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Avian senses, immunology, and welfare

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Avian senses, immunology, and welfare

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Editorial: Avian senses, immunology, and welfare

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Editorial on the Research Topic
Avian senses, immunology, and welfare

Introduction

Ensuring the adaptive success and welfare of birds across diverse ecosystems depends on understanding the intricate relationships between their physiology including how they sense the environment. Insights from avian immunology reveal how birds respond to disease challenges and other stressors, crucial for improving health outcomes especially in light of increasing global demand for poultry products. Birds encounter a myriad of natural and human-driven challenges that disrupt their internal balance, triggering responses across sensory, immune and other physiological systems, all essential for survival and optimal performance. While extensive research has explored these areas independently, a comprehensive understanding requires synthesizing insights across these domains. The overarching aim is to compile and merge findings from avian sensory biology, behavior and immunology to reveal how birds respond to environmental challenges and stressors, enhancing welfare and fostering their survival in diversely challenging ecosystems.

A total of 14 papers are part of this collection: one perspective, two reviews and 11 research papers. The contributions have been grouped under two themes.

1. Avian Senses and Environmental Adaptation

The first theme of this Research Topic highlights the relevance of the senses in the adaptation of wild and domestic birds to the diversity of habitats they are exposed ranging from the wilderness to farms and urban settings. The sense of taste has been largely

undermined for many years partially caused by the lack of taste papillae in the avian tongue in contrast to mammals. The review from Niknafs et al. is a comprehensive update on the scientific knowledge to date about the anatomy and physiological function of taste across domestic and wild avian species. The review illustrates how birds have adapted taste sensory systems to dietary profiles in avian herbivores, insectivores, omnivores or strict carnivores amongst others. However, bitter taste in avian species is still an understudied field. A second avian taste manuscript presents the results on the highly developed bitter taste sensitivity in zebra finches (Kumar et al.). In this paper Kumar and co-authors investigate the bitter activation profiles of three zebra finch receptors Tas2r5, -r6, and–r7. In this work the well-developed bitter taste of the zebra finch shows to be particularly tuned to detect cucurbitacin I suggesting a prominent ecological role of this compound for this bird.

The perspective article by Loxdale proposes that the bright, contrasting feather colors of certain European birds may act as warning signals to deter predators, possibly indicating chemical defenses. Such coloration, potentially mimicking toxicity, could allow these birds to avoid predation or gain time to escape, as seen with the avoidance of Eurasian magpies by some predators. Fossesca et al. have presented a review aiming at explaining the scientific evidence of the impact of anthropogenic noise on birds. The review explains effects of noise on the avian auditory processing and discusses species-specific behavioral and physiological responses. Finally, a research article written by Kimball et al. studies vigilance against predators in songbirds. The article uncovers that in breeding season female songbirds maintain full focus on vigilance towards predators.

2. Avian Physiological Resilience and Welfare, and Advances in Avian Immunology and Health.

This second theme is composed by nine original research articles, which explore from the microbiota-gut interaction or the interface of physiology and behavior in the context of avian welfare, to exploring pathological scenarios from an immunological perspective. Aylward et al. indicate that modern broiler chickens face challenges in balancing growth with immune responses, especially around 2 weeks post-hatch. This period is critical as muscle accretion accelerates, leaving them particularly vulnerable to disease challenges due to inadequate resource allocation between growth and immunity. In their paper, Nolin et al. expose that the microbiome is both likely affected by host divergent genetic selection and that it exerts influence on host antibody response by various mechanisms. Li et al. findings indicate that dietary chlorogenic acid (CGA) significantly mitigates the disruption of the ileac barrier, as well as reduces oxidative damage and inflammation caused by high stocking density environment in broiler chickens. As a result, CGA enhances ileac integrity and promotes the presence of beneficial intestinal bacteria.

The fact that an IgY-Fc receptor (FcRY) is a critical IgY receptor that regulates the IgY uptake from the maternal blood circulation into the yolk of avian species, further indicating that the two steps of maternal–newly-hatched IgY transfer are controlled by a single receptor, is described in detail by Okamoto et al. Weston et al. show that natural antibodies are a pool of relatively distinct immunoglobulins, and that antigen specificity may affect interpretation of natural antibody function and comparative immunology.

Three papers deepen in pathological scenarios: Majeed et al. findings suggest that natural Intraepithelial lymphocytes (IEL) with innate and innate-like functions might play a critical role in the host response during subclinical necrotic enteritis, potentially conferring protection against *C. perfringens* infection; Milby-Blackledge et al. data show that mucosal immune responses in broiler chickens after *Salmonella* Typhimurium (ST) infection had increased pro-inflammatory cytokines, chemokines, and colony-stimulating factors indicating influx of immune cells. An unexpected rise in interleukin-10 suggests an immuno-regulatory role. Additionally, elevated vascular endothelial growth factor (VEGF) levels imply potential tissue repair and angiogenesis in ST-infected birds. Last but not least, Mo et al. center on the morphological characterization and cytokine response of chicken bone marrow-derived dendritic cells to infection with highly pathogenic and low pathogenic avian influenza viruses.

Behaviour and welfare interactions are explored by Lundgren and Løvlie reporting promising finding as it shows that higher tryptophan levels can help reduce fearfulness in a non-invasive way, such as through diet rather than injections, making it practical for larger settings. However, given the complexity of the serotonergic system, further research is needed to fully understand its role in influencing behavior in poultry, particularly in production environments.

In conclusion, a wide variety of contributions totalizing 14 items address two main themes: 1) The Avian Senses and Environmental Adaptation and 2) Avian Physiological Resilience and Welfare including Advances in Avian Immunology and Health. The first theme focuses on avian senses and their interaction with the environment. One of the main conclusions is the discovery of the role of the taste system on the adaptation to different dietary regimes and ecosystems. The bitter taste system plays a crucial role in this process by recognizing potential harmful chemicals present in the environment such as in the zebra finch. In addition, the Research Topic showcases how bright feather coloration may serve as a predator deterrent and how anthropogenic noise affects avian behavior. The second theme emphasizes the challenges faced by modern broiler chickens in balancing growth and immune responses, particularly during critical growth phases. Research highlights the role of genetics and diet in influencing gut health and microbiota, along with the importance of natural antibodies and maternal transfer mechanisms. Additionally, insights into pathological scenarios reveal significant immune responses to infections and the interplay between inflammatory processes and protective mechanisms. The exploration of behavior and welfare underscores the potential of dietary interventions to reduce fearfulness in poultry, pointing to the need for further investigation into the underlying biological mechanisms. Overall, this Research Topic of studies contributes valuable knowledge to our understanding of Avian Senses, Immunology, and Welfare.

Author contributions

FN: Writing–original draft, Writing–review and editing. RD: Writing–review and editing. MF: Writing–review and editing. ES: Writing–review and editing. SN: Writing–review and editing. RA: Writing–review and editing. ER: Writing–original draft, Writing–review and editing.

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Effects of dietary chlorogenic acid on ileal intestinal morphology, barrier function, immune factors and gut microbiota of broilers under high stocking density stress

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Aims: The purpose of this research was to assess the effect of chlorogenic acid (CGA) in the diet on ileac structure, barrier function, immunological state, and microbial profile of broiler chickens in a high stocking density (HD) environment.

Methods: Four hundred and seventy-six male AA broiler chickens were randomly split into four groups, two with a normal stocking density (ND) of fourteen birds per m^2 and two with a high stocking density of twenty-two birds per m^2. Each of the treatments consisted of five replicates. One of the two ND and HD groups received the usual feed, while the other two were given at 1.5 g/kg CGA as part of their dietary regimen.

Results: The ND CGA group showed a greater increase in villus height and villus height/crypt depth compared to the ND group at 35 and 42 days. The HD group experienced a greater elevation in villus height due to CGA supplementation than the HD group across days 28, 35, and 42. At day 42, the HD group saw a decline in *OCLN* and *ZO-1* mRNA expression in the ileum, but CGA was able to restore them. The HD group experienced a greater rise in *OCLN* mRNA than the control HD group when supplemented with CGA. The expression of *TNF-α, IL-1β*, and *IL-6* in the ileum was higher in the HD group, and CGA supplementation enhanced this effect. The HD group experienced a greater rise in *IL-10* mRNA expression than

Abbreviations: CD, ileum crypt depth; CGA, chlorogenic acid; CLA, conjugated linoleic acid; CLDN1, Claudin-1; CLDN2, Claudin-2; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GSH-Px, glutathione peroxidase; HD, high density stocking; HD CGA, HD+CGA group; H&E, hematoxylin and eosin staining; IL-1β, interleukin-1β; IL-6, interleukin-6; IL-10, interleukin-10; LEfSe, LDA effect size; ND, normal density stocking; ND CGA, ND+CGA group; OCLN, occludin; OUT, operational taxonomic unit; PCoA, principal coordinates analysis; PFA, paraformaldehyde; SCFAs, short-chain fatty acids; TM, melting temperature; TNF-α, tumornecrosis factor-alpha; TRIZOL, total RNA extraction reagent; VH, ileum villus height; VH/CD, ratio of ileum villus height to ileum crypt depth; ZO-1, zonula occludens-1.

the control group following the administration of CGA. The HD group showed reduced alpha diversity and an increase in detrimental microbes such as *Turicibacter* and *Shigella* in the gut compared to the ND group, while the HD CGA group saw a reduction in *Turicibacter, Shigella*, and other harmful microbes. These findings reveal that HD stress suppressed the growth of ileac villi, decreased the expression of tight-junction genes, amplified the expression of inflammatory genes, and disturbed the gut microbiota, ultimately leading to increased intestinal permeability.

Conclusion: We conclude that when chickens are given dietary CGA, the disruption of the ileac barrier and increased oxidative damage and inflammation due to HD stress are reduced, which increases ileac integrity and the presence of beneficial intestinal bacteria.

KEYWORDS

high stocking density stress, chlorogenic acid, antioxidant capacity, ileac barrier function, microbial community, broilers

1 Introduction

In recent years, the poultry industry has seen a major increase in production of broilers worldwide to meet the demands of a growing population. This extensive development has not only improved quality of life, but has had a substantial positive effect on national economies (Li et al., 2019). As broilers have become more important as a meat source, it would be more cost-effective to increase the stocking density (SD) in order to produce more meat per unit area (Thaxton et al., 2006). Nevertheless, there is a growing emphasis on animal health and welfare concerns, which are closely linked to SD (Vanhonacker et al., 2009; Wang et al.,2019). SD varies from country to country with 45–54 kg/m^2 being the norm in the Netherlands, 40 kg/m^2 in the United Kingdom, 41.5 kg/m^2 in the United States, and 30–36 kg/m^2 in Switzerland (Nasr et al., 2021). A recommended SD, in consideration of animal welfare, is thought to be beneficial for broiler production and high product quality (Simitzis et al., 2012). High stocking density (HD) has been shown to cause oxidative stress and has been linked to diminished production in broilers, as well as an increased likelihood of health problems (Dozier et al., 2005). Consequently, many strategies have been implemented to improve production of broiler chickens under HD (Abd El-Hack et al., 2020; Sugiharto, 2022). It has been widely accepted that dietary supplementation with certain antioxidants is a viable and convenient solution to reduce HD stress.

As the site in the digestion of starches and fats, the ileum harbors a highly varied microbial population, making intestinal homeostasis a critical element in maintaining the health of broilers (Stanley et al., 2014). HD stocking can cause oxidative stress (OS), which can damage ileal structures by shortening the villi, deepening the recesses, and depleting mucosal epithelial cells, leading to severe disruption of ileal integrity (Tan et al., 2010). Research has demonstrated that OS can dramatically decrease the expression of *occludin* (OCLN), *claudin-1* (CLDN1), and *zonula occludens-1* (ZO-1) in the broiler intestine (He et al., 2016; Zhang et al., 2017), along with an increase in the expression of *interleukin-6* (IL-6) (Quinteiro-Filho et al., 2017) and *tumor necrosis factor-α* (TNF-α) (He et al., 2019) in the intestinal mucosa, and a decrease in *interleukin-10* (IL-10) mRNA expression in the intestine (Quinteiro-Filho et al., 2017). Despite this, it is still unclear if OS caused by HD has a negative effect on ileal homeostasis.

Chlorogenic acid (CGA), a bioactive dietary polyphenol, is esterified by quinic acid and caffeic acids, which are found in green coffee bean extract (Tajik et al., 2017). CGA is widely used in many Chinese herbal medicines, derived from botanical sources like *Dendranthema grandiflora* (*L. chrysanthemum*), *F. lonicerae* (*Flos lonicerae*) and *Eucommia ulmoides* (*E. ulmoides*) (Leiss et al., 2009). It has been documented that CGA has a range of pharmacological effects in animals, which can augment development, strengthen immunity, and modify the gut microbiota by enhancing bioactivities such as antioxidant (Liang and Kitts, 2015), anti-inflammatory (Vukelić et al., 2018), antibacterial (Zhang et al., 2020), and antiviral (Gamaleldin Elsadig Karar et al., 2016), and by regulating lipid metabolism (Sung et al., 2015). In light of current regulations to limit the generation of antimicrobial resistance by eliminating addition of antibiotics to feed, as well as restrictions on resistance in livestock and poultry breeding, the future of animal husbandry appears to be returning to the use of safe natural plant products with similar medicinal properties (Jiang and Xiong, 2016; Vizzier Thaxton et al., 2016).

Our prior research has demonstrated that chickens experienced a decrease in weight gain and feed intake due to HD stress at 28, 35, and 42 days. Despite this, administering 1.5 g/kg of CGA proved to be effective in enhancing both body weight and average daily gain in the HD chickens. These data presented points to the potential of CGA supplementation in chicken diets to counteract the effects of HD stress on chicken productivity (Liu et al., 2023). Consequently, we forecast that CGA boosts efficacy from HD stress by fortifying ileum wellness and augmenting advantageous bacteria. The main goal of this study was to assess the influence of supplementing the feed with CGA on the ileal structure, protective capability, immune system, and gut bacteria of broilers under HD stress conditions at 28, 35, and 42 days of age.

2 Materials and methods

2.1 Statement of ethical treatment

The Care and Use of Experimental Animals Committee of the Henan University of Science and Technology (HUST) (AW20602202-

TABLE 1 Ingredients and nutrient levels in the basal diet.

Ingredient (g/kg)	Starter (1–21 days)	Grower (22–42 days)
Corn	527.9	577.8
Soybean meal	368.9	300.0
Zea gluten meal	0	24.3
Soybean oil	40.0	40.0
Sodium chloride	3.0	3.0
Choline chloride	3.0	2.6
Vitamin premix[b]	0.3	0.3
Trace element premix[a]	2.0	2.0
Stone powder	12.2	11.7
Dicalcium phosphate	19.1	16.2
DL-Methionine	2.7	1.1
L-Lysine	0.4	0.45
Wheat bran	20.0	20.0
Total	1,000	1,000
Metabolic energy (MJ/kg)	12.4	13.0
Crude protein	211.8	198.4
Lysine	11.4	10.5
Methionine	4.9	4.8
Calcium	10.2	8.5
Available P	4.5	4.2
Total P	6.9	6.3
Threonine	7.7	2.2
Analyzed content		
Calcium	10.2	8.5
Total P	6.8	6.2
Calcium: Total P	1.50	1.37

[a]Trace element premix is provided as per kg of feed: 8 mg copper ($CuSO_4 \cdot 5H_2O$); 80 mg iron ($FeSO_4$); 100 mg manganese ($MnSO_4 \cdot H_2O$); 0.15 mg selenium (Na_2SeO_3); 0.35 mg iodine (KI).
[b]Vitamin premix per kg feed: VA, 9500 IU, VD, 362.5 μg, VE, 30 IU, VK, 32.65 mg, VB1 2 mg, VB6 6 mg, VB12 0.25 mg, biotin 325 μg, folic acid 1.25 mg, pantothenic acid 12 mg, niacin 50 mg.
[c]Calculated nutrient concentrations.

1-3) gave its consent to the experimental protocol of this study in 2020. The experiments conformed to their regulations for the humane treatment of animals.

2.2 Animals and experimental design

Four hundred and seventy-six healthy one-day-old male Arbor Acres broiler breeders were obtained from the Henan Quanda Poultry Breeding Co., Hebei, China, and experiments were carried out at the Animal Research Unit of HUST. The chickens were inspected upon arrival to detect illness or physical problems. Chickens were housed with an automated system for ventilation, temperature, humidity, and illumination regulation. At the beginning of the experiments, the temperature of the feeding room was held at 33°C ± 1°C for 1 week, then gradually lowered by 1°C-2°C per week until it reached a final temperature of 25°C ± 2°C by the 42nd day. The room humidity was maintained at 60%–70 %, and lights were kept on for 23 h daily and turned off from 7 to 8 p.m. On the seventh day, five replicates each of healthy broilers, with an average weight of 138.5 ± 2.2 g, were randomly distributed among four different experimental treatments: normal stocking density of 14 birds/m^2 (ND), high stocking density of 22 birds/m^2 (HD), and dietary supplementation with CGA at 1.5 g/kg in the ND CGA and HD CGA groups. To guarantee that the density stayed the same for each sampling date, three extra sets were prepared for every density group.

2.3 Diet composition and CGA supplementation

The main ration for broilers consisted of the corn-soybean meal pellet feed, with the nutritional information listed in Table 1. The supplemental CGA (98% pure) was provided by Changsha Staherb Natural Ingredients Co., Ltd. (Changsha, China). The amount of CGA used here (1.5 g per kg of feed) was in line with the conditions of published studies, and CGA was continually present during the experiment (Liu et al., 2023). An unlimited supply of food and water was made available.

2.4 Sample collection

Two chickens were randomly selected from each replicate and humanely euthanized by inhalation of carbon dioxide. The entire digestive apparatus was swiftly extracted and placed in an icy stainless-steel container and washed with pre-chilled saline solution. The ileum, positioned between Meckel's diverticulum and the ileocaeco-colic junction, was carefully dissected using sterile forceps and scissors. Approximately 5 cm of the intestine was cut from the middle of the organ and a front portion of ileal approximately 1.5 cm was taken and treated with 4% paraformaldehyde (PFA) for hematoxylin and eosin (H&E) staining and further examination of intestinal structure from 28, 35, 42 days of age, while the remaining part was quickly frozen in liquid nitrogen and stored at −80°C for mRNA expression analysis. At 42 days old, five birds from each treatment were sampled for their ileac contents, which were then immediately placed in a sterile container, frozen with liquid nitrogen, and kept at −80°C for microbial analysis. The entire sampling process was completed in a quarter of an hour.

2.5 Intestinal morphology

The tissue samples fixed in 4% PFA were dehydrated with a graded series of ethanol solutions (70%, 96%, and 100%), embedded in paraffin, and 5-μm sections were cut and mounted on a slide. Each section was dewaxed with 100% xylene and then rehydrated. The intestinal tissues were H&E-stained for 8 min, rinsed for 10 s with 1% HCl in ethanol, extensively washed with deionized water, and restrained with eosin for 1 min. Stained slides were washed with deionized water for 6 min, dehydrated with ethanol, cleared in xylene, and allowed to dry overnight. The sections were visualized using a scanner (Pannoramic MIDI, Hungary) for better examination of intestinal morphology and accurately measuring changes in villi. The height of the villi was determined by calculating the distance from the apex to the crypt entrance, and the crypt depth was ascertained by determining the distance from the crypt base to the crypt opening. The average height of villi and depth of crypts was determined by computing the mean values. Case Viewer was used to measure the heights of villi and depths of crypts, and calculate the ratio of villus height to crypt depth (villus height/crypt depth, V/C).

2.6 Determination of intestinal mRNA expression by quantitative real-time PCR (qRT-PCR)

Total RNA was isolated from ileal tissues with TRIzol reagent. RNA quality and quantity were established by spectrophotometer (Nanodrop 2000C, Thermo Fisher). The A260/A280 ratio of 1.9-2.0 showed that the RNA was of suitable quality for mRNA determinations. The process of reverse transcribing RNA into cDNA was completed using the universal SYBR qPCR master mix, and a real-time fluorescence quantitative PCR reaction was then performed using the HiScript III RT SuperMix for qPCR kit (Takara Biotechnology Co., Ltd., Tokyo, Japan). The PCR was conducted using a CFX96 thermocycler (Bio-Rad Touch, Bio-Rad) with a 20 μL PCR reaction composed of 2 μL of template cDNA, 10 μL of SYBP master-mix, 0.4 μL of each primer, and 7.2 μL of dd H_2O. Table 2 provides the primer information of the gene to be tested, while GAPDH was employed as the reference genes. The PCR amplification was followed by the visualization of the melting curve. The PCR amplification was followed by a visualization of the melting curve. The $2^{-\triangle\triangle Ct}$ relative quantitative method was employed to determine relative gene expression.

2.7 16s rRNA sequencing of ileal microorganisms

The ileac contents (100 mg) were processed with the QIAamp Fast Stool Mini Kit (Qiagen, Hilden, Germany) to isolate microbial DNA, which was stored at −80°C. The DNA concentration was determined with a Qubit® 3.0 fluorometer, and its integrity was checked by electrophoresis on a 2 % agarose gel. The V3 and V4 hypervariable regions of the 16S rRNA genes were amplified using the primers: 341F—CCTACGGRRBGCASCAGKVRVGAAT, and 806R–GGACTACNVGGGTWTCTAATCC. Adapters were attached to the ends of the amplicons to produce indexed libraries suitable for sequencing on an Illumina Miseq sequencer. The accuracy of the DNA library's concentration was checked using the Qubit® 3.0 fluorometer. The Illumina MiSeq was loaded with 10 nM DNA libraries which were multiplexed in accordance with the manufacturer's guidelines (Illumina, San Diego, CA, United States). The raw reads that were acquired were combined into continuous sequences based on the overlaps between them, and any low-quality or inadequate sequences were rejected. The software search (ver 1.9.6) was employed to align the obtained sequences into operational taxonomic units (OTUs) against the Silva 123 database, at a pre-clustered sequence identity of 97%. Venn diagrams were constructed using R (version 3.1.1) in order to highlight the shared and distinct OTUs across the four groups. QIIME (version 1.7.0) was employed to investigate the rarefaction curve, alpha diversity, and beta diversity. The Chao1, Shannon, and Simpson indices revealed the alpha diversity, while principal coordinate analysis (PCoA) was utilized to demonstrate the beta diversity. The non-parametric ANOSIM test was employed to evaluate the differences between groups. The ribosomal database program classifier was applied to assign taxonomic levels down to the genus level, including the kingdom, phylum, class, order, and family, with 80% certainty.

TABLE 2 Primer sequences of target genes.

Genes[a]	Forward primer (5′-3′)	Reverse primer (5′-3′)	Length	TM[b]°C	Accession No
OCLN	ACGGCAGCACCTACCTCAA	GGGCGAAGAAGCAGATGAG	123	51.7	XM_025144247.2
CLDN1	CATACTCCTGGGTCTGGTTGGT	GACAGCCA TCCGCA TCTTCT	100	51.3	NM_001013611.2
CLDN2	CCTACATTGGTTCAAGCATCGTGA	GATGTCGGGAGGCAGGTTGA	131	50.3	NM_001277622.1
ZO-1	CTTCAGGTGTTTCTCTTCCTCCTC	CTGTGGTTTCA TGGCTGGATC	144	51.5	XM_021098886.1
TNF-α	GAGCGTTGACTTGGCTGTC	AAGCAACAACCAGCTA TGCAC	176	55.4	NM_214022.1
IL-1β	ACTGGGCA TCAAGGGCTA	GGTAGAAGA TGAAGCGGGTC	154	55.6	NM_214005.1
IL-6	GCTGCGCTTCTACACAGA	TCCCGTTCTCA TCCA TCTTCTC	203	55.4	NM_204628.1
IL-10	AGAAATCCCTCCTCGCCAAT	AAATAGCGAACGGCCCTCA	121	51.2	NM_001004414.2
GAPDH	TGCTGCCCAGAACATCATCC	ACGGCAGGTCAGGTCAACAA	142	50-60	NM_204305

[a]Primer sequences of OCLN, CLDN1, CLDN2, ZO-1, TNF-α, IL-1β, IL-6, IL-10, and GAPDH.
[b]TM, melting temperature.

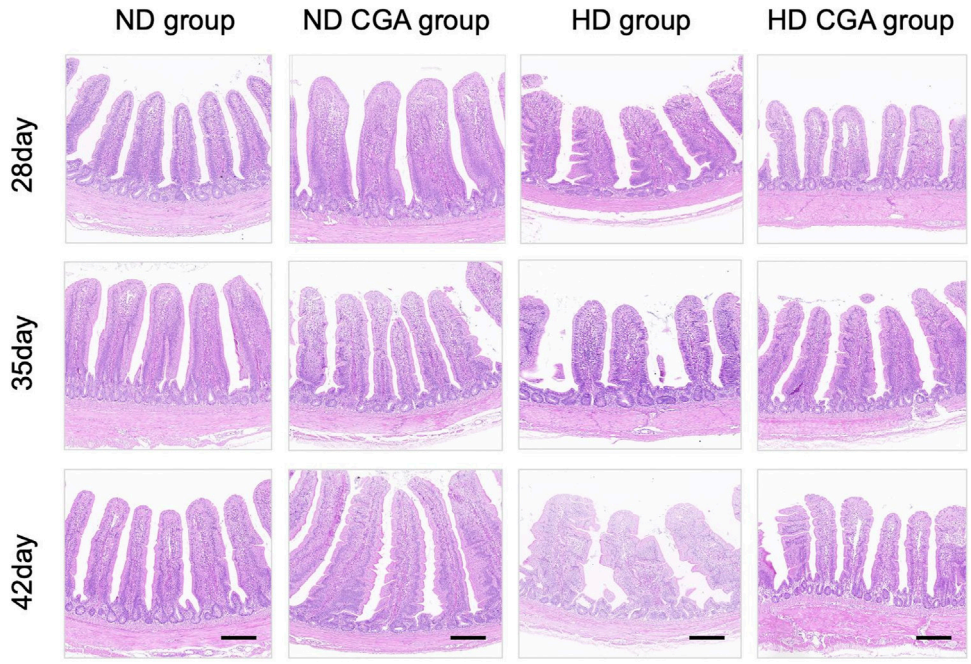

FIGURE 1
Effects of CGA on ileum morphology of broilers under HD stress. ND group, normal stocking density + basal diet; ND + CGA group, normal stocking density + basal diet +0.15% CGA; HD group, high stocking density + basal diet; HD + CGA group, high stocking density + basal diet +0.15% CGA. Scale bar = 100 μm.

The sequence data from our investigation were deposited in the NCBI SRA database (Acc. No. PRJNA916381).

2.8 Statistical analysis

The data were analyzed for adherence to a normal distribution using the SPSS statistical package (ver. 20.0 for Windows, SPSS Inc., Chicago, IL, United States). A one-way ANOVA was conducted to compare the data between groups, and Tukey's multiple comparison procedure was applied when the differences were deemed statistically significant. This statistical analysis only allows for the four treatments to be compared on the same day of the experiment. No discrepancies were observed in the data from different days. The SEM was used to express the results, with $p < 0.05$ being indicative of a significant difference and $0.05 \leq p < 0.1$ showing a notable distinction. The figures were created using GraphPad Prism 9 (GraphPad Software Inc., San Diego, CA, UnitedStates).

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TABLE 3 Effects of CGA on Ileac morphology of Broilers under HD stress.

Parameter	Days	Dietary treatment[a]				SEM	*p*-value
		ND	ND + CGA	HD	HD + CGA		
VH (μm)	28	747.83^{b}	874.31^{a}	680.47^{c}	725.17^{bc}	19.09	<0.01
	35	841.75^{b}	942.93^{a}	731.68^{c}	804.60^{b}	19.42	<0.01
	42	893.57^{b}	986.23^{a}	761.88^{c}	881.35^{b}	18.09	<0.01
CD (μm)	28	73.90	70.17	84.42	80.88	2.96	0.321
	35	91.18	82.42	96.80	97.98	3.68	0.113
	42	101.38	96.03	111.88	103.17	3.01	0.202
VH/CD	28	7.48	11.50	8.00	8.38	0.59	0.551
	35	9.37^{b}	12.22^{a}	6.80^{b}	8.21^{b}	0.59	<0.01
	42	8.98^{b}	10.65^{a}	6.38^{c}	8.40^{b}	0.41	<0.01

[a]ND, group, normal stocking density + basal diet; ND + CGA, group, normal stocking density + basal diet +0.15% CGA; HD, group, high stocking density + basal diet; HD + CGA, group, high stocking density group + basal diet+ 0.15% CGA, group. Values with different letters within the same row are indicative of statistically significant differences ($p < 0.05$, Tukey's HSD, test after one-way ANOVA). The statistical model used does not allow for a comparison between different days' data.

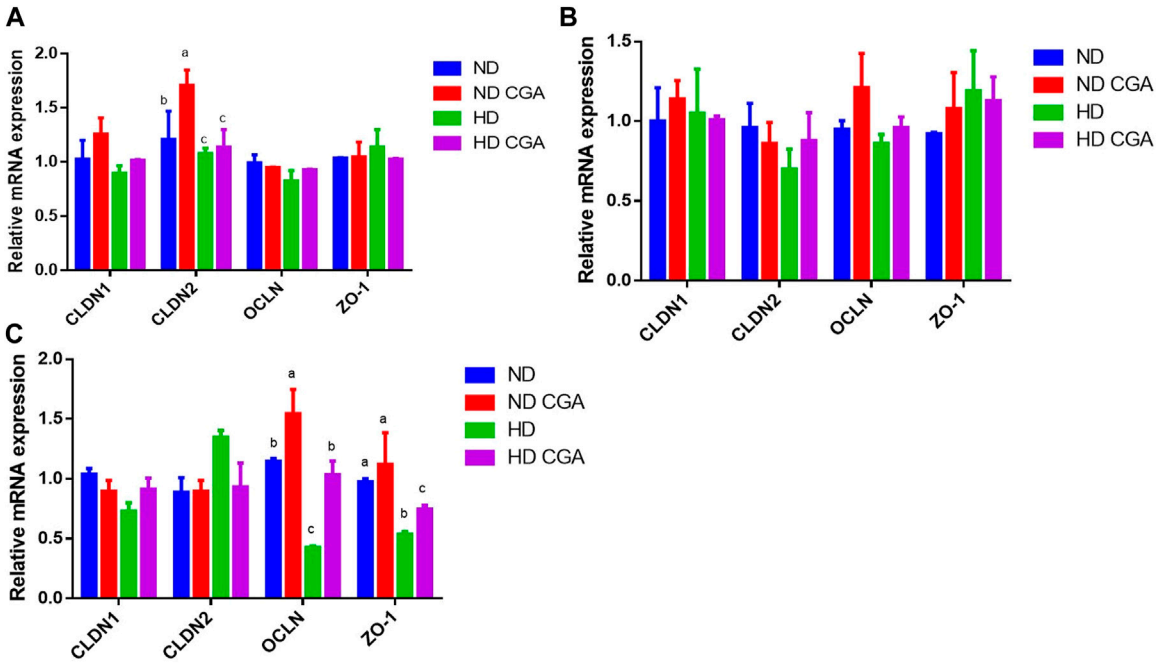

FIGURE 2
Effects of CGA on ileum mRNA expression of tight junction genes in broilers under HD stress. ND group, normal stocking density + basal diet; ND + CGA group, normal stocking density + basal diet +0.15% CGA; HD group, high stocking density + basal diet; HD + CGA group, high stocking density + basal diet +0.15% CGA. **(A)** Relative mRNA expression at day 28. **(B)** Relative mRNA expression at day 35. **(C)** Relative mRNA expression at day 42. Each vertical bar represents the mean ± SEM ($n = 10$). Values with different letters within the same row are indicative of statistically significant differences ($p < 0.05$, Tukey's HSD test after one-way ANOVA).

3 Results

3.1 Effect of CGA on intestinal morphology of HD chickens

The height of the villi, the crypt depth, and the V to C ratio are shown in Figure 1 and Table 3. On days 28, 35, and 42, the HD broilers had a significantly lower villus height than the NDs ($p < 0.01$), and the ND CGA birds had a higher villus height than the NDs ($p < 0.01$). On days 35 and 42, the HD CGA birds had a higher villus height than the HDs ($p < 0.01$). On days 35 and 42, the V/C was higher in the ND CGA group than in NDs ($p < 0.01$), and on day 42, the V/C was higher in the HD CGA broilers than in the HDs ($p < 0.01$).

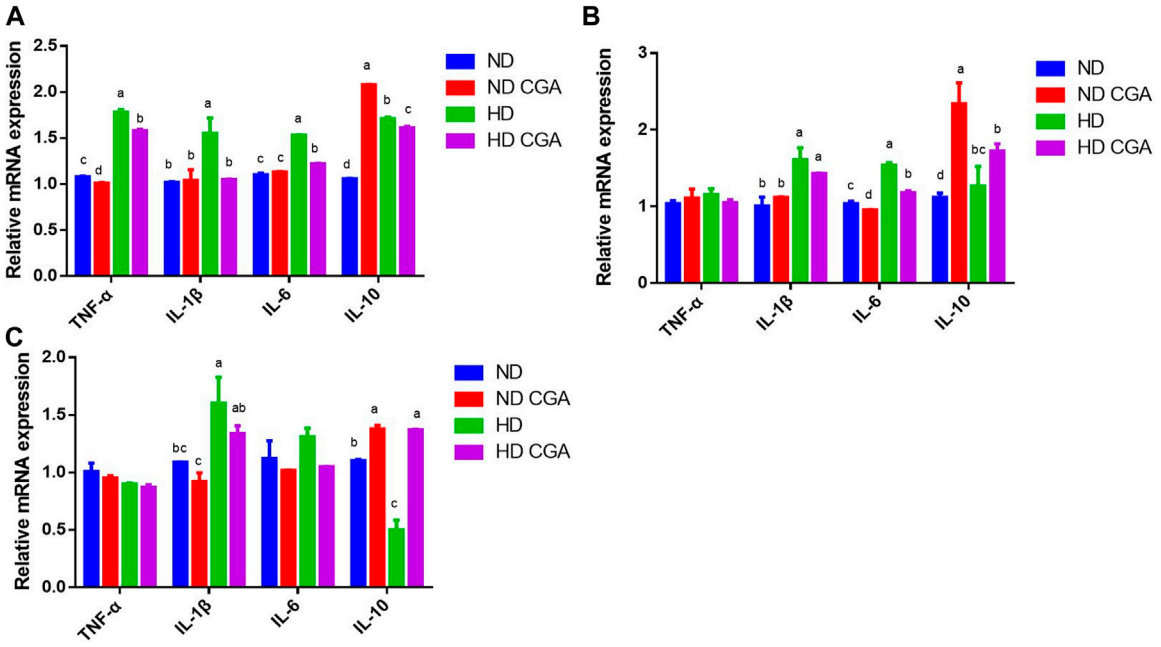

FIGURE 3
Effects of CGA on ileum mRNA expression of immune factors in broilers under HD stress. ND group, normal stocking density + basal diet; ND + CGA group, normal stocking density + basal diet +0.15% CGA; HD group, high stocking density + basal diet; HD + CGA group, high stocking density + basal diet +0.15% CGA. **(A)** Relative mRNA expression at day 28. **(B)** Relative mRNA expression at day 35. **(C)** Relative mRNA expression at day 42. Each vertical bar represents the mean ± SEM (n = 10). Values with different letters within the same row are indicative of statistically significant differences ($p < 0.05$, Tukey's HSD test after one-way ANOVA).

3.2 Effect of CGA on tight junction gene expression in ileum from HD chickens

The relative mRNA levels in the ileum of the different groups are shown in Figure 2. The expression of *CLDN-2* was lower ($p < 0.05$) in the HD broilers compared to the ND birds on day 28. The HDs showed a reduced ($p < 0.05$) expression of *OCLN* and *ZO-1* on day 42 when compared to the NDs. On day 28, the ND CGA group had a higher ($p < 0.05$) expression of *CLDN-2* than the ND group. The ND CGA group had a greater *OCLN* expression ($p < 0.05$) than the ND birds on day 42. *OCLN* expression was upregulated ($p < 0.05$) in the HD CGAs compared to the HDs on day 42.

3.3 Effect of CGA on inflammatory factors in ileum from HD chickens

The mRNA expression of ileal inflammatory cytokines in each group is shown in Figure 3. The expression of *TNF-α*, *IL-1β*, *IL-6*, and *IL-10* was upregulated ($p < 0.05$) in HD broilers compared to NDs at day 28, and decreased ($p < 0.05$) in the HD CGA birds compared to the HDs. The mRNA levels of the inflammatory factor genes, *IL-1β*, *IL-6*, and *IL-10* were significantly upregulated ($p < 0.05$) in HD broilers compared to NDs on day 35, and the expression of *IL-6* was lower ($p < 0.05$) in HD CGA than in HD. *IL-1β* was upregulated ($p < 0.05$) in the HD broilers relative to the NDs on day 42, and the expression of *IL-10* was higher in the HD CGAs ($p < 0.05$) compared to HD chickens without CGA.

3.4 Effect of CGA on microbial composition in ileum from HD chickens

Distinctive populations of ileal microorganisms were seen among the four groups (Figure 4). The Venn diagram shows 239 OTUs that were common to all four groups, while the ND, ND CGA, HD, and HD CGA groups were found to contain 1,009, 1,149, 967, and 922 distinct OTUs, respectively. In the ileum of ND, ND CGA, HD, and HD CGA groups, 492, 611, 459, and 397 distinct OTUs were counted, respectively in Figure 4A.

The samples' species reduction rates are depicted in Figure 4B. The dilution curve can be used to evaluate the accuracy of sequencing data and give an indication of the diversity of species present in the samples. When the sample size from each group exceeds 35,000, the graph tends to level off. The data indicate that the sequencing level is nearly maximized, the greatest diversity is present, and additional data will only yield a few additional species (OTUs). The sequences demonstrate the capacity to accurately portray the microbial population of a natural habitat and can be utilized for data analysis.

The PCoA (Figure 4C) showed that the community composition of the ND group was significantly different from the HD and ND CGA groups, and the difference was quite substantial. In addition, the ND CGA group had a more concentrated distribution than the other two groups. It is evident from Figures 4D–F that the α-diversity index in each group is close to 1, indicating that the data of each group accurately reflect the true ileal microflora composition. The Simpson and Chao bacterial richness and α-diversity indices of HD birds were

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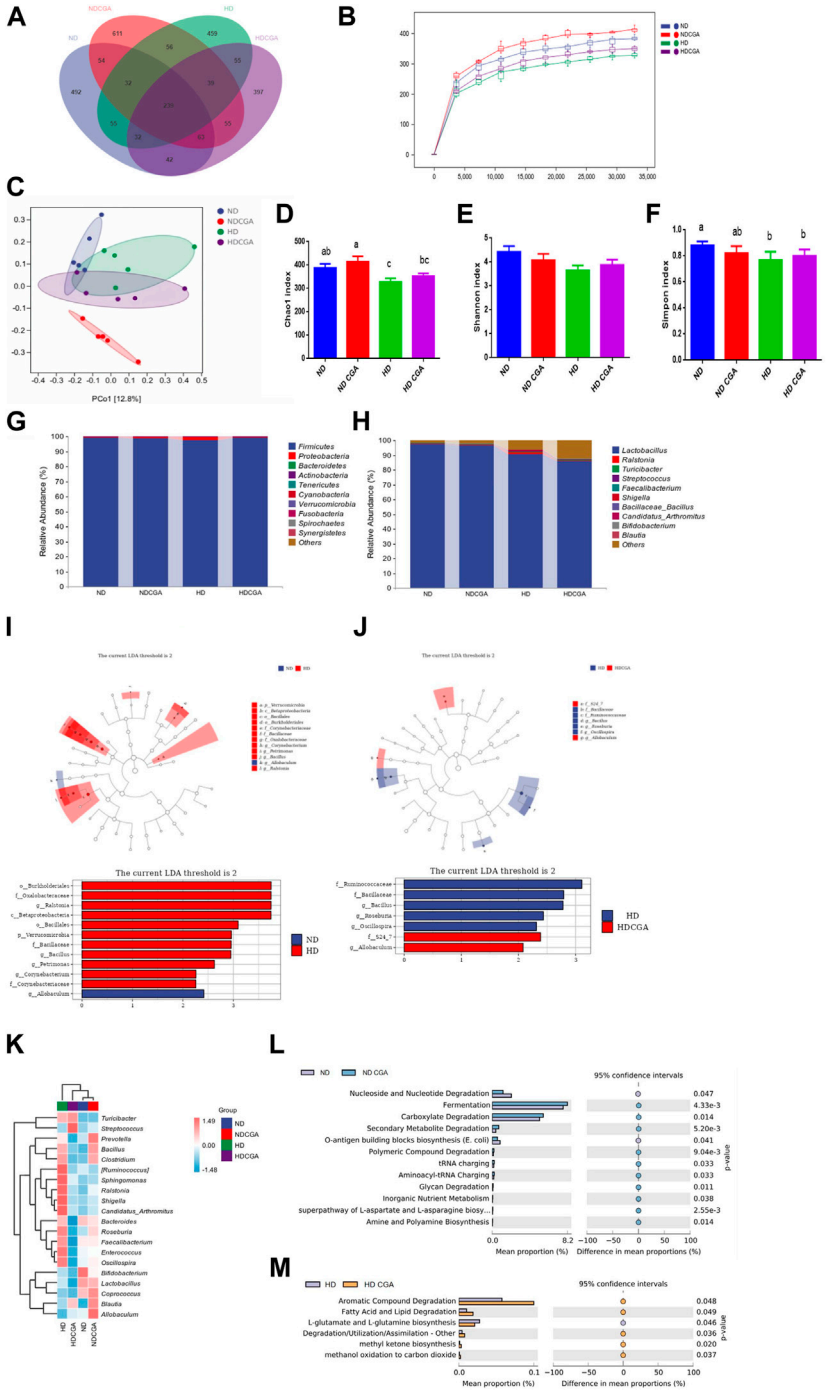

FIGURE 4
The impact of CGA consumption on the bacterial population in the ileum of chickens from the ND, HD, ND CGA, and HD CGA groups. **(A)** Venn diagram showing unique and shared numbers of genera predicted. **(B)** Rarefaction Curve. **(C)** Two-dimensional OTU abundance based principal coordinate analysis (PCoA) of ileac microbiota. D-F. CGA was found to increase caecal microbial alpha diversity as measured by Simpson and Chao1 indicators. **(G)** Microbial composition at the phylum level. **(H)** Microbial composition at the genus level. **(I-J)** Leaf and bar plots obtained by linear discriminant analysis effect size (LEfSe) analysis showed differences in the abundance of broiler fecal microbes. **(K)** A graphical representation of the range of species present in the top twenty genera in each sample. Pink represents positive correlation and blue indicates negative correlation. **(L-M)** COG functional classification and differences in COG abundance.

significantly reduced ($p < 0.05$) compared to ND, as indicated by the decline in single species indices.

Analysis of the taxonomic units indicated that Firmicutes was the dominant phylum among the four groups (Figure 4G; Table 4), making up 99.06%, 98.68%, 97.63%, and 99.00% of the populations, respectively, while Proteobacteria and Bacteroidetes accounted for 0.33%, 0.70%, 1.77%, and 0.55%, and 0.28%, 0.32%, 0.21%, and 0.24%, respectively. The Proteobacteria count for the HD group was

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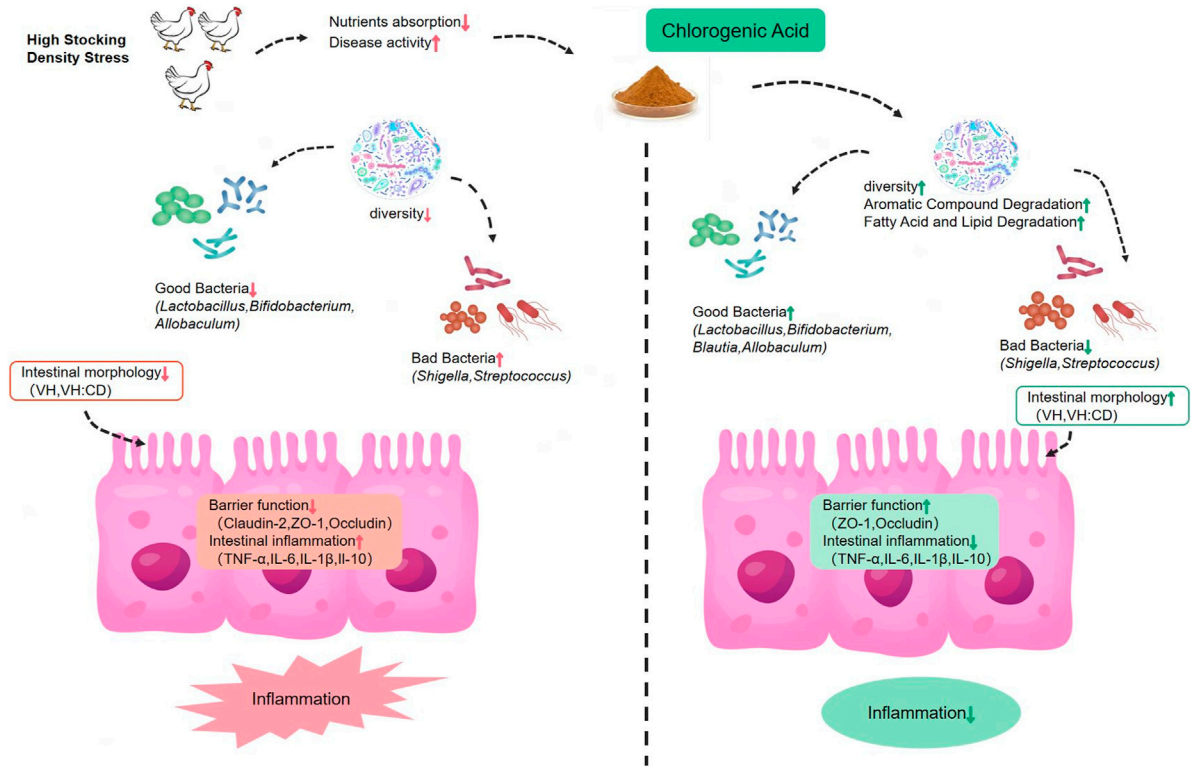

FIGURE 5
Systematic analysis of the effects of dietary chlorogenic acid on inflammatory index, intestinal barrier function and intestinal microflora in broilers under high stocking density stress.

TABLE 4 Composition of ileum microbiota of broilers at phylum level.

Microorganism	Dietary treatment[a]				SEM	*p*-value
	ND	ND + CGA	HD	HD + CGA		
Firmicutes	99.06%	98.68%	97.63%	99.00%	0.012	0.160
Proteobacteria	0.33%^{c}	0.70%^{b}	1.77%^{a}	0.55%^{b}	0.011	0.039
Bacteroidetes	0.28%^{b}	0.32%^{a}	0.21%^{b}	0.24%^{b}	0.008	0.042
Actinobacteria	0.12%	0.11%	0.17%	0.12%	0.006	0.091
Fusobacteria	0.11%	0.14%	0.13%	0.06%	0.004	0.327
Cyanobacteria	0.01%	0.02%	0.01%	0.01%	0.001	0.057

[a]ND, group, normal stocking density + basal diet; ND + CGA, group, normal stocking density + basal diet +0.15% CGA; HD, group, high stocking density + basal diet; HD + CGA, group, high stocking density group + basal diet+ 0.15% CGA, group. Values with different letters within the same row are indicative of statistically significant differences ($p < 0.05$, Tukey's HSD, test after one-way ANOVA).

significantly higher and the Bacteroidetes count was significantly lower than in NDs ($p < 0.05$). The numbers of Proteobacteria and Bacteroidetes in the ileum of broilers in the HD CGA group was significantly higher than in the HDs ($p < 0.05$). Cyanobacterial abundance was likely affected by CGA, but the difference was statistically non-significant ($p = 0.057$). The predominant genera in the ileum of the four groups as shown in Figure 4H and Table 5 were *Lactobacillus* (97.62%, 96.48%, 90.84%, 86.02%), with minor amounts of *Ralstonia* (0.17%, 0.47%, 1.12%, 0.32%), *Turicibacter* (0.02%, 0.03%, 0.45%, 0.32%), *Streptococcus* (0.12%, 0.11%, 0.28%, 0.16%), *Faecalibacterium* (0.15%, 0.15%, 0.17%, 0.10%), *Shigella* (0.02%, 0.06%, 0.35%, 0.03%), Bacillaceae *Bacillus* (0.02%, 0.20%, 0.18%, 0.05%), *Candidatus arthromitus* (0.02%, 0.07%, 0.30%, 0.06%), *Bifidobacterium* (0.13%, 0.09%, 0.04%, 0.08%), and *Blautia* (0.08%, 0.10%, 0.08%, 0.09%). The ileac *Lactobacillus* and *Bifidobacterium* count in HD birds was significantly lower than those of ND ($p < 0.05$). The HD birds had a significantly larger number of *Turicibacter*, *Streptococcus*, and *Shigella* than the ND ($p <$

TABLE 5 Composition of ileum microbiota of broilers at genus level.

Microorganism	Dietary treatment[a]				SEM	*p*-value
	ND	ND + CGA	HD	HD + CGA		
Lactobacillus	97.62%[a]	96.48%[a]	86.02%[b]	90.84%[b]	0.156	0.046
Ralstonia	0.17%	0.47%	1.12%	0.32%	0.008	0.251
Turicibacter	0.02%[c]	0.03%[c]	0.45%[a]	0.32%[b]	0.007	0.026
Streptococcus	0.12%[b]	0.11%[b]	0.28%[a]	0.16%[b]	0.002	0.047
Faecalibacterium	0.15%	0.15%	0.17%	0.10%	0.001	0.256
Shigella	0.02%[b]	0.06%[b]	0.35%[a]	0.03%[b]	0.004	0.031
Bacillaceae_*Bacillus*	0.02%	0.20%	0.18%	0.05%	0.001	0.086
Candidatus_Arthromitus	0.02%	0.07%	0.30%	0.06%	0.003	0.301
Bifidobacterium	0.13%[a]	0.09%[a]	0.04%[b]	0.08%[ab]	0.003	0.035
Blautia	0.08%	0.10%	0.08%	0.09%	0.001	0.353

[a]ND, group, normal stocking density + basal diet; ND + CGA, group, normal stocking density + basal diet +0.15% CGA; HD, group, high stocking density + basal diet; HD + CGA, group, high stocking density group + basal diet+ 0.15% CGA, group. Values with different letters within the same row are indicative of statistically significant differences ($p < 0.05$, Tukey's HSD, test after one-way ANOVA).

0.05). The HD CGA broilers had fewer *Turicibacter*, *Streptococcus*, and *Shigella* compared to the HD group ($p < 0.05$), however, the other dominant bacterial genera were not distinguishable among the four groups.

The LEfSe results (Figures 4I, J) indicated that Burkholderiales, Oxalobacteraceae, *Ralstonia*, and Betaproteobacteria were the major bacterial groups present in HD chickens compared to ND. The HD CGA broilers featured *S24-7* and *Allobaculum* as their primary colony members and biomarkers, which was different from the HD group. To illustrate the variation in species and their abundance trends among the groups, the top twenty genera in terms of relative abundance were grouped according to genus and visualized in a heat map (Figure 4K). The ND group was significantly populated with *Bifidobacterium*, *Lactobacillus*, and *Coprococcus*, while *Allobaculum*, *Prevotella*, *Blautia*, and *Coprococcus* were highly represented in ND CGA. The HD birds were populated with *Shigella*, *Ruminococcus*, *C. arthromitus*, and *Sphingomonas*; *Streptococcus*, *Turicibacter*, and *Blautia* were present in increased numbers in HD CGA birds. A comparison between the ND and ND CGA groups revealed that the ND CGA dataset possessed a higher level of annotation richness than the ND ($p < 0.05$), with tRNA charging, fermentation, and degradation of carboxylate, secondary metabolites, and polymeric compounds, being the most common annotations (Figures 4L, M). Comparison between the HD and HD CGA datasets, showed that the HD CGA group had a notably higher number of annotations ($p < 0.05$) than the HD. The most common annotations were aromatic compound degradation, lipid degradation, degradation/utilization/assimilation, methyl ketone biosynthesis, and methanol oxidation to carbon dioxide.

4 Discussion

Our previous research has verified that HD stress can significantly hinder the growth and development of broilers, causing physical damage to the jejunum and increasing the level of inflammation, and altering the microbial composition of the jejunum. Nevertheless, supplementing feed with CGA can mitigate the oxidative stress caused by HD stress, thus promoting the growth and development of broilers (Liu et al., 2023). Consequently, the HD stress could lead to damage in other parts of the digestive tract. The structure of the intestines is pivotal in preserving intestinal health (Pourabedin et al., 2014). The small intestine's digestive and absorptive capabilities can be gauged by looking at the intestinal villus height, crypt depth, and the ratio of villus height to crypt depth (Farahat et al., 2021), which provide a comprehensive evaluation of the organ's functioning. Therefore, we studied the effects of CGA supplementation on chickens dealing with HD stress by looking at the morphological changes in their intestines over the course of their growth. The results indicated that HD had a more detrimental effect on the villus height of the ileum; nevertheless, CGA was able to lessen the detrimental impact. This suggests that CGA can preserve the healthy development of intestinal villi and avert HD stress. The work of Zhang et al. (2018) demonstrated that inclusion of CGA in the diet of piglets had a beneficial impact on the intestine structure of weaned piglets, leading to a significant increase in small intestinal villus height as well as the V/C ratio of the jejunum and ileum. The study found that CGA supplementation significantly increased the ileum villus height and V/C ratio, and strengthened the intestinal barrier of weaned rats exposed to lipopolysaccharide (Ruan et al., 2014).

The poultry intestine is a complex structure comprised of four distinct parts: physical, chemical, immune, and microbial, that act as a barrier between the animal body and the outside environment (Xiao et al., 2017). The physical barrier acts as a protective shield between the intestinal lumen and the internal environment. When chickens are subjected to stress from crowding, the connections between intestinal jejunal epithelial cells can be weaken, thus allowing a greater number of molecular pathogens and pathogenic bacteria to pass through and stimulate the intestinal immune system (Matricon et al., 2012). Our findings demonstrated

that exposure to HD stress caused a considerable decline in the *Occludin* and *ZO-1* mRNA levels in the ileum of broilers, thus disrupting the intestine's ability to act as a barrier. Many investigations have determined that stress has a detrimental effect on *Occludin* and *ZO-1* in the intestinal mucosa of poultry (Varasteh et al., 2015; Zhang et al., 2017). CGA in the diet of broilers led to a marked increase in *Occludin* and *ZO-1* mRNA levels in the ileum, and successfully averted the disruption of the barrier's integrity; this shows that CGA has a beneficial effect in reinforcing the mechanical barrier of the intestine. The immune system of the intestinal mucosa is integral to the onset and progression of intestinal inflammation. Cytokines play key roles in an animal's immune response and defensive mechanisms (Li et al., 2014). An imbalance in the levels of pro-inflammatory and anti-infective cytokines, such as IL-6, IL-1β, and TNF-α in the intestinal mucosal immune system can lead to an impaired immune response and the development of intestinal inflammation (Delgado et al., 2015). Our research has revealed that HD stress can increase intestinal inflammation by altering the expression of inflammatory factors like IL-6, IL-1β, and TNF-α. Another investigation confirmed that HD stress resulted in the induction of the pro-inflammatory cytokines, IL-1β and TNF-α (Bai et al., 2022). Our research indicated that HD stress could alter the intestinal immune balance from an anti-inflammatory to a pro-inflammatory state after 28, 35, and 42 days. Despite this, the incorporation of CGA in the diet was successful in diminishing the presence of inflammatory elements. It appears that CGA has the potential to alleviate inflammation and reduce the pressure of HD.

The intestine not only serves as the site for digestion and nutrient uptake, but is also the body's most extensive immune organ (Adedokun and Oloejede, 2019). It is essential to sustain a healthy gut for the successful breeding of poultry (Hafez and Attia, 2020). Current research has shown that the composition of the gut microbiome can significantly affect the wellbeing of poultry as well as their productivity (Tan et al., 2010). The intestinal microbial population is an indispensable part of the body, instrumental in maintaining homeostasis not only by facilitating metabolic processes like digestion, nutrient uptake, and energy regulation, but also by preserving the intestinal barrier, and regulating the nervous, endocrine, and immune systems (Ruan et al., 2014).

The present study revealed that CGA supplementation led to a significant increase in microbial diversity in ileac samples from HD-stressed chickens, as demonstrated by the Simpson and Chao1 indices, as well as the PCoA analyses that indicated that the ND CGA group had a dense and uniform bacterial population structure. This type of diversity, referred to as alpha diversity, encompasses the richness, variety, and evenness of species found in a single habitat. Research has demonstrated that CGA has the capability to alter the intestinal microbiota of animals leading to a greater variety of microbial species (Chen et al., 2021). A study found that CGA could counteract the dextran sodium sulfate (DSS)-caused decline in gut microbial diversity in mouse feces, as well as boost the number of *Lactobacillus*, implying that CGA might be able to safeguard the colon from DSS-induced damage by raising the variety of microbes in the gut (Zhang et al., 2019). Research has indicated that Bacteroidetes and Sclerenchyma bacteria located in the digestive system of animals are essential for metabolizing various substances (Ruan et al., 2014). Schreuder et al. (2019) found that the majority of phyla present in the feces of laying hens were Firmicutes and Bacteroidetes. The results of this research demonstrated that, on a phylum level, the gut bacteria were primarily Firmicutes and Proteobacteria. The numbers of Bacteroidetes and Firmicutes fluctuated in response to how effectively energy was acquired from the food consumed, causing an increase in the proportion of Bacteroidetes relative to Sclerenchyma, which could enhance the growth of animals (Turnbaugh et al., 2006). The ND CGA group exhibited a greater prevalence of Bacteroidetes at the phylum level relative to the ND group. Some studies revealed that the inclusion of CGA in the diet of chicken increased Bacteroidetes and decreased Firmicutes in the gut, suggesting that CGA may be able to combat intestinal inflammation (Wang et al., 2019). These conclusions are similar to those found in a previous study (Liu et al., 2023) providing evidence that dietary CGA lessened the impact of HD stress on the gut microbiota of broilers, ultimately leading to better intestinal health.

The presence of *Lactobacillus* in the intestine at the genus level can diminish the adhesiveness of bacterial pathogens like *Escherichia coli* and *Salmonella* to the intestinal wall and improve an animal's immunity (Wang et al., 2021). Song and others determined that levels of *Lactobacillus* in the digestive tract of chickens were reduced when the birds were stressed (Song et al., 2013). In the current study, HD stress was also seen to reduce the numbers of *Lactobacillus* in the gastrointestinal tract of broilers. The bacterium *Shigella* is the primary cause of bacillary dysentery. A major indication of *Shigella* infection in chickens is the presence of diarrheal stools, as well as ulceration and inflammation of the intestinal lining. Infants and juveniles are particularly vulnerable to *Shigella* infection (Ashkenaz, 2004). Our results demonstrated a large decrease in the prevalence of *Shigella* in HD chickens fed CGA, from 0.35% with no CGA to 0.03% in the HD CGA group, suggesting that CGA can impede the escalation of detrimental bacteria due to HD and boost intestinal health. Bifidobacteria generate a significantly greater amount of lactic and acetic acid than lactobacilli, and bifidobacteria also have a part to play in keeping the gastrointestinal barrier stable, influencing local and systemic immune responses, hindering the penetration of pathogens, and aiding the conversion of indigestible dietary components into beneficial molecules (Cisek and Binek, 2014). Some studies revealed that stress caused a decrease in the numbers of viable lactobacilli and bifidobacteria in the small intestine of broilers (Song et al., 2014). Our research showed that when broilers were subjected to HD, the populations of *Lactobacillus* and *Bifidobacterium* in the ileal digest decreased, while *Streptococcus* and *Shigella* populations increased. The damaging alterations to intestinal microbiota could be a factor in the stress-induced damage to intestinal morphology and permeability associated with raising chickens under HD conditions. *Blautia* may be beneficial in reducing the negative effects of HD stress on the gut microbe populations in chickens because of its capacity for producing short chain fatty acids (SCFAs) (Delgado et al., 2015). In our work, the ND group was found to have a higher prevalence of *Allobaculum* than the HD group, according to LEfse analysis. *Allobaculum* is a genus of Firmicutes that produces high levels of butyrate and efficiently utilizes glucose in the digestive tract. Our data also showed that increased *Allobaculum* levels were associated with higher yields of SCFAs, particularly butyrate (Balakrishnan et al., 2021). In comparison to HD chickens, the HD CGA group was populated with a greater abundance of S24-7 and *Allobaculum*, and

these two bacterial species have been linked to the promotion of health (Kumar et al., 2019; Cheng et al., 2021).

The results of PICRUSt's predictive analysis indicated that the inclusion of CGA in feed can bolster the ability of resident bacteria to ferment or break down carboxylates, degrade secondary metabolites and polymeric compounds, and charge tRNAs. The microorganisms within the digestive tract are essential for carrying out various metabolic processes. Most creatures, including chickens, do not possess enzymes for carbohydrate metabolism, such as glycoside hydrolase, polysaccharide lyase, and carbohydrate esterase (Yeoman et al., 2012). The inability of chickens to process fiber, starch, cellulose, and pectin would require the addition of enzymes to provide adequate nutrition (Jha and Mishra, 2021), but a better way to solve this problem is through the use of gut microbiota. Microorganisms in the intestines aid the animal body in breaking down and assimilating substances that are difficult to digest, creating nutrients that can be utilized (Krajmalnik-Brown et al., 2012). As already noted, some bacteria can ferment carbohydrates to produce short-chain fatty acids, which inhibit pathogens and serve as sources of nourishment and energy for host organisms (den Besten et al., 2013). Many research studies have demonstrated that the host's capacity to take up ions such as calcium, magnesium and iron is largely enabled by the presence of SCFAs such as acetate, propionate, and butyrate (Yeoman et al., 2012). Consequently, incorporating CGA into broiler feed could enhance the capacity of HD broilers to overcome stress and absorb nutrients to produce energy for growth. Dietary CGA promotes the breakdown of aromatic compounds, fatty acids, and lipids, the production of methyl ketones, and the oxidation of methanol to carbon dioxide as well as other types of degradation, utilization, and assimilation (Upadhyay and Mohan Rao, 2013).The presence of certain bacteria, such as *lactobacilli*, *enterococci*, *bifidobacteria*, *Clostridium* spp. and *Bacteroides* spp. in the gut, can lead to the degradation of bile acids, making it more difficult for fats to be broken down, absorbed, and stored (Begley et al., 2006). In addition, intestinal bacteria also synthesize fatty acids including conjugated linoleic acid (CLA), which is essential for maintaining the health of humans and animals. Research has shown that supplementation with CLA can lead to a heightened level of catalase activity in the liver of chickens, which may be correlated with a decrease in the fat content of the animals (Rahman et al., 2001). To summarize, administering chlorogenic acid to HD-stressed broilers can help optimize fat metabolism and decrease the amount of fat in their bodies.

5 Conclusion

As depicted in Figure 5, our findings demonstrate that HD stress can result in a reduction of villi size and the V/C ratio in the ileum, decreased expression of tight junction mRNAs, increased expression of pro-inflammatory cytokines, reduced gut microbial diversity, and an increase in the presence of potentially harmful bacteria such as *Turicibacter* and *Shigella*. The supplementation of broiler feed with CGA has been found to enhance the morphology of the ileal intestinal tissue of HD broilers, upregulate the mRNA of tight junction, and reduce the expression of pro-inflammatory cytokines, as well as improve the microbiome composition in the ileum which the phylum-level analysis revealed a decrease in Proteobacteria, and a higher Firmicutes/Bacteroidetes ratio. The presence of *Bacillus* and *Blautia* could be augmented at the genus level, thus allowing for effective regulation of the gut microbiome and improvement in the intestinal health of broilers.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/, No. PRJNA916381.

Ethics statement

The animal study was reviewed and approved by The animal protocol was approved by the Institutional Animal Care and Use Committee (IACUC) of Henan University of Science and Technology, and experimental procedures followed IACUC guidelines.

Author contributions

Investigation, Y.L. (Yu-qian Li), Y.L. (Yan-hao Liu) and X.H.; methodology, X.H., H.T. and K.L.; software, Y.L. (Yu-qian Li), H.T. and K.L.; formal analysis, Y.Z., D.B. and W.Z.; data curation, Y.L. (Yu-qian Li), X.H. and Y.Z.; writing the original draft, Y.L. (Yu-qian Li), Y.Z. and D.B.; 477 reviewing and editing, D.B., K.I., W.Z., C.Z. and B.Z.; supervision, Y.M.; and project administration, Y.M. All authors have read and approved this submitted manuscript version.

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Conflict of interest

The 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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Are some brightly coloured European wild birds toxic?

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Some colourful European wild birds display contrasting bright colours. These are often striking black and white or primary and secondary colours, and with the same basic plumage livery shared by both sexes. These contrasting colours are, one assumes, very obvious to predators when these birds forage diurnally, or continue other aspects of their life history, including courting, nest building and rearing their brood. Here, I posit that such birds may be displaying aposematic warning colouration, possibly enhanced by chemical noxious substances in their flesh and/or feathers, as is already known in certain bird species, including colourful as well as cryptic species. The warning colouration may be Müllerian or Batesian in nature, or maybe is a ruse to suggest to predators that they are in some way noxious, and thus to be avoided. Even if not actually noxious as such, this may give the intended prey time to escape. Certainly, birds like the very obviously blue-black and white patterned Eurasian magpie, *Pica pica*, are largely avoided by the Eurasian sparrowhawk, *Accipiter nisus*, although this of course could be a size-related avoidance, as other larger raptorial birds do predate it. These various possibilities are discussed in the present article.

KEYWORDS

aposematic, Batesian mimicry, birds, Müllerian mimicry, plumage, predators, toxicity, warning colouration

Introduction

As is well-established (Cott, 1940), many examples of cryptic camouflage occur in the natural world, with some animals mimicking stones, flowers, twigs and branches, or the ground itself. Such camouflaged animals abound in many groups, especially insects, but also including fish, amphibians, reptiles, mammals and birds. They do so either to escape predation or in the case of predators, to capture prey. For example, the Eurasian stone-curlew, *Burhinus oedicnemus* (L.), disappears against the dry, often stony ground on which it lives and nests. Similarly, the tawny frog mouth, *Podargus strigoides* (Latham) of Australia, appears to be a part of a branch on a tree, until that is, it opens its mouth wide to catch a flying insect.

Many animals also show aposematic warning colouration: bright contrasting colours, such as yellow and orange, yellow and red or black and white, often displayed as sudden flash colouration, as in the hind wings of moths like the red underwing moth, *Catocala nupta* (L.) (Lepidoptera: Erebidae; Rothschild and Lane, 1960). This is to warn would-be attackers that they are to be avoided; that they are in some way chemically defended, either with noxious chemical sprays (e.g., skunks), poisonous venom (e.g., certain snakes like the coral snake), toxic skin or flesh (e.g., certain tropical frog species), or the warning is a ruse (Cott, 1940; Baker and Parker, 1979; Howse, 2014, 2021; Hedley and Caro, 2022). Perhaps then it is not surprising that certain bird species also apparently display warning colours, except that we have perhaps often failed to recognise the true purpose of such colourful plumage. We assess such birds as being harmless and hence ignore these warning signs, as they are apparently not a threat to us. But this may well

not be true of other, smaller creatures like Passerine birds, reptiles and predatory arthropods which see – and duly take notice of – such signals (Howse, 2014, 2021, 2022).

Are some birds toxic including European species?

So is it true? Do some bird species, including European species, show warning colours and signals and are they potentially toxic if eaten, not only by us but by other animals? It is known that a few tropical birds display warning colours and are toxic (Baker and Parker, 1979; Hedley and Caro, 2022). One of the best examples is the hooded pitohui, *Pitohui dichrous* (Bonaparte) of New Guinea (Figure 1A), a medium-sized songbird with rich chestnut and black plumage, which contains a range of batrachotoxin (BTX) compounds (extremely potent cardio- and neurotoxic steroidal alkaloids found in certain species of beetles, birds, and frogs) in its skin, feathers and other tissues (cf. Bodawatta et al., 2023 and references therein). These are obtained from its diet, a mix of fruit, seeds and insects and other arthropods. The sequestered toxins in its feathers and flesh are used by the bird to provide it with some degree of protection against predators, probably mainly birds and snakes, since only one larger predatory mammal, a marsupial, exists in New Guinea (Leary et al., 2016). Both sexes have similar plumage. The toxins are especially concentrated in the breast and belly feathers, such that these not only protect the adult birds, but may also rub off on eggs and young in the nest (Dumbacher et al., 2000; Yeung et al., 2022). That such warning colouration is shown by other, poisonous pitohuis in New Guinea (Jønsson et al., 2008), as well as bird species of other genera, e.g., the blue-capped ifrit, *Ifrita kowaldi* (De Vis) (Dumbacher et al., 2000; Bodawatta et al., 2023) appears to be a case of convergent evolution leading to Müllerian mimicry, i.e., gaining additional protection by mimicking other contrastingly, brightly coloured noxious/venomous animals (Cott, 1940; Baker and Parker, 1979; Hedley and Caro, 2022). The Hooded Pitohui's defence is reinforced by a strong odour (Dumbacher et al., 1992). Its livery is also apparently mimicked by other un-related, non-toxic birds, these birds hence showing Batesian mimicry,[1] where a non-toxic animal mimics a toxic or dangerous model organism (cf. Cott, 1940).

With this in mind, it occurred to me whist watching the wild birds coming to the bird table and feeders in my garden in North Devon, SW England that the many European Goldfinches, *Carduelis carduelis* (L.) (Figure 1B) that visit with their red, black and white face masks, black and white wing and tail feathers and bright yellow flash colouration on the wings, may be displaying warning colouration (Loxdale, 2022; cf. also Howse, 2014, p. 85). In the same article, I also surmised that other common garden birds like the Great Spotted woodpecker, *Dendrocopos major* (L.), tits, e.g., Great tit, *Parus major* L., and even wagtails, e.g., Pied wagtail, *Motacilla alba* L. (Figures 1C–E), may also be showing warning colouration. If their feathers and flesh are to some degree toxic to would-be predators, these could be further examples of avian Müllerian mimicry. On the other hand, if the birds are not toxic, then such examples could be evidence of Batesian mimicry. Besides the pitohuis and blue-capped ifrit of New Guinea, other birds from other taxa and geographical regions are known to have toxic flesh as a result of sequestering toxins from their seed and insect diet. These include the

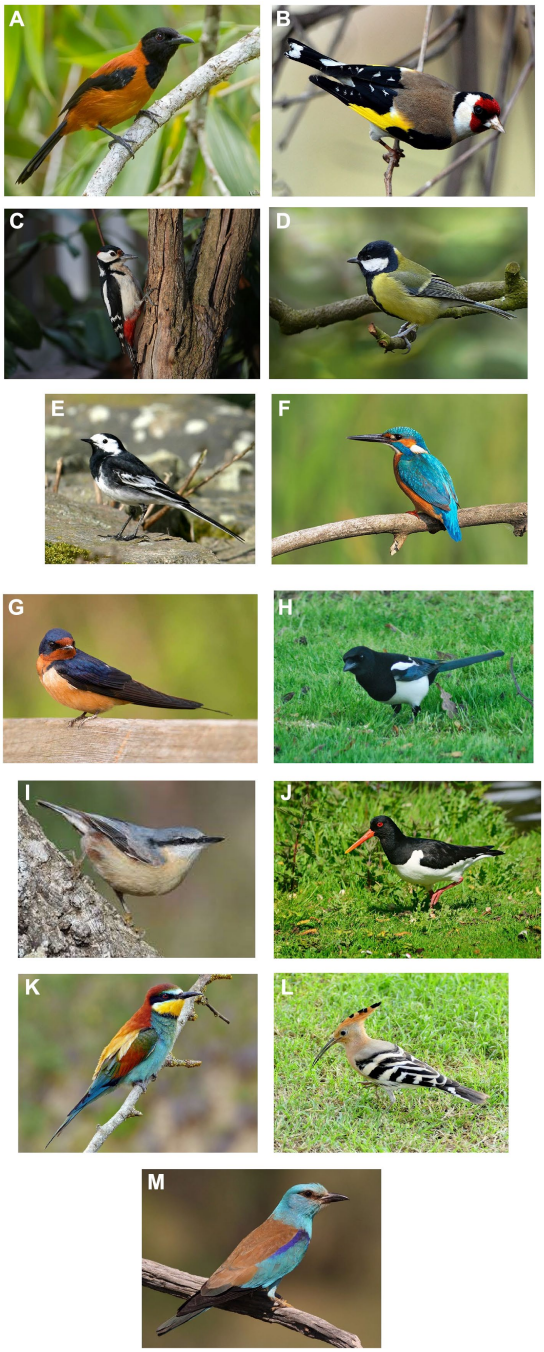

FIGURE 1
(A-M): Left to right, from top: **(A)** Hooded Pitohui; **(B)** Goldfinch; **(C)** Great Spotted Woodpecker; **(D)** Great Tit; **(E)** Pied Wagtail; **(F)** Kingfisher; **(G)** Barn Swallow; **(H)** Eurasian Magpie; **(I)** Eurasian Nuthatch; **(J)** Eurasian Oystercatcher; **(K)** European Bee-eater; **(L)** Hoopoe; **(M)** Roller. Photo credit: **(A)** eBird,;Frédérick Pelsy **(B)** eBird, Eitan Altman; **(C)** Pixabay, Kurt Bouda; **(D)** Pixabay, Christiane; **(E)** Nature Spot, David Nicholls; **(F)** Pixabay, Timo Schlüter; **(G)** iStock, Trevor Jones; **(H)** NautreSpot, Barbara Cooper; **(I)** eBird, Santiago Caballero Carrera; **(J)** Pixabay, Mabel Amber; **(K)** eBird, Josep del Hoyo; **(L)** Pixabay, Harald Landsrath; **(M)** eBird, Rafael Merchante.

1 https://en.wikipedia.org/wiki/Hooded_pitohui

cryptically-coloured European or Common quail, *Coturnix coturnix* (L.) (Korkmaz et al., 2011) and the Ruffed grouse, *Bonasa umbellus* (L.) of North America (Causey, 2020). Goldfinches consume seeds and feed insects to their young, whilst the diet of tits and wagtails is largely insectivorous. Herbivorous insects like caterpillars and aphids, the principal food of many small insectivorous Passerine birds, are known to harbour toxins from their plant-based diet (Opitz and Müller, 2009). Cott (1947, p 489) considers these to be a likely source of some of the toxins found in distasteful birds.

In addition to the aforementioned European garden birds, other species may be displaying warning colouration related to Müllerian or Batesian mimicry, and like the Hooded pitohui and goldfinch, show a lack of sexual dimorphism in plumage colouration. In this light, the Eurasian kingfisher, *Alcedo atthis* (L.), Barn swallow, *Hirundo rustica* L., Eurasian magpie, *Pica pica* (L.), Eurasian nuthatch, *Sitta europaea* L. (Pallas), Eurasian oystercatcher, *Haematopus ostralegus* L., and the rare visitors to northern Europe, including the British Isles, the European bee-eater, *Merops apiaster L.*, Eurasian hoopoe, *Upupa epops* L., and Roller, *Coracias garrulus* L. come to mind (Figures 1F–M). There are probably other gaudy species also that fit the category of noxious species, as Cott (1947) implies. Perhaps we just do not see it, but other animals do, notably would-be predators. These are suitably impressed enough, if only momentarily, to generally avoid these birds when they come across them, allowing the intended prey to escape. Certainly, the largely insectivorous hoopoe, which shows aposematic warning colouration in the form of contrasting black and white striped wings and tail, and a striking black and white-tipped orange crest surmounting its salmon pink head and upper torso (Figure 1L), is known to have anti-predator chemical defences in the nest. The uropygial gland of the incubating and brooding female produces a foul-smelling liquid, as do the glands of nestlings, these secretions being rubbed into the plumage. The secretion smells like rotting meat and is used to deter predators and parasites and possibly act as an antibacterial agent (Martin-Platero et al., 2006). The secretions stop just prior to the young leaving the nest (Fry, 2003).

Discussion and conclusions

An excellent pioneering insight into the edibility of different bird species was provided in the 1940s by Cott (1947). This concerned a 4-year comparative quantitative study of the attractiveness and acceptability of the flesh, especially the breast meat, of a range of bird species inhabiting Egypt and the Lebanon, whilst the author was stationed out there in the military during World War 2. Most of the birds tested in the study (38 spp.) were directly shot. Their edibility was assessed by exposing the flesh of the various birds tested (a lump 5–7 mm. in diameter) to the predations of the Oriental hornet, *Vespa orientalis* L. (Hymenoptera: Vespidae), an omnivorous social insect. The number of insects attending the flesh over a given 5-min period was assessed visually and the data collated and represented both graphically and in tabular form (cf. Cott, 1947 for further details).

A major finding of this study was that there was a clear inverse relationship between edibility and visibility: the more cryptic species were generally more desirable to the hornets than the more visible species. The latter often appeared in broad daylight and were seen to display aposematic warning colouration to a greater or lesser degree, depending on the species concerned. To do this, Cott prepared a list of bird species vs. edibility (E), arbitrarily giving each species an edibility ranking of 1–20, where 20 was the most edible. Also, a list of species vs. visibility (V) with a ranking of 1–10, where 10 was the most visible (see his Table LVIII, p. 414 and Figure 30, p. 437). He stated that visibility is in many ways dependent on the habitat that the particular bird species inhabits (desert, semi-arable, arable), some species of course showing severe habitat fidelity (e.g., those highly adapted species inhabiting deserts). Other brightly coloured species (e.g., Barn swallow, *H. rustica*), are seen to move between habitats, especially when water is present (oases, streams and rivers, etc.). Cott made the additional point that some visible species like the bee-eater are not so visible when they are seen against – say – a background of mixed vegetation, whist, as we now know, some birds have hues not necessarily visible to the human eye, i.e., ultraviolet (Cuthill et al., 2000; Withgott, 2000; see below). Lastly, he suggested that birds with pale underparts are actually showing a certain degree of crypsis as they move around the habitat, especially one assumes with the sky above them.

So the picture that emerges is generally clear, but with some level of variation associated with it. Certainly, some anomalies in the rankings are apparent. Of the aforementioned species inhabiting Europe as residents and tested by Cott (1947), i.e., Kingfisher (Mediterranean form); Barn swallow, Goldfinch *Carduelis carduelis* ssp. *niediecki* Reichenow (Middle-Eastern form), Great tit, *Parus major* ssp. *terrae-sanctae* Hartert. (Palestinian form), Pied (White) wagtail, or very occasional visitors from Africa, i.e., the bee-eater [Cott tested the closely related and colourful species, the Little bee-eater, *Merops orientalis cleopatra* (Nicoll)], and the closely related and similarly marked hoopoe subspecies, *Upupa epops major* (Brehm; Cocker and Mabey, 2005), these were found to have high V and low E rankings, respectively, as shown in Table 1. Although not directly tested in the various feeding experiments, he cites Neumann (1905) concerning the edibility of the Great spotted woodpecker, *D. major*, describing it as "*coarse, and retaining some of the repulsive smell of the bird itself, even when roast, so that it does not make pleasant eating*" (cf. p. 283 in Neumann, Band 4, pp. 432). Interestingly, one of the most edible of the 38 species tested by Cott was the Wryneck, *Jynx torquilla* (L.), a very cryptically-camouflaged species with a visibility and edibility ranking of V = 1 and E = 20, respectively. The odour and edibility of the nuthatch was not tested nor seemingly by other authors subsequently, and therefore awaits such investigation.

