood. Specifically, Myt-5D Hematology controls (normal control) from ORPHEE SA (CH-1228 Geneva/Pla-les-Quates SWITZERLAND) were used.

Statistical Analysis

Statistical analyses were performed using JASP version 0.10.2. Behavioral data were analyzed considering the cage-mate mice as non-independents. Rest of the analyses were done considering each mouse as independent. Body mass and body mass proportion were compared using repeated measures (RM) Analyses of Variance (ANOVA) and Chasing behavior and Time under the feeder were analyzed by one-way ANOVA since the normality assumptions (sphericity and the equality of variances) were respected. Kruskal-Wallis non-parametric analysis was applied for ACTH levels and Total number of *Pushes* since the criteria of normality were not assumed in these cases. Whenever a significant difference between groups was found, the Bonferroni correction was applied in a *post-hoc* derived from the main analysis. The significance level in all cases was $p < 0.05$.

RESULTS

On the second day of CR, intensely aggressive behavior (26) was observed in two cages (six mice) of the group *Restricted unrelated* males. There was fierce fighting between animals, with blood and several skin injuries. This forced us to apply the ethical protocol and, as was mentioned above under *Method*, the nine animals of the group *Restricted unrelated* males were discarded from the experiment and CR was interrupted to avoid further fighting and possible injuries to mice.

Body Mass and Body Mass Proportion

Figure 1 shows body masses for the five groups of mice (*ad libitum unrelated male and female, Restricted unrelated female, and Restricted related male and female*) throughout the 23 days of the experiment (for statistical analyses, first day was treated as a covariate factor). Application of RM-ANOVA here confirms the sphericity and equality of variances (Levene Fs < 2.03, df $= 42$; $p > 0.09$). Results showed a significant main effect of Group (ANOVA Between Subjects Effect: F = 131.517, df = 5; $p = 0.001$; $\eta^2 = 0.79$) and the interaction between Group and Day (ANOVA Within Subjects Effect: F = 15.171, df = 105; $p = 0.001$; $\eta^2 = 0.078$). However, the variable Day did not exert significant main effect (ANOVA Within Subjects Effect: F = 1.553, df = 21; $p = 0.054$; η^2 Magnitude of the effect = 0.002). Analysis of the interaction (keeping the first day as a covariate factor) shows that *ad libitum* Females have a significant higher body mass than Restricted related Female since D3 to D23 (Bonferroni $p = 0.001$), Restricted unrelated Female since D4 to D23 (Bonferroni $p = 0.001$) and Restricted related Male D4 (Bonferroni $p = 0.001$), D5 (Bonferroni $p = 0.002$) and since D6 to D23 (Bonferroni $p = 0.001$). *Ad libitum* Males have a significant higher body mass than Restricted related Female D3 (Bonferroni $p = 0.003$), D5 (Bonferroni $p = 0.002$), and since D6 to D23 (Bonferroni $p = 0.001$) except D15 (Bonferroni $p = 0.079$). *Ad libitum* Males have a significant higher body mass than Restricted unrelated Female and Restricted related Male D3 (Bonferroni $p = 0.011$), and since D5 to D23 (Bonferroni $p = 0.001$). Restricted related Male have a significant less body mass than Restricted related Female Days D15 (Bonferroni $p = 0.04$), D17 (Bonferroni $p = 0.02$) and D21 (Bonferroni $p = 0.006$). The rest of the days no differences between these groups were significant. Restricted related Male have a significant less body mass than Restricted unrelated Female only days D19 (Bonferroni $p = 0.048$) and D21 (Bonferroni $p = 0.005$). Finally, Restricted related Female and Restricted unrelated Female did not have any significant difference in their body mass. This result shows the effectiveness of CR in male and female mice.

Regarding body mass proportions, after first checked the sphericity and equality of variances (Levene D1-D23 Fs < 2.178; $p > 0.08$), RM ANOVA showed that Group (ANOVA Between groups effect: F = 0.315, df = 5; $p = 0.832$; $\eta^2 = 0.02$) and Day (ANOVA Within groups effect: F = 0.315; df = 22; $p = 0.401$; $\eta^2 = 0.004$) variables had no effect, and nor did their interaction (F = 0.286; df = 110; $p > 0.8$; $\eta^2 = 0.019$). This means that body mass proportions were similar throughout the days of the experiment.

Pushes

Figure 2 shows the number of *pushes* under the feeder for *Restricted* unrelated and *Restricted related* mice. Due to the total absence of activity under the feeder when food was administered, the *ad libitum* groups were eliminated for analysis. A one-way ANOVA of the mean of the sum of pushes for each group (Unrelated female, Related female and Related males) throughout the days of the experiment showed a violation of the equality of variances (Levene F = 6.557; $p < 0.04$). The Kruskal-Wallis analysis showed significant differences between groups (K-W = 6.489, df = 5; $p < 0.04$). Comparing groups while applying the Kruskal-Wallis analysis shows a higher number of *pushes* in the group of unrelated females than in Related females and Related males (K-W = 3.857, df = 2; $p = 0.05$). No differences were found in the number of *pushes* between Related females and Related males (K-W = 2.33, df = 2; $p = 0.12$). Subsequent analysis of the cumulative frequencies of the pushes over the days showed for Restricted unrelated females ($p = 0.029$) and Restricted related males ($p = 0.005$) a significant linear component was observed (no stabilization of the number of pushes over the days). Quadratic or cubic component was not significant ($p > 0.09$). However, this linear, quadratic or cubic component was not significant for the group of sister females Restricted related females ($p > 0.06$).

Chasing Behavior and Time Under the Feeder

Analysis of the total chasing behavior through the 23 days was done for Restricted unrelated and Restricted related mice after the absence of activity in the *ad libitum* groups. Application of ANOVA here confirms the sphericity and equality of variances (Levene F = 0.703, df = 2; $p = 0.532$). Analysis showed no significant effect of Group (One Way ANOVA: F = 0.290, df = 2; $p = 0.748$. $\eta^2 = 0.088$). Similar, for the time under the feeder was done for Restricted unrelated and Restricted related mice. Due to the total absence of activity under the feeder when food was administered, the *ad libitum* groups were eliminated for analysis. Application of ANOVA here confirms the sphericity and equality of variances (Levene F = 1.697, df = 2; $p = 0.261$). Analysis showed no significant effect of group (One Way ANOVA: F = 1.395, df = 2; $p = 0.318$. $\eta^2 = 0.318$).

Adrenocorticotropic Hormone Levels

Figure 3 shows the adrenocorticotropic hormone values obtained for the five groups. A one-way ANOVA revealed the violation of the equality of variances (Levene F = 3.329; $p < 0.03$). Kruskal-Wallis analysis shows the absence of significant differences between groups (K-W = 0.721, df = 5; $p > 0.9$). Additional analysis of the magnitude of the effect using the Cohen's *d* shows values between the groups negative or under 0.2 (lower effect). Only the differences between the restricted related female with the restricted related male and restricted no-related female had a Cohen's *d* of 0.242 and 0.221, respectively. This

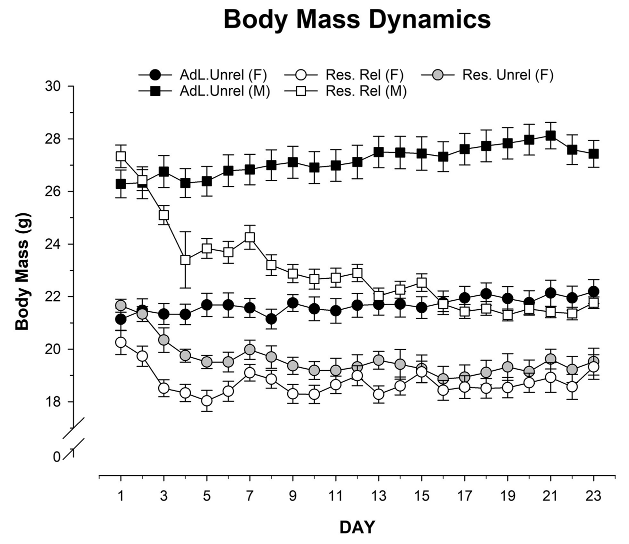

FIGURE 1 | Mean (±SEM) body mass (g) throughout the 23 days (D) in *ad libitum* unrelated female and male (Adl.Unrel.), restricted unrelated female (Res. Unrel.) and restricted related female and male (Res.Rel) mice. Results showed the significant main effect of the variable group and the interaction between group and day. However, the variable day did not exert a significant main effect. Analysis of the interaction (keeping the first day as a covariate factor) shows that *ad libitum* Females have a significant higher body mass than restricted related female (days 3–23), restricted unrelated female and restricted related male (days 4–23). *Ad libitum* males have a significant higher body mass than restricted related female (days 3, 5, 6–14, 16–23). *Ad libitum* males have a significant higher body mass than restricted unrelated female and restricted related male (days 3, 5–23). Restricted related male have a significant less body mass than restricted related female (day 15, 17, 21). Restricted related male have a significant less body mass than restricted unrelated female (days 19, 21).

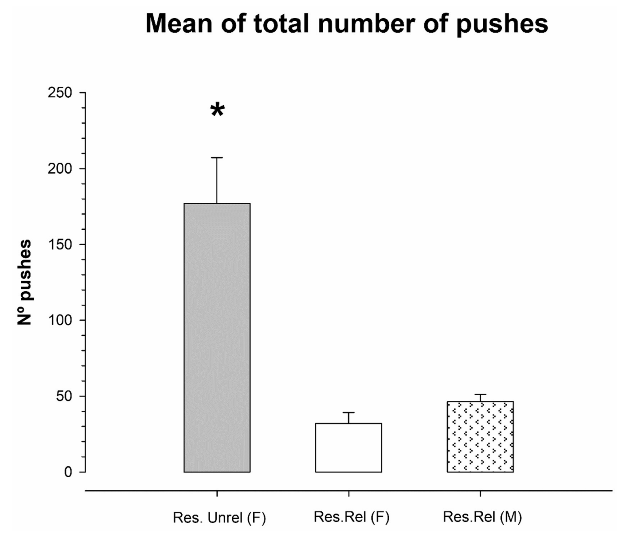

FIGURE 2 | Mean (±SEM) of total number of *pushes* during the 23 days in restricted unrelated female (Res.Unrel) and restricted related female and male (Res Rel) mice. Restricted unrelated females showed a higher number of *pushes* than restricted related females and males as indicated by (*) sign.

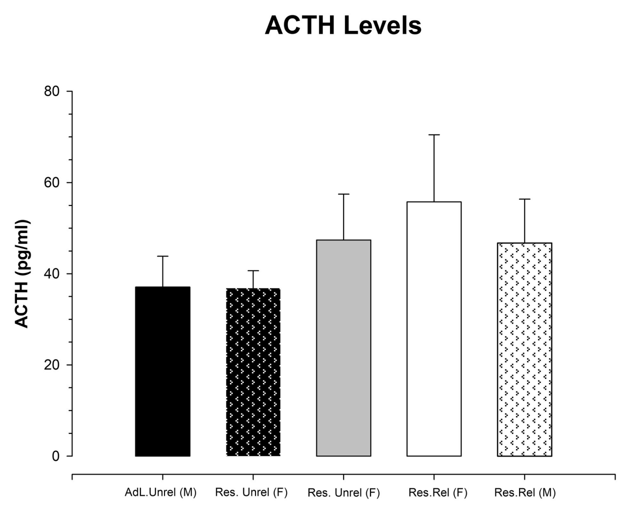

FIGURE 3 | Mean (±SEM) of adrenocorticotropic hormone concentration (pg/mL) in *ad libitum* unrelated female and male (Adl.Unrel), restricted unrelated female (Res Unrel) and restricted related female and male (Res.Rel) mice collected on the 23rd day of the experiment. No significant differences between groups are observed.

means that the CR procedure produced no significant increases in adrenocorticotropic hormone levels in any group.

DISCUSSION

The first result that requires comment is the impossibility of applying CR in unrelated male mice, contrary to the lack of aggressive behavior observed in unrelated rats under CR conditions (34). This data supports the greater aggressiveness previously observed in mice (42), and particularly in BALB/C mice (22, 43, 44). This greater aggressiveness in males toward other males (45) made CR impossible among unrelated males. However, in littermates, and in unrelated female mice the aggressiveness does not appear and neither does clear a type of considered cooperative social behavior such as the time under feeder of all the animals at the same time. This possible absence of social behavior (measured in our experimental conditions) can also be interpreted as a sign of less complex empathy in mice. Although mice display empathy (27) and social behavior (46), it seems that under CR conditions this social and cooperative behavior does not appear even though the mice lived together after weaning for 7–8 weeks. Groups of littermates showed lower levels of aggressiveness, confirming previous observations in mice (47) and this was demonstrated in the non-aggressive behavior observed under CR conditions in male and female mice. In the case of unrelated females, the correct development of CR conditions was facilitated by levels of aggressiveness that were lower than usual among non-pregnant females (45). However, an analysis of the *pushes*, shows a higher total number of pushes in unrelated female mice under CR conditions than in related female and male mice. This was an unexpected result. In previous research, aggressiveness implies another kind of behaviors more violent (attacks, bites or squeaks). In our experiment, this kind of behavior was only showed in the restricted unrelated mice, but was not observed in the rest of the restricted groups. Only chasing behavior was observed and the differences between groups were no significant between restricted unrelated female and the restricted female and male littermates. Differences were observed only in pushing. The *pushes* observed in unrelated female mice did not imply aggressive behavior, causing (sometimes) other mice to shuffle or fall. Perhaps this low aggressive behavior is explained considering that it was observed in females. Another possible explanation for this behavior could be the 7–8 weeks that unrelated female mice were housing together. This previous cohabitation and the grouping right after weaning might perhaps mitigate this aggressive response that have been observed in restricted unrelated males (47). These data open the possibility to considerer other variables such as time of cohabitation apart and not only the strain or the characteristics of grouping (22, 35). Likewise, consideration of the possible influence of environmental changes on the induction of aggressive behaviors (e.g., as the observed in transportation to research facilities) or housing conditions (42) should be noted.

These positive effects have been observed in the adrenocorticotropic hormone analysis. An absence of significant differences between groups, which could be interpreted as an indirect measure of the absence of alterations in stress levels (36, 37, 48). It is also true that samples were taken at the end of 23 days under CR conditions, and these levels could therefore actually be associated with other biomarkers. In this respect, the possible role played by orexin has been studied as a neuropeptide that might connect prolonged food restriction periods, aggressiveness and social behavior (46, 49–51). The long time period between the CR and the adrenocorticotropic measure has perhaps have produced an adaptation as probably other biomarkers such as feeding times, usually done in the dark period [for a review see (52)]. It might be interesting to further investigate in this area, to clarify not only the aggressive response associated to CR in unrelated mice, but also changes in biomarkers. However, in our study the objective was just to evaluate the viability of applying long CR in mice despite its inherent aggressiveness (22).

Lastly, and regarding the effectiveness of CR, while there was a 21% reduction in body mass in related males under CR relative to *ad libitum* males, which is consistent with previous results in rats (34, 53, 54), body mass reduction associated with CR in females was lower (13%). This result can be attributed to differences observed between female and male metabolism (55, 56).

CONCLUSIONS

Our results have important implications, particularly in relation to the difficulties attached to long-term CR in unrelated male mice. In that context, and based on the results observed in terms of aggressiveness, the best option when implementing CR in mice would be to group-house littermates.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by The University of Granada's Research Ethics Committee and the Junta de Andalucía, Consejería de Agricultura, Ganadería, Pesca y Desarrollo Sostenible approved the experimental protocol with reference number 09/08/2019/137.

AUTHOR CONTRIBUTIONS

CP, IM, JZ, and CC: study concept and design. CP and IM: acquisition of data. CP, LR-L, AV-Á, and IM: analysis and interpretation of data and statistical analysis. IM: drafting of the manuscript. CP, LR-L, AV-Á, IM, JZ, and CC: critical revision of the article for important intellectual content. JZ and CC: obtained funding. All authors had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

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REFERENCES

1. Claassen V. Neglect factors in pharmacology and neuroscience research: biopharmaceutics, animal characteristics, maintenance, testing conditions. In: Houston SP, editor. *Techniques in the Behavioural and Neural Sciences*, Vol 12. Amsterdam: Elsevier (1994).
2. Speakman JR, Mitchell SE. Caloric restriction. *Mol Asp Med.* (2011) 32:159–221. doi: 10.1016/j.mam.2011.07.001
3. Bertrand HA, Herlihy JT, Ikeno Y, Yu PB. Diatery restriction. Yu BP, editor. In: *Methods in Aging Research.* CRC Press. Boca Raton (1999). p. 271–300.
4. Keenan KP, Ballan GC, Dixit R, Soper KA, Laroque P, Mattson BA, et al. The effects of diet, overfeeding and moderate dietary restriction

on Sprague-Dawley rat survival, disease and toxicology. *J. Nutr*. (1997) 127:851S−6S. doi: 10.1093/jn/127.5.851S
5. Fernandes G, Venkatraman JT, Turturro A, Attwood VG, Hart RW. Effect of food restriction on life span and immune functions in long-lived Fischer-344 X Brown Norway F-1 rats. *J Clin Immunol*. (1997) 17:85–95. doi: 10.1023/A:1027344730553
6. MacDonald R. Some considerations for developing diets for mature rodents used in long-term investigations. *J Nutr*. (1997) 127:847S−50S. doi: 10.1093/jn/127.5.847S
7. Masoro EJ. Food restriction in rodents: an evaluation of its role in the study of aging. *J Gerontolog*. (1988) 43:B59–66. doi: 10.1093/geronj/43.3.B59
8. Rehm S, Nitsche B, Deerberg F. Non-neoplastic lesions of female virgin Han:NMRI mice, incidence and influence of food restriction throughout life span. I: Thyroid. *Lab Anim*. (1985) 19:214–23. doi: 10.1258/002367785780893601
9. Weindruch R, Walford RL, Fligiel S, Guthrie D. The retardation of aging in mice by dietary restriction: longevity, cancer, immunity and lifetime energy intake. *J Nutr*. (1986) 116:641–54. doi: 10.1093/jn/116.4.641
10. Yu BP. Aging and oxidative stress: modulation by dietary restriction. *Free Radic Biol Med*. (1996) 21:651–68. doi: 10.1016/0891-5849(96)00162-1
11. Niraula A, Sheridan JF, Godbout J. Microglia priming with aging and stress. *Neuropsychopharmacology*. (2017) 42:318–33. doi: 10.1038/npp.2016.185
12. Levay EA, Govic A, Penman J, Paolini AG, Kent S. Effects of adult-onset caloric restriction on anxiety-like behaviour in rats. *Physiol Behav*. (2017) 92:889–96. doi: 10.1016/j.physbeh.2007.06.018
13. Mantis J, Fritz CL, Marsh J, Heinrichs SC, Seyfried TN. Improvement in motor and exploratory behavior in Rett syndrome mice with restricted ketogenic and standard diets. *Epilep Behav*. (2009) 15:133–41. doi: 10.1016/j.yebeh.2009.02.038
14. Riddle MC, McKenna MC, Yoon YJ, Pattwell SS, Santos PM, Casey BJ, et al. Caloric restriction enhances fear extinction learning in mice. *Neuropsychoparmacology*. (2013) 38:930–7. doi: 10.1038/npp.2012.268
15. Hendriksen H, Bink DI, Vergoosen DL, van Slobbe ES, Olivier B, Oosting R. Food restriction does not relieve PTSD like anxiety. *Eur J Pharmacol*. (2015) 753:177–82. doi: 10.1016/j.ejphar.2014.10.060
16. Yanai S, Okaichi Y, Okaichi H. Long-term restriction causes negative effects on cognitive functions in rats. *Neurobiol Aging*. (2004) 25:325–32. doi: 10.1016/S0197-4580(03)00115-5
17. Sabau RM, Ferkin M. Food restriction affects maternal behavior provided by female meadow voles (microtus pennsylvanicus). *J Mamm*. (2013) 94:1068–76. doi: 10.1644/13-MAMM-A-060.1
18. Govic A, Levay EA, Kent S, Paolini AG. The social behavior of male rats administered an adult-onset calorie restriction regimen. *Phys Behav*. (2009) 96:581–5. doi: 10.1016/j.physbeh.2008.12.012
19. Lister RG, Hilakivi LA. The effects of novelty, isolation, light and ethanol on the social behavior on mice. *Psychopharmacology*. (1988) 96:181–7. doi: 10.1007/BF00177558
20. Peters DP. Effects of prenatal nutritional deficiency of affiliation and aggression in rats. *Physiol Behav*. (1978) 20:359–62. doi: 10.1016/0031-9384(78)90313-X
21. Whatson TS, Smart J. Social behaviour of rats following pre- and early postnatal undernutrition. *Physiol Behav*. (1978) 20:749–53. doi: 10.1016/0031-9384(78)90301-3
22. Lidster K, Owen K, Browne WJ, Prescott MJ. Cage aggression in group-housed laboratory male mice: an international data crowdsourcing project. *Sci Rep*. (2019) 9:15211. doi: 10.1038/s41598-019-51674-z
23. Thorsell A, Slawecki CJ, El Khoury A, Mathe AA, Ehlers CL. The effects of social isolation on neuropeptide Y levels, exploratory and anxiety-related behaviors in rats. *Pharmacol Biochem Behav*. (2006) 83:28–34. doi: 10.1016/j.pbb.2005.12.005
24. Mähler M, Berard M, Feinstein R, Gallagher A, Illgen-Wilcke B, Pritchett-Corning K, et al. FELASA recommendations for the health monitoring of mouse, rat, hamster, guinea pig and rabbit colonies in breeding and experimental units. *Lab Anim*. (2014) 48:178–92. doi: 10.1177/0023677213516312
25. Balcombe JP. Laboratory environments and rodents' behavioural needs: a review. *Lab Anim*. (2006) 40:217–35. doi: 10.1258/002367706777611488
26. Crawley JN. *Social Behavior Tests for Mice*. (2007). Available online at: http://ccsystems.us/v2mag/L018.pdf (accessed August 5, 2014).
27. Keum S, Shin HS. Genetic factors associated with empathy in humans and mice. *Neuropharmacology*. (2019) 159:107514. doi: 10.1016/j.neuropharm.2019.01.029
28. Smith BM, Yao X, Chen KS, Kirby ED. A larger social network enhances novel object location memory and reduces hippocampal microgliosis imaged mice. *Front Aging Neurosci*. (2018) 10:142. doi: 10.3389/fnagi.2018.00142
29. Strasser A, Skalicky M, Hansalik M, Viidik A. The impact of environment in comparison with moderate physical exercise and dietary restriction on BDNF in the cerebral parietotemporal cortex of aged sprague-dawley rats. *Gerontology*. (2006) 52:377–81. doi: 10.1159/000095117
30. Boggiano MM, Cavigelli SA, Dorsey JR, Kelley CEP, Ragan CN, Chandler-Laney PC. Effect of cage divider permitting social stimuli on stress and food intake in rats. *Physiol Behav*. (2008) 95:222–8. doi: 10.1016/j.physbeh.2008.04.025
31. Yamada K, Ohki-Hamakazi H, Wada K. Differential effects of social isolation upon body weight, food consumption, and responsiveness to novel and social environment in bombesin receptor subtype-3 (BRS-3) deficient mice. *Physiol Behav*. (2000) 68:555–61. doi: 10.1016/S0031-9384(99)00214-0
32. Madra M, Zeltser LM. BDNF-Val66Met variant and adolescent stress interact t promote susceptibility to anorexic behavior in mice. *Transl Psychiatry*. (2016) 6:e776. doi: 10.1038/tp.2016.35
33. Zhang F, Yuan S, Shao F, Wang W. Adolescent social defeat induced alterations in social behavior and cognitive flexibility in adult mice: effects of developmental stage and social condition. *Front Behav Neurosci*. (2016) 10:149. doi: 10.3389/fnbeh.2016.00149
34. Moneo M, Martín Zúñiga J, Morón I. Caloric restriction in grouped rats: aggregate influences on behavioural and hormonal data. *Lab Anim*. (2017) 51:490–7. doi: 10.1177/0023677216686805
35. Swenson KL, Gatt DM, Valdar W, Welsch CE, Cheng R, Chester EJ, et al. High-resolution genetic mapping using the mouse diversity outbred population. *Genetics*. (2012) 190:437–47. doi: 10.1534/genetics.111.132597
36. Ehrhart-Bornstein M, Bornstein SR. Cross-talk between adrenal medulla and adrenal cortex in stress. *Ann NY Acad Sci*. (2008) 1148:112–7. doi: 10.1196/annals.1410.053
37. Möstl A, Palme R. Hormones as indicators of stress. *Domest Anim Endocrinol*. (2002) 23:67–74. doi: 10.1016/S0739-7240(02)00146-7
38. Friard O, Gamba M. BORIS: a free, versatile open-source event-logging software for video/audio coding and live observations. *Methods Ecol Evol*. (2016) 7:1325–30. doi: 10.1111/2041-210X.12584
39. Blanchard DC, Blanchard RJ. Ethoexperimental approaches to the biology of emotion. *Ann Rev Psychol*. (1988) 39:43–68. doi: 10.1146/annurev.ps.39.020188.000355
40. Bolivar VJ, Ganus JS, Messeret A. The development of behavioral abnormalities in the motor neurondegeneration (mnd) mouse. *Brain Res*. (2002) 937:74–82. doi: 10.1016/S0006-8993(02)02470-8
41. Silverman P. *Animal Behaviour in the Laboratory*. New York, NY: Pica Press (1978).
42. Webers EM, Dallaire JA, Gaskill BN, Pritchett-Corning KR, Garner JP. Aggression in group-housed laboratory mice: why can't we solve the problem? *Lab Anim*. (2017) 46:157–61. doi: 10.1038/laban.1219
43. Cambona K, Dos-Santos Coura R, Grocc L, Carbona A, Weissmann D, Changeuxd JP, et al. Aggressive behavior during social interaction in mice is controlled by the modulation of tyrosine hydroxylase expression in the prefrontal cortex. *Neuroscientie*. (2010) 171:840–51. doi: 10.1016/j.neuroscience.2010.09.015
44. Gaskill BN, Stottler AM, Garner JP, Winnicker CW, Mulders GB, Pritchett-Corning KR. The effect of early life experience, environment, and genetic factors on spontaneous home-cage aggression-related wounding in male C57BL/6 mice. *Lab Anim*. (2017) 46:176–84. doi: 10.1038/laban.1225
45. Albert DJ, Jonik RH, Walsh ML. Hormone-dependent aggression in male and female rats: experiential, hormonal, and neural foundations. *Neurosc Biobehav Rev*. (1992) 16:177–92. doi: 10.1016/S0149-7634(05)80179-4
46. Abbas G, Shoji H, Soya S, Hondo M, Miyakawa T, Sakurai T. Comprehensive behavioral analysis of male Ox1r-/- mice showed implication of orexin receptor-1 in mood, anxiety and social behavior. *Front Behav Neurosc*. (2015) 9:324. doi: 10.3389/fnbeh.2015.00324
47. Mendl M, Paul ES. Parental care, sibling relationships and the development of aggressive-behavior in 2 lines of wild house mice. *Behavior*. (1991) 116:11–41. doi: 10.1163/156853990X00347

48. Veenema AH, Meijer OC, de Kloet RE, Koolhaas JM, Bohus BG. Differences in basal and stress-induced HPA regulation of wild house mice selected for high and low aggression. *Horm Behav*. (2003) 43:197–204. doi: 10.1016/S0018-506X(02)00013-2
49. Kotz M. Integration of feeding and spontaneous physical activity: role of orexin. *Phys Behav*. (2006) 88:294–301. doi: 10.1016/j.physbeh.2006.05.031
50. Sato N, Tan L, Tate K, Okada M. Rats demonstrate helping behavior toward a soaked conspecific. *Anim Cogn*. (2015) 18:1039–47. doi: 10.1007/s10071-015-0872-2
51. Sucajtys-Szulc E, Turyn J, Goyke E, Korczynska J, Stelmanska E, Slominska E, et al. Differential effect of prolonged food restriction and fasting on hypothalamic malonyl-CoA concentration and expression of orexigenic and anorexigenic neuropeptides genes in rats. *Neuropeptides*. (2010) 44:17–23. doi: 10.1016/j.npep.2009.11.005
52. Jensen TL, Kiersgaard MK, Sørensen DB, Mikkelsen LF. Fasting of mice: a review. *Lab Anim*. (2013) 47:225–40. doi: 10.1177/0023677213501659
53. Gedik CM, Grant G, Morrice PC, Wood SG, Collins AR. Effects of age and dietary restriction on oxidative DNA damage, antioxidant protection and DNA repair in rats. *Eur J Nutr*. (2005) 44:263–72. doi: 10.1007/s00394-004-0520-0
54. He XY, Zhao XL, Gu Q, Shen JP, Hu Y, Hu RM. Calorie restriction from a young age preserves the functions of pancreatic β cells in aging rats. *Tohoku J Exp Med*. (2012) 227:245–52. doi: 10.1620/tjem.227.245
55. Chowen JA, Argente-Arizon P, Freire-Regatillo, Argente J. Sex differences in the neuroendocrine control of metabolism and the implication of astrocytes. *Front Neuroendoc*. (2018) 48:3–12. doi: 10.1016/j.yfrne.2017.05.003
56. Link JC, Reue K. Genetic basis for sex differences in obesity and lipid metabolism. *Ann Rev Nutr*. (2017) 37:225–45. doi: 10.1146/annurev-nutr-071816-064827

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Welfare Assessment of 30 Dairy Goat Farms in the Midwestern United States

Melissa N. Hempstead[1†], Taylor M. Lindquist[1], Jan K. Shearer[1], Leslie C. Shearer[1], Vanessa M. Cave[2] and Paul J. Plummer[1]*

[1] Veterinary Diagnostic and Production Animal Medicine, College of Veterinary Medicine, Iowa State University, Ames, IA, United States, [2] Knowledge and Analytics, AgResearch Ltd., Ruakura Research Centre, Hamilton, New Zealand

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***Correspondence:**
Paul J. Plummer
pplummer@iastate.edu

†Present address:
Melissa N. Hempstead,
AgResearch Ltd., Grasslands Research Center, Palmerston North, New Zealand

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Dairy goat animal welfare assessment protocols have been developed and conducted in Europe and the United Kingdom for dairy goats; however, there are no published reports of large-scale welfare assessment for dairy goats on farms in the Midwestern United States (US). Therefore, the objective of this study was to perform welfare assessment of lactating dairy goats and identify the most prevalent welfare issues on 30 farms across the Midwestern US. Thirty dairy goat farms (self-selected) were enrolled in the study if they shipped milk for human consumption (regardless of herd size). The number of lactating does on each farm ranged from 34 to 6,500 goats, with a median number of 158 lactating does (mean ± SD: 602 ± 1,708 lactating does). The protocol used was developed from available literature on goat welfare assessment but modified for use in the Midwestern US. Observations were made without handling the animals and included 22 animal-based indicators evaluated at the group- and individual-level. The observations were conducted during ∼3–5 h during a milking session (either morning or afternoon) and time in the home pen. Principal components analysis (PCA) was carried out on the welfare assessment data from each farm. The first two dimensions of the PCA explained 34.8% of the variation. The PCA biplot indicated correlations between indicators. The most prevalent conditions observed across the 30 farms included any knee calluses (80.9%), any claw overgrowth (51.4%), poor hygiene (14.9%), skin lesions (8.9%), poor hair coat condition (8.3%) and any ear pathology (8.0%). These results are the first to provide the Midwestern US dairy goat industry with information to improve commercial dairy goat welfare.

Keywords: animal welfare, animal husbandry, welfare assessment, well-being, goat, caprine, dairy

INTRODUCTION

Defining animal welfare is difficult because there are multiple interpretations (1). An early interpretation of animal welfare was formulated by the Farm Animal Welfare Council, named the "Five Freedoms," and outlined the basis of acceptable levels of welfare (i.e., freedom from hunger or thirst, discomfort, pain, injury or disease, fear and distress and the freedom to express normal behaviors (2). Since then, other viewpoints have been developed such as the "three overlapping dimensions" of welfare where an animal's quality of life relates to basic health and functioning, affective states, and natural living (3), or the "Five Domains" model, whereby an animal experiences good welfare if its nutritional, environmental, health, behavioral, and mental (i.e., affective state) needs are met (4). However, regardless of how animal welfare is defined, the development of an on-farm monitoring system or welfare assessment protocol, which encompasses multiple indicators of welfare can be developed and utilized for small ruminants (5).

Early research on development of protocols to assess welfare at the farm-level for dairy goats evaluated multiple animal-based indicators of welfare and highlighted the major welfare issues across 24 farms in the UK (6) and 30 farms in Norway (7). Since then, the European Animal Welfare Indicators Project (AWIN) developed a science-based, step-wise welfare assessment protocol for species (including goats, sheep, horses, donkeys, and turkeys) that had until then, been largely excluded from welfare assessment projects such as Welfare Quality® (8). Welfare Quality®, a large-scale science-based European program designed to assess the welfare of cattle, swine, and poultry used a framework consisting of 4 key principles (i.e., good feeding, housing and health, and appropriate behavior), with 12 criteria (e.g., absence of prolonged hunger, comfort around resting, expression of social behavior) (9). AWIN was based on the same such principals and criteria as Welfare Quality® as they are considered necessary to cover all aspects of animal welfare (8). Some examples of animal-based indicators of welfare used by AWIN include hair coat and body condition, fecal soiling, udder asymmetry, overgrown claws, and lameness (10). Development and testing of the AWIN protocol for dairy goats has since demonstrated valid, reliable, and feasible animal-based indicators of welfare in a European setting (11–15). However, to the authors' knowledge, no such on-farm welfare assessment protocols have been designed for, or undertaken on dairy goats in the Midwestern US.

In the US, there are welfare assessments of commercial swine [see review by (16)], poultry [see review by (17, 18)], dairy cattle (19) and turkey (20) farms. However, welfare assessment data for dairy goats in the US is scarce. In 2020, there were ∼440,000 dairy goats in the US, and of those, 135,000 (∼31%) were populated in the Midwestern region comprising Minnesota, Iowa, Wisconsin, and Illinois (21). Dairy goat welfare assessment data can help inform producers on areas of deficiency and consequent improvement, promotion of good welfare policies, and can add to the growing body of science-based research on welfare assessment of dairy goats worldwide.

The objective of this study is to perform welfare assessment of dairy goats and identify the most prevalent welfare issues on 30 farms across the Midwestern United States (US).

MATERIALS AND METHODS

This study was approved by the Institutional Animal Care and Use Committee at Iowa State University prior to data collection (Protocol number: IACUC-18-341).

Farm Recruitment

Advertising material was distributed to farms by a milk company operating in the Midwestern region on our behalf. Additionally, farms were visited by study personnel (with a feed representative) and advertising material was distributed directly to farm owners. Participation was incentivized by receipt of compensation associated with participation on the study. Once 30 dairy goat farm owners had voluntarily completed an online application form (Smartsheet Inc., Bellevue, WA), their farms were enrolled in the study if they shipped milk for human consumption (regardless of herd size) and were situated within the Midwestern states: Minnesota, Iowa, Wisconsin, and Illinois. Farm owners were asked to complete a survey independently of on-farm assessment, which focused on farm owner attitude to goat behavior and welfare, husbandry practices, goat-specific information and other details of the farm (Hempstead et al., unpublished data).

Protocol Development

The protocol was developed from the available literature on goat welfare assessment (5, 10, 22) including assessment protocols that had been used previously (6–8, 12, 14). The protocol was designed for use on adult lactating does and comprised 22 animal-based indicators of welfare at the individual- (9 indicators; **Table 1**) and group-level (13 indicators; **Table 2**) that were decided for inclusion by a small committee of veterinary practitioners and an animal scientist.

Sampling periods included (1) assessments of individuals in the milking parlor during routine milking and (2) group assessments, which were carried out in the home pen. The order of these sampling periods (i.e., at milking or the home pen), depended on whether a morning or afternoon milking session was attended. Within each sampling period, the indicators were assessed in the same order for each farm (**Tables 1**, **2**); for example, if the morning milking session was observed (between 0400 and 0700 h), then the group-level assessment took place following milking. However, if an afternoon milking session was observed (between 1400 and 1800 h), the group-level assessment was carried out prior to milking. The separate sampling periods were chosen in order to facilitate multiple farm visits within 1 day. The time of feed distribution relative to assessment of the home pen was not recorded. Observations were performed without animal handling. Indicators were excluded if they (i) required laboratory analysis, or specific instruments to be used on the animal (e.g., stethoscope, thermometer), (ii) were overly time consuming and could not be carried out on the day of observation (i.e., requiring post-observation video analysis), (iii) were reported to have low prevalence [e.g., oblivion, abnormal lying (12)], or (iv) necessary training was not available [e.g., qualitative behavior analysis (7, 12)]. Resource-based indicators that provided information on environmental conditions such as space allowance per goat and bedding material were collected.

The initial protocol was tested over multiple visits to a local farm in Iowa over a 2 week period. Two observers tested the protocol in the milking parlor and home pen to ensure the definitions accurately reflected the observations made, and the length of time required to perform the assessments. Where differences in the results between observers were observed, further training was provided to improve agreement on subsequent visits.

TABLE 1 | Descriptions of the *individual-level* welfare indicators and the order of which they were assessed for the dairy goat welfare assessment protocol.

