NUTRITIONAL AND ENVIRONMENTAL MODULATION OF THE ENDOCRINE SYSTEM: EFFECTS ON METABOLISM AND GROWTH

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NUTRITIONAL AND ENVIRONMENTAL MODULATION OF THE ENDOCRINE SYSTEM: EFFECTS ON METABOLISM AND GROWTH

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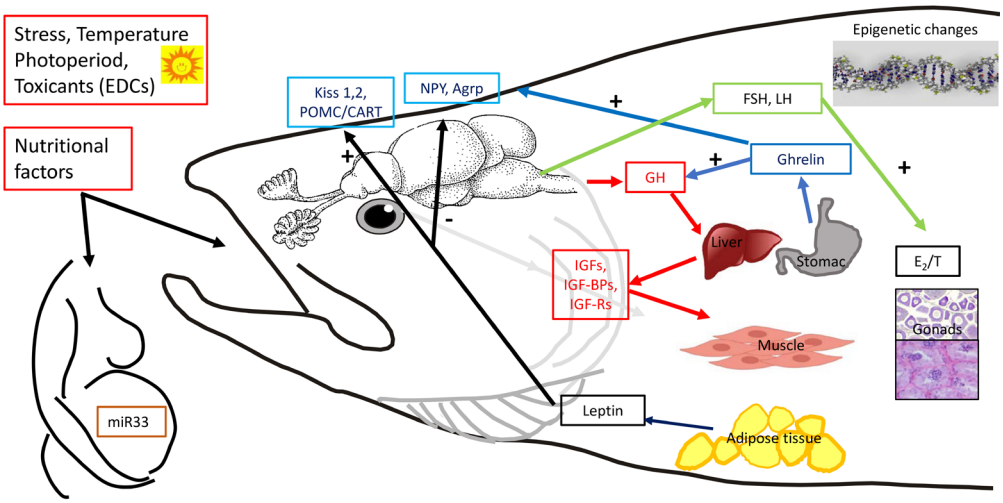

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The present eBook is the result of the Frontiers Research Topic entitled "Nutritional and environmental modulation of the endocrine system: effects on metabolism and growth". It contains 12 chapters, comprising 7 original research articles, 3 reviews, and 2 minireviews. The objective of the Research Topic was to provide a multidisciplinary approach of cutting-edge research on metabolism and growth aiming to address key questions about the interplay between nutritional, environmental or other external factors (i.e. temperature or pollutants) and signals modulating feed intake with the endocrine system, regulating these processes. Evidences about the molecular principle behind the complex interactions of all these factors on the control of the endocrine and nervous systems regulating the metabolic process are presented. The knowledge provided by this eBook focusing in cells, model organisms and farmed species, have highlighted the importance of dietary and environmental factors, and their interactions with the endocrine system to regulate growth and metabolism.

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EDITORIAL
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Editorial: Nutritional and Environmental Modulation of the Endocrine System: Effects on Metabolism and Growth

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Keywords: growth, metabolism, endocrine disruptors, feed factors, appetite regulation

Editorial on the Research Topic

Nutritional and Environmental Modulation of the Endocrine System: Effects on Metabolism and Growth

Metabolism and growth are under the control of the endocrine system that, working in cooperation with the nervous system, regulates these functions. The objective of this Research Topic was to provide a multidisciplinary approach of cutting-edge research on metabolism and growth in different experimental models, including farmed species. The Research Topic contains 12 contributions, comprising 7 original research articles, 3 reviews, and 2 minireviews. These works include a wide range of cellular and *in vivo* models, methodological and conceptual approaches. The Topic focused on recent research conducted in the field of metabolism and growth, and aimed to address key questions about the interplay between nutritional, environmental, or other external factors (i.e., temperature or pollutants) and the endocrine system, as well as the modulation of signals involved in the control of feed intake, regulating these processes.

From fish to mammals, the growth hormone (GH)/insulin-like growth factor I (IGF-I) axis is the major endocrine system stimulating growth (1, 2), indicating a strong evolutionary conservation. GH regulates growth directly, but also indirectly through induced production of IGF-I, mostly in the liver, but also in peripheral tissues, where this growth factor exerts paracrine and autocrine actions. In the current Topic, Pérez-Sánchez et al. reviewed the evolution of the GH, prolactin, and somatolactin family of peptides and their sub-functionalization in marine fish species, and revisited the direct and indirect effects of GH and IGF-I on growth and development. In addition to growth, the GH/IGF-I system controls other physiological functions including metabolism and mineral balance, and, to properly grow and meet metabolic demands, hormones produced from the brain and peripheral tissues contribute regulating feeding (3, 4). The GH/IGF-I system and the hormones controlling appetite are regulated by both, internal and external signals. The internal signals inform about the metabolic status of the organism and in relation to that, Pérez-Sánchez et al. commented on sirtuins, new markers informing of energy status that can modulate the anabolic actions of GH. In this line, Björnsson et al. evaluated the impact of energy reserves on GH resistance and circulating GH-binding protein levels in rainbow trout (*Oncorhynchus mykiss*) of different genetic background, demonstrating that initial body reserves affect whether or not GH resistance is acquired under catabolic physiological conditions. In another study in pregnant women treated with *Lycium barbarum* L. polysaccharides (LBLP), a relation between insulin resistance and secretion, lipid profiles, and miR-33 levels was reported, showing the beneficial effects of LBLP improving the symptoms of gestational diabetes mellitus (Yang et al.).

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Moreover, the close relationship between growth and reproduction has been discussed in a manuscript focusing on the low levels of leptin associated with the overexpression of GH in transgenic common carp (*Cyprinus carpio*) and the resulting delay in puberty onset (Chen et al.).

Among the external signals modulating growth and metabolism, diet availability and composition are the most notable. In line with this, one review within the Topic has explored the nutritional factors that through influencing the endocrine system control feeding and growth in fish, describing the nutrients as well as the brain and peripheral hormones that regulate food intake (Bertucci et al.). Secondly, the recent knowledge achieved concerning the interaction of stress with the different neurosensing mechanisms and peripheral endocrine signals such as leptin that regulate food intake in fish have been revised (Conde-Sieira et al.). Then, considering the effects of specific nutrients on metabolism, the physiological and epigenetic changes induced by methionine restriction have been reviewed encompassing both, mammalian and fish models (Neslund Latimer et al.). Next, specifically in gilthead seabream (*Sparus aurata*), the effects of dietary supplementation with the aminoacid derivative creatine on muscle growth and quality, have been explored (Ramos-Pinto et al.). Increasing dietary creatine enlarged dorsal cross-sectional muscle area and modulated the expression of genes related to muscle growth (i.e., myogenic regulatory factors, MRFs) and texture (i.e., protein degradation), although further studies are required to better clarify these effects in fish.

Considering environmental signals, two articles within the Topic have explored the effects of temperature on metabolism and growth in different fish species. Sanhueza et al. reported in Atlantic salmon (*Salmo salar*), that a wide thermal range is associated with significant increases in gene transcription of circadian-clock related genes and monoamines hormone levels, which decrease aggressive behavior, and positively influence stress, welfare, and growth, whereas a restricted thermal range showed the opposite effects. Moreover, since climate change is a major challenge that humanity is facing nowadays, a study investigated in gilthead sea bream, the effects of three rearing temperatures (19, 24, and 28°C) on growth and lipid metabolism using *in vivo* and *in vitro* approaches (Balbuena-Pecino et al.). Increasing temperatures caused unfavorable musculoskeletal growth conditions due to reduced expression of GH/IGF-I system members and specific MRFs genes. Moreover, to cope with the increased energy needs, lipid metabolism was induced in the muscle although not efficiently.

Besides abiotic factors, environmental contaminants are known to cause adverse health effects, impairing not only reproduction but also metabolism and development in wildlife and humans, since there is evidence that endocrine disruptive chemicals (EDCs) can interact with a variety of hormones and/or hormone receptors, exerting actions as agonists or antagonists (5, 6). In the present Topic, the effects of EDCs on lipid metabolism and health and their potential "transgenerational obesogenic effects" have been revised, focusing on the modifications occurring at hepatic level in different animal models (Maradonna and Carnevali). Moreover, the effects of several EDCs individually and in mixture were tested on male fathead minnows (*Pimephales promelas*) through analysis of liver transcriptomes by expression microarrays and subsequent quantitative PCR, identifying distinct modes of action for the different chemicals as well as pinpointing a number of new biomarkers that can potentially be used for environmental screening (Zare et al.).

Taken together, these studies have demonstrated the effects of dietary and environmental factors on metabolic processes, and the complex interactions of all these factors on the control of the endocrine system regulating growth and metabolism mainly in model organisms and farmed species.

AUTHOR CONTRIBUTIONS

OC and EC drafted and critically reviewed the article.

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REVIEW
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Somatotropic Axis Regulation Unravels the Differential Effects of Nutritional and Environmental Factors in Growth Performance of Marine Farmed Fishes

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The Gh/Prl/Sl family has evolved differentially through evolution, resulting in varying relationships between the somatotropic axis and growth rates within and across fish species. This is due to a wide range of endogenous and exogenous factors that make this association variable throughout season and life cycle, and the present minireview aims to better define the nutritional and environmental regulation of the endocrine growth cascade over precisely defined groups of fishes, focusing on Mediterranean farmed fishes. As a result, circulating Gh and Igf-i are revitalized as reliable growth markers, with a close association with growth rates of gilthead sea bream juveniles with deficiency signs in both macro- or micro-nutrients. This, together with other regulated responses, promotes the use of Gh and Igf-i as key performance indicators of growth, aerobic scope, and nutritional condition in gilthead sea bream. Moreover, the sirtuin-energy sensors might modulate the growth-promoting action of somatotropic axis. In this scenario, transcripts of *igf-i* and *gh* receptors mirror changes in plasma Gh and Igf-i levels, with the *ghr-i/ghr-ii* expression ratio mostly unaltered over season. However, this ratio is nutritionally regulated, and enriched plant-based diets or diets with specific nutrient deficiencies downregulate hepatic *ghr-i*, decreasing the *ghr-i/ghr-ii* ratio. The same trend, due to a *ghr-ii* increase, is found in skeletal muscle, whereas impaired growth during overwintering is related to increase in the *ghr-i/ghr-ii* and *igf-ii/igf-i* ratios in liver and skeletal muscle, respectively. Overall, expression of insulin receptors and *igf* receptors is less regulated, though the expression quotient is especially high in the liver and muscle of sea bream. Nutritional and environmental regulation of the full Igf binding protein 1–6 repertoire remains to be understood. However, tissue-specific expression profiling highlights an enhanced and nutritionally regulated expression of the *igfbp-1/-2/-4* clade in liver, whereas the *igfbp-3/-5/-6* clade is overexpressed and regulated in skeletal muscle. The somatotropic axis is, therefore, highly informative of a wide-range

of growth-disturbing and stressful stimuli, and multivariate analysis supports its use as a reliable toolset for the assessment of growth potentiality and nutrient deficiencies and requirements, especially in combination with selected panels of other nutritionally regulated metabolic biomarkers.

Keywords: growth hormone, insulin-like growth factors, insulin-like growth factor binding proteins, growth hormone receptors, insulin and IGF receptors, sirtuins, oxygen availability, energy status

EXPANSION OF Gh/Prl/Sl FAMILY

The fish growth hormone (Gh) and prolactin (Prl) family was initially expanded with the discovery of somatolactin (Sl) in olive flounder (1) and Atlantic cod (2). Thereafter, Sl has been identified, purified or recombinantly produced from a multitude of teleost species, including chum salmon (3), sole (4, 5), gilthead sea bream (6, 7), goldfish (8), eel (9), rainbow trout (10), and European sea bass (11, 12). Sl has also been identified in primitive fishes, such as the white sturgeon and the West African lungfish (13), which suggests that the ancestors of Gh and Sl were present before the divergence of lobe-finned fishes (*Sarcopteygii*) and ray finned fishes (*Actinopterygii*). Thereafter, the *sl* gene was duplicated in the basal teleost tetraploidization (3R), giving rise to *slα* and *slβ*, which were first identified in zebrafish (14) and Atlantic salmon (15). However, the *slβ* gene was lost from the lineage, leading to spiny-rayed fishes (*Acanthomorpha*), so it is not found in the most diverse and species-rich group of modern teleosts (16). Conversely, Prl2, the last member of the fish Gh/Prl/Sl family, has been identified in almost all non-mammalian vertebrate species ranging from cartilaginous fishes to tetrapods (17, 18). Despite this, both the *sl* and *prl2* genes were lost twice independently in tetrapods: once at the base of the amphibian lineage and once early in mammalian evolution (16, 19).

At a closer look, proteins of the Gh/Prl/Sl family share a common genomic organization with five to six exons in almost all fish species examined, including cyprinids, salmonids, flat fishes and Perciformes (20–22). Another characteristic feature is the cysteine residues involved in disulfide bridges, one linking distant parts of the polypeptide chain and another forming a loop close to the C-terminus that are strictly retained through vertebrate evolution. However, an additional N-terminal disulphide loop appeared in the Sl and Prl/Prl2 branches, prior to the divergence of bony fishes from the lineage leading to tetrapods, and thereafter the specific *sl* and *prl2* deletion events occurred between lungfish and amphibian lineages (23). In this context, the precise timing of gene losses and early duplication events is difficult to establish, though analyses of phylogeny and conserved synteny suggest that the *gh* and *prl/prl2* genes arose likely from a local duplication before 1R tetraploidization, and a second local gene duplication within the same time window gave rise to *sl* (16, 24) (see **Figure 1**).

Synteny approaches also reveal that the Gh receptors (Ghr) and Prl receptors (Prlr) are located on the same chromosome, probably as the result of an early local gene duplication (25). By contrast, the Sl receptors (Slr), identified as belonging to the Ghr-i clade, might have arisen much later in the 3R tetraploidization. This would be an example of subfunctionalization, where the named Ghr-i and Ghr-ii might have evolved for differential ligand binding preferences for Sl or Gh, respectively. However, in contrast to the ligand binding study in masu salmon (26), subsequent studies in black sea bream (27), and zebrafish (28) did not reveal differences between Ghr-i and Ghr-ii in terms of ligand binding affinities. Moreover, Ghr-i preferentially binds Gh rather than Sl in Japanese eel (29) and trout (30) binding assays. It appears, therefore, that the observed fish species differences in Ghr binding are more likely to be due to the different natures of Gh/Sl preparations or other factors that are hitherto unknown. Moreover, Bergan-Roller and Sheridan (31) pointed out that one Ghr would be more responsive for transmitting lipolytic or stress signals of Gh/Sl, while the other Ghr subtype would be more active in transmitting growth-promoting signals. In this context, it is of relevance to define clear patterns of Ghr expression in combination with changes in other growth- and metabolic-related factors and, more importantly, how the somatotropic axis is affected as a whole by the advent of new rearing systems and diet formulations for the intensification of fish farming in the 2030 horizon. In this regard, the aim of this minireview is to update the environmentally-mediated changes in fish somatotropic axis activity, linking them with a more accurate phenotyping of fish nutritional and metabolic status through the development and productive cycles. Attention is focused on marine fishes with special emphasis on gilthead sea bream (*Sparus aurata*) as the most important farmed fish of the Mediterranean aquaculture.

FISH Gh/Sl SUB-FUNCTIONALIZATION

Life-history decisions are not fixed and often depend on critical size and sufficient energy at a specific stage ("opportunity window") several months prior to biological transformations. For instance, the decisive factor in salmonids to become smolts or sexually mature is linked to growth and fat depositions at mid-summer and spring (32, 33). Thus, plasma Gh levels in most fishes, including gilthead sea bream, peak at late spring and early summer, whereas the peaks of Prl and Sl are delayed to mid-summer and autumn, respectively (20, 34). Therefore, Gh, Prl and Sl are differentially regulated on a seasonal basis, but also in response to energy availability (**Figure 2**). Certainly, circulating Sl increases transitorily with fasting in gilthead sea bream, but,

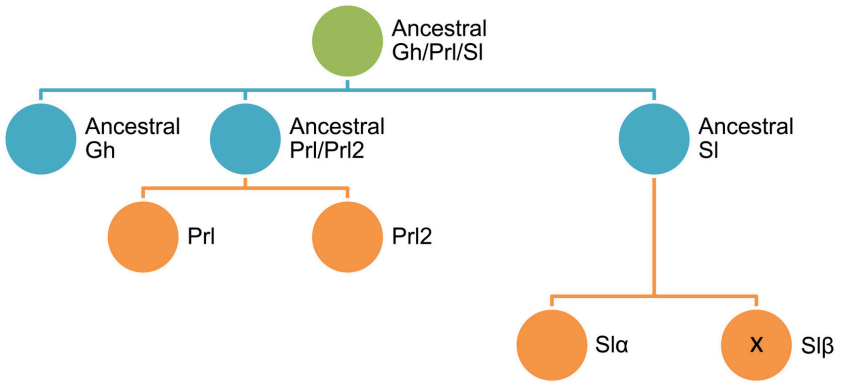


FIGURE 1 | Schematic representation of the diversification of Gh/Prl/Sl family in fishes.

opposite to Gh, a persistent increase in plasma Sl is found with the increase in adiposity in overfed or old animals (35, 36). This supports a close association between Sl and lipostatic signals in fishes and gilthead sea bream in particular. In agreement with this, murine leptin is able to stimulate the *in vitro* secretion of pituitary Sl as part of the nutritional mechanisms driving the onset of puberty in European sea bass (37), though the seasonal pattern of Sl is more erratic in European sea bass than in gilthead sea bream, and it is difficult to infer a characteristic Sl pattern when PIT-tagged animals are considered individually (38). In any case, studies in gilthead sea bream highlight that recombinant Sl triggers a transient inhibition of feed intake, a satiety effect that is related to decreases in the respiratory quotient (CO_2 output per O_2 uptake) (12, 36). This pattern of gas exchange suggests the activation of lipid catabolism, which is consistent with the Sl inhibition of the hepatic activity of acetyl-coenzyme-A carboxylase (12). In salmonids, a role of Sl in energy mobilization during reproduction, acute stress or exhaustive exercise (39–42) has also been proposed. Therefore, Sl may help to expedite growth-reproductive processes following replenishment of fat stores and/or mediate the adaptation to fasting until the lipolytic action of Gh and/or other endocrine factors is fully accomplished. Contrary to this, other authors point out that the skin-color regulation is the only definite role of Sl so far demonstrated in fishes and medaka in particular (43). This still remains under debate, but the consensus is that Sl does not enhance *in vivo* or *in vitro* the production of insulin-like growth factors (Igfs) (12, 44, 45).

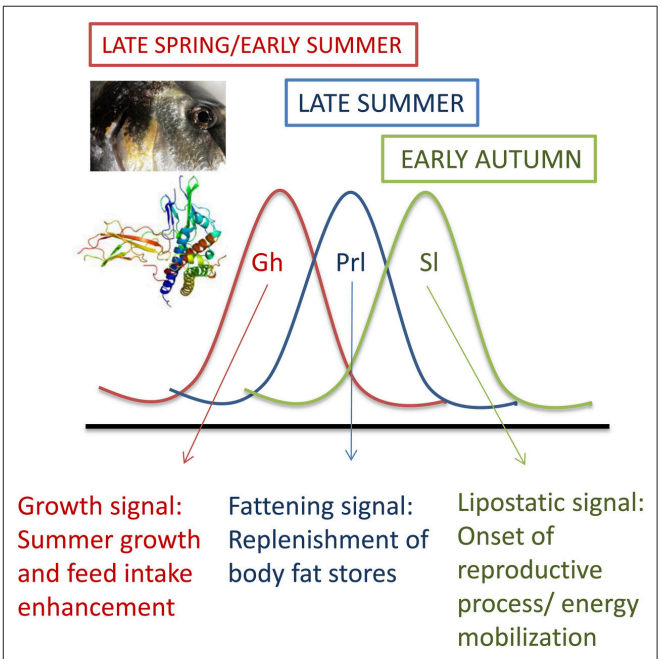


FIGURE 2 | Seasonal patterns of plasma levels of Gh, Prl, and Sl in juveniles of gilthead sea bream. Adapted from Pérez-Sánchez et al. (20).

GH-DEPENDENT GROWTH

Pituitary cells producing GH are the main source of this hormone in the body, and its circulating concentration is barely detectable following hypophysectomy or impaired somatotroph differentiation in humans and birds (46). Hence, pituitary GH is largely responsible for the endocrine actions of GH during perinatal and postnatal growth, and according to the classical somatomedin hypothesis GH is released into the systemic circulation and then transported to target tissues to act via specific receptors to regulate growth and metabolism, directly or indirectly through the induction of hepatic or locally produced IGFs (47). However, this concept changed in the last quarter of twentieth century with the evidence of a widespread distribution of GH in most extrapituitary tissues, including neuronal, reproductive, immune, and gastrointestinal tissues, which may be independent on the pituitary-specific transcription factor-1 (PIT-1) (48). Indeed, pituitary GH expression does not occur in early embryonic or fetal development, whereas extrapituitary GH is expressed much earlier, prior to its ontogenic appearance in pituitary somatotrophs. In other words, as highlighted in previous reviews (49–52), embryonic or early fetal growth is independent of pituitary GH in higher vertebrates, and

the persistence of extrapituitary GH through the life cycle is indicative of paracrine or autocrine roles for locally-produced GH. Indeed, the functional relevance of this extrapituitary Gh in postnatal growth would vary among vertebrates. For instance, the decreased expression of extrapituitary *GH* is not related to dwarf phenotypes in humans (51), whereas interactions between systemic and locally produced GH may contribute to explain the inconsistent relationship of circulating GH and growth in chickens (49).

As in birds and humans, extrapituitary Gh has been detected in immunorelevant and reproductive tissues of gilthead sea bream, trout and Japanese eel (53–55). The *gh* gene is transcribed and translated early during fish larval development before or after pituitary differentiation (29, 56, 57). Moreover, the main components of the somatotropic axis (Gh, Ghrs, Igfs) are produced as soon as transcription starts in fish embryos (58), and binding studies revealed a high concentration of actively transcribed Ghrs on the head of gilthead sea bream larvae a few days after hatching, which is consistent with their allometric growth. All this evidence together suggests a direct action of Gh rather than systemic effects via hepatic Igfs during early life stages (59), but later in life this complex trade-off would have evolved in a different manner within each group of vertebrates.

CIRCULATING GH AND IGF-I

Growth and IGF-I Association

IGFs are the primary mediators of the growth-promoting effects of GH, operating in an autocrine, paracrine and endocrine manner (60). They affect many biological processes, including protein synthesis and turnover, cell proliferation and differentiation, and cell apoptosis and tissue maintenance, which make IGFs good candidates as growth indexes. Unlike GH and insulin, IGFs are not stored or released in pulses, and clearance rates are retarded by the action of a suite of IGF-binding proteins (IGFBPs), a feature that allows relatively constant levels of IGFs in the blood. However, as reviewed by Beckman (61), the correlation between Igf-i and growth ranges from tight to non-discernible in fishes, probably reflecting a changing and sometimes confounding scenario. Thus, ration size, circulating Igf-i and growth rates are often positively correlated (62–64), though the Igf system appears to have a notable inertia after extended fasting or feed-restriction periods.

Overall, fast-growing fish strains also share an enhanced somatotropic axis activity (65, 66). However, the relationship between Igf-i and growth is largely affected by a wide-range of endogenous and exogenous factors, including gender, developmental and maturity state, photoperiod, temperature and salinity as well as stress and disease condition, which makes the Igf-i and growth relationship variable over season and productive cycles (61). Accordingly, Igf-i is a reliable growth index over precisely defined groups of fishes, but conservative approaches are needed for comparisons of Igf-i and growth rates across experiments within and between different fish species. For instance, the true effects of different photoperiods on growth and plasma Igf-i level are difficult to discern in trout and salmon (67–69). Likewise, some seasonal delay exists between growth rates and plasma Igf-i level over the productive cycle of gilthead sea bream (34), though circulating Igf-i continues to be perceived as one of the most reliable markers of growth performance in a wide range of fishes, including the hybrid striped bass (70), juvenile lingcod (71), or Nile tilapia (72). Furthermore, a concordant Igf-i and growth relationship has been reported combining data from juveniles of three Mediterranean farmed fishes (European sea bass, gilthead sea bream, common dentex) reared in the same indoor experimental facilities (35). A relatively high degree of concordance was also found for the decreased growth rates and circulating level of Igf-i in 1-, 2-, and 3-year old gilthead sea bream (73). Likewise, the recovery of plasma Igf-i during refeeding highly reflects the increase in weight gain during the phase of compensatory growth (74). More recently, the circulating level of Igf-i was concordant with the growth-promoting effects of moderate exercise in juveniles of gilthead sea bream (75, 76). Also in gilthead sea bream, a linear increase in growth rates and circulating Igf-i was reported in fingerlings in response to a single dose of recombinant bovine GH (77). However, these growth/endocrine patterns highly differ from those found in salmonids and, for the same or even higher growth rates, the circulating concentrations of Gh and Igf-i are often 3–10 times lower in trout juveniles than in Mediterranean farmed fishes (78).

Fish-species differences in the regulation of the Gh/Igf system also apply to the osmoregulatory action of Gh, which may act synergistically with cortisol and Igf-i to enhance the hypo-osmoregulatory ability, mainly in salmonid species. Thus, anadromous migrations from rivers to hyperosmotic environments stimulates the somatotropic axis for rapid growth and triggers different osmoregulatory actions related to the development of preparatory mechanisms for seawater entry (79). In this regard, Gh action can be mediated by Ghrs to stimulate Igf-i production not only in liver, but also in osmoregulatory organs (gills, kidney, and intestine), which in turn orchestrates ion and water movements to preserve or achieve a new steady state of plasma osmolality, as reported in Sakamoto et al. (80). However, confounding results have been reported in non-salmonid species. In several tilapia species (*Oreochromis mossambicus* and *O. niloticus*), no significant differences have been reported in pituitary *gh* expression or in Gh plasma levels in juvenile or larval stages in freshwater-, brackishwater- or seawater-acclimated fishes (81–83), whereas other studies suggest a role of Gh during osmotic acclimation as a result of modifications in plasma Gh level after hyperosmotic transfer (84–86). In eels, no changes in plasma Gh have been reported with transfer from freshwater to seawater (87, 88). In meager and gilthead sea bream, a clear increase in pituitary *gh* transcripts is found in hyperosmotic environments (89, 90). This response is maintained during acclimation to isosmotic salinity in silver sea bream (91, 92), black sea bream (93) or gilthead sea bream (94), which in turn would trigger the improvement of growth rate (95) through changes in the pentose phosphate pathway or synthesis of stress proteins (91, 93, 96). The ultimate mechanisms remain to be established, but importantly, microarray gene expression profiling of liver, gills and hypothalamus after hypo- or hyperosmotic challenges

identified more than 750 differentially expressed genes in gilthead sea bream, with three major clusters of overlapping canonical pathways corresponding to energy metabolism, oxidative stress and cell and tissue architecture (97).

Sirtuin Energy-Sensing and GH/IGF-I

Differences in key performance indicators necessarily reflect different uses of nutrients and energy, as voluntary feed intake and growth are limited by the capacity to preserve the redox balance (98–100). In that sense, animals with enhanced feed intake and growth rates are able to grow efficiently in a cellular milieu with enhanced risk of oxidative stress, and the differential regulation of sirtuins (SIRTs) contributes to the readjustment and preservation of metabolic homeostasis. These enzyme deacetylases use NAD^+ as cofactor and couple the acetylation status of histone and non-histone substrates with the energy status of the cell via NAD^+/NADH ratio. SIRTs are virtually ubiquitous through all kingdoms of life, and the number of family members increases with the organismal complexity: prokaryotes have one to two family members, fission yeast three, worms four, flies five and higher vertebrates (including fishes) seven (101, 102). This, together with different cellular locations (nuclear, SIRT1 and SIRT6-7; cytoplasmic, SIRT2; mitochondrial, SIRT3-5) offers the possibility of complementary but also non-redundant and tissue-specific energy-sensing mechanisms strongly influenced by nutrient availability, energy demand and tissue-specific metabolic capabilities (103–105).

The ultimate mechanisms by which changes in *SIRT* expression and activity modulate the action of metabolic hormones and the GH/IGF system remains poorly studied. However, studies in mice have found that *in vivo* knockdown of hepatic *Sirt1* restores the fasting-induced decrease in serum IGF-I and enhances the GH-dependent increase in IGF-I (106). Knockdown of *Sirt1* in mice enhances the acetylation and GH-induced tyrosine phosphorylation of STAT5, indicating that SIRT1 negatively regulates GH-dependent IGF-I production via deacetylation of transcription factor STAT5. Additionally, SIRT1 acts at the brain level as a link between somatotropic signaling and calorie restriction (107), and brain-specific *Sirt1* knockout mice have dwarfism and reduced plasma GH and IGF-I (108), displaying similar phenotypes to those of long-lived mutant mice (109). By contrast, SIRT1 activation with resveratrol suppresses GH synthesis in pituitary rat cells by reducing PIT-1 availability to the *Gh* promoter via the transcriptional suppression of *Creb* (110). Unlike this apparent controversy, the net effect of SIRT1 activation continues to be the suppression of the GH/IGF tonus, which would serve to drive a decreased energy demand for growth purposes in a cellular milieu with a reduced availability of metabolic fuels.

Relatively less is known about the regulation of *sirts* in fish, but recent studies in gilthead sea bream highlight that short-term fasting does not alter significantly the *sirt1* gene expression in liver, whereas the expression pattern for other *sirt* isotypes (*sirt2-6*) is an overall suppression linked to reduced energy demand for hepatic lipogenesis (102, 111). In contrast, gilthead sea bream strains that regularly perform better than other genetically different strains have a reduced hepatic *sirt1* expression, in combination with a more active feeding behavior and Gh/Igf-i system, resulting in enhanced growth and higher circulating Igf-i (112). The expression of *sirt* genes is also highly regulated by energy demand in the white skeletal muscle of gilthead sea bream, and *sirt2* mRNA is clearly upregulated in fast growing fish, whereas the mitochondrial *sirt5* emerges as a more responsive element during forced fasting (102) or natural fasting when comparisons are made between fishes of different size during the cold season (unpublished results). In both cases, this occurs in combination with the enhancement of the lipolytic machinery and reduced energy wastage, evidenced by the downregulation of muscle mitochondrial uncoupling proteins and changes in the expression of many markers of lipid metabolism and oxidative phosphorylation, as was also highlighted by the muscle microarray gene expression profiling of fishes fed at maintenance ratio (113).

For comparative purposes, it is relevant that physiological studies in humans have revealed a regulatory role of SIRT2 in muscle stem cell proliferation and differentiation (114–116). Moreover, single-nucleotide polymorphisms of *Sirt2* have been associated with different body size traits in Qinchuan cattle (117). Adiposity is also a main factor affecting *SIRT* expression, and SIRT6 activity is depressed in the adipose tissue of obese human patients (118). In this way, it is likely that the concurrent upregulation of *sirt5* and *sirt6* in the adipose tissue of our fast-growing fish strains orchestrate a lean phenotype, resulting in a reduced adipose tissue mass with an increased mobilization of fatty acids toward skeletal muscle and liver. All of the above evidences a complex metabolic crosstalk between Sirt-energy sensors and the Gh/Igf system, with perhaps the double aim of (i) avoiding or minimizing the loss of muscle protein mass during stages of negative energy balance and (ii) precisely tuning the growth energy-demanding processes of organisms to the exogenous supply and availability of metabolic fuels (see **Figure 3**). However, we are far from fully understand this complex picture, and current studies aiming to highlight whether tissue-specific differences in fish Sirt profile result from nutritional, genetic or epigenetic sources of variation as key players of genome stability during environmental stimuli and stress response are underway, as reviewed by Bosch-Presegué and Vaquero (119).

Gh/Igf Responsiveness in Hypoxic and Crowded Fishes

Animals overexpressing *Gh* combat oxidative stress less efficiently than normal and dwarf mice (120, 121). In the same way, *gh*-transgenic fishes have a lower capacity to manage a hypoxic environment efficiently (122, 123), though paradoxically early studies in mammals indicate that circulating Gh can be increased by either the increase in O_2 requirements or the reduction of O_2 availability (124). A possible explanation is that regulation of circulating Gh level in hypoxic animals mirrors the changing energy needs rather than the availability of metabolic fuels. In this way, circulating Gh increased markedly in salmonids submitted to maximum swimming (125, 126). The same response has been found in gilthead sea bream, and

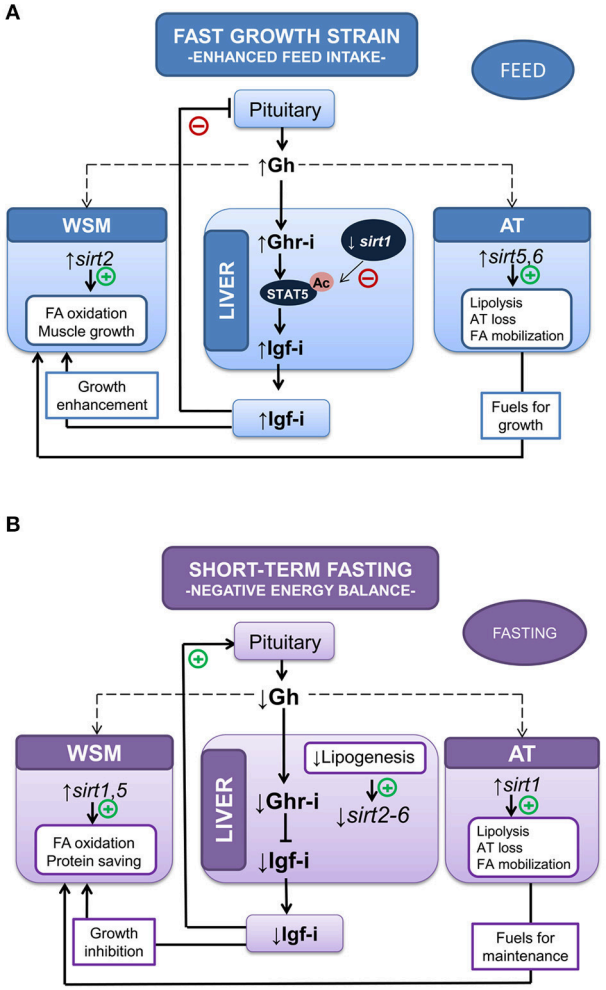


FIGURE 3 | Metabolic crosstalk between Sirtuin-energy sensors and the Gh/Igf system in liver, white skeletal muscle (WSM) and adipose tissue (AT) in two different experimental models: **(A)** accelerated growth in fishes with enhanced feed and **(B)** negative energy balance (10-day-fasted fishes). Adapted from Simó-Mirabet et al. (102, 112).

plasma Gh levels are of predictive value of the aerobic scope in swimming test chambers, as circulating Gh before exercise is a surrogate marker of critical speed (swimming speed that could theoretically be maintained indefinitely without exhaustion) (127). The opposite is also true, and circulating Gh is lowered after acute or chronic confinement exposure in a wide range of fishes, including gilthead sea bream (128–130), trout (131), Atlantic salmon (132), and Nile tilapia (133). However, as first pointed out by Pickering et al. (131), hypoxia, crowding and water quality induce confounding effects that include the activation of the hypothalamic-pituitary-interrenal axis (HPI-axis) and the overall downregulation of hepatic *igf* and *ghr* genes, concomitant with growth inhibition and lowered plasma Igf-i in both crowded and hypoxic fishes (130, 134).

Fine adjustments of metabolism machinery also take place at the mitochondrial transcriptional level, and experimental evidence reveals a reduced production of energy and reactive oxygen species (ROS) when fishes are crowded (135) or exposed to multiple stressors that mimic daily aquaculture operations (136). In that sense, the capacity of fish to efficiently manage the allostatic load (defined as the maintenance of internal homeostasis through changes of a number of stress mediators) by readjustments of O_2-carrying capacities and metabolic suppression are of high value to finally reach the internal equilibrium in a hypoxic-challenging scenario, which would prime a reduced production/accumulation of toxic byproducts from anaerobic metabolism (137–139). In hypoxic gilthead sea bream, this metabolic suppression is exemplified by the overall depression of catalytic, assembly, and regulatory enzyme subunits of complex I, II, III, and V of the mitochondrial respiratory chain, which is concurrent with the upregulation of catalytic and regulatory elements of complex IV (last electron donor to oxygen acceptor) (134). This dualism offers the possibility of a reduced but more efficient mitochondrial respiration during exposure or recovery from severe hypoxia, as has been shown, at least in part, in fishes fed with seaweed extracts as dietary surplus to protect against oxidative stress (140). Most of these metabolic readjustments, linked with endocrine disturbances (130, 134), have been reported after exposure to severe hypoxia (18–19% oxygen saturation, 20–22°C) for 4 h under steady-state conditions, but most analyzed parameters require lasting periods to be responsive when the water O_2 concentration is fixed close to limiting oxygen saturation (LOS), defined as the threshold level where regulatory mechanisms are no longer sufficient to maintain the O_2 consumption rate with changes of the level of O_2 saturation (141, 142). Thus, juveniles of gilthead sea bream reared at 40% O_2 saturation and 20–22°C exhibit a reduced, but active feeding behavior, that is adjusted to meet dietary O_2 demands according to the oxystatic theory (143). This allows fishes to grow efficiently at slower rates, which is consistent with a total or partial recovery of control plasma levels of cortisol and Igf-i regardless of a persistent hypersomatotropic state that would reflect an enhanced demand for metabolic fuels (**Figure 4**). These endocrine signatures clearly indicate the different dynamics of the HPI and the Gh/Igf system in response to severe and moderate hypoxia, which merits consideration for metabolic phenotyping of farmed fishes in a scenario of increasing temperatures and global change.

Nutritional Background and Gh/Igf Status

The current stagnation of fish meal (FM) and fish oil (FO) production from wild fisheries limits further growth of aquaculture (144). In the meantime, the most immediate alternatives are plant products, which have been used for years in salmonids and marine fish feeds to reduce the reliance of European aquaculture on marine fishery resources. Thus, most carnivorous farmed fish, including European sea bass (145) and gilthead sea bream (146, 147), can be successfully reared with plant-based diets containing <10% marine feedstuffs. Moreover, complete replacement of FO by vegetable oils is feasible when the theoretical requirements for phospholipids and n-3 long-chain polyunsaturated fatty acids (LC-PUFA) are met by the lipids contained in FM (148–150), but the concomitant

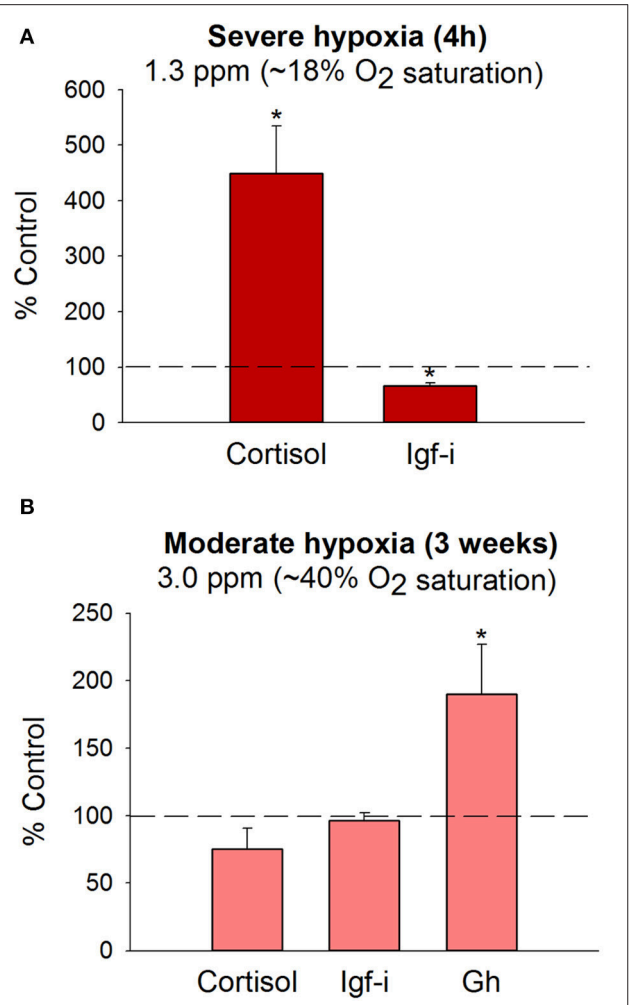


FIGURE 4 | Plasma hormone levels in juveniles of gilthead sea bream following exposure to severe **(A)** or moderate hypoxia **(B)** at 20–22°C. Bars indicate changes in plasma cortisol, Gh and Igf-i levels in comparison to normoxic fishes (>85% O_2 saturation). Data are presented as the mean ± SEM (n = 10–20). Asterisks indicate statistically significant differences ($P <$ 0.05, t-test). Adapted from Martos-Sitcha et al. (134) and Magnoni et al. (140).

FM/FO replacement continues to be challenging despite the important research efforts within the framework of PEPPA (2001–2004), AQUAMAX (2006–2010), and ARRAINA (2012–2016) EU projects for meeting the nutrient requirements of European farmed fish in terms of growth, health, and welfare criteria. In that respect, important progress has been made in salmonid and non-salmonid fishes on the diagnosis of nutrient deficiencies or the re-evaluation of nutritional requirements of vitamins, minerals, and other key nutrients by means of surrogate markers resulting from blood biochemistry and hematology profiling, histopathological and organo-somatic index scoring or measures among others of enzyme activities or vertebral mineral concentrations (151–155).

Often, plant-based diets have an impact on the gastro-intestinal transcriptome, mucus intestinal proteome or intestinal microbiome, but the use of feed additives (e.g., butyrate) helps to preserve the wild phenotype of fish fed FM/FO-based diets, improving the disease outcomes of gilthead sea bream challenged with bacteria or intestinal parasites (156–158). Historically, the somatotropic axis has also been used as an endocrine marker of the effectiveness of alternative feed formulations to support maximum growth. A first study combining measures of circulating Gh, Igf-I, and Igfbps with those of Gh-binding and *gh* and *igf* transcripts was conducted by Gómez-Requeni et al. (78) to investigate the physiological consequences of the partial or total replacement of FM by plant ingredients in trout juveniles. Likewise, measurements of circulating Gh and Igf-i in combination with those of transcripts of *ghrs*, *igfs*, *igfbps,* and other growth-related markers, including molecular chaperones, myogenic factors, energy sensors, and markers of protein turnover, lipid metabolism, oxidative phosphorylation (OXPHOS) and mitochondria respiration uncoupling, are highly informative for a wide-ranging assessment of growth performance; first with the replacement of FM by plant proteins (159) and second with the combined and maximized replacement of FM and FO by plant proteins and vegetable oils (146, 160–162). This results in differences in growth rates with a high degree of concordance of growth and growth-promoting factors between trials (9–11 weeks) conducted during the summer growing period with juvenile fishes of the same strain (Atlantic fish strain) and class of size (14–16 g initial body weight).

The above observations further support a consistent growth output for a given Igf-i concentration that makes feasible meta-analysis of data from highly controlled experiments. In that sense, the evolutionary past of living animals is marked by periods of undernutrition that are characterized by protein-calorie deficiencies or vitamin and mineral deficiencies and even starvation, which makes survival dependent on the body's ability to mobilize energy stores (163). Since GH plays a key role in mobilizing energy (lipolytic role non-dependent of IGFs) its elevated circulating levels in the setting of low IGF-I confers a metabolic advantage during undernutrition periods. Therefore, a state of GH resistance is highly conserved through the evolution of fishes and higher vertebrates as part of the adaptive response to inappropriate nutritional conditions. This reflects a reduced responsiveness of target tissues to the anabolic GH action, which may be due to receptor or postreceptor defects in the transmission of GH signaling. Thus, diets deficient in tryptophan resulted in reduced growth and plasma Igf-i in juveniles of the hybrid striped bass (164). Hevrøy et al. (165) also found that lysine-enriched diets resulted in a significant increase in muscle protein deposition and hepatic *igf-i* mRNA in Atlantic salmon. Likewise, methionine availability modulates the expression of genes involved in the Gh/Igf response and protein turnover, further affecting growth performance in trout (166). Previous studies in trout indicate that plasma Gh level is not affected by either methionine or taurine supplementation, and even more, methionine excess has been associated with decreased plasma Igf-i in fishes fed FM-free diets (167). This apparent discordance indicates the complexity of endocrine growth regulation within and between fish species. However, it is noteworthy that recent studies in gilthead sea bream highlight a close linear relationship between growth and circulating levels of Gh and Igf-i in fishes

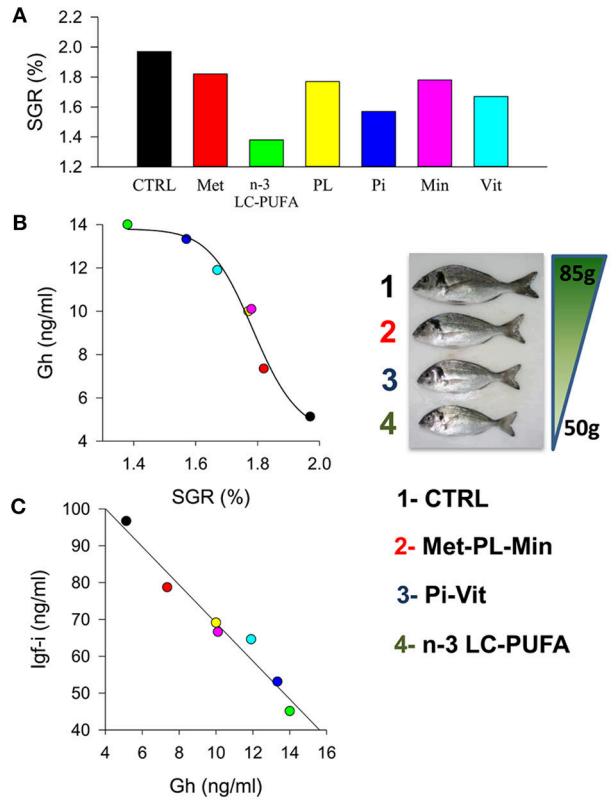


FIGURE 5 | (A) Effect of nutrient deficiencies on the specific growth rates (SGR) in juveniles of gilthead sea bream fed to visual satiety for 13 weeks from May to July. **(B)** Curvilinear correlation between SGR and plasma Gh levels. Insert image shows fish size differences at the end of trial. **(C)** Correlation between plasma Gh and Igf-i levels. Colors indicate each experimental group: CTRL, control (black); Met, methionine (red); n-3 LC-PUFA, n-3 long-chain polyunsaturated fatty acids (green); PL, phospholipids (yellow); Pi, phosphorus (blue); Min, minerals (pink); Vit, vitamins (cyan). Adapted from Ballester-Lozano et al. (152).

fed semipurified diets formulated for deficiencies in methionine, n-3 LC-PUFA, phospholipids, phosphorus, vitamins, or minerals (**Figure 5**). This association between growth and Gh/Igf-i is also concordant with that found in juveniles fed practical diets with varying degrees of FM and FO replacement. Therefore, the somatotropic axis highly reflects the drawback effects on growth performance due to a reduced nutritive value of the main dietary protein or lipid sources, but also in response to specific nutrient deficiencies mimicking those induced by enriched plant-based diets.

More recently, it was found that the growth-promoting action of medium-chain fatty acids in juveniles of gilthead sea bream fed FO-based diets resulted in a slight increase in the Igf-i/Gh ratio (168). In this way, the combined use of circulating Igf-i and the Igf-i/Gh quotient have been revitalized as reliable key indicators of growth performance in fishes, highly reflecting the magnitude of a wide range of nutritional and growth derangements. Certainly, circulating Gh and Igf-i are either the up- or downstream factors of the Gh/Igf system, but they are informative of the intensity of growth impairment rather than the nature or origin of disturbing processes. Hence, as indicated below, measures of transcripts of the main actors of the somatotropic axis alone or in combination with other markers of protein mass accretion and cellular stress are required for a refined diagnosis of a given nutrient deficiency or even for the re-evaluation of species-specific nutrient requirements.

REGULATION AT THE TRANSCRIPTIONAL LEVEL

Effects of Nutrition and Season on *ghrs*

Fish *ghrs* were first cloned and sequenced in goldfish (169), turbot (170), and sparid fishes (171, 172). Nucleotide sequences encoding for fish *ghrs* were also available in coho and masu salmon at the end of the last century, and several authors suggested a divergent evolution of salmonid and non-salmonid *ghrs*. However, as first pointed out by Saera-Vila et al. (173), duplicated fish *ghrs* are actively transcribed in trout, European sea bass or gilthead sea bream and they are more similar to each other than to *Ghrs* of tetrapods. Searches for *ghrs* in fish genome databases also confirmed their occurrence in almost all the analyzed fishes, but unfortunately the initial controversy over *ghr* phylogeny has made the nomenclature of fish *ghrs* somewhat confusing. To solve this issue, the convention for fish *ghr* nomenclature is the clade *ghr* type I (also written type 1 or *slr* in masu salmon) for the initially described *ghr* of non-salmonid fishes, while the clade *ghr* type II (also written type 2) corresponds to the initially described *ghr* isotype of salmonids [see Reindl and Sheridan (60)], where the chromosomal location of duplicated sequences of *ghrs* of type I and II are consistent with the generation of paralogous blocks in the salmonid tetraploidization (174).

Most of what we know about GH signaling comes from mammalian species and cell lines, but intracellular signaling pathways are generally well conserved, and many commercially-available antibodies developed to target mammalian signaling molecules also detect orthologs in piscine species (175). This opens new research opportunities, and several attempts have been made to support the possibility that a given Ghr subtype might be responsible for transmitting the lipolytic action of Gh, while the other Ghr subtype would be more active in transmitting the growth-promoting action of Gh (31). To our knowledge, more work is needed to establish these explicit links, though it is well-recognized that Gh differentially activates disparate signaling pathways when stimulating growth through Jak-sat, Pi3k-akt, and Mapk (176), and lipolysis through Plc-pkc, MapK, and Hsl (177–179). Meanwhile, the differential and tissue-specific regulation of *ghr* subtypes by nutritional and environmental factors becomes especially clear in farmed gilthead sea bream, in which hepatic transcripts of *ghr-i* (in a low extend *ghr-ii*) mirror changes in growth rates, plasma Igf-i level, and hepatic *igf-i* transcription through development and production cycles, indicating a prominent role of Ghr-i rather than Ghr-ii in the systemic growth-promoting action of Gh (73, 146, 160). Moreover, the hepatic *ghr-i*/*ghr-ii* gene expression ratio

remains mostly unaltered during seasonal changes of growth rates. However, this gene expression ratio is upregulated in white skeletal muscle from 2 to 3 in summer to more than 4 in winter. This gene expression ratio is also highly regulated at the nutritional level, and fishes fed unbalanced plant-based diets or semipurified diets formulated for specific nutrient deficiencies downregulate hepatic *ghr-i*, decreasing the *ghr-i*/*ghr-ii* ratio from 1.8 to 2 to <1 (146, 152, 160). Likewise, in white skeletal muscle, the *ghr-i*/*ghr-ii* ratio decreases from 2.5 to 3 to close to 1 in fishes with signs of nutrient deficiencies, but this molecular feature seems to be the result of the counterregulatory upregulated expression of *ghr-ii*. Conversely, impaired growth during overwintering is not able to alter significantly the hepatic *ghr-i*/*ghr-ii* mRNA ratio regardless of the overall depressed gene expression during the cold season (see **Figure 6**).

Insulin/Igf System: Evolutionary Prospect

Insulin, IGF-I and IGF-II are structurally similar and they are derived from a common ancestral molecule through a series of gene duplications and mutations (180). Fishes are the first group in the vertebrate tree in which there is evidence of distinct insulin and Igf molecules and receptors (181). However, certain cross-interaction between ligands and receptors of insulin and Igfs occurs. This is especially evident for Igf-ii, which exerts its mitogenic action through insulin and Igf-i receptors (182, 183). Additionally, important differences regarding receptors specificity and abundance through the evolution have been reported. Indeed, fish Igf-i receptors show a higher degree of specificity than insulin receptors (184, 185), and binding studies in cardiac and skeletal muscle highly support that the number of insulin receptors is lower than the number of Igf-i receptors in fishes, amphibians and reptiles (186–188), whereas the opposite is found in birds and mammals (189, 190). Nevertheless, at the expression level, the relative abundance of insulin receptors in juveniles of gilthead sea bream fed practical diets from early life stages is higher than initially expected. This is especially evident in liver, where the expression quotient ratio for insulin receptors and Igf receptors remains almost equal along seasons. The same trend, but less evident, is found in white skeletal muscle, where the expression level of insulin receptors is almost equal to that of the *igf-i* receptor in both summer and winter, and higher than that of the *igf-ii* receptor in summer (see **Figure 7**). Since gilthead sea bream is an euryhaline, eurythermal, and protandrous hermaphrodite fish, it is tempting to speculate that the increase in the insulin receptor/*Igf* receptor expression ratio with organismal complexity from ectotherms to endotherms also applies to a fish with a well-recognized growth and metabolic plasticity, and improved resilience to aquaculture stressors (112, 135, 136, 191).

igf-ii/*igf-i* Expression Ratio

A growing body of evidence from more than 25 years indicates that IGFs play key roles in the growth and development of mammals and chickens. As a result of multiple transcription initiation sites and alternative splicing, the *Igf-i* gene gives rise to different transcripts in higher vertebrates (192, 193), encoding for several Igf-i precursor polypeptides. The biological significance of these splice variants still remains under debate, although a differential expression profile has been reported in response to varying conditions and pathologies (194, 195) and potentially different bioactivity of the Igf-i isoforms is suggested. Multiple forms of Igf-i have been also detected in fishes, including gilthead sea bream (196–199). This functional plasticity is not found in IGF-II, which is considered a primary growth factor for embryonic and fetal growth (200, 201), while IGF-I is required for achievement of maximal postnatal growth (202). Indeed, postnatally elevated levels of *Igf-ii* transcripts fail to rescue the dwarfism of *Igf-i*-deficient mice (203), and *Igf-ii* transcripts decrease quickly during the postnatal development of mice and rats (204). However, substantial *IGF-II* amounts are found later in life in humans and in a wide-range of fishes, including common carp (205), trout (206), Nile tilapia (207), channel catfish (208), and gilthead sea bream (209). Overexpression of *igf-ii* is especially evident in extrahepatic tissues: in gilthead sea bream, the *igf-ii*/*igf-i* expression ratio ranges from 0.5 in liver and 3–9 in skeletal muscle, adipose tissue, gills and brain to 75–150 in heart, intestine and gonads. In these studies, *igf-i* measures considered the expression of the totality of transcripts given the retention of the core mature peptide in all Igf-i precursors (159).

Regarding in depth *igf-ii* expression, compensatory increases in skeletal muscle *igf-ii* mRNA also occurs in juveniles of gilthead sea bream fed FO-free diets to counteract, at least in part, the suppressed growth and expression of hepatic *igfs* (160). However, more recent data using fishes fed semipurified diets formulated for nutrient deficiencies indicate that this type of muscle response is perhaps more informative of deficiencies in vitamins rather than n-3 LC-PUFA. The *igf-ii*/*igf-i* expression ratio is also sensitive to seasonal changes in growth rates, and the apparent winter suppression of muscle *igf* expression is more evident for the lowest-expressed *igf* gene. The *igf-ii*/*igf-i* ratio in juvenile fishes varies from 10 in summer to 3–5 in winter. By contrast, the hepatic *igf-ii*/*igf-i* ratio remains almost unaltered and near 0.5 over season in both well and malnourished fishes. This is because the two hepatic *igf* transcripts are similarly regulated when facing different environmental and nutritional stimuli (see **Figure 6**). The regulation of *igf* expression quotient is, therefore, different from that reported for *ghrs*, as the expression of *ghr-i* is more regulated in liver than in skeletal muscle, whereas *ghr-ii* and *igf-ii* appear more regulated at the muscle local level, as part of some kind of compensatory growth response. This finding helps to clarify the different functions of *ghr* and *igf* duplicated genes, helping to refine the molecular signatures of a given growth and nutritional condition through production cycles.

Fish *igfbp* Repertoire

The ancestral *Igfbp* gene was duplicated in tandem during an early stage of vertebrate evolution to produce a pair of *Igfbp*s that gave rise in subsequent genome duplication events the two *Igfbp* clades of modern vertebrates (*Igfbp-1/-2/-4*; *Igfbp-3/-5/-6*) (210–213). Additionally, the third and fourth round of whole-genome duplications created the corresponding paralog pairs. The resulting number of Igfbp subtypes is thus variable between fish lineages, but always higher than in mammals and other vertebrates. For example, zebrafish have retained nine actively

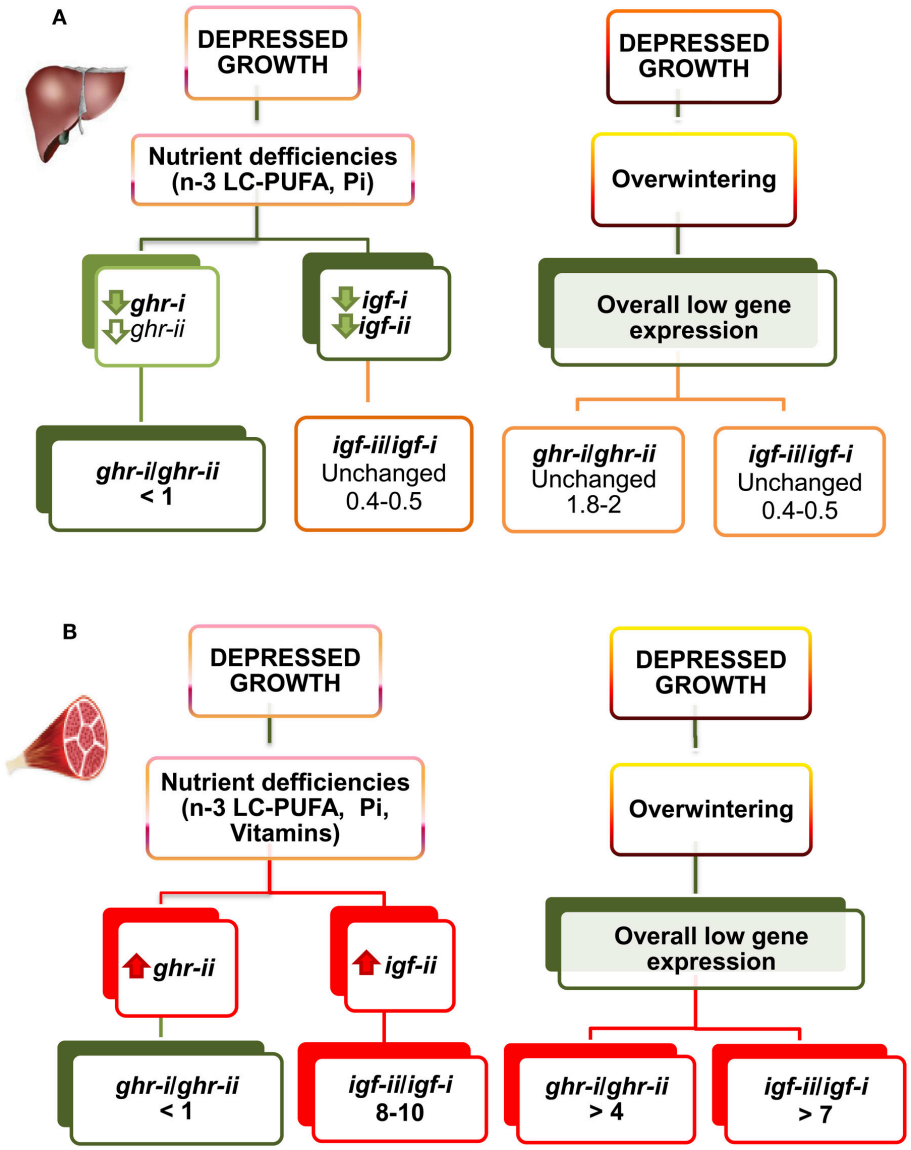


FIGURE 6 | Regulated gene expression of *igfs* and *ghrs* by diet composition and season in liver **(A)** and skeletal muscle **(B)** of gilthead sea bream juveniles. The direction of change is represented by color (red, increase; green, decrease; orange, unchanged). For full details of gene expression profiling see **Tables S1–S6**. Adapted from Benedito-Palos et al. (146, 160) and Ballester-Lozano et al. (152).

transcribed genes that include *igfbp-3a* and paralog pairs of *igfbp-1*, *-2*, *-5*, and *-6* (214–217). Atlantic salmon possesses 22 unique *igfbp* genes with 11 paralog pairs of *igfbp-1a*, *-1b*, *-2a*, *-2b*, *-3a*, *-3b*, *-5a*, *-5b*, *-6a*, and *-6b*. Common carp retains 17 *igfbp* genes including *igfbp-2a* and paralog pairs of *igfbp-1a*, *-1b*, *-2b*, *-3a*, *-5a*, *-5b*, *-6a*, and *-6b* (218). Likewise, searches in the gilthead sea bream genome database (http://nutrigroup-iats.org/seabreamdb) have identified 11 *igfbp* genes, covering the full *igfbp-1* to *-6* repertoire with paralog pairs of *igfbp-1*, *-2*, *-3*, *-5*, and *-6*. These findings evidence a different and perhaps divergent evolution of the *igfbp* repertoire in fish species, though high quality reference genomes, with all genes properly annotated and assembled into corresponding chromosomes, are required to confirm this idea. At this stage, to avoid nomenclature confusion, new and previous *igfbp* sequences of gilthead sea bream are annotated, and uploaded to GenBank, according to the proposed nomenclature of salmonids. The identity of the annotated *igfbp* sequences has been corroborated by phylogenetic analyses, which evidenced the differential expansion of *igfbp* genes within salmonids, carp, northern pike and gilthead sea bream (**Figure 8**).

Regarding the expression profile, the clearest pattern is the different tissue-specific profile of *igfbps* in liver and skeletal muscle. Of note, transcripts of the *igfbp-1/-2/-4* clade are highly represented in the liver tissue of gilthead sea bream juveniles: *igfbp-2b* and *igfbp-4* comprise more than 70 and 20% of *igfbp*

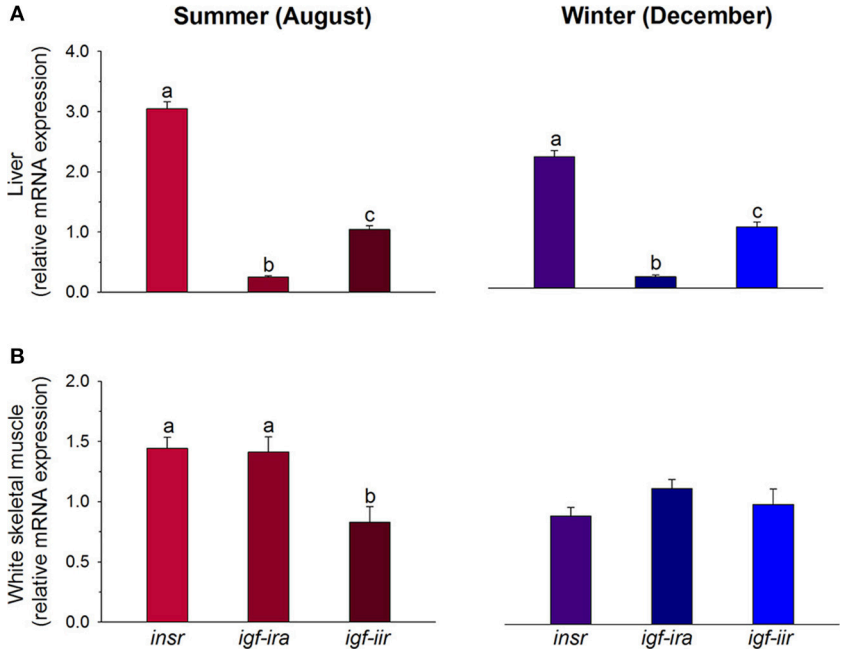


FIGURE 7 | Regulated gene expression of receptors of insulin (*insr*), *igf-i* (*igf-ira*), and *igf-ii* (*igf-iir*) in liver **(A)** and skeletal muscle **(B)** of gilthead sea bream juveniles during summer (red colors) and winter (blue colors). For each tissue and season, data values (mean ± SEM, $n = 6–7$) are normalized to the expression level of *igf-iir* (arbitrary value of 1). Different superscript letters indicate significant differences ($P < 0.05$; ANOVA followed by Student-Newman–Keuls test). Data derived from samples from Benedito-Palos et al. (146).

mRNAs, respectively. In contrast, the *igfbp-3/-5/-6* clade is overrepresented in skeletal muscle, and *igfbp-3* and *igfbp-4* transcripts make up 90% of mRNAs coming from *igfbp* genes. This clear divergence of expression patterns for the two *igfbp* clades is also a characteristic feature of Atlantic salmon in liver and muscle tissues (213). By contrast, this dichotomy is not retained by the adipose tissue where a relatively high expression level is found for both *igfbp-4* (*igfbp-1/-2/-4* clade) and *igfbp-5b* (*igfbp-3/-5/-6* clade) genes in gilthead sea bream (**Figure 9**). In blood, the presence of different Igfbps within a molecular range of 20–50 kDa has been reported by Igf-binding assays in various fish species but, unlike in mammals, Igfbp-2 and not Igfbp-3 is emerging as the major blood Igf-i carrier in fish species (219), which is consistent with the elevated expression pattern of *igfbp-2* genes in the liver tissue of this group of vertebrates.

More controversial are the main roles of each IGFBP as a growth-promoting or inhibiting factor through their IGF- or non-IGF-mediated effects. Recently, this has been extensively reviewed (210, 218), though it remains difficult to draw a general conclusion since most of the physiological roles, when conserved, differ across species and physiological contexts. Moreover, the lack of substantial phenotypes when *Igfbp-3*, *-4*, and *-5* were knocked out together in mice suggests a high degree of functional redundancy and/or genetic compensatory mechanisms (220), which reflects versatile modes of regulation in response to specific stressful or aberrant conditions. In any case, a large body of evidence in higher vertebrates and fasted, refed, or *gh*-transgenic fishes supports a main role of Igfbp-1 as a negative regulator of teleost growth. For instance, knockdown of *igfbp-1* alleviates the hypoxia-induced growth retardation and development delay in zebrafish, whereas overexpression of *igfbp-1* causes growth and developmental retardation under normoxia (221). Along the same lines, we found that hepatic *igfbp-1a* expression remains mostly repressed in grow-out gilthead sea bream during both summer and winter periods (**Figures 10A,B**). Mixed results exist on the regulation of *Igfbp-2* genes. Mouse embryos overexpressing *Igfbp-2* show a reduced growth rate, likely through reduced IGF availability (222). By contrast, work in Atlantic salmon highlights an increase in circulating Igfbp-2 in response to Gh (218), while early studies in zebrafish reported that Gh inhibits *igfbp-2* expression (223). As in salmonids, data on *igfbp-2b* expression in gilthead sea bream support a growth-promoting action of Igfbp-2, which is substantiated by its seasonal expression pattern and its depressed expression in fishes with signs of essential fatty acid deficiencies (**Figure 10C**). On a seasonal basis, the expression pattern of *igfbp-4*, recognized as a growth-promoting factor in salmonids (218), is similar to that of *igfbp-2b* at a lower expression level, but importantly the regulation of *igfbp-4* appears especially sensitive to nutrient deficiencies in essential fatty acids and phosphorus (**Figures 10A–C**).

Igfbp-3 seems to have less of a common role among fish lineages. Indeed, studies in zebrafish, flounder and yellowtail reported a significant increase in the expression level of *igfbp-3* in liver and/or muscle in response to fasting (224–226), which may act to restrict Igf signaling. By contrast, studies in trout and Atlantic salmon have reported no changes in the expression of *igfbp-3* in response to fasting (63, 218), whereas the muscle

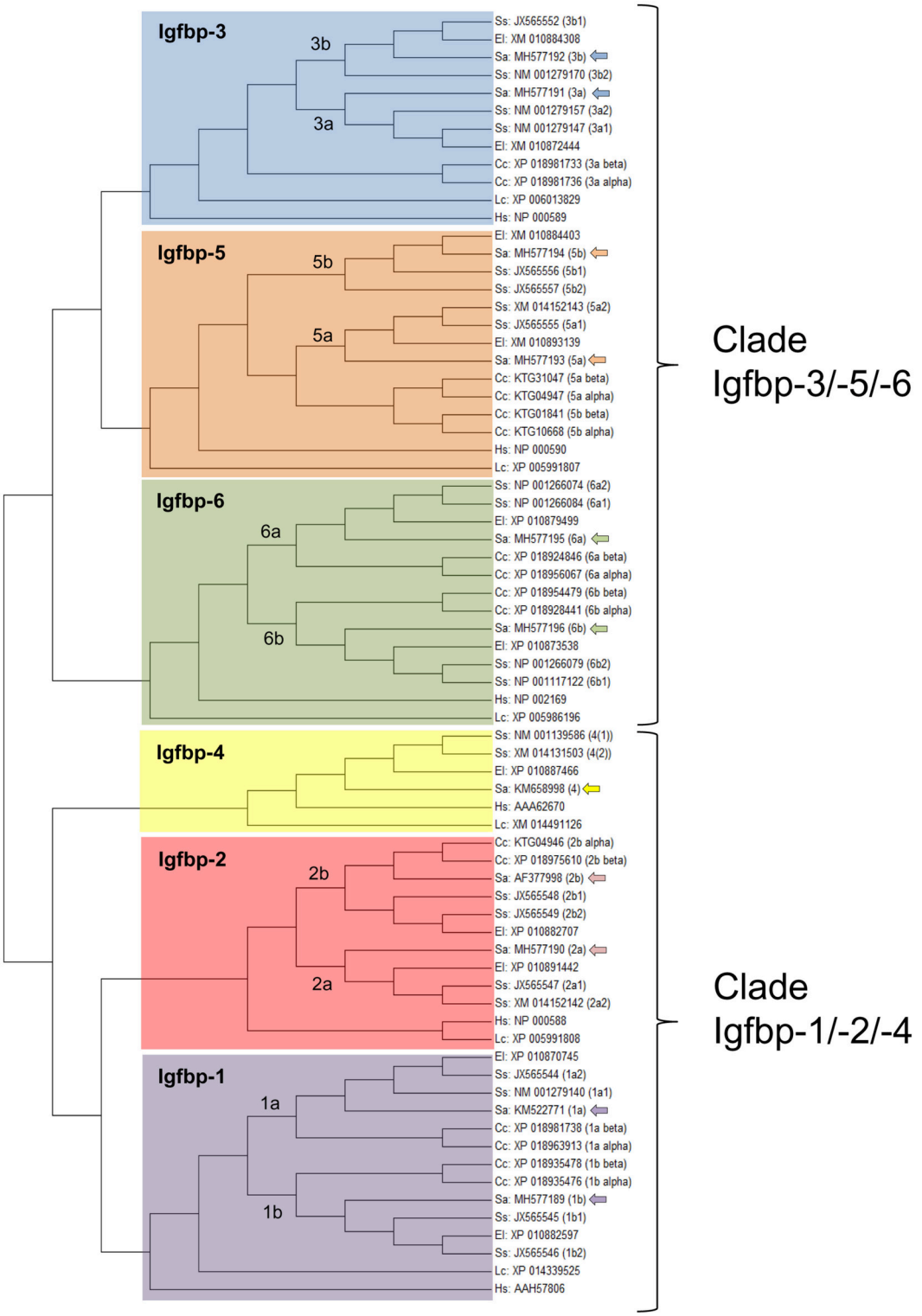


FIGURE 8 | Phylogenetic analysis of the Igfbp family, evidencing the two Igfbp modern vertebrate clades and the expansion of the family in teleosts. The unrooted tree was constructed by means of Mega 6.0 using the maximum likelihood method, with the amino acid alignment of 72 Igfbp sequences from Human, *Homo sapiens* (Hs); Comoran coelacanth, *Latimeria chalumnae* (Lc); northern pike, *Esox lucius* (El); carp, *Cyprinus carpio* (Cc); Atlantic salmon, *Salmo salar* (Ss); and gilthead sea bream, *Sparus aurata* (Sa). GenBank accession numbers are given for all sequences. Duplication events in teleosts are shown. The 11 gilthead sea bream sequences, including *igfbp-4* and paralog pairs of *igfbp-1*, *-2*, *-3*, *-5,* and *-6* are marked with arrows.