TABLE 1 Relative visibility and edibility rankings of birds assumed to display aposematic warning colouration and to have intermediate or low (bold numbering) edibility, as originally tested by Cott (1947).

Species name/ ranking	Visibility	Edibility
Kingfisher	8	**6**
Barn swallow	9	**6**
Goldfinch	7	12
Great tit	7	10
Pied wagtail	8	9
Bee-eater	3	17
Hoopoe	9	**4**

Key: visibility ranking 0–10, where 10 = most visible; edibility ranking 0–20, where 20 = most edible. The intermediate or low edibility values are in bold type face.

In a further series of experiments, edibility tests were performed with domestic cats, *Felis catus* L. and humans. In the case of cats, Cott (1947) states that (p. 455):

> Neither the number of experiments carried out nor the number of species tested is sufficient to yield a detailed comparison between food preferences of cat and hornet. The available evidence does appear, however, to show a general agreement on the likes and dislikes of these two unrelated meat-eaters.

With humans, rather than direct tests of edibility, he cites various published sources. He concludes that:

> When we turn to compare the preferences of man, cat and hornet, unfortunately the available evidence only covers seven genera, namely *Chloris, Passer*, *Merops*, *Streptopelia*, *Parus, Upupa*, and *Ceryle* – in which the same or closely allied species were tested by all three meat-eaters. It is a striking fact that in none does the evidence suggest a considerable difference in the edibility rating: this concurrence of taste appears all the more remarkable when found, as here, in three creatures so utterly differing in organisation and habits.

In a follow-up study performed in Zambia, Cott and Benson (1969) continued investigation of the relative palatability vs. visibility of birds using 200 species belonging to 57 families, tested using a panel of human volunteers. Many of these birds were "*not normally eaten by man*," but again an inverse relationship was found between edibility and visibility. Interestingly, the authors noted that the palatability of the very visibly marked Lilac-breasted roller, *Coracias caudatus* L. was given a moderate value of 6.1 (range 2 = inedible to 9 = excellent), whereas its visibility was scored at 5 on a scale of seven grades ranging from 1 (highly cryptic) to 7 (highly conspicuous; Cott and Benson, 1969).

Lastly in this context, Götmark (1994) re-analysed Cott's data and showed that in 30 South European passerines there was, irrespective of sex, a negative correlation between visibility/conspicuousness and edibility. Similarly, in 87 non-passerine birds from southern Africa, a similar trend was noted across species, including closely-related species. However, in 105 female passerines tested, also from southern Africa, visibility/conspicuousness were negatively correlated with edibility, but only significantly so in relation to conspicuousness. Conversely, male breeding plumage conspicuousness of these species was not negatively correlated with edibility in any analysis. Thus, in such sexually dimorphic species, visibility and conspicuousness may, in males, be sexually selected, or signal other aspects of prey unprofitability rather than being related to edibility as such.

From these various studies and analyses, there is sometimes an obvious and clear negative association between visibility/conspicuousness and edibility, whereas in other studies and tests, including using live raptorial birds and either stuffed prey species or their flesh, the correlation is not clear. Even so, on the basis that nothing in nature exists if it has not a purpose (or once had) à la Dobzhansky (1973), then an explanation for bright colours in sexually monomorphic birds needs to be found, or at least posited.

This is something that Weldon and Rappole (1997) have investigated further by asking field and museum ornithologists which bird species they considered odorous (from intact or skinned exemplars) or unpalatable (from cooked birds). Likewise, Dumbacher and Pruett-Jones (1996) review the available evidence, direct and apocryphal, as to the nature and extent of avian toxins amongst different bird species representing widely different taxa, which they duly tabulate in terms of their odour or palatability. These studies show that some of the aforementioned bird groups are malodourous or unpalatable, including species of woodpecker, kingfisher, tits and the Eurasian oystercatcher, the last having a very prominent black and white livery and a crimson bill, pink legs and feet (Neumann, 1905; Dumbacher and Pruett-Jones, 1996; Figure 1J). Empirical studies need to be performed to test this hypothesis further and determine the chemical nature of the toxins involved for the various species. Very recently, Yeung et al. (2022) have reviewed the nature of the toxicity of some well-known poisonous birds, such as the pitohui and hoopoe, including the structure of the toxins themselves (cf. also Bartram and Boland, 2001 and Ligabue-Braun and Carlini (2015) in this respect).

Of the aforementioned colourful, sexually monomorphic birds displaying aposematic warning colouration, what would be of further interest is to ascertain whether this is Müllerian or Batesian in nature (in this context, cf. Table 1 in Hedley and Caro, 2022). The fact that these birds fly and feed in daytime in full sight of would-be predators suggests that they do indeed possess a certain degree of toxicity. Birds such as the Goldfinch may not be especially toxic (according to Cott's scale of edibility the species is intermediate; Table 1), but as with the harmless scarlet king snake, *Lampropeltis elapsoides* (Holbrook) of the eastern and south eastern USA, a Batesian mimic of the deadly coral snake, *Micrurus fulvius* (L.), it is a false aposematic mimic. Their plumage livery might be enough to deter many, if not most, predators, but not all (Unprofitable Prey Hypothesis, UPH; cf. Hedley and Caro, 2022).

Raptorial birds, including Eurasian sparrowhawk, Peregrine falcon, *Falco peregrinus* Tunstall, Goshawk, *Accipiter gentilis* (L.), Merlin, *Falco columbarius* L. and Hobby, *Falco subbuteo* L. are known to predate some or all of the following prey species: Goldfinch, Barn swallow, Great tit, Pied wagtail, Magpie and Great spotted woodpecker (Owen, 1932; Selås, 1993; Zawadzka and Zawadzki, 2001; López-López et al., 2009; Ivanovskij and Sidorovich, 2018). In the USA, the American Oystercatcher, *Haematopis palliates* Temminck, which has a similar livery to the Eurasians species, is predated by skunks and racoons and some larger birds, including raptors (Hardin, 2014). However, according to Uttendörfer (1939; cited in Cott, 1947), of 43,211 prey items taken by Eurasian sparrowhawk, only 5% comprised Great tit and 5% Barn swallow (cf. Cott, 1947, p. 491 for further details). In the case of the Eurasian magpie, a strikingly obvious diurnal feeder, it is rarely, if ever, attacked by the Sparrowhawk (Owen, 1932), but is regularly attacked by the larger Goshawk in urban areas of Germany (Rutz, 2004), Peregrines in Spain (López-López et al., 2009) and Common Buzzard, *Buteo buteo* (L.) in the northeast of Ireland (Rooney and Montgomery, 2013). As quoted in Cott (1947; p. 482), the flesh of the magpie as eaten by one person was described as being "*pretty awful*." In a quantitative study by Götmark (1997) carried out in Scandinavia in which he tested stuffed Eurasian magpies and Eurasian jay, *Garrulus glandarius* (L.) susceptibly to attacks by Goshawk, he found the attack rate was similar, and overall, the tests suggested frequency dependent selection of the prey birds by the predator. The author concluded that the livery of Eurasian magpies is not aposematic in nature and that whilst it may increase the likelihood of predation, may instead favour sexual or social selection. Despite this

conclusion, the bird is very obvious, with a colour scheme normally associated with warning colouration in other animals like the Common European adder, *Vipera berus* (L.) and Striped skunk, *Mephitis mephitis* (Schreber) of North America.

In other experiments (Götmark and Unger, 1994) involving stuffed birds, in this case, Pied wagtail paired with cryptic Meadow pipit, *Anthis pratensis* (L.) and Great Spotted woodpecker paired with cryptic (brown) female Blackbird, *Turdus merula* L., the attacks by avian predators (mostly Accipiters and filmed with automatic cameras) on the wagtail and pipit were similar, whereas the blackbird were much more frequently attacked than the woodpecker. To test the palatability of woodpecker vs. blackbird, the authors also conducted experiments feeding flesh of these birds directly to captive falcons. No clear evidence was found that woodpeckers are especially distasteful to these particular avian predators. A stuffed bird is of course not supplying all the potential deterrents that a living bird may do, i.e., unpleasant odour and/or unpalatable or toxic flesh.

Perhaps, as with the North American Blue Jay, *Cyanocitta cristata* (L.) during a first encounter with the Monarch butterfly, *Danaus plexippus* (L.) (Lepidoptera: Nymphalidae), the larvae of which feed on *Asclepias curassavica* L. (Gentianales: Apocynaceae) which contain toxic cardiac glycosides (Brower et al., 1967), naïve predatory birds and mammals may initially attack such prey bird species. However, if these are too big to easily subdue or if subdued and eaten, have an unpleasant aftertaste or cause an unpleasant reaction, then the predator soon learns to avoid them. Conjecture yes, but then again, how does one account for such obvious livery and diurnal foraging behaviour of these colourful birds, which overtly display their presence in the landscape? Because both sexes show the same or largely the same bright plumage (e.g., Great spotted woodpecker), this is probably not for purposes of sexual display and reproduction (Loxdale, 2022). Therefore, it does not involve a trade-off between negative selection incurred by predators due to the visibility and "showiness" of the male vs. crypsis and greater survival of the female, as seen in sexually dimorphic birds like the Common Chaffinch, *Fringilla coelebs* L. and Eurasian Bullfinch, *Pyrrhula pyrrhula* (L.) (cf. Götmark, 1993 for conflicting experimental evidence for this assumption).

Other aspects of interest when considering the putative palatability – or not – of the aforementioned colourful bird species is whether they are universally toxic to would-be predators, or only to some of them, which may have evolved a certain tolerance to the innate toxins. Besides the fact that different species of raptor have different food preferences and diet breadth, even known noxious birds such as the hoopoe are predated by some raptors in some locations (e.g., Peregrine in eastern Spain; López-López et al., 2009). The fact that the Eurasian Magpie, whilst being shunned by many predatory birds, is taken by Buzzard, Goshawk and Peregrine, perhaps relates to the fact that these are larger than the Sparrowhawk and can thus subdue it more easily. It is also possible that the prey is not uniformly toxic, and the predator selects the flesh that is less so, or they have become tolerant to the toxins the species contain, as has the Peregrine to those harboured by Hoopoe. The abundance of potential prey (availability) must also play some part in whether or not a particular predator is likely to tackle a given prey species, as shown experimentally in other predator–prey scenarios (Blouin, 1990; Marples, 1993; Hossie et al., 2021).

That we see the birds, the topic of this article, as very obvious within the environment, also does not mean that they are universally seen as such. For a start, as aforementioned, birds can see into the UV spectrum, which means that their perception of these colours and hues may be somewhat different than those we humans or other mammals/animals perceive. Furthermore, with any discussion concerning aposematically coloured animals vs. cryptic ones, this has to be seen in context to the habit they are living in, in turn affected by environmental conditions of habitat complexity, prevailing light conditions and weather/climate. Thus for example, it is known that distance-dependent factors are associated with the perception of putative prey by would-be predators (Barnet and Cuthill, 2014; Barnett et al., 2018). There are in addition, trade-offs between prey obviousness in terms of aposematism (and hence the possibly of predation by naïve birds) and crypsis, such that an intermediate state is found to be adaptively favoured (Tullberg et al., 2005 in the case of swallowtail butterfly larvae, *Papilio machaon* L.). In further support of this idea, Loeffler-Henry et al. (2023) suggest that species (here amphibians) also display an intermediate stage between crypsis and bright colouration involving facultative exposure of concealed colours (e.g., under the belly) when attacked. In this way, they gain the best of both worlds in terms of crypsis and warning defence. Ultimately, however, if the selective pressure is great enough, the costs of producing full-on warning colouration, perhaps with concomitant changes in warning behaviour, must outweigh the intermediate strategy in terms of adaptive and hence survival strategy.

Another aspect worthy of consideration is the variation in the toxicity of the prey and how this might affect predation. Speed et al. (2012) argue that over the course of the evolution, toxin variation is of adaptive significance and depends on the dose of toxin borne by a particular species geno-phenotype in relation to their abundance in the population, hence leading to the frequency-dependent selective response of predators. In a similar vein, Endler and Mappes (2004), using mathematical modelling, explore the effects of predators on prey in relation to frequency-dependent, frequency-independent, and negative frequency-dependent predation. Their main conclusion is that "*weak signalling of aposematic species can evolve if predators vary in their tendency to attack defended prey*," again presumably in relation to the cost benefits of producing the toxin with accompanying warning signalling, chemical and behavioural. Less effort in responding to predators in terms of signals/signalling may also be adaptively advantageous.

Certainly, in the case of the presently discussed colourful birds, the fact that they are predated by some of the common bird predators like sparrowhawk and goshawk, despite showing clear aposematic warning coloration, argues that these particular predators, especially naïve ones, may indeed be functioning on a frequency-depended basis. Over time, they learn of the non-profitability of such prey, and avoid them, If this were not so, why do such bright warning colours persist, suggesting that they have been positively selected over the course of evolution. This does not help much in determining whether such colouring really is Müllerian or Batesian in nature, but probably it is the former, a broad warning signal, as with venomous wasps and bees. If it were Batesian, then models would have to be identified, and none are obvious.

An additional factor to consider in the context of the attack vs. avoidance of aposematically-coloured birds includes social learning. Thus individuals of conspecific/congeneric predator species, or even those of very different taxa, rapidly learn of the effect on their fellow predators when these attack a particular kind of prey, e.g., discarding

the prey or, if eaten, vomiting it up shortly afterwards (Hämäläinen et al., 2020), as in the Blue Jay/Monarch butterfly scenario.

Lastly, in all discussion of the obviousness of a potential prey item within its habit, aspects of habitat matching and/or disruptive coloration must be considered (Cott, 1940). This topic is discussed by Stevens and Merilaita (2009), who revise earlier categorisations, especially by Thayer (1909) and Cott (1940), and re-assesses the range of principles and sub-categories that govern the phenomena involved (cf. Stevens and Merilaita, 2009 for details; also Honma et al., 2015 and Kang et al., 2015). Only in light of these various factors and influences can the true nature of the colourful livery of the birds be more fully understood.

In conclusion, the available evidence strongly suggests that some familiar European birds are displaying aposematic warning colouration and as such, may be noxious-toxic to would-be predators, although this is not to say that naïve birds may initially predate them, but learn to avoid them in future. The mimicry could well be Müllerian in nature, i.e., a broad warning signal, but may also be a ruse to some extent (so-called "cheats"; Speed et al., 2012), as in the case of the Goldfinch, possibly making some predators (but clearly not all, i.e., some Accipiters) wary of attacking the bird in the first place. If this is so, the gaudy livery, rather than being of no purpose, and apparently not involved in sexual selection by the female since both sexes share the same or nearly the same livery, argues that the plumage colouring is of selective advantage (Endler and Mappes, 2004; Speed et al., 2012). It is unlikely, but not impossible, that in some species with low toxicity status, the mimicry is Batesian in nature, but what the exact model might be remains conjectural.

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.

Author contributions

The author confirms being the sole contributor of this work and has approved it for publication.

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Conflict of interest

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

Publisher's note

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Effect of estradiol and predator cues on behavior and brain responses of captive female house sparrows (*Passer domesticus*)

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The presence of predators can cause major changes in animal behavior, but how this interacts with hormonal state and brain activity is poorly understood. We gave female house sparrows (*Passer domesticus*) in post-molt condition an estradiol (n = 17) or empty implant (n = 16) for 1 week. Four weeks after implant removal, a time when female sparrows show large differences in neuronal activity to conspecific vs. heterospecific song, we exposed birds to either 30 min of conspecific song or predator calls, and video recorded their behavior. Females were then euthanized, and we examined neuronal activity using the expression of the immediate early gene (IEG) ZENK to identify how the acoustic stimuli affected neuronal activation. We predicted that if female sparrows with estradiol implants reduce neuronal activity in response to predator calls as they do to neutral tones and non-predatory heterospecifics, they would show less fear behavior and a decreased ZENK response in brain regions involved in auditory (e.g., caudomedial mesopallium) and threat perception functions (e.g., medial ventral arcopallium) compared to controls. Conversely, we predicted that if females maintain auditory and/or brain sensitivity towards predator calls, then female sparrows exposed to estradiol would not show any differences in ZENK response regardless of playback type. We found that female sparrows were less active during predator playbacks independent of hormone treatment and spent more time feeding during conspecific playback if they had previously been exposed to estradiol. We observed no effect of hormone or sound treatment on ZENK response in any region of interest. Our results suggest that female songbirds maintain vigilance towards predators even when in breeding condition.

KEYWORDS

predator playback, anti-predator behavior, immediate early gene, ZENK, *Passer domesticus*

1 Introduction

Predation risk often induces measurable changes in animal behavior, and animals often respond to predator calls differently than other types of sounds. For example, in response to predator calls, black-capped chickadees (*Poecile atricapillus*) attempted to recruit nearby conspecifics to mob predators, frequently moved from location to location, and did not ruffle their feathers (Congdon et al., 2016). Researchers interpreted these responses as preparation to attack, to elude the predator, and to reduce the risk of being seen in high-threat conditions, respectively. Increased freezing and vigilance in response to predator cues are commonly

observed across taxa (Beani and Dessí-Fulgheri, 1998; Mesquita and Young, 2007; Rahlfs and Fichtel, 2010). In the chickadee brain, exposure to predator vocalizations induced neural activation in areas involved in fear and emotion (AMV and NCL) (Hobbs, 2015; Zanette et al., 2019). These differences in brain activity may be correlated with behavioral response to predators, but this has rarely been investigated outside of mammalian systems (but see Cross et al., 2013). Further, we do not fully understand how an animal's underlying hormonal state affects anti-predator behaviors and neuronal response to predators.

Previous research has shown that hormonal state can affect both the production of and the neural response to breeding signals like mating calls. Efficient auditory communication requires the rapid recognition of salient signals and the ability to filter other sounds. The preference for conspecific vs. heterospecific song has been well characterized in songbirds, and conspecific-selective neuronal activity has been found in auditory nuclei like the caudomedial mesopallium (CMM) and the caudomedial nidopallium (NCM) (Mello et al., 1992; Louder et al., 2019). Sex steroids may influence auditory processing by affecting both auditory and social behavior brain regions (e.g., the medial ventral arcopallium) (Maney and Pinaud, 2011), by altering the auditory brainstem response (Caras et al., 2010), or even by acting on the ear directly (Noirot et al., 2009). Estradiol increases the range of frequency sensitivity of the hearing organs of some animals, likely increasing female sensitivity to male vocalizations (Sisneros and Bass, 2003; Ronald et al., 2018), Estradiol can directly modulate the selectivity of neural substrates due to the abundance of estrogen production throughout the avian brain (Vahaba and Remage-Healey, 2018) or via circulating estrogen produced in the ovary (Maney et al., 2007), and there is a high density of estrogen receptors in many relevant brain nuclei (Bernard et al., 1999) as well as in the auditory brainstem and ear (Henry and Lucas, 2009). For example, female songbirds in breeding condition increase neuronal activity in response to conspecific song compared to frequency-matched tones in several brain regions of the social behavior network (Maney et al., 2008; Maney and Pinaud, 2011; O'Connell and Hofmann, 2011). House sparrows with experimentally-increased estradiol showed decreased neuronal activity to heterospecific compared to conspecific song in brain regions involved in auditory perception (Lattin et al., 2017b). Additionally, when plasma estradiol reached breeding levels, activity in the auditory forebrain of female white-throated sparrows (*Zonotrichia albicollis*) selected for conspecific song, suggesting that processing of auditory conspecific signals is seasonally regulated by sex steroids (Sanford et al., 2010). However, past studies have focused solely on comparisons between conspecific song and neutral acoustic stimuli (i.e., tones or the song of other songbirds). What is unknown is whether female songbirds in breeding condition also "tune out" the calls of predator species like hawks and owls that might present a danger to them; i.e., do females maintain sensitivity to predatory stimuli while in breeding condition? It is undeniably important to maintain vigilance towards predation no matter the time of year. However, several studies have reported increased depredation of female songbirds during the breeding season (Slagsvold and Dale, 1996; Götmark et al., 1997; Post and Götmark, 2006), which could, in part, be explained by a decrease in auditory and neuronal sensitivity to predator calls.

In this experiment, we were interested in testing two competing hypotheses: 1) Hormonal state affects neuronal response to predator cues; 2) Hormonal state does not affect neuronal response to predator cues (hereafter referred to as the alternative and null hypotheses, respectively). To test these hypotheses, we examined both behavior and protein expression of the immediate early gene (IEG) ZENK as a measure of neuronal activity in captive female house sparrows (*Passer domesticus*) exposed to predator calls or male conspecific song. ZENK is often used to assess neuronal responses to acoustic stimuli in songbirds (Maney et al., 2008; Lynch et al., 2012; Rivera et al., 2019), therefore we interpret ZENK immunoreactivity as neuronal activity. The caudomedial nidopallium (NCM, involved in auditory perception), caudomedial mesopallium (CMM, involved in auditory memory), medial ventral arcopallium (AMV, involved in threat perception), caudal hippocampus (cHP, involved in integrating sensory and emotional responses), apical hyperpallium (HA, involved in behavioral flexibility), and caudolateral nidopallium (NCL, involved in decision making) all increase neuronal activity in response to aversive or threatening conditions (Rose and Colombo, 2005; Cross et al., 2013; Dai et al., 2018; Brito et al., 2019; Zanette et al., 2019; Kimball et al., 2022). These regions are therefore ideal candidates to respond to predator calls. Because estradiol influences auditory processing, we simulated breeding condition in half of the females with estradiol implants and the other half received empty implants as a control.

To understand how reproductive status modulates responsiveness to predator cues relative to conspecific cues, we first analyzed behavior during acoustic exposures. Based on previous literature, we expected to see higher levels of freezing in female sparrows exposed to predator calls compared to females exposed to male conspecific song. However, if estradiol-treated females showed reduced responsiveness to predator calls, we predicted less freezing behavior in this group compared to predator-exposed females receiving empty implants. We then analyzed neural responses. If females in breeding condition maintain sensitivity to predator calls, we predicted female sparrows exposed to estradiol would have no difference in ZENK response in any region of interest regardless of playback type (i.e., predator or conspecific calls). If female sparrows exposed to estradiol decrease responsiveness to predator calls, as previously observed with heterospecific calls and neutral tones, we predicted they would have lower ZENK response in brain regions involved in auditory perception (e.g., NCM and CMM) compared to estradiol-treated females hearing male sparrow song. We also expected to see less ZENK response in regions involved in aversive responses and threat perception (AMV, NCL, cHP, and apical hyperpallium) in estradiol-treated sparrows hearing predator calls compared to females with empty implants. We predicted no difference in ZENK response between conspecific and predator playback in sparrows receiving empty implants because the NCM and CMM showed increased selectivity towards conspecific signaling only when plasma estradiol reached breeding levels (Maney et al., 2006; Sanford et al., 2010).

2 Materials and methods

2.1 Study subjects

Adult female house sparrows (n = 33) were captured using mist nets at bird feeders in East Baton Rouge Parish between June and August 2020. Sparrows were doubly housed in cages in a vivarium at Louisiana State University with unlimited access to mixed seeds, grit, a vitamin-rich food supplement (Purina Lab Diet), and water. Sparrows also had access to a variety of perches and a dish of sand for dustbathing. Sparrows were maintained at natural day length (13L:11D) for a minimum of 4 weeks to acclimate to the captive environment before implant surgeries began. Although females were kept on long days, all females molted in captivity and females in the control group had small ovaries (data not shown), suggesting females were photosensitive but not photostimulated before receiving implants. Animals were collected under a Louisiana State Scientific Collecting Permit and all experimental procedures approved by the Louisiana State University Institutional Animal Care and Use Committee under protocol 96-2018. We used approved methods for bird capture, transport, husbandry, and surgery as specified in the Ornithological Council's Guidelines to the Use of Wild Birds in Research (Fair et al., 2010), and approved methods of euthanasia for laboratory animals as specified in the 2020 American Veterinary Medical Association Guidelines for the Euthanasia of Animals.

2.2 Implant surgeries

All sparrows received subcutaneous implants in the skin of the back (n = 17 estradiol, n = 16 control). Estradiol implants consisted of silastic medical-grade tubing packed with crystalline 17-beta-estradiol (Sigma-Aldrich, St. Louis, MO, United States), sealed at both ends with a silicone adhesive. Control implants were empty. The size of implants (15 mm long, 2 mm outer diameter) was the same as used in a previous experiment (Lattin et al., 2017b), which were shown to significantly increase ovary size (437.6 ± 102.5 mg) compared to females with empty implants (13.4 ± 12.3 mg). Estradiol implants also significantly increased levels of circulating estradiol concentrations to (2.5 ± 0.7 ng/ml) compared to females with empty implants (0.16 ± 0.05 ng/ml) and 4 weeks after implant removal (1.1 ± 0.8 ng/ml) (Lattin et al., 2017b).

For implant surgeries, sparrows were anesthetized with inhaled isoflurane (4% induction, 3.5%–2% maintenance), and maintained at a surgical plane of anesthesia. Depth of anesthesia was assessed using toe pinch, breathing rate, and palpebral reflex, and we used a heating pad under a sterile surgical pad to maintain body temperature. Birds were given subcutaneous ketoprofen (5 mg/kg) as a pre-emptive analgesic, a small incision was made in cleaned and disinfected skin between the shoulder blades, an implant was inserted, and the incision site closed with Vetbond (3M, Maplewood, MN, United States). The following day, all birds were monitored to ensure proper healing and check implant placement and were given a second dose of ketoprofen to minimize discomfort. Implants remained in place for 1 week and were then removed using a similar procedure to implant insertion: isoflurane anesthesia (4% induction, 3.5%–2% maintenance), ketoprofen, an incision in cleaned and disinfected skin, removal of the implant using sterile forceps, and the incision site closed with Vetbond. Sparrows were checked again the day after implant removal to ensure proper healing and given a final dose of ketoprofen. Four weeks later, we conducted playback experiments. We used this time course because in a previous house sparrow study, greater effects of estradiol treatment on brain responses to conspecific vs. heterospecific playback were observed during this later time point than during the first week implants were in place (Lattin et al., 2017b), suggesting that the full effects of estradiol on the brain and auditory perception can take several weeks to fully develop.

2.3 Playback trials

The evening before playback trials, females were individually housed in smaller test cages with access to multiple perches, food, grit, and water. For playback trials, females were rapidly transported one at a time to an acoustically isolated testing room and exposed in individual trials to either a unique 30 min playlist of several different male house sparrows singing with a few calls (n = 8 control females, n = 8 estradiol-treated females) or a unique mix of calls from local predators: Barn owls (*Tyto alba*), Eastern screech owls (*Megascops asio*), Great horned owls (*Bubo virginianus*), Broad-winged hawks (*Buteo platypterus*), Cooper's hawks (*Accipiter cooperii*), Red-shouldered hawks (*Buteo lineatus*), Red-tailed hawks (*Buteo jamaicensis*), American kestrels (*Falco sparverius*), Loggerhead shrikes (*Lanius ludovicianus*), and Mississippi kites (*Ictinia mississippiensis*) (n = 8 control females, n = 9 estradiol-treated females). Therefore, treatment groups were as follows: n = 8 for Empty + Predator, n = 8 for Empty + Sparrow, n = 9 for Estradiol + Predator, and n = 8 for Estradiol + Sparrow. A female sparrow heard, on average, 31.7 ± 1.5 (range: 30–35) different male house sparrow sound files during conspecific playbacks or 58.5 ± 2.9 (range: 53–63) different predator sound files during predator playbacks. All 11 predator species were represented at least once in each predator playback playlist, and each had on average five different songs or calls per sound file. Sound files were obtained from the Macaulay Library (Cornell Lab of Ornithology, Ithaca, NY, United States) and the Borror Laboratory of Bioacoustics (The Ohio State University, Columbus, OH, United States). Each bird heard a unique playlist of different sound files played in a randomized order. No other sparrows were present in the testing room during trials. Loudness was standardized to 60 dBA from bird to speaker using a sound level pressure meter and we video recorded female behavior during the playback trials using a Logitech C615 portable webcam. A researcher began recording videos after transporting the individual cage, and then started the playback and immediately exited the room.

Previous work in songbirds has shown that IEG proteins peak ~90 min after stimulus exposure (Goodson et al., 2005). Therefore, after the 30 min playback behavior trials, females were transported to a dark quiet room for 60 min before being deeply anesthetized with ketamine (80 mg/kg) and xylazine (20 mg/kg), doses shown to be appropriate for house sparrows (Muresan et al., 2008). Once animals were in a surgical plane of anesthesia, they were transcardially perfused with ice-cold heparinized saline and 0.1 M phosphate buffer containing 4% paraformaldehyde sequentially.

Euthanasia was confirmed using rapid decapitation and brains extracted.

2.4 Behavioral analysis

Thirty-minute video recordings were analyzed using BORIS v 7.10.2 (Friard and Gamba, 2016). One individual's video was lost due to a recording malfunction, therefore final sample sizes for behavior were as follows: n = 8 for Empty + Predator, n = 8 for Empty + Sparrow, n = 9 for Estradiol + Predator, and n = 7 for Estradiol + Sparrow. An ethogram was created to associate keys with point-type behaviors, which were discrete behaviors with no duration (movement, beak wiping, feather ruffling, and calling), and state-type, which were behaviors with duration (preening and feeding). We were interested in any behaviors that might have been altered by threatening stimuli. Movement was classified as a hop, flight, jump, or any time both feet came off the ground. Behaviors like foot adjustments, shuffling, stretching, and head bobbing were not considered movements. Beak wiping was counted when an individual wiped its beak on an object in the cage, usually a perch. One bout of beak wiping was considered when at least 2 s occurred between subsequent wipes (Lattin et al., 2017a). Calls were classified as one bout of vocalizations (e.g., chirp or rattle). Feather ruffling was classified as an event when an individual would puff up its feathers and ruffle quickly. Preening was classified as any time an individual would pull on their feathers with their beak or spread oil from their preen glands. Finally, feeding was classified when the bird perched on the food dish in the cage and fed. For each individual, the total number of occurrences were recorded for each behavior and total duration was recorded for state-type behaviors and means and standard deviations were calculated for each treatment group. Videos were watched without sound to classify all behaviors except for calls, so the observer was blind to playback type (predator vs. male sparrow), and these videos were watched by the same observer to ensure consistency (intra-observer coefficient of variation from watching 4 videos twice: movement = 0.5%, feeding duration = 1.1%). A separate observer watched all videos with the sound on to be able to accurately quantify sparrow calling behavior. Both observers were blind to bird treatment (estradiol vs. empty implant). Beak wiping, feather ruffling, calls, and preening were infrequently observed (each occurred in 25% or fewer of videos) and were not included in the final behavior analysis.

2.5 Immunohistochemistry and ZENK quantification

Brains were post-fixed in 4% paraformaldehyde phosphate buffer for 24 h at 4°C, then soaked in 0.1 M phosphate buffer containing 30% sucrose for cryoprotection. After sinking (~2 days), brains were flash-frozen in powdered dry ice and stored at −80°C until sectioning. Brains were cut at −20°C in the coronal plane in 40 µm sections using a ThermoFisher NX50 cryostat. Starting at striatum, triplicate sections were collected in wells containing cryoprotectant (0.2 M phosphate buffer, 15 M PVP, 1.5 M sucrose, and 0.5 M ethylene glycol in distilled water) and stored at −20°C until the day of immunohistochemistry.

Brain regions were identified based on visible landmarks. Apical hyperpallium (HA) sections were taken when Area X was still visible and approximately ~120 µm before the first appearance of the lateral septum. We used caudal dorsomedial hippocampal sections where the cerebellum first became visible and the mesopallium began to disappear. We targeted medial ventral arcopallium (AMV) based on the visibility of the cerebellum and arcopallium. Sections used for caudolateral nidopallium (NCL) were 40 µm after AMV sections, in a pallial area where we have confirmed the presence of dense basket fiber staining for tyrosine hydroxylase in house sparrows, consistent with NCL in other songbird species (von Eugen et al., 2020). Caudomedial mesopallium (CMM) was taken ~40–80 µm before and caudomedial nidopallium (NCM) was taken ~40–80 µm after the appearance of field L2. For each region we ran immunohistochemistry for all 33 animals in the same assay on the same day.

Sections were stained for ZENK as done previously (Kimball et al., 2022). Briefly, sections were washed 3 times in Tris-buffered saline (TBS, pH 7.6), incubated in 0.5% hydrogen peroxide for 30 min, washed again 3 times in TBS, and blocked with 10% normal horse serum (Vector Laboratories, Burlingame, CA, United States of America) in 0.3% Triton in TBS (TBS-T) for 1 h. After washing 3 more times in TBS, sections were moved out of mesh well inserts and incubated with a monoclonal mouse anti-ZENK antibody (1: 500 in TBS-T and 1% normal horse serum; antibody 7B7-A3) donated by Dr. David Keays, Research Institute of Molecular Pathology in Vienna, Austria and raised against an N-terminal fragment 260 amino acids in length (1–260) of rock pigeon ZENK (Nordmann et al., 2020). Sections were incubated for ~20 h at 4°C. After washing 3 times in TBS, sections were then incubated at room temperature for 1 h in biotinylated horse anti-mouse IgG (Vector Laboratories) diluted 1:500 in TBS-T, followed by three more washes in TBS-T. Sections were incubated in avidin–biotin horseradish-peroxidase complex (Vectastain ABC, Elite kit, Vector) at a concentration of 1:100 for 1 h and washed twice in TBS. Sections were visualized with DAB (Sigma Fast-DAB), mounted onto slides, dehydrated in ethanol, cleared in HemoDe (Scientific Safety Solutions, Keller, TX, United States), and cover-slipped using Permount (Electron Microscopy Sciences, Hatfield, PA, United States).

We measured immunopositive cell density for ZENK in all six regions of interest. Images of each region were captured using an Olympus TH4-100 microscope with a ×20 objective lens using consistent lighting for each photo. We quantified four sections per individual, with each section including right and left hemispheres unless the hemisphere was damaged during staining, measured the area that was quantified, calculated density (cells/mm^2), and averaged the cell density for each individual in each region. One to two images per hemisphere were captured for smaller regions (cHP, CMM, NCM, NCL, and AMV), and three images were taken of the HA (Figure 1). Individuals were excluded from the following regions if more than two out of the four sections had unclear staining due to artifact, or if tissue was too torn to properly quantify cell density: cHP: n = 1 for Estradiol + Predator, n = 1 for Estradiol + Predator; HA: n = 1 for Empty + Predator, n = 2 for

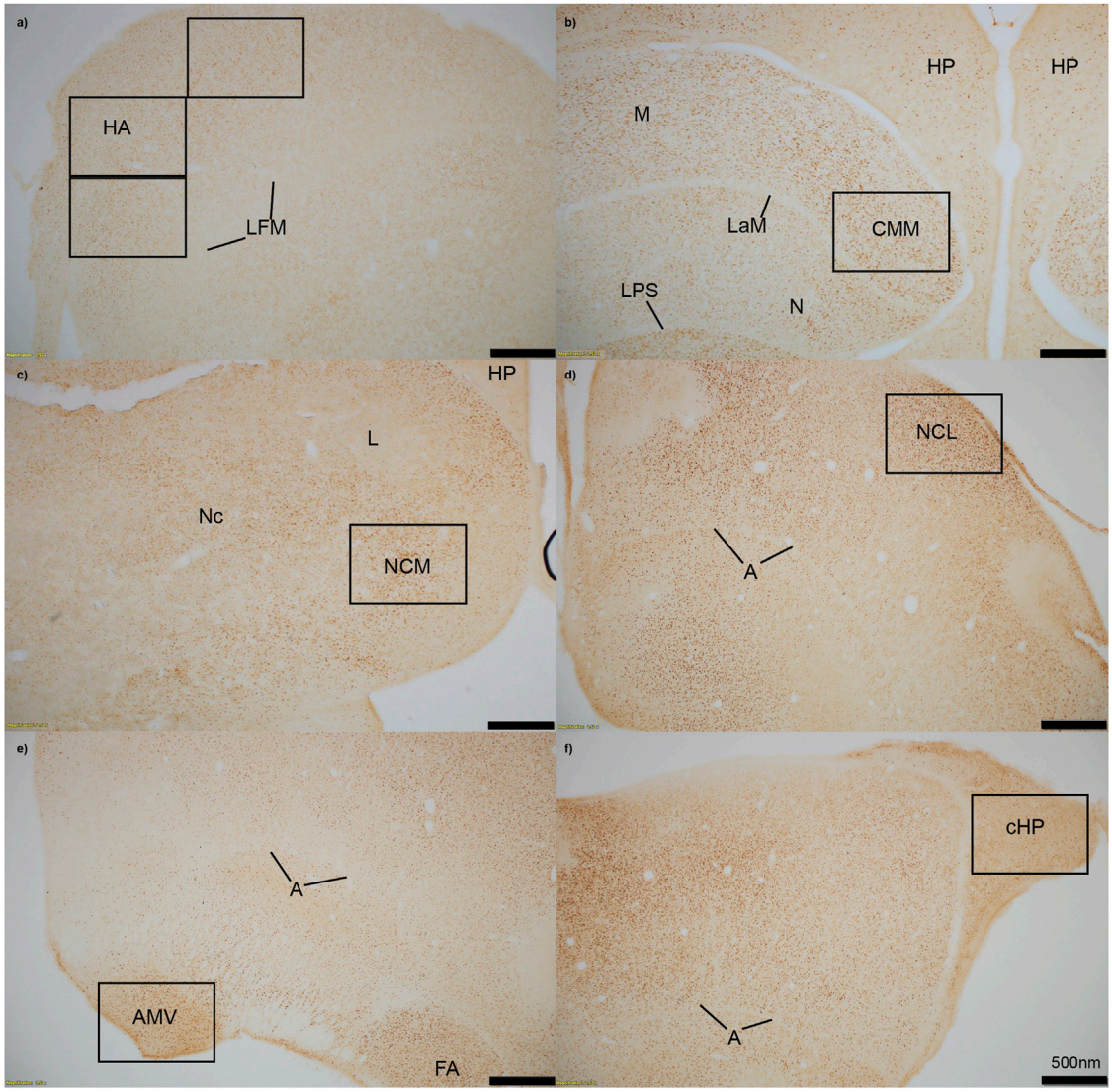

FIGURE 1
Immediate early gene ZENK staining in six regions of interest at 4x magnification. **(A)** HA = apical hyperpallium, **(B)** CMM = caudomedial mesopallium, **(C)** NCM = caudomedial nidopallium, **(D)** NCL = caudolateral nidopallium, **(E)** AMV = medial ventral arcopallium, **(F)** cHP = caudal hippocampus. Quantification of active neurons in each region occurred in black boxes, which approximately represent 20x magnification. Stereotaxic coordinates are not labelled because there is currently no atlas for the house sparrow brain. A = arcopallium, FA = tractus fronto-arcopallialis, HP = hippocampus, L = field L, LaM = lamina mesopallialis, LFM = lamina frontalis suprema, LPS = lamina pallio-subpallialis, M = mesopallium, N = nidopallium, Nc = nidopallium caudale.

Empty + Sparrow, n = 1 for Estradiol + Predator; NCM: n = 1 for Empty + Sparrow, n = 1 for Estradiol + Predator, n = 2 for Estradiol + Predator; CMM: n = 1 for Empty + Predator, n = 1 for Estradiol + Predator, n = 2 for Estradiol + Sparrow.

We used ImageJ (Schneider et al., 2012) to measure immunopositive cell density in each image using a procedure adapted from Mischler et al. (2017). All images were cropped to include only the region of interest, and the area of each region was measured. We then converted each image to 8-bit grayscale, increased the contrast, and used the threshold tool to make immunopositive nuclei white against a black background. We then used the count function to quantify the number of immunopositive nuclei and calculated staining density. Image analysis was done by two observers blind to treatment (estradiol vs. empty and predator vs. sparrow).

2.6 Data analyses

We used JMP Pro 16.0 (SAS Institute) for all behavior and IEG analyses, and all individuals were included in each analysis (n = 33), unless data was missing (as described above). We first ran generalized linear models assessing the effect of hormone and sound treatments on behavior. Movement was Box-Cox transformed (Box and Cox, 1964), which fits variables to a normal distribution by creating a unique transformation

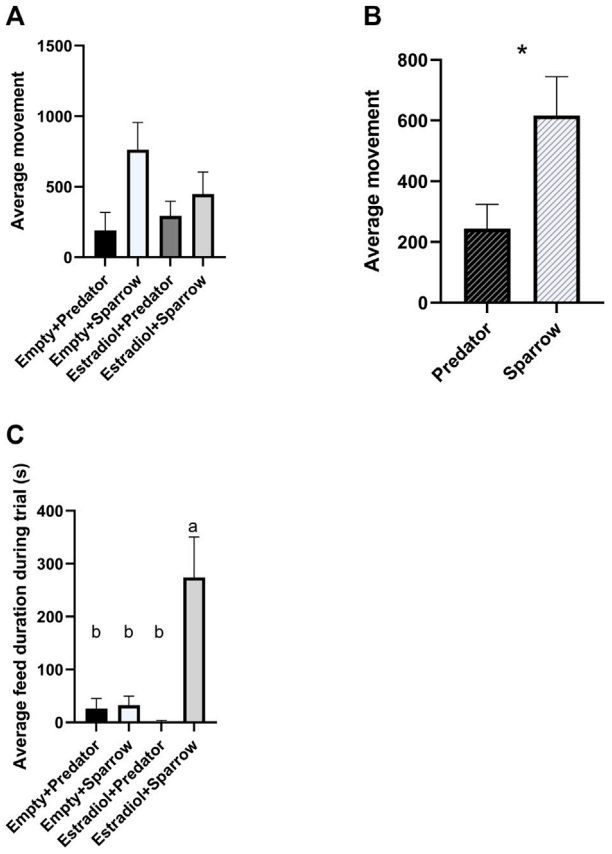

FIGURE 2
Female house sparrows moved less when exposed to predator calls **(A and B)** and fed more when treated with estradiol and exposed to conspecific song **(C)**. Behaviors were quantified for 30 min during playback. "Average movement" in panels a refers to the average number of hops and flights for females in that group. Sample sizes for panels a and c were n = 8 for Empty + Predator, n = 8 for Empty + Sparrow, n = 9 for Estradiol + Predator, and n = 7 for Estradiol + Sparrow. In panel b, sample sizes were n = 17 for Predator and n = 15 for Sparrow sound treatment groups. In panel c, different letters indicate $p < 0.05$. Error bars represent standard error. *$p < 0.05$.

calculation from the given raw data. The following equation was produced:

$$\frac{(\text{Movement} + 1)^{0.255} - 1}{0.00889}$$

Box-Cox transformation cannot be run on values of zero and below, therefore we added 1 to movement data prior to transformation. We used two separate generalized linear models to analyze sparrow behavior, with either movement or feed duration as the dependent variable and hormone treatment, sound treatment, and a hormone treatment * sound treatment interaction as fixed effects.

We next ran models assessing the effect of hormone and sound treatments on ZENK immunoreactivity in each region of interest (six models total), with ZENK density (cells/mm^2) as the dependent variable and hormone treatment, sound treatment, and a hormone treatment * sound treatment interaction as fixed effects. In cases where there was a significant effect of treatment in behavior or IEG analyses, we compared treatment groups using ANOVA and Tukey's HSD tests. For all models, Bartlett's test indicated equal between-group variances. Normal quantile plots showed right-skewed data; therefore, all models were fit to an exponential distribution.

To investigate any possible associations between neuronal activity, movement, and feeding duration, we ran Spearman's rank-order correlations. Spearman's rank-order coefficients were calculated for average ZENK activity in the 6 brain regions of interest and the 2 behaviors, for a total of 12 correlations (e.g., ZENK activity in the AMV and movement). *p*-values in all analyses were corrected for multiple testing using the Holm-Bonferroni method (Holm, 1979).

3 Results

3.1 Behavior

We found a significant overall effect of sound treatment on movement (Figure 2A; $X^2_1 = 5.98$, $p = 0.015$), where female sparrows exposed to predator calls moved less than females exposed to male sparrow calls (Figure 2B; ANOVA: $F_{1,30} = 9.71$, $p = 0.004$). Movement was not affected by hormone treatment ($X^2_1 = 0.95$, $p = 0.33$) and there was no interaction between hormone and sound treatment ($X^2_1 = 1.56$, $p = 0.21$). For feeding duration, there was a significant overall effect of sound treatment (Figure 2C; $X^2_1 = 10.36$, $p = 0.0013$), and a significant interaction between hormone and sound treatment (Figure 2C; $X^2_1 = 8.41$, $p = 0.0037$). Females exposed to estradiol implants and sparrow vocalizations spent more time feeding compared to all other treatment groups (Figure 2C; Tukey's HSD: all $p < 0.0079$). There was also a significant overall effect of hormone treatment on feed duration (Figure 3C; $X^2_1 = 4.67$, $p = 0.031$); however, this became non-significant after Holm-Bonferroni correction.

3.2 ZENK activity

There was a significant effect of hormone treatment on ZENK activity in the HA (Figure 3C; $X^2_1 = 5.07$, $p = 0.024$), NCL (Figure 3D; $X^2_1 = 4.45$, $p = 0.035$), and CMM (Figure 3F; $X^2_1 = 4.59$, $p = 0.032$); however, after Holm-Bonferroni correction for multiple testing all *p*-values became non-significant (all $p > 0.05$). We did not detect an overall effect of sound treatment (all $p > 0.17$), or an interaction between hormone and sound treatment (all $p > 0.66$) in any region of interest, nor did we find an effect of hormone treatment in the AMV, cHP, and NCM (Figures 2A,B,E; all $p > 0.18$). There were no significant correlations between ZENK activity in any of the brain regions of interest and movement or feeding (all Spearman's coefficients <0.09, all $p > 0.12$).

4 Discussion

The overall goal of this research was to understand the effect of hormonal state on behavior and brain activity in response to breeding and predation signals. Female songbirds in breeding

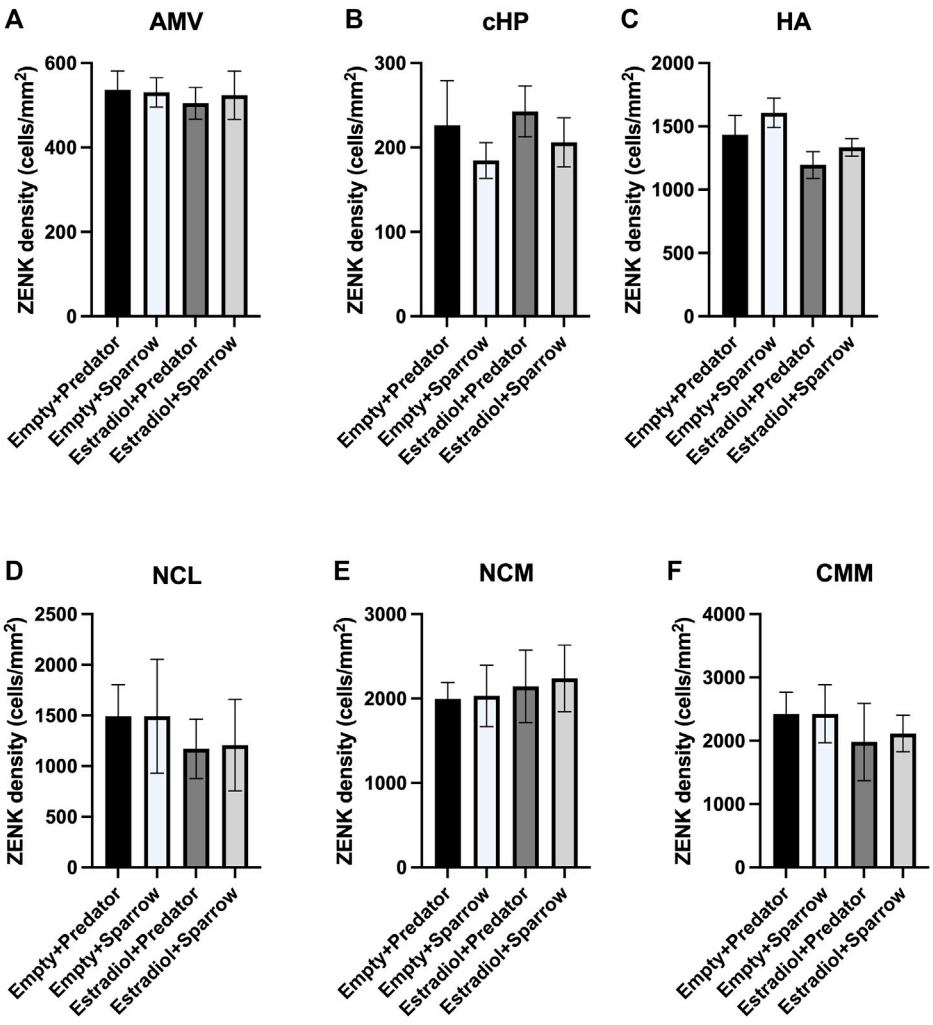

FIGURE 3
ZENK expression of female sparrows did not differ in response to predator or conspecific vocalizations. Sample sizes were n = 8 for Empty + Predator, n = 8 for Empty + Sparrow, n = 9 for Estradiol + Predator, and n = 8 for Estradiol + Sparrow. See Methods for data point losses. **(A)** AMV = medial ventral arcopallium, **(B)** cHP = caudal hippocampus, **(C)** HA = apical hyperpallium, **(D)** NCL = caudolateral nidopallium, **(E)** NCM = caudomedial nidopallium, and **(F)** CMM = caudomedial mesopallium. Error bars represent standard error.

condition were previously shown to have higher neuronal activity in response to conspecific song than to neutral tones (Maney et al., 2008; Sanford et al., 2010) and heterospecific song (Lattin et al., 2017b), but there had been no direct comparisons to sounds encoding potential threats. We hypothesized that female songbirds exposed to estradiol may also selectively "tune out" predator calls in favor of conspecific song, but we did not find support for this hypothesis. We instead found support for the null hypothesis, finding that neuronal activity did not differ in females hearing predator calls and male sparrow song, regardless of estradiol exposure. These data suggest female sparrows do not decrease their sensitivity to predator vocalizations while they are in breeding condition, and that this is not an explanation for the previously observed increase in depredation of breeding female songbirds (Slagsvold and Dale, 1996; Götmark et al., 1997; Post and Götmark, 2006). Thus, our data suggest there is no trade-off between females' auditory sensitivity toward mates and threats, and conspecific and predator cues both elicit a strong increase in neuronal activity in several brain regions.

We predicted that female sparrows exposed to predator calls would show more freezing behavior compared to sparrows exposed to conspecific song. Our findings supported this prediction: female sparrows exposed to predator playback moved less than females exposed to male sparrow song, regardless of hormone treatment. This is likely due to sparrows freezing to reduce attention to themselves while a predator is nearby, as seen in previous avian studies (Borchelt and Ratner, 1973; Quinn and Cresswell, 2005). Freezing behaviors are a common antipredator strategy of many wild animals in response to direct predator signals or the alarm calls of conspecifics (Beani and Dessí-Fulgheri, 1998; Rahlfs and Fichtel, 2010; Bedore et al., 2015). Other antipredator behaviors include vigilance and escape; however, the type of antipredator behavior exhibited may depend on the type of predator signal. For example,

red-legged partridges *(Alectoris rufa)* exposed to taxidermy models of terrestrial and aerial predators froze more in response to aerial predators and were most vigilant in response to terrestrial predators (Binazzi et al., 2011). Thus, we may have observed freezing (characterized as less movement in our study) due to the inclusion of aerial predator calls in our recordings.

We gave sparrows estradiol implants to simulate breeding condition. Consistent with previous studies using estradiol implants in female house sparrows (Lattin et al., 2017b), we did not observe copulation solicitation behaviors, one of the main mating behaviors studied in female birds (Searcy and Capp, 1997). However, sparrows that previously had estradiol implants spent more time feeding when exposed to conspecific song. This contradicts many rodent studies where increasing estradiol decreases feeding, and, further, that ovariectomized rodents increase feeding (Butera, 2010). However, estradiol implants increase feeding in Holstein heifers (Lammers et al., 1999), and estradiol-treated female sparrows have high circulating glucose levels, demonstrating that estradiol increases energy mobilization in house sparrows (Lattin et al., 2017b). Altogether, these studies suggest a relationship between estrogen levels and feeding behavior, but the direction of the relationship may vary by species. This relationship may be driven by reproductive strategy and seasonality: both heifers and songbirds are seasonal breeders, whereas laboratory rodents are not. Additionally, we only observed increased feeding behavior in females previously exposed to estradiol hearing conspecific song, not in those hearing predator calls. House sparrows increase their latency to feed in the presence of a predator signal (Seress et al., 2011); therefore, females in breeding condition may suppress feeding behavior in the presence of predator calls to increase vigilance. A trade-off between feeding and vigilance behavior is commonly observed across different species (Dill and Fraser, 1984; Poysa, 1987; Pueta et al., 2016).

Although female sparrows displayed different behavioral responses to predator and conspecific playback, we did not observe differences in ZENK activity in any of our regions of interest. We would typically expect that large differences in behavior would co-occur with differences in neuronal activity in parts of the brain involved in perceiving and responding to threats (e.g., AMV, cHP), which has been seen in other studies (Cross et al., 2013; Zanette et al., 2019; Freeman et al., 2020). Behavioral discrimination of conspecific calls has been positively correlated with activation of specific brain regions in rodents, but not in many other species (Sadananda et al., 2008; Schwarting et al., 2018), and we did not find any significant correlations between ZENK activity in any of our brain regions of interest and movement or feeding behavior. One possible explanation for these results is that regions we did not examine in this study may have been important in responding to predator cues, and these unexamined regions might have differed in ZENK expression. Other possible regions of interest could include the paraventricular nucleus and lateral septum, both of which are involved in regulating emotional and hormonal responses to stressful stimuli (Nagarajan et al., 2014; Smulders, 2021; 2017; Iyer and Tole, 2020). Alternatively, we may have seen differences in IEG activation in response to predator and conspecific playbacks if had we examined expression of a different IEG, for example, c-Fos. Several studies have found differences in the expression of different IEGs in response to the same stimulus (Sewall and Davies, 2017; Kimball et al., 2022). We chose ZENK because it is commonly used to assess neuronal activity in songbirds in response to acoustic stimuli (Maney et al., 2003; Rivera et al., 2019); however, future studies should consider using multiple IEGs to examine the brain's response to positive, neutral, or aversive auditory cues. Lastly, the inclusion of a neutral frequency-matched tone treatment or silent treatment group may have showed differences in brain responses to conspecific or predator vocalizations relative to neutral tones or silence; however, we were specifically interested in females' responses to predators relative to conspecifics, so we did not include this type of control.

In conclusion, female sparrows altered their behavior in response to breeding hormones and predator calls, specifically showing increased freezing behavior in response to potentially threatening sounds. Additionally, estradiol-treated female sparrows did not selectively "tune out" predator calls in the same way they can "tune out" neutral tones or heterospecific bird song (Maney et al., 2008; Sanford et al., 2010; Lattin et al., 2017b). These results are compelling because they suggest that different types of sounds may be perceived–and responded to–differently by breeding females, depending on the information content and valence of these sounds. Specifically, sparrows maintain high neuronal responses to signals directly related to reproduction and survival. Together, these behavioral and neural findings indicate that female sparrows are vigilant and responsive to the threat of predation regardless of their breeding condition.

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.

Ethics statement

The animal study was reviewed and approved by Louisiana State University Institutional Animal Care and Use Committee under protocol 96-2018.

Author contributions

CL designed the experiments. MK, CH, KC, KS, TK, and CL conducted the experiments. MK and CH analyzed the data. MK wrote the paper. MK, CH, KC, KS, TK, and CL reviewed and edited the paper.

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Conflict of interest

The 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.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2023.1172865/full#supplementary-material

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The avian taste system

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Taste or gustation is the sense evolving from the chemo-sensory system present in the oral cavity of avian species, which evolved to evaluate the nutritional value of foods by detecting relevant compounds including amino acids and peptides, carbohydrates, lipids, calcium, salts, and toxic or anti-nutritional compounds. In birds compared to mammals, due to the relatively low retention time of food in the oral cavity, the lack of taste papillae in the tongue, and an extremely limited secretion of saliva, the relevance of the avian taste system has been historically undermined. However, in recent years, novel data has emerged, facilitated partially by the advent of the genomic era, evidencing that the taste system is as crucial to avian species as is to mammals. Despite many similarities, there are also fundamental differences between avian and mammalian taste systems in terms of anatomy, distribution of taste buds, and the nature and molecular structure of taste receptors. Generally, birds have smaller oral cavities and a lower number of taste buds compared to mammals, and their distribution in the oral cavity appears to follow the swallowing pattern of foods. In addition, differences between bird species in the size, structure and distribution of taste buds seem to be associated with diet type and other ecological adaptations. Birds also seem to have a smaller repertoire of bitter taste receptors (T2Rs) and lack some taste receptors such as the T1R2 involved in sweet taste perception. This has opened new areas of research focusing on taste perception mechanisms independent of GPCR taste receptors and the discovery of evolutionary shifts in the molecular function of taste receptors adapting to ecological niches in birds. For example, recent discoveries have shown that the amino acid taste receptor dimer T1R1-T1R3 have mutated to sense simple sugars in almost half of the living bird species, or SGLT1 has been proposed as a part of a T1R2-independent sweet taste sensing in chicken. The aim of this review is to present the scientific data known to date related to the avian taste system across species and its impact on dietary choices including domestic and wild species.

KEYWORDS

avian species, gustatory system, taste receptors, diet, preference

1 Introduction

Birds are the most diverse group of vertebrates with more than 10,000 species (Pimm et al., 2006). Birds compared to mammals have often been referred to as having a poor taste acuity based on low numbers of taste buds (e.g., 767 in chicken compared to 7,902 in humans), less saliva production, and rapid transit of food through the oral cavity (Klasing, 1998; Roura et al., 2013). This undermining of the avian taste not only neglects the millions of years of evolution of the chemical senses, but it also underestimates the

existence of a highly developed taste (gustatory) system crucial for the adaptation of birds to a diverse range of ecosystems and dietary regimes. Evolutionary analyses based on taste receptor genes showed that the pillars of gustation were developed in vertebrates before the separation of teleost fish and tetrapods which ultimately led to birds and long after to mammalians. In addition, sweet and umami related taste receptors have remained highly conserved in vertebrates including humans (Shi and Zhang, 2006). Intriguingly, it appears that the entire avian clade evolved based on the early loss of sweet taste receptor (T1R2). However, soon after the loss, sugar detection ability was evolved by shifting umami taste receptors (T1R1-T1R3) to sweet sensor in songbirds comprising almost half of all current bird species (Toda et al., 2021). In fact, in two-choice assays, nectarivorous and non-nectarivorous songbirds strongly preferred sucrose solution over water. Thus, regardless of their diet, songbirds have the ability to perceive sweet taste (Toda et al., 2021).

Taste perception plays a key role in sustaining the adequate consumption of a balanced diet. In the wild, dietary choices reflect the innate drive to achieve and maintain nutritional homeostasis (Roura and Navarro, 2018). This, in turn, implies the existence of a network of nutrient receptor/sensors covering physiological functions starting with the control of appetite, dietary selection and overall feed intake (Roura et al., 2019). Thus, the main role of the taste system in the oral cavity is to perceive dietary nutrients and assess the nutritional quality of the meal. The avian sense of taste has been tuned to distinguish at least five and up to seven groups of tastants (or nutrients) including amino acids, fatty acids, salts, acids, bitterants and potentially simple carbohydrates and calcium (Roura et al., 2013; Niknafs and Roura, 2018).

The avian taste system has been studied by researchers from two perspectives. Firstly, understanding the biological and ecological aspect of taste mainly done by ornithologists. These scientists have investigated a wide range of wild and domesticated birds. Bath (1906) was among the pioneers of this area investigating the taste system in blackbird, barn swallow, mallard, flamingo, budgerigar, oystercatcher, European greenfinch, and common (European) starling. Secondly, the poultry scientist's perspective trying to understand agricultural and economic implications of the avian taste system. Poultry species such as chicken, turkey, quail, and duck have been the main interest. These scientists try to understand how the taste system plays a role in feed intake regulation, opening opportunities for using non-conventional feedstuff to the poultry industry (Roura et al., 2013).

This review article will focus on the description of the avian taste system including a brief historical perspective, fetal development, anatomical structure and function, oral-brain axis, molecular mechanisms eliciting taste and behavioral aspects linked to oral nutrient sensing in avian species. Some of the diversities observed between bird species relevant to the taste system will be highlighted. It is noted that the description of taste types (sweet, umami, bitter, salty, and sour) is necessarily based in anthropomorphic descriptions. Admittedly, the taste qualities elicited by taste receptor ligands is unknown in non-human animals. This review is based on the assumption that homology in taste receptor genes relates to homology in the type of the taste perceived.

2 A brief historical perspective on the discovery of the avian taste system

The study of the avian taste system has been ongoing at least since the 19th century. However, the first traceable scientific study failed to identify taste papillae or other anatomical structures such as taste buds known to exist in mammalian tongues (Merkel, 1880). In 1903, Elliott Coues in the fifth edition of his book, *Key to North American Birds*, laid out the importance of avian taste in food choice and the involvement of chorda tympani and cranial nerves in sensing taste (Coues, 1903). However, there was no mentioning of taste buds in birds. The discovery of avian taste buds should probably be credited to Eugen Botezat in 1904 (Botezat, 1906). Soon after, a topographical study on taste buds across several bird species was published by Bath (1906). After these first discoveries, no searchable scientific research on the avian taste system was published until the end of World War II. Some of the early reports after World War II on chickens showed the existence of a small number of only eight taste buds in the oral cavity (Lindenmaier and Kare, 1959). Consequently, a consensus that taste in birds did not have the functional relevance that it had in mammals dominated the scientific community until recent times (Niknafs and Roura, 2018).

A more accurate understanding of the relevance of the avian taste system started with the work published by Berkhoudt (1985) and especially by Ganchrow and Ganchrow (1985), who reported 70 and 316 taste buds in the chicken oral cavity, respectively. Ganchrow and Ganchrow reported that 69% of the buds were on the palate and not on the tongue like in mammals. These findings not only confirmed some of the earlier observations in wild birds reported by Bath (1906) but were also a turning point that triggered novel interest in the sense of taste in chickens. Some of the research published in the following years illustrated that birds had the ability of making dietary choices based on taste to a similar or higher accuracy than mammals (Matson et al., 2000). From the behavior point of view, a series of studies published by Van Heezik et al. (1983) and Gerritsen et al. (1983) demonstrated strong evidence that taste cues are used by shorebirds including Sanderling (*Calidris alba*), Dunlin (*C. alpina*), Purple Sandpiper (*C. maritima*), and Red Knot (*C. canutus*) to locate their prey and regulate their foraging behavior.

The advent of the genomic era triggered a positive boost on the appreciation of the taste system in avian species. In 2004, the first bird species genome, the chicken, was sequenced and released (Hillier et al., 2004). Analyzing the chicken genome revealed a full repertoire of taste receptor (TR) genes but also the lack of the mammalian sweet taste receptor T1R2 and a smaller number of bitter taste receptors (T2R), consisting of only three members compared to 25 in humans (Shi and Zhang, 2006). In addition, taste buds were historically studied using microscopic methods and the focus of the studies was mainly on tongue (Ganchrow and Ganchrow, 1985). However, recently molecular and immunohistochemistry (IHC) approaches along with expanding the search to palate led recently to the discovery of a plethora of taste buds in the oral cavity of chickens. IHC enables researcher to detect sensory cells using florescent antibodies resulting in identifying taste buds that have been missed using microscopic visualization. Rajapaksha et al. (2016) reported 767 taste buds in

chicken, 66% of which on the palate and the rest on the base of the oral cavity. In the past few years, fascinating aspects of the avian taste system have been discovered which highlight the evolutionary relevance of the avian taste system to adapt to dietary requirements in several bird species (Baldwin et al., 2014; Toda et al., 2021; Cockburn et al., 2022). Looking to the future, the recent release of the whole genome sequence of 363 bird species in 2020 and the initiative to sequence the genome of all the 10,000 bird species (B10K project: https://b10k.genomics.cn) will provide the opportunity to further understand the role of taste system in avian biology and evolution (Feng et al., 2020).

3 Comparative anatomy and development of the avian taste system

One of the most characteristic body structures that differs amongst bird species is the beak/bill. Beaks have been classified to reflect the adaptation to feeding regimes. Similarly, the avian tongue is also highly variable in length and shape adapted to food collection, manipulation, and swallowing (Berkhoudt, 1992). Beaks and tongues and their anatomical structure and function are highly associated with the topographic distribution of taste buds in the oral cavity in avian species (Berkhoudt, 1992; Kudo et al., 2008). For example, Crole and Soley (2015) demonstrated that taste buds are strategically located in the non-pigmented oropharynx in *Dromaius novaehollandiae*, enabling the bird to sample the food during ingestion.

3.1 Embryonic taste development

In chickens, taste bud development begins at early stages of embryonic development, and the rapid formation of taste buds occurs during the last stages between days 17 and 21 (Ganchrow and Ganchrow, 1985). In chicken embryos, beak and tongue can be differentiated by day 8, at the same time, mandibular salivary glands start developing from mucosal stem cells concluding on day 16 (Hamilton, 1953; Hamburger and Hamilton, 1992). In contrast, taste buds start emerging later at day 17 in the base of the epithelium forming spherical cluster of cells in the lower beak (Ganchrow and Ganchrow, 1987). These first buds reach the surface of the epithelium by day 19 when taste pores become distinguishable (Ganchrow and Ganchrow, 1987). On embryonic day 20, basal and perigemmal cells in the taste buds are recognizable (Ganchrow and Ganchrow, 1989). By the time of hatch at day 21, taste buds continue to elongate to an ovoid shape and almost all buds' pores are opened to the oral cavity with no spherical shapes remaining (Ganchrow and Ganchrow, 1987). Before hatching, the embryonic taste system is responsive to stimuli such as quinine, fructose, HCl, NaCl, and KCI (Vince, 1977). At hatch, taste buds are fully functional and responsive to taste stimuli. There is a fast increase in the number of taste buds during embryonic day 17 and 18 and reaches 80 taste buds by day 19 (Cheled Shoval et al., 2022). While some data suggest that the total number of taste buds has been reached before hatching, some other have shown that the taste system continues to grow and mature reaching the peak by day 3 post-hatching (Ganchrow and Ganchrow, 1987; Ganchrow et al., 1995; Rajapaksha et al., 2016). These inconsistencies between research groups may indicate potential differences in the development of taste buds between breeds and sexes (Liu et al., 2018).

3.2 Oral topographic distribution of taste buds

Unlike mammals, the avian tongue is not a major sensory organ. The lingual epithelium is often keratinized and does not contain differentiated appendices or organelles such as taste papillae (Elner et al., 2005). Most taste buds in birds are located on the soft and glandular epithelia of the palate (Erdoğan and Iwasaki, 2014). In addition, taste buds appear in clusters around salivary ducts as it has been shown in many avian species including chicken, sparrow, kingfisher, spotted owl, pigeon hawk, western sandpiper, dunlin, and parrot (Nalavade and Varute, 1977; Elner et al., 2005). The presence of abundant saliva in the oral cavity is crucial to facilitate the sensing of food compounds by reaching the taste buds and getting in physical contact with the taste receptors. The production of saliva is variable depending on feeding strategies being best developed in birds that consume dry diets such as granivores or insectivores (Beltman and Kare, 1961). However, avian species have often been described as having limited saliva secretion when eating (Klasing, 1998). Thus, pattern of location of taste buds surrounding salivary glands guarantees an efficient use of the saliva for taste sensing (Kurosawa et al., 1983).

The topographic distribution of taste buds in the avian oral cavity has been related to feeding behaviors and ingestion routes reflecting the main role of the tongue in food collection and swallowing (Martin, 2017). Three distribution patterns can be differentiated related to tongue functions (King and McLelland, 1984; Klasing, 1998):

1 Type I relates to tongues adapted to swallowing. These tongues are characteristically short and non-protrusile. This type has been described in chicken, pigeon, pelicans, cormorants, ostriches, or cassowaries. For example, the chicken tongue is keratinized in the tip and central body but not in the back towards the pharynx (Berkhoudt, 1992). Tongue keratinization seems incompatible with sensory properties. The process of swallowing involves pecking the food between beak tips before moving it intraoropharyngeal with the tongue pressing the food against the taste buds on the upper palate. These movements optimize the contact of food particles with the saliva and taste sensory cells (Fowler, 1991; Van den Heuvel and Berkhoudt, 1997). The taste bud distribution in chicken (Figure 1) seems to be consistent with the swallowing process. In brief, the major location of taste buds in this group is the upper palate (almost 70%), but they are also found in the strips of soft oral mucosa on both sides (5%), at the back (5%), and base (20%) of the tongue (Bath, 1906; reviewed by Berkhoudt, 1992; Rajapaksha et al., 2016). However, there is a wide range of diversity even within this category. For example, the Eurasian collared dove (*Streptopelia decaocto*) and chicken

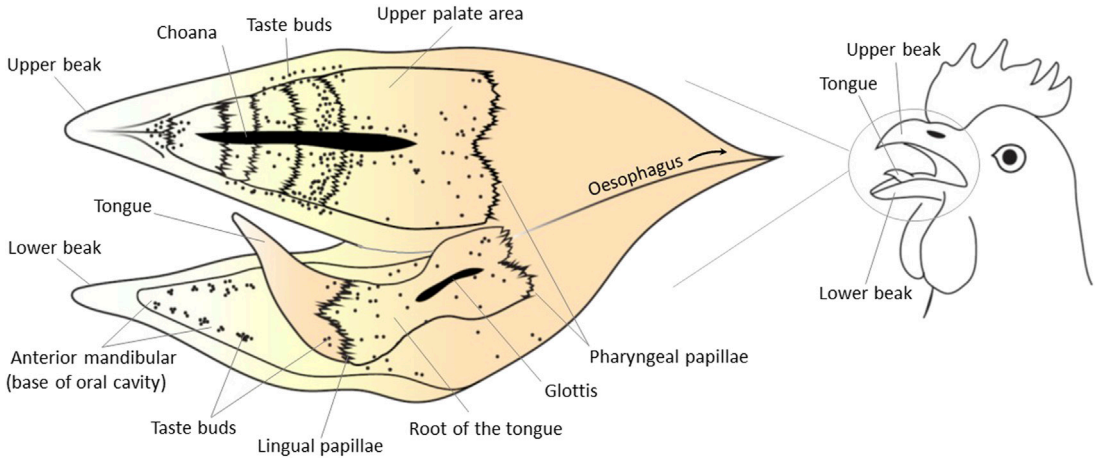

FIGURE 1
Topographical distribution of taste buds in the chicken oral cavity. The schematic view of the internal surface of the oral cavity is shown as a flat surface with the upper and lower surfaces next to each other in an "open book"-type view. Black dots represent taste buds. Lingual and/or pharyngeal papillae refer to mechanical (and not taste) organelles. The tip of the tongue is keratinized and does not contain taste buds. The main density of taste buds is in the upper palate. Other parts of the oral cavity in chickens with presence of taste buds include the root of the tongue and oropharynx, the base of the tongue and the soft oral mucosa in the mandible on both sides of the tongue. Taste buds gather in groups of 1–10 to form clusters, and these clusters are broadly distributed on the palate and the base of the oral cavity mainly around salivary ducts. This figure was created based on the data published by Kudo et al. (2008), Rajapaksha et al. (2016) using Adobe Illustrator 24.0.

have similar tongue anatomy, but taste buds and salivary glands are present in the lingual epithelia in Eurasian collared dove. This has been claimed to be an adaptation to an herbivorous feeding style. The body and root of the tongue and the laryngeal mound contain ovoid-shaped taste buds (El-Mansi et al., 2021).

2 Type II refers to tongues adapted for food manipulation. These tongues are usually not very protrusile and can be subdivided into three subtypes: a) thick and muscular tongues as required for extraction of seeds from cones and husks such as in parrots, finches, and crossbills; b) tongues with extensive sharp papillae to hold slippery preys such as in fish-eating birds or raptors; or c) tongues rich in thread-like papillae for aquatic filter feeding such as in waterfowls. In ducks, for example, the wide and fleshy tongue leaves no space free in the sides of the mandible mucosa and no taste buds are present there. In contrast, additional rostral fields of taste buds are located in the mandible under the tongue tip and in the palate and in both posterior and anterior parts of the oral cavity and include pressure buds in the tip of the bill and on the roof of the oral cavity (Berkhoudt, 1977; Fowler, 1991). In Mallards, taste buds occur not only on the tongue but also in the mouth floor and the bill tip. This suggests the ability of Mallards in distinguishing taste cues just by holding food materials between the bill tips without bringing them further into the oral cavity (Zweers and Wouterlood, 1973; Martin, 2017).

3 Type III has been identified for birds with long beaks and tongues. Tongues in this group are adapted to food collection being long and protrusile and functioning as probes. This is the case for woodpeckers, hummingbirds, honeyeaters, and lorikeets with tongues collecting sap, insects, or nectar. Species of this group have some areas at the back of their tongue and oropharynx containing high number of taste buds (Bath, 1906). A detailed and systematic study on oral distribution of taste buds in this group is missing.

3.3 Taste sensory cell types and comparative taste bud structure

According to Berkhoudt (1992) avian taste buds consist of four types of cells including sensory cells (light cells in the electron microscope with a low-density cytoplasm and high number of vesicles), supporting cells (dark cells with a dense cytoplasm and less vesicles), follicular cells, and basal cells (Figure 2). Ganchrow and Ganchrow (1987) also found four different cells in chicken taste buds labelling as light, intermediate, dark, and basal cells. The light was identified as sensory cells, and dark and intermediate cells as supporting cells. In mammals, these different cells are commonly known as Type I, II, III, and IV (Chaudhari and Roper, 2010; Roper and Chaudhari, 2017). These four types are probably the equivalent of dark cell, light cell, intermediate cell, and basal cell, respectively (Figure 2). Type I and III in mammals would be the equivalent of the supporting cells in chicken's buds (Kurosawa et al., 1983). Differences in cell types between avian and mammals is possibly related to the adaptation to nutrition requirements, feeding habits and pattern of ingesting food in birds.

In chickens, 55% of the cells in the taste buds belong to the Type I cell group referred to as dark cells (Ganchrow et al., 1998). These cells have a function similar to glial cells in the central nervous system where they clear the neurotransmitters from the extracellular environment, thus, terminating the signal transmission (Chaudhari and Roper, 2010). In addition, these cells are involved in the homeostasis of K^+ in the buds and the transduction of salty taste (Vandenbeuch et al., 2008; Dvoryanchikov et al., 2009).

The light cells are equivalent to the Type II cells in mammals. They express the G-protein coupled receptors (GPCR) known to mediate sweet, umami, and bitter taste in humans (Roper and Chaudhari, 2017). Any single cell of this type expresses the

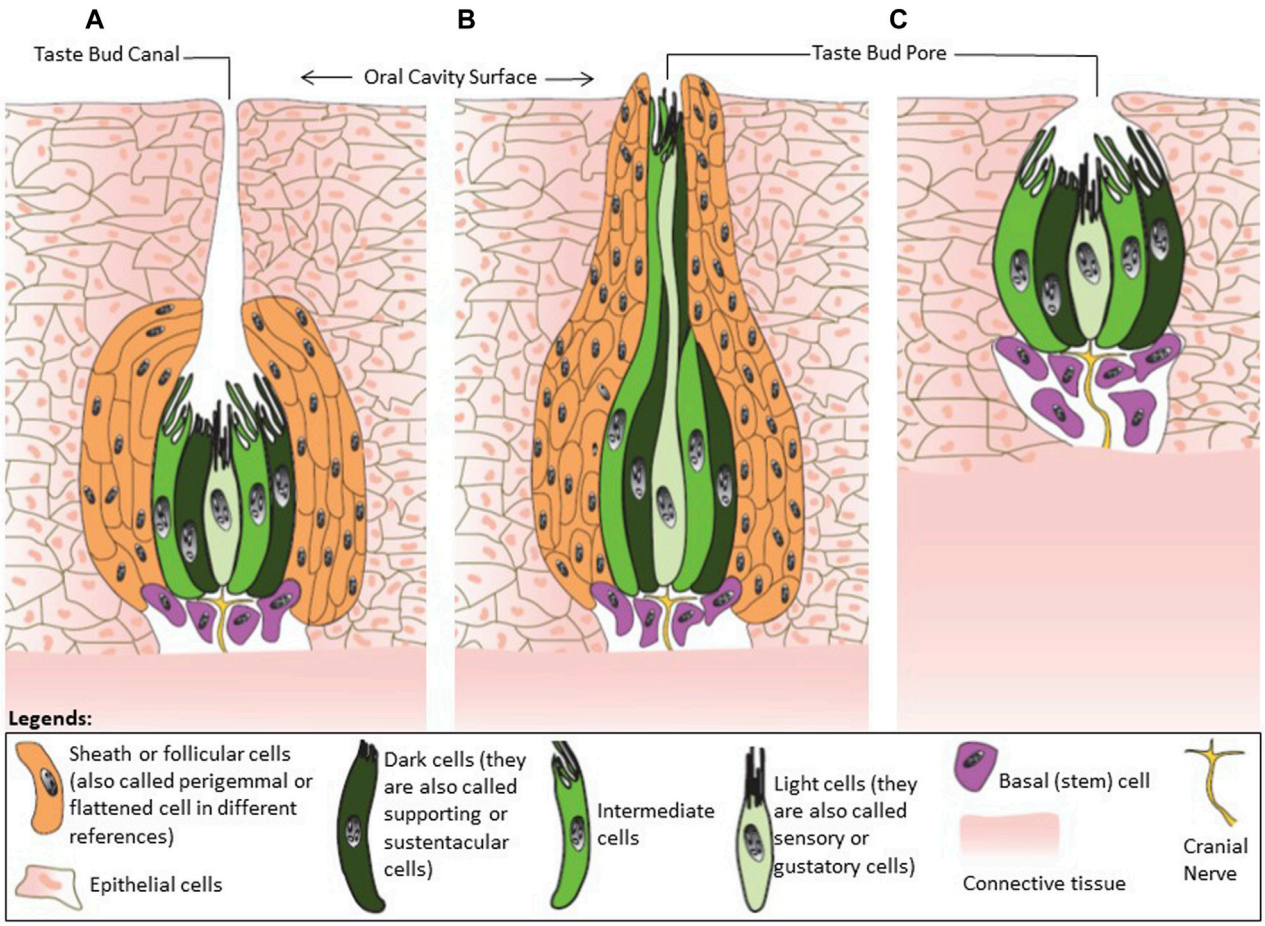

FIGURE 2
Classification of avian taste buds. **(A)** Type I is an ovoid taste bud enwrapped by follicular cells. **(B)** Type II have an elongated shape. **(C)** Type III has no follicular cells. This figure was created based on the descriptions from Bath (1906), Rowland et al. (2015) using Adobe Illustrator 24.0.

receptors to convey only one taste type, i.e., either sweet, or bitter, or umami (Nelson et al., 2001).