Order	Welfare indicator	Description
1	Ear pathology	
	Ear tear	Complete or partial tear of the pinna.
	Missing ear tag	A hole in the pinna from a missing ear tag.
	Infected ear tag	Ear tag with evidence of infection (e.g., swelling, pus).
	Frostbitten ears	Any amount of pinna is missing (appears as a straight cut).
2	Ocular discharge	Moist (or dry) fluid from the eye(s) that is clear or colored fluid, thick, or runny.
3	Nasal discharge	Moist (or dry) fluid from the nostril(s) that is clear or colored fluid, thick, or runny.
4	Skin lesion	Any broken skin, abscess or ulceration (fresh or in the process of healing, i.e., crust). Regions that were observed for skin lesions included the head or neck, and the rump or thigh. Fully re-epithelialized tissue was excluded.
5	Knee callusing	
	Mild	Thickened skin (with hair loss) covered *part* of the knee. The score of the worst knee was recorded. Knees were not scored if calluses were not clearly visible (i.e., too dirty).
	Severe	Thickened skin covered the *entire* knee (with hair loss) and may have had broken skin. The score of the worst knee was recorded. Knees were not scored if calluses were not clearly visible (i.e., too dirty).
6	Poor hygiene	The presence of any fecal material (or dirt) on the hind quarters (i.e., rump, thigh, rear legs, udder) that can be dry or moist. Goats that kidded recently (i.e., visible afterbirth or blood) were not scored.
7	Fecal soiling	Presence of feces around the anus or sides of the tail. Goats that kidded recently (i.e., visible afterbirth or blood) were not scored.
8	Udder asymmetry	One side of the udder was >25% longer than the other side (from the udder attachment to udder floor; excluding teat).
9	Overgrown claw	Only the rear claws were assessed.
	Mild	Overgrowth beyond the triangular shape of the claw, but no change in hoof conformation. The score of the worst claw was recorded.
	Severe	Extreme claw overgrowth with loss of the triangular shape and conformational changes of the hoof, which may include weight bearing on the heel. The score of the worst claw was recorded.

Both sides of the animal were assessed for all indicators.

On-Farm Assessments

Assessments were performed by a single assessor between March and August 2020. The assessor wore the same colored clean coveralls and used disposable boot covers and gloves between farms. Observations were manually recorded using a tablet (10.2″ iPad, 8th Generation, Apple Inc., Cupertino, CA) equipped with data collection software (REDCap, Vanderbilt University, Nashville, TN). Due to equipment malfunction after seven farm visits, data was then recorded onto printed record sheets and then manually entered onto REDCap software after completion of the farm visit.

The temperature and humidity were measured 10 min after arrival to the pens using a temperature and humidity logger (WD-20250-42; Digi-Sense, Vernon Hills, IL). Temperature and humidity ranged from −7.6 to 34.7°C with an average of 21.4°C (SD: 10.2) and 20.7% rh (relative humidity) to 80.6% rh with an average of 51.5% rh (SD: 13.7), respectively.

Intra-observer reliability was completed pre- and post-observation and was assessed by scoring 50 images of goats collected prior to farm visits (with some images collected during farm visits) and then re-examined. Percentage agreement for pre- and post-observation reliability (respectively) was as follows: 98% for ear pathology (pre- and post-observation), 94 and 98% for ocular discharge, 96 and 98% for nasal discharge, 96 and 98% for skin lesion, 92 and 90% for knee callusing, 97% for hygiene (pre-observation reliability not completed due to lack of images of goats with poor hygiene), 98% and 100% for fecal soiling, 98% and 94% for udder asymmetry, 92 and 94% for overgrown claw, 100% for horn growth (pre- and post-observation), 98 and 90% for poor hair coat condition, and 90 and 94% for body condition. Inter-observer reliability was not conducted for some indicators (e.g., queuing behavior, thermal stress, kneeling, and lameness) that showed low occurrence rates or were difficult to photograph.

Group Assessments

The number of pens (and animals) assessed was determined at each farm visit and depended on the number of lactating goats on farm (**Table 3**). All pens that housed <230 lactating does were observed unless the farm had more than 600 lactating does. In this case, either one pen of goats was observed or as many pens that could be evaluated in a 2 h period. After observing all pens on the farm, the assessor chose the pen(s) to be assessed based on being representative of the farm and containing mobile, and lactating goats (i.e., not the sick pen). Note that pens were selected in this way on only three farms. The group assessments took place in the goat barn after a short acclimatization period of ∼5 min. Depending on the number of animals in each pen, the group-level assessments of the goats were observed for up to 2 h. Due to inconsistencies in recording of the durations of animal observations at each farm, this information will not be reported. During this period, the assessor moved slowly along the outside rail of each pen recording observations. Once outside pen observations were complete, the assessor entered the pen and began the latency to approach test; this involved moving to a predetermined location adjacent to a pen wall and while remaining motionless and without making eye contact with the

TABLE 2 | Descriptions of the *group-level* welfare indicators and the order of which they were assessed for the dairy goat welfare assessment protocol.

Order	Welfare indicator	Description
1	Queuing at feed rack(s)	Goat standing behind another goat at the feed rack(s) within 1 m with head oriented toward the feed rack(s) during feeding time. The number of goats queuing was counted over 16 min (scan sample every 2 min).
2	Queuing at drinking place(s)	Goat standing behind another goat at the drinking place(s) within 1 m with head oriented toward the drinking place. The number of goats queuing was counted over 16 min (scan sample every 2 min) during feeding time.
3	Horn growth	
	Horn	Horn(s) with normal growth. Horns with the tip mechanically removed were included.
	Scur	Soft, partially formed horn that is not attached to the underlying frontal bone.
4	Poor hair coat condition	Dull, rough, and shaggy hair coat that may be longer on some parts of the body than others.
5	Thermal stress	
	Cold	Cramped posture (arched back) raised hair along the neck and spine (i.e., horripilation), limited movement and may include shivering. Goats that were involved in agonistic interactions (with associated horripilation) were excluded.
	Heat	Accelerated respiration rate, open mouth panting with or without drool from the lips.
6	Kneeling	Transitions between lying and standing were excluded.
	In pen	Knees touching the pen floor at the *lying area* (≥5 s/bout).
	At feed rack(s)	Knees touching the pen floor at the *feed rack* (≥5 s/bout).
7	Latency to approach test	Time taken for a goat to contact any part of a novel person in the pen (including clipboard). The assessor moved to a predetermined location in the pen, usually with their back to the wall or gate. The test ended after a non-contact time of 5 min.
8	Body condition	
	Overweight	Hip and pin bones were difficult to identify and the line between them was convex.
	Underweight	Hip and pin bones were prominent and the line between them was concave.
9	Lameness	Abnormal gait and curvature of the spine that may have included head nodding (bobbing). Goats were encouraged to walk by the assessor. Those that did not stand or had any obvious injuries were excluded.

goats. Once stationary the assessor started a stopwatch and the time taken (in seconds) for the first goat to contact any part of the assessor (including recording devices) was recorded. The assessor then moved slowly throughout the pen assessing body condition and lameness. All goats within the pen were made to walk, except those that did not stand or had obvious injuries and were excluded from lameness scores. The assessor avoided contact with the goats as much as possible.

Individual Goat Observations

The number of does on each farm assessed at the individual-level depended on the number of lactating does and is presented in **Table 3**. When the number of lactating does was <230, all does were assessed. For farms that had more than 230 does, the assessor observed as many does as could be observed in a 2 h period.

The assessor moved slowly between each goat, making sure to observe both sides of the head and neck region at the front of the goat and the dorsal view of the legs and both sides of the rump at the back of the goat.

Data Management and Statistical Analysis

The data was exported from REDCap software as a comma-separated values file and used with Excel (Microsoft Corporation, Redmond, WA). The data has been presented as a mean with standard error (SE) or median with interquartile range (IQR), where appropriate. The individual- and group-level data was calculated as the number of animals displaying each indicator out of the number of animals observed per farm.

The individual assessment data from one farm was excluded from analysis as the goats were not individually observed in the milking parlor due to logistical constraints. In some instances, milking parlor layout prevented observations from being recorded (e.g., rotary parlors prevented the front and back end of the goats from being observed of the same animal) and consequently some individual assessment data were not collected on three farms. Body condition scoring and lameness data were excluded from one farm as it could not be assessed as the pen was spread across multiple buildings making clear identification of goats difficult.

A principal component analysis (PCA) biplot (based on a correlation matrix) was used to explore the relationships between the farms, and their characteristics with respect to the welfare assessment variables. Missing data (4% of the dataset) was imputed using the mean value of the variable. Heat and cold stress data were excluded from the PCA due to the variation in seasons (i.e., temperature) across farms over the study period.

RESULTS

Welfare assessment was performed on 30 farms in the Midwestern US and the characteristics of those farms are presented in **Table 4**. The number of goats assessed individually and at the group-level was 4,777 goats and 6,593 goats, respectively. The number of lactating goats ranged from 34 to 6,500 goats, with a median herd size of 158 goats (IQR = 80.8; mean ± SE: 533.9 ± 243.3 goats). The individual-level welfare assessment data are presented in **Table 5** and the group-level

TABLE 3 | The number of total pens and lactating does on-farm, the number of pens assessed (and number of does within pens) and does individually assessed in the milking parlor on each farm.

Farm	Total pens on farm	Total lactating does on farm	Number of		
			Pens assessed	Does within pen(s) assessed	Does in milking parlor assessed
1	1	36	1	36	36
2[a]	9	6,500	1	168	510
3[d]	1	70	1	70	67
4[b]	5	179	5	172	179
5[c]	1	142	1	142	139
6	2	178	2	178	178
7[d]	1	110	1	140	110
8	5	857	5	857	158
9	1	1,000	1	1,000	243
10	3	128	3	128	128
11	2	140	2	140	140
12	2	172	2	172	172
13	2	125	2	125	125
14	3	207	3	207	207
15	3	227	3	227	227
16	1	180	1	180	180
17[b]	7	157	7	151	157
18[e]	1	266	1	266	NA
19[c]	3	34	3	78	34
20	3	158	3	158	158
21	2	700	1	322	246
22	2	118	2	118	118
23[c]	1	180	1	185	180
24	2	91	2	92	92
25[d]	2	121	2	162	121
26	12	3,960	2	440	216
27[d]	5	204	5	204	179
28	1	145	1	145	145
29	3	144	3	144	144
30	1	187	1	187	187

[a]includes separate observations of the front of 257 goats and the rear of 253 goats as the front and back of the same animal could not be observed; [b]some goats were not observed during pen assessment; [c]non-lactating goats were housed in the pen with lactating goats; [d]some goats were not observed in the milking parlor; [e]individual assessments in the milking parlor were not carried out.

welfare assessment data are presented in **Table 6**. The average latency for goats to approach the assessor was 33.6 ± 12.0 s (mean ± SE), with a range of 2.0 s to 300.0 s (note that the test ended at 300 s).

Results of a PCA biplot on the welfare assessment data from each farm are shown in **Figure 1**. The overall welfare state of the goats on each farm was described using 19 animal-based indicators (latency to approach test, and heat and cold stress were not included). The first 2 dimensions of the PCA (PC-1 and PC-2) explain 34.8% of the variation. For each variable, the direction of its biplot axis is indicated by an arrow. Axes of welfare indicators that are close to one another (and in the same direction) indicate these variables are positively correlated (e.g., severe claw overgrowth and poor hygiene); axes with arrows in opposing directions indicate negative correlations (e.g., overweight and horns), and perpendicular axes indicate no correlation (e.g., ocular discharge and any ear pathology). The individual farms are represented by points. The predicted value of a welfare indicator for a farm is given by projecting the point onto the axis (i.e., drawing a perpendicular line from the point to the axis). Thus, farms that cluster together (e.g., Farms 17 and 19) are predicted to have similar characteristics with respect to the welfare indicators, and those far apart (Farms 17 and 29) are predicted to be dissimilar.

Farms with a high number of goats that have horn growths (scurs or horns), ear pathologies, fecal soiling, poor coat condition, are underweight, and kneel at feed racks are on the right side of **Figure 1** (e.g., Farms 3, 7, 10, 11, 24, and 29). Conversely, farms with a low number of goats with these welfare issues are scattered on the left side of **Figure 1** (e.g., Farms 1, 17,

TABLE 4 | Characteristics of 30 dairy goat farms in the Midwestern United States.

Farm characteristics	Value
Breeds (No. of farms)*	
Saanen	28
Alpine	28
American LaMancha	18
Anglo-Nubian	11
Toggenburg	10
Oberhasli	6
Sable	2
Kiko	1
Feed space/goat (mean ± SE; min-max; ft)	1.0 ± 0.1 (0.3–1.8)
Total space allowance/goat (mean ± SE; min-max; ft^2/goat)	108.3 ± 43.7 (14.0–1282.0)
Indoor (mean ± SE; min-max; ft^2/goat)	29.3 ± 4.8 (7.4–132.7)
Outdoor (mean ± SE; min-max; ft^2/goat)	78.8 ± 41.2 (0–1178.7)
Type of feed (No. of farms)*	
Hay	27
Grain/concentrate	27
Fermented forage (e.g., silage)	7
Total mixed ration	3
Fresh cut grass	2
Corn	1
Bedding material	
Straw (No. of farms, %)	24 (80.0)
Corn husks (No. of farms, %)	3 (10.0)
Soy fodder (No. of farms, %)	1 (3.3)
Straw, wood shavings, corn husks (No. of farms, %)	2 (6.7)
Milking procedure	
Mechanical (No. of farms, %)	28 (93.3)
Hand-milking (No. of farms, %)	2 (6.6)
Access to outdoor space (No. of farms, %)	22 (73.3)
Outdoor space surface	
Earthen (No. of farms, %)	19 (86.4)
Pasture (No. of farms, %)	13 (59.1)
Concrete (No. of farms, %)	6 (27.3)
Rock (No. of farms, %)	2 (9.1)
No. of permanent staff (mean ± SE; min-max)	6.3 ± 0.9 (1.0–25.0)

**farms provided more than one type of feed and raised more than one breed of goat.*

and 19). Farms scattered near the top of **Figure 1** have a high number of goats that are lame, have severe claw overgrowth, perform queuing at the drinking place, experience heat stress, poor hygiene, severe knee callusing and skin lesions, but a low number of goats with that experience cold stress, have nasal discharge, perform kneeling in the pen and queuing at the feed rack (e.g., Farms 4, 9, 12, and 24).

DISCUSSION

The objective of this study was to perform welfare assessment of dairy goats on 30 farms across the Midwestern US and identify the most prevalent welfare issues. Based on the results of our study, the most prevalent welfare issues observed were knee callusing, claw overgrowth, poor hygiene, skin lesions, poor hair coat condition, and ear pathologies. The collected data was processed and then provided to the producers in the form of benchmarking reports. These reports contained the range of values across farms, the median value, and each farms' average for the welfare indicators. Thus, producers were able to visualize their farms' comparative success (or failure) to the other farms in the study. It was hypothesized that provision of benchmarking reports would encourage producers to alter their farm practices to improve goat welfare in the areas identified as being deficient in comparison to the other farms. Farm visits to conduct secondary welfare assessment and evaluate the effect of the benchmarking reports was delayed due to COVID-19 restrictions on travel.

On-farm welfare assessment of dairy goats has been previously conducted in Europe (7, 12, 14), the United Kingdom (6), and more recently, Mexico (23); however, to the authors' knowledge, these are the first data on dairy goat welfare assessment on farms across the Midwestern US. In 2017, Europe produced 15% of global dairy goat milk production, compared with 4% from the Americas (24). There are differences (and similarities) that exist between North American and European dairy goat industries and associated farming practices (e.g., intensive vs. semi-intensive farming, breeds raised, pain management for painful husbandry practices). In Europe, dairy goat production is highly specialized for milk production likely associated with the higher demand for goat milk products; whereas dairy goat production is comparatively less well-developed, and relatively small by global standards in the US (24). Information on dairy goats in the US is limited due to the viewpoint that goats are a minor species in comparison with cattle, creating issues for farmers, veterinary practitioners, and policy makers (24). Although there are large-scale, commercial dairy goat farms in operation (e.g., 9,000-goat herds), the majority are still small (25). Recent data from the National Animal Health Monitoring Survey (NAHMS), Goat Study 2019 shows that the average herd size across the US is approximately 20 goats (26). For a review of recommendations on dairy goat kid husbandry practices under intensive production systems in Canada, US and France please refer to Bélanger-Naud and Vasseur (64).

Mild or moderate knee calluses are a common occurrence among dairy goats [99.3% of 575 goats (7)], and can reflect the type(s) of surface or amount of bedding available, but it is the severity of knee callusing (i.e., thickness, full width of the knee, broken skin) that may be a welfare concern. Severe knee calluses can be indicative of excessive kneeling, insufficient or inadequate bedding (discussed later) and may be associated with lameness (6). However, the PCA in the present study, showed a negative correlation between severe knee callusing and kneeling in the pen (and only a weak positive correlation with lameness). Additionally, kneeling at the feed rack appeared to show no relationship with severe knee calluses (or kneeling in the pen). This result contradicts our assumption that increased time spent on the knees would result in knee calluses. Anzuino et al. (6) reported that 79.2% of 24 farms in the UK had goats kneeling at the feed trough, but that this was not correlated with

TABLE 5 | *Individual-level* welfare indicators observed for 4,524 goats on 30 farms across the Midwestern United States during on-farm welfare assessment at milking.

Indicator	Number of		Variation in indicator occurrence (% of goats) across farms		
	Goats (%)	Farms (%)	Median	IQR	Maximum
Any ear pathology	361 (8.0)	23 (85.2)$^{\Psi}$	6.5	1.5–12.7	38.6
Frostbitten ears	151 (3.3)	17 (65.4)$^{\Psi}$	1.1	0–3.1	29.3
Torn ears	94 (2.1)	17 (65.4)$^{\Psi}$	1.1	0–3.6	6.7
Missing ear tags	110 (2.4)	18 (69.2)$^{\Psi}$	0.8	0–3.4	11.7
Infected ear tags	6 (0.1)	5 (19.2)$^{\Psi}$	0.5	0–2.5	3.9
Ocular discharge	132 (2.9)	22 (81.5)*	1.6	0.8–3.8	17.6
Nasal discharge	313 (6.9)	25 (92.6)*	3.5	1.4–8.5	38.5
Skin lesions$^{\eta}$	427 (8.9)	26 (96.3)*	10.4	4.5–14.5	40.0
Any knee callusing$^{\Omega}$	3,657 (80.9)	26 (100)$^{\Psi}$	96.8	82.8–99.1	96.8
Mild$^{\Omega}$	2,516 (55.7)	26 (100)$^{\Psi}$	63.8	53.4–75.4	63.8
Severe$^{\Omega}$	1,141 (25.2)	26 (100)$^{\Psi}$	29.2	14.9–41.6	53.1
Poor hygiene (dirty)$^{\Omega}$	674 (14.9)	24 (82.8)$^{¥}$	9.6	3.3–23.7	43.1
Fecal soiling$^{\Omega}$	157 (3.5)	24 (82.8)$^{¥}$	1.6	0.5–6.3	20.9
Udder asymmetry$^{\Omega}$	147 (3.3)	24 (82.8)$^{¥}$	2.9	1.2–4.8	11.1
Any claw overgrowth$^{\Omega}$	2,325 (51.4)	28 (96.6)$^{¥}$	48.6	20.6–75.6	98.3
Mild$^{\Omega}$	1,527 (33.8)	28 (96.6)$^{¥}$	30.0	14.8–46.6	67.8
Severe$^{\Omega}$	798 (17.7)	21 (72.4)$^{¥}$	6.6	0–28.7	69.9

Total number of goats observed for indicators: $^{\Omega}$ *= 4,520,* $^{\eta}$ *= 4,777.*
Data was excluded from:1 farm$^{¥}$*, 3 farms*, 4 farms*$^{\Psi}$*.*

TABLE 6 | *Group-level* welfare indicators observed for 6,593 goats on 30 farms across the Midwestern United States during on-farm welfare assessment.

Indicator	Number of		Variation in indicator occurrence (% of goats) across farms		
	Goats (%)	Farms (%)	Median	IQR	Maximum
Queuing at feed rack(s)$^{\Omega}$	247 (6.8)	22 (75.9)*	5.0	0.4–11.7	35.6
Queuing at drinking place(s)$^{\Omega}$	73 (2.0)	14 (50.0)$^{¥}$	0.0	0.0–2.9	11.1
Horn growth					
Horns	79 (1.2)	11 (36.7)	0	0–0.9	16.0
Scurs	365 (5.5)	28 (93.3)	2.9	1.8–8.0	24.2
Poor hair coat condition	545 (8.3)	30 (100)	6.9	4.9–12.1	27.3
Thermal stress					
Cold stress	4 (0.1)	2 (6.7)	0	0	4.3
Heat stress	243 (3.7)	12 (40)	0	0–3.6	50.8
Kneeling					
In pen	17 (0.3)	8 (26.7)	0	0–0.2	2.3
At feed rack(s)	43 (0.7)	11 (36.7)	0	0–0.6	5.7
Body condition					
Overweight	256 (3.9)	26 (92.9)$^{¥}$	4.1	1.7–5.7	15.2
Underweight	264 (4.0)	26 (92.9)$^{¥}$	2.8	1.0–9.2	22.9
Lameness	99 (1.2)	22 (75.9)*	1.2	0.5–2.3	4.3

Total number of goats observed for indicators: $^{\Omega}$ *= 3,606.*
Data was excluded from: 1 farm, 2 farms*$^{¥}$*.*

lameness. Although observing kneeling behavior on farms is a valid and feasible indicator of discomfort at the feed trough, whether it has good intra- and inter-reliability remains unknown (10). In the present study, the assessor observed the goats in the home pen for up to 2 h, which may not have been enough time to adequately sample kneeling behavior. Further, the assessor observed the goats during two different time periods (i.e., before or after milking), which may affect our ability to directly compare

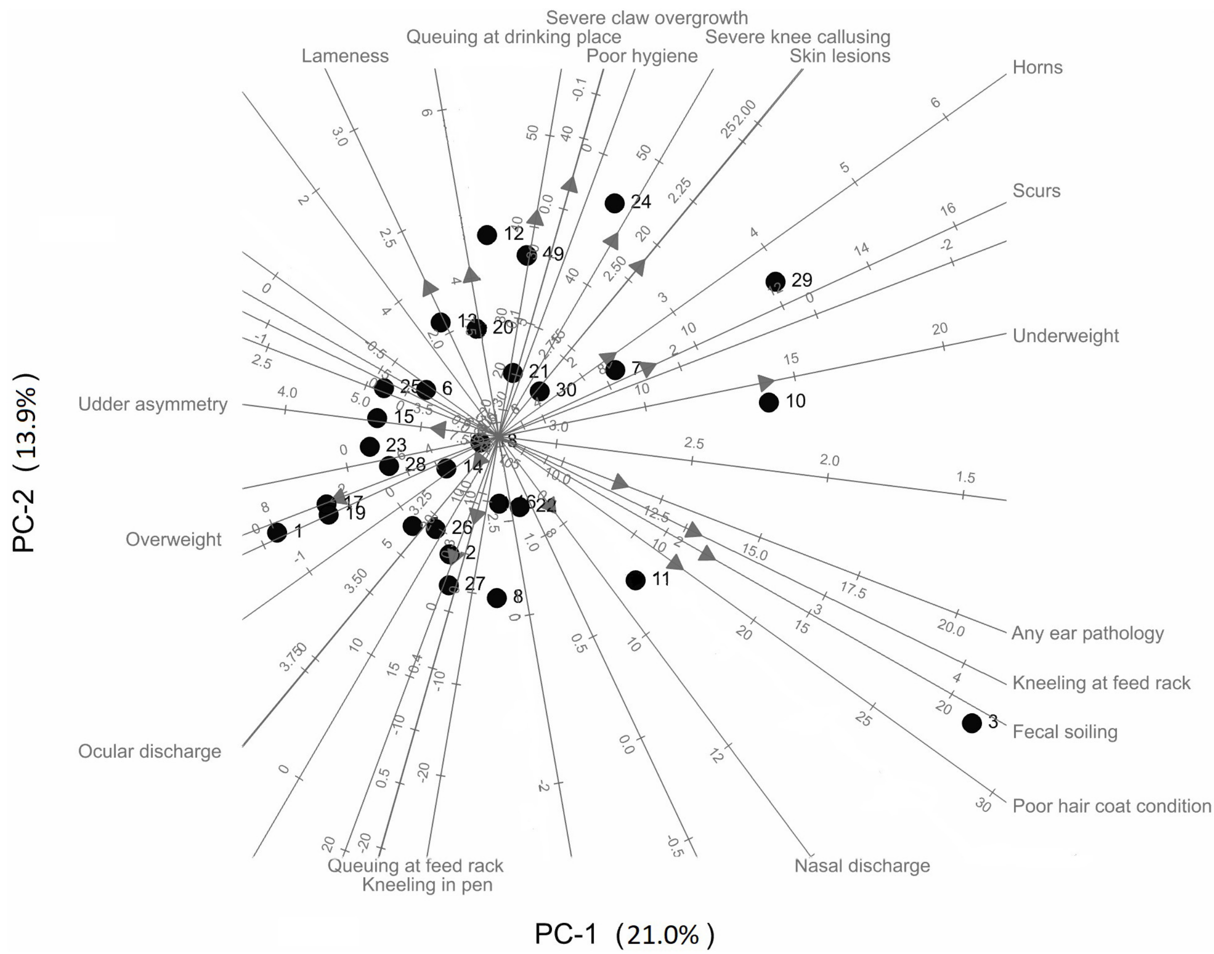

FIGURE 1 | Principal components biplot of welfare indicators of dairy goats across 30 farms in the Midwestern United States.

differences, but was utilized for feasibility in relation to assessing multiple farms per day. We observed mild knee calluses in just over half of the animals assessed with a further 17.7% of goats with severe knee calluses. Severe knee calluses have been reported previously and range from 8.9 to 18.3% (6, 12). The relatively high proportion of goats with severe knee calluses in the present study may be associated with bedding-related factors such as type, depth, dirtiness, or wetness of the bedding. The majority of the farms in this study used straw bedding, similar to those involved in the study of Anzuino et al. (6), which demonstrated that severe knee calluses were positively correlated with dirty limbs. Bedding that is wet, dirty or with poor drainage can increase the risk of developing skin lesions in swine (27) and dairy cattle (28, 29). Cows bedded on sand presented lesions of lower severity and were less dirty than those bedded on straw (30). Future research on the effect of bedding or lying surfaces on hock or knee calluses or skin lesions for goats is required to improve bedding management and goat welfare.

Severely overgrown claws typically result from a lack of wear of the claw or insufficient foot trimming. To reduce the risk of welfare problems such as lameness, which correlates with claw overgrowth (6, 31, 32), trimming should be undertaken at least twice yearly in intensive farms, where movement is limited (10). In the present study, we observed relatively low rates of severe claw overgrowth (17.7%), compared with previous studies, which ranges from 16.8 to 55.5% (6, 7, 12, 14, 32). Anecdotally, producers may be hesitant to perform frequent claw trimming as they believe that this encourages growth. More research is required demonstrating the benefits of regular foot trimming practices in preventing welfare issues such as lameness (discussed below). In addition, the provision of abrasive surfaces in the home pen or parlor that may encourage natural hoof wear should be considered. Further, environmental enrichment (e.g., rocks) can improve welfare outcomes by allowing for expression of natural behavior although not validated.

Hygiene or cleanliness is considered a valid indicator of welfare in dairy cows (29, 33), poultry (34) and goats (6, 12). Goats generally prefer not to lie in wet bedding, and goat feces is dryer than cattle; therefore, goats with poor hygiene may be indicative of poor environmental cleanliness and management practices (e.g., inadequate bedding management) (10). At high ambient temperatures (e.g., 38.0–39.5°C), goats generally show increased water intake and experience diuresis (35), which may result in a wetter environment; therefore, a higher ambient temperature may explain the increased rates of dirtiness with heat stressed goats. Increased lying duration has been reported in goats experiencing high ambient temperatures and with restricted water supply (36). A further explanation for a relationship between heat stress and poor hygiene is that to reduce the negative effects of heat stress, goats may lie in wet bedding to increase heat loss. Cows spend less time lying down during periods of heat stress to expose more body surface area for evaporative cooling (37); however, cows will actively avoid wet bedding to reduce the effects of conductive heat loss when experiencing cold temperatures (38). Observations of poor goat hygiene range from 2.4 to 36.4% (6, 7, 12, 14). In the present study, we observed 14.9% of goats had poor hygiene. It is important to note that the definition used in the present study included the presence of any fecal material (or dirt) and therefore the number of animals in the study with poor hygiene may be over-represented. The wide variation in the amount of goats with poor hygiene observed across studies may be associated with how the body areas were classified; for example, whether separate anatomical areas were hygiene scored (6, 12) or collective regions were scored together (i.e., rump, thighs, udder, and rear legs) as has been done in the present study.

Poor hair coat condition has been demonstrated as a reliable and valid indicator of welfare in goats; goats with poor hair coat condition had lower body condition (underweight), mineral deficiencies, presence of ectoparasites, and higher prevalence of abnormal lung sounds (11). Poor hair coat condition can be defined as uneven or shaggy and matted, that is frequently longer than normal, whereas a normal coat is shiny, smooth and adheres to the body's surface (11). We observed 8.3% of goats with poor hair coat condition, which is far lower than the reported ranges in Europe of 22.9 to 24.1% (12, 14). The comparatively lower rate of poor hair coat condition is likely associated with differences in sampling methodology. Battini et al. (12) and Can et al. (14) selected the pens with the worse welfare conditions (e.g., high stocking density, horned and hornless animals together, limited access to resources), which likely captured a greater number of animals with poor hair coat condition, compared with the present study, which used a different strategy.

Ear pathologies were observed on farms in the present study. The most common ear pathologies were characterized as damage associated with ear tags (either missing or torn ears), and frostbite. The majority of the farms involved in this study used ear tags as a form of identification (18/30; Hempstead et al., unpublished data). Incorrect placement of ear tags that are not in the center of the ear may result in inflammation or ear tears (6, 39). Ear tags may be ripped out as goats move their heads in and out of the feed troughs. In the present study, 2.1% of 4,524 (94 goats) goats had ear tears, which is in line with Anzuino et al. (6), who reported that 6.2% of 1,520 (∼94 goats) goats had ear tears. Frostbitten ears are generally the result of extended exposure to low temperatures when the animals are first born. Care must be taken to ensure newborns are dried (especially the ears and feet) shortly after birth, and/or by moving newborn kids to temperature controlled environments to reduce the incidence of frostbitten ears (40). The extent of pain or discomfort associated with ear tears and frostbite is not well-understood and requires further investigation.

Skin lesions such as abscesses, swellings, or broken skin and hair loss can be indicative of many health issues including caseous lymphadenitis (CL), or other dermal skin infections, ectoparasites and tissue injury from animals with horns, or environmental structures (40–42). There is a wide range of prevalence rates of skin lesions from 0.3 to 35.5% (6, 7, 12, 14), and our data appears to be on the lower end of the range (8.9%); this may have multiple explanations. First, there were differences in research methodologies between studies: skin lesions were categorized into anatomical regions of the body in earlier studies, whereas we evaluated skin lesions together without specifying the location on the body. Sampling strategies across studies also differed as we observed the goats in the parlor at the speed they were milked, whereas Can et al. (14) and Battini et al. (12) observed the goats restrained whilst in the pens. The best location for assessing skin lesions on dairy goats requires further validation. Second, there are likely differences in management practices such as utilization of a vaccination program for CL, minimization of pen structures that can cause skin lesions (e.g., protruding wire or sharp objects), treatment for ectoparasites or disbudding practice (discussed later).

Body condition scoring evaluates the level of muscle and fat development and is a reliable and valid method of monitoring fluctuations in fat reserves (10, 43, 44). A numerical rating scale of 5 points is commonly used across ruminant species (7, 45, 46). Until recently, the most accurate form of body condition scoring goats involved palpation of the lumbar and sternum regions due to differences in the amount of visceral and subcutaneous fat deposits with other species (47); however, valid and reliable BCS can be conducted from observations of the rear of the animal either in person or from digital photos (43, 44), which removes the need for individual restraint. Furthermore, identification of animals experiencing extreme nutritional deficiencies (e.g., overweight/too fat or underweight/too thin), compared with assigning a score (i.e., from 1 to 5), may reduce the time required and hence improve on farm feasibility and reliability (10). Underweight animals may have decreased feed intake where their energy expenditure exceeds nutritional status, which may reflect an inadequate feed supply or increased energy output, whereas overweight animals are generally the result of overfeeding or excessive confinement (5). In the present study, the amount of underweight and overweight goats appeared similar (4.0 and 3.9%, respectively), indicating that feed management is an area of potential improvement for farm managers. However, some caution should be taken when interpreting our results as due to the sampling strategy (i.e., sampling animals in the home pen where animals are free to move around), some animals may have been missed or counted twice. Other studies have reported overweight goats ranging from 2.7 to 18.2% and underweight goats ranging from 3.4 to 13% (6, 12, 14). The PCA shows that there was a positive correlation between underweight, fecal soiling, and poor hair coat condition, which may be associated with disease. Paratuberculosis or Johne's disease is a chronic wasting disease that affects ruminants and causes persistent diarrhea, progressive weight loss and may lead to death (48, 49).

Disbudding is a common husbandry procedure carried out to prevent horn growth that can result in injuries [see review by (50)]. If incomplete disbudding is performed (i.e., not enough horn bud tissue removed), then scurs will likely result. Scurs are partial horn regrowth's that are not fused to the frontal bone of the skull. Animals that have been disbudded unsuccessfully and have scur development or not disbudded at all and have horns, can have injurious interactions with conspecifics (51). Furthermore, horned and hornless goats show differences in their behavior toward each other, in that horned goats display more threat behavior compared with hornless goats, which attack others more frequently (52, 53). Previously reported rates of scurs range from 6.4 to 12.7% (6, 12, 14) and a single study reported 1.5% of goats assessed (∼23 of 1,520 goats) were not disbudded and had horns (6). We observed scurs and horns at a rate of 5.5 and 1.2%, respectively, which showed a positive correlation in the PCA. Together, these results demonstrate firstly, the difficulty in preventing horn regrowth in goats, and secondly, deficiencies in adequate training and practice of the operators performing disbudding, which is an area gaining attention for dairy calves (54, 55), but is still required for the dairy goat industry. In addition, extended iron application can cause brain injury in goat kids (56), which may mean that disbudding operators use less application time than required to adequately destroy the horn buds to avoid brain damage. Therefore, alternatives to cautery disbudding that reduce or eliminate pain and brain injury should be investigated.

Lameness is a debilitating condition that is associated with pain (57) and is a common issue on dairy goat farms with a range of 9.1 to 24% (6, 31, 32) and 1.7 to 3.1% in the UK and Europe, respectively (7, 12, 14). Lameness can be caused by multiple factors including overgrowth of claws (with or without conformational changes of the hoof) associated with infrequent hoof trimming or lack of natural wear, or diseases such as interdigital dermatitis, foot rot, foot lesions or caprine arthritis encephalitis (31, 32, 58). Furthermore, lameness is a useful behavioral indicator of pain in sheep (59, 60) and cattle (57, 61, 62), but studies on pain associated with lameness in goats are limited. Scoring systems for evaluating lameness in goats typically use a 4-point scale (7, 31, 63). Although, more recently, Deeming et al. (65) developed a 5-point scoring system to identify initial signs of lameness in goats (i.e., uneven gait) allowing for early intervention. Gait scoring individual animals was impractical in the present study due to the high number of animals observed, therefore only goats that were obviously lame were quantified. We observed a relatively low number of lame goats (1.2%), compared to the other studies described. Apparent differences in lameness rates across studies may be associated with different management practices, such as frequency of hoof trimming, the availability of hard surfaces or outdoor spaces to encourage natural wear of claws and how lameness was evaluated (10). Anzuino et al. (6) assessed lameness whilst the goats were exiting the milking parlor, whereas the other studies, including the present study, assessed lameness in the pens, where the soft bedding material may have concealed those goats with minor or moderate lameness (6). Additionally, the use of level surfaces (i.e., flat) for gait scoring may provide the most accurate reflection of lameness (57), which may not always be present. Another factor affecting the rates of lameness observed in the present study is that due to the sampling strategy (as for BCS), some animals may have been missed or counted repeatedly due to sampling in the home pen with animals able to freely move around.

We acknowledge that our study was not without limitations. To our knowledge there were no publicly available databases of dairy goat farms within the Midwestern US that we could access, thereby preventing random selection of farms. Therefore, farms included in this study were self-selected meaning that the data collected may not be representative of the wider dairy goat population in the Midwest US as a whole. However, our study was able to provide useful education resources and information on goat well-being for those producers that were involved. In follow-up visits, we can evaluate whether the benchmarking reports affected dairy goat well-being. We acknowledge that there was likely an effect of how the data was collected in separate sampling periods on our results; for example, queuing behavior was observed prior to milking on some farms and following milking on others and motivation to access the feed rack was likely affected. Further, the time of feed distribution relative to assessment of the home pen was not recorded, which may have also influenced the level of queuing behavior observed as fresh feed was likely not fed out at the same time across farms. Ideally, all assessments would have been completed at the same time of the day across farms, but this was not possible in the present study due to logistical restraints of time and personnel. The amount of time that the goats were observed in the pen was not recorded consistently, however, these times generally differed between farms, due to the difference in the number of animals on each farm. This likely affected the number of animals across farms observed for the various behavioral indicators assessed (e.g., queuing, kneeling). In addition, the difference in time spent in the milking parlor observing individual goats likely impacted on our results, as goats that were slower to enter the milking parlor for some reason (e.g., less dominant, sick, or injured), may have been missed. There is need for a more standardized protocol in relation to observations around feeding times and morning or afternoon milking sessions as outlined above. Future studies on welfare assessment are required that utilize a greater sample of goat farms (than the present study) and those that are randomly selected, to achieve a more accurate reflection of areas of dairy goat welfare deficiency in the Midwestern US.