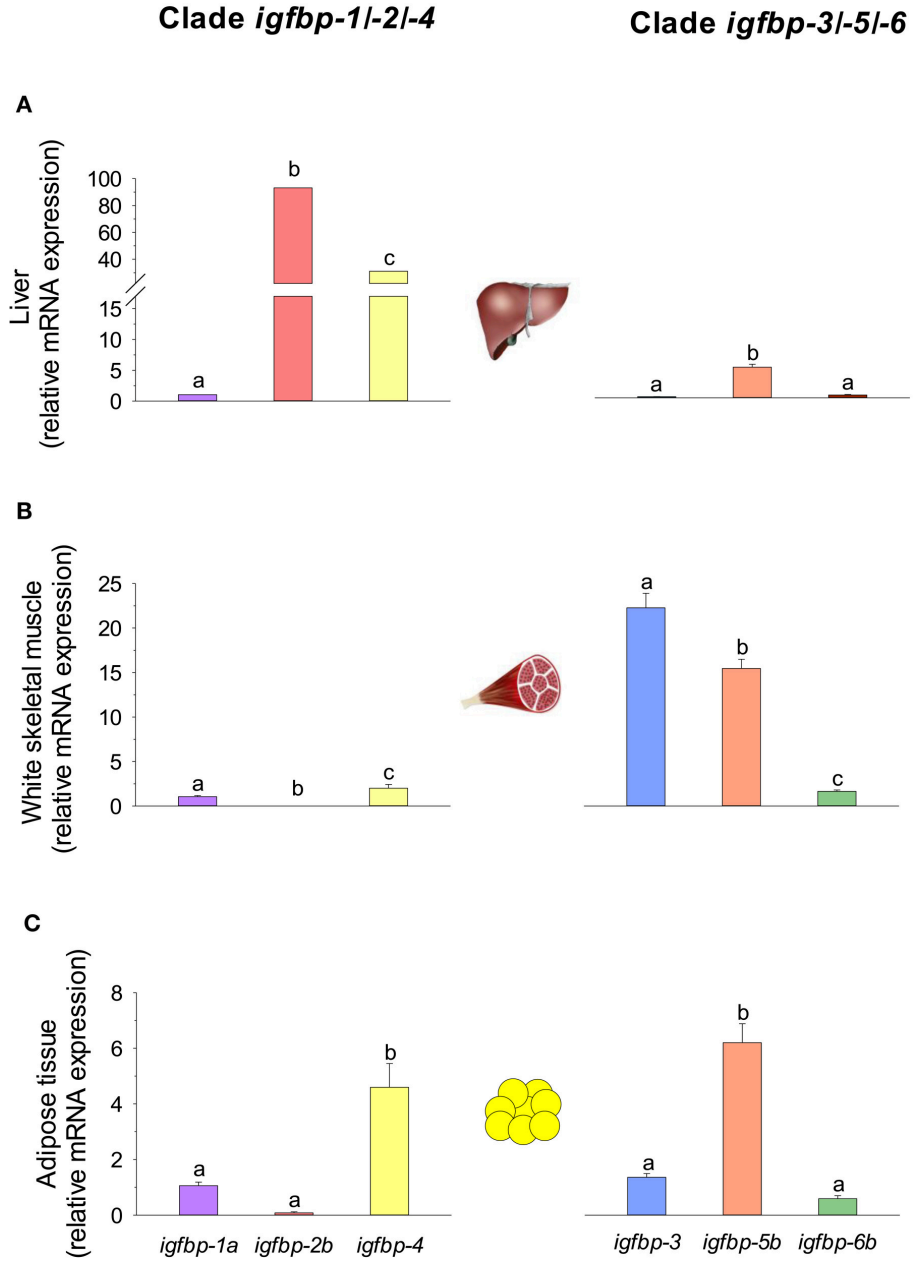


FIGURE 9 | *igfbp* gene expression profile in **(A)** liver, **(B)** white skeletal muscle, and **(C)** adipose tissue of juveniles of gilthead sea bream. RT-qPCR of tissue total RNA was conducted as previously reported (134). Analyzed genes of clade *igfbp-1/-2/-4* were *igfbp-1a*, *igfbp-2b,* and *igfbp-4*. For clade *igfbp-3/-5/-6*, primers for *igfbp-3* detected both *igfbp-3a* and *-3b* paralogs, and expression levels of *igfbp-5b* and *igfbp-6b* were analyzed. For each tissue, data values (mean ± SEM, $n = 8–9$) are normalized to the expression level of *igfbp-1a* (arbitrary value of 1). In each tissue and clade, different superscript letters indicate significant differences ($P < 0.05$; ANOVA followed by Student-Newman–Keuls test).

expression of *igfbp-3a1* is enhanced by *gh*-transgenesis in coho salmon (227), which is consistent with a growth-promoting action in salmonids. To make matters more complicated, in gilthead sea bream, the expression of muscle *igfbp-3* is elevated not only during the summer growth enhancement but also during growth impairments due to phosphorus deficiencies, which supports both growth-promoting and -inhibiting roles depending on the physiological context. In contrast, the main role of Igfbp-5b is to promote growth in response to changes in gene expression with the season and nutritional status (**Figures 10D–F**). Overall, the available evidence for Igfbp-5 supports a growth-promoting function in salmonids, but again functional divergence has been reported across species and physiological contexts [see García de la Serrana and Macqueen

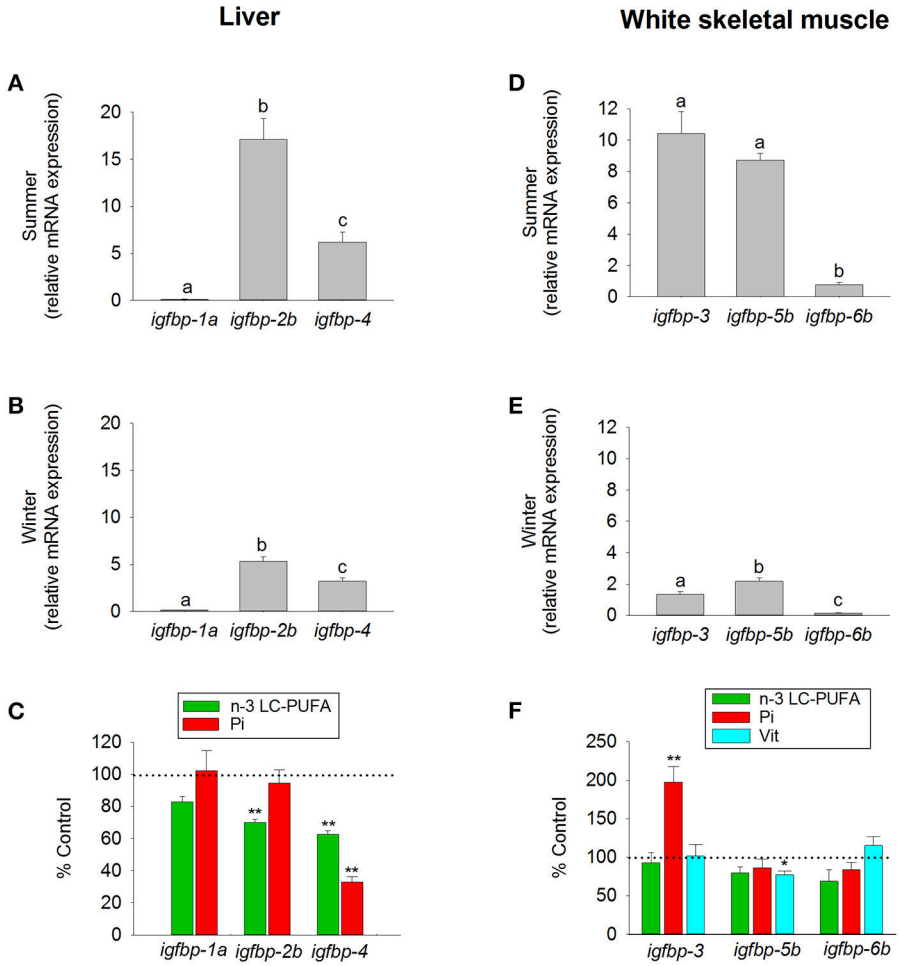


FIGURE 10 | Tissue-specific regulation of *igfbp* by season and diet in juveniles of gilthead sea bream. Liver expression of *igfbp-1a*, *igfbp-2b,* and *igfbp-4* (clade *igfbp-1/-2/-4*) was analyzed in fishes during **(A)** summer, **(B)** winter, and **(C)** after a dietary challenge with a diet deficient in n-3 LC-PUFA (green bars) or phosphorous (Pi, red bars). White skeletal muscle expression of *igfbp-3* (primers detected both *igfbp-3a* and *-3b* paralogs), *igfbp-5b,* and *igfbp-6b* (clade *igfbp-3/-5/-6*) was analyzed in fishes during **(D)** summer, **(E)** winter, and **(F)** after a dietary challenge with a diet deficient in n-3 LC-PUFA (green bars), phosphorus (Pi, red bars) or vitamins (Vit, cyan bars). RT-qPCR of tissue total RNA was conducted as previously reported (134). Data are represented as the mean ± SEM ($n = 6–7$). For each tissue and season **(A,B,D,E)**, different superscript letters indicate significant differences ($P < 0.05$; ANOVA followed by Student-Newman–Keuls test). Asterisks in **(C,F)** indicate significant differences compared with paralog expression in fishes from group fed a control diet (*t*-test, $^*P < 0.05$; $^{**}P < 0.001$). Data derived from samples from Benedito-Palos et al. (146) and Ballester-Lozano et al. (152).

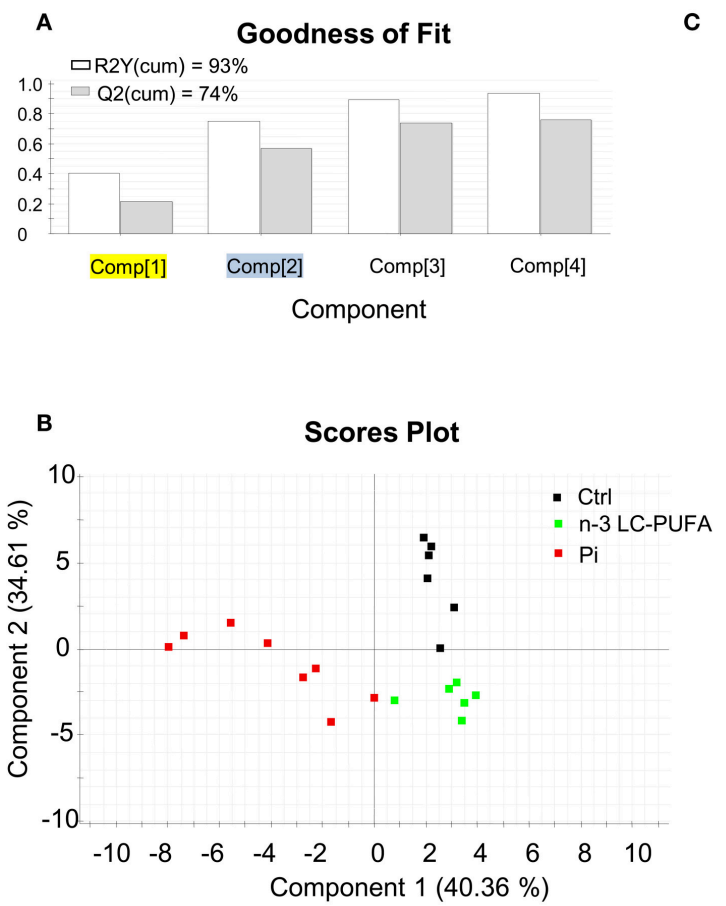
(218)]. Conversely, Igfbp-6 is emerging as a growth-inhibitory factor, though this Igfbp is particularly understudied, and it is difficult to draw overarching conclusions. However, taking into account its relatively low expression in the liver and skeletal muscle of well-nourished fishes, the proposed role for Igbp-6b in gilthead sea bream is closer to a growth-inhibiting rather than a growth-promoting action.

CONCLUDING REMARKS AND PROSPECTS

The GH/IGF system plays a key role in the endocrine cascade of growth, and overall changes at the protein and mRNA levels closely reflect differences in growth performance through development and production cycles. However, this relationship varies within and across fish species and physiological contexts from tight to non-discernible correlations due to the actions of a wide range of endogenous and exogenous factors. Despite this, changes in circulating GH and IGF-I levels continue to be one of the most robust markers of growth performance through vertebrate evolution. This is especially relevant in fish species since gene expansion by local or whole-genome duplications offers the possibility of a complex gene subfunctionalization and/or acquisition of novel functions. This is evidenced at the ligand level for Gh and Sl and at the receptor level for the *ghr-i* and *ghr-ii* genes, which are differentially regulated in liver and skeletal muscle, helping to distinguish stressful and growth disturbances due to overwintering or malnutrition as a result of changes in feed intake, protein and lipid feedstuffs or any

other specific nutrient. Close links between oxygen availability, energy status and the somatotropic axis are also now emerging via Sirts, which are potential markers of informing of energy status and can modulate the anabolic action of Gh by inhibiting Ghr signaling. The differential regulation of *igf-i* and *igf-ii* genes in liver and skeletal muscle also offers the possibility of a more refined analysis of growth potentiality and nutritional condition. Igfbps are emerging as highly regulated components of the Gh/Igf system, though the puzzle is far from complete because their tissue-specific regulation has not been established for all paralogs across fish species and different physiological conditions. Thus, further research is needed to combine the search for a robust, highly specific set of biomarkers for a given growth derangement, which may have an impact later in life by means of different epigenetic mechanisms, involving changes in DNA methylation and histone acetylation, among others (228).

The combination of conventional and different -omic approaches (functional genomics, proteomics, metabolomics, metagenomics) have been gaining acceptance as an option to assess the nutritionally and environmentally mediated effects on the growth performance, metabolic homeostasis, stress responsiveness and health condition of farmed fishes. However, at the present stage, a reliable diagnostic should combine measures of conventional biomarkers (e.g., data on blood biochemistry and histopathological scoring) with molecular signatures of the different components of the Gh/Igf system in addition to other related markers of growth and cell differentiation and proliferation, protein breakdown, protein folding and assembly, inflammatory/anti-inflammatory response, energy sensing, OXPHOS, mitochondrial respiration uncoupling, and lipolytic/lipogenic activity. In our hands, a key point for the simultaneous gene expression profiling of all the genes included in our growth PCR-array is the operation



C

Genes, VIP > 1	
igf-i	1.84
igfbp-4	1.82
ghr-i	1.73
igfals	1.71
igf-ii	1.65
cpt1a	1.63
sirt4	1.59
der-1	1.52
il-6rb	1.50
grp-170	1.50
sco1	1.44
ctsl	1.44
capn1	1.43
igf-ii/igf-i	1.37
il-1r2	1.35
grp-94	1.25
il-1r1	1.25
igfbp-2b	1.23
ghr-il/ghr-ii	1.21
capn3	1.12
ctsd	1.07
il-8	1.07
nd2	1.01
pcna	1.00

FIGURE 11 | Discriminant analysis (PLS-DA) of liver molecular signatures of fishes fed nutrient deficient diets. Relative expression data of the 73 genes included in the array can be found in **Table S1**. **(A)** Cumulative coefficients of goodness of fit (R^2, white bars) and prediction (Q^2, gray bars) by each component; the two first components explained 74.97% of total variance. **(B)** PLS-DA score plot of acquired data from dietary challenged individuals along the two main components. Individuals fed the phosphorus deficient diet (Pi, red squares) are separated along the first component, and component 2 separates individuals fed the control (Ctrl, black squares) and LC-PUFA-deficient diets (green squares). **(C)** Ordered list of markers by variable importance (VIP) in the projection of PLS-DA model for group differentiation. Markers with VIP values >1 after the first or second component are highlighted in yellow and blue, respectively. Data derived from samples from Ballester-Lozano et al. (152).

of the analytical platform by handling robots, resulting in a minimal variation between technical replicates. By means of multivariate analysis, this offers the possibility to identify at a high level of confidence the most responsive tissues and biomarkers in animals facing a given stressful rearing condition. This is exemplified herein by comparing the molecular signatures of liver and skeletal muscle of fishes with signs of nutrient deficiencies in n-3 LC-PUFA, phosphorus, or vitamins (**Figures 11**, **12**). These three types of nutrient deficiencies were chosen because they are considered the most constraining factors

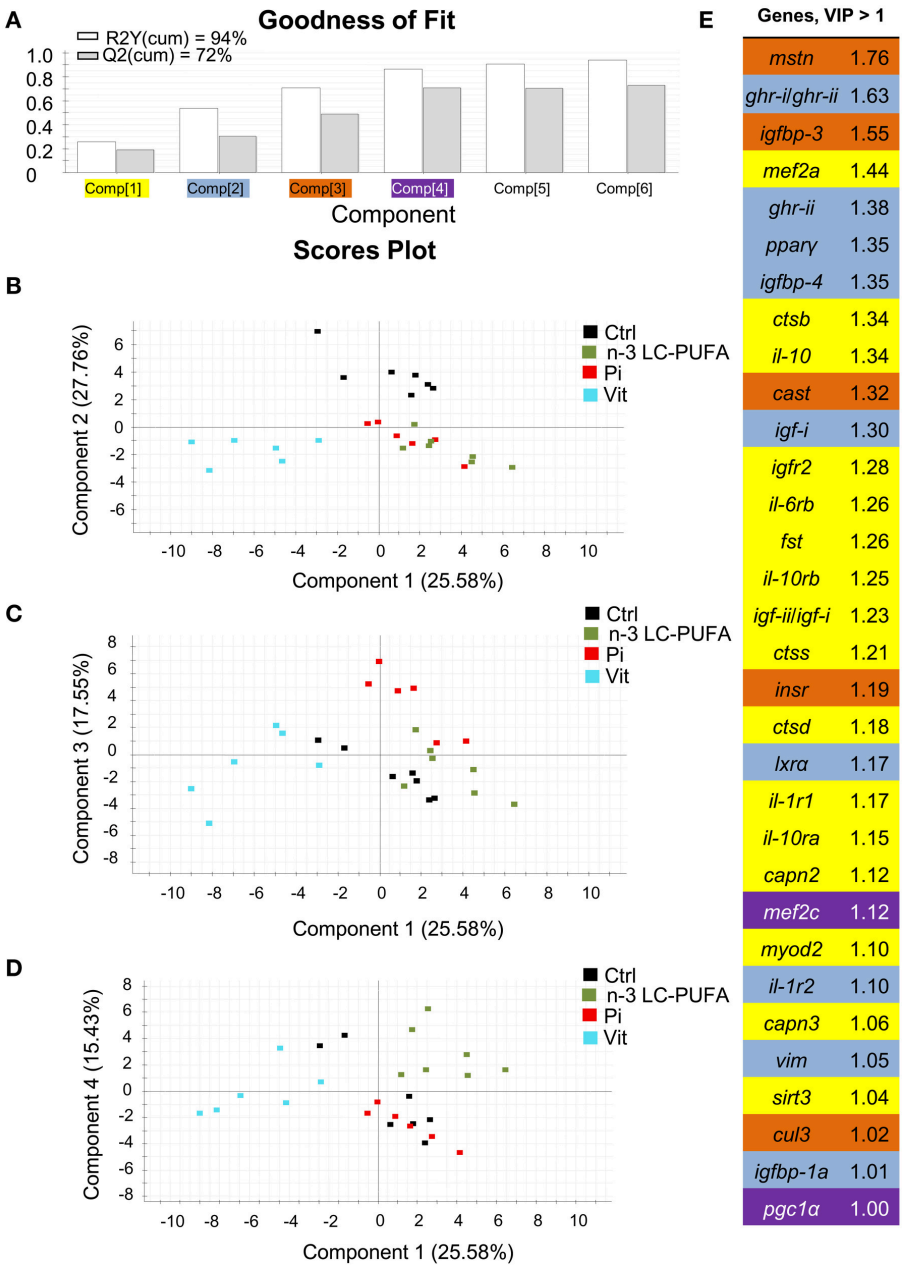


FIGURE 12 | Discriminant analysis (PLS-DA) of skeletal muscle molecular signatures of fishes fed nutrient deficient diets. Relative expression data of 84 genes can be found in **Table S2**. **(A)** Cumulative coefficients of goodness of fit (R^2, white bars) and prediction (Q^2, gray bars) by each component; 86.32% of total variance is explained by four components. **(B)** PLS-DA score plot of acquired data from dietary-challenged individuals along components 1 and 2. Individuals fed the vitamin-deficient diet (Vit, cyan squares) are separated along the first component. **(C)** PLS-DA score plot of acquired data from dietary-challenged individuals along components 1 and 3. Component 3 separates individuals fed the phosphorus-deficient diet (Pi, red squares). **(D)** PLS-DA score plot of acquired data from dietary-challenged individuals along components 1 and 4. Component 4 separates individuals fed the LC-PUFA-deficient diet (green squares). **(E)** Ordered list of markers by variable importance (VIP) in the projection of PLS-DA model for group differentiation. Markers with VIP values >1 after the first, second, third and fourth components are highlighted in yellow, blue, orange and purple, respectively. Data derived from samples from Ballester-Lozano et al. (152).

of FM/FO replacement by alternative plant ingredients in marine farmed fishes. Using this approach, discriminant analysis (PLS-DA) is able to explain more than 93% of the variance (R) and to predict more than 72% of the total variance (Q). Thus, from this meta-analysis, it is conclusive that the liver tissue is especially responsive to deficiencies in essential fatty acids or phosphorus, whereas skeletal muscle is emerging as the main target tissue for the diagnosis of vitamin deficiencies. This is supported by variable importance (VIP) analysis, which highlights the different contribution of the 73–84 genes analyzed in our growth-arrays. This is just one example of what can be done with this type of approach, helping to establish the normal range of variance of highly informative biomarkers as a function of developmental stage and nutritional background. This procedure is based on the ARRAINA-derived biomarkers (229), and current research is taking advantage of this knowledge within the PerformFISH and GAIN H2020 EU Projects to validate, at the pilot scale and farm levels, new rearing systems and diet formulations for European aquaculture intensification. How all this is affected by nutrition and genome interactions is, however, a major challenge in efficiently managing aquaculture breeding programs and producing more robust farmed fishes, fed sustainable diets in a changing environment. In this regard, integrative studies on fish endocrinology can help to establish the best phenotype and the normal range of reference for different growth-promoting factors in animals with different nutritional and environmental backgrounds, allowing us to re-evaluate the nutritional status and nutrient requirements. How this can also include other criteria, such as functional microbiota, requires more research, but the endocrine system can help explain the now-emerging distal effects of intestinal microbiota on productive traits other than those more directly related to intestinal health. For instance, a growing body of evidence is pointing out that microbiota modulates host circulating Igf-i levels (230), a feature that seems to be conserved in fishes (231), and probably mediated by microbiota production of short-chain fatty acids. However, studies in fishes relating the composition of the core microbiota to a wide range of endogenous and exogenous factors are still in their infancy.

AUTHOR CONTRIBUTIONS

JP-S: writing, reviewing, and conception; PS-M, FN-C, JM-S, EP, AB-N, and LB-P: writing; JC-G: writing and reviewing.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2018.00687/full#supplementary-material

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The Impact of Initial Energy Reserves on Growth Hormone Resistance and Plasma Growth Hormone-Binding Protein Levels in Rainbow Trout Under Feeding and Fasting Conditions

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The growth hormone (GH)–insulin-like growth factor I (IGF-I) system regulates important physiological functions in salmonid fish, including hydromineral balance, growth, and metabolism. While major research efforts have been directed toward this complex endocrine system, understanding of some key aspects is lacking. The aim was to provide new insights into GH resistance and growth hormone-binding proteins (GHBPs). Fish frequently respond to catabolic conditions with elevated GH and depressed IGF-I plasma levels, a condition of acquired GH resistance. The underlying mechanisms or the functional significance of GH resistance are, however, not well understood. Although data suggest that a significant proportion of plasma GH is bound to specific GHBPs, the regulation of plasma GHBP levels as well as their role in modulating the GH–IGF-I system in fish is virtually unknown. Two *in vivo* studies were conducted on rainbow trout. In experiment I, fish were fasted for 4 weeks and then refed and sampled over 72 h. In experiment II, two lines of fish with different muscle adiposity were sampled after 1, 2, and 4 weeks of fasting. In both studies, plasma GH, IGF-I, and GHBP levels were assessed as well as the hepatic gene expression of the growth hormone receptor 2a (*ghr2a*) isoform. While most rainbow trout acquired GH resistance within 4 weeks of fasting, fish selected for high muscle adiposity did not. This suggests that GH resistance does not set in while fat reserves as still available for energy metabolism, and that GH resistance is permissive for protein catabolism. Plasma GHBP levels varied between 5 and 25 ng ml^{-1}, with large fluctuations during both long-term (4 weeks) fasting and short-term (72 h) refeeding, indicating differentiated responses depending on prior energy status of the fish. The two opposing functions of GHBPs of prolonging the biological half-life of GH while decreasing GH availability to target tissues makes the data interpretation difficult, but nutritional regulatory mechanisms are suggested. The lack of correlation between hepatic *ghr2a* expression and plasma GHBP levels indicate that *ghr2a* assessment cannot be used as a proxy measure for GHBP levels, even if circulating GHBPs are derived from the GH receptor molecule.

Keywords: adiposity, fasting, salmonid, growth hormone-binding protein, growth hormone resistance

INTRODUCTION

As in mammals, the growth hormone (GH)–insulin-like growth factor I (IGF-I) system is the major endocrine system simulating growth in salmonids (1), indicating a strong evolutionary conservation. Indeed, mammalian and salmonid data are in agreement on all key aspects of the GH–IGF-I system. The GH receptor (GHR) is found in most tissues, with the highest density in the liver (2–4). GH can thus stimulate tissue growth directly, but does so also indirectly through GH-induced production of IGF-I in most tissues, where it exerts paracrine regulation of growth and metabolism through its IGF-I receptor (IGF-IR) (1, 5). In the liver, GH stimulates IGF-I secretion into the circulation, where it acts as an endocrine stimulator, especially of skeletal growth (6) as well as acting as a negative-feedback signal on GH secretion (7). In mammals as well as fish, both hormones also regulate important aspects of metabolism through augmentative effects on protein accretion and counteractive effects on glucose and lipid utilization (1, 8, 9).

Fasting normally leads to increased plasma GH levels while IGF-I levels decrease, an endocrine condition defined as acquired GH resistance (10, 11). While early studies in mammals and fish suggested that downregulation of the GHR was the key underlying mechanism (11, 12), it now appears that acquired GH resistance is largely due to inhibition of the JAK–STAT pathway for GH signaling (13–15). Irrespective of the causal mechanisms, one of the major endocrine consequences of GH resistance is the decreased hepatic secretion and thus plasma levels of IGF-I (16). Under catabolic conditions such as fasting, plasma IGF-I levels may decrease independently of the onset of GH resistance, as hepatic IGF-I secretion is stimulated by circulating nutrient levels, which decline during fasting (17). As plasma IGF-I exerts negative-feedback inhibition on pituitary GH secretion (8, 18), decreased IGF-I levels during fasting leads to increased GH secretion and plasma GH levels (19, 20).

Specific binding proteins have been identified and characterized for both GH and IGF-I and therefore, the endocrine regulation of physiological processes by the GH–IGF-I system can be modulated by both growth hormone-binding proteins (GHBPs) and IGFBPs. Much functional information has been obtained on the roles of the multiple IGFBPs, both in mammals and fish (21), while much less is known about the regulatory function of the GHBPs. Despite mammalian data indicating that about 50% of plasma GH is bound to specific, high-affinity GHBP (22–24), its role and impact on GH bioavailability in humans is still unclear (25), as is the functional importance of GHBPs in the GH–IGF-I system in fish.

In mammals, with the exception of rodents (26), the circulating GHBP is the extracellular domain of the GHR protein, which is released into the circulation through proteolytic cleavage of the membrane-bound GHR (27). Thus, the GHR molecule has a double functional role in the GH–IGF-I system; conveying the endocrine GH signal to the target cells as well as being the substrate for plasma GHBP production (28). Mechanistically, observed changes in hepatic GHR density and/or GHR gene expression may indicate changes in tissue sensitivity to GH, and/or changes in GHBP production and plasma GHBP levels.

Sohm et al. (29) provided the first evidence for GHBPs in rainbow trout (*Oncorhynchus mykiss*) plasma using GH binding and cross-linking assays as well as immunoprecipitation and presented semi-quantitative data indicating that plasma GHBP levels increase 2 days after seawater transfer. Similar methodological approach was used to demonstrate the existence of GHBPs in plasma of goldfish (30) and Chinese sturgeon (31). Subsequently, through the use of GHR-transfected CHO cells, Liao et al. (32) demonstrated that fish GHBP stems from the extracellular domain of the membrane-bound GHR, as in non-rodent mammals. Recently, the first immunoassay for non-mammalian vertebrate GHBPs was established and validated for rainbow trout and Atlantic salmon [*Salmo salar* (33)], providing the first ever quantitative data on circulating GHBPs in fish, where plasma GHBP levels were indicated to increase following seawater transfer of Atlantic salmon smolts (33).

The aims of this study were to explore the relation between initial energy balance and the onset of acquired GH resistance by comparing rainbow trout with high and low muscle adiposity during fasting. Furthermore, to gain insights into the regulatory roles of the GH–IGF-I system in rainbow trout in regard to energy balance by elucidating possible roles of plasma GHBPs in functional modulation of the GH–IGF-I system. To achieve these aims, plasma GHBP levels as well as plasma GH and IGF-I levels were measured, together with quantitative analysis of hepatic *ghr2a* mRNA expression, in two separate studies in which the energy balance and nutritional conditions of rainbow trout were manipulated. In experiment I, rainbow trout were fasted for 4 weeks and then refed over 72 h. In experiment II, two selectively bred strains of rainbow trout which differ in muscle and visceral adiposity were studied under feeding conditions as well as during a 4-week fasting period.

MATERIALS AND METHODS

Fish, Holding Conditions, and Design of Experiment I

Rainbow trout ($n = 116$) with a mean body weight (BW) of 146 g and body (fork) length (BL) of 24.7 cm were obtained from a local fish farm, Antens Laxodling AB, outside Gothenburg, Sweden. This stock has been maintained in Swedish aquaculture for generations, but with no directed breeding-selection program. At the animal facilities at the Department of Biological and Environmental Sciences, University of Gothenburg, the fish were randomly distributed among 12 circular 150 l fiberglass tanks, supplied with running, aerated fresh water and acclimated for 2 weeks. Water temperature was 12°C and photoperiod was 12L:12D. The fish were fed manually *ad lib* once a day. After the acclimation period, fish in six tanks were fasted for 4 weeks (FA group) while fish in six tanks were fed *ad lib* during this period (AL group). At the end of the 4-week feeding/fasting period, at time designated as 0 h (t_{0h}), eight fish from each group were sampled. Then, both AL and FA fish were fed *ad lib* and sampled after 2, 7, 24, and 72 h (t_{2h}, t_{7h}, t_{24h}, and t_{72h}). Between each sampling, all fish were fed *ad lib* to guarantee maximal feed availability. At each sampling time, eight fish of each treatment regime were sampled,

four from two replicate tanks. To minimize disturbance, at least 24 h were allowed to pass before fish were sampled again from a previously sampled tank. The fish were anesthetized with metomidate (12 mg l^{-1}), killed by a blow to the head and sampled, see below.

Two of the AL fish sampled at t_{0h} had empty gastrointestinal (GI) tract and a low condition factor (CF) similar to that of the FA group. It was concluded that they had not been actively feeding and were eliminated from the study. Data on leptin endocrinology obtained from this study have been presented in Johansson and Björnsson (34).

Fish, Holding Conditions, and Design of Experiment II

Two divergent rainbow trout lines have been established through a breeding program with muscle adiposity as a selection criterion; a fat line (FL) with high muscle lipid content and a lean line (LL) with low muscle lipid content (35). This study was carried out on fish from the seventh generation of this breeding program These FL fish had double the muscle adiposity of the LL fish, which on the other hand had higher visceral fat content than the FL fish (36). The study was carried out at the PEIMA-INRA aquaculture research facility in Brittany, France. On April 15th, 2014, eight tanks were stocked with FL fish (mean BW 238 g) and eight tanks with LL fish (mean BW 262 g). The water volume of these outdoor tanks was 1.8 m^3, water flow 3 m^3 h^{-1} and oxygen levels >6.0 mg l^{-1}, under ambient photoperiod and temperature conditions, which rose gradually from 10.6 to 13.5°C over the course of the study from mid-April to early June.

When fed, the fish were given size 5 pellets[1] by automatic feeders five times daily. The ration was adjusted weekly based on size and temperature, and increased from about 1.16 to 1.25% BW day^{-1} over the study.

After a 3-week acclimation period, a 4-week feeding/fasting experiment was initiated, encompassing four different experimental feeding regimes involving 0, 1, 2, and 4 weeks of fasting. Thus, the 0-week groups were fed throughout, the 1-week groups were fed for 3 weeks followed by 1 week of fasting, the 2-week groups were fed for 2 weeks, and then fasted for 2 weeks, and the 4-week groups were fasted throughout. Each feeding regime included duplicate tanks of FL as well as LL fish. To enable sampling of all fish after 28 days, the experimental feeding regimes were initiated 1 day apart and then sampled 1 day apart 4 weeks later.

For each feeding regime, 20 fish of each line were sampled, 10 from each of the duplicate tanks. The fish were netted and placed in a lethal dose (160 mg l^{-1}) of isoeugenol (ScanAqua). When ventilation ceased, the fish were sampled, see below.

Data from this experiment on peripheral leptin endocrinology and energy stores have been published in Johansson et al. (36) and on central leptin signaling in Gong et al. (37).

Sampling

Sampling was initiated by measurements of BW and BL, after which blood was drawn from the caudal vessels into a heparinized syringe. Blood was kept on ice for <15 min before centrifuged, the obtained plasma frozen in aliquots on dry ice and kept at −80°C until analysis. The liver was dissected out and weighted (LW), after which about 1 g piece was placed in aluminum foil, immediately frozen in liquid nitrogen and kept at −80°C until analysis. The whole GI tract was dissected out and weighted after which all food was removed from both stomach and intestine and it weighted again as visceral weight (VW).

Analyses

Plasma GH and IGF-I Analysis

Plasma GH was analyzed with radioimmunoassay (RIA), using anti-GH antibodies specific for salmonids. The method has been described by Björnsson et al. (38) and evaluated for rainbow trout.

IGF-I was extracted from plasma as described by Shimizu et al. (39) and analyzed using a 2-day RIA protocol described by GroPep Ltd.[2] with some modifications. Salmon/trout IGF-I (GroPep) was used for iodination and standards. Antibodies against barramundi IGF-I, obtained from GroPep, were made in rabbits by Agrisera.[3] Iodination was carried out using chloramine-T, with 0.5% BSA added to the RIA buffer. Microliters of the extracted neutralized samples were diluted 1:4 with RIA buffer, and 100 µl samples and standards were analyzed. Anti-barramundi IGF-I rabbit serum was used at a final dilution of 1:42,000 in the assay tubes, and the assay ^{125}I-IGF-I solution was adjusted to 5,000 cpm per 50 µl solution. The antigen–antibody complex was precipitated with anti-rabbit IgG (R0881), and gamma globulin (I 8140) from Sigma[4] and 3% polyethylene glycol (PEG 6000) After incubation, the samples were centrifuged at 3,200 rpm for 60 min, aspirated and the pellets counted in a gamma counter.

Plasma GHBP Analysis

Plasma GHBP was analyzed using a 3-day competitive, non-equilibrium RIA, described and validated for rainbow trout by Einarsdottir et al. (33). Briefly, a GST-tagged recombinant extracellular part of the Atlantic salmon GHR subtype 1 (sGHR1) was used as standards and iodinated with the chloramine-T method. Antibody against the extracellular part of the sGHR1 (anti-sGHR1) was produced in rabbits by Agrisera against a 15 amino acid synthetic peptide conjugated to keyhole limpet hemocyanin. The rabbit anti-GHR1 serum was affinity purified with the antigen coupled to the stationary phase. The sGHR1 isoform, described by Benedet et al. (40), corresponds to the rainbow trout GHR2a isoform as it is defined by Reindl and Sheridan (3) and was previously termed GHR1, and the synthesized amino acid sequence used to raise the anti-sGHR1 is near-identical between the two species.

Hepatic Growth Hormone Receptor 2a (*ghr2a*) Gene Expression Analysis

Total RNA was extracted from 30 mg liver using RNeasy® Plus Mini Kit (Qiagen, Hilden, Germany). For the tissue homogenizing,

[1] www.aqua.legouessant.com.

[2] http://gropep.com.

[3] http://agrisera.com.

[4] http://sigmaaldrich.com.

TissueLyser II (Qiagen) was used in Study I, with each tube containing a 5 mm ∅ stainless steel bead (Qiagen), and in Study II, the Precellys®24 homogenizer (Bertin Technologies, France) was used. RNA quantity and quality were assessed using the NanoDrop Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA) by absorbance at 260 and 280 nm. To check the RNA quality with another method, random samples were assessed from Study II using the Experion Automated Electrophoresis System (Bio-Rad Laboratories, Sundbyberg, Sweden). Total RNA (1 µg) was reverse-transcribed to cDNA using iScript™ cDNA Synthesis Kit on a MyCycler Thermal Cycler (Bio-Rad). The GHR isoform analyzed has earlier been termed GHR1 [e.g., Ref. (9, 14)], but has since been redefined as GHR2a (3). The gene expression of the rainbow trout *ghr2a* and reference gene, elongation factor 1α (*elfl1*α) was analyzed by quantitative PCR (qPCR) using the reagent, SsoAdvanced™ Universal SYBR® Green supermix (Bio-Rad), in a CFX Connect™ real-time cycler (Bio-Rad). The primer sequences were listed in **Table 1**, and purchased from Eurofins MWG Synthesis GmbH, Ebersberg, Germany.

All samples were analyzed in duplicate using 5 µl qPCR reagent, 0.5 µl of each primer (final concentration 500 nM), and 4 µl cDNA template (10 ng cDNA) in a total volume of 10 µl. The qPCR reaction involved 40 cycles using a dissociation temperature of 95°C for 10 s and annealing and elongation in the same step at 60°C for 30 s. The quantification cycle number (C_q) was used to calculate the gene expression for each sample. No non-specific products or primer-dimers were co-amplified with the specific product. Both target gene (*ghr2a*) and reference gene (*elf1*α) was amplified with efficiencies near 100%.

Ethical Permits

Experiment I was carried out at a certified animal facility at the Department of Biological and Environmental Sciences, University of Gothenburg, under license 85-2012 by the Ethical Committee for Animal Research in Gothenburg. Experiment II was carried out at Pisciculture Expérimentale INRA des Monts d'Arrée, which is approved for animal experimentation through license C29-277-02 in accordance with the European Communities Council Directive 86/609/EEC, and carried out under the official license 29-036 of Dr. Labbé Laurent.

Calculations and Statistics

Condition factor was calculated as $CF = (BW \times BL^{-3}) \times 100$. Liver somatic index (LSI) was calculated as $LSI = (LW \times BW^{-1}) \times 100$, and visceral somatic index (VSI) was calculated as $VSI = (VW \times BW^{-1}) \times 100$. For the calculations, all weights (BW, LW, and VW) are expressed in grams, and BL is expressed in centimeters.

The relative hepatic expression level of the *ghr2a* gene was calculated using the formula of ratio (target/reference) = $2^{Cq\ (reference) - Cq\ (target)}$.

The data from experiments I and II were statistically analyzed using two-way ANOVA, establishing the significance of the main effects (feeding regime and time in experiment I; line and time in experiment II) as well as the interaction between the main effects. When main effects were found to be statistically significant, *post hoc* analysis was conducted using Fisher's least significant differences. The statistical analysis was carried out using the IBM SPSS Statistics version 25 software package.

RESULTS

Experiment I

At the end of the 4-week fasting period (t_{0h}), the FA fish had significantly lower BW, CF, LSI, and VSI than the AL fish (**Table 2**). Furthermore, in comparison with the AL fish, the FA fish had elevated plasma GH levels (**Figure 1A**), suppressed plasma IGF-I (**Figure 1B**), while plasma GHBP levels (**Figure 1C**) and hepatic *ghr2a* expression was similar between the groups (**Figure 1D**).

Refeeding differentially affected the various components of the GH–IGF-I system. Thus, plasma GH levels of the FA fish were elevated over the AL fish at 8 h, after which plasma GH levels were similar in both groups (**Figure 1A**). Conversely, plasma IGF-I levels of the FA fish were depressed at 8 h, after plasma IGF-I levels were similar between the groups (**Figure 1B**). Plasma GHBP levels oscillated. While neither feeding regime nor time significantly affected GHBP levels, the interaction of the main effects was significant (**Figure 1C**). The hepatic *ghr2a* expression remained similar between the groups over the 72 h refeeding period (**Figure 1D**).

Correlation analysis of plasma GH, IGF-I, and GHBP levels as well as hepatic *ghr2a* expression shows no significant correlation among these parameters (data not shown).

Experiment II

Plasma levels of GH, IGF-I, and GHBP, as well as hepatic *ghr2a* expression in the LL and the FL fish are shown in **Figure 2**. Plasma GH levels were significantly elevated in LL fish during fasting, being significantly elevated after 2 and 4 weeks, while plasma

TABLE 1 | Primer nucleotide sequences used for quantitative PCR analysis of rainbow trout hepatic growth hormone receptor gene 2a (*ghr2a*) and reference gene *elf1*α in experiments I and II.

Gene	Primer	Sequence (5′–3′)
ghr2a[a]	GHR2aFwOm	TGGGAAGATGAGTGCCAGACT
	GHR2aReOm	CACAAGACTACTGTCCTCTGTTGG
*elf1*α	EFa-f	CAAGGATATCCGTCGTGGCA
	EFa-r	ACAGCGAAACGACCAAGAGG

[a]*The ghr2a gene was earlier termed ghr1, see Ref. (3).*

TABLE 2 | Body weight (BW), condition factor (CF), liver somatic index (LSI), and visceral somatic index (VSI) of rainbow trout fed *ad lib* (AL group; $n = 6$) or fasted (FA group; $n = 7$) for 4 weeks in experiment I and sampled before onset of refeeding.

Group	BW (g)	CF	LSI (%)	VSI (%)
Fed *ad lib* (AL)	227.4 ± 26.6	1.33 ± 0.08	1.26 ± 0.14	1.55 ± 0.11
Fasted (FA)	146.3 ± 5.2*	1.06 ± 0.04*	0.62 ± 0.06*	1.04 ± 0.07*

Data are presented as means ± SEM.

**Statistical significant differences between groups at the $p < 0.05$ level.*

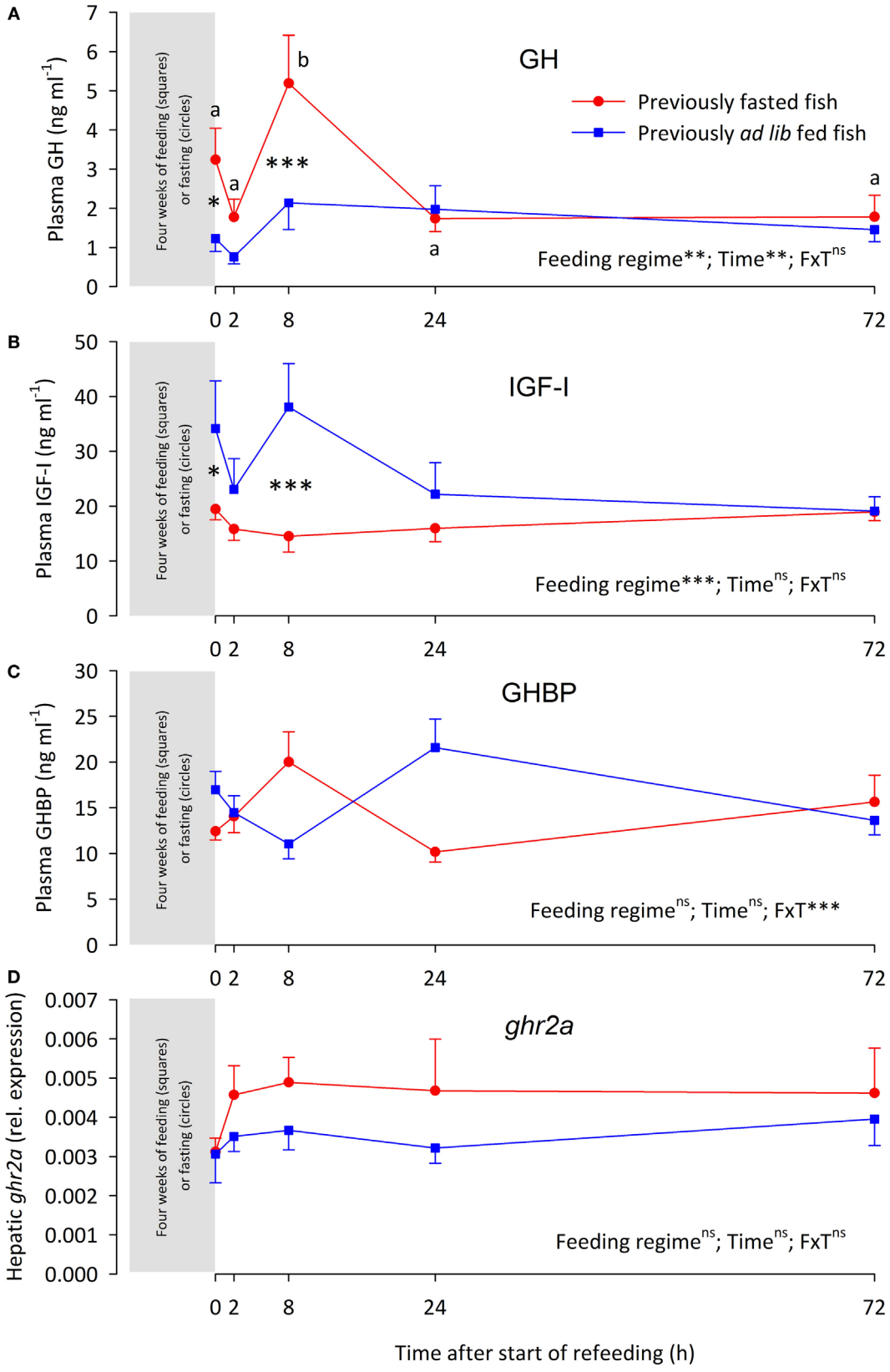

FIGURE 1 | Continued

FIGURE 1 | **(A)** Plasma growth hormone (GH) levels, **(B)** plasma insulin-like growth factor I (IGF-I) levels, **(C)** plasma growth hormone-binding protein (GHBP) levels, and **(D)** hepatic expression of the growth hormone receptor gene 2a (*ghr2a*) in rainbow trout fed (■) or fasted (●) for 4 weeks (sampled at time 0 h) after which both groups were fed over a 72 h "refeeding" period. Data are presented as means ± SEM. Two-way ANOVA results on main effects (feeding regime and time) as well as the interaction between the main effects (F × T) are indicated in the panels as being non-significant (ns, $p > 0.05$) or significant at the levels of $^{**}p < 0.01$ and $^{***}p < 0.001$. *Post hoc* analysis was carried out if main effects were significant. For "Feeding regime," differences are indicated as $^{*}p < 0.05$ or $^{***}p < 0.001$. For "time," significant differences are indicated by different letters, lower case for fasted fish.

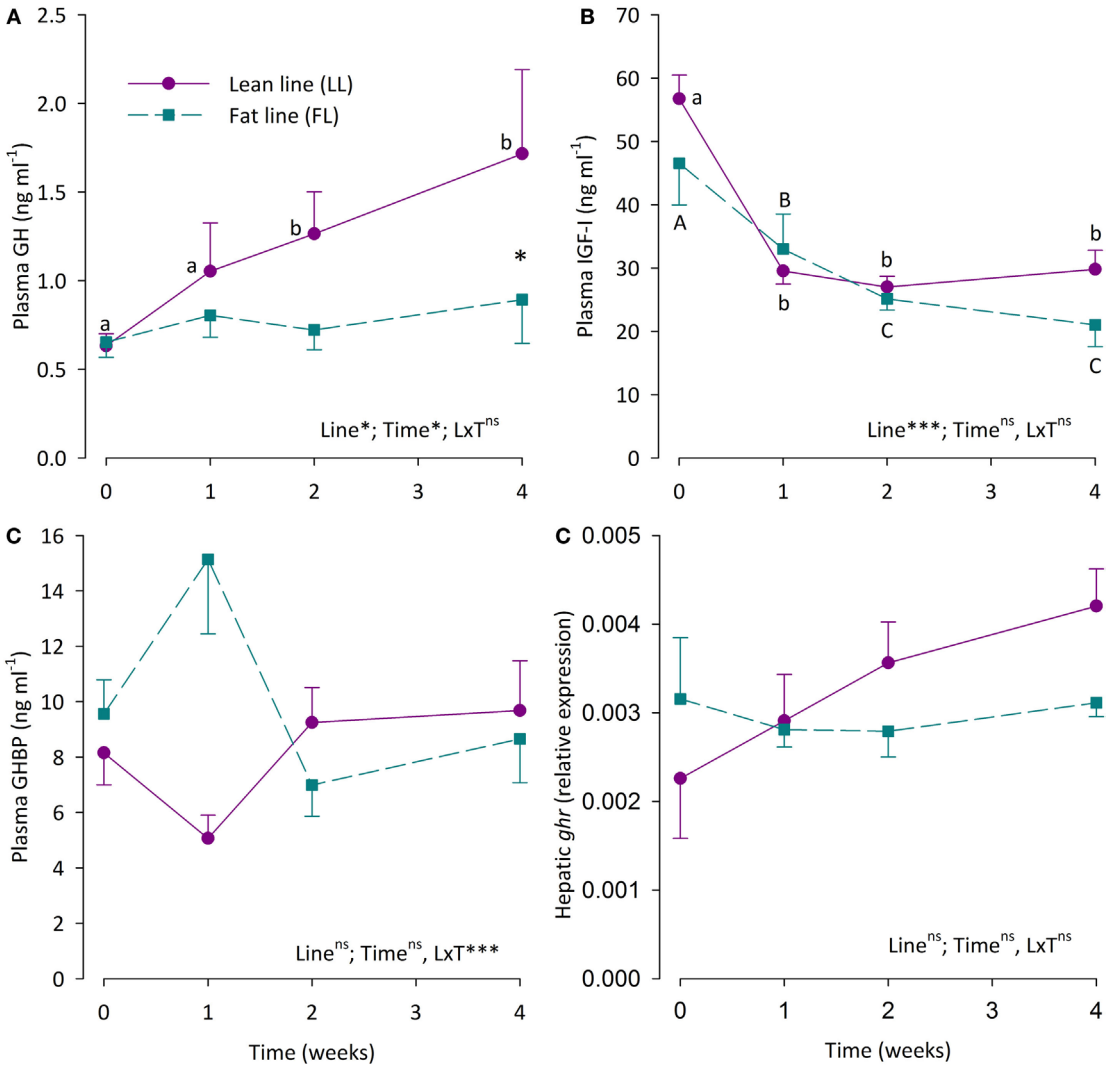

FIGURE 2 | **(A)** Plasma growth hormone (GH) levels, **(B)** plasma insulin-like growth factor I (IGF-I) levels, **(C)** plasma growth hormone-binding protein (GHBP) levels, and **(D)** hepatic expression of the growth hormone receptor gene 2a (*ghr2a*) in fat line (FL, ■) and lean line (LL, ●) rainbow trout over a 4-week fasting period. Data are presented as means ± SEM. Two-way ANOVA results on main effects (strain and time) as well as the interaction between the main effects (S × T) are indicated in the panels as being non-significant (ns, $p > 0.05$) or significant at the levels of $^{*}p < 0.05$, or $^{***}p < 0.001$. *Post hoc* analysis was carried out if main effects were significant. For "Strain," differences are indicated as $^{*}p < 0.05$. For "Time," significant differences are indicated by different letters, lower case for LL fish and upper case for FL fish.

GH levels of the FL fish were not affected by fasting. Thus, after 4 weeks of fasting, the LL fish had significantly higher plasma GH levels than the FL fish. Plasma IGF-I levels decreased successively in a similar manner in both fish groups during fasting and were significantly lower than pre-fasting levels already after 1 week of fasting. Plasma GHBP levels oscillated. While neither fish line nor time significantly affected GHBP levels, the interaction of the main effects was significant. Relative hepatic *ghr2a* gene expression did not differ statistically between the fish lines or different fasting periods.

Correlation analysis of plasma GH, IGF-I, and GHBP levels as well as hepatic *ghr2a* expression shows no significant correlation among these parameters (data not shown).

Energy reserves as reflected in LSI, VSI, and muscle fat content are given in **Table 3**. LSI was similar between FL and LL fish and decreased significantly during the first week of fasting after which it did not decrease further in either group. VSI was higher in LL than FL fish throughout the 4-week fasting, during which the VSI declined successively in both groups. Muscle lipid levels were higher in FL than LL fish both initially and after 1-week fasting. However, while muscle lipid levels did not change in the LL fish throughout the fasting period, they declined continuously in the FL group.

DISCUSSION

This study allows examination of two important aspects of the GH–IGF-I system in rainbow trout, i.e., the functional importance of GH resistance in energy mobilization during fasting, and whether plasma GHBPs are exerting a modulating effect on the GH–IGF-I system during fasting and refeeding. Furthermore, the study explores the short-term responses of the GH–IGF-I system during refeeding.

GH Resistance and Energy Mobilization in Rainbow Trout During Fasting

In this study, the rainbow trout which have been breeding-selected for high muscle adiposity for seven generations (FL fish of experiment II) do not, in contrast to the other rainbow trout studied, enter a state of acquired GH resistance during the 4-week fasting, as they maintain normal GH levels. This is most likely linked to the high, initial energy reserves of these fish, allowing them to mobilize lipids throughout the 4-week fasting period. As described in Johansson et al. (36), while visceral lipids were mobilized to similar extent in both fish lines [VSI %, 0 → 4 weeks of fasting; FL: 8.24 → 5.71 (−30.7%), LL: 9.67 → 7.09 (−26.7%)], the FL fish were able to continuously mobilize muscle fat over the 4-week fasting period. By contrast, the LL fish did not have enough initial muscle fat reserves to mobilize significant amounts of fat [muscle lipid content %, FL 7.31 → 3.26 (−52.4%), LL: 3.90 → 3.43 (−12.1%)]. Thus, during the fasting period, the LL fish have probably activated protein-dominated catabolism, which occurs in vertebrates only when the lipid levels have reached a critical threshold, with proteins being the fuel of last resort during starvation (41). As GH resistance is the key endocrine mechanism permitting protein catabolism (42), the GH resistance in the LL fish may act as a permissive mechanism, allowing the fish to meet metabolic demands during fasting through protein catabolism when carbohydrate and lipid reserves are depleted.

Although the GH part of the GH–IGF-I system is functioning normally in the FL fish, with normal hepatic *ghr2a* expression and normal plasma GH levels, the IGF-I part of the system is suppressed during fasting. This is likely to be linked to some aspects of the catabolic state the fish are in, as IGF-I expression and plasma levels in both fish and mammals are strongly affected by nutritional status, with fasting leading to depressed IGF-I activity (43–46).

The elevated plasma GH levels during fasting in the fish of experiment I (FA group) and the LL fish of experiment II are a response frequently observed during fasting in salmonids (47–50). Together with declining plasma IGF-I levels, this indicates that the fish are entering a state of acquired GH resistance, a state typically observed under catabolic conditions such as fasting (11, 42) in various vertebrate groups (44), including fish (17).

It has been hypothesized that the primary mechanism for this condition to develop is a downregulation of the hepatic GHR, leading to decreased plasma IGF-I levels and thus decreased IGF-I feedback inhibition of pituitary GH secretion, resulting in elevated plasma GH levels (12, 51). Thus, this hypothesis postulates that fasting-induced elevation of plasma GH levels is be due to an increased pituitary GH secretion rate as well as decreased hepatic clearance rate of the hormone. However, the GH resistant rainbow trout in this study shows no indication of hepatic GHR downregulation after 4-week fasting as *ghr2a* mRNA levels remain unchanged. Although care should be taken not to overinterpret gene expression data as they are not a very reliable indicator of protein abundance (52), the current data do not support this hypothesis. The present data are in agreement with a study by Norbeck et al. (9) in which hepatic *ghr2a* (then termed GHR1) expression was unaffected by 2-week fasting. However, that study also included rainbow trout fasted for 6 weeks, at which time-point the hepatic *ghr2a* expression was suppressed. By contrast, rainbow trout fasted for 30 days in a study by Gabillard et al. (14) had significantly elevated hepatic *ghr2a* mRNA abundance. Thus, data on the GH–IGF-I system in rainbow trout during fasting diverge in terms of the effects

TABLE 3 | Energy-related physical characteristics of fat line (FL) and lean line (LL) rainbow trout of experiment II under normal feeding conditions (0-week fasting), and after 1, 2, and 4 weeks of fasting.

Fasting (weeks)	LSI (%)		VSI (%)		Muscle lipid content (%)	
	FL	LL	FL	LL	FL	LL
0	1.23 ± 0.05^{A}	1.16 ± 0.03^{A}	$8.24 \pm 0.31^{A,**}$	9.67 ± 0.23^{A}	$7.31 \pm 0.72^{A,**}$	3.90 ± 0.54^{A}
1	0.85 ± 0.02^{B}	0.88 ± 0.04^{B}	$7.02 \pm 0.39^{B,**}$	8.22 ± 0.24^{B}	$5.10 \pm 0.84^{A,B,**}$	3.06 ± 0.39^{A}
2	0.79 ± 0.05^{B}	0.81 ± 0.03^{B}	$6.69 \pm 0.16^{B,C,**}$	$7.95 \pm 0.22^{B,C}$	$4.58 \pm 0.58^{B,C}$	2.99 ± 0.40^{A}
4	$0.75 \pm 0.03^{B,**}$	0.89 ± 0.03^{B}	$5.71 \pm 0.26^{C,**}$	7.09 ± 0.37^{C}	3.26 ± 0.22^{C}	3.43 ± 0.62^{A}

These include liver somatic index (LSI), visceral somatic index (VSI), and muscle lipid content.
*Statistical significant differences between groups are indicated as *p < 0.05 or **p < 0.01.*
Statistical significant differences (p < 0.05) over time are indicated with different superscript letters.

on hepatic *ghr* expression. In mammals, starvation can decrease GHR levels while malnutrition such as protein deficiency rather appears to inhibit the post-GHR signaling pathways (10, 13, 46). This indicates that the severity of the fasting/starvation episode will affect the outcome in terms of hepatic GHR expression and density. As both water temperatures and initial energy reserves will influence the temporal severity of fasting in fish, these factors may lead to the divergent hepatic *ghr* expression observed in the rainbow trout [this study; (9, 14)].

While speculative, as GH secretion and clearance rates have not been assessed in this study, it appears likely that in the absence of hepatic GHR downregulation, elevated GH levels in fasting fish are primarily due to increased pituitary GH secretion rate.

Regulation of Plasma GHBP Levels During Fasting and Refeeding

In fish as in most mammals, GHBPs are principally generated through proteolysis of the full-length GHR (32, 53, 54), rather than through alternative splicing of the *ghr* gene, as in rodents (55, 56). However, truncated *ghr* genes encoding for the extracellular GHR domain have been identified in both early and late vertebrates such as sea lamprey (57) and human (53) as well as zebrafish (58), representing an alternative production pathway for plasma GHBPs.

The high GHR density in the salmonid liver (2, 25) makes it a likely organ source for plasma GHBP levels. It could thus be suggested that correlation existed between *ghr2a* levels and plasma GHBP levels in the rainbow trout. This study clearly demonstrates that this is not the case, as also has been observed in humans (25), indicating that hepatic *ghr* expression is not a reliable predictor of plasma GHBP levels. Such lack of correlation is not surprising, as the circulating GHBP levels are dependent on the posttranslational cleavage of the extracellular domain of the GHR, an enzymatic mechanism which is independently regulated, making the *ghr*–GHBP link even less direct (59).

In both experiments, plasma GHBP levels fluctuated with time in such a way that while no main effects of feeding regime (experiment I) or fasting (experiment II) were found, there was significant interaction between the main effects. This indicates differentiated regulation of plasma GHBP levels, both during fasting and refeeding, based on the prior energetic status of the fish established through feeding regime in experiment I and breeding selection in experiment II.

Thus, the current GHBP data are complex and make it hard to propose a defined regulatory role for circulating GHBPs in the endocrine GH–IGF-I system in rainbow trout. This echoes conclusions from mammalian studies. By binding GH, plasma GHBPs prolong the biological half-life of the hormone, but at the same time decrease availability of GH to target tissues through competing GHR ligation and limit the free GH levels. These two opposing mechanisms through which GHBPs affect GH kinetics has made it hard to establish the role of GHBPs and their impact on GH bioavailability in mammals, including humans (25).

However, it appears likely that plasma GHBP levels in the rainbow trout are to some extent regulated by nutritional factors, as seen in the rat (60). Furthermore, the relatively elevated GHBP levels in FL fish after 1-week fasting, concomitant with low plasma GH levels, suggest that the GH-endocrinology has been altered during the genetic selection for high muscle adiposity, and the FL fish may represent an "obesity" phenotype (37), similar as seen in obese humans with low GH and high GHBP plasma levels (61, 62).

Short-Term Impact of Refeeding on the GH–IGF-I System

The initiation of refeeding after 4-week fasting of rainbow trout in experiment I leads to relatively rapid (2–24 h) changes in plasma levels of GH, IGF-I and GHBP, i.e., the components of the GH–IGF-I system which had previously been affected by the fasting. This suggests that the GH–IGF-I system is rapidly readjusting, and that a shift in the endocrine regulation of growth and energy balance from catabolic to anabolic conditions is completed within 72 h of the onset of refeeding. The present hormonal data are in line with earlier data on "corrective" shifts in plasma GH and IGF-I levels during refeeding of rainbow trout (9, 14) and fine flounder (20), even if these studies indicate that while plasma GH levels reach "normal" pre-fasting levels within days, it may take as long as 2 weeks for plasma IGF-I levels to normalize.

As the hepatic *ghr2a* expression was unaffected by the 4-week fasting and was similarly unaffected by refeeding, the gene expression of this GHR isoform does not appear to be a major regulatory component of the GH–IGF-I system, even if downregulation (63) as well as upregulation (14) of this gene has been reported during fasting of salmonids.

Conclusion and Future Perspectives

The causal mechanisms and functional significance of acquired GH resistance during fasting in fish has received limited attention. This study provides a novel experimental model. It shows that manipulation of energy reserves, such as through breeding selection for high muscle adiposity, can affect whether or not acquired GH resistance sets in during a period of fasting. As the physiological function of GH in salmonids as in other vertebrates is to favor protein synthesis over break-down, the functional significance of GH resistance during fasting is likely to allow protein catabolism to proceed when lipid stores are depleted. In this context, the link between GH resistance and protein catabolism needs to be studied further. It can, e.g., be hypothesized that if the FL fish had been fasted for longer than 4 weeks, at which point they had little or no muscle fat reserves left to mobilize, the fish would develop GH resistance and enter a starvation phase of muscle protein break-down.

Although this study provides novel data on plasma GHBP levels in salmonids and non-mammalian vertebrates in general, current understanding on mechanisms regulating GHBP levels as well as the functional significance of plasma GHBPs as modulators of the GH–IGF-I system is still severely lacking. This study demonstrates that analysis of hepatic *ghr2a* expression does not provide a useful proxy measure for plasma GHBP levels, as correlation between these parameters is lacking. Thus, direct measurements of circulating GHBPs appear necessary, and thus, the RIA established by Einarsdottir et al. (33) is a major step forward.

The current data indicate that GHBP may be nutritionally regulated and could possibly act as a temporary modulator of GH action during postprandial periods and short-term fasting, but further studies are clearly needed in this area. Future studies on fish should, e.g., explore the activity and regulation of the proteases responsible for GHBP production to elucidate if they represent an important regulatory mechanism.

ETHICS STATEMENT

Experiment I was carried out at a certified animal facility at the Department of Biological and Environmental Sciences, University of Gothenburg, under license 85-2012 by the Ethical Committee for Animal Research in Gothenburg. Experiment II was carried out at Pisciculture Expérimentale INRA des Monts d'Arrée, which is approved for animal experimentation through license C29-277-02 in accordance with the European Communities Council Directive 86/609/EEC, and carried out under the official license 29-036 of Dr. Labbé Laurent.

AUTHOR CONTRIBUTIONS

MJ, NG, IE, and BB designed and carried out the experiments. IE and MJ carried out the sample assays and data analysis. IE, BB, and NG are responsible for the writing.

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Polysaccharide IV from *Lycium barbarum L*. Improves Lipid Profiles of Gestational Diabetes Mellitus of Pregnancy by Upregulating ABCA1 and Downregulating Sterol Regulatory Element-Binding Transcription 1 *via* miR-33

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Lycium barbarum L. (LBL) has beneficial effects on gestational diabetes mellitus (GDM) but the related mechanism remains unclear. Polysaccharides of LBL (LBLP) are the main bioactive components of LBL. miR-33, ATP-binding cassette transporter A1 (ABCA1) and sterol regulatory element-binding transcription 1 (SREBF1) affect lipid profiles, which are associated with GDM risk. LBLP may exert protective against GDM by affecting these molecules. Four LBLP fractions: LBLP-I, LBLP-II, LBLP-III, and LBLP-IV were isolated from LBL and further purified by using DEAE-Sephadex column. The effects of purified each fraction on pancreatic beta cells were comparatively evaluated. A total of 158 GDM patients were recruited and randomly divided into LBL group (LG) and placebo group (CG). miR-33 levels, lipid profiles, insulin resistance and secretory functions were measured. The association between serum miR-33 levels and lipid profiles were evaluated by using Spearman's rank-order correlation test. After 4-week therapy, LBL reduced miR-33 level, insulin resistance and increased insulin secretion of GDM patients. LBL increased the levels of ABCA1, high-density lipoprotein cholesterol (HDL-C) and reduced miR-33, SREBF1, low-density lipoprotein cholesterol (LDL-C), total cholesterol (TC), triglyceride (TG), and malondialdehyde. Homeostatic model assessment of β-cell function and insulin resistance was lower in LG than in CG, whereas homeostatic model assessment of β-cell function and insulin secretory function was higher in LG than in CG. There was a strong positive association between miR-33 level and TG, or TC and or LDL-C, and a strong negative association between miR-33 level and HDL-C. The levels of miR-33 had negative relation with ABCA1 and positive relation with SREBF1. ABCA1 has negative relation with TG, TC, and LDL-C and positive relation with HDL-C. Inversely, SREBF1 had positive relation with TG, TC, and LDL-C and negative relation with HDL-C. The main bioactive compound LBLP-IV of LBL increased insulin secretion of beta cells and the levels of ABCA1, and reduced miR-33 levels and SREBF1 in beta

cells. However, LBLP-IV could not change the levels of these molecules anymore when miR-33 was overexpressed or silenced. LBLP-IV had the similar effects with LBL on beta cells while other components had no such effects. Thus, LBLP-IV from LBL improves lipid profiles by upregulating ABCA1 and downregulating SREBF1 *via* miR-33.

Keywords: *Lycium barbarum L.*, gestational diabetes mellitus, ATP-binding cassette transporter A1, sterol regulatory element-binding transcription, miR-33

INTRODUCTION

Gestational diabetes mellitus (GDM), a special type of diabetes, is caused by multiple factors with genetic predisposition (1) and endocrine metabolic diseases (2, 3). GDM is defined as the different glucose intolerance that occurs within the first trimester of pregnancy. It is estimated that the incidence of GDM represents average 3–8% of all pregnancies (4). GDM incidence will continue to increase due to the changes of lifestyle and living conditions. Compared with other types of diabetes mellitus, GDM not only affects their own health status and but also increases the risk of postpartum diabetes. Previous study showed that the cumulative incidence of type 2 diabetes mellitus was 6 weeks to 28 years in postpartum women with GDM (5). GDM has negative effects on future generations, including neonatal death (6), stillbirth (7), perinatal mortality (8), preeclampsia (9), large fetus (10), neonatal jaundice (11), low blood sugar (12), low calcium (13), and so on. However, the pathogenesis of GDM is still not fully understood. Many countries have invested much money on the research programs of GDM, including GDM pathogenesis, impact factors and diagnostic criteria. Living environment (14), family history (15), pregnancy (16, 17), low birth weight (18), prepregnancy obesity (19), and dietary imbalance (20, 21) are common risk factors of GDM. Balanced nutrition and appropriate physical labor and exercise are the main methods for preventing the occurrence of GDM (22).

Medical treatment is still the main method for GDM therapy (23, 24). However, the safety or efficacy of the medicine remains unclear in pregnancy (25, 26). It is necessary to find natural medicine with a fewer side effects. *Lycium barbarum L.* (LBL) is a deciduous woody perennial plant primarily in the Ningxia Hui Autonomous Region (Ningxia, China) (27). Polysaccharides of LBL (LBLP) are the main bioactive components (28, 29). LBLP-IV administration has been reported to control the animal model with diabetes. LBLP-IV may be a potential therapeutic agent in diabetic treatment (30). However, the molecular mechanism for the effects of LBLP-IV on diabetes remains unknown. MicroRNA is short, single-stranded RNA molecules with 22 nucleotides in length. MiR-33 can regulate lipid metabolism (31), which is associated with GDM. There is much evidence linking miR-33 to lipid metabolism by targeting ATP-binding cassette transporter A1 (ABCA1) and sterol regulatory element-binding transcription 1 (SREBF1) (32). ABCA1 is the cholesterol efflux regulatory protein, which regulates cholesterol efflux and phospholipid homeostasis (33). SREBP are the transcription factors, which bind to the sterol regulatory element and repressed its expression, including ABCA1 gene (34). ABCA1 (35) and SREBF1 (36) affect lipid metabolism too. Thus, the polysaccharides may improve lipid profiles by affecting ABCA1 and SREBF1 levels *via* miR-33. However, the miR-33-related molecular mechanisms for the functions of LBL on GDM patients are still unclear.

In this study, we want to explore the effects of LBL on GDM patients by investigating serum lipid profiles and related molecules. Changes in the miR-33, ABCA-1, and SREBF1 expressions, as well as insulin sensitivity and blood insulin and resistin levels, were also assessed. Meanwhile, the bioactive compound from LBL was isolated and its effects on β cell were measured.

MATERIALS AND METHODS

LBL Polysaccharides Isolation

Lycium barbarum L. leaves were purchased from Ningxia, China and LBL polysaccharides were isolated according to an early report (37). Fifty-microgram LBL leaves were crushed to fine powder and extracted in triplicate by using 1 l distilled water for 1 h at 90°C. The filtrated solution was concentrated by using a rotary evaporator (RE-52A, Shanghai Woshi Co., Shanghai, China) at 55°C, and precipitated by adding fourfold volume of anhydrous ethanol. LBLP were washed three times with anhydrous ethanol and acetone after being centrifuged at 3,000 rpm for 15 min, and then and finally lyophilized. The crude protein was removed by using the Sevag method (38) and decolorized with the macroreticular resin AB-8 (Cangzhou Resin Company, Cangzhou, Hebei, China). Final polysaccharides were isolated by using a DEAE SephadexA-25 column (Amersham Pharmacia Biotech, Piscataway, NJ, USA) equilibrated with distilled water for one day. Individual polysaccharide was eluted with distilled water, 0.1 and 0.2 M NaCl at 0.8 mL/min. The polysaccharide fractions were collected at 2 min/tube using an automatic collector (Beijing Xinhuizeao Technology Co., Ltd., Beijing, China), then the collected solution was dialyzed (MWCO 3600, Sigma) and lyophilized finally.

The homogeneity of isolated polysaccharide was determined by high-performance gel-permeation chromatography (HP-GPC) (Dionex, Sunnyvale, CA, USA). Twenty-microliter sample solution was performed at a flow rate of 0.5 mL/min (distilled water and 100, 200 mM NaCl) as a mobile phase. The columns were calibrated with T-series dextran (T-10, 40, 70, 500, 2,000), and the molecular weight of polysaccharides was confirmed by referencing to a calibrated curve.

Participants

All protocols were approved by ethical committee of our hospital (Approval no. 201602X4), and the study was carried out according to the principles described in the World Medical Association

Declaration of Helsinki (39). All subjects gave written informed consent in accordance with the Declaration of Helsinki. From April 2016 to May 2016, a total of 158 women diagnosed with GDM were collected at our hospital. All the pregnant women met the diagnostic criteria of GDM *via* a 2-h 75-g oral glucose tolerance test according to an earlier report (40).

Including Criteria

Pregnant women were age 18–40 years; the patient had singleton pregnancy and no previous GDM; pregnant women had an impairment of their glucose tolerance according to the results of a 2-h 75-g oral glucose tolerance test; the patients were diagnosed with GDM from 26 to 30 weeks of gestation.

Excluding Criteria

The patients were smokers and or alcohol drinkers; some condition and or medication that affected glucose levels; the patients were unwilling to follow the prescribed diet. The patients had cardiac, dizziness, and related diseases; the patients had obvious abnormal clinical findings.

GDM Patients Grouping

After screening *via* inclusion and exclusion criteria, 158 patients were evenly and randomly assigned into a LBL group (LG, received 10-mg LBL daily) and a control group (CG, received 10-mg placebo daily) (**Figure 1**).

Blood Glucose (BG) Measurement

Five-milliliter blood is either directly sucked into a vacuum test tube from a vein of each patients. Serum was isolated by centrifugation at 10,000 rpm for 5 min. The concentration of BG was measured by using glucose oxidase (41). Unified quality control standards were used for all the 26–30-week pregnancy with GDM. Fasting blood glucose (FBG) was measured in the morning *via* centrifugation after taking venous blood from each subject, and then dissolved in two pairs of bottles filled with water.

Biochemical Analysis

The concentrations of glucose and HbAlc were measured after taking 2-h 75 g oral glucose. The concentration of BG was measured by using glucose oxidase (41). Serum HbA1c levels were measured by was measured by HPLC (D-10 Dual Program; Bio-Rad, Hercules, CA, USA). High- and low-density lipoprotein-cholesterol (HDL-C and LDL-C) were measured by using an Olympus AU 600 auto-analyzer (Olympus Optical Co. Ltd., Schimatsu-Mishima, Japan). Triglyceride (TG) levels were measured a Technicon RA-500 analyzer (Bayer, Etobicoke, ON, Canada). Basal BG and FBG levels were examined by ABL 800FLEX blood gas analyzer (Midland, ON, Canada). Serum resistin was measured by using the resistin ELISA kit from Phoenix Pharmaceuticals (Belmont, CA, USA) according to manufacturer's instructions. Serum basal insulin and fasting insulin (FINS) were tested by radioimmunoassay (Linco, Seaford, DE, USA). Homeostatic model assessment of β-cell function and insulin resistance (HOMA-IR) and homeostatic model assessment of β-cell function and insulin secretory function (HOMA-IS) were measured by using the following equations: HOMA-IR = FBG × FINS/22.5 and HOMA-IS = 20 × FINS/(FBG − 3.5), respectively.

Measurement of Serum Lipid Profiles

Two-milliliter serum was separated from whole blood by allowing the blood to just let stand. A lipid profiles is closely associated with the risk of GDM (42–44). Lipid profiles were measured by using the same method in Section "MiR-33 Silencing." Malondialdehyde (MDA) level was measured by using a MDA detection kit (A003; Nanjing Jiancheng Bioengineering Institute, Nanjing, China). Lipid indexes were measured before and after 4-week experiment.

Cell Culture

Gestational diabetes mellitus represents the major diabetes, and β-cell dysfunction plays an important role in the development and progression of the disease. The components purified by

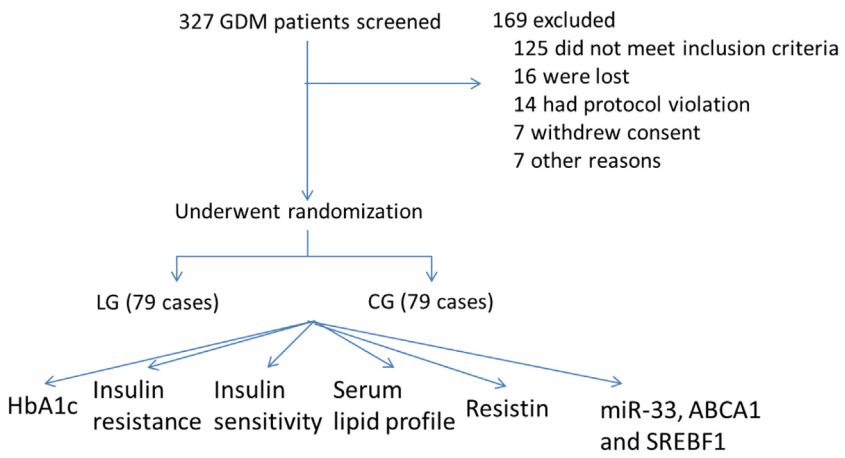

FIGURE 1 | Flowchart of the study population. ABCA1, ATP-binding cassette transporter A1. SREBF1, sterol regulatory element-binding transcription. The whole period was 4 weeks.

DEAE-Sephadex A-25 column, were measured by using human pancreatic carcinoma cell SW1990, which was purchased from cell bank, CAS (Shanghai, China). The cell lines were cultured in RPMI-1640 at 37°C with 5% CO_2. After 3-day culture, the cell concentrations were adjusted to 1×10^5 cells/mL and 100 μL cells were transferred to each cell of 96-cell plate, treated with 10 μg/mL different fractions and further cultured for three days under the same situation.

MiR-33 Silencing

Lipofectamine™ 2000 (Invitrogen, Waltham, MA, USA) was used to transfect islet β-cell line SW1990 with miR-33 RISC complex. 10^5 SW1990 cells were seeded in six-well plates. Scrambled control siRNA was carried out (Beijing Dingguo Changsheng Biotechnology, Beijing, China). miR-33 (GenBank No., NR_029507.1) four target siRNA sequences: UGUGGUGCAUUGUAGUUGCAUUGCA, U G G U G C A U U G U A G U U G C A U U G C A U G , GCAUUGUAGUUGCAUUGCAUGUUCU and CAUUGUAGU UGCAUUGCAUGUUCUG. The transfection with 40 nM siRNA was performed in DMEM (with 1% L-glutamine, 10% FCS and without penicillin/streptomycin) for 72 h.

The Effects of LBL Fractions on Insulin Secretion in SW1990 Cells

SW1990 were seeded in 24-well plates (1×10^5 cells/well), treated with different fractions of LBL and cultured for 3 days to investigate insulin secretion. Insulin level was measured by using an insulin ELISA kit (Thermo Fisher Scientific Inc., Cleveland, OH, USA). After the determination of bioactive components, LBL was then used for clinical trials.