The intermediate cells are equivalent to the mammalian Type III cells. They account for the lowest number of cells in the buds and are characterized by the synaptic junctions with neural fibers (also referred as pre-synaptic cells). These cells receive signals from Type II cells amplifying the response to a broad range of tastants (Tomchik et al., 2007; Chaudhari and Roper, 2010).

The basal cells are at the bottom of the taste buds, and they are undifferentiated cells. These cells are the precursors of the other three types of cells in the buds (Roper and Chaudhari, 2017).

The number of cells required to form a taste bud in avian species does not differ significantly from the numbers observed in mammals based on comparative size. The size of taste buds (height × width) in some granivore and insectivore such as pigeon, swallow, woodpecker, and greenfinch are about 75 μm × 44 μm. Omnivore birds such as chickens, sparrow, and starling have bigger taste buds (114 μm × 32 μm) (Bath, 1906). In humans and pigs, taste buds are 79 μm × 39 μm and 93 μm × 36 μm, respectively. Avian taste buds have been classified into three groups based on histological structure and are represented in Figure 2 (Bath, 1906; Botezat, 1906; Berkhoudt, 1992; Rowland et al., 2015; Cheled Shoval et al., 2022):

The type I are ovoid-shaped taste buds enwrapped by follicular cells (Figure 2. I). Chickens, pigeons, and songbirds have Type I taste buds. In chickens, there is a long canal (tubule) in the taste buds ending with a pore at the surface of the epithelium. This canal is a feature that has not been observed in any mammalian species known to date.

The type II are elongated and narrow taste buds with the follicular cells protruding into the epithelial surface (Figure 2. II). Examples of avian species presenting Type II taste buds are ducks and waders.

The type III are mammalian-like rounded taste buds lacking follicular cells (Figure 2. III). Parrots are an example of avian species with Type III taste buds.

The diversity in taste bud types across avian species has been associated with the adaptation to available foods and the food patterns summarized in Table 1 (Rowland et al., 2015). Bird species feeding mainly on dry foods such as insectivores such as European starlings, have developed small taste buds (123 × 38 μm). In contrast, aquatic birds (e.g., ducks) appear with the largest taste buds (130 × 60 μm). As shown in Table 1, omnivore birds such as

TABLE 1 Food consumption patterns and the taste system of birds.

Diet type[a]	Taste bud number, type, and other features	Number of T2Rs	Behavioral response to different tastants	References
Insectivores	European starling	White-throated sparrow: 13	Great tit: Preference for sucrose. Large variability for chloroquine diphosphate but consumes prey secreting defensive bitter compounds	Toda et al. (2021), Espaillat and Mason (1990), Hämäläinen et al. (2020), Bath (1906)
	200, Type I, height × width of taste buds is 123 × 38 μm	Bar-tailed Trogon: 12		
	Blue tit	Rifleman: 9	European starling: Avoidance from tannic solution	
	24, Type I	European starling: 7		
Carnivores	European kestrel: unknown, Type I, height × width of taste buds is 148 × 45 μm	Owl: 2	Overall, this category of birds accepts sugary solutions	Abumandour and El-Bakary (2017), Espaillat and Mason (1990), Werner et al. (2008), Cheled-Shoval et al. (2017b), Bath (1906)
		Falcon: 2	Passerine: Preference for Alanine and MSG	
Granivores	Pigeon	Pigeon: 1	Canary: Preference for sucrose	Toda et al. (2021), Matson et al. (2004), Bath (1906)
	59, Type I, height × width of taste buds is 93 × 62 μm	Zebra finch: 8		
	Bullfinch	Medium ground finch: 11	Cockatiels: Avoidance from quinine	
	42, unknown			
Omnivores	Chicken	Chicken: 3	Overall, this category of birds accepts sugary solutions	Crole and Soley (2015), Cheled Shoval et al. (2022), Niknafs et al. (2022), Duncan (1962), Balog and Millar (1989), Brand et al. (2022), SpillariViola et al. (2008), Rajapaksha et al. (2016), Wang and Zhao (2015), Berkhoudt (1977), West et al. (2022)
	767, Type I, height × width of taste buds is 114 × 32 μm, and more than 66% of the taste buds on the palate and the rest on the base of the oral cavity	Duck: 4	Chicken: Preference for Alanine, Calcium, long chain fatty acid, and salt at 85-10 mM. Avoidance from quinine, acidic or alkalic solution at high concentration	
	Duck: Mallard	Turkey: 4	Ostrich: Preference for salt at 14/g/kg of feed	
	375, Type II, 130 × 60 μm unknown, Type II	Kea: 2	Blackbirds: Avoidance from tannic solution	
	Japanese Quail: Turkey	Crow: 10	Muscovy duck: taste cues affected tactile foraging behavior	
	62, unknown 200, unknown			
	Emu: unknown, Type I, height × width of taste buds is 96 × 51 μm			
Frugivores	Parrot		Overall, this category showed higher preference for hexose monosaccharide compared to sucrose	Rio et al. (1988), Martinez del Rio and Stevens (1989), Napier et al. (2013)
	350, Type III			
Piscivores		Dalmatian Pelican: 2		Davis et al. (2010), Wang and Zhao (2015)
		Great Crested Grebe: 2		
Molluscivores	Waders and Flamingos: unknown, Type II	American Flamingo: 2		Davis et al. (2010), Wang and Zhao (2015)
Nectarivores		Anna's Hummingbird: 10	Hummingbirds, sugarbirds, sunbirds, honeyeater, white eye, and bulbul: Preference for sugar solutions	Toda et al. (2021), Jackson et al. (1998), Clark et al. (2015), Toda et al. (2021)

T2Rs, Bitter taste receptors; MSG, monosodium glutamate.
[a]Klasing, 1998.

emu (96 × 51 μm) have smaller taste buds than carnivore species such as European kestrel (148 × 45 μm). In general, the average size of taste buds in birds seem to be bigger than in mammals except for ruminant species. The size might be related to the number of cells shaping the taste bud.

There are a few additional differences between mammalian and avian taste buds that include the life span and the embryonic tissues of origin. The average life span of chicken's taste buds is 3–4 days whereas in laboratory rodents is around 10–12 days (Ganchrow et al., 1994). Thus, taste bud's basal (stem) cells in birds undergo a more rapid development to meet the high turnover (Liu et al., 2018; Wang et al., 2019a). In addition, it has been reported that taste bud cells are not derived from neural crests but from mesenchymal cells with high migratory properties in chickens. In

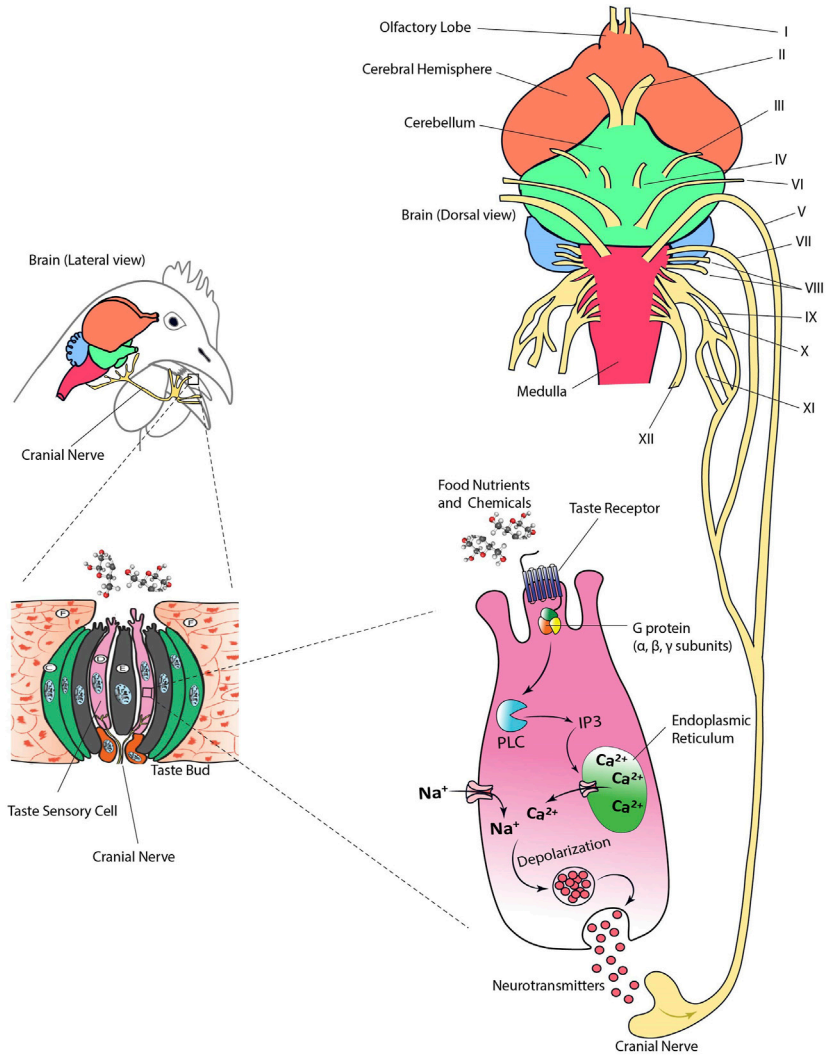

FIGURE 3
Taste transduction via cranial nerves in birds. Cranial nerves (CN) V, VII, IX, X, and XI transmit taste information from the taste buds to the brain. Sensory ganglia are not shown. Figure was created using Adobe Illustrator 24.0.

contrast, taste bud cells in mammals are from epithelial origin (Witt et al., 2000; Niknafs and Roura, 2018; Roura and Foster, 2018; Yu et al., 2021).

3.4 Neuroanatomy of the avian taste system

Nutrients and other chemical compounds present in food such as glucose, amino acids, minerals, and organic acids come into contact with taste sensory cells in the oral cavity. Dietary nutrients bind to the TRs and transmembrane channels triggering a cascade of biochemical reactions. These reactions trigger a signal resulting in the release of neurotransmitters and the excitation of cranial nerves that ultimately will stimulate the gustatory cortex of the brain (Figure 3). There are 12 cranial nerves (CN) in birds of which V, VII, IX, X, and XI are involved in transmitting taste information (Gentle, 1983; Clippinger et al., 1996; Orosz, 1996; Orosz and Bradshaw, 2007; Roper, 2007; Clark et al., 2015).

3.4.1 CN V (Trigeminal Nerve)

This nerve has 3 main branches (V1, V2, and V3). The V3 branch is innervating sensory information from mucosa and skin at the lower bill and rictus (Orosz and Bradshaw, 2007). It also transmits gustatory (taste) information from the taste buds in the floor of the oropharynx to the nucleus solitaries of the medulla in the central nervous system (Orosz, 1996).

3.4.2 CN VII (Facial Nerve)

It has been described that the facial nerve controls muscles responsible for facial expression in mammals. However, there is a poor development of facial expression with only one muscle, the mandibular depressor, innervated by CN VII in birds (Orosz and Bradshaw, 2007). In addition, CN VII is involved in transmission of taste information from the tongue to the nucleus solitaries of medulla in the central nervous system. The chorda tympani which is a branch of CN VII transmits information from the taste buds close to the anterior mandibular salivary glands. Most

of the glands in the head including salivary glands, which are key elements in taste perception, receive parasympathetic intervention from CN VII (Berkhoudt, 1980; Gentle, 1984; Ganchrow et al., 1986).

3.4.3 CN IX, X, XI (Glossopharyngeal Nerve)

These nerves are bundled together leaving the ventrolateral medulla. CN IX, X, and XI gather taste information from the posterior buccal and pharyngeal areas in avian species. CN IX and VII convey the signals from lingual taste buds and taste perception (Clippinger et al., 1996).

4 Taste sensing: physiology and feeding behavior

The taste receptors (TRs) involved in nutrient sensing are highly conserved in vertebrate animals and can be traced as far back as Dinosauria (Baldwin et al., 2014). The taste qualities that nutrients and other compounds may elicit in birds can only be inferred from the taste qualities known in humans (i.e., sweet, umami, bitter, fatty, salty, and sour). In birds, the family 1 taste receptors (T1R1 and T1R3) mediate the taste of amino acids and sugars, this is, umami and sweet taste in humans, respectively. The Family 2 referred to as the T2Rs, are associated with bitter perception. In addition, it seems that umami and bitter compounds stimulate different parts of the brain in chickens. Umami tastants triggered higher neural response (measured by c-Fos activity) in the right hemisphere of the nucleus taeniae of the amygdala, while bitter tastant created higher response in the left hemisphere (Protti-Sánchez et al., 2022).

The intracellular biomarkers specific to taste sensory cells originally identified in mammalian species (i.e., laboratory rodents or humans) are also highly conserved in birds. For example, the α subunit of the G-protein α-gustducin has been extensively used as a biomarker of taste sensory cells in chickens (Rajapaksha et al., 2016; Venkatesan et al., 2016) (Figure 3). Other cytosolic compounds identified as part of the taste transduction cascade in birds include PLC-β2 and TRPM5 (Witt et al., 1999; Venkatesan et al., 2016; Yoshida et al., 2018; Yoshida et al., 2019). In addition, vimentin has been identified as a taste sensory cell biomarker in chickens (Katsumoto et al., 1990). Vimentin is an intermediate filament protein involved in development of taste bud cells (Witt et al., 2000). This protein regulates cell migration and mechanics, and its expression is an indicator of transition from epithelial to mesenchymal cell (Sivagurunathan, et al., 2022). Thus, vimentin and α-gustducin seem to be specific to taste receptor cells in birds. However, recent data in chickens has shown that T1R3 is mainly expressed in vimentin-positive cells, while T2Rs were expressed in vimentin-negative cells indicating the specificity of taste-type marker of the filament (Yoshida et al., 2021a).

In recent years, significant progress has been made on how macronutrients such as carbohydrates, proteins, lipids, and calcium activate chemosensory mechanisms in the avian taste system (Niknafs and Roura, 2018). Each macronutrient has been related to one taste-type such as the umami to amino acids and peptides, sweetness to simple carbohydrates (sugars) and fatty to free fatty acids. However, the qualification of fatty taste in humans is still controversial (let alone in birds). Overall, birds appear to have an acute sense of taste allowing for the discrimination of dietary macronutrients (Roura et al., 2013).

4.1 Umami taste

Dietary protein and some amino acids are essential nutrients across most vertebrate species (Alagawany et al., 2020; Li and Wu, 2023). It appears that the ancestral T1R family of receptors evolved after the separation of vertebrates and invertebrates preceding the emergence of terrestrial animals. In particular, the heterodimer T1R1-T1R3 has become the principal dietary amino acid receptor in fish, amphibians, avian and mammalian species (Oike et al., 2007). In humans, the heterodimer was identified as the TR responsible for eliciting umami (savory) taste (Nelson et al., 2001). All avian species require dietary sources of amino acids such as meat, grains, or insects. Thus, it is likely that an umami-like taste has been developed in birds to identify food rich in amino acids (Rowland et al., 2015). The T1R1-T1R3 heterodimer has been found in all birds studied to date across all feeding styles including carnivores (e.g., falcon), piscivores (e.g., sea birds), micro-faunivores (e.g., some ducks), insectivores (e.g., swift, flycatcher), omnivores (e.g., quail), herbivores (e.g., some ducks), and granivores (e.g., chicken, finches, pigeons) (Shi and Zhang, 2006; Roura et al., 2008; Zhao et al., 2011). However, in addition to amino acids, the T1R1-T1R3 sensor in songbirds has mutated to sense simple sugars consistent with a feeding strategy specialized in sugar-rich food resources (Baldwin et al., 2014; Toda et al., 2021; Cockburn et al., 2022). The latter has been further elaborated in Section 4.2. The sensitivity of T1R1-T1R3 to amino acids and sugars changes across different bird species. Alanine, lysine, arginine, asparagine, valine, serine, and glycine trigger the T1R1-T1R3 heterodimer more than other amino acids (Cockburn et al., 2022).

Interestingly, L-alanine showed the highest affinity for the chicken T1R1-T1R3 consistent with the responses reported in other avian species like the swift, or other vertebrates such as the medaka fish or the mouse (Baldwin et al., 2014; Yoshida et al., 2022). Behavioral studies in passerines and chickens also showed robust taste preferences for alanine. Both red-winged blackbirds and starlings preferred Alanine solution at ≥0.7% concentrations (Espaillat and Mason, 1990; Werner et al., 2008; Niknafs et al., 2022). From a nutritional point of view, it is relevant to note that alanine is a non-essential amino acid (not required in feeds) since it can be synthesized sufficiently from metabolic precursors in all eukaryotic cells. Another non-essential amino acid eliciting robust preferences in birds is glutamic acid or monosodium glutamate (MSG). Glutamic acid is the most abundant amino acid in animal tissues, particularly muscle fibers (Dalle Zotte et al., 2020). The threshold of detecting MSG in chicken was reported at 300 mM indicating a lower sensitivity than humans with mean detection threshold of 1.22 mM (Cheled-Shoval et al., 2017b; Lim et al., 2022). However, some researchers have observed low preference for umami solution (Yoshida et al., 2021a). This has been associated with the findings that T1R1-T1R3 heterodimer is barely formed in chickens since T1R1 and T1R3 were only co-expressed in 5% of the taste sensory cells (Yoshida et al., 2021a). However, further data is needed to support such claim since the heterodimer was shown to be

responsive to alanine and serine in a chicken cell model (Baldwin et al., 2014).

In addition to T1R1-T1R3, other amino acid or peptide receptors such as mGluR1, mGluR4, GPR92, CaSR, GPR139, and GPRC6A have also been reported to be functional in the avian oral cavity (Baldwin et al., 2014; Cheled-Shoval et al., 2014; Niknafs et al., 2018). For example, CaSR is expressed in chicken taste buds and its activity increased responding to alanine, tryptophane, and phenylalanine (Omori et al., 2022). However, low extracellular calcium negatively affected the activation of CaSR by these amino acids (Omori et al., 2022). Furthermore, CaSR may play an essential role in sensing calcium which is discussed in Section 4.4.

4.2 Sweet taste

Many different bird species rely on simple (i.e., glucose, fructose, sucrose) or complex (i.e., starch) carbohydrates as the main dietary energy sources including frugivore, granivore, and nectarivore species (Klasing, 1998). The main sensor for simple carbohydrates (sugars) was identified as the heterodimer T1R2-T1R3 in mammals which in humans has been described to mediate sweet taste perception (Zhao et al., 2003; Meyers and Brewer, 2008). However, it appears that the T1R2 gene has been lost in avian species (Shi and Zhang, 2006; Zhao et al., 2011; Lagerstrom et al., 2006). Birds descended from carnivorous theropod dinosaurs who had no essential needs for carbohydrates (Padian and Chiappe, 1998). Thus, as it has been also shown in mammalian carnivores, the loss of T1R2 in the avian ancestor would have been related to the adaptation of the taste system to a carnivore feeding regime (Nei et al., 2008). The loss of the sweet taste receptor T1R2 may have played a key role in the early evolution of avian species in adapting to ecological niches. However, subsequent evolution of some avian species drifted to feeding patterns involving sugar-rich sources (e.g., nectar or sweet fruits) as the principal nutrient sources which, in turn, resulted in the development of high preferences for sugars in hummingbirds, sugarbirds and sunbirds (Jackson et al., 1998; Clark et al., 2015; Toda et al., 2021). These findings suggest that alternative T1R2-independent mechanisms for sugar detection have evolved in these species. A series of mutations occurred in the umami taste receptor that shifted the sensitivity of the T1R1-T1R3 dimer to carbohydrate ligands in these avian species (Baldwin et al., 2014; Toda et al., 2021). Such shift conferred the ability to perceive sweet taste in almost half of the living bird species (i.e., songbirds) in the absence of T1R2 (Toda et al., 2021). Baldwin and co-workers (2014) discovered that hummingbirds regained sweet taste perception by mutating both T1R1-T1R3 subunits. The mutated receptor in hummingbirds strongly responded to sucrose, fructose, glucose, sorbitol, erythritol and the artificial sweetener sucralose while showing a loss of affinity to amino acids compared to chickens or swifts (Baldwin et al., 2014). The regaining of the ability to taste simple carbohydrates (sweet taste) has been shown to be stable despite frequent transitioning in some bird species from and to nectar feeding (Toda et al., 2021).

Interestingly, the concentration and ratios between sucrose, glucose and fructose in nectar varies greatly among flowers and seems to be relevant to determine species-specific preferences (Honda et al., 2010). Within nectarivore bird species, different preferences have been related to differences in the efficiency of intestinal hydrolysis and osmolality (Leseigneur and Nicolson, 2009; Medina-Tapia et al., 2012; Martínez del Rio et al., 2015; Koutsos et al., 2016). Frugivore birds exhibit lower appetites for sucrose compared to hexose monosaccharides, seemingly due to the lack of sucrase and/or the lower absorption rates (Rio et al., 1988; Martinez del Rio and Stevens, 1989; Napier et al., 2013). This variations in the type of sugar preferences have been associated with the pollinator role of birds which would influence nectar composition, concentration, and volume as an example of pollinator-plant co-evolution (Lotz and Schondube, 2006; Johnson and Nicolson, 2008). As in mammals (Sclafani et al., 1987), sex differences relevant to sugar appetites have been reported in nectarivores (Espaillat and Mason, 1990; Markman et al., 2006). Gut transit time of sucrose in Palestine sunbirds was longer in males (50 min) than females (30 min) (Markman et al., 2006). This may be linked to differences in taste sensitivity, energy requirements, and digestive capacity between male and female birds.

Insectivorous, omnivorous, and carnivorous birds reportedly accept sugary solutions. However, sensitivity and thresholds tests have not been performed in most avian taxa (reviewed by Clark et al., 2015; Rowland et al., 2015). The threshold of detecting sucrose in chicken was reported at 1 M indicating less sensitivity of chickens compared to humans which was reported to be 6.8–10.2 mM (Cheled-Shoval et al., 2017b; Petty et al., 2020). Studies testing glucose, fructose, sucrose, and artificial sweeteners such as saccharin in chickens have been contradictory (recently reviewed by Cheled-Shoval et al., 2017a; Rowland et al., 2015). Since the chicken T1R1-T1R3 dimer has high affinity for alanine and serine and no affinity for sugars, it is tempting to speculate that chickens may have a T1R-independent sweet perception such as in laboratory rodents where the sweet sensing of glucose or maltose originate in starch hydrolysis. The apical membrane of taste buds has been associated with disaccharidase activities and the transmembrane Sodium-Glucose Transporter 1 (SGLT1) (Sukumaran et al., 2016). Chickens show an intact mammalian-like disaccharidase-SGLT1 system which may also account for the T1R2-independent glucose and galactose sensing mechanism in the oral cavity (Higashida et al., 2022). Another possible mechanism could be related to the activity of SGLT1 in extra-oral tissues such as intestine. In mice, for example, it has been shown that preference for sucrose over artificial sweetener was mediated by duodenal neuropod cells (Buchanan et al., 2022). Neuropod cells differentiate between sugar and sweetener by eliciting different neurotransmission pathways using SGLT1 and sweet taste receptors (Buchanan et al., 2022).

4.3 Bitter taste

In birds as in mammals, the sensing associated with the activation of family two taste receptors (T2R) plays a primary defense function to prevent the ingestion of potential toxic compounds presumably by eliciting bitterness or a similar unpleasant sensation, showing behavior responses like head shaking, beak wiping and tongue and beak movements (Gentle, 1978). Birds learn to use the distastefulness associated with bitter compounds as a signal of toxicity (Skelhorn and Rowe, 2010). Such deterrence to bitter tastants (e.g., quinine, D-pulegone or garlic oil)

has been used to protect crops and horticulture or to protect birds from toxic pesticide applications (Mastrota and Mench, 1995; Hile et al., 2004; Clapperton et al., 2012). Blackbirds and European starlings avoided the consumption of tannic solutions (0.5%–5%, which is in the range found in fruits and grains) over distilled water inferring they could perceive a bitter/unpleasant taste or smell (Espaillat and Mason, 1990). Starlings were more sensitive to tannic acid solution (0.5%–1%) compared to blackbirds (Espaillat and Mason, 1990). This may be reflecting the difference in their feeding habits meaning starlings consume less of foods containing tannin.

Bitter compounds relate to many different chemical categories including alkaloids (e.g., glycoalkaloids present in tubers, fruits, and seeds), isoprenoids (e.g., terpenes found in plants and insects), or phenylpropanoids (such as polyphenols present in fruits and cereals). Interestingly, some of these bitter compounds have beneficial biological functions such as antimicrobial, antioxidant, or digestive enhancing activities (Bernards, 2010). In fact, for non-toxic compounds there is a biphasic response to bitterness consisting of a first innate aversion followed by an adaptive behavior and acceptance as reported in insectivores, granivores and nectarivores (Fink and Brower, 1981; Marples and Roper, 1997; Johnson et al., 2006; Skelhorn, 2015). In nectarivores, bitter compounds may selectively encourage or discourage the consumption of specific plant nectars. For example, phenolic-rich dark nectar from *Aloe vryheidensis* attracted dark-capped bulbuls while repelling sunbirds (Johnson et al., 2006). In the case of great tits (*Parus major*), the large variability in their perception threshold for chloroquine diphosphate (0.01 to 8 mmol/L) did not impact their foraging choices regarding the consumption of preys secreting the bitter compound as a defensive mechanism. The energy status (body condition) and not the bitter compound seemed to be the main driver of prey consumption (Hämäläinen et al., 2020). The threshold of detecting quinine in chicken was reported at 0.3 mM indicating a similar sensitivity level to mammals (Cheled-Shoval et al., 2017b). However, the *in-vivo* threshold is often higher than the *in-vitro* threshold (Cheled-Shoval et al., 2017a).

Avian species have a wide range of the bitter taste receptor repertoires. The range goes from one T2R in the turkey to up to thirteen T2R in the white-throated sparrow (Table 1), indicating a dynamic role in evolution to adapt to new environments (Davis et al., 2010). Such variation may have had evolutionary implications for bird species. In episodes of expansion, large number of receptors allow the birds to detect and discriminate a wider range of bitter compounds, with higher feeding specialization in a particular ecological niche. In contrast, during contraction episodes of the T2R repertoire, there is a reduced ability to identify different bitter substances (Dong et al., 2009; Davis et al., 2010). It has also been speculated that the loss of T2Rs in birds, such as in chickens, is related to the overall reduction in genome size with no subsequent occurrence of gene expansion (Go, 2006). Wang et al. (2019b) stated that higher number of T2Rs in Anna's hummingbird compared to its two close insectivorous relatives (chuck-will's widow and chimney swift) suggested increased sensitivity to bitter nectars. Bitter and potentially toxic nectars probably played a key role in developing specificity of pollinators (Wang et al., 2019b). A positive correlation between the number of avian T2Rs and the abundance of toxins in their diets has been reported. Insects normally secret bitter compounds as a defense mechanism; thus, this could be a reason for higher number of T2Rs in insectivorous birds compared to others (Wang and Zhao, 2015).

The short T2R repertoire in chickens consists of three widely tuned T2Rs (T2R1, T2R2 and T2R7) capable of responding to most compounds known to be bitter to humans (Li and Zhang, 2013; Roura et al., 2013; Behrens et al., 2014; Cheled-Shoval et al., 2015; Hirose et al., 2015; Kawabata et al., 2019; Yoshida et al., 2019). Chicken T2Rs were mainly expressed in vimentin-negative taste cells suggesting there might be a different downstream molecule involved in transmitting bitter sensation compared to mammals (Kawabata and Tabata, 2022). Chickens were found to respond to quinine at a similar detection threshold than humans and rodents which is between 0.1 and 0.3 mM (Cheled-Shoval et al., 2017b). Similarly, cockatiels (*Nymphicus hollandicus*) have a quinine sensitivity comparable to humans and superior to other mammals (Matson et al., 2004). Behrens et al. (2014) concluded that a low number of functional T2R genes in birds is possibly compensated by a wider tuning specificity and a high ligand affinity (Behrens et al., 2014).

In chickens, T2Rs are expressed at least in three different oral tissues: the palate, the base of the oral cavity and the posterior tongue (Yoshida et al., 2019). However, oral taste-bud numbers differ between breeds. Broiler chickens (genetically selected for meat production) have a larger number of buds, and these buds are linked to a higher sensitivity to quinine compared to egg producing breeds (Kudo et al., 2010). Age is also associated with bitter sensitivity. Young chicks show more sensitivity to bitterness and higher expression of T2R compared to adult chickens (Ueda and Kainou, 2005; Dey et al., 2018). Concurrently, it appears that bitter taste sensitivity can be affected by genetic selection in broiler chickens. Modern broiler chickens compared to their ascendants showed higher sensitivity to bitterants (Yoshida et al., 2021b).

4.4 Salty taste

The intracellular and extracellular concentrations of electrolytes are critical for life and therefore tightly regulated. In birds, the main dietary cationic minerals are sodium and potassium, while the major anionic electrolyte is chloride. Salt (NaCl) is particularly critical with both deficiency as well as excess consumption being lethal. The presence of nasal salt gland in marine birds enable them to tolerate relatively high levels of NaCL by safely removing them from the body. In contrast, high NaCl solutions (i.e., 2% or more) are toxic to birds without salt glands (Mason and Clark, 2000). Salt sensing has been shown in many different types of birds, but thresholds may vary significantly across species. In non-marine birds the taste of salt triggers two divergent behavioral responses, depending on the concentration of the food and the sodium status of the animal. High concentrations are aversive, while low concentrations show high preference particularly in a sodium deficient status (Meyer et al., 1986). For example, chickens show preference for salt solutions between 85 and 100 mM, while reject solutions of 250 mM or higher (Duncan, 1962; Balog and Millar, 1989). Also, in a free-choice assay, ostrich chicks, provided with a range of flavored feed (salt, sweet, sour, bitter), significantly preferred salt-added feed (14 g/kg) over other flavors as well as the control (Brand et al., 2022). In this study,

the control feed (mainly consist of barley, maize, and soybean meals) contained 4 g/kg of salt, and the highest level tested was 34 g/kg. Other studies on preference for salt solutions include blackbirds, European starlings, cockatiels, and pigeons and ranged from 0.1%–1% (Espaillat and Mason, 1990; Nakajima and Onimaru, 2006). It is possible that salt taste mechanism in birds is similar to mammals. Unlike other taste qualities that have a dedicated cells in taste buds, salt taste detection is mediated by multiple sensory cells. Also, salt taste requires movement of sodium into the taste cell facilitated by ENaC channel. The sodium influx depolarizes the cell resulting in neurotransmitter ATP release and electrical signal to the central nervous system (reviewed by Taruno and Gordon, 2023).

4.5 Sour taste

Acidity in foods relates to the concentration of H^+ ions released during the ingestion process in the oral cavity. Sour foods are often associated to bacterial or yeast fermentation. Thus, the associated presence of potential pathogens in foods evokes a protective rejection response. However, the response to acidic foods depends on the avian species and age (Mason and Clark, 2000). Studies in chickens have shown that, overall, there is a tolerance for medium acidic or alkaline solutions but strong avoidance for extreme acid or alkaline solutions including organic acids such as citric acid (Fuerst and Kare, 1962; Gentle, 1972; Balog and Millar, 1989; SpillariViola et al., 2008). For example, in a two-choice test of control feed *versus* feed supplemented with 6% citric acids, birds consumed 35% less from the citric acid added feed (Balog and Millar, 1989). The main sour taste receptor is thought to be the dimer transmembrane proton channel Otopetrin-1 (OTOP1) that is highly selective for hydrogen ions in mammals (Teng et al., 2019; Zhang et al., 2019). The protein coding gene has also been mapped for many avian species and can be found in genomic databases such as NCBI. However, further functional evidence of this receptor in avian species is currently lacking. In a recent study, chickens' OTOP1 showed a similar response to extracellular acids compared to humans (Tian et al., 2023). Interestingly, OTOP1 in chickens, humans, and four other vertebrates responded to alkaline pH 9.0, suggesting that this gene is also an alkali-activated channel (Tian et al., 2023).

4.6 Calcium taste

Calcium is the most challenging mineral in bird diets, being the most limiting nutrient in bird reproduction (Reynolds and Perrins, 2010). Firstly, birds may require calcium not only for bone formation but also for eggshell production. Secondly, it is a common behavioral trait in birds to provide calcium-rich feeds to their chicks immediately after hatching (Reynolds and Perrins, 2010). Thirdly, calcium requirement is extremely variable during the bird lifespan. Variations in dietary calcium requirements may increase up to 20-fold during oviposition in some avian species (Klasing, 1998). Lastly, many foods available to birds are likely to be deficient in calcium. For example, the amount of calcium in seeds (granivores) or insects (insectivores) is insufficient for egg laying forcing female birds to select calcium-rich foods as a supplement (Graveland and van Gijzen, 1994). It is important to note that calcium metabolism is tightly associated with phosphorus and vitamin D metabolism involving intestine, kidneys, and bones in avian species. Deficiency of circulating calcium increases parathyroid hormone resulting in bone resorption, renal excretion of phosphorus, and increased intestinal absorption of calcium (Li et al., 2016). Preference for calcium-rich foods such as snail or mollusc shells is particularly apparent in the evening allowing the acid conditions in the gizzard to dissolve the calcium source and spare the mobilization of bone during the overnight eggshell formation (Houston et al., 1995; Graveland, 1996). Thus, a physiological mechanism to taste dietary calcium seems particularly essential in birds.

On the one hand it seems speculative to define a sense of calcium taste in birds. On the other hand, birds show a high preference for diets containing high amounts of calcium (Reynolds and Perrins, 2010). Calcium-driven foraging by laying birds has been widely reported for several species including pheasants (Sadler, 1961), vultures (Mundy and Ledger, 1976), great tits (Graveland and Berends, 1997), and geese (Campbell and Leatherland, 1983). Leeson and Summers (1978) offered laying hens with diets containing two levels of calcium (131 vs. 4.7 g/kg) and protein and energy (107 g CP and 7.28 MJ/kg vs. 191 g CP and 12.82 MJ/kg) and a control diet (30 g/kg Ca, 171 g CP, 11.69 MJ/kg). They observed 7% less feed intake and better shell quality in birds receiving the former diets compared to the control in choice feeding experiment. It was suggested by the authors that reduced feed intake was linked to an specific appetite for calcium (Leeson and Summers, 1978). Similarly, broiler chickens were able to adjust the consumption of a calcium supplement to the calcium level in feed according to growth requirements (Wood-Gush and Kare, 1966; Joshua and Mueller, 1979; Wilkinson et al., 2014). For example, Wilkinson et al. (2014) provided broilers with two complete diets containing different levels of calcium (5 and 10 g/kg) and access to a separate source of calcium (CaCO3), and they found that broilers fed with diet containing 5 g/kg of calcium consumed significantly higher amount of CaCO3. The Calcium Sensing Receptor (CaSR), a GPCR related to some amino acid and calcium sensing in mammals, is expressed in chicken's oral tissue (Kawabata et al., 2018; Omori et al., 2022). Also, using cell model and Ca^{2+} imaging, Omori et al. (2022) demonstrated that extracellular calcium and magnesium activate chicken CaSR. Thus, it seems plausible that the CaSR functions as a calcium sensing receptor in birds. However, further studies are needed to demonstrate if ligands of chicken CaSR can elicit behavioral responses in chickens or other avian species. Elucidating the mechanism of calcium taste in avian species will have important implications on feed intake regulation, reproductive and feeding behaviors, and egg and meat production.

4.7 Fatty acid taste

Similar to the calcium oral sensing, it is also speculative to define a sense of fatty taste in birds based on only indirect evidence such as choice feeding tests and the existence of the fatty acid (FA) receptors in the oral cavity. Behavioral studies regarding FA sensing in chickens have consistently shown preferences for long-chain FA-supplemented feeds (Furuse et al., 1996; Mabayo et al., 1996;

Vermaut et al., 1997; Palomar et al., 2020). Different fat sources at inclusion rate of 6% with different amounts of free FA and various level of unsaturated to saturated FA ratios were added to laying hens' diet. Results showed a higher preference for higher free FA and a stronger preference for saturated than unsaturated FA (Palomar et al., 2020). In addition, chickens did not show significant preferences for oleic acid (a mono-unsaturated FA) in choice feeding tests (Kawabata et al., 2021). In contrast, other FA including poly-unsaturated omega-3 eicosapentaenoic and docosahexaenoic acids, and omega-6 arachidonic acid, activated the FFAR4 in chickens (Kawabata et al., 2022). Furthermore, birds like mammals appear to differentiate the sensing of short from medium or long chain FA. Taken together, these findings suggest that FA sensing is likely as relevant in birds as it is in mammals.

Free fatty acid receptors (FFAR) 2, 3, 4, and FA transporter CD36 are expressed in the oral cavity of chickens (Colombo et al., 2012; Sawamura et al., 2015; Kawabata et al., 2019). Each receptor responds to different fatty acid chain lengths. FFAR2 and FFAR3 respond to short while FFAR4 to long chain FA (Roura et al., 2019). In contrast, it appears that the two-medium chain FFAR identified in mammals (FFAR1 and GPR84) are missing in the chicken genome (Meslin et al., 2015). This warrants further investigations particularly with the recent release of whole genome sequence of hundreds of avian species (Feng et al., 2020).

5 Conclusion and future directions

Birds have a well-developed gustatory system exquisitely adapted to ecological niches, nutritional needs, and available food sources. The ability to sense different tastes begins before hatching and the rapid developments occurring during the peri-hatching period. Generally, it seems that birds compared to mammals have more diversity in terms of anatomical structures of taste buds with at least three identified types. Also, the distribution patterns of taste buds in avian species compared to mammals are more diverse and mainly located on palates instead of tongue. The interchange between umami and sweet tastes have played a key role in evolutionary process of avian species. Genetic mutations in umami receptors (T1R1-T1R3) have granted many bird species including songbirds the ability to sense sweet taste despite the loss of the relevant receptor (i.e., T1R2). Such genetic change has resulted in allowing avian species to develop unique feeding strategies (such as in hummingbirds). Although birds compared to mammals generally have lower number of bitter taste receptors, their receptors can detect a wider range of compounds. This may reflect higher diversity of natural diets available to birds.

Deeper knowledge of the avian taste sense, anatomical structure and post-ingestion consequences will improve our understanding of the feeding behavior and nutrient requirements of domestic and wild birds. Some of the data reviewed has shown the importance of revisiting previous findings. For example, the latest study reported 767 taste buds in the chicken oral cavity. This represents a high sensing capacity similar (when not superior) to mammalian species and clearly debunking previous assumptions and scientific reports inferring no or lower taste sensitivities in birds. More importantly, the recent release of genome sequences of hundreds of bird species as a part of the Bird 10,000 genomes (B10k) sequencing project will have great implications for not only studying individual birds but facilitating functional and taxonomical comparison between species. The availability of genomic sequences of taste receptors in different bird species provides the opportunity to study molecular structures and functions of taste receptors. Also, the genetic changes can be identified and accurately linked to dietary habits and ecological adaptations in avian species. Overall, this review brings together strong evidence suggesting the importance of taste system in avian species and fundamental differences compared to mammals. Large diversity of species and wide range of dietary habits in birds could be strongly linked to their taste system.

Author contributions

SN: literature review, designing, and writing the manuscript, artwork. MN: literature review, partial writing of the manuscript. ES: editing, literature review. ER: editing, designing, and partial writing of the manuscript. All authors contributed to the article and approved the submitted version.

Conflict of interest

The 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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Increased dietary 5-hydroxytryptophan reduces fearfulness in red junglefowl hens (*Gallus gallus*)

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Our production animals typically suffer poor welfare, which can be revealed by measuring the affective state these animals are in. Negative affective state is linked to poorer welfare, and can be measured as fearfulness. While continuing to research how to improve animal welfare, a compliment to reduce negative affective state could therefore be to reduce individuals' fearfulness, similar to how negative affective states are medicated in humans. A proposed mechanism for this is via the monoaminergic systems. This is based on previous studies across species that have linked the serotonergic system and fear-related behaviour. We here aimed to experimentally manipulate the serotonergic system in red junglefowl hens (*Gallus gallus*), the main ancestor of all domesticated chickens. We measured fearfulness as latency remaining immobile in a tonic immobility test, and did so both before and after our experimental manipulation. We set out to experimentally manipulate the serotonergic system via sub-chronic dietary treatment of 5-hydroxytryptophan (the precursor to serotonin). Our dietary manipulation of 5-hydroxytryptophan significantly reduced measured fearfulness in the manipulated hens, while latency in tonic immobility did not significantly change in our unmanipulated, control hens. This finding is promising since it indicates that increased tryptophan levels can be used to reduce fearfulness. Additionally, our result suggests that this can be done non-invasively via food (instead of injections), thus presenting a potentially feasible manipulation also for larger settings. Nevertheless, the serotonergic system is complex and its role in modulating behaviour in the fowl should be explored further to evaluate our findings, and more directly explored also in a production setting.

KEYWORDS

animal welfare, chickens, poultry, serotonin, tonic immobility

1 Introduction

Animals across a range of species typically suffer poor welfare in an industrial setting. Poor welfare can be measured by estimating the affective state (i.e., emotion, mood, Mendl et al., 2010) of individuals, where individuals with poorer welfare often are in a more negative affective state (e.g., Harding et al., 2004; Forkman et al., 2007). For example, negative affective state measured as fearfulness (Jones, 1996; Forkman et al., 2007) increased when animals are housed in poorer conditions (e.g., Hansen et al., 1993), and is linked to higher stress hormone levels (e.g., de Haas et al., 2012). Further, being in a negative affective state can enhance negative impacts of poor welfare (Jones, 1996). Despite that animal welfare

research focuses on improving animal welfare, the problems remain (Nicol, 2020). Therefore, while continuing to work on external factors that can improve animal welfare, underlying mechanisms which in turn can improve the affective state of animals, should also be explored. This route is very commonly used to improve human affective state, when medicating and reducing depression and anxiety (e.g., Hamon and Blier, 2013). Such an approach can also be successfully used to alter affective state in animals (e.g., Hymel and Sufka, 2012; reviewed by Neville et al., 2020).

Monoamines (i.e., neurotransmitters) have broadly, and across species, repeatedly been shown to affect a range of behaviour. Focusing on serotonin, this monoamine can directly or indirectly affect behaviour of relevance to animal welfare (e.g., Coppens et al., 2010; Stracke et al., 2017; Abbey-Lee et al., 2018; 2019), and experimental alteration of serotonin has successfully reduced fearfulness or related behaviours (e.g., several fish species, Winberg and Thörnqvist, 2016; mice, *Mus musculus*; Foltran et al., 2020; Mediterranean field crickets, *Gryllus bimaculatus*; Lundgren et al., 2021). Hence, due to the conserved nature of the monoaminergic systems (Gunnarsson et al., 2008), manipulation of the serotonergic system may alter fearfulness also in agricultural species.

We here focus on monoaminergic manipulation of fearfulness in chickens. The domestic chicken (*Gallus gallus domesticus*) is today the most numerous bird in the world and one of our most intensely farmed animals, with billions raised yearly for meat and egg production (reviewed by, e.g., Nicol, 2015; Pizzari, 2016). These chickens suffer from a variety of severe welfare problems, such as cannibalism, feather- and vent pecking (Lambton et al., 2015; Nicol, 2015). To measure their welfare, estimating level of fearfulness is commonly used to describe their negative affective state (Forkman et al., 2007; Laurence et al., 2012). Also in chickens is the serotonergic system linked to a range of behaviour (e.g., Shea et al., 1991; Dennis et al., 2013; de Haas and van der Eijk, 2018; Birkl et al., 2019), including fear-related behaviour (e.g., Hicks et al., 1975; Hennig, 1980; Phi Van et al., 2018). Therefore, with the extended aim to improve their welfare, we specifically investigate if experimental manipulation aimed to target the serotonergic system could reduce fearfulness in chickens.

Previous work on manipulations of the serotonergic system in both fowl and other species have primarily been done via drug injection (e.g., Hennig, 1980; Foltran et al., 2020; Lundgren et al., 2021) or through drinking water (Dulawa et al., 2004). Both of these approaches have their drawbacks: while injection is fast and the amount of drug given is precise, injections can cause stress to test subjects (Morton et al., 2001), may need repeated injections to keep stable levels (Hennig, 1980), and is thus impractical to large number of individuals. Administering through the drinking water has minimal stress effect on the test subject and is practical for larger number of individuals, but dosing of the drug and time of dosing will be less precise. Considering these drawbacks, we opted for an alternative approach in administering the drug in a non-invasive way, by manipulating the food given.

We chose to focus on alteration of 5-hydroxytryptophan levels (5-HTP), since this is the precursor of serotonin (where the amino acid tryptophan is metabolised into 5-HTP, which quickly is metabolised into serotonin, e.g., O'Mahony et al., 2015). We did so by experimentally manipulating the amount of 5-HTP given in feed. Increase of dietary tryptophan has resulted in higher plasma and turnover levels of serotonin in the brain of chickens (e.g., van Hierden et al., 2004b) and other animals (e.g., Winberg et al., 2001). Alteration of tryptophan in domestic fowl has previously shown to affect behaviour, such as feather pecking (Birkl et al., 2019) and aggression (Shea et al., 1991). Using a dietary manipulation allowed us to control the amount of drug each individual was given, whilst not having potentially stressful effects of injections. However, chickens have the ability to store food in their crop (Svihus, 2014), and may therefore not digest the manipulated food immediately. Thus, drug administration via food has a similar drawback as administration through water in that the timing of the dose will be less precise. As a way to get around this, we chose to study the sub-chronic effect (i.e., longer than acute, but shorter than chronic, Dulawa et al., 2004) and dose our chickens for several days in sequence.

We used this dietary manipulation of the serotonergic system on red junglefowl (*Gallus gallus*) hens. The red junglefowl is a growing model species for research on animal behaviour and animal welfare, and is behaviourally and cognitively similar to its descendant the domesticated chicken (reviewed by Garnham and Løvlie, 2018). Further, the red junglefowl is the main common ancestor of all domestic chickens (Fumihito et al., 1994), which should enable our findings to be general for chickens broadly and not limited to certain strains of fowl (e.g., broilers, layers).

2 Materials and methods

2.1 Animals and housing

In October and November 2021, red junglefowl hens (n = 48) from a larger, pedigree-bred population maintained at Linköping University (see Sorato et al., 2018 for further details) were used for this study. These hens came from 23 parental pairs (where none were half-siblings), with 1–4 from each parental pair. All hens from the cohort were used, and these were raised and housed together throughout life. Hens took part in the study between 55 and 58 weeks of age (i.e., when sexually mature). To facilitate identification, all hens were wing-tagged as chicks with unique numbers. Hens were divided up in two groups: treatment (n = 24) and control (n = 24), with chickens from families with multiple offspring represented in both groups. During this experiment, hens were housed together with roosters in female-male pairs in enclosures (60 cm × 45 cm × 50 cm, L x W x H) containing a perch, shelter, sawdust, and a laying/brooding area, light (6:30 a.m. to 6:30 p.m.) and with *ad libitum* access to commercial poultry feed and water. The experiment was carried out in accordance with Swedish ethical requirements (Linköping Ethical Committee, ethical permit number 288-2019).

2.2 Experimental set-up

All hens were tested individually and had previously taken part in behavioural studies and were used to human presence and handling (Rubene and Løvlie, 2021; Garnham et al., 2022a). During testing, all hens were exposed to a tonic immobility test

(see below) twice: once on the day before the experimental dietary manipulation began and once on the day after the final dose (i.e., 5 days later).

2.3 Experimental dietary manipulation

Based on previous work (Donato et al., 2015), our experimental manipulation used 5-hydroxytryptophan (5-HTP, Sigma-Aldrich), the precursor to serotonin. 5-HTP was diluted in Phosphate buffered saline (PBS, Sigma-Aldrich), and mixed together with cottage cheese at the time of dosing. Cottage cheese was used as it is an attractive food source for the hens, and it was easily mixed with the 5-HTP-mixture. One day prior to testing, each hen was weighed (to the nearest gram) and the dosage of 5-HTP received was calculated to 30 mg of 5-HTP per kg of body weight (based on Donato et al., 2015). Our females weighed between 722 and 1,103 g, with the average weight of 877 g. This resulted in doses ranging between 21.66 and 33.09 mg, with the average dose of 26.43 mg for our hens. Twenty-four hens were individually fed 7 g cottage cheese mixed with the drug for 5 consecutive days at around 4 p.m. Control hens (n = 24) were given the same handling as above, but were fed cottage cheese (7 g) mixed with only PBS. All females ate all cottage cheese given to them, on all 5 days. Due to logistical constraints, we were here only able to use one dose of 5-HTP for our treatment.

2.4 Measuring fearfulness

Hens were individually exposed to a tonic immobility test, a test commonly used in poultry research to measures fearfulness, where longer latency immobile describes more fearful individuals (Gallup, 1979; Hennig, 1980; Jones, 1986; Forkman et al., 2007). Each hen was tested by the same observer (KL) to minimize any differences in handling. We followed the protocol routinely used by our group (e.g., Favati et al., 2015; Zidar et al., 2017; Garnham et al., 2019). To induce tonic immobility, the observer placed the hen on her back in a cradle and gently held her down with one hand over the chest and the other hand over her head, whilst avoiding eye contact with the hen. After 15 s, the observer lifted his hands and tonic immobility was considered induced if the hen remained on her back for at least 3 s. Fearfulness was measured as the time (in seconds) a hen took until she stood upright again after tonic immobility had been induced, with longer latency used as a measure of higher level of fearfulness (Forkman et al., 2007). If a hen did not enter tonic immobility after a maximum of three attempts to induce this, she was given a tonic immobility latency of 0 s (this only happened to one hen prior to treatment, and eight after). Hens that did not come out of tonic immobility within 600 s were given a latency of 600 s (this only happened to four hens prior to treatment, and three hens after).

2.5 Statistical analyses

R version 4.0.1 (R Core team, 2020) was used for statistical analyses. The measure of fearfulness (i.e., latency to stand, in seconds) did not follow the assumptions needed for parametric statistics. Therefore, non-parametric statistical tests were used. To test for any unintended differences between later treatment and control hens, a Mann-Whitney U test was used by comparing fearfulness of our 5-HTP treated hens and control hens (i.e., comparing data from two independent samples), prior to the experimental manipulation was carried out. To test for the effect of our 5-HTP manipulation, two separate Wilcoxon matched pair tests were used to compare fearfulness of the same individual between first and second trial of the tonic immobility test (hence for hens in the treatment group: before and after manipulation) for hens in the treatment and control group, separately. Further, the difference in fearfulness between first and second trial was compared between hens in the treatment and control groups, by running a generalized liner model with Poisson distribution with Δfearfulness (change in latency to stand per hen over the two trials, calculated as latency in first test occasion minus latency in second test occasion) as the response variable with type of treatment (5-HTP vs. control; categorical variable) as a fixed effect. To fit model assumptions, Δfearfulness was transformed according to the formula: transformed = Δfearfulness + 491 + 1, as some values were negative (i.e., when a hen increased her latency to stand). Transformation of Δfearfulness resulted in that the lowest value was 1. The statistical significance of the fixed effect was assessed based on a 95% credible interval (CI) around the mean (β), and considered to be significant when the 95% CIs did not overlap zero.

3 Results

Prior to our experimental manipulation, there was no significant difference in fearfulness between the later 5-HTP-treated hens and our control hens (5-HTP-treated: n = 24, mean ± SE = 247.71 ± 46.71; control: n = 24, mean ± SE = 277.91 ± 51.67, W = 321.5, p = 0.49; Figure 1).

However, our 5-HTP manipulation significantly lowered fearfulness in the 5-HTP-treated hens (before manipulation: n = 24, mean ± SE = 247.71 ± 46.71; after manipulation: n = 24, mean ± SE = 117.46 ± 34.28, W = 386.5, p = 0.04, Figure 1), while no such significant decrease was observed in our control hens (first test occasion: n = 24, mean ± SE = 277.91 ± 51.67; second test occasion: n = 24, mean ± SE = 190.37 ± 41.44, W = 343.5, p = 0.25, Figure 1). Further, this reduction in fearfulness was confirmed to only be significant in our 5-HTP manipulated hens, by our generalized linear model (Δfearfulness, 5-HTP-treated: n = 24, mean ± SE = 130.25 ± 43.18; Δfearfulness, control: n = 24, mean ± SE = 87.54 ± 52.84; β = −0.07, 95% CIs = −0.09, −0.05).

4 Discussion

We set out to test if fearfulness could be reduced via dietary manipulation of the underlying monoaminergic systems, specifically focusing on the serotonergic system. We did this by enhancing food given to individual red junglefowl hens with 5-hydroxytryptophan, for 5 consecutive days (i.e., we did a sub-chronical manipulation). We here show that this relatively short feed manipulation reduced the latency treated hens remained in tonic immobility, a common measure of fearfulness in poultry (where shorter latencies implies

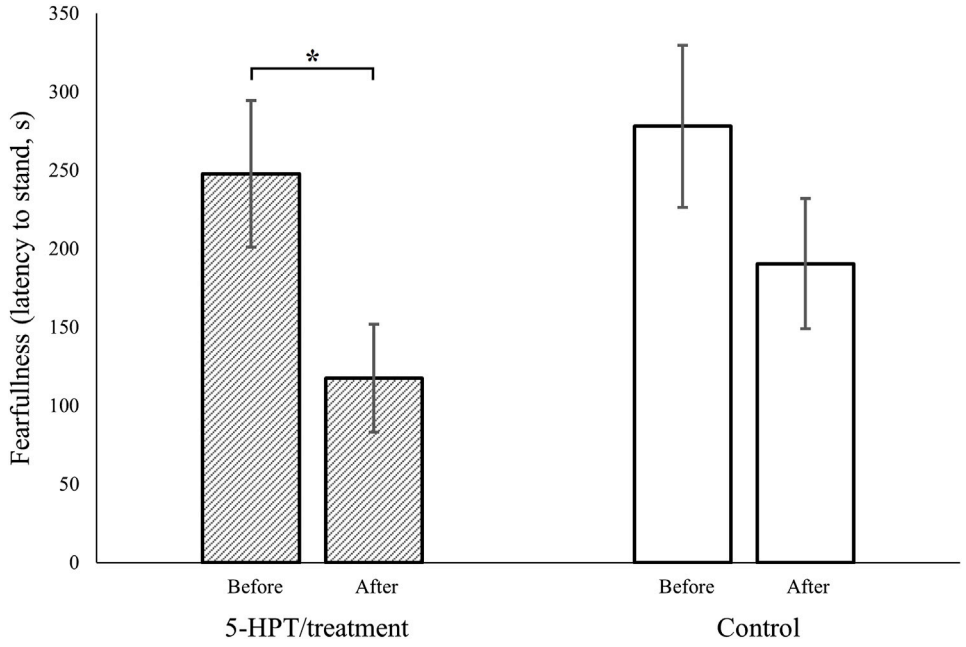

FIGURE 1
Fearfulness (latency to stand, in seconds, after induction of tonic immobility) in red junglefowl hens was reduced after intended serotonin manipulation (via increased dietary 5-HTP, 5-hydroxytryptophan, the precursor to serotonin, dashed columns), while no such significant reduction was observed in our control hens (empty columns). Before vs. After refer to first and second trial in a tonic immobility test, respectively. Columns present mean, bars present SEM, * symbolizes $p < 0.05$, and that the 95% credible intervals did not overlap zero.

less fearful individuals). In our control hens were fearfulness also somewhat reduced in the second test, although not as strongly as what we observed for our experimental hens, and not significantly. The observed reduction in fearfulness in our control hens therefore confirms that habituation to the test can reduce latencies in immobility (e.g., Ratner and Thompson, 1960), and that the effect we observed in our experimental birds was mainly explained by our dietary manipulation.

We used 5-hydroxytryptophan to experimental manipulate feed given to our treatment hens. 5-hydroxytryptophan is a metabolite of tryptophan, which in turn is metabolised rapidly into serotonin (O'Mahony et al., 2015). Our finding that a manipulation set out to affect the serotonergic system affected behavioural responses, is confirmed across taxa (e.g., several fish species, Winberg and Thörnqvist, 2016; mice; Foltran et al., 2020; Mediterranean field crickets; Lundgren et al., 2021). When focusing on previous manipulation of the serotonergic system affecting fear-related behaviours, that is also confirmed across vertebrate species (e.g., humans, Hindi Attar et al., 2012; chickens; Nicol, 2015; mice; Waider et al., 2019). These findings seem to be general, independent of method of manipulation. For example, in mice, manipulated serotonin levels via injection or diet gave similar reduction in levels of brain serotonin (Foltran et al., 2020). It is promising that the use of a non-invasive method produces similar results to also more precise, invasive methods, as this should ease translation of the method to a more industrial setting.

Tryptophan is an essential amino acid obtained only through food. Poultry feed already have some percentage tryptophan (e.g., van Hierden et al., 2004b; Barua et al., 2021), and this percentage can relatively easily be enhanced further (e.g., Winberg et al., 2001), thus should be possible to increase commercially. However, there are several aspects that should be clarified before this commercial step can be taken. Firstly, it should be confirmed that dietary manipulation of 5-HTP indeed increased serotonin levels in treated hens (as shown previously in chickens, van Hierden et al., 2004b), and whether this resulted in increased peripheral serotonin levels (in plasma), and/or in the central nervous system (brain). The vast majority of serotonin (ca 95%) is synthesised in the gut and circulated in the blood (see reference in, e.g., Yan et al., 2018). The synthetic cascade is similar in the gut as in the brain (tryptophan—5-hydroxytryptophan–serotonin). However, since peripheral serotonin cannot cross the brain-blood barrier (Mann et al., 1992), serotonin synthesis in the gut and in the brain has two separate pathways and the function of serotonin can differ in the two separate networks. Across vertebrates, serotonin in both networks can affect aspects of relevance to welfare (O'Mahony et al., 2015), and to poultry welfare more specifically (e.g., feather pecking, van Hierden et al., 2004a; van Hierden et al., 2004b; de Haas and van der Eijk, 2018). Yet, the role of serotonin does not need to be parallel in plasma and brain. For example, peripheral serotonin levels were unaltered, while serotonin levels in the brain were affected by comparing broilers given probiotic diet vs. control birds (Yan et al., 2018). How and why our dietary manipulation affected behaviour, is currently unknown, and investigation of whether plasma or brain serotonin levels changed could give hint to the underlying mechanism through which our dietary manipulation acted. Further, even if the underlying mechanism to observed behavioural alteration is known, further work needs to consider

that serotonin manipulation via food will not result in individual dosing. Thus, both the preferred dose, and the acceptable span of levels individuals obtained dependent on differential food intake, must be explored further. This is because dose response curves of serotonin and responses to the same fearfulness test we here used, the tonic immobility test, shows that doses can produce quite varying and non-linear responses (Hennig, 1980). In addition, alteration of feed can cause amino acid imbalances. Further, high levels of serotonin can cause serotonin syndrome in which a range of abnormal behaviours can be observed, such as head weaving, hyperactivity and twitching (reviewed by, e.g., Haberzettl et al., 2013).

On a slightly different note, yet of relevance for animal welfare, manipulation of serotonin levels in domestic fowl can reduce feather pecking (e.g., van Hierden et al., 2004a; de Haas and van der Eijk, 2018). Feather pecking is a large welfare problem in the poultry industry, and serotonin manipulations can reduce certain aspects of feather pecking (van Hierden et al., 2004b). Therefore, further work on how to enhance serotonin levels could have positive influences on chicken welfare for multiple reasons. However, the underlying mechanisms for fearfulness and feather pecking are still not fully understood, and warrant future research.

We here used a well-established behavioural test of fearfulness commonly used on poultry (not only chickens, but, e.g., quail, Jones et al., 1991). Further, the use of latency to come out of immobility after induction of tonic immobility is used as a measure of fearfulness also across other animals (reviewed by, e.g., Hennig, 1980). Yet, this measure does not always correlate with other aspects of fearfulness, or positive welfare. Longer latencies in tonic immobility has been linked with other measures of fearfulness (reviewed by, e.g., Forkman et al., 2007), but also with stress (Zulkifli et al., 1998; Kozak et al., 2019). Thus, what exactly the tonic immobility test is measuring is still debated (reviewed by, e.g., Humphreys and Ruxton, 2018). Nevertheless, the test seems to capture negative affective state. We recently showed, in the same population of junglefowl here used, that latency to remain in tonic immobility was moderately positively correlated with responses to ambiguous, intermediate cues in a cognitive judgement bias test (Garnham et al., 2022b). A judgement bias test is widely used as one of the very few confirmed methods to measure positive affective state of animals (reviewd by, e.g., Lagisz et al., 2020; Neville et al., 2020). That a low fearfulness correlated with a higher positive affective state is promising in that what we here have shown may translate to not only "less bad welfare," but also improved welfare. However, this should be directly tested by manipulation of the serotonergic system and measure positive affective state, suggestively by the use of a judgement bias test. Suggesting such a link is also our previous findings that responses to a judgement bias test is linked to variation in the monoaminergic systems (Zidar et al., 2018; Boddington et al., 2020). In the same population as here used, we previously shown that brain gene expression of the DRD1 (dopamine receptor D1, another important neurotransmitter of the monoaminergic systems) gene, was positively correlated with positive affective state measured in a judgement bias test (Boddington et al., 2020). In layer hens, brain turnover rate of dopamine was positively correlated with positive affective state measured in a judgement bias test (Zidar et al., 2018). On the other hand, in a smaller sample of individuals from the same population of red junglefowl here used, no correlation was observed between brain gene expression of a range of serotonergic genes and latency remaining in tonic immobility (Lundgren et al., 2021). Thus, the relationship between negative and positive affective state, and its underlying mechanisms have to be explored further.

Clarification of the link between responses to tonic immobility and measured positive affective state in a judgement bias test is relevant both as it may establish how negative and positive affective states are linked, but also if tonic immobility test can be used instead of judgement bias tests. Judgement bias tests are both time consuming and take a lot of handling of the animals, as the values of negative and positive cues need to be learned (see, e.g., Zidar et al., 2018; Garnham et al., 2019). The use of a tonic immobility test is thus more time efficient, although this too needs individual handling and testing of birds. When the relationship between dietary manipulation of underlying monoaminergic systems is better understood, and if reduction in feather pecking is produced as a side-effect of serotonin enhancement, visual inspection of flocks with regard to feather pecking could perhaps act as a group-level measure of altered affective state. However, this is currently only a speculation.

Overall, the relationship between negative and positive affective states in chickens and other animals needs to be better described. We recently showed that the relationship between responses to tonic immobility and responses in the judgement bias test depends on whether chicks or adults were tested (e.g., the relationship was only found in chicks, and not in adults, Garnham et al., 2022b). This suggests that before generalizing to different ages and strains of fowl, the generality of our findings should be investigated further since our results may differ for broilers (i.e., chicks) vs. layers (i.e., adults). Further, as a first step, we here only tested females. In our population, we have previously observed no or weak effects of sex on responses to the tonic immobility test (e.g., Favati et al., 2015; Zidar et al., 2017; Lundgren et al., 2021; Garnham et al., 2022b). However, sex effects in responses to monoamine manipulation on behaviour also warrant future exploration.

5 Conclusion

We have here shown that a relatively short (5 days) experimental manipulation of diet with the aim to alter the serotonergic system of red junglefowl hens, the main ancestor of all domesticated chickens, reduced how fearful these were, measured in a tonic immobility test. Our manipulation was non-invasive through 5-hydroxytryptophan enhanced food given to the birds. Tryptophan, the precursor of serotonin (and from which 5-hydroxytryptophan in turn is metabolised), is already part of commercial chicken feed. Although still in its early stage, these aspects together produce promising results for a hopefully practical method to reduce fearfulness of also industrial birds, which in turn may improve their animal welfare. Future work should evaluate tryptophan enhanced food in industrial settings, together with the drug dosage needed to produce satisfactory levels of animal welfare.

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.

Ethics statement

The animal study was approved by the Linköping ethical committee, Sweden. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

KL and HL conceived and developed the experiment, KL carried out data collection, KL analysed the data in discussion with HL, KL wrote the manuscript in collaboration with HL. HL funded the research. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The 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.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2023.1213986/full#supplementary-material

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The silent assumption of the masking hypothesis: avian auditory processing and implications for behavioral responses to anthropogenic noise

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Anthropogenic noise and its impact on wildlife has recently received considerable attention. Research interest began to increase at the turn of the century and the number of publications investigating the effects of anthropogenic noise has been growing steadily ever since. Songbirds have been a major focus in the study of anthropogenic noise effects, with a significant portion of the literature focusing on the changes in singing behavior in noise. Many of these studies have found increases in the amplitude or frequency of song, or changes in the temporal patterning of song production, putatively due to the masking effects of noise. Implicit in the masking hypothesis is the assumption that all species process sounds in noise similarly and will therefore be subject to similar masking effects. However, the emerging comparative literature on auditory processing in birds suggests that there may be significant differences in how different species process sound, both in quiet and in noise. In this paper we will (1) briefly review the literature on anthropogenic noise and birds, (2) provide a mechanistic overview of how noise impacts auditory processing, (3) review what is known about the comparative avian auditory processing in noise, and (4) discuss the implications of species level differences in auditory processing for behavioral and physiological responses to anthropogenic noise.

KEYWORDS

critical ratios, masked thresholds, birds, traffic noise, hearing

The effects of anthropogenic noise on birds

With increasing urbanization across the globe, the threat of anthropogenic noise pollution to animals will increase over time (Brumm and Horn, 2019). Commonplace sounds found in areas of human settlement, including traffic and industrial noises, can have adverse effects on the behavior and health of wildlife (Barber et al., 2010). In accordance with the growing threat, research interest on the topic has steadily risen since the turn of the century, with publications including keywords such as "traffic noise" having increased at a greater rate than the total output of scientific papers.

Anthropogenic noise produces a wide array of negative effects in animals. Across species, anthropogenic noise leads to reduced rates of survival (Simpson et al., 2016), greater physiological stress responses (Kleist et al., 2018), poorer offspring success (Nedelec et al., 2017; Zollinger et al., 2019), and decreased cognitive performance (Maes and De Groot, 2003; Stansfeld and Matheson, 2003; Osbrink et al., 2021). Additionally, noise has been shown to have a number of adverse effects on acoustically-mediated behaviors. For instance, noise can lead to decreased anti-predator responses (Jung et al., 2020), lower foraging success (Mason et al., 2016; Halfwerk and van Oers, 2020), and increased time spent on vigilance (Meillère et al., 2015), presumably to compensate for diminished acoustic detection of threats. Perhaps the best studied effects of noise on acoustically-mediated behaviors are the impacts of noise on songbird communication.

Songbirds have long been a model for acoustic communication and therefore became a natural model for understanding how communication is affected by noise, both natural and anthropogenic (Brumm and Naguib, 2009; Naguib, 2013; Wiley, 2017). Anthropogenic noise can overlap signals produced by songbirds, which results in masking of these signals (Slabbekoorn and Peet, 2003). This masking is a problem both from a sender and a receiver perspective, although most research has focused on the impacts of noise on the production of the signal by the sender. Senders may benefit from a variety of strategies to enhance signal detectability including moving closer to the intended receiver or relocating to a higher perch (Dabelsteen et al., 1993; Mathevon et al., 1996; Mathevon et al., 2005), producing louder vocalizations (Brumm and Todt, 2002; Brumm, 2004), or producing signals during times when noise levels are lower (Fuller et al., 2007; Dominoni et al., 2016). Additionally, anthropogenic masking noise can result in frequency shifts in vocal signals (Brumm and Slabbekoorn, 2005; LaZerte et al., 2016), although there is some evidence to suggest that these pitch-shifts are a byproduct of singing at a higher amplitude which may be more effective in enhancing signal transmission (Nemeth and Brumm, 2010; Brumm and Zollinger, 2011). Receivers can employ similar strategies to senders and have additional options, such as changing the orientation of the head (Dent et al., 1997; Dooling and Leek, 2018). However, not all strategies are available to all species (Zollinger et al., 2017).

Some tactics to reduce the impact of noise seem to be relatively conserved across species, while others vary considerably in their employment. For instance, moving to a higher song post to enhance signal detectability is found in many species (see Mathevon et al., 2005; Barker and Mennill, 2009; Sprau et al., 2012), implying that this tactic may be effective for enhancing signal detection in noise regardless of the sensory biases of a particular species (Polak, 2014; Halfwerk et al., 2018). Pitch shifting also appears to be a common, (Bermúdez-Cuamatzin et al., 2011; Seger-Fullam et al., 2011; LaZerte et al., 2016; Roca et al., 2016; Halfwerk et al., 2018), but not universally employed tactic (Roca et al., 2016; Zollinger et al., 2017) and there appear to be constraints on the degree of pitch shifting that can occur. There is substantially more variability in whether species are able to switch to a new song type to reduce the effects of noise (Slabbekoorn and den Boer-Visser, 2006; Dowling et al., 2012). Some species appear to increase song switching in noise, while others reduce switching between song types, perhaps to increase redundancy of information (Brumm and Slater, 2006). Some species are known to alter the timing of song production on both relatively short (e.g., seconds to minutes; Brumm et al., 2009; Francis et al., 2011) or long-term (e.g., time of day; Fuller et al., 2007; Dominoni et al., 2016; Dorado-Correa et al., 2016) times scales. It is not yet clear how universally these time shifting tactics are employed, although they appear to be inaccessible to at least some species (Yang and Slabbekoorn, 2014).

Many of the behavioral responses to noise are thought to be a result of the masking effects of noise, although noise may also be aversive or distracting (Chan et al., 2010; Morris-Drake et al., 2016; Zhou et al., 2019). In all cases, the response to noise is mediated through the detection of noise by the auditory system and the effect of noise on the processing of signals of interested. For instance, signal parameters including frequency and duration have been shown to enhance auditory perception of signals by great tits (*Parus major)* in anthropogenic noise (Pohl et al., 2009; Pohl et al., 2012; Pohl et al., 2013). Yet, despite an understanding of how senders respond to noise and a growing understanding of how receivers behaviorally respond to signals in noise, there is surprisingly little comparative work on auditory processing of signals in noise for birds, songbirds in particular. Indeed, many papers on signal production and/or communication in anthropogenic noise that have invoked the masking hypothesis focus only on the relative overlap of the signal and noise in frequency, either incorrectly assuming that all species are processing signals in noise in an identical manner or ignoring the issue of auditory processing entirely. Notably, though, species can vary significantly in their auditory processing abilities in quiet and, although less explored, in noise. Therefore, differences in behavioral response to noise may be mediated by species-specific variation in the ability of the auditory system to process signals in noise. These variations, however, are rarely considered at the comparative level. Here we review the comparative literature on avian auditory processing in noise, focusing primarily on masked thresholds and critical ratios, as the greatest comparative literature exists for these metrics. However, these are relatively simplistic metrics and future work on more complex metrics of auditory processing in noise, including comodulation and spatial masking release, dip-listening, and the impact of signal features on detection and discrimination in noise, in a comparative framework would be particularly valuable (see Klump and Langemann, 1995; Dent et al., 1997; Dent et al., 2009; Pohl et al., 2009; Pohl et al., 2012; Pohl et al., 2013).

How the auditory system deals with noise

The cochlea in birds responds to sound based on many of the same general peripheral neural encoding principles found in mammals (reviewed in Gleich and Manley, 2000). Briefly, the avian cochlea in birds is tonotopically organized, with each location along the length of the hair-cell epithelium (basilar papilla in birds) tuned to a different characteristic frequency (CF, Table 1). Spectral analysis of sound, due to this tonotopic response, allows separation of competing sounds based on differences in frequency content, and therefore provides an important foundation for processing signals in noise as discussed below. In contrast to basic similarities in peripheral neural processing, the anatomy of the avian cochlea shows only a slight curvature along its longitudinal axis, while the mammalian cochlea has a prominent coiled structure, and the avian hair-cell epithelium is comparatively short and broad with up to thirty hair cells across the width in low-CF apical regions (Takasaka and Smith, 1971). Moreover, rather than distinct populations of specialized inner and outer hair cells as found in mammals, birds show a hair-cell gradient across the width of the epithelium from "tall" to "short" morphology. Tall hair cells are taller than they are wide, are located closer to the epithelial edge overlying the auditory-nerve ganglion, and are innervated by peripheral axons of afferent myelinated auditory-nerve fibers *via* ribbon synapses (Fischer, 1994), similar to mammalian inner hair cells. Short hair cells receive primarily unmyelinated efferent innervation and are implicated in cochlear nonlinearity and amplification like mammalian outer hair cells (Manley et al., 1989). Several aspects of tall/inner hair-cell innervation by afferent nerve fibers are shared between birds and mammals: each afferent neuron makes synaptic contact with a single tall hair cell through a peripheral axon terminating in a ribbon synapse, and has its soma located in the auditory-nerve ganglion (called spiral ganglion in mammals) at short distance from the hair cells. Central axons of auditory-nerve fibers bundle together to exit the cochlea and provide the primary input to the ascending auditory pathway.

Perhaps surprisingly, in light of peripheral anatomical differences, auditory-nerve fiber response properties in birds are similar in most respects to those reported in mammals (Sachs et al., 1974; Manley et al., 1985). Avian auditory-nerve fibers show irregular spontaneous discharge activity in the absence of sound, of variable rate (typically up to 100 spikes per second) across fibers and perhaps with an inverse relationship to the threshold at CF (Salvi et al., 1992). Average discharge rate increases with increasing sound level over a limited dynamic range before saturating at the maximum discharge rate. In response to tones of varying frequency and level, auditory-nerve fibers in birds show V-shaped threshold tuning curves with CFs (i.e., the frequency of the tuning curve tip) distributed across the frequency range of hearing sensitivity. Notably, measures of frequency selectivity such as Q10, defined as the CF divided by the bandwidth of the tuning curve 10 dB above the threshold at CF, are similar to if not slightly sharper than observed in typical mammalian species (reviewed in Gleich and Manley, 2000). These measures of frequency selectivity are of significance because this spectral analysis in the peripheral auditory system is a key mechanism facilitating detection and processing of signals in noise, and is the foundation of auditory filtering models (see below). Regarding temporal response properties, auditory-nerve fibers in birds show prominent phase locking to tone frequencies up to several kHz (Sachs et al., 1974; Manley et al., 1985; Gleich and Narins, 1988; Köppl, 1997), as well as to amplitude fluctuations in the envelopes of complex sounds (Gleich and Klump, 1995). All of the patterns described above are commonly seen in mammals. Indeed, the main differences in avian peripheral physiology compared to mammals appear to be tuning curves that are constrained to lower CFs (due to filtering properties of the avian middle ear system), slightly higher spontaneous and maximum discharge rates (perhaps associated with higher average body temperature in birds; Sachs et al., 1974), and higher thresholds for tone frequencies presented below CF (i.e., no tuning curve tails as commonly observed in mammalian auditory-nerve-fiber responses; Manley et al., 1985).

The frequency tuning of auditory neurons arises from tonotopy of the hair-cell epithelium within the cochlea and is the physiological manifestation of now classic band-pass auditory filtering models for hearing in noise (Fletcher, 1940; Glasberg and Moore, 1990). In this model framework, a narrowband signal such

TABLE 1 Glossary of terms and abbreviations.

Term	Description
Auditory Evoked Potentials (AEP)	Gross electrophysiological technique for assessing auditory processing (generally in the periphery and brainstem).
Auditory Threshold	Amplitude (typically dB SPL) at which an acoustic stimulus is just detectable.
Auditory Filter	Overlapping bandpass filters that perform spectral decomposition of broadband sounds in the auditory periphery.
CF	Characteristic frequency.
Critical Ratio	The ratio of the signal to noise spectrum levels at the masked threshold. The critical ratio is typically assumed to be insensitive to the level of the masking noise.
K'	Response efficiency. The signal-to-noise ratio of the auditory filter at threshold expressed in dB.
Masked Threshold	Amplitude (typically dB SPL) at which an acoustic stimulus is just detectable in a noise masker (typically white noise).
SNR	Signal to noise ratio.
Spectrum Level (dB/Hz)	Sound level in 1 Hz wide bands. For pure tones amplitude and spectrum level are equivalent.

as a tone is detected in a competing noise "masker" based on the response of a single auditory filter centered on the tone frequency. For the classic critical-band model, which we considered first, detection of a tone in noise is based on an increase in the output level of the filter compared to a reference condition for which only the noise is presented. Detection is possible because for a tone of sufficiently high signal-to-noise ratio, addition of the tone to fixed-level noise increases the overall energy level at the output of the filter. Importantly, the sensitivity of the system for detecting the tone in noise depends crucially on the bandwidth of the auditory filter. These basic principles are illustrated in Figure 1, which in panel A shows input–output functions of auditory-filter models with bandwidths ranging from 100 to 1600 Hz. The threshold "critical ratio" occurs at the signal-to-noise ratio (SNR), expressed relative to the noise spectrum level, above which the distribution of energy-level values observed across the tone-plus-noise stimulus trials (vertical error bars) sufficiently exceeds the distribution observed across the noise-alone trials (horizontal band height). Crucially, auditory filters of greater bandwidth passes more total noise energy than narrow filters, which consequently increases the threshold critical ratio above which addition of the tone to the noise masker appreciably increases filter output. The resulting dependence between filter bandwidth and critical ratio is illustrated in Figure 1B. It is based on the above relationships between auditory filter bandwidth and masked threshold that critical ratios have been used for decades now as a first approximation for peripheral tuning bandwidths. Behavioral critical ratios in humans and many nonhuman animal species increase with increasing tone frequency (see further discussion below), in general agreement with typical increases in neural tuning bandwidth for higher CFs.

The critical-band model discussed above provides only a starting point for understanding auditory processing in noise, as organisms clearly have access to other neural cues than those related to output energy from single auditory filters. Adding a tone to noise introduces temporal fine structure at the output of auditory filters, which can be encoded through neural phase locking (Costalupes, 1985; Henry and Heinz, 2012), and alters the statistics of the amplitude envelope at the filter output as well (Kohlrausch et al., 1997; Wang et al., 2021). Furthermore, neurons along the central auditory pathway can combine information across peripheral auditory-filter channels to perform signal-processing tasks in noise. For example, European starlings (*Sturnus vulgaris*) show improved detection of tones in noise when amplitude fluctuations are coherent across frequency bands of the masker rather than independent (Klump and Langemann, 1995). This phenomenon, known as comodulation masking release, has been widely studied in humans and is not explainable by the critical-band model. Consistent with limitations of the basic critical-band model, auditory behavioral studies show that birds perceive sound in ways inconsistent with its predictions, as also found in humans. For the basic task of tone-in-noise detection for which overall stimulus is randomly varied ("roved") across trials, budgerigars (*Melopsittacus undulatus*) show essentially no change in sensitivity compared to fixed-level listening conditions (Henry et al., 2020; Henry and Abrams, 2021). This result echoes findings of human studies (Richards, 1992; Leong et al., 2020) but not the critical-band model, which in its basic form predicts large threshold shifts (impaired performance) for the roving-level condition. Follow-up analyses of trial-by-trial error patterns in budgerigars using decision-variable correlations showed that while behavioral responses were related to some extent with trial-by-trial variation in stimulus energy level, behavioral choices were also strongly associated with envelope cues (Henry et al., 2020; Henry and Abrams, 2021), also echoing human findings (Mao et al., 2015). For tone-in-noise detection, the envelope cue is a flattening of the

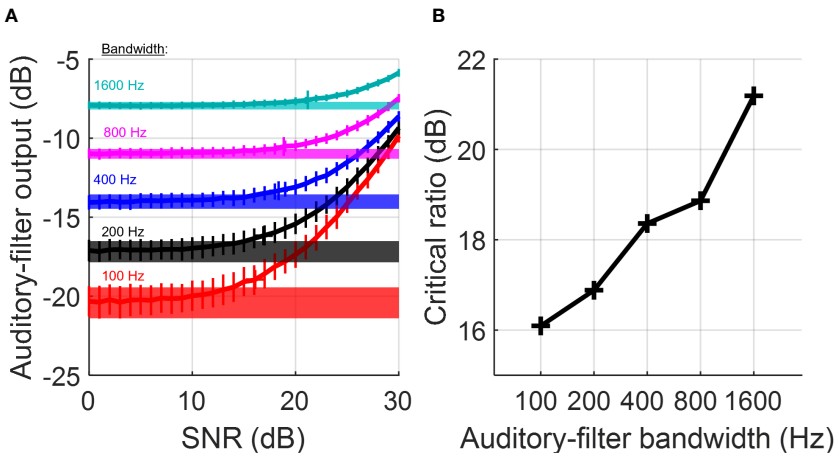

FIGURE 1
Dependence of tone-in-noise sensitivity on auditory-filter bandwidth. Input–output relationships of auditory-filter simulations **(A)** showing filter output (mean ± SD) as a function of stimulus signal-to-noise ratio (SNR) for filter bandwidths ranging from 100–1600 Hz (color-keyed text). Thick horizontal bands indicate filter output for noise alone (mean ± SD). The threshold "critical ratio" is defined as the SNR (re. noise spectrum level) above which the distribution of filter output between tone-plus-noise trials and noise alone differ by ≥1.5 SD. Critical ratios for tone-in-noise detection **(B)** increase for greater auditory filter bandwidths. Simulations were conducted in MATLAB using 12th order Butterworth filters, stimulus duration of 0.2 s, tone frequency of 2000 Hz, and 200 randomly generated stimuli per SNR.

envelope at the output of the auditory filter that occurs when a tone is added to the noise masker (Kohlrausch et al., 1997).