In conclusion, our developed protocol for evaluating dairy goat welfare on farm in the Midwestern US identified areas of deficiency including knee calluses, claw overgrowth, poor hygiene, skin lesions, poor hair coat condition and ear pathologies. Further, using this protocol to assess a combination of welfare indicators, we have identified farms that may require changes to husbandry practices or the environment in order to improve goat welfare. The results of this research can be used by producers to improve dairy goat welfare and by researchers to continue evaluating welfare assessment on-farm in the Midwestern US.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The animal study was reviewed and approved by Institutional Animal Care and Use Committee at Iowa State University (Protocol number: IACUC-18-341). Written informed consent was obtained from the owners for the participation of their animals in this study.

AUTHOR CONTRIBUTIONS

MH, TL, JS, LS, and PP: conceptualization and methodology. MH and VC: formal analysis. MH and TL: investigation. MH: writing—original draft preparation. MH, TL, JS, LS, VC, and PP: writing—review and editing. PP and JS: funding acquisition. All authors contributed to the article and approved the submitted version.

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REFERENCES

1. Stafford JK. Introduction. In: Stafford K, editor. *Livestock Production in New Zealand*. Palmerston North: Massey University Press (2016). p. 8–10.
2. FAWC. *The Five Freedoms*. Farm Animal Welfare Council press release, London (1992).
3. Fraser D, Weary DM, Pajor EA, Milligan BN. A scientific conception of animal welfare that reflects ethical concerns. *Anim Welf.* (1997)6:187–205.
4. Mellor DJ, Reid CSW. Concepts of animal well-being and predicting the impact of procedures on experimental animals. In: Baker RM, Jenkin G, Mellor DJ, editors. *Improving the Well-being of Animals in the Research Environment.* Adelaide, SA: ANZCART (1993). p. 3–18.
5. Caroprese M, Casamassima D, Rassu SPG, Napolitano F, Sevi A. Monitoring the on-farm welfare of sheep and goats. *Ital J Anim Sci.* (2009) 8:343–54. doi: 10.4081/ijas.2009.s1.343
6. Anzuino K, Bell NJ, Bazeley KJ, Nicol CJ. Assessment of welfare on 24 commercial UK dairy goat farms based on direct observations. *Vet Rec.* (2010) 167:774–80. doi: 10.1136/vr.c5892
7. Muri K, Stubsjoen SM, Valle PS. Development and testing of an on-farm welfare assessment protocol for dairy goats. *Anim Welf.* (2013) 22:385–400. doi: 10.7120/09627286.22.3.385
8. AWIN (2015). *AWIN Welfare Assessment Protocol for Goats*.
9. Blokhuis HJ, Veissier I, Miele M, Jones B. The Welfare Quality® project and beyond: safeguarding farm animal well-being. *Acta Agric Scand A Anim Sci.* (2010) 60:129–40. doi: 10.1080/09064702.2010.523480
10. Battini M, Vieira A, Barbieri S, Ajuda I, Stilwell G, Mattiello S. Invited review: animal-based indicators for on-farm welfare assessment for dairy goats. *J Dairy Sci.* (2014) 97:6625–48. doi: 10.3168/jds.2013-7493
11. Battini M, Peric T, Ajuda I, Vieira A, Grosso L, Barbieri S, et al. Hair coat condition: a valid and reliable indicator for on-farm welfare assessment in adult dairy goats. *Small Ruminant Res.* (2015) 123:197–203. doi: 10.1016/j.smallrumres.2014.12.009
12. Battini M, Barbieri S, Vieira A, Stilwell G, Mattiello S. Results of testing the prototype of the AWIN welfare assessment protocol for dairy goats in 30 intensive farms in Northern Italy. *Ital J Anim Sci.* (2016) 15:283–93. doi: 10.1080/1828051X.2016.1150795
13. Battini M, Barbieri S, Waiblinger S, Mattiello S. Validity and feasibility of Human-Animal Relationship tests for on-farm welfare assessment in dairy goats. *Appl Anim Behav Sci.* (2016) 178:32–9. doi: 10.1016/j.applanim.2016.03.012
14. Can E, Vieira A, Battini M, Mattiello S, Stilwell G. On-farm welfare assessment of dairy goat farms using animal-based indicators: the example of 30 commercial farms in Portugal. *Acta Agric Scand A Anim Sci.* (2016) 66:43–55. doi: 10.1080/09064702.2016.1208267
15. Can E, Vieira A, Battini M, Mattiello S, Stilwell G. Consistency over time of animal-based welfare indicators as a further step for developing a welfare assessment monitoring scheme: The case of the Animal Welfare Indicators protocol for dairy goats. *J Dairy Sci.* (2017) 100:9194–204. doi: 10.3168/jds.2017-12825
16. Pairis-Garcia MD, Johnson AK, Azarpajouh S, Colpoys JD, Rademacher CJ, Millman ST, et al. The U.S. swine industry: historical milestones and the future of on-farm swine welfare assessments. *CAB Rev Perspect Agric Vet Sci Nutr Nat Resour.* (2016) 11:025. doi: 10.1079/PAVSNNR201611025
17. Blatchford AR. Animal behavior and well-being symposium: poultry welfare assessments: current use and limitations. *J Anim Sci.* (2017) 95:1382–7. doi: 10.2527/jas2016.0957
18. Meyer MM, Johnson AK, Bobeck EA. Development and validation of broiler welfare assessment methods for research and on-farm audits. *J Appl Anim Welf Sci.* (2020) 23:433–46. doi: 10.1080/10888705.2019.1678039
19. Stull CL, Reed BA, Berry SL. A comparison of three animal welfare assessment programs on California dairies. *J Dairy Sci.* (2005) 88:1595–600. doi: 10.3168/jds.S0022-0302(05)72828-9
20. Marchewka J, Estevez I, Vezzoli G, Ferrante V, Makagon MM. The transect method: a novel approach to on-farm welfare assessment of commercial turkeys. *Poult Sci.* (2015) 94:7–16. doi: 10.3382/ps/peu026
21. USDA. *Sheep and Goats*. National Agricultural Statistics Service (NASS), Washington, DC (2020).
22. Miranda-de la Lama GC, Mattiello S. The importance of social behaviour for goat welfare in livestock farming. *Small Ruminant Res.* (2010) 90:1–10. doi: 10.1016/j.smallrumres.2010.01.006
23. Silva Salas MÁ, Mondragón-Ancelmo J, Jiménez Badillo MR, Rodríguez Licea G, Napolitano, F. Assessing dairy goat welfare in Mexican intensive or semi-intensive farming conditions. *J Dairy Sci.* (2021) 104:6175–84. doi: 10.3168/jds.2020-19557
24. Miller BA, Lu CD. Special Issue — CURRENT status of global dairy goat production: an overview. *Asian Australas J Anim Sci.* (2019) 32:1219–32. doi: 10.5713/ajas.19.0253
25. Lu CD, Miller BA. Special Issue — current status, challenges and prospects for dairy goat production in the Americas. *Asian Australas J Anim Sci.* (2019) 32:1244–55. doi: 10.5713/ajas.19.0256
26. USDA. How is the U.S. goat industry growing? In: *NAHMS Goat Study 2019*. Fort Collins: USDA, Animal and Plant Health Inspection Service Fort Collins (2020), 1–2.

27. Kilbride A, Gillman C, Ossent P, Green L. Impact of flooring on the health and welfare of pigs. *In Pract.* (2009) 31:390–5. doi: 10.1136/inpract.31.8.390
28. Weary DM, Taszkun I. Hock lesions and free-stall design. *J Dairy Sci.* (2000) 83:697–702. doi: 10.3168/jds.S0022-0302(00)74931-9
29. Andreasen SN, Forkman B. The welfare of dairy cows is improved in relation to cleanliness and integument alterations on the hocks and lameness when sand is used as stall surface. *J Dairy Sci.* (2012) 95:4961–7. doi: 10.3168/jds.2011-5169
30. Norring M, Manninen E, de Passillé AM, Rushen J, Munksgaard L, Saloniemi H. Effects of sand and straw bedding on the lying behavior, cleanliness, and hoof and hock injuries of dairy cows. *J Dairy Sci.* (2008) 91:570–6. doi: 10.3168/jds.2007-0452
31. Hill NP, Murphy PE, Nelson AJ, Mouttotou N, Green LE, Morgan KL. Lameness and foot lesions in adult British dairy goats. *Vet Rec.* (1997) 141:412–6. doi: 10.1136/vr.141.16.412
32. Christodoulopoulos G. Foot lameness in dairy goats. *Res Vet Sci.* (2009) 86:281–4. doi: 10.1016/j.rvsc.2008.07.013
33. Hughes J. A system for assessing cow cleanliness. *In Pract.* (2001) 23:517. doi: 10.1136/inpract.23.9.517
34. Saraiva S, Saraiva C, Stilwell G. Feather conditions and clinical scores as indicators of broilers welfare at the slaughterhouse. *Res Vet Sci.* (2016) 107:75–9. doi: 10.1016/j.rvsc.2016.05.005
35. Olsson K, Josäter-Hermelin M, Hossaini-Hilali J, Hydbring E, Dahlborn K. Heat stress causes excessive drinking in fed and food deprived pregnant goats. *Comp Biochem Physiol A Physiol.* (1995) 110:309–17. doi: 10.1016/0300-9629(94)00186-W
36. Kaliber M, Koluman N, Silanikove N. Physiological and behavioral basis for the successful adaptation of goats to severe water restriction under hot environmental conditions. *Animal.* (2016) 10:82–8. doi: 10.1017/S1751731115001652
37. Tucker CB, Jensen MB, de Passillé AM, Hänninen L, Rushen J. Invited review: lying time and the welfare of dairy cows. *J Dairy Sci.* (2021) 104:20–46. doi: 10.3168/jds.2019-18074
38. Fregonesi JA, Veira DM, Von Keyserlingk MAG, Weary DM. Effects of bedding quality on lying behavior of dairy cows. *J Dairy Sci.* (2007) 90:5468–72. doi: 10.3168/jds.2007-0494
39. Smith MC, Sherman DM, editors. Fundamentals of goat practice. In: *Goat Medicine*. 2nd ed. Ames, IA: Wiley-Blackwell (2009). p. 3–20.
40. Smith MC, Sherman DM, editors. Skin. In: *Goat Medicine*. 2nd ed. Ames, IA: Wiley-Blackwell (2009). p. 27–53.
41. Smith MC, Sherman DM. Dehorning and descenting. In: *Goat Medicine*. 2nd ed. Ames, IA: Wiley-Blackwell (2009). p. 723–31.
42. Windsor AP. Control of caseous lymphadenitis. *Vet Clin N Am Food Anim Pract.* (2011) 27:193–202. doi: 10.1016/j.cvfa.2010.10.019
43. Ferguson JD, Azzaro G, Licitra G. Body condition assessment using digital images. *J Dairy Sci.* (2006) 89:3833–41. doi: 10.3168/jds.S0022-0302(06)72425-0
44. Vieira A, Brandão S, Monteiro A, Ajuda I, Stilwell G. Development and validation of a visual body condition scoring system for dairy goats with picture-based training. *J Dairy Sci.* (2015) 98:6597–608. doi: 10.3168/jds.2015-9428
45. Wildman EE, Jones GM, Wagner PE, Boman RL, Troutt HFJr, Lesch TN. A dairy cow body condition scoring system and its relationship to selected production characteristics. *J Dairy Sci.* (1982) 65:495–501. doi: 10.3168/jds.S0022-0302(82)82223-6
46. Munoz CA, Campbell AJD, Hemsworth PH, Doyle RE. Evaluating the welfare of extensively managed sheep. *PLoS ONE.* (2019) 14:e0218603. doi: 10.1371/journal.pone.0218603
47. Mcgregor B, Butler K. Relationship of body condition score, live weight, stocking rate and grazing system to the mortality of Angora goats from hypothermia and their use in the assessment of welfare risks. *Aust Vet J.* (2008) 86:12–17. doi: 10.1111/j.1751-0813.2007.00249.x
48. Clarke JC. The pathology and pathogenesis of paratuberculosis in ruminants and other species. *J Comp Pathol.* (1997) 116:217–61. doi: 10.1016/S0021-9975(97)80001-1
49. Chacon O, Bermudez LE, Barletta RG. Johne's disease, inflammatory bowel disease, *Mycobacterium paratuberculosis*. *Ann Rev Microbiol.* (2004) 58:329–63. doi: 10.1146/annurev.micro.58.030603.123726
50. Hempstead MN, Waas JR, Stewart M, Sutherland MA. Goat kids are not small calves: species comparisons in relation to disbudding. *Anim Welf.* (2020) 29:293–312. doi: 10.7120/09627286.29.3.293
51. Waiblinger S, Schmied-Wagner C, Mersmann D, Nordmann E. Social behaviour and injuries in horned and hornless dairy goats. In: Kofer J, Schobesberger H, editors. *Proceedings of the XVth International Congress of the International Society for Animal Hygiene*. Vol. 1. Brno; Vienna: Tribun EU s.r.o. (2011). p. 421–2.
52. Aschwanden J, Gygax L, Wechsler B, Keil NM. Social distances of goats at the feeding rack: influence of the quality of social bonds, rank differences, grouping age and presence of horns. *Appl Anim Behav Sci.* (2008) 114:116–31. doi: 10.1016/j.applanim.2008.02.002
53. Hillmann E, Hilfiker S, Keil NM. Effects of restraint with or without blinds at the feed barrier on feeding and agonistic behaviour in horned and hornless goats. *Appl Anim Behav Sci.* (2014) 157:72–80. doi: 10.1016/j.applanim.2014.05.006
54. Winder CB, LeBlanc SJ, Haley DB, Lissemore KD, Godkin MA, Duffield TF. Comparison of an online learning module to hands-on training in teaching a cautery disbudding technique for dairy calves including cornual nerve block application. *Can Vet J.* (2017) 58:735–40.
55. Winder CB, LeBlanc SJ, Haley DB, Lissemore KD, Godkin MA, Duffield TF. Comparison of online, hands-on, and a combined approach for teaching cautery disbudding technique to dairy producers. *J Dairy Sci.* (2018) 101:840–9. doi: 10.3168/jds.2017-13217
56. Hempstead MN, Shearer JK, Sutherland MA, Fowler JL, Smith JS, Smith JD, et al. Cautery disbudding iron application time and brain injury in goat kids: a pilot study. *Front Vet Sci.* (2021) 7:568750. doi: 10.3389/fvets.2020.568750
57. O'Callaghan KA, Cripps PJ, Downham DY, Murray RD. Subjective and objective assessment of pain and discomfort due to lameness in dairy cattle. *Anim Welf.* (2003) 12:605–10.
58. Smith MC, Sherman DM, editors. Musculoskeletal system. In: *Goat Medicine*. 2nd ed. Ames, IA: Wiley-Blackwell (2009). p. 85–162.
59. Ley SJ, Waterman AE, Livingston A. A field study of the effect of lameness on mechanical nociceptive thresholds in sheep. *Vet Rec.* (1995) 137:85–7. doi: 10.1136/vr.137.4.85
60. Dolan S, Kelly JG, Monteiro AM, Nolan AM. Up-regulation of metabotropic glutamate receptor subtypes 3 and 5 in spinal cord in a clinical model of persistent inflammation and hyperalgesia. *Pain.* (2003) 106:501–12. doi: 10.1016/j.pain.2003.09.017
61. Hernandez JA, Garbarino EJ, Shearer JK, Risco CA, Thatcher WW. Comparison of milk yield in dairy cows with different degrees of lameness. *J Am Vet Med Assoc.* (2005) 227:1292–6. doi: 10.2460/javma.2005.227.1292
62. Dyer RM, Neerchal NK, Tasch U, Wu Y, Dyer P, Rajkondawar PG. Objective determination of claw pain and its relationship to limb locomotion score in dairy cattle. *J Dairy Sci.* (2007) 90:4592–602. doi: 10.3168/jds.2007-0006
63. Mazurek M, Marie M, Desor D. Potential animal-centred indicators of dairy goat welfare. *Anim Welf.* (2007) 16:161–4.
64. Bélanger-Naud S, Vasseur E. Graduate Student Literature Review: current recommendations and scientific knowledge on dairy goat kid rearing practices in intensive production systems in Canada, the United States, and France*. *J Dairy Sci.* (2021) doi: 10.3168/jds.2020-18859. [Epub ahead of print].
65. Deeming LE, Beausoleil NJ, Stafford KJ, Webster JR, Zobel G. Technical note: the development of a reliable 5-point gait scoring system for use in dairy goats. *J Dairy Sci.* (2018) 101:4491–7. doi: 10.3168/jds.2017-13950

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SYSTEMATIC REVIEW
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A Systematic Review on Validated Precision Livestock Farming Technologies for Pig Production and Its Potential to Assess Animal Welfare

Yaneth Gómez[1]*, Anna H. Stygar[2], Iris J. M. M. Boumans[3], Eddie A. M. Bokkers[3], Lene J. Pedersen[4], Jarkko K. Niemi[2], Matti Pastell[5], Xavier Manteca[1] and Pol Llonch[1]

[1] Department of Animal and Food Science, Universitat Autònoma de Barcelona, Barcelona, Spain, [2] Bioeconomy and Environment, Natural Resources Institute Finland (Luke), Helsinki, Finland, [3] Animal Production Systems Group, Wageningen University and Research, Wageningen, Netherlands, [4] Department of Animal Science, Aarhus University, Tjele, Denmark, [5] Production Systems, Natural Resources Institute Finland (Luke), Helsinki, Finland

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Edward Narayan, The University of Queensland, Australia

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***Correspondence:**
Yaneth Gómez
yanethrocio.gomez@uab.cat

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Several precision livestock farming (PLF) technologies, conceived for optimizing farming processes, are developed to detect the physical and behavioral changes of animals continuously and in real-time. The aim of this review was to explore the capacity of existing PLF technologies to contribute to the assessment of pig welfare. In a web search for commercially available PLF for pigs, 83 technologies were identified. A literature search was conducted, following systematic review guidelines (PRISMA), to identify studies on the validation of sensor technologies for assessing animal-based welfare indicators. Two validation levels were defined: internal (evaluation during system building within the same population that were used for system building) and external (evaluation on a different population than during system building). From 2,463 articles found, 111 were selected, which validated some PLF that could be applied to the assessment of animal-based welfare indicators of pigs (7% classified as external, and 93% as internal validation). From our list of commercially available PLF technologies, only 5% had been externally validated. The more often validated technologies were vision-based solutions ($n = 45$), followed by load-cells ($n = 28$; feeders and drinkers, force plates and scales), accelerometers ($n = 14$) and microphones ($n = 14$), thermal cameras ($n = 10$), photoelectric sensors ($n = 5$), radio-frequency identification (RFID) for tracking ($n = 2$), infrared thermometers ($n = 1$), and pyrometer ($n = 1$). Externally validated technologies were photoelectric sensors ($n = 2$), thermal cameras ($n = 2$), microphone ($n = 1$), load-cells ($n = 1$), RFID ($n = 1$), and pyrometer ($n = 1$). Measured traits included activity and posture-related behavior, feeding and drinking, other behavior, physical condition, and health. In conclusion, existing PLF technologies are potential tools for on-farm animal welfare assessment in pig production. However, validation studies are lacking for an important percentage of market available tools, and in particular research and development need to focus on identifying the feature candidates of the measures (e.g., deviations from diurnal pattern, threshold levels) that are valid signals of either negative or positive animal welfare. An important gap identified are the lack of technologies to assess affective states (both positive and negative states).

Keywords: PLF, sensor, validation, welfare, sows, piglets, fattening pigs

INTRODUCTION

Animal welfare comprises three components (1): natural living, affective states, and basic health and functioning. Natural living corresponds to the ability of animals to live according to their behavioral needs. An affective state refers to animal's emotions and moods, which can go from negative (e.g., depressed) to positive (e.g., pleasure). Basic health deals with the normal biological functioning and fitness of animals.

These three components of animal welfare can be measured by indicators based, primarily on the animal, but the surrounding environment can also provide useful information. Animal-based indicators provide a more direct measure of the welfare of the animal compared with resource-based indicators. As an example, to assess the absence of prolonged hunger, Welfare Quality® (WQ) (2), one of the most spread animal welfare assessment protocols, uses the body-condition score as an animal-based indicator. However, in the absence of a reliable animal-based indicator for assessing the absence of prolonged thirst, a resource-based indicator such as water supply, is used, which can only inform about an aspect of the environment animals live in.

Knowledge on the welfare of pigs is important for producers (3) and consumers (4). As an example, for producers, poor health or the presence of damaging behavior such as tail biting negatively impact growth performance (5, 6). Diseases and injuries might urge producers to increase the use of antibiotics (7). Regarding consumers, animal welfare is considered as an important aspect of product quality (8), and studies indicate their willingness to pay for pork produced with enhanced welfare (9–11). Goods produced under improved welfare conditions can be communicated to consumers by certification schemes and associated labeling. Most animal welfare labels related to pig farming in Europe have requirements concerning resource-based welfare indicators such as a space allowance, provision of bedding and enrichment, and minimum transportation time (12). However, animal-based indicators have gained more attention, especially after the WQ protocols were published. For example, most pig welfare labels consider mother-offspring interaction through setting a minimum weaning age (e.g., Mehr tierwohl in Germany, Beter Leven in Netherlands, and Bedre Dyrevelfærd in Denmark).

At present, an adequate assessment of farm animal welfare requires a substantial amount of time and effort. Furthermore, current welfare assessment protocols have some other limitations. To mention a few, they do not contain all three components of animal welfare (1), often lack animal-based indicators, focus on expressing the welfare status at group (farm) level instead focusing on the individual (13), and are largely based on human observation (14), which might imply some subjective judgements (15). This means that current protocols provide a limited picture of the welfare of animals throughout their life, restricting the capacity for early detection welfare problems as well as overall life-time welfare.

The use of monitoring technology in animal production systems to optimize farming processes and reduce human workload, often called precision livestock farming (PLF), is growing. According to Berckmans (15), the objective of PLF is to provide the farmers with tools for online and continuous monitoring of the status of the animals and their environment. These tools may therefore help in decision-making and management of the herd (16). Moreover, PLF could contribute with relevant information related to animal welfare in an easier and quicker manner, making continuous welfare assessments more feasible.

Different sensors exist to measure features of individual pig behavior, and/or physical conditions (e.g., accelerometers, microphones, cameras) (17). PLF can add value for the welfare assessment of animals by (1) allowing individual or sub-group tracking, (2) avoiding stressful procedures involving an animal handling during assessment (e.g., by body weight measurements using video cameras instead of manual weighing), and (3) allowing real-time monitoring. In addition, allows implementing early-warning signals of suboptimal status of the animals, to prevent welfare problems (18). PLF technologies have some limitations though. Technologies are created by humans, who set limits for specific problem detection (e.g., tail biting), so could also be burdened with certain subjectivity (18, 19). Also, as demonstrated in large-case studies for sensor profitability in dairy farmers, investment in PLF technologies might not necessary lead to economic gain (20, 21). In addition, not all PLF tools have an automatic alert, making a gap between the time of problem detection and the potential intervention of the staff. Reliability of data management could be considered a further limitation, since it is carried out by the PLF manufacturing company, which in fact are the data owners. To improve transparency, evaluation on the PLF tools performance by external bodies is essential.

A procedure for validation in the real operation environment of a technology is required before it is transferred to the market (22). Validation is the procedure for evaluating the performance of a technology contrasted with a gold standard to know if it achieves satisfactory prediction accuracy of a measured trait (23). For instance, how well a thermal camera detects fever, compared with a standard thermometer, or how well an automatic feeding system can detect feeding behavior. This validation procedure should be performed internally (on a sample of individuals during the system building), but also externally (on different individuals than those used during building phase) (24). For the sake of transparency, buyers (i.e., farmers) need to know the exact features of the technology they are buying and how accurate they monitor a given condition. It is preferable that the external validation need is carried out by independent bodies.

To the best of our knowledge, an overview of existing PLF technologies that potentially can be used for pig welfare assessments and the validity and reliability of these technologies, is still lacking. The aim of this review is to explore market available PLF technologies that are potentially applicable in commercial pig production, and to review (1) their ability to contribute to longitudinal welfare assessment, and (2) their state of validation. This review focus on technologies that have been validated (either internal or external) and which results have been published.

MATERIALS AND METHODS

Search for Commercially Available Technologies

A web search to identify commercially available PLF systems for pigs was conducted by using Google search engine by one researcher (YG), between February and April 2020. Search terms included pigs (and related words such as sows, piglets), and different technologies known to monitor animal-based welfare indicators for pigs. Technologies provided by a wide range of suppliers were scanned. More specifically, the search criteria included the following animal categories: *(pig)*, *(piglet)*, *(weaner)*, *(fattener pig)*, *(sow)*, and the technology using one of the following terms: *(automatic drinker OR automatic waterer)*, *(automatic feeder)*, *(electronic feeding station)*, *(activity sensor OR activity monitor)*, *(RFID)*, *(GPS)*, *(thermal camera)*, *(infrared thermometer)*, *(automatic weigh scale)*, *(sorting scale)*, *(weight camera)*, *(body condition score sensor OR automatic body condition score)*, *(body condition camera)*, *(lameness sensor)*, *(automatic lameness detection)*, *(pressure mat OR force sensor)*, *(automatic behavior analyzer)*, *(image-based behavior analyzer)*, *(body-temperature sensor)*, *(automatic sound analysis)*, *(cough sensor OR cough monitor)*. No boolean operators were applied, except OR boolean, as Google does not allow the use of * to automatically fill the search term to include related words. The example search looked as follows: *pig automatic weigh scale OR automatic weigher*.

The first five pages (50 hits) of results in each search were reviewed. Only commercially available technologies were selected for further review, excluding prototypes or devices in the building phase. If required, technology providers were approached to clarify the stage of development. Information on a sensor name, provider name, internet link, sensor type, aim, and provider country were summarized. Information regarding the production phase that the technology is applicable or designed for, was also specified.

Literature Search

Following the Preferred Reporting Items for Systematic Reviews and Meta-Analysis (PRISMA) guidelines (25), a literature search was conducted by one researcher (YG), and verified by a second researcher (AS). The search was focused on finding external validation studies on PLF technologies for pig welfare. In addition, the obtained data set (studies reporting different validation levels) was used for checking internal validation to find potential technologies for pig welfare monitoring that are not yet externally validated.

The literature search was conducted through Web of Science and Scopus databases, between the 1st of June and the 31st of July 2020. Search terms included: different phases in the production cycle of pigs, terms regarding validation, types of sensor or their commercial names. Besides, some animal-based welfare indicators were included as search terms, including body temperature, body weight, and locomotion as physical condition indicators; activity, feeding, drinking and vocalizations as behavioral indicators; and cough and lameness as physiological indicators. Search terms related to individual recognition and animal location in the pen were also included.

Search terms were applied to title, abstract and keywords as follows:

(pig OR sow OR weaner OR piglet OR fattenn)*
AND
(validat OR evaluat* OR assess* OR test*)*
AND (one of the following search combinations)

1. *(accelerometer), ((“activity sensor” OR “motion sensor” OR “locomotion sensor” OR “infrared motion” OR (activity AND automat*))*
2. *((position* AND sensor) OR rfid OR “tracking system”)*
3. *((vision AND camera) OR “image analysis”)*
4. *((thermistor OR infrared) OR (body temperature) AND (monitor* OR detect* OR sensor)*
5. *((scale* AND weigh*) AND automat*)*
6. *(“body condition scor*” AND sensor OR automat*)*
7. *((“feeding behavior*” OR “feeding behavior*”) AND sensor)*
8. *(“feeding station” OR “feed* meter” OR “water meter” OR “automatic feeder”)*
9. *(“drinking behavio*” AND monitoring)*
10. *((sound AND sensor) OR (cough AND detect*))*
11. *(respiratory AND distress AND monitor)*
12. *((sound AND sensor) OR (vocali* AND detect*))*
13. *((gait OR lameness OR lame*) AND (sensor OR “image analy*” OR image OR automat* OR mat OR “pressure mat” OR “pressure sensor” OR “force plate*”))*

NOT (*review OR beef OR sheep OR survey OR goat* OR hors* OR pipeline OR genom* OR “wild boar” OR “swine model” OR “porcine model”*).

To make sure that all technologies identified in the first search were checked for validation, an additional search of literature using the name of identified commercial sensors in Google (**Supplementary Table 1**) was performed. An example of search criteria for “FLIR T300” technology was: *pig OR piglet OR weaner OR fattener OR sow FLIR T300*.

Inclusion and Exclusion Criteria

Only peer-reviewed articles, written in English and published between January 2000 and July 2020 were considered. Articles related to welfare assessment in species other than domesticated

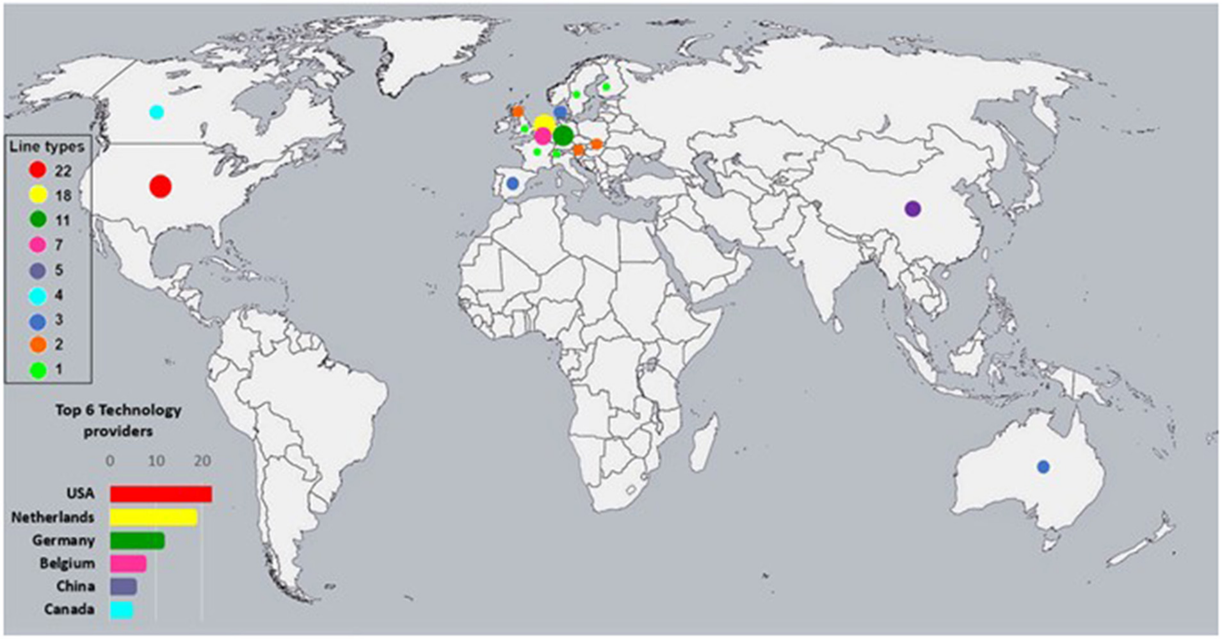

FIGURE 1 | Countries of origin of commercially available PLF technologies with potential use in pig welfare assessment. For companies with multiple locations, address of the headquarter was used. Some companies have operations in more than one country.

TABLE 1 | Commercially available Precision Livestock Farming technologies categorized by the sensor type and measured trait.

Type of technology		**Animal-based measure**	**Number of identified technologies**		**% over total commercial solutions (*n* = 83)**	
Load cells and flow meters	Force plates	Gait attributes	2	Load cells with RFID 18	22%	45%
	Load cells	Feed intake	3			
	Flow meter	Water intake	2			
	Load cells/Flow meter		1			
	Feeder/drinker	Feed/water intake	5			
	Scale	Body weight	5			
	Feeder/drinker/RFID	Feed/water intake/body weight	15	Load cells without RFID 19	23%	
	Scale/RFID	Body weight	4			
Cameras		Body weight	14	22	26%	
		Behavior and activity	8			
Thermal cameras		Body temperature	10		12%	
Microphones		Cough	2	5	6%	
		Animals sounds	3			
Accelerometers		Activity	4		5%	
Body temperature devices	Contact-temperature device	Body temperature	2		2%	
	Pyrometer	Body temperature				
Photoelectric sensors		Lameness	2		2%	
GPS		Location	1		1%	
RFID		Individual identification and tracking	1		1%	

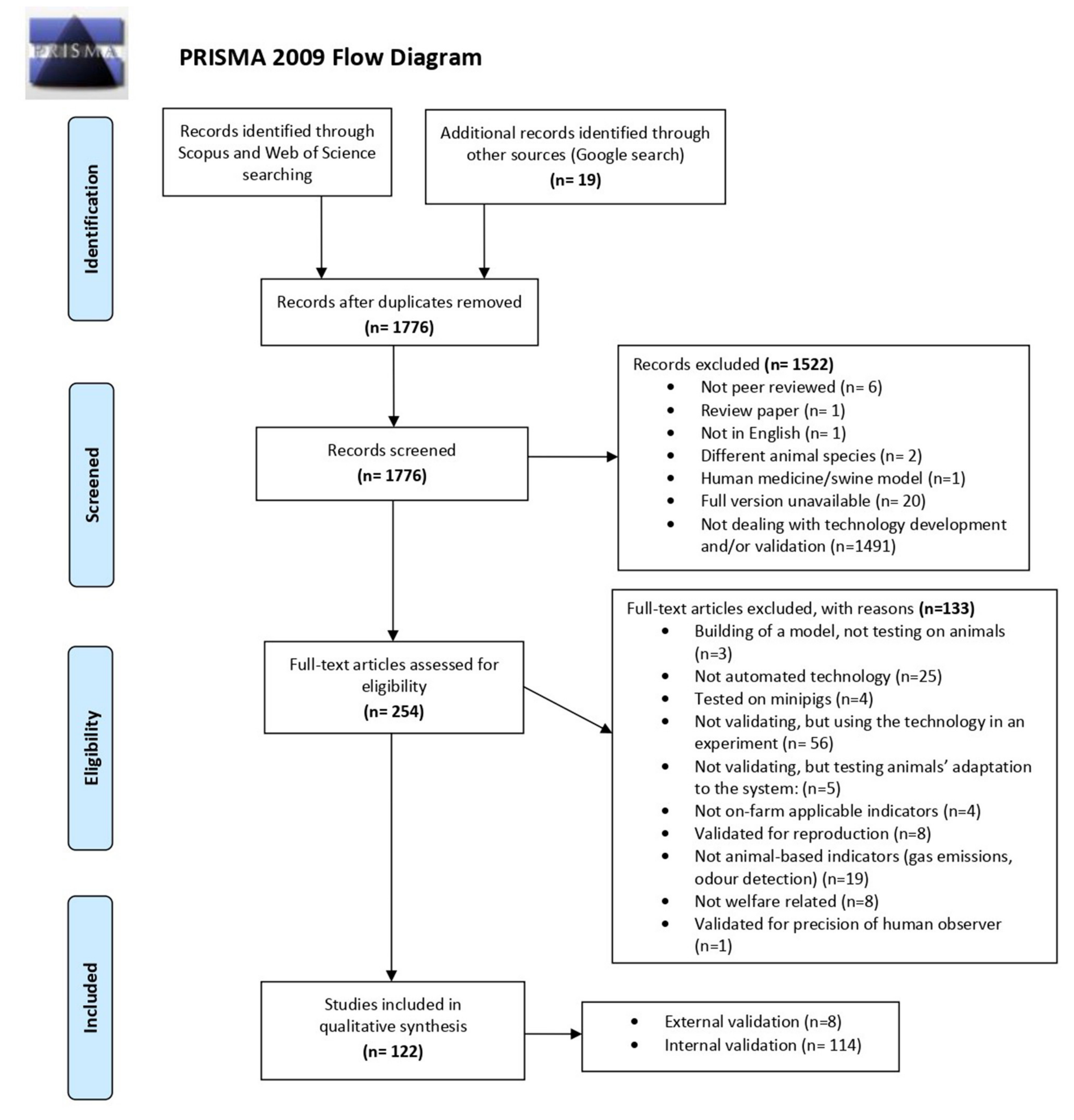

FIGURE 2 | Modified PRISMA flow diagram (25) with the systematic review search strategy and study selection.

pigs (*Sus scrofa*) were excluded. Studies not addressing technology development or validation, as well as studies using a PLF technology, but not testing its performance or validating it, were also excluded.

Only articles addressing automated and on-farm applicable PLF technologies were included in this review. Studies testing on pigs not meant for farm practices (e.g., minipigs) were excluded. Articles neither dealing with aspects directly related to animal welfare (such as estrus detection) nor with animal-based welfare indicators (e.g., environmental measurements such as climatic aspects) were excluded. Duplicates were also removed from the data set.

Selected studies were grouped based on the type of PLF technology [accelerometers, photoelectric sensors, RFID (Radio

Frequency Identification), load-cells, flow meters, microphones, cameras, thermal cameras, infrared (IR) thermometers, pyrometers]. The final data set included sample size, production phase, and the relevant animal-based indicator(s).

Study Classification

A gold standard is defined as a criterium by which given tool was evaluated (26, 27). In the conducted review, there were three possible options:

1) tool was validated against a human observer,
2) tool was validated against other tool with well-defined performance record,
3) tool was validated based on its ability to detect change in animal behavior or physical condition during planned experiment.

As in Stygar et al. (28), a similar review but focusing on dairy cattle PLF technologies to monitor animal welfare, and based on Altman et al. (24), we defined the following levels of validation:

1) External self-validation: studies where the system was evaluated using a fully independent data set, meaning that data was collected from different herds not used for system development. Validation was conducted by either one scientist, at least, involved in the technology development or by someone representing the company who owns the technology.
2) External independent validation: studies where the technology was validated using a fully independent data set, from different herds than those used for technology development, and research was conducted by independent scientists with no relationship with the company that owns the technology.
3) Internal validation: studies where the technology was validated using the same data set as for technology building, or where the commercial name of the technology was not specified, or the origin of the validation data set was unknown.