Quantitative RT-PCR Analysis

Blood samples were obtained from GDM patients. Serum was isolated from blood samples within 2 h. Total RNA was extracted by using a miRNeasy Serum/Plasma Kit (QIAGEN Sciences, Germantown, MD, USA). Finally, 2-μg RNA was obtained from 1-mL serum. MiR-33 (GenBank No. NR_029507.1), forward primer: 5′-GTCCGTGGTGCATTGTAGT-3′; reverse primer: 5′-GTGCAGGGTCCGAGGT-3′. U6 (GenBank No. NR_004394.1), forward primer: 5′-TTGGTGCTCGCTTCGG CA-3′; reverse primer: 5′-GTGCAGGGTCCGAGGT-3′. U6 snRNA was used as an internal control. ABCA1 (GenBank No. AB055982.1), forward primer: 5′-ATTGTGGCTCGCCTGTTCT C-3′; reverse primer: 5′-TAGACTTTGGGAGAGAGAGG-3′. SREBF1 (GenBank No. NM_001005291.2), forward primer: 5′-TGAGCTCCTCTCTTGAAGCC-3′; reverse primer: 5′-GTAG CCTAACACAGGGGTGG-3′; Beta actin (GeanBank No. HQ154074.1, as a loading control), forward primer: 5′-TCCAG CCTTCCTTCCTGGGC-3′; reverse primer: 5′-GCCAGGG TACATGGTGGTAC-3′. qRT-PCR was conducted by using an Applied Biosystems 7300 Real-time PCR System. 1-μL RT products were added to 20-μL reaction volume including 0.5-μL sense primer and reverse primer, 1-μL SYBR® Green Real-Time PCR Master Mixes (Thermo Fisher Scientific, Waltham, MA, USA), and one-unit Taq [Takara Biotechnology (Dalian) Co., Ltd., Dalian, China]. The reaction was carried out by using the following parameters: 94°C for 5 min, followed by 45 cycles of 94°C for 20 s and 65°C for 1 min. After the reaction, the CT was calculated *via* threshold settings. The ratio of uterine sarcoma serum miRNA and healthy subjects was presented by using $2^{-\Delta G}$, in which $\Delta G = C_{T\ cancer} - C_{T\ normal}$.

Western Blot Analysis

SW1990 cell lines were treated with cocktail and lysed *via* a freezing and thawing method. Meanwhile, serum samples were also prepared for Western Blot analysis. Twenty-five microgram proteins were separated by 12% SDS-PAGE and transferred to a PVDF membrane, which was blocked by non-fat milk for 30 min. The membranes were incubated with the antibodies ABCA1 (ab66217) and SREBF1 (ab28481), Beta actin (ab6276, as a loading control), goat polyclonal secondary antibody to rabbit IgG-H&L (HRP) (ab6721, Abcam, Cambridge, MA, USA). With X-ray film exposure, the expression of ABCA1 and SREBF1 was detected *via* Quantity One software.

Statistical Analysis

Results were presented as the mean ± SEM. Paired student's *t*-test was used to compare the differences between two groups. Spearman's rank-order correlation test was used to test the relationship between two variables. There were statistically significant differences if $P < 0.05$.

RESULTS

Characterization of LBL

Four main polysaccharides were isolated from LBL after the purification of DEAE-Sephadex A-25 column (**Figure 2A**), which was accordant with an earlier report (37). The isolated components were further confirmed by HP-GPC under the conditions that produced masses predicted for LBLP I (**Figure 2B**), LBLP II (**Figure 2C**), LBLP III (**Figure 2D**), and LBLP IV (**Figure 2E**) were 55.2, 94.0, 241.3, and 418.0 kDa, respectively.

Baseline Characters

Table 1 shows the clinical characters were similar between two groups. The mean ages of were at age of 30.1 ± 5.4 in LG and 29.5 ± 4.3 in CG. The statistical difference was insignificant for baseline demographic and metabolic characteristics of the patients between two groups ($P > 0.05$).

LBL Consumption Improves Biochemical Parameters and Lipid Pattern

Table 2 shows LBL-reduced insulin resistance and increased insulin sensitivity and secretory function when compared with the CG group ($P < 0.05$). LBL increased the level of HDL-C and reduced the levels of TG, total cholesterol (TC), and LDL-C ($P < 0.05$). The statistical differences were significant for lipid profiles between two groups ($P < 0.05$). **Table 3** shows that LBL consumption reduced the serum levels of TG, TC, LDL-C, and MDA while increased the level of HDL-C after 4 weeks. The statistical difference for the changes in the body weight of the patients was insignificant between two groups (LG, 64.9 ± 8.4;

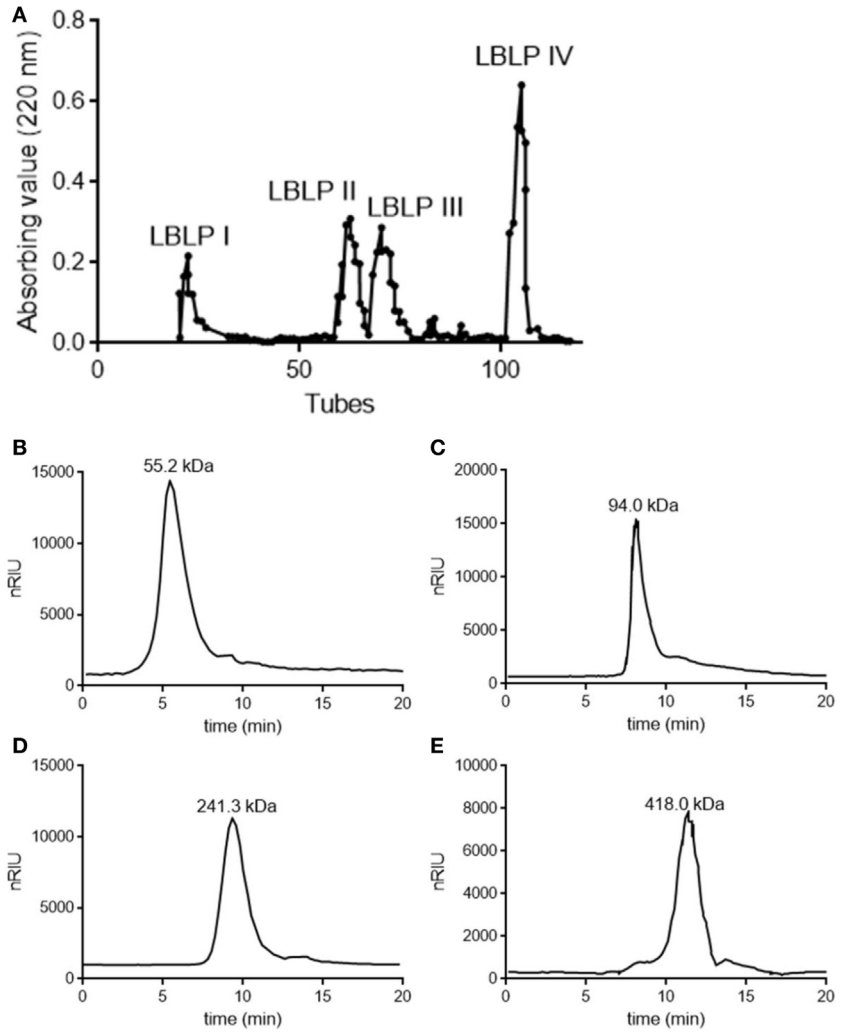

FIGURE 2 | The polysaccharides of the extracts of *Lycium barbarum L*. (LBL) are purified by using a DEAE SephadexA-25 column. **(A)** There are four main polysaccharides [polysaccharides of LBL (LBLP) I, II, III, and IV] in the extracts of LBL **(B)** High-performance gel-permeation chromatography (HP-GPC) analysis of LBLP I. **(C)** HP-GPC analysis of LBLP II. **(D)** HP-GPC analysis of LBLP III. **(E)** HP-GPC analysis of LBLP IV.

CG, 68.7 ± 11.3, $P > 0.05$) after 4-week therapy. The values of HOMA-IR were lower in LG than in CG after therapy when compared with before therapy, whereas the values of HOMA-IS was higher in LG than in CG ($P < 0.05$). All the results suggest that LBL consumption significantly improves lipid patterns of GDM patients, reduces the HOMA-IR and increases the HOMA-IS.

LBL Consumption Reduces Serum miR-33 Level and Relative mRNA Level of SREBPF1, and Increases the Level of ABCA1

The statistical difference was insignificant for relative mRNA levels of miR-33 (**Figure 3A**), ABCA1 (**Figure 3B**), and SREBF1 (**Figure 3B**) between two groups ($P > 0.05$). The serum levels of miR-33 and SREBF1 were decreased while ABCA1 level was increased in LBLG when compared with CG after 4-week LBL consumption (**Figures 3A,B**, $P < 0.05$). The results suggest that long-term LBL consumption can affect GDM by reducing the serum mRNA levels miR-33 and SREBF1, and increasing the mRNA level of ABCA1.

LBL Consumption Significantly Reduces Relative Protein Levels of SREBPF1 and Increases the Level of ABCA1

The statistical difference was insignificant for relative protein levels of ABCA1 and SREBF1 among four groups (**Figure 3C**, $P > 0.05$). The protein level of SREBF1 was decreased and the level of ABCA1 was increased in LG when compared with CG after 4-week LBL consumption (**Figure 3C**, $P < 0.05$). The results suggest that long-term LBL consumption can affect GDM by reducing protein level of SREBF1, and increasing the protein level of ABCA1.

TABLE 1 | Baseline demographic and metabolic characteristics of GDM (gestational diabetes mellitus of pregnancy) subjects.

Characteristics of patients	LG ($n = 79$)	CG ($n = 79$)	t/χ^2	*P*-value
Age (years)	30.1 ± 5.4	29.5 ± 4.3	0.23	0.64[a]
Race, *n* (%)				
Han Zhu	64	65	0.04	0.84[b]
Manchu	10	9	0.06	0.81[b]
Mongolians	4	4	0.13	0.72[b]
Tibetans	1	1	0.51	0.48[b]
Body weight (kg)	68.3 ± 10.2	67.9 ± 11.5	0.97	0.12[a]
BMI (kg/m^2)	28.4 ± 4.9	27.7 ± 5.3	0.86	0.25[a]
Insulin (mIU/L)	20.6 ± 2.3	20.5 ± 2.6	0.23	0.69[a]
HbA1c (%)	6.9 ± 1.7	6.7 ± 1.9	0.72	0.58[a]
FBG (mmol/L)	8.3 ± 1.1	8.4 ± 1.2	0.84	0.32[a]
2hPG (mmol/L)	14.9 ± 2.5	14.1 ± 3.2	0.60	0.55[a]
TG (mmol/L)	2.8 ± 1.2	2.7 ± 1.4	0.19	0.81[a]
TC (mmol/L)	5.9 ± 1.4	5.7 ± 1.6	0.24	0.40[a]
HDL-C (mmol/L)	1.3 ± 0.3	1.4 ± 0.5	0.20	0.56[a]
LDL-C (mmol/l)	3.7 ± 1.0	3.9 ± 1.2	0.18	0.72[a]
Resistin (ng/mL)	15.1 ± 4.6	15.2 ± 3.7	1.24	0.15[a]
HOMA-IR	6.4 ± 3.4	6.6 ± 3.5	1.90	0.26[a]
HOMA-IS	66.1 ± 36.7	68. 3 ± 27.4	1.55	0.10[a]

One hundred and fifty-eight patients were assigned into an LBL group (LG, received 10 mg LBL daily) and a control group (CG, received 10-mg placebo daily). There is not significant statistic difference at P > 0.05.

[a]*Paired t-test.*

[b]*Chi-square test.*

BMI, body mass index. HbA1c, hemoglobin A1c. FBG, fasting blood glucose. 2hPG, 2 h postprandial plasma glucose. TC, total cholesterol. TG, triglyceride; HDL-C, high-density lipoprotein cholesterol. LDL-C, low-density lipoprotein cholesterol; HOMA-IR, homeostatic model assessment of β-cell function and insulin resistance; HOMA-IS, homeostatic model assessment of β-cell function and insulin sensitivity.

MiR-33 Level Is Associated with Lipid Components

Spearman's Rank-Order Correlation Test showed that the increase in relative level of miR-33 resulted in the increase in the concentrations of TG (**Figure 4A**), TC (**Figure 4B**), and LDL-C (**Figure 4D**) and decrease in the concentration of HDL-C (**Figure 4C**). There was a strong positive association between miR-33 level and TG, or TC and or LDL-C, and a strong negative association between miR-33 level and HDL-C ($P < 0.05$). These results suggest there is a strong association between serum miR-33 level and lipid components.

Effects of LBLP IV on Insulin Secretion

As shown in **Figure 5**, LBP IV increased insulin secretion from 20.6 ± 2.6 ng/mL (basal levels) to 52.7 ± 6.8 ng/mL. Under the same situations, LBLP I, II, and III could not cause significant changes for insulin secretion in SW1990 cells. The results suggest that LBLP IV may be the major bioactive ingredient of LBL for the therapy of GDM patients.

LBLP IV from LBL Significantly Reduces miR-33 Level, and Relative mRNA Level of SREBPF1, and Increases the Level of ABCA1

To explore the specific function of LBL components, four polysaccharides were purified and tested to their effects on human pancreatic carcinoma cell SW1990. Real time qRT-PCR showed that LBLP I could not affect miR-33 levels (**Figure 6A**, $P > 0.05$)

TABLE 2 | Parameters changes for antidiabetic activity in both groups.

Parameters	LG ($n = 79$)			CG ($n = 79$)			*P*-values (LG *via* CG)
	Before	After 4 weeks	*P*–values	Before	After 4 weeks	*P*-values	
FBG (mmol/L)	8.3 ± 1.1	8.0 ± 1.3	0.21	8.4 ± 1.2	8.3 ± 1.2	0.17	0.32
2hPG (mmol/L)	14.9 ± 2.5	14.0 ± 3.2	0.16	14.1 ± 3.2	13.9 ± 3.4	0.27	0.30
HbAlc (%)	6.9 ± 1.7	6.4 ± 1.9	0.08	6.7 ± 1.9	6.5 ± 1.6	0.41	0.29
Insulin (mIU/L)	20.3 ± 2.5	52.7 ± 6.8	0.05	20.7 ± 2.4	20.4 ± 2.5	0.34	0.57
Resistin (ng/mL)	15.1 ± 4.6	12.1 ± 3.2	0.02	15.2 ± 3.0	14.5 ± 4.1	0.14	0.03
HOMA-IR	6.4 ± 3.4	5.8 ± 3.1	0.04	6.6 ± 3.5	6.4 ± 2.9	0.17	0.04
HOMA-IS	66.1 ± 36.7	74. 4 ± 21.3	0.03	68. 3 ± 27.4	70.34 ± 14.2	0.24	0.04

One hundred and fifty-eight patients were assigned into an LBL group (LG, received 10-mg LBL daily) and a control group (CG, received 10-mg placebo daily).

TABLE 3 | Comparison of lipid pattern in GDM patients before and after therapy.

		Total lipids (g/L)	TG (mmol/L)	TC (mmol/L)	HDL-C (mmol/L)	LDL-C (mmol/L)	MDA (mmol/L)
Before	LG	11.6 ± 1.3	2.8 ± 1.2	5.9 ± 1.4	1.3 ± 0.3	3.7 ± 1.0	1.7 ± 0.3
	CG	11.2 ± 1.1	2.7 ± 1.4	5.7 ± 1.6	1.4 ± 0.5	3.9 ± 1.2	1.6 ± 0.2
	P-value	0.45	0.81	0.40	0.56	0.72	0.84
4 weeks	LG	8.2 ± 0.7	1.8 ± 1.2	4.6 ± 1.1	1.6 ± 0.4	3.0 ± 1.2	0.9 ± 0.2
	CG	10.9 ± 1.0	2.6 ± 1.4	5.5 ± 1.0	1.2 ± 0.3	3.9 ± 1.3	1.6 ± 0.4
	P-value	0.02*	0.01*	0.02*	0.01*	0.01*	0.01*

One hundred and fifty-eight patients were assigned into an LBL group (LG, received 10-mg LBL daily) and a control group (CG, received 10-mg placebo daily).

**P < 0.05 via CG.*

MDA, malondialdehyde.

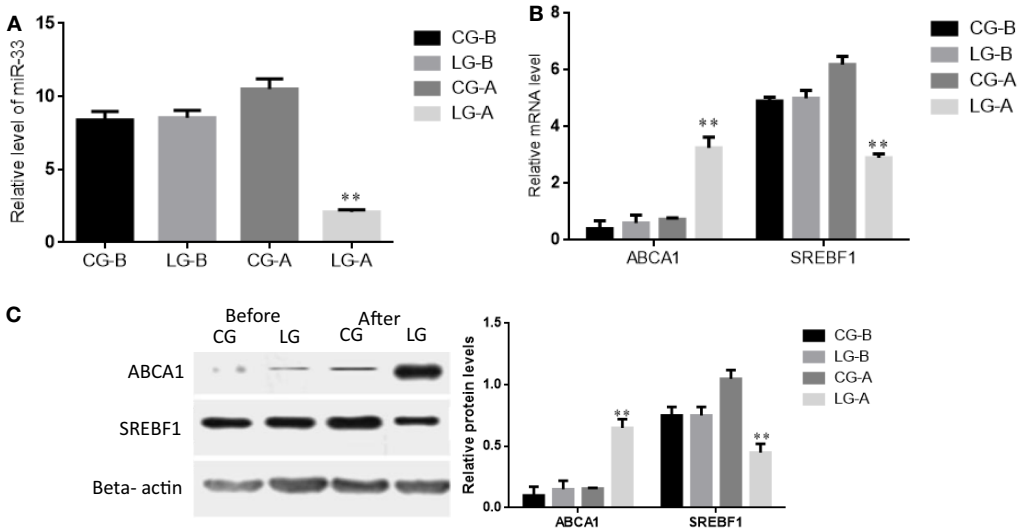

FIGURE 3 | *Lycium barbarum L*. (LBL) consumption significantly reduces miR-33 level and the level of sterol regulatory element-binding transcription 1 (SREBPF1) and increases the level of ABCA1 in gestational diabetes mellitus (GDM) patients. **(A)** The effects of LBL on miR-33 level. **(B)** The effects of LBL on relative mRNA levels of ABCA1 and SREBF1. LG-B, the GDM patients before receiving LBL treatment. CG-B, the GDM patients before receiving placebo. LG-A, the GDM patients after receiving 4-week LBL treatment. CG-A, the GDM patients after receiving 4-week placebo. **(C)** LBL consumption significantly reduces protein level of SREBPF1 and increases the level of ABCA1 in GDM patients. All data were presented as mean values ± SD. There were statistically significant differences if $^*P < 0.05$ vs. a control group.

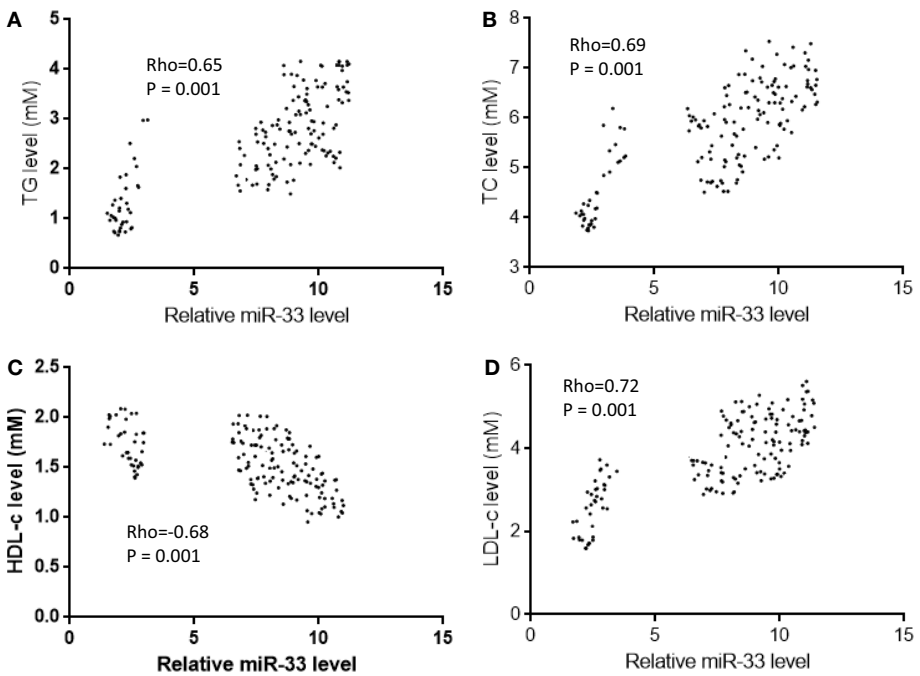

FIGURE 4 | The analysis of spearman's rank correlation coefficient for the relationship between serum miR-33 level and serum lipid profiles. **(A)** The relationship between serum miR-33 level and triglyceride (TG) concentrations. **(B)** The relationship between serum miR-33 level and total cholesterol (TC) concentrations. **(C)** The relationship between serum miR-33 level and high-density lipoprotein cholesterol (HDL-C) concentrations. **(D)** The relationship between serum miR-33 level and low-density lipoprotein cholesterol (LDL-C) concentrations. Spearman's Rho is used to measure the strength of association between two variables, where the value *r* falls between 0.5 and 1 means a strong positive correlation and the value *r* falls between −1 and −0.5 means a strong negative correlation.

while LBLP II and III increased miR-33 level, and LBLP IV and LBL reduced miR-33 level significantly ($P < 0.05$) when compared with controls. On the other hand, LBLP I, II and III reduced while LBL and LBLP IV increased relative mRNA levels of ABCA1 (**Figure 6B**, $P < 0.05$). Comparatively, LBL and LBLP IV reduced more relative mRNA levels of SREBF1 than other polysaccharides

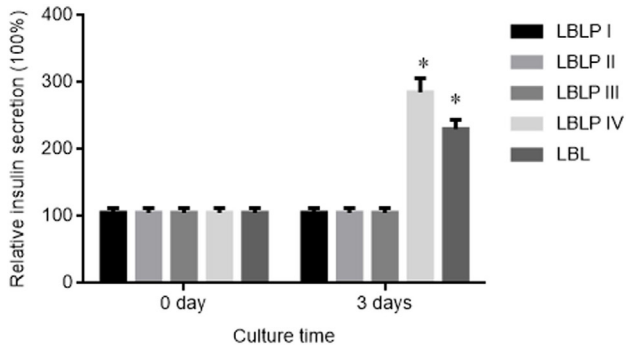

FIGURE 5 | Polysaccharides of *Lycium barbarum L*. (LBL) activate insulin secretion in SW1990 cells. Values were represented as the means ± SD. from eight experiments. There were statistically significant differences if $^*P < 0.05$. LBL, the extracts of *LB*L with four main polysaccharides (LBLP I, II, III, and IV).

(**Figure 6B**, $P < 0.05$). There were no changes for miR-33 levels when the cells were transfected with scrambled miRNA when compared with the cells without transfection (**Figure 6C**). In the similar cases, there were no changes for ABCA1 and SREBF1 levels when the cells were transfected with scrambled miRNA when compared with the cells without transfection (**Figure 6D**). By contrast, miR-33 levels reached the highest level or the lowest level, and were almost same in all groups when the gene was overexpressed (**Figure 6E**) or silenced (**Figure 6G**). All the results suggest that LBLP IV from LBL significantly reduces miR-33 level, and relative mRNA level of SREBF1, and increases the level of ABCA1. LBLP IV could not affect the levels of ABCA1 and SREBF1 anymore when miR-33 was overexpressed (**Figure 6F**, $P > 0.05$) or silenced (**Figure 6H**, $P < 0.05$). The levels of miR-33 had negative relation with ABCA1 and positive relation with SREBF1 (**Figure 6**). ABCA1 has negative relation with TG, TC, and LDL-C and positive relation with HDL-C (**Figure 6**; **Table 3**). Inversely, SREBF1 had positive relation with TG, TC, and LDL-C and negative relation with HDL-C (**Figure 6**; **Table 3**). The results suggest LBLP IV affect the levels of ABCA1 and SREBF1 by regulating miR-33 levels.

LBLP IV from LBL Significantly Reduces Relative Protein Level of SREBPF1, and Increases the Level of ABCA1

Real-time qRT-PCR showed that LBLP I, II, and III reduced while LBL and LBLP IV increased protein levels of ABCA1 (**Figure 7A**, $P < 0.05$). Comparatively, LBL and LBLP IV reduced relative protein levels of SREBF1 whereas LBLP I, II and III increased the levels of SREBF1 (**Figure 7A**, $P < 0.05$). All the results suggest that LBLP IV and LBL significantly reduce relative protein level of SREBF1, and increase the level of ABCA1. There were no changes for relative protein level of SREBF1 and ABCA1 when the cells were transfected with scrambled miRNA when compared with the cells without transfection (**Figures 7A,B**). LBLP IV could not affect the levels of ABCA1 and SREBF1 anymore when miR-33 was overexpressed (**Figure 7C**, $P > 0.05$) or silenced (**Figure 7D**, $P > 0.05$). The results suggest LBLP IV affects the protein levels of ABCA1 and SREBF1 by regulating miR-33 level.

DISCUSSION

Lycium barbarum L. has been reported to have potential anti-inflammatory (45) and anticarcinogenic applications (46), and attenuate lipid peroxidation (47), and diverse health protecting benefits (48). Furthermore, LBLP IV can treat diabetic rats and it can be developed as a potential dietary therapeutic agent in the treatment of diabetes (30). Present findings demonstrate that the LBLP IV is the major compound in LBL and shows significant antidiabetic activities for GDM. More importantly, LBLP IV promotes the insulin secretion (**Figure 5**), which is beneficial for GDM patients. LBLP IV has been found to reduce serum level of miR-33.

Hepatic mRNA and protein expression of lipid-related genes have been reported to be associated with miRNAs (49). The administration of LBL significantly reduced serum TC and TG levels but increased the HDL-C content (**Table 3**). The mRNA and protein expression level of ABCA1 were upregulated and SREBF1 was down-regulated (**Figures 6** and **7**). Furthermore, the expression levels of miR-33, which directly modulate ABCA1 and SREBF1, which indirectly regulates fatty acid synthase (FAS) (50, 51). The repression of miR-33 is a possible molecular mechanism of the hypolipidemic effects of LBLP IV in the liver. Compared with LBLP IV, the three compounds (LBLP I, II, and III) of LBL cannot reduce serum level of miR-33, which regulate ABCA1 and SREBF1, and closely associated with lipid metabolism.

Although LBLP IV was proven to be a bioactive compound from LBL, it could not be made on a large scale. Thus, LBL was still used in subsequent experiment in GDM patients. The changes of biochemical composition indicated that LBL consumption reduced insulin resistance, increased insulin secretory function (**Table 2**; **Figure 5**) and improved a lipid profiles (**Table 3**). Notably, LBL showed a better result after 4 weeks. In contrast, long-term consumption of LBL polysaccharides significantly ameliorates diabetes, including the improvement of general well-being and the decrease of the levels of HbA1c (52), FBG (53), and body mass index (BMI) (54). Similarly, LBL has the functions for controlling the levels of BMI and BG.

The reasons for the functions of LBL are complex. According to Chinese theory, LBL can transfer the strength between deficiency and excess from different parts of human body, including upper and lower limbs, internal organs and environment. Full-body and cooperation among different organs are the main ideas of LBL. Differentiation and development genes were repressed by embryonic stem cell-enriched miRNAs, which maintained the stem cell state. MicroRNA level has been reported to be affected by vitamin C (55), polyphenols (56), flavone (57), and polysaccharide (58). Composition analysis of LBL showed that polysaccharides were rich. The results suggest LBLP IV improves antidiabetic capabilities of GDM patients.

Polysaccharides of LBL IV reduced serum level of miR-33, which regulated ABCA1 and SREBP1. The latter two molecules affected lipid metabolism. Thus, LBLP IV improved lipid profiles

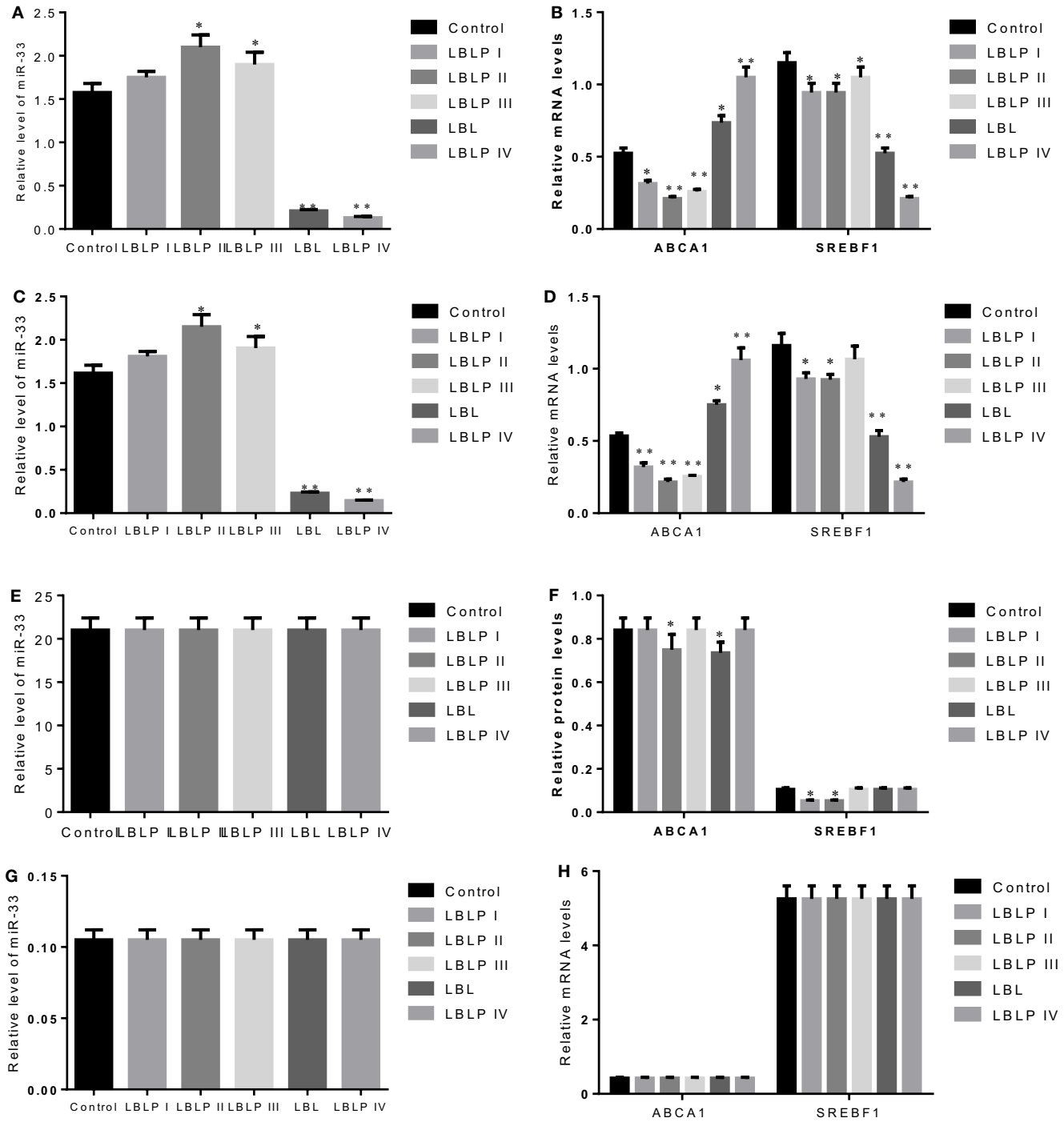

FIGURE 6 | LBLP IV reduces miR-33 level and mRNA level of SREBPF1 and increases the level of ABCA1 in human pancreatic carcinoma cell SW1990. **(A)** The effects of different polysaccharide on miR-33 level. **(B)** The effects of different polysaccharides on relative mRNA levels of ABCA1 and SREBF1. **(C)** The effects of scrambled miRNA on miR-33 level. **(D)** The effects of scrambled miRNA on relative mRNA levels of ABCA1 and SREBF1. **(E)** The effects of miR-33 overexpression on miR-33 level. **(F)** The effects of miR-33 overexpression on relative mRNA levels of ABCA1 and SREBF1. **(G)** The effects of miR-33 silence on miR-33 level. **(H)** The effects of miR-33 silence on relative mRNA levels of ABCA1 and SREBF1. All data were presented as mean values ± SD. There were statistically significant differences if $^{*}P < 0.05$ and $^{**}P < 0.001$ vs. a control group.

may by affecting serum miR-33. To approve that, miR-33 was overexpressed and silenced, and the levels of ABCA1 and SREBF1 were significantly changed too (**Figures 6** and **7**). However, the LBLP IV treatment could not change these molecules anymore. The results suggest that LBLP IV improves lipid profiles by regulating the levels of ABCA1 and SERBF1 *via* miR-33.

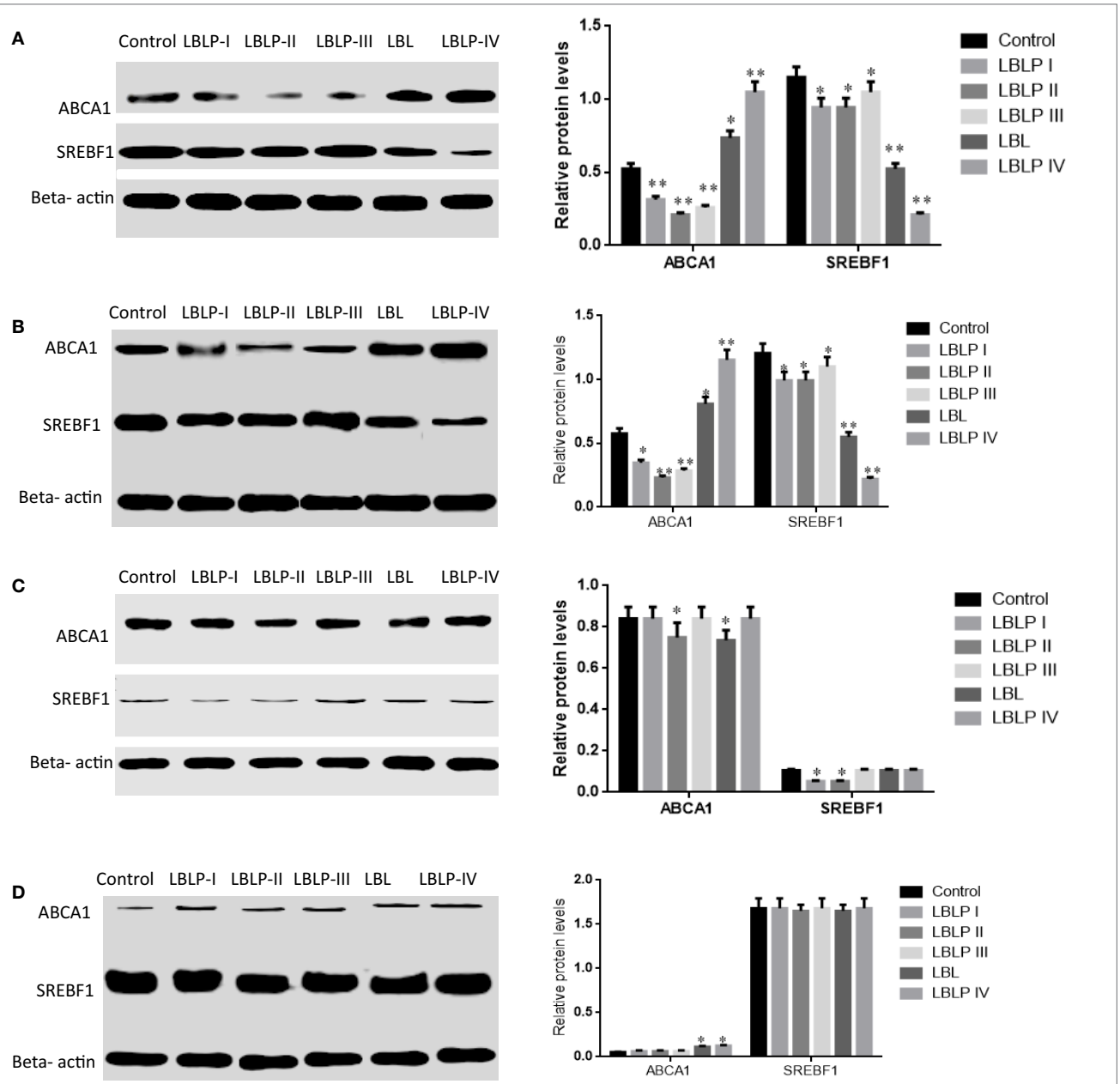

FIGURE 7 | Polysaccharides of *Lycium barbarum* L. (LBLP) IV reduces protein level of sterol regulatory element-binding transcription 1 (SREBPF1) and increases the level of ABCA1 in human pancreatic carcinoma cell SW1990. **(A)** The effects of different polysaccharide on relative protein levels of ABCA1 and SREBF1. **(B)** The effects of scrambled miRNA on protein levels of ABCA1 and SREBF1. **(C)** The effects of miR-33 overexpression on protein levels of ABCA1 and SREBF1. **(D)** The effects of miR-33 silence on protein levels of ABCA1 and SREBF1. All data were presented as mean values ± SD. There were statistically significant differences if $^{*}P < 0.05$ and $^{**}P < 0.001$ vs. a control group.

The present findings showed that LBLP IV reduced the level of SREBF1 *via* miR-33. The variants of SREBF1 have been found to be discreetly associated with hyperglycemia because of the reduction in insulin sensitivity. SREBF1 is a mediator of insulin action and can affect normal insulin secretion (59). Moreover, the SNP of SREBF1 is closely related to insulin resistance (60). SREBF1 also regulates resistin expression (61). Resistin regulates insulin secretion and glucagon from beta or alpha cells, and pancreatic islets (62). Thus, LBLP IV treatment will affect insulin secretion, HOMA-IR and resistin levels. On the other hand, overexpression of SREBP1 will increase fatty acid synthesis and triacylglycerol accumulation (63) and regulate fatty acid oxidation by activating acetyl coenzyme a carboxylase 2 (64). Although the decrease of SREBF1 can be caused by LBLP-II and –III since the oligosaccharides also regulate miR-33 and the close relationship occurs between miR-33 and SREBF1, the decreased degree was still lower than that caused by LBLP-IV. The result will lead to no significant difference for the changes of lipid profiles when compared with controls.

In the past decades, the multitarget of miRNA has caught much interest. miRNA has become a critical factor for regulating lipoprotein (65). Lipid metabolism is a main cause of GDM (66) and there is increasing evidence that miRNA plays an important role in lipid metabolism (67). miRNA can control LDL-C level by regulating TR4 expression in ox-LDL-induced macrophages, and thus affect lipid accumulation (68). miRNA also control LDL-C level by regulating the genes, which are associated with very LDL secretion, cholesterol synthesis, and LDL receptor. Interestingly, several of these miRNAs are located in genomic loci associated with abnormal levels of circulating lipids in humans. MiRNA is a potential drug potential for affecting cholesterol and TG levels in patients (69).

However, the exact molecular mechanism for the effects of LBLP IV on the level of miR-33 remains unknown. There may be the following possible mechanisms: (1) some factors promote microRNA expression by binding miRNA precursor *via* stem-loop recognition (70). LBLP IV may promote microRNA expression by binding miR-33 precursor *via* stem-loop recognition. (2) The relation between amplification and deletion of miRNA binding sites, 3′ UTR length, and miRNA expression has been reported (71). Thus, the site can also be explored to detect the interaction between LBLP IV and miR-33. (3) There is the evidence of the miRNA promoter modification may be a critical determinant of overexpression of miRNA. Restored the hypermethylated promoter can decrease target mRNA and proteins levels (72). Oligosaccharide has an epigenetic effect on gene expression by inhibiting the de-methylation of a "CpG" island within the promoter (73).

There were some limitations to the present study: (1) SW1990 is derived from a spleen metastasis of a grade II pancreatic adenocarcinoma derived from the exocrine pancreas. It is not a representable for a GDM model. A better clinical sample should be applied in this case for analysis. For example, placenta is responsible for transporting nutrients, gasses, and cytokines to the fetu, and eliminate wastes. Thus, normal placental development is very important for the fetus and mother. Trophoblast are the main cells of placenta and primary mouse placental trophoblast cells will be a useful tool to study placental development trophoblast at specific stages of pregnancy (74). Further work shows that Serotonin (5-HT) transporter (SERT) can affect 5-HT concentration in placenta. In GDM, free plasma 5-HT levels are increased because the 5-HT uptake is remarkably reduced, which is caused by impairment in translocating SERT to cell surface. Regular expression of SERT of trophoblast will be beneficial to alleviate GDM-associated complications (75). By using human placenta, the changes of functional SLC6A4 polymorphisms have been found to be associated with long-term outcomes of infants exposed to GDM (76). Insulin signaling is often required for maintaining normal function of SERT on cytoplasma membrane of the trophoblast in placenta. The findings from clinical samples demonstrate that in GDM-associated defect on insulin receptor would change 5-HT uptake rates (77). (2) LBL consumption should be performed in a larger population since the shows little side effects. (3) LBLP IV is the major ingredient in LBL but it is still difficult to determine other components of LBL, which must be determined in the future studies. (4) LBLP IV could not be produced on a larger scale from LBL and LBL was still used in the present study. (5) Some conclusions needed to be confirmed by using broad samples, since only blood serum samples were used in this case. Further work is highly demanded to address these issues.

CONCLUSION

Long-term LBL consumption was beneficial for improving some symptoms of GDM. However, LBL may have a lot of good or bad effect on GDM because of without the complete examinations for its effects on all aspects or symptoms of GDM. The rehabilitate functions of LBLP IV from LBL may improve lipid profiles. The changes for the level of TG, TC, HDL-C, LDL-C, and MDA also increase antioxidant activity of GDM patients. Furthermore, LBLP IV in LBL plays an important role in antidiabetic activities in GDM patients. Further work is highly demanded to make sure LBL consumption as non-pharmaceutical intervention for preventing the risk or progression of GDM.

ETHICS STATEMENT

All protocols were approved by ethical committee of our hospital (Approval no. 201602X4), and the study was carried out according to the principles described in the World Medical Association Declaration of Helsinki. All subjects gave written informed consent in accordance with the Declaration of Helsinki.

AUTHOR CONTRIBUTIONS

SY and LF performed the experiments. LS, WJ, and HP designed the experiment and analyzed all data. RL wrote the article. All authors approved the final submission in this journal.

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Growth Hormone Overexpression Disrupts Reproductive Status Through Actions on Leptin

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Growth and reproduction are closely related. Growth hormone (GH)-transgenic common carp exhibit accelerated growth and delayed reproductive development, which provides an amenable model to study hormone cross talk between the growth and reproductive axes. We analyzed the energy status and reproductive development in GH-transgenic common carp by using multi-tissue RNA sequencing, real-time-PCR, Western blotting, ELISA, immunofluorescence, and *in vitro* incubation. The expression of *gys* (glycogen synthase) and *igfbp1* (insulin-like growth factor binding protein) as well as blood glucose concentrations are lower in GH-transgenic carp. *Agrp1* (agouti-related protein 1) and *sla* (somatolactin *a*), which are related to appetite and lipid catabolism, are significantly higher in GH-transgenic carp. Low glucose content and increased appetite indicate disrupted metabolic and energy deprivation status in GH-transgenic carp. Meanwhile, the expression of genes, such as *gnrhr2* (gonadotropin-releasing hormone receptor 2), *gthα (*gonadotropin hormone, alpha polypeptide), *fshβ* (follicle stimulating hormone, beta polypeptide), *lhβ* [luteinizing hormone, beta polypeptide] in the pituitary, *cyp19a1a* (aromatase A) in the gonad, and *cyp19a1b* (aromatase B) in the hypothalamus, are decreased in GH-transgenic carp. In contrast, pituitary *gnih* (gonadotropin inhibitory hormone)*, drd1* (dopamine receptor D1)*, drd3* (dopamine receptor D3), and *drd4* (dopamine receptor D4) exhibit increased expression, which were associated with the retarded reproductive development. Leptin receptor mRNA was detected by fluorescence *in situ* hybridization in the pituitary including the pars intermedia and proximal pars distalis, suggesting a direct effect of leptin on LH. Recombinant carp Leptin protein was shown to stimulate pituitary *gthα*, *fshβ*, *lhβ* expression, and ovarian germinal vesicle breakdown *in vitro.* In addition to neuroendocrine factors, we suggest that reduced hepatic leptin signaling to the pituitary might be part of the response to overexpression of GH and the resulting delay in puberty onset.

Keywords: common carp, growth hormone, reproductive regulation, energy balance, leptin

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INTRODUCTION

Growth and reproduction are closely related, and there is cross talk between the endocrine systems controlling these fundamental processes in vertebrates (1–3). Examples of the clinical manifestations of the growth–reproduction interaction include delayed onset of puberty and ovarian problems in women with growth hormone (GH) insufficiencies (4). GH hypersecretion in acromegaly can also be

associated with menstrual disturbances and reduced fertility (5). In normal animals, GH can stimulate testicular spermatogenesis and ovarian hormone synthesis (6, 7) or indirectly affect gonad development by stimulating the expression of insulin-like growth factor 1 (IGF1) (8). As GH participates in hormone synthesis, ovulation, growth and renewal of follicles, oocyte maturation, spermatogenesis, sperm motility, and other aspects of reproductive development, it may be considered as a co-gonadotropin (9).

Paradoxically, reproduction in GH-transgenic fish is often reduced or disrupted to some extent. Stable GH-transgenic fish lines, including tilapia (*Oreochromis niloticus*) (10), coho salmon (*Oncorhynchus kisutch*) (11), loach (*Misgurnus mizolepis*) (12), and common carp (*Cyprinus carpio*) (13) have been established as fast-growing genetically modified organisms for potential human consumption. After decades of controversy, in 2015 the Federal Department of Agriculture in the US approved the first GH-transgenic Atlantic salmon (*Salmo salar*) as a legally edible animal. This is a milestone for transgenic animal industrialization, yet challenges remain. Beside increased growth, GH-transgenic fish species exhibit reduced courtship and spawning (14, 15), reduced sperm quantity and ovarian size (10, 16), and reduced nest loyalty, quivering frequency, and spawning participation (17).

Rahman (10) and Bessy (14) were among the first to report increased energy allocation to somatic growth rather than gonad development for reduced gonadal size and reproductive potential in GH-transgenic fish. We also reported on delayed sexual maturation and decreased gonadal size of fast-growing GH-transgenic common carp (18). In that study, high levels of GH were shown to directly inhibit luteinizing hormone (LH) production and release through GH receptors in pituitary gonadotrophs to suppress reproductive processes. This provided the first proposal of a mechanism whereby GH overexpression in transgenic fish could affect reproduction. However, the exact mechanism linking altered energy allocation, somatic growth, and suppressed reproduction in GH-transgenic animals remains to be elucidated.

The present study was therefore conducted using RNA sequencing of hypothalamus, pituitary, gonad, liver from 5-month-old GH-transgenic common carp, and their wild-type counterparts. An association between decreased *leptin* expression and the overexpression of GH in the transgenic animals led us to investigate the role of leptin. This is especially relevant given that leptin plays critical roles in the regulation of body weight by inhibiting food intake and stimulating energy expenditure. We determined that recombinant carp Leptin directly stimulates both pituitary gonadotropin subunit expression and ovarian germinal vesicle breakdown (GVBD). In GH-transgenic common carp, decreased *leptin* expression is therefore one link between altered energy status and disrupted reproduction.

MATERIALS AND METHODS

Experimental Fish

The female and male GH-transgenic common carp (Yellow river strain) used in the study were from our TG2 line, carrying the grass carp (*Ctenopharyngodon idellus*) GH gene (13). GH-transgenic and non-transgenic common carp were derived from the same non-transgenic mother and were reared at Guanqiao Experimental Depot, Wuhan, China. Ten individuals of each group were sampled, and the body weights were measured. Five fish were sampled in each group for RNA sequencing. Ten fish were sampled for qPCR and serum hormone analysis. The gonadosomatic index (GSI; gonad weight/body weight × 100%) was also calculated for each individual. The Animal Care and Use Committee of the Institute of Hydrobiology approved all procedures.

RNA Isolation, Library Construction, and Sequencing

Tissue samples of the hypothalamus, pituitary, liver, and gonad were collected from five GH-transgenic and non-transgenic common carp in 5-month-old at puberty developmental stage, respectively. Total RNA was isolated using the Trizol reagent (Invitrogen, USA), according to the manufacturer's protocol. All of the samples had an RNA integrity number value greater than 8. Sequencing libraries were generated using the NEB Next Ultra RNA library prep kit from Illumina (New England Biolabs, USA), according to the manufacturer's protocol. Libraries were sequenced on an IlluminaHiseq™ 2000 platform and 150 bp paired-end reads were generated. The transcriptome raw data are available at http://www.ncbi.nlm.nih.gov/bioproject/337990.

Data Analysis

Clean data (clean reads) were obtained by removing reads with adaptors, reads with unknown sequence more than 5% or low quality reads which had more than 50% QA ≤ 15 bases, by using in-house Perl scripts. All further analyses were performed using only the cleaned, high-quality data. *De novo* transcriptome assembly was carried out using the Trinity program (19) with optimized k-mer length of 25. On the other hand, the clean reads were also mapped to the common carp (Heilongjiang strain) reference mRNA database[1] using SOAPaligner/soap2. Two base mismatches were allowed in the mapping process, total mapped reads were calculated, and the mapped regions were counted. HTSeq software was used to count the number of reads mapped to each gene. The normalized gene expression level was separately calculated as reads per kilobase of mRNA per million of mapped reads (RPKM) for each library.

The transcriptomic data included expressed genes in hypothalamus, pituitary, gonad, and liver of both female and male individuals of GH-transgenic and non-transgenic carp. There were in total 16 libraries constructed and sequenced. The mixed library was constructed following *de novo* assembly processes, including 172,823 unigenes, which was far more than the estimated gene number of common carp genome. The clean reads were also mapped to the reference mRNA database of common carp. The average percentage of total mapped reads was 38.09% and that of unique mapped reads was 32.71% (Tables S1 and S2 in Supplementary Material). The common carp database was compared to the *de novo* assembled reference database using the Basic Local Alignment Search Tool.[2] We

[1] http://www.carpbase.org (Accessed: September 27, 2015).
[2] https://blast.ncbi.nlm.nih.gov/Blast.cgi (Accessed: October 08, 2015).

then assembled a new reference database that included unique sequences of Yellow River common carp strain and excluded any redundancies. This improved database containing 52,327 genes was used as the reference database for further analysis. The average percentage of total mapped reads was improved to 53.83% and that of unique mapped reads was improved to 47.55% (Table S3 in Supplementary Material). The following differential expression analysis was based on the improved reference database.

Differential Gene Expression Analysis

The expression level of each gene was estimated by RPKM values. The 16 libraries were named NTHF, NTHM, THF, THM, NTPF, NTPM, TPF, TPM, NTLF, NTLM, TLF, TLM, NTGF, NTGM, TGF, and TGM where NT and T indicates non-transgenic (NT) or transgenic (T) groups. The four tissues were hypothalamus (H), pituitary (P), liver (L), and gonads (G) in female (F) and male (M) common carp. Data sets were subjected to a series of comparisons between GH-transgenic and non-transgenic groups. The number of differentially expressed genes (DEGs) identified is listed in Table S4 in Supplementary Material. Detailed information of all DEGs is shown in Table S5 in Supplementary Material. Since we focused on the delayed gonadal development in both female and male GH-transgenic carp, the genes changed both in female and male individuals were filtered using Venn diagram analysis (Figure S1 in Supplementary Material). Detailed information of DEGs in both male and female of different tissues is shown in Table S6 in Supplementary Material.

Differential expression analysis was performed using the DESeq package (20). The resulting *P*-values were adjusted using Benjamini and Hochberg's method for controlling the false discovery rate. Genes with an adjusted *P*-value less than 0.05 were considered to be differentially expressed. Gene ontology (GO) enrichment analysis of DEGs was implemented by the GOseqR package (21) with gene length bias correction. For hypothalamus of GH-transgenic carp and non-transgenic carp, annotated genes were categorized into cellular component and molecular function (Figure S2 in Supplementary Material). Pituitary DEGs were categorized into molecular function categories. In the liver of GH-transgenic and non-transgenic carps, the DEGs were categorized into cellular component, molecular function, and biological processes. In gonad, the DEGs were categorized into cell component change. The detailed list is attached in Table S7 in Supplementary Material.

The KOBAS software (22) was employed to test the statistical enrichment of DEGs in Kyoto Encyclopedia of Genes and Genomes database (KEGG[3]) pathways. The KEGG terms with corrected *P*-values less than 0.05 were considered significantly enriched. In order to identify possible biochemical pathways that DEGs were involved in, KEGG analysis was carried out to understand the common changed pathways that enriched in both male and female GH-transgenic carp. There are 81 significantly enriched terms in hypothalamus, pituitary, liver, and gonad which are listed in Table S8 in Supplementary Material.

[3]http://www.kegg.jp/ (Accessed: November 15, 2015).

To further investigate reproductive development in GH-transgenic carp, significant DEGs in tissues common to both females and males were identified and annotated. We filtered those DEGs exhibiting a $\log_2$ fold change ≥ 1 to include those with a RPKM value higher than 10 in at least one sample and those genes reported to be involved in either endocrine control of gonadal development or energy regulation. These DEGs are listed in Table S8 in Supplementary Material.

Validation of DEGs by qPCR

In order to confirm the reliability of the data obtained by RNA-seq, 15 DEGs were selected for validation using qPCR. The primers are listed in Table S9 in Supplementary Material. The RNA samples from an independently repeated study were used for reverse transcription. qPCR was carried out on a Bio-Rad fluorescence quantitative PCR instrument (CFX96 Touch™). Each qPCR mixture contained 0.8 μL sense and reverse primers, 1 μL template, 10 μL 2 × SYBR mix (TOYOBO, Japan), and 7.4 μL ddH_2O. Three replicates were conducted for each sample, and β-actin gene was used as an internal control. The program for qPCR was as follows: 95°C for 10 s, 40 cycles of 95°C for 5 s and 60°C for 20 s. Relative expression level was calculated using the $2^{-\Delta Ct}$ method. All data are given as mean ± SD of three replicates.

In Situ Hybridization and Immunofluorescence

The leptin receptor *(lepR)* probes for *in situ* hybridization were made using the primer pair (5′-TTATCTAATCATCCAGTGC-3′; and 5′-TAATACGACTCACTATAGGGCGGAGAACGGTCGA-GTA-3′), following validated protocols (23). *In situ* hybridization for *lepR* combined with immunocytochemical localization of Lhβ (antibody FMU-cGTHIIβ9) in non-transgenic female common carp (8 months old) was performed as previously described (18).

Determination of Serum Hormone Concentrations

Blood was collected from GH-transgenic and non-transgenic common carp (5 months old, $n = 7$–15). Serum samples were obtained by centrifugation at 3,000 × *g* for 15 min at 4°C. The Gh ELISA system, with the assay range of 1.56–50 ng/mL, was developed and validated in our previous studies (24). The estradiol ELISA kit (#582251) was purchased from Cayman Chemical (USA). Igf1 (CSB-E12122Fh) and Igf2 (CSB-EL011088FI) ELISA kits were purchased from Cusabio Life Science (Wuhan, China) (25). The hormone analyses followed the manufacturer's instructions.

In Vitro Incubation with Recombinant Leptin

Full-length cDNA of common carp *leptin* was inserted into the pMXB10b vector (New England Biolabs, Beijing, China), and the recombinant construct was transformed into DE3 competent cells. The leptin protein fused to a chitin binding domain was expressed in the bacteria following induction with IPTG. After incubation with 0.3 mM IPTG at 30°C for 6 h, the bacteria were harvested and disrupted by sonication (Scientz-IID, Ningbo,

China). The bacterial lysate was passed through a chitin column, and the anchored fusion protein was then incubation with 50 mM DTT, to cleave and harvest the recombinant common carp Leptin protein. The purity of the recombinant protein was confirmed by polyacrylamide gel electrophoresis (Figure S3 in Supplementary Material).

Pituitary glands were removed from non-transgenic common carp (5 months old). After being washed three times, the pituitaries were cut into small pieces with scissors and equally transferred into 24-well plates for the following incubation. To determine the effects of Leptin on pituitary *gthα*, *fshβ*, and *lhβ* gene expression, carp pituitary fragments were treated with recombinant Leptin. The dosage of Leptin was chosen according to serum Leptin concentration (~10^{-9} M) we determined in this study and reported in other animals (26–28). Pituitary fragments were harvested at 30 min, 1 h, and 2 h after incubation. Levels of *gthα*, *fshβ*, and *lhβ* were quantified by real-time PCR. Data presented in this study were the results of at least three independent experiments and were expressed as fold change relative to the controls.

GVBD Assay

Zebrafish ovarian follicles were isolated and incubated following an established protocol (29). Briefly, gravid female zebrafish were deeply anesthetized with 0.01% tricaine methanesulfonate solution (Sigma, USA) for 2 min and sacrificed. Ovaries were washed three times in PBS, and the individual ovarian follicles were separated without damaging the follicle cell layers. Full-grown follicles (550–650 μm in diameter) were selected and randomly distributed into a 24-well plate (20–30 follicles per well) and treated with recombinant Leptin protein. The follicles were incubated for 4–16 h and scored at each time point for %GVBD.

RESULTS

Body Weights and GSI in GH-Transgenic Carp

The body weights of GH-transgenic carp were 1.8- and 1.9-fold higher than female and male non-transgenic animals, respectively (**Figures 1A,C**). The GSI in GH-transgenic carp decreased by 2.5- and 9.8-fold compared with non-transgenic carp (**Figures 1B,D**).

Differential Gene Expression in GH-Transgenic Carp

Analysis of the DEGs in hypothalamus revealed that four terms involved in signal transduction and signaling molecules and interaction were enriched. These included neuroactive ligand receptor interaction, the Jak-STAT signaling pathway, cytokine–cytokine receptor interaction, and cell adhesion molecules (Table S7 in Supplementary Material). The gene with the highest expression (with the highest RPKM value) in wild-type is *cyp19a1b*, or aromatase b, which is the estrogen synthesis enzyme found in radial glial cells in the teleost brain. Expression of *cyp19a1b* was downregulated more than twofold (with $\log_2$Ratio (T/NT) −1.2 for female and −1.3 for male common carp, Table S8 in Supplementary Material) in the hypothalamus of GH-transgenic carp. Hypothalamic *slα* (somatolactin alpha) was also decreased in GH-transgenic fish. Genes that were upregulated in the

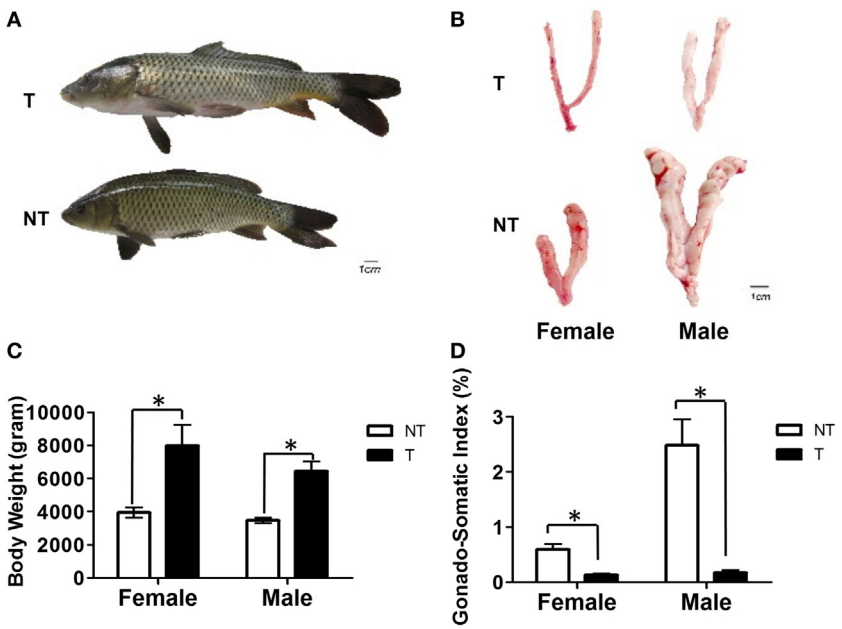

FIGURE 1 | Comparison of body growth and gonadal development between non-transgenic (NT) and GH-transgenic (T) common carp of 5 months. **(A)** Comparison of morphology of NT and T common carp. **(B)** Comparison of the gonad morphology of NT and T common carp. **(C)** Comparison of body weight of female and male NT and T common carp. **(D)** Comparison of gonadal-somatic index between NT and T common carp of females and males. Samples were collected at 5 months of age. Values are represented as means ± SEM (n = 10–15) at each sampling time and analyzed by Student's t-test between NT and T. Asterisks indicate statistically significant differences compared within NT and T at $P < 0.05$.

hypothalamus of GH-transgenic carp include the growth-related transcriptional factor *stat1* (signal transducer and activator of transcription 1), *stat2* (signal transducer and activator of transcription 2), and *irs2* (insulin receptor substrate 2) (Table S8 in Supplementary Material).

In the pituitaries of GH-transgenic carp, the expression levels of genes encoding hormones such as *gh*, *gthα*, *fshβ*, and *lh* β exhibited downregulation, while *prl* (prolactin) was upregulated (**Figure 2**). These genes are related to the GO terms neuroactive ligand–receptor interaction pathway and endocrine system. Growth-related genes in the pituitary that were differentially expressed in GH-transgenic carp include upregulated *slα*, *tshr* (thyroid stimulating hormone receptor), *sstr2* (somatostatin receptor 2), *sstr3* (somatostatin receptor 3), *socs1* (suppressor of cytokine signaling 1), *socs3b* (suppressor of cytokine signaling 3b), *socs5a1* (suppressor of cytokine signaling 5a1), *socs5* (suppressor of cytokine signaling 5), *slα*, *irs2* (insulin receptor substrate 2), *sst* (somatostatin), *lepR*, and *lepRl* (leptin receptor long isoform), and downregulated *gh* and *igfbp1* (insulin-like growth factor binding protein 1) (Table S8 in Supplementary Material). These genes are related to the Jak-STAT signaling and cytokine–cytokine receptor interaction signaling pathways. In the pituitary, reproduction-promoting genes such as *gnrhr2* (gonadotropin-releasing hormone receptor 2), *fshβ*, *lhβ*, *cyp19a1b*, and *gabar* (gamma-aminobutyric acid receptor-associated protein-like) were downregulated, while reproduction-inhibiting genes such as *drd1* (dopamine receptor D1) and *drd3* (dopamine receptor D3) were upregulated in GH-transgenic carp. Genes related to circadian rhythms such as *clock1a*, *clock2*, and *per2* were upregulated while *cyclinG2* was downregulated in the pituitaries of GH-transgenic carp. Markers for reactive oxygen species such as *ho* (heme oxygenase 1) and *grik4* (glutamate receptor ionotropic, kainate 4) were also upregulated in the GH-transgenic group.

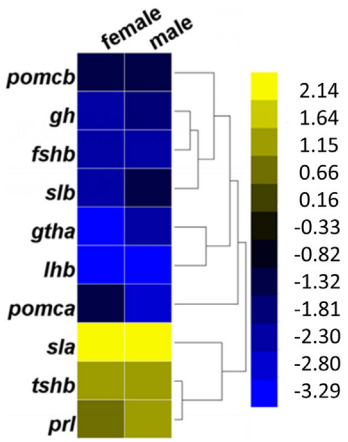

FIGURE 2 | Changed expression pattern of genes in GH-transgenic (T) common carp compared with non-transgenic (NT) common carp. Fold change of pituitary hormone-encoded genes in female and male T common carp compared with NT animals. Yellow represented for upregulation, dark blue and dark yellow for nearly no expression change, and blue for downregulation.

In the liver, most DEGs were related to the GO terms of lipid metabolism, amino acid metabolism, and carbohydrate metabolism (Table S7 in Supplementary Material). We found that the hepatic expression levels of *erβ1* (estrogen receptor beta1), *ho*, *desaturase*, and *fabp7* (fatty acid binding protein 7) were increased in GH-transgenic carp while those of *leptinI*, *ghrelin*, *epo* (erythropoietin), *gys*, *igfbp1* decreased (Table S8 in Supplementary Material). All these genes are associated with the lipid metabolism signaling pathway, which is also related to reproductive development.

In the gonad, DEGs in both male and female were associated with organismal systems including the immune system, development, endocrine system, and digestive system. For specific genes related to reproductive and gonadal development, *cystatin* and *zp3* expression were upregulated, whereas *trypsin*, *npsn* (nephrosin precursor), *igf3*, and *cyp19a1a* expression were down-regulated in the GH-transgenic carp gonads (Table S8 in Supplementary Material).

Validation of RNA-Seq Data

To validate the RNA-Seq data, 18 DEGs were selected for qPCR analysis. These genes included *fshβ*, *lhβ*, *gthα*, *gh*, *mch* (melanin-concentrating hormone), *gnih*, *pkm* (pyruvate kinase), *gnrh3*, *drd1*, *drd2*, *drd3*, *drd4*, *gnrhr1*, *gnrhr2*, *cyp19a1a*, *cyp19a1b*, *igf3*, and *leptinI* (Table S10 in Supplementary Material). As shown in Table S10 in Supplementary Material, the direction of change in expression of all 18 DEGs as determined by qPCR was consistent with the RNA-seq results. The Pearson product-moment correlation coefficient for results obtained from qPCR and RNA-seq for the 18 genes in both sexes was $R = 0.835$ ($P < 0.0001$), which confirmed the reliability and accuracy of the RNA-seq data.

The levels of Gh, estradiol, Igf1, and Igf2 were quantified using ELISA. The results showed that serum Gh levels were significantly increased in GH-transgenic carp (**Figure 3A**). Serum estradiol levels were lower in GH-transgenic female carp, while there were no effects of GH-transgenesis in males (**Figure 3B**). There was no difference in Igf1 and Igf2 levels in GH-transgenic carp compared with non-transgenics ($P > 0.05$) (**Figures 3C,D**). Serum glucose levels were decreased in both female and male GH-transgenic carp compared with non-transgenic animals (**Figure 3E**). As determined using Western blotting, pituitary Gh, Lhβ, and Gthα subunit levels were lower in GH-transgenic carp (Figure S4A in Supplementary Material), whereas Prl levels were higher in GH-transgenic carp than non-transgenic carp (Figure S4A in Supplementary Material). Semi-quantitative PCR showed that *gnrhr1* was expressed mainly in hypothalamus, whereas *gnrhr2*, *gnrhr3*, and *gnrhr4* mainly in pituitary (Figure S4B in Supplementary Material). These results also support the reliability of the RNA-seq data.

Effects of Recombinant Leptin on Pituitary Gonadotropin Subunit Expression and Ovarian Follicular Maturation *In Vitro*

We found that *lepR* is expressed in the pituitary and is upregulated in GH-transgenic carp (Table S10 in Supplementary Material).

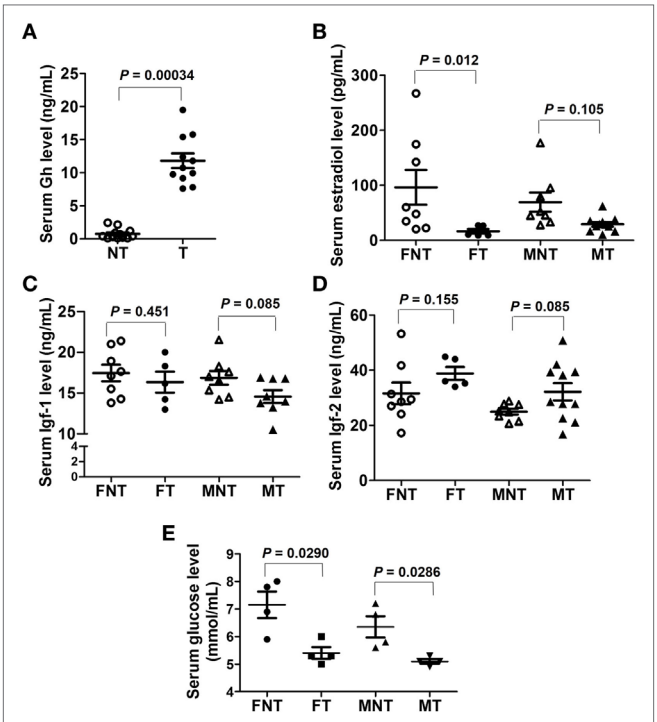

FIGURE 3 | Serum levels of **(A)** Gh, **(B)** estradiol, **(C)** Igf1, **(D)** Igf2, and **(E)** glucose contents in female (F) and male (M) non-transgenic (NT) and GH-transgenic (T) common carp. The results obtained were analyzed within sex by independent sample *t*-test. The *P*-values are indicated on the graph.

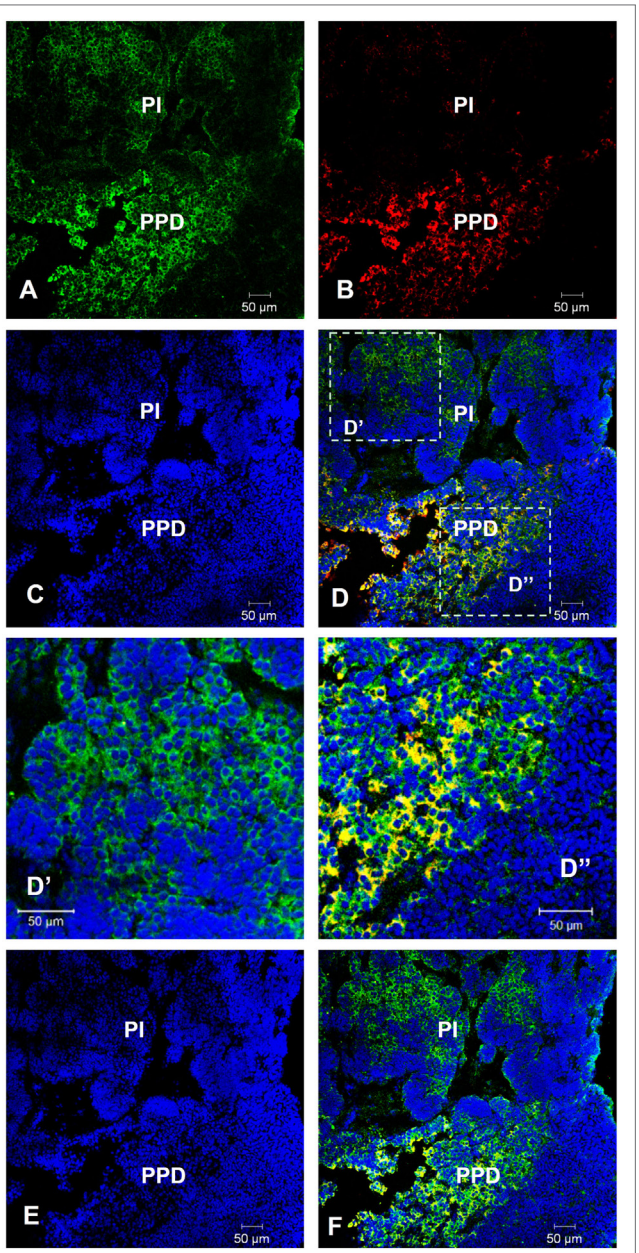

FIGURE 4 | Co-localization of *lepR* and Lh in non-transgenic common carp pituitary. *In situ* hybridization of *lepR* (green) and immunofluorescence of Lh (red) in 8-month-old female **(A–D,D′,D″)** and male **(E,F)** common carp pituitary. Yellow-orange color indicates colocalization. PI, pars intermedia; PPD, proximal pars distalis. Scale bar, 50 μm.

Given its importance in both body weight regulation and reproduction (30, 31), we therefore focused our attention on leptin. The *lepR* mRNA was detected by fluorescent *in situ* hybridization in the pars intermedia and proximal pars distalis (PPD) of the female pituitary. The *lepR* signal in PPD was co-localized with Lhβ (**Figure 4**).

To further study whether there is a direct effect of Leptin on reproductive processes, we first tested the effects of the recombinant common carp Leptin protein on pituitary *gthα*, *fshβ*, and *lhβ* expression using real-time PCR (**Figure 5**). Two-way ANOVA was used to determine the concentration and time-dependent effects of Leptin on expression of the gonadotropin subunits. For *gthα*, the concentration × time interaction was significant ($P = 0.01$). While the main effects of increasing Leptin on *gthα* were not statistically significant ($P = 0.22$), there was an overall increase in expression of *gthα*, with time ($P = 0.009$). Only at the concentration of 10^{-8} M did Leptin increase ($P < 0.05$) *gthα* at 2 h (**Figure 5A**). For *fshβ*, the concentration × time interaction was significant ($P = 0.01$). Levels of *fshβ* increased with time ($P = 0.0002$) and with concentration of leptin ($P < 0.0001$). The 10^{-9} M dose increased *fshβ* up to 1 h of incubation. Leptin concentrations of 10^{-8} M were the most effective at enhancing *fshβ* from 30 min to 2 h of incubation (**Figure 5B**). The overall pattern of expression of *lhβ* resembled that of *fshβ*. For *lhβ*, the concentration × time interaction was significant ($P = 0.0007$). Levels of *lhβ* increased with time ($P = 0.0018$) and concentration of Leptin ($P < 0.0017$). The 10^{-9} M dose of Leptin increased *lhβ* only at 1 h of incubation. For 10^{-8} M Leptin, *lhβ* was increased at both 1 and 2 h of incubation (**Figure 5C**).

Recombinant common carp Leptin also stimulated zebrafish follicle maturation as assessed using the *in vitro* GVBD assay (**Figure 6**). Two-way ANOVA was used to determine the concentration and time-dependent effects of Leptin on GVBD. Both time ($P < 0.0001$) and Leptin concentration ($P < 0.0001$) increased GVBD over the 4–16 h incubation period. Although

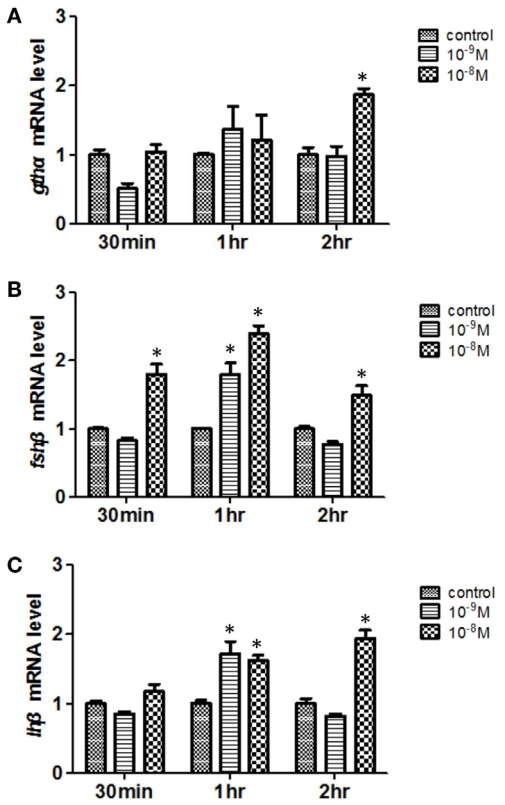

FIGURE 5 | Effects of Leptin on **(A)** *gthα*, **(B)** *fshβ*, and **(C)** *lhβ* expression in 5-month-old female non-transgenic carp pituitary cells *in vitro*. Pituitary cells were exposed to Leptin for 30 min, 1, and 2 h. The expression of the target genes (mean ± SEM) were determined by real-time PCR and were expressed as fold change normalized to the controls. The results obtained were analyzed by independent sample *t*-test compared with controls. Asterisks indicate statistically significant differences at $P < 0.05$.