Another important limitation of the critical-band model is the inability to explain masking in the modulation domain, which is masking of amplitude-modulated signals by envelope fluctuations of similar frequency that occur in the noise background (Bacon and Grantham, 1989; Houtgast, 1989). Human listeners show band-pass modulation masking patterns consistent with a modulation filterbank processing strategy (Hose et al., 1987; Dau et al., 1997a; Dau et al., 1997b; Ewert et al., 2002) thought to occur in the central nervous system. Similar "modulation tuning" of neural responses has also been observed at the forebrain level in the mynah bird (Hose et al., 1987). Neural recordings at the midbrain processing level in zebra finches and budgerigars show band-pass modulation transfer functions in response to sinusoidally amplitude-modulated stimuli (Woolley and Casseday, 2005; Henry et al., 2016) that could potentially support perceptual modulation filtering. This aspect of hearing in noise could be especially important for avian species that communicate with amplitude and/or frequency modulated signals in environments with rapid envelope fluctuations. Studying masking in the modulation domain, temporal integration, and spectro-temporal masking effects such as comodulation masking release in a broad comparative context should be an important priority for future research, because it can provide much needed clarity into the evolution of auditory processing mechanisms for hearing in noise.

Comparative avian auditory processing in noise

Although we have a detailed understanding of how noise affects auditory processing in a few model organisms (see above) and a growing body of work on comparative avian auditory processing, there is still substantially less known about avian auditory processing in noise in a comparative framework. There have generally been two methodological approaches taken for comparative auditory processing work: psychophysics and gross electrophysiology, specifically auditory evoked potentials. Other techniques that have been employed to study auditory processing, such as single unit electrophysiology in the auditory nerve or auditory forebrain have been employed almost exclusively in model organisms, with a few exceptions, and we will not cover those here.

Psychophysical experiments provide a comprehensive, whole-organism response to acoustic stimuli and the results of these types of experiments may most closely mirror the responses of free-living birds to sound. However, the approach has limitations as well. Psychophysical experiments are sensitive to the motivation and learning abilities of individual subjects, are limited to subjects that can be sufficiently rewarded with access to food, and require prolonged housing in a laboratory setting. Historically, the results of such studies were based on a relatively limited number of subjects (typically 1–4 in critical ratio experiments, see Supplementary Table S1) and the same subjects were often used in several experiments. Additionally, repeated training on detection and discrimination tasks may result in an overestimate of detection and discrimination abilities in free-living birds that may have more demands on their attention. The training paradigm may also select for individuals that are not representative of the species.

Auditory evoked potentials can be employed for a diverse range of species, as only temporary laboratory housing is required, effectively increasing the breadth of comparative work. However, this technique represents only the subcortical processing of auditory information and is the summed response of all neural units responding to sound (Henry et al., 2017). Some aspects of audition, such as auditory thresholds, measured *via* auditory evoked potentials (AEPs) have been shown to correlate well with single unit and psychophysical measurements in a variety of avian taxa (Brittan-Powell et al., 2002; Gall et al., 2011; McGrew et al., 2022). However, AEP estimates of auditory thresholds are generally elevated relative to psychophysical thresholds and correlations are strongest in the frequency range of greatest sensitivity. For other aspects of auditory processing, such as temporal resolution, frequency resolution, and auditory processing in noise, there are very few species for which multiple methodological approaches have been taken and therefore the relationship between the peripheral processing and whole organism response is largely unknown. AEP estimates of auditory processing, therefore, are best used as relative comparisons among species, rather than absolute estimates of audition.

Noise can affect the detection, discrimination and recognition of acoustic information and these effects are a product of both peripheral processing and higher order cognitive functions (Dooling and Leek, 2018). However, most of our comparative understanding of avian audition comes from estimates of absolute sensitivities in quiet anechoic chambers. Although these absolute sensitivities are not fully sufficient to understand auditory processing in noise, they may provide some guidance on which species may be most affected by noise. Anthropogenic noise is largely concentrated at lower frequencies, although as the amplitude of noise increases there are concomitant changes in the upward spread of excitation (Slabbekoorn et al., 2018). This suggests that in moderate levels of noise species with lower peak frequencies of sensitivity or with greater sensitivity overall may be more subject to the effects of masking, as noise is more likely to overlap with their regions of best sensitivity and exceed their thresholds.

Absolute auditory thresholds have been estimated for many dozens of species spanning most of the major taxonomic clades using both psychoacoustic techniques (see Dooling et al., 2000; Crowell et al., 2016; Dooling and Blumenrath, 2016) and auditory evoked potentials (Pytte et al., 2004; Brittan-Powell et al., 2005; Lohr et al., 2013; Henry et al., 2017; Crowell et al., 2015; Beatini et al., 2018; McGee et al., 2019; Larsen et al., 2020). The shape of the audibility curve (i.e., audiogram) is broadly similar across birds. Peak sensitivity lies somewhere between 1 and 6 kHz for most avian species, with a steep increase in thresholds at frequencies above and below the range of peak sensitivity. However, differences do emerge both across and within clades that may influence the relative sensitivity to noise. Very broadly, the audibility curves for larger-bodied bird species, including members of the Anseriformes,

Galliformes, and some Accipitriformes, are typically shifted away from higher frequencies relative to the audibility curves of smaller-bodied species, such as those of the order Passeriformes (reviewed in Dooling et al., 2000; Crowell et al., 2015; Crowell et al., 2016; McGee, 2019). However, there are also substantial differences among species that are likely a product of behavior and ecology. For instance, Northern gannets (*Morus bassanus*) have among the highest thresholds (i.e., low sensitivity) of any species measured with the AEP method and a peak sensitivity of only 1.7 kHz, lower than any other seabird tested (Crowell et al., 2015). This poor auditory sensitivity may be a result of their plunge diving foraging methods, high natural levels of noise in their environments, and relatively short distances over which they typically communicate. Northern gannets also have a small and thickened tympanic membrane, which may reduce damage during plunge diving at the expense of auditory sensitivity (Crowell et al., 2015). Masking noise, therefore, may have relatively little effect on this species except at very high amplitudes. On the other hand, barn owls (*Tyto alba;* psychophysical method Konishi, 1973; Dyson et al., 1998; AEP method Thiele and Koppl, 2018) and Northern saw-whet owls (*Aegolius acadicus;* AEP method Beatini et al., 2018) have the among the lowest thresholds for birds, as well as an extended range of peak sensitivity from 1.6 to 7.1 kHz. This again, is likely a reflection of the behavior and ecology of the animals. Barn owls and Northern saw-whet owls can hunt in total darkness, localizing prey by sound, which requires enhanced auditory sensitivity relative to other species (Payne, 1971; Mason et al., 2016). Barn owls and Northern saw-whet owls both have asymmetric ears and complete facial ruffs that likely enhance sound detection and localization (Norberg, 1977; Knudsen and Konishi, 1979; Moiseff, 1989). Although thresholds have not been determined psychophysically for Northern saw-whet owls, they have been shown behaviorally to have excellent sound localization abilities (Frost et al., 1989). The extremely low thresholds suggest that even very low amplitude masking noises may have an effect on the detection abilities of owls (Mason et al., 2016).

Although the differences in audibility curves are more subtle between species in the same order, differences in the frequency range of best sensitivity have been shown in free-living passerines (reviewed in Henry et al., 2017). These differences are often, but not always perfectly, aligned with the peak energy in their vocalizations. For example, Velez et al. (2015) found that song complexity, rather than habitat or peak frequency of vocalizations, best predicted the high frequency sensitivity of nine songbird species. Therefore, estimates of sensitivity to masking that rely solely on vocal analysis may be misleading, as auditory sensitivity and vocal energy are not always perfectly correlated.

The comparative information available for auditory processing in noise is more limited than for absolute sensitivity. There are several ways in which we can assess the effects on noise on auditory processing, but here we will focus primarily on the effect of noise on detection limits (i.e., thresholds), as we have the most information for this metric. This is a relatively simplistic way of approaching auditory processing in noise. To fully understand the effects of auditory processing on communication in noise requires a comparative understanding of masking in the modulation domain, temporal integration, spectro-temporal masking effects such as comodulation masking release, dip-listening in modulated maskers and spatial masking release. Currently, however, this information is available for only a small number of avian species (e.g., Klump and Langemann, 1995). Thus, we have largely limited our review to metrics available for a larger number of species, although we also briefly discuss several types of masking release and encourage comparative work that extends to these more nuanced aspects of auditory processing in noise.

We can assess the effects of noise on detection limits with masked thresholds and critical ratios. Masked thresholds are produced using procedures identical to those for producing audiograms, with the addition of a noise masker (typically white noise). When the masker is wideband white noise and the signal is a pure tone, the detection threshold for the tone expressed in dB relative to the spectrum level of the noise is known as the critical ratio. On average, critical ratios for avian species are approximately 22 dB at 0.5 kHz and 29 dB at 4 kHz. However, there is also a fair amount of variation across species in both the magnitude of their critical ratios and the shape of the critical ratio by frequency function. For instance, at 1 kHz critical ratios range from 21 dB in wild-caught hooded crows (*Corvus corone cornix;* Jensen and Klokker, 2006) to 42 dB in red-winged blackbirds (*Agelaius phoeniceus*) and brown-headed cowbirds (*Molothrus ater;* Hienz and Sachs, 1987). Many of the other songbird species have critical ratios in the 24–28 dB range at 1 kHz (Supplementary Table S1; Okanoya and Dooling, 1987; Langemann et al., 1998; Okanoya and Dooling 1988; Lohr et al., 2003; Noirot et al., 2011). This suggests that at 1 kHz, a frequency at which there is often substantial anthropogenic noise, blackbirds and cowbirds might be much more susceptible to the effects of noise masking than other species.

Different patterns emerge at 4 kHz, a frequency generally considered less susceptible to masking at all but the highest anthropogenic noise levels. Critical ratios range from 20.5 dB in budgerigars (Dooling and Saunders, 1975) on the low end and 34–37 dB for canaries (*Serinus canaria*; Okanoya and Dooling, 1987), blackbirds and cowbirds on the high end. Many of the songbirds lie in between, for instance a critical ratio of 29 dB for song (*Melospiza melodia*) and swamp sparrows (*Melospiza georgiana*) (Figure 2, Supplementary Table S1). European barns owls had critical ratios of 23.5 dB at 4 kHz and 27.5 dB at 6.3 kHz (Dyson et al., 1998). While these frequencies are not usually considered as susceptible to masking as lower frequencies, barn owls have an absolute threshold of −14.2 dB SPL at 6.3 kHz (Dyson et al., 1998), thus masking of low amplitude prey noise at 6.3 kHz could occur even with relatively small amounts of masking noise.

On average, the critical ratios of birds appear to increase at a rate of 2–3 dB per octave. However, there is substantial variation in the shape of the critical ratio by frequency function. Generally, these functions fall into one of three categories: (1) The standard increase of 2–3 (and up to 5.1) dB per octave, (2) relatively flat functions and (3) relative flat functions with sharp increases at high frequencies. In some cases (functions 2 and 3) the critical ratio initially appears to decrease as the function moves towards the best frequency for each species, before increasing rapidly at higher frequencies. Species that appear to follow the 2–3 dB increase per octave pattern include

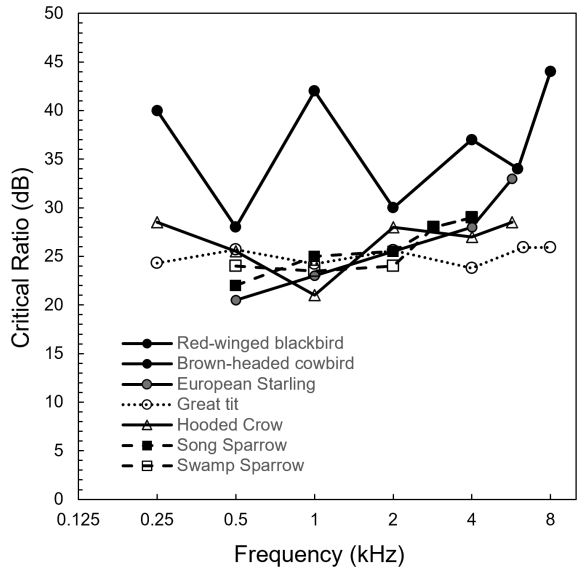

FIGURE 2
Critical ratio by frequency functions for all wild-caught passerine songbirds which have been studied to date. Not included are critical ratios of lab-bred or purchased songbirds. Data from Hienz and Sachs, 1987; Okanoya and Dooling, 1988; Langemann et al., 1998; Jensen and Klokker, 2006. Data were taken from the texts or table when available, or extrapolated to the nearest 0.5 dB from figures.

canaries, zebra finches (*Taeniopygia guttata*), European starlings (Langemann et al., 1995), song sparrows, swamp sparrows (Okanoya and Dooling, 1987), and domestic pigeons (*Columba livia domestica*; Hienz and Sachs, 1987). Species with relatively flat critical ratio by frequency functions include hooded crows (Jensen and Klokker, 2006), red-winged blackbirds, brown-headed cowbirds (Hienz and Sachs, 1987), and great tits (Langemann et al., 1998). Orange-fronted conures (*Aratinga canicularis*; Wright et al., 2003) and parakeets (a.k.a. budgerigars; Dooling and Saunders, 1975; Saunders et al., 1979; Okanoya and Dooling, 1987; Hashino et al., 1988) show relatively flat or slightly decreasing critical ratios as frequency increases towards best frequency, with a rapid increase in critical ratio at or above 5 kHz. Note that for all these psychoacoustic measurements of critical ratios, the sample sizes are quite small, so we don't have a good estimate of the variation among individuals in critical ratios, nor the ability to statistically compare them.

Critical ratios have also been estimated for three species (budgerigars, canaries, and zebra finches) using auditory evoked potentials (Noirot et al., 2011). Generally, the AEP method results in critical ratios that are 18–23 dB higher than those measured psychophysically. Additionally, the shape of the critical ratio by frequency function appears to largely reflect the functions determined psychophysically, although the magnitude of differences between the two methods seems to be larger outside of the region of best sensitivity. The critical ratios measured *via* the AEP method appear to initially decrease or remain fairly flat as the frequency increases towards best frequency and then critical ratios increase as the stimulus frequency increases past best frequency. We have found similar critical ratio by frequency functions in three additional passerine species: black-capped chickadees, tufted titmice, and white-breasted nuthatches (Chou et al., unpublished). Additionally, in Northern saw-whet owls we have found a relatively flat critical ratio by frequency function, with a rapid increase in critical ratio at higher frequencies (Desai et al., submitted), a shape that largely mirrors the critical ratio function in barn owls (Dyson et al., 1998).

Attempts to measure the auditory processing of more complex signals in noise and conversely the processing of signals in more complex noise have covered a much narrower range of species, typically limited to traditional avian model species (e.g., zebra finch, canaries, budgerigars, and starlings). For instance, Lohr et al. (2003) measured detection and discrimination thresholds for natural contact call stimuli of zebra finches and canaries in flat and traffic shaped noise. Generally, they found that as the bandwidth of the calls increased and as the level of masking noise increased, so did the thresholds. Thresholds for discrimination were also higher than thresholds for detection, suggesting the active space of signals may be much smaller than previously estimated in communication scenarios where the signals need to be discriminated. Pohl et al. (2009) similarly found that great tit detection thresholds were impacted by the features of both signal elements and noise. Thresholds were lowest in quiet and increased in woodland and urban noise, with the highest thresholds in dawn chorus noise. Detection thresholds were lower for signal elements with narrower bandwidths (Pohl et al., 2009) and longer durations (Pohl et al., 2013). Additionally, higher frequency songs increased detection and discrimination of songs in urban noise for great tits (Pohl et al., 2012). Dooling and Blumenrath (2016) found that the critical ratios of budgerigars and canaries for simple stimuli in constant flat noise were similar to those in temporally fluctuating anthropogenic noise, suggesting that standard critical ratios could be reasonably applied to models of signal active spaces.

Birds may benefit also from maskers that are spatially segregated from the signal of interest or are modulated in amplitude (Klump and Langemann, 1995; Dent et al., 1997; Langemann and Klump, 2001; Dent et al., 2009). Moreover, there is reason to believe that species may differ in the degree of unmasking that is experienced for different signals and maskers. For instance, spatially separating a pure tone signals from white noise maskers by 90° or 180° can result in 8–9 dB of masking release in budgerigars (Dent et al., 1997). However, on a more naturalistic task – detecting a zebra finch vocalization in short duration maskers resembling songs or choruses – budgerigars did not experience a significant release from masking (an average of 1.6 dB) when signal and masker were segregated by 90°. Zebra finch performance improved (an average of 6.8 dB of masking released) at 90°, but showed no improvement when signal and masker were segregated by 180° (Dent et al., 2009). When maskers were presented continuously zebra finches experienced greater spatial release from masking (average 29 dB) than budgerigars (average of 20 dB), suggesting that species may differ significantly in the amount of spatial release from masking they experience for a given signal. Modulation of masker amplitude may also impact signal detection. Starlings experience an average of 11.8 dB of comodulation masking release when signals are presented in

coherently modulated noise compared to unmodulated noise, with the greatest masking release in broader band maskers (Klump and Langemann, 1995). Additionally, starlings have been shown to experience up to 28 dB of masking releasing when target stimuli are presented during the troughs of coherently modulated maskers compared to targets presented during the peaks or in incoherently modulated maskers (Langemann and Klump, 2001). The greatest masking release occurred when the masker was spread across multiple auditory filters, rather than confined to the one auditory filter. Although the temporal modulation of noise is likely to be important in signal detection, it is not yet clear how much variation exists across species in the degree of masking release experienced in modulated maskers.

There have been some attempts to investigate the effects of noise on the processing of more complex signals using the AEP method. For instance, Goller et al. (2022) presented tones, amplitude modulated, and frequency modulated signals to bald eagles (*Haliaeetus leucocephalus*) and golden eagles (*Aquila chrysaetos*) in either white or pink noise. They found that noise had a substantial effect on less dynamic signals (i.e., those with less modulation) but that processing of more modulated signals was less effected by the addition of noise. This suggests that species with dynamic signals may experience less masking than those with more static signals, although this is somewhat at odds with the findings of Lohr et al. (2003) and Pohl et al. (2009) that broader bandwidth signals were more susceptible to masking.

Finally, some features of auditory processing (i.e., critical bands and auditory filter bandwidth) that can be determined directly with single unit techniques cannot be directly measured with psychophysical or auditory evoked potential techniques. Here the effects of noise masking are not the primary interest, rather noise-masked signals are used to suppress responses from adjacent cochlear partitions. The psychophysical estimates of critical bands appear to correspond well to the (4dB) bandwidth of tuning curves (i.e., auditory filter bandwidths) determined from single units in starlings (Langemann et al., 1995) and frequency representation on the basilar papilla (octaves mm^{-1}) is correlated with critical ratios across several species (Gleich and Langemann, 2011), suggesting that masking effects of noise may be related to the frequency selectivity of the auditory periphery. Additionally, the output of an auditory filter model correlates well with perceptual distances between song exemplars in two noise conditions in great tits (Pohl et al., 2012), further suggesting that auditory filter widths may provide some insight into signal processing in noise.

Auditory filter bandwidths have also been determined for several species from both psychophysical and AEP tone thresholds in notched-noise maskers. Psychophysical estimates of auditory filter bandwidths in starlings increase in absolute width with increasing center frequency and correspond well with other methods of determining filter width (Marean et al., 1998). Auditory filter bandwidths have been estimated *via* AEPs for wild-caught Carolina chickadees, tufted titmice, white-breasted nuthatches, dark-eyed juncos (*Junco hyemalis*), brown-headed cowbirds, red-winged blackbirds, and house sparrows (*Passer domesticus*) (Gall and Lucas, 2010; Henry and Lucas, 2010a; Henry and Lucas, 2010b; Henry et al., 2011; Gall et al., 2013). In nearly all of these species filter bandwidth increases with increasing center frequency, with the exception of dark-eyed juncos, in which auditory filter bandwidth decreased slightly with increasing center frequency. Generally, open habitat species tended to have broader filters than woodland species (Henry and Lucas, 2010b). There is some evidence that there is sex-specific seasonal variation in auditory filter width in house sparrows, with females having narrower auditory filters than males during the breeding season, with no sex difference during the non-breeding season (Gall et al., 2013). Narrower filter bandwidth may increase neural synchrony to temporal fine structure in noise, based on studies of sensorineural hearing loss in the mammalian auditory system (Henry and Heinz, 2012).

As discussed above, filter bandwidth might affect the processing of signals in noise, with wider filters generally assumed to result in poor signal in noise processing, although this is likely an oversimplistic view. The notched-noise method also allows for the estimation of the efficiency or signal-to-noise ratios of the filters (K'), which do not always directly correspond to filter bandwidth. For instance, although white-breasted nuthatches have fairly narrow filters, they had a higher K' than other woodland and open habitat species, suggesting that they may be more susceptible to masking than other species. Additionally, female chickadees had narrower auditory filters than males, but a higher K' suggesting less efficient extraction of signals from noise (Henry and Lucas, 2010a). On the other hand, brown-headed cowbird females had both narrower auditory filters and lower K' than males, suggesting greater frequency selectivity and improved signal-in-noise detection (Gall and Lucas, 2010). Together, the comparative literature suggests that different species, and perhaps even different individuals within the same species, may differ in their ability to detect and discriminate signals in noisy backgrounds, although there is much we have left to learn. Therefore, we must consider not only the signals and the noise spectrum when attempting to understand the effects of anthropogenic noise on communication and other acoustically-mediated behaviors, but also the relative auditory sensitivity of different species and their ability to extract signals in environmental noise. In the next section, we speculate on the effects that variation in auditory processing abilities might have on the behavioral responses of different species to noise.

Linking auditory processing to behavior in noise

The evolution of communication systems has been shaped by the natural noise of the environment (Klump, 1996; Wiley, 2017). Natural noise differs in amplitude and spectral and temporal properties across habitats, which can influence both the evolution of the signals and the properties of the auditory system of different species (Brumm and Naguib, 2009). The perceptual properties and signal structure of a given species may evolve to optimize signal detection under natural noise conditions (Wiley, 2017). The amplitude, spectrum, and timing of anthropogenic noise can vary significantly from natural noise and with sound source, so the

relative impact of anthropogenic noise on each species will depend on particulars of the environment. However, if we consider a single case, we can see the effect that even moderate noise might have on signal detection. Nemeth and Brumm (2010) found city noise to have a spectrum level of approximately 23 ± 7.5 dB/Hz at 1 kHz, while forest noise had a spectrum level of only 6 dB/Hz. The critical ratio represents the signal level relative to this noise level that is needed for detection. Therefore, we can determine the signal spectrum level needed for detection by adding the critical ratio to the spectrum level of the noise for a given frequency. Under this noise paradigm, the introductory notes in the songs of red-winged blackbirds and brown-headed cowbirds would need to reach an amplitude of 65 dB at 1 kHz for detection at even a relatively short range in anthropogenic noise, while song sparrows, swamp sparrows, and great tits would need a signal of only 45.5–48 dB.

However, even this doesn't tell the full story. While red-winged blackbirds and brown-headed cowbirds have very similar auditory processing (Hienz and Sachs, 1987; Gall et al., 2011; Figure 2) they differ dramatically in the spectral content of their songs and their reproductive strategies. Red-winged blackbirds have vocalizations with peak energy concentrated between 2 and 4 kHz, corresponding to their best sensitivity. Brown-headed cowbirds, have songs with a similar structure, but with much lower and higher frequency components in their songs (Lowther, 2020). These low amplitude elements are likely to be masked by anthropogenic noise, while the higher frequency components may largely evade anthropogenic noise masking. Red-winged blackbirds have high amplitude vocalizations that they use for advertisement and territory defense. The high amplitude of their vocalizations may be sufficient to overcome relatively high levels of masking, particularly at short distance, although the active space of signals is likely to be reduced. Brown-headed cowbirds, on the other hand, are brood parasites and males typically vocalize at a short distance from females. Even so, the high critical ratios and low frequency spectral content of brown-headed cowbirds may lead to significant masking of some components of song, even in low level noise. Both species typically pair vocalizations with a visual display, which may compensate for or enhance the detection of vocal components in noise (Peek, 1972; Cooper and Goller, 2004; Ríos-Chelén et al., 2015; Magdaleno et al., 2022).

Red-winged blackbirds typically nest in freshwater marshes and prairies, which can be subject to low frequency masking by wind noise, although they are adaptable and can also be found nesting near roadside ditches and in urban parks (Yasukawa and Searcy, 2020). Song sparrows (Arcese et al., 2020) and swamp sparrows (Herbert and Mowbray, 2020) can be found in similar habitats to red-winged blackbirds, but have much lower critical ratios. Song sparrows have greater sensitivity and lower critical ratios than swamp sparrows at frequencies above 1 kHz and below 4 kHz and also have relatively greater spectral energy in their songs at these frequencies (Okanoya and Dooling, 1988). Both species have peak spectral energy in their songs at 4 kHz, with swamp sparrow broadcast song containing relatively greater spectral energy above 4 kHz. This suggests that song sparrow song is more likely to be masked by anthropogenic noise, which contains greater energy at lower frequencies. Moreover, low thresholds at these frequencies can enhance detection under quiet conditions, but may also result in masking of signals with lower amplitude noise. However, song sparrows also have lower critical ratios between 1 and 4 kHz, as well as more complex signals, which may enhance signal detection in noise. Song sparrows have also been shown to increase visual displays (Akçay and Beecher, 2019) and pitch shift songs in anthropogenic noise (Wood and Yezerinac, 2006), which could also enhance communication.

The SNR required for discrimination has been shown to be approximately 3dB greater than the SNR required for detection (Brenowitz, 1982; Lohr et al., 2003). Song sparrows have more complex songs and less redundancy compared to swamp sparrows, which may favor lower critical ratios. Additionally, song sparrow song is largely composed of tonal elements (i.e., spectral; Herbert and Mowbray, 2020), which may be better processed in narrower auditory filters (Henry and Lucas, 2010b) and therefore produce lower critical ratios. However, swamp sparrow song is trilled, with repeated frequency modulated elements (i.e temporal characteristics; Yasukawa and Searcy, 2020). These temporal qualities may be better processed in broader auditory filters (Henry et al., 2011), which in turn could result in elevated critical ratios. Further complicating the issue are the soft or warbled songs of the two species. The warbled or soft songs of song sparrows are more complex and have a greater spectral range than broadcast songs (Anderson et al., 2008). Swamp sparrow soft songs, on the other hand have less complexity and more repeated components (Ballentine et al., 2008). Here, again, the swamp sparrow songs are likely more detectable in noise than the song sparrow songs, given only the characteristics of the songs, but the lower thresholds and critical ratios of the song sparrow may compensate for these differences. Taken together, this suggests that there is significant complexity in determining whether species level differences in auditory processing in noise are likely to affect communication, due to significant differences in the spectral, temporal, and amplitude characteristics of vocalizations and background masking noise.

There are other contexts, however, in which auditory processing in noise may play an important role in determining whether some species are disproportionately affected by anthropogenic noise. For instance, red-winged blackbirds, song sparrows, and swamp sparrows are often found nesting in similar habitats and may be subject to similar nest predators (Arcese et al., 2020; Herbert and Mowbray, 2020; Yasukawa and Searcy, 2020). Acoustic detection of approaching nest or adult predators may be best achieved by species with the lowest critical ratio, favoring song sparrows over swamp sparrows in habitats with greater noise. Indeed, elevated levels of noise have been shown to increase vigilance and decrease foraging, presumably due to a diminished capacity to detect acoustic cues of approaching predators (Sweet et al., 2002). Differences in critical ratios might also be important in the detection of signals from heterospecifics, either through eavesdropping on anti-predator communication during the breeding season, or for interspecific communication in mixed species flocks. Differences in critical ratios may also be important for species that rely on acoustic information for prey detection. For instance, Northern saw-whet owls, short-eared owls (*Asio flammeus*) and long-eared owls (*Asio otus*, Senzaki et al., 2016) have impaired prey detection under even relatively low levels of anthropogenic noise, which may be due to elevated critical ratios in owls at frequencies corresponding to prey rustling sounds (Dyson et al., 1998).

The role of auditory processing in species level response to anthropogenic noise is still largely unclear. Although only one component of a complex set of interacting factors, there is reason to believe that species level differences may result in differential capacities of species to thrive in environments with anthropogenic noise. We recommend that future work expand the number of species for which we have information on auditory processing in noise, expand the scope of the stimuli and approaches taken in a comparative context, and explicitly consider the auditory processing abilities of species when investigating their behavioral responses to noise.

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MG conceptualized the review. MF, TC, MG, and KH contributed to the writing, editing, and preparation of the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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Supplementary material

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Bitter taste receptors of the zebra finch (*Taeniopygia guttata*)

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Despite the important role of bitter taste for the rejection of potentially harmful food sources, birds have long been suspected to exhibit inferior bitter tasting abilities. Although more recent reports on the bitter recognition spectra of several bird species have cast doubt about the validity of this assumption, the bitter taste of avian species is still an understudied field. Previously, we reported the bitter activation profiles of three zebra finch receptors Tas2r5, -r6, and −r7, which represent orthologs of a single chicken bitter taste receptor, Tas2r1. In order to get a better understanding of the bitter tasting capabilities of zebra finches, we selected another Tas2r gene of this species that is similar to another chicken Tas2r. Using functional calcium mobilization experiments, we screened zebra finch Tas2r1 with 72 bitter compounds and observed responses for 7 substances. Interestingly, all but one of the newly identified bitter agonists were different from those previously identified for Tas2r5, -r6, and −r7 suggesting that the newly investigated receptor fills important gaps in the zebra finch bitter recognition profile. The most potent bitter agonist found in our study is cucurbitacin I, a highly toxic natural bitter substance. We conclude that zebra finch exhibits an exquisitely developed bitter taste with pronounced cucurbitacin I sensitivity suggesting a prominent ecological role of this compound for zebra finch.

KEYWORDS

bitter taste receptor, calcium mobilization assay, avian, food selection, chemosensation

1 Introduction

The sense of taste in animals is required to guide food selection towards nutritive and non-toxic food items (Behrens and Meyerhof, 2018). In general, the vertebrate taste system is equipped with receptive proteins for sweet, salty, umami (the taste of L-amino acids, in case of human mostly L-Glu), sour and bitter (Chaudhari and Roper, 2021). Sour and salty tastes are mediated by the ion channels otopetrin-1 (Tu et al., 2018; Teng et al., 2019; Zhang et al., 2019) and, as a likely candidate sensor, ENaC [(Chandrashekar et al., 2010), but *cf.* (Bigiani, 2020; Lossow et al., 2020; Vandenbeuch and Kinnamon, 2020)]. The remaining taste modalities, sweet, umami and bitter are transduced by G protein-coupled receptors (GPCR) of the taste 1 receptor and taste 2 receptor families [gene symbols: TAS1R1-3 in human, Tas1r1-3 in mouse, other species frequently T1R; TAS2R (number), Tas2r (number), T2R (number)] (Behrens and Meyerhof, 2016). The three taste 1 receptor genes form 2 different heteromers constituting the sweet taste receptor, which consists of TAS1R2 and TAS1R3 subunits and the umami taste receptors with a TAS1R1/TAS1R3 composition (Hoon et al., 1999; Max et al., 2001; Montmayeur et al., 2001; Nelson et al., 2001; Li et al., 2002; Nelson et al., 2002). The bitter taste receptors constitute the taste 2 receptor family, which

differs grossly in size between species, ranging from 0 to more than 100 (Behrens and Meyerhof, 2018). The various taste receptor genes are expressed in the oral cavity in specialized cells, which are combined to taste buds (Chaudhari and Roper, 2021). Apart from the oral cavity, taste receptor gene expression has been reported in numerous non-gustatory tissues such as the gastrointestinal tract and the respiratory system (Wang et al., 2020; Martens et al., 2021; Behrens and Lang, 2022). Although in general five basic taste modalities act in concert to assess the nutritional quality of food, a considerable number of animals have lost some taste modalities (Antinucci and Risso, 2017). A well known example is the pseudogenization of the sweet taste receptor in all bird species, although several bird clades with a high demand for sweet tasting nutritional resources such as nectar, achieved the subsequent modification of the umami taste receptor for sweet compound detection (Baldwin et al., 2014; Toda et al., 2021; Cockburn et al., 2022). In the past, it was believed that birds possess an inferior sense of taste, an assumption that was supported by the loss of the sweet tasting ability as well as a small number of bitter taste receptors (Niknafs and Roura, 2018). However, the demonstration of re-gained detection of sweet substances (Baldwin et al., 2014), the finding that the bitter taste receptor repertoires of birds are not generally small as some birds possess a number of intact bitter taste receptor genes matching that of mammals (Davis et al., 2010) and the demonstration that even small bitter taste receptor repertoires can compensate their low number by extraordinary tuning breadths (Behrens et al., 2014), has caused some re-thinking. The first demonstration of profound bitter tasting capabilities in birds was achieved by the functional expression and bitter compound profiling of chicken, turkey and zebra finch bitter taste receptors (Behrens et al., 2014). Despite the low number of only three functional chicken bitter taste receptors, about half of all bitter compounds known to activate human TAS2Rs are detected by the three chicken bitter taste receptors, since each of them responds to a large array of bitter compounds. Similarly, the 2 turkey receptors were found to be broadly tuned, suggesting that a low number of bitter taste receptors alone is not an indication for inferior bitter taste. The same study investigated of in total seven zebra finch Tas2rs the functional properties of three zebra finch Tas2rs that are relatively recent paralogs of chicken Tas2r1. It turned out that the 3 zebra finch receptors each recognize a smaller number of bitter substances compared to the single chicken Tas2r, the Tas2r1, and in fact all three zebra finch receptors together detect as many bitter compounds as the single chicken Tas2r1 (Behrens et al., 2014). Thus, an expanded Tas2r repertoire may allow the development of more specialized receptors. To investigate if the lower tuning breadth of zebra finch Tas2rs might represent a general feature for birds with an elevated number of putatively intact bitter taste receptor genes or if this observation is only true for rather recently evolved paralogs, we screened another zebra finch receptor from a phylogenetically distant branch, the zebra finch Tas2r1. Next, we compared our screening results with chicken, turkey and other zebra finch receptors to assess similarities and differences.

2 Materials and methods

2.1 Chemicals

Absinthin and Parthenolide were available from previous studies (Brockhoff et al., 2007). Other reagents were purchased as follows: Amarogentin from ChromaDex; Limonin from Apin Chemical; Quassin from CPS Chemie and all other bitter tastants from Sigma-Aldrich.

2.2 Database mining and construction of the Tas2r phylogenetic tree

Database mining has been described in a previous study (Behrens et al., 2014). The phylogenetic tree was generated by the Maximum-Likelihood method. The amino acid sequences of the 7 zebra finch bitter taste receptors, the 3 chicken Tas2rs, the 2 turkey Tas2rs, and a turtle (*Pelodiscus sinensis*) bitter taste receptor serving as outgroup were aligned using MAFFT version 7.

2.3 Cloning of zebra finch Tas2r cDNA

The cDNA of zebra finch tgTas2r1 was synthesized by MWG Operon and subcloned into vector pcDNA5FRT (Invitrogen). Sequence analysis to verify the integrity of constructs was done by double stranded Sanger sequencing (MWG Eurofins).

2.4 Immunocytochemistry

The immunocytochemical staining experiment was mainly done as published previously (Behrens et al., 2021; Ziegler and Behrens, 2021). Briefly, HEK 293T-Gα16gust44 cells grown on poly-D-lysine-coated glass cover slips were transiently transfected with expression constructs coding for zebra finch Tas2r1, -r5, -r6, and –r7. For a negative control, empty expression vector was transfected. On the next day, cells were washed with 37°C warm PBS and fixated with icecold methanol and acetone (1:1, v/v). After washing with PBS at room temperature and blocking buffer (PBS, 5% normal horse serum, 0.5% Triton X-100) treatment, mouse anti-HSV was added at a dilution of 1:15,000 in modified blocking buffer (PBS, 5% normal horse serum, 0.2% Triton X-100). Following thorough rinses with PBS at room temperature, anti-mouse Alexa Fluor488 diluted 1:2,000 in modified blocking buffer was applied for 1 h to facilitate detection of the C-terminally added hsv-epitope. After additional rinses with PBS, cellular nuclei were stained with DAPI, cells were rinsed again with PBS and, finally deionized H_2O and embedded in Dako mounting medium. Confocal laser scanning microscopy (Zeiss LSM 780) was used to obtain images.

2.5 Screening of bitter compounds

The construct of Tas2r1 was transiently transfected in HEK 293T cells stably expressing the G protein chimera Gα16gust44

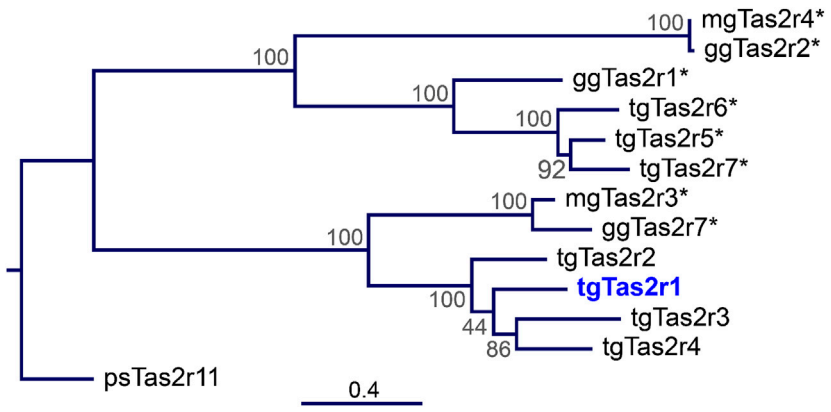

FIGURE 1
Phylogenetic tree of zebra finch, chicken, and turkey Tas2r. The tree was generated by the Maximum-Likelihood method. The amino acid sequences of the 7 zebra finch bitter taste receptors (tgTas2r1-7), the 3 chicken Tas2rs (ggTas2r1, -r2, -r7), the 2 turkey Tas2rs (mgTas2r3 and –r4), and a turtle bitter taste receptor serving as outgroup (Tas2r11 from *Pelodiscus sinensis*) were aligned using MAFFT version 7. The scale bar at the bottom indicates the phylogenetic distance of peptide sequences. The node numbers represent branch support. Blue, Tas2r1 deorphaned here; asterisks, previously deorphaned Tas2rs.

(Ueda et al., 2003) using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. HEK 293T-Gα16gust44 cells were cultured in DMEM supplemented with 10% fetal calf serum (FCS) and glutamine. For cultivation of transfected cells as well as functional calcium imaging analyses, the protocols were described previously (Behrens et al., 2021; Lang et al., 2022a; Lang et al., 2022b). The chemicals used for the screening, which was performed between 2014 and 2015, were selected based on chemical diversity from a compound library of substances known to taste bitter to humans. Almost all bitter compounds that were previously tested on bird and frog receptors (41 of 46) (Behrens et al., 2014) were included and expanded by a set of additional compounds. Two different dilutions of test substances in C1-buffer were applied to the cells. Firstly, the highest applicable compound concentration (based on solubility and/or the highest concentration not leading to receptor-independent calcium signals) used previously (Meyerhof et al., 2010; Lossow et al., 2016). Secondly, a 10-fold lower concentration. Calcium signals of Tas2r1 transfected cells and empty vector (negative control) transfected cells were recorded and compared.

2.6 Recording and calculations

Cells were seeded, transfected, and stimulated as described for the screening procedure. For the determination of the dose-response relationship of tgTas2r1 with cucurbitacin I, the averaged signal amplitudes were plotted against the logarithm of the compound concentrations. Calculation and plotting was done using SigmaPlot software as published before (Behrens et al., 2021; Lang et al., 2022a; Lang et al., 2022b).

3 Results

In contrast to the previously investigated 3 zebra finch tgTas2rs, tgTas2r5, -r6, and –r7, which share a clade with chicken ggTas2r1, zebra finch receptor tgTas2r1 does not belong to these closely related receptor clusters (Figure 1). Instead, tgTas2r1 is a representative of a separate clade, which may be the ortholog of ggTas2r7. This makes this receptor highly interesting for functional studies.

To confirm the expression of tgTas2r1, we performed immunocytochemical staining experiments to visualize epitope-tagged receptor proteins along with cell nuclei. For comparisons, we included constructs coding for the previously characterized zebra finch receptors tgTas2r5, -r6, and –r7 (Behrens et al., 2014) and as a negative control empty vector (=mock) transfected cells (Figure 2).

The immunocytochemical experiment confirmed the successful expression of tgTas2r1 in HEK 293T-Gα16gust44 cells. The apparent expression rate reached by transient transfection with tgTas2r1 is 7.9% ± 1.6%, which is lower than the rates observed for tgTas2r5, -r6, and –r7. Nevertheless, the clear detection of receptor tgTas2r1 in the cell line allowed the functional screening as the next step.

Using a functional heterologous expression assay, we screened tgTas2r1 with in total 72 substances of our bitter compound library. We observed receptor responses of tgTas2r1-expressing HEK 293T-Gα16gust44 cells with 7 bitter compounds (Figure 3). The newly identified 7 activators of the previously orphan receptor tgTas2r1 were further assessed for the relative fluorescence changes (ΔF/F) induced by the agonists at two different concentrations (Figure 4A).

The substance cucurbitacin I resulted in the highest signal amplitudes, whereas absinthin, amarogentin, colchicine, erythromycin and limonin resulted in considerably lower signal amplitudes. Denatonium benzoate activated tgTas2r1 only slightly. In order to investigate the cucurbitacin I responsiveness of tgTas2r1 in more detail, we monitored a full dose-response relationship (Figure 4B). Due to substantial receptor-independent artefacts at cucurbitacin I concentration above 0.3 mM (*cf.* plot of empty vector transfected cell signals), we were not able to deduce an EC_{50}-concentration. The threshold concentration (defined as the lowest concentration of receptor expressing cells exhibiting

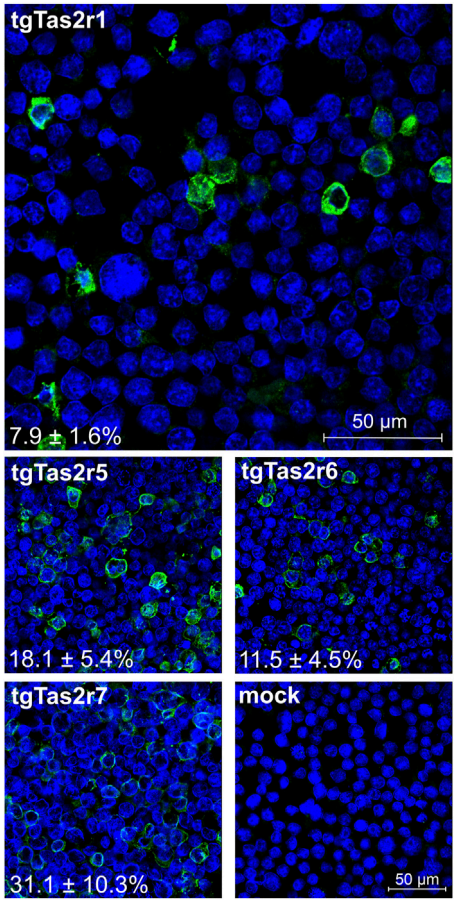

FIGURE 2
Immunocytochemistry of tgTas2r expression after transient transfection. Expression of tgTas2r1 in Gα16gust44 cells was monitored by confocal laser scanning microscopy using an antiserum against the hsv-epitope attached to the receptor C-terminus (green) and DAPI to visualize nuclei (blue). For comparison also expression constructs coding for tgTas2r5, -r6, and −r7 were analyzed. The specificity of receptor detection is demonstrated by the lack of green signals in identically treated empty vector transfected cells (mock). The constructs used for transient transfection and the determined expression rates (in % ± SD) are labeled in the corresponding panels. The average expression rates were based on the counting of 479–521 cells (nuclei) from 3 representative images by 3 independent persons. Scale bars are shown in the upper and bottom right panels.

statistically significant different fluorescence changes compared to identically treated empty vector transfected cells) was 0.0009 mM for cucurbitacin I.

The response rate of tgTas2r1 with 7 activators among the screened 72 substances (~10%) suggests that this receptor belongs to the group with intermediate tuning breadths. The majority of compounds that were screened positive represent natural bitter compounds. Two of the natural new agonists, cucurbitacin I and colchicine, are quite powerful toxins targeting cytoskeletal structures of cells (Wang et al., 2017; Angelidis et al., 2018) which is in good agreement with the function of bitter taste receptors as warning sensors.

Interestingly, the agonist profile discovered for tgTas2r1 shows nearly no overlap with previously identified activators for tgTas2r5, -r6, and –r7, thus increasing the coverage of potentially harmful bitter substances in the habitat of zebra finch (Table 1). With four common agonists, tgTas2r1 exhibits the largest overlap with the agonist spectrum of chicken ggTas2r7, a very broadly tuned generalist receptor. A similar extent of agonist overlap is observed between tgTas2r1 and turkey mgTas2r3, although this agonist set is not identical to the overlap between tgTas2r1 and ggTas2r7.

3 Discussion

In the present manuscript, we characterized the activation profile of the zebra finch bitter taste receptor Tas2r1. Unlike the previously examined receptors Tas2r5, -r6, and –r7 that are orthologs of chicken Tas2r1 (Behrens et al., 2014), the zebra finch Tas2r1 belongs to a different branch of bird Tas2rs othologous to chicken Tas2r7 and turkey Tas2r3 (Figure 1). Our data show that tgTas2r1 clearly complements the bitter agonist spectrum of zebra finch (Table 1). Of the 7 substances identified, only a single, denatonium benzoate, is also activating other already investigated tgTas2rs. Of the 29 bitter activators summarized in table 1, 23 activate at least one chicken receptor, 19 activate turkey Tas2rs, and 15 activate zebra finch receptors. Hence, even though chicken and turkey possess fewer functional bitter taste receptors, they cover a broader bitter agonist spectrum. This may be due to zebra finch lacking representatives in the chicken Tas2r2/turkey Tas2r4 clade (Behrens et al., 2014) and (Figure 1), or the gap in the zebra finch bitter recognition spectrum might also be covered by the 3 tgTas2rs not investigated at present.

Despite its broad tuning for chemically diverse agonists, tgTas2r1 seems to be rather selective. Whereas the sesquiterpene lactone absinthin activates the receptor, the other sesquiterpene lactones included in the screening, parthenolide and picrotoxinin, exhibited no agonistic properties. Of the screened alkaloids, only colchicine induced responses. Moreover, all identified agonists belong to different chemical classes. The, by far, most pronounced response of tgTas2r1 was observed with the substance cucurbitacin I (Figures 3, 4). The apparent potency and efficacy of this ligand suggests an important role of this and related compounds in the ecology of zebra finch. Cucurbitacins are toxic compounds mostly found in plants of the cucurbitaceae family which include also well known edible varieties such as cucumber, zucchini and pumpkin (Kaushik et al., 2015). Birds can get in contact with cucurbitacins either directly by feeding on the plants or indirectly by insects, mostly beetles, sequestrating cucurbitacins as defense from predators (Rowell-Rahier et al., 1995; Gillespie et al., 2003). Although data on cucurbitacin sensitivity of birds are scarce, it was shown that European starlings, which belong to the same order of passerine birds like zebra finches, strongly avoid this compound class (Mason and Turpin, 1990). This observation is likely predictive also for an avoidance behavior of zebra finch, which has been shown to possess taste buds within the oral cavity (Heidweiller and Zweers, 1990). Another interesting observation made in chicken is the increase of bitter taste receptor gene expression by perinatal administration of bitter substances such as quinine (Cheled-Shoval et al., 2014). This may allow birds to adjust their bitter taste sensitivity to the occurrence of particular bitter plants in their individual habitats.

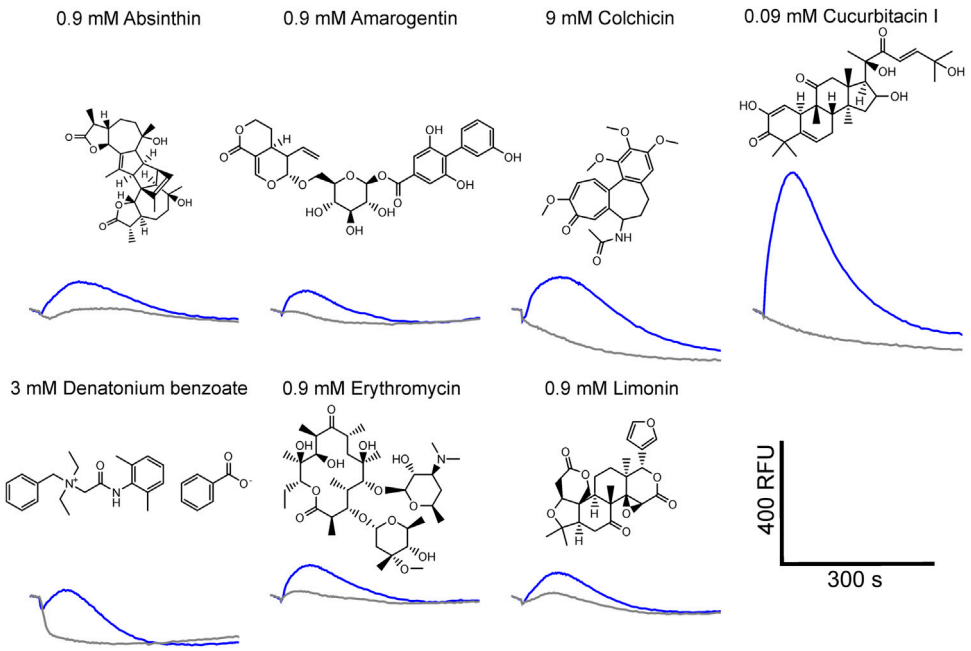

FIGURE 3
Fluorescence traces of tgTas2r1 expressing cells stimulated with bitter activators. The tgTas2r1 expression construct was transiently transfected in HEK 293T-Gα16gust44 cells and challenged with 72 compounds of a bitter compound library. The fluorescence traces of compounds eliciting responses (blue traces) are shown together with the corresponding substance concentrations. The traces were averaged from one representative experiment performed in duplicate wells. Empty vector transfected and identically treated cells were used as negative controls (gray traces). Scale bar, bottom right.

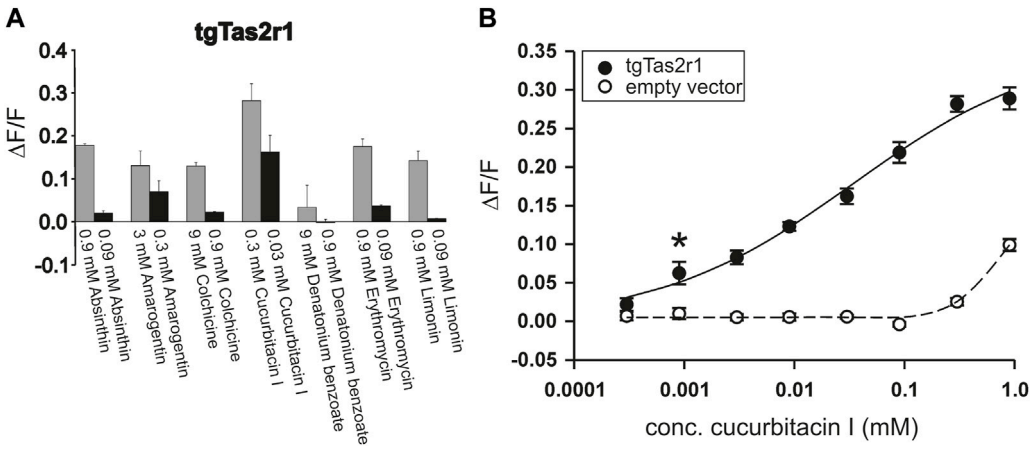

FIGURE 4
Functional screening of zebra finch bitter taste receptor tgTas2r1. tgTas2r1 was transiently transfected in HEK 293T-Gα16gust44 cells and screened with in total 72 natural and synthetic bitter compounds by calcium imaging. **(A)** Each compound resulting in the stimulation of a tgTas2r1 in a prescreening was tested in two different concentrations. The responses upon stimulation with the maximal concentration not leading to unspecific cellular responses, are shown by gray bars. The black bar representing lower concentration of compounds (1:10 dilution). The *y*-axis shows the relative fluorescence changes (ΔF/F ± SD), and the *x*-axis is labeled with activating bitter compounds. **(B)** Dose-response relationship of tgTas2r1 with cucurbitacin I. The data are derived from two independent experiments each performed with 8 technical replicates for each concentration of receptor transfected and 4 corresponding technical replicates for empty vector transfected cells. The relative changes in fluorescence of receptor expressing cells (ΔF/F ± SEM, black circles, solid line) did not show clear signal saturation at concentrations (≤0.3 mM) with absent or tolerable receptor-independent artefact signals (*cf.* empty vector control, open circles, broken line). The asterisk indicates the threshold concentration defined as the lowest concentration at which receptor-transfected cells show a significant higher signal ($p < 0.05$) than empty vector transfected cells.

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TABLE 1 Activation of avian Tas2rs by bitter substances.

No.	Receptor substance	tgTas2r1	tgTas2r5	tgTas2r6	tgTas2r7	ggTas2r1	ggTas2r2	ggTas2r7	mgTas2r3	mgTas2r4
1	Absinthin	•						●		
2	Amarogentin	•						●	•	
3	Andrographolide			•	•			●	•	
4	Azathioprine					●				●
5	Caffeine						●			●
6	Camphor			•	•					
7	Carisoprodol							●	•	
8	Chloramphenicol		•			●	•	●	•	●
9	Chloroquine		●			●				
10	Chlorpheniramine				•	●	•	•		•
11	Colchicine	•						●	●	
12	Coumarin					•	●			●
13	Cucurbitacin I	●								
14	Cycloheximide				•			•		
15	Denatoniumbenzoate	•	•		●					
16	Diphenhydramin					•				
17	Diphenidol		•	●		●	●	●	●	●
18	Erythromycin	•						●	●	
19	Gingkolide A							•	•	
20	Limonin	•							●	
21	Nicotin					•				

(Continued on following page)

TABLE 1 (*Continued*) **Activation of avian Tas2rs by bitter substances.**

No.	Receptor substance	tgTas2r1	tgTas2r5	tgTas2r6	tgTas2r7	ggTas2r1	ggTas2r2	ggTas2r7	mgTas2r3	mgTas2r4
22	Parthenolide						●	•	•	●
23	Picrotoxinin					•		●	●	
24	PTC		•							
25	Quassin							●		
26	Quinine sulphate					●	●	●	•	●
27	Saccharin								●	
28	(−)-α-Thujone							●	●	
29	Yohimbine						•	●	•	

The bitter compounds shown to activate zebra finch (tgTas2r), chicken (ggTas2r), and turkey (mgTas2r) bitter taste receptors are listed. The signal amplitudes are divided in 3 categories, weak (●, ΔF/F < 0.2), medium (●, ΔF/F 0.2–0.4), and high (●, ΔF/F > 0.4).

Depending on the plant, the plant part ingested and some seasonal variations, cucurbitacin levels can be considerable. For example, the plant *Trichosanthes cucumerina L.*, used for traditional medicine in India, contains between 1.7 and 37 mg/kg of total curcurbitacines which equals roughly 3–70 µmol/kg (Attard et al., 2011). As we observed a threshold concentration as low as 0.9 µM with cucurbitacin I, it appears safe to assume that zebra finch are well equipped to recognize cucurbitacins at biologically relevant concentrations. The rather low cucurbitacin I LD_{50}-concentration of 5 mg/kg body weight observed for mice (Gry et al., 2006) would translate to 1.6 g of the fruits containing the highest cucurbitacin concentration for zebra finch, which weigh only about 12 g. This suggests the necessity for sensitive aversive reactions in zebra finch.

In summary, our data confirm that, based on Tas2r function, birds do not exhibit inferior bitter taste abilities and that bitter taste perception, as in almost all other vertebrate species, fulfills a key role for their survival.

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.

Ethics statement

Ethical approval was not required for the studies on humans in accordance with the local legislation and institutional requirements because only commercially available established cell lines were used. Ethical approval was not required for the studies on animals in accordance with the local legislation and institutional requirements because only commercially available established cell lines were used.

Author contributions

MB conceptualization and design of the study. PK, UR, TL, and SK performed and analyzed experiments. MB, PK, and SK wrote the first draft of the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The 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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Differences in the microbiome of the small intestine of Leghorn lines divergently selected for antibody titer to sheep erythrocytes suggest roles for commensals in host humoral response

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For forty generations, two lines of White Leghorn chickens have been selected for high (HAS) or low (LAS) antibody response to a low dose injection of sheep red blood cells (SRBCs). Their gut is home to billons of microorganisms and the largest number of immune cells in the body; therefore, the objective of this experiment was to gain understanding of the ways the microbiome may influence the differential antibody response observed in these lines. We achieved this by characterizing the small intestinal microbiome of HAS and LAS chickens, determining their functional microbiome profiles, and by using machine learning to identify microbes which best differentiate HAS from LAS and associating the abundance of those microbes with host gene expression. Microbiome sequencing revealed greater diversity in LAS but statistically higher abundance of several strains, particularly those of *Lactobacillus,* in HAS. Enrichment of microbial metabolites implicated in immune response such as lactic acid, short chain fatty acids, amino acids, and vitamins were different between HAS and LAS. The abundance of several microbial strains corresponds to enriched host gene expression pathways related to immune response. These data provide a compelling argument that the microbiome is both likely affected by host divergent genetic selection and that it exerts influence on host antibody response by various mechanisms.

KEYWORDS

microbiome, antibody response, VA tech HAS and LAS, machine learning, immunogenetics

Introduction

Gut microbes are integral to intestinal physiology. Necessary for maintaining homeostatic balance between roles of nutrient absorption and pathogen response; resident microbes train the intestine to tolerate commensals while recognizing and responding to pathogens (Shulzhenko et al., 2011; Oakley et al., 2014). The accessibility

of next-generation tools, such as 16S ribosomal RNA sequencing, has facilitated better characterization of chicken microbiomes and correlated associations between commensal microbes and bird health and physiology (Benson et al., 2010; Oakley et al., 2014; Al-Marzooqi et al., 2020). Research is emerging which further connects host humoral response to vaccines and the commensal microbiota (Zimmermann and Curtis, 2018; Gonçalves et al., 2021; Jordan, Carding, and Hall, 2022).

Lines of White Leghorns have been divergently selected for high or low antibody response 5 days post injection with intravenous sheep red blood cells (Siegel and Gross, 1980). These lines, HAS and LAS, have been under constant divergent selection for over 40 generations and are valuable models for avian immunology and genetics, however the majority of the phenotypic differences remain unexplained (Gehad et al., 1999; Dorshorst, Siegel, and Ashwell, 2011; Lillie et al., 2017). Fecal 16S sequencing of HAS and LAS, along with associated lines where selection was relaxed, HAR and LAR, show selection associated differences in microbial abundance (L. Yang et al., 2017). Understanding how selection affects changes in microbiome composition and abundance is important in exploring the host microbe relationship. Equally important however, is unravelling ways in which the resident microbes may influence host phenotype.

In addition to simply characterizing the resident microbes, there is much interest in functional analysis to better explain host-microbe interactions. For amplicon-based sequencing, functional analysis is inferentially determined using software tools such as Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) (Douglas, Beiko, and Langille, 2018). Functional profile inference provides more information to better understand what molecules may be involved in host-microbe interactions, however this approach has obvious limitations both in terms of incomplete information due to dependence on previously sequenced microbial genomes as well as understanding how microbial gene products may affect host physiology. More integrative approaches are necessary to make accurate associations between microbes and host molecular responses.

Machine learning is revolutionizing omics data analysis (Lin and Lane, 2017; Leite et al., 2018; Oh et al., 2021). Predictive algorithms make it possible to identify patterns in large data sets and make relevant associations. This is useful in microbiome data analysis because the abundance of specific microbes can identify microbial "signatures" specific to, and able to differentiate between, two or more groups. This analysis can complement conventional methods to define which microbes best characterize a phenotypic group. Additionally, once microbial signatures are identified, that information can be used as the "classifier" for subsequent machine learning with other data. We have RNA sequencing data for the jejuna segments from which the microbes for 16S sequencing were obtained (Nolin Shelly et al., 2023). Machine learning utilizing microbial signatures with gene expression data, enables the identification of patterns of gene expression which best predict microbe abundance. By determining what molecular pathways are altered by genes differentially expressed in chickens with specific microbial signatures we are then able to make associations of host gene expression with commensal microbiomes. The integration of microbial data, with genetic line and gene expression data becomes a novel means to further explain how microbes may influence underlying host physiology and in turn host phenotype.

Materials and methods

Animal work

Eggs from the 40th generations of lines HAS and LAS were obtained from Virginia Polytechnic Institute and State University and co-incubated until hatch. At hatch, all chicks were tagged for line identification and randomly transferred to battery cages such that chickens from both lines were raised together for the duration of the experiment. At 46 days of age, six chickens from each line were randomly selected, euthanized, and intestinal content samples collected from the duodenum, jejunum, and ileum for microbial DNA isolation. Corresponding intestinal tissue samples were collected for RNA isolation. This time point was selected because it is the traditional age of selection and the age corresponding to that of a cohort of chickens which had been injected with SRBC 5 days prior. All animal research was done in accordance with the North Carolina State University Institutional Animal Care and Use Committee.

Nucleic acid isolation, quantitation, and sequencing

Microbial DNA was isolated from intestinal contents using the QiaAmp DNA Stool Mini Kit and RNA was isolated from each jejunum sample using the RNEasy mini kit (Qiagen, Hilden, Germany) using the manufacturer's protocols. RNA and microbial DNA quantity were measured, and purity was assessed using the NanoDrop ND2000 spectrophotometer (Thermo Fisher Scientific, Waltham MA). Five hundred nanograms of each sample for 16S rRNA sequencing targeting the V3-V4 hypervariable region were sent to the University of North Carolina Microbiome center for library preparation, barcoding, and pyrosequencing on the Roche 454 sequencer. Two micrograms of RNA from each sample were taken to the North Carolina State University Genomics Sciences Laboratory for library preparation and sequencing on the Illumina HiSeq 2,500 sequencer.

Sequencing data analysis

CLC Genomics Workbench (Qiagen, Hilden, Germany) was used for all sequencing and statistical analyses. Statistical comparisons use a generalized linear model and calculate a *p*-value as well as false discovery rate *p*-value (FDRp) and Bonferroni correction to correct for multiple testing.

16S amplicon sequencing data was analyzed using the CLC Microbial Genomics Module. Sequence reads passing quality control were grouped into operational taxonomic units (OTUs) based on 97% sequence similarity to reference database SILVA SSU 99 version 138.1 (Pruesse et al., 2007; Quast et al., 2012; Yilmaz et al., 2014; Glöckner et al., 2017) and representative sequences were selected from each OTU for taxonomic assignment. Metrics were calculated

for alpha (within line) diversity using total number of OTUs and beta (between line) diversity using weighted unifrac. Differences in OTU abundance between HAS and LAS were considered significant at $p \leq 0.05$. Additionally, weighted unifrac permutational multivariate analysis of variance (PERMANOVA) was run to investigate line by tissue differences and were also considered significant at $p \leq 0.05$.

The microbial genomics module also contains a tool for inferring functional microbial profiles utilizing PICRUSt2 (Douglas et al., 2020). By importing a table containing Kmer frequency profiles with term multipliers and associated 16S copy numbers, the software can produce approximate functional profiles for mapped OTUs. This tool was used to create functional profiles as represented by KEGG (Kyoto encyclopedia of genes and genomes) (M. Kanehisa and Goto, 2000; Kanehisa, 2019; Kanehisa et al., 2021) identifiers present for HAS and LAS. Differential abundance of microbial molecules was calculated using the same GLM tool for determining OTU abundance. Due to the large number of functional molecules as well as the inferential nature of the data, the most conservative cut-off, Bonferroni correction ≤0.05 was considered significant. Differentially abundant KEGG molecules were entered into the KEGG orthology database and analyzed for module enrichment between lines.

CLC Genomics Workbench version 11 (Qiagen, Hilden, Germany) was used for RNA sequencing analysis as described previously (Nolin Shelly et al., 2023). High quality RNA sequencing reads were mapped to the Galgal6 reference genome (GCA_000002315.5) and differentially expressed genes were determined between lines, with FDRp ≤0.05 considered statistically significant.

Machine learning

Waikato Environment for Knowledge Analysis suite (WEKA) (Hall et al., 2009) was used for Machine learning. Three algorithms were used: support vector machines, artificial neural networks, and a decision tree. Algorithms were validated using two cross validation methods, a %-split and a K-fold stratified hold-out. A % split cross validation randomly splits the data into training or test. In our experiment we used a 66%–34% split, wherein 66% of the data was assigned to be the training set and the remaining 34% used to test. Sample groups are equally represented in the test and training sets. The K-fold stratified hold-out works by randomly dividing the data into K datasets, where K-1 datasets are used for training with the remaining 1 "hold-out" dataset as the test set. For our experiment, 6 is the number of biological replicates for each line/tissue, so K = 6. This algorithm runs sequentially 6 times, such that each dataset is the test set only once and included in the training set the remainder; performance is given as the average for the 6 runs. Performance for all machine learning is calculated as the average for the three algorithms and two validation methods.

To utilize machine learning to associate microbes with genetic line and later host gene expression, first we ran the algorithms to determine which jejunal microbes were most predictive of line. We started by running the algorithms with all OTUs to determine the predictive performance using the entire dataset. Next the OTUs were ranked by combining the entropy based InfoGainAttribute ranker function in WEKA (Li et al., 2004) with the *p*-value which was calculated for differential abundance for each OTU. A portion of OTUs were then removed (those with the highest *p*-value and lowest entropy) and the algorithms were re-run with the smaller set of OTUs. This process was repeated until the optimal microbial signatures were determined for highest prediction performance. This is known as reduction of data dimensionality and allows for identifying the optimal microbial "signature" for line prediction. Once these microbes were identified, they were each used as the sole attribute for running the algorithms to determine their individual predictive performance. Microbes with <75% correct prediction performance were eliminated from further analysis, as were those exclusively present in only one line.

These remaining OTUs were divided into groups by their relative abundance as high, low, or absent, or if the microbe was present in all samples as high, medium or low, as shown in Table 1. Abundance could then be used as the classifier and each sample, previously identified as HAS# or LAS#, was now identified by relative OTU abundance. OTU counts greater than the mean (for all present samples) were classified as high and those less than the mean as low, samples which did not contain any OTU counts were obviously grouped as absent. For microbes which were present in all samples, high was calculated as the mean OTU counts +50%, low as mean −50%, and medium were counts in between. Dividing the abundances for the microbes into three groups was necessary to avoid samples being classified solely by line. RNA sequence data for each sample was then used as the attributes with microbial abundance as the classifiers for machine learning in order to determine the gene expression patterns which predict microbe abundance. As statistical analysis of differential gene expression could not be performed based on microbial abundance, the InfoGainAttribute ranker function in WEKA (Li et al., 2004) was used to rank the genes for purposes of reducing data dimensionality as before. The optimal gene lists for each microbe were run in Ingenuity Pathway Analysis (IPA, Qiagen, Hilden, Germany) to determine enriched host pathways.

Negative controls for machine learning were also run for line prediction and microbial abundance. Datasets were randomized, wherein each sample identifier was randomly assigned to the data for a different sample, this allows for disassociation between classifier and attributes. Ten random datasets were used for each comparison and the average performance was calculated for each of the ten runs. Given two groups, HAS *versus* LAS 50% correct prediction would indicate random chance, whereas for microbial abundance with three classifiers, it would be ~33%. If the negative controls performance is close to that of random probability, we can be confident that true machine learning occurred in our experimental data sets.

Results

Microbial diversity and abundance

A total of 2,543 OTUs were identified, however those with less than 10 total OTU counts were filtered out, leaving 450 OTUs remaining for further analysis. LAS had the slightly more diverse microbiota of the two lines, with 350 OTUs present *versus* 304 OTUs

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TABLE 1 Explanation of how samples were identified by microbial abundance as, absent (A), low (L), medium (M), or high (H).

OTU	HAS1	HAS2	HAS3	HAS4	HAS5	HAS6	LAS1	LAS2	LAS3	LAS4	LAS5	LAS6
1	A	L	A	A	A	A	H	H	H	L	L	H
2	M	H	H	H	M	M	L	L	L	L	L	L
3	M	H	H	H	M	M	M	L	L	L	L	L
4	M	H	H	H	M	M	M	M	L	L	M	M
5	L	A	A	A	L	L	H	H	L	A	L	L
6	L	H	H	H	H	M	M	L	L	L	M	L
7	L	H	L	H	H	L	L	A	L	A	L	L
8	H	L	H	H	H	L	A	L	L	A	A	L
9	L	L	H	H	H	A	A	A	L	L	L	A

present in HAS. There was overlap of 204 OTUs common to both lines, leaving a substantial percentage of the microbiota unique to one line or the other, see Figures 1A–F. As previously reported (Yang et al., 2017), *firmicutes* were the most abundant phyla in both lines, as well as for all intestinal segments, accounting for >80% of all microbes in our samples, followed by *actinobacteria*, *cyanobacteria*, and *proteobacteria* as shown in Figure 2. Within the *firmicutes*, lactic acid bacteria of the closely related genera *Lactobacillus*, *Ligilactobacillus*, and *Limosilactobacillus* are by far the most dominant for HAS and LAS, both in terms of relative abundance (>95%) as well as number of individual OTUs. Each intestinal segment also had a microbial signature unique from the others. At the order level, the duodenum had the most diversity, followed by the ileum, and very little diversity was found in the jejunum. These data are shown in Figure 3.

Alpha diversity box plots in Figure 4 further illustrate the microbial richness of LAS vs. HAS, as the total number of OTUs observed for LAS samples are greater than those of HAS for each intestinal segment. Additionally, while rarefaction curves are not shown, the diversity captured in HAS seems thorough, whereas additional sequencing in LAS may have identified additional OTUs.

Beta diversity, variation between samples, as shown by principal coordinate analysis in Figure 5 illustrates how the samples cluster by tissue and line. In general, it seems that within line microbial differences became less prominent while between line differences became more prominent from anterior to distal segments, given that samples cluster somewhat more closely in the ileum and jejunum than in the duodenum, particularly so for the HAS line.

Statistical differences in abundance

We found 206 OTUs which differed in abundance between lines, irrespective of tissue, at FDRp ≤0.05. Of those, 65 OTUs were observed in both lines. In contrast to most of the data where LAS had exhibited more diversity only 18 of the common OTUs were more abundant in LAS while 47 were more abundant in HAS as shown in Figure 6. Additionally, 100 of the 206 OTUs only present in one line were found exclusively in HAS while 41 were only found in LAS.

PERMANOVA revealed statistically significant line by tissue differences. The jejunum and ileum were significantly different between lines, though there was not a significant difference between the duodenum of HAS *versus* LAS. Within line, significant differences were observed between HAS duodenum and jejunum, and between LAS jejunum and ileum.

Functional analysis

Functional analysis identified 1385 KEGG molecules which were differentially abundant between lines; 704 more abundant in LAS *versus* 681 more abundant in HAS. Using the KEGG mapper tool for each list of molecules revealed enrichment of modules for different types of metabolic pathways between lines. Of the top 10 modules in HAS five were associated with biosynthesis of molecules fatty acid, lysine, pyrimidine, riboflavin, and coenzyme A. Four were involved in the 3-Hydroxypropionate bi-cycle, glycolysis, antimicrobial resistance, and ATPase activity. Conversely, LAS had three modules involved in degradation: AMP, GMP, and phenylacetate. Three modules were enriched for reductive carbon conversion, two were involved in menaquinone biosynthesis, and one in NAD biosynthesis. LAS and HAS both had enriched modules for C5 isoprenoid, though HAS was enriched for the mevalonate pathway whereas the non-mevalonate pathway was enriched in LAS.

Machine learning and host pathway analysis

Figure 7 summarizes the machine learning workflow. In the jejunum, the combination of the top twenty OTUs identified via machine learning resulted in an average predictive performance of 98.61%, that is to say the algorithms were able to identify patterns in the abundance data for these microbes which could be used to accurately classify the samples as being from the HAS line or LAS line on average 98.61% of the time. The randomized datasets performed at an average of 45%, which is very close to the 50% expected by random probability and assured us that "true" machine learning occurred. Of those top twenty OTUs, eleven had average individual predictive performance >75%, however two were only present in the LAS line. The nine remaining OTUs which were

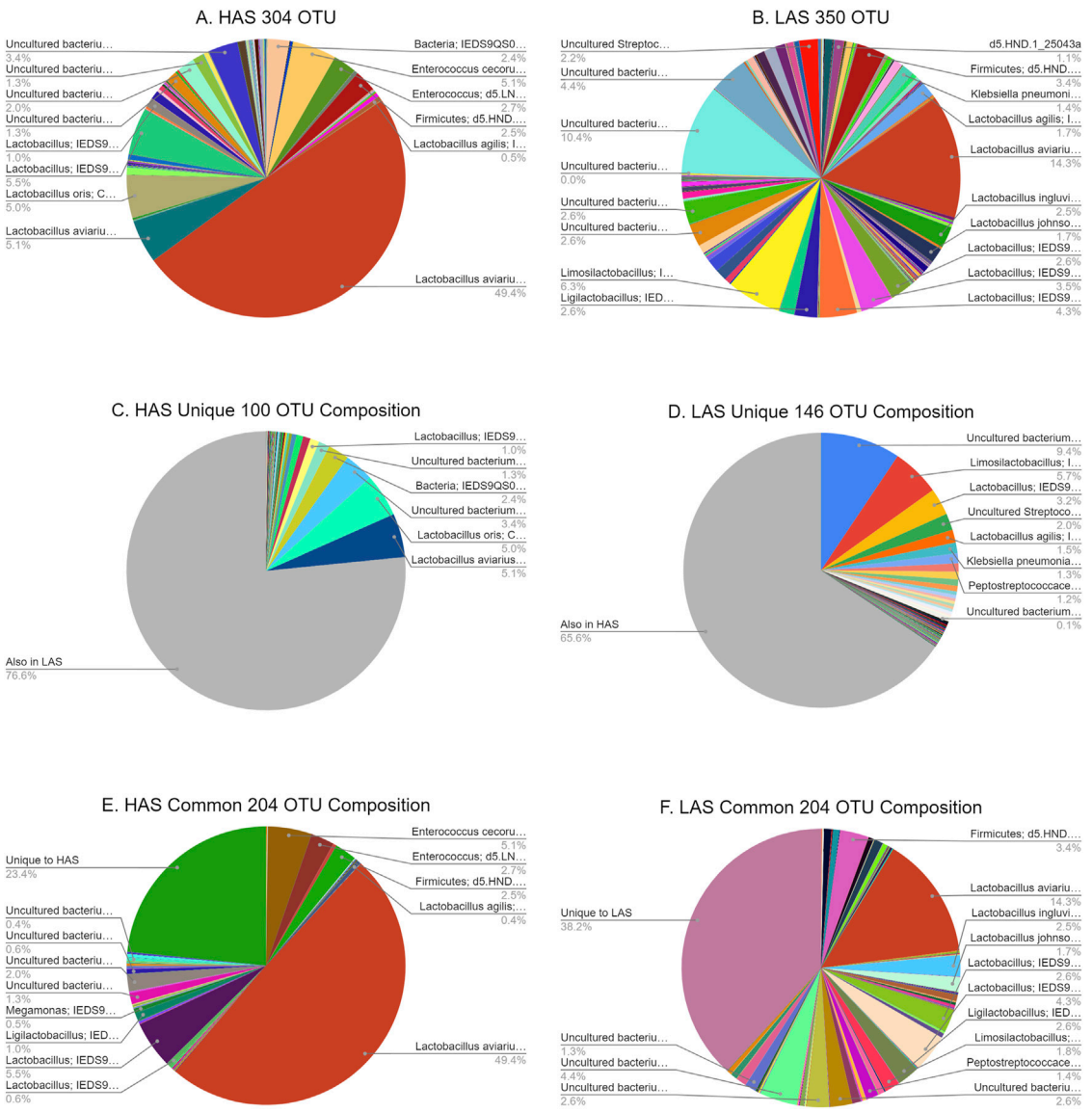

FIGURE 1
(A–F) Relative OTU composition of HAS and LAS small intestine.

both predictive of line and present in at least one sample from each line included eight uncultured strains of the genus *Lactobacillus* identified as EU776306.1.1423, AY958858.1.1540, EU460910.1.1421, EU774852.1.1423, EU774852.1.1423, IEDS9QS05FX4Q0, sp. HM218868.1.1554, and HM218952.1.1524, one species of *Lactobacillus crispatus*: EU559595.1.1562, and one of the genus *Enterococcus* EU459393.1.1420 (also uncultured). These OTUs were subsequently used to identify host gene expression associated with the relative abundance of each microbe, which were used as input for pathway enrichment analysis.

Each OTU dataset was run independently using the three machine learning algorithms and two cross validation methods. The relative abundance of microbes was used as the classifier (absent, low, medium, or high) and the predictive dataset attributes were composed of the gene expression data in transcripts per million. RNA was sequenced from the jejunum tissue samples, the contents from which the microbial DNA was isolated. The list of genes whose expression patterns most accurately predicted relative OTU abundance was determined for each of the nine OTUs and used for pathway enrichment analysis. Predictive performance for each optimized gene list was between 67% and 90%. Genes lists varied from 35–500 and IPA was able to map >60% of those in each list. The randomized negative control dataset performances were between 27% and 38%, close to the expected 33.3% indicative of random chance. The results of machine learning and IPA are summarized in Table 2, and all metrics for the machine learning algorithm runs with optimized feature sets can be found in Supplementary Table S1.