For determining the validation level within the literature search, the technology and the validation location were identified. The technologies were identified by looking for their commercial names or papers describing its development phase (prototypes). Studies where the specific location of herd was not mentioned (for example due to privacy concerns), but clearly used different herds than for system building, were included as external validation level.

RESULTS

Commercially Available Technologies

All PLF technologies with a potential link to animal-based pig welfare assessment are listed in the **Supplementary Table 1.** In total 83 technologies were found, based on 10 different types of sensors, from 46 different providers whose headquarters are located in 17 countries. **Figure 1** shows the origin of the commercially available technologies. Most of the providers are located in the United States of America ($n = 22$), the Netherlands ($n = 18$), and Germany ($n = 11$), followed by Belgium ($n = 7$), China ($n = 5$), and Canada ($n = 4$). Location of providers was identified in a minor extent in other countries (including Spain, Australia, Slovakia, Scotland, Austria, Switzerland, Turkey, Sweden and England).

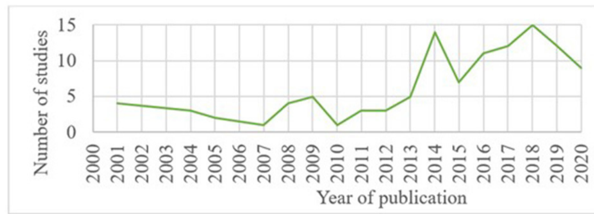

FIGURE 3 | Temporal distribution of validation studies on PLF technologies included in this review, with potential use in pig welfare assessment.

As summarized in **Table 1**, load-cells based and vision-based technologies were the largest groups of identified technologies. Thermal-image technology was the third most common type of sensor. Remaining identified technologies included microphones, accelerometers, body temperature devices, photoelectric sensors, GPS (Global Positioning System), and RFID (for animal tracking). Most of the identified commercial tools can be used for different pig production phases, however, some are targeted at a specific production phase. Of the commercially available technologies, 39% was used for fattening pigs, 33% for sows, and 28% for piglets and weaned piglets. Load-cells based and vision-based body-weight tools are mainly used for fattening pigs. No technologies exclusively developed or adjusted for piglets and weaners were found.

Literature Search on Validation Trials

The literature search through databases provided 2,463 results. Nineteen studies used the commercial names of technologies identified in the web search. After removing duplicates and applying the inclusion and exclusion criteria, 111 studies remained. The PRISMA flow diagram in **Figure 2** describes the stages of studies selection process and reasons for exclusion.

As illustrated in **Figure 3**, the number of publications on PLF internal and external validation increased over the last decade. Neither the internal nor the external validation studies followed any particular pattern of temporal distribution of the publications. Only eight (7%) of the 111 selected studies, fulfilled the external validation criteria, whereas 103 (93%) were classified as having an internal validation (**Figure 4**). Within the internal validation studies, 23 (22%), did not meet the criteria for external validation, but could be included as internal validation. In 18 of those 23 studies, the name or origin of the sensor was not provided; it was therefore impossible to identify its commercial availability or development stage. This applied to nine studies with camera-based technologies (29–37), three studies on load-cells [a drinker (38), a scale (39), and a force plate (40)], two on RFID (41, 42), two on accelerometer (including one on accelerometer and microchip for body-temperature) (43, 44), one study on microphone (45), and one on load cells with RFID (46). In the other five of those 23 studies, the origin or location of the herds used, or the origin of the

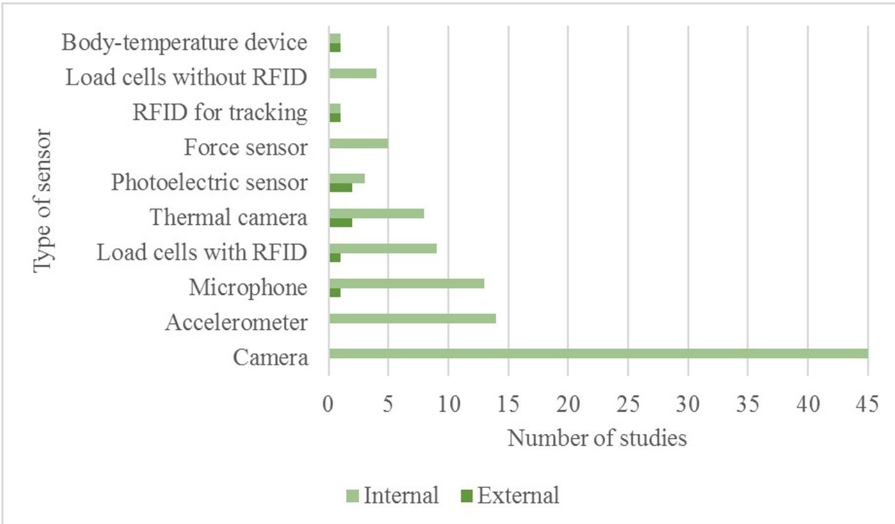

FIGURE 4 | Number of studies classified as internal or external validation for different sensor categories.

TABLE 2 | Number of peer-reviewed validation studies on sensor technologies used in pig production, categorized by sensor type and validation level (internal or external).

Type of sensor	Number of internal validation studies	Number of external validation studies	Total number of validation studies
Camera	45	0	**45**
Load-cells	With RFID- 8 (Feeders-9 Drinker-1)	With RFID- 1 (Feeder-1)	With RFID- **10** (Feeders-10 Drinker-1)
	Without RFID- 7 (Force plates-5 Scales-2)	Without RFID- 0	Without RFID- **7** (Force plates-5 Scales-2)
Accelerometer	14	0	**14**
Microphone	13	1	**14**
Thermal camera	8	2	**10**
Photoelectric sensors	3	2	**5**
Flow meters	2	0	**2**
RFID	1	1	**2**
Non-contact body-temperature sensors	Infrared thermometer- 1	Pyrometer- 1	Infrared thermometer- **1** Pyrometer- **1**

The bold numbers indicates the total sum of the number of internal and external validation studies on each type of sensor. In brackets: the specific sensors included in each sensor type category.

sensor was not described [two studies on thermal cameras (47, 48), one on load cells with RFID in a feeding station (49), one on cameras (50), and one on microphone (51)]. From the obtained list of commercially available PLF technologies, 14% were validated in some identified papers of literature search (12 of 83 technologies), of which 5% corresponded to external validation (52–55).

An overview of internal and external validation studies can be found in **Table 2**. Most internal validation studies concerned camera-based technologies, followed by load-cells based technologies. The next most frequent validated type of sensors were accelerometers and microphones, followed by thermal-cameras, photoelectric sensors, flow meters, and RFID (for animal tracking). The less common validated technologies were non-contact body-temperature sensors (infrared thermometers, and pyrometer). All validation studies, together with performance indicators, are described in detail in **Supplementary Table 2**.

Regarding the productive phase of animals used for the studies, the most frequently used pigs were fatteners (51 studies), followed by sows (28 studies), and weaners (21 studies). Sensors for piglets and gilts were less frequent (eight and five studies, respectively). In our results on commercial search, no PLF solution developed or adapted exclusively for piglets or weaners was identified. However, research on PLF solutions for piglets exists, as studies on cameras, thermal cameras, feeders with RFID, microphones, photoelectric sensors, pyrometers and RFID for tracking were identified using young pigs (from birth to 10 weeks old or up to 70 days old) as target animals. Five studies used pigs in general, not specifying the productive phase. Sample size used in the selected studies are illustrated in **Figure 5**. Some

TABLE 3 | Studies on externally validated (independent or self-validated) sensor technologies with potential use in pig welfare assessment, specifying the sensor type, commercial name, the animal-based indicator assessed and its evaluation level (individual or group).

Technology name	Indicator	Reason of use (monitored trait)	Evaluation level	Nr of validation trials	Used sensors	Independent validation[a]	Self-validation[b]
OPTEX RX-40QZ	Activity and posture-related behavior	Active and/or passive (without distinguishing on activity type)	Group	1	Photoelectric	(56)	
STREMODO (commercially unavailable)	Physical condition	Stress vocalization (due to handling)	Group	1	Microphone		(57)
FLIR E5 thermal imaging camera	Physical condition	Body temperature	Individual	2	Thermal camera	(53)	
FLIR ThermoCAM S60	Physical condition	Body temperature	Individual			(54)	
FIRE	Physical condition	Body weight	Individual	1	Load cells and RFID	(52)	
	Feeding and drinking behavior	Feed intake (kg)					
Pyrometer Optris	Physical condition	Body temperature	Individual	1	Pyrometer	(55)	
Prototype system	Feeding and drinking behavior	Feeding behavior, feeding time and/frequency	Individual	1	RFID		(58)
Standing lying sensor	Activity and posture-related behavior	Posture change (between lying, standing and sitting)	Individual	1	Photoelectric	(59)	

[a]*External independent validation—validated using independent data set (different animals and herd than for technology building) and co-authors were not involved in technology development.*
[b]*External self-validation—validated using independent data set (different animals and herd than for technology building) and was developed and validated by at least one the same co-author (based on the authorship of papers) or have been validated by at least one co-author representing a company providing a technology.*

patterns were observed in relation to the size of the samples and validated technology. The smallest sample size (including samples of <10 animals) was used in studies validating cameras (eight studies), accelerometers (five studies), microphones (three studies), RFID for tracking (one study), and force plates (one study). However, most of the studies on accelerometers (11 out of 14 studies) and force plates (four out of five studies) were conducted using sample sizes smaller than 24 animals. Automatic feeders and drinkers, with or without RFID, and sorting scales systems, were mostly validated in studies using sample sizes from 55 to more than 1,000 animals. Three studies on drinkers with RFID validated the technology using samples between 25 and 30 animals. Studies validating accelerometers, force plates, cameras, microphones and RFID for tracking of animals used samples sizes from 3 to more than 500 animals. Studies on thermal cameras and photoelectric sensors also were performed using varied ranges of sample sizes (from 11 to 297 animals). In the external validation studies sample sizes were between 20 and 63 animals.

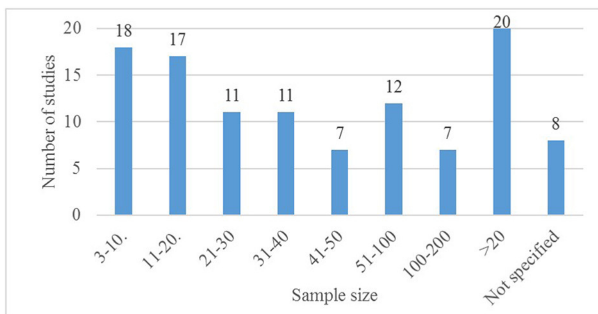

FIGURE 5 | Sample size (the number of animals) used for external or internal validation in the reviewed studies.

Validation Studies and Technologies for Welfare Assessment in Pigs

External Validation Studies

Table 3 summarizes the externally validated (self-validation and independent validation) technologies with potential use for pig welfare assessment.

Measured Traits and Technologies in Internal and External Validation Studies

Table 4 provides an overview of technologies tested to monitor different welfare indicators related to pig production. Validated traits were grouped in following categories: activity and posture-related behavior, feeding and drinking behavior, other behaviors, physical condition, and health-related traits.

Activity and Posture-Related Behavior

We identified five sensor types (cameras, accelerometers, photoelectric sensors, thermal cameras, and RFID) that were used for activity measurement (**Table 4**). The following traits were monitored: general motion activity (active, inactive state), walking (number of steps, identified as separate behavior), tracking (identifying location or number of animals in this location), postural state and transition between states (lying, standing and sitting), as well as general motion activity and tracking (studied in relation to thermal comfort). We identified

TABLE 4 | Summary of internally and externally validated technologies to monitor different pig welfare indicators, classified by monitored trait and sensor type.

Indicator	Reason of use (monitored trait)	Technologies tested
Activity and posture-related behavior	Active and/or passive (without distinguishing on activity type)	Accelerometer (60–65) Photoelectric sensor (56)[a], (66–68) Camera (30, 69–71)
	Lying	Camera (72–76) Accelerometer (43, 60–62, 77, 78)
	Standing	Camera (72–74, 79) Accelerometer (43, 62, 77)
	Sitting	Camera (72–74) Accelerometer (43, 77)
	Kneeling	Camera (73, 74)
	Posture state and transitions between states (e.g., between lying and standing)	Photoelectric sensor (59)[a] Accelerometer (78) Camera (74)
	General motion activity and tracking (related to thermal comfort)	Camera (34, 76, 80) Accelerometer (78) Thermal camera (81)
	Walking (number of steps)	Accelerometer (61)
	Tracking (identifying location or number of animals)	Camera (32, 82–84) RFID (41)
Feeding and drinking behavior	Feed intake (kg)	Load cells with RFID (52)[a], (49, 85)
	Feeding time and/or frequency	RFID (42, 58, 86)[a], (87, 88) RFID and environment temperature and humidity sensors (46) Camera (37, 73, 74) Accelerometer (61, 64)
	Hunger stress identification	Thermal camera (89) Microphone (90–92)
	Nursing, suckling	Camera (37)
	Drinking time and/or frequency	RFID (93) Accelerometer (64) Camera (37, 73, 74, 94–97)
	Thirst stress identification	Thermal camera (89) Microphone (90, 92)
Other behavior	Nest- building behavior	Accelerometer (98)
	Aggressive behavior	Camera (99–104) Accelerometer (64)
	Cascade defense (freezing and startle duration)	Camera (105)
	Rooting	Accelerometer (61)
	Mounting behavior	Camera (97, 106)
	Tail biting	Camera (50, 107) Water flow meter and environment temperature sensor (108)
	Exploratory behavior	Accelerometer (64)
	Playing behavior	Camera (96)[b] Accelerometer (64)[c]
Physical condition	Gait attributes	Load cells [force plates, (35, 40, 109–111)] Camera and accelerometer (112), Camera (113)
	Cough detection	Microphone (45, 51, 114, 115)
	Body weight	Camera (29, 33, 36, 116–122) Load cells (scales) with RFID (52)[a] Load cells (scales) (39, 123)
	Muscle score	Camera (124)
	Body temperature	Thermal camera (48, 53)[a], (54)[a], (125, 126) Pyrometer (55)[a]
	Stress (e.g., due to heat or cold, pain, fear)	Microphone (57, 90)[a], (91, 92, 127–130) Thermal camera (89)

(Continued)

TABLE 4 | Continued

Indicator	Reason of use (monitored trait)	Technologies tested
Health-related traits	Lameness and claw lesions detection	Accelerometer (131) Camera (35) Thermal camera (132)
	African Swine Fever (sign: changes in activity level)	Camera (31) Accelerometer and microchip for body temperature (44)
	Influenza A virus (signs: fever) and changes in activity level	IR thermometer (133)
	Respiratory disease	Thermal camera (47, 134) Microphone (135, 136)
	General health problems	RFID (137)
	Diarrhea	Water flow meter (38)

[a] *External validation study.*
[b] *Water base play.*
[c] *Use of manipulating material.*

five sensor types (cameras, accelerometers, photoelectric sensors, thermal cameras, and RFID) that were used for activity measurement. Studies on accelerometers were mostly developed for sows, to classify postures and activity. Several studies validating the use of image analysis for postural states monitoring were found. For activity traits related to tracking (individual recognition and pen location), two types of sensors were identified: cameras and RFID. General motion activity and tracking related to thermal comfort (clustering behavior) was assessed using thermal-imaging.

Feeding and Drinking Behavior

Five types of technologies were identified for monitoring feeding and drinking behavior: RFID (feeders and drinkers), cameras, accelerometers, thermal cameras, and microphones (**Table 4**). Measured traits were: feed intake, feeding and drinking frequency and duration, stress related to hunger or thirst, as well as nursing and suckling behavior. Sows' nursing behavior was monitored using cameras (37). The estimation of stress conditions related to hunger and thirst was assessed by vocalizations (90–92) using microphones and via skin temperature using thermal cameras (89), applying different stressors to the animals. Feed intake was monitored using RFID in an electronic feeding station (52, 85). Evidence suggests that the performance of RFID feeders for monitoring feeding behavior is negatively affected by accumulation of debris under the feed trough, the large number of pigs per feeder space and pen space allowance (52). Therefore, frequent recalibration of the device is needed. Other studies validated feeding stations for monitoring individual daily feed intake (49). RFID systems were also validated for registering feeding and drinking patterns of individual growing-finishing pigs (58, 86, 93). Drinking patterns can be monitored using video analysis for evaluating visits to the drinker and contact time (94, 95), and for distinguishing drinking from drinker-playing behavior (96). Cameras for the identification of behavior of sows were used for identifying feeding and drinking behavior in the farrowing crate (37, 73, 74), as well as in group-housed sows (97).

Other Behavior

For monitoring other behavior, accelerometers, cameras, and water flow meters were used (**Table 4**). Cameras were the most often tested for monitoring other behavior ($n = 12$), followed by accelerometers ($n = 5$). Cameras were used for assessing behavior as a predictor of tail biting outbreak (restlessness) (107), as well as for recognizing high and medium aggression events based on image detection of motion and acceleration (as displacement in image) (100–103). Accelerometers were used for assessing movement associated to nest-building (98) and aggression (64). Water flow meters have been used in a study for predicting tail biting outbreaks by combining the frequency of use of water points and ambient temperature (108). Image analysis was also used for recognizing movement and location associated to walking, running, exploring, playing, nursing, feeding, urinating and mounting. Image methods for analyzing low tail posture as an early warning of tail biting have been studied (50). None of the vision-based tools have been externally validated (see **Table 2**).

Physical Condition

The following technologies were identified for monitoring physical condition: load cells (force plates, scales), load cells with RFID, cameras, microphones, thermal cameras, and pyrometer (**Table 4**). Measured traits included gait attributes (weight distribution on legs, gait characteristics, axial body movements trajectory during walking), cough, body temperature, stress (e.g., due to heat or cold, pain, fear), body weight as well as muscle score (loss in muscle condition is associated to acute and chronic diseases, and affects strength, immune function, and wound healing). Body weight was the most studied attribute, followed by stress and gait characteristics. Cameras were frequently used to assess body weight. One study tested the potential of depth-image analysis to evaluate axial body movements trajectory during walking, as an early indicator of lameness (113). Microphones were applied for evaluating the features of stress vocalizations, applying stressors such as handling, cold, heat, pain, hunger and thirst (eight studies) and for cough detection. Load cells (force plates) were applied for gait characteristics assessment. Thermal image was used for assessing body-temperature as an alternative of rectal temperature measurement. Also, the usefulness of thermal image to assess piglets' stress by measuring body-temperature changes when applying stressors (cold, pain, hunger, thirst) was tested (89). One study was found using a pyrometer for continuously measuring body-temperature, showing negative validation results (55). Load cells (scales) with and without RFID were validated for assessing body weight.

Health-Related Traits

Seven technologies were identified for assessing health-related traits: cameras, accelerometers, infrared thermometer, thermal cameras, microphones, RFID, and water flow meters (**Table 4**). The following health-related traits were assessed: lameness, claw lesions, detection of signs of disease associated to African Swine fever (decrease in activity), as well as Influenza A virus (fever), respiratory disease, diarrhea, and general health problems. Respiratory disease was the most frequent studied health-related trait (four studies), followed by body-temperature to detect fever (three studies), and lameness (three studies). Acceleration in combination with body-temperature data was tested for generating early alerts of disease (44). Acceleration was also applied for lameness detection based on sows' postures (131). Thermal imaging for assessing health problems was applied in three studies: one for detecting inflammation related to lameness in pregnant sows (132), and two for respiratory disease assessment (measuring skin-temperature at chest level for detecting lung tissue damage) (47, 134). One study tested the use of infrared thermometry for fever detection (133). Microphones for cough detection to identify sick pigs was applied in two studies. Moreover, RFID data were used to detect deviations in individual pigs' feeding patterns to point diseases or other disturbances, correlating it with the Welfare Quality® protocol assessment (looking for skin, ear and tail lesions, soiling, abnormalities in body condition, respiration, locomotion, bursitis, lameness, or diarrhea) (137). Finally, water usage data from flow meters have been tested as an early indicator of potential presence of diseases at group level, demonstrating that changes in diurnal drinking patterns of pigs can predict, for example, a diarrhea outbreak before clinical signs show up (38).

DISCUSSION

The aim of this review was to explore existing PLF technologies that potentially can contribute to measure animal-based welfare indicators of pigs and investigate their validation status. There is a substantial number of PLF tools (83 in our commercial list) in the market that can be potentially used to assess animal-based indicators of pig welfare. However, only a limited number of technologies have been internally validated, and only four market available technologies were externally validated (two thermal cameras, one pyrometer for monitoring body-temperature, and one RFID feeding station for monitoring feed intake and body weight) (52–55). Through this review, we identified important gaps in terms of validation on commercially available sensors. PLF tools that can identify stress due to hunger and thirst (90–92) have been found in the literature search but not in the commercial search. Similarly, tools that can assess play (64, 96), exploratory behavior (64), and aggressive behavior (64, 99–104) as well as models trained for recognizing specific diseases, such as African swine fever (31, 44), are not yet commercially available. The combination of different sensors as part of the same PLF solution was identified in several studies of the literature search, but they were not found as commercial solutions in our list (except for the combination of RFID and load cells, and accelerometers with body temperature sensors attached to ear tags).

Initially, we were searching for externally validated tools. However, only eight tools with external validation records were found. Therefore, the obtained data set has been used to find out which technologies have potential to contribute to pig welfare monitoring, but are not yet externally validated. Among the market available PLF tools, only 14% were found in validation studies. However, it needs to be noted that information obtained from the market is an overview of available PFL tools, as only products with websites and commercial information in English were included. Besides, several solutions may have been left out of the list, as we excluded technologies not addressing animal-based welfare indicators, or without direct involvement with animal welfare, for instance, those measuring reproductive parameters [e.g., (138–141)], or animal identification, such as facial recognition (142, 143).

Also, the literature search on validation studies may not have included all relevant PLF technologies for measuring animal-based welfare indicators. The reason for this was the choice of search criteria. Our search criteria specified the type of sensor applied to title, abstract and keywords. For this reason, some studies which mention sensor type only in material and methods section were omitted. This was the case for one study on image-analysis, one on water meters and one on load-cells (144–146) for instance. Pen fouling outbreaks, which can cause health problems due to poor hygiene, can be predicted by analyzing lying behavior using machine learning (144) and drinking patterns from water meters (146). The usefulness of load-cells to detect abnormalities in growth patterns of pigs at group level has been proved, even if the animals are not individually identified, by measuring the initial body weight, average daily gain and daily fluctuations in body weight parameters (145). One of the exclusion criteria was to remove articles not dealing with animal-based welfare indicators. Hence, all papers that use environmental data for welfare monitoring [e.g., (147), using ambient temperature data] were excluded.

The recent development of certain technologies, such as computer vision based technologies (analysis of static images and video, 2D, 3D and thermal-imaging) begin to appear on the market (148). In our review, vision-based PLF was the type of technology that could have potentially assessed the largest number of animal-based welfare indicators. However, most studies using computer vision for monitoring measures related to animal welfare assessment still report some need of improvement. For instance, in automatic body-weight detection, there is a need for development of algorithms accounting for the effect of gender and genotype (118), and the refinement of algorithms on automatic detection rate of pig boundaries (116). Similarly, for lameness detection, some reports have suggested the need for algorithms refinement to increase sensitivity and reliability (113), and the need to incorporate additional elements to the system, such as infrared lights (35). None of the reviewed systems were externally validated.

Performance of Validated and Commercially Available PLF Technologies, and Its Potential for Pig Welfare Assessment

According to our results there are no guidelines on the reporting of performance information in PLF validation studies. For that reason, the differences in performance measures reported by validation studies were not used as exclusion criteria. To be considered valid and feasible in commercial conditions, the performance of a technology should be tested in multiple practical scenarios, in different types of production systems and with different housing environments. In the reviewed studies, external validation was only performed in 7% of studies. Low number of validation studies can be explained by: (i) insufficient reporting (e.g., lack of information on validation place), (ii) low scientific interest (e.g., reluctance of scientific journals to publish validation studies on tool not applied for research), (iii) high costs and labor intensity of data collection, (iv) reluctance to publish negative results, and (v) the recent development of certain technologies. According to our results, validation trials for commercial purposes were less common than for research purposes, and it could be due to time and resources requirements for validation. Besides, market available PLF technologies for pigs are mostly calibrated by the providers, and its precision and reliability on data management is assumed by them without an independent validation (**Table 3**). The fact that PLF companies perform validation trials themselves, and could obtain negative results without reporting these, has to be considered as an important reason for reluctance of dissemination.

Concerning the quality of reporting, external validation requires specific information on the location of the trials, the name of validated device, software provider, and studied population, knowledge about the origin of the animals, if the test procedure was applied in commercial or experimental conditions, and clear information on which golden standard was used for validation and how it was measured. Information gaps in reporting were found in 22% of studies, for which reason were classified as internal validation studies. Few examples are the study of Petry et al. (48), and Guarino et al. (51), which despite reporting their results under laboratory and practical conditions, presented lacks of information in materials and methods (regarding used animals, and study location, respectively).

In addition, internal validation studies with samples smaller than 10 or 20 animals were very frequent (validating some cameras, accelerometers, microphones, RFID for tracking, and force plates). It was observed that larger samples (above 20 animals) were mainly used in studies validating feeders and drinkers with or without RFID, and sorting scales. According to Royston and Altman (23), an appropriate validation sample is required to provide a reasonably accurate estimate of a measure and to avoid the risk of false negatives. Thus, studies with limited sample sizes could have low validity and are inconclusive (23). However, at present, a standardized parameter is not known for what could be considered a reasonable sample size (depending on the type of technology to be validated). A remarkable lack was found regarding technologies developed or with adapted algorithms for young pigs exclusively. Thus, there is an important concern in regard to the usefulness of PLF for monitoring the welfare of young animals.

As stated by Stygar et al. (28), in the case of the dairy cow industry, devices used for the official recording of milk (such as milk samplers) must comply with the requirements of the International Organization for Standardization (ISO) to obtain the certification, and must be tested for approval by the International Committee for Animal Recording and Analysis (ICAR) (149). Recommendations on proper validation procedures for PLF technologies for pig industry are still lacking.

There is a constant development of PLF technologies to offer solutions for animal production including animal welfare. Despite the lack of external validation for the majority of technologies, the link between the feature measured by a sensor and the state of the animal in terms of welfare is not always clear. For instance, camera-based motion detection is often mentioned as a tool for welfare assessment. However, few studies have demonstrated a clear link between features of motion and specific animal welfare problems, such as lameness (113), or specific diseases (31). The performance of identified types of PLF for monitoring animal-based welfare indicators and measured traits in validation studies will be described below. The types of sensors are listed in descending order, according to the number of validation studies compiled for each. **Supplementary Table 2** shows full information on the validation results of each study.

Camera-Based Technologies

Internal validation of the vision-based technologies in many cases reported very promising results with accuracy above 95% [e.g., (30, 33, 58, 73, 74, 93, 95, 97, 107, 145–147)]. However, none of the outstanding performance results for vision-based monitoring have been confirmed by external validation. Image-analysis has been used for assessing sows' postures, such as standing, lying, and sitting (73), evaluating lying patterns in group-housed pigs responding to thermal conditions of the pen (76, 80), detecting animals' location (32, 84), distinguishing drinking and drinker-playing behavior (96), identifying feeding behavior (37, 73, 74, 97), recognizing aggression events (100–103) and tail biting (50), estimating body weight (36, 118, 119), and detecting African swine fever (31).

Changes in animals' postures can be used as health indicators (31). Although the assessment of certain postures (sitting, kneeling) is not very accurate using vision based technologies (72), it is possible to distinguish standing active behaviors, such as feeding or walking, against resting patterns (30, 69). Lying posture, predicted by image-analysis, can indicate health problems. For instance, resting duration and frequency changes due to diseases (31), lesions and stressful situations, are at the same time associated to damaging behavior outbreaks (50, 107). Similarly, resting can be used to extrapolate maternal ability of sows, as it is associated to nursing behavior (37), and thermal comfort in the pen (34). Lying posture can also be used for assessing diurnal activity patterns of the animals (79). Image technologies that detect locomotion and axial body-movement are promising tools for assessing lameness, an important welfare issue (113), especially in sows (35).

Image based technologies are also able to accurately assess drinking behavior and water usage, which are acknowledged to be crucial for pig welfare (73, 74). Vision-based technologies have great potential of assessing animal welfare by continuously monitoring behaviors of pigs, which can be used to detect changes and deviations in normal behavioral patterns related to animals' affective state (150). Some specific features such as the posture of the tail can provide useful information in relation to tail biting outbreaks or can even be related to the affective state of the pig (150). Besides, computer vision can provide information on behavioral changes such as interactions between individuals, allowing the detection of aggressive events and affiliative behaviors as nursing and playing (37). The use of image-analysis to evaluate the cascade defense has been validated in just one study, however, it still shows the potential of this tool to assess fear and stress-associated conditions (105).

Body weight detection, individual recognition, behavior and activity tracking are the most frequent uses of commercial image PLF technologies. According to Wurtz et al. (148), one of the difficulties of camera-based technologies is to monitor animals at individual level. Nevertheless, results on studies validating vision algorithms for individual identification and location, seem to be promising (84). Image-based individual recognition is not invasive, and can be used in real-time, helping to overcome some of the limitations of RFID systems (stress to the animals when attaching an RFID tag, and time requirements to the farmer in attaching and reading). Current protocols, such as Welfare Quality® (2), assess the nutritional state of animals by the body-condition. Image-analysis seems to be a promising tool to improve the assessment of the nutritional status continuously, by monitoring the body size (117, 122). Compiled results on camera-based systems in farm conditions for pig weight estimation, show potential of these tools for reducing the need of human-animal interaction, reducing stress associated to an unfamiliar human presence (118, 119, 124). Besides, camera-based PLF allows to monitor specific situations as farrowing, and the detection of the number of piglets in the farrowing pen, which has been studied to prevent perinatal asphyxia and piglets' crushing (83).

Load Cells and Flow Meters

Flow meters are discussed in the same section with load-cells, as its application for welfare assessment is strongly related to monitoring of feed-intake. Load-cells also include force plates.

Scales without individual identification have been used for body weight measurement. Reported deviations are around 1 kg at group (39) and individual level (123). In combination with RFID, load-cells systems (electronic feeding stations), could estimate body weight with a percentage error of 3% (52), showing less accuracy than an ordinary scale. Monitoring the feed intake by measuring the feed weight in an electronic station with RFID was found to reach a 90% accuracy (85). An overestimation of 1.1% of feed intake has been found in one study (49). Feeding patterns (time and frequency) of individual growing-finishing pigs can be analyzed by combining RFID and load-cells, reaching an accuracy of 97% (58, 86). RFID data for measuring the drinking behavior of individual pigs, showed 93% of accuracy (93).

Load-cell technologies allow to monitor body weight and growing patterns at group level. When working with RFID, load-cells can monitor feeding and drinking patterns and growing performance at individual level, overcoming one of the challenges that cannot be achieved by current welfare protocols, which can only monitor these aspects at group level. Although a normal growth pattern may have little predictive value in terms of good animal welfare, growth deviations or retardations have been used to identify health issues and other welfare problems (137). Automatic feeders with RFID are a promising technique to understand animals' requirements and anticipate welfare problems based on feeding patterns deviations, allowing the implementation of corrective measures and thus improving animal health and welfare (46).

Force Plates

Lameness is a frequent and important welfare problem, because of the intense pain it causes, the disadvantages that it brings in terms of access to food and water (151, 152). Also, in the normal housing conditions of a pig farm, which mainly use slatted floor (151), may only exacerbate the problem. Due to stocking density, and subjectivity of observations, the usual visual diagnosis of lameness is challenging. The most affected animals often lose feeding times, and consequently body condition decreases, which gets the attention of farm staff, and that is when observation is usually performed. Early diagnosis of lameness can prevent the associated high culling and mortality rates, especially for sows (152). Force plates are accurate for evaluating gait characteristics and detecting lameness even at an early stage (40). Several validation studies confirm their potential (35, 40, 109–111, 153). Different features have been extracted and validated using visual observation as a gold standard (109, 110, 153). Weight distribution of legs (percentage of weight, ratio between the weights applied by contralateral legs, weight shifting, amplitude of weight bearing and weight removing) significantly correlated with the golden standard (CV = 5.22%) (111). Weight shifting frequency and the ratio between the weights applied by contralateral legs performed the best in terms of identifying lame individuals (109).

Flow Meters

The use of flow meters to assess drinking patterns and water usage, have proved useful for prediction of several welfare conditions, such as presence of disease (38), and tail biting outbreaks (108). Performance of warning algorithms based on deviations from expected diurnal pattern in water consumption, showed that the algorithms were capable to predict a diarrhea outbreak 1 day before presentation of clinical signs (38).

Accelerometers

Accelerometers have been used to classify postures and activity with a performance for detecting and classifying activity ranging from 75 to 100% (60–64, 77). By classifying postures and activity nest-building behavior can be monitored to predict farrowing time with an accuracy of 86% (98). Acceleration data have also been used to detect lameness based on sow postures with an accuracy of up to 93% (131). Acceleration in combination with body temperature data was tested for generating early alerts of disease, reaching 97% of sensitivity and 89% of specificity (44).

Deviations in activity pattern might point out to health issues (44) and lameness (131). Accelerometers can therefore provide useful information, but the application on pig farms will be limited because sensors have to be attached to individual pigs, implying handling stress. For instance, accelerometers may be embedded in ear tags, which requires the perforation of an animal's ear for placement. Another alternative is the attachment of the accelerometer on the animal's back or leg, but ensuring that the device remains in place can lead to complications. Besides, the maintenance of a device attached to the pigs' bodies could be difficult under farm conditions, as it can motivate other pigs' chewing behavior in response to novelty of an object (154). Short battery life of wearable sensors is also a limitation of its applicability on farm. However, optimization of power consumption and battery life are currently being improved (65). For lameness detection, accelerometers can be mainly relevant to be used in sows.

Microphones

Microphones accuracy for assessing and classifying vocalizations was >73% (eight out of nine validation trials studies). One sound-analysis algorithm reached an accuracy of 98% distinguishing stress vocalizations associated to pain, using duration and intensity of vocalization signal as a gold standard (91). The detection of vocalizations related to hunger, thirst, cold and heat conditions (ranged from 69 to 71%) (91). Cough detection for localization of sick pigs at barn level using microphones, reached an interval of confidence of 95% (135). It was also found an accuracy from 73 to 93% for correct identification ratio of sick pigs cough sounds (136).

Sound analysis has been used for detecting coughing pigs. Coughing is a sign of respiratory problems or at least of poor climate conditions (dust, ammonia). Measuring coughing is therefore a relevant indicator contributing to animal welfare assessment, although it cannot be done at the individual level. Furthermore, if stress and pain related vocalizations can be reliably identified, it could also be used to further welfare aspects such as stress assessment and fighting events, for instance. Distress vocalizations induced by hunger, or extreme thermal discomfort seem to be more difficult to classify than vocalizations due to pain (91). Future research is needed in a larger vocal spectrum of vocal signals, not only to assess negative welfare aspects but also for assessing positive welfare.

Thermal Cameras

Thermal cameras are mainly used for remote sensing of body temperature (17). Body temperature is relevant in relation to animal welfare because over certain thresholds it can evidence hyperthermia or hypothermia and may also reflect fever. Besides, thermal imaging seems to be a promising tool for monitoring physiological responses as inflammation related to lameness (132), and animals' distribution responding to housing thermal conditions (81). Additionally, thermal imaging can be a promising tool for assessing acute stress events by body temperature changes (89). Thermal image for predicting stress in piglets, reached accuracies of 50, 86, 91, and 100% when stress was related to pain, hunger, thirst, and cold, respectively (89). Thermal cameras for assessing animals' space distribution (clustering behavior) in function of body temperature and radiated temperature, was validated showing a significant correlation between clustering and temperature response (81). Also, the correlation between thermal image measurements and rectal temperature was high ($r = 0.80$) (126). Inflammation related to lameness in pregnant sows was also detected using thermal imaging, showing significant correlation between mean upper metatarsal temperature and sows' parity (132). Therefore, thermal imaging allowed to differentiate between lame and non-lame sows, and to detect temperature differences in the affected leg. Hence, the welfare problem resulting from the pain caused by the inflammation associated with lameness (151), can be detected by thermal imaging. Thermal imaging at chest level for the diagnosis of lung tissue alterations associated with *Actinobacillus pleuropneumoniae* infection, by measuring the body temperature at chest level, reached a specificity of 100% (134).

Photoelectric Sensors

Photoelectric sensors, the only sensor group with external validation records for activity measurements, showed a precision lower than 90% (56, 66). The potential of these sensors to detect position changes in puerperal sows showed 64% of sensitivity and 88% of specificity (59). For monitoring activity levels in pigs, photoelectric sensors detected movement in <1 s (67).

Photoelectric sensors can detect movement and therefore provide useful information about the activity level and postural transitions, which contribute to welfare assessment. According to Besteiro et al. (56), these sensors work better with recently weaned piglets and assessing play than feeding behavior. As the body-weight of animals increases, the coverage area of the photoelectric sensor decreases, resulting in less precise measurements. In contrast, the detection of intense activity is more precise than non-intense activity (56).

RFID

RFID technology used for individual recognition of multiple pigs at the same RFID reader in the pen, can reach an accuracy of 92% (41). It has been demonstrated that the use of two RFID tags instead of one, increased the accuracy up to 97% (58, 86). Deviations in feeding patterns as an indicator of disease, monitored by RFID data, have showed an accuracy of 97%, and a precision of 71% (137).

RFID is used for individual identification, and this is essential if we want to opt for an increasingly individualized welfare evaluation. RFID is very useful in combination with many other devices such as scales and automatic feeders and drinkers. RFID allows to track animals' location. It may offer additional practical applications such as monitoring social interaction as a possible transmission path for diseases (155), as the contact intensity and length between individuals may be an indicator for disease transmission (156).