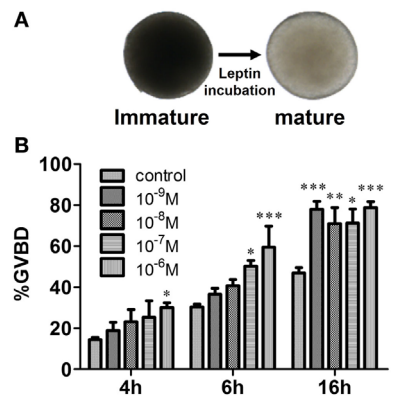

FIGURE 6 | Effects of recombinant carp Leptin on zebrafish oocyte maturation. **(A)** Fully growth follicles were treated with recombinant carp Leptin. The follicles became mature (transparent) after germinal vesicle breakdown (GVBD). **(B)** The % follicles undergoing GVBD was scored and assessed. Each value represents the mean ± SEM of three independent experiments.

the interaction of time × concentration was not significant ($P = 0.26$), lower Leptin concentrations were less effective over short incubation times, and all doses of Leptin enhanced GVBD at 16 h.

DISCUSSION

In the present study, we uncovered a novel mechanism for energy allocation between growth and reproduction in GH-transgenic fish. Common carp, tilapia, and coho salmon that carry a GH-transgene have enhanced growth and delayed reproductive development (10, 14, 18). Such genetically modified fish are amenable models to study interactions between the endocrine axes controlling growth and reproduction. Unfortunately, most published studies have only speculated that increased energy allocation to somatic growth rather gonad development is the reason for reduced reproductive performance in transgenic fish (10, 14). Our data indicate that in addition to neuroendocrine factors, reduced hepatic leptin may be part of the response to overexpression of GH and the resulting delayed in puberty onset.

Leptin May Act as a Sensor for Energy Status to Regulate Reproductive Development in GH-Transgenic Common Carp

The DEGs in GH-transgenic compared to non-transgenic common carp were related to feeding, growth, metabolism of glucose and lipids, and reproductive function. Growth-enhanced GH-transgenic common carp exhibit increased appetite, which was previously surmised to be driven at least partially by increased *AgRP1* expression (32). In this study, we found the reduced expression of *gys* (glycogen synthase) and *igfbp1*, which was accompanied by decreased blood glucose level in GH-transgenic carp. Improved appetite in GH-transgenic carp could be driven by low glucose (33–35). On the other hand, decreased fat and energy content (32, 36), together with improved appetite and metabolic rate (32, 37, 38) in GH-transgenic fish, reflects an energy-deprived status. Under low glucose conditions, lipids can be used as energy sources. We observed that the lipid metabolism pathway was activated in the livers of GH-transgenic common carp. In particular, elevated pituitary *slα* in GH-transgenic carp may be important because somatolactin stimulates lipid catabolism in fish (39, 40). Increased hepatic *fas* (fatty acid synthase) expression in the GH-transgenic carp could also be indicative of accelerated fatty acid synthesis.

Notably, GH-transgenic common carp exhibit decreased hepatic *leptin* expression level, positively related to decreased fat content, as previously reported (41). Thus, leptin might play an important role in energy sensing. On the other hand, leptin is suspected to have a role in reproductive development in teleost fish. In sexual mature Atlantic salmon, the expression level of *leptin* is higher than in immature animals (31). In mammals, it is known that leptin affects both pituitary and gonadal functions (42). In addition, the effect of leptin on the activation of GnRH neurons is suspected to be indirect (43). We found that levels of

pituitary *gthα*, *fshβ*, and *lhβ* mRNAs were stimulated by Leptin *in vitro*. We show that carp LH cells express the leptin receptor as reported in other fish (44, 45). Moreover, we demonstrate that recombinant Leptin stimulates GVBD in zebrafish. Together these results indicate that a GH–leptin–gonadotropin axis at least partially mediates the cross talk between growth and reproduction in fish.

Other Factors Are Linked to Reduced Reproductive Performance in GH-Transgenic Common Carp

Our previous study reported that elevated Gh production in GH-transgenic common carp suppressed pituitary Lh content and serum Lh levels (18). Zhou et al. (46) first demonstrated the intrapituitary autocrine/paracrine regulation between gonadotrophs and somatotrophs in grass carp pituitary cells and found that Gh inhibits Lh secretion *in vitro*. *In vivo* in the GH-transgenic carp model, GH regulates reproductive development through paracrine effects whereby Gh inhibits pituitary *gthα*, *fshβ*, and *lhβ* expression and Lh secretion (18).

Our data suggest that Gh may also negatively regulate reproductive processes at other levels of the hypothalamic–pituitary–gonadal axis. In cyprinid fish, GnRH3 is the main form stimulating Lh release (47). In the present study, the expression of *gnrh3* did not change in GH-transgenic common carp, while the expression of *gnrhr2*, the mRNA encoding for the GnRH receptor mediating the effects of GnRH3 in the pituitary, decreased significantly. Previously, we demonstrated that GH-transgenic carp have reduced pituitary sensitivity because coinjection of a GnRH agonist and dopamine agonist was not very effective at increasing *lhβ* and *fshβ* expression in GH-transgenic versus wild-type carp (18). Thus, the downregulation of *gnrhr2* caused by Gh is a contributing factor to reduced gonadotropin production. RNA sequencing and targeted PCR indicate that expression of several dopamine receptor subtypes were increased in the GH-transgenic carp. Increased expression of *drd1*, *drd3*, and *drd4* was confirmed by PCR. It has long been known that in goldfish (*Carassius auratus*), DA neurons innervate the anterior pituitary and stimulate Gh secretion *via* D1 receptors (48). It is unknown how Gh may regulate *drd1* and how it may relate to decreased expression of endogenous pituitary Gh noted in this study of GH-transgenic carp. On the other hand, there is clear evidence that DA acting *via* the pituitary DA D2 receptor is a critical inhibitor of gonadotropin synthesis and secretion in many teleosts (49, 50). Therefore, it is important to note that expression of *drd2* was not significantly different (only 1.2-fold) in the pituitaries of GH-transgenics. On the other hand, *drd3* and *drd4b* are members of the D2-like family, and they were significantly upregulated. A lesser known inhibitory effect of DA *via* D1 receptors has been reported for goldfish (51), such that a DA D1 antagonist potentiates glutamatergic stimulation of Lh release. Moreover, DA D3- and D4-selective drugs can inhibit GnRH-stimulated LH release from cultured tilapia pituitary cells (52). Perhaps, Gh also enhances dopaminergic inhibition of LH in GH-transgenic carp by increased DA receptor expression. Increased expression of hypothalamic *gnih* was accompanied by increased *gnihr* in pituitary in GH-transgenic common carp. In reproductively active adult goldfish (53) and carp (54), GnIH-3 is a strong inhibitor of LH.

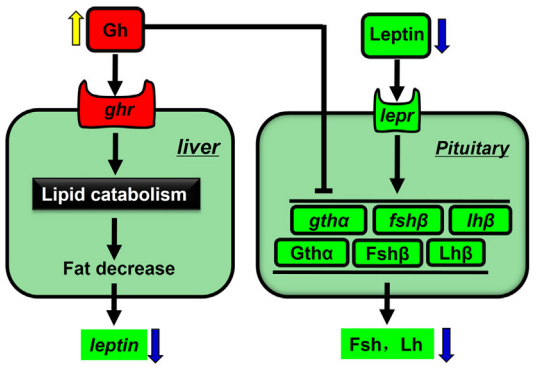

FIGURE 7 | Simplified model for the influence of growth hormone (GH) overexpression on disruption of reproduction in common carp. Genes and proteins that are upregulated in GH-transgenic carp are shown in red, while those downregulated are shown in green. Black arrows indicate stimulation, and black blunt-end lines indicate suppression.

The suppressed expression of gonadotropin subunits in the pituitary and decreased Lh and Fsh concentrations on the blood were accompanied by significantly reduced blood estradiol in females and a tendency for the same in males. It is known that physiological concentrations of estradiol exert strong positive feedback effects at the pituitary to enhance GnRH-induced Lh secretion in goldfish of both sexes with intact gonads (2, 55). The decrease in estradiol may therefore result in a reduced stimulation of *gthα*, *fshβ*, and *lhβ*.

The expression of *igf3* in the female and male gonads decreased significantly, which might also be related to the delayed gonadal development in GH-transgenic carp. Igf3 is a gonadal insulin-like growth factor expressed specifically in follicular and Sertoli cells (56, 57). It functions to promote oocyte maturation in females (58) as well as spermatogonial proliferation in the testes (59).

CONCLUSION

Overexpression of Gh leads to increased somatic growth and altered glucose and lipid metabolism in common carp. We observed that hepatic leptin, pituitary gonadotropin subunit expression was decreased in GH-transgenic carp with reduced gonadal growth. This led us to explore the link between reduced leptin and suppressed reproductive processes. We found that recombinant carp Leptin stimulates gonadotropin subunit expression and induced ovarian GVBD *in vitro*. This provides evidence for a GH–leptin–gonadotropin axis that may mediate the cross talk between growth and reproduction in GH-transgenic fish (**Figure 7**).

AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: WH, MC, and ZZ. Performed the experiments: JC and MC. Analyzed the data

and interpreted the results: MC, JC, AZ, and MS. Contributed reagents/materials/analysis tools: WH, BT, YL, and YW. Wrote and revised the paper: JC, MC, VT, and WH.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at https://www.frontiersin.org/articles/10.3389/fendo.2018.00131/full#supplementary-material.

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Nutrient Regulation of Endocrine Factors Influencing Feeding and Growth in Fish

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Endocrine factors regulate food intake and growth, two interlinked physiological processes critical for the proper development of organisms. Somatic growth is mainly regulated by growth hormone (GH) and insulin-like growth factors I and II (IGF-I and IGF-II) that act on target tissues, including muscle, and bones. Peptidyl hormones produced from the brain and peripheral tissues regulate feeding to meet metabolic demands. The GH-IGF system and hormones regulating appetite are regulated by both internal (indicating the metabolic status of the organism) and external (environmental) signals. Among the external signals, the most notable are diet availability and diet composition. Macronutrients and micronutrients act on several hormone-producing tissues to regulate the synthesis and secretion of appetite-regulating hormones and hormones of the GH-IGF system, eventually modulating growth and food intake. A comprehensive understanding of how nutrients regulate hormones is essential to design diet formulations that better modulate endogenous factors for the benefit of aquaculture to increase yield. This review will discuss the current knowledge on nutritional regulation of hormones modulating growth and food intake in fish.

Keywords: food intake, growth, nutrients, fish, aquaculture

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INTRODUCTION

Physiological processes in fish, as well as in other vertebrates, are subject to complex regulatory mechanisms that act in response to both internal and external signals (1–4). Signals provided by the environment, along with internal cues are sensed and centrally integrated, providing information about the metabolic status. This enables fish to determine whether conditions are ripe to feed, grow, reproduce and save energy (4, 5). Among the external signals that can influence feeding behavior and growth in fish, one of the most important is food (6, 7). Both food availability and food composition exert a critical control of these processes, primarily by acting on the hormones in charge of their endocrine control. The main aim of this review is to summarize the recent advances on the role of feeding and fasting, as well as of dietary macro- and micronutrients, on the regulation of appetite- and growth-regulating hormones in fish. A better understanding of the effects of feeding status and diet composition on the expression and release of those hormones could be beneficial to determine the effects of a specific diet or feeding regime on fish health, growth, and development, which could be crucial in aquaculture. This review will also aim to identify gaps in knowledge and directions for future research regarding this important topic.

NUTRIENTS AND THEIR IMPORTANCE IN FISH

Nutrients are organic compounds involved in biochemical reactions that produce energy and are constituents of cellular biomass (8). They are divided into two broad groups: macronutrients and micronutrients. Macronutrients are classified into carbohydrates (CHO), proteins and lipids, and are needed in relatively large amounts since they are the primary source to generate the energy organisms require to survive, grow, and reproduce. These nutrients can be stored within the body for later use, or be utilized, leading to the somatic growth of the animal (9). The micronutrients comprise of vitamins and minerals and are needed in smaller quantity, although they have several critical roles in cellular processes. The nutritional requirements regarding the composition and proportion of different nutrients present in the diet vary among species and within species, is determined by various extrinsic and intrinsic factors such as environmental conditions, stage of life cycle, sex, and reproductive state (10). The importance and main roles of each nutrient in fish metabolism are summarized in **Table 1**.

Organisms coordinate their growth and development with nutrient level fluctuations in their environment, and therefore they must be able to sense their internal and external nutrient levels (24). In mammals, sensing mechanisms regulate specific processes such as food intake, hormone secretion, and energy expenditure to maintain energy homeostasis (25–27). The sensing of nutrients may occur directly or indirectly. In the first case, the nutrient molecule binds to its sensor. In the second case, nutrient abundance is detected through a related molecule (8). These detection processes occur in both central and peripheral tissues in fish. At the central level, the brain integrates metabolic information related to nutrient availability, satiety/hunger signals and hormones related to adiposity. As a result of such signal integration, a response is generated in peripheral tissues aiming to modulate the metabolism (28, 29). At the periphery, metabolic regulation by the sensing systems occurs directly or indirectly through the action of endocrine effectors (28).

HORMONES REGULATING FOOD INTAKE

The regulation of food intake involves the integration of exogenous and endogenous factors to supply the energy necessary to support biological processes. Such regulation is achieved by the endocrine system, which secretes hormones and regulates the activity of the cells by transferring information between the organs. The major organs that secrete hormones involved in regulating appetite are the brain (hypothalamus) and gastrointestinal tissues. The following section will provide a brief description of the main hormones with a critical role in feeding regulation.

Brain Hormones/Neuropeptides

Neuropeptide Y (NPY)

Neuropeptide Y, a 36 kDa amino acid protein, belongs to the NPY family of peptides, which also includes the pancreatic polypeptide (PNP) and peptide tyrosine (PYY). All these peptides share a common three-dimensional structure composed of polyproline coil and an amphipathic helix. The structure of the neuropeptide Y family is tightened by hydrophobic interactions between prolines and the helix (30). NPY is mainly secreted by the neurosecretory cells in the hypothalamus and is abundantly expressed in the brain, pituitary, spleen, gastrointestinal tract, kidney, testis and smooth muscles (31, 32). In teleosts, NPY has been shown to play an essential role in stimulating feeding [see Volkoff (33) for review].

Agouti Related Protein (AgRP)

AgRP is a 128 amino acid neuropeptide released by the NPY/AgRP neurons, and is an endogenous antagonist of melanocortin receptors 3 and 4 (MC3R and MC4R) (34). It is mainly expressed in the brain, but it is also found in several peripheral tissues, including ovary, muscle and ventral skin (34). AgRP receptor is highly localized at the site of the paraventricular nucleus, the dorsal motor nucleus of the vagus nerve and also in the raphe nucleus, all areas that are highly involved in energy homeostasis. AgRP acts as an orexigenic factor in fish, by antagonizing the activity of MC4R (33).

Proopiomelanocortin (POMC)

POMC is a 267 amino acid peptide secreted by the hypothalamic neurons located in the arcuate nucleus, as well as the corticotropic cells of the anterior pituitary, the melanotropic cells of the pars intermedia and skin melanocytes (35). In vertebrates, precursors of POMC has three domains, namely N-terminal pro-γ-melanocyte stimulating hormone (MSH), adrenocorticotropic hormone (ACTH) and C-terminal β-lipotropin, which are cleaved by the action of prohormone convertases. The most important of these derivatives are α-MSH, which plays a vital role in suppressing feeding by acting as an agonist at the anorectic MC4R (33), and ACTH, which regulates the secretion of glucocorticoids from the adrenal glands (36, 37).

Cocaine- and Amphetamine-Regulated Transcript (CART)

CART was isolated from rat striatum upon injection of cocaine and amphetamine, two psychomotor stimulants (38). In goldfish, two forms of CART precursor exist, namely, CARTI that encodes a 117 amino acid pro-CART, and CART-II which encodes a 120 amino acid pro-CART (39). Both CART precursors have been reported to be abundantly expressed in brain, pituitary and also in other peripheral tissues such as eye, interrenal tissues, and gonads in goldfish (39). CART exerts multiple physiological functions in fish, including the inhibition of appetite (39), regulation of the stress response (40) and energy balance (41).

Orexins

Orexins/hypocretins consist of two orexins, orexin-A, and orexin-B, both cleaved from the same precursor, prepro-orexin (42, 43). In fish, both the prepro-orexin RNA and the peptides A and B have been shown to be abundant in the hypothalamus (44), as well as in the gastrointestinal tract (33). Two heptahelical G-protein coupled receptors are known to mediate orexin functions. Orexins have been reported to have a significant role in increasing feeding behavior and locomotor activity (33), and they were also

TABLE 1 | The role of principal macronutrients in fish metabolism.

Nutrient	Major role in fish	Importance for fish metabolism	References
Carbohydrates (CHO)	Stored as glycogen that can be mobilized to satisfy energy demands when necessary. In fish, CHO seems to play only a minor role compared to lipids and proteins. Fish are in general unable to rapidly lower circulating glucose levels following a glucose load or a high CHO meal, thus leading to the interpretation that fish are glucose-intolerant.	Great importance to the metabolism of all fish species, since they act as an oxidative substrate to some cells and tissues. Control of glucose levels exists in tissues relying on glucose such as the brain. Numerous studies carried out in recent years have also demonstrated the existence of glucose sensing mechanisms in such tissues.	(7, 11–14)
Lipids	Storage and provision of metabolic energy in the form of ATP provided through the β-oxidation of fatty acids. They also play an important role as precursors for the synthesis of many hormones and in the formation of cell membranes.	Together with their constituent fatty acids, they are the main nutrients playing important functions as sources of metabolic energy for growth and reproduction. In fish diet, they are particularly important the *n*-3 and *n*-6 unsaturated fatty acids ("omega 3" and "omega 6"), which are not synthesized by animals, and therefore must be supplied in the diet.	(15–21)
Proteins	Amino acids are an essential component of the diet of all animals. Fish require a balanced combination of the 20 amino acids, of which 10 are not synthesized by fish and adequate amounts must be provided through their diet. These essential amino acids are methionine, arginine, threonine, tryptophan, histidine, isoleucine, lysine, leucine, valine and phenylalanine.	Proteins and amino acid requirements differ between fish species since they develop different roles. Some proteins are enzymes, catalyzing a wide range of chemical reactions; other proteins have essential functions in muscle contraction, the transport of specific molecules or as structural elements. In carnivorous fish, proteins also have an important role as a source of energy.	(9, 21, 22)
Vitamins	Are necessary for normal fish growth and health. They are usually not synthesized by fish and must be supplied in the diet. Vitamin deficiency results in scoliosis, dark coloration and/or, most commonly, reduction in growth rate.	Are highly important in the proper functioning of the metabolism since some of them are enzymatic co-factors.	(20)
Minerals	Fish can absorb many minerals directly from the water through their gills and skin, allowing them to compensate in some degree, mineral deficiencies in their diet. Some are incorporated into the bones, while others have a major function in acid-base balance, electron transfer or the maintenance of cell homeostasis.	They can be divided into two groups based on the quantity required in the diet. Macro-minerals such as sodium, chloride, potassium or phosphorous, regulate osmotic balance and are integrated into the skeletal structure. Micro-minerals are required in small amounts as components in enzyme and hormone systems.	(20, 21, 23)

implicated in the regulation of sleep, energy homeostasis and circadian cycle (45).

Melanin-Concentrating Hormone (MCH)

Melanin-concentrating hormone (MCH), a 17 amino acid cyclic peptide, was initially isolated from the brain of chum salmon (46). Two genes, MCH1 and MCH2, have been identified in zebrafish and pufferfish (47). The MCH receptor was identified as the GPCR SLC-1, later termed as MCH-R1. MCH-R1 couples to different G-proteins and plays an essential role in activating different signaling pathways. The MCH-R1 is preferentially expressed in the brain, particularly in the hypothalamus, areas of the cortex, arcuate and ventromedial nuclei, and olfactory lobes. MCH mainly acts on the melanophores regulating the color change and also lightens the skin in fish. Besides this, MCH seems to have a role in the regulation of feeding, although such a function is still unclear in fish [see Volkoff (33) for review].

Nesfatin-1

Nesfatin-1 is an 82 amino acid peptide originally isolated from the rat hypothalamus and is encoded in the nucleobindin-2 (NUCB2) gene (48). It is proposed that NUCB2 is cleaved by the prohormone convertases into three different peptides, namely nesfatin-1 (82 amino acids), nesfatin-2 (85–163 amino acids) and nesfatin-3 (166–396 amino acids), respectively. Of all these peptides, nesfatin-1 has been shown to have biological activity. The 30-amino acid mid-segment of nesfatin-1 is considered as the bioactive core and has been shown to affect appetite, the hypothalamus-pituitary-ovarian axis, and to modulate intracellular $Ca2^{+}$ signaling in mammals (49). Among non-mammals, nesfatin-1 has been studied in various fish species, including goldfish (50), Ya-fish (51), and rainbow trout (52). In goldfish, nesfatin-1-like immunoreactivity has been found in the hypothalamus, particularly in the nucleus lateralis tuberis (NLT) (50). Exogenous administration of nesfatin-1 has been shown to cause anorectic actions in goldfish (50).

Hormones Primarily Arising From Peripheral Tissues

Ghrelin

Ghrelin has been identified in numerous fish species. It consists of 28 amino acids in mammals and has 12 to 25 amino acids in fish depending on the species. The gene encoding the protein was identified within the chromosome 3 and consists of four exons. Ghrelin exerts its physiological functions by binding to the growth hormone secretagogue receptor-1a (GHS-R1a/ghrelin receptor) (53). This peptide is mainly released in the stomach

(or its equivalent in stomachless species), although other tissues have been shown to synthesize the hormone, particularly the hypothalamus (54). The central role described for ghrelin is its potent orexigenic role (33), but there are other well-known physiological roles for this hormone, including the regulation of GH release from the pituitary, a role in energy balance regulation, and cardiovascular effects, among others (55–57).

Cholecystokinin (CCK)

CCK is a peptide characterized by a C-terminal tetrapeptide sequence. The structure of pro-CCK consists of three sulfated tyrosine residues, which play a crucial role in the activation of CCK receptors (58). CCK binds to two receptor subtypes, CCK-A receptor (CCK1) and CCK-B receptor (CCK2), which are primarily localized at the site of the gastrointestinal tract and the brain (59). In fish, CCK and its cleavage sites suggest that the precursor protein (pro-CCK) is processed into octapeptides and are fully sulfated. CCK plays an essential role in the regulation of feeding, influences digestion and activates satiety signals (60). In goldfish, acute administration of CCK resulted in the suppression of food intake (61), likely by acting on NPY and orexin-A (62).

Peptide YY

Peptide YY, a member of the Y family of peptides, is a 36 amino acid gut-brain hormone that is known to have anorectic actions in goldfish (63). PYY is released from the endocrine cells of ileum and colon (64), and binds to the NPY receptor 1 which is abundantly expressed in the brain and gut of fish. Similar to NPY, PYY plays an essential role in signaling between the enteric nervous system and central nervous system in fish (31, 65).

Glucagon-Like-Peptide-1 (GLP-1)

GLP-1, another anorexigenic intestinal peptide, belongs to the family of glucagon-like peptides encoded in the preproglucagon gene. In fish, the pancreas synthesizes glucagon and GLP-1, while the intestine secretes oxyntomodulin (66), and all of these peptides are processed from the proglucagon in the nervous system and intestine. GLP mRNAs have been identified in several teleosts, and its receptor (GLP-1R) has been successfully cloned in zebrafish and goldfish (67). Apart from reducing food intake, GLP-1 has been involved in gastric emptying and plays an important role in regulating liver glycogenolysis and gluconeogenesis (66).

Leptin

Leptin is a 16 kDa protein encoded in chromosome 7. While mammalian leptin is mainly produced by the adipose tissue, the liver appears to be the main leptin production site in fish (68). The structure of leptin resembles that of growth hormone, belonging to the family of tetrahelical cytokines. In fish, this hormone has been shown to affect adipogenesis (57), and to increase lipolysis while reducing lipogenesis in liver (69). Besides this, leptin decreases food intake in several fish species (33), likely by stimulating the anorexigenic neuropeptides POMC and CART (70).

HORMONAL REGULATORS OF GROWTH

Regulation of growth in fish, as well as in most of the vertebrates, is coordinated by the GH-IGF system (71). A summary of each component of the GH-IGF system as well as their principal function related to growth is presented in this section.

Growth Hormone

Growth hormone (GH) is an endocrine regulator of many physiological processes in vertebrates. In fish, GH is involved in almost all physiological processes including osmotic balance, lipid, protein and CHO metabolism, reproduction and growth. Moreover, studies have indicated that GH also affects behavioral aspects, such as appetite (72) and foraging (73) in rainbow trout and transgenic Atlantic salmon, respectively. In fish, GH is released from the adenohypophysis in response to hypothalamic signals, and exerts its effects on target tissues (74).

Growth Hormone Receptors

In fish cells, GH binds to its receptors GHR-I and GHR-II (growth hormone receptor I and II, respectively) to exert its biological actions (75). GH receptors (GHRs) are widely distributed in tissues, but the primary expression is in the liver (or hepatopancreas). In that tissue, the important response to GH binding its receptors is the release of the insulin-like growth factor I (IGF-I). In other tissues, GHRs also mediate the growth-promoting effects of GH, although the liver is still the place in which GHRs have a significant role in the somatic growth regulation.

Insulin-Like Growth Factors

As GHRs, IGF-I and IGF-II are expressed in several tissues, but the main expression is in fish liver. Both factors play a key role in the promotion of cellular proliferation and differentiation in vertebrates (76–78). These and other biological functions of IGFs are mediated by binding to specific transmembrane receptors, present in fish as well as in mammals (79). Apart from growth, IGF-I has also been associated with fish metabolism, development, reproduction and osmoregulation in seawater (74). The IGF-II mRNA has been detected in the liver as well as in the brain, heart, kidney, gills, gastrointestinal tract, pancreatic islets, skeletal muscle and gonads of fish (74). The widespread gene expression of *IGF-II* detected by RT-qPCR in both juvenile and adult fish contrasts the findings in mammals, in which its expression seems to be relevant only during the early stages of development (80). A role in metabolism regulation by IGF-II was demonstrated in muscle cells from trout (81), indicating that this factor could act as a metabolic hormone in fish. There is evidence that GH regulates *igf-II* gene in all tissues in fish (82–84), while GH most likely regulates only the expression of the *igf-I* gene in vertebrates. This situation makes fish an excellent model to study species-specific differences in the growth system.

NUTRITIONAL REGULATION OF HORMONES MODULATING FOOD INTAKE IN FISH

Feeding

The nutritional status is an important modulator of appetite-regulating hormones in fish. In this respect, several central and peripheral appetite regulators are affected by a single meal, showing periprandial fluctuations in their expression and/or secretion levels. Brain hormones showing such changes in fish include NPY (85, 86), orexin (87–90), CART (87, 88, 91, 92), and nesfatin-1 (93). In general terms, appetite-stimulating or orexigenic factors, including NPY (85, 86), and orexin (89, 90), have been shown to display periprandial changes characterized by higher expression levels before or at mealtime, and lower levels post feeding, suggesting that they are hunger signals. By contrast, appetite inhibiting or anorexigenic signals, such as CART (91, 92), were found to be mainly upregulated after a meal, thus acting as a postprandial satiety signal.

As for peripheral appetite-regulating hormones, the most studied in terms of periprandial fluctuations have been ghrelin, CCK, PYY, and leptin. Circulating levels of ghrelin (in its acylated form) were found to rise pre-prandially in goldfish (94), supporting the role of acyl-ghrelin as a meal initiator in this teleost. Consistently, Unniappan et al. (95) described a significant decrease in total ghrelin in circulation after a meal in goldfish, although this postprandial decrease in plasma ghrelin levels seems to be species-specific as it was detected neither in rainbow trout (96) nor in Mozambique tilapia (97). Ghrelin has also been shown to display periprandial fluctuations in terms of gene expression, although different profiles have been described depending on the species [Atlantic cod, (90); gibel carp, (98); goldfish, (94, 95); Mozambique tilapia, (97, 99); *Schizothorax prenanti*, (100); zebrafish, (101)]. The periprandial changes of CCK have been only studied in terms of its mRNA expression. Although species-specific changes were observed (88, 89), periprandial variations of *cck* expression are mainly characterized by an increase in mRNA levels after a meal in the intestine and/or the hypothalamus [dourado, (87); *Schizottorax prenanti*, (102)]. A similar periprandial profile was described for *pyy* in the hypothalamus of the Mexican blind cavefish (89) and *Schizottorax prenanti* (103), in the brain of goldfish (63), and in the brain and gut of Siberian sturgeon (104). Finally, leptin seems only to vary postprandially, although such variations are species- and tissue-specific. Thus, postprandial increases in *leptin* mRNA expression has been observed in the brain and liver of the mandarin fish (105), in the liver of goldfish (106) and in the visceral adipose tissue of the Atlantic salmon (107), but not in the brain of goldfish (106), the brain and intestine of pacu (88), or the liver of *Schizothorax prenanti* (102).

Food Deprivation

Food deprivation has been shown to regulate the secretion and expression of appetite-regulating hormones. **Table 2** includes a summary of the available studies within the literature and shows the main effect of fasting periods of different duration on the circulating levels and the mRNA expression of the main appetite regulators in several fish species. In general terms, as expected, fasting has been found to upregulate the levels of orexigens and decrease the levels of anorexigens, but several exceptions have been observed depending on the duration of the fasting period, the tissue analyzed and the species (see **Table 2** for results and references). In general an upregulation of orexigens and the GH-IGF system should result in an increase in growth. However, without a complete profile of the redundant endocrine milieu, such conclusions are not valid. In addition, a major limitation of many of the studies is that only mRNA expression was determined. Without understanding more about the peptide synthesis in its major tissue sources, and its levels in circulation, it is difficult to reach conclusions on the effects of nutrient status on these hormones.

Diet Composition

Another nutritional aspect influencing the appetite-regulating hormones is the composition of diets. This is of great importance as there is significant interest in fisheries and aquaculture in modulating fish growth and reproduction by altering diet and/or endocrine milieu. Therefore, it is important to understand the dietary regulation of hormones, as they have remarkable effects on both reproduction and growth. Although the literature available on this is not very large, several studies have described that altering the macronutrient (i.e., carbohydrates, proteins, and fat) composition of the diet has significant effects on the secretion and/or expression of appetite-regulating hormones in fish.

Carbohydrates (CHO)

Few studies have been performed in fish describing the effects of CHO on appetite regulators. In 2002, Narnaware and Peter described that feeding goldfish CHO-enriched diets significantly reduces NPY mRNA levels in brain areas (175). Additionally, this macronutrient has been shown to upregulate *preproghrelin* mRNA expression in the pituitary (176). An *in vitro* assay performed to test the effects of glucose on the expression of appetite-regulating hormones revealed that exposure of goldfish intestinal fragments to different concentrations of this monosaccharide leads to a downregulation of *nucb2/nesfatin-1* mRNAs and an upregulation of *preproghrelin* mRNA expression (177).

Proteins

High-protein diets also modulate important appetite-regulating hormonal systems. A study performed in sea bream revealed that fish fed on high protein diets has higher mRNA expression levels of *preproghrelin* and *cck* than those fish fed on diets containing a lower amount of protein (178). In goldfish, feeding diets enriched in this type of macronutrient results in a significant increase in *nucb2/nesfatin-1* expression in the pituitary, and a significant decrease in *nucb2/nesfatin-1* mRNAs in the gut and in *preproghrelin* mRNAs in the liver (176). Accordingly, expression of both *nucb2/nesfatin-1* and *preproghrelin* was reduced by direct exposure of goldfish intestinal and hepatopancreas fragments to L-tryptophan (177). Very recently, Volkoff et al. demonstrated that the replacement of dietary fish protein with soy protein does not produce major changes in the expression of *cart*, *orexin*, *cck*,

TABLE 2 | Effects of fasting periods of different duration on the circulating levels and the mRNA expression of the main appetite-regulating hormones in several fish species.

	Short-term fasting (1–6 days)			**Mid-term fasting (7–29 days)**			**Long-term fasting (≥30 days)**		
	Circulating levels	**Brain expres.**	**Periph. expres.**	**Circulating levels**	**Brain expres.**	**Periph. expres.**	**Circulating levels**	**Brain expres.**	**Periph. expres.**
NPY		Atlantic salmon = (108) Blunt snout bream ↑ (109) Goldfish ↑ (110)	Blunt snout bream = (109)		Blunt snout bream ↑ (109) Brazilian flounder ↑ (85) Coho salmon ↑ (111) Cunner ↓ (112) Nile tilapia ↑ (113) Platyfish = (114) *Schizothorax prenanti* ↑ (115) Tiger puffer ↑ (116) Winter flounder ↑ (117) Winter skate ↑ (118) Yellowtail ↑ (119) Zebrafish↑ (120)	Blunt snout bream = (109)		Rainbow trout = (121)	
AgRP		Atlantic salmon ↓ (108) Goldfish ↑ (34) *Schizothorax prenanti* ↓ (122) Sea bass ↓ (123)			Common carp ↓ (124) Goldfish ↑ (34) *Schizothorax prenanti* = (122) Sea bass ↑ (123) Zebrafish ↑ (125)			Rainbow trout = (121)	
POMC		Flatfish ↑ (126)			Goldfish = (127) Rainbow trout = ↑ (128) Zebrafish = (125) Zebrafish ↓ (129)			Rainbow trout ↑ (121)	
CART		Atlantic salmon ↓ (108) Catfish ↓ (130) Dourado = (87) Goldfish ↓ (39) Siberian sturgeon ↑ (92) Zebrafish ↓ (131)			Atlantic cod ↓ (132) Channel catfish ↓ (133) Common carp ↓ (124) Cunner ↓ (112) Goldfish ↓ (134) Pacu ↓ (88) Platyfish ↓ (114) Red-bellied piranha ↓ (135) *Schizothorax prenanti* ↓ (91) Siberian sturgeon↑ (92) Winter flounder ↓ (117) Winter skate = (118)			Rainbow trout = (121)	
Orexin		Dourado ↑ (87) Zebrafish = (136)			Blind cave fish ↑ (89) Cunner ↓ (112) Goldfish ↑ (134) Pacu ↑ (88) Platyfish ↑ (114) Red-bellied piranha ↑ (135) Zebrafish ↑ (136, 137)				
MCH		Starry flounder ↑ (138)			Atlantic code ↑ (139) Barfin flounder ↑ (140) Hammerhead shark = (141) *Schizothorax prenanti* ↑ (142) Winter flounder ↑ (143) Zebrafish ↑ (144)				

(Continued)

TABLE 2 | Continued

	Short-term fasting (1–6 days)			Mid-term fasting (7–29 days)			Long-term fasting (≥30 days)		
	Circulating levels	**Brain expres.**	**Periph. expres.**	**Circulating levels**	**Brain expres.**	**Periph. expres.**	**Circulating levels**	**Brain expres.**	**Periph. expres.**
Nesf-1		Zebrafish = (93)	Goldfish ↑ (50) Ya-fish ↑ (51) Zebrafish = (93)		Goldfish ↓ (50) Ya-fish ↓ (51) Zebrafish ↓ (93)	Goldfish ↑ (50) Ya-fish ↑ (51) Zebrafish ↓ (93)			
Ghrelin	Atlantic salmon ↑ (145) Tilapia = (97)	Blunt snout bream ↑ (109) Grass carp ↑ (146) *Schizothorax prenanti* = (100) Zebrafish = (147) Zebrafish ↑ (148)	Atlantic salmon ↑ (149) Atlantic salmon ↓ (145) Blunt snout bream ↑ (109) Chinese perch ↓ (150) Gibel carp = (98) Grass carp ↑ (146) *Schizothorax davidi* ↑ (151) *Schizothorax prenanti* = (100) Sea bass = (152) Tilapia = (97) Zebrafish ↓ (153) Zebrafish ↑ (148)	Atlantic salmon = (145) Goldfish = (93) Hybrid striped bass ↑ (154) Rainbow trout = (96) Tilapia = (155) Tilapia ↑ (97)	Blunt snout bream ↑ (109) Goldfish = ↑ (93, 156) Grass carp ↑ (146) Red-bellied piranha = (157) Zebrafish = (147) Zebrafish ↑ (148)	Atlantic salmon = (145) Blunt snout bream ↑ (109) Gibel carp ↑ (98) Goldfish ↑ (93, 156) Grass carp ↑ (146) Red-bellied piranha ↑ (157) *Schizothorax davidi* ↑ (151) Sea bass ↑ (152) Tilapia = (97) Zebrafish ↑ (148)			
CCK		Blunt snout bream ↓ (109) Dourado = (87) Grass carp ↓ (158) *Schizothorax prenanti* ↓ (102)	Atlantic salmon = (149) Blunt snout bream ↓ (109) Dourado = (87) Grass carp ↓ (158) *Schizothorax prenanti* ↓ (102) White sea bream ↑ (CCK-1), ↓ (CCK- 2) (159) Yellowtail ↓ (160) Zebrafish ↓ (153)		Blind cave fish = (89) Blunt snout bream ↓ (109) Grass carp ↓ (158) Pacu = (88) Red-bellied piranha = (135) *Schizothorax prenanti* ↓ (102) Winter skate = (118) Platyfish ↓ (114) Cunner ↓ (112)	Blunt snout bream ↓ (109) Grass carp ↓ (158) Pacu ↓ (88) Platyfish ↓ (114) Red-bellied piranha = (135) *Schizothorax prenanti* ↓ (102) Winter flounder ↓ (117) Winter skate ↑ (118)	Cunner ↓ (112)		
PYY		Goldfish ↓ (63) Siberian sturgeon ↓ (104) *Schizothorax prenanti* ↓ (103)	Siberian sturgeon ↓ (104) Atlantic salmon = (149) Yellowtail ↑ (160)		Blind cave fish = (89) Goldfish ↓ (63) Nile tilapia ↓ (113) Red-bellied piranha = (135) Siberian sturgeon ↓ (104) *Schizothorax prenanti* ↓ (103)	Nile tilapia ↓ (113) Red-bellied piranha ↓ (135) Siberian sturgeon ↓ (104)			

(Continued)

TABLE 2 | Continued

	Short-term fasting (1–6 days)			Mid-term fasting (7–29 days)			Long-term fasting (≥30 days)		
	Circulating levels	**Brain expres.**	**Periph. expres.**	**Circulating levels**	**Brain expres.**	**Periph. expres.**	**Circulating levels**	**Brain expres.**	**Periph. expres.**
Leptin		Mandarin fish ↑ (105)	Common carp = (161) Mandarin fish ↑ (105) *Schizothorax prenanti* ↓ (102)	Fine flounder ↑ (162, 163) Rainbow trout ↑ (164) Tilapia ↑ (1)	Goldfish = (106) Orange-spotted grouper = (165) Pacu = (88) Red-bellied piranha = (157) Tilapia = (166)	Common carp = (161) European sea bass ↑ (167) Goldfish = (106) Green sunfish ↓ (168) Orange-spotted grouper ↑ (165) Pacu = (88) Rainbow trout ↑ (169) Red-bellied piranha ↓ (157) *Schizothorax prenanti* ↓ (102) Striped bass ↓ (170) Tilapia ↑ (1) White-clouds Mountain minnow ↑ (171) Zebrafish ↓ (172)	Fine flounder = (163) Rainbow trout = (121)		Arctic charr ↑ (173) Atlantic salmon = (107) Eel = (174) Rainbow trout ↑ (121)

= *levels not altered;* ↑ *levels upregulated;* ↓ *levels downregulated.*

and *leptin* in the pacu (88). Similarly, varying the diet lysine to arginine ratio has been described to not significantly modify the expression of *npy* and *cck* in juvenile cobia (86). Leucine reduced leptin secretion from the adipocytes of food-restricted rainbow trout (57).

Lipids

Intake of fat-enriched diets has been described to reduce the gene expression of *npy* in the goldfish telencephalon-preoptic area (175), and to increase the mRNA levels of *nucb2/nesfatin-1* in the hypothalamus and liver, and of *preproghrelin* in the pituitary of goldfish (176). Treatment of goldfish intestine with different fatty acids *in vitro* revealed that fatty acids, in general, downregulate NUCB2/nesfatin-1 in the intestine, but only the longer and highly unsaturated fatty acids inhibit preproghrelin (177). Jönsson and coworkers showed that rainbow trout fed a normal-protein/high-lipid diet tends to have higher plasma ghrelin levels than those fed a high-protein/low-lipid diet (96). Apart from the above-mentioned studies, which were all carried out in adult fish, few studies have been performed at larvae or post-larvae state to study the effects of the replacement of the dietary fat source on the expression of metabolic hormones. Bertucci and coworkers demonstrated that the replacement of dietary fish oil with sunflower oil leads to a decrease in *nucb2/nesfatin-1* mRNA expression in pejerrey larvae (179). Additionally, it was described that Senegalese sole post-larvae fed with diets containing soybean oil have higher *cart1* and *cck* mRNA levels in the brain, but lower peripheral *cck* levels than larvae fed cod liver oil (180).

NUTRITIONAL REGULATION OF THE GH-IGF SYSTEM AND ITS INFLUENCE ON FISH GROWTH

Nutritional Status

The main environmental factor that regulates the GH-IGF system is the nutritional status (181). **Table 3** summarizes the effects of fasting on the expression of components of the GH-IGF system in different fish species**.** During fasting, growth ceases, and energy is mobilized from tissues to support metabolism. This is mainly mediated by changes in the GH-IGF system: plasma levels of GH generally increase while plasma levels of IGFs decrease (182). These changes are explained by a phenomenon known as liver GH resistance, which is characterized by the fact that hepatocytes become resistant to GH, resulting in decreased IGF production despite elevated GH (197). These changes in GH-IGF system during fasting could be adaptive jn response to the

TABLE 3 | Effects of fasting periods of different duration on the circulating levels and the mRNA expression of the GH-IGF system endocrine components in several fish species.

	Short-term fasting (1–6 days)			**Mid-term fasting (7–29 days)**			**Long-term fasting (≥30 days)**		
	Circulating levels	**Brain/Pit expres.**	**Periph. expres.**	**Circulating levels**	**Brain/Pit expres.**	**Periph. expres.**	**Circulating levels**	**Brain expres.**	**Periph. expres.**
GH	Chinook salmon = (182) Tilapia ↑ (97)			Channel catfish = ↑ (183) Chinook salmon ↑ (182) Coho salmon ↑ (76, 184) Fine flounder ↑ (162) Rainbow trout ↑ (185–187) Tilapia ↑ = (97, 188, 189)	Channel catfish = ↑ (183) *Cichlasoma dimerus* = (190) Crucian carp ↓ (191) Grouper ↑ (192) Tilapia ↑ (188)				
IGF-I	Chinook salmon = ↓ (182) Tilapia ↓ (97)		Chinook salmon = ↓ (182)	Channel catfish ↓ (183) Chinook salmon ↓ (182) Coho salmon ↓ (184) Rainbow trout ↓ (185) Tilapia ↓ (97, 188, 189, 193)		Atlantic salmon = (145) Channel catfish ↓ (183) Chinook salmon ↓ (182) Coho salmon *Cichlasoma dimerus* = ↓ (190) ↓ = (76) Crucian carp ↓ (191) Grouper ↓ (192) Tilapia ↓ (188, 189, 193) Yellowtail = (194)	Brown trout ↓ (195) Masu salmon ↓ (196)		Masu salmon = (196)
IGF-II						Atlantic salmon = ↓ (145) Tilapia = (193)			

= levels not altered; ↑ levels upregulated; ↓ levels downregulated

high GH plasmatic levels together with low levels of IGF-I (and also insulin) induces lipolysis making fatty acids available to peripheral tissues (197). Using this mechanism, fish are capable to usually maintain its internal functions during fasting, avoiding somatic growth.

Studies on the effects of feed quantity are available regarding the GH-IGF system. In general, increased feed ration results in inverse changes in GH-IGF system hormones, compared to fasting (182, 198–200). This state in which plasmatic GH levels are low and IGFs are high is correlated with an increase in the somatic growth rate of fish (200). However, if feed ration size is considerable, plasmatic GH levels are high, and GHRs and IGFs levels remain low. This probably diminishes feed utilization for growth (199).

Diet Composition

CHO

Diet composition is another important factor regulating the GH-IGF system and somatic growth (197). Several works have been carried out in the past years aiming to determine the effect of dietary carbohydrates, especially glucose, on GH in fish. Rodgers et al. (201) demonstrated that when tilapia pituitaries were incubated in the presence of varying glucose concentrations, the quantity of GH released is inversely related to glucose concentration in the culture media. However, Riley et al. (202) found no changes in pituitary GH mRNA or plasma GH in response to intraperitoneal (IP) glucose injection. In the liver, IP glucose treatment significantly elevates the levels of GHSR mRNAs. Although the IGF-I mRNA expression was not

altered by the IP glucose injection, the IGF-I plasma levels were significantly reduced in tilapia (202). In an *in vitro* assay carried out by our group (203), it was found that incubation of goldfish hepatopancreas with different glucose concentrations significantly increases the expression of *ghr-I*, *ghr-II*, *igf-I,* and *igf-II* mRNAs at 4 h. The increase in GHR and GH mRNAs caused by glucose could be related its insulinotropic effect, as such outcomes were demonstrated in mammals (204). IGFs were postulated as regulators of glucose uptake in fish (205), and in mammals (206), likely through the modulation of GLUT-1 glucose transporter (207). GH has been shown to have a hyperglycemic effect in several species of fish, and it is glycogenolytic, glycolytic and gluconeogenetic in some tissues, including the liver, brain, and gill (208). Therefore, IGFs seem to play a role in maintaining the balance between GH and insulin to promote normal carbohydrate metabolism.

Proteins

Dietary protein seems to regulate the hepatic IGF-I expression and secretion, as it was demonstrated in mirror carp (209) and Nile tilapia juveniles (210). In both cases, authors found a significant correlation between dietary protein levels and hepatic IGF-I mRNA expression. Moreover, Qiang et al. (210) found that the increase in the dietary protein content not only increases the IGF-I mRNA expression but also increases the plasmatic IGF-I levels. These authors also reported a significant correlation between the fish-specific growth rate (SGR) and the IGF-I plasmatic levels and/or IGF-I hepatic mRNA expression. In gibel carp (211), it was found that an increase in dietary protein levels leads to an increase in SGR and hepatic IGF-I mRNA levels, although a decrease in both parameters was observed with extremely high protein levels in the diet. Moreover, Tu et al. (211) reported that GH mRNA expression in pituitary shows the opposite trend compared to hepatic IGF-I mRNA. Pérez-Sánchez et al. (199) found that gilthead seabream fed on low protein diets had significantly higher GH, but lower hepatic GH binding and lower IGF-I levels than fish fed on higher protein diets. This situation resembles the one discussed previously in which fish under fasting shows diminished IGFs production and elevated GH. Therefore, low and very high dietary protein levels may influence the GH-IGF axis through the same mechanism as fasting, possibly by direct control of pituitary hormone secretion by circulating nutrients.

Lipids

Dietary lipids have also been shown to modulate fish growth. In several fish species, an increase in the dietary lipid content or a decrease in the protein/lipid ratio was shown to have a negative effect on growth, as reported for the Senegalese sole (212), the turbot (213), and the flounder (214). Although a few studies show the effect of dietary lipids on fish growth performance, there is little knowledge on their effect on the regulation of the GH-IGF system. In Senegalese sole, it was demonstrated that an increase in dietary lipid content increases the hepatic IGF-I mRNA expression, and this was inversely correlated with the somatic growth (215). In pejerrey, an increase from 10 to 21% in the dietary lipid amount generates a decrease in the hepatopancreatic GHR-II mRNA expression, while no changes in somatic growth were found (216). In largemouth bass, the effects of different carbohydrates/lipids (CHO/L) ratios on the GH-IGF system, as well as on the somatic growth, were studied. Authors found that CHO/L ratios from 0.32 to 2.36 significantly upregulate GH mRNA expression and downregulate IGF-I mRNA expression. Higher ratios did not exert any further effects on them. A positive correlation between hepatic IGF-I mRNA levels and specific growth rates with varying dietary CHO/LIP ratios was found (217). All these findings indicate that dietary lipid level can differentially regulate the growth endocrine axis, at least at the transcription level. That can be directly associated with the role of these hormones in regulating lipid homeostasis, and particularly with the direct lipolytic effects of GH and the promotion of tissue growth by IGF-I. Moreover, results presented here also indicate that hepatic IGF-I mRNA as well as its plasma abundance could be a reliable index to assess growth and nutritional fitness.

Several studies have been focused on the effect of fatty acids present in dietary lipids and in the replacement of dietary fish oil by vegetable oil on fish growth. In most of the cases, an increase in the dietary amount of fatty acids from vegetable sources causes an increase in GH mRNA expression and/or circulating levels, while diminishes the IGF-I plasmatic and GHR-I mRNA levels (179, 203, 218). The hepatic mRNA expression of GHR-II and IGF-II seems to be constitutive and not affected by dietary fatty acids in sea bream (218), while in pejerrey the replacement of dietary fish oil by sunflower oil increases their mRNA expression (179). As a general conclusion, large replacements of high-unsaturated fatty acids by low-unsaturated fatty acids in fish diet lead to the GH-IGF system pattern to resemble one observed during fasting.

Micronutrients

The effect of micronutrients on the expression of GH-IGF system components was not extensively studied. To the best of our knowledge there is only one work reporting an effect of vitamins on *igf-I* expression (219). In this, the authors found that 4% of dietary vitamins generate an increase in the IGF-I mRNA expression in sea bass larvae after 38 days of the experiment, compared with diets containing lower or higher amounts.

FUTURE PERSPECTIVES

The role of nutrition as a potent modulator of the endocrine system governing feeding behavior and growth in fish is described in this review. Aquaculture research is a topic of increasing interest due to the demand for sustainable food production. That makes it essential to summarize the knowledge generated in the last past years in order to identify the weak points and to determine the direction in which new studies should be focused. As described here, food availability and feed composition are two of the most influent external signals modulating appetite-regulating and growth modulatory hormones. Future research should, therefore, be focused on tapping into this knowledge, expanding it in depth, and exploring its use to enhance feeding and growth in fish. Such approaches will eventually lead to increased yield in cultured fish.

It is also interesting to note the species-specific differences in the effect of feeding on the expression of appetite-regulating hormones and the GH-IGF system. Such differences could be related to the feeding behavior of specific fish species, as the carnivorous, herbivorous and omnivorous fish tend to fall into three different groups of responses. This fact also reflects the flexibility of metabolic systems, considering that the same components present in all fish species could generate distinct responses from the same food stimuli. Therefore, it is critical to bear in mind that results obtained from one species of fish might not apply to another.

As a final consideration, it would be interesting to broaden our knowledge of the crosstalk between hormones regulating food intake and those regulating growth. For instance, GH and IGF-I not only regulate somatic growth but can at the same time modulate lipid and carbohydrate metabolism, respectively (220). Others, including ghrelin, could stimulate food intake (221), and also GH release from pituitary (222, 223). Likewise, it has been shown that leptin directly regulates the expression of IGFs and GHRs in fish hepatopancreas (224). These are a few examples of the crosslinks mentioned above, that serve to highlight the close interaction that exists between hormones controlling food intake and growth to ensure the proper growth and development of fish. Thus, comprehensive approaches to determine both metabolic and growth regulatory hormone responses to nutritional challenges are more desirable from an aquaculture perspective. Future research must focus to identify gaps in knowledge, including the ones identified above. In addition, the use of alternate feed ingredients, use of endogenous feeding and growth regulatory factors as feed additives, and employing hormones using targeted molecular and cellular approaches should be explored to modulate growth rate and yield in cultured species.

AUTHOR CONTRIBUTIONS

JB prepared larger sections and complied the manuscript. AB, LS, JR, and CV wrote smaller sections. SU provided the idea, outline, provided feedback, and corrected the manuscript.

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REVIEW
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Stress Effects on the Mechanisms Regulating Appetite in Teleost Fish

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The homeostatic regulation of food intake relies on a complex network involving peripheral and central signals that are integrated in the hypothalamus which in turn responds with the release of orexigenic or anorexigenic neuropeptides that eventually promote or inhibit appetite. Under stress conditions, the mechanisms that control food intake in fish are deregulated and the appetite signals in the brain do not operate as in control conditions resulting in changes in the expression of the appetite-related neuropeptides and usually a decreased food intake. The effect of stress on the mechanisms that regulate food intake in fish seems to be mediated in part by the corticotropin-releasing factor (CRF), an anorexigenic neuropeptide involved in the activation of the HPI axis during the physiological stress response. Furthermore, the melanocortin system is also involved in the connection between the HPI axis and the central control of appetite. The dopaminergic and serotonergic systems are activated during the stress response and they have also been related to the control of food intake. In addition, the central and peripheral mechanisms that mediate nutrient sensing capacity and hence implicated in the metabolic control of appetite are inhibited in fish under stress conditions. Finally, stress also affects peripheral endocrine signals such as leptin. In the present minireview, we summarize the knowledge achieved in recent years regarding the interaction of stress with the different mechanisms that regulate food intake in fish.

Keywords: fish, stress, HPI axis, HSC axis, appetite suppression, food intake regulation, hypothalamic integration

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INTRODUCTION

In the last years, a great effort has been made in order to describe the mechanisms that regulate food intake in fish (1–3). The inhibition of food intake is a generic response in fish submitted to acute or chronic stress conditions recovering the appetite when the stressor disappears (4–7). In the complex network that regulates appetite, the hypothalamus acts as a central integrator of the endogenous peripheral and central afferent signals informing about the energetic and nutritional status of the animal combined with the incoming external information (1). In the present mini-review, we aimed to describe the existing knowledge about different mechanisms through which stress interacts with food intake control in fish (**Figure 1**).

STRESS AXIS ACTIVATION AND ITS IMPLICATION ON APPETITE REGULATION

Stress in fish is an adaptive behavior whose final goal is to reach internal homeostasis after an external disturbance. The response to stress in teleost fish starts with the recognition of an external stimulus (harmful or hazardous) promoting the activation of several neuroendocrine pathways affecting fish physiology (8). Two main pathways have been reported in teleost fish that are activated by a stress stimulus, on one side the hypothalamus-sympathetic system-chromaffin tissue (HSC) involved in the immediate release of catecholamines (CA), epinephrine (E), and norepinephrine (NE) to blood stream and, on the other side, the hypothalamus- pituitary-interrenal axis (HPI). Thus, when fish are submitted to stress, the circulatory levels of CA increase quickly and recover the normal values in a short period of time (minutes) (5, 9, 10).

The control of cortisol synthesis under stress is directly caused by the activation of HPI axis (5). After recognition of a stress stimulus by central nervous system (CNS), corticotropin releasing factor (CRF) is released from hypothalamus stimulating the synthesis and release of adrenocortitropic hormone (ACTH) in the corticotrophs cells of the anterior pituirary gland. Once in the blood stream, ACTH reaches the steroidogenic cells in the interrenal tissue inducing the activation of the melanocortin 2 receptor and therefore stimulating the signaling cascade of synthesis and release of glucocorticoids (11–14). Other neuropeptides that play an important role in the activation of the HPI axis are thyrotropin-releasing hormone (TRH) and arginine vasotocin (AVT), produced and released in hypothalamus, and urotensin I (UI) which is produced in the brain and in terminal segments of the caudal neurosecretory system. TRH, AVT, and UI stimulate the production of cortisol in the interrenal cells directly (UI) or indirectly through promotion of ACTH (UI and AVT) and alpha-melanocyte-stimulating hormone (α-MSH; TRH) (15–19).

In order to understand the anorexigenic effect of the activation of HPI axis, some studies were carried out in fish, suggesting that CRF, UI, AVT, proopiomelanocortin (POMC) via α-MSH, and cortisol play an inhibitory effect on food intake (11, 20–24). The anorectic effect of stress seems to be mainly mediated by CRF, which is expressed in the telencephalic area, preoptic area, and tuberal hypothalamus (25, 26), matching up with the areas involved in the regulation of food intake. Moreover, several stress stimulus such as hypoxia, handling or isolation, which have a clear anorectic effect, increase the mRNA expression of CRF in those areas (11). The activation of CRF neurons seems to be mediated by α-MSH (among others) through melanocortin 4 receptor (MC4R). In fact, CRF neurons are innervated by melanocortin terminals (27). Thus, once POMC hypothalamic cells are excited, α-MSH is released from the neuronal terminal to the cleft and binds to MC4R receptor in the preoptic area, where is highly expressed, in line with CRF production. Particularly, stress conditions seems to elevate the levels of α-MSH (28), which is directly related to food intake inhibition (29, 30). This is suggesting that the anorexigenic effect of α-MSH involves activation of CRF neurons (30, 31).

In addition, pharmacological experiments demonstrate that CRF and its neuropeptide counterpart UI evoke an anorexigenic effect in teleost fish. UI and CRF are generally recognized as key regulators of the anorexigenic stress response in vertebrates (11) where central injections of UI elicit an anorexigenic effect even more potent than the effect promoted by CRF treatment (22). Furthermore, the effect of this treatment is blocked by the administration of an antagonist of CRF and UI receptor suggesting that these neuropeptides are directly related to the food intake inhibition (32, 33). In addition to this, CRF and UI regulate the presence of ACTH, which works downstream of the melanocortin system (27) and is the main promoter of cortisol release (16, 34, 35). It has been reported that chronic and acute cortisol treatments exert a potent anorexigenic effect in fish (36, 37). Furthermore, cortisol seems to be also involved in maintaining the anorectic response in teleost, with specific glucocorticoid receptors acting as mediators (38). In contrast, in some fish species as goldfish, low levels of cortisol administration have a stimulatory effect on food intake (39) suggesting a different effect of cortisol depending on the species and/or stress severity.

MONOAMINERGIC SYSTEMS UNDER STRESS AND THEIR EFFECTS ON FOOD INTAKE

When fish are submitted to a stress stimulus, several neuronal networks are altered. For instance, serotonergic and dopaminergic systems are fast activated as a consequence of the stress (9). Experiments carried out in rainbow trout demonstrated that the monoaminergic activity in the brain is altered under different kind of stress. For instance, episodes of acute stress stimulated the monoaminergic activity in the forebrain (9, 14). Additionally, some studies evidenced the anorexigenic effect of serotonin and dopamine by using agonist/antagonist of their respective receptors or by stimulation of the monoaminergic activity (4, 40–43).

Moreover, treatments that increase the serotonergic activity also increase the expression of POMC and decrease the expression of neuropeptide Y (NPY) and agouti-related peptide (AgRP) (32, 40, 44). Particularly, the activation of hypothalamic serotonin $5HT_{2c}$-like receptors (theoretically located in POMC neurons) has an inhibitory effect on food intake in rainbow trout (22, 40, 45).

On the other side, central administration of dopamine induced a decreased gene expression of AgRP whereas there was no effect on POMC and NPY gene expression (43). In the same way, oral treatments with L-Dopa (which is the dopamine precursor) induced an increase of NPY expression but no effects were observed for AgRP and POMC expression in sea bass hypothalamus whereas food intake was inhibited by this treatment (4). These results suggest that the inhibitory effect of dopamine on food intake is not mediated by these hypothalamic neuropeptides. However, the presence of dopamine in fish

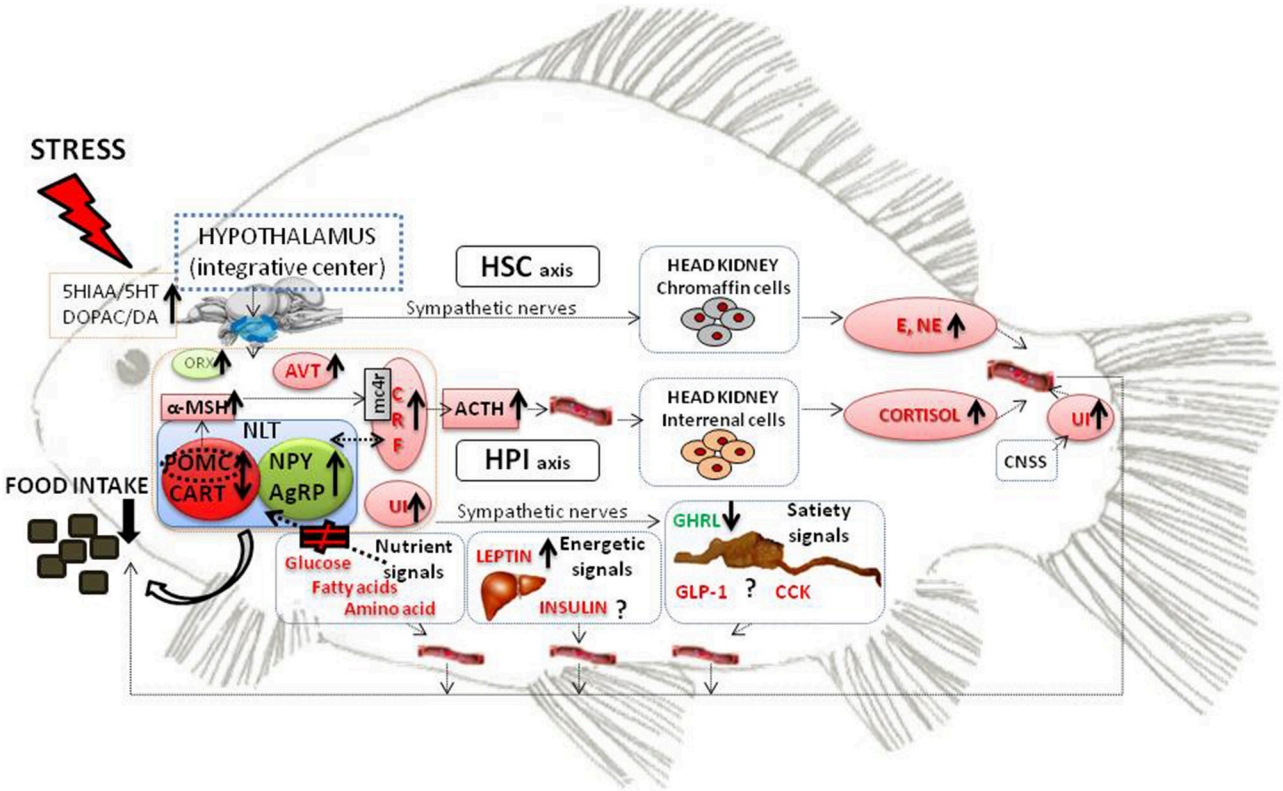

FIGURE 1 | Schematic model representing the main components of the HPI and HSC axis that are activated during the physiological stress response and could be involved in the reduced food intake response under stress conditions. The main factors involved in the homeostatic control of food intake in teleost fish are also represented. Black thick arrows indicate enhanced or decreased levels under stress conditions. Under stress conditions, there is an increase of the dopaminergic and serotonergic activity in the fish brain. HPI and HSC axis are activated leading to the increase of several components with known anorexigenic properties (in red). On the other hand, orexigenic components (in green) are increased (neuropeptides NPY and AgRP) or decreased (ghrelin) under stress conditions. Furhermore, nutrient signals are not properly sensed by the hypothalamus. All this information is integrated in the hypothalamus resulting in decreased food intake. ACTH, adrenocortitropic hormone; AgRP, Agouti-related peptide; AVT, arginine vasotocin; CART, cocaine- and amphetamine-related transcript; CCK, cholecystokinin;CNSS, caudal neurosecretory system; CRF, corticotrophin releasing factor; DA, dopamine; DOPAC, 3,4-dihydroxyphenylacetic Acid; E, epinephrine; GHRL, ghrelin; GLP1 glucagon like peptide 1; HPI, hypothalamus- pituitary-interrenal axis; HSC, hypothalamus-sympathetic system-chromaffin tissue; NE, norepinephrine; NLT, nucleus lateral tuberal; NPY, neuropeptide Y; ORX, orexin; POMC, pro-opio melanocortin; UI, Urotensin I; 5HIAA, 5-hydroxyindole-3-acetic acid; 5HT, serotonin.

hypothalamus induces an up regulation of CRF expression, suggesting that the effect of the activation of dopaminergic system under stress conditions on food intake regulation could be mediated by hypothalamic CRF neurons (4).

Furthermore, fish that were previously adapted to a fixed feeding schedule and then subjected to a 7 days fasting period showed increased mRNA abundance of TPH (tryptophan hydroxylase) and TH (tyrosine hydroxylase), which are limitant enzymes of 5HT and DA synthesis. This effect might relate to a feeding/searching behavior instead of the effect of both monoamines on food intake regulation (46).

THE EFFECT OF STRESS ON NUTRIENT SENSING MECHANISMS CONTROLLING FOOD INTAKE

Nutrient availability can be detected in specialized cells of the organism that possess mechanisms to detect changes in nutrient levels (47). In the last years, nutrient sensing mechanisms have been described in central and peripheral areas of fish, particularly rainbow trout, responding to changes in levels of glucose (48, 49), fatty acids (50), and amino acids (51). The activation of these systems in central areas, especially hypothalamus, relates to the control of food intake through changes in the gene expression of appetite-related neuropeptides (49–53). These nutrient sensing mechanisms are in some way inhibited under stress conditions in fish. Thus, rainbow trout submitted to chronic stress by high stocking density do not respond properly to different glycemic conditions in terms of food intake levels and in the activities/values of parameters involved in glucosensing activity (49, 54). In response to stressors, hypothalamic glucosensing is muted and the regulation of appetite becomes independent of circulating glucose levels. In addition, the decreased gene expression of orexigenic peptides such as NPY and the increased gene expression of anorexigenic factors such as cocaine and amphetamine-related transcript (CART) and POMC observed in hyperglycaemic control fish

is not observed in hyperglycaemic fish submitted to crowding stress (52). Furthermore, the expression of these appetite-related neuropeptides in normoglycemic stressed fish are not correlated with a decreased appetite (which would correlate with an increased anorexigenic/decreased orexigenic potential). Altogether, these findings would suggest that the effect of stress on the inhibition of food intake is more related to the loss of the central glucosensing capacity than to a direct regulation of the expression of appetite-related neuropeptides (49, 52). However, the expression of CRF increased in stressed fish irrespective of the glycemic condition (52). In addition, the increase in CRF induces changes in the main parameters related to the glucosensing capacity in rainbow trout hypothalamus. These results suggest that CRF could be involved in the inhibition of the hypothalamic control of food intake by glucosensing mechanisms under stress conditions (55). Stress may also relate to fatty acid sensing mechanisms regulating appetite in fish (47). It has been reported in rainbow trout that the counter-regulatory response to a induced decrease of circulating fatty acid levels is associated with the activation of HPI axis (56, 57). As for the possible effect of stress in the control of food intake exerted by amino acid sensing mechanisms there are no available studies in fish yet.

Moreover, in the last recent years, the regulation of food intake by nutrient sensing mechanisms was reported in fish hypothalamus to be mediated by transcription factors like phosphorylated cAMP response-element binding protein (CREB), forkhead box01 (Fox01), and/or brain homeobox transcription factor (BSX), which would induce variations in the anorexigenic/orexigenic potential in accordance with the presence of nutrients (3). Since stress is known to modulate the expression of the main neuropeptides involved in food intake control in fish, an effect of stress on these transcription factors in fish may not be discarded and clearly deserve to be assessed.

INTERACTION OF STRESS WITH ENDOCRINE APPETITE-RELATED SIGNALS

In mammals, insulin and leptin are considered adiposity signals. These peripheral hormones are released to the bloodstream in a way proportional to the white adipose tissue abundance, thus informing the brain about the long-term energetic status of the organism and inducing anorexia (58). In fish, despite this "lipostatic theory" is not so evident since these hormones are more related to glucose homeostasis (59), they have an anorectic effect (60, 61).

Under stress conditions, energy reserves are mobilized to cope with increased metabolic demand, and, therefore, energetic signals related to appetite may be affected. Few studies are available in fish addressing the effect of stress on these energy signals.

Although the role of insulin in food intake regulation in fish is not entirely clear (2), evidence exists regarding an anorexigenic effect of this hormone. Thus, intraperitoneal injection of insulin in rainbow trout decreased food intake whereas isletectomy in goby induced hyperphagia (2). In mammals, it is known that increased glucocorticoid levels results in elevated insulin levels, which leads to Cushing's syndrome associated with type-2 diabetes (62–64). However, there are no available studies in fish regarding the interaction between stress and insulin.

As for leptin, in spite of the low conservation of its gene sequence among animal groups, a similar role of this hormone as energy modulator through mobilization of energy stores during the stress response has been reported in fish as in mammals (60, 65). It has been suggested a synergistic regulation of cortisol and leptin release, with cortisol inducing the leptin release, which in turn elicits the mobilization of energy reserves by stimulating glycogenolysis and gluconeogenesis (60). In fish, elevated cortisol levels induced an increase in hepatic mRNA levels of leptin (37). In turn, ACTH-induced cortisol and ACTH released by CRF induction decrease in the presence of leptin (21, 37, 60, 66). Under hypoxia, mRNA levels of leptin and its receptors in common carp liver increase while food intake decrease, suggesting that leptin is involved in food intake regulation and in the endocrine effects of the stress response (21).

Some studies reported that stress decreases the secretion and availability of the hormones related to the growth hormone (GH)-insulin growth factor-1 (IGF-1) system, the main endocrine growth regulator with implications on food intake regulation (67–69). Thus, the decrease in plasma GH levels has been related to the initial increase of cortisol levels under stress conditions (68). On the other hand, IGF-I levels decreased in plasma of Atlantic salmon exposed to stressful elevated temperatures (69).

Other endocrine signals related to regulation of food intake in teleost fish are also affected by the presence of a stressor. Thus, Pavlidis et al. (70) reported increased levels of orexin (an orexigenic hormone) in high-grade long-term stressed fish, but this hormone seems related to promotion of wakefulness and activity in the presence of stress (70, 71).

In general, ghrelin has been reported to be orexigenic in fish as is in mammals (72–74) by stimulating the expression of NPY and orexin (73, 74). However, the effect of ghrelin on food intake regulation in fish is controversial and depends on fish species, size or via of administration. Thus, some studies reported that ghrelin exerted an anorexigenic effect in fish probably through interaction with CRF neurons (75). In mammals, central ghrelin may play a role in the regulation of food intake during stress (76). In fish, the presence of elevated cortisol levels resulted in a decrease in ghrelin levels in plasma of rainbow trout (77) and tilapia (78). However, in the presence of stress, increased mRNA abundance of ghrelin and its receptors were observed in brain areas of tilapia (79). Ghrelin has been suggested to mediate the decrease in food intake levels in fish under prolonged stress conditions. Moreover, there is not a relationship between ghrelin levels and food intake when fish are submitted to a brief stress, suggesting that other central mechanisms may regulate appetite under short-term stress conditions (79). Accordingly, Cortés et al. (80) reported increased levels of ghrelin mRNA in

zebrafish brain after handling stress, suggesting that ghrelin is not mediating the anorexigenic effects of stress or that a rapid counterregulation may occur in response to this anorexigenic effect since increased levels of mRNA of NPY and AgRP were found in hypothalamus in parallel (80). Finally, other appetite-related hormones like cholecystokinin, peptide YY and glucagon-like peptide 1 have anorexigenic properties in fish (1–3) but their interaction with stress response has not been studied yet.

EFFECT OF STRESS ON THE HYPOTHALAMIC INTEGRATION OF THE APPETITE-RELATED SIGNALS

The appetite related signals arriving to the brain are integrated by the hypothalamus which responds in consequence by delivering orexigenic or anorexigenic neuropeptides that eventually induce a decrease or increase in food intake (3). As in mammals, the hypothalamus of fish appears to contain two neuronal populations, AgRP/NPY neurons whose activation resulted in increased food intake (orexigenic potential) and POMC/CART neurons whose activation resulted in decreased appetite (anorexigenic potential). There are several studies in fish addressing the response in hypothalamus of these neuropeptides under stress conditions. Thus, in the presence of stress, increased mRNA levels of hypothalamic NPY were reported in zebrafish submitted to handling stress (80) and rainbow trout under high stocking density (38, 52) or no changes were found in tilapia under both crowding and handling stress (79). In the same way, increased mRNA levels of NPY were found in goldfish after cortisol administration (39), in accordance with that reported in mammals where glucocorticoid treatment increase NPY levels (58). Furthermore, in other brain areas, NPY is also altered under stress conditions. Thus, 24 h treatment with cortisol reduced NPY mRNA abundance in telencephalon but not in hypothalamus of tilapia (78) whereas increased NPY occurred in preoptic area and forebrain in socially subordinate rainbow trout (81). In the case of AgRP, its gene expression increased 15 min after acute handling stress in zebrafish (80). However, these results regarding NPY/AgRP abundance are in contrast with the decreased food intake during stress suggesting that other neuronal population should be mediating the anorectic effect of stress (80). This might relate to the connection of NPY neurons with CRF neurons (39).

Regarding the anorexigenic peptides, CART and POMC mRNA levels are elevated under acute stress conditions in zebrafish (80), but no changes or decreased levels were found in rainbow trout after chronic crowding stress (38, 52). Other studies also found decreased POMC levels in sole under high stocking density (82, 83). These results suggest a complex response depending on the species and the type of stress. Furthermore, the interaction of POMC with stress may be more complex because this peptide is the precursor of other peptides belonging to the melanocortin system that is involved in the activation of the HPI axis as commented previously.

CONCLUSIONS AND PERSPECTIVES

Evidence obtained so far in fish suggest the existence of a complex network through which different components of the stress axis may interact with the mechanisms involved in the regulation of food intake. Despite achievements of recent years, the knowledge regarding these mechanisms is very limited, and basically restricted to the direct actions of cortisol and CRF on neuropeptide expression, the inhibition of glucosensing mechanisms, increased forebrain monoamines, and the interaction with several endocrine systems involved in food intake regulation such as leptin or ghrelin. However, further research is required to address the involvement of the components of the HPI and HSC axis at all levels of food intake control including information arriving from the gastrointestinal tract (impact on chemoreceptors, mechanoreceptors and the flow of information, through nervous system or synthesis and release of gastrointestinal hormones), endocrine systems, and nutrient sensors. Further input is also required to assess the way in which the integration of such information in specific neurons of hypothalamus is altered by different components of the stress axis. Besides elucidation of direct mechanisms, the impact of other factors must be also addressed. These may include the early life stress-effects since it is known in mammals that perinatal stress affects mechanisms regulating food intake at the long term (84). The impact of diet composition in the interaction between food intake control and stress response is also a topic that deserves further research. Finally, almost all studies carried out in fish regarding this subject relate to homeostatic regulation of food intake (3). However, stress might affect not only the mechanisms related to homeostatic control of appetite but also the mechanisms related to hedonic regulation of food intake as in mammals where stress induces increased food intake in animals fed with lipid-enriched meals (85).

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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MINI REVIEW
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Physiological and Molecular Mechanisms of Methionine Restriction

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Methionine restriction (MR) has been studied extensively over the last 25 years for its role in altering metabolic hallmarks of disease. Animals subjected to MR, display changes in metabolic flexibility demonstrated by increases in energy expenditure, glucose tolerance, and lifespan. These changes have been well characterized in a number of model systems and significant progress has been made in understanding how hepatic fibroblast growth factor 21 links MR to several components of its metabolic phenotype. Despite these advances, a complete understanding of mechanisms engaged by dietary MR remains elusive. In this review, we offer a brief history of MR and its known mechanisms associated with stress, metabolism, and lifespan extension. We consider the role of epigenetics in the response of animals to MR and propose a novel epigenetic pathway involving the regulation of microRNAs during MR.

Keywords: microRNA, methionine restriction, stress, physiological, caloric restriction, mechanisms

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INTRODUCTION

Methionine restriction (MR) is known to produce lifespan extension and has been more recently investigated for its role in improving metabolic health. While much is known about the resulting phenotype following MR, a complete picture of mechanisms involved in MRs metabolic phenotype is less clear. At a molecular level, methionine metabolism creates *S*-adenosylmethionine (SAM), which acts as a master methyl donor. Methylation of cytosines in promoter regions of genes can regulate gene expression by controlling binding of transcription factors (1). Changes in methionine availability through dietary MR have the potential to decrease these available methyl donors and thus change transcriptional status of certain genes. One of these potential genes is fibroblast growth factor 21 (FGF21) which has been implicated in the metabolic responses to MR (i.e., increased energy intake and expenditure as well as increased insulin sensitivity) (2, 3). While increases in circulating and hepatic FGF21 are responsible for certain aspects of MRs metabolic phenotype, FGF21$^{-/-}$ mice did not exhibit changes in transcriptional responses to MR in the liver (2). This FGF21 independent mechanism could potentially be linked to epigenetic changes that take place during MR in the liver when there is a reduction in methyl donors.