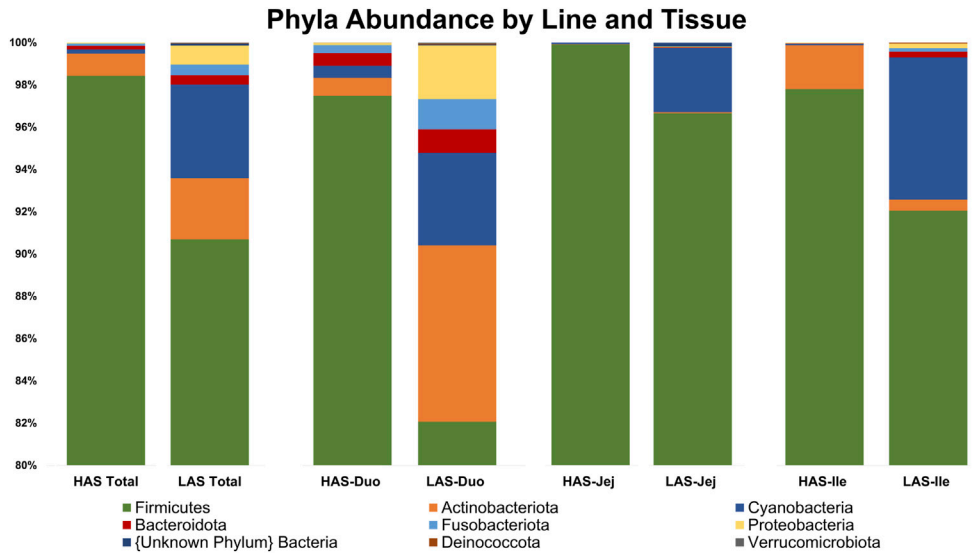

FIGURE 2
Relative abundance of the top 20% of microbes at the Phylum level in HAS and LAS total and by intestinal segment.

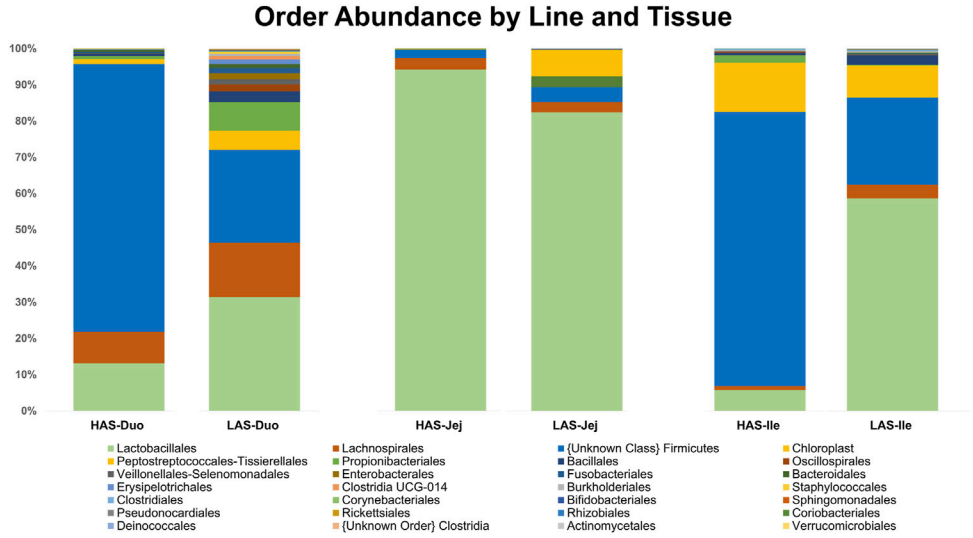

FIGURE 3
Relative abundance of microbes at the Order level in HAS and LAS by intestinal segment.

Discussion

Forty generations of divergent selection have resulted in a greater than six-fold difference in 5 day post injection antibody titer to sheep red blood cells between the HAS and LAS lines. Previous research has identified differences which help explain the phenotype, including their major histocompatibility B-haplotypes, as well as other genetic loci identified via pooled resequencing and quantitative trait loci mapping of an advanced intercross line (Gehad et al., 1999; Dorshorst, Siegel, and Ashwell, 2011; Lillie et al., 2017). However, much of the variation in antibody response has yet to be elucidated. The microbiome has become a key area of immunology research, and given that commensal microbes are thought to coevolve with their host (Zhao et al., 2013; Meng et al., 2014; Yang et al., 2017; Koskella and Joy, 2020), and influence host humoral response to vaccines (Zimmermann and Curtis, 2018; Gonçalves et al., 2021) the microbiomes of HAS and LAS likely play a role in their differential antibody response phenotypes.

Sequencing the small intestinal microbiome of HAS and LAS has demonstrated an association between genetic selection and microbial composition and diversity. The microbiome of the small intestine were distinctly different between lines HAS and LAS, wherein LAS exhibited greater microbial diversity than

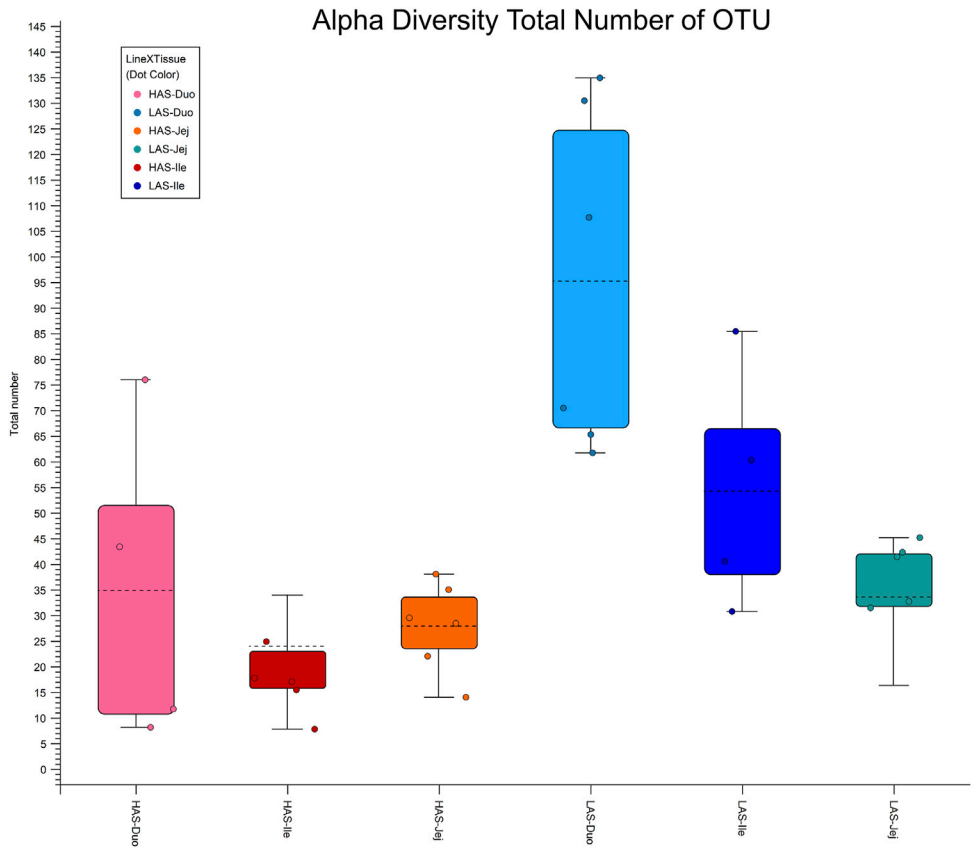

FIGURE 4
Alpha diversity plot total number of OTUs for duodenum, jejunum and ileum in lines HAS and LAS.

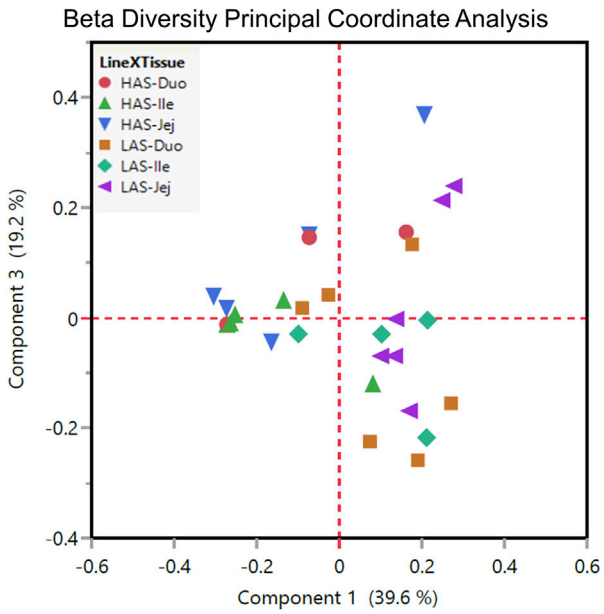

FIGURE 5
Beta diversity plot principal coordinate analysis for all HAS and LAS duodenum, jejunum, and ileum sample.

HAS, in agreement with previous fecal microbiome research (Zhao et al., 2013). Genetic selection for high or low antibody response to SRBCs appears to have resulted in an inverse relationship with commensal microbe diversity. While LAS exhibited greater numerical diversity, it is also important to note that from a statistical standpoint HAS displayed an increased abundance of more microbial strains. The discordant observances in statistical *versus* numerical abundance may be attributed to greater within line diversity in LAS than HAS. That is to say, HAS may have more microbes which differ statistically from LAS, but that is because LAS samples differ more from one another than HAS samples do. This is further supported by the results of the alpha diversity analysis, where the diversity captured in the HAS samples appears more comprehensive.

Lactobacillus, being a predominant genus in both lines, warrants additional exploration. The species/strains of lactobacilli present in one line are often significantly reduced or entirely absent in the other and it is important to consider the additive effects of small differences in microbial abundance and composition that may contribute to host physiology. Studies of feeding different probiotic *Lactobacillus* strains result in various degrees and types of immunostimulant (Perdigón, Fuller, and Raya, 2001). One of the main ways microbes exert an effect on host immune response in the gut is via fermentation products such as lactic acid, and the short

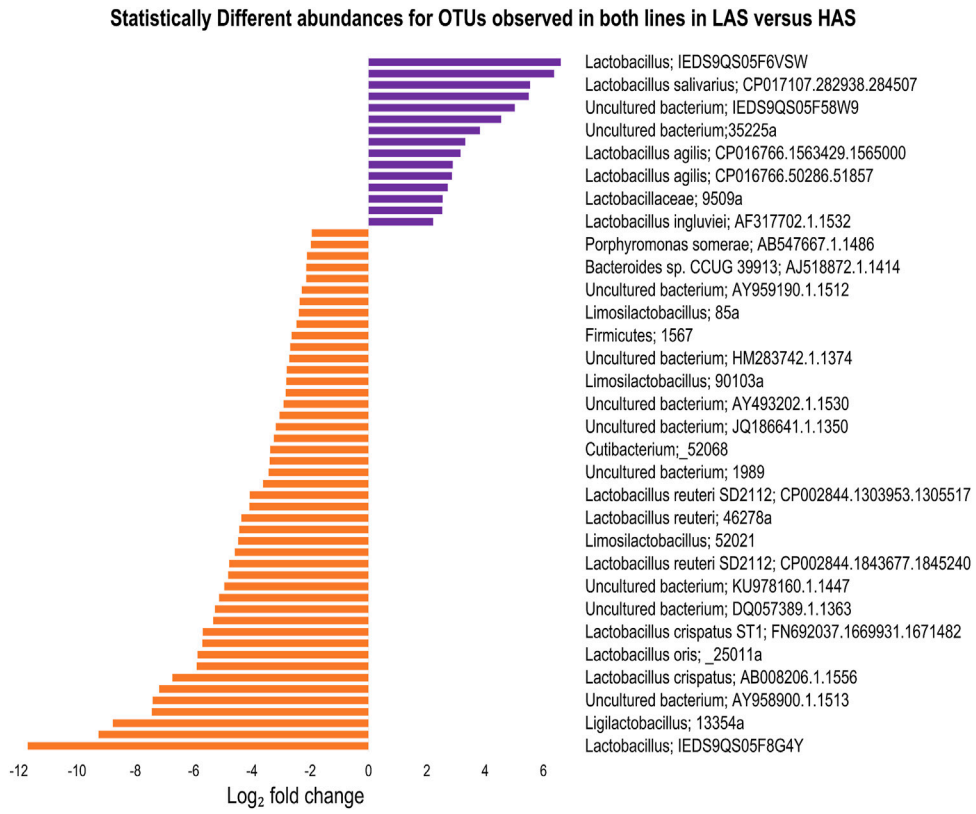

FIGURE 6
Statistically different microbes between HAS and LAS for OTUs observed in both lines in any intestinal segment.

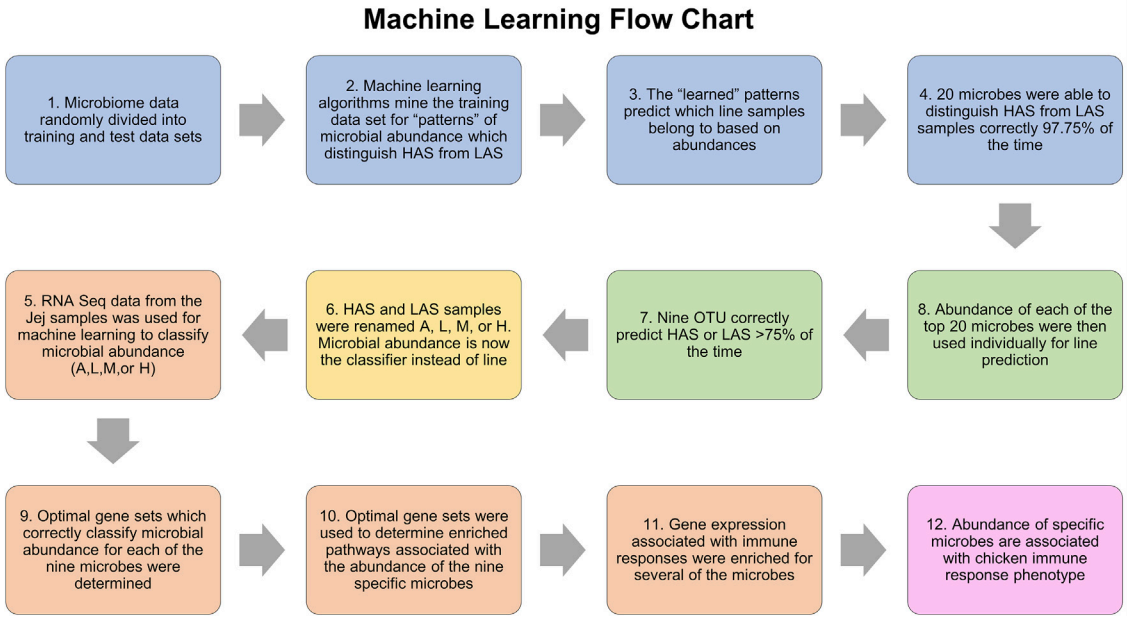

FIGURE 7
Flowchart of the machine learning process.

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TABLE 2 Functional pathway enrichment for genes associated with most predictive microbes.

Taxa	% Correct line prediction	Top ave % performance for RNASeq	# Genes for maximum performance	#IPA mapped genes	Top enriched pathways
Ent_UnCul_EU459393.1.1420	90	82	339	244	Neuroinflammation Signaling Pathway, Antigen Presentation Pathway, Virus Entry via Endocytic Pathways
LB_crispatus_EU559595.1.1562	83	85	35	22	Flavin Biosynthesis IV, Tetrapyrrole Biosynthesis II, Selenocysteine Biosynthesis II
LB_UnCul_EU776306.1.1423	78	78	325	253	Glycolysis I, Colanic Acid Building Blocks Biosynthesis, Heme Degradation
LB_UnCul_AY958858.1.1540	88	74	349	264	Gnaq Signaling, fMLP Signaling in Neutrophils, IL-1 Signaling
LB_UnCul_EU460910.1.1421	76	88	83	58	Synaptogenesis Signaling Pathway, Neuregulin Signaling, UDP-N-acetyl-D-galactosamine Biosynthesis II
LB_UnCul_EU774852.1.1423	79	85	500	444	Role of Macrophages, Fibroblasts and Endothelial Cells in Rheumatoid Arthritis, ERK/MAPK Signaling, Role of Osteoblasts, Osteoclasts and Chondrocytes in Rheumatoid Arthritis
LB_UnCul_IEDS9QS05FX4Q0	76	90	228	186	Phagosome Maturation, Antigen Presentation Pathway, Colanic Acid Building Blocks Biosynthesis
LB_UnCul_sp.HM218868.1.1554	79	75	312	219	WNT/B-catenin Signaling, Neuroinflammation Signaling Pathway, Stearate Biosynthesis I, (SLC25A51)
LB_UnCul_HM218952.1.1524	78	67	45	40	NADH Repair, Salvage Pathways of Pyrimidine Deoxyribonucleotides, Insulin Secretion Signaling Pathway

chain fatty acids (SCFA) (Kim, 2018). As its name suggests, *Lactobacillus* is responsible for producing lactic acid and while some strains can produce short chain fatty acids or manipulate the microbial environment leading to SCFA production by other microbes (H. Li et al., 2020) there is a negative correlation between *Lactobacillus* and SCFA content, particularly butyrate, in the small intestine of poultry (H. Yang et al., 2018; Du et al., 2020; Ranjitkar et al., 2016). Butyrate, and to a lesser degree propionate, have been reported to inhibit dendritic cells, T helper cells, and B cells (Millard et al., 2002; Singh et al., 2010; Arpaia et al., 2013; Trompette et al., 2014; Sanchez et al., 2020) all of which would contribute to diminished immunoglobulin production. If the intestinal microbiome composition of HAS favors a reduction in SCFA producing bacteria compared to LAS, it could help explain the enhanced antibody response to sheep red blood cells observed in that line. Conversely, while intestinal lactic acid concentration has not been studied specifically in regard to antibody response, there are data to indicate that it may contribute positively. Cell culture experiments with supplemented lactic acid resulted in an increase in antibody production (Kromenaker and Frirdrich, 1994). Children suffering from rotavirus induce diarrhea given oral probiotic *Lactobacillus gg* exhibited an increase in antibody secreting cells (Kaila et al., 1992) and broilers fed probiotic *Lactobacillus bulgaris* had increased antibody titer to Newcastle's Disease Virus vaccination (Apata, 2008). Additionally lactic acid bacteria are currently being investigated as a useful vector for vaccines (Szatraj, Szczepankowska, and Chmielewska-Jeznach, 2017). Whether it is a result of the higher levels of lactic acid, or some other metabolite produced by *Lactobacillus*, the antibody response to SRBCs observed in HAS is likely influenced by the increased abundance of commensal *Lactobacillus*.

Other microbial metabolites can also stimulate host physiology. Modules for microbial riboflavin and lysine biosynthesis were more enriched in HAS compared to LAS, which have been shown to impact immune cell function and antibody response. Antibodies to *Salmonella pullorum* were observed to be impaired in animals with diets deficient in riboflavin (Panda and Combs, 1963) and influenza vaccines containing riboflavin adjuvants resulted in increased antibody titers (Quintilio et al., 2016). Additionally, microbes have been shown to present riboflavin metabolites to the MHCI

related protein (MR1) of mucosal associated invariant T-cells (Treiner et al., 2003; Toubal et al., 2019). Lysine deficiency has been associated with reduced antibody response to Newcastle disease in broilers, and chickens on diets with supplemented lysine were found to have an increase in antibody titer (Chen, Sander, and Dale, 2003; Faluyi et al., 2015). Future studies may include full metagenomic sequencing to better characterize the spectrum of gut microbial metabolites which may impact chicken antibody response.

Machine learning allows for the addition of host pathway analysis related to microbe abundance. These data further support the involvement of commensal microbes in antibody response as the abundance of the microbial strains most predictive of host line are associated with enriched expression of host genes involved in antigen presentation, inflammation, and immune cell responses. Future studies may look to better determine how these specific microbes may be impacting the molecular pathways identified using this integrated machine learning approach.

By taking an integrated approach which includes 16S microbiome sequencing, functional microbiome analysis, and machine learning to characterizing the small intestinal microbiome of genetically selected lines, HAS and LAS we have been able to demonstrate differences which may influence antibody response to SRBCs. In agreement with the results of fecal microbiome sequencing (Yang et al., 2017) we saw an increase in microbial diversity in LAS birds and an increase in *Lactobacillus* abundance in HAS birds. In addition to characterizing the microbiota of the separate small intestinal segments *versus* the combined microbiota of feces, another novel aspect of our experiment is that the chickens in our study had not been injected with sheep red blood cells, thus the differences we observe are native and not influenced by antigen exposure. Based on what has been published with regard to *Lactobacillus* and microbial metabolites, our results support a role for the involvement of the associated gut microbiome with the high and low antibody response to SRBCs in these divergently selected lines. Including functional microbiome data has allowed for the inferential identification of enriched microbial modules, the products of which may influence host phenotype. Finally, machine learning allows for identifying specific microbe strains whose abundance accurately predicts line (HAS or LAS) and enables associating those microbial abundances with host gene expression data to further explore the host-microbe relationship to host antibody response. This is to our knowledge the first time these different data types have been integrated together in this way via the use of machine learning algorithms and offers a novel approach to better understanding host physiology with regard to microbiota composition.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/, GSE206804 https://www.ncbi.nlm.nih.gov/, GSE240908.

Ethics statement

The animal study was approved by North Carolina State University Institutional Animal Care and Use Committee. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

SN: Conceptualization, Formal Analysis, Investigation, Methodology, Software, Writing–original draft, Writing–review and editing. CA: Conceptualization, Project administration, Resources, Supervision, Writing–review and editing. PS: Resources, Writing–review and editing.

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Conflict of interest

The 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.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2023.1304051/full#supplementary-material

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Natural intraepithelial lymphocyte populations rise during necrotic enteritis in chickens

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Intraepithelial lymphocytes (IEL) reside in the epithelium at the interface between the contents of the intestinal lumen and the sterile environment of the lamina propria. Because of this strategic location, IEL play a crucial role in various immunological processes, ranging from pathogen control to tissue stability. In mice and humans, IEL exhibit high diversity, categorized into induced IEL (conventional CD4 and CD8αβ T cells) and natural IEL (TCRαβCD8αα, TCRγδ, and TCR^{neg} IEL). In chickens, however, the subpopulations of IEL and their functions in enteric diseases remain unclear. Thus, we conducted this study to investigate the role of IEL populations during necrotic enteritis (NE) in chickens. At 14 days of age, sixty-three Specific-pathogen-free (SPF) birds were randomly assigned to three treatments: Control (sham challenge), *Eimeria maxima* challenge (EM), and *Eimeria maxima* + *Clostridium Perfringens* (*C. Perfringens*) co-challenge (EM/CP). The EM and EM/CP birds were infected with *Eimeria maxima* at day 14 of age, and EM/CP birds were additionally orally inoculated with *C. perfringens* at days 18 and 19 of age. Birds were weighed at days 18, 20, and 26 of age to assess body weight gain (BWG). At 20 days of age (1 day-post *C. perfringens* infection; dpi), and 26 days of age (7 dpi), 7 birds per treatment were euthanized, and jejunum was harvested for gross lesion scores, IEL isolation, and gene expression. The EM/CP birds exhibited subclinical NE disease, lower BWG and shorter colon length. The Most changes in the IEL populations were observed at 1 dpi. The EM/CP group showed substantial increases in the total number of natural IEL subsets, including TCRαβ$^+$CD4$^-$CD8$^-$, TCRαβ$^+$CD8αα$^+$, TCRγδ$^+$, TCR^{neg} and innate CD8α (iCD8α) cells by at least two-fold. However, by 7 dpi, only the number of TCRαβ$^+$CD4$^-$CD8$^-$ and TCRαβ$^+$CD8αα$^+$ IEL maintained their increase in the EM/CP group. The EM/CP group had significantly higher expression of proinflammatory cytokines (IL-1β and IFN-γ) and Osteopontin (OPN) in the jejunum at 1 dpi. These findings suggest that natural IEL with innate and innate-like functions might play a critical role in the host response during subclinical NE, potentially conferring protection against *C. perfringens* infection.

KEYWORDS

necrotic enteritis, *Clostridium perfringens*, mucosal immunity, intraepithelial lymphocytes, gene expression, intestine, chickens

1 Introduction

Necrotic Enteritis (NE) presents a significant challenge for the poultry industry, resulting in substantial financial losses of approximately $6 billion annually (1). This disease can manifest in two forms: clinical infection, causing high mortality rates, and subclinical infection, leading to reduced performance (2). Both forms negatively impact the health and profitability of birds in the poultry industry. In the USA, Antibiotic Growth Promoters (AGPs) have been routinely used in poultry operations since 1951 to control NE and other enteric infections (3). However, due to the concerns about antibiotic resistance and the emergence of superbugs, numerous countries, particularly Europe have imposed restriction on AGP usage in poultry (4). These restrictions have resulted in a resurgence of NE and have promoted numerous research efforts to identify feasible alternatives for its control (5–7). Research into antibiotic alternatives has yielded potential strategies for NE control, although none have been as effective as AGPs (6). Therefore, a more comprehensive understanding of various components within the chicken enteric immune system and their interactions with *C. perfringes* is needed. One promising avenue is studying intraepithelial lymphocytes (IEL) response to NE. IEL are located within the intestinal epithelium and represent one of the first lines of defense in encountering enteric infections (8).

Our current knowledge regarding the role of IEL primarily derives from studies conducted in mice and humans, with limited research focused on chickens. These cells have been recognized as an essential arm of mucosal immunity in the intestine, playing a crucial role in maintaining intestinal homeostasis by tolerating food particles and microbiota while eliminating pathogens (9). The IEL populations are composed of diverse subsets of immune cells, with the majority of being T cells (90%) expressing either TCRαβ or TCRγδ receptors (8, 9). These T cells can be further categorized into induced and natural IEL (8). Induced TCR^+ IEL, including conventional CD4 and CD8αβ T cells, are activated in the periphery upon encountering their specific foreign antigens prior to entering the IEL populations. In contrast, natural IEL ($TCR\alpha\beta^+CD8\alpha\alpha^+$ and $TCR\gamma\delta^+$ subsets) migrate directly to the intestinal epithelium upon their generation (10). The frequencies of T lineage IEL vary across the small intestine and colon in both humans and mice (9–11). In humans, there is a higher frequency of TCRαβ+ IEL compared to TCRγδ+ IEL in the small intestine and colon (12, 13). Conversely, the mouse small intestine has nearly equal proportions of both populations, with a shift toward TCRαβ+ IEL in the colon. In both human and mouse, the colon maintains a higher proportion of $TCR\alpha\beta^+CD4^+$ IEL than the small intestine (13). However, the opposite is observed for $TCR\alpha\beta^+CD8\alpha\beta^+$ IEL. In mice, 20-50% of TCRαβ+ IEL in the intestine can be TCRαβ+CD8αα+, while in humans, this population constitutes less than 1% of TCRαβ+ IEL (11). Finally, approximately 10% of IEL are non-T cells known as TCR^{neg} IELs, which include innate lymphoid-like cells (14–16), intracellular CD3, and iCD8α (17, 18).

In the chickens, the presence of IEL was first reported by Lawn and his colleagues in 1988, using light and electron microscopy (19). Their findings indicated that the majority of chicken IEL are T-lineage lymphocytes. Subsequent later studies, while confirming the majority of IEL are T cells, demonstrated that natural killer (NK) cells also make up a significant portion of IEL (20, 21). Moreover, the role of IEL subsets mediating chicken responses to enteric diseases was limited to $CD4^+$, $CD8^+$, and $TCR\gamma\delta^+$ T cells, which increased in frequency and changed their gene expression profiles during *Eimeria* infection (22–24). However, the response of subsets to NE has not been investigated. Therefore, the main aim of this study is to characterize IEL subsets using flow cytometry during *C. perfringens*-induced NE in SPF chickens. We employed a co-infection approach involving *Eimeria maxima* followed by *C. perfringens* to induce NE. *Eimeria* parasites are recognized as the primary predisposing factor for NE due to their ability to disrupt the integrity of the intestinal epithelium (25, 26). This disruption leads to the secretion of mucus and leakage of nutrients into the intestinal lumen, creating an environment that attracts *C. perfringens* to the small intestine and facilitates colonization (27, 28). Our results indicate that the numbers of natural IEL subsets, including $TCR\alpha\beta^+CD4^-CD8^-$, $TCR\alpha\beta^+CD8\alpha\alpha^+$, $TCR\gamma\delta^+$, TCR^{neg}, and $iCD8\alpha^+$, increased during an early *C. perfringens* infection. Additionally, *C. perfringens* infection induced expression of OPN, IL-1β, and IFN-γ genes at 1 dpi (day-post C. *perfringens* infection).

2 Materials and methods

2.1 Ethics statement

The animal in the research trial was approved by The Ohio State University's Institutional Animal Care and Use Committee (IACUC 2022A00000026) and conducted in compliance with the guidelines and regulations.

2.2 Husbandry, experimental design, and performance

Sixty-three SPF birds were placed in a single-floor pen with a sawdust litter in an environmentally controlled house (Supplementary Figure 1). The birds were divided into three groups (control, EM, and EM/CP) of 21 birds each and placed in three separate pens. On day 14 of age, the control group underwent a mock challenge with phosphate buffer saline (PBS, Quality Biological), while the EM and EM/CP groups were orally challenged with $5x10^3$ sporulated oocysts/ml/bird of *Eimeria maxima* (provided generously by Dr. Lisa Beilke). On days 18 and 19 of age, the EM/CP group was orally inoculated with *C. perfringens* strain type A (CP1 [$netB^-$] (29), provided by Dr. Lisa Beilke) at a concentration of $1x10^8$ CFU/ml, while the EM group was administered PBS. Monitoring for signs of illness, mortality, and body weight was carried out starting from day 18 of age and continued throughout the duration of the experiment. Body weight gain (BWG) was calculated from days 18 to 20 and days 18 to 26 of age. At day 20 (1dpi) and 26 (7dpi) of age, 7 birds per group were euthanized through CO_2 asphyxiation for sample collections.

2.3 Lesion scores and intestinal length

At 1 dpi, the jejunum-ileum junction was examined for NE lesions using 0-4 scale system (30), where 0 denotes no gross lesions, 1 signifies a thin-walled or friable intestine, 2 indicates focal necrosis or ulceration, 3 means large patches of necrosis, and 4 represents a severe, extensive necrosis. Additionally, the lengths of the duodenum, jejunum, ileum, and colon were individually measured for each bird.

2.4 IEL isolation and flowcytometry

At 1 and 7 dpi, approximately 10 cm segment of the jejunum was subjected to mechanical disruption, following a previously established protocol (31). In brief, the intestinal tissues were thoroughly rinsed with PBS and longitudinally opened to remove mucus and fecal matters. The tissue was cut into approximately 1 cm pieces and agitated in PBS supplemented with 5% chicken serum (Sigma-Aldrich, St. Louis, MO, USA), 2 mM EDTA (Quality Biological, Gaithersburg, MD, USA), and 2 mM dithiothreitol (Sigma-Aldrich, USA) at 150 rpm, 37°C for 45 minutes. Subsequently, the supernatant was passed through a gauze column, and the IEL fraction was enriched from the supernatants using a discontinuous 40/70% Percoll density gradient (Cytiva, Marlborough, MA, USA). The recovered cells were incubated for 5 minutes with ACK buffer (Quality Biological, USA) to lyse red blood cells and resuspended in a staining buffer. The number of live cells was counted using the trypan blue exclusion method. Then, the immune cells were stained with fluorochrome-conjugated anti-chicken CD45 SPRD (LT40), CD4 PE-CY7 (CT-4), CD3 AF547 (CT-3), TCRγδ FITC (TCR-1), CD8α AF700 (CT-8), and CD8β PE (EP42) antibodies (Southern Biotech, Birmingham, AL, USA). To differentiate between live and dead cells, we further stained cells with ghost viability dye-Red 510 (Tonbo Biosciences, San Diego, CA, USA). The frequency of stained cells was acquired using a flow cytometer BD FACSCanto II (BD Biosciences, Franklin Lakes, NJ, USA), and data analysis was carried out using FlowJo v10.8.1 software (BD Biosciences, USA). Figure 1 shows the gating strategy for IEL subsets. Data was reported as the number of cells per gram of tissue.

2.5 RNA isolation and gene expression

At 1 and 7dpi, about 1 cm from the distal portion of the jejunum tissues were collected in RNA later and stored in -80° C until further analysis. RNA was isolated from jejunum tissues using the Monarch® Total RNA Miniprep Kit (New England Biolabs®, Ipswich, MA, USA) according to the standard protocol. Subsequently, cDNA was synthesized from the RNA using the LunaScript® RT SuperMix Kit (New England Biolabs®, USA). Quantitative real-time PCR was then conducted on a Bio-Rad CFX connect machine using Luna® Universal qPCR Master Mix (New England Biolabs®, USA) for OPN, IL-1β, IFN-γ, TGF-β, and TNF-α genes. The primer sequences are shown in Table 1. The cycle threshold (Ct) for each gene was normalized to the housekeeping gene, GAPDH. Relative fold change was calculated in comparison to the control group using the $2^{-\Delta\Delta Ct}$ method (32).

2.6 Statistical analysis

Data analysis was performed using GraphPad PRISM v10.0.3 software (GraphPad, Boston, MA, USA). Flow cytometric data and lesion scores underwent analysis through a one-way non-parametric test (Kruskal-Wallis), followed by Dunn's test. For BWGs, intestinal length, and gene expression, One-way ANOVA was conducted, followed by the Dunnett test. Multiple comparison tests were employed to distinguish means between groups. The

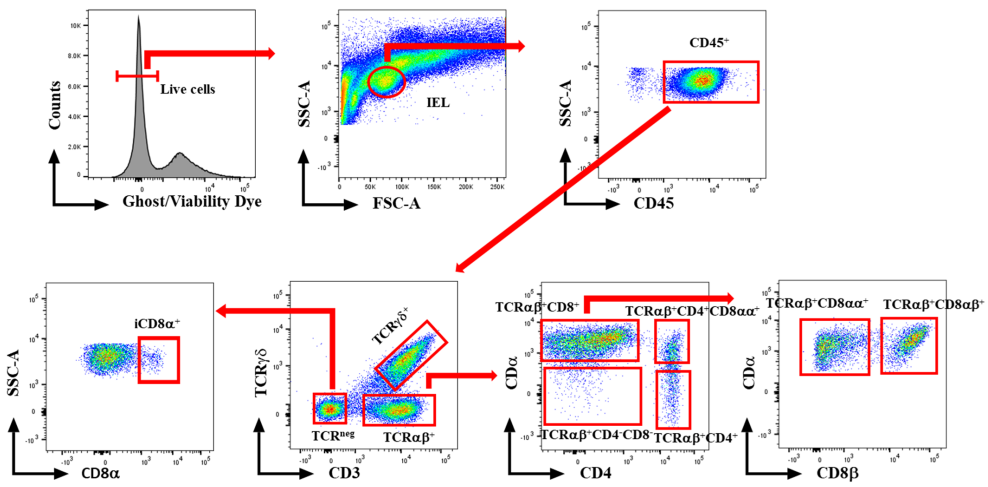

FIGURE 1
Gating strategy for flowcytometric analysis of IEL populations in jejunum of chickens. Cells were stained with ghost Red 510 and anti-chicken CD45 SPRD, CD4 PE-CY7, CD3 AF547, TCRγδ FITC, CD8α AF700, and CD8β PE antibodies. Gates were created in FlowJo.

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results were expressed as mean ± standard error of the mean (SEM), with statistical significance set at $P < 0.05$.

3 Results

3.1 *Emeria Maxima* and *C. perfringens* co-infection induced mild NE disease

Throughout the course of *C. perfringens* infection, no instances of mortality or morbidity were recorded in any of the groups. Body weights were documented for each group on days 18, 20, and 26 of age. Body weight gain (BWG) was calculated for two intervals (18-20 and 18-26). In both intervals, the EM/CP group exhibited significantly lower BWG ($P < 0.01$) compared to the non-infected control group (Figure 2A). However, BWG was comparable between the EM/CP and EM groups. Only at 18-20 interval, the EM showed significantly reduced BWG ($P < 0.01$) compared to the control group. At 1 dpi, the EM/CP group displayed the highest lesion scores (score 1) in the jejunum-ileum junction compared to the control and EM groups (Figure 2B).

Colon length shortening is commonly associated with intestinal inflammation and is considered a biological marker in various colitis models in mice (33–36). Hence, we investigated whether NE led to alterations in the length of intestinal sections at 1 dpi (Figure 2C). The EM/CP and EM groups exhibited a significantly longer jejunum length and a shortened colon length compared to the control group ($P<0.05$, and $P < 0.01$, respectively). However, the jejunum and colon lengths were comparable in the EM/CP and EM groups. Additionally, there were no discernible changes in the lengths of the duodenum and ileum between the groups. These findings suggest that *Emeria Maxima* and *C. perfringens* co-infection induced a mild or subclinical NE disease under our experimental conditions.

3.2 Natural IEL increases during the early incidence of NE

To explore the involvement of Intraepithelial Lymphocyte (IEL) populations during NE disease, we examined the IEL subpopulations in jejunum tissues following *C. perfringens* infection at 1 and 7 dpi. Figure 1 outlines the gating strategy applied for flow cytometric analysis of IEL subsets. At 1 dpi, there were no alterations in the number of induced IEL populations ($TCR\alpha\beta^+CD8\alpha\beta^+$, $TCR\alpha\beta^+CD4^+$, $TCR\alpha\beta^+CD4^+CD8\alpha\alpha^+$) across all groups (Figure 3). However, there were notable increases in the number of jejunal natural IEL subpopulations after *C. perfringens* infection at 1 dpi (Figures 4-6). The EM/CP groups demonstrated over a two-fold increase in the number of $TCR\alpha\beta^+CD8\alpha\alpha^+$ cells compared to the control and EM groups (Figure 4). The number of $TCR\alpha\beta^+$ IEL lacking the expression of CD4 and CD8 receptors significantly ($P<0.01$) increased in the EM/CP group compared to the control group only.

In mice, TCRγδ T cells constitute a small fraction of circulating lymphocytes in the blood and peripheral tissues (37), and these cells make up about 40-70% of the total IEL population, with 80% of TCRγδ cells expressing CD8αα homodimers (38). Chickens, on the other hand, have a substantial fraction of TCRγδ T cells in the blood and various tissues, including the intestine (39). In the current study, the total number of TCRγδ cells was significantly ($P<0.05$) increased by twofold in the EM/CP group compared to the other two groups (Figure 5). Because intestinal TCRγδ T cells in chickens express CD8αα homodimers or CD8αβ heterodimers (40), we further analyzed the $TCR\gamma\delta^+$ IEL subpopulations in the jejunum based on their expression of CDαα and/or CDαβ receptors. The EM/CP group had more $TCR\gamma\delta^+CD8\alpha\alpha^+$ and $TCR\gamma\delta^+CD8\alpha\beta^-$ cells compared to the non-infected control group, while their numbers of $TCR\gamma\delta^+CD8\alpha\alpha^+$ and $TCR\gamma\delta^+CD8\alpha\beta^+$ cells were increased compared to the EM group. Then, we examined a

TABLE 1 List of sequences of primers used for quantitative real-time PCR.

Gene	Accession NO.	Primer sequence (5′-3′)	Amplicon size (bp)
OPN	NM_204535.4	F: AAGAGGCCGTGGATGATGATG	254
		R: ATCCTCAATGAGCTTCCTGGC	
IL-1β	XM_015297469.1	F: CCCGCCTTCCGCTACA	66
		R: CACCARGCACTTCTGGTTGATG	
IFN-γ	NM_205149.1	F: GCTCCCGATGAACGACTTGA	63
		R: TGTAAGATGCTGAAGAGTTCATTCG	
TNF-α	MF000729.1	F: CCCATCCCTGGTCCGTAAC	77
		R: ATACGAAGTAAAGGCCGTCCC	
TGF-β	NM_001318456.1	F: GCCGACACGCAGTACACCAAG	54
		R: GCAGGCACGGACCACCATATTG	
GAPDH	NM_204305	F: CCTAGGATACACAGAGGACCAGGTT	64
		R: GGTGGAGGAATGGCTGTCA	

OPN, osteopontin or Spp-1; IL-1β, interleukin 1beta; IFN-γ, interferon gamma; TNF-α, tumor necrosis factor alpha; TGF-β, tumor growth factor beta; GAPDH, Glyceraldehyde 3-phosphate dehydrogenase; F, forward; R, reverse.

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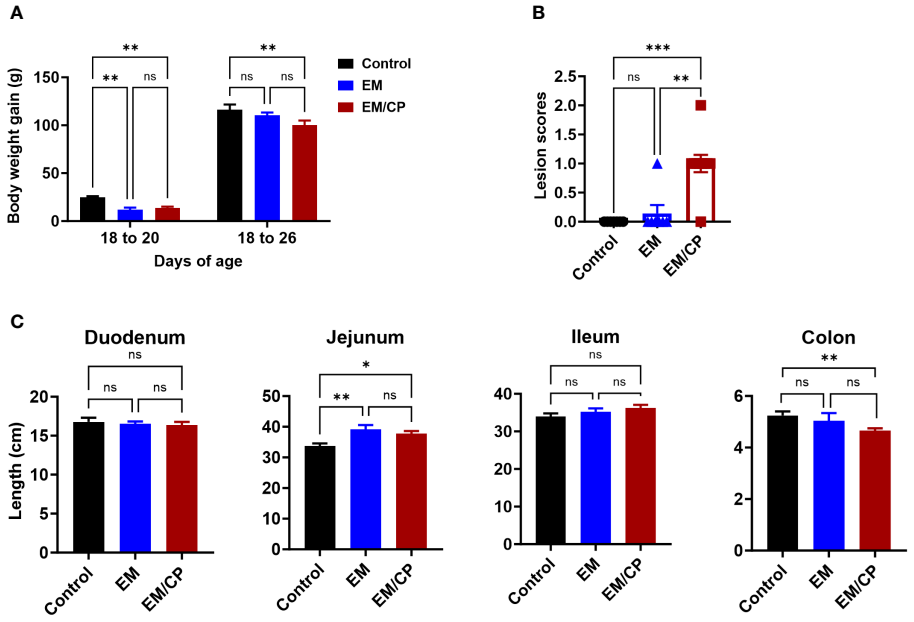

FIGURE 2
Eimeria challenge followed by *C. Perfringes* infection induced mild NE disease. **(A)** Body weight gain per gram from day 18-20 and 18-26 of age. One-way ANOVA and Dunnett tests. **(B)** Gross lesion scores of jejunal-ileum junctions at 1 dpi (day 20 of age). Control group received PBS. EM group infected with *Eimeria maxima*; at day 14 of age. EM/CP group infected with Eimeria maxima at day 14 of age and *C. perfringens* at days 18 and 19 of age. One-way Kruskal-Wallis and Dunn's tests. Each dot represents an individual bird. **(C)** Length of intestinal sections in cm at 1 dpi (day 20 of age). One-way ANOVA and Dunnett tests. ns, non-significance, *P<0.05; **P<0.01; ***P<0.001.

fraction of non-T cell IEL that do not express CD3 receptor, TCR^{neg} IEL (Figure 6). There was a significant surge in the number of TCR^{neg} IEL in jejunum following *C. perfringens* infection compared to the other groups. Among TCR^{neg} cells, the number of $iCD8\alpha^+$ cells increased in the EM/CP group compared to the EM group but not to the control group at 1dpi.

At 7dpi, there were no changes in the number of most IEL subpopulations in the jejunum, except for $TCR\alpha\beta^+CD4^-CD8^-$ and $TCR\alpha\beta^+CD8\alpha\alpha^+$ cells (Figure 7). The EM/CP group maintained a significant greater number of these cells compared to the control group. These results indicate that natural IEL might play a critical intestinal defense during the early stage of NE.

3.3 OPN expression in the jejunum is associated with early *C. perfringens* infection

OPN is a pleiotropic cytokine encoded by the Spp-1 gene and plays a crucial role in various biological processes. Our previous findings in mice indicate that OPN is primarily secreted by iCD8α cells in the intestine, and together, they serve as important mediators of IEL homeostasis by providing survival and proliferation signals for most IEL populations (31, 41). Observing an increase in the number of iCD8α cells in the jejunum after *C. perfringens* infection at 1 dpi, we investigated whether the expression of the OPN gene is associated

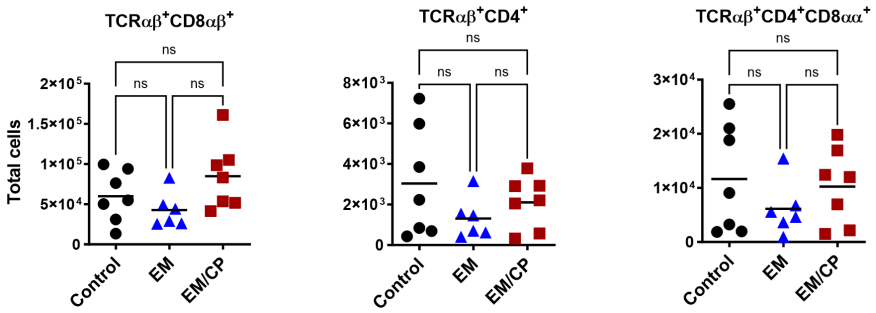

FIGURE 3
The Number of induced IEL was not impacted by NE disease at 1 dpi. Control group received PBS. EM group infected with *Eimeria maxima*; at day 14 of age. EM/CP group infected with *Eimeria maxima* at day 14 of age and *C. perfringens* at days 18 and 19 of age. $TCR\alpha\beta^+$ cells are $CD45^+CD3^+$ $TCR\gamma\delta^-$. Total cell number/gram of jejunum. One-way Kruskal-Wallis and Dunn's tests. Each dot represents an individual bird. Black bar depicts mean value. ns, non-significance.

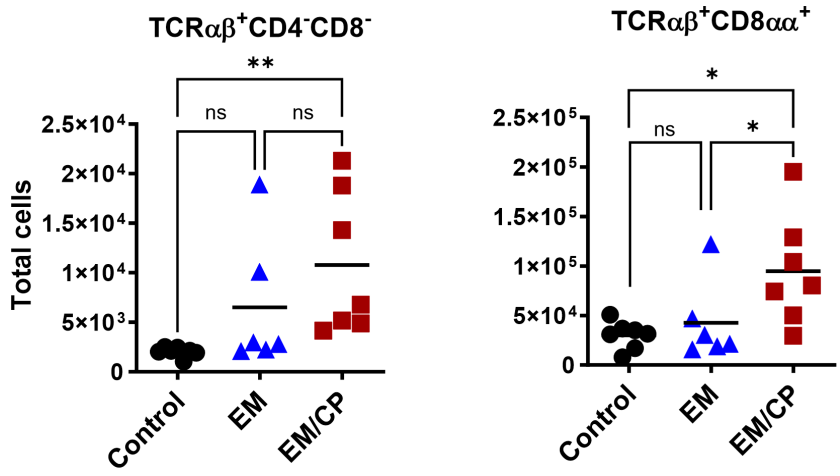

FIGURE 4
The number of natural TCRαβ$^+$CD8αα$^+$ IEL increased following NE disease at 1 dpi. Control group received PBS. EM group infected with *Eimeria maxima*; at day 14 of age. EM/CP group infected with *Eimeria maxima* at day 14 of age and *C. perfringens* at days 18 and 19 of age. TCRαβ$^+$ cells are CD45$^+$CD3$^+$ TCRγδ$^-$. Total cell number/gram of jejunum. One-way Kruskal-Wallis and Dunn's tests. Each dot represents an individual bird. Black bar depicts mean value. ns, non-significance; *P<0.05; **P<0.01.

with the rise in the number of iCD8α cells. At 1 dpi, the expression of OPN mRNA in the jejunum of the EM/CP group was significantly upregulated by 15 and 2 folds compared to the control (P<0.001), and the EM groups (P<0.05), respectively (Figure 8). There was no change in the expression of OPN between groups at 7 dpi. Additionally, we assessed the expression of different inflammatory and anti-inflammatory cytokines. Similar to the OPN pattern, the expressions of IFN-γ, and IL-1β genes were significantly upregulated in the EM/CP group compared to the control (P<0.0001) and EM (P<0.05) groups at 1 dpi, but not at 7 dpi (Figure 8). In addition, The EM group displayed significant upregulation of IFN-γ and IL-1β genes at 1 dpi only. On the other hand, there were no changes in the expression of TGF-β and TNF-α genes between groups at all time points.

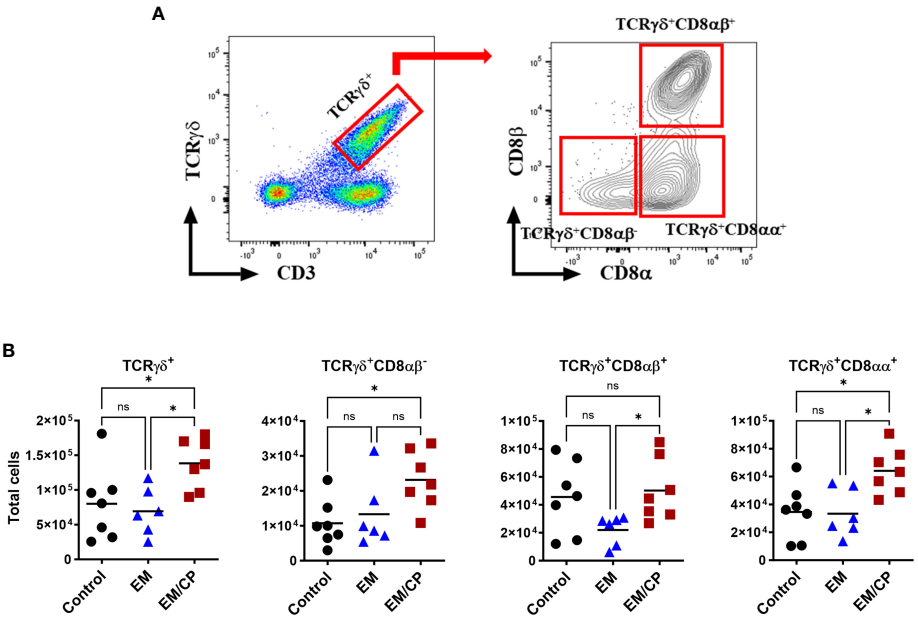

FIGURE 5
The number of natural TCRγδ IEL subsets increased following NE disease at 1 dpi. **(A)** Gating strategy used to differentiate TCRγδ IEL subpopulations. **(B)** The number of TCRγδ IEL subsets/gram of jejunum. Control group received PBS. EM group infected with *Eimeria maxima*; at day 14 of age. EM/CP group infected with *Eimeria maxima* at day 14 of age and *C. perfringens* at days 18 and 19 of age. TCRγδ$^+$ cells are CD45$^+$CD3$^+$ TCRγδ$^+$. One-way Kruskal-Wallis and Dunn's tests. Each dot represents an individual bird. Black bar depicts mean value. ns, non-significance; *P<0.05.

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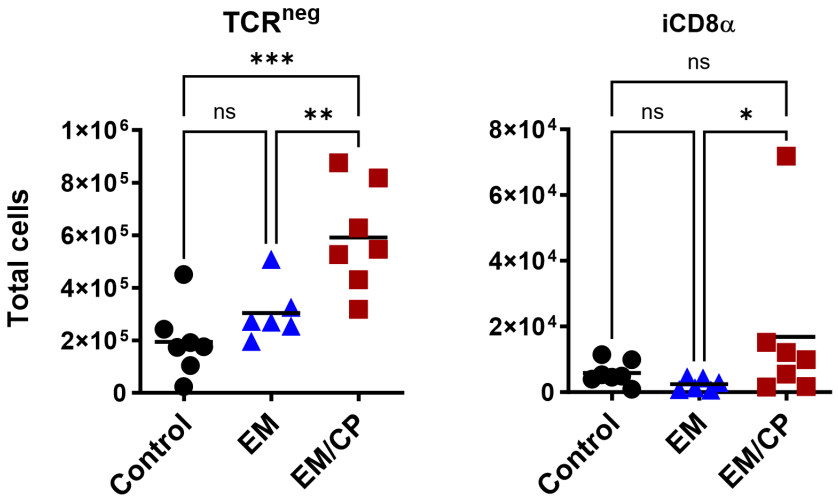

FIGURE 6
The number of natural TCR^{neg} IEL induced following NE disease at 1 dpi. Control group received PBS. EM group infected with *Eimeria maxima*; at day 14 of age. EM/CP group infected with *Eimeria maxima* at day 14 of age and *C. perfringens* at days 18 and 19 of age. TCR^{neg} cells are CD45^{+}CD3^{-}. Total cell number in jejunum/gram. One-way Kruskal-Wallis and Dunn's tests. Each dot represents an individual bird. Black bar depicts mean value. ns, non-significance; *P<0.05; *P<0.05; **P<0.01; ***P<0.001.

4 Discussion

C. perfringens is an opportunistic bacterium known to trigger NE outbreaks in broiler chickens, particularly in the presence of predisposing factors such as stress, immunosuppression, an imbalanced diet, and intestinal damage caused by *Eimeria* infection (26). The outcomes of NE pathogenicity can manifest as either clinical infection, characterized by a high mortality rate and severe intestinal damage, or subclinical infection, associated with low mortality, mild intestinal damage, malnutrition, and reduced body weight gain (30, 42). In our experimental conditions, *C. perfringens* infection induced subclinical NE disease, as evidenced by the absence

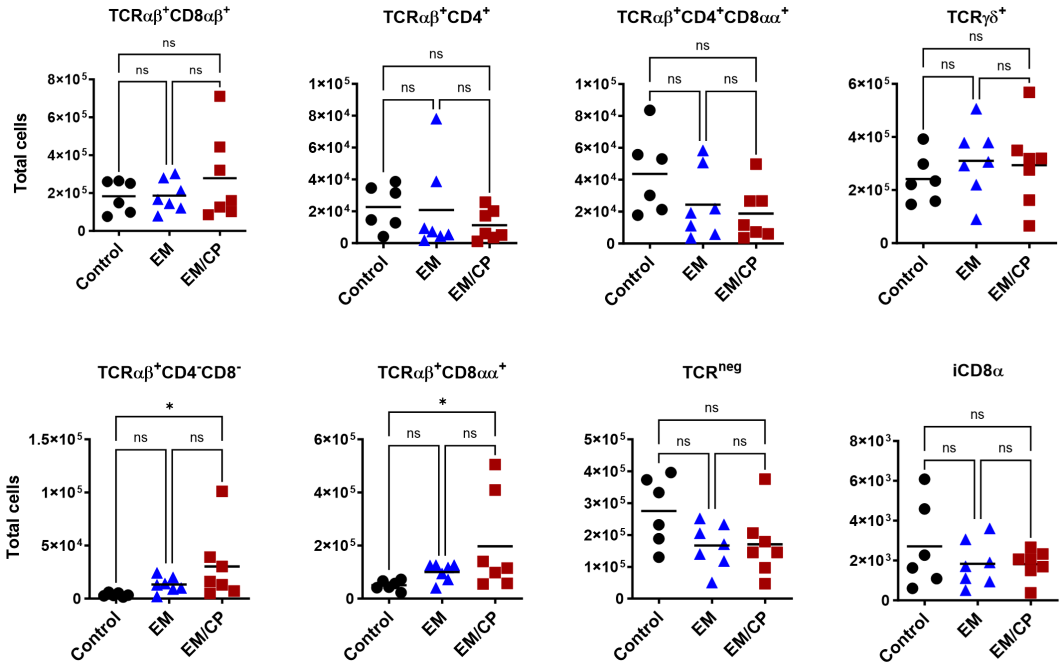

FIGURE 7
The changes in IEL subsets in the jejunum at 7dpi, were limited to TCRαβ^{+}CD4^{-}C8^{-} and TCRαβ^{+}CD8αα^{+} cells. Control group received PBS. EM group infected with *Eimeria maxima*; at day 14 of age. EM/CP group infected with *Eimeria maxima* at day 14 of age and *C. perfringens* at days 18 and 19 of age. Total cell number/gram of tissue. One-way Kruskal-Wallis and Dunn's tests. Each dot represents an individual bird. Black bar depicts mean value. ns, non-significance; *P<0.05.

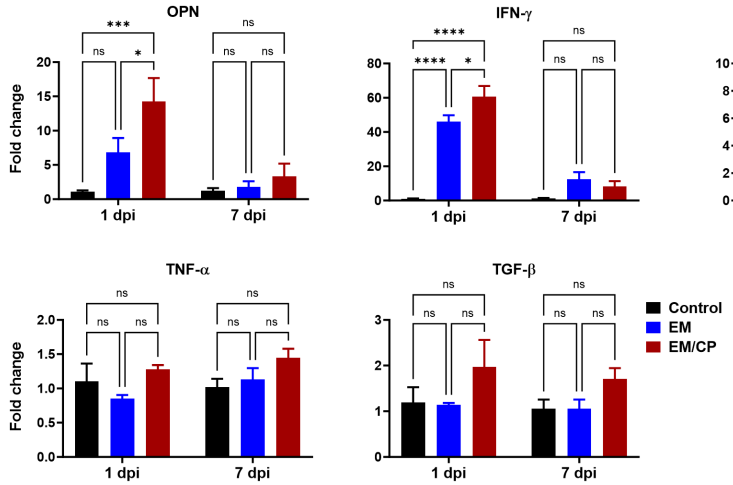

FIGURE 8
Gene expression of selected genes in jejunum following NE disease at 1dpi and 7dpi. Control group received PBS. EM group infected with *Eimeria maxima*; at day 14 of age. EM/CP group infected with *Eimeria maxima* at day 14 of age and *C. perfringens* at days 18 and 19 of age. OPN, osteopontin or Spp-1; IL-1β, interleukin 1beta; IFN-γ, interferon gamma; TNF-α, tumor necrosis factor alpha; TGF-β, tumor growth factor beta. One-way ANOVA and Dunnett tests. Fold change presented as mean ± SEM. ns, non-significance; *P<0.05; ***P<0.01; ****P<0.001.

of mortality, reduced BWGs, and mild intestinal damage (average lesion scores = 1; Figure 2). Although the EM/CP group exhibited the lowest BWGs, these gains were not statistically significant compared to EM group. This lack of significance could be attributed to factors such as the chicken background (SPF layer chicks in this study), their age, and virulence of *C. perfringens* strain (25). At 14 days of age, birds might be more susceptible to EM infection with $5x10^3$ oocysts, which conceals the differences in BWGs between EM/CP and EM groups (43). The signs of subclinical NE were accompanied with upregulation of proinflammatory cytokine genes IL-1β and IFN-γ, but not TNF-α at 1 dpi, which is necessary for the activation of innate immune cells such as macrophages (Figure 8). It is common in murine models of colitis that intestinal inflammation alters colon length (33–36). Our data indicated that *C. perfringens* infection shortened the colon length at 1 dpi (Figure 2C), suggesting potential colonic inflammation. Interestingly, mice infected with porcine *C. perfringens* displayed a shortened colon length in an infectious dose-dependent manner (44).

Intestinal IEL which are interspersed at the basolateral side of epithelial cells, represent the primary immunological defense against microbial invaders. To deepen our understanding of host-pathogen interactions during NE disease, we performed a flow cytometric analysis of IEL compartments in the jejunum following *C. perfringens* infection at 1 and 7 dpi. During the early stage of infection at 1 dpi, the EM/CP-infected group exhibited substantial numbers of natural IEL subpopulations, including TCRαβ$^+$CD4$^-$CD8$^-$, TCRαβ$^+$CD8αα$^+$, TCRγδ$^+$, and TCR^{neg} cells in the jejunum compared to the control and EM groups (Figures 4–6). The first three cell types are subsets of T cells known as "innate-like T cells," capable of interacting with non-classical MHC molecules and, therefore, do not require antigen presentation for their functions (45, 46). The TCRαβ$^+$CD4$^-$CD8$^-$ cells generated in the thymus, migrate directly to the intestinal epithelium, where they gain expression of CD8αα homodimers to become TCRαβ$^+$CD8αα$^+$IEL (47–49). TCRαβ$^+$CD8αα$^+$ cells are exclusively part of the IEL compartment, constituting 20-50% and less than 1% of T-cell IEL in mice and humans, respectively (11, 50). Our current study is the first to report the presence of TCRαβ$^+$CD8αα$^+$ IEL in chickens, which constituted 20-30% of total T cell IEL. While the exact role of TCRαβ$^+$CD8αα$^+$ IEL in mucosal immunity is not fully understood, there is some evidence suggesting they have immune regulatory functions through the expression of TGF-β3, lymphocytes activation gene-3, and fibrinogen-like protein 2 (51). Therefore, the TCRαβ$^+$CD8αα$^+$ IEL protect immune compromised mice against colitis development following the adoptive transfer of CD4$^+$CD45RB^{hi} T cells (52). The robust increase in the number of natural TCRαβ$^+$CD8αα$^+$ IEL in EM/CP groups at 1 dpi and their potential regulatory function, might explain relatively low lesion scores and mild signs of NE disease.

Another significant alteration in natural IEL was the increase in the number of TCRγδ$^+$ cells following *C. perfringens* infection at 1 dpi (Figure 5). Previous research has reported a high frequency of TCRγδ+ cells in the cecal tonsils following infection with a virulent-*C. perfringens* strain, suggesting these cells play a crucial role in mediating anti-*C. perfringens* immunity in chickens (53). Furthermore, ex-vivo stimulation of the small intestinal TCRγδ$^+$ cells with *C. perfringens* increased the frequency of these cells (54). In the current study, the majority of jejunal TCRγδ$^+$ IEL are expressing either CD8αα homodimers or CDαβ heterodimers, identifying two-distinct subsets of IEL (40). In contrast to mice and humans, TCRγδ$^+$CD8αβ$^+$ cells are particularly interesting because they are unique to the chicken IEL compartment, and their role in mucosal immunity is yet to be elucidated. Our data indicated an induction of TCRγδ$^+$CD8αα$^+$ IEL in EM/CP groups compared to all other groups, and the number of TCRγδ$^+$CD8αβ$^+$ IEL increased in the EM/CP group compared to the EM group at 1 dpi. The main function of TCRγδ$^+$ IEL is to protect the intestinal epithelium by inhibiting an early invasion of resident and pathogenic microorganisms and maintaining intestinal homeostasis by limiting excessive inflammation and tissue damage during infection

(55, 56). TCRγδ$^+$ IEL produce a variety of effector molecules such as pro-inflammatory cytokines (IFN-γ and TNF-α), anti-inflammatory cytokines (TGF-β and IL-10), wound healing factors (TGF-β, prothymosin β4, and keratinocyte growth factor), cytotoxic enzymes (granzymes), and antimicrobial peptides (11). In *Salmonella*-infected chickens, both TCRγδ$^+$CD8αα$^+$ and TCRγδ$^+$CD8αβ$^+$ cells express IFN-γ mRNA, suggesting these cells can promote the phagocytic capability of macrophage to clear infection (57). Therefore, the upregulation of INF-γ expression (Figure 8) and augmentation of TCRγδ$^+$ subsets in the jejunum of EM/CP group imply the essential role of TCRγδ$^+$ IEL in enhancing the bactericidal function of macrophages at the early infection stage.

The third cell group of natural IEL that experienced a significant increase following *C. perfringens* infection at 1 dpi, was TCR^{neg} cells (Figure 6). These cells belong to the innate immune cells and are not fully characterized in chickens. Previous studies have identified NK cells as a distinct TCR^{neg} IEL population in chickens during embryonic and early life (20, 21). In mice and humans, several subsets of TCR^{neg} IELs have been identified and characterized with vital functions in the mucosal immunity (8). TCR^{neg} IEL are composed of innate lymphoid cells expressing NK receptors (NKP46, NK1.1, and NKP44) (15–17), and lymphocytes expressing intracellular CD3γ. The later lymphocytes are further divided according to the expression of CDαα into intracellular CD3 (CD8α$^-$) and iCD8α (CD8α$^+$) (18, 19). The iCD8α cells produce a diverse array of effector molecules, such as monocyte chemotactic protein 1 (MCP-1), IFN-γ, and OPN, and possess antigen processing and presentation, cytotoxicity, and phagocytosis, highlighting their crucial role during early immune responses (19). Our results indicate, for the first time, that chickens harbor iCD8α cells in the intestine and their number increased following *C. perfringens* infection (Figure 6), suggesting that these cells might confer protection against *C. perfringens*, similar to their protective role against *Citrobacter rodentium* infection in mice (19).

Our previous results in mice indicated that iCD8α cells through OPN mediate the homeostasis of IEL subpopulations by promoting their survival in a CD44-dependent manner, proliferation, and migration to intestinal epithelium (31). For example, OPN-knockout mice exhibit a reduction in the numbers of TCRαβ$^+$CD4$^+$, TCRαβ$^+$CD4$^+$CD8αα$^+$, TCRαβ$^+$CD8αβ$^+$, TCRαβ$^+$CD8αα$^+$, and TCRγδ$^+$ IEL in the small intestine and colon compared to wild-type mice. Moreover, OPN sustains proper expression of Foxp3 on regulatory T cells in the intestine and therefore, protect against colitis (31). In the present study, induction of subclinical NE was associated with upregulation of OPN mRNA level and high number of iCD8α IEL in the jejunum (Figure 8). These findings underscore the importance of iCD8a cells and OPN in intestinal health and warrant further investigation of their roles during mucosal immune responses.

In summary, in this report, we provide evidence supporting the immunological significance of natural IEL subsets, specifically TCRγδ$^+$, TCRαβ$^+$CD8αα$^+$, and TCR^{neg}, including iCD8a cells, in the early immune response of chickens to NE. Furthermore, we report, for the first time, that chickens harbor fractions of TCRαβ$^+$CD8αα$^+$ and iCD8a cells in IEL compartments, suggesting their potential role in conferring protection against *C. perfringens* infection. However, the precise functions and mechanisms of these IEL populations in the context of intestinal inflammation in chickens warrant further investigation.

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 approved by The Ohio State University's Institutional Animal Care and Use Committee. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

SM: Data curation, Investigation, Methodology, Writing – original draft. SH: Data curation, Investigation, Methodology, Resources, Writing – review & editing. BS: Data curation, Investigation, Methodology, Writing – review & editing. LB: Methodology, Writing – review & editing. AN: Conceptualization, Formal Analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing – review & editing.

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Conflict of interest

The 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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Supplementary material

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Serum cytokine profile of neonatal broiler chickens infected with *Salmonella Typhimurium*

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The avian immune system responds to *Salmonella* infection by expressing cytokines and chemokines. We hypothesized that the immune status of *Salmonella* Typhimurium (ST) challenged neonatal broilers would differ from the uninfected treatment. The objective of this experiment was to evaluate 12 cytokines. Day of hatch male chicks were randomly allocated into a control or ST challenged group. At day three of age, sterile diluent or 5.0 × 10^8 CFU of ST was given orally to each chick. Blood was obtained 24 h post challenge and serum separated for later analysis (n = 30 chicks/treatment). Significant ($p \leq 0.05$) increases in ***pro-inflammatory cytokines***-interleukin-6 (IL-6), IL-16, and IL-21; ***anti-inflammatory cytokines***- IL-10; ***chemokines***-regulated on activation, normal T cell expressed and secreted (RANTES), macrophage inflammatory protein-1β (MIP-1β), and MIP-3α; ***colony stimulating factors***-macrophage colony-stimulating factor (M-CSF); and ***growth factors***-vascular endothelial growth factor (VEGF) were observed in the serum of the challenged chicks when compared to the control. No significant differences were observed in IL-2, interferon gamma (IFNγ), and IFNα. These data indicate the detection of mucosal immune responses in broiler chickens following ST infection. The heightened levels of pro-inflammatory cytokines, chemokines, and colony stimulating factors align with known inflammatory mechanisms, like the influx of immune cells. However, the elevation of IL-10 was unexpected, due to its immunoregulatory properties. Notably, the rise in VEGF levels is compelling, as it suggests the possibility of tissue repair and angiogenesis in ST infected birds.

KEYWORDS

immune response, immunoassay, poultry, serum cytokine, *Salmonella Typhimurium*

1 Introduction

Salmonellosis is a zoonotic disease caused by *Salmonella* enterica serovars resulting in an estimated 1.4 million cases of foodborne illness and 400 deaths in the United States annually (CDC, 2022). There are more than 2,600 *Salmonella* enterica serovars, but less than 100 of these serovars are known to cause human salmonellosis (USDA-FSIS, 2021). *Salmonella* Typhimurium (ST), *S.* Enteritidis (SE), and *S.* Kentucky (SK) are the three most common isolates found in contaminated poultry products (Van Immerseel et al., 2005; Kumar et al., 2019; Pineda et al., 2021). Poultry infected with paratyphoid *Salmonella* are

typically asymptomatic but can continually shed into the environment causing cross-contamination of carcasses and morbidity in humans (USDA-FSIS, 2021).

Salmonella are facultative anaerobes that can survive in low oxygen, warm, and humid environments (Parkin et al., 2012; Kurtz et al., 2017). Many *Salmonella* infections occur after ingestion of food or water contaminated with the pathogen (Griffin and McSorely, 2011; Pham and McSorely, 2015; Kurtz et al., 2017). Transmission in poultry is typically via the fecal-oral route (Wigley, 2014; Pham and McSorely, 2015). Once ingested, the organism may adhere to intestinal cells through the pilli or fimbriae where they can colonize the small intestine (Vegad and Katiyar, 2002; Dar et al., 2017). *Salmonella* can then be transported to lymphoid tissues of the gastrointestinal tract and are disseminated throughout the bloodstream (Griffin and McSorely, 2011; Pham and McSorely, 2015). Inflammatory processes will occur and lead to increased expression of cytokines along with an influx of heterophils and monocytes (Wigley, 2014; Swaggerty et al., 2019).

Cytokines are low molecular weight cell signaling proteins that are secreted by immune cells to support and regulate inflammation. They are produced by a variety of cells and play critical roles in inflammation, acute phase protein production, chemotaxis, cell proliferation, and differentiation (Kogut, 2000; Zhong et al., 2019). Pro-inflammatory cytokines are produced by activated macrophages, T helper 1 (Th_1) cells, and dendritic cells and are involved in the upregulation of immune cells at inflammatory sites (Wigley and Kaiser, 2003; Rothwell et al., 2012; Kaiser and Staheli, 2014). Anti-inflammatory cytokines are a series of immunoregulatory molecules that control the pro-inflammatory responses (Kogut, 2000; Rothwell et al., 2004). Chemotactic cytokines, or chemokines, play a major role in recruiting lymphocytes to the site of inflammation (Mohammed et al., 2007; Sun et al., 2012). They are functionally divided into the CC and CXC superfamilies. These groups are determined by the positioning of cysteine residues (Kirkaldy et al., 2003; Kogut et al., 2005). Colony stimulating factors are a family of growth factors involved in hematopoiesis, while the family known as growth factors are reported to play important roles in cell proliferation, migration, and differentiation during tissue repair and regeneration (Kogut, 2000; Murphy and Weaver, 2017).

The present study examined multiple cytokines during a *Salmonella* infection. We hypothesized that the immune profile of ST challenged chicks would differ from uninfected neonatal broilers. The objective of the current report was to characterize the immune profile of ST infected broilers by evaluating twelve serum cytokines.

2 Materials and methods

2.1 Experimental design

All experimental procedures were approved by the Texas A&M University Institutional Animal Care and Use Committee (IACUC 2016-0270 and 2019-0171) and the Institutional Biosafety Committee (IBC 2016-112 and 2019-073). Serum samples were used from a previous study (Zhao, 2021). Briefly, day-of-hatch, non-vaccinated male broiler chicks were placed on clean pine shavings in two ABSL-2 floor pens with an environmentally controlled and age-appropriate climate to ensure uniformity. Birds were fed a balanced unmedicated starter diet that met or exceeded industry recommendations for nutrition (Cobb-Vantress, 2018). Upon arrival, chick tray papers were cultured to confirm that the chicks were *Salmonella* negative. After 3 days of acclimation, chicks were orally challenged with 0.5 mL of sterile tryptic soy broth (TSB) or 5.0×10^8 CFU of ST in TSB to ensure a successful challenge of all chicks. The level of colonization of ST-infected chicks was 7.23 ± 0.74 $\log_{10}$ CFU/g of cecal contents compared to 0 $\log_{10}$ CFU/g of cecal contents observed in the control group. Twenty-four hours post-challenge, blood samples were collected from euthanized birds by cardiac puncture. Blood was collected, kept at room temperature for 2 h, and centrifuged at 2,000 x g for 10 min at 4°C. Serum was separated and transferred into 2 mL aliquots and stored at −80°C for future use. A total of 30 serum samples per treatment were analyzed.

2.2 Serum preparation

Serum samples were thawed at room temperature the morning of the experiment. Once unfrozen, serum samples were centrifuged at 10,000 x g for 15 min at 4°C to remove particulates.

2.3 Cytokine assays

A workflow of serum preparation and the cytokine procedure is shown in Figure 1. The study was conducted with the Luminex MAGPIX® System (EMD Millipore Corp., Millerica, MA, United States). A MILLIPLEX® Chicken Cytokine/Chemokine Panel (EMD Millipore Corp.) was used to quantify 12 different analytes. Interferon alpha (IFNα), interferon gamma (IFNγ), interleukin 2 (IL-2), interleukin 6 (IL-6), interleukin 10 (IL-10), interleukin 16 (IL-16), interleukin 21 (IL-21), macrophage inflammatory protein-1 beta (MIP-1β), macrophage inflammatory protein-3 alpha (MIP-3α), regulated on activation, normal T cell expressed and secreted (RANTES), macrophage colony-stimulating factor (M-CSF), and vascular endothelial growth factor (VEGF) were evaluated. Detailed definitions of each cytokine are explained in Table 1. The assay was run according to the manufacturer's instructions with standards, samples and quality controls in duplicate. Premixed antibody immobilized beads, quality controls, wash buffer, and the serum matrix were prepared prior to use. Overnight incubation with shaking at 4°C (16–18 h, 500 rpm) occurred and a handheld magnetic separation block (EMD Millipore Corp.) was used during the plate washing steps.

2.4 Data analysis

Individual microbeads were identified and quantified based on fluorescence signals. Data from the beads were analyzed via Luminex® xPONENT® Acquisition Software (Luminex Corp., Austin, TX, United States) and then exported to the Belysa™ Analysis Software (EMD Millipore Corp.) for further examination. A detection target of 50 beads per region was

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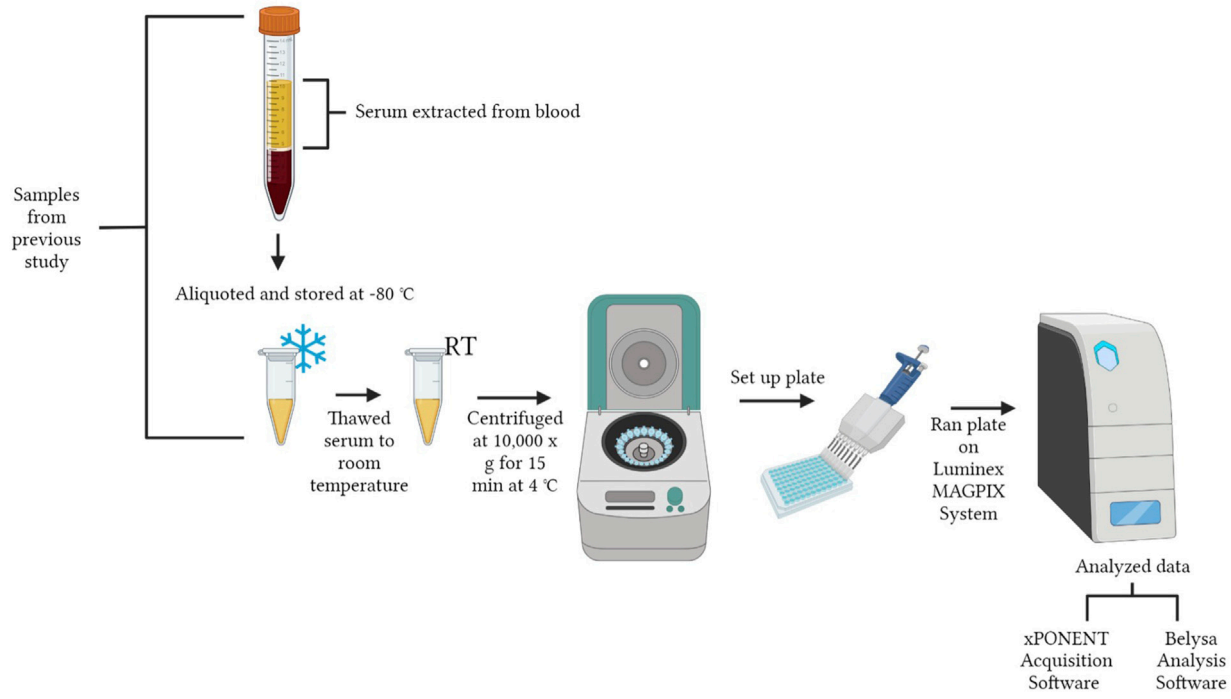

FIGURE 1
Workflow of experimental design. The figure was created in BioRender.com by Allison Milby-Blackledge.

TABLE 1 Analytes observed, where they are produced, their function and type of cytokine response.

Analyte	Produced By	Response	Function
IFNα	Neutrophils, Dendritic Cells, Macrophages	Anti-inflammatory	Anti-viral; MHC I expression
IFNγ	Natural Killer Cells, T cells (Th_1)	Pro-inflammatory	Macrophage activation; Ig class switching; anti-viral; anti-tumor
IL-2	T cells (Th_1)	Pro-inflammatory	T cell growth and proliferation; macrophage activation; B cell growth
IL-6	T cells, B cells, Macrophages, Fibroblasts	Pro-inflammatory	Acute phase protein production; B and T cell activation; hematopoiesis
IL-10	Monocytes/Macrophages, T cells, B cells	Anti-inflammatory	Downregulate pro-inflammatory responses; tissue homeostasis
IL-16	B cells, Mast Cells, Eosinophils, Macrophages, Fibroblasts	Pro-inflammatory	Chemotactic activity; MHC II expression; induce inflammation
IL-21	Natural Killer Cells, T cells (Th_1 or Th_2)	Pro-inflammatory	Regulate proliferation; immunomodulatory; effector functions of B, T, natural killer, and dendritic cells
M-CSF	Bone Marrow, Fibroblasts, Endothelial Cells	Colony Stimulating Factor	Regulates growth, differentiation, and activation of monocytes/macrophages
MIP-1β/CCL4	Monocytes/Macrophages	Chemokine	Aid in the release of pro-inflammatory cytokines; chemotaxis of neutrophils and lymphocytes
MIP-3α/CCL20	Lymphoid Tissues	Chemokine	Chemotactic activity to epithelial cells surrounding lymphoid tissues
RANTES/CCL5	T cells, Monocytes, Platelets	Chemokine	Recruit leukocytes to inflammatory sites; activation and proliferation of natural killer cells
VEGF	Macrophages, Tumor Cells, Platelets	Growth Factor	Promote growth of new blood vessels

All analyte definitions are comprised from avian immunology literature apart from Janeway's Immunobiology, ninth Edition.
Kogut, 2000; Wigley and Kaiser, 2003; Min and Lillehoj, 2004; Mohammed et al., 2007; Coble et al., 2011; Yang et al., 2011; Rothwell et al., 2004; Rothwell et al., 2012; Kaiser and Staheli, 2014; Murphy and Weaver, 2017; Al-Khalaifah and Al-Nasser, 2018; Van der Eijk et al., 2019.

inputted into the software; standards, quality controls, and sample wells with bead counts of less than 36 were excluded. The Belysa™ software was used to examine the standard curve. Raw data was transferred from the Belysa™ Analysis Software to Microsoft Excel (Microsoft, Redmond, WA, United States) for statistical analysis and removal of outliers.