Non-contact Body-Temperature Sensors

Non-contact body-temperature sensors revealed its limited usefulness as an alternative to sensing temperature measurement. The one study found on pyrometer for continuously measuring pigs' body temperature showed that the performance was not reliable (55). Under fever-induced situations, comparing vaginal thermometer data and pyrometer data in the orbital area of animals in time periods from 0.25 to 5 h, a positive correlation was found only in a third of the sample. The longer the measuring period, the fewer animals showed a significant correlation. Similar conclusions were obtained from testing the accuracy of infrared thermometers for body-temperature measurement, compared to rectal temperature as a gold standard (133). Several authors conclude that environmental conditions such as ambient temperature, sunlight, air movement, barn and pen configuration, and stocking density, have a significant impact on the reliability of infrared thermometry to assess body-temperature in pigs (133, 157).

Trends and Gaps in PLF Technologies for Pig Welfare Assessment

To increase transparency of animal production, there is a need for reliable data on the welfare of farmed animals. This information is above all important for the animals as if used in a proper way it can improve their lives. It can also assist both consumers and producers to make decisions from an informed perspective. For the sake of the animals and production efficiency, producers need to monitor the health and welfare status of animals. It may be done with reliable and up-to-date information as early-warning systems, before implementing corrective and timely measures. Consumers are demanding clear information about farm animal welfare to assist them in identifying and choosing enhanced welfare-friendly products. Recent advances in sensor technologies increasingly allow systematic and automated monitoring of several indicators that inform about the welfare status of farm animals. This data could be transformed to useful information for consumers as labeling.

However, there is a need to identify and select the most appropriate indicators and the relevant PLF technologies to assess them. This review, is the first of its kind, spotting relevant technologies that can assist on this task. Nevertheless, we identified some challenges and gaps that need to be addressed. To date, welfare has been based on focal assessments, and as information is mainly applicable to the day in which the evaluation is carried out, a limited picture of welfare status of animals is provided. PLF technologies dispense continuous welfare information using both behavioral (e.g., activity) and physiological indicators (e.g., body temperature and weight), which could yield a continuous and systematic assessment at different stages of their life, and in the future, may revolutionize the way animal welfare is evaluated. This may allow to investigate deviations from normality at the individual level, leading to one welfare appraisal which is predominantly animal-based, and that is less dependent on environmental-based indicators. Deviations from "normal" patterns at individual level will account for individual differences rather than trying to understand "average" animals. Information on the evolution of animal behavior and welfare throughout an animal's lifetime and throughout the chain may facilitate understanding of factors impairing or promoting it. This understanding of animal welfare will further be reinforced by accessibility to large data sets, only available with the integrated automatic and systematic assessment.

There is thus a need for an integration of the different aspects of animal welfare (i.e., health, nutrition, comfort, affective state and natural behavior) into relevant information that could assist stakeholders to make decisions. The combination of sensors may provide more relevant information than if taken separately as animal-based indicators can be related. For instance, the use of one activity sensor may alert when an animal stops moving, which could be a sign of different health problems (e.g., lameness, disease), but if the activity sensor is combined with a thermal camera informing about body temperature, the welfare information delivered can be much more precise. In order to cover these needs, block chain technology has been judged useful to integrate information throughout the entire production chain and monitor welfare at different stages of the animal's lives (158, 159).

Market availability and validation records of sensor technologies dedicated to animal-based welfare monitoring in dairy industry has been recently conducted (28). There are clear differences between dairy and pig industry when it comes to market availability, type of sensors used and validation records. It seems that the pig industry is behind dairy regarding sensor availability (and validation), especially when population numbers are compared [pigs around 677.6 millions of heads (160) against to 270 million oh head in dairy cattle (161)]. Looking on the nature of production, pigs are mostly kept in groups, and very often are not individually identified. Since lifespan of a productive pig is limited (excluding sows), individual identification is relatively expensive and more difficult to manage (145, 162). Nevertheless, individual identification allows for a more specific picture of any sub-optimal state of well-being, which is not captured by group averages (46, 58, 85, 86, 93, 145). There is a difference in the investment on individual animals' identification in function of their productive objectives. Sows are more commonly identified by RFID tags than fattening pigs, especially in farms using electronic feeding stations, as their productive lifespan is longer. In fattening pigs, group monitoring is more common as it reduces the costs of assessment. This might be a reason why some technologies, which would have great potential for health and welfare monitoring, are so scarcely represented on the market.

Based on market analyses, it is clear that availability of vision-based monitoring for pigs are greater than in cattle production. It could be due to cost concerns (163). For example, in order to monitor the body weight of fattening pigs, few technologies could be considered. Using a weight sorting system based on load cells and RFID requires substantial investment on farms and might only be feasible in newly constructed farms (164), while cameras can be installed also to already operating systems with potentially less financial input. Interestingly, neither of those systems are validated externally.

In conclusion, existing PLF technologies are potential tools for on-farm animal welfare assessment in pig production. A variety of animal-based welfare indicators can be monitored on an individual scale, continuously and in real time, using PLF. These tools had demonstrated potential for yielding a continuous and systematic assessment at different stages of animals' lives, overcoming some difficulties and gaps of current welfare assessment protocols. Thus, in the future, PLF may revolutionize the way animal welfare is assessed and informed. However, validation studies are lacking for an important percentage of market available solutions, and in particular, research and development need to focus on identifying feature candidates of the measures (e.g., deviations from diurnal pattern, threshold levels etc.) that are valid signals of either negative or positive animal welfare. An important gap identified are the lack of technologies to assess affective states (both positive and negative states).

In this review, tools were validated against three possible golden standards: human observer, other tool with well-defined performance record, or based on the tool's ability to detect change in animal behavior or physical condition during planned experiment. The need for an established protocol for the validation procedures of PLF technologies can be noticed, as the measurements presented in the performance reports are very heterogeneous.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

AUTHOR'S NOTE

This study is conducted within ClearFarm project which aim is to co-design, develop, and validate a software platform powered by the integration of PLF technologies, to assess animal welfare throughout the production chain, and to provide this information to all parts of it.

AUTHOR CONTRIBUTIONS

YG, AS, and PL conceived the idea of this manuscript. YG defined and performed the literature search and the company search. YG and AS conducted the literature screening. YG wrote the paper with input from AS, PL, JN, MP, EB, IB, LP, and XM. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

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REFERENCES

1. Fraser D, Weary DM, Pajor EA, Milligan BN. A scientific conception of animal welfare that reflects ethical concerns. *Anim Welf.* (1997) 6:187–205.
2. Welfare Quality®. *Welfare Quality® Assessment Protocol for Pigs.* Welfare Quality Consortium, Lelystad (2009). p. 182. Available online at: http://www.welfarequalitynetwork.net/media/1018/pig_protocol.pdf
3. Jääskeläinen T, Kauppinen T, Vesala KM, Valros A. Relationships between pig welfare, productivity and farmer disposition. *Anim Welf.* (2014) 23:435–43. doi: 10.7120/09627286.23.4.435
4. Clark B, Panzone LA, Stewart GB, Kyriazakis I, Niemi JK, Latvala T, et al. Consumer attitudes towards production diseases in intensive production systems. *PLoS ONE.* (2019) 14:e0210432. doi: 10.1371/journal.pone.0210432
5. Homola JJ, Loftin CS, Survey G, Fish MC, Cammen KM, Sciences M, et al. Impact of health challenges on pig growth performance, carcass characteristics and net returns under commercial conditions. *Transl Anim Sci.* (2019) 2:50–61. doi: 10.1093/tas/txx005
6. Sinisalo A, Niemi JK, Heinonen M, Valros A. Tail biting and production performance in fattening pigs. *Livest Sci.* (2012) 143:220–5. doi: 10.1016/j.livsci.2011.09.019
7. Stygar AH, Chantziaras I, Toppari I, Maes D, Niemi JK. High biosecurity and welfare standards in fattening pig farms are associated with reduced antimicrobial use. *Animal.* (2020) 14:2178–86. doi: 10.1017/S1751731120000828
8. Thorslund CAH, Aaslyng MD, Lassen J. Perceived importance and responsibility for market-driven pig welfare: literature review. *Meat Sci.* (2017) 125:37–45. doi: 10.1016/j.meatsci.2016.11.008
9. Denver S, Sandøe P, Christensen T. Consumer preferences for pig welfare – can the market accommodate more than one level of welfare pork? *Meat Sci.* (2017) 129:140–6. doi: 10.1016/j.meatsci.2017.02.018
10. Xu L, Yang X, Wu L, Chen X, Chen L, Tsai FS. Consumers' willingness to pay for food with information on animal welfare, lean meat essence detection, and traceability. *Int J Environ Res Public Health.* (2019) 16:3616. doi: 10.3390/ijerph16193616
11. Cornish AR, Briley D, Wilson BJ, Raubenheimer D, Schlosberg D, McGreevy PD. The price of good welfare: does informing consumers about what on-package labels mean for animal welfare influence their purchase intentions? *Appetite.* (2020) 148:104577. doi: 10.1016/j.appet.2019.104577
12. Sørensen JT, Schrader L. Labelling as a tool for improving animal welfare-the pig case. *Agriculture.* (2019) 9:1–13. doi: 10.3390/agriculture9060123
13. Czycholl I, Büttner K, Beilage EG, Krieter J. Review of the assessment of animal welfare with special emphasis on the "Welfare Quality® animal welfare assessment protocol for growing pigs." *Arch Tierzucht.* (2015) 58:237–49. doi: 10.5194/aab-58-237-2015
14. Rutherford KMD, Donald RD, Lawrence AB, Wemelsfelder F. Qualitative behavioural assessment of emotionality in pigs. *Appl Anim Behav Sci.* (2012) 139:218–24. doi: 10.1016/j.applanim.2012.04.004
15. Berckmans D. Automatic on-line monitoring of animals by precision livestock farming. *Livest Prod Soc.* (2006) 1:287–94. doi: 10.3920/978-90-8686-567-3
16. Guarino M, Norton T, Berckmans D, Vranken E, Berckmans D. A blueprint for developing and applying precision livestock farming tools: a key output of the EU-PLF project. *Anim Front.* (2017) 7:12–7. doi: 10.2527/af.2017.0103
17. Benjamin M, Yik S. Precision livestock farming in swinewelfare: a review for swine practitioners. *Animals.* (2019) 9:133. doi: 10.3390/ani9040133
18. Matthews SG, Miller AL, Clapp J, Plötz T, Kyriazakis I. Early detection of health and welfare compromises through automated detection of behavioural changes in pigs. *Vet J.* (2016) 217:43–51. doi: 10.1016/j.tvjl.2016.09.005

19. Tullo E, Fontana I, Diana A, Norton T, Berckmans D, Guarino M. Application note: labelling, a methodology to develop reliable algorithm in PLF. *Comput Electron Agric.* (2017) 142:424–8. doi: 10.1016/j.compag.2017.09.030
20. Steeneveld W, Hogeveen H, Oude Lansink AGJM. Economic consequences of investing in sensor systems on dairy farms. *Comput Electron Agric.* (2015) 119:33–9. doi: 10.1016/j.compag.2015.10.006
21. Steeneveld W, Vernooij JCM, Hogeveen H. Effect of sensor systems for cow management on milk production, somatic cell count, and reproduction. *J Dairy Sci.* (2015) 98:3896–905. doi: 10.3168/jds.2014-9101
22. Wieringa R. Empirical research methods for technology validation: scaling up to practice. *J Syst Softw.* (2014) 95:19–31. doi: 10.1016/j.jss.2013.11.1097
23. Royston P, Altman DG. External validation of a Cox prognostic model:principles and methods. *BMC Med Res Methodol.* (2013) 13:33. doi: 10.1186/1471-2288-13-33
24. Altman DG, Vergouwe Y, Royston P, Moons KGM. Prognosis and prognostic research: validating a prognostic model. *BMJ.* (2009) 338:b605. doi: 10.1136/bmj.b605
25. Moher D, Liberati A, Tetzlaff J, Altman DG, Altman D, Antes G, et al. Preferred reporting bibitems for systematic reviews and meta-analyses: the PRISMA statement. *PLoS Med.* (2009) 6:e1000097. doi: 10.1371/journal.pmed.1000097
26. Reitsma JB, Rutjes AWS, Khan KS, Coomarasamy A, Bossuyt PM. A review of solutions for diagnostic accuracy studies with an imperfect or missing reference standard. *J Clin Epidemiol.* (2009) 62:797–806. doi: 10.1016/j.jclinepi.2009.02.005
27. Gold R, Reichman M, Greenberg E, Ivanidze J, Elias E, Tsiouris AJ, et al. Developing a new reference standard. *Acad Radiol.* (2010) 17:1079–82. doi: 10.1016/j.acra.2010.05.021
28. Stygar AH, Gómez Y, Berteselli GV, Dalla Costa E, Canali E, Niemi JK, et al. How precise is precision livestock farming? A systematic review on commercially available and validated sensor technologies for welfare assessment of dairy cattle. *Front Vet Sci.* (2020) 8:634338. doi: 10.3389/fvets.2021.634338
29. Cang Y, He H, Qiao Y. An intelligent pig weights estimate method based on deep learning in sow stall environments. *IEEE Access.* (2019) 7:164867–75. doi: 10.1109/ACCESS.2019.2953099
30. Chung Y, Kim H, Lee H, Park D, Jeon T, Chang H-H. A cost-effective pigsty monitoring system based on a video sensor. *KSII Trans Internet Inf Syst.* (2014) 8:1481–98. doi: 10.3837/tiis.2014.04.018
31. Fernández-Carrión E, Martínez-Avilés M, Ivorra B, Martínez-López B, Ramos ÁMÁM, Sánchez-Vizcaíno JMJM. Motion-based video monitoring for early detection of livestock diseases: the case of African swine fever. *PLoS ONE.* (2017) 12:e0183793. doi: 10.1371/journal.pone.0183793
32. Navarro-Jover JM, Alcañiz-Raya M, Gómez V, Balasch S, Moreno JR, Grau-Colomer V, et al. An automatic colour-based computer vision algorithm for tracking the position of piglets|Sistema de visión artificial basado en el color, para el seguimiento de la posición de lechones. *Spanish J Agric Res.* (2009) 7:535–49. doi: 10.5424/sjar/2009073-438
33. Parsons DJ, Green DM, Schofield CP, Whittemore CT. Real-time control of pig growth through an integrated management system. *Biosyst Eng.* (2007) 96:257–66. doi: 10.1016/j.biosystemseng.2006.10.013
34. Shao B, Xin H. A real-time computer vision assessment and control of thermal comfort for group-housed pigs. *Comput Electron Agric.* (2008) 62:15–21. doi: 10.1016/j.compag.2007.09.006
35. van Riet MMJMMJ, Vangeyte J, Janssens GPJGPJ, Ampe B, Nalon E, Bos EJE-J, et al. On-Farm claw scoring in sows using a novel mobile device. *Sensors.* (2019) 19:1473. doi: 10.3390/s19061473
36. White RPP, Schofield CPP, Green DMM, Parsons DJJ, Whittemore CTT. The effectiveness of a visual image analysis (VIA) system for monitoring the performance of growing/finishing pigs. *Anim Sci.* (2004) 78:409–18. doi: 10.1017/S1357729800058811
37. Yang A, Huang H, Zheng B, Li S, Gan H, Chen C, et al. An automatic recognition framework for sow daily behaviours based on motion and image analyses. *Biosyst Eng.* (2020) 192:56–71. doi: 10.1016/j.biosystemseng.2020.01.016
38. Madsen TNTN, Kristensen ARAR. A model for monitoring the condition of young pigs by their drinking behaviour. *Comput Electron Agric.* (2005) 48:138–54. doi: 10.1016/j.compag.2005.02.014
39. Schinckel AP, Einstein ME, Miller D. Evaluation of a method to analyze pig live weight data from animal sorting technologies. *Prof Anim Sci.* (2005) 21:50–8. doi: 10.15232/S1080-7446(15)31166-9
40. McNeil B, Díaz JC, Bruns C, Stock J, Millman S, Johnson A, et al. Determining the time required to detect induced sow lameness using an embedded microcomputer-based force plate system. *Am J Anim Vet Sci.* (2018) 13:59–65. doi: 10.3844/ajavsp.2018.59.65
41. Porto SMC, Arcidiacono C, Cascone G, Anguzza U, Barbari M, Simonini S. Validation of an active RFID-based system to detect pigs housed in pens. *J Food, Agric Environ.* (2012) 10:468–72.
42. Gertheiss J, Maier V, Hessel EF, Staicu A-M. Marginal functional regression models for analyzing the feeding behavior of pigs. *J Agric Biol Environ Stat.* (2015) 20:353–70. doi: 10.1007/s13253-015-0212-7
43. Thompson R, Matheson SMSM, Plötz T, Edwards SA, Kyriazakis I. Porcine lie detectors: automatic quantification of posture state and transitions in sows using inertial sensors. *Comput Electron Agric.* (2016) 127:521–30. doi: 10.1016/j.compag.2016.07.017
44. Martínez-Avilés M, Fernández-Carrión E, López García-Baones JM, Sánchez-Vizcaíno JM. Early detection of infection in pigs through an online monitoring system. *Transbound Emerg Dis.* (2017) 64:364–73. doi: 10.1111/tbed.12372
45. Van Hirtum A, Berckmans D. Objective recognition of cough sound as biomarker for aerial pollutants. *Indoor Air.* (2004) 14:10–5. doi: 10.1046/j.1600-0668.2003.00195.x
46. Cross AJ, Rohrer GA, Brown-Brandl TM, Cassady JP, Keel BN. Feed-forward and generalised regression neural networks in modelling feeding behaviour of pigs in the grow-finish phase. *Biosyst Eng.* (2018) 173:124–33. doi: 10.1016/j.biosystemseng.2018.02.005
47. Loughmiller JA, Spire ME, Dritz SS, Fenwick BW, Hosni MH, Hogge SB. Relationship between mean body surface temperature measured by use of infrared thermography and ambient temperature in clinically normal pigs and pigs inoculated with Actinobacillus pleuropneumoniae. *Am J Vet Res.* (2001) 62:676–81. doi: 10.2460/ajvr.2001.62.676
48. Petry A, McGilvray W, Rakhshandeh ARR, Rakhshandeh ARR. Technical note: assessment of an alternative technique for measuring body temperature in pigs. *J Anim Sci.* (2017) 95:3270–4. doi: 10.2527/jas.2017.1566
49. Bruininx EM, Van Der Peet-Schwering CM, Schrama JWW, Den Hartog LAA, Everts H, Beynen ACC. The IVOG® feeding station: a tool for monitoring the individual feed intake of group-housed weanling pigs. *J Anim Physiol Anim Nutr.* (2001) 85:81–7. doi: 10.1046/j.1439-0396.2001.00305.x
50. D'Eath RB, Jack M, Futro A, Talbot D, Zhu Q, Barclay D, et al. Automatic early warning of tail biting in pigs: 3D cameras can detect lowered tail posture before an outbreak. *PLoS ONE.* (2018) 13:e0194524. doi: 10.1371/journal.pone.0194524
51. Guarino M, Jans P, Costa A, Aerts J-MM, Berckmans D. Field test of algorithm for automatic cough detection in pig houses. *Comput Electron Agric.* (2008) 62:22–8. doi: 10.1016/j.compag.2007.08.016
52. Faltys GL, Young JM, Odgaard RL, Murphy RB, Lechtenberg KF. Technical note: validation of electronic feeding stations as a swine research tool. *J Anim Sci.* (2014) 92:272–6. doi: 10.2527/jas.2013-6808
53. Farrar KL, Field AE, Norris SL, Jacobsen KO. Comparison of rectal and infrared thermometry temperatures in anesthetized swine (*Sus scrofa*). *J Am Assoc Lab Anim Sci.* (2020) 59:221–5. doi: 10.30802/AALAS-JAALAS-19-000119
54. Sykes DJJ, Couvillion JSS, Cromiak A, Bowers S, Schenck E, Crenshaw M, et al. The use of digital infrared thermal imaging to detect estrus in gilts. *Theriogenology.* (2012) 78:147–52. doi: 10.1016/j.theriogenology.2012.01.030
55. Schmidt M, Ammon C, Christian Schön P, Manteuffel C, Hoffmann G, Schön PCPC, et al. The suitability of infrared temperature measurements for continuous temperature monitoring in gilts. *Arch Anim Breed.* (2014) 57:1–12. doi: 10.7482/0003-9438-57-021
56. Besteiro R, Rodríguez MR, Fernández MD, Ortega JA, Velo R. Agreement between passive infrared detector measurements and human observations of animal activity. *Livest Sci.* (2018) 214:219–24. doi: 10.1016/j.livsci.2018.06.008

57. Schön PC, Puppe B, Manteuffel G. Automated recording of stress vocalisations as a tool to document impaired welfare in pigs. *Anim Welf.* (2004) 13:105–10.
58. Maselyne J, Saeys W, De Ketelaere B, Mertens K, Vangeyte J, Hessel EFEF, et al. Validation of a high frequency radio frequency identification (HF RFID) system for registering feeding patterns of growing-finishing pigs. *Comput Electron Agric.* (2014) 102:10–8. doi: 10.1016/j.compag.2013.12.015
59. Mainau E, Dalmau A, Ruiz-de-la-Torre JL, Manteca X. Validation of an automatic system to detect position changes in puerperal sows. *Appl Anim Behav Sci.* (2009) 121:96–102. doi: 10.1016/j.applanim.2009.09.005
60. Cornou C, Lundbye-Christensen S, Kristensen ARARARAR. Modelling and monitoring sows' activity types in farrowing house using acceleration data. *Comput Electron Agric.* (2011) 76:316–24. doi: 10.1016/j.compag.2011.02.010
61. Escalante HJ, Rodriguez SV, Cordero J, Kristensen AR, Cornou C. Sow-activity classification from acceleration patterns: a machine learning approach. *Comput Electron Agric.* (2013) 93:17–26. doi: 10.1016/j.compag.2013.01.003
62. Marcon M, Meunier-Salaün MC, Le Mer M, Rousselière Y. Accelerometer technology to perform precision feeding of pregnant sows and follow their health status. in *Precision Livestock Farming 2017 - Papers Presented at the 8th European Conference on Precision Livestock Farming, ECPLF 2017*. Nantes, (2017). p. 666–73.
63. Oczak M, Maschat K, Berckmans D, Vranken E, Baumgartner J. Can an automated labelling method based on accelerometer data replace a human labeller? - Postural profile of farrowing sows. *Comput Electron Agric.* (2016) 127:168–75. doi: 10.1016/j.compag.2016.06.013
64. Rodriguez-Baena DS, Gomez-Vela FA, García-Torres M, Divina F, Barranco CD, Daz-Diaz N, et al. Identifying livestock behavior patterns based on accelerometer dataset. *J Comput Sci.* (2020) 41:101076. doi: 10.1016/j.jocs.2020.101076
65. Liu LS, Ni JQ, Zhao RQ, Shen MX, He CL, Lu MZ. Design and test of a low-power acceleration sensor with Bluetooth Low Energy on ear tags for sow behaviour monitoring. *Biosyst Eng.* (2018) 176:162–71. doi: 10.1016/j.biosystemseng.2018.10.011
66. Von Jasmund N, Wellnitz A, Krommweh MS, Büscher W. Using passive infrared detectors to record group activity and activity in certain focus areas in fattening pigs. *Animals.* (2020) 10:792. doi: 10.3390/ani10050792
67. Ni JQJ, Liu S, Radcliffe JS, Vonderohe C. Evaluation and characterisation of Passive Infrared Detectors to monitor pig activities in an environmental research building. *Biosyst Eng.* (2017) 158:86–94. doi: 10.1016/j.biosystemseng.2017.03.014
68. Besteiro R, Arango T, Rodríguez MR, Fernández MD, Velo R. Estimation of patterns in weaned piglets' activity using spectral analysis. *Biosyst Eng.* (2018) 173:85–92. doi: 10.1016/j.biosystemseng.2017.06.014
69. Ott S, Moons CPHPH, Kashiha MAA, Bahr C, Tuyttens FAMAM, Berckmans D, et al. Automated video analysis of pig activity at pen level highly correlates to human observations of behavioural activities. *Livest Sci.* (2014) 160:132–7. doi: 10.1016/j.livsci.2013.12.011
70. Kashiha MA, Bahr C, Ott S, Moons CPH, Niewold TA, Tuyttens F, et al. Automatic monitoring of pig locomotion using image analysis. *Livest Sci.* (2014) 159:141–8. doi: 10.1016/j.livsci.2013.11.007
71. Matthews SG, Miller AL, Plötz T, Kyriazakis I. Automated tracking to measure behavioural changes in pigs for health and welfare monitoring. *Sci Rep.* (2017) 7:17582. doi: 10.1038/s41598-017-17451-6
72. Zheng C, Zhu X, Yang X, Wang L, Tu S, Xue Y. Automatic recognition of lactating sow postures from depth images by deep learning detector. *Comput Electron Agric.* (2018) 147:51–63. doi: 10.1016/j.compag.2018.01.023
73. Leonard SMM, Xin H, Brown-Brandl TMM, Ramirez BCC. Development and application of an image acquisition system for characterizing sow behaviors in farrowing stalls. *Comput Electron Agric.* (2019) 163:104866. doi: 10.1016/j.compag.2019.104866
74. Lao F, Brown-Brandl T, Stinn JP, Liu K, Teng G, Xin H. Automatic recognition of lactating sow behaviors through depth image processing. *Comput Electron Agric.* (2016) 125:56–62. doi: 10.1016/j.compag.2016.04.026
75. Nasirahmadi A, Edwards SASASA, Matheson SMSM, Sturm B. Using automated image analysis in pig behavioural research: assessment of the influence of enrichment substrate provision on lying behaviour. *Appl Anim Behav Sci.* (2017) 196:30–5. doi: 10.1016/j.applanim.2017.06.015
76. Nasirahmadi A, Richter U, Hensel O, Edwards S, Sturm B. Using machine vision for investigation of changes in pig group lying patterns. *Comput Electron Agric.* (2015) 119:184–90. doi: 10.1016/j.compag.2015.10.023
77. Ringgenberg N, Bergeron R, Devillers N. Validation of accelerometers to automatically record sow postures and stepping behaviour. *Appl Anim Behav Sci.* (2010) 128:37–44. doi: 10.1016/j.applanim.2010.09.018
78. Thompson RJRJ, Matthews S, Plötz T, Kyriazakis I. Freedom to lie: how farrowing environment affects sow lying behaviour assessment using inertial sensors. *Comput Electron Agric.* (2019) 157:549–57. doi: 10.1016/j.compag.2019.01.035
79. Kim J, Chung Y, Choi Y, Sa J, Kim H, Chung Y, et al. Depth-based detection of standing-pigs in moving noise environments. *Sensors.* (2017) 17:2757. doi: 10.3390/s17122757
80. Nasirahmadi A, Hensel O, Edwards SAA, Sturm B. A new approach for categorizing pig lying behaviour based on a Delaunay triangulation method. *Animal.* (2017) 11:131–9. doi: 10.1017/S1751731116001208
81. Cook NJJ, Bench CJJ, Liu T, Chabot B, Schaefer ALL. The automated analysis of clustering behaviour of piglets from thermal images in response to immune challenge by vaccination. *Animal.* (2018) 12:122–33. doi: 10.1017/S1751731117001239
82. Ahrendt P, Gregersen T, Karstoft H. Development of a real-time computer vision system for tracking loose-housed pigs. *Comput Electron Agric.* (2011) 76:169–74. doi: 10.1016/j.compag.2011.01.011
83. Oczak M, Maschat K, Berckmans D, Vranken E, Baumgartner J. Automatic estimation of number of piglets in a pen during farrowing, using image analysis. *Biosyst Eng.* (2016) 151:81–9. doi: 10.1016/j.biosystemseng.2016.08.018
84. Nilsson M, Herlin AHH, Ardö H, Guzhva O, Aström K, Bergsten C. Development of automatic surveillance of animal behaviour and welfare using image analysis and machine learned segmentation technique. *Animal.* (2015) 9:1859–65. doi: 10.1017/S1751731115001342
85. Jiao S, Tiezzi F, Huang Y, Gray KA, Maltecca C. The use of multiple imputation for the accurate measurements of individual feed intake by electronic feeders. *J Anim Sci.* (2016) 94:824–32. doi: 10.2527/jas.2015-9667
86. Maselyne J, Saeys W, Briene P, Mertens K, Vangeyte J, De Ketelaere B, et al. Methods to construct feeding visits from RFID registrations of growing-finishing pigs at the feed trough. *Comput Electron Agric.* (2016) 128:9–19. doi: 10.1016/j.compag.2016.08.010
87. Reiners K, Hegger A, Hessel EFEF, Böck S, Wendl G, Van den Weghe HFAHFA. Application of RFID technology using passive HF transponders for the individual identification of weaned piglets at the feed trough. *Comput Electron Agric.* (2009) 68:178–84. doi: 10.1016/j.compag.2009.05.010
88. Adrion F, Kapun A, Eckert F, Holland EME-M, Staiger M, Götz S, et al. Monitoring trough visits of growing-finishing pigs with UHF-RFID. *Comput Electron Agric.* (2018) 144:144–53. doi: 10.1016/j.compag.2017.11.036
89. da Fonseca FN, Abe JM, de Alencar Nääs I, da Silva Cordeiro AF, do Amaral FV, Ungaro HC. Automatic prediction of stress in piglets (*Sus Scrofa*) using infrared skin temperature. *Comput Electron Agric.* (2020) 168:105148. doi: 10.1016/j.compag.2019.105148
90. da Silva Cordeiro AF, de Alencar Nääs I, Oliveira SRM, Violaro F, de Almeida ACM, Neves DP. Understanding vocalization might help to assess stressful conditions in piglets. *Animals.* (2013) 3:923–34. doi: 10.3390/ani3030923
91. da Silva JP, de Alencar Nääs I, Abe JM, da Silva Cordeiro AF. Classification of piglet (Sus Scrofa) stress conditions using vocalization pattern and applying paraconsistent logic Eτ. *Comput Electron Agric.* (2019) 166:105020. doi: 10.1016/j.compag.2019.105020
92. Moi M, Nääs IA, Caldara FR, Paz ICLA, Garcia RG, Cordeiro AFS. Vocalization data mining for estimating swine stress conditions. *Eng Agric.* (2014) 34:445–50. doi: 10.1590/S0100-69162014000300008
93. Maselyne J, Adriaens I, Huybrechts T, De Ketelaere B, Millet S, Vangeyte J, et al. Measuring the drinking behaviour of individual pigs housed in group using radio frequency identification (RFID). *Animal.* (2016) 10:1557–66. doi: 10.1017/S1751731115000774
94. Kashiha M, Bahr C, Haredasht SA, Ott S, Moons CPH, Niewold TA, et al. The automatic monitoring of pigs water use by cameras. *Comput Electron Agric.* (2013) 90:164–9. doi: 10.1016/j.compag.2012.09.015

95. Zhu W-X, Guo Y-Z, Jiao P-P, Ma C-H, Chen C. Recognition and drinking behaviour analysis of individual pigs based on machine vision. *Livest Sci.* (2017) 205:129–36. doi: 10.1016/j.livsci.2017.09.003
96. Chen C, Zhu W, Steibel J, Siegford J, Han J, Norton T. Classification of drinking and drinker-playing in pigs by a video-based deep learning method. *Biosyst Eng.* (2020) 196:1–14. doi: 10.1016/j.biosystemseng.2020.05.010
97. Zhang Y, Cai J, Xiao D, Li Z, Xiong B. Real-time sow behavior detection based on deep learning. *Comput Electron Agric.* (2019) 163:104884. doi: 10.1016/j.compag.2019.104884
98. Oczak M, Maschat K, Berckmans D, Vranken E, Baumgartner J. Classification of nest-building behaviour in non-crated farrowing sows on the basis of accelerometer data. *Biosyst Eng.* (2015) 140:48–58. doi: 10.1016/j.biosystemseng.2015.09.007
99. Šustr P, Špinka M, Cloutier S, Newberry RC. Computer-aided method for calculating animal configurations during social interactions from two-dimensional coordinates of color-marked body parts. *Behav Res Methods Instruments Comput.* (2001) 33:364–70. doi: 10.3758/BF03195390
100. Chen C, Zhu W, Liu D, Steibel J, Siegford J, Wurtz K, et al. Detection of aggressive behaviours in pigs using a RealSence depth sensor. *Comput Electron Agric.* (2019) 166:105003. doi: 10.1016/j.compag.2019.105003
101. Chen C, Zhu W, Ma C, Guo Y, Huang W, Ruan C. Image motion feature extraction for recognition of aggressive behaviors among group-housed pigs. *Comput Electron Agric.* (2017) 142:380–7. doi: 10.1016/j.compag.2017.09.013
102. Oczak M, Viazzi S, Ismayilova G, Sonoda LTLT, Roulston N, Fels M, et al. Classification of aggressive behaviour in pigs by activity index and multilayer feed forward neural network. *Biosyst Eng.* (2014) 119:89–97. doi: 10.1016/j.biosystemseng.2014.01.005
103. Lee J, Jin L, Park D, Chung Y. Automatic recognition of aggressive behavior in pigs using a kinect depth sensor. *Sensors.* (2016) 16:631. doi: 10.3390/s16050631
104. Viazzi S, Ismayilova G, Oczak M, Sonoda LTT, Fels M, Guarino M, et al. Image feature extraction for classification of aggressive interactions among pigs. *Comput Electron Agric.* (2014) 104:57–62. doi: 10.1016/j.compag.2014.03.010
105. Statham P, Hannuna S, Jones S, Campbell N, Robert Colborne G, Browne WJ, et al. Quantifying defence cascade responses as indicators of pig affect and welfare using computer vision methods. *Sci Rep.* (2020) 10:8933. doi: 10.1038/s41598-020-65954-6
106. Nasirahmadi A, Hensel O, Edwards SA, Sturm B. Automatic detection of mounting behaviours among pigs using image analysis. *Comput Electron Agric.* (2016) 124:295–302. doi: 10.1016/j.compag.2016.04.022
107. Li YZ, Johnston LJLJ, Dawkins MSMS. Utilization of optical flow algorithms to monitor development of tail biting outbreaks in pigs. *Animals.* (2020) 10:323. doi: 10.3390/ani10020323
108. Larsen MLV, Pedersen LJ, Jensen DB. Prediction of tail biting events in finisher pigs from automatically recorded sensor data. *Animals.* (2019) 9:458. doi: 10.3390/ani9070458
109. Conte S, Bergeron R, Gonyou H, Brown J, Rioja-Lang FC, Connor L, et al. Measure and characterization of lameness in gestating sows using force plate, kinematic, and accelerometer methods. *J Anim Sci.* (2014) 92:5693–703. doi: 10.2527/jas.2014-7865
110. Mohling CMM, Johnson AKK, Coetzee JFF, Karriker LAA, Abell CEE, Millman STT, et al. Kinematics as objective tools to evaluate lameness phases in multiparous sows. *Livest Sci.* (2014) 165:120–8. doi: 10.1016/j.livsci.2014.04.031
111. Sun G, Fitzgerald RF, Stalder KJ, Karriker LA, Johnson AK, Hoff SJ. Development of an embedded microcomputer-based force plate system for measuring sow weight distribution and detection of lameness. *Appl Eng Agric.* (2011) 27:475–82. doi: 10.13031/2013.37063
112. Grégoire J, Bergeron R, D'Allaire S, Meunier-Salaün M-CC, Devillers N. Assessment of lameness in sows using gait, footprints, postural behaviour and foot lesion analysis. *Animal.* (2013) 7:1163–73. doi: 10.1017/S1751731113000098
113. Stavrakakis S, Li W, Guy JH, Morgan G, Ushaw G, Johnson GR, et al. Validity of the Microsoft Kinect sensor for assessment of normal walking patterns in pigs. *Comput Electron Agric.* (2015) 117:1–7. doi: 10.1016/j.compag.2015.07.003
114. Chedad A, Moshou D, Aerts JMM, Van Hirtum A, Ramon H, Berckmans D. Recognition system for pig cough based on probabilistic neural networks. *J Agric Eng Res.* (2001) 79:449–57. doi: 10.1006/jaer.2001.0719
115. Zhao J, Li X, Liu W, Gao Y, Lei M, Tan H, et al. Dnn-hmm based acoustic model for continuous pig cough sound recognition. *Int J Agric Biol Eng.* (2020) 13:186–93. doi: 10.25165/j.ijabe.20201303.4530
116. Buayai P, Piewthongngam K, Leung CK, Saikaew KR. Semi-automatic pig weight estimation using digital image analysis. *Appl Eng Agric.* (2019) 35:521–34. doi: 10.13031/aea.13084
117. Fernandes AFA, Dórea JRR, Fitzgerald R, Herring W, Rosa GJM. A novel automated system to acquire biometric and morphological measurements and predict body weight of pigs via 3D computer vision. *J Anim Sci.* (2019) 97:496–508. doi: 10.1093/jas/sky418
118. Kashiha M, Bahr C, Ott S, Moons CPH, Niewold TA, Ödberg FO, Berckmans D. Automatic weight estimation of individual pigs using image analysis. *Comput Electron Agric.* (2014) 107:38–44. doi: 10.1016/j.compag.2014.06.003
119. Wongsriworaphon A, Arnonkijpanich B, Pathumnakul S. An approach based on digital image analysis to estimate the live weights of pigs in farm environments. *Comput Electron Agric.* (2015) 115:26–33. doi: 10.1016/j.compag.2015.05.004
120. Condotta ICFSICFS, Brown-Brandl TMTM, Silva-Miranda KOKO, Stinn JPJP. Evaluation of a depth sensor for mass estimation of growing and finishing pigs. *Biosyst Eng.* (2018) 173:11–8. doi: 10.1016/j.biosystemseng.2018.03.002
121. Jun K, Kim SJ, Ji HW. Estimating pig weights from images without constraint on posture and illumination. *Comput Electron Agric.* (2018) 153:169–76. doi: 10.1016/j.compag.2018.08.006
122. Wang K, Guo H, Ma Q, Su W, Chen L, Zhu D. A portable and automatic Xtion-based measurement system for pig body size. *Comput Electron Agric.* (2018) 148:291–8. doi: 10.1016/j.compag.2018.03.018
123. Schinckel AP, Wastell M, Einstein ME, Hubbs JT, Preckel PV. Evaluation of alternative methods to analyze pig body weight data from animal sorting technologies. *Prof Anim Sci.* (2009) 25:654–62. doi: 10.15232/S1080-7446(15)30774-9
124. Alsahaf A, Azzopardi G, Ducro B, Hanenberg E, Veerkamp RF, Petkov N. Estimation of muscle scores of live pigs using a kinect camera. *IEEE Access.* (2019) 7:52238–45. doi: 10.1109/ACCESS.2019.2910986
125. Lu M, He J, Chen C, Okinda C, Shen M, Liu L, et al. An automatic ear base temperature extraction method for top view piglet thermal image. *Comput Electron Agric.* (2018) 155:339–47. doi: 10.1016/j.compag.2018.10.030
126. Feng Y-Z, Zhao H-T, Jia G-F, Ojukwu C, Tan H-Q. Establishment of validated models for non-invasive prediction of rectal temperature of sows using infrared thermography and chemometrics. *Int J Biometeorol.* (2019) 63:1405–15. doi: 10.1007/s00484-019-01758-2
127. Cordeiro AFDS, Nääs IDA, Oliveira SRDM, Violaro F, De Almeida ACM. Efficiency of distinct data mining algorithms for classifying stress level in piglets from their vocalization. *Eng Agric.* (2012) 32:208–16. doi: 10.1590/S0100-69162012000200001
128. Vandermeulen J, Bahr C, Tullo E, Fontana I, Ott S, Kashiha M, et al. Discerning pig screams in production environments. *PLoS ONE.* (2015) 10:e0123111. doi: 10.1371/journal.pone.0123111
129. Cordeiro AFDS, Nääs IDA, da Silva Leitão F, de Almeida ACM, de Moura DJ. Use of vocalisation to identify sex, age, and distress in pig production. *Biosyst Eng.* (2018) 173:57–63. doi: 10.1016/j.biosystemseng.2018.03.007
130. Moura DJ, Silva WT, Naas IA, Tolón YA, Lima KAO, Vale MM. Real time computer stress monitoring of piglets using vocalization analysis. *Comput Electron Agric.* (2008) 64:11–8. doi: 10.1016/j.compag.2008.05.008
131. Scheel C, Traulsen I, Auer W, Müller K, Stamer E, Krieter J. Detecting lameness in sows from ear tag-sampled acceleration data using wavelets. *Animal.* (2017) 11:2076–83. doi: 10.1017/S1751731117000726
132. Amezcua R, Walsh S, Luimes PH, Friendship RM. Infrared thermography to evaluate lameness in pregnant sows. *Can Vet J.* (2014) 55:268–72. doi: 10.13140/RG.2.2.11670.80968
133. Bowman ASS, Nolting JMM, Workman JDD, Cooper M, Fisher AEE, Marsh B, et al. The inability to screen exhibition swine for influenza A virus using body temperature. *Zoonoses Public Health.* (2016) 63:34–9. doi: 10.1111/zph.12201