In this review, we explore the mechanisms of MR that could drive these epigenetic responses. One possible mechanism that has emerged as a factor in MR phenotypes is microRNAs (miRNAs). Studies have shown that MR alters miRNA expression levels in rainbow trout myosatellite cells (4, 5) and bone structures of mice (6), supporting a proposed connection between MR and miRNAs. This review will discuss the history and phenotypes associated with MR, as well as the role for miRNAs in MR phenotypes.

METHIONINE RESTRICTION

Dietary interventions have been studied for many decades in fields from nutrition to healthy aging. The most investigated intervention has been caloric restriction (CR), which has been shown to increase lifespan and health span (7). While CR, defined as a decrease of 30–60% from *ad libitum* feeding, is a well-known and effective treatment, first demonstrated in 1935 (8), compliance is often low. Alternatives to CR such as protein restriction (*de novo* amino acid restriction) (9, 10) have been used in patients with renal failure and more recently in mice as a lifespan extension strategy (11). Following studies on protein restriction, Segall et al. began to explore the effects of long-term tryptophan restriction in rats (12–14). While this proved to be a lifespan extension strategy hypothesized to work by decreasing levels of serotonin in the brain, early mortality of animals was a significant problem and led to abandonment of this strategy (15, 16). Orentreich et al. were the first to propose MR as an alternative therapy to increase lifespan (17). Early on, it was established that the effects seen during MR were not due to *de novo* CR, but rather some other mechanism. Orentreich et al. pair-fed control diets based on amounts consumed by rats receiving MR diets to control for differences in food consumption and size to investigate whether the extension in lifespan was due to CR rather than MR. These pair-fed animals had no impairment in growth, meaning the modest reduction in feed intake by the MR animals did not account for the extension in lifespan. Further investigation revealed that MR animals weighed less but consumed more food per gram than their age matched counterparts (i.e., they were slightly hyperphagic).

To identify whether there is genetic variation in the MR response, Zimmerman et al. evaluated MR diets in three diverse strains of rat; Brown Norway, Sprague-Dawley, and Wistar Hannover (18), and demonstrated comparable increases in median lifespan in all of these strains. Miller et al. were the first to report increased lifespan in (BALB/cJ × C57BL/6J) F1 mice on MR diets. In addition, these MR mice displayed lower amounts of plasma Insulin-Like Growth Factor-1 (IGF-1), insulin, glucose, and thyroxine (T4) (19), suggesting novel markers of an endocrine response. These markers were validated in F344 rats fed an MR diet, where MR rats had lower levels of glucose and leptin with corresponding increases in adiponectin, triiodothyronine (T3), and daily energy expenditure when compared to pair-fed controls (20). In the same study, a separate cohort of MR animals were subjected to an oral glucose tolerance test to test the hypothesis that MR preserves insulin action with age. The MR animals responded in a similar fashion to the oral glucose tolerance test as rats fed a control diet at 0, 23, and 72 weeks, although MR animals showed a marked decrease in area under the curve for insulin at both 23 and 72 weeks, demonstrating a preservation of insulin action with age (20). Furthermore, these rats displayed decreased accumulation of visceral fat that, in combination with the endocrine response, supports MR as a strategy to extend lifespan in rodents.

In addition, data support the role of decreased fat deposition, preserved insulin sensitivity, and disruption of the lipogenic/lipolytic balance in adipose tissue as mediators of MRs metabolic phenotype (20–22). This balance was shown to be distrupted by MR through a cycle of increased lipolysis and increased lipogenesis *in vitro* hypothesized to lead to decreases in adipose tissue in older F344 rats (21). In addition, a study found that young and mature animals, as well as obesity prone Osborne–Mendel rats, on a MR diet exhibited long-term increases in energy expenditure and uncoupling protein-1 (UCP-1) in both brown and white adipose tissue. This change in UCP-1 was also accompanied by decreased leptin and increased adiponectin proposing a remodeling of the adipose tissue during MR (23). Authors suggested that MR acts to increase energy expenditure and decrease fat deposition by lowering metabolic efficiency during the night when increases in lipogenesis typically occur (23). These changes explain the extension of healthspan observed in these animals with a complete mechanism for MRs ability to decrease metabolic efficiency (i.e., hyperphagia with decreased growth) (21–24). A recent study detailed that animals introduced to the diet either postweaning or at 80% of mature size had differential effects of hyperphagia (~50 and ~20% increase in energy intake per unit body weight in juvenile and adults, respectively) indicating that the MR diet has differential effects on energy intake and expenditure depending on age (25).

The work detailed thus far has focused on the metabolic effects of MR in mice and rats, which occupies much of the current literature. However, work has also been done in the unique teleost fish model, the rainbow trout; *Oncorhynchus mykiss*. Methionine requirements of these fish are well-known due to their economically important aquaculture status (26–29) and MR has been studied in part due to the implementation of naturally methionine-deficient plant-based diets. In addition, rainbow trout are naturally glucose intolerant making them a potential model for studying the glucose-intolerant phenotype associated with human type-II diabetes and metabolic syndrome (30). In rainbow trout, MR, paired with carbohydrate-enriched diets, was shown to reduce genes associated with *de novo* fatty acid synthesis and reduce circulating plasma glucose 6 h postprandial (30); however, these results must be interpreted cautiously as the design of the diets included no methionine (i.e., methionine deprivation), but adequate cysteine levels. On the other hand, consistently, juvenile rainbow trout fed a formulated MR diet over 6 weeks displayed transcriptional markers of GCN2/eIF2α activation mirroring the effects observed in mammals (31). Conversely, brood stock females fed a similar MR diet for 6 months prior to spawning had reduced triacylglycerol levels with increased total cholesterol and LDL-cholesterol, opposing the cholesterol lowering effects observed in mammals (32). In addition, 48 h of MR *in vitro* in trout hepatocytes resulted in greater levels of glucose uptake due primarily to an increase in the sodium-glucose transporter 2 (33). While the literature is less extensive on the metabolic effects associated with MR in teleosts, it is clear that there is at least a partial conservation of the MR phenotype.

PHYSIOLOGICAL MECHANISMS OF MR

Lifespan Extension

A proposed mechanism of lifespan extension associated with CR is a decreased rate of mitochondrial reactive oxygen species

generation (mitROS) (34). It has been shown that some long-lived species have markedly lower rates of mitROS production compared to their short-lived counterparts (35, 36). Similarly, animals on a MR diet display lower rates of mitROS production and less oxidative damage of mtDNA in both the heart and liver (37). Previous studies have also established that neither carbohydrate restriction nor lipid restriction can replicate the phenotypes seen in CR, PR, and MR (38, 39). This information leads to the hypothesis that changes in mitROS production during both CR and PR could be attributed in part to MR. MR-induced reductions in mitROS production in the heart occurred primarily at complex I, previously established to be the main complex targeted in CR (40). However, in liver MR reduced mitROS in complexes I/III, while CR appears to reduce mitROS only at complex I (41). It should be noted that the studies done on mitROS production during MR had similar effects at both 40 and 80% MR. Since the time of publication, it has been shown that most physiological effects of MR only occur at 80% (42) so the results must be interpreted cautiously.

An additional potential mechanism in the life span response to MR includes an enhanced capacity for a cellular autophagic response and the concomitant acidification of the vacuole (43). Studies done in *Saccharomyces cerevisiae* (Bakers yeast) utilized three different strains of yeast which varied in their ability to synthesize methionine (100% capability, moderate impairment, no *de novo* synthesis). The authors found that lifespan extension was achieved in both the moderate and devoid strains in normal media with the devoid strain demonstrating decreased survival in media with a 90% reduction in methionine. This extension in lifespan was found along with a sharp increase in autophagy followed by acidification of the vacuoles. Additionally, when key genes essential to autophagy were deleted (ATG 5,7,8) this increase in lifespan was ameliorated (43), providing some support for a relationship between autophagic capacity and lifespan, at least in yeast [full review Ref. (44)], although this mechanism has yet to be investigated in MR mammals.

Metabolic Changes

Two mechanisms related to the sensing of amino acid deficiency may partially explain the metabolic adaptations that take place during MR (**Figure 1**). A decrease in methionine leads to an increase in uncharged tRNAs that can activate the general control non-derepressible 2 (GCN2) kinase (45, 46), leading to metabolic adaptation during MR. However, GCN2 may not be indispensable for the response as metabolic adaptation can also take place through a noncanonical PKR-like endoplasmic reticulum kinase (PERK)/NRF2 pathway (47). Both of these mechanisms are significant for the MR-induced integrated stress response (ISR) that contributes to cellular homeostasis and regulation of the physiological response. They converge through their mutual regulation of eukaryotic initiation factor-2α (eIF2α) phosphorylation and the subsequent effects on translational capacity *via* eIF2 and synthesis of the active full-length activating transcription factor 4 (ATF4). Active ATF4 is defined as an amino acid sensor (48) and master regulator (49) of metabolism central for controlling expression of genes associated with the stress response, including genes related to lipid metabolism (50), autophagy (51), and maintenance of oxidative stress (52). However, recent evidence indicates that MR restriction can induce ATF4 independent of the eIF2 pathway (53), suggesting that additional mechanisms may be regulating MR-induced ATF4 response.

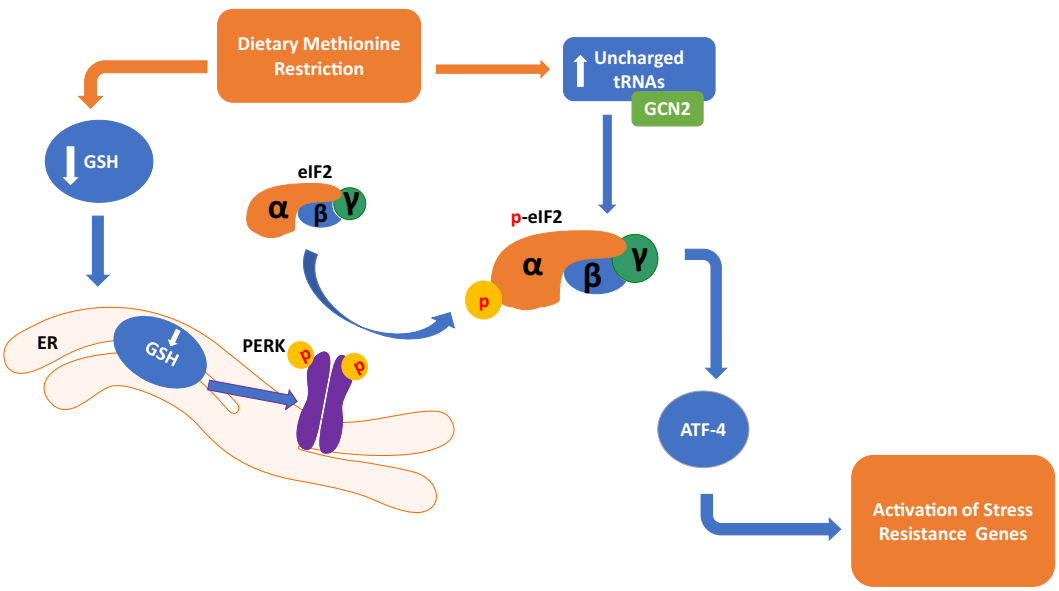

FIGURE 1 | Dietary methionine restriction has been shown to occur through two distinct pathways in the cell. In the endoplasmic reticulum, a reduction of Glutathione (GSH) causes an activation of the PKR-like endoplasmic reticulum kinase (PERK), which activates eukaryotic translation initiation factor 2a (eIF2a). In the cytoplasm, a buildup of unchanged tRNAs activates general control non-derepressible 2 (GCN2) which in turn also activates eIF2a. Both of these pathways converge in the activation of activating transcription factor 4 (ATF4) and activation of genes that respond to stress.

Molecular Mechanisms of MR

Epigenetics

Nutrigenomics is a rapidly evolving area of research that identifies how nutrition and diet interact with the genome and affect gene expression (54). Epigenetic regulation includes methylation of cytosines within genes or regulatory regions and can activate, but more commonly inhibit, gene expression. Epigenetic regulation also includes histone modifications as methylation, phosphorylation, and acetylation that affect chromatin structure and transcriptional activation. Regulation of methylation status is predominantly controlled by *de novo* methyltransferases and demethylases that acquire their methyl groups from the downstream methionine metabolite, SAM, converting it to *S*-adenosylhomocysteine (SAH).

Age-related changes in the epigenome are implicated in the aging process (55) and although it is established that the epigenome responds to nutrient signals, a direct link between the aging response and nutrient restriction-induced epigenetic effects has yet to be clearly established (56). However, recent evidence indicates that age-related methylation drift, which is hypothesized to be a determinant of mammalian lifespan (57), is attenuated by CR in rhesus monkeys and mice and affects gene expression (58).

Epigenetic effects of protein or MR have largely emphasized consumption during gestation and subsequent effects on offspring development. This concept of "nutritional programming" actually supports dietary supplementation of methyl donors like folate, choline, and methionine to improve offspring health and growth potential, and the extent to which this response is regulated by differential methylation of genes critical for development remains unclear (59). There is, however, a small body of work examining the epigenetic response to MR as it relates to aging. *In vitro* findings indicate that physiologically relevant concentrations of methionine in cell culture media alter the SAM/SAH ratio and methylation status of H3K4trimethyl, leading to regulation of gene transcription (60). The methionine levels in the serum of humans exhibit enough variation during MR to alter histone methylation (60). Similar findings were observed in adult (but not young) mice in which short-term (12 weeks) MR (0.12%-MR, 0.84%-CD) decreased SAH concentration and increased global DNA methylation in liver, although the opposite was observed in adipose tissue (61). These studies collectively support a role for changes in epigenetic marks by MR, although additional research is needed to further characterize the gene-specific responses.

MicroRNAs

MicroRNAs are small endogenous 18–22 base pair nucleotide sequences (62) that play roles in mediating posttranscriptional gene silencing in mammals, plants, and invertebrate organisms (63, 64). They are synthesized from primary miRNAs by two enzymes Drosha in the nucleus and Dicer in the cytoplasm (65) and are bound by Argonaute subfamily of proteins before integration into the RISC complex. After integration miRNAs have the ability to post transcriptionally regulate gene expression through the formation of RNA duplexes (62). The miRNA profile changes with age and numerous miRNAs that are affected by age are also regulated by CR (66), suggesting these regulatory RNA molecules are associated with CR-induced extension of lifespan. Many of these miRNAs associated with the IGF-1 PI3K/AKT mTOR signaling pathways are important for nutrient sensing and subsequent physiological response during CR.

Recent evidence also supports a role for miRNAs in the MR phenotype in teleosts and mammals. Rainbow trout myosatellite cells exposed to methionine-deficient cell media regulate miRNAs that reduce their capacity for differentiation (miR-133a, miR-206, and miR-210) (4). Rainbow trout fed an MR (0.775%) diet for 4 weeks had lower levels of miR-133a in the skeletal

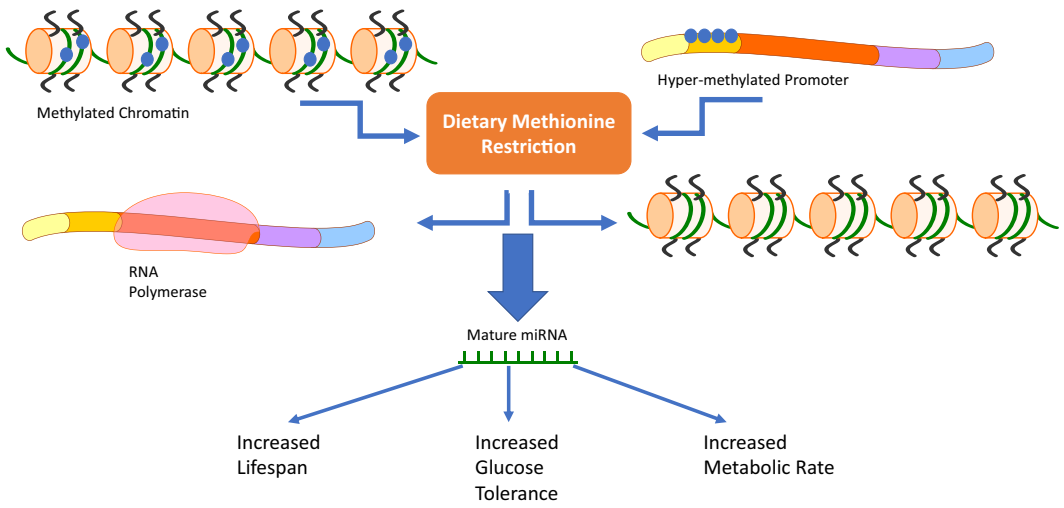

FIGURE 2 | Our review suggests that a third pathway should be added to the already known mechanisms of methionine restriction (MR). Changes in chromatin methylation or methylation status of DNA promoters due to dietary MR has the ability to activate or repress microRNAs (miRNAs) involved in the cells response to MR. These distinct changes could then be related to MRs phenotype characterized by increased lifespan, glucose tolerance, and metabolic rate.

muscle at 4 weeks and increased glucose tolerance following a glucose challenge at 8 weeks (5). Mice consuming an MR diet (0.12%) exhibit increased expression of miR-133a, miR-335-5p, and miR-204 in the bone marrow as well as miR-31 in the plasma and liver (5, 6, 67, 68). Although these studies did not directly investigate the aging response, they do indicate that miRNAs are regulated by methionine availability and potentially play a role in the metabolic phenotype.

While miRNAs have traditionally been known to repress transcription, evidence suggests that cells undergoing an ISR, such as those under MR, may activate miRNAs to regulate a unique suite of mRNAs to maintain homeostasis (69). As previously mentioned, a potential mechanism of MR is through the GCN2 amino acid deprivation pathway and the PERK/NRF2 (**Figure 1**) pathway. Both of these kinases phosphorylate eIF2α, which activates ATF4 and induces the formation of stress granules (70) containing miRNAs, mRNA targets, and Argonaute proteins (71). Similar to MR, leucine deficiency activates GCN2/ATF4 (72), which increases expression of miRNA-212-5p that subsequently reduces lipid accumulation (72) and enhances gluconeogenesis (73). Although the effect of leucine restriction on lifespan is opposite that of MR, activation of GCN2/ATF4 and subsequent regulation of specific miRNAs appears to be a common response to cellular stress induced by amino acid restriction.

While the notion of miRNA involvement in the MR phenotype is new, it is well-known that a few miRNAs regulate cellular stress response in other situations. miR-211 is known as a pro-survival miRNA during ER stress activated by the PERK pathway (74). In this circumstance, miR-211 is used to attenuate the amount of the pro-apoptotic transcription factor *chop* and keep the cell from initiating apoptosis during periods of short-term ER stress. miR-122 causes a repression of the cationic amino acid transporter-1 under normal conditions. Under varying conditions of stress, this miR can de-repress the mRNA transcript and cause it to be localized to the polysomes (75). For a full review of how miRNAs are regulated during stress responses, see Leung and Sharp (76). Because MR is known to activate the PERK pathway without induction of ER stress (47) the connection between MR and miRNAs must be interpreted carefully and warrants further study.

CONCLUSION AND FUTURE PERSPECTIVES

The evidence presented clearly shows MRs role in altering metabolic phenotypes in both mammals and teleosts. The activation of miRNAs during MR provides a potential link between changes in methylation and the ISRs in cells. Studies utilizing rainbow trout myosatellite cells *in vitro* and juvenile rainbow trout *in vivo* (4, 5) have shown that methionine can regulate the level of expression of miRNAs in teleosts (**Figure 2**). Studies done in mammalian systems have also shown that miRNAs are differentially regulated in the plasma, liver, and bone marrow of MR mice (6). This review explores mechanisms responsible for the MR phenotype including miRNAs, amino acid starvation, and stress response pathways.

Future research should investigate miRNAs in circulation (77) during MR. miRNAs found to be altered during MR (78) can then be analyzed for their role in controlling muscle-specific transcription factors like MyoD and myogenin (79) and can also be explored in other tissues to observe conservation of function during stress responses in tissue (80). Indeed, the master regulator of many of these mechanisms may lie in epigenetic changes that occur during MR. These changes have been hinted at in previous literature (60, 61) but have not yet been fully explored.

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Dietary Creatine Supplementation in Gilthead Seabream (*Sparus aurata*) Increases Dorsal Muscle Area and the Expression of *myod1* and *capn1* Genes

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Creatine (Cr) is an amino acid derivative with an important role in the cell as energy buffer that has been largely used as dietary supplement to increase muscle strength and lean body mass in healthy individuals and athletes. However, studies in fish are scarce. The aim of this work is to determine whether dietary Cr supplementation affects muscle growth in gilthead seabream (*Sparus aurata*) juveniles. Fish were fed *ad libitum* for 69 days with diets containing three increasing levels of creatine monohydrate (2, 5, and 8%) that were compared with a non-supplemented control (CTRL) diet. At the end of the trial, the fast-twist skeletal muscle growth dynamics (muscle cellularity) and the expression of muscle-related genes were evaluated. There was a general trend for Cr-fed fish to be larger and longer than those fed the CTRL, but no significant differences in daily growth index (DGI) were registered among dietary treatments. The dorsal cross-sectional muscle area (DMA) of fish fed Cr 5 and Cr 8% was significantly larger than that of fish fed CTRL. The groups supplemented with Cr systematically had a higher relative number of both small-sized ($\leq$20 μm) and large-sized fibers ($\geq$120 μm). Dorsal total fibers number was highest in fish fed 5% Cr. In fish supplemented with 5% Cr, the relative expression of myogenic differentiation 1 (*myod1*) increased almost four times compared to those fed the CTRL diet. The relative expression of calpain 3 (*capn3*) was highest in fish fed diets with 2% Cr supplementation, but did not differ significantly from those fed the CTRL or Cr 5%. The *myod1* gene expression had a positive and significant correlation with that of *capn1*, *capns1a*, and *capn3* expression. These results suggest that the observed modulation of gene expression was not enough to produce a significant alteration in muscle phenotype under the tested conditions, as a non-significant increase in muscle fiber diameter and higher total number of fiber was observed, but still resulted in increased DMA. Additional studies may be required in order to better clarify the effect of dietary Cr supplementation in fish, possibly in conjunction with induced resistance training.

Keywords: calpains, creatine supplementation, muscle growth and differentiation, myogenesis, myogenic differentiation 1 (*myod1*), myogenic regulatory factors (MRFs)

INTRODUCTION

In the last two decades, the amount of captured fish has stagnated, whereas fish produced in aquaculture has been increasing (1). Several seabream species are farmed worldwide due to their savory meat and to meet its growing consumption trend. Among Sparidae, the gilthead seabream (*Sparus aurata*, L.) is one of the most important farmed fish species in the Mediterranean region with an estimated production of 160.563 tons in 2016 (2, 3).

Skeletal muscle represents 40–60% of the fish body mass and represents the edible part of the fish (filet). High growth performance and flesh quality are crucial for the success of the aquaculture industry. It is known that consumers show a preference for fresh fish with a firm texture (4). Several studies have reported the relationship between the muscle fiber size and the firmness of the flesh (5–7). In Atlantic salmon, Johnston et al. (8) demonstrated that the firmness and the color of a smoked filet were positively correlated with the muscle fiber density. Likewise, in gilthead seabream, flesh firmness positively correlated with both the fiber density and the number of small fiber but showed a negative correlation with skeletal muscle diameter (9). Nutrient availability is one of the most important factors influencing the muscle growth performance in fish. Therefore, the need to establish the most favorable rearing conditions, to produce robust fish that grow fast and have a texture able to fulfill consumer's expectations, is of major importance for the farming industry.

Creatine (Cr) is an amino acid derivative naturally synthesized in vertebrates from methionine, glycine, and arginine (10). It combines with inorganic phosphate to form phosphocreatine (PCr), which is mainly stored in skeletal muscle (∼95%) (11, 12). Importantly, Cr is a physiological compound and is a part of the ATP/PCr phosphate energy system. PCr is a donor of phosphate to ADP for energy production and is controlled by creatine kinase (CK) that catalyzes the reversible reaction of the energy transfer pathway known as the CK/PCr energy shuttle, which provides immediate replenishment of ATP via high-energy phosphate compounds (13). Since skeletal musculature is a high-energy demand tissue, Cr plays an important role in muscle fibers as an energy buffer and also acts indirectly on muscle growth and strength by increasing the energy availability.

In humans, Cr analogs have proved to display important biological activities acting synergistically with some pharmaceutical formulations available in the market (11). In addition, it is well known that the oral ingestion of Cr-rich items, such as meat and fish, or via dietary supplements, will increase the whole body Cr pool (14). Studies have shown that Cr ingestion in humans can significantly increase the amount of physical work that can be performed, and hence, the athletes use Cr as a performance-boosting supplement (11, 12, 14). Currently, Cr supplementation in humans, in conjunction with heavy training exercise, was found to increase type I and II muscle fiber area, satellite cell number, myonuclei concentration, and type I and II myosin heavy chain (mhc) mRNA transcripts and protein content (15–18). Recent studies have also found that when subjects boost their muscle Cr levels via supplementation, they also increase the secretion of growth hormone (gh) and the expression of IGF-I at rest with no additional effect of exercise (19, 20). In fish, the effects of Cr on muscle growth have been poorly evaluated, but gh plays an important role in protein synthesis via the interaction with the growth hormone receptor (*ghr*) on the cell membrane (21), which are regulated during starvation and refeeding of rainbow trout (22). Gh induces muscle growth by modulating the expression of several genes belonging to the myostatin (*mstn*), atrophy, *gh*, and IGF systems as well as myogenic regulatory factors (MRFs). The IGF system, a major hormone axis regulating the cellular dynamics of muscle growth, directly stimulates cell proliferation, differentiation, and hypertrophy, and inhibits muscle atrophy. Such effects on muscle are mediated by the specific binding with IGF1 receptor (IGFR1) (23). In mice, previous studies showed that ablation of the IGF-1 receptor in skeletal muscle resulted in smaller myofibers (24). In rainbow trout, fasting and refeeding induced a coordinated regulation of IGF-I, IGFBP-5, and IGFBP-rP1 in muscle, and were suggested to be strongly involved in myogenesis resumption. Willoughby and Rosene (17) hypothesized that increased *mhc* gene expression induced by Cr supplementation is mediated by MRFs, which are transcription factors (*myod*, *myf5*, *mrf4*, and myogenin) that regulate myogenesis. In fact, *mrf4* level was increased after Cr intake in combination with resistance training. Increased *mrf4* and myogenin protein were further correlated to muscle CK mRNA expression (25). Safdar et al. (26) showed that short-term Cr supplementation for 10 days in young men increases the expression of numerous genes involved in osmotic regulation, glycogen synthesis and degradation, cytoskeletal remodeling, proliferation and differentiation of satellite cells, repairs and replication of DNA, RNA transcriptional control, and cell death. Furthermore, Young and Young (27) suggested that the beneficial effects of Cr supplementation in rat muscle mass and strength are due to an enhanced ability to train, rather than a direct effect on muscle. Hence, the potential anabolic effects of Cr might depend on the adjustment of workout intensity during training.

Although the majority of Cr research is focused in humans, its effect on other mammalian species meat quality has also been studied. Cr supplementation in pork diets prior to slaughter seems to affect the post-mortem muscle metabolism (pH decline in the muscle) and to improve the pork quality (28). The importance of the Cr system in fish still remains to be largely unknown, although, according to Borchel et al. (29), Cr metabolism differs between mammals and rainbow trout. It has been demonstrated that fish muscle has higher Cr content than that of mammals (30). McFarlane et al. (31) found that exogenous Cr supplementation (dietary or injected) did not alter rainbow trout muscle Cr levels, but during a fixed velocity sprint test, increased endurance was concomitantly observed with Cr intake. The short time frame of this study (7 days) associated with a too low dose to detect similar changes as seen in humans, given the lower metabolic rates of these poikilotherms, might explain the lack of Cr uptake in supplemented fish (31).

Relatively, less information is available on the Cr system of fish, and the effects of its dietary supplementation on muscle cellularity have never been evaluated before. The present study aims to contribute to a better understanding of the effects

of dietary Cr supplementation levels on *S. aurata* juvenile's muscular growth. A comprehensive approach was undertaken based on the histological parameters (cellularity of the fast twitch muscle) and molecular biology techniques (relative expression of muscle-related genes).

MATERIALS AND METHODS

Experimental Diets

A practical commercial-based diet, i.e., a control (CTRL), was formulated (49% protein and 23 kJ.g^{-1}) to fulfill the known nutritional requirements of the gilthead seabream (**Table 1**). Three experimental diets were formulated by adding 2, 5, and 8% Cr monohydrate (Sigma, Ref. C3630) to the CTRL diet. All diets were manufactured by SPAROS (Olhão, Portugal). The main ingredients were pulverized (below 250 μm) in a micropulverizer hammer mill (Hosokawa Micron Ltd., United Kingdom) and mixed in a double-helix mixture (TGC Extrusion, France) to attain a basal mixture (no oils were added at this stage). All diets were extruded (pellet size 5.0 mm) by means of a pilot-scale twin-screw extruder CLEXTRAL BC45 (Clextral, France) with a screw diameter of 55.5 mm and temperature ranging 105°-110°C. Upon extrusion, all batches were dried in a convection oven (OP 750-EF, LTE Scientifics, United Kingdom) for 2 h at 60°C and left to cool at room temperature. The Cr was mixed with fish oil fraction according to each target concentration (2, 5, and 8%) and added under vacuum coating conditions in a Pegasus vacuum mixer (PG-10VCLAB, DINNISEN, The Netherlands) to the respective mixture.

TABLE 1 | Ingredients and proximate composition of the control (CTRL) diet*.

Ingredients	%
Fishmeal LT[a]	10.00
Fishmeal 60[b]	10.00
Porcine blood meal	5.00
Soy protein concentrate[c]	10.00
Wheat gluten[d]	10.00
Corn gluten[e]	7.25
Rise protein concentrate	3.50
Soybean meal[f]	10.00
Rapeseed meal	4.00
Wheat meal	12.00
Fish oil[g]	14.50
Vit & Min Premix[h]	0.15
Soy lecithin[i]	2.00
Antioxidant	0.40
Dicalcium Phosphate[j]	0.50
L-Lysine[k]	0.50
DL-Methionine	0.20
PROXIMATE COMPOSITION	
Dry Matter (%)	95.39 ± 0.04
Crude protein (%DM)	49.28 ± 0.14
Lipid (%DM)	20.37 ± 0.31
Ash (%DM)	8.39 ± 0.06
Gross energy (kJ/g DM)	23.43 ± 0.07

[a] *Peruvian fishmeal LT: 71% crude protein, 11% crude fat, EXALMAR, Peru.*
[b] *Fish by-products meal: 540 g Kg^{-1} CP, 80 g kg^{-1} CF, COFACO, Portugal.*
[c] *Soycomil P: 65% CP, 0.7% CF, ADM, The Netherlands.*
[d] *VITEN: 85.7% CP, 1.3% CF, ROQUETTE, France.*
[e] *GLUTALYS: 61% CP, 8% CF, ROQUETTE, France.*
[f] *Solvent extracted dehulled soybean meal: 47% CP, 2.6% CF, SORGAL, Portugal.*
[g] *Henry Lamotte Oils GmbH, Germany.*
[h] *PVO40.01 SPAROS standard premix for marine fish, PREMIX Lda, Portugal.*
[i] *Yelkinol AC (65% phospholipids): 750 g Kg^{-1} CF,ADM, The Netherlands.*
[j] *Dicalcium phosphate: 18% phosphorus, 23% calcium, Fosfitalia, Italy.*
[k] *L-Lysine HCl 99%: Ajinomoto Eurolysine SAS, France.*
* *Experimental diets (Cr 2, 5, and 8%) were formulated by adding 2%, 5% and 8% Cr monohydrate (Sigma, Ref. C3630) to the CTRL diet.*

Animal Growth Conditions

The current trial was conducted by trained scientists (following FELASA category C recommendations) and according to the European Economic Community animal experimentation guidelines on the protection of animals used for scientific purposes from the European directive 2010/63/UE at Ramalhete, CCMAR facilities (Centre of Marine Sciences of Algarve).

Triplicate groups of 24 gilthead seabream (initial body weight: 173 ± 2.4 g) were randomly distributed in 500 L tanks and were hand-fed *ad libitum* with each experimental diet twice a day (except Sundays) for 69 days. Sea water was supplied at 2 L/min (mean temperature 23.3°C ± 0.90; mean salinity 37 ± 0.39 ppm) in a flow through system with artificial aeration (mean dissolved oxygen above 5 mg.L^{-1}). All physical and chemical water parameters were evaluated during the experiment to ensure the levels within the recommended limits for the species.

Sampling

At the end of the experimental trial, all fish were deeply anesthetized in an aqueous solution of MS-222 (Sigma, Switzerland) and individually weighted to calculate the daily growth index [DGI = 100 × (FBW$^{1/3}$-IBW$^{1/3}$)/trial duration (days)]. Six fish from dietary treatment were also measured for total standard length (cm) and sacrificed by decapitation under a cork board on ice. Their fins were cut and fish were softly scaled on both sides. A cross-sectional filet with skin (2–3 mm thick) was taken immediately before the dorsal fin position—filet A (**Figure 1a**). The dorsal area of each filet was then quickly photographed (with scale reference) and properly labeled, for later determination of the cross-sectional area. Four representative samples (a-c) of fast-twist muscle (0.5 × 0.5 cm) were collected from the right part of the filet (**Figure 1B**), immediately placed in a cryoprotective embedding medium—OCT (Thermo Scientific™ Shandon™ Cryomatrix™), and snap frozen in isopentane cooled by liquid nitrogen. Samples were then stored at −80°C for later morphometric evaluations.

A second cross-sectional filet (**Figure 1A**), filet B, was taken and 2–3 g of fast-twist muscle (right filet) was taken and stored in RNA*later*™ solution (Sigma-Aldrich, USA) overnight at 4°C. The excess solution was then discarded and the samples were stored at −80°C for posterior molecular biology analysis.

Analytic Methods

Morphometric Procedures

The morphometric study was done using an interactive image analysis system (Olympus Cell*Family), working with a live-image captured by CCD-video camera (ColorView Soft Imaging

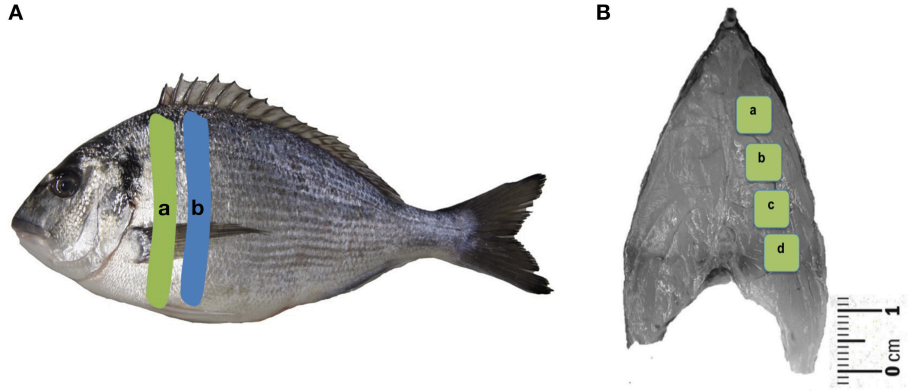

FIGURE 1 | (A) Filet sampling area for histology parameters (a) and for molecular biology analysis (b) and **(B)** dorsal areas (a–d) selected for muscle cellularity evaluation.

System, Olympus) and a light microscope (BX51, Olympus, Japan). Muscle total dorsal muscular area (DMA) (mm^2) was computed by the software after demarcating the physical limits of the whole dorsal section without considering any red muscle area. These measurements were based on the photo taken at the sampling time.

Transversal fast-twist muscle sections from each block (a-d) were cut at 7 μm in a cryostat CM 1950 (Leica Microsystem GmbH, Wetzlar, Germany) and mounted on polysine adhesion slides. The sections were stained with haematoxylin–eosin (Merk, Whitehouse Station, NJ, USA) before placing a cover slip and left to dry. The relative number (density) of fast-twist muscle fibers per unit area N_A($n°/mm^2$) was estimated as follows: N/area $= \Sigma$ N(fibers)/Σ [a (sampled field)], where ΣN (fibers) is the total number of fibers counted over the sampled fields in the sections (a–d), and "a" is the total area of the fiber counting fields. The total number of fast-twist muscle fibers per dorsal cross-section (N) was estimated as follows: N (fibers) = N_A (muscle fibers) × DMA (muscle), where N_A is the number of fast-twist muscle fibers per unit area (mm^2) and DMA the dorsal cross-sectional muscle area. From each fish, the physical limits of a minimum of 700 white muscle fibers (from the four blocks a-d) were circumscribed using a 20x objective to determine the mean fiber area [$\bar{a}$ (μm^2)]. The corresponding mean diameter was calculated assuming that all the fibers were circular.

RNA Extraction and cDNA Synthesis

White muscle samples were disrupted with a PureZol solution (Bio-Rad Laboratories) using Precellys® 24 lysis/homogenizer (Bertin Technologies, France). Total RNA was extracted using the Ilustra RNAspin Mini RNA isolation kit (GE Healthcare UK Limited), including an on-column DNAse I digesting step, according to the manufacturer's instructions. RNA quantification and quality were evaluated by absorbance at 260 and 280 nm using the Take3 Micro-Volume plate (Take3, Biotek, Germany) and the Gen 5 software (BioTek, USA), and the values were within the expected ratio of 1.8–2.2, indicating high RNA purity. RNA integrity was verified by the banding pattern of 28S:18S ribosomal RNA in 1% TAE (w/v) agarose gel electrophoresis stained with GelRed (Biotium, Hayward CA, USA).

For complementary deoxyribonucleic acid (cDNA) synthesis, 750 ng of total RNA was transcribed for all samples, with the iScript™ Reverse Transcription Supermix for real-time polymerase chain reaction (RT-qPCR) (Bio-Rad Laboratories) in a final volume of 20 μL following the manufacturer's instructions and stored at −80°C.

Real Time PCR Analysis

Primers used for qPCR had been previously published (**Table 2**) and were synthesized by STABVida (Portugal). The qPCR reactions were performed in iQ5 Real-Time PCR Detection System (Bio-Rad), using SsoFast EvaGreen Supermix (Bio-Rad Laboratories), and prepared to a final volume of 20 μl, with a final primers concentration of 300 nM, according to the manufacturer's instructions. Thermal cycling for these experiments occurred under the following conditions: initial step at 95°C for 30 s, followed by 40 cycles of denaturation at 95°C for 5 s, and plus annealing/extension (annealing temperatures in **Table 2**) for 10 s.

Then the melting curve analysis was performed to verify the amplicon purity and size, with a dissociation protocol from 65° to 95°C followed by gel electrophoresis. Five-point standard curves constructed with 5-fold serial dilutions of pooled cDNA were used for qPCR efficiency calculation. All samples were performed in duplicated and always included a negative control to confirm the absence of contamination. To evaluate the relative transcript levels, the $2^{-\Delta\Delta CT}$ method was used with *β-actin* and *rpl27α* as the best housekeeping genes out of three, estimated by geNorm® software, to provide the most reliable normalization. The PCR efficiency for target genes ranged from 85 to 110%.

Statistical Analysis

Statistic evaluation of the data was accomplished by one-way analysis of variance (ANOVA). All data were checked for

TABLE 2 | List of specific primers used for real time PCR.

	Primer Sequence 5′-3′	Annealing T.(°C)	Accession number	Reference
TARGET GENES				
mstn	**F:** GTACGACGTGCTGGGAGACG	60	AF258448.1	(32)
	R: CGTACGATTCGATTCGCTTG			
myod2	**F:** CACTACAGCGGGGATTCAGAC	60	AF478568	(32)
	R: CGTTTGCTTCTCCTGGACTC			
mrf4	**F:** CATCCCACAGCTTTAAAGGCA	60	JN034421	(32)
	R: GAGGACGCCGAAGATTCACT			
myogenin	**F:** CAGAGGCTGCCCAAGGTCGAG	68	EF462191	(32)
	R: CAGGTGCTGCCCGAACTGGGCTCG			
myf5	**F:** TGTCTTATCGCCCAAAGTGTC	64	JN034420	(32)
	R: CTACGAGAGCAGGTGGAGAACT			
myod1	**F:** GTTTTGTTCCAGGCGGTCT	60	AF478569	(33)
	R: GCTGGTGTCGGTGGAGAT			
mhc	**F:** AGCAGATCAAGAGGAACAGCC	60	NM131404	(33)
	R: GACTCAGAAGCCTGGCGATT			
capn1	**F:** CCTACGAGATGAGGATGGCT	58	AM951595.1	(34)
	R: AGTTGTCAAAGTCGGCGGT			
capn2	**F:** ACCCACGCTCAGACGGCAAA	61	FM152855.1	(34)
	R: CGTTCCCGCTGTCATCCATCA			
capns1a	**F:** CGCAGATACAGCGATGAAAA	56	AM962179.1	(34)
	R: GTTTTGAAGGAACGGCACAT			
capns1b	**F:** ATGGACAGCGACAGCACA	56	ERP000874	(34)
	R: AGAGGTATTTGAACTCGTGGAAG			
capn3	**F:** AGAGGGTTTCAGCCTTGAGA	56	FG262721.1	(34)
	R: CGCTTTGATCTTTCTCCACA			
igfr-1a	**F:** TCAACGACAAGTACGACTACCGCTGCT	60	KJ591052	
	R: CACACTTTCTGGCACTGGTTGGAGGTC			
igfr-2	**F:** ACATTCGGGCAGCACTCCTAAGAT	60	KM522776	
	R: CCAGTTCACCTCGTAGCGACAGTT			
ghra	**F:** ACCTGTCAGCCACCACATGA	60	AF438176	
	R: TCGTGCAGATCTGGGTCGTA			
REFERENCE GENES				
β-actin	**F:** TCCTGCGGAATCCATGAGA	60	X89920	(34)
	R: GACGTCGCACTTCATGATGCT			
rpl27α	**F:** AAGAGGAACACAACTCACTGCCCCA	68	–	(35)
	R: GCTTGCCTTTGCCCAGAACTTTGTAG			
18S	**F:** CGAGCAATAACAGGTCTGTG	60	–	(36)
	R: GGGCATGGACTTAATCAA			

For each gene, the annealing temperature and the gene bank accession number, whenever available, are indicated.

normality and homogeneity of variance, by using the Shapiro-Wilk and the Levene test, respectively. Data transformation [log(x) and arcsin(x)] was applied when homogeneity and normality of the variables were not achieved. A non-parametric test (Kruskal-Wallis H-test) was performed, if these assumptions where still not achieved. A pair-wise Mann–Whitney U-test was used for *post-hoc* multiple comparisons. Where significant main effects were identified by ANOVA, individual means were compared using *Tukey HSD* multiple comparison test. A significance of $p < 0.05$ was applied to all statistical tests. A Spearmen's rank correlation coefficient (ρ) test was applied to all variables. Correlation was considered significant at the bilateral levels of 0.05 (*) or 0.01 (**). All tests were run with IBM SPSS statistics software (SPSS ver.22.0; Chicago, USA).

The evaluation of expression stability for the three reference genes was performed using the statistical application geNorm® (https://genorm.cmgg.be).

RESULTS

Muscle Growth

During the experimental period, no mortalities were registered and all fish reached the commercial size (>250 g). There was a general trend for Cr-fed fish to be larger and longer than

those fed the CTRL, but without differing significantly (**Table 3**). Condition factor (K), used as an index of the productivity in fish growth, ranged from 2.3 to 2.4 with no significant differences between treatments, nonetheless was higher in fish fed the highest Cr inclusion. No significant differences in daily growth index (DGI) were registered among the dietary treatments.

The dorsal muscular area (DMA) of fish fed with Cr 5 and Cr 8% was significantly larger than that of fish fed with CTRL and Cr 2% diets ($P < 0.05$; **Table 3**). Dorsal total fiber number was highest in fish fed with 5% Cr, but no significant differences could be perceived among dietary treatments. The mean diameter of fast-twist fibers had a tendency to increase with Cr supplementation, whereas fiber density showed an inverse trend (**Table 3**). In addition, the distribution of skeletal fast-twist fiber diameters showed no significant diet-induced differences (**Figure 2B**). Muscle fiber diameter ranged from $<20\,\mu m$ to a maximum of $160\,\mu m$ (**Figures 2A,B**). The groups supplemented with creatine systematically had a higher relative number of both small-sized ($\leq 20\,\mu m$) and large-sized fibers ($\geq 120\,\mu m$) (**Table 3**).

Relative Expression of Target Genes

In fast-twitch muscle, the expression of *myod1*, *capn1*, and *capn3* was significantly affected by the dietary treatments, whereas other myogenic genes (*myod2*, *myf5*, *mrf4,* and *myog*) and biomarkers of muscle structure, function, and growth (*igfr-1a, igfr-2, mhc*, *mstn, capns1a, capns1a,* and *capn2*) were not significantly changed (**Figures 3**, **4**). In fish supplemented with 5% Cr, the relative expression of *myod1* increased almost four times compared with those fed with the CTRL diet ($P = 0.045$; **Figure 3A**). The *mrf4* had the very same trend of *myod1* but changes were not significant. The relative expression of *ghr*-1 increased almost three times in fish fed with 5% Cr compared with those fed with 2% Cr ($P = 0.041$; **Figure 3H**) but did not differ significantly from other treatments. The relative expression of both *myf5* and *myog* tended to decrease with increasing Cr supplementation but without statistical significance. In addition, the expression of calpain 1 (*capn1*) increased significantly in fish fed with Cr 2 and Cr 5% ($P = 0.005$; **Figure 4A**). On the other hand, fish fed with 8% Cr showed a similar *capn1* expression to those fed with the CTRL diet. The relative expression of *capn3* was highest in fish fed with 2% Cr supplementation but did not differ significantly from those fed with the CTRL or Cr 5%. Fish fed with Cr 8% diet had the lowest *capn3* expression.

To better understand the possible relationship between the relative expression of muscle-related genes and the muscle cellularity, a Spearman rank order correlation was performed with all parameters (**Table 4**). The expression of the majority of the genes was not significantly correlated with muscle phenotype. However, a positive correlation was found between *mstn* and fiber diameter ($P = 0.664$), whereas *myog* expression levels were negatively correlated with DMA ($P = -0.622$). Interestingly, the expression of several genes implicated in myogenesis was significantly correlated with the expression of genes from the calpain family. Both *myod* paralogs in muscle (*myod1* and *myod2*) were positively correlated with almost all the genes from the calpain family analyzed herein (**Table 4**). The *myod1* gene had a positive and significant correlation with *capn1*, *capns1a*, and *capn3* expression. Similarly, *myod2* showed a strong positive correlation with *capn1* ($\rho = 0.727$), *capns1a* ($\rho = 0.643$), *capn2* ($\rho = 0.594$), and *capn3* ($\rho = 0.762$) expressions. *Myf5* was also significantly correlated with *capn2* ($\rho = 0.769$) and *mrf4* with *capn1* ($\rho = 0.790$) expression (**Table 4**).

DISCUSSION

Cr supplementation has been used for many years by athletes to promote body mass growth and to improve their training resistance. A relatively large number of scientific studies have associated with the increased lean body mass to Cr supplementation combined with strength training (15, 16, 37); however, it is still not very clear whether the Cr supplementation *per se* is enough to promote such effects (38). Studies concerning the effect of Cr, although widely disseminated with regard to humans and mammal species, are extremely scarce in teleost fish. This study has been conducted to evaluate the potential of Cr supplementation to improve gilthead seabream muscle growth and the possibility of tailoring filet quality to fulfill the consumers' expectations.

The present results show that Cr supplementation does not seem to be very effective in promoting body mass increase in gilthead seabream, as fish final weight and DGI were not significantly improved after 69 days of feeding. Similarly, a short-term (7 days) dietary Cr supplementation did not significantly affect the specific growth rate (% body weight change d^{-1}) in juvenile rainbow trout (31). Nevertheless, the present study shows that the supplementation of Cr up to 5% in diets for gilthead seabream resulted in a significant increase of fish DMA. This was associated with a concomitant increase in muscle fiber diameter (muscle hypertrophy), mainly due to increased number of large-sized fibers ($\geq 120\,\mu m$) and higher total number of fiber in those fish. It is well-known that the skeletal muscle cellularity (i.e., the number, diameter, and density of fibers) is the main determinant of muscle texture both in raw and cooked filet, and is directly related with fish growth potential (4). In gilthead seabream, previous studies showed that flesh firmness was positively correlated with both the fiber density and the number of small fiber, and negatively correlated with skeletal muscle diameter (9). The present results suggest that in gilthead seabream, the dietary Cr supplementation *per se* significantly increased the DMA but was not enough to promote significant effects on the muscle fiber cellularity after a 69-day feeding period. Although fish muscle Cr and PCr levels are less susceptible of manipulation than human muscle stores, either by dietary supplementation or injection (31), a longer feeding period or the conjugation with resistance training might further result in a significant stimulus to growth but could also have a negative impact on flesh texture parameters due to increased muscle fiber diameters. Further studies are required to clarify such potential effects. In spite of the differences regarding the metabolism of Cr between mammals and fish (29), it has been demonstrated that Cr supplementation associated with exercise resulted in muscle thickness improvement in young athletes (39). However,

TABLE 3 | Growth performance and muscle cellularity of gilthead seabream juveniles fed CTRL, Cr 2, 5, and 8% diets*.

	Diets			
	CTRL	**Cr 2%**	**Cr 5%**	**Cr 8%**
Final Weight (g)	272.14 ± 18.92	274.98 ± 17.36	291.29 ± 23.60	288.32 ± 29.32
Length (cm)	22.75 ± 0.90	22.86 ± 0.39	22.96 ± 0.73	22.92 ± 0.45
Condition factor (K)	2.31 ± 0.13	2.26 ± 0.27	2.28 ± 0.31	2.43 ± 0.24
DMA (mm^2)	771.83 ± 46.99^b	798.44 ± 71.69^b	933.04 ± 22.16^a	899.51 ± 82.98^a
Fiber Density (N/mm^2)	170.47 ± 12.94	166 ± 18.80	166.55 ± 21.14	149.18 ± 12.32
Dorsal total fiber number x1000	131.40 ± 10.57	132.98 ± 22.66	150.75 ± 16.61	134.37 ± 16.55
Diameter of fibers (μm)	69.06 ± 2.38	69.59 ± 4.62	70.75 ± 2.67	73.71 ± 3.70
Fibers ≤20μm (%)	1.49 ± 1.12	1.99 ± 1.52	1.91 ± 1.68	1.65 ± 1.29
Fibers ≥120μm (%)	8.97 ± 1.74	9.19 ± 3.36	9.35 ± 1.81	12.19 ± 3.52

**Values represent the mean ± standard deviation (n = 6). Mean values within a row with different letters (a, b) represent significant differences between diets (P < 0.05). DMA, dorsal cross-sectional muscle area.*

the controversy regarding this subject persists; for instance, in a work with rats conducted by Aguiar et al. (40), dietary Cr supplementation did not significantly affect fiber hypertrophy neither when used alone nor when the rats were subjected to resistance training.

The growth potential of fish is intrinsically dependent on post-natal hyperplasia and hypertrophy dynamics in muscle tissue, which is controlled by signaling pathways involving the growth hormone (*gh*)/insulin-like growth factor (*igf*) system. *Gh* and *igfs* stimulate somatic growth through binding their corresponding receptors (*gh* and *igfrs*, respectively) that are widely distributed among different tissues, including muscle, and are influenced by the nutritional status of fish (22, 41). Previous studies reported that in humans, high Cr supplementation enhanced GH secretion, mimicking the response of a strong exercise, which might result in acute body weight and strength gain probably due to the indirect anabolic property of Cr (41). In addition, Cr supplementation at rest increased the muscular expression of the insulin-like growth factors that are extremely important growth-promoting agents (20). In fish, the impact of dietary supplementation of Cr in the GH/IGF system has never been reported before. In the present study, an up-regulation of *ghra* was observed in fish fed with 5% Cr diet (the relative expression increased by 110%), the gene that has prominent role in the systemic growth-promoting action of Gh (42, 43), whereas both *igfr-1a* and *igfr-2* remained unaffected. Vélez et al. (44) also reported an up-regulation of *ghra* in the muscle of gilthead sea fingerlings as the effect of rBGH treatment, suggesting that the GH anabolic effects may be induced in this tissue directly through the activation of this receptor.

Previous studies showed that a higher number of small-sized fibers are associated with higher growth potential (4, 45), which in turn depend on the proliferation and differentiation of the myogenic progenitor cells (MPCs, equivalent to mammalian satellite cells) that are responsible for controlling the expression of muscle-related genes. Myogenic activity is regulated by the differential expression of MRFs, which are transcription factors involved in the proliferation and differentiation of MPCs (46). The *myod* (myoblast determination factor) and *myf5* are primary MRFS involved in the specification and proliferation of myoblasts to form the MPC population. These cells, after activation and proliferation, will enter the differentiation process that will result in myotube formation and enlargement, involving the expression of the secondary MRFs *(myog and mrf4)* (46). There is a lack of surveys dedicated to the effect of dietary Cr on vertebrate's myogenic program. In the present study, the *myod1* relative expression significantly increased concomitantly with Cr dietary supplementation. The highest expression was observed in fish fed 5% Cr, suggesting that an increase in myoblast recruitment was occurring. During the muscle differentiation process in adult fish, such new cells fuse to form additional fibers or are absorbed by the existing fibers as they expand in diameter (hypertrophic growth) (47).

The currently observed up-regulation of *myod1* in gilthead seabream fed diets supplemented with 2–5% Cr was paralleled with a significant increase in DMA (*myod1* relative expression increased 167%, whereas DMA increased 21 % in fish fed with 5% inclusion of Cr in relation to the control diet). This was probably due to the concomitant increase in total number and size of muscle fibers. Moreover, *myod2* transcripts levels have not only showed lower levels compared with those of *myod1* but also were not significantly affected by dietary Cr. Similarly, in gilthead seabream, a differential expression of *myod1* and *myod2* was observed in amino acid-deficient media (48), also suggesting a differential nutritional regulation of the two *myod* paralogs. According to Tan and Du (49), the two non-allelic *myoD* genes are functional in seabream adult skeletal muscles and their expression is regulated differently: MyoD1 is expressed in both slow and fast muscles, whereas MyoD2 is specifically expressed in fast muscles (49). Campos et al. (50) have previously shown that in Senegalese sole larvae, *myod1* was correlated with fiber diameter, but not *myod2.* Moreover, in the present study, only *mstn* evidenced a negative correlation with fiber diameter. Overall, this indicates that the observed nutritional regulation can vary depending on the fish species and the stage of myogenesis of the muscle under study. Aguiar et al. (51) found a strong correlation between the muscle fiber CSA and the expression of *myod* in an experiment of resistance training in rats. The authors argued that this factor is more involved in the control of muscle mass than in fiber-type transitions

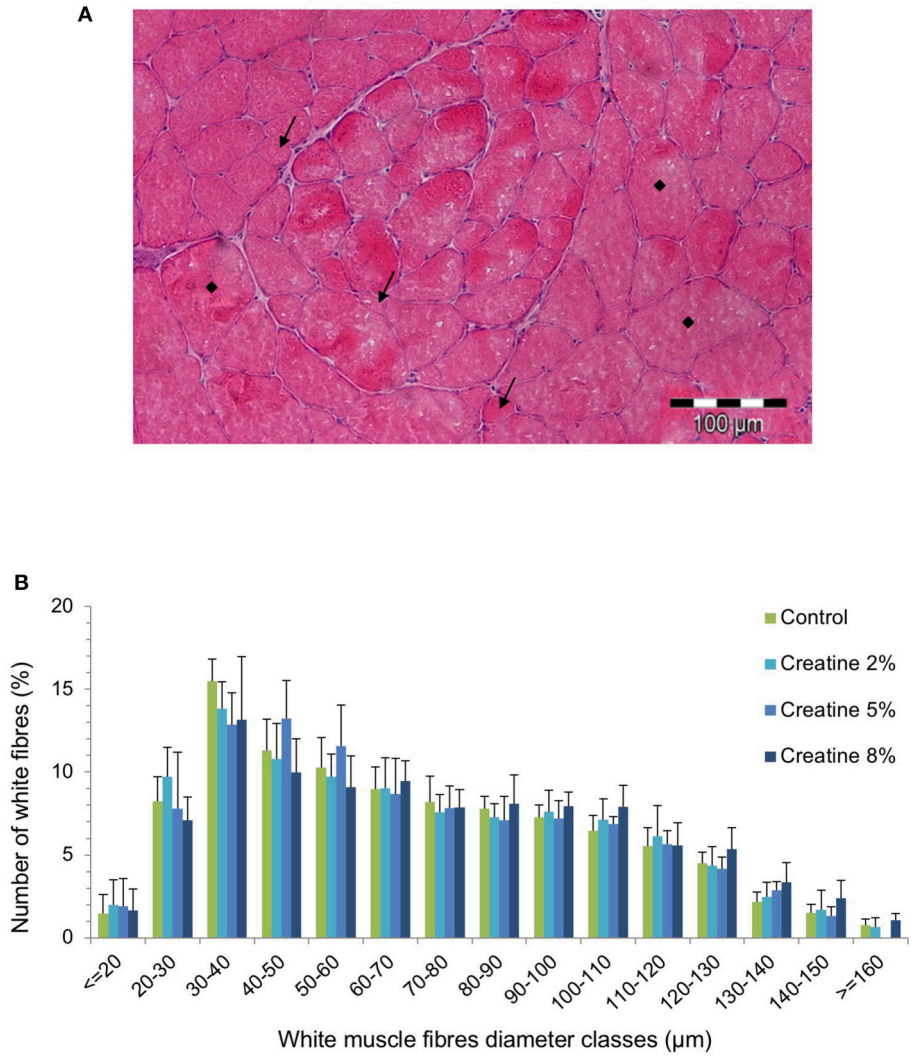

FIGURE 2 | Cross section of skeletal white muscle in a juvenile gilthead seabream fed 5% Creatine diet, showing newly (i.e., small [arrow]) recruited muscle fibers between older (i.e., large ■) muscle fibers **(A)**, and white muscle fiber diameter classes of juveniles fed juveniles fed the experimental diets for 69 days ($n > 700$ fibers) **(B)**. Error bars indicate the standard error of the mean for each treatment ($n = 6$).

(51). Accordingly, Siqin et al. recently explored the relationships among muscle fiber-type composition, diameter, and MRFs expression in different skeletal muscles, they also suggested that MRFs expression patterns were relatively stable with the changes in fiber-type composition and increases in fiber size resulting from mutually interacting processes during muscle development (52). Furthermore, Deldicque et al. (53) identified a major signaling cascade by which Cr promotes the differentiation program of C_2C_{12} cells, via p38 MAPK and ERK1/2 pathway, which may increase the expression of transcription factors (i.e., *myod* and *mef2*) capable of regulating the activation and differentiation of satellite cells. Studies in humans reported an increase in the expression of both myogenin and *mrf4* levels after Cr supplementation in conjunction with resistance training, which were strongly correlated with muscle Cr kinase mRNA expression (25), but other studies did not observe any significant changes in myogenin expression (54). In our study, *mrf4* expression tended to increase with Cr supplementation (43% increase in fish fed with Cr 5% compared with those fed with the CTRL diet), but *myog* expression even showed a downward trend in relative expression from Cr 2 to Cr 8%, which could foresee a decrease in fiber differentiation. Nevertheless, myostatin expression was positively correlated with muscle fiber diameter, although no clear trend could be perceived in its expression level in fish fed with increasing Cr levels. Both myogenin and myostatin are known to control myoblast differentiation and fusion that lead to the formation of myofibrils in several species (46). However, in Senegalese sole fed with different dietary, lipid diets *mstn* was negatively correlated with the percentage of large-sized fibers and with fish DMA (55). Data from previous works in aged mice reported a similar behavior and myostatin inhibitors having significant positive effects on muscle fiber size and mass

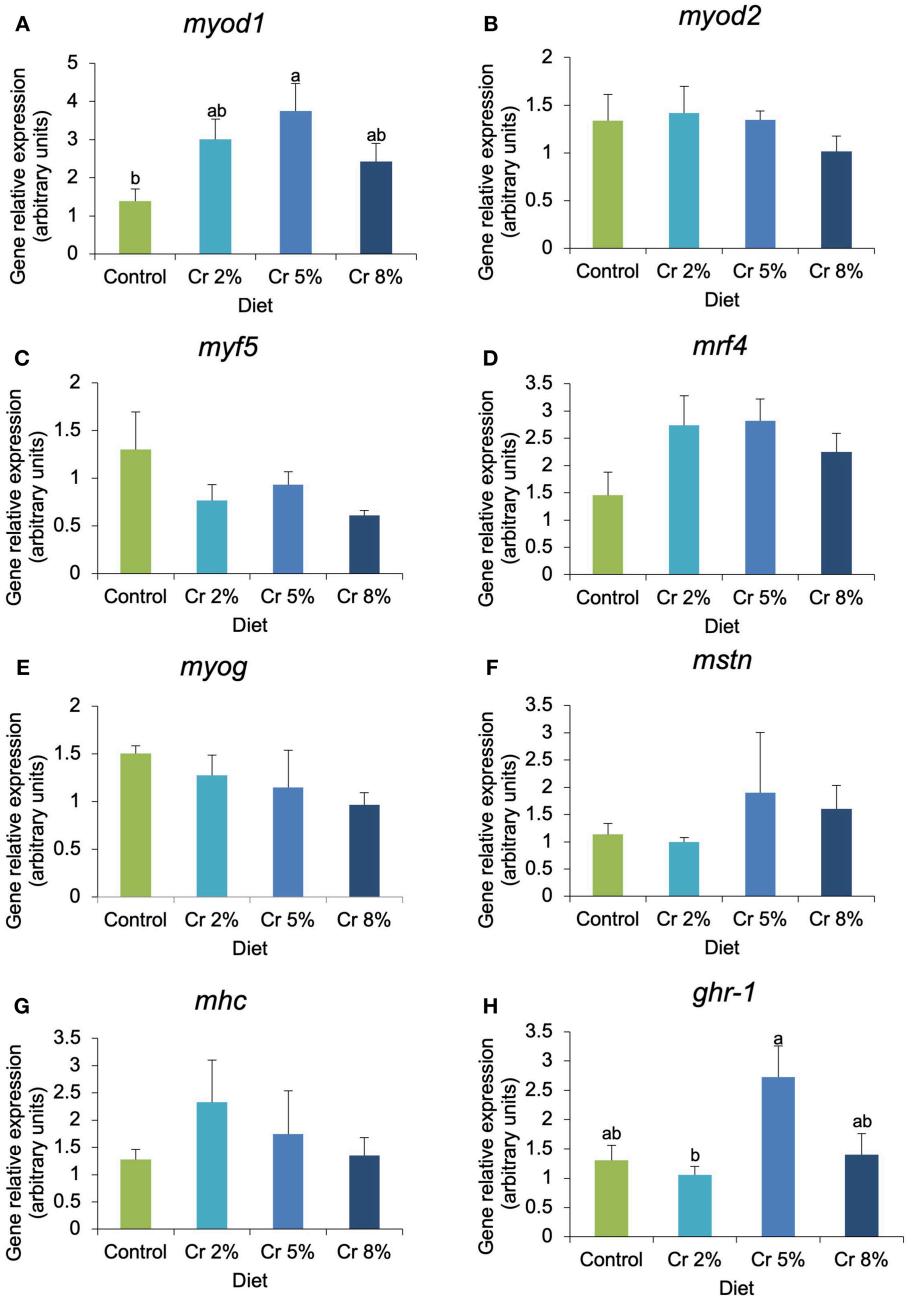

FIGURE 3 | (A–H) Relative expression of myogenic genes and markers of muscle structure and function of gilthead seabream juveniles fed the control and the experimental diets (2, 5, and 8% creatine). Different letters indicate significant differences between groups. $P < 0.05$. Error bars indicate the standard error for each treatment ($n = 6$).

(56, 57). Although recognized for repressing skeletal muscle growth through inhibiting both muscle cell hypertrophy and hyperplasia, in fish, recent studies suggested that *mstn1* seems to inhibit muscle cell proliferation, but not its differentiation (58). Thus, further research is needed to better clarify the effects of dietary Cr in *mstn*-associated muscular behavior.

The expression of several genes implicated in myogenesis was significantly correlated with the expression of genes from the calpain family. Calpains are a group of non-lysossomal Ca^{2+}-dependent cysteine proteases involved in cell cycle progression, myoblasts fusion, muscle protein turnover and growth, cell mobility, and cell degradation (59). Although in fish the role of calpains remains controversial, these proteases are generally associated with flesh tenderization and with the post-mortem changes occurring in muscle (60). They act in synergy with cathepsins to contribute to a rapid proteolysis of muscle proteins

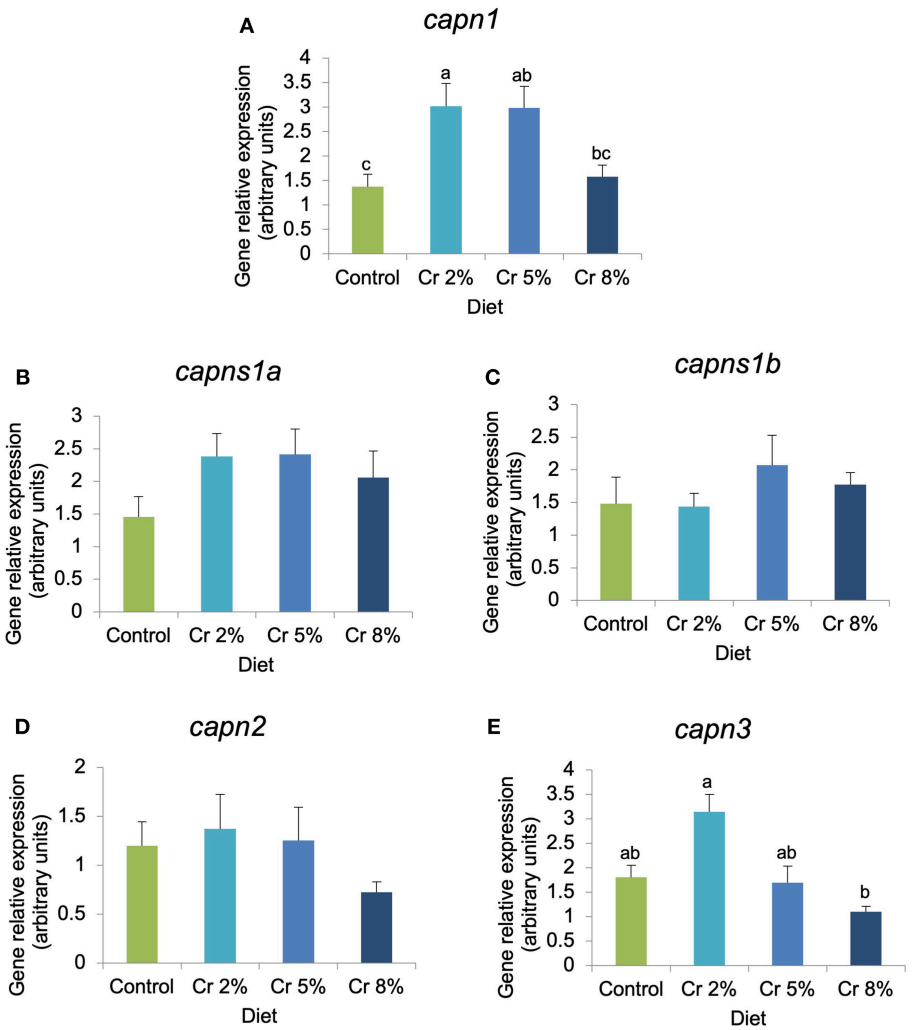

FIGURE 4 | (A–E) Relative expression of genes involved in proteolysis in gilthead seabream juveniles fed the control and the experimental diets (2, 5, and 8% creatine). Different letters indicate significant differences between groups. $P < 0.05$. Error bars indicate the standard error for each treatment ($n = 6$).

and associated flesh softening during post-mortem storage of meat. *Capn1* and *capn2* regulate physiological processes like myoblast fusion, and *capn3* is known to play an important role in skeletal muscle homeostasis and protein turnover (35). Previous studies with gilthead seabream showed that the expressions of *capn1* and *capns1a* were inversely correlated with muscle texture, suggesting that they may serve as potential genetic markers of flesh quality (34). In salmonids, calpain activity also influences the filet quality but did not seem to substantially function in active muscle turnover (61). In cattle and sheep, a strong correlation has been observed between *capn3* expression levels and meat tenderness (shear force measurements), but no direct evidence could link *capn3* levels with fish (34) or porcine muscle texture (62–64).

In the present study, the relative expression of both *capn1* and *capn2* showed an overall increase with Cr supplementation up to 5%, and the expression of calpain 1 (*capn1*) increased significantly in fish fed with Cr 2 and Cr 5% in comparison with the CTRL group. Moreover, the *capn1* gene not only had a positive and significant correlation with *myod1* but also with *mrf4*, suggesting an important role in myoblast proliferation and fusion in response to Cr supplementation. A strong positive correlation among *capn3* and both *myod1* and *myod2* was also observed in the fast skeletal muscle of gilthead seabream juveniles fed with Cr-supplemented diets. In fish, information regarding the function of calpains on myogenesis is extremely scarce and its involvement in the regulation of MRFs remains largely unknown. However, in gilthead seabream, calpains were shown to be very important during the proliferation phase of early myogenesis, decreasing progressively with development (65). This suggests an anabolic aspect of calpains mainly involved in disassemble of sarcometric structure during muscle remodeling and cell fusion. This is supported by the present findings where a concomitant upregulation of *capn1, capn2,* and *myod* was observed in fish fed with Cr up to 5% resulting in increased myoblast proliferation and fiber hypertrophy. Notwithstanding, previous studies using

TABLE 4 | Correlations between gene expression and muscle growth parameters (DMA and fiber diameter) in gilthead seabream juveniles.

	DMA	Fiber diameter	*capn1*	*capns1a*	*capn2*	*capn3*
myod1	NS	NS	$\rho = 0.804^{**}$	$\rho = 0.650^{*}$	NS	$\rho = 0.580^{*}$
myod2	NS	NS	$\rho = 0.727^{**}$	$\rho = 0.643^{**}$	$\rho = 0.594^{*}$	$\rho = 0.762^{**}$
myf5	NS	NS	NS	NS	$\rho = 0.769^{**}$	NS
mrf4	NS	NS	$\rho = 0.790^{**}$	NS	NS	NS
mstn	NS	$\rho = 0.664^{*}$	NS	NS	NS	NS
myog	$\rho = -0.622^{*}$	NS	NS	NS	NS	NS

NS, not significant. Significance levels set at $P < 0.05$ (*) and $P < 0.01$ (**).
DMA, dorsal cross-sectional muscle area.

μ-calpain (*capn1*) knockout mice reported an increase in size and number of fast-twitch glycolytic muscle fibers, indicating that mice with *capn1* suppressed exhibit an increased capacity to accumulate and maintain protein (i.e., proteins associated with muscle regeneration) in their skeletal muscle, and a decrease in *myod* expression, suggesting less muscle regeneration (66). Studies using C_2C_{12} cells further demonstrated that *capn3* is involved in the myogenic differentiation process, affecting the establishment of the reserve cells pool by decreasing the transcriptional activity of the myod via proteolysis without affecting the other MRFs (67). However, it was shown unlikely that myod function within myotubes was affected due to the presence of high levels of myod. In fish, the proliferation of MPC continues largely after the juvenile stage contrarily to what is observed in higher vertebrates where hyperplasia stops after birth (33). It is, hence, probable that distinct processes may be involved in the regulation of the satellite cell compartment among species. In juvenile seabream, the activity of the myod or the capn3 levels was not evaluated, but the increased expression of *myod1* in fish fed with Cr suggests an activation and differentiation of cells that resulted in increased number and size of muscle fibers. Moreover, the upregulation of *capn3* might have increased muscle proteolysis in Cr-treated gilthead seabream juveniles, but the observed increase in dorsal muscle area also suggests a concomitant increase in protein synthesis probably resulting from the upregulation of *myod1*. Further studies are still required to fully understand the proteolytic system in fish and its implication on the myogenic program.

CONCLUSIONS

From this study, we can conclude that the dietary Cr supplementation in gilthead seabream juveniles resulted in a significant increase in fish DMA. Dietary Cr *per se* significantly affected the expression of some genes related with myogenesis (*myod1*) and others involved in muscle texture and proteolysis (*capn1*), contributing to their upregulation in fish fed up to 5% Cr. Nevertheless, this modulation of gene expression was not enough to produce a significant alteration in muscle phenotype under the tested conditions because a non-significant increase in muscle fiber diameter and higher total number of fiber was observed, but still resulted in increased DMA. Additional studies may be required in order to better clarify the effect of dietary Cr supplementation in fish, possibly in conjunction with the induced resistance training. Moreover, supplementation during teleost's early life stages, where muscle growth is more pronounced (nutritional programming), and evaluation of filet yield and textural properties in commercial-sized fish need further research.

AUTHOR CONTRIBUTIONS

LV and PR conceived and designed the study. LR-P, GL, VS, LC, and DS performed all laboratorial work and collected data. LR-P and GL drafted the manuscript. All authors contributed to the interpretation and discussion of the data. The final version of the manuscript was approved by all the authors.

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Thermal Modulation of Monoamine Levels Influence Fish Stress and Welfare

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Fish are ectotherm organisms that move through different thermal zones according to their physiological requirements and environmental availability, a behavior known as thermoregulation. Thermoregulation in ectothermic animals is influenced by their ability to effectively respond to thermal variations. While it is known that ectotherms are affected by thermal changes, it remains unknown how physiological and/or metabolic traits are impacted by modifications in the thermal environment. In captivity (land-based infrastructures or nets located in the open sea), fish are often restricted to spatially constant temperature conditions within the containment unit and cannot choose among different thermal conditions for thermoregulation. In order to understand how spatial variation of temperature may affect fish welfare and stress, we designed an experiment using either restricted or wide thermal ranges, looking for changes at hormonal and molecular levels. Also, thermal variability impact on fish behavior was measured. Our results showed that in Atlantic salmon (*Salmo salar*), a wide thermal range (ΔT 6.8°C) was associated with significant increases in monoamines hormone levels and in the expression of clock genes. Aggressive and territoriality behavior decreased, positively affecting parameters linked to welfare, such as growth and fin damage. In contrast, a restricted thermal range (ΔT 1.4°C) showed the opposite pattern in all the analyzed parameters, therefore, having detrimental effects on welfare. In conclusion, our results highlight the key role of thermal range amplitude on fish behavior and on interactions with major metabolism-regulating processes, such as hormone performance and molecular regulatory mechanisms that have positive effects on the welfare.

Keywords: confinement, fish husbandry, behavior, HPI-axis, thermoregulation

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INTRODUCTION

Nowadays, there is considerable public debate regarding welfare of fish kept in captivity, specifically under aquaculture conditions. This debate has also raised interest in the scientific community, leading to precise the meaning of welfare in fish through the study of their emotional and cognitive capacities and how captivity may influence these parameters. In this context, studying the dynamics

of social interactions in captive fish is of high relevance for understanding the behavioral biology of a particular species as well as for optimizing its production and avoid negative impacts of culture procedures (1–3). Social interactions are crucial for the development of specific traits, which rely upon a succession of particular interactions among individuals, leading to the development of hierarchies (4). Among those interactions, the result of aggressive encounters has been described as a key factor determining the establishment of dominant or subordinated individuals (5), and for both, aggressive encounters imply an intense social stress (6, 7).