2.5 Statistical analysis

Statistical analysis was performed using JMP Pro 15 (SAS Institute Inc., Cary, NC, United States). Data were analyzed via a Student's t-test and compiled into Microsoft Excel for further analysis and presented as mean ± the standard error of the mean (SEM). Substantial outliers that exceeded two standard deviations from the mean (SD) were removed. A p-value of less than 0.05 was considered statistically significant.

3 Results

The levels of cytokines, chemokines, colony-stimulating factors, and growth factors were quantitatively detected. Differences in n may be seen between the control and ST treatments of each analyte due to variation from low bead counts, concentrations below the readable limit, and outliers.

As shown in Figure 2, we observed that ST-challenged neonatal broilers after 24 h post-infection had significant increases in ***pro-inflammatory cytokines***- IL-6 (p = 0.0025), IL-16 (p = 0.0196), and IL-21 (p = 0.0066). No significant differences were observed in IL-2 (p = 0.1778) and IFNγ (p = 0.1316). It is interesting to note that IL-6 displayed a 2.04 fold increase in the ST treatment compared to the control.

The concentration of ***anti-inflammatory cytokine***- IL-10 (p = 0.0047) was significantly higher in the ST treatment than in the control. No significant differences were observed on IFNα (p = 0.5044) concentrations in both groups. Both cytokines in this group were above the minimum detectable concentrations set forth by the manufacturer.

All ***chemokines*** tested in this experiment: RANTES (p = 0.0002), MIP-1β (p = 0.0019), and MIP-3α (p = 0.0050), exhibited significant increases in the ST group compared to the control group.

The mean concentration of the ***colony stimulating factor***-M-CSF in the ST treatment was significantly higher than the control group (p = 0.0239) with a 1.26 fold increase in the ST chicks compared to control chicks.

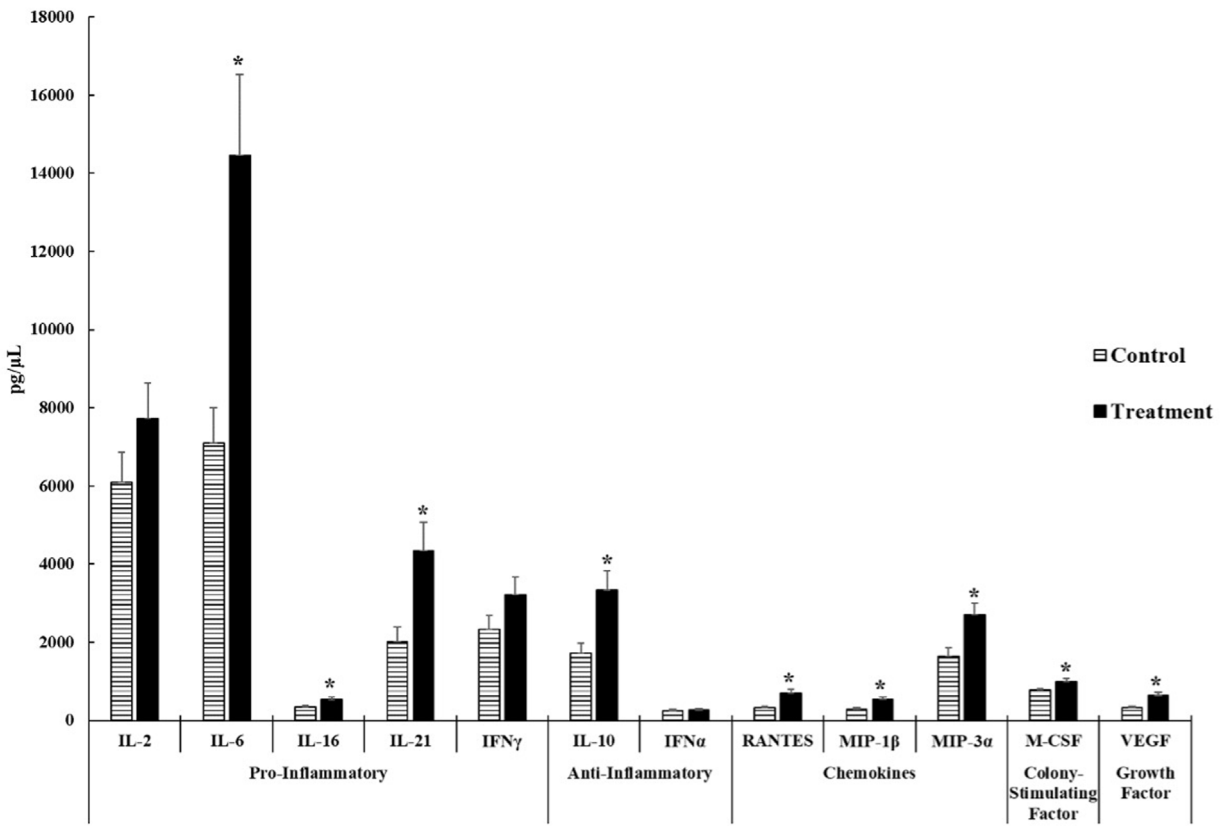

FIGURE 2
Cytokine and chemokine analysis of the serum of control and *Salmonella* Typhimurium (ST) challenged chicks. Day-old chicks were divided into control and ST treatment groups. Birds in the treatment group were challenged with 5.0×10^8 CFU of ST. The levels of cytokines, chemokines, growth factors, and colony-stimulating factors were analyzed. The target sample size (n) was 30 samples. However, n differs between control and ST treatments of each analyte due to variation from low bead counts, concentrations that were below the readable limit, as well as outliers causing the actual n to range from 23 to 27 samples for the control group and 26 to 29 samples for the ST treatment group. An asterisk (*) indicates concentrations that are considered significant differences at $p \leq 0.05$ compared to the control group.

The mean concentration of the ***growth factor***- VEGF (p = 0.0022) was significantly increased in ST-infected chicks compared to the control. There were 24 control samples and 29 ST-infected samples that were viable.

4 Discussion

Cytokines represent a diverse array of secreted proteins that serve as vital communication tools among all cells throughout the body (Eckmann and Kagnoff, 2001). Data is limited when it comes to systematic protein expression in poultry. Protein concentration although generally more conserved than mRNA levels across species, is not widely used to show cytokine levels in poultry (Jiang, et al., 2023). This study was performed to better understand the role the immune system has on combatting ST infection in chickens, herein we report the significant upregulation of protein expression in nine out of the twelve serum cytokines analyzed in ST-infected chicks.

In poultry *Salmonella* infections, intestinal colonization can cause inflammation, specifically to the distal ileum and ceca of birds (Broz et al., 2012; Dar et al., 2017). This can cause rapid expression of pro-inflammatory cytokines in newly hatched chicks (Withanage et al., 2004). Interleukin 6 is a common pleiotropic cytokine associated with *Salmonella* infection (Kaiser et al., 2000). It is a cytokine that stimulates acute phase protein production, inflammation, B cell proliferation and differentiation (Wigley and Kaiser, 2003; Nishimichi et al., 2006; Al-Khalaifah and Al-Nasser, 2018). Many studies performed on mammalian species after *Salmonella* infection or LPS challenge have been evaluated (Franchi, 2011; Abos et al., 2016; Suksungworn et al., 2020; Huang, 2021). Previous studies on mice support our findings of the significant increases in IL-6 protein concentrations following ST infection (Mathur et al., 2012; Febriza et al., 2020). However, many experiments in poultry focus on mRNA levels (Shebl et al., 2010). For example, our study differs from Pineda et al.'s study, where no significant differences in IL-6 or IFNγ mRNA expression were observed in the broiler cecal tonsils or liver when inoculated with SK, ST, and SE (Pineda et al., 2021). However, our data correlates to Kaiser and associates *in vitro* study, when specifically looking at IL-6 (Kaiser et al., 2000). This study focused on primary chick kidney cells (CKC) to determine the levels of pro-inflammatory cytokines against ST and SE. Interleukin-6 mRNA levels were significantly increased in the ST-infected CKC compared to controls (Kaiser et al., 2000). In the current study, no significant differences were observed in IL-2 which is responsible for T-cell proliferation and activation of macrophages or the antiviral type II interferon, IFNγ (Wigley and Kaiser, 2003). Although parameters were not the same (2 h of contact time with the cells instead of 24 h challenge to the chick), our findings contradict what is found in the literature, as mRNA expression of IFNγ and IL-2 were significantly downregulated nearly fivefold when exposed to ST compared to the control (Kaiser et al., 2000). Interleukin 16 is generated by B cells, epithelial cells, macrophages, mast cells, fibroblasts, and eosinophils. It is considered an inflammatory cytokine in chickens that induces lymphocyte chemotaxis (Min and Lillehoj, 2004; Kaiser and Staheli, 2014). Cytokines have been reported to be expressed in the serum following a *Salmonella enterica* challenge (Song et al., 2020). Our data not only correlates to this statement but also is similar to Swaggerty et al.'s experiment, where the addition of antioxidants in breeder hen diets were used to protect against SE in the progeny. In Swaggerty et al.'s study, cytokine and chemokine production was measured in the serum using the MILLIPLEX® Chicken Cytokine/Chemokine Panel and analyzed using the Luminex 200 xMAP Technology. Chicks from the control fed hens had increased levels of IL-16 in the serum (Swaggerty et al., 2023). Since chicks in the protected biofactors and antioxidant (P(BF + AO)) feed additive-fed hen group had a 32.6 percent reduction of SE, the increase in IL-16 levels could be because the immune system is having to work harder to fight off infection which could also be linked to why we are seeing an increase in our ST group (Swaggerty et al., 2023). Interleukin 21 is a cytokine of the cell mediated immune system with immunomodulatory properties. It regulates proliferation, differentiation, and effector functions on T cells, B cells, and natural killer cells (Murphy and Weaver, 2017). In the present study, increases (p = 0.0066) of IL-21 concentrations were observed in the ST treatment, indicating the involvement of immune cells. Although interesting, the body of literature for avian IL-21 is limited to a characterization paper (Rothwell et al., 2012) and the effect that *Mycoplasma synoviae* and lentogenic Newcastle disease virus (NDV) coinfection have on gene expression of chick embryos where it was concluded that IL-21 mRNA expression of the liver and spleen were significantly downregulated and the mRNA expression of the thymus was significantly upregulated in NDV-infected embryos (Bolha et al., 2013).

Anti-inflammatory cytokines act as immunoregulatory molecules that return the immune system to baseline or inhibit the Th_1 response (Opal and DePalo, 2000; Kogut and Arsenault, 2017). Interleukin 10 is primarily produced by T cells, B cells, and monocytes/M2 macrophages (Chuang et al., 2016; Murphy and Weaver, 2017). Much of our understanding regarding the role of IL-10 in infectious disease originates from observations in the murine model. It is generally recognized that decreased IL-10 levels can promote resistance to primary infection, while elevated levels can increase susceptibility to intracellular pathogens (Rothwell et al., 2004). Although some poultry studies demonstrate reduced IL-10 gene expression, post-ST or SE challenge (Fasina et al., 2008; Redmond et al., 2009; Crhanova et al., 2011), our findings correlate to previous research conducted in ST-infected mice where increased IL-10 production results are indicative of B cells and T cells promoting and developing immune tolerance induced by ST (Salazar et al., 2017).

Macrophages can produce a small heparin-binding class (molecules that bind growth factors and promote tissue repair) of cytokine that acts as a chemoattractant to bring leukocytes to infected tissues, better known as chemokines (Martino et al., 2012). The observed outcomes of the current study demonstrated significant increases in MIP-1β (CCL4), MIP-3α (CCL20), and RANTES (CCL5), which suggest the movement of immune cells toward areas of inflammation (Hughes and Nibbs, 2018). Macrophage inflammatory protein-1 beta is an inflammatory

chemokine secreted by chicken monocytes/macrophages that induces a pro-inflammatory response and chemotactic migration of heterophils and lymphocytes (Yang et al., 2011; Van der Eijk et al., 2019). A previous investigation performed by Kogut, He, and Kaiser that centered on examining the activation of chicken heterophils triggered by lipopolysaccharide (LPS), showcased the generation of CXC and CC chemokines, notably MIP-1β, in response to infection (Kogut et al., 2005). Our current findings are consistent with the literature, implying that these outcomes indicate the ability of chemokines to guide heterophil recruitment to the inflammation site (Kogut et al., 2005). Macrophage inflammatory protein-3 alpha is a chemokine that recruits dendritic cells during an inflammatory response (Aziz et al., 2016). It is produced by activated epithelial cells and attracts T cells to the site of inflammation (Akahoshi et al., 2003). Reports of MIP-3α in poultry *Salmonella* infection are limited. Recent studies of MIP-3α expression are mainly found in human medicine (Aziz et al., 2016) However, a study performed by Withanage and others, showed increased expression of the MIP family chemokines in the ileum and cecal tonsils of ST infected birds. Although this is not specific to MIP-3α, their results correlate to what is seen in the current study and they speculated that the MIP family chemokines are involved in the recruitment of T cells to the intestines and protective immunity to the host (Withanage et al., 2005). The chemokine RANTES, acts as a pro-inflammatory chemokine, attracts T cells and basophils to the site of inflammation, and induces natural killer cell proliferation and activation (Maghazachi et al., 1996; Coble et al., 2011). Coble and others measured a significant increase in RANTES mRNA levels in broilers infected with *Salmonella* compared to hens (Coble et al., 2011). Dorner and associates found that when looking at the initial expression of all of these chemokines combined in the murine immune system, the role of chemoattractants MIP-1β and RANTES paired with MIP-1α amount to coactivation of macrophages; and when these chemokines are then paired with IFNγ, they act as cell mediated cytokines used by the natural killer cells to bridge components of the Th_1 response (Dorner et al., 2002).

Colony stimulating factors are a family of growth factors involved in the development, proliferation, and survival of hematopoietic cells (Kogut, 2000). In this experiment, M-CSF is the colony-stimulating factor of interest, which is derived from bone marrow fibroblasts and endothelial cells. Macrophage-colony stimulating factor is a growth factor that regulates the development, proliferation, and differentiation of macrophages (Kogut, 2000; Al-Khalaifah and Al-Nasser, 2018). Previous research has shown that *Salmonella* can cause impairment to M-CSF-induced macrophage recruitment and decreases levels of M-CSF secreted by epithelial cells in mice (Zhang et al., 2014). However, M-CSF has not been widely observed in poultry *Salmonella* infections and data to compare to this study are limited. Other researchers have found M-CSF-like activity in the chicken embryo specifically the egg yolk, amniotic fluid, and chorioallantoic fluid during development (Shao et al., 1994; Shao et al., 1996). Levels in these studies were downregulated after incubation of the egg. These results contradict what is viewed in the current experiment where M-CSF was significantly upregulated in ST infected broilers compared to control broilers. In another study conducted by Sakurai et al., the administration of M-CSF was found to control antigen-specific immune responses due to increases in B cells, natural killer cells, and the activation of murine macrophages for tumor killing (Sakurai et al., 2008). Our results in the current study showed that M-CSF could be an analyte of interest for further research on *Salmonella* infection of poultry, because it could be involved in regulating macrophage and monocyte populations or myeloid cells (Ushach and Zlotnik, 2016).

Growth factors are biologically activated molecules that are secreted in response to cell proliferation, migration, and differentiation during tissue repair and regeneration (Stone et al., 2022). Vascular endothelial growth factor is specifically involved in promoting endochondral ossification and angiogenesis (Zhang et al., 2013; Murphy and Weaver, 2017). Vascular endothelial growth factor is not a typical cytokine of interest for *Salmonella* infection. Previous research uses VEGF for exploring avian diseases like tibial dyschondroplasia (TD; Zhang et al., 2013; Huang et al., 2017), tumor angiogenesis (Duffy et al., 2013), and increasing angiogenesis in chicken embryo membranes (Fernandez and Bonkovsky, 2003). In the current study, we saw significant increases of this growth factor in ST infected broiler chicks compared to the control. We are unsure why VEGF was upregulated in *Salmonella* infected poultry, however, it is plausible that it could be linked to possible tissue repair and blood vessel growth during ST infection.

Our research characterized the immune profile of ST infected neonatal broilers. As we know, immune cells are an important part of the avian lymphatic system, they move systemically through the blood looking for foreign invaders, or they can reside and function in lymphoid tissues (Farber, 2021). Inflammation is a complex process that occurs in response to foreign bodies. It recruits cells from the blood and lymph to infected tissues and produces cytokines (French et al., 2020). After testing serum cytokine levels, these data suggest that the anti-inflammatory properties of IL-10 and the pivotal role IL-6 has in T cell and B cell mediated immunity, could be associated with the switch from cell mediated immunity to humoral immunity (Tan and Coussens, 2007; Choy and Rose-John, 2017; Huang, 2021). Increased concentrations of IL-10 could also be linked to developing tolerance to ST colonization by preventing damage to the host without affecting pathogen numbers (Opal and DePalo, 2000; Dorner et al., 2002; Tan and Coussens, 2007; Kogut and Arsenault, 2017). Furthermore, the increase in VEGF could potentially be linked to promoting restoration of damaged tissues and angiogenesis in ST infected birds based on previous research performed in TD (Zhang et al., 2013; Huang et al., 2017). Although 24 h post-infection is typically viewed as premature for the onset of humoral immunity, elevated IL-6, IL-10, and chemokine levels are compelling and we believe future trials will allow us to further examine these cytokines by using antibody titers to determine B cell development and immune reactions occurring in the body. We also believe that further investigation into cell populations and their involvement with cytokine expression could support the current data that is being presented.

Data availability statement

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

Ethics statement

The animal study was approved by Institutional Animal Care and Use Committee (IACUC 2016-0270 and 2019-0171) and Institutional Biosafety Committee (IBC 2016-112 and 2019-073). The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

AM-B: Data curation, Formal Analysis, Investigation, Methodology, Resources, Validation, Visualization, Writing–original draft, Writing–review and editing. YF: Writing–review and editing, Conceptualization, Methodology, Resources, Supervision. DZ: Conceptualization, Methodology, Resources, Supervision, Writing–review and editing. LB: Writing–review and editing. CL: Data curation, Resources, Software, Supervision, Writing–review and editing. MM: Data curation, Resources, Software, Supervision, Writing–review and editing. JB: Writing–review and editing. MF: Conceptualization, Funding acquisition, Methodology, Project administration, Resources, Supervision, Writing–review and editing.

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Conflict of interest

Authors CL and MM were employed by the company Millipore Sigma.

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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FcRY is a key molecule controlling maternal blood IgY transfer to yolks during egg development in avian species

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Maternal immunoglobulin transfer plays a key role in conferring passive immunity to neonates. Maternal blood immunoglobulin Y (IgY) in avian species is transported to newly-hatched chicks in two steps: 1) IgY is transported from the maternal circulation to the yolk of maturing oocytes, 2) the IgY deposited in yolk is transported to the circulation of the embryo via the yolk sac membrane. An IgY-Fc receptor, FcRY, is involved in the second step, but the mechanism of the first step is still unclear. We determined whether FcRY was also the basis for maternal blood IgY transfer to the yolk in the first step during egg development. Immunohistochemistry revealed that FcRY was expressed in the capillary endothelial cells in the internal theca layer of the ovarian follicle. Substitution of the amino acid residue in Fc region of IgY substantially changed the transport efficiency of IgY into egg yolks when intravenously-injected into laying quail; the G365A mutant had a high transport efficiency, but the Y363A mutant lacked transport ability. Binding analyses of IgY mutants to FcRY indicated that the mutant with a high transport efficiency (G365A) had a strong binding activity to FcRY; the mutants with a low transport efficiency (G365D, N408A) had a weak binding activity to FcRY. One exception, the Y363A mutant had a remarkably strong binding affinity to FcRY, with a small dissociation rate. The injection of neutralizing FcRY antibodies in laying quail markedly reduced IgY uptake into egg yolks. The neutralization also showed that FcRY was engaged in prolongation of half-life of IgY in the blood; FcRY is therefore a multifunctional receptor that controls avian immunity. The pattern of the transport of the IgY mutants from the maternal blood to the egg yolk was found to be identical to that from the fertilized egg yolk to the newly-hatched chick blood circulation, via the yolk sac

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membrane. FcRY is therefore a critical IgY receptor that regulates the IgY uptake from the maternal blood circulation into the yolk of avian species, further indicating that the two steps of maternal–newly-hatched IgY transfer are controlled by a single receptor.

KEYWORDS

maternal immunity, avian species, immunoglobulin, IgY, Fc receptor, egg yolk, FcRY

Introduction

Maternal immunity, a passive immunoglobulin (Ig) transfer, plays a key role in neonates to prevent infections during the developmental stages of their immune systems. In birds, IgY, functionally homologous to mammalian IgG, is transported from the hen to the newly-hatched chick in two steps (1–3); in the first step, maternal IgY is transported to the yolk of developing oocytes, while in the second step, the deposited IgY is transported from the yolk to the circulation of the embryo via the yolk sac membrane during late embryonic development. The second step relies on an IgY-Fc receptor, FcRY, the functional homolog of the mammalian neonatal Fc receptor, FcRn (4, 5). During the first step, IgY is believed to be selectively transported into the oocytes by receptor-mediated uptake. However, the receptor responsible for maternal blood IgY transfer into yolks has not been identified.

A study on the structural requirements of IgY for egg yolk transport is vital to identify the receptor responsible for IgY transfer in the first step. Intravenously-injected IgY and its Fc fragments are incorporated into egg yolks of laying hens more than Fab and F(ab') fragments (6). Additionally, IgY-Fc mutants with an amino acid residue substituted at the interface of the Cυ3–Cυ4 domain produce large differences in uptake into the egg yolk (7, 8). These results indicate that a receptor, which recognizes specific regions of the IgY-Fc domain, is involved in the first step of maternal IgY transfer.

FcRY was isolated from the chicken yolk sac membrane as the receptor responsible for the second step (4, 9). FcRY is a member of the mannose receptor family, with 180 kDa as its transmembrane form (10). FcRY has 55% homology to mammalian (human) phospholipase A_2 receptor, but the function is similar to mammalian FcRn. FcRY has IgY-Fc-binding capacity due to a conformational change at ~pH 6.0 and releases IgY above pH 7.4 (11). An experiment using FcRY-expressing cell lines has shown that FcRY transcytoses IgY from the apical to the basolateral site (12). Until now, it has been proposed that FcRY is not involved in the first step of maternal IgY transfer (13, 14) from observations of human IgG (hIgG) transport into avian egg yolks. Human IgG is believed to be transported into egg yolks by the same mechanism of avian IgY, but hIgG does not bind to FcRY (4). However, nobody has examined whether hIgG presents the same transport ability as IgY. IgY-deficiency in bursectomized chickens displayed an enhanced ability to transport IgY into the egg yolks and microarray analysis revealed an increased expression of FcRY in the ovarian follicles (15). These results suggest that FcRY may be the receptor responsible for IgY transport in the first, as well as the second step.

The present study investigated the hypothesis that FcRY was a key molecule in regulating IgY transport from the maternal circulation to the egg yolk. This is the first direct comparison of yolk-transport amounts of IgY and hIgG and includes a detailed localization of FcRY in ovarian follicles of chickens and quail. To clarify whether the IgY/FcRY interaction produces the yolk-transport amounts in the first step, the relationship between the binding properties of IgY-Fc mutants/FcRY and the transport ability of the IgY-Fc mutants into the yolk was examined. Finally, *in vivo* FcRY function in maternal IgY transfer was examined by neutralizing FcRY-binding ability to IgY.

Materials and methods

Reagents and conventions

All chemicals were of analytical grade. IgY amino acid residues were numbered according to the system of Suzuki and Lee (16).

Recombinant proteins

An expression vector containing chicken FcRY, lacking the membrane-spanning domain (the residues at 36–1396) from the template vector (donated by Dr. Pamela J. Bjorkman, California Institute of Technology), named secretory FcRY, was constructed.

The cDNA encoding the quail IgY (qIgY) υ-heavy chain was isolated from a quail splenocyte cDNA library (8). The gene encoding the Fc regions (H chain residues at 231–568) was isolated by polymerase chain reaction (PCR) from the template cDNA, and the PCR product was ligated into the *Kpn*I-*Xho*I cloning

Abbreviations: cIgY, chicken IgY; DIG, digoxigenin; FcRn, neonatal Fc receptor; GC, granulosa cell; hIgG, human IgG; Igs, immunoglobulins; IgG, immunoglobulin G; IgY, immunoglobulin Y; qIgY, quail IgY; WT, recombinant wild-type IgY-Fc.

site of pSecTag2, a mammalian expression vector (Invitrogen, Carlsbad, CA), with a C-terminal 6 × His tag. The constructed expression vector was used to synthesize recombinant quail wild-type IgY-Fc (designated WT). The IgY-Fc mutants (Y363A, G365A, G365D, G365S, and N408A) were generated using QuickChange II Mutagenesis Kit (Stratagene, Santa Clara, CA) or the KOD -Plus- Mutagenesis Kit (Toyobo, Osaka, Japan) according to the manufacturer's instructions. Another IgY-Fc and its mutants, lacking the His tag, were also generated by inserting a stop codon in front of the His tag codon. The sequences of the mutant genes were verified by an ABI3130 sequencer (Applied Biosystems, Foster City, CA) or by Eurofins Genomics Inc. (Tokyo, Japan).

The generated secretory FcRY and IgY-Fc constructs were then transiently transfected into CHO-S cells using FreeStyle™ MAX reagent (Invitrogen) or the ExpiCHO™ Expression System (Thermo Fisher Scientific, Waltham, MA), following standard protocols. The expressed proteins were purified by His Spin Trap affinity columns, according to the manufacturer's instructions (GE Healthcare, Waukesha, WI). The IgY-Fc lacking the His tag was purified using HiTrap™ NHS-activated HP columns (GE Healthcare), immobilized with rabbit anti-qIgY (17). The purification and molecular mass of the secretory FcRY and IgY-Fc were analyzed using SDS-PAGE under reducing and non-reducing conditions. Gels were stained with 0.5% Coomassie Brilliant Blue R-250 (CBB). The concentrations of the proteins were determined by measuring absorption at 280 nm (A_{280}), with 1.4 as the extinction coefficient (A_{280}/1.4 = concentration in mg/mL). Following the analyses, the formations of standard curve were checked by an original enzyme-linked immunosorbent assay (ELISA) system detecting qIgY (17).

The purified proteins were labeled with digoxigenin (DIG) using a DIG protein labeling kit (Roche Diagnostics, Indianapolis, IN) as necessary, according to the recommendations of the manufacturer.

Antibodies against FcRY

To obtain a specific antibody against FcRY, the purified secretory chicken FcRY was used as an antigen. The rabbits were immunized by Eurofins Genomics Inc. The acquired blood serum was mixed with 3 M ammonium sulfate for the precipitation of antibodies. The pellets of antibodies were resuspended in Tris-buffered saline (TBS) and dialyzed overnight against TBS at 4°C.

For the isolation of the FcRY-specific antibody, affinity chromatography consisting of a HiTrap™ NHS-activated HP column immobilized with the secretory chicken FcRY was used. The acquired antibodies were dialyzed against phosphate-buffered saline (PBS) (pH 7.4), and they were used for both FcRY detection and neutralizing FcRY binding activity.

Experimental birds and their management

Commercial female Japanese quail (*Coturnix japonica*) and White Leghorn-type commercial chickens (*Gallus gallus*; Julia Light®) were purchased from a local hatchery (Cyubu-kagaku-shizai, Nagoya, Japan). Female quail of a closed colony strain (WE, White Egg shell) was supplied by the National BioResource Project - Chicken and Quail, Nagoya University (Nagoya, Japan). All birds were maintained individually with free access to water and a commercial diet (Quail: Uzura-super®; Toyohashi Feed Mills, Toyohashi, Japan, Chicken: S-seven; Nosan Co., Kanagawa, Japan). The photoperiod was set at 16L:8D during the experiment. The room temperatures were controlled at 24 ± 2°C. Ten- to 30-week-old quail and 64-week-old chickens laying consistently were used for the experiments and their egg production was recorded daily. The animal care was in total compliance with the guidelines of the Nagoya University Policy on Animal Care and Use (2012030901, 2019031204, AGR2019044, A210510, A220021).

Intravenous injection of Igs and IgY-Fc mutants to laying chicken and quails and preparation of egg yolk extract

The quail laying regularly were injected intravenously with 20 μg/100 g BW of DIG-labeled chicken IgY (cIgY; Rockland, Philadelphia, PA), DIG-labeled hIgG (Sigma-Aldrich, St. Louis, MO), DIG-labeled IgY-Fc, and DIG-labeled IgY-Fc mutants dissolved in PBS (20 μg Igs/200 μL). Each chicken laying regularly was also injected intravenously with 100 μg/1,500 g BW of DIG-labeled cIgY or DIG-labeled hIgG dissolved in PBS (100 μg Igs/200 μL). All laid eggs were collected for 6 or 7 days after the injection and stored at 4°C until analysis. The egg yolk was separated from egg white to measure the incorporated proteins. Egg yolk extracts containing IgY and the incorporated proteins were prepared as described previously (7). The final solution was used for the determination of the incorporated protein concentrations using ELISA to detect conjugated DIG. Blood samples were collected 3 h post-injection. The concentration of the injected Igs in serum was determined using ELISA.

In ovo injection of IgY-Fc mutants

At day zero of incubation, a small hole was drilled in the eggshell of fertilized eggs (Japanese quail) using a 27G needle. An amount of 150 μL of albumen from the eggs was discarded. Following this, the DIG-labeled IgY-Fc mutants, WT, Y363A, G365A, and hIgG at 17 μg diluted in 25 μL saline with 1% (v/v) 100 × penicillin–streptomycin solution (FUJIFILM Wako, Osaka, Japan) were injected into the egg yolks. After the injection, 20 μL of 100 × penicillin–streptomycin solution (diluted in PBS) was injected into the albumen of the eggs. The hole was sealed with scotch tape. The eggs were incubated at 37.6°C with 55 to 60% relative humidity and turned once per 1 h until day 15 of incubation. Immediately after hatching at day 18, chicks were intraperitoneally administered with anesthetic agents (0.75 μg/kg BW Medetomidine hydrochloride, 4 μg/kg BW Midazolam, 5 μg/kg BW Butorphanol tartrate) diluted in 80 μL of saline. Blood samples were collected from the heart. The concentrations of injected Igs in the serum were determined using ELISA.

Injection of neutralizing antibodies in laying quail

To neutralize FcRY binding activity, each regularly-laying quail was injected intravenously with 1 mg/100 g BW of FcRY-specific antibody and 20 μg/100 g BW of DIG-labeled WT for the tracer antibody, diluted in 500 μL of saline. All laid eggs were collected for 5 days after the injection and stored at 4°C until analysis. Blood samples were collected at 1- and 3-h post-injection. The concentrations of the injected DIG-labeled WT and endogenous qIgY in serum were determined using ELISA.

ELISA

The concentration of native cIgY in the egg yolk extracts was measured using a commercial Chicken IgG ELISA quantitation kit (Bethyl Laboratories, Montgomery, TX), according to the manufacturer's instructions. The concentrations of native qIgY in blood samples were measured using an original ELISA (17). The concentrations of DIG-labeled Igs in the egg yolk extracts and in blood samples were measured using an original ELISA detecting conjugated DIG (18).

Western blotting analysis

The expression pattern of FcRY was examined using western blotting analysis. Each tissue sample from the chickens [white ovarian follicle, theca layer, and granulosa cell (GC) layer of yellow ovarian follicle] was homogenized with lysis buffer. The egg yolk and tissue homogenate samples (equivalent to 2 or 5 μg of protein) were separated using SDS-PAGE under reducing conditions. The blotting PVDF membranes were first incubated with rabbit anti-chicken FcRY antibody (1:2,500) or rabbit anti-human GAPDH (1:1,000; Santa Cruz Biotechnology, Dallas, TX) and then with HRP-conjugated goat anti-rabbit IgG antibody (1:2,000; Cell Signaling technology, Danvers, MA). The membranes were visualized using a chemiluminescence detection method (SuperSignal® West Dura Extended Duration Substrate; Thermo Fisher Scientific) with a charge-coupled device camera (AE-6960/C; Atto, Tokyo, Japan).

Visualization of FcRY in ovarian follicles

The FcRY expression in ovarian follicles was visualized using immunohistochemistry and immunofluorescence detection. The chickens were anesthetized with pentobarbital, and perfused intracardially with heparinized physiological saline for 5 min. The ovarian follicle (F3) from the chicken was then fixed with Mildform® (FUJIFILM Wako). The quail were euthanized by decapitation. The ovarian follicle (F4) was removed and fixed with Mildform®. All samples were embedded in paraffin.

The paraffin-embedded samples were sliced into 3-μm sections using a microtome. The sections were incubated overnight with rabbit anti-chicken FcRY antibody (1:500 in blocking buffer) at 4°C. Similar concentrations of serum-derived rabbit IgG (FUJIFILM Wako) were used for isotype control. In absorption control, 50 μL of secretory FcRY (5 μg/mL) was added to the anti-chicken FcRY primary antibody and incubated overnight at 4°C before adding to the sections. After washing, the sections were incubated with biotin-conjugated goat anti-rabbit IgG and then with avidin-biotinylated HRP complex (PK4001; Vector Laboratories, Newark, CA). Finally, the sections were visualized with diaminobenzidine solution (Dako, Glostrup, Denmark) followed by counterstaining with hematoxylin. The sections were observed under an upright microscope (BX51; Olympus, Tokyo, Japan) equipped with a charge-coupled device camera (DP20; Olympus) and microscope software (DP2-BSW ver. 2.2; Olympus). Images were captured at 40 × objective lens or at 100 × oil-immersion objective lens.

Using immunofluorescence detection, the samples were sliced into 5-μm sections. To observe FcRY, the sections were treated overnight with rabbit anti-chicken FcRY antibody (1:125) at 4°C. After washing, the sections were incubated with biotin-conjugated goat anti-rabbit IgG (BA-1000; Vector Laboratories, 1:250) in blocking buffer for 1 h in a dark room. The sections were then washed and incubated with Dylight®488-conjugated streptavidin (SA-5488; Vector Laboratories, 1:100 in PBS) for 30 min in a dark room. To observe the vascular endothelial cells of quail, a quail vascular endothelial cell marker, QH1 (AB_531829; DSHB, 1:125) and AlexaFluor® 568-conjugated anti-mouse IgG (ab175473; Abcam, Cambridge, UK, 1:1,000) were used as the primary and secondary antibodies, respectively. Subsequently, all sections were quenched and DAPI-stained using the Vector® TrueView® Autofluorescence Quenching Kit with DAPI (SP-8500; Vector Laboratories) for 5 min in a dark room. The sections were sealed and examined with a confocal laser scanning microscope (FV1000-D; Olympus) equipped with operational software (FV10-ASW). Images were captured at 100 × oil-immersion objective lens. The acquired images were adjusted for contrast using Fiji ImageJ ver. 1.53c (19). The outlines of cells in the ovarian follicle were traced on the basis of the differential interference contrast image and the fluorescence signal.

Visualization of IgY-Fc mutants in ovarian follicles

Deposition and infiltration of the injected WT and Y363A mutants into ovarian follicles were visualized using a standard avidin-biotinylated HRP complex method. DIG-labeled WT and Y363A mutants (400 μg) were injected intravenously into laying quail. After 0.5 h, the quail were euthanized by decapitation and the ovarian follicles were removed and fixed with Mildform®. The third largest ovarian follicle (F3) was paraffin-embedded, and the samples were sliced with a microtome into 5-μm sections. To detect the perivitelline layer, the sections were incubated overnight with rabbit anti-qZP3 IgG (1:200; donated by Dr. Tomohiro Sasanami, Shizuoka University, Japan) in a blocking buffer at 4°C and then

with tetra methyl rhodamine isothiocyanate (TRITC)-conjugated goat anti-rabbit IgG in a blocking buffer for 1 h. To examine the deposition of IgY-Fc mutants, they were incubated overnight with monoclonal anti-DIG mouse IgG (1:700) at 4°C after the second blocking step, and then treated with biotinylated anti-mouse IgG1 (1:400). They were then treated with Fluorescein Avidin DCS (1:250) for 1 h. Finally, the sections were loaded with fluorescent mounting medium and observed using a fluorescence microscope (BZ-X; Keyence, Osaka, Japan).

IgY-binding activities against FcRY

Copper-coated 96 well plates (Pierce™ Copper Coated High-Capacity Plates, Thermo Fisher Scientific) were incubated with 100 µL/well of the secretory FcRY with His tag (10 µg/mL) in PBS with 0.05% Tween 20 and 20 mM MES/HEPES (pH 4.0–7.4) for 60 min at room temperature. The plates were washed and loaded with 150 µL/well of blocking solution (pH 4.0–7.4) containing 1% (v/v) bovine serum albumin (Sigma-Aldrich) for 30 min. After washing, the plates were incubated with the native cIgY, qIgY, or IgY-Fc mutants (1.1-1,111 nM, pH 4.0–7.4) at 100 µL/well for 60 min. In the competitive binding assay, the plates were incubated with the DIG-labeled qIgY (27.8 nM final concentration) and subsequently loaded with several concentrations of IgY-Fc mutants as a competitor at pH 6.0 for 90 min. After washing, a basic solution (pH 8.0) was added to liberate bound Igs from the FcRY. The concentrations of Igs in the collected solutions were determined using ELISA.

To assess the ability of the FcRY-specific antibody to neutralize IgY binding activity, graded amounts of FcRY-specific antibody and native cIgY (111.1 nM final concentration) were reacted with the plates immobilized with FcRY; IgY bound to FcRY was then determined using ELISA.

Bio-layer interferometry

Binding of Igs to biosensor surfaces was evaluated using BLI on BLItz™ (45–5000; Sartorius, Goettingen, Germany) in advanced kinetics mode, following the manufacture's protocol. PBS buffer (0.05% Tween 20 and 20 mM MES at pH 5.5 or 20 mM HEPES at pH 7.4) was used in initial baseline step, and PBS buffer supplemented with 0.5% BSA was used in the later steps. All solutions were filtered using 0.2-µm syringe filters. Before measurements, Ni-NTA sensor chips (Sartorius) were hydrated using PBS (20 mM MES at pH 5.5 or 20 mM HEPES at pH 7.4) for 10 min. Chicken FcRY (400 nM) was immobilized on the sensor chip via a His tag in the loading step (180 s). After the baseline step, the sensor chips were dipped into antibody solutions (50–600 nM) in the association step (120 s). The sensor chips were subsequently soaked with dissociation buffer (120 s). For each subsequent run, a new biosensor was prepared (biosensors were not re-used). Data analysis and fitting (1:1) were achieved using BLItz Pro Software 1.0.

Statistical analyses

The data were analyzed using one-way analysis of variance (ANOVA). The mean values were compared using Turkey–Kramer's test or Dunnett's test. Comparisons between two groups were performed using unpaired-Student's *t*-tests. All error bars are expressed as the mean ± standard deviation (SD), and differences between means were considered to be significant at $p < 0.05$. Statistical analysis was performed with BellCurve for Excel (ver. 3.21) or GraphPad Prism 9.4.0 (GraphPad Software, Inc., La Jolla, CA, USA).

Results

Comparison of human IgG and avian IgY uptake into egg yolks

If FcRY is the receptor responsible for blood IgY transfer into yolks, uptake of blood hIgG into yolks must be extremely low compared to that of avian IgY. To test this, the uptake of intravenously injected DIG-labeled hIgG or DIG-labeled cIgY into egg yolks was compared. Because the uptake of injected cIgY into egg yolks was comparable to that of injected qIgY in quail (18), cIgY was used here as a tracer. In chickens and quail, the uptake of cIgY into egg yolks were markedly higher than that of hIgG (Figures 1A, C). Only small or statistically insignificant differences were observed in blood concentrations of cIgY and hIgG collected 3 h after the injection (Figures 1B, D), suggesting small or no influence of blood clearance on their uptake. These results suggest a possibility that FcRY facilitates IgY-specific transfer into egg yolks.

Avian FcRY is expressed in capillary endothelial cells of the ovarian follicle

Immunohistochemical analysis showed that FcRY was expressed in a variety of tissues, including ovarian follicles (Figure 2B), the liver, kidney, spleen, jejunum, and the embryonic yolk sac membrane (Supplementary Figure 1, Supplementary Table 1). In both chicken and quail ovarian follicles, FcRY signals were detected in the internal theca layers, close to the GC layer (Figures 2A, B-i, ii). Absorption of the FcRY antibody by recombinant FcRY completely eliminated these signals to the same level as the isotype control (Figure 2B-iii-vi). Western blotting analysis showed that FcRY protein, with a molecular mass of 180 kDa, was expressed in both the developing white ovarian and the maturing yellow ovarian follicles (Figure 2C). Similar to the immunohistochemical analysis, FcRY was expressed in the theca layer but not in the GC layer.

The ends of the blood capillaries in the internal theca layer of the ovarian follicle are distributed throughout the surrounding region, close to the basement membrane (Figure 2A). It was hypothesized that FcRY in the theca layer of ovarian follicles would be expressed at the terminal capillaries and transport IgY

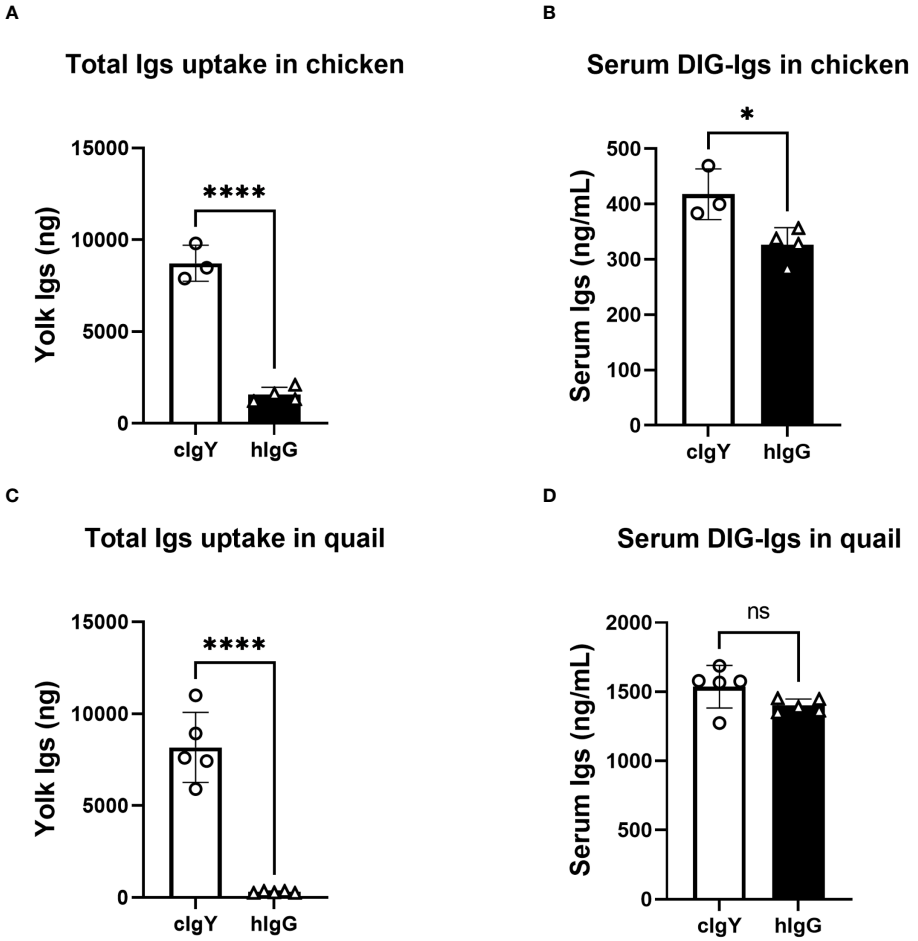

FIGURE 1
Uptake of exogenously-injected avian IgY into egg yolks is markedly higher that of human IgG. **(A, B)** Chickens were intravenously injected with 100 µg/1,500 g BW of DIG-labeled cIgY or DIG-labeled hIgG dissolved in PBS (n = 3 or 4). All eggs laid were collected for 7 d after the injection and uptake into the egg yolks was measured using ELISA **(A)**. The blood samples were collected 3 h after injections and concentrations in the serum were measured **(B)**. **(C, D)** Quail were intravenously injected with 20 µg/100 g BW of DIG-labeled cIgY or DIG-labeled hIgG dissolved in PBS (n = 5). All eggs laid were collected for 7 d after the injection and total uptake into the egg yolks was measured **(C)**. The blood samples were collected 3 h after injections and concentrations in the serum were measured **(D)**. Vertical bar indicates mean ± standard deviation (SD). Statistically significant p values were determined using unpaired Student's *t*-tests: $*p < 0.05$, $****p < 0.0001$.

to the internal layers of the ovarian follicles. Confocal microscopy analysis indicated an FcRY signal in a line near the basement membrane of the theca layer (Figure 2D-i; green). The QH1 signal, a quail endothelial cell marker, was detected throughout the theca layer, but intensively in the internal theca layer (Figure 2D-ii; magenta). The FcRY signals were colocalized with QH1 (Figure 2D-iii, iv), suggesting that FcRY was present in the vascular endothelial cells.

Transport efficiency of IgY-Fc mutants into egg yolks and newly-hatched chicks

Recombinant IgY-Fc and its mutants can be used to clarify whether the IgY/FcRY interaction controls maternal IgY transfer. IgY-Fc mutants substituting one amino acid residue for alanine were generated in a previous study and have variable abilities for transport into yolks when injected into laying birds (8). Analysis of the transport efficiency and binding properties of IgY-Fc mutants can elucidate the involvement of FcRY in maternal IgY transfer.

The uptake of the injected quail IgY-Fc mutants into quail egg yolks was analyzed during the first step (Figure 3A top): G365A was detected at two-fold higher concentrations in the egg yolks compared to WT, whereas Y363A was undetected (Figure 3B); the blood concentration of WT was slightly higher than that of G365A and Y363A (Figure 3C). These results indicate that the blood clearance of the mutants did not account for the differences in their uptake into the egg yolk.

To examine whether the transport pattern of the mutants in the first step was identical to the pattern in the second step, the IgY-Fc mutants and hIgG were injected into yolks of fertilized quail eggs and incubated, followed by measurement of the transferred mutants in blood of newly-hatched chicks (Figure 3A bottom). The G365A concentration in blood was 1.5-fold higher than that of the WT, whereas the Y363A concentration was less than 3% of the WT; hIgG was undetectable (Figure 3D).

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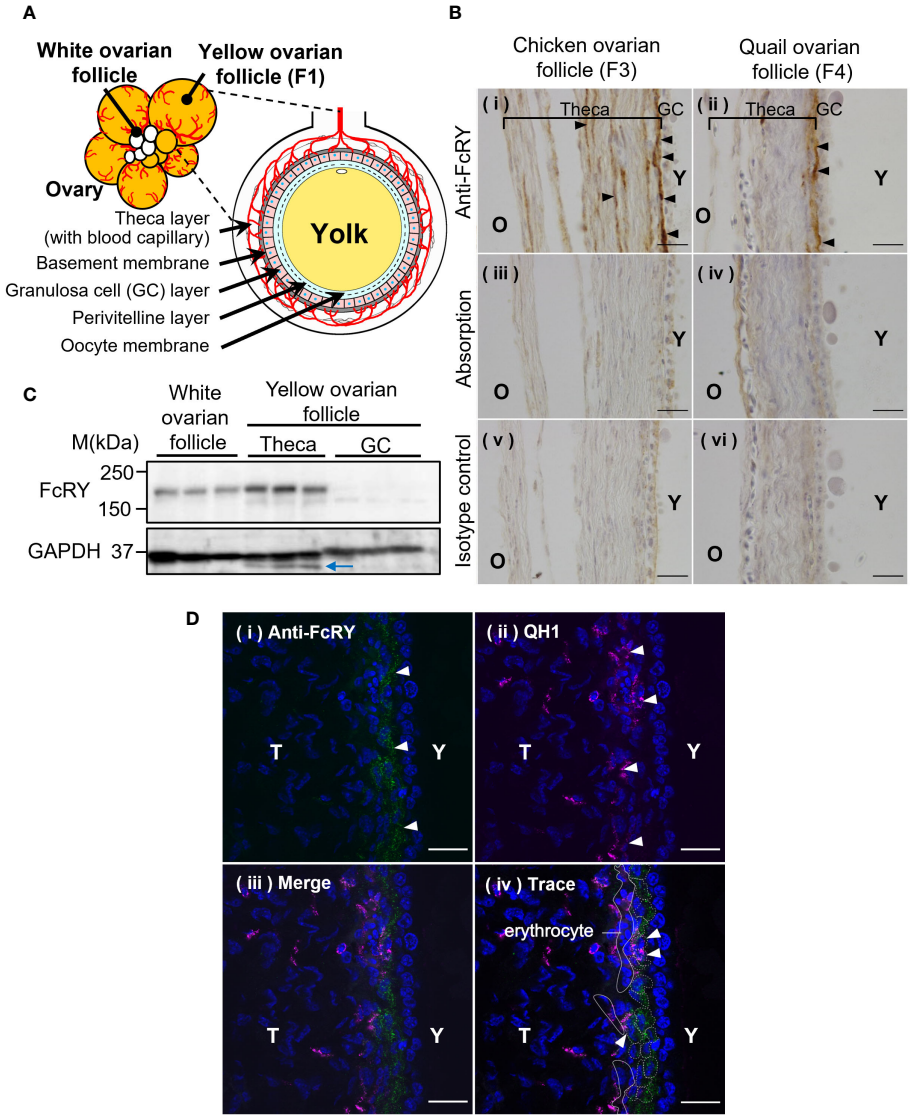

FIGURE 2
Avian FcRY is expressed in the capillary endothelial cells of ovarian follicles. **(A)** Graphical illustration of the ovarian follicle of laying birds. **(B)** Detection of FcRY in a chicken ovarian follicle (F3) and a quail ovarian follicle (F4) using immunohistochemical analysis (× 100). The sections were stained with anti-FcRY antibody. In the absorption control, the anti-FcRY antibody was incubated with recombinant secretory FcRY overnight at 4°C before adding it to the sections. Scale bars, 20 µm. **(C)** Detection of FcRY in the chicken yolk sac, white (small), and yellow (large) ovarian follicles using western blotting analysis (n = 3). Multiple surface layers of yellow ovarian follicles were separated into the theca layer and the GC layer. The blue arrow indicates the non-specific binding bands. **(D)** Confocal images of quail ovarian follicle stained with anti-FcRY antibody (green) and with QH1, a quail endothelial cell marker (magenta; original magnification, ×100). The nuclei were stained with DAPI (blue). Scale bars, 20 µm. GC, granulosa cell; T, theca layer; Y, yolk.

Overall, the transport patterns of the IgY-Fc mutants and hIgG from the yolk sac to the embryonic blood circulation was consistent with that of the injected IgY-Fc mutants and hIgG into the quail egg yolks, suggesting that the same receptor may contribute to both IgY transport in the first and second steps.

Localization of the injected DIG-labeled WT and Y363A in quail ovarian follicles was visualized using a fluorescent microscope. WT (green) accumulated in the yolks of oocytes on the inside of the qZP3 signal (marker of perivitelline layer, red; Figure 3E-i–iii). In contrast, Y363A was detected outside of the qZP3 signal and GC layer (Figure 3E-iv–vi). These results suggest that there is a key molecule regulating the uptakes of IgY-Fc and its mutants in the internal theca layer of the ovarian follicle, where FcRY is localized.

Binding activities of IgY-Fc mutants to FcRY immobilized in the 96-well plate

We hypothesized that the binding activity of IgY-Fc and its mutants to FcRY controls their uptake into egg yolks. FcRY is known to bind to IgY below pH 6.0 and dissociate above pH 7.4 (4). Consistent with previous studies, at pH 6.0, both chicken and quail

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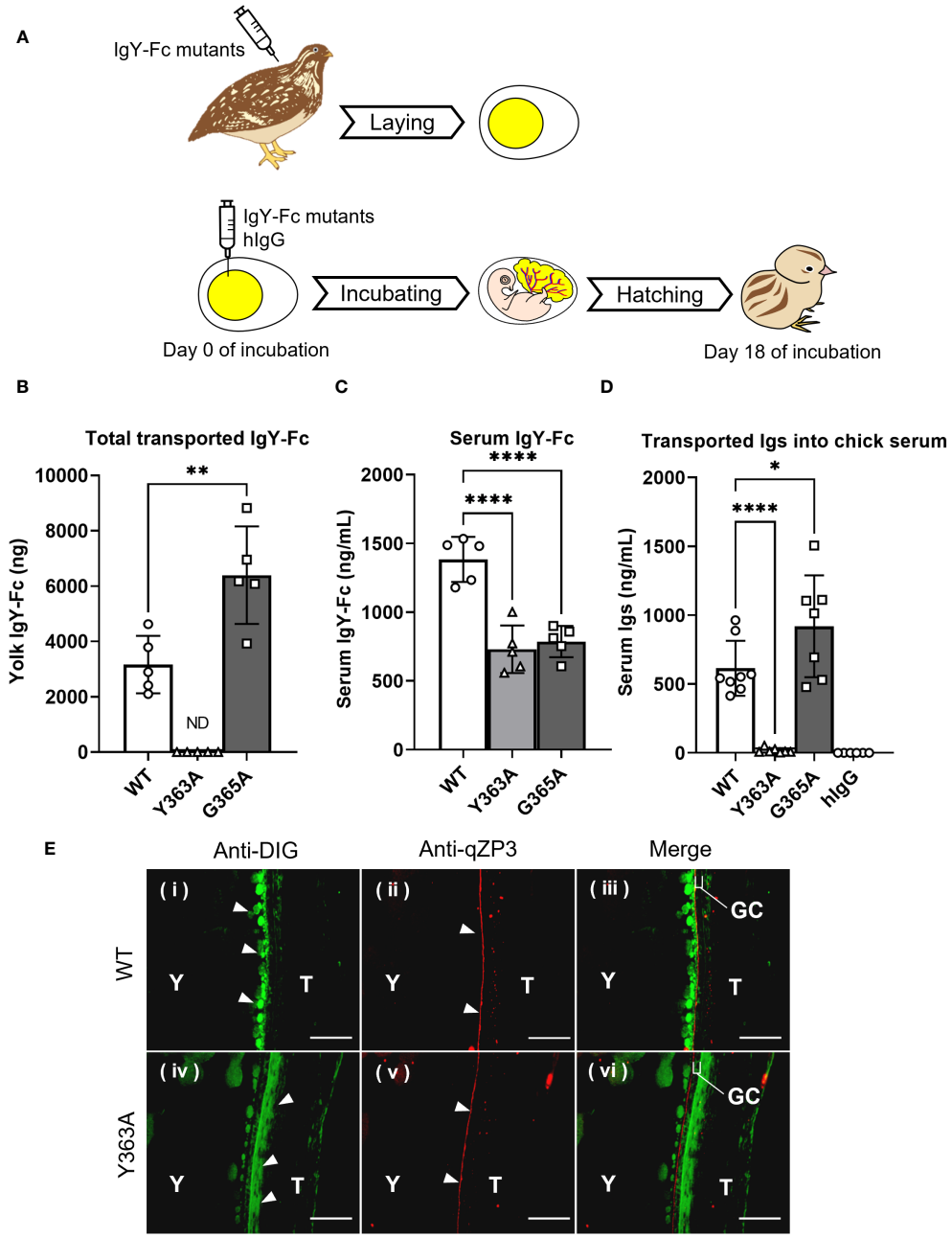

FIGURE 3
Variable uptakes of IgY-Fc mutants with a single amino acid substitution into egg yolks and newly-hatched chicks. **(A)** Schematic representation of the experimental approach from injection to sampling. **(B, C)** Quail were intravenously injected with 20 µg/100 g BW of DIG-labeled recombinant quail IgY-Fc (WT, Y363A, G365A; n = 5). All laid eggs were collected for 6 d after the injection and the total uptake into the egg yolks was measured using ELISA **(B)**. The blood samples were collected 3 h after injections and concentrations in the serum were measured **(C)**. **(D)** The egg yolks of fertilized quail eggs were injected with 17 µg of DIG-labeled quail IgY-Fc (WT, Y363A, G365A) and DIG-labeled hIgG (n = 6–9). After hatching, the blood samples of newly-hatched chicks were collected, and the concentrations of injected Igs in each sample were detected using ELISA. Vertical bar indicates mean ± standard deviation. Statistically significant p-values were determined using unpaired Student's *t*-tests in **(B)** or analysis of variance with Dunnett's multiple comparisons in **(C, D)**, $*p < 0.05$, $**p < 0.01$, $****p < 0.0001$. **(E)** Quail were intravenously injected with 400 µg of DIG-labeled quail WT or Y363A, and the third largest ovarian follicles were collected 0.5 hour after injection. The sections were prepared from the ovarian follicles, and then they were incubated with anti-DIG antibody (green) to detect the WT or Y363A. The perivitelline membrane was detected using anti-qZP3 (red; original magnification, ×40). Scale bars, 40 µm. GC, granulosa cell layer; T, theca layer; WT, recombinant quail wild-type IgY-Fc; Y, yolk.

IgYs bonded to FcRY immobilized in the plate in a dose-dependent manner but failed to bind at all at pH 7.4 (Supplementary Figure 2). All the IgY-Fc mutants also bonded to FcRY in a dose-dependent manner when incubated at pH 6.0 (Figure 4A). Compared to WT, G365A bonded more avidly to FcRY; Y363A bonded more strongly to FcRY than WT and G365A, while none of the mutants bonded at pH 7.4 (Supplementary Figure 3A). Further examination of the binding to FcRY at graded pH (pH 4.0–7.4) showed that all mutants

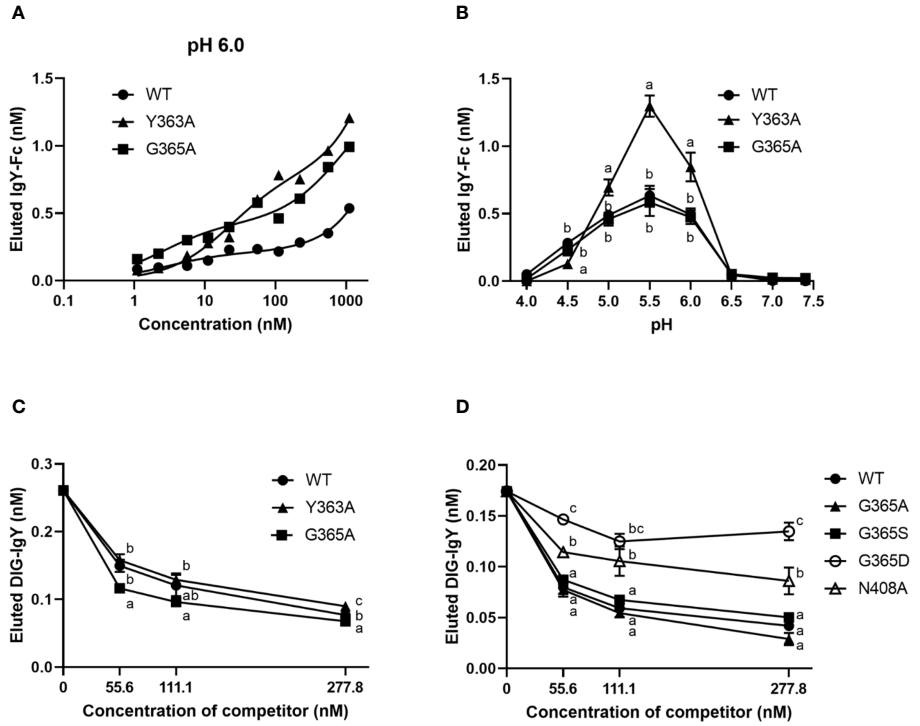

FIGURE 4
The binding activity of IgY-Fc mutants to FcRY is consistent with the transport of these mutants to egg yolks. **(A)** Copper-coated 96 well plates were incubated with the secretory FcRY (10 µg/mL). The plates were then incubated with the quail IgY-Fc mutants at pH 6.0 (1.1–1,111 nM; n = 1). After washing, a basic solution of pH 8.0 was added to liberate the bound mutants from the FcRY. The concentrations of IgY-Fc mutants in the collected solutions were determined using ELISA. **(B)** pH-dependent binding activity of quail IgY-Fc mutants (55.6 nM) to FcRY at pH 4.0–7.4 (n = 3). **(C, D)** DIG-labeled chicken IgY (27.8 nM final concentration) was incubated with an FcRY-immobilized plate with the graded concentrations of IgY-Fc mutants as a competitor at pH 6.0 (n = 3). The concentration of the DIG-IgY bound to FcRY was detected using ELISA. The results are shown as mean ± standard deviation. The letters a, b, and c within the same concentration represent significant differences at $p < 0.05$, as determined using analysis of variance with Turkey–Kramer's multiple comparison test. WT, recombinant quail wild-type IgY-Fc.

bonded most strongly to FcRY at pH 5.5 (Figure 4B). Approximately twice as much Y363A was found to bind strongly at pH 5.0–6.0 than WT and G365A.

The competitive ability of each mutant was compared using DIG-labeled IgY. All mutants inhibited DIG-IgY binding to FcRY in a dose-dependent manner (Figures 4C, D). Y363A showed a similar inhibition activity to WT, whereas G365A more effectively inhibited the DIG-IgY binding to FcRY than WT. G365S, which has similar ability to transport IgY into egg yolks as G365A (8), also strongly inhibited the binding of DIG-IgY (Figure 4D). G365D and N408A, which are not usually transported to egg yolks (8), were substantially less potent in inhibiting DIG-IgY binding than the WT. These results indicate that the binding activity of IgY-Fc mutants to FcRY was consistent with the transport pattern of these mutants to egg yolks.

Binding affinities and kinetic parameters of IgY-Fc mutants to FcRY using the BLI system

To analyze binding properties and stability in more detail, the binding affinities and kinetic parameters (association/dissociation rate) between the mutants and FcRY were determined using a BLI system. At pH 5.5, all mutants bonded to FcRY in a dose-dependent manner (Figures 5A–C), but Y363A bonded more strongly to FcRY (Figure 5D). At pH 7.4, binding of IgY and its mutants to FcRY markedly decreased (Supplementary Figures 3B-E). Their K_D, k_a, and k_d values were calculated using BLItz-specific software (Table 1). The K_D of IgY was 275 nM, which was comparable to that obtained previously using surface plasmon resonance (4). G365A showed a greater association rate (2.2-fold) and stronger binding affinity than WT (K_D; 197 nM of G365A vs. 333 nM of WT). However, Y363A had a lower dissociation rate (approximately 1.5-fold) and stronger binding affinity than WT (K_D; 65 nM of Y363A vs. 333 nM of WT). These results suggest that G365A binds to FcRY more efficiently than the WT, and that Y363A, once bound to FcRY, hardly ever dissociates.

Inhibition of FcRY by neutralizing antibodies and its effect on IgY transport into egg yolks in laying birds

To obtain direct evidence that FcRY was responsible for IgY transport to the egg yolk, we generated FcRY-specific antibodies. When FcRY antigen was administered to different rabbits, two lots of the antibodies against FcRY with different neutralizing activities

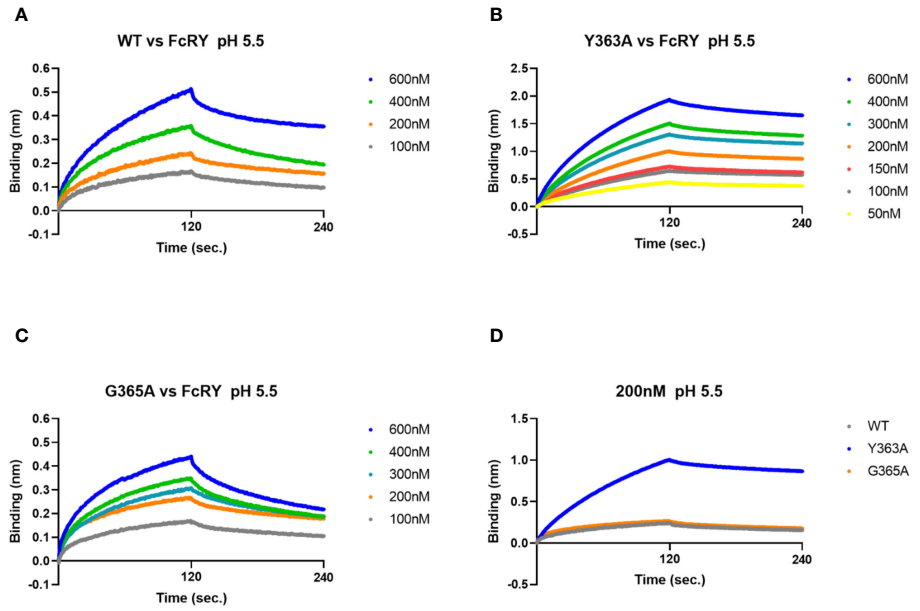

FIGURE 5
The G365A mutant with a strong efficiency of transport to the egg yolk has a higher binding affinity to FcRY than to the WT. **(A–C)** FcRY (400 nM) was immobilized on the sensor chip. The sensor chips were dipped into WT **(A)**, Y363A **(B)**, or G365A **(C)** at 50–600 nM under pH 5.5 in association steps (120 s). The sensor chips were subsequently soaked with dissociation buffer (120 s). **(D)** Comparison of the binding among IgY-Fc mutants (WT, Y363A, and G365A) at 200 nM to FcRY. WT, recombinant quail wild-type IgY-Fc.

were obtained. Both antibodies neutralized IgY binding to FcRY in a dose-dependent manner (Figure 6A). The first neutralizing antibody (called Strong Ab) efficiently and strongly inhibited IgY binding compared to the second neutralizing antibody (called Weak Ab).

Next, each neutralizing antibody was intravenously co-administered with DIG-labeled IgY-Fc (WT) tracer to the laying quail. Prior to the experiment, we confirmed both neutralizing antibodies did not interact with IgY by Western blotting and immunohistochemical analyses. In quail treated with Strong Ab, the tracer uptake in the yolks was reduced to approximately 20% of that in the control rabbit IgG (Figure 6B). The blood tracer concentration collected 3 h after the administration showed a substantial reduction to about 30% of the control (Figure 6C). Similarly, the total IgY concentration in the blood was also reduced to approximately 65% (Figure 6D). In quail treated with Weak Ab, the tracer transported to the yolks was also reduced to ~60% (Figure 6E). However, no differences were observed in the blood tracer concentrations (Figure 6F) or the total blood IgY concentrations (Figure 6G). Thus, the inhibition of FcRY by the Weak Ab reduced IgY uptake in egg yolks with minimal impact on blood IgY concentrations. These results indicate that FcRY controls maternal IgY transfer to the yolks in avian species.

Discussion

The results of this study are the first to demonstrate that avian FcRY plays a key role in IgY transfer from maternal blood circulation to egg yolks in maturing oocytes of laying birds. Our main findings were: 1) the majority of FcRY in ovarian follicles was expressed in capillary endothelial cells of the internal theca layer, 2) binding properties of IgY-Fc mutants/FcRY closely matched the transport ability of the IgY-Fc mutants into the egg yolk, 3) the injection of neutralizing antibodies against FcRY reduced IgY uptake into egg yolk in the hen. These results support the hypothesis that maternal–newly-hatched IgY transfer in the two steps is regulated by a single receptor, FcRY, in avian species. The

TABLE 1 K_D, k_a and k_d values at pH 5.5 derived from BLItz.

IgY or IgY-Fc (concentration; nM)	K_D (nM)	k_a (1/Ms)	k_d (1/s)	R^2
qIgY (100, 200, 300, 400, 600)	275	3.5×10^{4}	9.5×10^{-3}	0.977
WT (100, 200, 400, 600)	333	1.9×10^{4}	6.5×10^{-3}	0.982
Y363A (50, 100, 150, 200, 300, 400, 600)	65	2.3×10^{4}	1.5×10^{-3}	0.999
G365A (100, 200, 300, 400, 600)	197	4.2×10^{4}	8.2×10^{-3}	0.985

Table includes the binding affinity of qIgY and IgY-Fc mutants to FcRY calculated the sensorgrams in Figures 5A-C using BLItz Pro Software.

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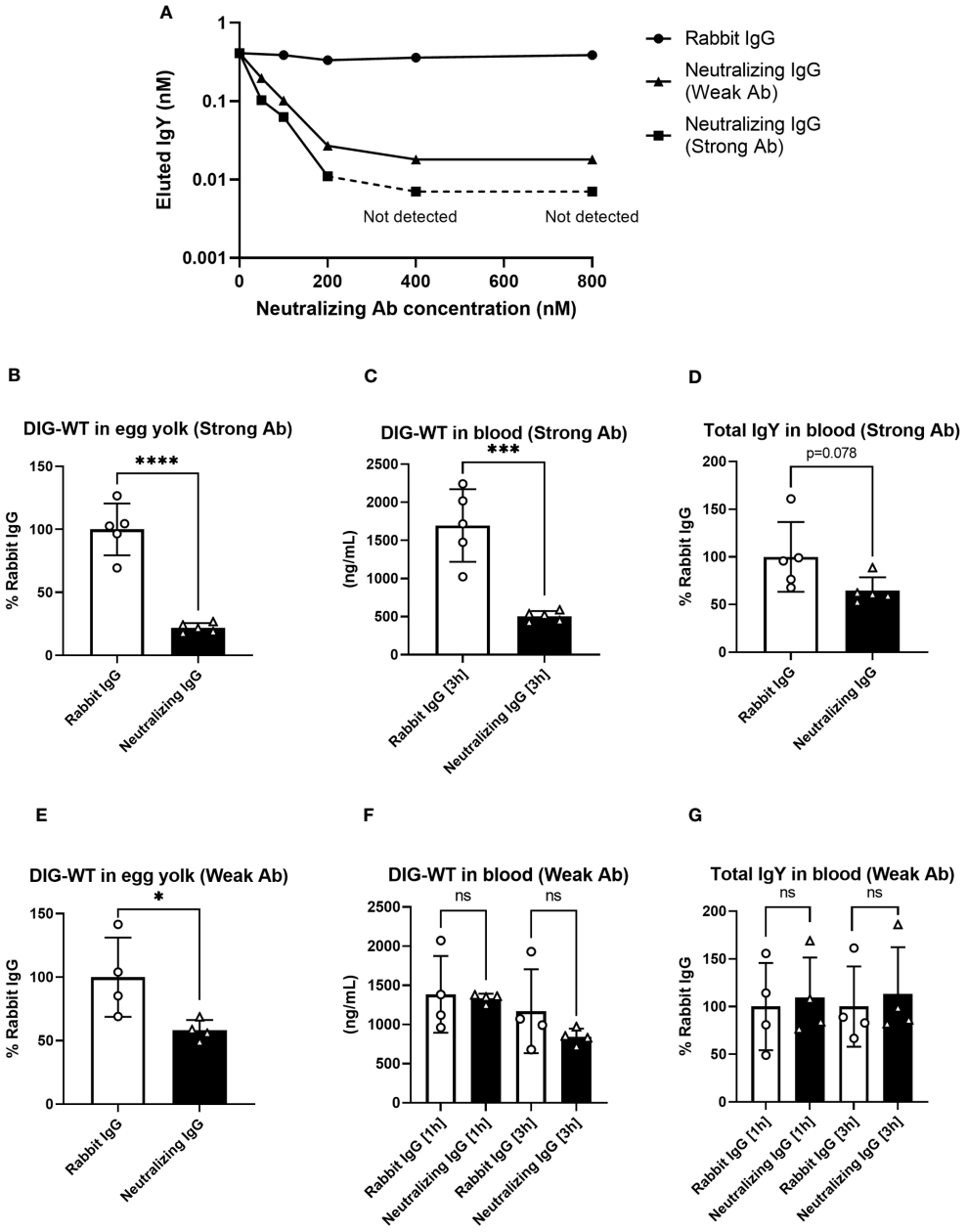

FIGURE 6
The injection of neutralizing antibodies against FcRY reduces IgY transport into the egg yolks of laying quail. **(A)** Graded amounts of the neutralizing antibodies against FcRY were added to the FcRY-immobilized 96 well plate with IgY (111.1 nM) at pH 6.0. After washing, a basic solution of pH 8.0 was added to liberate the bound IgY from the FcRY. The concentrations of IgY in the collected solutions were determined using ELISA. The values of the strong Ab at 400 nM and 800 nM were not detected using ELISA. **(B–G)** The regularly laying quail (n = 4, 5) were injected intravenously with 1 mg/100 g BW of FcRY-specific neutralizing antibodies (**B–D**, Strong Ab; **E–G**, Weak Ab) and 20 μg/100 g BW of DIG-labeled WT both diluted in 500 μL of saline. All eggs that were laid were collected for 5 d after injections and the concentration of DIG-WT in egg yolks were measured using ELISA **(B, E)**. Blood samples were collected 1- and 3-h post-injection. The concentrations of the injected WT **(C, F)** and total IgY **(D, G)** in serum were determined using ELISA. Vertical bars indicate mean ± standard deviation. Statistically significant p-values were determined using unpaired Student's *t*-tests: **p* < 0.05, ****p* < 0.001, *****p* < 0.0001. WT, recombinant quail wild-type IgY-Fc.

FcRY-dependent IgY transport system to the egg yolks may be a basic mechanism, broadly applicable to maternal immunity in reptiles as well as avian species.

Analysis of the FcRY/IgY complex structure by cryoelectron microscopy showed that FcRY is in close contact with IgY Cυ4 domain and its surroundings (11). The LYI (362–364 at Cυ3) and HEAL motif (550–553 at Cυ4) within the interface of Cυ3 and Cυ4 domain are conserved well in transportable Igs into egg yolks (20, 21). The substitution of one amino acid residue in the region to other amino acids markedly impacts the uptake of mutants into egg yolks (7, 8). The G365A mutant with a high transport efficiency into egg yolks bonded strongly to FcRY, with much higher association and dissociation rates than WT (Figures 4, 5; Table 1). The G365 residue is next to α-helix from S358 to I364, and it constitutes an exposed

surface area of IgY-Fc (PDB ID 2W59). Predicted structure of the FcRY/WT complex by AlphaFold2 (22) provided an intermolecular hydrogen bond between the R841 residue in cysteine rich-domain 5 (CTLD5) of FcRY and the G365 residue of WT (Supplementary Figure 4C; Supplementary Table 2). This hydrogen bond was also observed in the FcRY/G365A complex (Supplementary Figure 4E). Another docking simulation by HADDOCK (23, 24) predicted that the A365 residue in the G365A mutant formed two hydrogen bonds to the R900 residue in FcRY (Supplementary Figure 5C; Supplementary Table 2), whereas the G365 residue of the WT formed only a single hydrogen bond with the R900 residue in FcRY (Supplementary Figure 5A). The results suggest that the G365A mutant strongly binds to FcRY via the formation of additional hydrogen bond. We speculate that, in the maternal body, the G365A mutant efficiently bind to FcRY in capillary endothelial cells, followed by the prompt release of G365A into extravascular ovarian tissues, thus resulting in a marked increase in G365A uptake in egg yolks. Several mutants (G365D and N408A), which are less likely to be transported to egg yolks, showed lower competitive ability against the binding of IgY to FcRY (Figure 4D). Variations in binding affinity between the FcRY and IgY-Fc mutant could explain the differences in the mutant uptake into egg yolks of laying birds.

In contrast, Y363A, a mutant with no ability to transport Igs into egg yolks, bonded strongly to FcRY, and its dissociation from FcRY was much slower than that of the WT and G365A mutant. Microscopic observations of Y363A injected into the ovarian follicles indicated its deposition in the vicinity of internal theca layer (Figure 3C-iv–vi). These results suggest that Y363A is strongly bound to the FcRY with less dissociation. The Y363 is positioned at the α-helix from S358 to I364, which are located in an exposed loop in close proximity to another loop of the Cυ4 domain (25). Side chain of the Y363 residue forms hydrogen bond with a side chain of the E551 at Cυ4 domain (PDB ID 2W59), and therefore contributes conformational stability of the interface of Cυ3 and Cυ4 domain. In support of this notion, the predicted Y363A mutant structure was dissimilar to that of the WT in terms of the angle between Cυ3 and Cυ4 domain (RMSD value 3.855; Supplementary Figure 4A), whereas the G365A mutant structure was nearly identical to that of the WT (RMSD value 0.470; Supplementary Figure 4B). Interestingly, the prediction results indicated that the both Y363 and A363 residues did not form any intermolecular hydrogen bonds to FcRY (Supplementary Figures 4C, D; Supplementary Table 2). In addition, the docking simulation by HADDOCK showed that the Y363A mutant bound to FcRY at distinct binding sites from those found in the WT and G365A mutant (Supplementary Figures 5A-C). We speculate that substitution of the Y363 to Ala may cause massive conformational change at Cυ3/Cυ4 domain, which might produce strong binding activity to FcRY (Figure 4B). However, the competitive ability of the Y363A mutant against IgY-FcRY binding was equivalent to that of the WT (Figure 4C). One plausible explanation is that the Y363A mutant gains binding ability to another FcRY region distinct from IgY- and WT-binding region on FcRY. Taken together, the Y363A mutant appears to have complex binding mode to FcRY dissimilar to other IgY-Fc mutants.

The precise mechanisms by which FcRY transports IgY using a pH-dependent binding property remain unclear. Immunofluorescent assays revealed that FcRY seemed to express intracellularly in capillary endothelial cells in internal theca layer of the ovarian follicle (Figure 2D). In mammals, maternal Igs are transported to the neonate via placenta and breast milk (26, 27). FcRn, which is structurally similar to class I major histocompatibility complex (MHC), is responsible for the transport of maternal IgG. The binding characteristic of mammalian FcRn are nearly the same as those of avian FcRY observed here. FcRn binds to the Fc region of IgG at the acidic pH, but dissociates from IgG at the physiological pH (26, 28). The strict pH dependence of the FcRn-IgG interaction is mediated by several amino acid residues in the Cγ2-Cγ3 region of IgG, and one of which is also conserved in cIgY (hIgG LMI251-253, cIgY LYI362-364) (29–33). Mammalian FcRn is localized in early and recycling endosomes of syncytiotrophoblast of the placenta (34, 35), and transports IgG from maternal circulating blood to fetal circulating blood (27, 36). The pH within early (pH ~6.0) and recycling endosomes (pH ~6.5) is mildly acidic (37, 38). It is quite convincing that all IgY-Fc mutants bound strongly to FcRY at pH 5.0–6.0, but not above pH 6.5 (Figure 4B). It is therefore possible that FcRY may localize within the endosomes of capillary endothelial cells like mammalian FcRn (Figure 7). Transported IgY from capillary endothelial cells in the theca layer has to pass through several membranes/layers of ovarian follicle (39). The basement membrane, one of the membranes just inside the theca layer, permits penetration of particles of < 40 nm (40, 41), thus IgY (< 20 nm) can move across the basement membrane. However, it is unclear how IgY is transported across other membranes/layers of ovarian follicle (granulosa cell layer, perivitelline layer, and oocyte membrane). FcRY at protein level was not detected in these layers except for theca layer (Figures 2C, D). Further research is needed to elucidate the mechanism of FcRY-dependent IgY transport in capillary endothelial cells and the subsequent transport pathway of IgY until incorporation in the yolks.