134. Menzel A, Beyerbach M, Siewert C, Gundlach M, Hoeltig D, Graage R, et al. Actinobacillus pleuropneumoniae challenge in swine: diagnostic of lung alterations by infrared thermography. *BMC Vet Res.* (2014) 10:199. doi: 10.1186/s12917-014-0199-2
135. Silva M, Ferrari S, Costa A, Aerts J-MM, Guarino M, Berckmans D. Cough localization for the detection of respiratory diseases in pig houses. *Comput Electron Agric.* (2008) 64:286–92. doi: 10.1016/j.compag.2008.05.024
136. Exadaktylos V, Silva M, Ferrari S, Guarino M, Taylor CJ, Aerts J-M, et al. Time-series analysis for online recognition and localization of sick pig (*Sus scrofa*) cough sounds. *J Acoust Soc Am.* (2009) 124:3803–9. doi: 10.1121/1.2998780
137. Maselyne J, Van Nuffel A, Briene P, Vangeyte J, De Ketelaere B, Millet S, et al. Online warning systems for individual fattening pigs based on their feeding pattern. *Biosyst Eng.* (2018) 173:143–56. doi: 10.1016/j.biosystemseng.2017.08.006
138. Manteuffel C, Hartung E, Schmidt M, Hoffmann G, Schön PC. Validation of individual parturition indicators for sows based on light barriers. in *Precision Livestock Farming 2015 - Papers Presented at the 7th European Conference on Precision Livestock Farming, ECPLF 2015*. Milan, (2015). p. 605–13.
139. Manteuffel C, Hartung E, Schmidt M, Hoffmann G, Schön PCPC. Towards qualitative and quantitative prediction and detection of parturition onset in sows using light barriers. *Comput Electron Agric.* (2015) 116:201–10. doi: 10.1016/j.compag.2015.06.017
140. Manteuffel C. Parturition detection in sows as test case for measuring activity behaviour in farm animals by means of radar sensors. *Biosyst Eng.* (2019) 184:200–6. doi: 10.1016/j.biosystemseng.2019.06.018
141. Oczak M, Maschat K, Baumgartner J. Dynamics of sows' activity housed in farrowing pens with possibility of temporary crating might indicate the time when sows should be confined in a crate before the onset of farrowing. *Animals.* (2020) 10:6. doi: 10.3390/ani10010006
142. Marsot M, Mei J, Shan X, Ye L, Feng P, Yan X, et al. An adaptive pig face recognition approach using Convolutional Neural Networks. *Comput Electron Agric.* (2020) 173:105625. doi: 10.1016/j.compag.2020.105386
143. Hansen MFMF, Smith ML, Smith LN, Salter MGMG, Baxter EM, Farish M, et al. Towards on-farm pig face recognition using convolutional neural networks. *Comput Ind.* (2018) 98:145–52. doi: 10.1016/j.compind.2018.02.016
144. Jensen DB, Larsen MLV, Pedersen LJ. Predicting pen fouling in fattening pigs from pig position. *Livest Sci.* (2020) 231:103852. doi: 10.1016/j.livsci.2019.103852
145. Stygar AH, Kristensen AR. Detecting abnormalities in pigs' growth – a dynamic linear model with diurnal growth pattern for identified and unidentified pigs. *Comput Electron Agric.* (2018) 155:180–9. doi: 10.1016/j.compag.2018.10.004
146. Dominiak KN, Hindsborg J, Pedersen LJ, Kristensen AR. Spatial modeling of pigs' drinking patterns as an alarm reducing method II. Application of a multivariate dynamic linear model. *Comput Electron Agric.* (2019) 161:92–103. doi: 10.1016/j.compag.2018.10.037
147. Jensen DB, Kristensen AR. Temperature as a predictor of fouling and diarrhea in slaughter pigs. *Livest Sci.* (2016) 183:1–3. doi: 10.1016/j.livsci.2015.11.007
148. Wurtz K, Camerlink I, D'Eath RB, Fernández AP, Norton T, Steibel J, et al. Recording behaviour of indoor-housed farm animals automatically using machine vision technology: a systematic review. *PLoS ONE*. (2019) 4:e0226669. doi: 10.1371/journal.pone.0226669
149. ICAR. *The Global Standard for Livestock Data. Section 11 - Guidelines for Testing, Approval and Checking of Milk Recording Devices*. Available online at: https://www.icar.org/Guidelines/11-Milk-Recording-Devices.pdf (2020).
150. Schrøder-Petersen DL, Simonsen HB. Tail biting in pigs. *Vet J.* (2001) 162:196–210. doi: 10.1053/tvjl.2001.0605
151. Jensen TB, Kristensen HH, Toft N. Quantifying the impact of lameness on welfare and profitability of finisher pigs using expert opinions. *Livest Sci.* (2012) 149:209–14. doi: 10.1016/j.livsci.2012.07.013
152. Heinonen M, Peltoniemi O, Valros A. Impact of lameness and claw lesions in sows on welfare, health and production. *Livest Sci.* (2013) 156:2–9. doi: 10.1016/j.livsci.2013.06.002
153. Pluym LMLM, Maes D, Vangeyte J, Mertens K, Baert J, Van Weyenberg S, et al. Development of a system for automatic measurements of force and visual stance variables for objective lameness detection in sows: SowSIS. *Biosyst Eng.* (2013) 116:64–74. doi: 10.1016/j.biosystemseng.2013.06.009
154. Day JEL, Kyriazakis I, Lawrence AB. An investigation into the causation of chewing behaviour in growing pigs: the role of exploration and feeding motivation. *Appl Anim Behav Sci.* (1996) 48:47–59. doi: 10.1016/0168-1591(95)01022-X
155. Will MK, Büttner K, Kaufholz T, Müller-Graf C, Selhorst T, Krieter J. Accuracy of a real-time location system in static positions under practical conditions: prospects to track group-housed sows. *Comput Electron Agric.* (2017) 142:473–84. doi: 10.1016/j.compag.2017.09.020
156. Maselyne J, Saeys W, Ketelaere B De, Briene P. How do fattening pigs spend their day? In: *Proceedings of the 6th International Conference on the Assessment of Animal Welfare at Farm and Group Level*. Clermont-Ferrand (2014). p. 157. doi: 10.3921/978-90-8686-798-1
157. Dewulf J, Koenen F, Laevens H, Kruif A. Infrared thermometry is not suitable for the detection of fever in pigs. *Vlaams Diergeneeskd Tijdschr.* (2003) 72:373–9.
158. Patelli N, Mandrioli M. Blockchain technology and traceability in the agrifood industry. *J Food Sci*. (2020) 85:3670–8. doi: 10.1111/1750-3841.15477
159. Kamilaris A, Fonts A, Boldv́ FXP. The rise of blockchain technology in agriculture and food supply chains. *Trends Food Sci Technol.* (2019) 91:640–52. doi: 10.1016/j.tifs.2019.07.034
160. Shahbandeh M. *Number of Pigs Worldwide From 2012 to 2020*. Statista (2020). Available online at: https://www.statista.com/statistics/263963/number-of-pigs-worldwide-since-1990/ (accessed December 4, 2020).
161. Shahbandeh M. *Number of Milk Cows Worldwide in 2019, by Country*. Statista (2020). Available online at: https://www.statista.com/statistics/869885/global-number-milk-cows-by-country/ (accessed December 4, 2020).
162. *Cost-Effectiveness Assesment of Improving Animal Welfare Standards in European Agriculture*. (2004). p. 4. Available online at: http://www.ganaderia.gob.do/index.php/component/k2/bibitemlist/category/146-articulos-tecnicos
163. Majewski E, Hamulczuk M, Malak-rawlikowska A, Gębska M, Harvey D. Cost-effectiveness assessment of improving animal welfare standards in the European Agriculture. In: *Selected Paper prepared for presentation at the International Association of Agricultural Economists (IAAE) Triennial Conference*. Foz do Iguaçu (2012). p. 22. doi: 10.22004/ag.econ.126741
164. Stygar AH, Dolecheck KA, Kristensen AR. Analyses of body weight patterns in growing pigs: a new view on body weight in pigs for frequent monitoring. *Animal.* (2018) 12:295–302. doi: 10.1017/S1751731117001690

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Using Expert Elicitation to Abridge the Welfare Quality® Protocol for Monitoring the Most Adverse Dairy Cattle Welfare Impairments

Frank A. M. Tuyttens[1,2], Sophie de Graaf[1,3], Sine Norlander Andreasen[4], Alice de Boyer des Roches[5], Frank J. C. M. van Eerdenburg[6], Marie J. Haskell[7], Marlene K. Kirchner[8], Luc. Mounier[5], Miroslav Kjosevski[9], Jo Bijttebier[1], Ludwig Lauwers[1,3], Wim Verbeke[3] and Bart Ampe[1]*

[1] Animal Sciences Unit, Institute for Agricultural and Fisheries Research (ILVO), Merelbeke, Belgium, [2] Department of Nutrition, Genetics and Ethology, Faculty of Veterinary Medicine, Ghent University, Merelbeke, Belgium, [3] Department of Agricultural Economics, Ghent University, Ghent, Belgium, [4] Department of Veterinary and Animal Sciences, University of Copenhagen, Frederiksberg, Denmark, [5] Université Clermont Auvergne, INRAE, VetAgro Sup, UMR Herbivores, Saint-Genès-Champanelle, France, [6] Department of Veterinary and Animal Sciences, Section of Animal Welfare and Disease Control, University of Copenhagen, Frederiksberg, Denmark, [7] Scotland's Rural College, Department of Population Health Sciences, Section Farm Animal Health, Faculty of Veterinary Medicine, Utrecht University, Utrecht, Netherlands, [8] Animal Behavior and Welfare, Animal and Veterinary Sciences, SRUC, Edinburgh, United Kingdom, [9] Animal Welfare Center, Faculty of Veterinary Medicine, Ss. Cyril and Methodius University in Skopje, Skopje, North Macedonia

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Edited by:
Edward Narayan,
The University of Queensland, Australia

Reviewed by:
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Pol Llonch,
Universitat Autònoma de Barcelona, Spain

***Correspondence:**
Frank A. M. Tuyttens
frank.tuyttens@ilvo.vlaanderen.be

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The Welfare Quality® consortium has developed and proposed standard protocols for monitoring farm animal welfare. The uptake of the dairy cattle protocol has been below expectation, however, and it has been criticized for the variable quality of the welfare measures and for a limited number of measures having a disproportionally large effect on the integrated welfare categorization. Aiming for a wide uptake by the milk industry, we revised and simplified the Welfare Quality® protocol into a user-friendly tool for cost- and time-efficient on-farm monitoring of dairy cattle welfare with a minimal number of key animal-based measures that are aggregated into a continuous (and thus discriminative) welfare index (WI). The inevitable subjective decisions were based upon expert opinion, as considerable expertise about cattle welfare issues and about the interpretation, importance, and validity of the welfare measures was deemed essential. The WI is calculated as the sum of the severity score (i.e., how severely a welfare problem affects cow welfare) multiplied with the herd prevalence for each measure. The selection of measures (lameness, leanness, mortality, hairless patches, lesions/swellings, somatic cell count) and their severity scores were based on expert surveys (14–17 trained users of the Welfare Quality® cattle protocol). The prevalence of these welfare measures was assessed in 491 European herds. Experts allocated a welfare score (from 0 to 100) to 12 focus herds for which the prevalence of each welfare measure was benchmarked against all 491 herds. Quadratic models indicated a high correspondence between these subjective scores and the WI ($R^2 = 0.91$). The WI allows both numerical (0–100) as a qualitative ("not classified" to "excellent") evaluation of welfare. Although it is sensitive to those welfare issues that most adversely affect cattle welfare (as identified by EFSA), the WI should be accompanied with a disclaimer that lists adverse or favorable effects that cannot be detected adequately by the current selection of measures.

Keywords: animal welfare, dairy cattle, integration, welfare assessment, compensation, aggregation, index

INTRODUCTION

A tool to correctly assess and monitor animal welfare is key to many initiatives to improve the welfare of livestock (1). Obviously, the characteristics of this monitoring tool depend on how it is to be applied. For example, the tool may be very elaborate, refined, high tech, and comprehensive if it is to be used in experimental animal welfare research or for in-depth assessments of a limited number of focal herds by a multidisciplinary team of highly trained specialists. The focus of the current study, however, is on a tool that is to be taken up widely by the food industry at large (e.g., for an animal welfare label on food products). For this type of application, the logistic feasibility, the costs, and the user-friendliness are major constraints. At the same time, as socioeconomic stakes can be high, decisions about the animal welfare status allocated to herds or food products ought to be transparent, non-disputable, and accepted as valid by the main stakeholders (e.g., farmer, auditor, retailer, consumer).

Balancing these logistic and scientific requirements is a huge challenge. As a multidimensional societal concept, the number of ways that the welfare of livestock can be affected positively or negatively, and how these effects can be assessed, is very diverse and almost endless. The scientific ambition to accurately document any small change in the status of any of these multiple animal welfare aspects is poorly compatible with the industry demand that the tool is cost efficient and easy to implement. Hence, choices will need to be made about which aspects of welfare to include and about the resolution by which these will be documented. These choices will be subjective to some degree because the conception of animal welfare is partly values based, and people differ in what they consider important or desirable for animals to have a good life (2).

Another characteristic of the monitoring tool that depends on the intended application concerns the need to aggregate the information from the individual welfare measures into an integrated, balanced overall welfare index (WI). Such aggregation may be redundant in case the tool is used to provide farm-specific feedback on how certain welfare problems in a herd could be addressed. However, it is essential for the purpose of the tool developed in this study, namely, to inform consumers about the general welfare status of the animals from which food is derived (1). In fact, aggregating data from various welfare measures into a WI reflecting the overall welfare status of the herd is one of the most difficult challenges in animal welfare science (3). As there is no "gold standard" for overall herd welfare, aggregating data on various welfare measures into an overall index again requires some degree of subjectivity (4).

Standardized methodologies for assessing the welfare of various categories of farm animals, including broiler chickens, laying hens, growing pigs, sows, veal calves, and dairy cattle, were developed in the European Welfare Quality® (WQ) project (5). The WQ protocols have been praised for being very comprehensive and for the implementation of a hierarchical approach to integrate data on a multitude of predominantly animal-based welfare measures enabling the assignment of farms or herds to one of the four overall welfare categories (not classified, acceptable, enhanced, and excellent). Although issues about consistency over time (6–9) and about reliance on complete and standardized farm/slaughterhouse records (10–12) have been raised, the WQ protocols have been criticized mainly with regard to the (i) the feasibility [mainly labor costs per farm, e.g., (11, 13)], (ii) the variable quality of the welfare measures included in the protocol (8, 10, 14), and (iii) the way these measures are aggregated into an overall WI (15–21). Indeed, uptake of the WQ protocols by the authorities and food industry at large for improving and better marketing of farm animal welfare has been below expectation. Although stakeholders have expressed interest in welfare monitoring of various types of farm animals, they have emphasized that the labor demand of about one farm or herd per day per certified assessor needs to be reduced. de Jong et al. (11) have addressed these industry concerns by proposing time-saving simplifications to the WQ broiler chicken protocol but—to our knowledge—no such modifications have been shown promising for the other protocols. This is particularly needed for the dairy cattle protocol as it takes up to 4.4–7.7 h to complete for a herd of 25–200 cows, respectively, excluding the time needed for making the appointment and for travel (22).

Criticisms on welfare measures often relate to their poor reliability, validity, or feasibility (10, 11, 13, 14). There is a growing consensus now that animal-based measures are preferred for directly assessing the outcome of the complex effects of the environment and management on the animal's actual state of welfare (1, 23, 24). Although one of the novel characteristics of the WQ protocols was the emphasis on animal-based measures, the WQ protocols also include resource- or management-based measures that have been criticized for describing the potential or risk for good or bad welfare rather than directly measuring the welfare status itself. The dairy cattle protocol, for example, relies on resource-based measures for assessing 3 of the 12 welfare criteria (water availability and cleanliness for the criterion absence of prolonged thirst, tethering for the criterion ease of movement, and pasture access for the criterion expression of other behaviors). It is particularly worrying that sensitivity analyses have revealed that a limited number of (often resource-based) measures seem to have a disproportionally large effect on the overall welfare categorization [e.g., 88% of the overall dairy cattle welfare categorization is predicted by water availability and cleanliness (17)], whereas some key (often animal-based) measures such as lameness and mortality have a negligible effect (16–18, 21). This appears to be an unwanted side effect of the very complex and hard-to-understand (and hence poorly transparent to most end-users) integration method, which was needed to aggregate so many measures of different scales with different thresholds.

Aiming for a wide uptake by the milk industry, in the current study, we revised and simplified the WQ dairy cattle protocol with a view to (i) drastically reduce the time needed to complete an assessment, (ii) make use of a minimal number of key animal-based measures, and (iii) transparently aggregate these measures into a continuous (and thus discriminative) WI. We describe and illustrate the steps in the development of this revised and simplified protocol for quantifying the level of herd welfare, albeit without claiming to be exhaustive. The WI is based upon the intuitively sensible method of Burow et al. (25) in which the relative weight of each welfare measure depends on its severity score (expert judgement of how severely a given welfare problem affects the welfare of an individual cow) multiplied by the herd prevalence for that measure. Moreover, we investigate the extent to which the integration method should allow compensation of poor scores with better scores. In some studies (4), it is argued that such compensation should be restrained, as good results on one aspect cannot compensate for poor scores on other aspects (e.g., having a good body condition score cannot compensate for being severely lame). Other studies, however, indicate that compensation between welfare aspects may be possible [reviewed by Leknes and Tracey (26)]. At present, there is little evidence that compensation reduction is warranted, let alone what type of compensation-reduction method best corresponds with expert opinion. The latter is examined in one of the proposed steps in this study. Some of the steps inevitably demand subjective decisions. These were based upon expert (defined as an animal scientist trained to use the WQ dairy cattle protocol) opinion, as considerable expertise about cattle welfare issues and about the interpretation, importance, and validity of the welfare measures was deemed essential. For this study we opted not to involve people without in-depth knowledge and expertise in dairy cattle welfare and the measures involved because of doubts about their ability to adequately balance the importance of different welfare measures. Indeed, the relative importance that ought to be allocated to a given welfare measure could depend on how exactly it is measured on-farm (e.g., selection of and size of the sample, to what extent confounding factors may influence the measures, objectivity of the measure). Moreover, it has been shown that detailed information on how data on welfare measures is collected on-farm can significantly influence the relative weights they are given by experts (27). Even for dairy cattle welfare experts, it can be a daunting task to make decisions about overall welfare status by integrating the scores of the various measures in such a way that the outcome reflects the range of what can be expected among real farms and allows realistic differentiation between these farms. Expert welfare scoring of herds was, therefore, based on a large database of WQ data that reflect a wide range of dairy herd types in Europe and thereby ensuring a substantial but realistic spread in observed values.

MATERIALS AND METHODS

Our approach to revise and simplify the WQ dairy cattle protocol involved five steps. The same steps can be used to revise and simplify the other WQ protocols or to add additional welfare measures if this would be deemed desirable. The first four steps inevitably require subjective decisions for which experts with knowledge of the WQ dairy cattle protocol were consulted. We emailed 31 researchers who were known to the authors, to our network, or to the Welfare Quality Network to have been trained to use the WQ dairy cattle protocol. These trained users were in turn asked to provide contact details of any additional animal welfare scientists who would be suitable (i.e., trained to use the WQ protocol). Fourteen declined the invitation to participate because they could not fill out the survey in time or did not respond. All experts who agreed to participate in the current study had experience with the WQ protocol for dairy cattle (i.e., were trained to perform the WQ protocol for dairy cattle and had used it to assess the welfare of dairy herds), were animal scientists, and had authored at least one peer-reviewed scientific paper about dairy cattle welfare involving the WQ protocol. Although we did not select for this, all participating experts were from Europe (the WQ protocols are used predominantly in Europe), and a total of eight nationalities were represented (British, Spanish, Macedonian, Dutch, Finnish, Austrian, German, and French). No experts whose input was used in the analyses were involved in creating the surveys.

Step 1 entails selecting animal-based welfare measures to be included in the protocol. At the core of Steps 2 and 3 is the WI. Based upon Burow et al. (25), the WI was constructed from perceived severity of welfare problems ("severity score") and observed prevalence of these welfare problems. The severity scores for the various welfare measures were determined in Step 2 by asking the experts to score how severely each of the selected welfare problems (that are quantified by the selected measures) impairs the welfare of an animal. The following formula forms the basis to integrate data on selected welfare measures into one score:

$$Welfare\ index\ score = \frac{1}{nm} \times \sum_{m=1}^{nm} Sm \times rPm$$

Here, n represents "number," m refers to "measure," S represents the "severity score," which ranged from 0 to 100, and rP refers to "relative prevalence," which is calculated as prevalence per herd/prevalence at 97.5th percentile of that measure among all herds in the EU database. In the proposed formula, rP rather than absolute prevalence was used so each herd covered the same possible spectrum for each measure. Prevalence of the 97.5th percentile was set as the maximum for each measure score, to prevent an extreme prevalence value of single measures from having a disproportionately large influence on the score. Therefore, herds with values equal to or higher than the 97.5th percentile were automatically given the maximum measure score. This allowed for a uniform method to determine thresholds for the different compensation-reduction methods (CRMs) that were tested. To achieve a score on a scale of 0 (very poor welfare)−100 (excellent welfare) and to test various CRMs, the formula was complemented as follows:

$$Welfare\ index\ score = \mathbf{100} - \frac{100}{Smax} \times \sum_{m=1}^{nm} Sm \times rPm \times \mathbf{Cm}$$

Here, Cm is the "compensation-reduction factor" for measure m (value between 1 and Cmax), and Smax is the sum of the products of Sm and the maximal compensation-reduction factor ($Smax = \sum_{m=1}^{nm} Sm \times Cmax$). To gain input for this formula, we performed two independent online surveys among

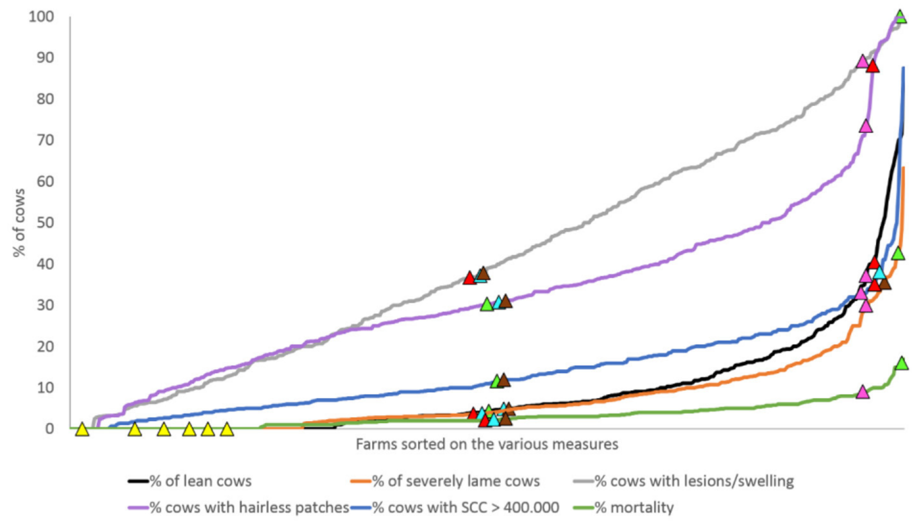

FIGURE 1 | One of the graphs presented to experts in the second survey showing the distribution of all herds in the database ($n = 491$) for the six selected measures. Colored triangles mark six (of the 12) focus herds.

the dairy cattle welfare experts. In Step 3, the WI is calculated, and correspondence with expert opinion is analyzed. Similarity between experts' welfare scores for several fictitious herds and integrated WI using the aforementioned formula with various CRMs is analyzed. Step 4 consists of interpreting the WI (what score indicates poor/good welfare). Step 5 comprises of checking to what degree the selected welfare measures are associated with factors that have the most severe impact on dairy cattle welfare. The five steps are elaborated below.

Step 1: Selecting Welfare Measures

Welfare measures were selected from the WQ protocol for dairy cattle (22). We used three criteria for selecting measures: (1) they ought to be animal-based, (2) it must be possible to express them as a percentage to allow using the proposed WI-formula, and (3) they must be considered as important for dairy cattle welfare by the experts. The importance of the measures was based upon an online survey where 17 experts ranked all WQ measures ($n = 27$) on importance for the overall welfare status of a herd of dairy cattle. Although the experts were presumed familiar with each of these measures, the precise methodology could be consulted in the WQ protocol for the assessment of dairy cow welfare (www.welfarequalitynetwork.net). It was mentioned to the experts that for ranking (*inter alia*) reliability, validity, perceived relevance, and prevalence may be considered. Subsequently, we compared compliance of these selected measures with the outcomes of published studies in which expert opinion had been used as well to rank cattle welfare measures on importance (25, 28–30). Hence, in theory, measures could have been added in the case that the literature search would have revealed important animal-based measures that had not passed our initial selection (but this was not the case in our study).

Step 2: Determining Severity Scores

To determine the severity scores for the selected measures, 14 of the same aforementioned 17 experts completed a second survey. In this second survey, they were asked to score how severely the welfare of an individual cow is affected by each of the six selected welfare impairments on a scale of 0 (totally not severe)—100 (extremely severe). The experts were informed that they may take (their perception of) both the degree and duration of suffering into account. In the ensuing Step 3, median severity scores were used in calculating the WI.

Step 3: Calculating WI and Testing Coherence With Expert Opinion

For checking correspondence between expert scores and aggregated WIs, in the subsequent part of the second survey, the 14 selected experts were presented with a graph showing the observed prevalence distribution of all selected welfare measures for 491 European herds that had been assessed using the WQ protocol (**Figure 1**). To reflect the current range present in Europe across various herding systems, existing WQ datasets were collated from seven European research institutes and included data from 10 countries [Macedonia, The Netherlands, France, Belgium, Scotland, Denmark, Romania, Northern Ireland, Spain, and Austria, more details in de Graaf et al. (20)]. In the graph, six "focus herds" were highlighted per expert (example: **Figure 1**; data shown in **Table 1**). These focus herds were fictitious but were based upon real herd data from the European dataset. In total, 12 focus herds were created to fit the following descriptions: (1) two herds that scored high in prevalence, taking the European dataset as a reference (indicating poor welfare) on all measures; (2) two herds that scored low (indicating good welfare) on all measures; (3) two herds that scored medium on one-half of the measures and high on the other half; (4) two herds that scored the other half of the measures medium and the other half high; (5) two herds that scored medium on all measures except for one (high for somatic cell count > 400,000), and (6) two herds that scored medium on all measures but high for one (high for severe lameness). High scoring measures in

TABLE 1 | Prevalences for the 6 selected dairy cattle welfare measures, for each of the 12 fictitious herds the experts ($n = 14$) allocated an integrated index score.

Herd	Measure scores	Very lean	Severely lame	Lesions and swellings	Hairless patches	SCC > 400,000	Mortality
1	All low[a]	0	0	3	3	0	0
2	All low[a]	0	0	0	0	0	0
3	All high[a]	46	33	92	92	38	10
4	All high[a]	37	30	90	74	33	9
5	Medium/high[a,b]	50	4	37	94	41	2
6	Medium/high[a,b]	41	4	37	88	35	2
7	High/medium[a,c]	4	32	92	30	11	10
8	High/medium[a,c]	4	44	100	30	11	16
9	Medium, high SCC[a]	4	4	37	30	35	2
10	Medium, high SCC[a]	4	4	37	30	38	2
11	Medium, high lameness[a]	5	34	39	30	11	2
12	Medium, high lameness[a]	4	33	37	30	11	2

[a] *Highest scores belonged to the top 5% of herds in the European dataset (n = 491), medium between 40 and 60%, and lowest scores were from the lowest 5% of herds.*
[b] *"% of too lean cows," "somatic cell count (SCC) > 400,000," and "nHP" were high.*
[c] *"% of cows with lesions," "% of cows with severe lameness," and "% of mortality" were high.*

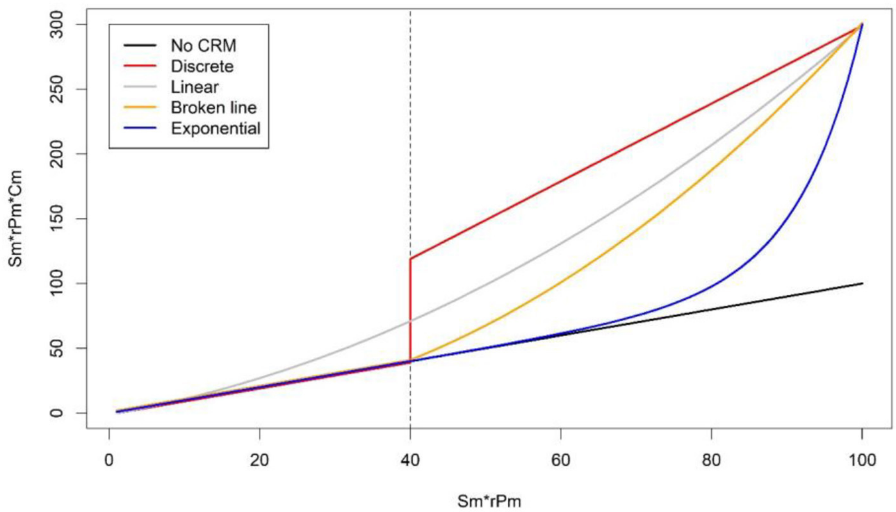

FIGURE 2 | Illustration of the compensation reduction methods (except Veto) tested in this study with a maximal compensation of 3 and a threshold of 40. No compensation reduction method (CRM, black line) results in the diagonal (value before and after compensation is the same). Discrete gives no compensation reduction for measures up to a certain threshold of Sm*rPm, above which the Sm*rPm score is multiplied with maximum fixed value Cm. For linear CRM, Cm increases linearly with an increasing Sm*rPm score of the welfare measures. The broken line CRM gives no compensation reduction for measures up to a certain threshold of Sm*rPm, above which Cm increases in a linear manner. Exponential CRM increases Cm exponentially with an increasing Sm*rPm score of the welfare measures.

the latter two mentioned herds were chosen randomly from the selected measures. Highest prevalence belonged to the top 5% for all welfare measures, medium between 40 and 60%, and lowest scores were from the lowest 5%. Each expert was presented with six focus herds, one of the two for each category (**Table 1**). Experts were asked to allocate a welfare score to each focus herd they were presented with using a tagged visual analog scale from 0 to 100. Tags were "Not Classified (<20)," "Acceptable (20–55)," "Enhanced (55–80)," and "Excellent (>80)," following WQ categorization (22). Each of the 12 focus herds was thus scored by six to eight experts. Subsequently, the degree of correspondence between expert scores and WI's were calculated with varying CRMs. One of the tested CRMs was "veto," where thresholds are defined for each measure above which a value cannot be compensated for. This is achieved by automatically attributing the worst possible welfare score to a herd, independent of the prevalence of other welfare problems. The other tested CRMs use various formulas to allocate increasingly more weight to worse scores on a certain measure. Tested formula in the current study were "Discrete," "Linear," "Broken line," and "Exponential" and are illustrated in **Figure 2**. In addition, scores were calculated without CRM ("no CRM"), thus allowing full compensation between measures as default.

For discrete, broken line, and veto CRM, a threshold at which compensation reduction starts needed to be determined. For all CRMs apart from veto, it also had to be determined what the maximum level of compensation reduction (Cmax) was. We checked which threshold value of S*rP (ranging between 5 and 70 in increments of 5) and which value for Cmax (set at between 1.5, 2, 3, 5, and 10) corresponded best with expert opinion based on model R^2. For the 20 models with the highest R^2, we calculated also the Akaike information criterion (AIC) and four additional metrics [root mean square error (RMSE), mean absolute difference, Liao's improved concordance correlation coefficient [ICCC, (31), and the Bland–Altman 95% limits of agreement [LOA, (32)] for quantifying the agreement between the model prediction and the experts' opinion. We ranked these 20 models according to the six agreement metrics and calculated the mean rank (giving equal weight to each of the six metrics). The model with the lowest mean rank was selected as the model (i.e., type of CRM) that provided the best fit with the opinion of the experts.

Statistical analyses were performed using the program R 3.2.2 (R Foundation for Statistical Computing, Vienna, Austria). Both linear and quadratic models were used to test correspondence between expert scoring and the integrated scores to determine if adding a CRM to the WI formula generated a better fit for varying thresholds and values of C. The Agreement Interval package was used to calculate the measures of agreement.

Step 4: Interpreting the WI

To interpret the WI scores in terms of bad/medium/good welfare, we asked the experts to score overall welfare for the 12 focus herds on a tagged visual analog scale with labels for four welfare categories following WQ categorization ("not classified" from 0 to 20, "acceptable" from 20 to 55, "enhanced" from 55 to 80, and "excellent" from 80 to 100). To extrapolate thresholds of these welfare categories, we (scatter) plotted the expert scores against the WI scores for the 12 fictitious herds and added the best fitting curve. We then identified the three points where the best-fitting curve intersects with the WQ thresholds of the scale on which the experts scored (expert scores 20, 55, and 80).

Step 5: Exhaustiveness Check

In Step 5, we assessed to what degree the selected measures are indicative of the "worst adverse effects" (factors that have the most severe impact) on dairy cattle welfare. For this end, we compared the selection of welfare measures with a list of worst adverse effects on dairy cattle welfare and associated animal-based welfare measures in a European Food Safety Authority (EFSA) report by Nielsen et al. (30). In this report, worst adverse effects were selected based upon several other EFSA reports (24, 33–37), Presi and Reist (38), Brenninkmeyer and Winckler (39), and expert opinion (**Table 2**).

TABLE 2 | Summary of which of the "worst adverse effects" for dairy cattle welfare are associated with the selection of welfare measures in the current study based upon Nielsen et al. (30).