Regarding aquaculture species, salmonids from the genus *Salmo* stand out among other major commercial fish species as they develop, either in nature or captivity, social hierarchies that rely on dominance and social rank, both having important consequences in the physiology and life history of individuals (8, 9). In captive fish, it has been observed that environmental modifications drive changes at hormonal and physiological levels, impacting the social stress and welfare (10). The molecular mechanisms underlying these changes have been widely studied in both endotherms and ectotherms. Among them, circadian regulator genes, monoamine neurotransmitters like serotonin (5-HT) and dopamine (DA) and hormones such as melatonin and cortisol have been demonstrated to play an important role in the stress response and social interaction (11–13). In addition, these molecules have been widely used as welfare and stress assessment indicators, as they are involved in homeostatic mechanisms responsive to allostatic challenges and aggressive behaviors (14, 15).

It has been extensively recognized that appropriate environmental enrichment, defined as a deliberate increase in environmental complexity, with the aim to reduce maladaptive and aberrant traits in fish reared in otherwise stimuli-deprived environments, is widely used in research and culture conditions as a tool for managing fearfulness, undesirable behaviors, stress and welfare of captive animal (16). Understanding the effects of the environmental thermal enrichment in the regulation of behavior and physiology is of high relevance for the identification of the biological mechanisms associated with stress and welfare, not only for captive fish, but also in wild fish under the thermal stress conditions associated to climate change. Given the considerations mentioned above, this study assessed how thermal enrichment can improve the welfare of *Salmo salar*. In our work, the paradigm of maintaining fish at a selected fixed temperature and in contrast, allowing the fish to choose among a broad range of temperatures, thus helping fish to develop natural behavior is discussed. We propose that fish aggressive behavior associated to an estricted thermal environment is driven by a neuroendocrine and molecular response. In order to test this hypothesis, we used a *Salmo salar* model to ask the following three questions:

- Can environmental thermal enrichment improve salmon health?
- How does thermal range amplitude affect fish hormone performance and, ultimately the behavior?

MATERIALS AND METHODS

Fish Husbandry and Experimental Conditions

All animal experiments conformed to international animal research regulations (the British Home Office Regulations. Animal Scientific Procedures Act 1986; care guidelines, EU 2010/63) and follow the guidelines for the use of laboratory animals established by the Chilean National Commission for Scientific and Technological Research (CONICYT), authorized by the Universidad de Concepcion Institutional Animal Care and Use Committee. Thermal experiments were carried out at the ThermoFish Lab, Biotechnology Center, University of Concepcion, Concepción, Chile. Fertilized eggs (n = 8,000) at the eyed egg stage from *Salmo salar* were obtained from AquaGen S.A. (Melipeuco, Chile) in December 2015. After arrival, the eggs were disinfected in iodophore (100 ppm) for 15 min. followed by a wash with clean water. Hatchery conditions were used as described by Boltaña et al. (17). Fish embryos were initially maintained in a temperature-controlled room (18°C). Two recirculating freshwater systems (210 × 150 × 90 cm) were used, with each system using UV-sterilized water and a flow rate of 5 m^3 h^{-1}. Each system contained three independent tanks (60 × 140 × 70 cm). The water temperature of each tank was measured twice per day (7 ± 0.7°C). Each tank was supplied with oxygen, with a saturation between 100 and 150% throughout the entire experiment. Dissolved oxygen was also measured daily and always remained above 9 mg/L^{-1}. Ammonia, nitrite and pH were measured twice per week. Total ammonia and nitrite concentrations in each tank were maintained under 0.05 and 0.01 mg L^{-1}, and pH remained at 8.0 ± 0.5. A 24-h dark cycle photoperiod was used until the embryos hatched. Water temperature, pH and dissolved oxygen were measured with a Multiparameter Water Quality Meter 9829 (Hanna Instruments®, Woonsocket, United States). Ammonia and nitrate / nitrite were measured with QUANTOFIX® test strips (MACHEREY-NAGEL, Dueren, Germany). Larvae were gradually acclimatized to a 12-h light: 12-h dark photoperiod cycle and after the yolk was fully absorbed (40 days post hatching, [dph]), water temperature was increased by 1°C per h until reaching the required thermal ranges. As a note, *S. salar* specimens were raised from first feeding (40 dph) to 5 months post hatching under a 12-h light: 12-h dark photoperiod to artificially reproduce the Autumn–Winter seasons, in correspondence with the annual cycle of species (18). First-feeding (f.f.) was initiated about 40 days after hatching. For the first 2 weeks after f.f., fish were fed according to appetite. Thereafter, they were fed at a daily rate corresponding to approximately 2.5% of bodyweight. Larvae were fed with biomar larvae diet (INICIO PLUS follow by PLUS 18%, Biomar, S.A., Puerto Montt, Chile) twice a day for 5 months. All experiments were performed in a temperature-controlled room (12°C). Fish were randomly assigned to two thermal treatment groups according to Boltaña et al. (19) and kept at a density of 10 kg/m^3. Temperature gradients for both groups were established using an external water jacket system set at different temperatures. This setup provided a continuous

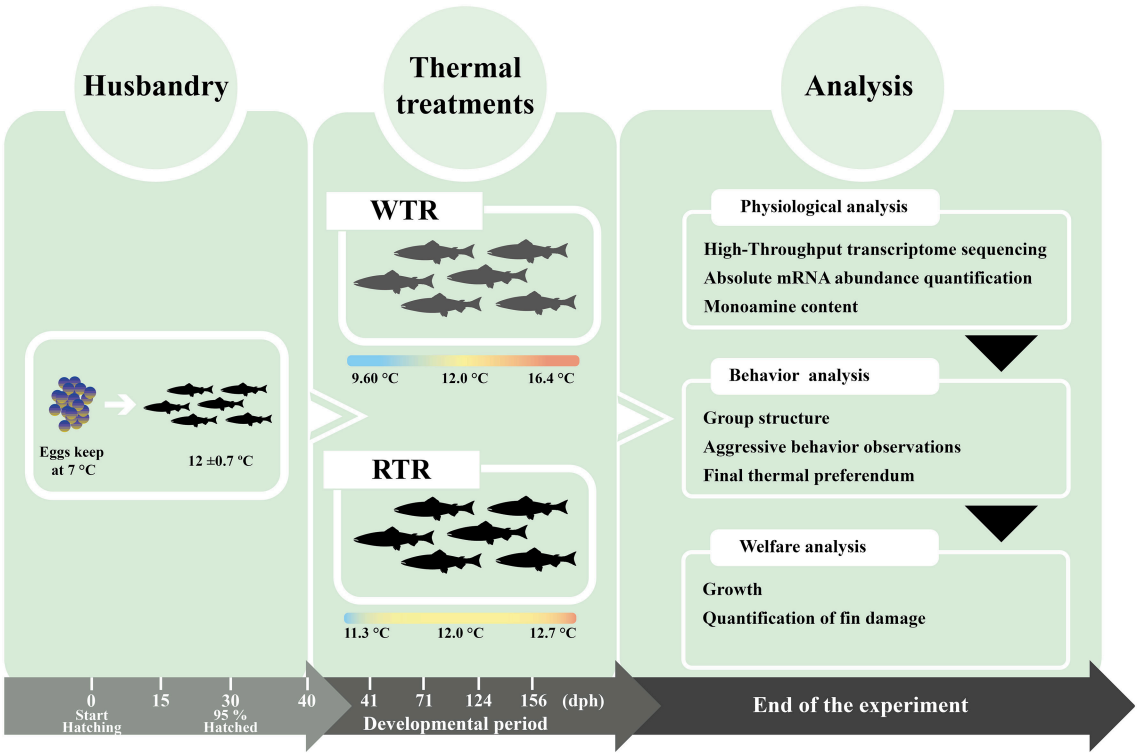

FIGURE 1 | Experimental design the diagram shows the thermal set-up treatments (restricted thermal range; ΔT 1.4°C and wide thermal range; ΔT 6.8°C) for *Salmo salar* rearing over a developmental period (156 dph).

vertical thermal gradient within the tanks, thus generating two treatment conditions (**Figure 1**): (1) wide thermal range (WTR), ΔT 6.8°C (Tmin 9.6°C to Tmax 16.4°C) from top to bottom, respectively, thereby mimicking a natural thermal gradient and (2) restricted thermal range (RTR), ΔT 1.4°C (Tmin 11.3°C to Tmax 12.7°C). Water temperature in the vertical column was recorded by thermal sensors located at different points within the water column (Thermocouple thermometer 53/54 II; Fluke® Corporation, Washington, United States). No significant differences were recorded in oxygen levels throughout the gradient. In the WTR, the temperature gradient was constantly maintained (i.e., 24/7). The gradient ranges were set based on (1) the natural thermal range of *S. salar*, which is between 7 and 22°C (20, 21) and (2) constant laboratory conditions that considered the most common land-based farming conditions. Eight individuals were randomly sampled per point, and tissues were stored in RNAlater® solution (Ambion, United States) at −80°C. To inspect the post morten analysis liver and kidney and gills of lived or dead fish were fixed in 10% buffered formalin for histopathological- and immune-histochemical (IHC) examination (data no shown).

Monoamine Analysis

For the hormone analysis, the brain and the whole body of three replicates per treatment (WTR and RTR) and time (41, 71, 124, and 156 dph) were snap-frozen in liquid nitrogen and conserved at −80°C. The brain and body content of melatonin, dopamine (DA), 3,4-dihydroxyphenylacetic acid (DOPAC, a major da metabolite), 5-HT, and 5-hydroxyindoleacetic acid (5-HIAA) were analyzed in triplicate by high performance liquid chromatography with electrochemical detection as previously described by Gesto et al. (22). Briefly, tissues were homogenized by ultrasonic disruption in 0.5 ml of mobile phase with the following composition: 85 mM sodium hydrogen phosphate, 0.72 mM octanosulfonic acid, 18% methanol, and adjusted to pH 3.0. Homogenates were centrifuged (16,000 × g for 10 min at room temperature) and prior to analysis supernatants were diluted 1:1 or 1:2 (supernatant/mobile phase). A 20-µl aliquot of each sample was injected into the HPLC system consisting of a Jasco PU2080 pump equipped with a Jasco AS-2057 autosampler, and an ESA Coulochem II detector (Bedford, MA, USA). The detection system included a M5011 ESA analytical cell with electrode potentials set at +20 and +300 mV, respectively. All separations were performed at room temperature at a flow rate of 8 ml/min. Acquisition and integration of chromatograms were performed by using the ChromNAV version 1.12 software (Jasco Corp.).

RNA Extraction, cDNA Synthesis and mRNA Abundance Quantification

Random fish (n = 24, four per tank) were sampled for each treatment (WTR and RTR) and time (41, 71, 124, and 156 dph) and subsequently snap-frozen in liquid nitrogen and conserved

at −80°C. Total RNA was extracted from brain and liver with the TRI Reagent® (0.5 mL; Sigma-Aldrich Missouri, United States) and quantified by absorbance at 260 nm. Only samples with an A260/280 ratio between 1.8 and 2.1, and an A260/230 ratio above 1.8 were used for reverse transcription. Purified RNA integrity was confirmed by agarose denaturing gel electrophoresis. cDNA was synthesized from total RNA (200 ng/μL) using the RevertAid H Minus First Strand cDNA Synthesis Kit (Fermentas, Waltham, MA, United States) according to the manufacturer's indications. RT-qPCR was performed using the StepOnePlus™ Real-Time PCR System (Applied Biosystems, Life Technologies, Carolina, United States), and each assay was run in triplicate using the Maxima SYBR Green qPCR Master Mix (2×) (Bio-Rad, Carolina, United States). cDNA used in qPCR assays was first diluted with nuclease free water (Qiagen, Hilden, Germany). Each qPCR mixture contained the SYBR Green Master Mix, 2 μl cDNA, 500 nmol/L each primer, and RNase free water to a final volume of 10 μl. Amplification was performed in triplicate on 96- well plates with the following thermal cycling conditions: initial activation for 10 min at 95°C, followed by 40 cycles of 15 s at 95°C, 30 s at 60°C, and 30 s at 72°C. A dilution series made from known concentrations of plasmid containing the PCR inserts was used to calculate absolute copy numbers for each of the genes examined (17). Real-time PCR assay was carried out in order to analyze the expression pattern in the liver of salmon for genes related to biosynthesis and function of cortisol (Steroid Acute Regulatory Proteins (*stara* and *starb*) and Glucocorticoid Receptor 1 (*gr1*) and in the brain for different clock genes (*bmal, clock, per1, cry2, aanat2, nr1d1;* see details **Supplementary Table 1**).

High-Throughput Transcriptome Sequencing: Library Construction and Illumina Sequencing

To drive this analysis the brain of nine individuals from each experimental set-up (WTR and RTR) time (14, 41, 71, 124, and 156 dph) was dissected, snap-frozen in liquid nitrogen and conserved at −80°C. RNA was individually isolated using Ribo-Pure™ Kit (Ambion®, United States) according to the manufacturer's instructions. The obtained RNA was subsequently treated with DNase I (Fermentas, Massachusetts, United States) to remove genomic DNA according to the manufacturer's protocol. RNA integrity number (RIN) was evaluated through the 2200 TapeStation (Agilent technologies, California, USA) using the R6K screen tape and reagents (Agilent Technologies, California, USA). Samples with RIN $\geq$ 8 and 260/280 ratio $\geq$1.8 were used for library construction. Total RNA from each condition (RTR and WTR) and time (41, 71 124, and 156 dph) were pooled ($n = 3$ individuals by time and treatment) and quantified with Qubit® 2.0 Fluorometer (Invitrogen, California, United States). Samples pools were prepared for Illumina sequencing using KAPA Stranded mRNA-Seq Kit (KapaBiosystems, Massachusetts, United States) according to the manufacturer's instruction. Libraries were analyzed on the 2200 TapeStation (Agilent technologies, California, United States) using D1000 screen tape and reagents (Agilent Technologies, California, United States) and quantified by qPCR using the Library Quantification Kit Illumina/Universal (KapaBiosystems, Massachusetts, United States) according to the manufacturer's instructions before pooling for sequencing on MiSeq (Illumina, Inc., California, United States) platform using a run of 2 × 250 paired-end reads at the Laboratory of Biotechnology and Aquatic Genomics, Interdisciplinary Center for Aquaculture Research (INCAR), Universidad de Concepción, Chile. The raw data for each sequenced developmental stage were separately trimmed by removing adaptor sequences, low quality sequences (quality score of 0.05), and sequences with lengths < 50 nucleotides, using the CLC Genomics Workbench software (Version 11.0.1, CLC Bio, Denmark). The cleaned reads from each developmental stage were used to analyze the differential expression between WTR and RTR groups, and the relative gene transcription levels for a panel composed of 28 transcripts associated with biosynthesis of monoamines and circadian clock. These sequences were used as references to map the reads of each dataset. Using the CLC Genomic Workbench software, values for the reads per kilobase per million mapped reads (RPKM) were separately calculated from the mapping sequences obtained for each developmental stage (two library replicates per stage). The settings used were a mismatch cost of 2, an insert cost of 3, a length fraction of 0.8 and a similarity fraction of 0.8. To visualize the results, a volcano graph showing differentially expressed genes between WTR and RTR groups was used (23). In addition, a hierarchical clustering of features was executed for the dataset, and a heatmap was constructed to plot significant differences in gene transcription between the developmental stages of *S. salar*.

Gene Ontology (GO-DAVID Analysis) and Interactome Analysis

Enrichment of specific gene ontology (GO) terms among the set of probes that are specific to challenges was assessed to correlate a specific set of mRNAs within a brain. In all GO analyses, Ensembl Gene Identifiers were tested using DAVID Bioinformatics Resources (https://david.ncifcrf.gov/home.jsp), (24, 25). Enrichment of each GO term was evaluated through use of the Fisher's exact test and corrected for multiple testing with FDR [pFDR < 0.05; (26)]. We applied a Bonferroni correction to account for multiple tests performed. Each gene set comprised of at least 4 transcripts that shared the same GO biological process or annotation term. The final GO enrichment analysis was carried out with the Cytoscape Cytoscape 3.5.1. (http://www.systemsbiology.org). Topological analysis of individual and combined networks was performed with Network Analyzer, and jActiveModules 2.2 was used to analyze network characteristics (27, 28). GO analyses were conducted with the Biological Network Gene Ontology (ClueGO, version 2.0) plugin (29) used for statistical evaluation of groups of proteins with respect to the current annotations available at the Gene Ontology Consortium (http://www.geneontology.org). In addition, we conducted a complementary analysis with ClusterMaker cytoscape plugin (30), using the MCL algorithm to search protein–protein interaction network modules derived from TAP/MAS (tandem affinity purification/mass spectrometry). This approach clustered the network into modules based on PE Score to indicate the

strength of the node association and given a fixed set of genes with high protein–protein affinity (interactome cluster nodes).

Group Structure and Behavioral Records

To analyse fish group structure, we captured images at each successive 30 min, resulting in 48 images. One group of 10 individuals of each tank (three groups for each thermal treatment) was used. The position of each fish in each image was mapped using its XY coordinates and determined by its center of mass using Image-Pro Plus 7 software (Media Cybernetics, Inc., Rockville, MD, United States). For group structure, we used three parameters to characterize each individual: the nearest neighbor distance (NND), the mean of inter-individual distances (D) and the variance of these inter-individual distances (V) as previously described by Colchen et al. (31). For each group of thermal treatment, the mean and SE of NND, D and V were calculated from 48 images. Aggressive behavior was recorded from the front and side of the tank. A total of 4 videos per tank were obtained. Ten minutes of each video recording were analyzed. Although experimental fish had been accustomed to and not disturbed by the presence of the observer, the first minute of each video was excluded to eliminate possible disquiet caused to the fish by the setting of the camera. Aggressive behavior was estimated by counting the number of aggressive acts as previously described Batzina and Karakatsouli (32) and Batzina et al. (33). Behavioral patterns observed and counted as one aggressive act were: (a) chasing without nipping or biting, (b) nipping without prior chasing, (c) biting without prior chasing, (d) chasing that ended up as nipping and (e) chasing that ended up as biting. Data refer to the whole fish group since fish were not marked and it was impossible to identify which one performed or received an attack. Group structure and behavioral records were analyzed for three consecutive days at the end of the experiment (154, 155, and 156 dph).

Quantification of Fin Damage

Digital photographs were taken at the end of the experimental period and fin damage was evaluated in every fish using the Relative Fin Index (RFI) as described by Bosakowski and Wagner (34). RFI has been suggested to allow reliable and objective measurement of the degree of fin damage (35) and it was obtained by dividing the maximum total fin length (longest fin-ray length from body) by the fork length in each individual fish. Pectoral, caudal and dorsal fins were measured and quantified using this index. Fin erosion was measured using an ordinal scale of 0, 1, 2, and 3, corresponding to no erosion (0% of fin eroded), mild erosion (1–24% of fin eroded), moderate (25–49% of fin eroded) and severe erosion (>50% of fin eroded), respectively (36, 37).

Growth Analysis

Random samples ($n = 10$ per group) were taken at 41, 71, 124, and 156 dph. Sampled fish were anesthetized with MS-222 (3-aminobenzoic acid ethyl ester; Sigma, Vienna, Austria). Body mass (W [g]) was measured using a microbalance Radwag WTB 200 (RADWAG®, Radom, Poland) and digital photographs were taken for body length measurement (TL [mm])using the Image-Pro Plus 7 software (Media Cybernetics, Inc., Rockville, MD, United States). The individual data of the effect of two-thermal treatments on the weight-length relationships were plotted together in order to examine whether there was any pattern in the way that W changes correlated with TL across group. Additionally, Fulton's condition factor (K) was calculated (38). In addition, the interindividual variance of weight was calculated by computing of coefficient of variation (CV) distributions based on individuals from each thermal group. CV value was calculated for each group by dividing the standard deviation of its weight by its average group weight and present as a CV percentage.

Final Thermal Preferendum

The final thermal preferendum analysis was carried out by recording the thermal behavior of fish in each experimental set-up (WTR and RTR). Three video cameras provided continuous monitoring of each tank. During the experiment, temperatures were recorded for 10 s every 15 min throughout 24 h (96 recorded events). Fish distribution inside the tank was monitored over time with video cameras and the number of fish in each compartment was counted manually from the images captured at each successive 15 min, resulting in 96 measurements per day. WTR was achieved with a mean difference in temperature of 6.8°C (Tmin 9.6°C to Tmax 16.4°C). RTR was achieved with a mean temperature of 12.0 ± 0.7°C (ΔT 1.4°C; Tmin 11.3°C to Tmax 12.7°C). All temperatures were recorded each day at the same time of the day.

Statistical Analyses

All data was tested for normality and variance homogeneity s using the Shapiro-Wilk's and Levene's test, respectively. When necessary, data was $\log_{10}$ transformed to achieve normality and homogeneity in all variances. For gene expression (absolute mRNA quantification by real-time polymerase chain reaction qPCR), monoamine content, relative fin index (RFI), total number of fish affected, quantification degree of fin damage, total length, body mass and Fulton's condition factor (K), data was analyzed using a two-way ANOVA followed by the Tukey HSD *post-hoc* test for multiple comparisons, using treatment (WTR and RTR) and sample time (41, 71, 124, and 156 dph), or fin (dorsal, perctoral and caudal) as independent variables. For aggressive behavior observations, group structure and body weight variation coefficients, the obtained data was analyzed using Student's *t*-test. Statistical analysis was undertaken using JMP 11.0.0 (SAS Institute, Cary, NC, United States) and results were considered significant when $p < 0.05$. Graphs were plotted with GraphPad PRISM v6.0 (GraphPad Software, Inc. California, United States).

RESULTS

Analysis of Brain Transcriptomes

To explore how thermal ranges drive changes in gene regulation, we profiled gene expression using RNA-Seq in the brain of salmon under two different thermal conditions, (1) wide thermal range (WTR), and (2) restricted thermal range (RTR). A total of

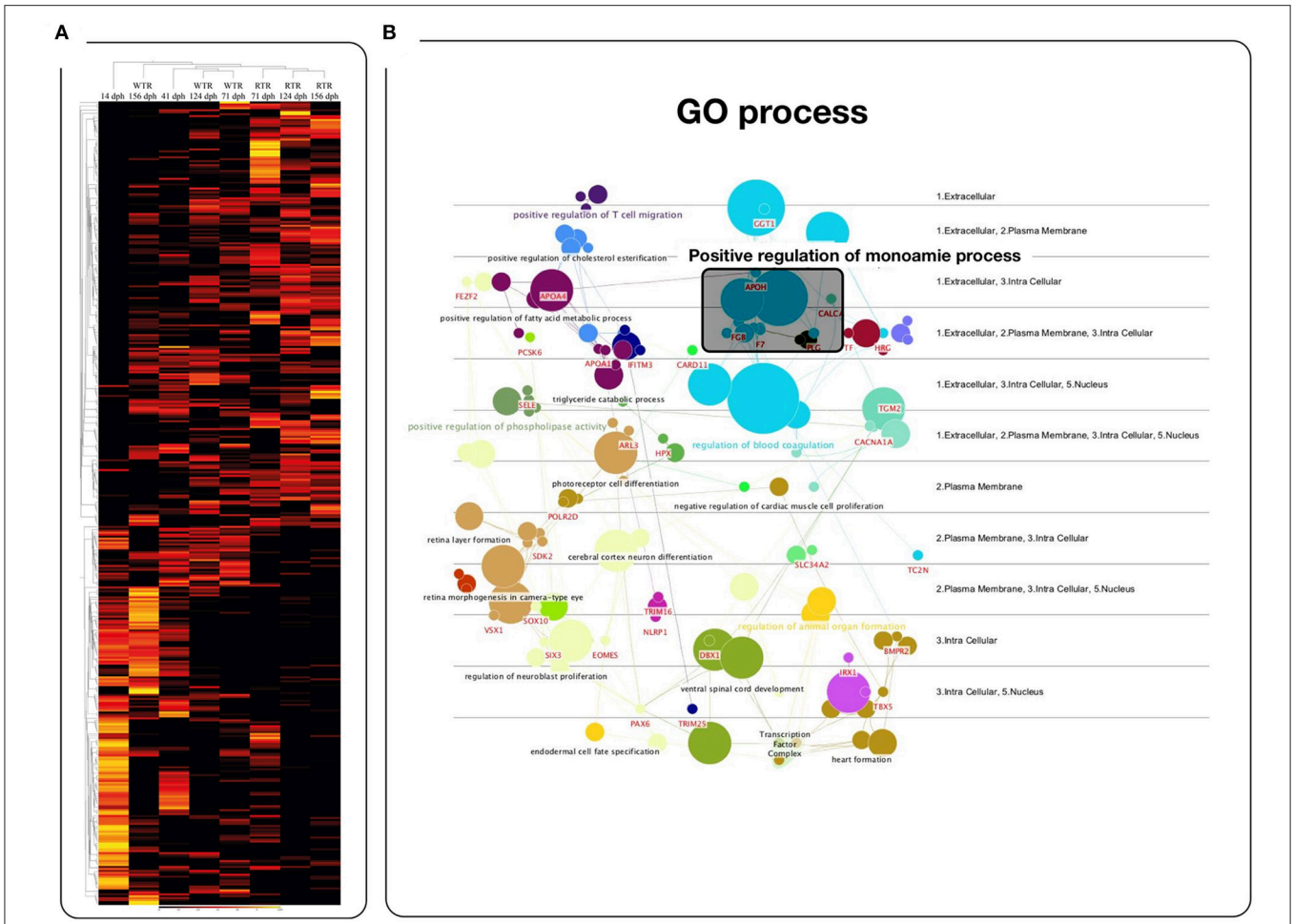

FIGURE 2 | Effects of the temperature on the gene expression levels. **(A)** Heatmaps of log2-transformed gene expression levels for rank-associated genes of each thermal range during the development. **(B)** Gene ontology enrichment analysis of 415 differentially expressed transcripts (GO DAVID and ClueGo Cytoscape Plugin) in *Salmo salar* brain during development (156 days post-hatching). Experimental groups are restricted thermal range (RTR, ΔT 1.4°C) and wide thermal range (WTR, ΔT 6.8°C). Transcript abundance is represented as color scales show relative transcript expression in RPKM values.

415 differentially expressed genes (DEGs) were obtained ($\log_2$ fold change $\geq |2|$; $P \leq 0.05$). The RPKM values of selected candidate genes were visualized in a heatmap based on a hierarchical clustering of features (CLC Genomic Workbench nomenclature) (**Figure 2**). Statistical analysis confirmed that thermal ranges modified the transcriptomes of Atlantic salmon, evidencing the formation of clusters of transcripts with different expression patterns. Our RNA-seq results show that genes related to the biosynthesis of dopamine and serotonin, such as *dcc* (124 and 156 dph), *tph1* (71 and 156 dph) and *tph2* (71 and 124 dph) were upregulated in the group of fish with access to thermal gradient (WTR), while genes involved in the degradation of monoamines, such as *monoamine oxidase a* (*maoa*) and *aldehyde dehydrogenase 2 family* (*aldh2*) were downregulated in the same group of fish at 156 dph. Genes related to the biosynthesis of melatonin such *aanat* (156 dph), *asmt* (124 dph) and *mtnr1b* (71 and 156 dph) were upregulated in WTR. Furthermore, the expression of clock genes *per1-2*, *nr1d1* and *cry1* were upregulated in the WTR group and only *arnt2* was upregulated in RTR group (**Figure 3**).

Absolute mRNA Abundance Quantification

We also profiled the expression of several genes related to the most commonly indicators of stress using absolute mRNA abundance quantification, which did not show large differences with the registered by the analysis of RNA-seq. Among these were cortisol biosynthesis related genes (liver samples) and clock genes (brain samples), important regulators of circadian rhythms, under two different thermal conditions (WTR and RTR). Both *stara* [two-way ANOVA; $F_{(3,\ 88)} = 3.249$; $p = 0.0496$] and *starb* [two-way ANOVA; $F_{(3,\ 88)} = 3.334$; $p = 0.0461$] showed significantly higher mRNA abundance in the restricted thermal range group (RTR) when compared to the wide thermal range group (WTR) from 71 dph onwards. *gr1* transcriptional levels were also higher in the RTR group; however, significant differences were registered only at 71 dph [two-way ANOVA;

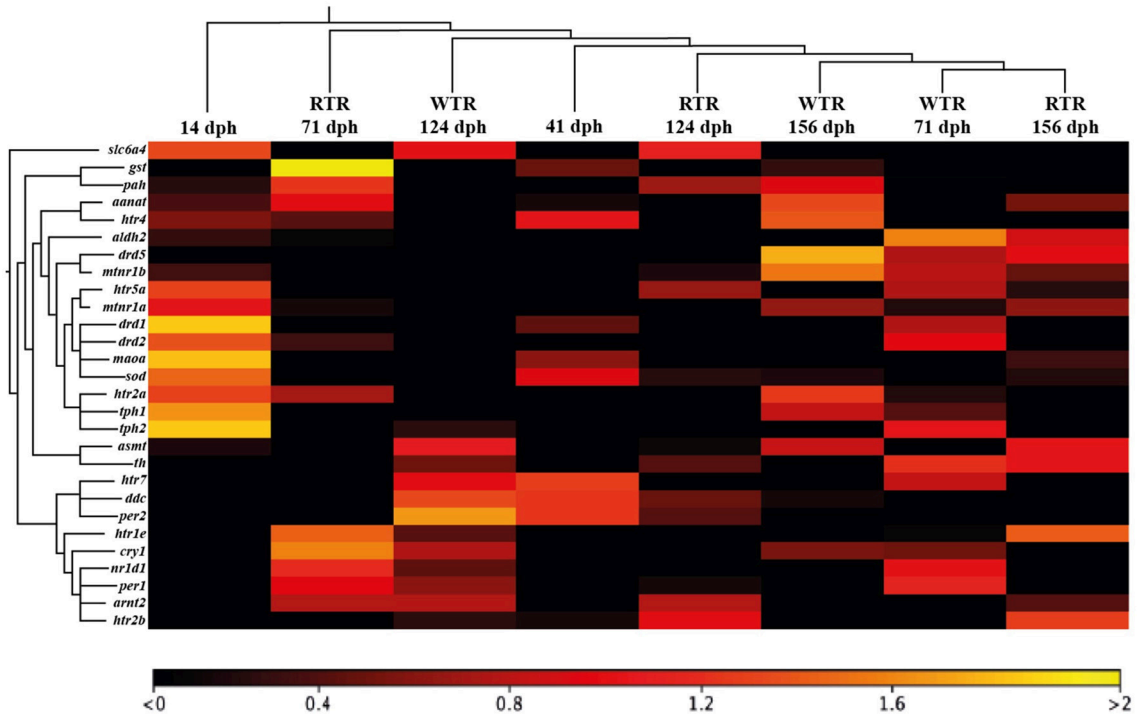

FIGURE 3 | Heatmap representing the expression profile of 28 differentially expressed genes in *Salmo salar* brain during development (156 days post-hatching). Experimental groups are restricted thermal range (RTR, ΔT 1.4°C) and wide thermal range (WTR, ΔT 6.8°C). Transcript abundance is represented as color scales show relative transcript expression in RPKM values.

$F_{(3, 88)} = 3.956$; $p = 0.0291$, **Figure 4A**]. We measured absolute mRNA abundance of four clock genes (*bmal, clock, per1* and *cry2*) in both thermal groups of fish (RTR and WTR). According to **Figure 4B**, in wide thermal range (WTR), *per*1 (124 and 156 dph) showed a significantly higher mRNA abundance [two-way ANOVA; $F_{(3, 88)} = 6.389$; $p = 0.0047$]. While, *bmal, clock* (156 dph) and *cry2* (124 and 156 dph) transcriptional levels were higher in the RTR group [two-way ANOVA; *bmal*, $F_{(3, 88)} = 4.014$; $p = 0.0263$; *clock*, $F_{(3, 88)} = 5.607$; $p = 0.008$ and *cry2*, $F_{(3, 88)} = 3.321$; $p = 0.0466$]. Additionally, *aant2* showed a significantly higher mRNA abundance [two-way ANOVA; $F_{(3, 88)} = 7.445$; $p = 0.0038$] in the wide thermal range group (WTR) at 71 and 124 dph when compared to the restricted thermal range group (RTR). For detail in *p*-values for each factor and the interaction see **Supplementary Table 2**.

Monoamine and Melatonin Content

To inspect hormone levels, we contrasted serotonin (5-HT) content, its metabolite 5-hydroxindoleacetic acid (5-HIAA) dopamine (DA) and its intermediate metabolite, 3,4-dihydroxyphenylacetic acid (DOPAC). Finally, melatonin content in each fish under both experimental set-ups (WTR and RTR) was assesed. The monoamine profile shows statistical differences the interaction of the thermal range (WTR-RTR) and the time (**Figure 5**, for detail in *p*-values for each factor and the interaction see **Supplementary Table 2**). No significant differences were registered between brain content of 5-HT in both groups at 41, 71, and 124 dph. However, at 156 dph, 5-HT content was significantly higher in the WTR group [two-way ANOVA; $F_{(3, 64)} = 4.210$; $p = 0.0327$]. The same pattern was observed for 5-HIAA onward 71 dph [two-way ANOVA; $F_{(3, 64)} = 3.565$; $p = 0.0444$]. When assessing dopamine (DA) levels in brains of fish from RTR and WTR group, and how this level changed over time, we observed significantly higher contents of DA in the WTR group at 71 and 124 dph when compared to RTR group [two-way ANOVA; $F_{(3, 64)} = 4.058$; $p = 0.0362$]. DA metabolite, 3,4-dihydroxyphenylacetic acid (DOPAC), was statistically higher in the WTR group at 156 dph. It is important to remark that both DA and DOPAC [two-way ANOVA; $F_{(3, 64)} = 1.670$; $p = 0.0213$] showed high levels in both experimental groups at 41 dph, with a significant decrease at 71 and 124 dph for DA and DOPAC, respectively. Similar patterns for 5-HT, DA, and their respective metabolites where observed in body monoamine content in both groups (**Figure 5** and **Supplementary Figure 1**). Similar to the monoamine content results, we also observed higher levels of melatonin in brains and body of the WTR group when compared to RTR group at 71 and 124 dph (**Figure 5** and **Supplementary Figure 1**).

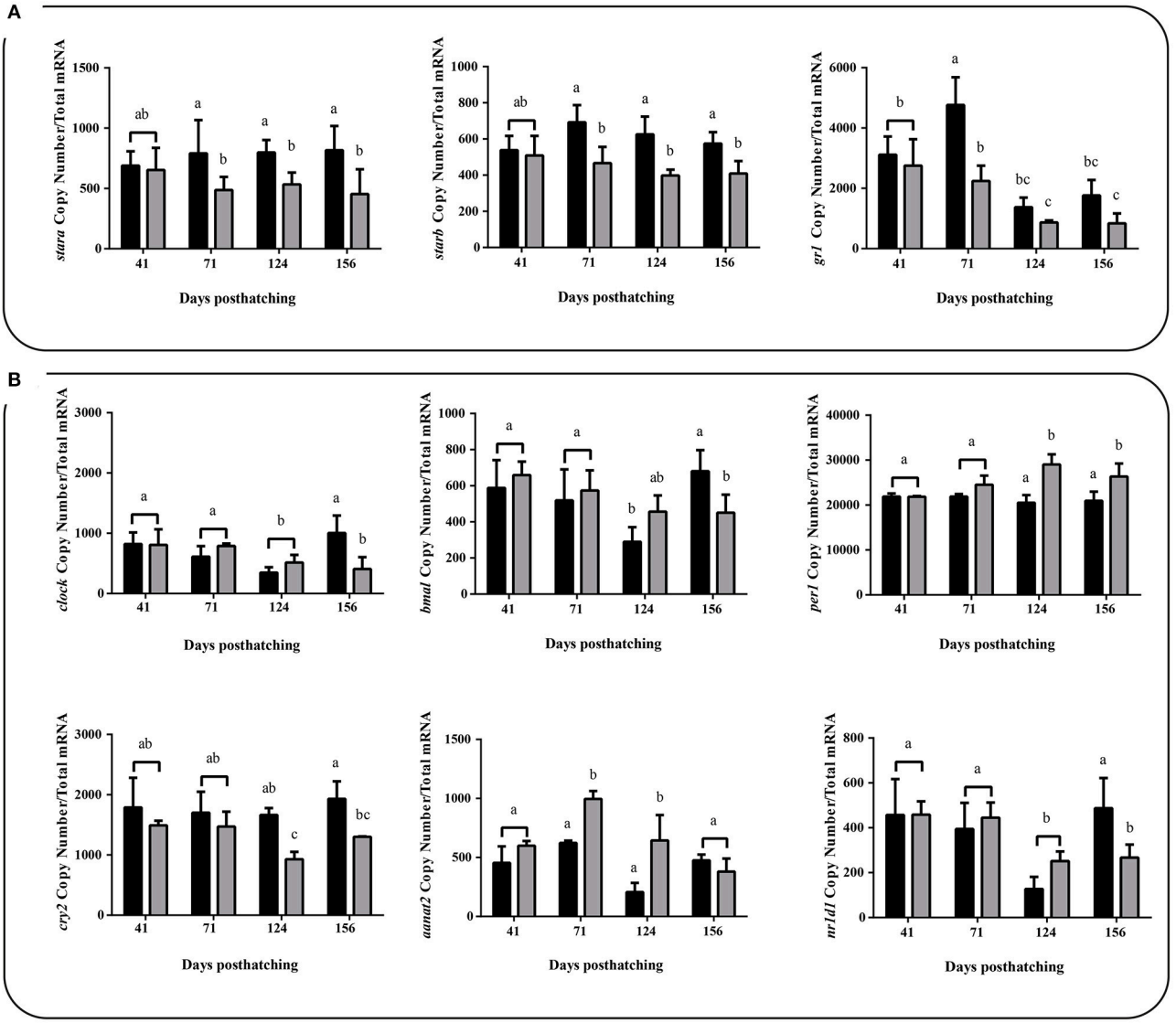

FIGURE 4 | Effect of thermal range in *Salmo salar* during development (156 days post-hatching). **(A)** Liver expression profiles for *stara*, *starb,* and *glucocorticoid receptors 1* (*gr1*). **(B)** Brain expression profiles for clock genes. Values are represented as the mean mRNA abundance ± SD. The experimental groups are: restricted thermal range (RTR, ΔT 1.4°C; black) and wide thermal range (WTR, ΔT 6.8°C; gray). Different letters denote significantly different mRNA levels between groups (2 way ANOVA; $p < 0.05$).

Group Structure and Behavioral Records

Group structure results show that in the wide thermal range group (WTR) the three parameters analyzed [nearest neighbor distance (NND), mean of inter-individual distances (D) and the variance of these inter-individual distances (V)] were significantly lower (Two-tailed Student's *t*-test, $p < 0.0001$), when compared to the individuals kept in a restricted thermal range (RTR), so that NND, D and V decreased around 50, 58, and 75%, respectively (**Figure 6a**). Statistical differences were observed between RTR and WTR groups regarding the number of aggressive encounters registered (Two-tailed Student's *t*-test, $p < 0.0001$; **Figure 6b**, **Supplementary Table 2**). Regarding behavior, in the RTR group we observed a mean of 23 aggressive encounters in the 10 min of video recording. In contrast, a significantly lower number of aggressive encounters was registered for the WTR group, with a mean of 7 encounters. Most aggressive encounters in the RTR group corresponded to chasing without nipping or biting, nipping without prior chasing and chasing that ended up as nipping, representing the 92% of all aggressive encounters (**Figure 6c**).

Growth Performance, Thermal Preferendum, and Fin Damage

Individuals kept in a restricted thermal range (RTR) and fish allowed to move freely in a wide thermal range (WTR) showed a mean Fulton's condition factor (K) of 1,10 ± 0,23, and

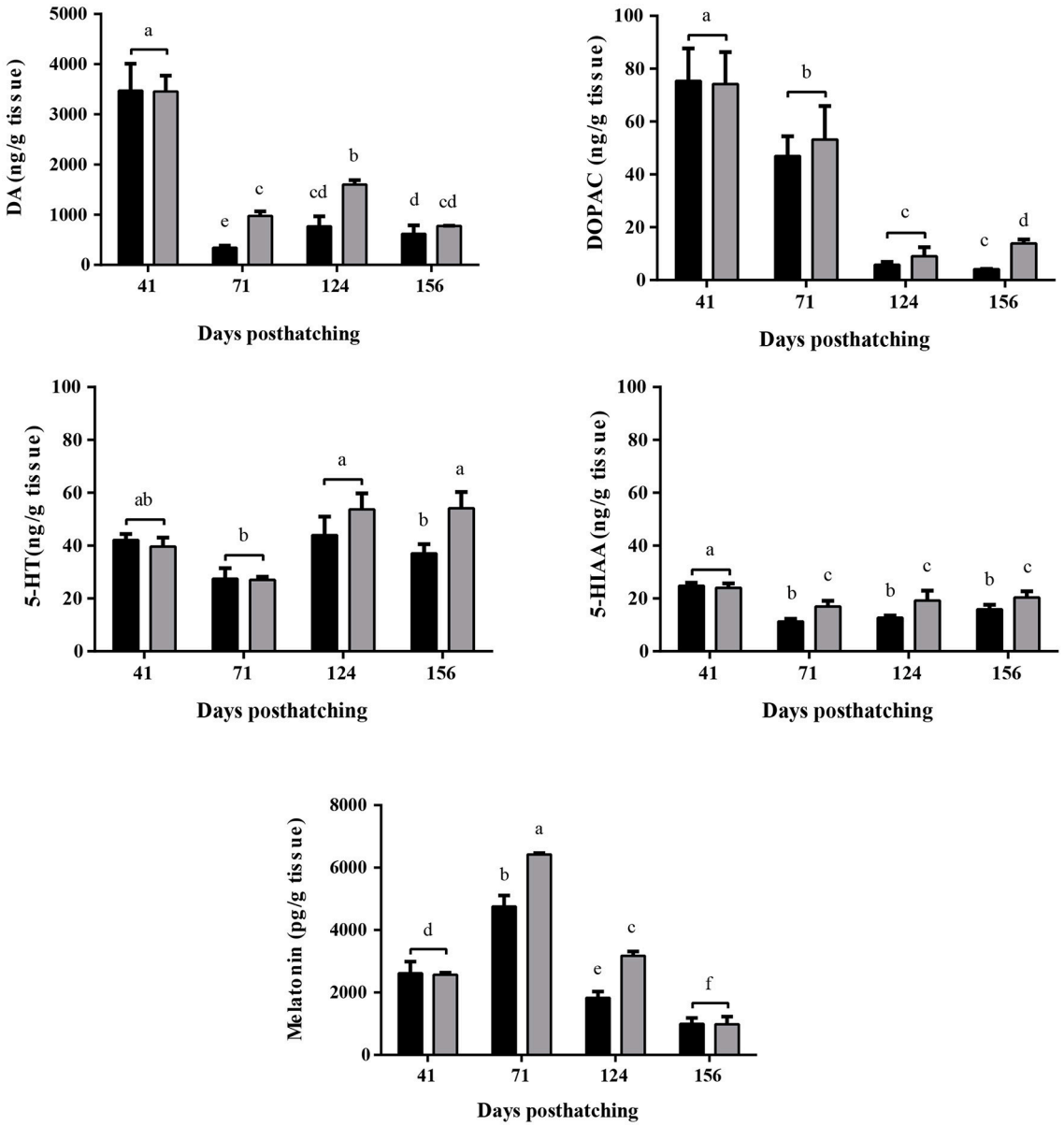

FIGURE 5 | Effect of thermal range in *Salmo salar* brain serotonin (5-HT), 5-hydroxyindoleacetic acid (5-HIAA), dopamine (DA), 3,4-dihydroxyphenylacetic acid (DOPAC) and melatonin concentrations during the development (156 days post-hatching). Values are represented as the mean ± SD. The experimental groups are restricted thermal range (RTR, ΔT 1.4°C; black) and wide thermal range (WTR, ΔT 6.8°C; gray). Different letters denote significantly different concentrations between groups (2 way ANOVA; $p < 0.05$).

1,09 ± 0,27, respectively (**Table 1**). Regarding the K index, as shown in **Figure 7C**, no significant differences were registered for fish growth, indicating that individuals from both groups had a proportional weight, in accordance to their respective body lengths [two-way ANOVA; $F_{(3, 16)} = 1.180$; $p = 0.349$; slope $F_{(1, 172)} = 0.91034$, $p = 0.3414$ and intercept $F_{(1, 173)} = 0.779304$, $p = 0.3786$, **Supplementary Table 2**]. In addition, when analysing growth trajectories, we observed that only the interaction between thermal range (WTR) and development time display significant differences when assesing both length and body mass [two-way ANOVA; $F_{(3, 232)} = 72.06$; $p < 0.0001$ and $F_{(3, 232)} = 37.00$; $p < 0.0001$ respectively, **Figures 7A,B**, for detail in p-values for each factor and the interaction see **Supplementary Table 2**]. We also calculated the coefficient of variation (CV) of weight in each thermal group. Interindividual variance of the RTR group was significantly higher when compared to WTR group (Two-tailed Student's t-test, $p < 0.0001$; **Figure 7D**). In addition, we observed considerable differences when comparing individuals kept in a restricted thermal range (RTR) and fish allowed to move freely in a wide thermal range

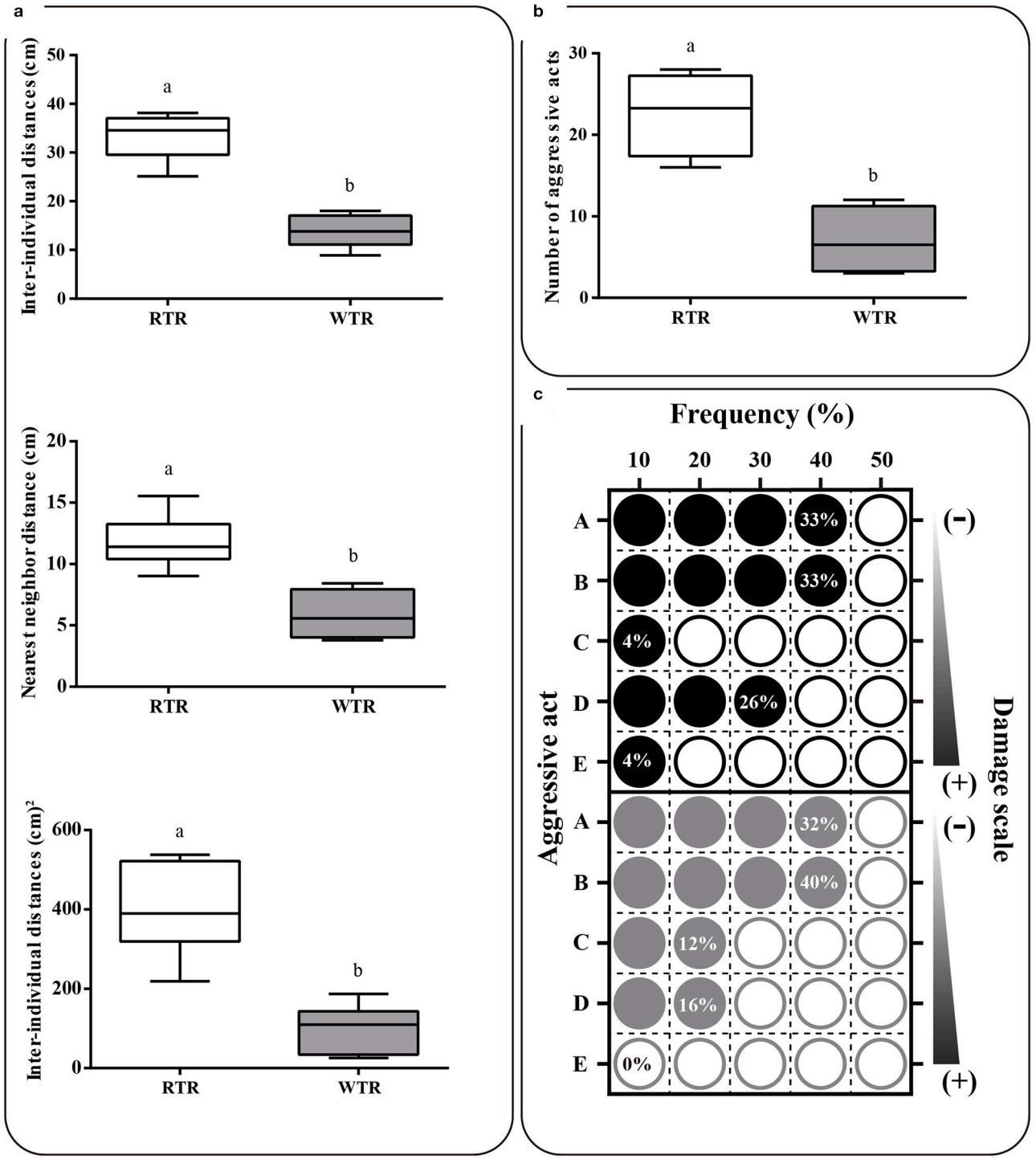

FIGURE 6 | Effect of thermal range in behavioral of *Salmo salar*. **(a)** Nearest neighbor distances, inter-individual distances, and variance of interindividual distances. **(b)** Number of aggressive acts **(c)** Matrix plots registered differences in frequency (%) and potential damage of each aggressive act. Behavioral patterns observed and counted as one aggressive act were: (A) chasing without nipping or biting, (B) nipping without prior chasing, (C) biting without prior chasing, (D) chasing that ended up as nipping and (E) chasing that ended up as biting. The experimental groups are: restricted thermal range (RTR, ΔT 1.4°C) and wide thermal range (WTR, ΔT 6.8°C). Different letters denote significant differences between groups (Two-tailed Student's *t*-test; $p < 0.05$).

(WTR) in parameters related to welfare. The behavioral analysis of the thermal preferendum (**Figure 8**) highlights that individuals placed in the thermal gradient tank constantly moved through the tank and did not prefer a specific zone (no statistical differences shown, two-way ANOVA **Supplementary Table 2**). These results suggest that the observed differences are not related with the fact that WTR fish spent more time in the warmer water layer in the tank. In concordance with the aforementioned results, fin damage analysis shows that although both RTR and WTR individuals display some level of body damage, the number of affected individuals was significantly higher in the RTR group, with around 80% of individuals affected for all dorsal, pectoral and caudal fins (**Figure 9**). In contrast, WTR affected individuals did not exceed the 40%, with no significant differences between dorsal, pectoral and caudal fin [two-way ANOVA; $F_{(2,\ 174)} = 6.391$; $p = 0.0021$; **Figures 9C,D**]. The RFI index was normalized dividing the maximum total fin length (longest fin-ray length from the body) by the fork length in each individual fish. The results observed in the RFI index show significant differences in the dorsal and caudal fins, being higher in the WTR individuals when compared to the RTR ones [two-way ANOVA; $F_{(2,\ 174)} = 39.98$; $p < 0.0001$; **Figure 9A**]. As previously described, we assessed the degree of fin erosion

TABLE 1 | Effect of thermal range in *Salmo salar* Fulton's condition factor.

Fulton's condition factor	RTR ($\bar{X} \pm$ SD)	WTR ($\bar{X} \pm$ SD)
41 dph	0.808 ± 0.162	1.090 ± 0.502
71 dph	1.311 ± 0.253	1.196 ± 0.242
124 dph	1.158 ± 0.088	1.129 ± 0.045
156 dph	1.129 ± 0.109	0.935 ± 0.138

ANOVA table	SS	DF	MS	F (DFn, DFd)	*p*-value
Thermal treatment * dph	0.195	3	0.065	$F_{(3,\ 16)} = 1.180$	0.349
dph	0.317	3	0.106	$F_{(3,\ 16)} = 1.914$	0.168
Thermal treatment	0.001	1	0.001	$F_{(1,\ 16)} = 0.021$	0.886
Residual	0.883	16	0.055		

Values are represented as the mean ± SD. The experimental groups are: restricted thermal range (RTR, ΔT 1.4°C) and wide thermal range (WTR, ΔT 6.8°C). Different letters denote significant differences between groups (2way ANOVA; $p < 0.05$).

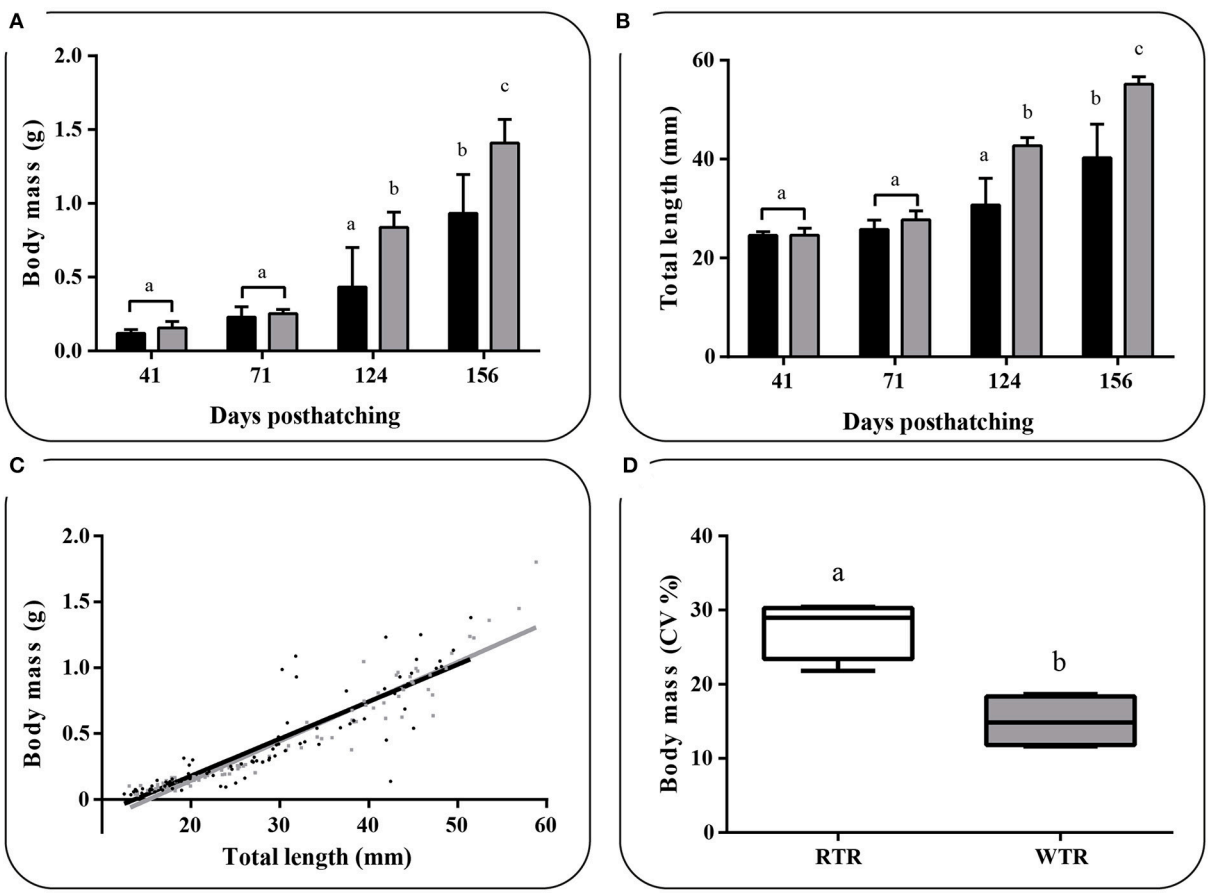

FIGURE 7 | Effect of thermal range in *Salmo salar* growth during the development (156 days post-hatching), expressed as **(A)** changes in body weight, **(B)** changes in body total length, **(C)** weight/length relationship and **(D)** Box and whiskers plots registered differences in coefficients of variation for body weight. Values are represented as the mean ± SD. The experimental groups were: restricted thermal range (RTR, ΔT 1.4°C; black) and wide thermal range (WTR, ΔT 6.8°C; gray). Different letters denote significant differences between groups (2 way ANOVA; Two-tailed Student's *t*-test; $p < 0.05$).

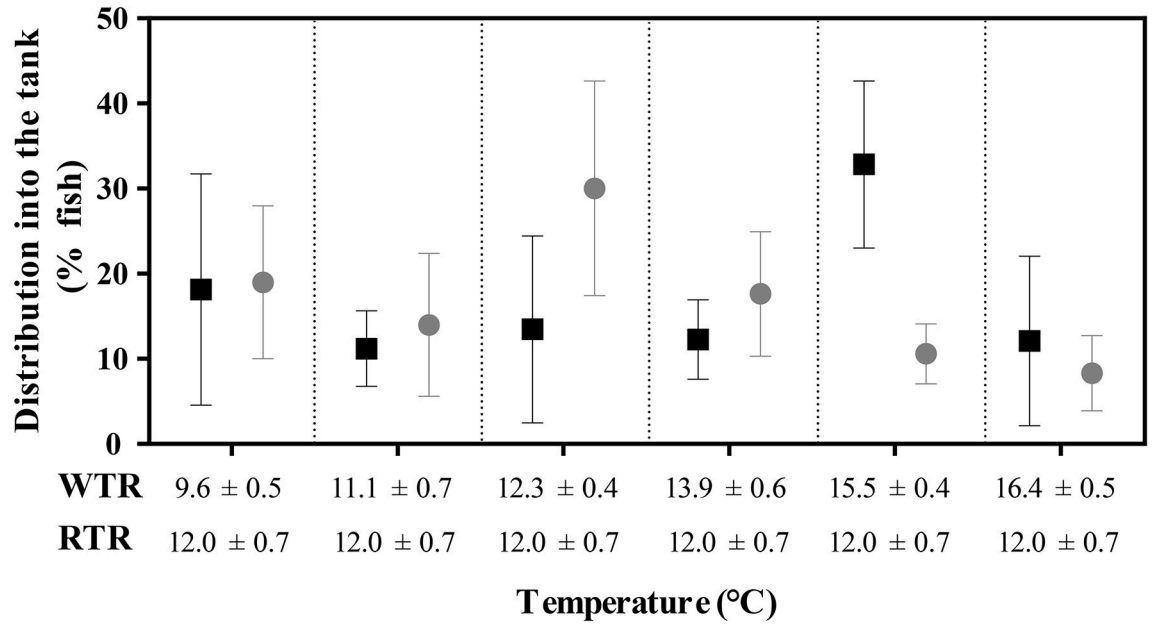

FIGURE 8 | Effect of thermal range in the final thermal preferendum of *Salmo salar.* Values are represented as the mean ± SD. The experimental groups are restricted thermal range (RTR; black) and wide thermal range (WTR; gray). Different letters denote significantly differents between groups (2 way ANOVA; $p < 0.05$).

using an ordinal scale. For all dorsal, pectoral and caudal fins, WTR groups showed approximately 80% of individuals with no erosion. In contrast, only a few individuals showed this degree in the RTR group. Significant erosion (degree 3) was observed in dorsal fins of the RTR group, with more than 80% of the individuals affected. In the same group, pectoral and caudal fin erosion was lower, with most individuals showing mild erosion (degree 1) [two-way ANOVA; $F_{(15, 696)} = 9952$; $p < 0.0001$, **Figure 9B**, for detail in *p*-values for each factor and the interaction see **Supplementary Table 2**].

DISCUSSION

Fish are reared in captivity for a number of different reasons, for example, food production, conservation, stock enhancement, angling, ornamental purposes and research (3). Life in captivity generally promotes maladaptive or unwanted traits compared to those animals adapted to wild conditions (39, 40). Fish farmers and researchers have discussed the mechanisms and methods able to avoid the occurrence of unappropriated characteristics in captive fish, especially when they are destined for release into nature (41), but also when fish are used as model organisms in laboratories (42). In addition, there is a rising public concern for the welfare of captive fish and their difficulty to develop natural behaviors (43). Fish welfare has been defined as the quality of life experienced by the animal itself, such experience is seen as the animal's qualitative assessment of fulfillment of their welfare needs (3, 44). Frequently, fins damage and higher growth rates (Fulton index) are increasingly being used as potential indicators of the welfare of fish (16, 45). While several aspects of fish welfare have received significant attention in the last decades, the question whether the thermal enrichment under captivity conditions impact on the stress and welfare is scarcely discussed in scientific literature. Our results imply, that introducing the thermal variation on the containment unit impact significantly on the hormone performance, allowing preferred behaviors and give animals the possibility to choose their environment. The present results suggest that fish kept at a restricted thermal environment display severe fin damage in terms of relative length and degree of erosion. In both thermal environments, fish moved freely through the tank and freely choose a temperature layer. This results are supported by the video records and data illustrated in **Figure 9** and suggest that the robust differences observed in the growth trajectories, hormone and molecular performance were due tothe thermal enrichment provided in the WTR tank and not due to a preference for a specific zone in the tank. Previous studies have reported that temperature can considerably affect fish growth (17), the present results propose that fish kept at a WTR show higher standards when compared to RTR ones, as far as body condition is concerned. Considering the present context, the aim of the current study was to investigate the degree of influence that spatial variation in temperature range has on *S. salar* welfare, stress or molecular underlying mechanisms. We hypothesized that fish response to spatially variable temperatures would involve temperature-dependent effects related to metabolic demands, such as growth, and hormone performance during development. Understanding the potential effects for this "bottom-up" interaction of thermoregulatory behavior with major biological processes is relevant for identifying reactions to dissimilar thermoregulatory ranges, particularly in the larger context of environmental enrichments of aquaculture or captivity conditions.

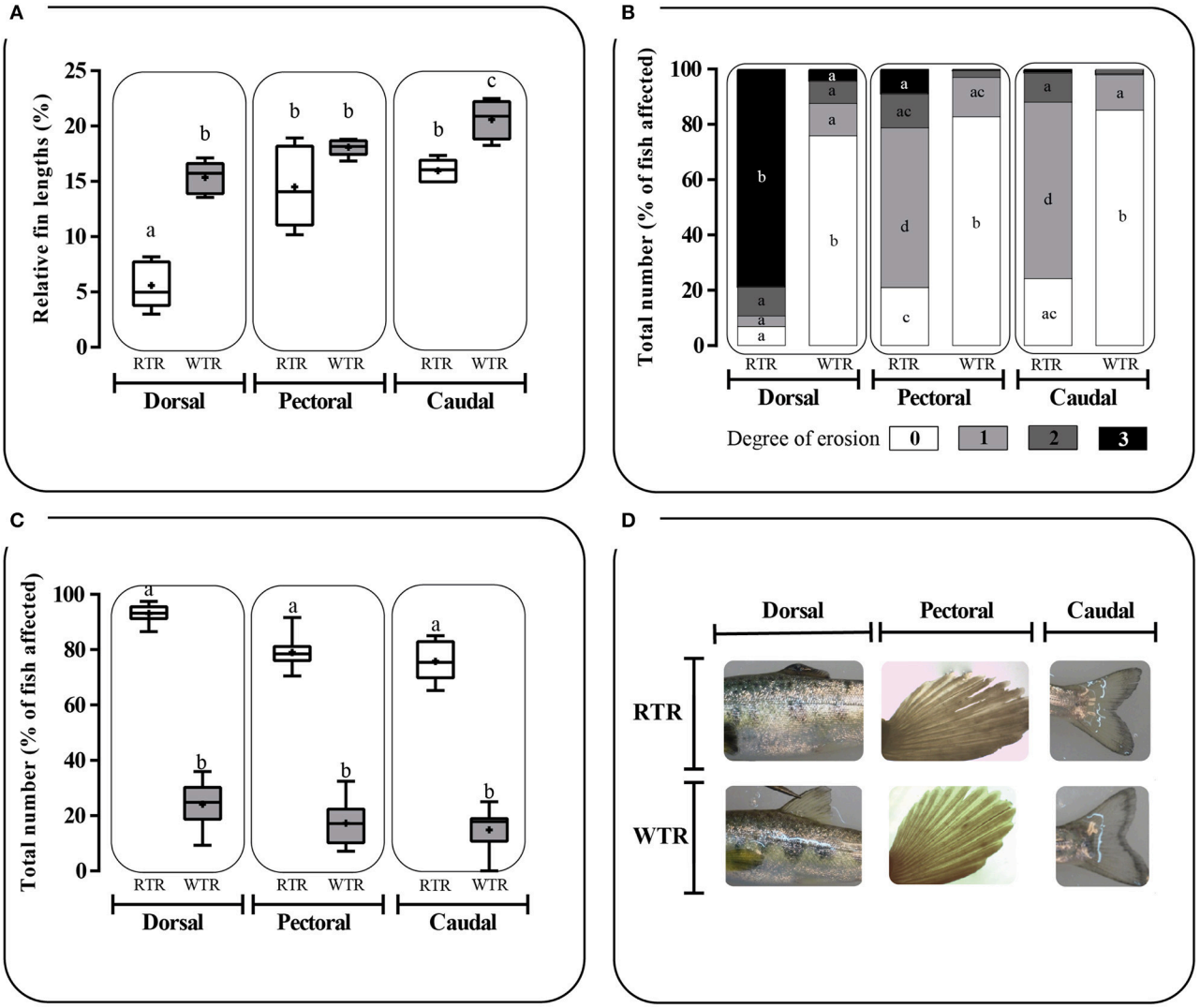

FIGURE 9 | Effect of thermal range in *Salmo salar* fin damage expressed as: **(A)** Relative Fin Index, **(B)** degree of fin erosion and **(C)** total number of fish affected in **(D)** dorsal, pectoral and caudal fin. Fin erosion was measured utilizing an ordinal scale of 0, 1, 2, and 3, corresponding to no erosion (0% of fin eroded), mild erosion (1–24% of fin eroded), moderate (25–49% of fin eroded) and severe erosion (>50% of fin eroded), respectively. Values are represented as the mean ± SD. The experimental groups are: restricted thermal range (RTR, ΔT 1.4°C; black) and wide thermal range (WTR, ΔT 6.8°C; gray). Different letters denote significant differences between groups (2 way ANOVA; $p < 0.05$).

The growth and the coefficient of variation (CV) is a parameter often impacted by temperature, a phenomenon known as growth depensation and defined as the increase in size distribution variance over time due to differential growth rates (46). In captivity, the growth depensation induce those which grow poorly to be socially subordinate (47). In the present study, through our novel experimental set-up we were able to determine key parameters associated with the establishment of social structure which might illustrate changes in the frequency of dominance/subordination interactions induced by the thermal environment (48). Similar results have been observed and show that deficient thermal environments affect inter-individual relations, promoting territorial and aggressive behaviors and consequently compromise fish welfare (31, 49). The present data might explain the growth depensation as a result of a higher frequency of dominance/subordination interactions, however, further studies will be required to fully test this hypothesis.

In culture or captivity conditions, the means to improve the welfare conditions might consist of choice the best species which are more amenable to a life in captivity, for example regarding aggressive behavior. The present study hypothesizes that the thermal enrichment is a key mechanism able to increase the welfare and decrease the aggressive behavior. The mechanism by which the temperature influence the stress and welfare have not been established, in contrast the physiological and

molecular bases for aggressiveness have been widely revised [e.g., Winberg et al. (34); Lepage et al. (50); Johnsson et al. (9); Beiderbeck et al. (51); Dahlbom et al. (6); Mogavero et al. (13); Winberg and Thörnqvist (52)]. Most of these studies suggest that neurotransmitters like serotonin, dopamine and melatonin hormone play a central role in regulating behavior, including aggressiveness or hyperactivity. The collective data suggest that the decreased level of dopamine and serotonin is correlated with an increase of aggressiveness or hyperactivity (12, 53). Our data are in concordance with the previous studies and show that serotonin, and the dopamine were significantly lower in the fish housing in thermal restricted environments (RTR) exhibiting more aggressive encounters and social conflicts.

Previous reports also have established the role clock related genes such as *per1-2*, *bmal1*, *cry1-2*, and *clock* in the modulation of monoamine oxidase a (MAOA), a key enzyme involved in the degradation of monoamines including dopamine or serotonin tightly linked with the regulation of aggressive behavior (54, 55). Specifically, it has been determined that a reduced gene expression of *per1* decreased the levels of dopamine (12). Regarding circadian rhythm control in the suprachiasmatic nucleus, synchronization is induced by serotonin activation, that is directly linked to the melatonin production (56). Our analysis show that the mRNAs linked with the circadian clock were juxtaposed regulated by both thermal environments. We registered a peak of both melatonin hormone and *per1* mRNA abundance in individuals rearing in environmental thermal enrichment (57). In mammals, *per1* mRNA also acts as a negative regulator of *clock* and *bmal* genes (57, 58), which might be explain the observed pattern of fish rearing in restricted thermal environments. This group of individuals (RTR) showed higher expression of *clock* and *bmal* in contrast to WTR ones, however, additional investigation is required as post-translation modifications may occur. Comparison of data sets obtained from RNA-Seq and Absolute-PCRs using the same set of samples showed a good correlation between gene expression profiles. However, some authors suggested that RNA-Seq had the best performance detecting low abundance transcripts or detecting isoforms that allow the identification of genetic variants (59). The performance difference between in the present work between both tools is an area of controversy in the scientific community. PCRs could use internal controls in order to obtain a high reproducibility when analyzing expression data by facilitating a choice between many types of transformation/normalization methods i.e., Efficiency Analysis informs us which methods to choose.

The responsiveness of the HPI-axis was also impacted by temperature. We observed that thermal enrichment provide a complex environment, which allows maximizing normal behaviors and minimizing stress-induced behaviors under captivity conditions (60, 61). Cholesterol is a rate-limiting metabolite as it is the source substrate for cortisol production, and *steroid acute regulatory protein* (*star*) plays the crucial role of transferring the hydrophobic cholesterol, across the aqueous barrier between the outer and inner mitochondrial membrane (62, 63). In cow, it has been demonstrated that mRNA abundance of *star* mirrors cortisol plasmatic levels (64, 65), and in salmon similar results have been observed during chronic stress (63, 66, 67). The present results show that fish under a restricted thermal range (RTR) are able to trigger the expression of mRNAs as *stara* and *starb* (68–70), suggesting that it can be associated to stress condition in contrast to the observed in wide thermal range fish (WTR), however additional experiments including plasmatic cortisol measures should be needed to robustly test this hypothesis. Regarding the glucocorticoid receptor (*gr*), an important mediator of the stress response at the tissue level, has been observed that salmonid fish have duplicate amino acid sequences "g*r1* and *gr2*" (71–73). The differential cortisol receptor subtype signaling may be defined by the kind of stressor (74, 75), tissue distribution and co-localization (71). In our experiment, the decrease observed for *gr1* mRNA abundance suggests a need for decreased cortisol signaling during development in the restricted thermal environment. This negative feedback of *gr1* gene expression has been previously observed in salmon, triggered by elevated plasma cortisol levels during chronical stress condition (70, 72, 76–79), but further analysis will be addressed to test this hypothesis.

In summary, our determinations that the spatial variation of temperature which never has been considered as an environmental/occupational enrichment is a key component to drive an increase the welfare relative to the reduction of physical and psychological monotony. The present results show that the thermal enrichment allows the induction of deep alterations in gene transcription of circadian-clock related genes as well as the modulation of hormone levels that drive a decrease in aggressiveness and territoriality. Our results highlight that the thermal range amplitude might influence health, stress and welfare condition of fish under farm conditions, thus making this study important for any aquaculture researcher, stakeholders and policy makers. The reported results should be contributing novel insights to a central question in endocrinology, and behavioral ecology, where spatial variation of temperature directly impact on the hormonal performance, growth and metabolism and finally on the welfare, consequently, researchers studying adaptation, climate change, behavioral ecology and immunology will be interested in this general phenomenon.

AUTHOR CONTRIBUTIONS

The study was conceived by SB. The behavioral experiments have been performed by NS and AA. JM analyzed the hormone data. RF analyzed the RNA-seq data. LT, AD, NS, BC, and JV have performed the gene expression analysis and provided extensive additional input. SB provided the funding acquisition. SB and LT drafted the manuscript with substantial contributions from all other authors.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2018.00717/full#supplementary-material

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Temperature Affects Musculoskeletal Development and Muscle Lipid Metabolism of Gilthead Sea Bream (*Sparus aurata*)

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World population is expected to increase to approximately 9 thousand million people by 2050 with a consequent food security decline. Besides, climate change is a major challenge that humanity is facing, with a predicted rise in mean sea surface temperature of more than 2°C during this century. This study aims to determine whether a rearing temperature of 19, 24, or 28°C may influence musculoskeletal development and muscle lipid metabolism in gilthead sea bream juveniles. The expression of growth hormone (GH)/insulin-like growth factors (IGFs) system-, osteogenic-, myogenic-, and lipid metabolism-related genes in bone and/or white muscle of treated fish, and the *in vitro* viability, mineralization, and osteogenic genes expression in primary cultured cells derived from bone of the same fish were analyzed. The highest temperature significantly down-regulated *igf-1, igf-2*, the receptor *igf-1ra*, and the binding proteins *igfbp-4* and *igfbp-5b* in bone, and in muscle, *igf-1* and *igf-1ra*, suggesting impaired musculoskeletal development. Concerning myogenic factors expression, contrary responses were observed, since the increase to 24°C significantly down-regulated *myod1* and *mrf4*, while at 28°C *myod2* and *myogenin* were significantly up-regulated. Moreover, in the muscle tissue, the expression of the fatty acid transporters *cd36* and *fabp11*, and the lipases *lipa* and *lpl-lk* resulted significantly increased at elevated temperatures, whereas β-oxidation markers *cpt1a* and *cpt1b* were significantly reduced. Regarding the primary cultured bone-derived cells, a significant up-regulation of the extracellular matrix proteins *on*, *op,* and *ocn* expression was found with increased temperatures, together with a gradual decrease in mineralization along with fish rearing temperature. Overall, these results suggest that increasing water temperature in this species appears to induce unfavorable growth and development of bone and muscle, through modulating the expression of different members of the GH/IGFs axis, myogenic and osteogenic genes, while accelerating the utilization of lipids as an energy source, although less efficiently than at optimal temperatures.

Keywords: thermal exposure, bone cells, white muscle, mineralization, GH/IGFs system, lipid catabolism

INTRODUCTION

Nowadays, society is facing one of the greatest world challenges: how to feed 9 thousand million people by 2050 in the context of global change and economic and financial uncertainty (1, 2). In this situation, aquaculture has a relevant role, satisfying the growing need of fish, and gilthead sea bream (*Sparus aurata*, L.) has become over the last 30 years one of the most important fish species farmed in the Mediterranean area (3). Besides food insecurity, climate change is also a major global challenge that concerns humanity. Warming of the climate system is unequivocal and particularly relevant for this study, the mean sea surface temperature may increase more than 2°C by the end of this century (4). Increased water temperature is known to directly influence several biochemical and physiological processes in ectothermic fish (5), including growth or metabolic rate (6).

Temperature can influence the growth hormone (GH)/insulin-like growth factors (IGFs) system, the main endocrine axis controlling growth in vertebrates. Previous studies have reported a link between environmental temperature and plasma levels of IGF-1 and GH, independently of the nutritional status (7, 8). The GH/IGFs system includes peptides (IGF-1 and IGF-2), IGF-1 receptors (IGF-1Ra and IGF-1Rb), as well as is composed of six IGF binding proteins (IGFBPs) that can exert different effects on IGFs function depending on the cellular context (9, 10). Interestingly, IGFBP-2 in teleosts is the main circulatory binding protein and shows a physiological regulation similar to the most relevant one in mammals, IGFBP-3 (11). Furthermore, elevated temperatures, along with other risk factors such as mineral and vitamin deficiencies, light regimes or fast growth, have been linked in fish with increased occurrence of skeletal anomalies (12). In fact, fast muscle growth can exert high mechanical pressure on the developing bone, hence, synchronicity between bone and muscle growth is required for proper musculoskeletal development [reviewed by Ytteborg et al. (13)]. The higher prevalence of vertebral deformities as a result of increased temperature during the early stages of development has been documented in gilthead sea bream (14), as well as in other species including *Solea senegalensis* (15), *Salmo salar* (16), or *Dicentrarchus labrax* (17). In the case of Sparids, the presence of abnormalities is more evident in larvae reared below 15°C and above 22°C (18), and recent studies have demonstrated in this species that thermal imprinting during embryogenesis causes long-term effects on bone physiology (19, 20). In this sense, the increase in temperature can be recognized as one important problem for aquaculture and animal welfare in a global climate change context.