Avian FcRY was first discovered in the yolk sac membrane during embryogenesis (4, 9). The uptake of yolk-deposited IgY through the yolk sac membrane dramatically increases in the last few days before hatching (1), which coordinates enhanced FcRY gene expression in the yolk sac membrane (unpublished data), implying that the yolk-deposited IgY transport to embryonic circulation is a brief event associated with rapid uptake of IgY. In contrast, deposition of yolk protein into ovarian follicles requires relatively long-term period over 2 months (42). Injection study of radio-labeled IgY into laying hens revealed that the injected IgYs were transported into yolks of both the small white ovarian follicles and large yellow ovarian follicles (43). In support of this, FcRY was expressed in both the white and yellow ovarian follicles in the present study (Figure 2C). Considering strong FcRY expression in the yolk sac membrane (Supplementary Figure 1, Supplementary Table 1), IgY-transport efficiency and capability of the second step via the yolk sac membrane would be much higher than those of the first step via the capillary endothelial cells in ovarian follicles.

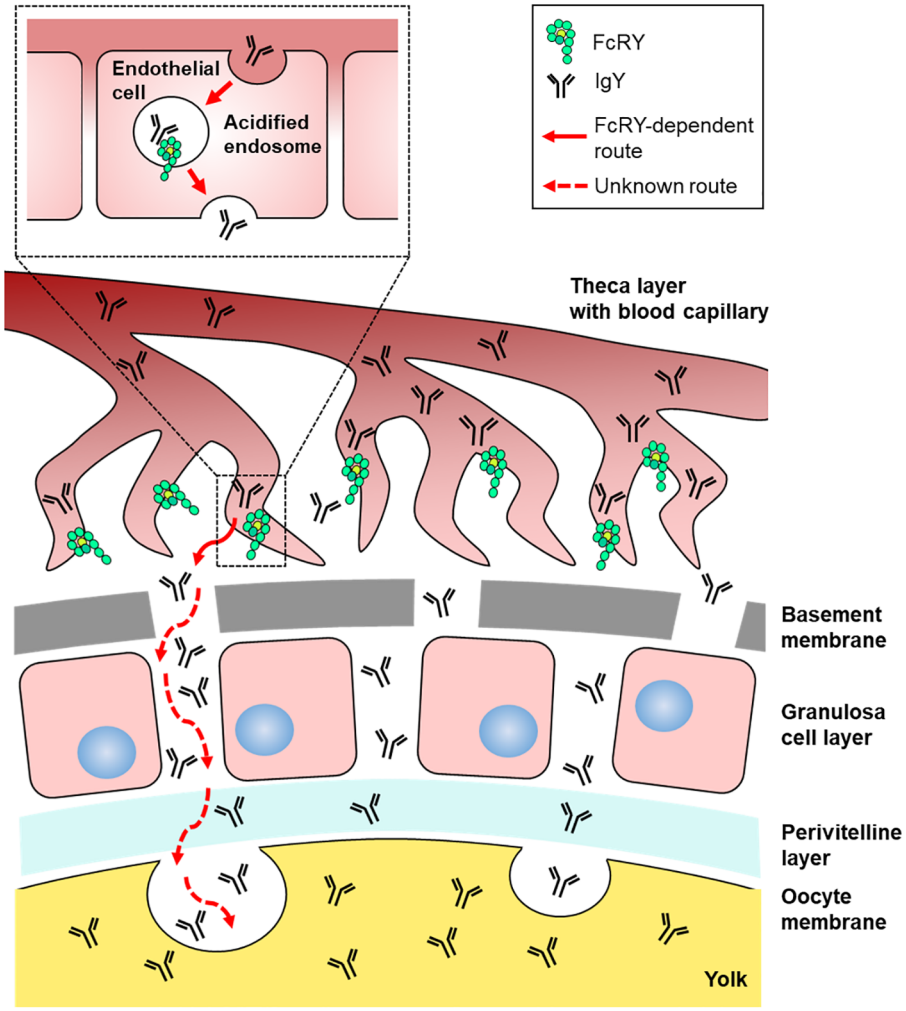

FIGURE 7
Graphical illustration of the IgY transport pathway in the ovarian follicle of laying birds. FcRY would localize within the acidified endosomes of capillary endothelial cells in the internal theca layer and transport IgY to the extravascular space. Transported IgY from capillary endothelial cells in the theca layer has to pass through several membranes/layers of ovarian follicle. The basement membrane, one of the membranes just inside the theca layer, permits penetration of particles, thus IgY can move across the basement membrane. It is unclear how IgY is transported across other membranes/layers of ovarian follicle (granulosa cell layer, perivitelline layer, and oocyte membrane). Finally, the IgY is incorporated into yolks probably due to endocytosis.

Another interesting finding was that the injection of neutralizing antibodies in laying quails markedly reduced not only IgY uptake into the egg yolks, but also the IgY concentration in the blood (Figures 6C, D). To date, no study has investigated the regulatory mechanism of blood IgY half-life in avian species. Our findings indicate that FcRY is also involved in prolongation of blood IgY half-life. Immunohistochemistry revealed that chicken FcRY is expressed in various cell types, such as the liver sinusoidal endothelial cells and splenic/intestinal/lung lymphocyte-like cells (Supplementary Table 1), which is where mammalian FcRn is expressed (44–46). Mammalian macrophages, monocytes, and vascular endothelial cells expressing FcRn are responsible for IgG recycling, which protects intracellular uptake of IgG from lysosomal degradation and transports it back into the bloodstream, increasing the half-life of blood IgG (47, 48). FcRn ablation results in substantially less blood IgG in mice (49). A polarized mammalian epithelial cell line expressing FcRY was shown to endocytose and recycle IgY (12). Thus, avian FcRY and mammalian FcRn are expected to be structurally distinct molecules but are functionally similar to the receptor controlling blood IgY-half life.

In conclusion, this study indicates that avian FcRY plays a major role in maternal blood IgY transfer into egg yolks. We found for the first time that FcRY was expressed in the capillary endothelial cells of ovarian follicles. Avian FcRY contributes to IgY recycling to extend the half-life of IgY in the bloodstream. The main limitation of this study is a lack of evidence that complete ablation of the *FcRY* gene inhibits transport of maternal blood IgY into egg yolks. Production of *FcRY* gene knockout/knockin birds using primordial germ cells and gene editing system would elucidate avian maternal immunity. The *FcRY* gene-modified birds also help find out novel immune functions of FcRY explaining the long half-life of IgY in blood. Finally, it would be

possible to provide a new strategy for increasing an amount of IgY in avian egg yolks and enhancing avian immunity by controlling the FcRY/IgY interaction. A careful consideration of the immune abnormality caused by the modified FcRY/IgY interaction is necessary to achieve that aim.

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.

Ethics statement

The animal study was approved by Nagoya University Policy on Animal Care and Use. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

MO: Investigation, Writing – original draft. RS: Investigation, Writing – review & editing. KoI: Investigation, Writing – review & editing. KD: Investigation, Writing – review & editing. FT: Investigation, Writing – review & editing. KO: Methodology, Writing – review & editing. TK: Methodology, Writing – review & editing. SM: Writing – review & editing. KeI: Methodology, Writing – review & editing. TY: Methodology, Writing – review & editing. KF: Writing – review & editing. MK: Writing – review & editing. FH: Writing – review & editing. AM: Project administration, Writing – original draft.

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Conflict of interest

The 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.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2024.1305587/full#supplementary-material

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Antigen specificity affects analysis of natural antibodies

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Natural antibodies are used to compare immune systems across taxa, to study wildlife disease ecology, and as selection markers in livestock breeding. These immunoglobulins are present prior to immune stimulation. They are described as having low antigen specificity or polyreactive binding and are measured by binding to self-antigens or novel exogenous proteins. Most studies use only one or two antigens to measure natural antibodies and ignore potential effects of antigen specificity in analyses. It remains unclear how different antigen-specific natural antibodies are related or how diversity among natural antibodies may affect analyses of these immunoglobulins. Using genetically distinct lines of chickens as a model system, we tested the hypotheses that (1) antigen-specific natural antibodies are independent of each other and (2) antigen specificity affects the comparison of natural antibodies among animals. We used blood cell agglutination and enzyme-linked immunosorbent assays to measure levels of natural antibodies binding to four antigens: (i) rabbit erythrocytes, (ii) keyhole limpet hemocyanin, (iii) phytohemagglutinin, or (iv) ovalbumin. We observed that levels of antigen specific natural antibodies were not correlated. There were significant differences in levels of natural antibodies among lines of chickens, indicating genetic variation for natural antibody production. However, line distinctions were not consistent among antigen specific natural antibodies. These data show that natural antibodies are a pool of relatively distinct immunoglobulins, and that antigen specificity may affect interpretation of natural antibody function and comparative immunology.

KEYWORDS

chickens, disease ecology, immunoglobulin, innate immunity, selection

1 Introduction

Natural antibodies (NAbs) are a class of immunoglobulins present in blood for which the immune system has had no prior stimulation (1–5) and which can function in the innate immune response (6–8). These immunoglobulins are variously described as polyreactive or having low antigen specificity (8–10). Natural antibodies are commonly measured using antigens that animals have not previously encountered such as keyhole limpet hemocyanin (KLH) or self-antigens like ovalbumin (OVA) (11–13). The NAbs may be directly protective by neutralizing pathogens (14–16), and they may facilitate the adaptive immune response by

supporting pathogen phagocytosis and antigen presentation (11, 17). Due to their defensive properties and their function as a bridge between the innate and adaptive compartments of the immune system, NAbs have been studied in the contexts of disease, ecology, evolution, and comparative immunology (15, 18–20). Experimental infections under laboratory conditions have shown that higher levels of NAbs are associated with lower infection intensities (21) and a higher probability of survival (1, 22). However, some NAbs appear inversely associated with higher survival probability (22). Studies of wildlife suggest that individuals with higher levels of NAbs have greater bactericidal capabilities (8, 18, 23, 24) and resistance to parasites (7, 25, 26). The NAbs appear to be correlated with other immunological factors (27–29) and vary among sexes (30), populations (31), life stages (32), food resources (33–35), and seasons (36). In animal agriculture, NAbs have been used as selection traits to enhance disease resistance (37–42). Finally, given that NAbs are thought to bridge innate and adaptive immune responses, they have been used to compare immune systems among different taxa (27, 28, 43, 44) and to explore tradeoffs in the evolution of immune systems (19, 30). Other than the mouse (*Mus musculus*), the chicken (*Gallus gallus*) is the most widely used animal model for the study of NAbs (1, 3, 6, 22, 27, 33, 39–42, 45–61). As early as 1923 natural antibodies were identified in chickens (53). Subsequently, NAbs have been linked to poultry survival (1, 45, 56) and pathogen resistance (39–41). The genetic basis for variation in NAbs has been explored using established genetic lines of poultry (6, 22, 40, 42, 47–49, 52, 55) and with modern genomics tools (62–66). The chicken has been a vital model for developing NAb assays used for wild bird species (25, 27, 28, 43). However, given the agricultural and economic importance of poultry, the primary application of NAb research on chickens has been in selective breeding for enhanced immune defense (39–42, 46, 49, 51, 58, 60).

Although some functional studies of NAbs are based on isolated B-1 cell lines in mice (18, 67–71), most studies of NAbs are based on measures of circulating immunoglobulins in serum or plasma (6, 18, 37, 38, 43). These measures are commonly done using one of two methods – (1) hemagglutination assay (HA) or (2) enzyme-linked immunosorbent assay (ELISA). Hemagglutination assays measure *in vitro* antibody binding and cross-linking of vertebrate red blood cells (72). In hemagglutination assays, antibodies bind to proteins on the surface of red blood cells, causing clumping (agglutination) of the red blood cells in a microtiter well (17, 72). The antibody titer is calculated based on a dilution series of sera or plasma incubated with a fixed concentration of red blood cells, and the experimenter determines the dilution point when agglutination fails to occur (3, 27). Hemagglutination assays are often employed in wildlife studies (23, 28, 30, 44) and are popular because they do not require special reagents or equipment (27). The ELISA uses a microtiter plate coated with a specific antigen and then filled and incubated with sera or plasma from the study animal. Antibodies in the sera/plasma that bind to the plate-affixed antigen can be quantified using a secondary antibody conjugated to a colorimetric enzyme or fluorescence marker detected by a spectrophotometer (17, 72, 73). The ELISA approach is frequently used in laboratory and livestock research (43, 74–76). Different antigens are used to measure NAbs with both methods. Hemagglutination assays may use blood cells from rabbits, sheep, chickens, or fish (27, 47–50, 77). Enzyme-linked immunosorbent assays may use antigens from bacteria (e.g., lipopolysaccharide), invertebrates (e.g., keyhole limpet hemocyanin), plants (e.g., phytohemagglutinin), or vertebrates (e.g., ovalbumin) (11, 51, 52).

These different methods measure NAbs binding to specific antigens, and it is unclear how these antigen-specific NAbs are related. Though NAbs are expected to be polyreactive (24, 78–81), studies of monoclonal NAbs reveal that NAbs do not bind equally well to all antigens. For example, Gunti et al. (8) showed that NAbs from a single B-1 clone exhibited wide variation in binding to different bacteria, including species from the same genus. Importantly, different NAbs can have unique (non-overlapping) ranges of antigen binding (82, 83). Baumgarth et al. (9, 84) argue that immunological defense by NAbs results from broad antigen reactivity of both individual NAbs and the natural antibody repertoire. These studies underscore that NAbs are not uniform and the NAb repertoire reflects a diverse pool of immunoglobulins with potentially unique but complimentary antigen specificities. Despite these insights, the majority of NAb studies are based on immunoglobulin measures using only one or two antigens (6, 19, 22, 27, 28, 30, 34, 42, 49, 51, 60, 74, 76, 85–97). This raises an important question – are NAbs measured with one antigen representative of the broader repertoire of NAbs? This is a vital question, given that many studies of NAbs infer immune function (14, 22, 27, 30, 76, 86, 91, 98), ecological relationships (25, 28, 34, 99), and evolution (19, 25, 42, 49, 97, 100) based on one or two NAb antigens. Additionally, the antigens used vary widely across studies (22, 27, 60, 89), which makes it difficult to synthesize results among different study systems.

To our knowledge, comparisons of circulating NAbs have not been reported in the literature. Here, we tested two related hypotheses regarding NAb specificity (1): Levels of antigen-specific NAbs are independent; (2) Antigen specificity affects comparison of natural antibodies among animals. We tested these hypotheses by measuring NAb levels among genetically distinct chicken breeds and selection lines, using different antigens with agglutination and ELISA methods. We discuss the potential importance of antigen specificity to the study of NAb function and comparative immunology.

2 Materials and methods

2.1 Poultry

Plasma was collected from female chickens in eight selection lines across three breeds: White Leghorn (WL), White Plymouth Rock (WPR), and Rhode Island Red (RIR). These three breeds are distinct, having been historically selected for different physical characteristics including feather color and eggshell color. Each was defined as a different breed over 100 years ago (101). The different lines within each breed have been separated from one another since the 1940s through artificial selection on egg production, body weight, and feed conversion efficiency (102). The Rhode Island Red breed is a cross of three breeds (Malay

Game, Leghorn, and Asian native) originating in Europe around 100 B.C (103, 104). The Leghorn breed originated in Italy in 400 B.C. and are classified as egg layers (103, 104) and are the most commonly utilized breed for white egg shell production. They exhibit a range of plumage colors, with white being the most common (104). The White Plymouth Rock was one of the breeds used to develop the modern commercial meat (broiler) bird in the early part of the 20th century (104). The Rhode Island Red and Plymouth Rock breeds are used for commercial brown egg production. All birds used in this study were from elite egg layer lines, selected for generations for multiple egg production related traits. These are all pure lines, not commercial cross production birds. They were maintained at the breeding facilities of Hy-Line International. The housing was in single cages, under standard production conditions with feed and water provided *ad libitum*.

2.2 Plasma samples

Blood was collected from birds in each line at ten weeks of age, and plasma samples were separated from blood by centrifugation at 240 x g for three minutes. After collection, plasma samples were split into aliquots for the different assays, then stored at -20°C until use. Sample sizes ranged from 98–106 birds per line with eight lines total across three breeds (two for Rhode Island Red, four for White Leghorn, and two for White Plymouth Rock), with a total of 813 samples for the ELISA assays, and sample sizes ranged from 10–25 birds per line, with a total of 119 samples for the rabbit blood cell hemagglutination (HA) assay.

2.3 Agglutination assay

Measures of NAbs binding to rabbit red blood cells (anti-rRBC NAbs) were determined using the hemagglutination assay (HA) (27, 28). A 1% rabbit blood cell (RBC) suspension was prepared using rabbit whole blood in Alsever's solution (catalog # RBA050, Hemostat Laboratories, Dixon, CA, USA). Briefly, RBCs in whole blood were washed four times with centrifugation at 241.5 x g for 5 min in phosphate-buffered saline (PBS, catalog# P3813, Sigma-Aldrich, St. Louis, MO, USA). Lysed red blood cells were removed with the supernatant after each wash. The washed cell concentration was measured using duplicate hematocrit capillary tubes following sample centrifugation at 0.004 x g for 1 min. Additional PBS was added to make a 1% red blood cell suspension with confirmation of the concentration via hematocrit. Each HA assay was done using a 96-well microtiter plate (CorningTM Clear Polystyrene 96-Well Microplates, catalog# 3795, Thermo Scientific, Rockford, IL, USA). Each row of wells tested plasma from a single bird, with 2x dilutions of the plasma from the first well (25 µl undiluted plasma) through the 11th well (1/1024 dilution of plasma in PBS; 25 µl total volume). Seven rows contained experimental samples (7 birds) and one row contained a positive control sample of pooled plasma. Column 12 wells contained 25µl PBS only, as a negative control. After plasma samples were distributed, 25µl of the 1% rabbit blood cell suspension were added to each well. The plates were sealed with Parafilm to prevent evaporation. Plates were placed on an orbital for 10 seconds and incubated in a 37°C water bath for 90 min. Plates were removed and placed at a 45-degree angle (on the long axis) for 20 min at room temperature to enhance the visualization of agglutination. We photographed and re-sealed each plate before incubation for an additional 70 minutes at a 45-degree angle at room temperature. We then photographed each plate again. Chicken samples were numerically coded and randomized across plates to avoid observer bias. The plates were scored blindly at the time of assay. The same researcher scored the photos of assays later to validate the original scores (27). Scores were recorded as the highest dilution (i.e., well number) at which agglutination occurred. Due to time constraints, we did not conduct HA assays on all plasma samples. A random subset of samples from each line of chicken breed was selected. The HA sample sizes matched or exceeded sample sizes reported in the literature for this assay (27, 28, 105–107). Sample sizes for the HA assay were Rhode Island Red (n=29), White Leghorn (n=59), and White Plymouth Rock (n=31) (Table 1).

2.4 Enzyme-Linked Immunosorbent Assay (ELISA)

We developed indirect ELISAs to quantify IgY NAbs binding to each of three antigens - keyhole limpet hemocyanin (KLH; Hemocyanin from *Megathura crenulate* (keyhole limpet), catalog #

TABLE 1 Sample sizes (n) of three breeds (Rhode Island Red, White Leghorn, and White Plymouth Rock) consisting of eight lines (RIR 1, RIR 2, WL 1, WL 2, WL 3, WL 4, WPR 1, and WPR 2) for assays (ELISA and HA) used to measure natural antibodies.

	Breed	n	Line	n
ELISA	*Rhode Island Red*	203	RIR 1	102
			RIR 2	101
	White Leghorn	402	WL 1	100
			WL 2	100
			WL 3	100
			WL 4	102
	White Plymouth Rock	208	WPR 1	106
			WPR 2	102
HA	*Rhode Island Red*	29	RIR 1	16
			RIR 2	13
	White Leghorn	59	WL 1	10
			WL 2	18
			WL 3	18
			WL 4	13
	White Plymouth Rock	31	WPR 1	14
			WPR 2	17

H7017–20MG, Sigma-Aldrich, St. Louis, MO, USA), phytohemagglutinin (PHA; Phytohemagglutinin-L, catalog # 11249738001, Sigma-Aldrich, St. Louis, MO, USA), and ovalbumin (OVA; Imject ™ Ovalbumin, catalog # 77120, Thermo Scientific, Rockford, IL, USA) using a single dilution approach (108–110). Each assay was optimized based on dilutions of antigen and plasma (i.e., checkerboard titration) with the following steps: (Step 1) 96-well clear microtiter plates (Immulon ® 4HBX Flat Bottom Microtiter® Plates, Thermo Scientific, Rockford, IL, USA) were coated with an antigen diluted in *coating buffer* (Carbonate-Bicarbonate Buffer, catalog # C3041–100CAP, Sigma-Aldrich, St. Louis, MO, USA), with 100µl of solution per well. Antigen concentrations ranged from 1x (stock solution) in row A to 1:2000 dilution in row H. The plate was incubated at room temperature on an orbital shaker for 1 hour. (Step 2) The plate was washed with *Tris buffer* (Tris Buffered Saline, with Tween ® 20, pH 8.0, catalog # T9039–10PAK, Sigma-Aldrich, St. Louis, MO, USA) three times. (Step 3) The wells were filled (300 µl/well) with *blocking buffer* (Pierce ™ Protein-Free Blocking Buffer, catalog # 37572, Thermo Scientific, Rockford, IL, USA) to cover any open binding surfaces on the plate. The plate was incubated at room temperature on an orbital shaker for 30 minutes. (Step 4) The plate was washed with *Tris buffer* three times. (Step 5) A pooled plasma sample was diluted in *sample buffer* (50 mM Tris buffered saline, pH 8.0, 1% BSA; Sigma Chemical # T6789; 10% Tween 20, Tween 20; Sigma Chemical P7949) and added to the plate (100µl/well) so that plasma concentrations ranged from 1x (undiluted) in column one to 1:2048 in column 12. The plate was incubated at room temperature on an orbital shaker for 1 hour. (Step 5) The plate was washed with *Tris buffer* three times. (Step 6) D*etection antibody* (anti-Chicken IgY – Fc Fragment Antibody with HRP conjugate; A30–104P, Bethyl Laboratories Inc., Texas, USA) was diluted in *sample buffer* 1:200,000 and added to all wells (100µl/well). The plate was covered with aluminum foil and incubated on an orbital shaker for 1 hour at room temperature. (Step 7) The plate was washed with *Tris buffer* three times. (Step 8) 100µl TMB (3,3',5,5'-Tetramethylbenzidine Liquid Substrate System, catalog # T8665–1L, Sigma-Aldrich, St. Louis, MO, USA) were added to each well and the plate was incubated on an orbital shaker for 15 min. (Step 9) We added 100µl of *stop solution* (BioFX ® 450nm Liquid Nova-Stop Solution for TMB Microwell Substrates, NC1026538, Thermo Scientific, Rockford, IL, USA) to each well. (Step 10) The plate was scanned at 450 nm with a spectrophotometer (Imark™ Microplate reader, Bio-RAD, Hercules, CA, USA) to determine the optical density (OD) in each well. Optical density correlates with the amount of antibody bound to the substrate antigen. We confirmed that OD values decreased as antigen dilution increased and as plasma dilution increased. This validated antibody-antigen binding in the assay. We compared the OD curves for the plasma dilutions among the different antigen concentrations. We selected the antigen concentration that yielded an OD curve with the steepest slope across plasma dilutions, because this reflected the antigen concentration most sensitive to changes in antibody concentration. Once the optimal concentration was determined for each antigen, the selected concentration was used for all sample assays. The selected antigen concentrations were KLH (1:40 dilution), PHA (1:500 dilution), and OVA (1:20 dilution). All assays used plasma samples diluted 1:100 and each sample was analyzed in triplicate wells (100µl/well). In addition, all assays included a positive control sample of pooled plasma and a negative control of *sample buffer* only. The OD value of the negative control (buffer only) was subtracted from all sample wells, and we determined the coefficient of variation (CV) for the optical densities of triplicate wells for each sample. For samples with a CV <20%, we averaged the triplicate well OD values to use in statistical analyses. Samples with CV values >20% were excluded from analyses. Sample sizes for ELISAs were as follows: Rhode Island Red (n=203), White Leghorn (n=402), and White Plymouth Rock (n=208) (Table 1).

2.5 Statistical methods

All sample data were standardized against positive controls by calculating the ratio of the sample value (S_i) to the value of the positive control (P) on the plate ($x_i = S_i/P$). This controlled any assay variation among different plates. We then normalized S/P values (x_i) of each assay by scaling from 0 to 1 with the following formula: $z_i = (x_i - \min(x))/(\max(x) - \min(x))$, where $x = (x_1,\ldots,x_n)$ and z_i is the i^{th} normalized data value. This was done to enable direct comparisons of assay results on a common scale. To analyze relationships between antigen-specific NAbs, we calculated Spearman's correlation coefficients for each pairwise combination of NAb assay antigens with the *psych* package in R (111). We expected that any correlated NAbs would reflect cross-reactivity of polyreactive NAbs or tightly linked production of antigen-specific NAbs. Conversely, uncorrelated NAbs would indicate antigen-specific NAbs are independent. We compared NAb levels among chicken breeds and lines for each antigen-specific NAb using Generalized Linear Models (GLM) with *stats package* in R (112). The GLM models were NAb_x level ~ Line + Breed, where NAb_x represents the normalized assay values for antigen x. *Post hoc* pairwise comparisons were evaluated using the *emmeans package* (113). Significant effects of chicken line or breed on NAb levels indicate genetic variation for NAb expression. Statistical differences in NAb levels among lines and breeds were qualitatively compared among assay antigens, to determine if antigen specificity affects comparisons of NAbs among genetically distinct animals.

3 Results

3.1 Correlations among antigen-specific NAbs

The correlation coefficients of antigen-specific NAb levels ranged from 0.10 to 0.31, indicating that antigen-specific NAb levels were independent (Figure 1). As a result, antigen-specific

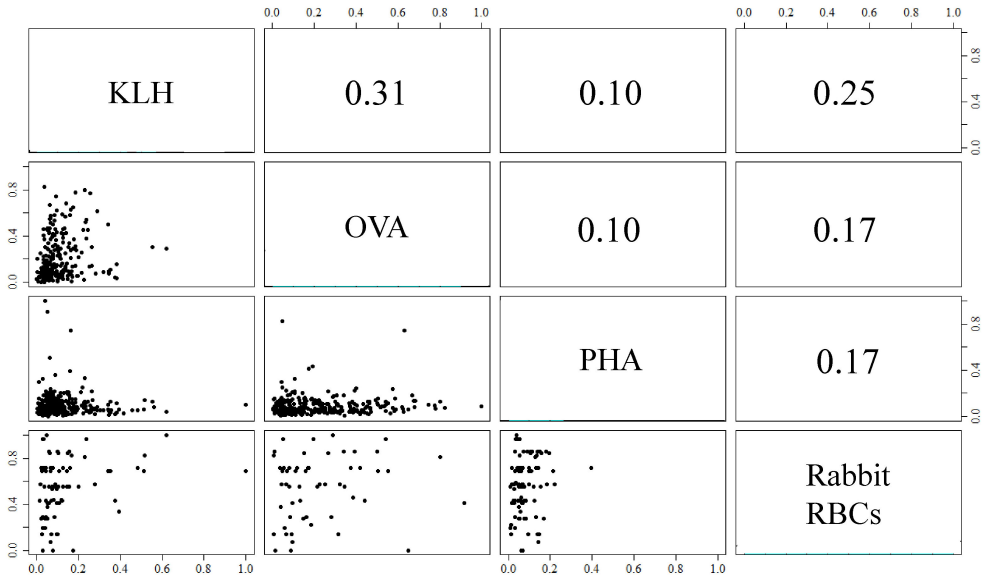

FIGURE 1
Pairwise correlations among levels of antigen-specific natural antibodies (NAbs) binding molecules ovalbumin (OVA), keyhole limpet hemocyanin (KLH), phytohemagglutinin (PHA), and rabbit red blood cells (RBCs). The NAb measures were normalized to a 0 to 1 scale for comparison. Antigen-specific NAb expression of individual birds is shown in scatter plots below the diagonal and Spearman's correlation coefficients of NAb expression above the diagonal.

NAbs were considered independent and unique subsets of the overall NAb repertoire.

3.2 Agglutination assays of NAbs among breeds and lines

The genetic line of chickens affected anti-rRBC NAbs (GLM, t=2.56, p=0.01), but there was no effect of breed (GLM, t=1.30, p=0.20) (Figure 2). White Leghorn Line 3 had lower levels of anti-rRBC NAbs compared to line White Leghorn Line 4 (GLM, t=3.20, p=0.04), but no other pairwise line differences were observed.

3.3 ELISAs of NAbs among breeds and lines

The genetic line and breed of chicken affected levels of NAbs measured in all three ELISAs (Figure 3). Among the poultry lines there was a difference in expression of NAbs binding KLH (GLM, t=3.41, p=0.001), OVA (GLM, t=4.32, p<0.0001), and PHA (GLM, t=3.80, p=0.0002). *Post hoc* pairwise comparisons of lines revealed significant differences between 5 pairs of lines in the KLH assay, 17 pairs of lines in the OVA assay, and 5 pairs of lines in the PHA assay (Table 2). Among the poultry breeds, there was a difference in the expression of NAbs binding KLH (GLM, t=2.27, p=0.02) and OVA (GLM, t=8.20, p<0.001), but no difference in the expression of NAbs binding PHA (GLM, t=1.55, p=0.12). *Post hoc* pairwise comparisons of breeds revealed significant differences between 2 pairs of lines in the KLH assay, 3 pairs of lines in the OVA assay, and no pairs of lines in the PHA assay (Table 3).

4 Discussion

We compared natural antibody levels among different breeds and selected elite lines of poultry using two methods (i.e. ELISA and HA) to test the hypotheses that (1) antigen-specific NAbs are independent and (2) antigen specificity affects comparisons of NAbs among different animals. Our data supported both hypotheses. Correlations among antigen-specific NAbs were absent or weak, indicating that the measured NAbs did not have strongly overlapping antigen binding (i.e., similar polyreactivity), nor were they tightly linked (i.e. co-regulated). We observed significant differences in levels of antigen-specific NAbs among chicken breeds and lines, reinforcing the concept that NAbs are germline-encoded (6, 22, 40, 42, 48, 49, 52, 55). However, breed and line effects were not consistent among antigen-specific NAbs. For example, all three breeds differed in levels of anti-KLH and anti-OVA NAbs, but not anti-rRBC nor anti-PHA NAbs. Relative differences were not consistent among antigen-specific NAbs. For example, White Leghorn chickens had lower levels of anti-OVA NAbs compared to Rhode Island Red chickens, but levels of anti-KLH NAbs were similar between the two breeds. Levels of anti-OVA NAbs were different among all eight lines. In comparison, anti-KLH and -PHA NAbs were different among six lines, and anti-rRBCs NAbs differed among only two lines. These data show that subsets of NAbs have some degree of unique (non-overlapping) antigen specificity and are not tightly linked. Genetic differences among chickens affect levels of antigen-specific NAbs, but these genetic effects (i.e., breed and line differences) were not consistent among antigen-specific NAbs. These results raise important questions about the diversity of immunoglobulins in the NAb

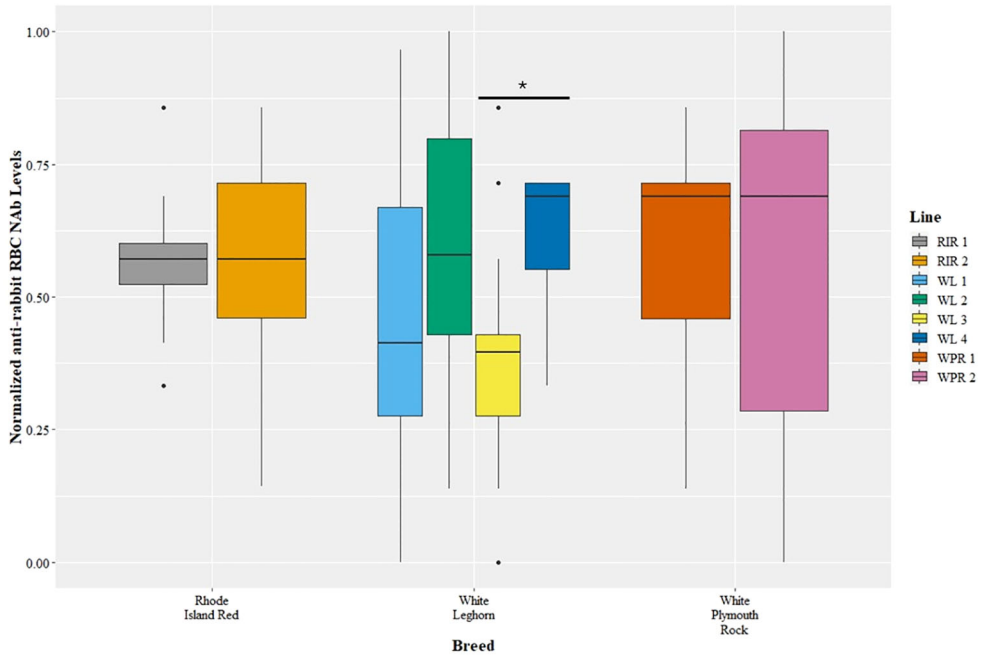

FIGURE 2
Levels of natural antibodies (NAb) binding rabbit red blood cells (rRBCs) were measured with hemagglutination assays. The anti-rRBC NAb levels were measured as the highest dilution of plasma that resulted in RBC agglutination. The anti-rRBC NAb levels were normalized to a 0 to 1 scale before comparison among breeds and lines. The NAb measures are shown for multiple breeds (x-axis) and lines (legend), with points representing outliers. There was a significant difference in anti-rRBC NAb level between lines WL3 and WL4 (*post hoc*, $p < 0.05$), but no differences among breeds.

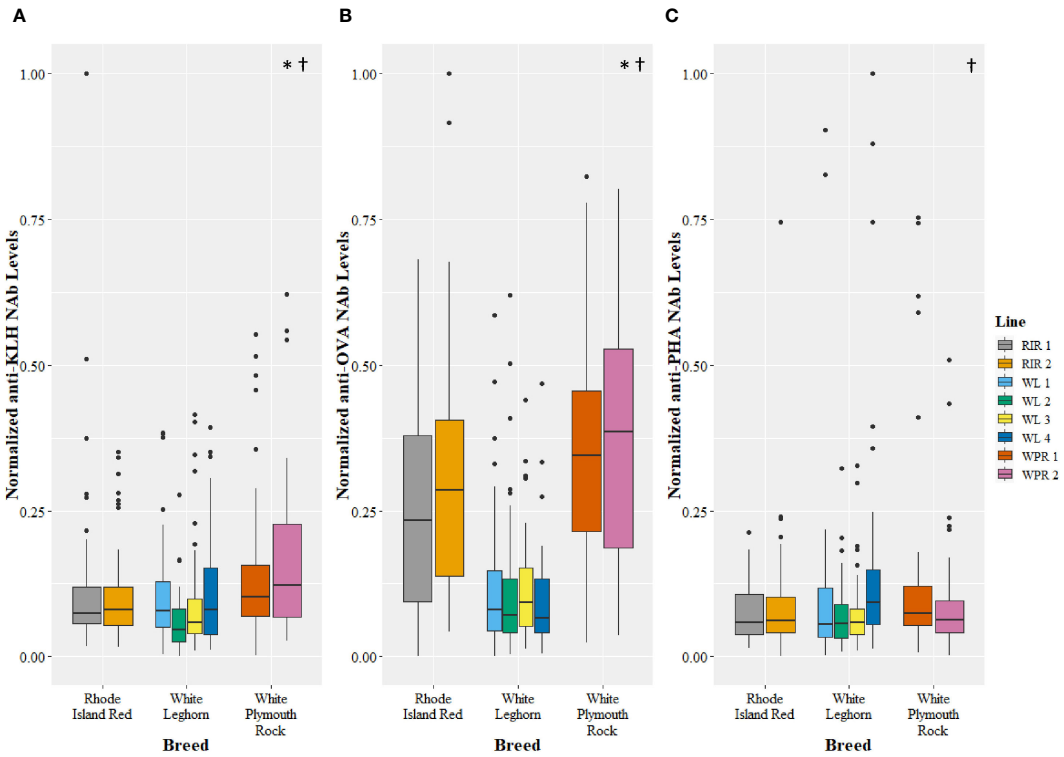

FIGURE 3
Levels of natural antibodies (NAb) binding keyhole limpet hemocyanin (KLH) **(A)**, ovalbumin (OVA) **(B)**, and phytohemagglutinin (PHA) **(C)** measured using enzyme-linked immunosorbent assays and recorded as optical density units with spectrophotometry. The optical density values were normalized to a 0 to 1 scale before comparison among breeds and lines. The antigen-specific NAb levels are shown for multiple breeds (x-axis) and lines (legend), with single points representing outliers. Symbols represent significant differences ($p<0.05$). The (*) symbol indicates a difference in NAb levels among breeds, and the (†) symbol indicates a difference in NAb levels among lines. See Tables 2, 3 for pairwise comparisons.

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TABLE 2 Pairwise comparisons of NAbs binding KLH, OVA, PHA, and rRBCs among poultry lines (RIR 1, RIR 2, WL 1, WL 2, WL 3, WL 4, WPR 1, and WPR 2).

	Contrast	Estimate	SE	df	t ratio	p value
KLH	*RIR 1 - WL 2*	0.066	0.019	459	3.409	0.0162
	WL 1 - WPR 2	-0.063	0.020	459	-3.098	0.0428
	WL 2 - WPR 1	-0.081	0.018	459	-4.423	0.0003
	WL 2 - WPR 2	-0.106	0.020	459	-5.206	0.0000
	WL 3 - WPR 2	-0.078	0.020	459	-3.898	0.0028
OVA	*RIR 1 - WL 1*	0.136	0.031	370	4.318	0.0005
	RIR 1 - WL 2	0.141	0.034	370	4.214	0.0008
	RIR 1 - WL 3	0.127	0.035	370	3.567	0.0096
	RIR 1 - WL 4	0.154	0.037	370	4.202	0.0009
	RIR 1 - WPR 2	-0.113	0.034	370	-3.323	0.0217
	RIR 2 - WL 1	0.192	0.031	370	6.269	$p<0.0001$
	RIR 2 - WL 2	0.198	0.033	370	6.027	$p<0.0001$
	RIR 2 - WL 3	0.183	0.035	370	5.257	$p<0.0001$
	RIR 2 - WL 4	0.210	0.036	370	5.851	$p<0.0001$
	WL 1 - WPR 1	-0.233	0.030	370	-7.738	$p<0.0001$
	WL 1 - WPR 2	-0.249	0.031	370	-8.024	$p<0.0001$
	WL 2 - WPR 1	-0.238	0.032	370	-7.386	$p<0.0001$
	WL 2 - WPR 2	-0.254	0.033	370	-7.676	$p<0.0001$
	WL 3 - WPR 1	-0.224	0.034	370	-6.520	$p<0.0001$
	WL 3 - WPR 2	-0.240	0.035	370	-6.823	$p<0.0001$
	WL 4 - WPR 1	-0.251	0.035	370	-7.080	$p<0.0001$
	WL 4 - WPR 2	-0.267	0.036	370	-7.367	$p<0.0001$
PHA	*RIR 1 - WL 4*	-0.060	0.016	658	-3.802	0.0039
	RIR 2 - WL 4	-0.049	0.016	658	-3.049	0.0489
	WL 2 - WL 4	-0.066	0.016	658	-4.234	0.0007
	WL 3 - WL 4	-0.062	0.015	658	-4.027	0.0016
	WL 4 - WPR 2	0.052	0.015	658	3.378	0.0175
rRBC	*WL 3 - WL 4*	-0.262	0.082	111	-3.188	0.0380

*tukey p-value adjustment for multiple comparisons.
Only significant comparisons are shown ($p<0.05$).

repertoire and the potential importance of antigen-specificity to NAb function and comparative immunology.

A hallmark of NAbs is polyreactivity (8, 9, 114) and binding to diverse antigens has been clearly demonstrated with studies using NAbs from monoclonal B-1 cells (7–9, 18, 24, 26). However, evidence in the literature illustrates that NAbs can have unique, non-overlapping ranges of antigen binding (8, 24) and structural diversity at the antigen binding site (114). Several reviews of NAbs have mentioned diversity of immunoglobulins in the NAb repertoire (11, 13) and this diversity is suggested to be essential to immune defense (9, 84). Despite this information, nearly all studies of NAbs are based on one or two antigens and the antigens used among studies are often different. Should we expect that measures of antigen-specific NAbs represent the broader NAb repertoire or reflect the activity of other natural antibodies? Evidence from the literature suggests that antigen-specific NAbs are not interchangeable.

In two notable studies, different red blood cells were used for hemagglutination assays of NAbs from the same animals. Bailey (53) observed that poultry sera agglutinated rabbit and rat red blood cells at a titer four times higher than the agglutination threshold for red blood cells from guinea pigs or frogs, and >20 times higher than

TABLE 3 Pairwise comparisons of NAbs binding KLH, OVA, PHA, and rRBCs among poultry breeds (Rhode Island Red, White Leghorn, and White Plymouth Rock).

	Contrast	Estimate	SE	df	t ratio	p value
KLH	*Rhode Island Red - White Leghorn*	0.027	0.012	464	2.265	0.0618
	Rhode Island Red - White Plymouth Rock	-0.033	0.014	464	-2.364	0.0480*
	White Leghorn - White Plymouth Rock	-0.060	0.012	464	-5.067	$p<0.0001$*
OVA	*Rhode Island Red - White Leghorn*	0.168	0.020	375	8.201	$p<0.0001$*
	Rhode Island Red - White Plymouth Rock	-0.075	0.023	375	-3.215	0.0040*
	White Leghorn - White Plymouth Rock	-0.243	0.020	375	-12.092	$p<0.0001$*
PHA	*Rhode Island Red - White Leghorn*	-0.014	0.010	663	-1.355	0.3654
	Rhode Island Red - White Plymouth Rock	-0.018	0.012	663	-1.546	0.2701
	White Leghorn - White Plymouth Rock	-0.004	0.010	663	-0.443	0.8975
rRBC	*Rhode Island Red - White Leghorn*	0.069	0.053	116	1.300	0.3980
	Rhode Island Red - White Plymouth Rock	-0.019	0.061	116	-0.306	0.9497
	White Leghorn - White Plymouth Rock	-0.088	0.052	116	-1.686	0.2150

*tukey p-value adjustment for multiple comparisons.
Significant differences are indicated by the * symbol ($p<0.05$).

the threshold for cells from sheep, turtle, or goat blood (53). Seto and Henderson (3) observed poultry sera agglutinated mouse red blood cells at a titer 1.5x higher than hamster red blood cells and 12x higher than blood cells from rabbits or sheep (3). The wide variation in NAb titers among different vertebrate red blood cells indicates that each assay measured a different antigen-specific natural antibody. This is perhaps unsurprising, given more recent analyses of the protein composition of red blood cells. Using quantitative mass spectrometry, Sae-Lee et al. (115) identified 1,944 distinct protein groups in human red blood cells, and Matei et al. (116) described multiple electrophoretic differences in red blood cell proteins among eight animal species, including sheep, rabbit, rat, and mouse. Molecular analyses of antigens used in NAb ELISAs also suggest significant structural diversity that could affect binding specificity. The KLH molecule, derived from the mollusk *Megathura crenulate*, is a ~390 kDa polypeptide with eight globular units (117). The OVA molecule, derived from egg white, is a 44.5 kDa glycoprotein with a tertiary structure (118, 119). Finally, a legume plant produces the PHA molecule, a 30.5 kDa glycoprotein with a quaternary structure (120). As with the agglutination assay, these molecular differences likely contribute to epitope variation among ELISAs. These antigen and epitope differences among NAb assays likely result in measurements of different subsets of NAbs (24, 70, 78, 121) which may affect analyses of NAbs relative to species differences, immune function, and ecology (27, 28, 30, 93).

Many studies have shown species and breed effects on levels of NAbs (6, 27, 28, 40, 42). However, to our knowledge, antigen specificity of NAbs has not been considered in comparative studies. Perhaps the best cited example of using NAbs as a comparative trait is Matson et al. (122). In that study, the authors compared levels of NAbs using agglutination assays with rabbit and trout red blood cells. Although the average NAb levels in the two assays were correlated ($R^2 = 0.62$), there were cases where species effects were not identical for anti-rabbit and anti-trout RBC immunoglobulins. For example, the Chilean pintail (*Anas georgica spinicauda*) and white-winged wood duck (*Cairina scutulata*) had similar titers of NAbs binding rabbit red blood cells but dissimilar titers of NAbs binding trout red blood cells. In contrast, South Georgia pintail (*Anas georgica georgica*) and black-bellied tree duck (*Dendrocygna autumnalis*) had the opposite pattern (122). Other studies have reported variation in NAbs among chicken breeds (22, 55), but breed effects are not identical among NAb antigens (40). These inconsistencies among antigen-specific NAbs may reflect complexity in the genetic control of natural antibody diversity (62–65, 114).

Natural antibodies are considered important to innate immune defense (9, 11, 13), but it remains unclear if antigen specificity of NAbs affects defense. Monoclonal NAbs do not bind uniformly to all pathogens (8, 24) and they do not neutralize different pathogens equally *in vitro* (24). This suggests that antigen specificity could affect defenses provided by NAbs. Animal studies with chickens and pigs have reported positive (41, 123) or absent (1, 56) relationships between antigen-specific NAbs and survival. In pigeons, Owen et al. (21) observed that some antigen-specific NAbs were predictive of bird resistance to internal and external parasites, but other antigen-specific NAbs were not. In addition, the authors showed that pigeons with a more diverse repertoire of antigen-specific NAbs were more resistant to parasites (21). These various studies suggest that antigen-specific NAbs provide defense against a restricted range of parasites or pathogens, reinforcing the idea that immune defense from NAbs relies on a diverse repertoire of these immunoglobulins (9, 84).

The mechanism(s) of defense by NAbs are not entirely understood and remain an active area of investigation (1–6, 11, 14–17, 54, 100, 106, 107). Although NAbs are germline-encoded and produced prior to infection, evidence suggests that production of NAbs can increase following immune stimulation (52, 54, 68,

106, 106, 110, 111). However, increased production of NAbs appears to be antigen specific. Some antigen-specific NAbs increase with immune challenge, but others remain unchanged (27, 52, 54, 106, 112, 113). In addition, relationships between NAbs, adaptive antibodies, and other immune effectors vary depending on antigen specificity (49, 83). Matson et al. (122) measured 13 immunological parameters that included NAbs, complement proteins, antimicrobial activity, and leukocyte counts from 10 species of waterfowl. The authors used principal component analysis as a statistical approach to group correlated variables. The NAb and complement levels were measured using the hemagglutination-hemolysis assay that combines a measure of complement protein activity (blood cell lysis) with NAbs that mediate the binding of complement to the target blood cells (i.e., classical complement pathway) (21, 24, 77). As discussed above, Matson et al. (122) used trout and rabbit red blood cells in separate assays of the same samples. The lysis (complement) and antimicrobial measures grouped into different PCs based on antigen. One PC showed a positive correlation with trout cell lysis and killing of *Staphylococcus aureus* bacteria. In contrast, a different PC correlated positively with rabbit cell lysis but negatively with the killing of *S. aureus*. In this example, relationships between NAbs, complement, and bacteria killing changed depending on NAb antigen specificity (100). These studies strongly suggest that antigen-specificity of NAbs affects interactions with pathogens and the defenses provided by these immunoglobulins. Accurate and comprehensive understanding of NAbs and immune function requires consideration of antigen specificity.

The data reported here reveal that natural antibodies in poultry are composed of a diverse repertoire of immunoglobulins. Our data align with previous studies of NAbs in chickens that show both breed and selection line affect natural antibody levels (6, 22, 40, 42, 47–49, 52, 55). Selective breeding of poultry has revealed that some antigen-specific NAbs are associated with pathogen resistance (39–41) and survival (1, 56). However, the mechanism(s) of these effects are unknown. Natural antibodies may provide important immune defenses in chickens or serve as relevant markers for selection. In either case, identifying the effects of antigen specificity could reveal more targeted strategies for improving traits in these important livestock.

5 Conclusions

Natural antibodies are important components of the vertebrate immune system, and these molecules have proven valuable in studies of immune function, ecology, evolution, and livestock selection. These molecules are defined as polyreactive with low specificity (11, 13), but evidence from our studies and the literature reveal that antigen-specific NAbs are independent and have unique interactions with pathogens and parasites (8, 24). Thus, antigen-specific NAbs are not interchangeable. A comprehensive understanding of NAbs requires consideration for functional differences between antigen-specific NAbs and characterization of immunoglobulin diversity in the NAb repertoire. This leads to the question of how researchers should proceed with measurements of NAbs? Natural antibodies should be appreciated as a group of diverse immunoglobulins rather than treated as a singular effector (9, 84). Going forward, we recommend three approaches to the study of NAbs. The first is to reconcile the similarities and differences among antigen-specific NAbs (114). For example, by combining western blot analyses and affinity chromatography, researchers may be able to identify how antigen-specific NAbs are related (46, 51, 124, 125). Second, researchers could use a panel of antigens to measure the NAb repertoire (21), or design ELISAs that utilize several antigens at once (e.g., multiplex ELISA) (21, 126–128). These approaches would yield more complete measures of the NAb repertoire. Finally, as biochemical and genetic resources become available for non-model animal species, researchers should endeavor to determine the cellular and molecular characteristics of NAbs in different animal species.

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 approved by WSU Institutional Animal Care and Use Committee. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

KW: Data curation, Formal analysis, Investigation, Visualization, Writing – original draft, Writing – review & editing. JF: Resources, Writing – review & editing. JO: Conceptualization, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Supervision, Validation, Visualization, Writing – review & editing.

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Conflict of interest

The 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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Publisher's note

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Morphologic characterization and cytokine response of chicken bone-marrow derived dendritic cells to infection with high and low pathogenic avian influenza virus

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Dendritic cells (DCs) are professional antigen-presenting cells, which are key components of the immune system and involved in early immune responses. DCs are specialized in capturing, processing, and presenting antigens to facilitate immune interactions. Chickens infected with avian influenza virus (AIV) demonstrate a wide range of clinical symptoms, based on pathogenicity of the virus. Low pathogenic avian influenza (LPAI) viruses typically induce mild clinical signs, whereas high pathogenic avian influenza (HPAI) induce more severe disease, which can lead to death. For this study, chicken bone marrow-derived DC (ckBM-DC)s were produced and infected with high and low pathogenic avian influenza viruses of H5N2 or H7N3 subtypes to characterize innate immune responses, study effect on cell morphologies, and evaluate virus replication. A strong proinflammatory response was observed at 8 hours post infection, via upregulation of chicken interleukin-1β and stimulation of the interferon response pathway. Microscopically, the DCs underwent morphological changes from classic elongated dendrites to a more general rounded shape that eventually led to cell death with the presence of scattered cellular debris. Differences in onset of morphologic changes were observed between H5 and H7 subtypes. Increases in viral titers demonstrated that both HPAI and LPAI are capable of infecting and replicating in DCs. The increase in activation of infected DCs may be indicative of a dysregulated immune response typically seen with HPAI infections.

KEYWORDS

chicken, innate immunity, dendritic cells, avian influenza, cytokines, interferon

1 Introduction

In recent years, avian influenza virus (AIV) has been one the leading causes of infection-based poultry mortality and morbidity. Prior to the 1990s, AIV outbreaks in domesticated poultry were rare, however ongoing outbreaks of highly pathogenic avian influenza (HPAI) have occurred globally for the past several years (1–3). The H5 A/goose/Guangdong/1996 (H5-Gs/Gd) lineage is responsible for most of the outbreaks, as the current clade 2.3.4.4b viruses appear to be highly adapted to migratory waterfowl (3). As a result of the adaptation, more spill over into domesticated poultry, mammals, and humans have been observed (4). High morbidity and mortality rates have led to reduced poultry production, embargoes on countries of origin, and increased expenses associated with vaccinating and controlling AIV within the global poultry industry (5, 6). In 2022, a total of 67 countries reported HPAI outbreaks, resulting in the deaths of 131 million poultry and wild birds (7, 8). In the U.S., the ongoing 2022-2024 HPAI H5N1 outbreak has resulted in the loss of over 60 million birds and $3 billion dollars in economic damages (1, 9).

Low pathogenic avian influenza (LPAI) viruses typically cause a mild disease in poultry that is restricted to the respiratory and intestinal tract because they contain a mono-basic cleavage site in the hemagglutinin (HA) protein that can only be cleaved by a few, localized cellular proteases (6, 10, 11). HPAI viruses contain a multi-basic cleavage site that allows for several common proteases to cleave the HA, which leads to a severe, systemic infection (6, 11). The rapid, multi-organ infection coupled with HPAI-specific dysregulated cytokine responses typically lead to death 1-6 days post infection in domesticated poultry (12). Early responses against viral infections are pre-dominantly mediated by host innate immunity, followed by migration of antigen-presenting cells (APC) and lymphocytes into the lymphoid tissues to initiate adaptive immune responses. Increased expression of pathogen recognition receptors (PRRs), interferons, pro-inflammatory cytokines, and chemokines are generally observed during the early stages of an AIV infection (13). PRRs, such as Toll-like receptors (TLRs) and MDA-5, sense viral RNAs and initiate inflammatory responses by releasing proinflammatory cytokines (14). A rapid induction of type 1 (interferon-alpha (IFN-α)) and type 2 (interferon-beta (IFN-β)) interferon leads to the upregulation of interferon stimulated genes (ISG)s, which are essential for an antiviral response. In particular, myxovirus resistance gene (Mx) is important because it promotes anti-AIV activity in various mammalian and avian species (14–17). The role of Mx is contested in chickens as there are conflicting reports of its effectiveness against HPAIV, however there is a known interaction between the viral nucleoprotein (NP) and Mx proteins (14, 17–20). Proinflammatory cytokines, including interleukin 6 (IL-6), interleukin 12 (IL-12), and interleukin 1 beta (IL-1β) upregulate inflammatory cytokine responses to limit infection, while anti-inflammatory cytokines such as IL-10 can inhibit expression of proinflammatory cytokines to down regulate the inflammation process (21).

Several AIV proteins have been implicated activating the necrotic and apoptotic cell death pathways (22). Necrosis is a passive, uncontrolled cell death, which typically causes an inflammatory reaction and affects surrounding cells, whereas apoptosis is an active, controlled cell death that does not affect surrounding cells (12, 22, 23). While both can occur during an infection, AIV proteins have been shown to block the Caspase-3 (Casp-3) and Caspase-8 (Casp-8) activation causing a shift from the apoptotic pathway to the necrotic pathway (24–30). The expression of these innate immune modulators drastically varies by virus strain, host, and target tissue making our understanding of immune response to AIV incomplete (12, 13).

APCs are crucial components of the primary immune response against pathogens and help bridge the innate and adaptive immune responses. Dendritic cells (DC) are professional APCs that play a central role as regulators of the adaptive immune response by interacting with T and B cells (13). Avian DC progenitors originate from hematopoietic stem cells in the bone marrow and translocate to non-lymphoid tissue where they become immature DCs (13, 31, 32). While immature DCs are capable of phagocytizing antigens, they are poor T-cell stimulators and lack proper antigen presentation capabilities. Upon activation, chicken DCs migrate to T-cell regions where they mature and upregulate several costimulatory molecules, including MHC-II, CD11c, CD40 and CD80. Mature DCs are specialized in antigen presentation to T cells (33). Recently, more emphasis has been put on understanding the immune modulation of chicken DC cells and their ability to combat disease.

Previous studies reported DCs could be grown *in vitro* by incubating chicken bone marrow (BM)-derived cells with chicken granulocyte-macrophage stimulating factor (GM-CSF) and chicken interleukin 4 (IL-4) (34, 35). In this study, we cultured bone-derived chicken dendritic cells (ck-BM-DC) and examined gene expression levels of IFN-α, TLR-3, TLR-7, MHC-I, IL-1β, IL-6, Mx, Casp-3, and Casp-8 pre/post infection with AIV. There are limited studies examining the interactions between chicken DCs and AIV (32, 36–38). The exact nature of how AIV infections affect DCs is largely unknown. We seek to determine whether active AIV replication can occur in DCs and if antigen processing occurs. In this study, we compared immune responses, morphological changes, and replication of ckBM-DCs following infection with contemporary H5 and H7 HPAI and LPAI viruses. A better understanding of how chicken antigen presentation occurs is needed, as the Gs/Gd lineage becomes entrenched in migratory waterfowl globally.

2 Methods

2.1 Chickens and chicken bone marrow dendritic cells isolation and culture

Four-week-old specific pathogen-free (SPF) white leghorn chickens were housed at the USDA-ARS U.S. National Poultry Research Center. The studies involving animals were reviewed and approved by the USDA-ARS U.S. National Poultry Research Center Institutional Animal Care and Use Committee (IACUC). All birds

used in these studies were cared for and handled in compliance with IACUC guidelines and procedures. ckBM-DCs were generated as previously described with minor modifications to the protocol (35). Briefly, following euthanasia with injected sodium pentobarbital using AVMA guidelines, femurs of chickens were removed and placed into 10 cm petri dishes containing 1X PBS with 1% antibiotics (Sigma-Aldrich, St. Louis, MO). Both ends of the femur bone were cut across the tops with sterile bone-scissors and a sterile iron wire was passed through and the bone marrow was flushed with sterile 1X PBS using 20 ml syringe with 16G needle. Marrow clusters were gently meshed through a 70 nm screen using a syringe plunger to obtain single-cell suspensions. Cell suspensions were overlaid with an equal volume of Histopaque 1119 (Sigma-Aldrich, St. Louis, MO) and centrifuged at 1200 g for 30 min at RT to remove red blood cells. Cells were collected and were washed three times in RPMI-1640 media (Thermo-fisher Scientific, Waltham, MA). After collection, cells were resuspended in 1X PBS and mixed 1:1 with trypan blue solution (Thermo-fisher Scientific, Waltham, MA) and checked under microscope using a hemacytometer for viable cells.

Cells were cultured in six-well plates at a concentration of 2×10^6 cells/ml at 41°C 5% CO_2 in RPMI-1640 supplemented with 10% chicken serum (Thermo-fisher, Waltham, MA), 1% L-glutamine, 1% non-essential amino acids and antibiotics (Gibco, Thermo-fisher, Waltham, MA) for 7 days. Different concentrations (0, 10, 25 and 50 ng/ml) of yeast-produced recombinant chicken IL-4 and chicken GM-CSF (Kingfisher, St Paul, MIM) were added to the medium to optimize culture conditions. Fresh complete medium was mixed with conditional media at a 3:1 ratio and added to the cells every 2 days. To induce maturation of bone-marrow cells into DCs, cells were stimulated with *Escherichia coli* LPS (500 ng/ml) (Thermo-fisher Scientific, Waltham, MA) for 30 hours. Images of the cells were taken at 30 hours using an EVOS 5000 (Invitrogen, Carlsbad, CA).

2.2 Virus propagation and inactivation

A total of 7 viruses, HPAI H5N2 A/chicken/Pennsylvania/1370/1983, H7N3 A/chicken/Jalisco/CPA1/2012 and LPAI H5N2 A/chicken/Pennsylvania/21525/1983, A/Cinnamon Teal/Mexico/2817/2006 (H7N3), A/turkey/Virginia/SEP-4/2009 (H1N1) and A/Turkey/Wisconsin/68 (H5N9) strains were propagated in the allantoic cavities of 9–11-day old SPF chicken eggs. Viral titers were determined as previously described (39). The H5N9 strain was inactivated with 0.01% β-propiolactone (BPL) overnight, followed by dialysis with sterile 1X PBS. Inactivation of the virus was tested by performing serial passages on eggs. After inactivation, the strain was labeled with FITC labeling kit according to manufacturer`s recommendation (Thermo-fisher, Waltham, MA.)

2.3 Morphology and phenotypic analysis

Cells were cultured for 6 days in the presence of different concentrations (0, 10, 25 and 50 ng/ml) of chicken GM-CSF and chicken IL-4. Cell morphology and cell growth were monitored daily. After stimulation with *Escherichia coli* LPS (500ng/ml) for 30 hours, images were taken to check change in cell morphology. Immunofluorescence labelling was performed to analyze the DC markers using FITC labeled mouse-anti-chicken MHC-II in combination with mouse anti-chicken CD40 (Bio-Rad, Hercules, CA), mouse anti-chicken CD11c (8F2, IgG2a) followed by incubation with a goat anti-mouse-Ig secondary Ab (Thermo-fisher Scientific, Waltham, MA). Primers for surface markers (MHC-II, CD40, CD11c, CD80, CD83 and CD86) were designed according to methods we have previously used (28).

2.4 Phagocytosis assay

Phagocytosis was assessed using FITC labeled inactivated H5N9 virus, and 0.5μm carboxylate modified fluorescent red latex beads (Sigma-Aldrich, St. Louis, MO). To explain briefly, non-stimulated ckBM-DCs were cultured for 6 days, followed by incubation with FITC-labeled inactivated H5N9 virus or chicken serum-opsonized red latex beads in RPMI-1640 medium at a density of 10^8 particles/ml at 41°C for 4 hours. Cells were washed five times with 1X PBS and visualized with immunofluorescence microscopy.

2.5 Immunofluorescence analysis

For sialic acid receptor staining, cells were fixed and stained by incubating FITC-labeled MAA (SA-α2,3-Gal) and TRITC-labeled SNA (SA-α2,6-Gal) for 1 hour at room temperature. Following 3 rinses in 1X PBS, cells were stained for 5 minutes with DAPI (Thermo-fisher Scientific, Waltham, MA). The immunofluorescence assays for virus nuclear protein (NP) detection were performed as previously described (40). Briefly, cells were infected with A/turkey/Virginia/SEP-4/2009 (H1N1) and A/Turkey/Wisconsin/68 (H5N9) virus at a MOI of 1 for 20 hours. Cells were then washed with 1X PBS twice, fixed and permeabilized with methanol. Viral antigens were detected with mouse-derived monoclonal antibody specific for a type A influenza virus nucleoprotein (developed at Southeast Poultry Research Laboratory, USDA), then stained with FITC-conjugated anti-mouse IgG antibody (Thermo-fisher Scientific, Waltham, MA).

2.6 Virus infection and analysis of cytokine expression by quantitative real-time RT-PCR

Cells were infected with either LPAI or HPAI H5N2 and H7N3 at a MOI of 1 in serum free DC medium for one hour with gentle agitation applied every 10 minutes. Cells were washed twice with 1X PBS and resuspended in DC medium containing 2% chick serum and incubated at 41°C 5% CO2. At 2, 8 and 24 hours post infection (hpi), supernatants were collected and stored at -80°C until titration. Virus titers are expressed as log10 50% embryo infectious dose (EID_{50}/ml) and HAU. Cells were harvested for RNA extraction at 8 hpi. Relative gene expression levels of IFN-α,

Mx, TLR-3, TLR-7, MHC-I, IL-1β, IL-6, Casp-3 and Casp-8 were evaluated by qRT-PCR as previously described (28).

2.7 Statistical analyses

Data are expressed as the mean ± standard error. Statistical differences were analyzed with Tukey one-way ANOVA using Prism 9 (GraphPad Co., San Diego, CA).

3 Results

3.1 Morphological characteristics of chicken bone marrow-derived DC

Morphological characteristics of ckBM-DC differed based on the levels of recombinant chicken GM-CSF and IL-4 (0, 10, 25, and 50 ng/ml) used. Untreated bone marrow cells displayed a rounded appearance, with follicle-like structures (cytoplasmic vacuoles) present within the cytoplasm (Figure 1A). Cells treated with 10 ng/ml or 25 ng/ml of GM-CSF and IL-4 retained a rounded appearance, but a few cells were observed to have some elongated morphology (Figures 1B, C). Cells treated with 50 ng/ml exhibited the greatest DC-like morphology by morphing into larger, elongated, and branched cells, as previously described (Figure 1D) (35). While no international consensus exists on how to determine units of activity for avian cytokines, 50 ng/ml of GM-CSF and 50 ng/ml IL-4 were used to maximize the number of cell aggregates in this study.

3.2 Maturation of ckBM-DC

To induce maturation of the ckBM-DCs, we stimulated cells with 500 ng/ml LPS on day 6 post culture for 30 hours. The cells were examined at different timepoints (0, 10, 20, and 30 hours) after the addition of LPS. At the 0 timepoint, cells displayed a veiled appearance, with small elongated branches on each cell (Figure 2A). After incubating with LPS for 10 hours, the DCs began displaying long and thin branch-like features, with a spiny or sheet-like appearance (Figure 2B). At the later timepoints, 20 and 30 hours, most of the cells developed the dendritic-like appearance (dendrites), indicating the presense of activated, mature DCs (Figure 2C, D).

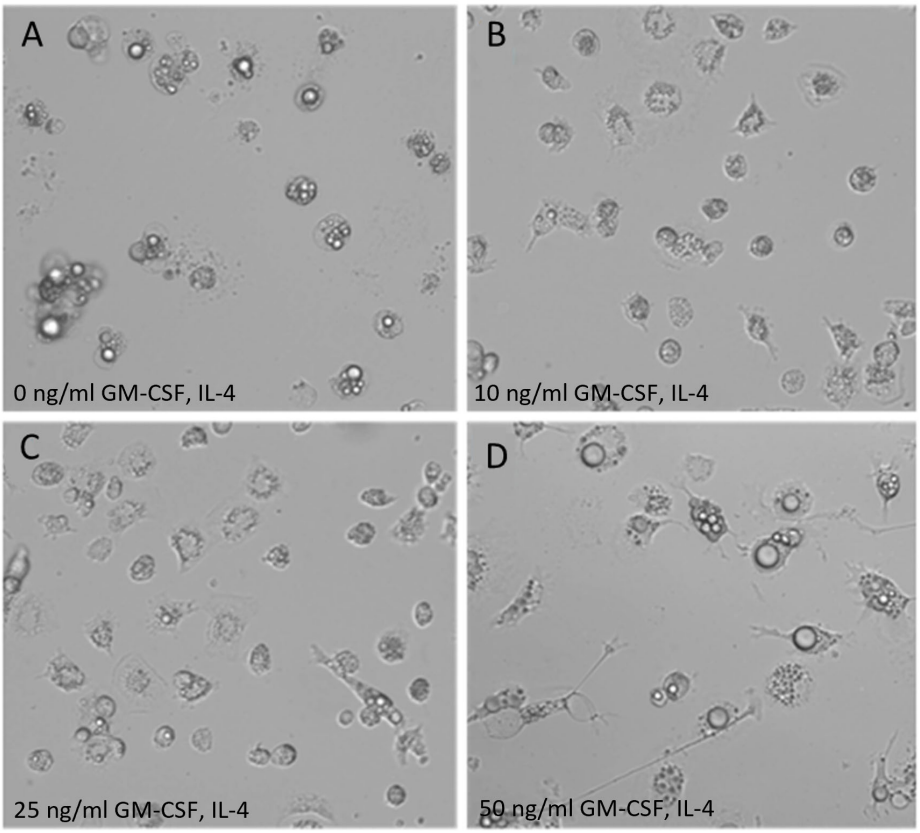

FIGURE 1
Morphology of ckBM-DC. Bone marrow-derived cells were cultured in the presence of different levels of recombinant chicken granulocyte-macrophage stimulating factor (GM-CSF) and recombinant chicken interleukin-4 (IL-4) for 6 days and dendrite formation was observed by microscopy. **(A)** 0 ng/ml GM-CSF + 0 ng/ml IL-4. **(B)** 10 ng/ml GM-CSF + 10 ng/ml IL-4. **(C)** 25 ng/ml GM-CSF + 25 ng/ml IL-4. **(D)** 50 ng/ml GM-CSF + 50 ng/ml IL-4. A representative image is shown for each concentration at 200x magnification.

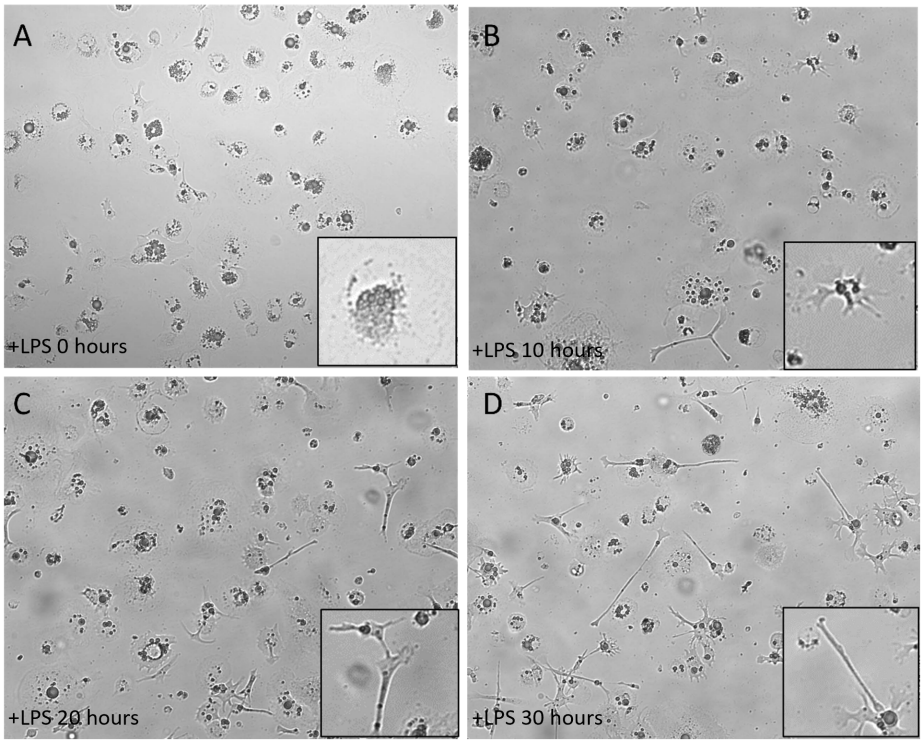

FIGURE 2
Morphology of immature ckBM-DC stimulated with LPS. Cells were cultured in the presence of 50 ng/ml GM-CSF + 50 ng/ml IL-4 for 6 days and then stimulated with LPS (500ng/ml). ckBM-DCs were observed by microscopy for 30 hours. Images show cells cultured at **(A)** 0 hours, **(B)** 10 hours, **(C)** 20 hours, and **(D)** 30 hours. A representative image is shown for each timepoint at 100x magnification. Differening levels of elongated dendrites are in black boxes.

3.3 Mature ckBM-DC cells share phenotypic similarities with mammalian DC cells

Dendritic cells co-exist in both immature and mature states. In mammals, immature dendritic cells are characterized by moderate or low-level expression of surface markers molecules such as MHC-II, CD11c, CD40, CD80, CD83 and CD86 and increase upon maturation (41). Immunofluorescence microscopy demonstrated that immature ckBM-DCs had some level of surface marker expression when stained with anti-chicken MHC-II (Figure 3A1), anti-chicken CD11c (Figure 3B1) and anti-chicken CD40 (Figure 3C1). After stimulation with LPS for 24 hours, expression level was increased in all 3 markers, MHC-II (Figure 3A2), CD11c (Figure 3B2) and CD40 (Figure 3C2). To quantify the level of surface marker expression, qPCR was used to determine the fold change of expression between immature and mature DCs. The level of surface marker expression was significantly enhanced in mature ckBM-DC cells, approximately 40-120-fold compared with their immature counterparts. The greatest change was observed with CD80, CD83 and CD86 (Figure 3B).

3.4 Immature ckBM-DCs retain the capability to phagocytosize foreign antigens

To test phagocytosis, 0.5 μm carboxylate modified fluorescent red latex beads and FITC labeled-inactivated H5N9 avian influenza virus particles were added to immature ckBM-DCs. The cells were able to phagocytose both the beads (Figure 4A) and viral particles (Figure 4B). The beads and virus were observed in the cytoplasm (Figures 4A, B).

3.5 AIVs are capable of infecting immature ckBM-DCs

To test whether immature ckBM-DCs can successfully be infected with AIV, immunofluorescence microcopy was performed to detect expression of SA-α2,3-Gal and SA-α2,6-Gal receptors on the DCs surface. Results demonstrated that both SA-α2,3-Gal (Figure 5A2) and SA-α2,6-Gal (Figure 5B2) receptors were extensively expressed in the immature DCs, however it

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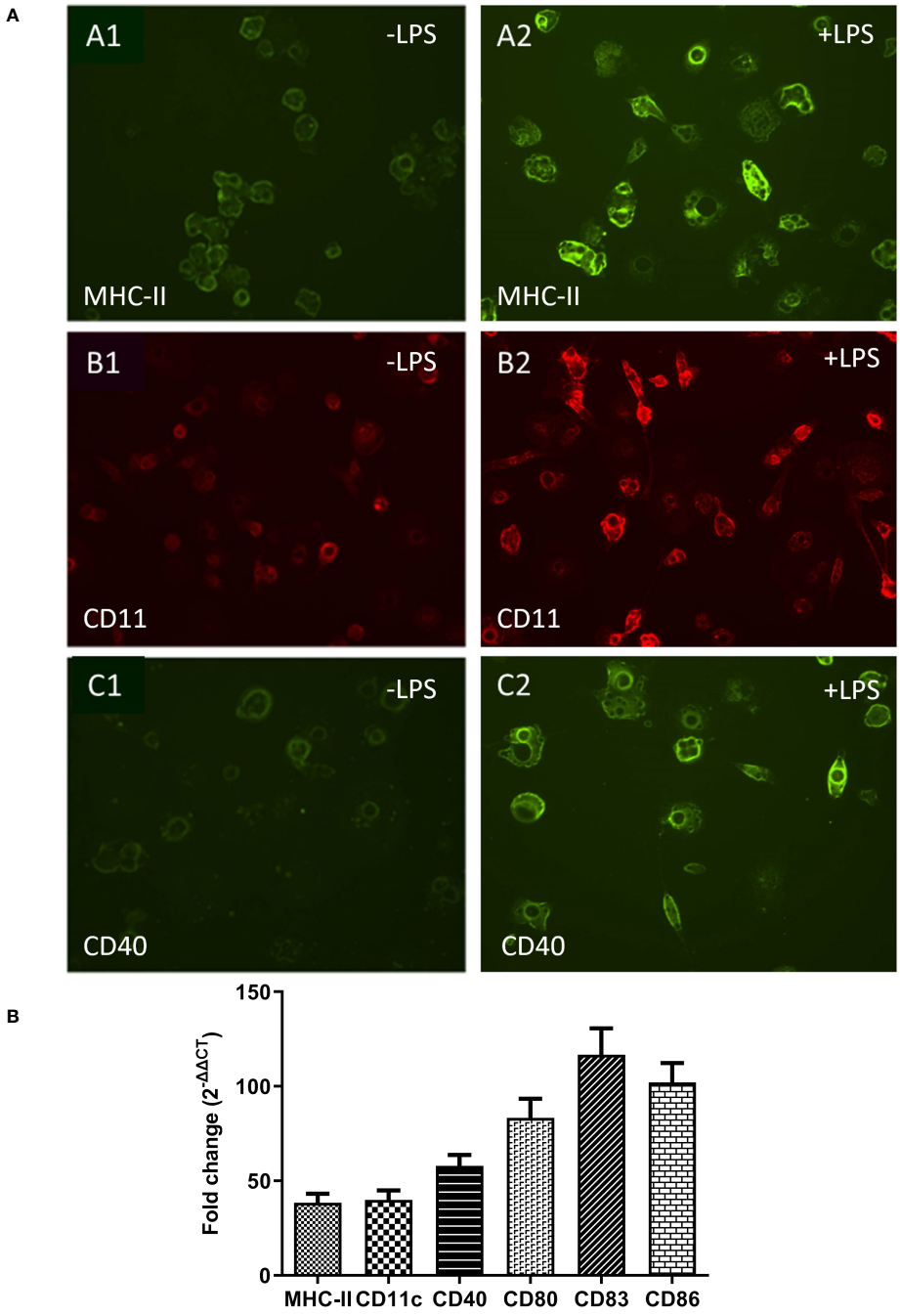

FIGURE 3
Comparative analysis of surface markers on immature and mature ckBM-DCs. Cells were cultured in the presence of 50 ng/ml GM-CSF + 50 ng/ml IL-4 for 6 days, and then stimulated with 500 ng/ml LPS for 30 hours. **(A)** Immature cells (-LPS) are on the left **(A1, B1, C1)** and mature cells (+LPS) are on the right **(A2, B2, C2)**. Immunofluorescence analysis was performed using a FITC labeled mouse-anti-chicken MHC-II antibody (green) **(A1, A2)**. Cells were also stained with mouse anti-chicken CD11c (B1, B2) (red) and mouse anti-chicken CD40 **(C1, C2)** followed by a goat-anti-mouse secondary (green). A representative image is shown for each at 100x magnification. **(B)** Cellular RNA was extracted to measure expression levels of surface markers, via qPCR, in ckBM-DCs. RNA was normalized using the Ck 28S house-keeping gene. The data is expressed as the fold change in mRNA levels between immature and mature ckBM-DCs for MHC-II, CD11c, CD40, CD80, CD83, and CD86. The data shown is a representative of three independent experiments. Error bars represent the standard error.