Adverse effects	Associated welfare measures
Foot disorders	Lameness, mortality, and lesions/swellings
Leg injuries	Lameness, lesions/swellings
Mortality (unassisted)	Mortality
Mortality (euthanasia)	Mortality
Exhaustion (prolonged metabolic demand)	Leanness, mortality, and lesions/swellings
Behavioral disruption—feeding (including social stress, pain, hunger, exhaustion, fear, and frustration)	Leanness, lameness
Behavioral disruption—rest (including too little rest, pain, and fear)	Lesions/swellings, lameness
Behavioral disruption—flooring/space (including fear, and pain)	Lesions/swellings, lameness
Thermal discomfort	No associations identified

RESULTS

Step 1: Selecting Welfare Measures

Highest median expert importance ranking for herd welfare was allocated to "lameness," "leanness," "mortality rate," and "integument alterations," which were therefore selected to be included in the protocol (**Figure 3**). The other measures among the top 10 ranked welfare measures were considered for inclusion as well: "time needed to lie down," "tied vs. loose housing," "disbudding/dehorning," "drinker space," "somatic cell count (SCC)," and "dystocia." Only one of the latter measures (SCC >400,000 as an indicator of mastitis) met all selection criteria. Lameness is measured in WQ using a gait score with categories "not lame," "moderately lame," and "severely lame" (22). As we needed indicators that can be expressed as a percentage, only severe lameness was used in the ensuing steps. Integument alterations consist of both hairless patches and lesions/swellings. As both may have different causes, we chose to separate the two in the ensuing steps of this study.

Step 2: Severity Scores

Median expert severity scores were highest for severe lameness (92, interquartile range = 90–97) and mortality (90, 69–100) followed by leanness (61, 50–71) and SCC > 400,000 (73, 43–80), and lowest for hairless patches (18–34) and wounds/swellings (40–58).

Step 3: Calculating the WI and Analyzing Coherence With Expert Scores

Welfare scores as indicated by the experts followed the patterns anticipated for the 12 focus herds (**Figure 4**). Herds 1 and 2, with a low prevalence for all measures, received a good score, while herds 3 and 4, with high prevalence for all measures (indicating poor welfare), received a bad score. Additionally, a high prevalence of the measure "severe lameness" while all other prevalences were medium (herds 11 and 12), lead to a lower expert score than when only "% of cows with SCC > 400,000" was high (herds 9 and 10), in line with the higher severity scores for lameness than SCC.

Quadratic models consistently achieved a higher R^2 than linear models. Using R^2 as a primary metric for agreement,

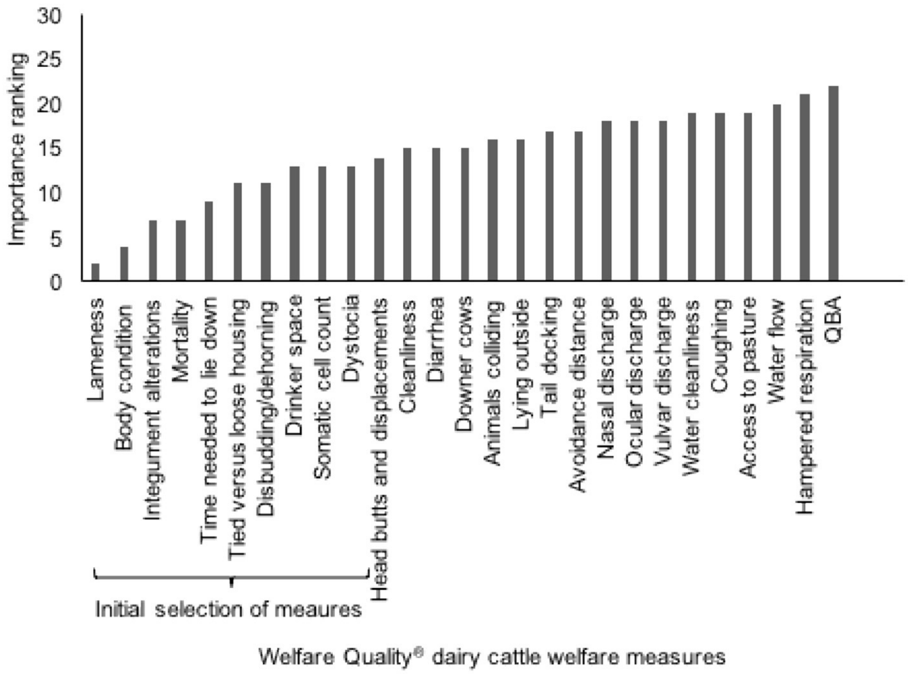

FIGURE 3 | Median importance ranking for all WQ measures as judged by 16 dairy cattle welfare experts. Initial selection of 10 welfare measures for the WI indicated using accolade.

the quadratic model with no CRM (i.e., full compensation) provided the best fit with the experts' scores ($R^2 = 0.91$, $F = 401.4$, **Figure 5**). For the 20 models with highest R^2, the full compensation model was ranked first for four other agreement metrics (AIC = 688.4, mean absolute difference = 18.23, RMSE = 24.46, LOA = 46.21) and third for ICCC (0.737). The mean rank for all six metrics was also lowest (i.e., best) for the full compensation model (rank = 1.29), followed by two discrete compensation reduction models (ranks = 2.57 and 3.64). We thus conclude that full compensation provides the best fit with expert opinion. As there is no evidence that a method of compensation reduction improves the fit with the expert scores, we can simplify the WI by removing Cm from the formula.

Step 4: Interpreting the WI

Based upon expert scores in the different welfare categories, thresholds for the category "Not classified" ranged from 0 to 46, for "Acceptable" from 46 to 77, for "Enhanced" from 77 to 93, and for "Excellent" from 93 to 100 (**Figure 5**).

Step 5: Exhaustiveness Check

The welfare measures that were selected are all mentioned in Nielsen et al. (30) as being associated with what they defined as being the "worst adverse effects" based on expert opinion and literature (**Table 2**). Some "adverse effects" and a single "worst adverse effect" (thermal discomfort) were not associated with any of our selection of measures.

DISCUSSION

Aiming for a better uptake by the milk industry, we followed five steps to develop a thoroughly revised and simplified version of the WQ protocol for monitoring the welfare of dairy cattle herds. The main focus was to improve the cost effectiveness of the protocol by collecting information on a limited number of key welfare indicators in a much shorter time. The time needed for a certified assessor to complete the protocol was reduced by a factor of 2–3. For example, using the estimated time needed to assess the various welfare measures listed in Welfare Quality (22, Table 12), an assessment of a herd of 100 cows takes approximately 6 h and 41 min with the original WQ protocol vs. 2 h and 42 min with our simplified protocol. Our simplified tool for monitoring and integrated labeling of dairy cattle welfare distinguishes itself from the original WQ protocol (and most other protocols) in four other important ways. First, the exclusive use of animal-based measures implies direct assessment of dairy cattle welfare (in contrast to the use of resource-based measures). Second, the simple and transparent integration formula for calculating overall welfare (WI) reduces the likelihood of unwanted side effects that are more likely to occur when using more complex aggregation procedures. The original WQ protocol is an example of a complex integration method that was innovative in its use of methods, where welfare measures are first integrated into 12 criteria scores and subsequently into 4 principle scores, which are then used to determine the overall welfare category (22). The welfare principles were separated to reflect different dimensions of welfare, and the complex integration methods were necessary

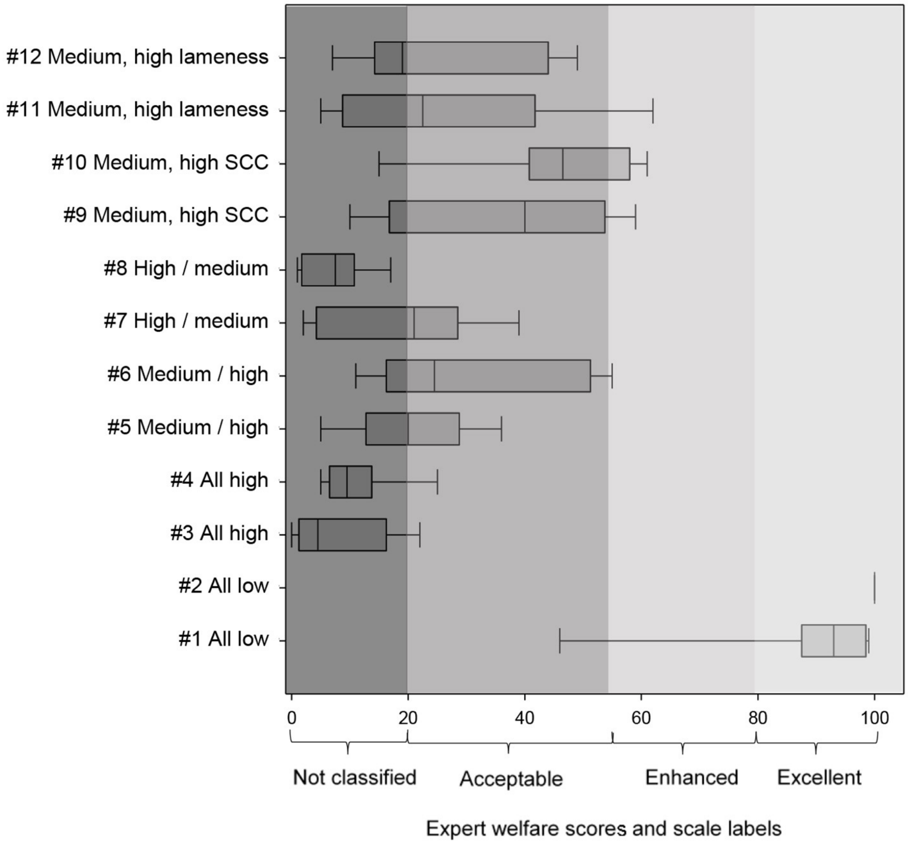

FIGURE 4 | Medians and interquartile range (box) of the welfare scores allocated by experts (n = 14) to the 12 focus farms (confer **Table 2**) using a 0–100 tagged Visual Analog Scale. Whiskers: data within 1.5 × the interquartile range. Higher scores imply better welfare. QBA, Qualitative Behavior Assessments. Highest scores belonged to the top 5% of herds in the European dataset (n = 491), medium between 40 and 60%, and lowest scores were from the lowest 5% of herds. [1]"% of too lean cows," "SCC > 400,000," and "number of hairless patches" were high, [2]"% of cows with lesions," "% of cows with severe lameness," and "% of mortality" were high.

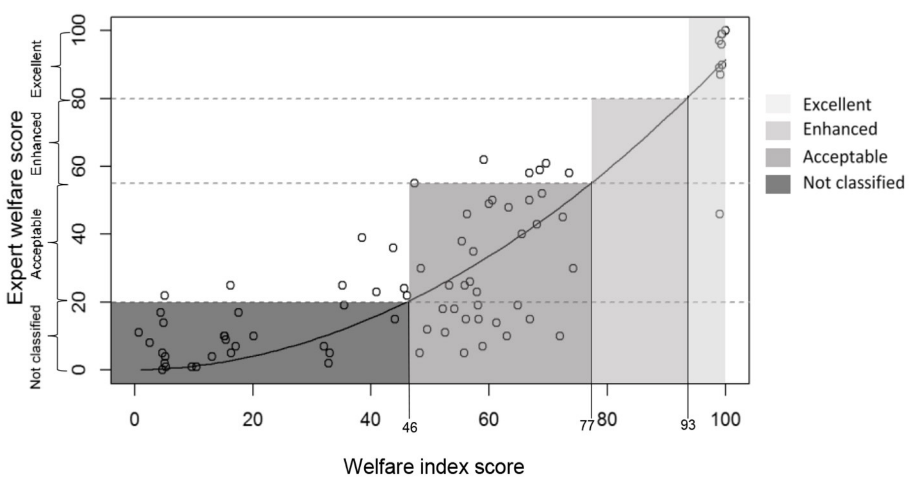

FIGURE 5 | Expert (n = 14) welfare scores of the fictitious herds (n = 12) plotted against the calculated WI scores of these herds using no CRM, with best fitting quadratic curve ($R^2 = 0.91$). Higher scores indicate better welfare; category thresholds determined using the expert scores are indicated underneath the x-axis.

to cope with the large number of measures included. However, an unintended consequence of the large number of measures and the method used to integrate them is that sensitivity of the overall welfare category to changes in individual welfare measures partly depends on the number of measures integrated into the criterion and principle scores (18, 21). For our revised protocol, we opted for a much simpler, but intuitively sensible and transparent, method of integration using a single formula in which the relative weights of the various measures directly reflect how severely they affect cattle welfare (as judged by the experts). Third, the WI was based upon, tested, and found to show high correspondence with expert opinion. Finally, the integrated WI being expressed on a continuous scale ensures a high degree of differentiation, which enables detection of relatively small differences between (or within) herds. This implies that even small improvements in individual measures will lead to (slightly) higher integrated scores. Such a high degree of sensitivity is likely more motivating for farmers to implement on-farm welfare improvements than a (categorical) WI, which changes only in response to very drastic improvements.

The formula we eventually used for calculating WI also directly reflects the experts' opinion of how severely cattle welfare is affected by the various welfare issues that are quantified by the selected animal-based measures because the compensation-reduction term could be removed. Models for none of the compensation-reduction methods produced a better fit with the overall welfare scores given to the focus herds by the experts when compared to applying no compensation reduction. This implies that our expert consultation provided no justification to insert additional terms for calculating the WI so that the lowest measure scores are given additional weight relative to the other measure scores. Hence, we recommend using the simplest formula for WI (i.e., without the Cm term, which is assumed to equal one). The simplification of the original WQ protocol into our WI has recently been shown to result in a better match with five other (i.e., non-WQ based) dairy herd welfare assessment metrics used in the Netherlands and with the consensus herd welfare score given by at least five dairy cattle veterinarians that visited the farms on a regular basis (40). These findings thus provide some support for an improved concurrent and consensus validity, respectively, of the WI as compared to the original WQ overall categorization.

The time reduction and simplification of the protocol inevitably comes at the expense of the comprehensiveness of the assessment. It should be borne in mind, therefore, that the aim of the revised protocol is not to detect *all* possible adverse or favorable effects on dairy cattle welfare, which we consider virtually impossible. Instead, we focused on an index that reflects the worst adverse effects on welfare (according to literature and experts). Incorporating an extensive list of welfare measures would complicate step 3 of the process (comparing expert scores with WIs) possibly leading to information overload. This occurs when people are unable to distinguish relevant from irrelevant information when presented with too much information (41, 42). As we are aware that our limited selection of measures is not sensitive to all possible adverse welfare effects, we strongly advice to use a disclaimer indicating which adverse effects may not be detected by the current selection of measures. This approach may be considered as more fair than claiming exhaustiveness, which, in our opinion, is close to impossible anyway. The proposed WI does enable detection of all the *worst* adverse effects on dairy cattle welfare according to Nielsen et al. (30). Nielsen et al. (30) selected the worst adverse effects from a list of adverse effects on dairy cattle welfare, based upon EFSA reports. Although this list was not assessed for comprehensiveness—this remains to be validated by future research—our current selection of welfare measures likely lacks sensitivity for documenting some additional (not-worst) adverse effects (i.e., reproductive disorders; thermal discomfort; pain, fear, and frustration; abomasal displacement; respiratory distress/pain; other adverse effects related to diseases and other adverse effects related to injuries). We note that the measures that were retained in the simplified protocol focus on the impairment of the health and physical condition of the animals. This focus partly reflects the approach in the original WQ protocol, which includes only a single animal-based measure that could (arguably) provide information on positive affective state, namely, the Qualitative Behavior Assessment (QBA). The experts, however, allocated the least importance to this measure, which probably reflects reservations about the reliability or validity of this measure. In our opinion, this reflects a more general problem in animal welfare monitoring that there is a need to develop feasible, reliable, and valid measures that better document the behavioral needs and (negative as well as positive) affective states of the animals. Indeed, Knierim et al. (43) also questioned whether health-centered welfare assessment protocols that are implemented in the dairy industry, such as the US-based FARM program (https://nationaldairyfarm.com/farm-animal-care-version-4-0/) or the UK-based AssureWel protocol (http://www.assurewel.org/dairycows) sufficiently take societal expectations into account, which often relate to naturalness for dairy cattle. Perhaps with the rapid advancements in the use of automated sensor technologies for monitoring livestock behavior and condition, such information may be incorporated in welfare assessment protocols in the future (44–46). Such measures could be added to the protocol by using the step-wise approach we proposed. Such additions would make the assessment more comprehensive and hedonic (47, 48) but at the expense of simplicity and logistic feasibility.

Experts were stimulated in the survey to take validity and reliability of the WQ measures into account for ranking of the welfare measures. However, it still may be questioned whether validity and reliability of all selected measures are truly adequate. For example, mortality rate is based on herd records of which reliability has barely been documented. As is the case for any welfare assessment protocol, it is important to strive for high reliability of the measures by training observers to achieve high test–retest, inter- and intraobserver reliability, and by unbiased sampling of animals.

Categorical differentiation between herds (i.e., welfare categorization) is useful to interpret the WI in terms of which scores indicate farms of poor or excellent welfare. In addition, such welfare categories may be used for labeling purposes to identify farms of varying welfare levels. In the current study, we determined thresholds based upon expert scores in the different welfare categories for the 12 focus herds. These thresholds are only indicative, given the limited number of herds and experts that they are based on.

Two main inputs were used in the current study: expert opinion and a European database of selected welfare measures' prevalence. As expert opinion was vital in the current study, we used stringent criteria to select experts. While this limited the number of experts who could participate, it also ensured adequate knowledge about dairy cattle welfare and the welfare measures concerned. Still, it would be relevant to test whether outcomes (in terms of selected welfare measures, severity scores, and correspondence with expert opinion) would be similar with a different composition or type of experts. Similarly, it would be interesting as well to test whether another setting [e.g., a workshop to achieve consensus like in Rodenburg et al. (27)] would affect the outcomes. Moreover, it could be argued that in order for the protocol to be perceived as being of high quality and hence be advocated by the industry, other stakeholders ought to have been involved in the selection of measures and the way these are integrated. We opted to base our current study on the opinion of scientific experts who are knowledgeable about the WQ measures for assessing cattle welfare, rather than on other stakeholders who do not have the same level of expertise (e.g., consumers) or who might have non-scientific motivations to bias the aggregation outcome in one way or another (e.g., milk industry). In our opinion, such in-depth knowledge was essential important for making well-informed decisions about which measures to retain, allocating the severity scores and allocating overall welfare scores to the focus herds. It could be verified in a follow-up study whether consumers and other stakeholders accept or refute the authority and outcome of these scientific expert judgements. If this would reveal important discrepancies, we would face the dilemma of increasing social acceptance either by adapting the protocol to better accord with stakeholder opinion or by better clarifying and explaining the decisions, outcomes, and credibility of the scientific experts to the stakeholders.

The second important, and rather unique, input used in the current study was the database containing prevalence data on the selected measures of 491 European dairy herds. This dataset allowed selected experts to benchmark results of the focus farms based on a wide range of data, which supported them in allocating welfare scores that can realistically be attained on commercial farms in Europe. Such a large database on other (non-WQ) measures where a uniform protocol was used may be hard to attain. As both the experts and the dataset were European, caution is required when applying the protocol to dairy herds in other parts of the world (where the welfare challenges for cattle may be different).

CONCLUSIONS

The stepwise approach employed in the current study led to thorough revision of the WQ protocol for on-farm monitoring of dairy herd welfare that is more user-friendly, more time efficient, and exclusively relies on key animal-based welfare measures (lameness, leanness, mortality, hairless patches, lesions/swellings, and somatic cell count) that are integrated into a highly differentiating, transparent, and continuous welfare index. In addition, the resulting WI is highly coherent with expert opinion. Although the reduction in the number of welfare measures reduces the comprehensiveness of the assessment, the current selection of six welfare measures are associated with all the worst adverse effects for dairy cattle welfare as identified by Nielsen et al. (30). Nevertheless, the integrated welfare index should be accompanied with a disclaimer that lists adverse and favorable effects that cannot be detected adequately by the current selection of measures. However, the proposed method is flexible such that measures can be replaced or added as deemed desirable by repeating the proposed steps.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements. Data recorded in the study is reported anonymously.

AUTHOR CONTRIBUTIONS

FT and SG wrote the paper. BA did the statistical analyses. WV, LL, JB, FT, and SG conceptualized the study. AB, FE, MH, MKi, LM, and MKj provided data. All authors have proof-read earlier manuscripts.

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REFERENCES

1. Blokhuis HJ, Jones RB, Geers R, Miele M, Veissier I. Measuring and monitoring animal welfare: transparency in the food product quality chain. *Anim.Welf.* (2003) 12:445–55.
2. Fraser D. Understanding animal welfare. *Acta Vet. Scand.* (2008) 50:S1. doi: 10.1186/1751-0147-50-S1-S1
3. Czycholl I, Büttner K, Beilage E, Krieter J. Review of the assessment of animal welfare with special emphasis on the Welfare Quality® animal welfare assessment protocol for growing pigs. *Arch Anim Breed.* (2015) 58:237–49. doi: 10.5194/aab-58-237-2015
4. Spoolder H, De Rosa G, Horning B, Waiblinger S, Wemelsfelder F. Integrating parameters to assess on-farm welfare. *Anim Welf.* (2003) 12:529–34.

5. Blokhuis HJ, Veissier I, Miele M, Jones B. The Welfare Quality® project and beyond: Safeguarding farm animal well-being. *Acta Agric Scan Section A-Anim Sci.* (2010) 60:129–40. doi: 10.1080/09064702.2010.523480
6. Kirchner MK, Westerath HS, Knierim U, Tessitore E, Cozzi G, Winckler C. On-farm animal welfare assessment in beef bulls: consistency over time of single measures and aggregated Welfare Quality® scores. *Animal.* (2014) 8:461–9. doi: 10.1017/S1751731113002267
7. de Graaf S, Ampe B, Tuyttens F. Assessing dairy cow welfare at the beginning and end of the indoor period using the Welfare Quality© protocol. *Anim Welf.* (2017) 26:213–21. doi: 10.7120/09627286.26.2.213
8. Czycholl I, Kniese C, Schrader L, Krieter J. How reliable is the multi-criteria evaluation system of the Welfare Quality® protocol for growing pigs? *Anim Welf.* (2018) 27:147–56. doi: 10.7120/09627286.27.2.147
9. Friedrich L, Krieter J, Kemper N, Czycholl I. Test-retest reliability of the 'Welfare Quality (R) animal welfare assessment protocol for sows and piglets'. Part 1. Assessment of the welfare principle of 'appropriate behavior'. *Animals.* (2019) 9:398. doi: 10.3390/ani9070398
10. Tuyttens FAM, Federici JF, Vanderhasselt RF, Goethals K, Duchateau L, Sans ECO, et al. Assessment of welfare of Brazilian and Belgian broiler flocks using the Welfare Quality® protocol. *Poult Sci.* (2015) 94:1758–176. doi: 10.3382/ps/pev167
11. de Jong IC, Hindle VA, Butterworth A, Engel B, Ferrari P, Gunnink H, et al. Simplifying the Welfare Quality® assessment protocol for broiler chicken welfare. *Animal.* (2016) 10:117–27. doi: 10.1017/S1751731115001706
12. Gocsik E, Brooshooft SD, de Jong IC, Saatkamp HW. Cost-efficiency of animal welfare in broiler production systems: A pilot study using the Welfare Quality® assessment protocol. *Agric Syst.* (2016) 146:55–69. doi: 10.1016/j.agsy.2016.04.001
13. de Vries M, Bokkers EAM, van Schaik G, Botreau R, Engel B, Dijkstra T, et al. Evaluating results of the Welfare Quality® multi-criteria evaluation model for classification of dairy cattle welfare at the herd level. *J Dairy Sci.* (2013) 96:6264–73. doi: 10.3168/jds.2012-6129
14. Knierim U, Winckler C. On-farm welfare assessment in cattle: validity, reliability and feasibility issues and future perspectives with special regard to the Welfare Quality® approach. *Anim Welf.* (2009) 18:451–8.
15. Tuyttens FAM, Vanhonacker F, Van Poucke E, Verbeke W. Quantitative verification of the correspondence between the Welfare Quality® operational definition of farm animal welfare and the opinion of Flemish farmers, citizens and vegetarians. *Livest Sci.* (2010) 131:108–14. doi: 10.1016/j.livsci.2010.03.008
16. de Vries M, Engel B, den Uijl I, van Schaik G, Dijkstra T, de Boer I, et al. Assessment time of the Welfare Quality® protocol for dairy cattle. *Anim Welf.* (2013) 22:85–93. doi: 10.7120/09627286.22.1.085
17. Heath CAE, Browne WJ, Mullan S, Main DCJ. Navigating the iceberg: reducing the number of parameters within the Welfare Quality® assessment protocol for dairy cows. *Animal.* (2014) 8:1978–86. doi: 10.1017/S1751731114002018
18. Buijs S, Ampe B, Tuyttens FAM. Sensitivity of the Welfare Quality® Broiler chicken protocol to differences between intensively reared indoor flocks: which factors explain overall classification? *Animal.* (2017) 11:244–53. doi: 10.1017/S1751731116001476
19. Czycholl I, Kniese C, Schrader L, Krieter J. Assessment of the multi-criteria evaluation system of the Welfare Quality® protocol for growing pigs. *Animal.* (2017) 11:1573–80. doi: 10.1017/S1751731117000210
20. de Graaf S, Ampe B, Winkler C, Radeski M, Mounier L, Kirchner MK, et al. Trained-user opinion about Welfare Quality measures and integrated scoring of dairy cattle welfare. *J Dairy Sci.* (2017) 100:6376–88. doi: 10.3168/jds.2016-12255
21. de Graaf S, Ampe B, Buijs S, Andreasen SN, Des Roches ADB, Van Eerdenburg, et al. Sensitivity of the integrated Welfare Quality® scores to changing values of individual dairy cattle welfare measures. *Anim Welf.* (2018) 27:157–66. doi: 10.7120/09627286.27.2.157
22. Welfare Quality®. *Welfare Quality® Assessment Protocol for Cattle.* Lelystad: Welfare Quality ® Consortium (2009).
23. Johnsen PF, Johannesson T, Sandøe P. Assessment of herd animal welfare at herd level: many goals, many methods. *Acta Agr Scand Section A-Animal Sci.* (2001) 51:26–33. doi: 10.1080/090647001316923027
24. European Food Safety Authority. Scientific Opinion on the use of animal-based measures to assess welfare of dairy cows. *EFSA J.* (2012) 10:2554–2. doi: 10.2903/j.efsa.2012.2554
25. Burow E, Rousing T, Thomsen PT, Otten ND, Sørensen JT. Effect of grazing on the cow welfare of dairy herds evaluated by a multidimensional welfare index. *Animal.* (2013) 7:834–42. doi: 10.1017/S1751731112002297
26. Leknes S, Tracey I. Pain and pleasure: masters of mankind. In: Kringelbach ML, and Berridge KC, editor. *Pleasures of the Brain*. New York, NY: Oxford University press (2010). p. 320–35.
27. Rodenburg TB, Tuyttens FAM, De Reu K, Herman L, Zoons J, Sonck B. Welfare assessment of laying hens in furnished cages and non-cage systems: assimilating expert opinion. *Anim Welf.* (2008) 17:355–61.
28. Whay HR, Main DCJ, Greent LE, Webster AJF. Animal-based measures for the assessment of welfare state of dairy cattle, pigs and laying hens: consensus of expert opinion. *Anim Welf.* (2003) 12:205–17.
29. Lievaart JJ, Noordhuizen JPTM. Ranking experts' preferences regarding measures and methods of assessment of welfare in dairy herds using Adaptive Conjoint Analysis. *J Dairy Sci.* (2011) 94:3420–7. doi: 10.3168/jds.2010-3954
30. Nielsen BH, Angelucci A, Scalvenzi A, Forkman B, Fusi F, Tuyttens FAM, et al. Use of animal based measures for the assessment of dairy cow welfare-ANIBAM. *EFSA External Sci Rep*. (2014) 11:659E. doi: 10.2903/sp.efsa.2014.EN-659
31. Liao JJ. An improved concordance correlation coefficient. *Pharm Stat.* (2003) 2:253–61. doi: 10.1002/pst.52
32. Altman DG, Bland JM. Measurement in medicine: the analysis of method comparison studies. *Statistician.* (1983) 32:307–17. doi: 10.2307/2987937
33. European Food Safety Authority. Scientific opinion on welfare of dairy cows in relation to behaviour, fear and pain based upon a risk assessment with special reference to the impact of housing, feeding, management and genetic selection. *EFSA J.* (2009) 7:1139. doi: 10.2903/j.efsa.2009.1139
34. European Food Safety Authority. Scientific opinion on welfare of dairy cows in relation to leg and locomotion problems based upon a risk assessment with special reference to the impact of housing, feeding, management and genetic selection. *EFSA J.* (2009) 7:1142. doi: 10.2903/j.efsa.2009.1142
35. European Food Safety Authority. Scientific opinion on welfare of dairy cows in relation to metabolic and reproductive problems based upon a risk assessment with special reference to the impact of housing, feeding, management and genetic selection. *EFSA J.* (2009) 7:1140. doi: 10.2903/j.efsa.2009.1140
36. European Food Safety Authority. Scientific opinion on welfare of dairy cows in relation to udder problems based upon a risk assessment with special reference to the impact of housing, feeding, management and genetic selection. *EFSA J.* (2009) 7:1141. doi: 10.2903/j.efsa.2009.1141
37. European Food Safety Authority. Scientific report on the effects of farming systems on dairy cattle welfare and disease. *EFSA J.* (2009) 7:1143. doi: 10.2903/j.efsa.2009.1143r
38. Presi P, Reist M. Review of methodologies applicable to the validation of animal based indicators of welfare. *EFSA Support Pub.* (2011) 8:171. doi: 10.2903/sp.efsa.2011.EN-171
39. Brenninkmeyer C, Winckler C. Relationships between animal welfare hazards and animal-based welfare measures. *EFSA Support Pub.* (2012) 9:253E−2. doi: 10.2903/sp.efsa.2012.EN-253
40. Van Eerdenburg FJCM, Hof T, Doeve B, Ravesloot L, Zeinstra EC, van der Staay FJ. The relation between hair-cortisol concentration and various welfare assessments of Dutch dairy farms. *Animals.* (2021) 11:821. doi: 10.3390/ani11030821
41. Malhotra NK. Information load and consumer decision making. *J Consum Res.* (1982) 8:419–30. doi: 10.1086/208882
42. Herbig PA, Kramer H. The effect of information overload on the innovation choice process: innovation overload. *J Consum. Mark.* (1994) 11:45–54. doi: 10.1108/07363769410058920
43. Knierim U, Winckler C, Mounier L, Veissier I. Developing effective welfare measures for cattle. In: *Understanding the Behaviour and Improving the Welfare of Dairy Cattle*, ed M. Endres. Philadelphia, PA: Burleigh Dodds Science Publishing (2021). p. 81–102. doi: 10.19103/AS.2020.0084.05

44. Molina FM, Marin CCP, Moreno LM, Buendia EIA, Marin DCP. Welfare Quality® for dairy cows: towards a sensor-based assessment. *J Dairy Res.* (2020) 87:28–33. doi: 10.1017/S002202992000045X
45. Hogeveen H, van der Voort M. Advances in precision livestock farming techniques for monitoring dairy cattle welfare. In: *Understanding the Behaviour and Improving the Welfare of Dairy Cattle*, ed M. Endres. Philadelphia, PA: Burleigh Dodds Science Publishing (2021). p. 103–22. doi: 10.19103/AS.2020.0084.06
46. Larsen MLV, Wang M, Norton T. Information technologies for welfare monitoring in pigs and their relation to Welfare Quality®. *Sustainability.* (2021) 13:692. doi: 10.3390/su13020692
47. Broom DM. Animal welfare: concepts and measurement. *J Anim Sci.* (1991) 69:4167–75. doi: 10.2527/1991.69104167x
48. Tannenbaum J. Ethics and animal welfare: the inextricable connection. *J Am Vet Med Assoc.* (1991) 198:1360–76.

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REVIEW
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The Use of the General Animal-Based Measures Codified Terms in the Scientific Literature on Farm Animal Welfare

Marta Brscic, Barbara Contiero, Luisa Magrin, Giorgia Riuzzi and Flaviana Gottardo*

Department of Animal Medicine, Production and Health, University of Padova, Legnaro, Italy

Background: The approach to farm animal welfare evaluation has changed and animal-based measures (ABM), defined as the responses of an animal or effects on an animal, were introduced to assess animal welfare. Animal-based measures can be taken directly on the animal or indirectly and include the use of animal records. They can result from a specific event or be the cumulative outcome of many days, weeks, or months. The objective of the current study was to analyze the use of general ABM codified terms in the scientific literature, the presence of their definitions, and the gap mapping of their use across animal species, categories, years of publication, and geographical areas of the corresponding author's institution. The ultimate aim was to propose a common standard terminology to improve communication among stakeholders. In this study, data models were populated by collecting information coming from scientific papers extracted through a transparent and reproducible protocol using Web of Science^{TM} and filtering for the general ABM codified terms (or synonyms/equivalents). A total of 199 papers were retained, and their full texts were assessed. The frequency of general codified ABM terms was analyzed according to the classification factors listed in the objectives. These papers were prevalently European (159 documents), and the most represented species was cattle. Fifty percent of the papers did not provide a definition of the general ABM terms, and 54% cited other sources as reference for their definition. The results of the study showed a very low penetration of the general codified ABM term in the literature on farm animal welfare, with only 1.5% of the papers including the term ABM. This does not mean that specific ABM are not studied, but rather that these specific ABM are not defined as such under a common umbrella, and there is no consensus on the use of terminology, not even among scientists. Thus, we cannot expect the stakeholders to use a common language and a standardized terminology. The recognition and the inclusion of ABM in the lists of commonly accepted abbreviations of scientific journals could be a first step to harmonize the terminology in the scientific literature.

Keywords: animal-based measure, animal welfare assessment, scientific literature, gap mapping, penetration level

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Edward Narayan,
The University of Queensland, Australia

Reviewed by:
Suzanne T. Millman,
Iowa State University, United States
Harry J. Blokhuis,
Swedish University of Agricultural Sciences, Sweden

***Correspondence:**
Marta Brscic
marta.brscic@unipd.it

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INTRODUCTION

The first animal welfare assessment schemes were developed in the 1990s, and they were introduced within the organic farming assurance protocols (1, 2). At that time, these assessment schemes relied mainly on resources and management-based parameters to evaluate relations between environmental conditions and animal welfare (3). The framework of the animal welfare assessment was the evaluation of the farming conditions, and end-users drew conclusions on animal welfare based on the estimated relation between these conditions and the extent that these fulfilled the needs of the animals. These needs were represented by the Five Freedoms and their provisions: freedom from hunger and thirst—by ready access to fresh water and a diet to maintain full health and vigor; freedom from discomfort—by providing an appropriate environment including shelter and a comfortable resting area; freedom from pain, injury, or disease—by prevention or rapid diagnosis and treatment; freedom to express normal behavior—by providing sufficient space, proper facilities, and company of the animal's own kind; and freedom from fear and distress—by ensuring conditions and treatment which avoid mental suffering. In the meantime, the animal welfare scientists started developing and testing for application on farm measures based on direct observation of the animals and on animal records. Several research groups promoted indeed the need of an integrated approach in which both resource/management and animal-based measures (ABM) are necessary to assess animal welfare in a holistic way (4, 5). This is, in particular, due to the fact that animal welfare was recognized as a multidimensional concept that includes both the physical and mental state of the animal and that the Five Freedoms were considered as defining ideal states rather than standards for acceptable welfare (6). Therefore, researchers have probably used ABM as tools to assess animal needs long before they were conceptualized and classified under the umbrella of the general ABM codified term. The ABM were aimed at measuring the welfare status of the animal by assessing the outcomes. Indeed they can show the outcome of integrated resource and management factors in the experience of the animal itself (7) and can therefore be a more valid measure of welfare (8). In dairy cows, for example, the approach changed in such a way that the direct assessment of the animal, by measuring the time needed to lie down, was preferred over measuring size, softness, and other characteristics of the cubicles as it was more valid in evaluating the real welfare state of the animal (9). The development of several valid ABM and their classification under a common terminology were the main achievements of the Welfare Quality project. After Welfare Quality, other research projects focusing on ABM were financed by the European Union (EU) within the 7th and Horizon 2020 framework programs. Most projects considered assessment of animal welfare on farm, either directly or retrospectively at slaughter (e.g., AWIN, AssureWel, PROHEALTH ClearFarm, different COST Actions, etc.). The European Food and Safety Authority (EFSA) considered the use of ABM in the assessment of animal welfare so relevant that it commissioned a statement in order to establish a common framework for future scientific opinions and to clarify some common issues on the terminology and integration of concepts (6). According to this statement, ABM are defined as the response of an animal or an effect on an animal used to assess its welfare. They can be taken directly on the animal or indirectly and include the use of animal records. They can result from a specific event, e.g., an injury, or be the cumulative outcome of many days, weeks, or months, e.g., body condition. Further pilot projects were commissioned by EFSA to point out the need for context-specific ABM as, for example, the case of small mountain dairy farms where ABM developed through the Welfare Quality project were not directly applicable to this context (10). Animal-based measures were also included in some EU animal welfare legislative acts, commentary documents from NGOs, and assurance schemes (e.g., those developed by AssureWel, RSPCA, Biobord, RedTractors).

The rationale behind this study comes from the evidence of the wide use of ABM by public institutions and governments, in dedicated EU projects, in a range of quality assurance schemes, in EU legislative acts for the protection of animals (11), and by animal protection NGOs and from the increasing awareness of the need for scientific validity of these measures. Therefore, the objective of the current study was to analyze the use of the general ABM codified terms in the scientific literature on farm animal welfare since their first conceptualization, along with the presence of their definitions and the gap mapping of their use across different animal species, categories (e.g., within cattle dairy, beef, calf), years of publication, and geographical areas of the institution of the corresponding author. The ultimate aim was to propose a common standard terminology to improve communication and facilitate the connections among stakeholders.