Cellular and molecular mechanisms for musculoskeletal development in teleost fish have been demonstrated to be similar than in mammals. Osteoblasts, as well as myocytes, arise from mesenchymal stem cells (MSCs), precursor cells that are also able to differentiate into other cell types like chondroblasts or adipocytes after the coordinated induction of key transcription factors expression. Recently, morphological and molecular characterization of a bone-derived cell culture of gilthead sea bream has been reported (20, 21), and the ability of those MSCs to differentiate into other cell types such as adipocyte-like cells has been demonstrated (22). However, these multipotent cells have not been deeply characterized at a structural/functional level. Concerning the main regulators of bone development at a transcriptional level, Runt-related transcription factor 2 (Runx2) is the one required for commitment toward the osteogenic lineage. Afterwards, osteoblasts express molecules of the extracellular matrix (ECM), which include structural fibers as collagen or fibronectin but also non-collagenous proteins that regulate mineralization of the ECM such as osteonectin (ON), osteopontin (OP), and osteocalcin (OCN) (20). In the case of muscle, abundant studies using a satellite cell model system have properly characterized the process of myogenesis in gilthead sea bream (23). The coordinated expression of myogenic regulatory factors (MRFs) is also required for myogenesis to properly occur. Among these transcription factors, Myf5 and MyoD are involved in myocytes activation and proliferation, whereas Myogenin and MRF4 act later allowing myotube formation and maturation (24).

In addition to growth, increased water temperature is also known to directly affect energy demand in ectotherms, and consequently to exert an impact in lipid metabolism and the use of fat depots (25). Lipids are an important energy source for fish skeletal muscle. Lipases such as lipoprotein lipase (LPL) and lipase A (LIPA) can provide fatty acids from triglycerides circulating in the form of chylomicrons and very low-density lipoproteins. Then, fatty acid transporters such as CD36 and FATP1, which are nutritionally and hormonally regulated in fish muscle (26, 27), facilitate the entry of these fatty acids into the cell. Endogenous stored triglycerides, when necessary, can also be hydrolyzed by other lipases as the hormone-sensitive lipase (HSL). Then, non-esterified fatty acids undergo β-oxidation in the mitochondrial matrix (28).

In this framework, the aim of the present study was to evaluate the effects of three increasing rearing temperatures (19, 24, and 28°C) in gilthead sea bream juveniles through an *in vivo*/*in vitro* approach. First, the *in vivo* expression of GH/IGFs axis-, osteogenic-, myogenic- and lipid metabolism-related genes in bone and/or white muscle was determined, and then, the *in vitro* development and expression of osteogenic genes in primary cultured bone-derived cells. All this performed to extend the knowledge of the possible impacts of global climate change on musculoskeletal growth and the physiology in this important aquaculture marine species.

MATERIALS AND METHODS

Animals and Experimental Trial

Gilthead sea bream juveniles (50 g body weight), were obtained from Piscimar fish farm (Andromeda Group, Burriana, Spain) and maintained at the animal facilities of the Faculty of Biology at the University of Barcelona (Spain). After 2 weeks acclimation period, fish were randomly distributed into three 200 L glass tanks (11 fish per tank and condition) under a 12 h light/12 h dark photoperiod, at room temperature (19 ± 1°C). The experiment was performed in January. Fish were daily fed *ad libitum* twice with a commercial diet (Skretting, Burgos, Spain). At the beginning of the trial, all three tanks started at the same temperature of 19°C and then, two of them went from 19 to 24°C

or 28°C, with a rate of Δ1°C each day following the protocol of Hevrøy et al. (6) with a 250 W thermostatic heater (EHEIM, Deizisau, Germany). Once the water temperature required was achieved, fish were held for 3 more days and sampled on the day fourth. A schema of the experimental trial is shown in **Figure 1**. The temperature of the tanks was registered with a precision thermometer (Sera®) three times a day to ensure the corresponding temperature was maintained. Before sampling, fish were fasted for 24 h and then were anesthetized (MS-222 150 mg/L) and subsequently sacrificed by a blow to the head. Samples of white muscle and vertebral bone were collected and immediately frozen in liquid nitrogen and stored at −80°C until further analyses, and small fragments of bone were also used to perform the primary cultures just after sampling as explained in section Primary Culture of Bone-Derived Cells.

All animal handling procedures were approved by the Ethics and Animal Care Committee of the University of Barcelona, in accordance with the guidelines of the European Union Council (86/609/EU), and the Spanish and Catalan Government assigned principles and legislation (permit numbers DAAM 6759 and 9336 for the *in vitro* and *in vivo* experiments, respectively).

Primary Culture of Bone-Derived Cells

Bone fragments of 10 fish per temperature condition were used per culture, each one considered an independent replicate, following the protocol of Capilla et al. (21). Briefly, the vertebras were removed, cleaned of all adherent tissues and washed twice in phosphate buffered saline with 1% antibiotic/antimycotic solution (A/A) prior to manually obtain with a scalpel small (<1 mm) fragments. After that, two digestions of 30 and 90 min, respectively, were done with 0.125% Type II collagenase in Hank's balanced salt solution at 18°C with gentle agitation. Next, the fragments were washed twice with Dulbecco's Modified Eagle Medium (DMEM) supplemented with 1% A/A solution and finally cultured in complete growth medium composed of DMEM supplemented with 10% fetal bovine serum and 1% A/A solution. Cells were seeded into 6 or 12-well plates and incubated at 23°C and 2.5% CO_2. Medium was changed every 2 days. As indicated in the following sections, the fragments were removed from the plates at different days after seeding in order to perform the corresponding assays with the cells attached. To investigate whether temperature could affect the phenotype and differentiation of bone-derived cells in culture, pictures at days 8 and 15 of development were taken with a Canon EOS 1000D digital camera. All cell-culture reagents were purchased from Sigma–Aldrich (Tres Cantos, Spain) and all plastic items were obtained from Nunc (Labclinics, Barcelona, Spain).

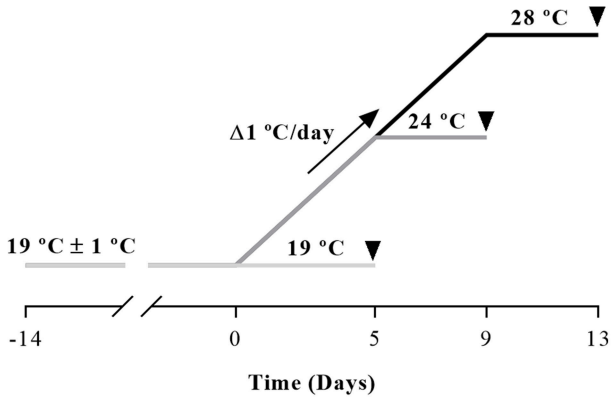

FIGURE 1 | Schematic design of the experimental trial. Gilthead sea bream juveniles were maintained 2 weeks at room temperature (19 ± 1°C) for acclimation before temperature was raised at a rate of 1°C/day with a 250 W thermostatic heater until achieving the desired 24 or 28°C. Fish were held for 3 days at each corresponding temperature and were sampled on the fourth day as indicated by the arrowheads.

Viability Assay

The methylthiazolyldiphenyl-tetrazolium bromide (MTT) assay was used to evaluate cell viability as previously done in gilthead sea bream bone cells (21) and muscle cells (29). This method was selected since comparative studies of different viability assays regularly used revealed that it is the most sensitive one in terms of detecting cytotoxicity (30). Briefly, on days 8 and 15 cell samples of two duplicate wells of the 12 well-plates were incubated for 3 h in DMEM with a final concentration of 5 mg/mL of MTT (Sigma-Aldrich). Then, cells were washed with phosphate buffered saline and resuspended in 150 μL of dimethyl sulfoxide (DMSO) per well. The viability values were obtained from the absorbance measured at 570 nm in duplicate 96-wells, with correction at 650 nm, using a microplate reader (Tecan Infinite 200). Data from day 15 cells are presented as fold change relative to each corresponding day 8 of culture ($n = 10$).

Mineralization Assay

Culture differentiation was evaluated according to mineralization of the ECM. The deposition of minerals was analyzed in day 20 cultured cells by alizarin red S (ARS) staining, following the protocol of Capilla et al. (21). Cells were fixed for 15 min with 10% formalin and stained with 2% ARS (pH 4.1–4.3) during 20 min. After washing excessive dye with water, quantification of the staining was done by means of acid extraction of the ARS stain with 10% acetic acid. The monolayer was then scrapped and transferred to a 1.5 ml tube. After vortex, the slurry was overlaid with mineral oil (Sigma–Aldrich), heated to 85°C for 10 min, cooled on ice and centrifuged at 16,000 g for 15 min. At this point, 10% ammonium hydroxide was added to the supernatant to neutralize the acid and finally, aliquots of the different samples were read at 405 nm in duplicate 96-wells, using a microplate reader (Tecan Infinite 200). Data are presented as optical density arbitrary units ($n = 10$).

Gene Expression Analyses

RNA Extraction and cDNA Synthesis

Total RNA was extracted from ∼100 mg of vertebral bone and white muscle tissues, or from cell samples of two duplicate wells of the 6 well-plates at day 15 with 1 mL of TRI Reagent Solution (Applied Biosystems, Alcobendas, Spain) following the manufacturer's instructions. Total concentration and purity were determined using a NanoDrop 2000 (Thermo Scientific,

Alcobendas, Spain), and integrity of the different samples was confirmed in a 1% agarose gel (w/v) stained with SYBR-Safe DNA Gel Stain (Life Technologies, Alcobendas, Spain). Next, 1,000 ng of total RNA were treated with DNase I (Life Technologies) to remove all genomic DNA, and reverse transcribed with the Transcriptor First Strand cDNA Synthesis Kit (Roche, Sant Cugat del Valles, Spain). The cDNA obtained was stored at −20°C for real-time quantitative PCR analyses (qPCR).

Real-Time Quantitative PCR

The mRNA transcript levels of the target genes plus three reference genes were examined in a CFX384^{TM} real-time system (Bio-Rad, El Prat de Llobregat, Spain). All the analyses were performed in triplicate wells using 384-well plates with 2.5 μL of iTaq Universal SYBR Green Supermix (Bio-Rad), 250 nM final concentration of forward and reverse primers (**Table 1**) and 1 μL of diluted cDNA for each sample, in a final volume of 5 μL. As described before (31, 32), reactions were performed with an initial activation step of 3 min at 95°C, 40 cycles of 10 s at 95°C and 30 s at 55–68°C (primer-dependent, see **Table 1**) followed by an amplicon dissociation analysis from 55 to 95°C at 0.5°C increase each 30 s. Before the analyses, a dilution curve with a pool of samples was run to confirm primer efficiency, specificity of the reaction, absence of primer-dimers, and to determine the appropriate cDNA dilution for each assay. Negative controls [no template control (NTC), no reverse transcriptase control (RTC) and MilliQ water (PCR)] were included and run in duplicate. The expression level of each target gene analyzed was calculated using the Pfaffl method (33), relative to the geometric mean of the two most stable reference genes determined for each tissue by the geNorm algorithm, both implemented in the Bio-Rad CFX manager 3.1. software.

Statistical Analysis

Data were analyzed using IBM SPSS Statistics v. 22 (IBM, Armonk, USA) and are presented as Mean ± SEM. Data normality and homoscedasticity were tested among temperature groups using Shapiro–Wilk and Levene's tests, respectively. Statistical significance was assessed by one-way analysis of variance (one-way ANOVA) followed by Tukey's *post-hoc* test. When homoscedasticity was not observed Dunnett T3 test was applied. Statistical differences were considered significant for all analysis when $p < 0.05$.

RESULTS

HSPs and Proliferation Marker Genes Expression in Bone and White Muscle Tissues

In bone, heat shock proteins, *hsp30* and *hsp90b* mRNA levels were similar among the three groups (**Figure 2A**), while the temperature of 24°C caused a significant increase in the gene expression of the proliferating cell nuclear antigen (*pcna*) respect to the other groups (**Figure 2B**).

In white muscle, *hsp30* remained also unaltered, but *hsp90b* gene expression was highest at 28°C (**Figure 2C**). Concerning *pcna*, a significant up-regulation at 24°C compared with the 19°C condition as in bone was observed (**Figure 2D**).

GH-IGFs Axis-, Osteogenic-, and Myogenic-Related Genes Expression in Bone and White Muscle Tissues

In bone, the mRNA levels of *igf-1*, *igf-2*, *igfbp-4, igfbp-5b,* and *igf-1ra* were significantly down-regulated at 28°C compared with the 19°C reared fish and, in most cases, compared to the 24°C condition as well (**Figures 3A,B**). Contrarily, *igfbp-1a, ghr-1, ghr-2,* and *igf-1rb* did not show differences among groups, although the former presented a tendency to gradually increase along with temperature. Concerning the osteogenic genes analyzed, none of them showed significant differences in response to temperature treatment under the experimental *in vivo* conditions tested (**Figure 3C**).

In white muscle, *igf-1* mRNA levels were lower at 28°C, although differences were only significant compared to 24°C-exposed fish (**Figure 3D**). Moreover, *igfbp-1a* showed the same pattern as observed in bone tissue, significantly increasing its expression along with temperature. *igf-2* and *igfbp-4* did not revealed differences among groups, and *igfbp-5b* was not detectable in this tissue. Furthermore, the levels of expression of *gh* and *igf-1 receptors* were unaltered for *ghr-1* compared with the group of 19°C but were significantly lower for *ghr-2* and *igf-1rb* at 24°C and for *igf-1ra* at 24 and 28°C (**Figure 3E**). With regards to the myogenic-related genes, the expression of *myod1* and *mrf4* was significantly lower in the 24°C-held fish compared to the other two groups, while *myod2* and *myogenin* mRNA levels were significantly higher in the fish kept at 28°C, and neither muscle growth inhibitor, *mstn1* nor *mstn2*, were affected by the rearing temperature (**Figure 3F**).

In addition, the expression of *igfbp-2b* was analyzed in both tissues, although none of them showed detectable levels.

Lipid Metabolism-Related Genes Expression in White Muscle Tissue

The fatty acid transporter *cd36* was significantly up-regulated with the temperature rise, showing the fish at 28°C the highest mRNA levels. The expression of *fatp1* remained unaltered, but *fabp11* was significantly enhanced at 24 and 28°C compared with the 19°C group (**Figure 4A**). Concerning lipases, although *hsl* mRNA levels were not different among groups, *lipa* and *lpl-lk* transcript levels were significantly increased in 28°C, or 24 and 28°C-exposed fish, respectively, compared with the 19°C group. In parallel to this, significant up-regulation of the lipase maturation factor (*lmf1*, an essential gene for the folding and assembly of LPL) was detected in the 28°C-reared fish (**Figure 4B**). Regarding β-oxidation markers, the gene expression of mitochondrial carnitine palmitoyltransferases (*cpt1a* and *cpt1b*) was significantly lower in juveniles maintained at 24 and 28°C when compared with fish reared at 19°C, while hydroxyacyl-CoA dehydrogenase *hadh* expression did not show significant differences among groups. Contrarily, the mitochondrial uncoupling protein *ucp2* showed significantly higher mRNA levels with increased temperature (**Figure 4C**).

TABLE 1 | Primers used in the qPCR analyses: sequences, melting temperatures (Tm), and GenBank accession numbers.

Gene	Primer sequences (5′-3′)	Tm, °C	Accession number
rps18	F: GGGTGTTGGCAGACGTTAC	60	AM490061.1
	R: CTTCTGCCTGTTGAGGAACCA		
ef1a	F: CTTCAACGCTCAGGTCATCAT	60	AF184170
	R: GCACAGCGAAACGACCAAGGGGA		
rpl27a	F: AAGAGGAACACAACTCACTGCCCCAC	68	AY188520
	R: GCTTGCCTTTGCCCAGAACTTTGTAG		
hsp30	F: GGTGACGGGAAAGAGA	60	GU60312
	R: CTGAGGAGGAGGTGCTGTTC		
hsp90b	F: TTCACGCATGGAAGAAGTTG	56	DQ012949
	R: GGTCCACCACACAACATGAA		
pcna	F: TGTTTGAGGCACGTCTGGTT	58	NM_131404.2
	R: TGGCTAGGTTTCTGTCGC		
igf-1	F: ACAGAATGTAGGGACGGAGCGAATGGAC	60	EF688016
	R: TTCGGACCATTGTTAGCCTCCTCTCTG		
igf-2	F: TGGGATCGTAGAGGAGTGTTGT	60	AY996778
	R: CTGTAGAGAGGTGGCCGACA		
igfbp-1a	F: AGTGCGAGTCCTCTCTGGAT	60	KM522771
	R: TCTCTTTAAGGGCACTCGGC		
igfbp-2b	F: CGGGCTGCTGCTGACATACG	60	AF377998
	R: GTCCCGTCGCACCTCATTTG		
igfbp-4	F: TCCACAAACCAGAGAAGCAA	68	F5T95CD02JMZ9K
	R: GGGTATGGGGATTGTGAAGA		
igfbp-5b	F: TTTCTCTCTCGGTGTGC	60	AM963285
	R: TCAAGTATCGGCTCCAG		
ghr-1	F: ACCTGTCAGCCACCACATGA	60	AF438176
	R: TCGTGCAGATCTGGGTCGTA		
ghr-2	F: GAGTGAACCCGGCCTGACAG	60	AY573601
	R: GCGGTGGTATCTGATTCATGGT		
igf-1ra	F: AGCATCAAAGACGAACTGG	55	KT156846
	R: CTCCTCGCTGTAGAAGAAGC		
igf-1rb	F: GCTAATGCGAATGTGTTGG	55	KT156847
	R: CGTCCTTTATGCTGCTGATG		
runx2	F: ACCCGTCCTACCTGAGTCC	60	JX232063
	R: AGAAGAACCTGGCAATCGTC		
fib1a	F: CGGTAATAACTACAGAATCGGTGAG	60	FG262933
	R: CGCATTTGAACTCGCCCTTG		
bmp2	F: GGAGAAGCAGCGTGGATTAAACACGAAT	65	AY500244
	R: GGCCTGCGCCTCAGTCCAAACATATT		
bmp4	F: CACGCCATTGTTCAGACACT	60	FJ436409
	R: GCCCTCCACTACCATTTCCT		
mgp	F: TGTGTAATTTATGTAGTTGTTCTGTGGCATCTCC	68	AY065652
	R: CGGGCGGATAGTGTGAAAATGGTTAGTG		
on	F: AGGAGGAGGTCATCGTGGAAGAGCC	68	AY239014
	R: GTGGTGGTTCAGGCAGGGATTCTCA		
op	F: AAAACCCAGGAGATAAACTCAAGACAACCCA	68	AY651247
	R: AGAACCGTGGCAAAGAGCAGAACGAA		
ocn	F: TCCGCAGTGGTGAGACAGAAG	56	AF048703
	R: CGGTCCGTAGTAGGCCGTGTAG		
pax7	F: ATGAACACTGTCGGCAACG	64	JN034418
	R: AGGCTGTCCACACTCTTGATG		

(Continued)

TABLE 1 | Continued

Gene	Primer sequences (5′-3′)	Tm, °C	Accession number
myf5	F: CTACGAGAGCAGGTGGAGAACT	64	JN034420
	R: TGTCTTATCGCCCAAAGTGTC		
myod1	F: TTTGAGGACCTGGACCC	60	AF478568.1
	R: CTTCTGCGTGGTGATGGA		
myod2	F: CACTACAGCGGGGATTCAGAC	60	AF478569
	R: CGTTTGCTTCTCCTGGACTC		
myogenin	F: CAGAGGCTGCCCAAGGTGGAG	68	EF462191
	R: CAGGTGCTGCCCGAACTGGGCTCG		
mrf4	F: CATCCCACAGCTTTAAAGGCA	60	JN034421
	R: GAGGACGCCGAAGATTCACT		
mstn1	F: GTACGACGTGCTGGGAGACG	60	AF258448.1
	R: CGTACGATTCGATTCGCTTG		
mstn2	F: ACCTGGTGAACAAAGCCAAC	60	AY046314
	R: TGCGGTTGAAGTAGAGCATG		
cd36	F: GTCGTGGCTCAAGTCTTCCA	60	-
	R: TTTCCCGTGGCCTGTATTCC		
fatp1	F: CAACAGAGGTGGAGGGCATT	60	-
	R: GGGGAGATACGCAGGAACAC		
fabp11	F: CATTTGAGGAGACCACCGCT	60	-
	R: ACTTGAGTTTGGTGGTACGCT		
hsl	F: GCTTTGCTTCAGTTTACCACCATTTC	60	EU254478
	R: GATGTAGCGACCCTTCTGGATGATGTG		
lipa	F: TACTACATCGGACACTCTCAAGGAAC	60	JQ308831
	R: GTGGAGAACGCTATGAATGCTATCG		
lpl-lk	F: CAGAGATGGAGCCGTCACTCAC	60	JQ390609
	R: TCTGTCACCAGCAGGAACGAATG		
lmf1	F: CGGCTGGACTGGCTCATGT	60	JX975718
	R: CTCACTCTGCTCGTAGGTCTGGAA		
cpt1a	F: GTGCCTTCGTTCGTTCCATGATC	60	JQ308822
	R: TGATGCTTTATCTGCTGCCTGTTTG		
cpt1b	F: CCACCAGCCAGACTCCACAG	60	DQ866821
	R: CACCACCAGCACCCACATATTTAG		
hadh	F: GAACCTCAGCAACAAGCCAAGAG	60	JQ308829
	R: CTAAGAGGCGGTTGACAATGAATCC		
ucp2	F: CGGCGGCGTCCTCAGTTG	60	JQ859959
	R: AAGCAAGTGGTCCCTCTTTGGTCAT		

F, forward; R, reverse. rps18, ribosomal protein s18; ef1a, elongation factor 1 alpha; rpl27a, ribosomal protein l27a; hsp30, heat shock protein 30; hsp90b, heat shock protein 90b; pcna, proliferating cell nuclear antigen; igf-1, insulin-like growth factor 1; igf-2, insulin-like growth factor 2; igfbp-1a, insulin-like growth factor binding protein 1a; igfbp-2b, insulin-like growth factor binding protein 2b; igfbp-4, insulin-like growth factor binding protein 4; igfbp-5b, insulin-like growth factor binding protein 5b; ghr-1, growth hormone receptor 1; ghr-2, growth hormone receptor 2; igf-1ra, insulin-like growth factor 1 receptor a; igf-1rb, insulin-like growth factor 1 receptor b; runx2, runt-related transcription factor 2; fib1a, fibronectin subunit 1a; bmp2, bone morphogenetic protein 2; bmp4, bone morphogenetic protein 4; mgp, matrix gla protein; on, osteonectin; op, osteopontin; ocn, osteocalcin; pax7, paired box 7; myf5, myogenic factor 5; myod1, myogenic differentiation 1; myod2, myogenic differentiation 2; myogenin, myogenin; mrf4, myogenic regulatory factor 4; mstn1, myostatin 1; mstn2, myostatin 2; cd36, cluster of differentiation 36; fatp1, fatty acid transport protein 1; fabp11, fatty acid binding protein 11; hsl, hormone sensitive lipase; lipa, lipase a; lpl-lk, lipoprotein lipase-like; lmf1, lipase maturation factor 1; cpt1a, carnitine palmitoyltransferase 1a; cpt1b, carnitine palmitoyltransferase 1b; hadh, hydroxyacil-CoA dehydrogenase; ucp2, uncoupling protein 2.

Characterization of the Bone-Derived Cells Culture

The effects of temperature during the differentiation of gilthead sea bream bone-derived cells are presented in **Figure 5A**. Morphologically, in all three groups, the cells showed at day 8 a spindle-like phenotype that changed to a polygonal one at day 15 as the cells differentiated spontaneously into osteoblasts. Moreover, deposits of minerals started to accumulate in the ECM, although mineral nodules were apparently in greater proportion in cells derived from the 19°C-reared fish compared to the other two groups. Besides, as shown in **Figure 5B**, significantly lower viability values were found in cells coming from fish reared at 24 and 28°C, compared with those at 19°C; and the same was observed concerning the deposition

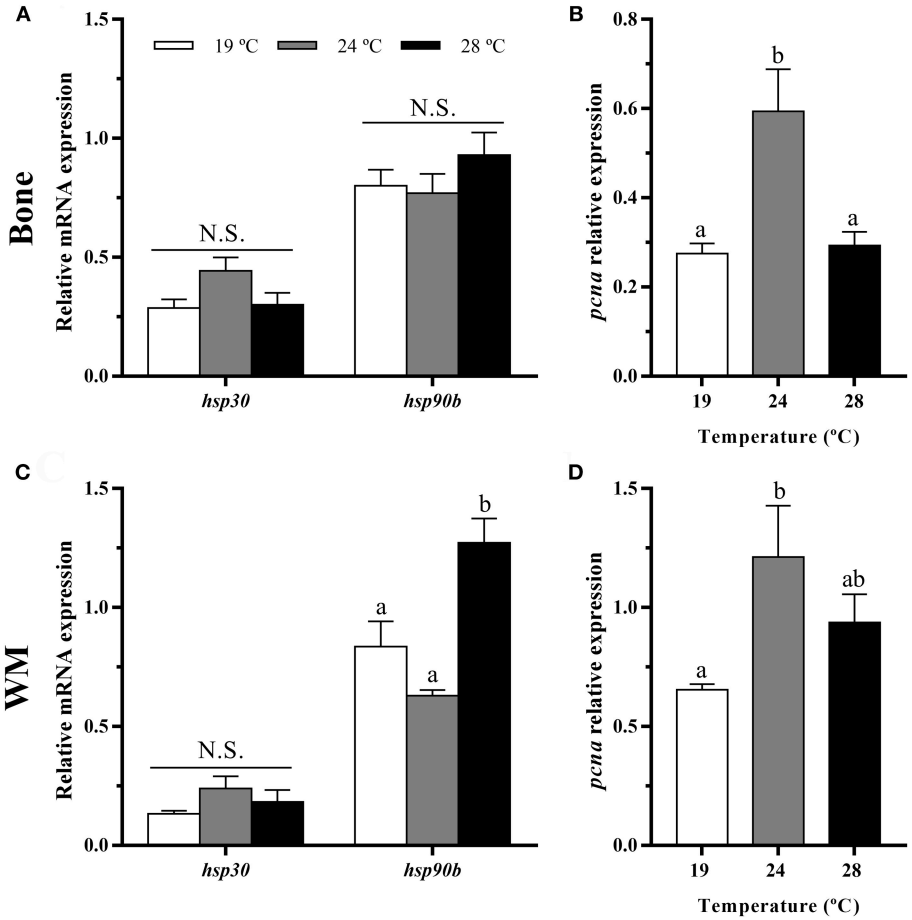

FIGURE 2 | *In vivo* effects of temperature over the gene expression of heat shock proteins and a proliferation marker in gilthead sea bream in **(A,B)** bone and **(C,D)** white muscle (WM). Relative mRNA expression normalized to *ef1a* and *rps18* of **(A,C)** *hsp30* and *hsp90b* and **(B,D)** *pcna*. Data are shown as Mean + SEM ($n = 8$). Significant differences among fish held at different temperatures were determined by one-way ANOVA and are indicated by different letters ($p < 0.05$). N.S., non-significant.

of minerals in the ECM (**Figure 5C**), which agreed with the visual observation.

HSPs and Proliferation Marker Genes Expression in Bone-Derived Cells

Exposure of gilthead sea bream juveniles to 24°C of temperature significantly increased the expression of *hsp30* and *hsp90b* transcript levels in bone-derived cultured cells compared to those coming from the other fish (**Figure 6A**). In addition, a significant down-regulation of the proliferation marker *pcna* gene expression was detected in the 28°C-cells compared with those obtained from 24°C-held fish (**Figure 6B**).

GH-IGFs Axis- and Osteogenic-Related Genes Expression in Bone-Derived Cells

The analysis of GH-IGFs system-related genes expression in cultured bone-derived cells revealed no differences among groups (**Figures 7A,B**). Regarding the expression of osteogenic genes, significant differences were neither observed for most of them but significant up-regulation of *on*, *op*, and *ocn* gene expression was found in cells coming from gilthead sea bream maintained at elevated temperatures, compared with those cells from 19°C-reared fish (**Figure 7C**). To corroborate the determination of the bone-derived cultured cells toward the osteogenic lineage, the gene expression of *pax7*, one of the main transcription factors shaping the fate of MSCs into the muscular lineage, was analyzed, resulting undetectable.

DISCUSSION

As existing literature reports, an increment of water temperature has been proved to be a valid approach to evaluate impact of global climate change on physiological responses in various fish species (6, 15, 25, 34, 35). Besides, we have recently described in gilthead sea bream temperature-dependent differential expression of genes involved in osteogenesis, indicating a

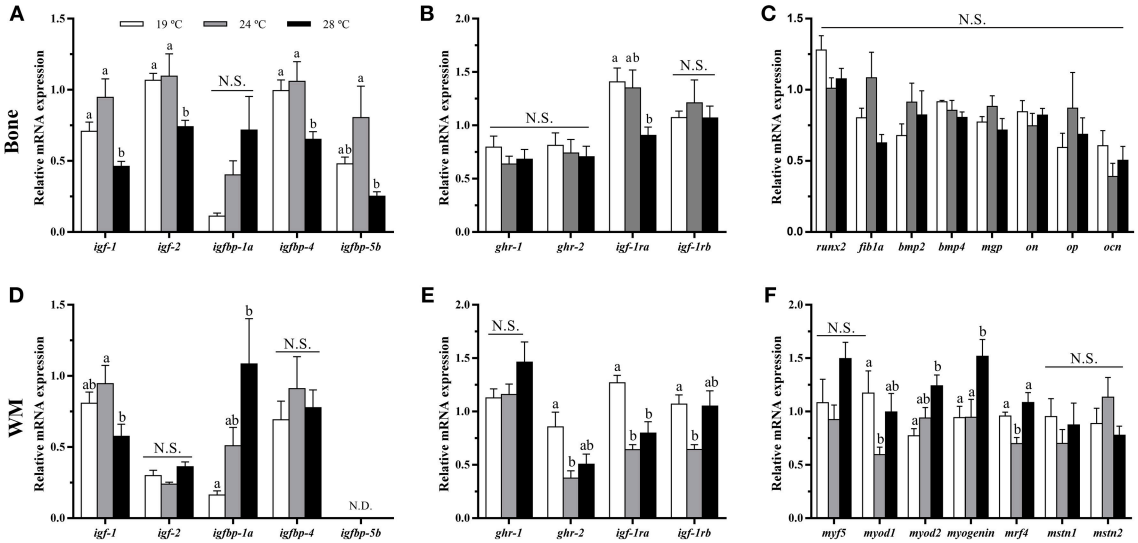

FIGURE 3 | *In vivo* effects of temperature over the expression of GH-IGFs axis-, osteogenic-, and myogenic-related genes in **(A–C)** bone and **(D–F)** white muscle (WM). Relative mRNA expression normalized to *ef1a* and *rps18* of **(A,D)** *igf-1*, *igf-2,* and *igf* binding proteins (*1a, 4,* and *5a*), **(B,E)** *gh* and *igf-1* receptors, **(C)** *runx2*, *fib1a*, *bmp2*, *bmp4*, *mgp*, *on*, *op,* and *ocn*, and **(F)** *myf5*, *myod1*, *myod2*, *myogenin*, *mrf4*, *mstn1,* and *mstn2.* Data are shown as Mean + SEM ($n = 8$). Significant differences among fish held at different temperatures were determined by one-way ANOVA and are indicated by different letters ($p < 0.05$). N.S., non-significant; N.D., non-detectable.

modulation of bone formation caused by this abiotic factor (20). In the current study, the aim was to characterize the effects of increased temperature in gilthead sea bream juveniles' musculoskeletal growth, muscle lipid metabolism and, in the *in vitro* development of primary cultured bone-derived cells to test the hypothesis that global climate change modulates the expression of key genes locally in bone and muscle, which might increase the occurrence of skeletal anomalies in this species.

Effects of Temperature on Cell Culture Development

The bone-derived cultured cells from gilthead sea bream vertebrae gave an initial homogenous population of cells. At the first stages, cells were mostly triangular as previously reported for this species (21) and mammalian bone marrow stem cells (36) and, up to day 8 there were no differences in morphology among the three temperature groups. At day 15, cell differentiation became more evident, with the change into a polygonal shape, characteristic of the osteoblast phenotype (36). These changes followed the profile also reported for gilthead sea bream either in primary cultured cells (21) or the osteoblast-like VSa16 cell line (37). This change in morphology together with the absent levels of *pax7* and the elevated expression of osteogenic-genes, confirmed that all three cultures performed in the present study were determined toward the osteogenic lineage. Nevertheless, the increase in temperature appeared to lead toward a disrupted, or at least retarded, osteogenic process, since a decrease in cell viability and mineralization was observed in bone cells derived from 24 to 28°C-held fish compared with the 19°C group. Reduced mineralization caused by a high-temperature treatment was also found by Ytteborg et al. (38) in Atlantic salmon vertebral tissue, supporting this hypothesis.

Effects of Temperature on *hsps* and *pcna* Gene Expression *in vivo* and *in vitro*

Although the gene expression of *hsp90b* was increased *in vivo* only in the muscle tissue under the highest temperature tested, the *in vitro* experiment showed direct evidence of water temperature up-regulating *hsp30* and *hsp90b*; thus, supporting the stressful condition induced to the animals. A similar response was reported for both genes in the same gilthead sea bream *in vitro* model when similar temperature changes were applied directly into the cells (20). Previous studies in larvae of sole and grass carp (*Ctenopharyngodon idella*) also described an increased expression of *hsp90* in response to a temperature rise (39, 40), as a protective mechanism against thermal stress. In fact, an increase in *hsp30* mRNA levels was observed only after 3 h of an *in vivo* temperature increase in rainbow trout (*Oncorhynchus mykiss*) (41). Similar activations of chaperones involved in protein folding have been reported in muscle tissue of gilthead sea bream (42) and rainbow trout (43) facing another stressful situation such as fasting.

The proliferation marker *pcna* showed the same pattern of expression *in vivo* in bone and muscle than *in vitro* in bone-cultured cells, with increased mRNA levels in 24°C-reared fish respect to the other groups. Thus, despite the cells showed reduced viability with the increase in temperature, this up-regulation of *pcna* could be considered a compensatory response, attempting these cells to recover from their initial heat stress-related situation. Accordingly, that could be considered a compensatory growth mechanism *in vivo*. Thus, it appears that 24°C would be the limiting temperature to properly

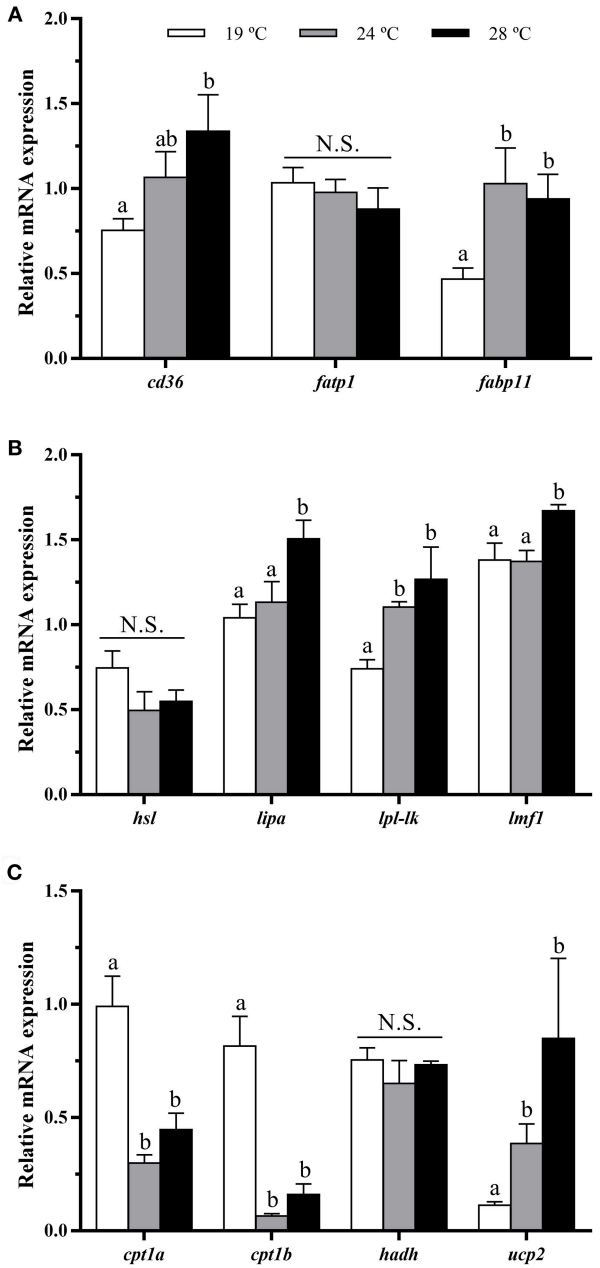

FIGURE 4 | *In vivo* effects of temperature over the expression of lipid metabolism-related genes in white muscle. Relative mRNA expression normalized to *rps18* and *rpl27a* of **(A)** fatty acid transporters *cd36*, *fatp1*, *fabp11*, **(B)** lipases *hsl*, *lipa* and *lp-lk*, *lmf1*, and **(C)** β-oxidation markers *cpt1a*, *cpt1b*, *hadh,* and *ucp2*. Data are shown as Mean + SEM ($n = 8$). Significant differences among fish held at different temperatures were determined by one-way ANOVA and are indicated by different letters ($p < 0.05$). N.S., non-significant.

grow gilthead sea bream, being the condition of 28°C fairly challenging. This is in agreement with the range of adequate rearing temperatures reported for this species (16–22°C) not causing significant harmful health effects and/or inducing skeletal malformations (14). Similarly, a previous study carried out in human osteosarcoma cells revealed that an increase in temperature outside optimum has a pronounced inhibitory effect on proliferation rate (44).

Effects of Temperature on GH/IGFs Axis-Related Genes Expression *in vivo* and *in vitro*

The expression of *igf-1* was significantly decreased in both bone and muscle tissues of 28°C-held fish when compared with fish reared at 24 or 19°C, while its expression was not detectable in cultured osteoblasts. IGF-1 plays an important role inducing not only muscle differentiation and hypertrophy, but also bone matrix production (16, 45). The down-regulating effect of high temperature on *igf-1* observed in gilthead sea bream has been also reported in muscle of different fish species (i.e., Atlantic salmon and southern flounder), as well as, in IGF-1 plasma levels (6, 46, 47), suggesting restricted growth. Notwithstanding, similar studies in rainbow trout showed contrarily, an increase in plasma GH and IGF-1 levels with high temperature (7, 8), overall suggesting that the response to temperature increase of the major growth factors in fish could be species-specific. Regarding *igf-2*, changes were not observed among groups in muscle and cultured bone cells, although the same response as *igf-1* was observed in the bone *in vivo*, indicating that this tissue appears to be more sensitive to changes in temperature. This data is in agreement with that observed previously in unresponsive rainbow trout muscle, both at mRNA and plasma levels (8); however, in Atlantic salmon, Hevrøy et al. (6) found that *igf-2* mRNA levels were significantly down-regulated in muscle and liver after 45 days of exposition to warm temperature but not after only 15 days. Thus, it cannot be excluded that a prolonged trial time could have affected the expression of this gene in the present study as well.

Concerning the GH and IGF-1 receptors, juveniles held at 24 and 28°C presented in muscle significantly lower levels of expression of *igf-1ra* than at 19°C; those reared at 24°C also had decreased *igf-1rb* and *ghr-2*, and in bone those at 28°C also showed reduced *igf-1ra* mRNA levels. Wargelius et al. (16) revealed that an increase in the gene expression of *igf-1ra* relates with an increase in bone density in Atlantic salmon. Thus, the decrease in *igf-1ra* expression observed in gilthead sea bream could lead to reduced mineralization in the long-term caused by the high rearing temperature, which would be in agreement with that observed by Ytteborg et al. (48) in the former species. Therefore, in this context, the results of the present study suggest that the GH/IGFs axis is influenced in gilthead sea bream by elevated temperature to locally decrease the expression of *ghrs*, *igfs,* and *igf-1rs* in bone and muscle in order to delay musculoskeletal growth. Interestingly, although differences exist between the *in vivo* and *in vitro* data, which could be due to modulation of the gene expression by systemic factors in the whole animal, the results obtained in the bone-derived cultured cells reflect this impaired situation as well.

With regards to the IGFBPs of major local action in the musculoskeletal tissues, the same increasing pattern in expression with temperature was observed concerning *igfbp-1a* in white

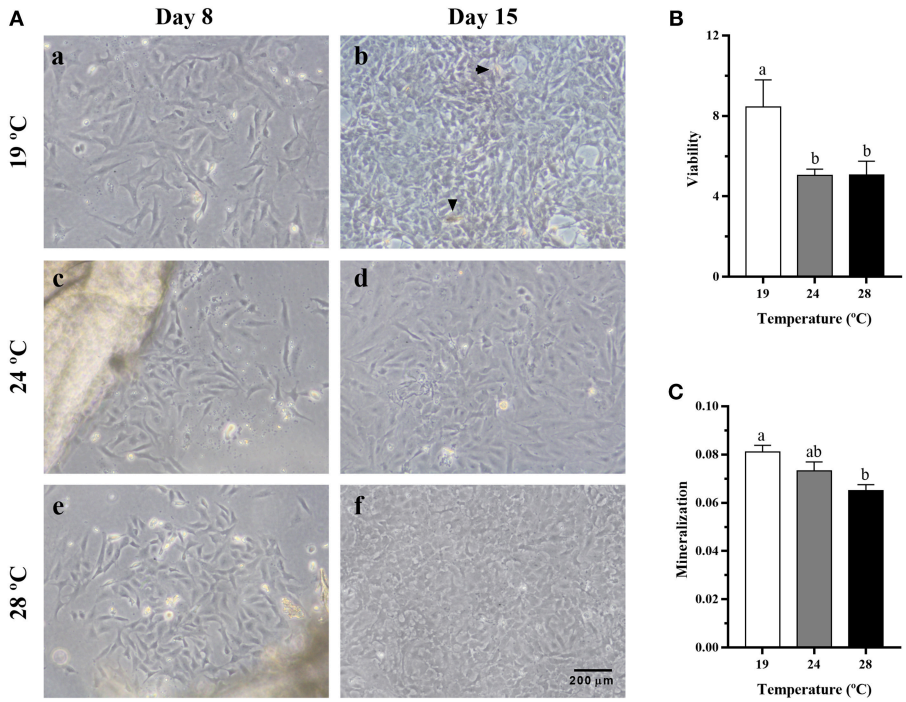

FIGURE 5 | (A) Representative images of cells derived from vertebrae bone of gilthead sea bream reared at different temperatures, at **(a,c,e)** day 8 and **(b,d,f)** day 15 of culture development. Magnification, 10x. Arrowheads indicate the presence of mineral nodules. **(B)** Quantification of viability in gilthead sea bream cultured bone-derived cells using an MTT assay presented as fold change of day 15 relative to day 8 of culture. **(C)** Quantification of mineralization in gilthead sea bream cultured bone-derived cells at day 20 determined by ARS staining. Data are shown as Mean + SEM ($n = 10$). Different letters among temperature groups indicate significant differences, calculated by one-way ANOVA ($p < 0.05$).

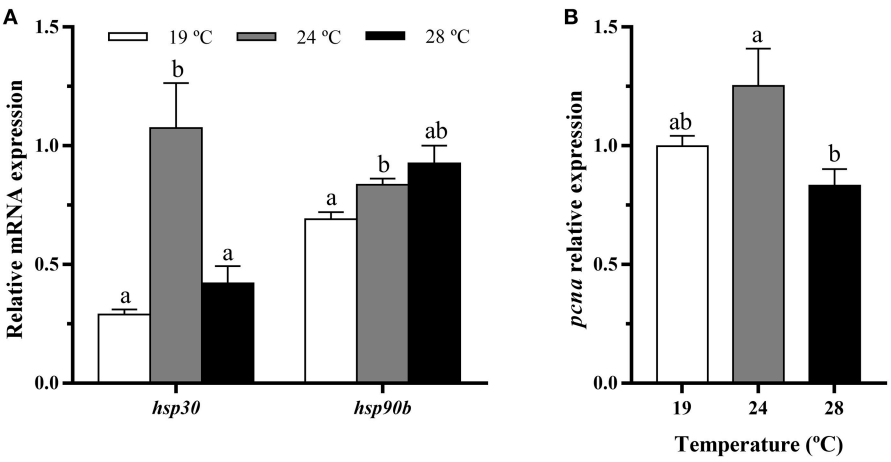

FIGURE 6 | *In vitro* effects of fish rearing temperature over the gene expression of heat shock proteins and a proliferation marker in bone-derived cells at day 15 of culture development. Relative mRNA expression normalized to *ef1a* and *rps18* of **(A)** *hsp30* and *hsp90* and **(B)** *pcna*. Data are shown as Mean + SEM ($n = 6$–7). Significant differences among temperatures groups gene were determined by one-way ANOVA and are indicated by different letters ($p < 0.05$).

muscle and bone, as previously found in Atlantic salmon muscle (6). Previous studies in zebrafish (*Danio rerio*) revealed that elevated expression of *igfbp-1a* limits cellular actions of IGF-1, being an important growth and developmental inhibitor (49). Moreover, this binding protein has been associated with stressful or negative conditions, since a strong relation with elevated serum cortisol levels has been reported (50, 51). In this context, the gradual increase of *igfbp-1a* along with temperature observed in this study, suggests impaired growth conditions in agreement with the reduced expression of *igfs* observed at

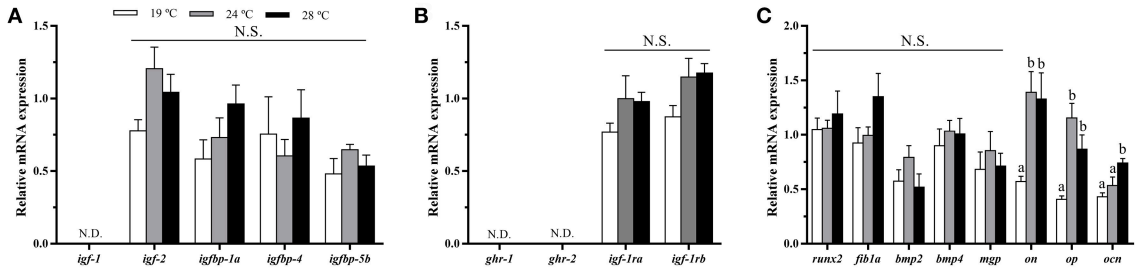

FIGURE 7 | *In vitro* effects of fish rearing temperature over the expression of GH-IGFs axis- and osteogenic-related genes in bone-derived cells at day 15 of culture development. Relative mRNA expression normalized to *ef1a and rps18* of **(A)** *igf-1*, *igf-2* and *igf* binding proteins (*1a*, *4,* and *5a*), **(B)** *gh* and *igf-1* receptors and **(C)** *runx2*, *fib1a*, *bmp2*, *bmp4*, *mgp*, *on*, *op,* and *ocn*. Data are shown as Mean + SEM ($n = 6–7$). Significant differences among temperatures groups were determined by one-way ANOVA and are indicated by different letters ($p < 0.05$). N.S., non-significant; N.D., non-detectable.

28°C. Furthermore, *igfbp-4* and *igfbp-5b* mRNA levels were also significantly decreased in bone tissue of 28°C-held fish, although remained unaltered in muscle and bone cells. In previous studies, these binding proteins have been reported as positive regulators of IGF-1 actions, with *igfbp-5b* promoting bone differentiation (45); and its mRNA levels being highly correlated with *igf-1* and *igf-2* in muscle (10, 32, 52, 53). Therefore, altogether, the down-regulation of *igfbp-4* and *igfbp-5b* in bone tissue and the increase of *igfbp-1a* in muscle of gilthead sea bream maintained at 28°C, indicates that such high temperature is an unfavorable condition, leading to reduced musculoskeletal growth and differentiation in this species.

Effects of Temperature on Osteogenic- and Myogenic-Related Genes Expression *in vivo* and *in vitro*

In the present study, although none of the osteogenic-related genes analyzed showed differences *in vivo*, increasing water temperature was sufficient to induce an up-regulating response on some genes involved in the mineralization of the ECM (*on*, *op,* and *ocn*) in the cultured bone-derived cells, suggesting that the thermal history can influence the developmental plasticity of the osteogenic process *in vitro*. Interestingly, the ECM glycoprotein ON has been reported as a heat shock protein having chaperone-like properties to prevent collagen denaturation (54, 55). Therefore, as it was observed in rainbow trout by Currie et al. (41) and in a previous study by our group using the same cellular system (20), the increase in *on* expression caused by changes in temperature could potentially represent an initial response of bone cells to stressful conditions. According to this, the increase in *on* mRNA levels may also suggest a negative effect for ECM production and mineralization, which agrees with the reduced number of deposited minerals in the ECM along with temperature in the bone-cultured cells of the present study. Moreover, the elevated expression of *op*, a well-known inhibitor of matrix mineral deposition (56) supports this improper mineralization of the bone when fish are maintained at high temperatures. However, the ability of OP to regulate this process depends on its state of phosphorylation (57), thus, further analyses should be done to confirm this hypothesis.

Concerning *in vivo* studies, after a long-term high-temperature treatment in Atlantic salmon, Ytteborg et al. (38) reported that the mRNA levels of *runx2* (the key transcription factor of osteogenesis), decreased when fish reached 15 g but not at 2 g of body weight; whereas other non-collagenic ECM molecules such as *ocn*, *on,* or *col1a1* were down-regulated already at the 2 g stage. These authors proposed that these results might suggest a defect in the late maturation of osteoblasts, which agrees with the lower mineral density and shorter length-height proportion observed in the vertebrae of these animals (38). It also agrees with the subsequent significantly increased incidence of malformations found in the fish reared at high temperature at body weights of 15 and 60 g. Moreover, the same authors observed, in an *in vitro* study with precursor muscle cells differentiated into osteoblasts and cultured at an elevated temperature, a reduced expression of *ocn* and *col1a1* (48). In gilthead sea bream, expression of osteogenic genes was modified by temperature in both embryo and larval stages, but in the juveniles, differences were only observed after producing a temperature challenge (20). In the same study, lower transcript levels of most of the osteogenic genes analyzed in cultured osteoblasts in response to a long-term treatment of increased temperature were reported. Overall, these data suggest that at a transcriptional level, the deleterious effects of temperature on bone development could depend on the time of exposure. Therefore, it cannot be discarded that a prolonged temperature treatment could have also affected the expression of osteogenic genes in gilthead sea bream *in vivo*, pointing out then that osteoblast differentiation and bone ECM mineralization could be impaired.

The coordinated expression of MRFs to properly control muscle development can be modulated by temperature in teleost fish, thus affecting muscle growth (42, 58). In the present study, *myod1* transcript levels were reduced in the fish maintained at 24°C, while *myogenin* and *mrf4* expression was highest in the 28°C-reared fish, suggesting potentially slackened cell proliferation but enhanced myocyte differentiation with the increase in water temperature, overall uncoupling the myogenic process. In this framework, with high rearing temperatures, gilthead sea bream musculoskeletal growth would not be under harmonic conditions, which could be leading in the long-term to increased prevalence of bone deformities.

Effects of Temperature on Muscle Lipid Metabolism-Related Genes Expression *in vivo*

The increase of temperature, up-regulated in the present work the muscle expression of the fatty acid transporter and binding protein *cd36* and *fabp11*, respectively, suggesting elevated fatty acid uptake and intracellular transport, upon high-temperature conditions. These data are in accordance with a recent study by Zoladz et al. (59), which reported enhanced protein expression of CD36 in rat skeletal muscle under hyperthermia. Nevertheless, the function of FABP11, which is probably an isoform restricted to fishes, is not completely known (60). With regards to the endothelial enzymes with a triglycerides lipase activity, gilthead sea bream juveniles held at 28°C presented significantly higher mRNA levels of *lipa* and *lpl-lk*, an exclusive fish lineage isoform of LPL (59). Regulation of the latter enzyme is far from being established yet, although it is known that in skeletal muscle of gilthead sea bream, changes in LPL-like are correlated with LMF1 (61), an endoplasmic reticulum membrane protein involved in the post-translational folding and assembly of LPL, among other proteins (62). This agrees with the results of this study, where *lmf1* mRNA levels were also increased as temperature was raised. Contrarily, the increase of temperature did not induce significant changes in *hsl* gene expression among groups, suggesting that fatty acids are mostly being uptaken by the muscle from circulating triglycerides, or non-esterified fatty acids provided by adipose tissue. Recent studies in Atlantic salmon and catfish (*Pelteobagrus vachellii*) demonstrated a reduction in hepatic triglycerides and relative viscera weight during exposure to elevated temperatures (6, 63), which illustrated that lipid metabolism may have increased, accelerating utilization of lipids as an energy source in peripheral tissues. In accordance with this, in rainbow trout exposed to high temperatures, endogenous lipid stores remained the most important energy source contributing up to 55% of total demand (64).

Concerning β-oxidation, an association between water temperature and fatty acid catabolism has been shown, but with inconsistent results in the literature. A recent study in Atlantic salmon showed an increase of β-oxidation in white muscle with increased temperature (25), while changes were not observed by Hevrøy et al. (47), and another study in rainbow trout reported increased capacities for oxidizing lipids at cold temperatures (65). In any case, it has been generally accepted that liver and red muscle, but not white muscle, are the most important tissues involved in fatty acid catabolism in fish; thus, an increase in water temperature has been shown to induce increased β-oxidation primarily in those tissues (66, 67). Other study in salmon found that elevation of water temperature was responsible for reduced β-oxidation in liver (47). These last data would be in agreement with the present study, where *cpt1a* and *cpt1b* were down-regulated at 24 and 28°C compared with the low-temperature group, considering the flux of β-oxidation is primarily determined by CPT1, which allows long chain fatty acids to enter into the mitochondria (25).

Notwithstanding, temperature can also affect mitochondrial uncoupling. The increase of temperature from 19 to 28°C led in gilthead sea bream muscle to the up-regulation of the uncoupling protein *ucp2*, indicating a higher proton leak, which is in agreement with previous studies in skeletal muscle in mammals (68, 69). UCPs are known to be activated not only by free fatty acids, but also by reactive oxygen species (70, 71). According to this, *ucp2* may play a role as a mechanism for attenuating the possible increase in reactive oxygen species associated with elevated temperature; therefore, overall indicating a less efficient use of fatty acids to obtain energy in this species, upon these environmental conditions.

CONCLUSIONS

To sum up, the present work reports that an increase in water rearing temperature from 19 to 24 and specially 28°C causes in gilthead sea bream juveniles unfavorable growth conditions for the musculoskeletal system due to reduced gene expression of members of the GH/IGFs system and specific MRFs. In white muscle as well, as energetic demand is increased along with temperature, the uptake of fatty acids is enhanced, although apparently, their use as an energy source is less efficient. In addition, the high temperatures applied *in vivo*, induced changes *in vitro* in the expression of several key osteogenic genes, suggesting reduced osteoblasts development and matrix production, consistent with the decrease observed in the deposition of minerals. Overall, the present study provides new insights into the possible impact of global climate change in this important Mediterranean species, demonstrating that temperature is a key environmental factor whose increase can lead to unbalanced muscle and bone growth, which should be considered to take preventive measures to reduce production losses and guarantee the sustainability and success of aquaculture.

AUTHOR CONTRIBUTIONS

MR-C and EC conceived and designed the experiments. SB-P, NR-H, and EV performed the experiments. JG, IN, MR-C, and EC contributed reagents and analysis tools. SB-P, NR-H, EV, JG, IN, MR-C, and EC drafted and critically reviewed the paper.

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REVIEW
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Lipid Metabolism Alteration by Endocrine Disruptors in Animal Models: An Overview

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Exposure to potential Endocrine Disrupting Chemicals (EDCs) pose a documented risk to both wildlife and human health. Many studies so far described declining sperm counts, genital malformations, early puberty onset, highlighting the negative impact on reproduction caused by the exposure to many anthropogenic chemicals. In the last years, increasing evidence suggested that these compounds, other than altering reproduction, affect metabolism and induce the onset of obesity and metabolic disorders. According to the "environmental obesogens" hypothesis, evidence exists that exposure to potential EDCs during critical periods when adipocytes are differentiating, and organs are developing, can induce diseases that manifest later in the life. This review summarizes the effects occurring at the hepatic level in different animal models, describing morphological alterations and changes of molecular pathways elicited by the toxicant exposure. Results currently available demonstrated that these chemicals impair normal metabolic processes via interaction with members of the nuclear receptor superfamily, including steroid hormone receptors, thyroid hormone receptors, retinoid X receptors, peroxisome proliferator–activated receptors, liver X receptors, and farnesoid X receptors. In addition, novel results revealed that EDC exposure can either affect circadian rhythms as well as up-regulate the expression of signals belonging to the endocannabinoid system, in both cases leading to a remarkable increase of lipid accumulation. These results warrant further research and increase the interest toward the identification of new mechanisms for EDC metabolic alterations. The last part of this review article condenses recent evidences on the ability of potential EDCs to cause "transgenerational effects" by a single prenatal or early life exposure. On this regard, there is compelling evidence that epigenetic modifications link developmental environmental insults to adult disease susceptibility. This review will contribute to summarize the mechanisms underlying the insurgence of EDC-induced metabolic alterations as well as to build integrated strategies for their better management. In fact, despite the large number of results obtained so far, there is still a great demand for the development of frameworks that can integrate mechanistic and toxicological/epidemiological observations. This would increase legal and governmental institution awareness on this critical environmental issue responsible for negative consequences in both wild species and human health.

Keywords: phthalates, zebrafish (*Danio rerio*), metabolic disorders, epigenetic, reproduction

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INTRODUCTION

The first definition of Endocrine disrupting chemicals (EDCs) was provided at the European Workshop on endocrine disruptors (EDs) hold in Weybridge UK, in 1996. "An endocrine disruptor is an exogenous substance that causes adverse health effects in an intact organism, or its progeny, secondary to changes in endocrine function." A further definition was also agreed, concerning potential EDs: "A potential endocrine disruptor is a substance that possesses properties that might be expected to lead to endocrine disruption in an intact organism."

EDCs, interfering with the endocrine (or hormonal) system, are tightly implicated in the global decline of metabolic health. This large and multifaceted family includes plasticizers as phthalates, bisphenols, industrial chemicals including alkylphenols, flame retardants, air pollutants, such as polycyclic aromatic hydrocarbons, pesticides, metals, and dioxins. Consumption of contaminated food and water and inhalation of airborne pollutants represent the major sources of human exposure to EDCs, significantly contributing to the onset of obesity (1) by inappropriately stimulating adipogenesis as well as perturbing lipid metabolism and energy balance (2). All those EDCs that inappropriately regulate and promote lipid accumulation and adipogenesis are defined "obesogens" (3). In the last years, with the addition of compounds able to affect lipid metabolism, the list of obesogenic compounds significantly enlarged. Exposure to EDCs, in fact, can directly increase the size/number of adipocytes or indirectly affect basal metabolic rate and hormonal control of appetite (4). The hypothalamic-pituitary-adrenal axis plays an important role in controlling appetite and satiety, stimuli regulated by a variety of monoaminoergic, peptidergic and endocannabinoid (EC) signals that can be generated in the digestive tract, adipose tissue and brain. All these signals are candidate targets of potential obesogenic EDCs. Lipids, which role has been considered crucial in many tissues including liver, fat and intestine are accumulated and stored till their use in case of energy needs (5). Many studies so far have been carried out to reveal the pivotal role of dietary lipid intake as a source of essential fatty acids governing energy balance, food intake, growth, reproduction and health. Dysregulation of lipid accumulation is at the basis of several metabolic syndromes, including Nonalcoholic Fatty Liver Disease (NAFLD) and hyperlipidemia (6). The windows of exposure to potential EDCs (e.g., fetal or early postnatal) is critical for the outcome of metabolic diseases and results particularly detrimental because of the permanent effects on obesity later in life.

Since the socio-economic burden of EDC-caused diseases in industrialized countries ranges between 50 and 300 billion €/year (7), research to increase the knowledge on the causal link between health effects and EDCs represents a great challenge for health care systems.

This review is aimed at providing a general overview on the endocrine mechanisms linking EDC exposure to lipid metabolism dysregulation in different experimental vertebrate models, from mammals to fish, also considering *in vitro* trials.

POTENTIAL MECHANISMS BY WHICH EDCs EXERT THEIR EFFECTS

To better understand how potential EDCs can dysregulate lipid metabolism leading to the onset of several health diseases, a brief overview of the mechanisms and of the main actors involved in the regulation of lipid synthesis and degradation will be given.

In the last decades, the study of a group of xenobiotic compounds known as peroxisome proliferators has led to the discovery of peroxisome proliferator-activated receptors (PPARs) as a novel subfamily of nuclear receptors (NRs) (8). They dimerize with retinoid X receptor (RXR) and bind to PPAR-responsive DNA regulatory elements controlling the expression of genes involved in adipogenesis, glucose, lipid, and cholesterol metabolism (9, 10). Similarly to the membrane Estrogen receptorα (ERα), recently it has been demonstrated that PPARs can activate a non-genomic, rapid signaling pathway (11, 12), but while several studies so far described the activation of the ER non-genomic, rapid pathway in response to potential EDCs (13–16), information regarding ability of pollutants to activate the PPAR-non genomic signaling is still lacking.

PPARs have a pivotal role in regulating metabolism, resulting the primary lipid sensors in vertebrates and being highly conserved between humans and zebrafish (17). Poly and mono unsaturated fatty acids (FA), eicosanoids and lipophilic hormones are PPAR natural ligands (18) with different affinity to PPAR isoforms and induce the expression of genes and enzymes involved in lipid metabolism. In addition to natural ligands, phthalates, plasticizers, certain herbicides, biocides organotins, perfluorooctanoic (PFOA) and perfluorooctanesulfonic (PFOS) acids, pharmaceuticals, halogenated derivatives of bisphenol A (BPA), the imidazole fungicide triflumizole, the fibrate class of hypolipidemic drugs, all listed as "Endocrine Disruptive Chemicals," interact with the above stated NR through a specific binding mechanism (19). The activation of RXR- PPARα dimer stimulates FA β-oxidation (20), while the RXR-PPARɤ heterodimer favors preadipocytes differentiation and regulates lipid biosynthesis and storage (21) (**Figure 1**). In most species, the major site of both lipolytic and lipogenic processes is the liver, where PPARα and β, other than regulating FA β-oxidation, have a key role in glucose storage, lipoprotein capture and inflammation reduction (22), while the activation of PPARɤ orchestrates adipocyte function and differentiation as well as lipid storage within adipocytes (9). In the liver, PPARα is abundantly expressed, whereas PPARβ and PPARɤ are expressed at lower levels. PPARα, being the major regulator of the hepatic response to fasting, induces the expression of a variety of genes involved in FA catabolism and ketogenesis (23). Consequently, fasting PPARβ knockout mice develop hepatic steatosis (23). In addition to PPARɤ, several genes regulate fat cell development and control, including CCAAT-enhancer-binding proteins (*c/ebp*), responsible for the secretion of adipokines, e.g., leptin and adiponectin, hepatic glucose metabolism, insulin sensitivity and inflammation (24). A side from PPARs and C/EBP, sterol regulatory element-binding proteins (SREBPs) are central players in lipid metabolism, controlling the expression of

genes important for lipid synthesis and uptake (25). In addition to the canonical functions in the transcriptional regulation of genes involved in lipid biosynthesis and uptake, SREBPs are also implicated in pathogenic processes including, inflammation, autophagy and apoptosis, and in this way, they contribute to the onset of several metabolic disorders (26). Activation of selected pathways responsible for adipogenesis and lipogenesis are summarized in **Figure 2**.

Once synthesized, lipids are stored within hepatocytes as source of energy. At the same time in these cells xenobiotic detoxification takes place. P450 enzymes are mainly implicated in this process via ligand-activated xenobiotic receptors, mainly aryl hydrocarbon receptor (AHR), constitutive androstane receptors (CAR) and pregnane X receptor (PXR). Recently, it was proposed that activation of these xenobiotic receptors is a triggering event of hepatic steatosis (30). To increase the knowledge regarding this aspect, it should be briefly considered the role of some signals involved in lipid regulation. In most species, triacylglycerol (TAG) is the main dietary component and lipoprotein lipase (LPL) is deputed to its hydrolyzation into non-esterified FA and 2-monoacylglycerol and their further storage in lipid droplets. It acts as "gate keeper" of FA uptake, working as a rate limiting enzyme in the provision of fatty acids to tissues (31). Intracellular transport of FA is performed by fatty acid binding proteins (FABP), which sequesters lipophilic compounds, regulates hepatocyte growth and transport them into mitochondria (32). Once stored in mitochondria, a set of genes involved in different aspects of lipid metabolism are activated (33). Fatty acid synthase (FAS) catalyzes the main pathway of lipogenesis, producing long saturated chain of carbon's atoms, finally stored in adipose tissues (34). Considering this brief state of art, it's clear that pivotal in this context is the ability of potential EDCs to interfere with/activate the PPAR cascade.

As novelty, recently, evidence emerged showing that one potential mechanism by which chemical exposure can influence lipid metabolism is through disturbance of circadian rhythms. While the circadian signals generated by clock genes produce metabolic rhythms, clock gene function is tightly coupled with fundamental metabolic processes, such as glucose and lipid metabolism (35). It has been demonstrated that the expression of clock genes, *Dec1, Dec2,* and *Bmal1*, is directly linked to energy metabolism, since *ppar* regulation is under the control of clock proteins. More specifically, DEC1 and DEC2 regulate adipogenesis by repressing the transcription of *pparɣ* (36).

In the last years, a direct link between the EC system and its role in the regulation of energy balance and in the onset of obesity emerged. ECs, differently from the protein hormone-based circuits and from the classical brain neurotransmitters, are synthesized and act locally and their effects are mediated by binding to surface receptors (37). Increasing evidence suggested that ECs bind and activate PPARs, being ECs fatty acids derivatives (38). It is likely that ECs, whose chemical structure is derived from arachidonic acid, might act not only through the classical type 1 and type 2 cannabinoid receptors (CB1 and CB2), the GPR55 orphan receptor and vanilloid type-1 receptor (VR1), but also through PPARs. Therefore, the binding between ECs and PPARs might mediate many of the biological effects of cannabinoids, including modulation of feeding behavior and lipid metabolism. Recent studies demonstrated the ability of potential EDCs to regulate the EC system. Among phthalates, the di-ethyl-hexyl-phthalate (DEHP) exerts its obesogenic action up-regulating hepatic *pparα, cb1,* and *srebp* levels and stimulating *de novo* FA synthesis and hepatic steatosis. This hepatic state may cause an inhibition of food intake stimulus up-regulating leptin, the typical sensor of the energy status, which, in the brain, may negatively control *cb1* and in turn reduce *srebp* gene expression (25).

EFFECTS OF EDCs EXPOSURE ON LIPID METABOLISM IN ANIMAL MODELS

Insights on *in vitro* and *in vivo* Exposure in

- **Mammalian Models**

  Increasing concern arose from the evidence that exposure to potential EDCs during critical periods, when adipocytes are differentiating and organs are developing, can induce effects, often as metabolic diseases, that manifest later in life. The correct functioning of the endocrine system has a central role in the organisms' health and its deregulation is directly responsible for the onset of many metabolic disorders, including obesity, NAFLD, and hyperlipidemia. The NAFLD, consisting of the excess triglyceride accumulation within hepatocytes, or steatosis, is considered the hepatic manifestation of obesity and metabolic syndromes. In addition, over the past four decades, research demonstrated that most of these metabolic diseases correlate, at least in part, with exposures to environmental chemicals (39). Potential EDCs can disrupt the normal hormonals level by inhibiting or stimulating the production of hormones or changing the way in which the hormones are transported to target tissues (4).

  This part of the review collects the most recent results relative to the *in vivo* or *in vitro* exposure of mammalian models to environmental chemicals.

  One of the first discovered synthetic PPAR ligand was tributyltin (TBT). Its exposure drives the differentiation of murine 3T3-L1 adipocytes *in vitro* and activates the RXR-PPARɣ-mediated pro-adipogenesis in liver and adipose tissue (40–42). In addition, an *in vivo* study, demonstrated that prenatal exposure to TBT, results in precocious lipid accumulation in adipose tissues and onset of hepatic steatosis in newborn mice (43). Adipogenesis was also promoted in human and mouse mesenchymal stem cells (MSCs) after a 14-days exposure to dibutyltin (DBT), the major TBT metabolite. More specifically, human MSCs resulted more responsive to the treatment than mouse MSC, with C/EBPα and PPARγ2, important signals in adipose differentiation and regulating each other through a positive feedback loop, resulting significantly up-regulated. In these cells, FABP4, fat-specific protein-27 (FSP27), and LPL were also over-expressed. DBT-induced adipogenic differentiation was abolished by the PPARγ antagonist T0070907, indicating that DBT was acting primarily through PPARγ (44). The

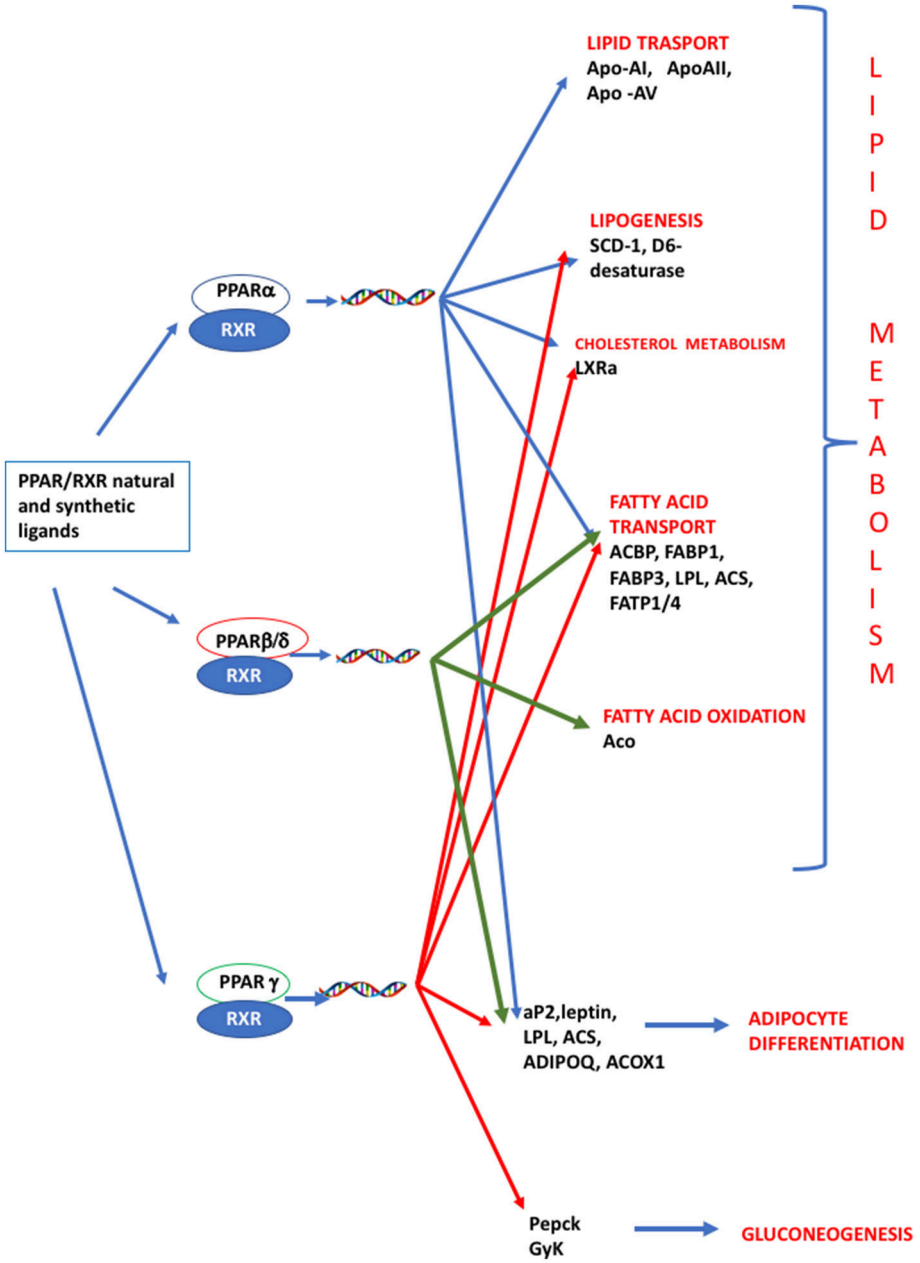


FIGURE 1 | PPAR signaling pathway. PPARs are nuclear hormone receptors that are activated by fatty acids and their derivatives. PPAR α, δ/β, ϫ, show different expression patterns in vertebrates. Each of them is encoded by a separate gene and binds fatty acids, eicosanoids and synthetic ligands. Key genes are reported. PPARα/RXR heterodimer activates the transcription of genes involved in lipid metabolism, including transport, lipogenesis, cholesterol metabolism and adipocyte differentiation. PPARβ/RXR heterodimers activate the transcription of signal involved in fatty acid transport, fatty acid oxidation, and signal triggering final adipocyte differentiation. PPARϫ/RXR heterodimers are involved in different steps of lipid metabolism and regulate the transcription of signal responsible for adipocyte differentiation and gluconeogenesis. ACBP, Acyl-CoA-binding protein; ACS, Acetyl-coenzyme A synthetase; ACO, andacyl-CoA oxidase; ACOX1, Peroxisomal acyl-coenzyme A oxidase 1; ADIPOQ, adiponectin; aP2, adipocyte fatty acid binding protein 2; Apo-AI, apolipoprotein A1; ApoAII, apolipoprotein AII; Apo-AV, apolipoprotein AV; FABP1, fatty acid binding protein 1; FABP3, fatty acid binding protein 1; FATP1/4, Fatty acid transport protein 1–4; GyK, glycerol kinase; LPL, lipoprotein lipase; LXRα, Liver receptor α; Pepck, phosphoenolpyruvate carboxykinase; SCD-1 stearoyl-CoA desaturase-1.

same authors observed an impairment of glucose tolerance, driven by a hypothalamic resistance to leptin rather than to a misfunctioning of Langerhans islet in mice perinatally exposed to DBT, suggesting that DBT, as already observed for many other potential EDCs, can contribute to the diabetes epidemic (45). In Sprague-Dawley rats fed on a sucrose-high fat diet, nonylphenol (NP) co-administration, increased both water and food intake, hepatic echogenicity and alteration of

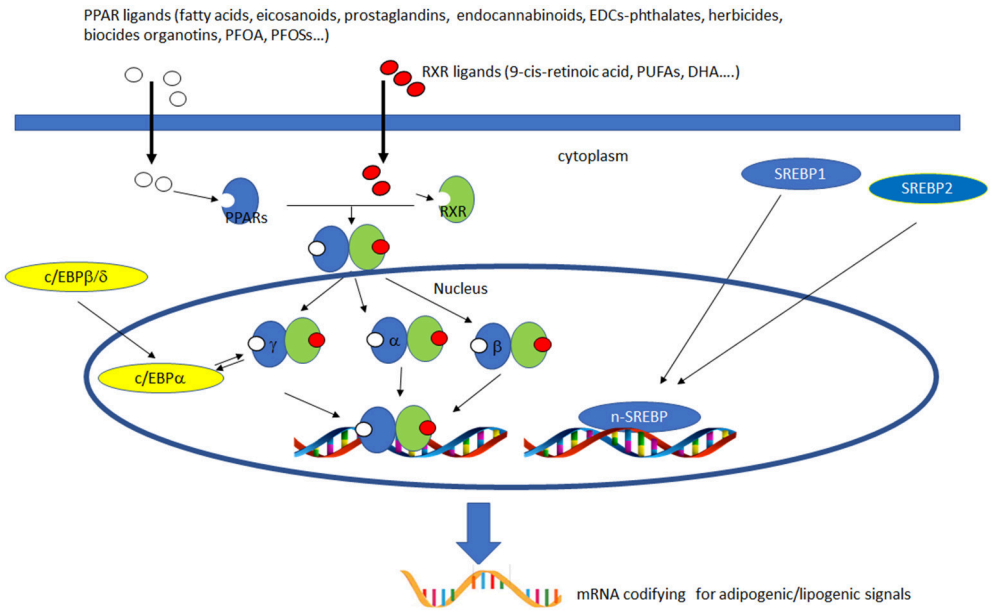


FIGURE 2 | Activation of lipogenic and adipogenic pathways. PPARs (α, β/δ, and γ) belong to the nuclear hormone receptor superfamily and are ligand-activated transcription factors activated by fatty acids, fatty acid derivatives (e.g., eicosanoids), endocannabinoids and potential EDCs. PPAR and RXR dimers form important transcription activators which upon binding PPAR response elements can modulate many important cell functions, e.g., PPARα-RXR dimers activate genes controlling peroxisome proliferation, fatty acid metabolism and lipid homeostasis; PPARγ-RXR dimers affect adipocyte differentiation. C/EBPs are a family of nuclear activators, transiently expressed very early during adipocyte differentiation. C/EBPβ/δ activate the expression of of C/EBPa. Furthermore, the expression of C/EBPa and PPARγ is sustained by apositive feedback loop. Both proteins cooperatively promote downstream adipocyte-related genes transcription. SREBPs are activators of the complete program of hepatic cholesterol and fatty acid synthesis. SREBP-1 preferentially activates genes of fatty acid and triglyceride metabolism, whereas SREBP-2 preferentially activates genes of cholesterol metabolism. SCAP transports SREBPs from the ER to the Golgi apparatus, where is cleaved by two proteases, Site-1 protease (S1P) and Site-2 protease (S2P). nuclear SREBP (nSREBP), translocates to the nucleus, where it activates transcription of multiple target genes. SREBP-2 responsive genes include those for the enzymes HMG-CoA synthase, HMG-CoA reductase, farnesyl diphosphate synthase, and squalene synthase. SREBP-1 responsive genes include those for ATP citrate lyase and acetyl-CoA carboxylase and fatty acid synthase, the fatty acid elongase complex, (27) stearoyl-CoA desaturase, and glycerol-3-phosphate acyltransferase (28) Finally both SREBP forms activate three genes required to generate NADPH, which is consumed at multiple stages in these lipid biosynthetic pathways (29).

several plasmatic aminotransferases. Hepatic macro-vesicular steatosis was found to be associated with congestion and dilation of central vein, inflammatory cell infiltration and up-regulation of genes involved in lipogenesis, e.g., *srebp-1C, fas*, and *ucp2* were described (46), suggesting that NP exposure exacerbates alcoholic fatty liver diseases.