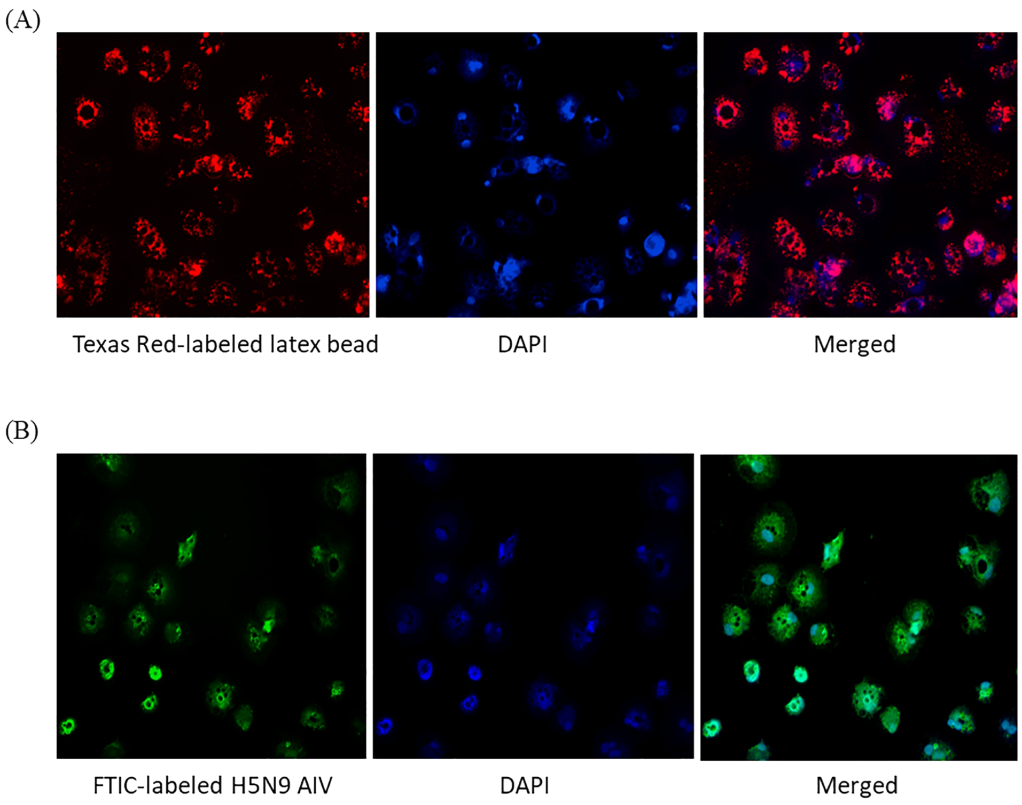

FIGURE 4
Functionality of immature ckBM-DCs. Cells were cultured in the presence of 50 ng/ml GM-CSF + 50 ng/ml IL-4 for 6 days. **(A)** ckBM-DCs were incubated with 0.5 µm carboxylate modified fluorescent red latex beads or **(B)** FITC labeled-inactivated H5N9 avian influenza virus for 4 hours. Following incubation cells were counterstained with DAPI, washed 5x with 1X PBS, and visualized by immunofluorescence microscopy. A representative image is shown for each at 100x magnification.

appears the SA-α2,3-Gal receptor was more prominent based on immunofluorescence (Figure 5A2). After infecting the cells with pandemic H1N1 (SA-α2,6-Gal preference) and H5N9 (SA-α2,3-Gal preference), immunofluorescence microscopy was performed using a AIV NP antibody to demonstrate the immature DCs could be infected with AIV (Figure 5C, D).

3.6 ckBM-DCs can be infected with both LPAIVs and HPAIVs

ckBM-DCs were infected with HPAIV and LPAIV (H5N2 and H7N3 subtypes) to determine the effect on cell morphology and viral replication. At 8 hpi, all infected cells underwent some degree of morphological change, more rounded cells were observed when infected with H7N3 compared to the H5N2 strains (Figure 6A). At 24 hpi, CPE was observed in the form of detached cells and changes in their morphology (rounding), regardless of subtype or pathogenicity (Figure 6A). There was little difference in severity of CPE between the H5N2 strains, but cells infected with HPAI H7N3 demonstrated more severe levels of CPE with larger number of detached cells compared to LPAI H7N3 (Figure 6A). In terms of viral growth, LPAI H5N2 demonstrated a titer of $10^{5.5}$ EID_{50}/ml at 24 hpi, compared to HPAI H5N3 which demonstrated a titer of $10^{4.8}$ EID_{50}/ml. In contrast, HPAI H7N3 demonstrated a higher titer compared to LPAI H7N3, with titers of $10^{6.5}$ EID_{50}/ml and $10^{4.8}$ EID_{50}/ml, respectively (Figure 6B). All virus titers increased by 3-5 logs between 2 and 8 HPI indicating AIV replicated in the ckBM-DCs (Figure 6B).

3.7 ckBM-DCs infected with HPAIVs demonstrates higher expression of immune markers compared to LPAIV

Several immune markers were examined post AIV infection. Increased expression of IFN-α and Mx genes, which are indicative of a viral infection, were increased in all groups (Figure 7). All HPAI infected cells expressed significantly higher levels of both IFN-α and Mx genes, compared to their LPAI counterparts, between 20-500-fold, depending on virus and gene (Figure 7A). Increased expression of TLR receptors and MHC-I were observed in both HPAI and LPAI groups, with HPAI groups demonstrating higher expression levels compared to LPAI groups with a 10-700-fold difference. (Figure 7B). Similar trends were observed in gene expression levels of proinflammatory cytokines IL-1β, IL-6 and apoptotic genes Casp-3 and Casp-8, in which all HPAIV infected cells demonstrating significantly higher levels of expression compared to LPAI groups, between 20-800-fold difference. (Figure 7C, D).

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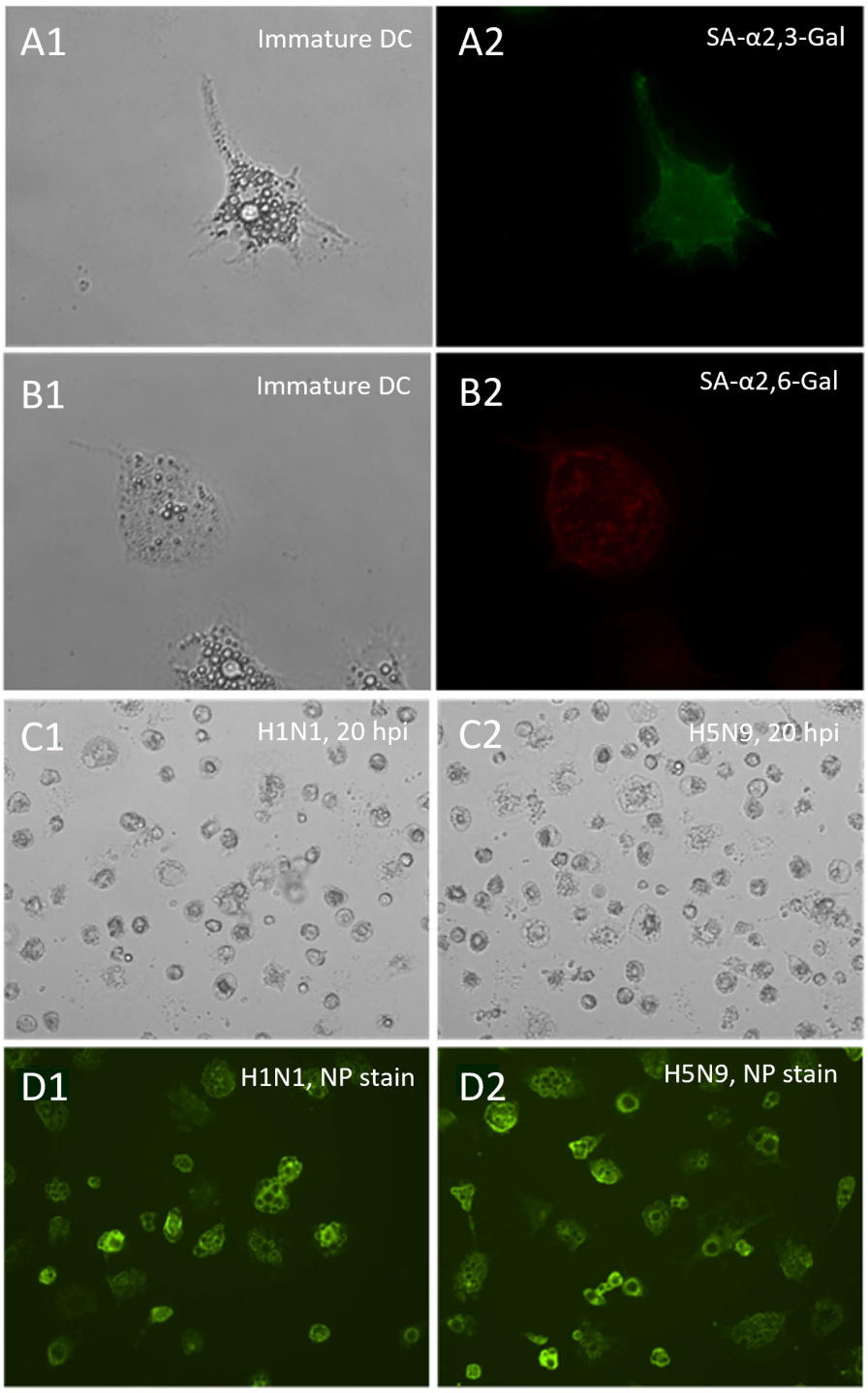

FIGURE 5
Distribution of sialic acid receptors on ckBM-DCs and susceptibility to pandemic H1N1 and H5N9 viruses. Cells were cultured in the presence of 50 ng/ml GM-CSF + 50 ng/ml IL-4 for 6 days. Immature ckBM-DCs **(A1, B1)** were stained with FITC-labeled MAA (SA-α2,3-Gal) **(A2)** or TRITC-labeled SNA (SA-α2,6-Gal) **(B2)** and visualized by immunofluorescence microscopy. CkBM-DCs were infected at an MOI of 1 with A/turkey/Virginia/SEP-4/2009 H1N1 (SA-α2,6-Gal preference) and A/turkey/Wisconsin/68 H5N9 (SA-α2,3-Gal preference). At 20 hpi, viral-infected cells, H1N1 **(C1)** and H5N9 **(C2)**, were washed 2x with 1X PBS, fixed with methanol, and observed by microscopy. Viral NP proteins, H1N1 **(D1)** and H5N9 **(D2)**, were detected using a mouse-anti-NP antibody followed by a FITC-conjugated anti-mouse IgG secondary **(D1,D2).** A representative image is shown for each at 200x **(A1,A2,B1,B2)** and 100x **(C1,C2,D1,D2)** magnification.

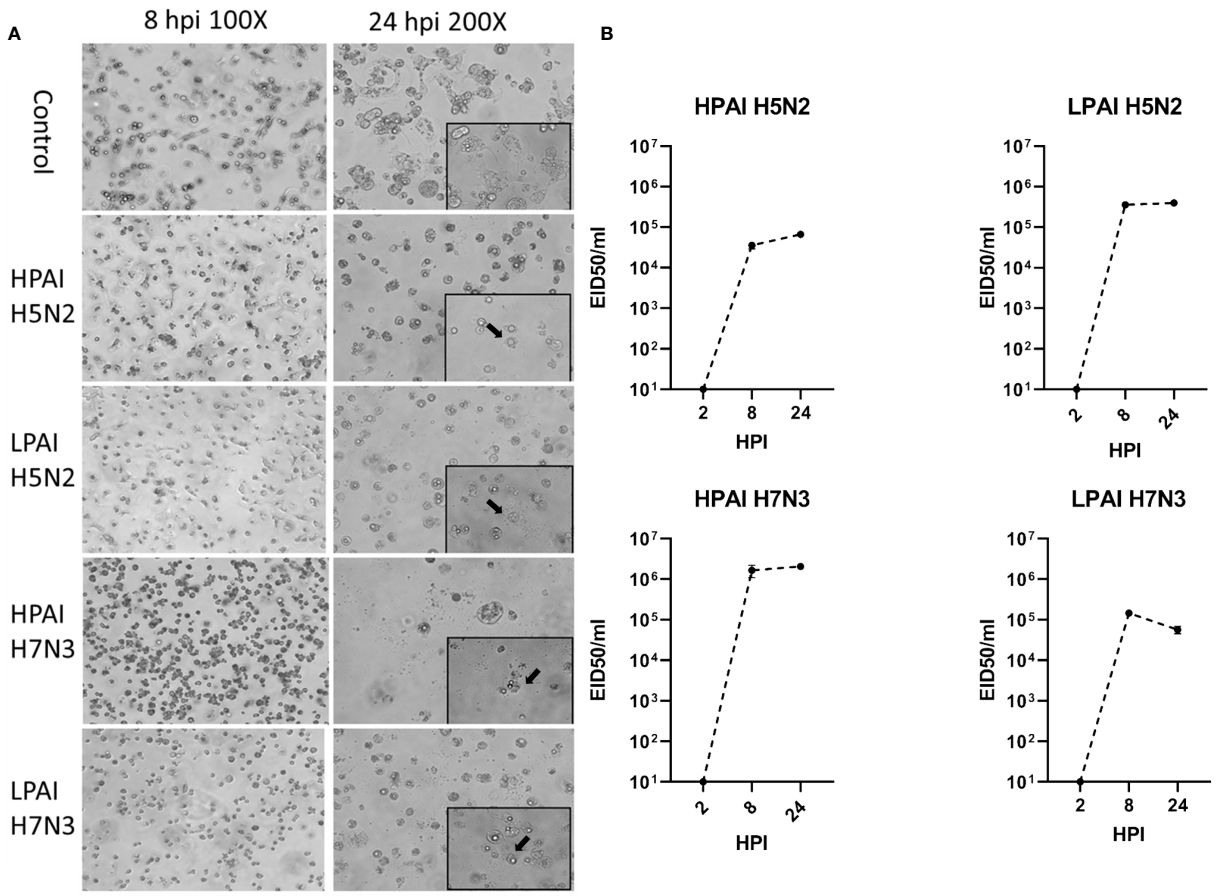

FIGURE 6
Change in morphology and growth of ckBM-DCs infected with LPAIV and HPAIV. Cells were cultured in the presence of 50 ng/ml GM-CSF + 50 ng/ml IL-4 for 6 days. Immature ckBM-DCs were infected at a MOI of 1 with LPAIV (A/Chicken/Pennsylvania/21525/1983 H5N2 and A/Cinnamon Teal/Mexico/2817/2006 H7N3) and HPAIV (A/Chicken/Pennsylvania/1370/1983 H5N2 and A/Chicken/Jalisco/CPA1/2017 H7N3) viruses. **(A)** CPE (black arrows) and cellular morphological changes were observed via microscopy at 8 and 24 HPI. A representative image is shown for each at 100x and 200x magnification. **(B)** Supernatants were obtained at 2, 8, and 24 HPI and viral titers were evaluated by EID50. The data shown is a representative of three independent experiments. Error bars represent the standard error of triplicate samples.

4 Discussion

The innate immune system plays a central role in detecting viral pathogens and mounting an early response by activating inflammatory and antiviral defense mechanisms. DCs are essential in bridging the gap between innate and adaptive immune responses because they process and present antigens to T cells and B cells. However, it is still largely unknown if AIV can directly infect ckBM-DCs and if infection causes morphological and physiological changes to the cells. This study established that ck DCs can be infected by AIV, and that viral growth occurs in them. We also demonstrated ck-BMDCs were able to phagocytosize viral particles as immature DCs. The ckBM-DCs were able to be infected by both HPAIV and LPAIV isolates. However, differences in cell morphology did exist between the virus strains and pathogenicity. LPAI H5N2 replicated better in ckBM-DCs than its HPAI counterpart. While the exact reason is not clear, it may indicate a delayed replication in DCs or be attributed to HPAI H5N2 lack of adaptation in cell culture. In contrast, HPAI H7N3 demonstrated more severe CPE at 8 hpi compared to the LPAI H7N3, suggesting some correlation with pathogenicity and CPE with H7 viruses. Viral titers also correlated with CPE and pathogenicity, with the titer of HPAI H7N3 demonstrating a 1.7 log difference in EID_{50}/ml titers compared to LPAI H7N3. The results are consistent with a previous study in which HPAI H7N1 showed better replication in chicken DCs compared to LPAI (32).

Controlled cell death is normally induced by apoptotic genes, during a viral infection up-regulation of related caspase genes is typically observed (23). In our study, Casp-3 and Casp-8 expression increasing in all infected groups, regardless of pathogenicity or subtype. However, DCs infected with HPAI viruses demonstrated significantly higher expression levels of Casp-3 and Casp-8 compared to their LPAI counterparts, indicating a correlation between caspase gene expression and pathogenicity. Studies have demonstrated AIV causes caspase-dependent apoptosis based on Casp-3 activation, which results in nuclear export of newly synthesized viral nucleoprotein (NP) and elevated virus replication. This suggests Casp-3 activation is a crucial event for AIV propagation and dissemination (42, 43). One study reported primary duck cells infected with LPAI H2N3 and classical H5N1

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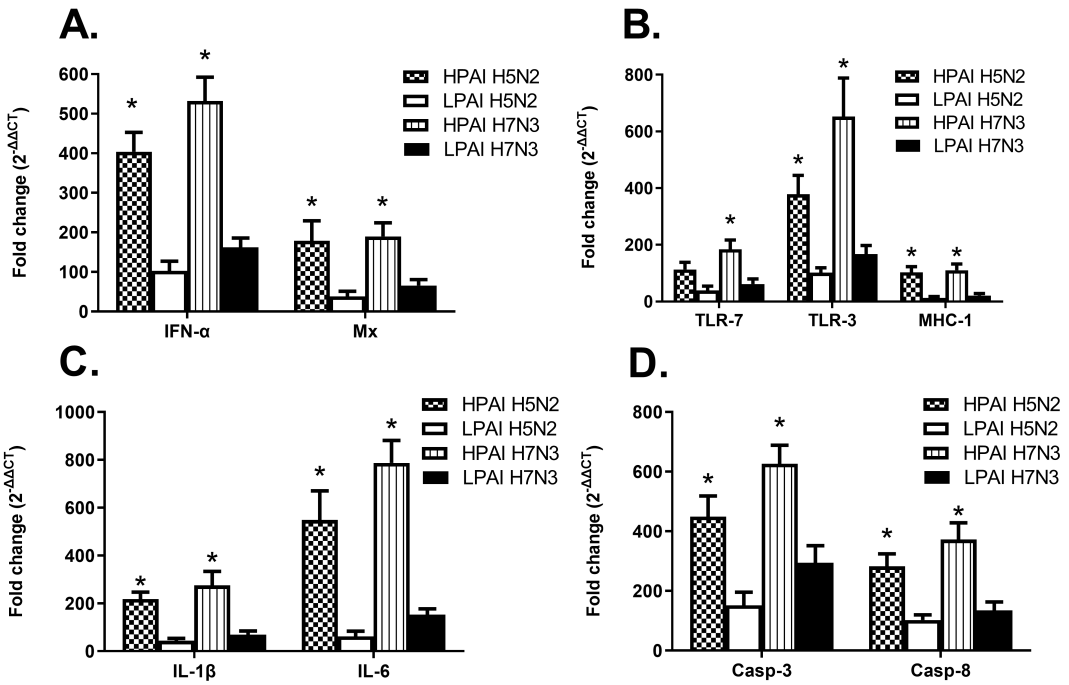

FIGURE 7
Cytokine expression levels of ckBM-DCs infected with HPAIV or LPAIV. Cells were cultured in the presence of 50 ng/ml GM-CSF + 50 ng/ml IL-4 for 6 days. Immature ckBM-DCs were infected at a MOI of 1 with LPAIV (A/Chicken/Pennsylvania/21525/1983 H5N2 and A/Cinnamon Teal/Mexico/2817/2006 H7N3) and HPAIV (A/Chicken/Pennsylvania/1370/1983 H5N2 and A/Chicken/Jalisco/CPA1/2017 H7N3) viruses. Cellular RNA was extracted 8 hpi to measure relative gene expression levels of IFNα, Mx, TLR-7, TLR-3, MHC-1, IL-1B, IL-6, Casp-3, and Casp-8 **(A–D)**. RNA was normalized using the Ck 28S house-keeping gene. The fold change ($2^{-\Delta\Delta CT}$) was determined by the comparison of infected ckBM-DCs to sham-infected ckBM-DCs. Tukey one-way ANOVA analysis was performed to determine significant differences between LPAIV and HPAIV infected ckBM-DCs. * indicates a significant difference ($p<0.05$). The data shown is a representative of three independent experiments. Error bars represent the standard error of triplicate samples.

strains underwent rapid cell death compared to primary chicken cells, both cell lines showed similar levels of viral RNA, but lower amounts of infectious virus were observed in the duck cells (44). Such rapid cell death was not observed in the same study with duck cells infected with a contemporary Eurasian H5N1 strain fatal to ducks, indicating the rapid apoptosis may be part of a mechanism of host resistance against AIV (45). An increased expression of caspase genes demonstrated in our study may further support the notion that AIV can induce cell death via Casp-3 and Casp-8.

During AIV infection, ssRNA and dsRNA are recognized by a specific group of PRRs. In this study, HPAIV infected cells demonstrated significantly higher expression levels of TLR-3 and TLR-7 compared to cells infected with LPAIV. However, the level of TLR expression did not correspond to the amount of viral load as the titers had mixed results between the HPAI and LPAI strains. Furthermore, the TLR-3 expression levels were significantly higher in HPAI H7N3 compared to HPAI H5N2s. One study reported that TLR-3 expression levels significantly increased at 4 hpi and 16 hpi with HPAI H7N1 infections, whereas the level of increase in HPAI H5N2s were more gradual (32). TLR-3 and TLR-7 closely interact with STAT-3, which is crucial for regulating cytokine-mediated responses, such as IL-6 to combat viral infections (46). One study reported STAT-3 expression was not adversely affected by LPAIV H3N2 in chicken cells, but expression levels were significantly decreased in chicken cells infected by HPAI H5N1 (45). In contrast, STAT-3 expression levels were significantly elevated in duck cells, indicating infection with the same H5N1 strain had a less adverse effect in duck cells. Thus, it can be speculated that differences in the cell signaling process, along with specificity of the strains, may affect cytokine responses.

Our results demonstrated that expression levels of proinflammatory related genes were higher in the HPAI groups in the early stages of infection, compared to LPAI groups. Geus et al. reported that levels of IFN-α were elevated in HPAI infected DCs and were maintained up to 24 hpi, compared to the LPAI infected DCs where most of the IFN-α expression occurred in only the early stages (47). Our study demonstrated that the expression levels of IFN-α and IL-6 genes in DCs were higher in the HPAI H7N3 group, compared to the HPAI H5N2 group, suggesting the ability to activate host innate responses may vary depending on the virus subtype and the host. Several studies have reported high levels of IL-6, IL-12 and IL-18 cytokine expression in the lungs and spleens of chickens infected with H5 HPAIVs while type 1 interferons were mostly present in the plasma and tissues (48–51). Another study reported similar amounts of viral RNA and cytokine expression levels following infection with HPAI and LPAI H7N1 in chickens (52). Kuribayashi et al. (2013), demonstrated that H7N1 strains can replicate more efficiently in chickens compared to H7N7, especially in the brain and are able to trigger excessive expression of inflammatory and antiviral cytokines, such as IFN-γ, IL-1β, IL-6, and IFN-α, in proportion to its proliferation. In contrast, another study reported that human-origin DCs infected with HPAI H7 resulted in delayed and

decreased expression of cytokines, including type 1 interferons, compared to other AIV subtypes (53). Thus, the difference of immune profiles of the host cell might be attributed to the specificity of the AIV. Furthermore, HPAI viruses may impair the regulatory activity of the TLR pathway, which is responsible for controlling the magnitude and duration of the inflammatory response, and lead to an uncontrolled immune response and cytokine storm. The acute uncontrolled innate immune response, which leads to overexpression of proinflammatory cytokines may be one of the causes for swift death in mammals infected with HPAI. Thus, one can speculate deregulation of these cytokines in chicken DCs may lead to multiple organ failure, as frequently seen in mammals.

Mx is a well-known antiviral protein, which can be induced by type 1 interferons (15). However, susceptibility to the inhibitory effects of Mx may vary by strain and host (14, 20, 54, 55). In this study, higher type 1 interferon expression levels were observed along with elevated expression of the Mx gene. However, despite the presence of elevated type 1 interferon and Mx expression levels, viral replication in DCs were not significantly inhibited. Rapid cell death and activation of caspase-dependent apoptosis did not appear to hinder the output of viral load. To date, the full complement of genes and their exact roles which contribute to antiviral properties are not well defined in chickens. However, one might speculate that the PRR dependent immune response may play a crucial role in mounting an antiviral defense, given the role of the TLR-7 and RIG-1 receptor signaling. For instance, it was shown that the presence of RIG-1 in cells stimulates expression of several key genes involved in innate immune responses that are crucial against viral infections, such as influenza (56).

Overall, we were able to demonstrate that AIV can infect and replicate in chicken DCs regardless of pathogenicity. HPAI subtypes trigger a significantly higher expression of various immune factors compared to LPAI subtypes, suggesting a dysregulation of the immune system. The increase in DC activation following infection may be indicative of the dysregulated immune responses typically seen with high pathogenic avian influenza infections.

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 approved by USDA-ARS U.S. National Poultry Research Center Institutional Animal Care and Use Committee (IACUC). The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

JM: Data curation, Formal analysis, Methodology, Validation, Visualization, Writing – original draft, Writing – review & editing. KS: Formal analysis, Conceptualization, Data curation, Methodology, Writing – original draft. KC: Conceptualization, Data curation, Formal analysis, Methodology, Writing – original draft. KB: Formal analysis, Visualization, Writing – review & editing. DK: Conceptualization, Formal analysis, Funding acquisition, Methodology, Resources, Supervision, Visualization, Writing – original draft, Writing – review & editing.

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Conflict of interest

Author KS was employed by company CSL Seqirus.

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.

Publisher's note

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Modern broiler chickens exhibit a differential gastrointestinal immune and metabolic response to repeated CpG injection relative to a 1950s heritage broiler breed

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The Athens Canadian Random Bred (ACRB) heritage broiler breed, which has not been selectively bred since the 1950s, is a point of comparison to the modern-day broiler and could highlight potential genetic-derived differences in immune responses. To observe the modern and heritage birds' immune responses in action, the innate immune ligand CpG oligonucleotides were administered at multiple time points through the birds' lives from the day after hatch to day 35 post-hatch. This study allowed for the observation of changes in metabolic and immune signaling in response to repeated injections of a known Toll-like receptor (TLR) ligand, CpG. Jejunum and cecal tonsil samples at multiple time points during grow out were collected and used for kinome array analysis to measure kinase activity in immunometabolic signaling pathways in the gut tissue. In addition cytokine gene expression was measured in these tissues. The modern birds' response to the treatment was more innate and showed evidence of metabolic energy shift. The heritage birds' response to the treatment was adaptive, with metabolic changes indicative of a well-regulated response. Overall, the results from this study suggest that modern broiler chickens do not adequately balance resources between growth and immune responses during an immune challenge, and this deficit is most evident around the 2-week post-hatch time point. This is a critical time for these birds, as their muscle deposition continues to accelerate, and they are vulnerable to disease challenges. Ideally, future work can clarify the reason for this response discrepancy in the modern broiler and therapeutic interventions to rescue this phenotype could be elucidated.

KEYWORDS

broiler, chicken, immune, CpG, metabolic

1 Introduction

The modern broiler chicken is the product of over 60 years of highly selective breeding based predominantly on production parameters. These birds have an extremely rapid rate of growth, particularly with respect to their breast muscle tissue, and are highly feed efficient. These attributes have contributed to the expansion of the poultry industry into one of the largest animal production industries in the United States, with an estimated value of 31.7 billion dollars in 2018 ("Poultry - Production and Value 2018 Summary 05/23/2019," 2018). Due to these phenomenal advancements in poultry genetics, chicken is the most popular meat consumed globally (Whitton et al., 2021). In addition to accelerated growth, however, the birds experience skeletal, cardiovascular, metabolic and immune issues, possibly as a byproduct of selectively breeding for growth (Cheema et al., 2003; Olkowski, 2007; Scheele, 1997). Due to the removal of growth promoting antibiotics from poultry feed formulations, broiler chickens are now left susceptible to disease challenges such as necrotic enteritis that the poultry industry has not had to contend with in the last 5 decades (Lanckriet et al., 2010; Kaldhusdal et al., 2016). Furthermore, the removal of antimicrobials from poultry production presents a human health and safety concern, as broiler chickens do not mount sufficient immune responses to clear *Salmonella* infections that may be reduced by antimicrobial administration (Abudabos et al., 2016; Vermeulen et al., 2017). It is necessary to understand and address the modern broilers' immune response to disease challenges from both an animal welfare standpoint as well as a human food safety standpoint.

To place the modern broiler's immune response in context, heritage broiler breeds provide a standard to which the modern birds can be compared. Heritage broiler breeds are commercial broiler production birds that have not undergone continued selective breeding for performance parameters. In this study, we use the Athens Canadian Random Bred (ACRB) heritage broiler breed, a pedigreed controlled commercial breed which has been managed at the University of Georgia to maintain the genetics comparable to those of 1958 (Hess, 1962; Collins et al., 2016). ACRB birds show more robust antibody immune responses than modern broilers, whereas modern broilers demonstrate cell-mediated immune responses (Cheema et al., 2003). ACRB birds are also better able to cope with stressors such as heat stress (Berrong and Washburn, 1998). Growth rate and body weight have been shown to impact immune parameters such as antibody responses, therefore it is interesting to observe and compare immune responses in a fast-growing and a slow-growing broiler (Yunis et al., 2000; 2002).

To study the differences in immune response between the heritage broiler and its modern counterpart, it is informative to observe the response to a known immunostimulatory treatment. CpG oligonucleotides (ODN) are synthetic oligonucleotides that induce the immunostimulatory effects of bacterial DNA by binding to the avian Toll-like receptor (TLR) 21 and initiating downstream signaling (Hemmi et al., 2000; Latz et al., 2004; Brownlie et al., 2009; Aylward et al., 2022). *In vitro*, CpG ODNs have been shown to cause increased mRNA expression of proinflammatory cytokines such as interleukin (IL)1β and interferon (IFN) γ and nitric oxide production in HD-11's, a chicken macrophage cell line (He et al., 2003). Furthermore, chickens and mice that are treated with CpG and then given a bacterial challenge, such as *Listeria monocytogenes* or *Salmonella* species are protected from infections and morbidity associated with these bacterial species (Krieg et al., 1998; Klinman et al., 1999; He et al., 2005). In the current study, modern and ACRB broilers were injected with CpG ODNs at multiple time points throughout the experimental period, with the first injection administered the day after hatch. Tissue samples were collected prior to and 24-h post-injection to track each broiler's response to the immunostimulant after repeated exposures.

To characterize the birds' responses to the CpG treatment, kinome arrays were utilized to assess kinase activity along immunometabolic signaling pathways (Johnson, et al., 2023). Kinases' phosphorylation of target proteins has a direct impact on downstream activation or inhibition of immune and metabolic signaling pathways. Kinome arrays provide a real-time snapshot of the function of kinases active in the tissue of interest. Kinome analysis of the cecal tonsil and jejunum at different timepoints throughout the grow-out period provide a picture of the birds'gut-associated immune and metabolic response to the repeated CpG treatment. qPCR was also performed to assess the mRNA expression of key inflammatory cytokines that are known to show altered expression after stimulation with CpG.

2 Materials and methods

2.1 Bird husbandry

Embryonic, day 16 post-lay Ross 308 eggs were acquired from Pedigree Chicks (Beaver Creek, PA) and were incubated in Jamesway incubators (Jamesway Incubator Co. Inc., Cambridge, ON, Canada) at 37.5°C and 60% humidity until day of hatch. Newly fertilized Athens Canadian Random Bred (ACRB) eggs were shipped from the University of Georgia Poultry Science Department (Athens, GA). These eggs were incubated in the same incubator conditions until day of hatch. On day of hatch chicks were removed from the incubators when dry and placed into one of eight colony houses on the University of Delaware campus farm (Newark, DE). The houses were equipped with clean pine shavings, bell waterers, and chick feeders, and the temperature was maintained at approximately 35°C for the first week of life. Feed and water were provided *ad libitum*. Birds were changed from starter feed to grower feed on day 12 and from grower feed to finisher feed on day 25.

2.2 Experimental design

Birds in two out of the total eight houses were assigned to one of four groups (modern broiler CpG treatment, modern broiler GpC control, ACRB CpG treatment or ACRB GpC control). GpC contains the same nucleotides as CpG but in an inverted, non-stimulatory, sequence (Ahmad-Nejad et al., 2002). The birds were given their first injection of either CpG or the control GpC as a 25-μg injection in 0.2 mL of 0.01 M sterile phosphate buffered saline (PBS, Sigma Aldrich, St. Louis, MO) 1-day post-hatch, this dose has been shown to be within the range necessary to elicit a measurable

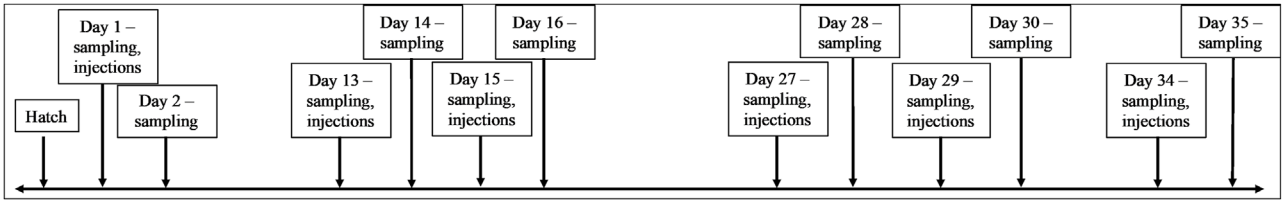

FIGURE 1
Timeline showing sampling and injection timepoints of trial.

immune response (Ahmad-Nejad et al., 2002). Prior to injections, 5 birds from each group were sacrificed via cervical dislocation and their tissues were collected for analysis. Twenty-4 hours later, five additional birds from each group were sacrificed and their tissues were collected. This pattern of sampling, injecting, and sampling again was repeated on days 13, 14, 15, 16, 27, 28, 29, 30 and 34 and 35 post-hatch (Figure 1). The tissues collected at each sampling time point included jejunal samples and cecal tonsil samples. The tissue samples reserved for kinome analysis were flash frozen in liquid nitrogen and stored at −80°C until used for analysis. The tissue samples reserved for RNA extraction and qPCR were preserved in ~1 mL of RNAlater, stored at 4°C overnight, then moved to −20°C until used for analysis.

2.3 Kinome array analysis

Jejunum and cecal tonsil tissue samples included in the analysis were from day 2, day 15, day 16, day 34, and day 35 post-hatch. The peptide array protocol using PepStar peptide microarrays from JPT Peptide Technologies GmbH (Berlin, Germany) was carried out as described previously (Arsenault, et al., 2017) and is summarized below with the listed modifications. Approximately 40 mg pieces of tissue were cut and homogenized by a Bead Ruptor homogenizer (Omni, Kennesaw GA) in 100 µL of lysis buffer (20 mM Tris–HCl pH 7.5, 150 mM NaCl, 1 mM Ethylenediaminetetraacetic acid (EDTA), 1 mM ethylene glycol tetraacetic acid (EGTA), 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM Na3VO4, 1 mM NaF, 1 µg/mL leupeptin, 1 g/mL aprotinin and 1 mM Phenylmethylsulphonyl fluoride). All chemicals were purchased from Sigma-Aldrich, Co. (St. Louis, MO) unless specified otherwise. Arrays were then imaged using a Tecan PowerScanner microarray scanner (Tecan Systems, San Jose, CA, USA) at 532–560 nm with a 580 nm filter to detect dye fluorescence. The kinome array images were gridded using the GenePix Pro seven software (Molecular Devices, LLC, San Jose, CA, USA), and the spot intensity signal was collected as the mean of pixel intensity using local feature background intensity calculation with the scanner saturation level set at 50%.

2.4 RNA extraction and qRT-PCR

RNA was isolated from the tissue samples (approximately 20 mg of tissue) using the Qiagen RNeasy MiniKit (Germantown, MD). The tissues were lysed in a 2 mL vial with 1.4 mm ceramic beads with 600 µL of buffer RLT using a Bead Ruptor 24 (Omni International, Kennesaw, GA) run on setting six for two cycles of 10 s. The isolated RNA was eluted into ultrapure water and stored at −80 °C until further analysis. RNA quality was verified by quantification on a NanoDrop 1,000 Spectrophotometer (Thermo Scientific, Waltham, MA) and gel electrophoresis of the RNA samples on an Invitrogen E-Gel EX 1% Agarose (Invitrogen, Carlsbad, CA). The RNA was used with the Applied Biosystems TaqMan RNA-to-CT 1-Step kit (Waltham, MA) following the protocol provided. The primers and probes for the housekeeping gene and cytokines of interest were ordered from Integrated DNA Technologies (Coralville, IA). PCR was performed on an Applied Biosystems 7900HT Real Time PCR System with a standard 96-well block. Each sample was run in triplicate.

2.5 Statistical analysis

Ross birds' weights were measured as a group on days 3–12 post-hatch and then individually on days 15–34 post hatch. When the weights were taken individually a one-way Student's t-test was used to compare the weights between the treatment and control birds and determine significant difference, and a p-value of <0.05 was considered significant. The ACRB birds were only weighed individually on days 29 and 34 post-hatch, at all other time points they were weighed as a group. When the ACRB birds were weighed individually, a one-way Student's t-test was again used to compare weights between treatment and control birds.

The kinome data were analyzed using the PIIKA two peptide array analysis software (http://saphire.usask.ca/saphire/piika/index.html) (Trost et al., 2013). The resulting data points were normalized to eliminate variance due to technical variation such as random variation in staining intensity between arrays or between blocks within an array. Variance stabilization normalization was performed. There were three biological replicates for each sample, and the normalized fluorescence data for the three replicates were averaged together. PIIKA two then performed one-tailed T tests to determine the statistical significance of the change in phosphorylation at each peptide fragment between the treatment and the control tissues, generating a fold change and a p-value. Peptides with fold changes with a p-value <0.05 were considered statistically significantly differentially phosphorylated between treatment birds' samples and control. In the rest of the

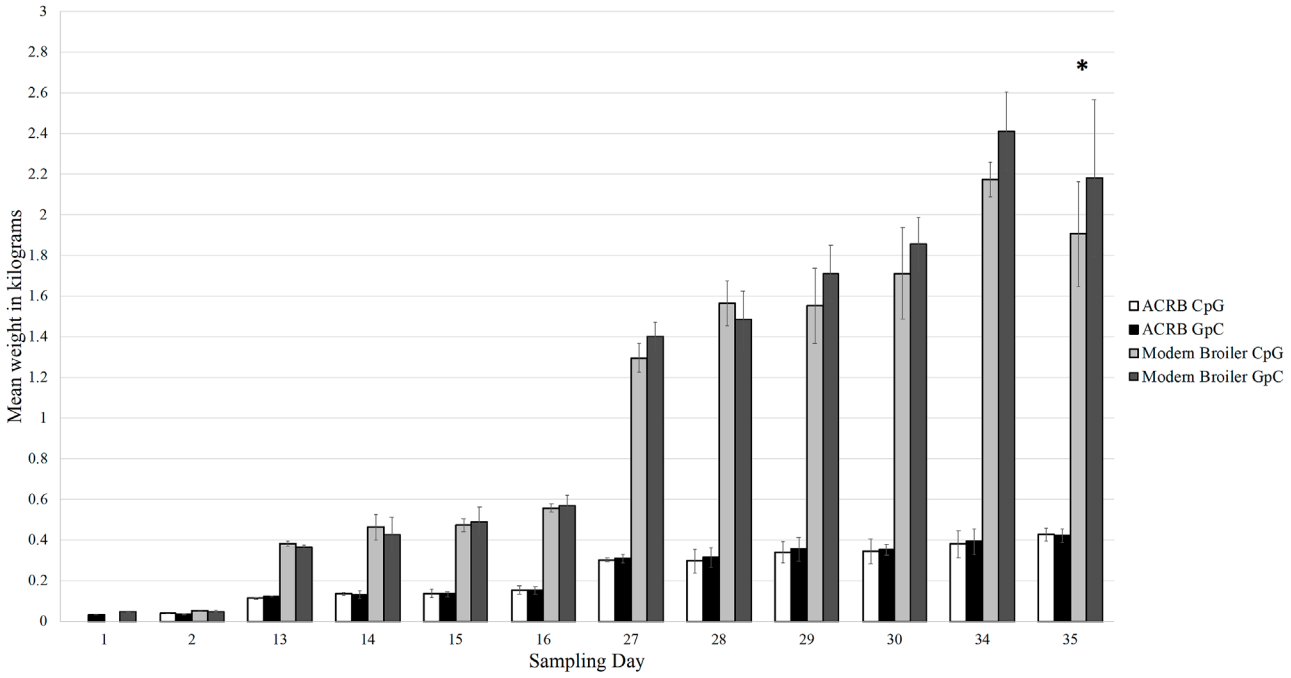

FIGURE 2
Bar graph showing the average weights of each bird type and treatment type at each sampling time point in the grow out period. The modern broilers, as expected, grew larger and faster than the ACRB birds. The difference in weights between the treatment and control birds was only significant in the modern broilers at day 35 post-hatch (denoted by the asterisk, $p < 0.05$). The difference in weight between the treatment and control ACRB birds was never significantly different from each other. The error bars indicate the standard deviation.

manuscript the word "significant" when used in reference to kinome data will refer to these statistically significantly differentially phosphorylated peptides. Significant peptides/protein data was input into the protein-protein interaction database STRING (Szklarczyk et al., 2015) for interaction analysis and KEGG pathway analysis (Kanehisa et al., 2016). For the PCR data analyses the raw CT values from the qRT-PCR were used in a ΔΔCT calculation to determine fold change of mRNA expression between treatment and control tissue samples. A one-way ANOVA was conducted to determine if there was a statistical difference in fold-change of each cytokine in each tissue by day. If there was a significant different, a Student's t-test with Bonferroni correction was performed to identify statistically significantly different fold changes.

3 Results

3.1 The modern broilers' final weight is significantly impacted by the CpG treatment, heritage birds' is not

Through the grow-out period of 35 days, the modern birds grew more rapidly than the ACRB birds and had higher final body weights, as expected. On day 35 post-hatch, the control modern broilers had an average body weight of 2.18 kg, and the CpG injected modern broilers had an average body weight of 1.91 kg. This was the only sampling timepoint at which the difference in body weights between the treatment and control birds was statistically significant (Figure 2). The ACRB birds' average body weight on day 35 post-hatch was 0.43 kg for both the control and treatment groups. At no point during the grow-out period was the difference in weights between control and treatment ACRB birds significant.

3.2 The significant peptides unique to the modern broiler's day 2 cecal tonsil show innate signaling, heritage broiler day 2 cecal tonsil shows adaptive signaling

To characterize the different broilers' response to the initial CpG injection, we compared the significant peptides in the CpG injected birds' day 2 post-hatch cecal tonsils to the control birds' in both the ACRB and the modern birds. We also repeated this comparison for the day 2 jejuna. When the day 2 ACRB and modern birds' treatment and control samples were compared, differences in the two strains' responses to the CpG treatment emerged. The list of significant peptides in the modern broiler day 2 cecal tonsils was compared to the list of significant peptides in the ACRB day 2 cecal tonsil, and this comparison was repeated for the jejuna samples. There were 58 significant peptides that were unique to the ACRB broilers' jejuna samples on day 2 (after the first injection) post-hatch, compared to 199 significant peptides unique to the modern broilers' jejuna samples (Supplementary Figure 1). When this analysis was performed using data from the cecal tonsil, there was not as great

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TABLE 1 The top 10 GO biological processes and KEGG pathways that were found via STRING analysis to be enriched within the set of significant peptides unique to the modern broiler day 2 cecal tonsils when compared to the list of significant peptides in the ACRB cecal tonsils.

Modern broiler day 2 cecal tonsil – GO terms				Modern broiler day 2 cecal tonsil – KEGG pathways			
pathway ID	pathway description	observed protein count	false discovery rate	pathway ID	pathway description	observed protein count	false discovery rate
GO.0016310	phosphorylation	30	3.81E-13	5200	Pathways in cancer	14	5.54E-08
GO.0007169	transmembrane receptor protein tyrosine kinase signaling pathway	25	5.95E-13	4152	AMPK signaling pathway	9	5.15E-07
GO.0006468	protein phosphorylation	26	8.64E-13	4010	MAPK signaling pathway	11	1.83E-06
GO.0006793	phosphorus metabolic process	35	9.72E-12	4015	Rap1 signaling pathway	10	2.20E-06
GO.0010646	regulation of cell communication	42	1.96E-11	4014	Ras signaling pathway	10	3.44E-06
GO.0023051	regulation of signaling	41	1.96E-11	4620	Toll-like receptor signaling pathway	7	1.59E-05
GO.0043085	positive regulation of catalytic activity	30	7.70E-11	4932	Non-alcoholic fatty liver disease (NAFLD)	8	1.59E-05
GO.0051128	regulation of cellular component organization	36	7.70E-11	4151	PI3K-Akt signaling pathway	10	0.000108
GO.0006796	phosphate-containing compound metabolic process	33	9.70E-11	5206	MicroRNAs in cancer	7	0.000129
GO.0051247	positive regulation of protein metabolic process	29	2.35E-10	30	Pentose phosphate pathway	4	0.000159

a disparity in the number of significant peptides unique to the ACRB birds' day 2 samples and the modern broilers' samples (107 and 99 respectively). To better understand the difference in each tissues' responses to the CpG treatment, the significant peptides unique to the ACRB and modern broiler tissue samples were analyzed using STRING (Szklarczyk et al., 2015) to identify GO Biological processes (GO Bio) and KEGG pathways (Kanehisa et al., 2016) significantly enriched within the set of peptides (Tables 1, 2).

In the list of pathways generated from significant peptides unique to the modern birds' cecal tonsils, the MAPK, Toll-like receptor, and PI3k-Akt signaling pathways are among the top ten enriched pathways identified. In the ACRB birds' cecal tonsils, however, the KEGG pathways enriched within the set of significant peptides unique to the ACRB day 2 cecal tonsil include more adaptive immune signaling pathways, such as the T and B cell receptor signaling pathways. In looking more closely at the proteins involved in the B and T cell receptor signaling pathways, there are indications of signaling activation or partial activation (Supplementary Table 1). The GO Bio terms enriched among the unique significant peptides unique to the ACRB day 2 cecal tonsil include the cellular response to organic substance (Table 2). Within the peptides enriched in this biological process, there are indications of cytoskeletal rearrangement, as well as lymphangiogenesis and in particular natural killer (NK) and innate lymphoid cell (ILC) development.

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TABLE 2 The top 10 GO biological processes and KEGG pathways that were found via STRING analysis to be enriched within the set of significant peptides unique to the ACRB day 2 cecal tonsils when compared to the list of significant peptides in the modern broilers' cecal tonsils.

ACRB day 2 cecal tonsil – GO terms				ACRB day 2 cecal tonsil – KEGG pathways			
pathway ID	pathway description	observed protein count	false discovery rate	pathway ID	pathway description	observed protein count	false discovery rate
GO.0010033	response to organic substance	37	1.14E-15	4660	T cell receptor signaling pathway	9	4.02E-09
GO.0071310	cellular response to organic substance	33	1.21E-15	4662	B cell receptor signaling pathway	8	4.02E-09
GO.0016310	phosphorylation	24	3.08E-12	4917	Prolactin signaling pathway	8	4.02E-09
GO.0070887	cellular response to chemical stimulus	32	3.08E-12	4014	Ras signaling pathway	11	5.34E-09
GO.0006468	protein phosphorylation	21	8.16E-12	4012	ErbB signaling pathway	8	1.10E-08
GO.0038095	Fc-epsilon receptor signaling pathway	13	8.16E-12	5205	Proteoglycans in cancer	10	6.74E-08
GO.0071495	cellular response to endogenous stimulus	23	8.16E-12	4722	Neurotrophin signaling pathway	8	9.52E-08
GO.0038093	Fc receptor signaling pathway	14	9.37E-12	4919	Thyroid hormone signaling pathway	8	9.52E-08
GO.0045087	innate immune response	22	1.02E-11	4010	MAPK signaling pathway	10	1.75E-07
GO.0071363	cellular response to growth factor stimulus	19	1.73E-11	5162	Measles	8	1.90E-07

3.3 Significant peptides unique to the modern broiler day 2 jejunum show pro-survival signaling, heritage birds' day 2 jejunum shows controlled innate immune response

In the ACRB birds' day 2 jejunum, the phosphorylation patterns of the significant peptides involved in the innate immune response GO Bio process and the Toll-like receptor/PI3k-Akt/Insulin signaling pathways indicate negative feedback and control of an immune response, specifically negative feedback of the antiviral response (Tables 3, 4). In the modern broiler's day 2 jejunum, conversely, much of the signaling activity corresponds to a pro-survival, non-apoptotic signaling, and immune signaling (Supplementary Table 2).

3.4 At day 15/16 heritage broilers have an adaptive immune response to CpG injection in their cecal tonsils, metabolic signaling predominates in modern broilers' cecal tonsils

Five birds from each group were sampled on day 15, then the remaining birds were given another injection of either CpG or GpC control as appropriate, and then five more birds from each group were sampled on day 16. The list of peptides that were significant prior to injection and the list of peptides significant after the injection for each tissue and bird type were compared to generate the Venn diagrams in Supplementary Figure 2. The CpG injection on day 15 led to a greater number of significant peptides in the modern broiler at day 16 in cecal tonsil than in the ACRB day 16 cecal tonsil (191 vs 83 respectively). The

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TABLE 3 The top 10 GO biological processes and KEGG pathways that were found via STRING analysis to be enriched within the set of significant peptides unique to the ACRB day 2 jejunum tissues' when compared to the modern broilers' significant peptides.

ACRB day 2 jejunum - GO Bio terms				ACRB day 2 jejunum - KEGG pathways			
pathway ID	pathway description	observed protein count	false discovery rate	pathway ID	pathway description	observed protein count	false discovery rate
GO.0006793	phosphorus metabolic process	19	0.00013	4620	Toll-like receptor signaling pathway	5	0.00181
GO.0016310	phosphorylation	15	0.00013	4910	Insulin signaling pathway	5	0.00333
GO.0006468	protein phosphorylation	13	0.000174	20	Citrate cycle (TCA cycle)	3	0.00492
GO.0006796	phosphate-containing compound metabolic process	18	0.000239	5164	Influenza A	5	0.00498
GO.0006955	immune response	15	0.000662	5020	Prion diseases	3	0.00633
GO.0009893	positive regulation of metabolic process	23	0.00173	1,200	Carbon metabolism	4	0.00676
GO.0010604	positive regulation of macromolecule metabolic process	20	0.00173	4014	Ras signaling pathway	5	0.0104
GO.0042325	regulation of phosphorylation	14	0.00173	4010	MAPK signaling pathway	5	0.0136
GO.0022407	regulation of cell-cell adhesion	8	0.00222	4621	NOD-like receptor signaling pathway	3	0.0136
GO.0045087	innate immune response	12	0.00222	5161	Hepatitis B	4	0.0136

most striking impact of the CpG injection on day 15 is that in the ACRB birds' day 16 cecal tonsil, the KEGG pathways enriched within the set of significant peptides unique to day 16 include adaptive and innate immune signaling pathways. In the modern birds' day 16 cecal tonsil, conversely, the pathways enriched in the significant peptides unique to the post-injection time frame indicate a more metabolic response to the injection (Supplementary Table 3)

To better understand the immune response to the CpG injection in the ACRB birds' cecal tonsils, the phosphorylation statuses of the peptides involved in all the top 10 KEGG signaling pathways enriched in the set of significant peptides unique to the day 16 cecal tonsil were examined. The phosphorylation patterns suggest potential T cell receptor signaling activation in the ACRB birds' day 16 cecal tonsil (Table 5). In the modern birds' day 16 cecal tonsil, however, the response to the CpG injection appears to be metabolic in nature. There is evidence of AMPK activation, increased insulin signaling, and mTOR inhibition in the modern birds' day 16 cecal tonsil (Table 5).

3.5 Modern broilers have enhanced immune signaling in the jejuna after treatment

In the jejuna samples, unlike the cecal tonsils, fewer significant peptides are unique to the day 16 post-injection time period than

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TABLE 4 The top 10 GO biological processes and KEGG pathways that were found via STRING analysis to be enriched within the set of significant peptides unique to the modern broilers' day 2 jejunum tissues' when compared to the ACRB birds' significant peptides.

Modern broiler day 2 jejunum – GO Bio terms				Modern broiler day 2 jejunum – KEGG pathways			
pathway ID	pathway description	observed protein count	false discovery rate	pathway ID	pathway description	observed protein count	false discovery rate
GO.0045087	innate immune response	55	4.34E-27	5200	Pathways in cancer	32	2.19E-21
GO.0050776	regulation of immune response	52	4.34E-27	4010	MAPK signaling pathway	25	9.20E-17
GO.0042325	regulation of phosphorylation	61	3.48E-26	4151	PI3K-Akt signaling pathway	28	9.20E-17
GO.0009893	positive regulation of metabolic process	95	8.32E-26	4722	Neurotrophin signaling pathway	18	1.82E-15
GO.0019220	regulation of phosphate metabolic process	65	8.32E-26	5205	Proteoglycans in cancer	21	6.52E-14
GO.0010604	positive regulation of macromolecule metabolic process	82	8.28E-25	5206	MicroRNAs in cancer	18	6.52E-14
GO.0031325	positive regulation of cellular metabolic process	84	1.91E-24	4015	Rap1 signaling pathway	20	1.89E-13
GO.0010033	response to organic substance	78	7.54E-24	4014	Ras signaling pathway	20	6.49E-13
GO.0001932	regulation of protein phosphorylation	56	8.96E-24	4620	Toll-like receptor signaling pathway	15	8.92E-13
GO.0002682	regulation of immune system process	59	1.79E-23	4910	Insulin signaling pathway	16	2.86E-12

are common to pre- and post-injection or unique to pre-injection (Supplementary Figure 3). In the ACRB birds, there is a nearly equal number of significant peptides that are unique to the pre-injection time period and the post-injection time period. The top ten enriched KEGG pathways among the significant peptides unique to the modern birds' post-injection jejuna samples show more indications of an adaptive immune response, however, the phosphorylation patterns show suppression of T and B cell receptor signaling activity (Table 6). In the ACRB birds, the significant peptides unique to the day 16 jejunum are more involved in metabolism and oxidative stress signaling, though there are indications of immune signaling as well (Table 6).

3.6 The response to the injection is similar in both bird strains

By the time the birds reach the end of the sampling period, days 34 and 35 post-hatch, the response to the CpG injection decreases, as there are fewer significant peptides that are unique to the post-injection timepoint in both the modern birds' and ACRB birds' cecal tonsils (Supplementary Figure 4). There is still a larger proportion of the total significant peptides unique to the post-injection timepoint in the modern birds than in the ACRB birds.

In the ACRB birds' cecal tonsils, there are only three to five proteins in the significantly enriched KEGG pathways generated

TABLE 5 Top 10 enriched KEGG pathways in the set of significant peptides unique to day 16 (post-injection) in the modern broiler birds' cecal tonsils (left) and in the significant peptides unique to day 16 (post-injection) in the ACRB birds' cecal tonsils (right).

Top 10 KEGG pathways enriched in the significant peptides unique to the modern bird day 16 cecal tonsil				Top 10 KEGG pathways enriched in the significant peptides unique to the ACRB day 16 cecal tonsil			
Pathway ID	Pathway description	Observed gene count	False discovery rate	Pathway ID	Pathway description	Observed gene count	False discovery rate
hsa04910	Insulin signaling pathway	24	3.20E-20	hsa04380	Osteoclast differentiation	8	8.57E-06
hsa04010	MAPK signaling pathway	25	2.23E-14	hsa04510	Focal adhesion	9	1.00E-05
hsa05169	Epstein-Barr virus infection	20	6.81E-13	hsa04660	T cell receptor signaling pathway	7	1.10E-05
hsa05200	Pathways in cancer	29	1.04E-12	hsa05206	MicroRNAs in cancer	8	1.10E-05
hsa04931	Insulin resistance	16	1.20E-12	hsa04664	Fc epsilon RI signaling pathway	6	1.28E-05
hsa05161	Hepatitis B	17	4.14E-12	hsa04670	Leukocyte transendothelial migration	7	1.28E-05
hsa04152	AMPK signaling pathway	16	4.29E-12	hsa05145	Toxoplasmosis	7	1.28E-05
hsa04922	Glucagon signaling pathway	15	4.78E-12	hsa04152	AMPK signaling pathway	7	1.48E-05
hsa04380	Osteoclast differentiation	16	5.28E-12	hsa04650	Natural killer cell mediated cytotoxicity	7	1.62E-05
hsa04211	Longevity regulating pathway	14	1.23E-11	hsa04151	PI3K-Akt signaling pathway	10	2.23E-05

from the list of unique significant peptides (Supplementary Table 4). Largely, the enriched pathways are involved in fatty acid oxidation, glucose metabolism, and T and B cell growth.

The significant peptides unique to the modern broiler's day 35 (post-injection) cecal tonsil are enriched for KEGG pathways including autophagy, osteoclast differentiation, and viral response associated pathways. The phosphorylation patterns in these signaling pathways indicate response to unfolded protein stress. The B cell receptor pathway is enriched within the set of peptides unique to day 35, but the signaling patterns are suggestive of inhibition.

By day 35, the signaling changes in both the ACRB and modern birds' jejuna are similar to each other. Among the top 10 enriched KEGG pathways in both tissues are the MAPK signaling pathway, the Insulin signaling pathway, Pathways in cancer, the Hepatitis B signaling pathway, and the PI3k-Akt signaling pathway (Tables 7, 8). There were also a similar number of significant peptides unique to the post-injection time point in the ACRB birds and the modern birds (105 and 117 respectively) (Supplementary Figure 5).

3.7 The cytokine expression data indicate initial robust response that tapers off

In both tissues and both bird types, the expression of proinflammatory cytokines is increased immediately following the first injection with CpG (Figure 3). There are differences in which cytokines are increased in each tissue, and the degree to which the expression increases. It is interesting that the increased cytokine expression continues to the day 15 post-hatch time point in the modern birds' cecal tonsil, but then following the injection on day 15 this expression drops off below the control birds. For all other tissues and time points in both birds the expression of each cytokine is similar to control or below control birds' levels. A one-way ANOVA was conducted to identify significant changes in fold change expression by day. In the modern broiler jejunum, the fold change in IFN-γ expression was significantly different on day 2 post-hatch compared to all other sampling time points. Similarly, in the modern birds' cecal tonsil the fold change in IL-1β expression

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TABLE 6 Top 10 enriched KEGG pathways in the set of significant peptides unique to day 16 (post-injection) in the modern broiler birds' jejunum (left) and in the significant peptides unique to day 16 (post-injection) in the ACRB birds' jejunum (right).

Top 10 KEGG pathways enriched in the significant peptides unique to the modern bird day 16 jejunum				Top 10 KEGG pathways enriched in the significant peptides unique to the ACRB day 16 jejunum			
#term ID	term description	observed protein count	false discovery rate	#term ID	term description	observed protein count	false discovery rate
hsa04660	T cell receptor signaling pathway	10	3.07E-09	hsa00010	Glycolysis/ Gluconeogenesis	8	6.71E-07
hsa04151	PI3K-Akt signaling pathway	14	1.31E-08	hsa04010	MAPK signaling pathway	13	6.71E-07
hsa04662	B cell receptor signaling pathway	8	5.45E-08	hsa04066	HIF-1 signaling pathway	9	6.71E-07
hsa04722	Neurotrophin signaling pathway	9	7.41E-08	hsa04662	B cell receptor signaling pathway	8	6.71E-07
hsa04152	AMPK signaling pathway	9	7.85E-08	hsa05200	Pathways in cancer	17	6.71E-07
hsa05200	Pathways in cancer	15	7.85E-08	hsa00052	Galactose metabolism	6	1.33E-06
hsa04910	Insulin signaling pathway	9	1.39E-07	hsa00500	Starch and sucrose metabolism	6	1.58E-06
hsa04510	Focal adhesion	10	1.97E-07	hsa04064	NF-kappa B signaling pathway	8	1.68E-06
hsa05161	Hepatitis B	9	1.97E-07	hsa05162	Measles	9	1.68E-06
hsa04620	Toll-like receptor signaling pathway	8	2.33E-07	hsa04620	Toll-like receptor signaling pathway	8	2.62E-06

was significantly different on day 2 post-hatch when compared with other sampling days. In the ACRB cecal tonsil, the fold change in IL-1β on day 2 post hatch was significantly different from that of other sampling days (day 15 post-hatch excepted). In the ACRB jejunum, the fold change in expression of IFN-γ was significantly different on day 2 post hatch from any other sampling day.

4 Discussion

The cecal tonsils are the largest organ in the avian gut-associated lymphoid tissue (GALT), and home to many lymphocyte populations from which cell-mediated and humoral immune responses could influence the immune system in the rest of the gastrointestinal tract. The signaling patterns in the modern broilers cecal tonsils on the day after the first injection were indicative of innate immune signaling as shown by the significantly enriched TLR signaling In the modern birds' day 2 cecal tonsil, there are indications of active TLR signaling; signaling proteins such as specific MAPKs, RAC1, and the phosphatase DUSP6 all show phosphorylation patterns that would result downstream of TLR ligation. When mononuclear cells are isolated from commercial broiler chicks' cecal tonsils and treated *in vitro* with TLR ligands, including LPS isolated from *E. coli* (*Escherichia coli*) O 111:B4 and CpG, the stimulation of the TLRs leads to increased mRNA expression of proinflammatory cytokines such as IFN-γ, IL-1β and IL-6 (Taha-abdelaziz et al., 2016). The broilers treated with CpG in this study showed similar increases in cytokine mRNA in their cecal tonsils after the first exposure. There are also indications of non-TLR specific innate signaling, such as activated receptors that would lead downstream to increased hematopoiesis of innate immune cells such as monocytes and dendritic cells. This signaling coupled with the increased proinflammatory cytokines suggest a primarily innate response occurred in the modern birds' cecal tonsils the day after the first treatment with CpG.

In the ACRB birds, conversely, two of the top 10 KEGG pathways significantly enriched among the peptides unique to the ACRB birds are the B cell receptor signaling pathway and the T cell receptor signaling pathway, the main cell types that constitute an adaptive immune response. The significantly differentially phosphorylated proteins are highly specific for either the B or T cell receptor signaling pathways. Zap70, for one, is a src-family kinase that is highly characteristic of T cell receptor signaling (Fischer et al., 2010), and it is uniquely significant in the ACRB birds' cecal tonsil. Bruton's tyrosine kinase (BTK) is another significant protein unique to the ACRB birds' cecal tonsil that is characteristic of B

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TABLE 7 Top 10 enriched KEGG pathways in the set of significant peptides unique to day 35 (post-injection) in the modern broiler birds' cecal tonsils (left) and in the significant peptides unique to day 35 (post-injection) in the ACRB birds' cecal tonsil (right).

Top 10 KEGG pathways enriched in the significant peptides unique to the modern bird day 35 cecal tonsil				Top 10 KEGG pathways enriched in the significant peptides unique to the ACRB bird day 35 cecal tonsil			
Pathway ID	Pathway description	Observed protein count	False discovery rate	Pathway ID	Pathway Description	Observed protein count	False discovery rate
hsa04140	Autophagy – animal	9	4.73E-07	hsa01200	Carbon metabolism	5	0.0022
hsa04380	Osteoclast differentiation	9	4.73E-07	hsa04910	Insulin signaling pathway	5	0.0022
hsa05160	Hepatitis C	9	4.73E-07	hsa05230	Central carbon metabolism in cancer	4	0.0022
hsa05167	Kaposi's sarcoma-associated herpesvirus infection	10	4.73E-07	hsa04146	Peroxisome	4	0.0024
hsa05200	Pathways in cancer	14	5.99E-07	hsa00520	Amino sugar and nucleotide sugar metabolism	3	0.0086
hsa04662	B cell receptor signaling pathway	7	7.21E-07	hsa04068	FoxO signaling pathway	4	0.0094
hsa05162	Measles	8	2.17E-06	hsa04213	Longevity regulating pathway - multiple species	3	0.012
hsa05169	Epstein-Barr virus infection	9	2.35E-06	hsa05206	MicroRNAs in cancer	4	0.012
hsa05161	Hepatitis B	8	2.73E-06	hsa00010	Glycolysis/ Gluconeogenesis	3	0.0127
hsa04066	HIF-1 signaling pathway	7	3.42E-06	hsa04920	Adipocytokine signaling pathway	3	0.0127

cell receptor signaling (Kurosaki and Kurosaki, 1997). Our data suggest that there are populations of B and T cells in the ACRB broilers' day 2 post-hatch cecal tonsil that potentially respond to the TLR21 stimulation.

There are also increases in proinflammatory cytokine mRNA in the ACRB birds' day 2 cecal tonsil, although only IL-1β and IFN-γ showed significant fold increases. IFN-γ is a proinflammatory cytokine that participates in the Th1 effector cell differentiation process and immune response (Morales-Mantilla and King, 2018). The increase in IFN-γ production along with T cell signaling suggest there is the possibility for this type of response in the ACRB birds' cecal tonsils. Pro-IL-1β needs to undergo cleavage by caspase 1 (Casp1) to become the final IL-1β protein (Denes et al., 2012). Casp1 is significantly dephosphorylated in the ACRB day 2 cecal tonsil, though this is at a site with no downstream implications of the phosphorylation yet elucidated, so the impact of this Casp1 phosphorylation is unknown.

In the modern broilers' jejunum on day 2 post-hatch, after the first injection of CpG, the signaling patterns indicate anti-apoptotic signaling as well as cell-cycle progression. Specifically, Caspase-3 is inhibited in the modern birds' jejunum. This caspase has been linked to neutrophil apoptosis during the contraction of an inflammatory response (Alvarado-Kristensson et al., 2004). Avian species have heterophils rather than neutrophils, but these cells have a similar function and phenotype to mammalian neutrophils. Potentially, their apoptosis processes could be controlled by a similar mechanism (Horn et al., 2012). This could be indicative of potentially increased heterophil longevity within the modern birds' jejunum as a response to the CpG. Modern broilers demonstrate robust cell-mediated immune responses (Cheema et al., 2003), and the inhibition of apoptosis and increased cell-cycle signaling observed may be indicative of that process.

In the ACRB birds, the significant peptides unique to the day 2 jejunum are suggestive of negative feedback and feedback inhibition of immune responses. TBK1 is phosphorylated at a site

TABLE 8 Top 10 enriched KEGG pathways in the set of significant peptides unique to day 35 (post-injection) in the ACRB broiler birds' jejunum (left) and in the significant peptides unique to day 35 (post-injection) in the modern broiler birds' jejunum (right).

Top 10 KEGG pathways in the significant peptides unique to ACRB birds' day 35 jejunum				Top 10 KEGG pathways in the significant peptides unique to modern broiler birds' day 35 jejunum			
Pathway ID	Pathway description	Observed protein count	False discovery rate	Pathway ID	Pathway description	Observed protein count	False discovery rate
hsa04010	MAPK signaling pathway	16	5.94E-10	hsa04010	MAPK signaling pathway	18	1.87E-11
hsa04380	Osteoclast differentiation	10	1.38E-07	hsa04910	Insulin signaling pathway	14	1.87E-11
hsa05230	Central carbon metabolism in cancer	8	2.08E-07	hsa05167	Kaposi's sarcoma-associated herpesvirus infection	15	2.08E-11
hsa05161	Hepatitis B	10	2.36E-07	hsa05161	Hepatitis B	13	1.77E-10
hsa04931	Insulin resistance	9	2.73E-07	hsa05200	Pathways in cancer	20	5.91E-10
hsa05200	Pathways in cancer	16	2.79E-07	hsa05215	Prostate cancer	11	6.95E-10
hsa04014	Ras signaling pathway	11	8.69E-07	hsa05169	Epstein-Barr virus infection	13	3.90E-09
hsa04151	PI3K-Akt signaling pathway	13	8.69E-07	hsa04151	PI3K-Akt signaling pathway	16	4.64E-09
hsa04910	Insulin signaling pathway	9	9.50E-07	hsa04657	IL-17 signaling pathway	10	5.76E-09
hsa04620	Toll-like receptor signaling pathway	8	1.64E-06	hsa05160	Hepatitis C	11	8.24E-09

that the kinase Lck would target for phosphorylation that would lead to inhibition of TBK1 as part of the negative feedback response (Liu et al., 2017). Lck is also phosphorylated on an activation site in these tissues, so negative feedback signaling as part of the antiviral response may be ongoing. Furthermore, NFIL3 is phosphorylated on a negative regulatory site (Table 6), and this protein is important for the development of ILCs and NK cells (Kostrzewski et al., 2018). It is interesting that in both the modern and ACRB broiler's day 2 jejunum the biggest change in cytokine expression after the first injection is a fold-increase in IFN- γ expression. CpG treatment is known to induce IFN-γ production in avian PBMCs, so this raises the question of which resident cells in the avian jejunum are producing this cytokine in response to the CpG treatment (He et al., 2003).

At the day 15/16 post-hatch experimental time point, the pattern seen earlier in the cecal tonsils is recapitulated, where the response in the ACRB birds mostly resembles adaptive immune signaling, however the response in the modern birds involves more metabolic signaling. Additionally, there is a larger number of significant peptides unique to the day 16 timepoint in the modern birds' cecal tonsil than the heritage bird. These data suggest that the impact of the CpG injection on day 15 was greater in the modern birds than in the heritage birds. In the ACRB birds' tissues at the post-injection time point there is partial activation along the T/B cell receptor signaling pathway and there is also activation of focal adhesion signaling pathways and cytoskeletal rearrangement. Focal adhesion and cytoskeleton rearrangement are important for T cell activation and signaling (Rozdzial et al., 1995) and activation of these pathways may further suggest T cell activation after the day 15 CpG injection. The metabolic state of immune cells has also been shown to directly influence their activation and differentiation (Shi et al., 2011; Macintyre et al., 2014). In the case of the ACRB birds' day 16 cecal tonsil, mTOR is significantly dephosphorylated on its activation sites, but the ribosomal protein S6 kinase beta-1 is significantly phosphorylated on an activation site (Table 8), where it would be phosphorylated by active mTOR (Saitoh et al., 2002). However, this phosphorylation can be accomplished by other kinases, such as PDK1, so it is still possible that mTOR is inactivated in these tissues. There is also evidence of TGF-β signaling ongoing in these tissues. The combination of TGF-β signaling, T cell receptor activation, and mTOR inhibition could be indicative of the generation of regulatory T cells (Ghiringhelli et al., 2005; Delgoffe et al., 2009). Tregs are important modulators of inflammatory responses, and especially so in the intestinal environment where there are large populations

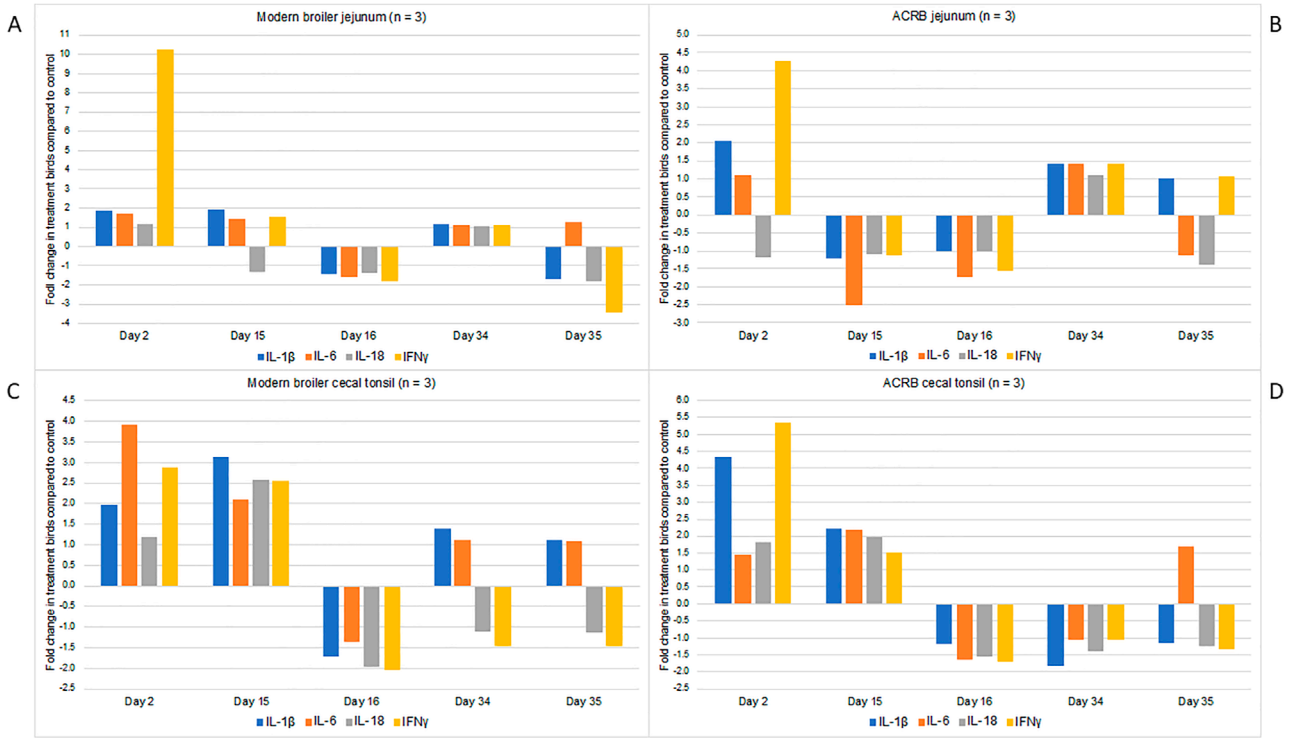

FIGURE 3
RNA was isolated from three individual birds' cecal tonsil and jejunum tissue samples and used for RT-PCR. Analyses were performed using primers for IL-1beta, IL-6, IL-18 and IFN-gamma. The data are presented here as fold-changes between the treatment birds and the control, i.e., numbers greater than one indicate a fold-increase, and negative numbers indicate a fold-decrease in treatment compared to control birds. Fold changes values between 0 and one were converted into the reciprocal negative number. **(A)** Shows gene expression data from the modern broiler jejunum tissue, **(B)** ACRB jejunum tissue, **(C)** modern broiler cecal tissue, and **(D)** ACRB cecal tissue.

of commensal bacteria and unwarranted inflammation could be damaging to the surrounding tissues. These data may indicate ACRB broilers' generating regulatory T cells to temper inflammation in response to the CpG treatment given on day 15 post-hatch. The PCR data support the potential for a regulatory environment, as there are no increases in expression of the pro-inflammatory cytokines in response to the day 15 injection.

In the modern birds' cecal tonsils after the day 15 injection metabolic signaling pathways are enriched in the significant peptides unique to the post-injection timepoint. Subunits of AMPK are significantly phosphorylated on target sites that play a role in AMPK localization and confer the potential for activation. Acetyl-CoA carboxylase is also significantly phosphorylated, which would be activated downstream of AMPK activity (Table 8). mTOR is also inhibited in these tissues, but there is activation of insulin signaling. These metabolic shifts indicate that there could be a need in these cells for increased cellular energy. AMPK is a master energy sensor in cells and is activated when cells sense a high AMP: low ATP ratio (Long and Zierath, 2006). Part of AMPK's response to low cellular energy is to inhibit processes that consume energy, such as protein synthesis, so AMPK inhibits mTOR activation. Raptor is a regulatory protein that controls mTOR activation and in these tissues it is significantly dephosphorylated at a site that would be phosphorylated by JNK1 in response to oxidative stress (Table 8) (Kwak et al., 2012). This JNK1 mediated phosphorylation of Raptor in response to stress would lead to mTOR activation, and the significant dephosphorylation of Raptor in the modern birds' day 16 cecal tonsils could indicate further inhibition of mTOR. The cytokine mRNA expression on day 16 supports the kinome data as the expression drops off considerably compared to the data from day 15 and drops below control.

In the modern birds' jejuna there is immune signaling enriched among the significant peptides unique to this time point (Table 8). This signaling is inhibitory and the cytokine data reflect that pattern as well (Figure 3). While the CpG has a more dynamic impact on signaling in the modern birds' cecal tonsil than the heritage birds', that is not the case in the jejunum.

By the end of the experimental period, the ACRB and modern broiler's responses to the injection are similar, with many of the same pathways enriched specifically in the post-injection timepoint in the jejunum samples. However, in the ACRB birds' cecal tonsils, uniquely, there is signaling that indicates increased fatty acid oxidation. Innate immune cells can undergo epigenetic changes in response to repeated exposure to non-specific pathogenic stimuli, and these changes can be linked to alterations in metabolism (Bekkering et al., 2018).

In summary, the modern broilers' jejunum, responded to the treatment with changes to metabolic signaling pathways (Table 5). In the ACRB, adaptive immune signaling was enriched specifically in their cecal tonsils (Table 4). ACRB jejuna also showed immune

signaling changes in response to the treatment, although there were additional changes in carbohydrate metabolism and indications of oxidative and metabolic stress. The modern broilers' cecal tonsils showed a consistently greater magnitude of response to the CpG treatment, with more significant peptides unique to the post-injection timepoint in the modern birds than the ACRB birds. After the first injection, both modern and ACRB broilers had increased mRNA expression of various pro-inflammatory cytokines in both their jejuna and cecal tonsils in the treatment birds compared to the control birds (Figure 3). After the subsequent injections, however, the cytokine mRNA expression did not exhibit large changes in expression between the treatment and control birds; indeed, the mRNA expression of the cytokines measured often decreased in the treatment birds compared to that of the control birds' post-injection.

5 Conclusion

Overall, the ACRB birds' responses to the repeated injections with CpG indicate initiation of adaptive immune responses early on, that then mature into regulatory responses. The modern birds' immune signaling indicates a more innate response initially, coupled with a metabolic shift in the cecal tonsil, the major lymphoid organ of the chicken GALT. Strong evidence for this is the reduced growth observed in the modern broilers while the ACRBs showed no detrimental effect on their growth from the repeated CpG injections.

The data presented in this study showcase the need to better understand the allocation of resources, and specifically energy in the modern broiler, a bird that is genetically programmed for rapid growth and yet needs the metabolic flexibility to support growth and effective immune functions. This balance is especially important during the time in broiler chickens' life when they are both experiencing accelerating growth and more susceptible to disease challenges, which coincide at approximately the 2-week post-hatch mark. Better understanding of this vulnerable time can potentially lead to therapeutic interventions to preserve bird health and efficiency in a post-antimicrobial industry.

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.

Ethics statement

The animal study was approved by Institutional Animal Care and Use Committee, University of Delaware. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

BA: Formal Analysis, Methodology, Visualization, Writing–original draft. CJ: Methodology, Visualization, Writing–review and editing. FP: Methodology, Writing–review and editing. RW: Conceptualization, Writing–review and editing. RA: Conceptualization, Methodology, Project administration, Writing–review and editing.

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Conflict of interest

Author RW was employed by Evonik Operations GmbH.

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.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2024.1473202/full#supplementary-material

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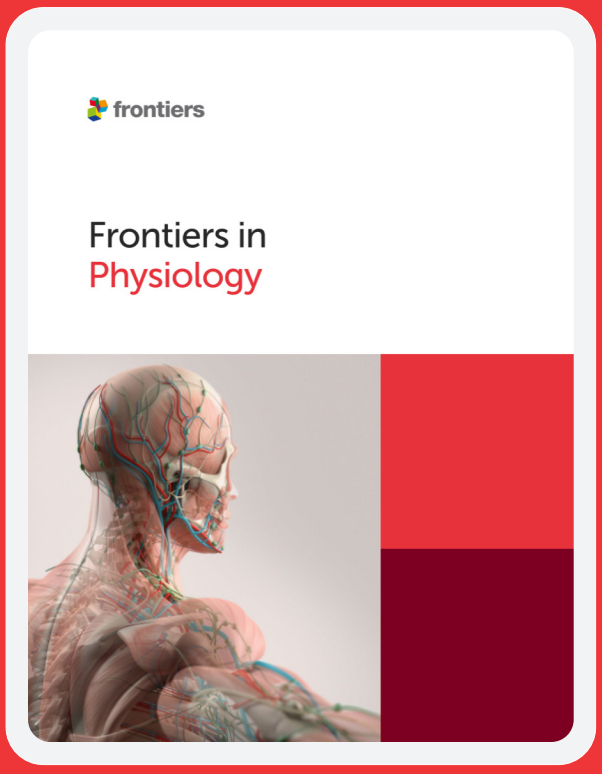

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