MATERIALS AND METHODS

Scientific Literature Search

A literature search protocol was set up by using Web of Science—© 2020 Clarivate Analytics to identify peer-reviewed papers that were written in English and that covered the topic of farm animal welfare. The search was performed in June 2020. The basic inclusion criteria for the selection of the peer-reviewed papers were as follows:

- Timespan = 1990–2019
- Search language = English
- Search topics = title, abstract, author keywords, keywords plus

The flow chart shown in **Figure 1** describes the search protocol that was developed through the following steps:

- selection of papers containing "animal welfare" OR "animal well-being" OR "animal wellbeing" (first search string)
- exclusion of papers dealing with "animal welfare", "animal well-being", or "animal wellbeing" in human-related studies. The exclusion criteria adopted were based on a search string that excluded "wild animal," "marine mammal", "pet animal", "laboratory animal", "companion animal", "zoo animal", "dog", "cat", "mouse", "mice", "rat", or "rodent" (second search string)

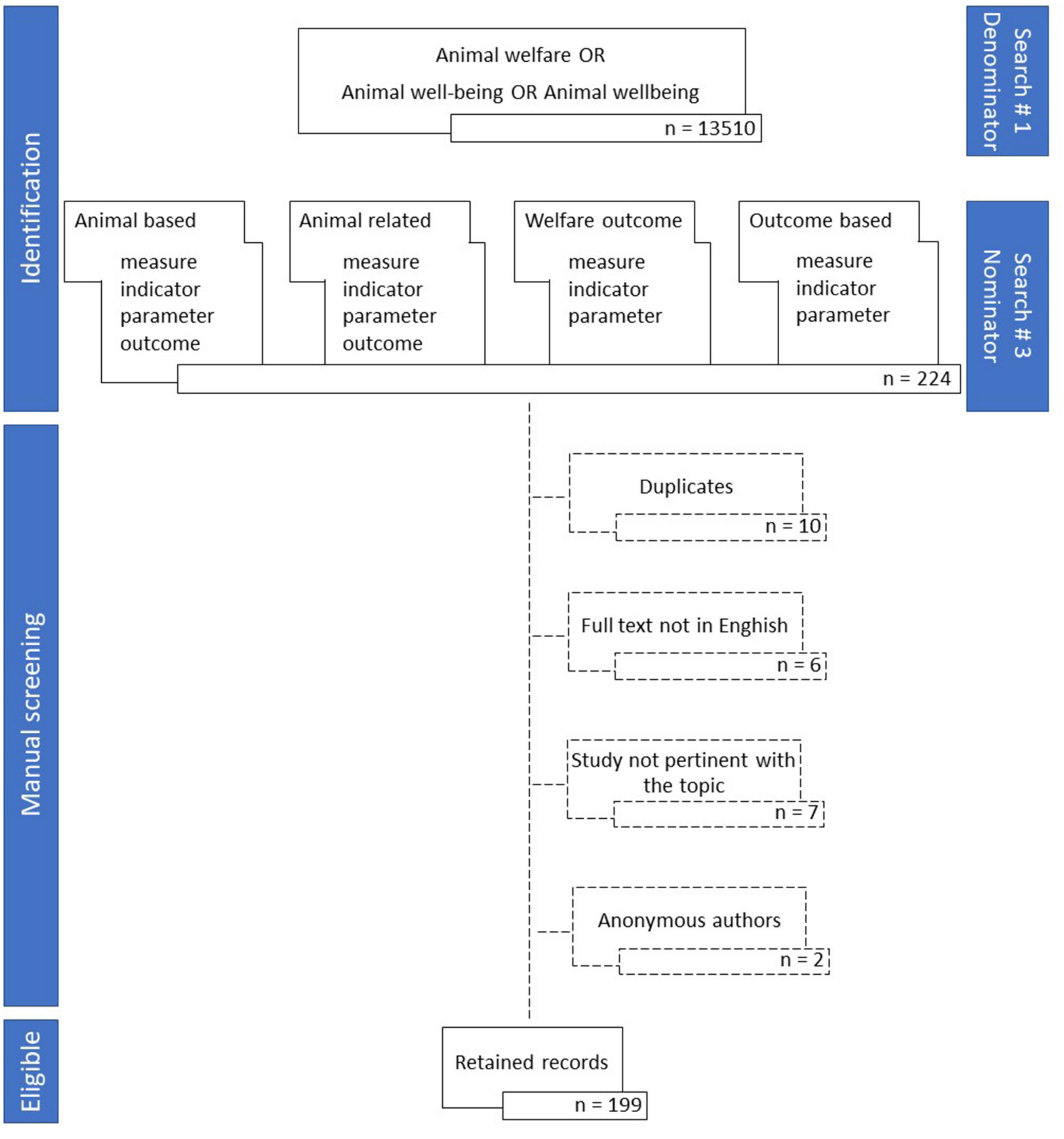

FIGURE 1 | Flow chart graphically representing the search protocol. The dashed line represents the papers excluded at manual screening.

- selection of papers containing the general codified ABM term or all the potential synonyms/equivalents of ABM (animal related, welfare outcome, and outcome based) considered by Gottardo et al. (11) within papers containing "animal welfare," "animal well-being," or "animal wellbeing." The search string applied was ("animal welfare" OR "animal well-being" OR "animal wellbeing) AND ((("animal based" OR animal-based) NEAR/3 measure*) OR (("animal based" OR animal-based) NEAR/3 indicator*) OR (("animal based" OR animal-based) NEAR/3 outcome*) OR (("animal based" OR animal-based) NEAR/3 parameter*) OR (("animal related" OR animal-related) NEAR/3 measure*) OR (("animal related" OR animal-related) NEAR/3 indicator*) OR (("animal related" OR animal-related) NEAR/3 outcome*) OR (("animal related" OR animal-related) NEAR/3 parameter*) OR (("welfare outcome" OR "outcome based" OR outcome-based*****) NEAR/3 measure*)

OR (("welfare outcome" OR "outcome based" OR outcome-based*) NEAR/3 indicator*) OR (("welfare outcome" OR "outcome based" OR outcome-based*) NEAR/3 parameter*)) (third search string). NEAR/x = finds records where the terms joined by the operator are within a specified number of words from each other. x is the maximum number of words that separates the terms [i.e., = ("animal based" NEAR/3 measure*) finds all the records where in a given sentence they are separated by no more than three words as in the case of "animal based welfare assessment measures"].

In line with the aim of this study, which is to standardize the terminology, we refer to:

- "animal welfare" for the broad first-level set of terms "animal welfare/animal well-being/animal wellbeing"
- "general ABM root term" for the second-level set of terms "Animal based/animal-based (AB), Animal related/animal-related (AR), Welfare Outcome (WO), and Outcome based/outcome-based (OB)"
- "general ABM ending term" for the third-level set of terms "Measure/Indicator/Parameter/Outcome"
- "general ABM codified term" for the combination of the "general ABM root term" and "general ABM ending term." From here onwards, we will use this categorization.

The retained records were then submitted to a manual screening that had different purposes. As graphically presented in **Figure 1**, the first manual screening was performed to clean the dataset, thus, to eliminate the records whose full text was not in English, duplicates, anonymous authors, and/or that were not pertinent to the topic. Once the eligible documents were retained, the full text was analyzed to classify each paper according to animal species and production category, type of study, scenario, and application in organic farming. The levels and the descriptions of the classification factors are reported in **Table 1**. The further analysis of the full text included (1) the identification of presence of one or more ABM general term (yes/no/not reported for each of the codified terms reported in **Figure 1**), (2) presence of a definition of the ABM general term provided by the authors (yes/no) or definition referring to a citation (yes/no), (3) in case the definition was referring to a citation, the reference was copied and pasted, and (4) presence of specific ABM in the full text, figures, or tables (yes/no) regardless of the specific ABM name, form, or unit [e.g., a study dealing with lameness was reported as including a specific ABM (yes), although it could include mild lameness, severe lameness, lameness prevalence, lameness scoring, percentages of lame animals within each score, and/or different scoring systems]. Reporting the specific ABM was not within the scope of this paper. The full texts were assessed by four assessors trained in a standard procedure to screen the papers and to fill in a shared Excel document with drop-down lists in order to have a common systematic criterion of evaluation and data collection. Each assessor evaluated individually an equal number of records. In case of doubts, the evaluators discussed among each other to make a final decision. The full list of retained documents is provided as **Supplementary Material**.

TABLE 1 | Factors used to classify the papers, the different levels, and the explanation of how each paper was classified.

Factor	Levels	Explanation
Animal species	Cattle Equine Fur animals Goat Poultry Rabbit Sheep Swine Other General	Each paper was classified according to the main farm animal species it dealt with, and each animal species was further classified according to the animal category (e.g., cattle was further subdivided in dairy, beef, and calf; swine was further subdivided in fattening pig, sow, piglet/other). A paper was classified as other if it dealt with other minor animal species or as general if it was of a general wide approach and not involving given animal species. A paper dealing with more than one species was classified in more than one class
Type of study	Methodological Research Assessment Other	Each paper was classified as methodological if it described a method applied or the development of a methodology (e.g., validation), as a research if it was an original applicative study with data produced by the research, as an assessment if it described an animal welfare assessment or its application, and as other if it did not fall in any of these classifications. A single paper was classified in more than one class if it considered more than one of the aspects listed
Scenario	On farm At slaughter During transport Not reported	Each paper was classified according to its scenario of application or with the scenario it dealt with: on farm, at slaughter, and/or during transport. A single paper was classified in more than one class if it considered more than one scenario
Organic farming	Yes No Not reported	Each paper was classified as dealing with organic farming (yes) if it included the application in organic farms (according to organic principles) or of it dealt with comparisons of conventional (no) vs. organic production systems (yes) and as not organic (no) if the application was on conventional farms and as not reported if it was not specified in the full text

Data were submitted to descriptive statistics by using Excel/STAT and considering publication year, animal species and category, scenario, and application in organic farming as classification factors. This approach was adopted also in order to carry out the gap mapping of the distribution of the ABM in the scientific literature across the different classification factors.

The gap mapping was carried out over the following subsequent steps: (1) identification of the problem area in the use of terminology related to the general ABM term, (2) definition of the goal for which at least animal welfare scientists could use a common terminology worldwide, (3) determination of the current use of the terminology within literature on animal welfare, (4) determination of a potential desired homogeneous use, and (5) identification of the gaps between the two uses.

An indication of how the use of terminology related to the general ABM term penetrated the scientific literature dealing with animal welfare was obtained by calculating the following two ratios:

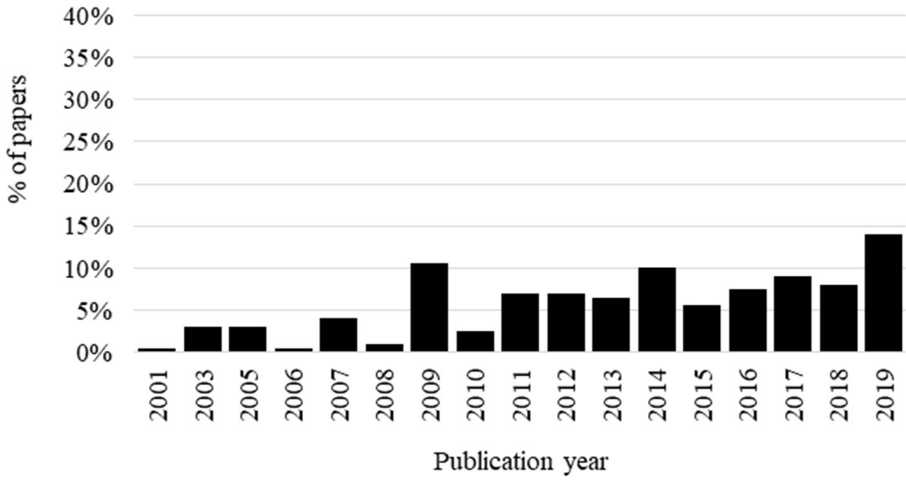

FIGURE 2 | Percentage of papers including a general animal-based measures codified term according to the publication year.

- the ratio between papers with the general ABM term (nominator) and the total number of papers on animal welfare (denominator) and
- the ratio between total citations and the number of papers for each general term: average number of citations per paper.

RESULTS AND DISCUSSION

The results of the literature search strings are reported in the flow chart in **Figure 1**, along with the number of records per each search combination. The final outcome of the set-up procedure described in section "Materials and Methods" identified 199 scientific papers that were retained for data collection and calculation of the penetration indexes. The ratio between papers with the general ABM term over the papers on animal welfare is 1.5%, and it is likely to indicate an overall low level of penetration of the ABM general term in the scientific literature on animal welfare. This result might not necessarily indicate a low use of specific ABM (e.g., body condition score, mortality, cleanliness, lameness) but rather that animal welfare scientists do not classify the specific ABM as such using a common terminology. Differences in the penetration of the use of the general ABM terms in the scientific literature were observed when analyzing the distribution of the papers in relation to the geographical area of the institution of the corresponding author. The majority of papers were from Europe (159 documents, 80%) followed by America (27 documents, 14%), Oceania (nine documents, 4%), and Asia (four documents, 2%). None of the documents, including the general ABM terms, were attributed to correspondence of African institutions. This overview is likely to reflect the fact that the European scientific community is more used to network for applications to project funding from EU, promoting a more homogeneous use of technical terminology. Once the general ABM term was conceptualized (5), several research groups probably started using it as an umbrella term. Indeed, as reported in **Figure 2**, the papers including the general ABM terms were published as of 2001, and the first paper referred to a European COST Action as funding source. The percentage of selected papers according to the publication year is reported in **Figure 2**. A peak was detected in 2009, when the Welfare Quality project ended with the publication of the welfare assessment protocols and most of the research groups active in the project published their papers. This could have determined an increasing trend in the subsequent years, with a new peak observed in 2019. The documents were published on 54 different scientific journals, making evident a scattered distribution of the general codified term also in journals that are not specialized in the animal welfare field. Eight journals published five or more papers containing the general ABM codified term (**Table 2**). Animal Welfare of the University Federation for Animal Welfare, a highly specialized journal, was the most represented in this list. The other journals have a more general approach, and they publish cross-cutting topics on animal science.

TABLE 2 | Number and percentage of papers including a general animal-based measures codified term published in the journals with more than five papers.

Journal	Number	Percentage (%)
Animal Welfare	54	27
Animals	16	8
Animal	15	7
Journal of Dairy Science	13	6
Italian Journal of Animal Science	11	5
Journal of Animal Science	7	4
Poultry Science	7	4

The retained peer-reviewed papers were classified as original research papers (63 documents, 32%), methodological studies (63 documents, 32%), assessments (57 documents, 29%), and/or as other if they could not be characterized within any of the above-mentioned macro groups (43 documents, 22%). We expected

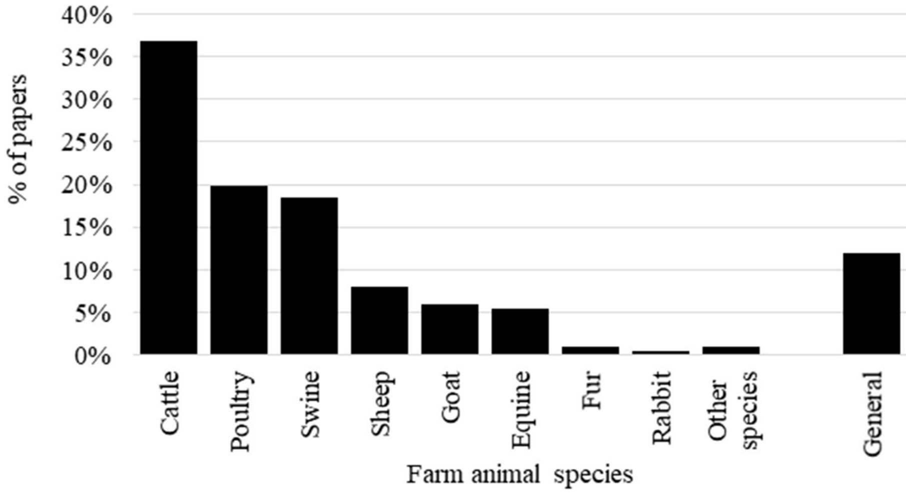

FIGURE 3 | Percentage of papers including a general animal-based measures codified term according to the animal species.

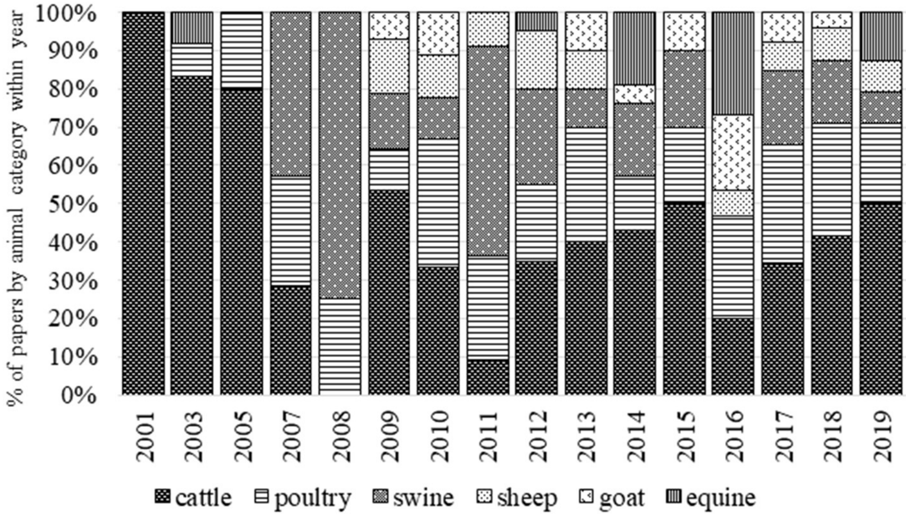

FIGURE 4 | Frequency distribution of papers including a general animal-based measures codified term according to the animal species within the year of publication.

a more homogenous use of terminology and a more frequent appeal to the general ABM terms in methodological and in assessments studies compared to original research papers. This expectation was not met by our results, and the frequency distribution did not show a high prevalence of specific types of papers, probably indicating that scientists are still exploring all these three aspects, the assessment scheme application (12, 13), research on ABM (14–16), or development of methodological aspects linked to ABM (17–19). However, listing a paper in only one of these classification groups was sometimes difficult.

The number and percentage of papers per animal species are reported in **Figure 3**. More than one species were included in nine papers, while 24 papers were of a general methodological approach (without any specific species analyzed). Cattle, poultry, and swine are the most represented species in the literature including a general ABM term. The first approach to the definition of the general ABM term was addressed in cattle in 2001 (**Figure 4**). Thus, it is likely that this terminology was largely used in literature on cattle, therefore causing this greater number of documents. Among cattle, the largest number of papers with ABM regarded dairy cows (67%), beef (11%), and calf (9%). Papers dealing with calves were on dairy calves, not veal. These results likely suggest that, in the past, cattle is the animal category that, in fact, has shown more need for ABM than other categories for the wide options of their housing and rearing systems (e.g., from pasture-based to indoor loose cubicle housing) in which the same evaluation method based on resource- and management-based measures was not directly applicable (5, 20). Indeed the literature on dairy cattle deals with the development of assessment protocols in small-scale mountain farms (21, 22),

TABLE 3 | Number and percentage of papers including a general animal-based measures codified term according to the different scenarios.

Scenario	Number	Percentage (%)
On farm	154	77
At slaughter	12	6
During transport	2	1
More than one scenario	11	6
Not reported	20	10

pasture-based systems (23, 24), or specific problems in indoor farms, e.g., lameness, mastitis, etc. (25–27). The second most represented group of species is poultry. Among poultry, broiler chicken (61%) and lying hens (39%) are involved in the vast majority of the papers, whereas duck, goose, and turkey are marginal (3% of the overall papers on poultry). This result does not mean again that specific ABM are not used in studies on these categories of animals, but only that they are not codified as such. Documents on poultry deal with studies on different husbandry systems including free-range and organic scenarios (28–30). As shown in **Figure 4**, papers on sheep, goat, and equine emerged in the timeframe that is subsequent to the outset of the AWIN project that aimed at developing assessment schemes for these species. As regards swine, the scientific literature focused mainly on fattening pigs and piglets (70%) rather than on sows and gilts. We expected a higher prevalence of papers on fattening pigs, which is likely due to the awareness of public opinion about tail docking and castration that promoted studies addressing the development of alternative production practices (31). However, the published papers including the general ABM term covered topics that were mainly on assessment scheme applications on farm, during transport, and at slaughter and the testing of intra- and inter-observer reliability (18, 32, 33). It is likely that the scientific studies on mutilations and pain management do not refer to the general ABM terms.

The number of papers with different scenarios of application is reported in **Table 3**. The large majority of the papers regarded on-farm studies. Among the 11 studies applied on more than one scenario, eight studies regarded activities on farm and at the slaughter, and three studies were applied during transport and at the slaughter. The frequency distribution of the scenarios is likely reflecting the fact that assessment schemes implying the use of ABM aim at evaluating the level of animal welfare on farm, regardless of the site of its application. Specific ABM developed to be used at slaughter may aim either at a retrospective evaluation of the welfare on farm (9, 34, 35) or during transport (36) or at assessing the welfare at the time of slaughter and related operations (37).

Organic farming systems are not significantly represented among documents retained in this study (four documents, 2%), whereas both organic and conventional systems were present in 19 documents (9.4%). The adoption of the organic system of production was not reported in almost 60% of the papers. This result does not meet our assumption that papers describing studies in organic production systems include the general ABM term since the first animal welfare assessment schemes were introduced within the assurances of organic farming. This might be due to the evidence that organic assurance schemes relied mainly on resources and management-based parameters as required, for example, by EU legislation (38).

Among the different general ABM root terms searched in the retained documents, AB was the most frequently used, followed by WO and OB, whereas AR was the less used one (**Table 4**). The most frequently used general ABM ending term was measure(s) followed by indicator(s), parameter(s), and outcome(s). Outcome was the less used term, which is likely due to the lower number of combinations with the general ABM root term for semantic reasons (e.g., WO and OB outcome). The matrix of the general ABM codified term made of combinations of roots (AB, AR, WO, and OB) and endings [measure(s), indicator(s), outcome(s), and parameter(s)] is reported in **Table 4**. The most frequent general ABM codified term used in combination was Animal based/animal-based measure(s), and this could be expected considering that the Welfare Quality project opted for this terminology in its outputs. On the other hand, Animal based/animal-based indicator(s) was the terminology preferentially used in the AWIN project. Cross-use of the terminology is not rare; indeed two or three general ABM codified terms were used in 38 (19%) and 3 (1%) papers, respectively, whereas still a single general codified term was used in the majority of papers (154 documents, 77%).

Further indicators of the level of penetration of the general ABM terms in the scientific literature, which reflect the interest by the scientific community toward this topic, could be the total citations and the average number of citations per paper. Total citation is the number of times that a paper was cited in other scientific publications from its publication year until 2019 when the current literature search was carried out. The total citations collected by the scientific corpus of the 199 retained papers was 2,983. The citation indicators according to the general ABM root terms are reported in **Table 4**. The total citations were greater for AB, although the average citations per paper were greater for AR. Four documents had more than 100 citations (5, 39–41). Fifty percent of the papers did not provide a definition of the general ABM terms, and 54% cited one or more other sources as reference for the definition of general ABM terms. The most cited papers as reference for the definition with more than five citations among the literature corpus used in this paper are reported in **Table 5**. The mostly cited document is Welfare Quality (9), and this could be expected considering the wide use of the general ABM term compared to the other potential synonyms/equivalents. Surprisingly, 67 papers (33.3%) did not provide any explicit definition nor references to other sources.

In conclusion, the results of this study showed that the general ABM terms are used in a very limited fraction of the literature on animal welfare. In the scenario of the 199 papers including a general ABM codified term, the Welfare Quality project had the greatest impact; thus, the considered terminology and the species and categories of its application were the most represented. Fur animals, rabbits, and other niche farm animals were poorly represented in the retained documents of this literature corpus which could be expected by the limited number of farms and the

TABLE 4 | Number of documents and percentage (in brackets), total citations, and average number of citations per paper according to the general animal-based measures (ABM) root, ending and codified terms.

		Papers in which the general ABM ending term is used[a]	General ABM root term[b]				Number of papers in which more than one term was used
			Animal based/animal-based (AB)	Animal related/animal-related (AR)	Welfare outcome (WO)	Outcome based/outcome-based (OB)	
Papers in which the general ABM root term is used[a]			172 (87%)	16 (8%)	25 (13%)	24 (12%)	
General ABM ending term	Measure(s)	139 (70%)	122 (71%)	3 (19%)	18 (72%)	21 (87%)	23
	Parameter(s)	48 (24%)	43 (25%)	10 (62%)	0 (0%)	0 (0%)	4
	Indicator(s)	74 (37%)	67 (39%)	6 (38%)	8 (32%)	3 (13%)	10
	Outcome(s)	9 (5%)	9 (6%)	0 (0%)			0
Total citations (TC)		2,682	417	382	521		
TC/number of papers		15.6	26.1	15.3	21.7		

[a] *Overall percentage expressed on the total number of 199 retained documents.*
[b] *Percentage expressed on 172 (AB), 16 (AR), 25 (WO), and 24 documents (OB), respectively.*

TABLE 5 | List of most cited papers (more than five times) as reference for the definition of the general codified animal-based measures term and number of documents in which they are cited in the corpus of the 199 retained papers.

	Number of documents
Welfare Quality® (9)	22
Whey et al. (39)	15
EFSA (6)	14
Main et al. (7)	8
Main et al. (42)	8
Johnsen et al. (43)	7
Webster et al. (44)	7
Blokhuis et al. (5)	6
Capdeville and Veissier (3)	6
Botreau et al. (45)	5
Keeling and Veissier (8)	5

localized productions. A different reason could support the fact that fish were almost absent, although there is a large number of farmed species and ABM are under development for aquatic organism. By looking at the source of the retained documents (journal of publication), it seems that the general codified ABM term is being used by experts involved in animal welfare studies, but it has also permeated scientists involved in animal production and other related topics where it could be expected that a common use of single specific measures is adopted, such as body condition, growth performance, cleanliness, and somatic cell count.

The implications of this study are linked to the fact that there is a huge amount of literature on animal welfare/well-being and a large body of literature that includes specific ABM (e.g., lameness, lesions, body condition, somatic cell count, mortality, etc.), but these specific ABM are not defined as such under a common umbrella, and there is no consensus on the use of terminology, not even among scientists. Thus, we could not expect stakeholders to use a common language and terminology. Going beyond the general terms, we expect that it could be even more difficult to have common names of specific ABM, which makes it even harder to define, standardize, or assess them for reliability, repeatability, reproducibility, robustness, feasibility, accuracy, sensitivity, specificity, and/or validity. In order to achieve the use of a common standard terminology in the future, some of the different possible ways forward could be their use by authorities in animal welfare legislations, by scientific societies dealing with animal behavior and welfare and animal sciences in general, and by NGOs, companies, and institutions involved in the development and application of quality assurance schemes. Moreover, the recognition and the inclusion of ABM in the lists of commonly accepted abbreviations of the scientific journals could be a first step to harmonize the terminology in the scientific literature.

AUTHOR CONTRIBUTIONS

All the authors contributed to data curation. FG, MB, and BC contributed to the conceptualization and writing of the original draft. BC performed the formal analysis. LM and GR contributed to the methodology. All authors contributed to the article and approved the submitted version.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2021.634498/full#supplementary-material

REFERENCES

1. Sundrum A, Andersson R, Postler G. *Tiergerechtheitsindex-200 1994. Ein Leitfaden zur Beurteilung von Haltungssystemen für Rinder, Kälber, Legehenen und Schweine*. Bonn: Verlag Köllen (1994). p. 211.
2. Bartussek H. A review of the animal needs index (ANI) for the assessment of animals' well-being in the housing systems for Austrian proprietary products and legislation. *Livest Prod Sci*. (1999) 61:179–92. doi: 10.1016/S0301-6226(99)00067-6
3. Capdeville J, Veissier I. A method of assessing welfare in loose housed dairy cows at farm level, focusing on animal observations. *Acta Agric Scand A Anim Sci*. (2001) 51:62–8. doi: 10.1080/090647001316923081
4. Edwards SA. Experimental welfare assessment and on-farm application. *Anim Welf*. (2007) 16:111–5.
5. Blokhuis HJ, Veissier I, Miele M, Jones B. The welfare quality® project and beyond: safeguarding farm animal well-being. *Acta Agric Scand A Anim Sci*. (2010) 60:129–40. doi: 10.1080/09064702.2010.523480
6. EFSA Panel on Animal Health and Welfare. Statement on the use of animal-based measures to assess the welfare of animals. *EFSA J*. (2012) 10:2767. doi: 10.2903/j.efsa.2012.2767
7. Main DCJ, Kent JP, Wemelsfelder F, Ofner E, Tuyttens FAM. Applications for methods of on- farm welfare assessment. *Anim Welf*. (2003) 12:523–8.
8. Keeling L, Veissier I. "Developing a monitoring system to assess welfare quality in cattle, pigs and chickens," in *Science and Society Improving Animal Welfare. Welfare Quality Conference Proceedings 17/18 November 2005* (Brussels), 46–50.
9. Welfare Quality. *Welfare Quality Assessment Protocol for Pigs (Sows and Pigets, Growing and Finishing Pigs)* (2009).
10. Zuliani A, Mair M, Kraševec M, Lora I, Brscic M, Cozzi G, et al. A survey of selected animal-based measures of dairy cattle welfare in the Eastern Alps: toward context-based thresholds. *J Dairy Sci*. (2018) 101:1428–36. doi: 10.3168/jds.2017-13257
11. Gottardo F, Contiero B, Brscic M. The use of animal-based measures to assess animal welfare in the EU - state of art of the last 10 years of activities and analysis of the gaps. Preparatory work. *EFSA Support Publ*. (2017) 12:902E. doi: 10.2903/sp.efsa.2015.EN-902
12. Kirchner MK, Westerath SH, Knierim U, Tessitore E, Cozzi G, Pfeiffer C, et al. Application of the Welfare Quality® assessment system on European beef bull farms. *Animal*. (2014) 8:827–35. doi: 10.1017/S1751731114000366
13. Battini M, Stilwell G, Vieira A, Barbieri S, Canali E, Mattiello S. On-farm welfare assessment protocol for adult dairy goats in intensive production systems. *Animals*. (2015) 5:934–50. doi: 10.3390/ani5040393
14. Sandgren CH, Lindberg A, Keeling LJ. Using a national dairy database to identify herds with poor welfare. *Anim Welf*. (2009) 18:523–32.
15. Sprenger M, Vangestel C, Tuyttens FAM. Measuring thirst in broiler chickens. *Anim Welf*. (2009) 18:553–60.
16. de Vries M, Bokkers EAM, van Schaik G, Engel B, Dijkstra T, de Boer IJM. Improving the time efficiency of identifying dairy herds with poorer welfare in a population. *J Dairy Sci*. (2016) 99:8282–96. doi: 10.3168/jds.2015-9979
17. Can E, Vieira A, Battini M, Mattiello S, Stilwell G. Consistency over time of animal-based welfare indicators as a further step for developing a welfare assessment monitoring scheme: the case of the Animal Welfare Indicators protocol for dairy goats. *J Dairy Sci*. (2017) 100:9194–204. doi: 10.3168/jds.2017-12825
18. Czycholl I, Grosse Beilage E, Henning C, Krieter J. Reliability of the qualitative behavior assessment as included in the welfare quality assessment protocol for growing pigs. *J Anim Sci*. (2017) 95:3445–54. doi: 10.2527/jas.2017.1525
19. Souza APO, Soriano VS, Schnaider MA, Rucinque DS, Molento CFM. Development and refinement of three animal-based broiler chicken welfare indicators. *Anim Welf*. (2018) 27:263–74. doi: 10.7120/09627286.27.3.263
20. EFSA Panel on Animal Health and Welfare. Scientific report on the effects of farming systems on dairy cow welfare and disease. *EFSA J*. (2009) 7:114r. doi: 10.2903/j.efsa.2009.1143r
21. Corazzin M, Piasentier E, Dovier S, Bovolenta S. Effect of summer grazing on welfare of dairy cows reared in mountain tie-stall barns. *Ital J Anim Sci*. (2010) 9:304–12. doi: 10.4081/ijas.2010.e59
22. Zuliani A, Romanzin A, Corazzin M, Salvador S, Abrahantes JC, Bovolenta S. Welfare assessment in traditional mountain dairy farms: above and beyond resource-based measures. *Anim Welf*. (2017) 26:203–11. doi: 10.7120/09627286.26.2.203
23. Armbrecht L, Lambertz C, Albers D, Gauly M. Assessment of welfare indicators in dairy farms offering pasture at differing levels. *Animal*. (2019) 13:2336–47. doi: 10.1017/S1751731119000570
24. Beggs DS, Jongman EC, Hemsworth PH, Fisher AD. The effects of herd size on the welfare of dairy cows in a pasture-based system using animal- and resource-based indicators. *J Dairy Sci*. (2019) 102:3406–20. doi: 10.3168/jds.2018-14850
25. Sadiq MB, Ramanoon SZ, Mossadeq WMS, Mansor R, Syed-Hussain SS. Association between lameness and indicators of dairy cow welfare based on locomotion scoring, body and hock condition, leg hygiene and lying behavior. *Animals*. (2017) 5:79. doi: 10.3390/ani7110079
26. Rouha-Mülleder C, Iben C, Wagner E, Laaha G, Troxler J, Waiblinger S. Relative importance of factors influencing the prevalence of lameness in Austrian cubicle loose-housed dairy cows. *Prev Vet Med*. (2009) 92:123–33. doi: 10.1016/j.prevetmed.2009.07.008
27. Hansson H, Szczensa-Rundberg M, Nielsen C. Which preventive measures against mastitis can increase the technical efficiency of dairy farms? *Animal*. (2011) 5:632–40. doi: 10.1017/S1751731110002247
28. Bassler AW, Arnould C, Butterworth A, Colin L, De Jong IC, Ferrante V, et al. Potential risk factors associated with contact dermatitis, lameness, negative emotional state, and fear of humans in broiler chicken flocks. *Poult Sci*. (2013) 92:2811–26. doi: 10.3382/ps.2013-03208
29. Bright A, Brass D, Clachan J, Drake KA, Joret AD. Canopy cover is correlated with reduced injurious feather pecking in commercial flocks of free-range laying hens. *Anim Welf*. (2011) 20:329–38.
30. Zaludik K, Lugmair A, Baumung R, Troxler J, Niebuhr K. Results of the Animal Needs Index (ANI-35L) compared to animal-based parameters in free-range and organic laying hen flocks in Austria. *Anim Welf*. (2007) 16:217–9.
31. Contiero B, Cozzi G, Karpf L, Gottardo F. Pain in pig production: text mining analysis of the scientific literature. *J Agric Environ Ethics*. (2019) 32:401–12. doi: 10.1007/s10806-019-09781-4
32. Pfeifer M, Eggemann L, Kransmann J, Schmitt AO, Hessel EF. Inter- and intra-observer reliability of animal welfare indicators for the on-farm self-assessment of fattening pigs. *Animal*. (2019) 16:1712–20. doi: 10.1017/S1751731118003701
33. Sørensen JT, Schrader L. Labelling as a tool for improving animal welfare-the pig case. *Agriculture*. (2019) 9:123. doi: 10.3390/agriculture9060123
34. Scollo A, Gottardo F, Contiero B, Mazzoni C, Leneveu P, Edwards SA. Benchmarking of pluck lesions at slaughter as a health monitoring tool for pigs slaughtered at 170 kg (heavy pigs). *Prev Vet Med*. (2017) 144:20–8. doi: 10.1016/j.prevetmed.2017.05.007
35. Magrin L, Brscic M, Armato L, Contiero B, Cozzi G, Gottardo F. An overview of claw disorders at slaughter in finishing beef cattle reared in intensive indoor systems through a cross-sectional study. *Prev Vet Med*. (2018) 161:83–9. doi: 10.1016/j.prevetmed.2018.10.018
36. Fàbrega E, Coma J, Tibau J, Manteca X, Velarde A. Evaluation of parameters for monitoring welfare during transport and lairage at the abottoir in pigs. *Anim Welf*. (2007) 16:201–4.
37. Grandin T. Auditing animal welfare and making practical improvements in beef-, pork- and sheep-slaughter plants. *Anim Welf*. (2012) 21:29–34. doi: 10.7120/096272812X13353700593400
38. European Commission. Commission Implementing Regulation (EU) 2020/464 of 26 March 2020 laying down certain rules for the application of Regulation (EU) 2018/848 of the European Parliament and of the Council as regards the documents needed for the retroactive recognition of pe. *Off J Eur Union*. (2020) 2–25. Available online at: http://data.europa.eu/eli/reg_impl/2020/464/oj
39. Whay HR, Main DCJ, Green LE, Webster AJF. Animal-based measures for the assessment of welfare state of diary cattle, pigs and laying hens: consensus of expert opinion. *Anim Welf*. (2003) 12:205–17.
40. Grandin T. Auditing animal welfare at slaughter plants. *Meat Sci*. (2010) 86:56–65. doi: 10.1016/j.meatsci.2010.04.022
41. Knierim U, Winckler C. On-farm welfare assessment in cattle: validity, reliability and feasibility issues and future perspectives with special regard to the Welfare Quality® approach. *Anim Welf*. (2009) 18:451–8.

42. Main DCJ, Whay HR, Leeb C, Webster AJF. Formal animal-based welfare assessment in UK certification schemes. *Anim Welf.* (2007) 16:233–6.
43. Johnsen PF, Johannesson T, Sandøe P. Assessment of farm animal welfare at herd level: many goals, many methods. *Acta Agric Scand A Anim Sci.* (2001) 30:26–33. doi: 10.1080/090647001316923027
44. Webster AJF, Main DCJ, Whay HR. Welfare assessment: indices from clinical observation. *Anim Welf.* (2004) 13:93–8.
45. Botreau R, Veissier I, Butterworth A, Bracke MBM, Keeling LJ. Definition of criteria for overall assessment of animal welfare. *Anim Welf.* (2007) 16:225–8.

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