Triclosan (TCS) exposure at non-cytotoxic concentrations can induce lipid accumulation by decreasing adipocyte protein 2 (*ap2*), *lpl*, and adiponectin (adipoq) gene expression, in human MSCs (47). Exposure of rats via oral gavage to DEHP (0.05, 5, 500 mg/kg) induces varying degrees of hepatic steatosis, associated with inflammation, lipid peroxidation, oedema of the liver cells and hepatic damage (48). Further *in vitro* studies, using HepG2 cells aimed at understanding the potential mechanisms involved in DEHP-induced toxicity. Results showed that DEHP promotes lipid accumulation in cells and alters the level of superoxide dismutase (SOD) and malondialdehyde (MDA) disrupting the balance of oxidative stress. Lipid accumulation in hepatocytes was promoted by the activation the SREBP-1c and PPARα-signaling pathway (49). The obesogenic effect of a chronic exposure to 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) [1 μg/kg body weight (bw)/week] of adult C57BL/6J mice from 10 to 42 weeks old, resulted obesogenic in adult mice (7% in males and 8% in females). A gender specific effect was observed in the fat mass distribution, in adipose tissue and in the hepatic triglyceride accumulation, with female resulting more susceptible to the exposure than males, providing evidence of the gender and multiorgan effects of dioxin (50). Other authors, using the same mice strain, demonstrated that the inhibition of the AHR prevents the diet–induced obesity and fatty liver (51), suggesting that TCDD toxic/obesogenic effects can be avoided by blocking AHR. In the same strain (C57BL/6J), the pubertal male, when orally administered with a cocktail of 10 mg/kg/body weight (bw) cypermethrin (CYP), 100 mg/kg/bw atrazine (ATZ) and 0.1 mg/kg/bw 17α-ethynyestradiol (EE2) for 4 weeks followed by a high-energy diet (HD) for 8 weeks, reported alteration of the hepatic levels of transcriptional factors including PPARα, PPARɤ, and SREBP1C and their target genes related to FA synthesis and oxidation, respect to control mice fed only a HD. The results showed that early-life-stage exposure to environmental EDCs affected the homeostasis of hepatic glucose and FA metabolism at adulthood (52). To simulate human environmental exposure

to BPA, 3T3-L1 pre-adipocytes were cultured for 3 weeks with 1 nM BPA. The exposure enhanced pre-adipocyte proliferation and anticipated the expression of master genes involved in lipid/glucose metabolism. Induced adipocytes are hypertrophic, displayed impaired insulin signaling and reduced glucose utilization concomitant to an increase of pro-inflammatory cytokine expression, supporting the hypothesis that BPA exposure, during sensitive stages of adipose tissue development, may cause adipocyte metabolic dysfunction and inflammation, thus increasing the risk of onset of obesity-related diseases late in life (53).

- **Amphibians**
  Few data are available describing the effects of potential EDCs exposure on lipid metabolism in amphibians. In 2006, Grün and collaborators described the effects of TBT exposure in *Xenopus*, showing its ability to activate RXR/PPARγ pathways and suggesting the evolutionary conservation of these signals among vertebrates. They further investigated the effects of the exposure to environmentally relevant low doses of TBT (1–10 nM), the RXR-specific ligands LG100268 and AGN195203 (10–100 nM), troglitazone (0.1–1 M), and E_2 (1–10 nM) on the developmental process of *X. laevis* tadpoles from stage 48 to metamorphosis. After TBT or RXR/PPARγ ligand exposure, they observed a dose-dependent increase in ectopic adipocyte formation around the gonads of both sexes. E_2 treatment did not induced evident effects on adipogenesis/lipogenesis (43).
- **Teleosts**
  To date, many studies documented the toxic response of fish exposed to environmental pollutants. Due to their physicochemical properties, most of toxic effects of organic compounds are dependent on their bioaccumulation in the lipids of aquatic organisms. Therefore, there is an increasing interest to investigate the gene expression as well as the presence and activity of proteins involved in FA metabolism.

A recent paper in zebrafish contributed to increase the knowledge on the effects of the exposure to environmental TBT concentrations on lipid metabolism. Exposure to 10 and 50 ng/L TBT from pre-hatch to 9 months of age, altered body weight, hepatosomatic index and hepatic triglyceride abundance in a gender and dose related manner, with male resulting more sensitive than female. Furthermore, in male, TBT significantly affected the transcription of key factors and enzymes involved in adipogenesis and lipogenesis (PPARγ, SREBP1, FASn, 11β-HSD2, C/EBPβ, and DGAT2). In female, hepatomegaly was observed, associated to a subtle, not significant adipogenic response in the transcription of genes (54). Differently to TBT, the exposure of zebrafish to triclosan (TCS), an antimicrobial agent, impaired mRNA expression levels of β-oxidation transcripts and lipid β-oxidation genes, including *pparα, cpt1, lpbe, cyp4a10*, and *aco* (55). Similarly, in adult male zebrafish, DEHP exposure disrupted metabolic processes in the liver, as demonstrated by alteration of five biological pathways: "FOXA2 and FOXA3 transcription factor networks," "Metabolic pathways," "metabolism of amino acids and derivatives," "metabolism of lipids and lipoproteins," and "fatty acid, triacylglycerol, and ketone body metabolism" (56).

In the same model, non-toxic concentrations of four different potential EDCs, TBT, tetrabrominated bisphenol A (TBBPA), tris (1,3-dichloroisopropyl) phosphate (TDCIPP) and benzophenone 3 (BP-3), selected on the bases of their ability to affect PPAR signaling, were added to rearing water from 9 to 14 dpf. All tested pollutants induced obesity, as visualized and quantified by fluorescent lipid staining in the trunk area between the gall bladder and the proximal intestine. In addition, exposed larvae were fed a standard diet, resulted in an even higher lipid accumulation than larvae fed a hypercaloric diet (HCD), suggesting that early exposure to toxicant, could affect fish metabolism later in life. Moreover, the ability of the above selected potential EDCs to affect the circadian rhythm was also demonstrated. Using a transgenic Tg (4xEbox:Luc) zebrafish larvae (57), a 24-h TBT exposure showed its ability to reduce the amplitude of oscillations and a prolongation of the period between maximum and minimum activity respect to transgenic-control fish. The period was even further prolonged by the TDCIPP. A loss of the characteristic oscillations was also observed in larvae exposed to TBBPA or BP-3. These authors concluded that clock activity could be modulated by excess fatty acids by activating PPARγ signaling (58) which specifically down-regulates the clock gene period1 (59) with a repression of the Clock/Bmal complex formation.

The ability of TBT and triphenyltin (TPT) to promote adipocytes differentiation was demonstrated in trout (60). Primary cultured adipocyte treated with TBT and TPT induced lipid accumulation and slightly enhanced PPARγ and C/EBPα protein expression, suggesting that the use of a primary adipocyte cell culture from this species is a valuable *in vitro* tool to estimate the capacity of different compounds and their synergism to interfere with adipocyte differentiation and lipid accumulation.

Several studies so far demonstrated that ECs are present in adipose tissue and other peripheral tissues involved in energy metabolism (i.e., liver and muscle), thus representing an additional clue to the understanding of adipose tissue functioning and possible onset of metabolic syndromes including obesity. Recent studies demonstrate the ability of pollutants to modulate the ECS (61, 62). In particular, in zebrafish, DEHP exerts its obesogenic action by up-regulating hepatic *pparα, cb1*, and *srebp* levels and by stimulating *de novo* FA synthesis and hepatic steatosis. This hepatic state may cause an inhibition of food intake stimulus by the up-regulation of leptin, the typical sensor of the energy status, which, in the brain, may negatively control *cb1* and in turn reduce *srebp* gene expression (63). Similar results were obtained after an acute exposure of adult zebrafish to BPA. The observed hepatosteatosis was associated with an increase in the liver of EC levels and variations of catabolic and anabolic enzyme levels. In the same study, acute and chronic exposure of HHL-5 cells to BPA, induced triglyceride accumulation in a CB1 dependent manner, suggesting that BPA induced hepatosteatosis in zebrafish and human hepatocytes is mediated by the up-regulation of EC system (64). Similarly, a 3 weeks chronic exposure of adults to three BPA concentrations (5, 10, and 20 μg/L), altered the expression of a number of genes involved in the EC control of metabolism in liver and brain, as well as that of endogenous ECs and EC-like mediators. These

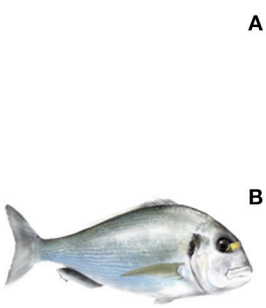
changes were associated with an increased presence of hepatic lipid vacuoles, without changes in food intake and appetite regulation (61). BPA exposure affected lipid metabolism in a non-monotonic dose-related fashion. The lowest dose of BPA increased the storage of TAGs and promoted FA synthesis, while the highest concentration promoted the *de novo* lipogenesis and cholesterologenesis (65). Moreover, a chronic BPA-exposure impacted the miRNome in adult zebrafish and established an epigenome more susceptible to cancer development. After a 3 weeks exposure to 100 nM BPA, in the liver, 6,188 mRNAs and 15 miRNAs were differently expressed ($q \leq 0.1$), uncovering signatures associated with NAFLD, oxidative phosphorylation, mitochondrial dysfunction and cell cycle, suggesting BPA potentiality to cause adverse health outcomes including cancer (66), and supporting previous studies evidencing the miRNA pivotal role in lipid synthesis, oxidation and related diseases (67). The expression of four miRNAs, miR-125b, miR-205, miR-142a, and miR-203a were significantly modulated by TCS in different experimental models both *in vivo* and *in vitro* (55), and resulted implicated in the downstream regulation of genes responsible for FA synthesis and metabolism. Moreover, in zebrafish, TCS was directly involved in the upstream regulation of miR-125b (68).

The results above described revealed the obesogenic effects of several potential EDCs, many of which are plasticizers and in the last years, the need to replace them, e.g., BPA and DEHP, with safer compounds, arose. Alternative candidates of DEHP included diethylene glycol dibenzoate (DGB) and di-isononylphthalate (DiNP). In a pilot study, a chronic exposure to five DGB concentrations (0.01; 0.1; 1; 10; 100 μg/L) affected hepatic lipid metabolism leading to increased lipid production and mobilization in a non-monotonic dose-related fashion. The lowest DGB concentrations (0.01 μg/L and 0.1 μg/L), increased *de novo* lipogenesis, cholesterol esters, TAG production and the possible conversion of lipids into apolipoprotein particles. A small reduction in *apoAla*, concomitant with the increase of apoliprotein mRNA codifying for a protein involved in very low-density lipoprotein and chylomicron production was also measured. Exposure to the highest concentrations (10 and 100 μg/L), increased *cebpa* levels, involved in adipocyte differentiation. Moreover, FT-IR analysis revealed that DGB exposure lead to changes in the biochemical composition of liver where the length of the aliphatic chains and phospholipid content increased (65).

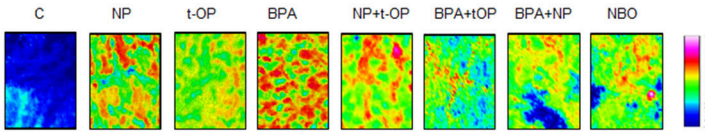
To date, one study described the role of DiNP in the onset of pathophysiologies in adult zebrafish. In addition to an impairment of oogenesis, which will be described later on, an up-regulation of orexigenic and hepatosteatotic signals together with a deregulation of the peripheral ECS and lipid metabolism was observed. At central level, a deregulation of ECS components (62), suggested that DEHP replacement with DiNP should be carefully evaluated.

Moving to marine teleosts, seabream resulted so far an excellent experimental model for ecotoxicological studies. In a feeding trial, juvenile seabream were fed a diet contaminated with BPA (5 mg/kg bw or 50 mg/kg bw BPA) or alkylphenolic contaminants, NP (5 mg/kg bw or 50 mg/kg bw NP) or *tert-octylphenol* (t-OP) (5 mg/kg bw or 50 mg/kg bw t-OP). The diet caused alteration of liver morphology, showing moderate-severe lipid accumulation, loss of the cord structure, ceroid accumulation and hydropic change in most of fed fish (69, 70). These findings prompted analysis of the expression of the major molecules involved in lipid metabolism: *ppars, fas, lpl*, and *hsl*. The modulation of the different signals strongly suggested that lipid accumulation within hepatocytes was associated to a decrease of lipid mobilization, thus causing hepatosteatosis as documented by histological analysis (70, 71).

Since most of studies have focused on the effects induced by the exposure to a single compound, recently the attention moved to the combined effects of mixtures of substances with dissimilar modes of action. In a further study, seabream were



A



B

Target gene	NP	t-OP	BPA	NP+ t-OP	BPA+ t-OP	BPA+NP	NBO
ppar α	/	/	/	/	/	/	/
ppary	+	+	+	+	/	+	/
lpl	/	+	+	/	+	+	/
hsl	-	-	/	/	-	/	/
fas	+	/	/	+	/	+	/
pepck	/	+	/	-	-	/	-

FIGURE 3 | Modulation of lipid content and metabolism in Seabream fed xenobiotics. **(A)** False color images of liver sections from C, NP, t-OP, BPA, and xenobiotic mixtures representing the topographical distribution of lipids. Adapted from Carnevali et al. (72). **(B)** mRNA variations in the different experimental groups. "+" upregulation, "–" downregulation, "/" no changes respect to control values. Experimental groups C, control fish receiving the commercial feed; NP, fed the commercial feed enriched with 5 mg/kg bw NP; t-OP, fed the commercial feed enriched with 5 mg/kg bw t-OP; BPA, fed the commercial feed enriched with 5 mg/kg bw BPA; NP + t-OP, fed the commercial feed enriched with 5 mg/kg bw NP + 5 mg/kg bw t-OP; BPA + t-OP, fed the commercial feed enriched with 5 mg/kg bw BPA + 5 mg/kg bw t-OP; BPA + NP, fed the commercial feed enriched with 5 mg/kg bw BPA + 5 mg/kg bw NP; NBO, fed the commercial feed enriched with 5 mg/kg bw NP + 5 mg/kg bw BPA + 5 mg/kg bw t-OP. Seabream picture by Dr. Marco Graziano http://tiktaalikillustrations.com.

fed on mixture of the above mentioned xenobiotics (NP, t-OP, BPA) and results evidenced that the administration of mixture of contaminants exerts a milder lipogenic effect, highlighting the contrasting/antagonistic interaction among chemicals (72) (**Figure 3**). In the same experimental model, a chronic exposure to two nominal concentrations of DGB (1 μg/L or 100 μg/L), demonstrated its action as PPARα agonist, resulting in a potential stimulation of key lipolytic genes and in a concomitant down-regulation of those involved in the ECS regulation (73). Using sea bream hepatocytes, the integration of *in silico* predictions with *in vitro* experiment results, evidenced the possible dose-relationship effects of diisodecyl phthalate (DiDP) exposure on PPAR:RXR-dependent gene expression pathways. Principal component analysis (PCA) showed the strength of relationship between transcription of most genes involved in FA metabolism and *ppar* mRNA levels. In particular, *fabp* was highly correlated to all *ppars* (74). Recently, in the same experimental model, it was demonstrated that dietary administration to BPA and DiNP altered the hepatic structure and the biochemical composition, increasing the presence of lipids and TAGs and decreasing phospholipids and glycogen abundance. In addition, the diet altered hepatic levels of ECs and EC-like mediators. These alterations were also associated to changes at the transcriptomic level of genes involved in lipid biosynthesis and ECS metabolism (75).

Marine medaka, *Oryzias javanicus*, male fish were exposed to 76 mg/L of BPA for 72 h to analyze the transcriptional responses of BPA exposure over four time-course by gene ontology enrichment analysis in terms of molecular functions. The most up-regulated transcripts belonged to lipid metabolism relevant genes, e.g., *apoA-IV, apoA-I,* long chain fatty acid CoA ligase (*acsl*), elongation of very long chain fatty acids protein (*elovl*), and *fabp*, showing that in medaka, BPA behaves in a similar way as in mammalian species (76).

Altogether, the results reported in this section, highlighted the common negative effects of the exposure to potential EDCs both in animal models and *in vitro* systems, providing a general overview on the toxicity of these environmental compounds, not depending from the route of exposure, via the food, via the water, perinatal, early in life or at adulthood.

TRANSGENERATIONAL EPIGENETIC INHERITANCE: FOCUS ON THE EFFECTS ON ADIPOGENESIS AND LIPID METABOLISM SIGNALS

Recent studies revealed that the increasing incidence of obesity, in addition to being due to bad life styles and occupational stress, could be also caused by exposure to xenobiotics and transmitted to subsequent/following generations on an epigenetic inheritance base (77). To be called transgenerational, expression of the trait has to persist for at least two or three generations (three mammals, two fish) after the initial exposure to the environmental agent (78, 79). This section will describe the epigenetic mechanisms underlying developmental plasticity and uncovering the existence of a mechanistic link between altered epigenetic gene regulation following an early toxicant exposure and potential onset of obesity later in the life. In general terms, evidences demonstrated that the obesogenic effects of potential EDCs are mediated by their ability to bind NRs. These receptors directly recruit methyl and acetyltransferase, thus altering epigenetic marks regulating gene expression (80). In this way, EDCs can modify chromatin states or the levels of DNA or histone methyltransferases (81). As stated above, one of the main target of obesogens is PPARγ, the master regulator of adipogenesis or its target-related genes. During the first developmental stages, PPARγ regulates the differentiation of MSCs into osteocytes or adipocytes. An *in vitro* exposure of 3T3-L1 preadipocytes cell line to TBT, resulted in an increase of the number of differentiated adipocytes associated with a global decrease of DNA methylation (82). The same cell line exposed for 8-days to brominated diphenyl ether 47 (BDE-47), differentiated into adipocytes and presented higher levels of *pparγ2, cebpα, cebpβ, cebpγ, srebf1a, lpl, Slc2a4, fabp4, Adipoq, G6pc, Lep, and Igf1*. Variation of *pparγ2* expression was associated with a decreased methylation of 3 CpG sites in promoter region (83). Moving to *in vivo* studies, in adipose-derived stem cells (ADSCs) isolated from white adipose tissue of C57BL/6J mice perinatally exposed to TBT by maternal gavage, increased lipid accumulation in differentiated adipocytes associated with an increase of early adipogenesis markers, *pparγ* and *fapb4*, were measured. In these genes, hypomethylation of promoter/enhancer region of *fapb4* but not of *pparγ2* were observed (82). Prenatally exposure of BALB/cByj mice (F1) to mixture of PAHs, displayed increased adult adipocyte size. The size alteration was also observed in F2-offspring and were associated in both F1 and F2, to general increased *pparγ, cox2*, and *cebpα* expression in adipose tissue. In both males and females of F1 and F2, a decreased methylation of 1 CpG site in *pparγ* promoter was detected, inversely correlating with *pparγ* expression (84). In a transgenerational study, outbred gestating female rats were transiently exposed to DDT and the F1 generation offspring bred to generate the F2 generation and F2 generation bred to generate the F3 generation. The F1 and F3 generation were aged and various pathologies investigated. The transgenerational transmission of disease was through both female (egg) and male (sperm) germlines. F3 generation sperm epimutations and differential DNA methylation regions of a number of obesity-related genes were induced by DDT. Interestingly, in this study, the authors concluded that male obesity is transmitted through the female germline and female obesity transmitted through the male germline (78). In a study using rats, females were daily intraperitoneally injected with methoxychlor from days 8 to 14 of gestation and then the onset of disease was evaluated in adult F1 and F3 generation progeny. Increase of obese rate was observed especially in the F3 and F4 generation demonstrating that female germline transmission of environmentally induced epigenetic transgenerational phenotypes is equally as stable as male germline transmission (85). In another study, gestating female rats were transiently exposed from days 8 to 14 of embryo gonadal sex determination to a plasticizer mixture containing BPA, DEHP and dibutylphthalate (DBP) and the

TABLE 1 | Main biological effects reported in different cell and animal models exposed to potential EDCs.

Environmental pollutant	Animal model	Biological effect observed	Bibliography
Benzophenone 3 (BP-3)	Zebrafish, *Danio rerio*	Obesity induction and alteration of circadian rhythms	(57)
Bisphenol A (BPA)	3T3-L1 pre-adipocytes	Adipocyte metabolic dysfunction and inflammation	(53)
	Zebrafish	Induction of TAG accumulation by up-regulation of ECS	(64)
	HHL-5 cells	Induction of TAG accumulation by up-regulation of ECS	(64)
	Zebrafish	Increased presence of hepatic lipid vacuoles, caused by alteration of the ECS	(61)
		Increased TAG storage and FA synthesis, *de novo* lipogenesis and cholesterologenesis promotion	(65)
		Evidence of the miRNome involvement in lipid synthesis, oxidation and related diseases	(67)
	Seabream, *Sparus aurata*	Hepatic lipid accumulation associated to a decrease of lipid mobilization	(70, 71)
		Alteration of hepatic structure lipids and TAG content and decreased phospholipids and glycogen abundance	(75)
	Marine medaka, *Oryzias javanicus*	Upregulation of *apo A-IV, apo A-I, Acsl1, Elovl, and fabp*	(76)
Brominated diphenyl ether 47 (BDE-47)	3T3-L1 pre-adipocytes	Increased levels of *Pparϫ2, Cebpα, Cebpβ, Cebpϫ, Srebf1a, Lpl, Slc2a4, Fabp4, Adipoq, G6pc, Lep, and Igf1*. *Transgenerational study*	(83)
Cypermethrin (CYP), atrazine (ATZ), 17α-ethynyestradiol (EE2)	C57BL/6J mice	Alteration of the hepatic levels of PPARα, PPARϫ, and SREBP1C	(52)
Dibutyltin (DBT)	Human MSCs	C/EBPα, PPARγ2, FABP4, FSP27, LPL upregulation	(44)
Dichlorodiphenyltrichloroethane (DDT)	Hsd:Sprague Dawley®™SD®™ Harlan	Obesity induction in males. *Transgenerational study*	(78)
Di(2-ethylhexyl) phthalate (DEHP)	HepG2 cells	Activation of the SREBP-1c and PPARα-signaling pathway	(49)
	Zebrafish	Alteration of FOXA2 and FOXA3 transcription factor networks', "Metabolic pathways," "metabolism of amino acids and derivatives," "metabolism of lipids and lipoproteins," and "fatty acid, triacylglycerol, and ketone body metabolism"	(56)
		Up-regulation of hepatic PPARα, Cb1, and SREBP levels, *de novo* FA synthesis and hepatic steatosis	(63)
	Sprague-Dawley rats	Hepatic steatosis, associated to inflammation, lipid peroxidation, oedema of the liver cells and hepatic damage	(48)
Di-isodecyl- phthalate (DiDP)	Seabream	PPAR-mediated regulation of *fabp*	(74)
DiNP	Zebrafish	Upregulation of orexigenic and hepatosteatosis signals, deregulation of the peripheral and central ECS and lipid metabolism	(62)
DiNP	Seabream	Alteration of hepatic structure lipids and triglycerides content and decreased phospholipids and glycogen abundance	(75)
Diethylene glycol dibenzoate (DGB)	Zebrafish	Increase of de novo lipogenesis, cholesterol esters, TAG production and potential conversion of lipids into apolipoprotein particles	(65)
	Seabream	PPARα agonist, stimulation of key lipolytic genes and downregulation of ECS	(73)
Methoxychlor	Sprague-Dawley rats	Obesity induction in females. *Transgenerational study*	(85)

(Continued)

TABLE 1 | Continued

Environmental pollutant	Animal model	Biological effect observed	Bibliography
BPA, DEHP and dibutylphthalate (DBP) mixture	Sprague-Dawley rats	Obesity induction. Transgenerational study	(86)
Nonylphenol (NP)	Sprague-Dawley rats	Hepatic *srebp-1C, fas* and *ucp2 upregulation*	(46)
	Wistar rats	Obesity induction, increased levels of cholesterol and leptin and alteration of the expression of genes involved in lipogenesis and adipogenesis. *Transgenerational study*	(87, 88)
	Seabream	Hepatosteatosis, alteration of lipid metabolism	(69, 71)
BPA, NP, *tert*-octylphenol (*t*-OP) Mixture	Seabream	Alteration of lipid metabolism	(72)
Polycyclic aromatic hydrocarbon (PAH) mixture	BALB/cByj mice	Increased *Pparϫ, Cox2, and Cebpα* expression in adipose tissue. *Transgenerational study*	(84)
RXR-specific ligands LG100268 and AGN195203	African clawed frog, *Xenopus laevis*	Ectopic adipocyte formation around the gonads	(43)
Tributyltin (TBT)	murine 3T3-L1 adipocytes	RXR-PPARϫ-mediated pro-adipogenesis in liver and adipose tissue	(40–42)
		Increase the number of differentiated adipocites. *Transgenerational study*	(82)
	C57BL/6 mice	Lipid accumulation in adipose tissues and onset of hepatic steatosis	(43)
		Increased lipid accumulation in differentiated adipocytes associated to an increase of early adipogenesis markers, Pparϫ and Fapb4. *Transgenerational study*	(82)
	African clawed frog	Activation of RXR/PPARϫ pathways	(43)
	Zebrafish	Male: increased body weight, hepatosomatic index, hepatic TAG abundance and expression of adipogenesis and lipogenesis genes *pparγ, srebp1, fasn, 11β-hsd2, c/ebpβ*, and *dgat2* Female: hepatomegaly with lack of response of signals involved in adipogenesis	(54)
		Obesity induction and alteration of circadian rhythms	(57)
	Trout (*Oncorhynchus mykiss*) Primary adipocyte culture	Promotion of adipocytes differentiation by enhancing PPARγ and C/EBPα protein expression	(60)
2,3,7,8-Tetrachlorodibenzodioxin (TCDD)	C57BL/6J mice	Sex specific modulation of mRNA levels involved in adipose tissue and hepatic metabolism, inflammation, xenobiotic metabolism and endocrine disruption	(50)
		AHR mediates obesity and fatty liver onset	(51)
Tetrabrominated bisphenol A (TBBPA)	Zebrafish	Obesity induction and alteration of circadian rhythms	(57)
t-OP	Seabream	Hepatosteatosis, alteration of lipid metabolism	(69, 71)
Triclosan (TCS)	Zebrafish	Upstream regulation of miR-125b	(68)
	Human MSCs	decreasing *aP2, lpl,* and *adipoq* gene expression	(47)
	Zebrafish	impaired mRNA expression levels of β-oxidation transcripts and lipid β-oxidation genes, including *pparα, cpt1, lpbe, cyp4a10*, and *aco*	(55)
Tyhriphenyltin (TPT)	Trout Primary adipocite culture	Promotion of adipocytes differentiation by enhancing PPARγ and C/EBPα protein expression	(60)
tris (1,3-dichloroisopropyl) phosphate (TDCIPP)	Zebrafish	Obesity induction and alteration of circadian rhythms	(57)

incidence of adult onset disease was evaluated in F1 and F3 generation rats. Obesity significantly increased in F3 rats (86). Epidemiological evidence showed that the developmental programming can be transferred to subsequent generation. The exposure to NP during critical windows of development, including fetal and/or early postnatal periods, can induce permanent alterations in adipose tissue and then obesity in mouse (87). NP action is mediated by ERα signaling pathway and the receptor deletion causes abdominal fat accumulation due to increased number and size of fat cells, increased levels of cholesterol and leptin and alteration in the expression of genes involved in lipogenesis and adipogenesis, in the two generation offspring's (88). Similarly to NP, it is well-known that BPA induces epigenetic modification (89, 90) and exerts its obesogenic action by binding ERα. The hormone-like-receptor complex bind the estrogen responsive element in the promoter of histone modifying methyltransferase EZH2 gene. After binding, several co-regulators are attracted and the up-regulation of the EZH2 levels increase H3K27 trimethylation (91). Recently Helsley and Zhou (92), reviewed the central role of PXR in lipid homeostasis. These evidences clearly suggest that although the research on obesogens mainly focuses on PPARs as master regulator, the involvement of other receptors, including steroid hormone receptor and PXR should be evaluated.

EDC EXPOSURE: INTERPLAY BETWEEN FAT/LIPID METABOLISM AND REPRODUCTION

Body energy reserves are gated with reproduction and are sensitive to different metabolic signals. Main actors of this tight relationship between energy homeostasis and fertility are represented by metabolic hormones (ghrelin and leptin) and neuropeptides (kiss1 and kiss2) (93, 94), that regulate the levels and the release of Gonadotropin-releasing hormone (GnRH) (95). Thus, full activation of the hypothalamic-pituitary-gonadal axis at puberty and its proper functioning later at adulthood critically depends on adequate energy stores (96). The identification of the adipokine leptin, which transfers information on the body's metabolic status to hypothalamic centers governing reproduction, represents an important step to understand the mechanisms regulating this interplay (97). On this regard, of particular interest was the finding that DEHP, is able to modulate leptin expression in the liver of zebrafish showing the potentiality of this plasticizer to impair the entire endocrine system linking fat storage to appetite, energy expenditure and reproduction (63).

Using a multidisciplinary approach, ranging from qPCR analysis, to histology and Fourier transform infrared imaging (FTIR), the effects of DiNP exposure were analyzed in zebrafish. Fish fecundity, oocyte growth, autophagic and apoptotic processes, as well as changes of morphological and biochemical composition of oocytes were investigated (98). Findings in zebrafish, provided evidence that exposure to DiNP adversely affects oocytes growth and maturation, leading to abnormal gonadal development and reproduction. More specifically, lipids, proteins and phosphate groups were significantly decreased in the ovaries of all the experimental groups and were associated to an alteration of vitellogenin, a phospholipoglycoprotein internalize by vitellogenic oocytes. These alteration of the macromolecular composition of oocytes and the decreased number of vitellogenic and mature eggs within the ovary could be responsible for the significant decrease of fecundity observed for all doses of DiNP (99). In Wistar rats receiving TBT for 15 days by gavage, metabolic dysfunctions and reproductive abnormalities were observed. The increase of leptin levels in obese rats, were negatively correlate with lower Kiss responsiveness, evidencing that TBT toxic effects may be either direct, on the reproductive axis, or indirect, by its abnormal metabolic regulation (100).

CONCLUSION

In conclusion, this review summarizing the most recent results describing the effects of potential EDC administration on metabolic health, clearly evidences the risk caused by the exposure to environmental chemicals (**Table 1**). A summary of the most common lipid metabolism alterations following their exposure has been presented. In addition, novel data regarding their ability to affect circadian rhythms as well as to up-regulate the expression of the ECS, in both cases leading to a remarkable increase of lipid accumulation, have been also reported. Finally, evidences of their"transgenerational obesogenic effects" following a prenatal or early life contamination have been discussed.

The integration of results suggests that hepatic steatosis, the first signal of the onset of several metabolic diseases, easily occurs following the exposure to environmental concentrations of pollutants, thus triggering an increase of FA synthesis or uptake and their decreased oxidation. Widespread pollutants including BPA, phthalates, PFCs, POPs, and TBT, targeting NR, induce FA synthesis in different animal models, highlighting the activation of a common pathway mediating the toxicity among species. The main evidence consists in the fact that liver also mediates xenobiotic metabolism which may increase oxidative stress and in turn impacts the correct FA metabolism. However, since few evidence still exists to characterize pathways and patterns leading to altered functional development, all these results should be considered and integrated by Environmental Agencies to propose novel biomarkers and innovative endpoints for the development of novel Organization for Economic Co-operation and Development (OEDC) test guidelines to screen chemical danger for metabolic functions.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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Differential Hepatic Gene Expression Profile of Male Fathead Minnows Exposed to Daily Varying Dose of Environmental Contaminants Individually and in Mixture

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Environmental contaminants are known to impair reproduction, metabolism and development in wild life and humans. To investigate the mechanisms underlying adverse effects of contaminants, fathead minnows were exposed to a number of endocrine disruptive chemicals (EDCs) including Nonylphenol (NP), bisphenol-A (BPA), Di(2-ethylhexyl) phthalate (DEHP), and a mixture of the three chemicals for 21 days, followed by determination of the liver transcriptome by expression microarrays. Pathway analysis revealed a distinct mode of action for the individual chemicals and their mixture. The results showed expression changes in over 980 genes in response to exposure to these EDC contaminants individually and in mixture. Ingenuity Pathway core and toxicity analysis were used to identify the biological processes, pathways and the top regulators affected by these compounds. A number of canonical pathways were significantly altered, including cell cycle & proliferation, lipid metabolism, inflammatory, innate immune response, stress response, and drug metabolism. We identified 18 genes that were expressed in all individual and mixed treatments. Relevant candidate genes identified from expression microarray data were verified using quantitative PCR. We were also able to identify specific genes affected by NP, BPA, and DEHP individually, but were also affected by exposure to the mixture of the contaminants. Overall the results of this study provide novel information on the adverse health impact of contaminants tested based on pathway analysis of transcriptome data. Furthermore, the results identify a number of new biomarkers that can potentially be used for screening environmental contaminants.

Keywords: environmental contaminants, biomarker, microarray, fathead minnow, immune system, lipid metabolism

HIGHLIGHTS

- A total of 980 transcripts were differentially expressed by more than two-fold in at least one of the EDC treatments relative to the untreated control.
- More than 50% of the differentially-expressed genes in each treatment were unique for that treatment.
- 18 genes were expressed in all four EDC treatments which represent potential biomarkers for initial screening of EDC contaminants.
- Pathway analysis of transcriptome data revealed a distinct mode of action for each individual chemical and their mixture. Our results also detected some similarities in molecular pathways regulated by each treatment.
- The EDC mixture induced the integrated stress response to a greater extent than any individual treatment through inhibition of the Acute Phase Response Signaling and activation of EIF2 Signaling.

INTRODUCTION

It is established that environmental contaminants cause adverse health effects in wildlife and humans. A number of environmental contaminants have hormone-like activity and are known as endocrine disrupting chemicals (EDCs). There is evidence that EDCs can interact with a variety of hormones and/or hormone receptors, and exert actions as agonists or antagonists. As a result, EDCs can disrupt the activity of hormones and alter normal physiological function at different levels [Reviewed in (1–3)]. Adverse effects of EDCs on the reproductive system can result from interaction with sex steroids or their receptors and other types of receptors, including aryl hydrocarbon receptor (AhR), Peroxisome proliferator-activated receptor (PPAR), liver X receptor (LXR), thyroid hormone receptors (TR), and retinoid X receptor (RXR). Thus, health impact of EDCs can result from altered gonadal development, gamete production (4–12), lower fertility, and reproductive success (13, 14), interfere with epigenetic mechanism (9, 15), altered growth (16, 17), morphology (18), metabolism (19, 20), lipid metabolism (18, 21), dysregulation of the central and peripheral endocannabinoid system (ECS) (21–23), Cardiac response and development (24, 25), DNA damage and cytotoxicity (26), neuronal development and behavior (27–29), as well as compromised immune system (3, 30) and stress performance (31). Furthermore, there is evidence that EDCs are able to increase progression of certain kinds of diseases, including obesity, diabetes, endometriosis, and hormone-dependent cancers [Reviewed in (32)].

Previous studies in our lab revealed the presence of organic contaminants downstream of treated municipal wastewater effluents and agricultural areas in Southern Alberta rivers (33–35). These studies provided evidence for female-biased sex ratio, intersex and altered gene expression in Longnose dace fish (*Rhinichthys cataractae*) in the areas containing environmental contaminants (33–35). Bisphenol A (BPA), Di(2-ethylhexyl) phthalate (DEHP), and nonylphenol ethoxylates (nonylphenol), were among the most abundant contaminants in the sites investigated. Due to their versatile uses in industry and in consumer products, these compounds are prevalent in the environment, including air soil and aquatic ecosystem, mainly through urban wastewater, industrial discharges and agricultural runoff (36–38). Reported concentration of BPA in the rivers range from below 1 to 21 μg/L. BPA can be detected as high as 72 μg/L in the municipal and paper manufacturing effluents (36, 39, 40) and in humans, ingestion due to migration to foods, beverages and infants' milk from plastic coating and bottles can reach as high as 100 μg/L (36). The NP level in surface water range from below 0.1 μg/L to 37.3 μg/L [reviewed in (41, 42)]. However, concentrations as high as 310 μg/L have been detected in surface water previously [Reviewed in (43)]. Phthalate concentrations reported range from 0.33 to 97.8 μg/L in surface water and up to 182 μg/L in sewage effluents [Reviewed in (44)]. Furthermore, a number of contaminants have hydrophobic properties and are absorbed by sediment particles. For this reason, concentrations are usually higher in sediments compared to the surface water (45, 46).

Previous studies demonstrated that exposure to low-dose (nanomolar) environmental concentrations of BPA, NP, and DEHP can alter the expression of a number of genes involved in the reproduction, gametogenesis, growth, and development (47), alter the steroid hormone levels, reduce the sperm quality (4, 5, 48), and dysregulate metabolite profile in goldfish (19). These compounds were also shown to impair the morphological development (49), disrupt hypothalamic neurogenesis and behavior in zebrafish (28).

It has been demonstrated that mixtures of contaminants with common mechanisms of action have the capacity to act in combination and exert their effects in an additive manner (50–52). The mixture effect is of particular importance when the concentration of each compound is below the threshold effect, but cumulative exposure to these chemicals can reach the effective dose to exert adverse effects (52–54). However, a number of studies have demonstrated that the effects of contaminants in mixture are synergistic rather than additive [[19. 49] for review see (52, 55–57)]. Previous studies demonstrated that mixture of BPA, NP and DEHP exert adverse effects on gene expression, metabolism and morphological development distinct from individual compounds in a non-additive manner (19, 49).

Using advanced analytical tools, an increasing number of compounds are detected in the ecosystem. However, many of these compounds are either unknown or have unknown toxicity (58). Therefore, development of effective biomonitoring tools with minimum animal would be desirable to address the risk factors associated with exposure to the contaminants. Although several techniques have been established successfully to screen for occurrence and biological effects of contaminants in the environment, their functionality is limited due to their specificity for particular classes of contaminants (59). Therefore, biomarkers, which are sensitive to various classes of contaminants, and also capable of providing information on biological outcomes of chemical exposure, would make valuable tools in ecotoxicology studies (59).

Despite considerable number of studies on environmental contaminant impacts on biological systems, and mechanisms underlying adverse effects of EDCs are less than clear [Reviewed

in (60–62)]. Study of the molecular mechanisms by which environmental contaminants exert their effects will help to understand how contaminant concentration and duration of exposure, and gene regulation are linked to the adverse physiological effects at the level of organism and ecosystem. In addition, this information can potentially improve risk assessment and may even help with the regulatory processes (63). Studies using "Omics" approaches (e.g., transcriptomics, metabolomics and proteomics) can provide valuable insight into broad adverse impact of contaminants on health and ecosystems [reviewed in (63)].

Transcriptomics can provide important information on global gene expression signatures of environmental contaminants and understanding of the molecular mechanisms underlying the impact of EDCs on aquatic organisms (29, 59, 63–65). Expression microarrays can be used to identify biological pathways and common regulators targeted by contaminants individually and in mixture [Reviewed in (66)]. A number of mathematical models has been developed to predict apical parameters from transcriptome data, which has shown to be consistent with results produced from traditional methods for chemical risk assessment (67, 68). Furthermore, using gene set enrichment analysis (GSEA) based tools make it possible to use non-human model organisms to make a potential connection between human disorders and environmental conditions that lead to differentially-expressed gene sets (69).

The objective of this study was to determine the impact of BPA, NP, and DEHP exposure on the hepatic expression profile of male fathead minnow. Liver is a key organ for storage and metabolism as well as playing a major role in reproductive processes by synthesizing lipoproteins such as vitellogenin (70). Fathead minnow (*Pimephales promelas*) is a native North American species, used widely as model organism in ecotoxicology studies and regulatory testing (71). In the present study, transcriptome profiles were investigated following exposure to three EDC contaminants individually and in mixture using an expression microarray. We sought to identify the biochemical pathways targeted by these environmental contaminants, which led us to generate testable hypotheses about upstream regulators, biological processes and biomarkers. This information can be applicable in biomonitoring of the environmental contaminants as well as investigating their adverse outcomes.

MATERIALS AND METHODS

Experimental Animals

Adult fathead minnows were purchased through Aquatic BioSystems, Inc., Colorado, US. The fish were acclimatized for 7 days prior to experimentation in the laboratory flow through glass tanks (49 L) at a constant temperature of 25°C and under a photoperiod of 16:8 L:D. Forty-five fish of mix gender per tank were selected randomly and used for each EDC treatment. Fish were fed the same amount of commercial diet of Nutrafin Max fish flakes, as recommended by the manufacturer. All protocols for maintaining and handling of fish were approved by the university animal care committee in accordance with the guidelines of the Canadian Council on Animal Care.

Exposure to Chemicals

Bisphenol A (4,4′-(propane-2,2-diyl) diphenol) (BPA, 239658), (Di(2-ethylhexyl) phthalate) (DEHP, 80030), and 4-nonylphenol (NP, 442873), were purchased from Sigma-Aldrich (Missouri, United States). To minimize bias, fish were distributed evenly and at random among five flow-through glass aquaria, and the treatments were randomly assigned to the experimental tanks. The flow-through glass tanks were supplied with activated carbon-filtered City of Calgary water (flow rate at 300 mL/min). Fish were exposed for 21 days based on the 21-day fish endocrine assay guidelines by EPA (72) in tanks treated with 100 μg/L of individual chemicals or a mixture of the three compounds (100 μg/L of each chemical). The control group was exposed to the same concentration of the vehicle (EtOH). These EDC compounds are among the most common and abundant environmental contaminants in various parts of the world including Alberta surface water and effluents (73). In order to study the compensatory and toxicity responses of animals to EDC exposure, the concentrations used for this study were within the higher range of the reported environmental levels [(44) Reviewed in (36, 43)]. The concentration of contaminants in the environment is variable throughout the day across geographic areas depending on the distance from the wastewater effluent discharge, flow rate or volume in the receiving rivers (74). Also, as the fish swim away from the wastewater effluent discharge they would expose to variable concentration of chemicals. In the present study, we created an experimental condition to expose fish to daily steady decline in chemical concentration using a flow-through system to address the variability in the contaminants concentrations in the river to some extent. Aquarium water was changed daily with fresh water followed by adding a nominal dose of contaminants (100 μg/L). Flow rate was adjusted to gradually reduce the concentration of chemicals by 50% every 4 h. This treatment procedure ensured exposure of fish to declining concentrations of chemical throughout the day as a result of the renewal of the water. Since the chemical levels were variable during the day, we did not measure their concentrations in the water. After exposure to the chemicals for 21 days, fish were anesthetized in 250 mg/L buffered tricaine methanesulfonate (MS-222, Sigma–Aldrich, MO, United States), euthanized, weighed and liver tissue from male fish were rapidly dissected, frozen in liquid nitrogen, and stored at −80°C for future studies. Dividing the weight of the gonads by the weight of the fish and then multiplying by 100 determined the gonadosomatic index (GSI) of each fish.

RNA Extraction

Total RNA was extracted from liver tissue using TRIzol Reagent (Invitrogen, CA, United States) according to the manufacturer's protocol (75) and treated with DNAse (Ambion, CA, United States). Total RNA was quantified verified using a NanoDropTM 1000 Spectrophotometer (Thermo Scientific, Delaware, United States) and OD 260/280 and 260/230 ratios were obtained to determine the purity of the RNA samples. A

portion of RNA isolated from each treatment was saved for qPCR sufficient for 12 replicates, and the rest of extracted RNA was pooled for the microarray experiments. Total RNA samples were run on a 1% agarose gel to confirm its integrity after isolation.

Microarray Experimental Design

We used two-channel (two-color) Agilent expression microarrays to determine the transcriptomes of male zebrafish livers exposed to BPA, DEHP, NP and a mixture of these three compounds. In this type of array compared to one-color microarray, the relative hybridization intensity of each treatment can be directly contrasted with the control sample (non-chemical treated group), which eliminates the variation due to the spot size or distribution pattern of each probe (76). Also performing the expression microarray experiments with dye swap reduces the technical variations (77). In expression microarray studies when a limited quantity of RNA is available, pooling RNA samples or preamplification of RNA samples can be utilized. However, studies have demonstrated the presence of amplification bias during cRNA synthesis (78) and also increased cross-hybridization in RNA-DNA hybridization on the expression microarrays (79). In this study, we pooled the RNA samples to generate adequate quantities to avoid amplification bias. We performed qPCR with 12 replicates to verify the expression microarray data of several relevant gene candidates.

Microarray Protocol

mRNA from 1 to 2 mg of pooled total RNA was extracted using oligo(dT) 25-cellulose beads (NEB, Massachusette, United States) in a Poly-Prep® Chromatography Column (BioRad, CA, United States). Isolated mRNA was quantified using a NanoDropTM 1000 Spectrophotometer (Thermo Scientific, Delaware, United States). Two microgram of mRNA was reverse transcribed using Oligo (dT)23 anchored primer (Sigma-Aldrich, Missouri, United States) and SuperScript II reverse transcriptase (Invitrogen CA, United States) and aminoallyl-dUTP (Sigma-Aldrich, Missouri, USA was incorporated into the cDNA during reverse transcription. Purified cDNA samples were then labeled with Cy™3 and Cy™5 [For detailed procedure see (80)]. Fathead minnow 8 × 15K Agilent expression microarrays were used for this study, in which gene-specific probes were designed by EcoArray (FL, United States) and manufactured by Agilent (CA, United States).

Microarray hybridization was carried out using a reference design, where each cDNA sample was compared to a reference (untreated male liver control). 1 μg of labeled cDNA of each treatment was co-hybridized onto the microarray with cDNA from the untreated control following the manufacturer's recommendations (Two-color microarray hybridization protocol; Agilent, CA, United States). Dye-swap normalization was performed to remove systematic dye intensity bias from expression microarray data.

The expression microarrays were scanned with an Axon GenePix® 4200A laser scanner (Molecular Devices, CA, United States) at 5 μm resolution using wavelengths of 532 nm for the Cy™5 and 625 nm for Cy™3 dyes. Microarray data quality was assessed by manual inspection. Axon GenePix® Pro 6.0 software (Molecular Devices, CA, United States) was used to generate the raw spot intensities of the expression microarrays.

Bioinformatics

LOWESS (Locally weighted Scatterplot Smoothing) normalization was performed on the raw expression microarray data using the R Bioconductor package LIMMA software. The average log2 ratios between the experimental channel and the control channel from the dye-swap experiments were calculated by *t*-test and corresponding *p*-values were obtained (81). Significant changes in gene expression were identified as having a $p < 0.001$. Hierarchical clustering was performed on differentially regulated transcripts ($P < 0.001$, and $> \pm 2$ fold changes) using Cluster 3.0 software (82). Java TreeView software was used for visualizing the heat map (83). In order to identify the common and unique genes that were significantly affected by chemical exposure, a Venn diagram was created using Venny 2.1 software (84).

We investigated the networks, pathways, regulators and functions associated with NP, BPA, DEHP, and mixture exposure from the expression microarray data using Ingenuity Pathway (IPA) analysis (Ingenuity Systems, CA, United States). Significantly differentially-regulated transcripts ($P < 0.001$, and $> \pm 2$ fold changes) were mapped to human homologs using the Entrez GeneID and along with their fold change information uploaded to IPA. The most significant biological functions and the top canonical pathways from the expression microarray data were identified. Based on the information from all the molecules and their relationships in the Ingenuity Knowledge Base, upstream regulators and downstream biological function and diseases associated with chemical exposure were predicted.

Z-scores and *p*-values were used to interpret the IPA results. Calculated Z-scores indicated whether it was likely that identified pathways, upstream regulators, and biological functions were activated or inhibited. *P*-values were calculated by the Fisher exact test to determine the significance of the overlaps between a set of focus genes in the treatment and a given process or pathway in IPA knowledge base. $P < 0.05$ were considered significant. Also, a comparison analysis among the four treatments was performed to determine the gene expression changes involved in certain molecular pathways of each EDC compound.

Quantitative Real-Time PCR (qPCR)

Total RNA was reverse transcribed using Applied Biosystems High Capacity cDNA Reverse Transcription Kit (Life Technologies, CA, United States) according to the manufacturer's protocol. The cDNA was stored at −20°C prior to qPCR.

We used qPCR to validate the expression microarray results and also to verify roles of the predicted upstream regulators and biological pathways obtained by IPA on observed dysregulation of the transcriptome. A number of genes were selected based on their function, microarray expression results and IPA pathway analysis. Specific primers were designed based on the genome sequences available in GenBank or provided by EcoArray Inc. (**Table 1**). Primer efficiency was determined by performing qPCR on a dilution series of cDNA. Primers with 90–105% efficiency were considered for use. PCR products were confirmed by sequencing (University of Calgary Core DNA Services) and

TABLE 1 | List of the primers used in gene expression analysis by quantitative Real-Time PCR.

GENE	Forward primer (5′-3′)	Reward primer (3′-5′)	A T
AR	TACCCTAACGTGCCCTGTGTGA	CCGCATCAAACCTGCCATCTGT	58
Cyp19a	TTGTGCGGGTTTGGATCAATGGTG	TTCCGATACACTGCAGACCCAGTT	55
Cyp1A	TGCCCTTGAGGAGCACATCAGC	CGTCGTCGTGGCTGTAGCG	58
CYP27B1	GAGTTCTACCGCTTTGGTCTC	GCTGTTGATGGACTGGATGA	56
DMRT1	CTCCTATTACAACCTCTACC	CTGGACCGGCGACCATTTCC	57
GAPDH	TGATGCTGGTGCCCTGTATGTAGT	TGTCCTGGTTGACTCCCATCACAA	57
GSTA	CTCTGATGCTGCAGGAGTTATT	GCTGCAGGAATTTGCTGATTT	55
IFIT2	TCATCTCCAACAGAGCTTCAC	CTTGTCATCCGGCTCTTTCT	56
IGF1	AACTCCACGATCCCTACGAG	CTTCTGATGGACCTCCTTACAGG	55
INSR	GAACTATACTGTGCGGATCAGAG	CCACACGTAGGTCCTTAACATAG	57
IRF7	CGCATCCTAGACAGCATTCA	CTGGTGCTGACGAAGACTTTA	56
LRP8	ATGAAGATGCGCCAGTCACA	TGCAGGTTGGAGGGTCTTTG	57
RBP4	CGATAACTACGCCATCCACTAC	AGGGTGTCGGGAGAATATGAAG	56
RSAD2	CAGGGCAAGAAGAGCCATTTA	GTAGGTGTTGATCACGGAGTTG	55
Vtg1	GAAGTGCGCATGGTGGCTTGTATT	AGCTGCCATATCAGGAGCAGTGAT	55

TABLE 2 | Gene expression changes in the liver of male fathead minnow response to exposure to NP, BPA, DEHP and the mixture.

Gene-expression changes	NP	BPA	DEHP	Mix
Total regulated genes (n)	316	335	381	247
Up-regulated genes [n (%)]	156 (49.4)	195 (58.2)	124 (32.5)	73 (29.6)
Down-regulated genes [n (%)]	160 (50.6)	140 (41.8)	257 (67.5)	174 (70.4)
Uniquely regulated genes [n (%)]	158 (49.4)	177 (52.8)	212 (55.6)	140 (56.7)
Commonly regulated genes [n (%)]	18 (5.6)	18 (5.4)	18 (4.7)	18 (7.2)
Regulated genes that overlap with mixture [n (%)]	45 (14.2)	46 (13.7)	73 (19.1)	–

melt curve analysis performed to ensure that the primers amplified a single product. The primer sequences and annealing temperatures are listed in **Table 1**.

qPCR reactions were carried out on a BIO-RAD CFX96 TouchTM Real- Time PCR Detection System using BIO-RAD IQTM SYBR® Supermix following the manufacturer's protocol (BIO-RAD, CA, United States). The conditions for each PCR reaction were as follows: 10 μL Supermix, 300 nM of each primer, 0.75 μL cDNA, and 8.65 μL ultrapure water (Life Technologies, CA, USA). The condition of qPCR amplification was as follows: initial denaturation at 95°C for 2 min, 40 cycles of denaturation at 95°C for 10 s and annealing/extension at a primer optimized temperature for 30 s. Reactions were carried out in triplicate to ensure consistency. Gene expression data was normalized by calculating the difference relative to the housekeeping gene. The main assumption for using a particular reference gene to normalize the data is that it should be stable with minimal variation following treatments. To maximize stability of the reporter gene, we tested the suitability of three reference genes β-actin, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and ribosomal protein subunit 18 (rps18) for stability as described previously (85). We found that β-actin and GAPDH were the least variable reference genes for normalization. To ensure the accurate normalization of the qPCR results, we used the mean of β-actin and GAPDH following the $2^{-\Delta\Delta Ct}$ method (86). One-way ANOVA followed by *post-hoc* Tukey's test using Prism 5 statistical software (GraphPad Software, Inc., United States) was carried out on the normalized expression data to identify significant expression changes ($p < 0.05$). All values were expressed as mean ± standard error.

RESULTS

In the present study, fathead minnows were exposed to 100 μg/L of NP, BPA, or DEHP or a mixture of three for 21 days to investigate hepatic gene expression changes associated with toxicity induced by these compounds. Based on the results of this study no treatment related mortality was observed. Body weight, gonadosomatic index (GSI) and hepatosomatic index (HIS) also were not significantly different between the treatments.

Gene Expression Profile in the Male FHM Liver Following NP, BPA, DEHP, and Mixture Treatment

There was a total of 980 transcripts differentially expressed by more than 2 folds ($p < 0.0001$) at least by one of these treatments compared to the control. Exposure to NP, BPA, DEHP and their mixture significantly modulated the expression of 316, 335, 381, and 247 target genes in the liver tissue relative to the control fish, respectively, (**Table 2**). Of these transcripts, 156, 195, 124, and 73

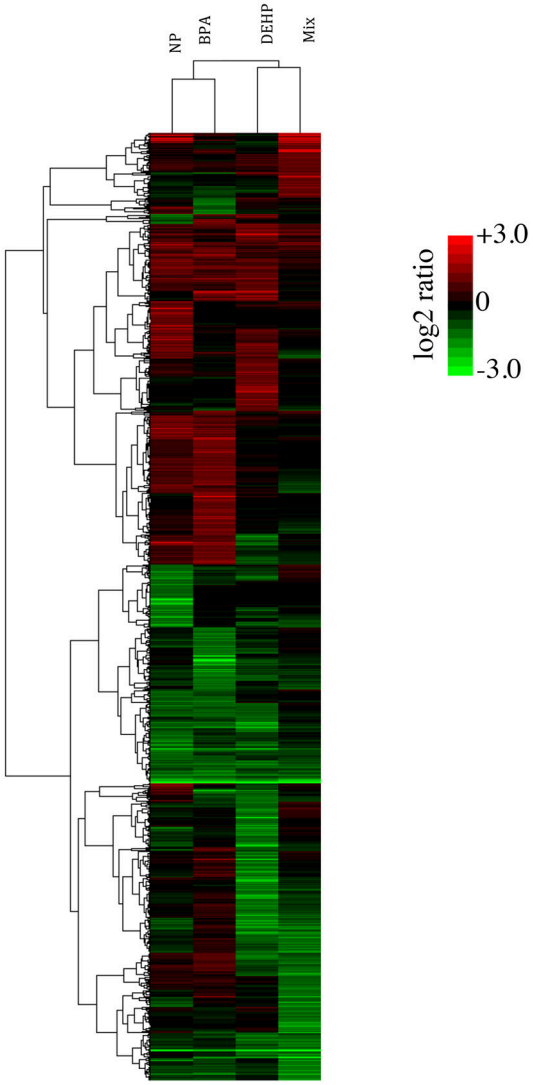

FIGURE 1 | Hierarchical cluster analysis (Pearson correlation) of differentially expressed-genes in the livers of male FHMs following 21 days exposure to 100 ug/L of NP, BPA, DEHP and their tertiary mixture ($p < 0.0001$; fold change > 2). Green color represents down-regulated genes, red color represents up-regulated genes, and black color represents no change in response to EDC treatment relative to the untreated control. Color bar indicates log2 expression ratios of EDC treatment relative to untreated controls.

were up-regulated relative to control while 160, 140, 257, and 174 were down-regulated by each treatment, respectively (**Table 2**).

To compare the global pattern of transcriptional response caused by individual contaminants and the mixture, we performed hierarchical cluster analysis of the 980 transcripts identified as significantly regulated after exposure (**Figure 1**). Each row in the heat map represents a gene, and each column represents a chemical treatment. The cluster analysis demonstrated that NP and BPA differentially-expressed genes clustered together and DEHP clustered with the mixture group. Despite the similarities, there is a clear difference among all four treatments, suggesting that each compound induces a distinct gene expression pattern compared to the mixture.

In **Figure 2**, the entire set of differentially-expressed genes was plotted in order of expression change (log2) for the mixture group and the order of the genes were kept the same for the other treatments. This graph revealed that while the mixture effects was additive for some of the transcripts, the majority of the genes was significantly altered only by the mixture. Also, a few transcripts were differentially expressed in response to individual compounds and appeared unaffected by the mixture. NP, BPA and DEHP only shared 14, 13.7, and 19.1%, respectively, of their differentially-expressed genes with the mixture (**Table 2**). Among the differentially-expressed genes in response to exposure to the mixture, the pattern of up and down-regulated genes was also different compared to the individual compounds.

The Venn diagram in **Figure 3** showed that few differentially-expressed genes were the same among the different EDC treatments. More than 50% of all the differentially expressed genes were uniquely affected by each treatment. There were only 43 transcripts commonly modulated by all three EDC compounds (**Figure 3A**) and among these 43 genes, just 18 of them overlapped with the mixture group (**Figure 3B**). Out of these 18 genes, four genes were up-regulated (**Figure 3C**) and 13 genes were down-regulated (**Figure 3D**) in all four treatment groups, and one gene was down-regulated in NP and BPA treated groups and up-regulate in DEHP and mixture group. Examples of some of these genes commonly down-regulated by all four treatments included Interferon Regulatory Factor 7 (IRF7), Radical S-adenosyl Methionine Domain Containing 2 (RSAD2), Similar to Interferon-inducible Protein IFI56 (IFIT2), and Synaptonemal Complex Protein 1 (SYCP1). In addition, Glutathione S-transferase A1 (GSTA1) is an example of the genes up-regulated in all four treatments. The biological function and cellular processes of these genes included DNA binding, metal ion binding and homeostasis, lipid binding, transport and metabolism, innate immune response, inflammatory response, cell division and differentiation, and carbohydrate metabolism (**Table S1**). Commonly regulated genes by all four treatments may represent candidate biomarkers of toxicity as they have the potential to response to a variety of chemical compounds with distinct mode of actions.

Pathway Analysis

We used IPA to investigate the biological pathways that were affected by exposure to individual compounds compared to the mixture exposure. Some of the most significantly affected pathways by mixture exposure included EIF2 Signaling, LXR/RXR Activation, PPAR/RXR Activation and Acute Phase Response Signaling pathways while PI3K/AKT Signaling and NRF2-mediated Oxidative Stress Response were mostly affected by individual chemicals (**Table 3**). Some examples of the identified upstream regulators included AhR, Hepatocyte growth factor (HGF), Androgen receptor (AR), Tuberous Sclerosis Complex 2 (TSC2), Apolipoprotein A-I (APOA1), Interferon Regulatory Factor 1(IRF1), and Colony Stimulating Factor 2 (CSF2) (**Table 4**). The molecular functions of these upstream regulators are detoxification, growth and metabolism,

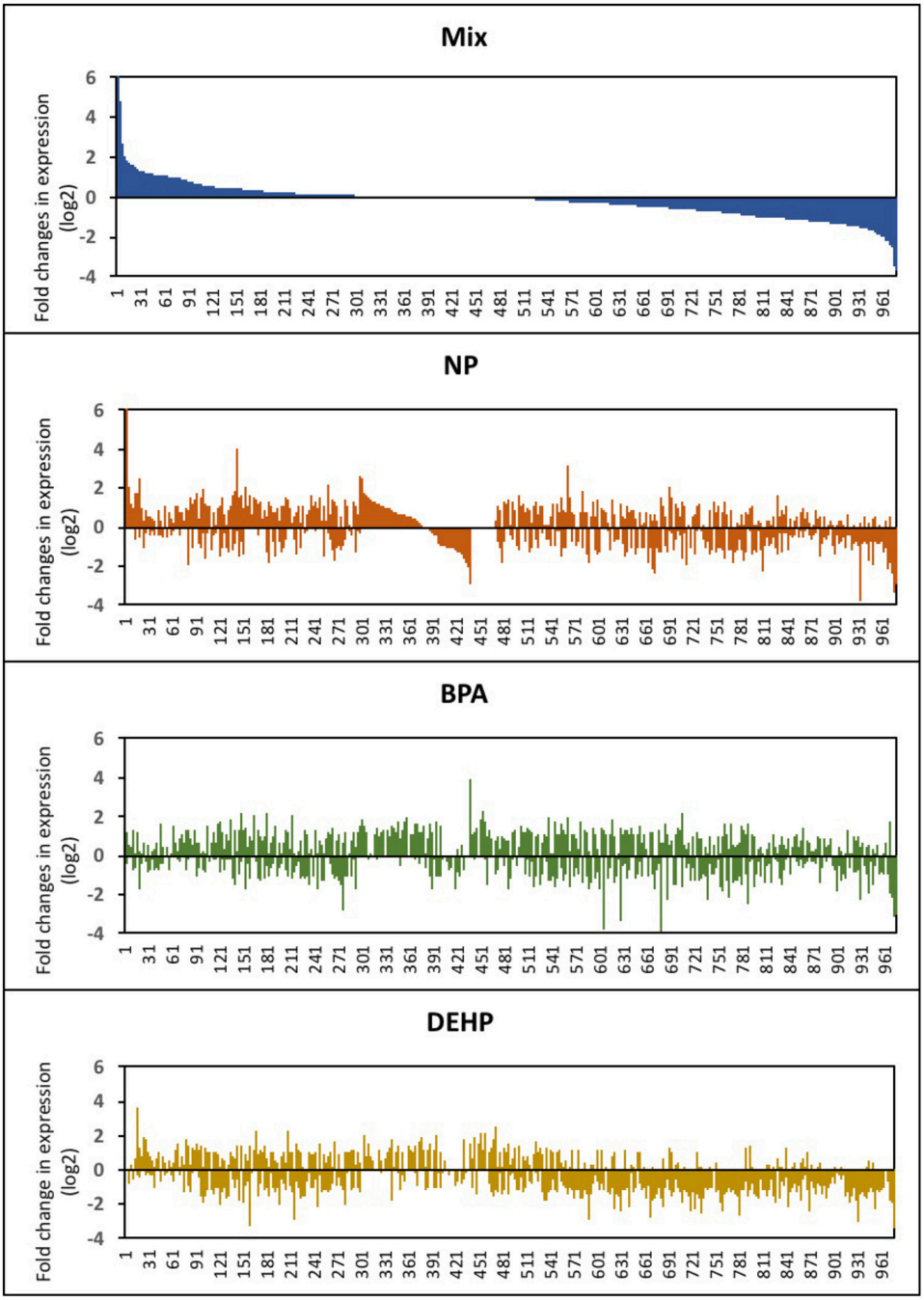

FIGURE 2 | Expression profile of male fathead minnow livers following exposure to NP, BPA, DEHP and the mixture of three compounds. Differentially-expressed genes are shown as the $\log_2$ fold change of expression in EDC-exposed male fish compared to untreated control fish. The gene order in the horizontal axis corresponds to the differential-expressed genes from the EDC mixture is kept consistent for the other three treatments.

reproduction, lipid metabolism, and immune system (**Tables S2–S5**). The potential diseases and biological outcomes that are associated with the observed over represented genes included, liver inflammation, binding and storage of lipids, steroid metabolism, cancer, apoptosis, and viral infection (**Tables S6**).

Quantitative Real-Time PCR

We used qPCR to validate the expression microarray results and also confirm some of the identified biological pathways and predicted upstream regulators that were hypothesized to be affected by these treatments from the pathway analysis. We selected a few candidate genes for qPCR confirmation based on

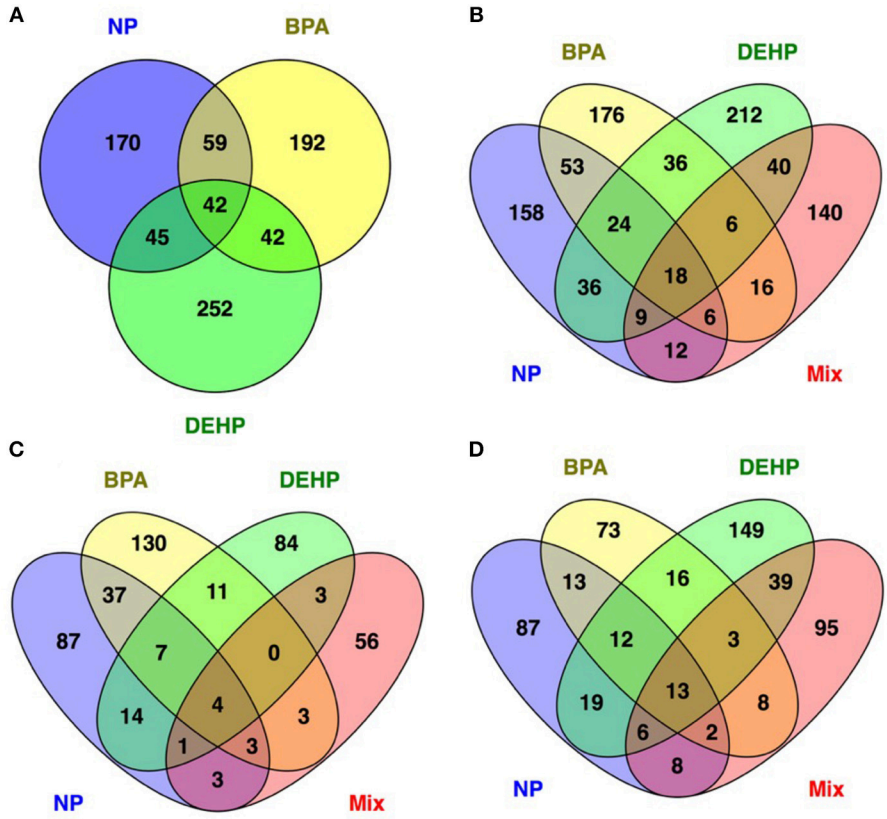

FIGURE 3 | Venn diagram showing the number of unique and common differentially regulated genes in male FHM hepatic tissue affected by NP, BPA and DEHP exposure **(A)** as well as mixture **(B)**. **(C,D)** show the upregulated and down regulated genes respectively. The Venn diagrams reveal 42 genes commonly modulated by individual treatments **(A)** and 18 genes regulated by all three compounds and their mixture **(B)**. Among these 18 genes 4 of them upregulated **(C)** and 13 of them inhibited **(D)** by all four treatments.

TABLE 3 | Canonical pathways and biological processes associated with significantly altered transcripts in fathead minnow liver after 21 days exposure to NP, BPA, DEHP and the mixture of the three chemicals using Ingenuity Pathways Analysis (IPA) and Fisher exact test ($P < 0.05$).

Canonical pathway	Z-Score			
	NP	BPA	DEHP	Mix
Acute phase response signaling	1.807	1.604	−1.807	−1.604
PI3K/AKT Signaling	−2.646	−1.342	−2.646	–
PPAR/RXR Activation	1.134	−1.633	2.121	1.633
Activation of IRF by Cytosolic Pattern Recognition Receptors	−1.342	−1.342	−2.236	−1
LXR/RXR Activation	1.706	−0.943	−1.225	−1.877
EIF2 Signaling	0.277	−1.508	1.387	2.496
IGF-1 Signaling	1.633	1.633	0.816	1.342
NRF2-mediated Oxidative Stress Response	−1.342	0.447	−2.236	1.342
Production of Nitric Oxide and Reactive Oxygen Species in Macrophages	0.535	0.302	−2.324	−1.387
Extrinsic Prothrombin Activation Pathway	0.816	−0.816	−1.342	−1.342
Prolactin Signaling	1.134	1.134	0.378	0.816
Growth Hormone Signaling	1.89	–	1.134	0.378
JAK/Stat Signaling	0.33	1.41	−0.33	1.13
ATM Signaling	2.0	–	1.0	–

A positive or negative Z-score indicates activation or inhibition of the pathway.

TABLE 4 | Predicted master regulators that might be associated with observed dysregulation in the male fathead minnow liver following exposure to NP, BPA, DEHP and the mixture of three compounds for 21 days.

Upstream regulators	Z-Score				Molecule function	
	NP	BPA	DEHP	Mix		
PXR ligandRXR	1.648	2.407	0.713	0.337	Detoxification	Detoxification
AHR	2.832	2.147	1.201	0.858	Adaptive and toxic response	
RXRA	1.047	2.231	−0.929	−0.01	Growth and development	Growth and metabolism
HGF	−1.424	−2.056	−3.094	−3.235	Hepatocyte growth factor, regulates cell growth	
Growth hormone	−0.295	−0.473	−2.173	−2.682	Stimulates growth, cell reproduction	
GHR	1.673	−0.447	2.4	0.946	Stimulates growth, cell reproduction	
PPARG	2.015	3.274	−0.929	−0.468	Metabolism and lipid uptake	
AR	−1.378	−0.684	−2.44	−1.459	Androgen receptor	Reproduction
FIGLA	1.534	2.396	1.534	1.534	Involved in folliculogenesis	
ER	1.691	0.228	0.036	−0.508	Nuclear receptor	
FSH	−0.041	−0.438	−1.616	−1.202	Gonadotropin hormone	
APOA1	–	–	1.94	−0.11	Apolipoprotein A-I	Lipid metabolism
APOA4	−1.29	−1.97	−0.21	−1.96	Apolipoprotein A-4 (equivalent to Vtg in oviparous)	
LDL	0.31	1.54	−1.79	−0.45	Low-density lipoprotein	
LDLR	−0.38	–	1.36	–	Low-density lipoprotein receptor	
ERBB2	−2.138	−2.494	−1.464	−2.11	Tyrosine kinases receptor, involved in breast cancer	
TSC2	2.219	2.219	2.219	−0.555	Involved in Tuberous sclerosis	
SASH1	−2.236	−2.236	−2.236	−2.236	Down-regulates in breast cancer cells	
STAT1	−1.29	−3.025	−1.721	−1.671	Cellular immunity, proliferation, apoptosis	
SP1	1.462	2.292	0.366	−0.623	Including cell differentiation, cell growth, apoptosis, immune responses	
DOCK8	−2.236	−2.236	−2.236	−2.236	Intracellular signaling	
IRF7	−2.506	−2.506	−2.913	−2.913	Immune respons	Immune response
CSF2	−2.182	−2.956	−2.525	−2.543	Immune response	
IL27	−1.725	−1.368	−2.439	−1.932	Immune response	
IFI16	−1.312	−1.373	−1.679	−1.129	Innate immune response	
IL1B	−1.447	−1.377	−1.023	−2.961	Immune response	
IL3	−2.068	−1.385	−2.068	−0.888	Immune response	
IFNAR1	−1.709	−2.432	−2.432	−2.432	Immune response, inhibits viral infection	
IRF1	−2.006	−3.243	−2.293	−2.248	Immune response	
IFNA2	−1.621	−2.283	−2.314	−3.144	Immune response, inhibits viral infection	
STAT	−0.708	−2.398	−0.145	−2.398	Cellular immunity, proliferation, apoptosis	
mir-15	1.741	2.207	1.741	−0.958	MicroRNA precursor, immune response	
Rosiglitazone	0.511	2.418	−2.998	−2.062	Insulin sensitizer, by binding to the PPAR	
Isobutylmethylxanthine	−0.73	2.376	−2.905	−3.132	Reduces inflammation and innate immunity	
Roscovitine	1.546	2.216	1.546	−0.492	Alter the growth phase or state within the cell cycle	Chemicals/drugs
Cadmium	2.88	1.767	−1.334	−1.182		
Bisphenol A	1.009	1.367	−0.174	−0.543		
Dihydrotestosterone	0.859	1.051	−2.135	−1.906	Sex steroid	
4-hydroxytamoxifen	2.578	0.133	1.165	0.209	Selective estrogen receptor modulator	

These regulators were predicted based on the significantly overrepresented genes in the dataset using Ingenuity Pathways Analysis (IPA) and Fisher exact test ($P < 0.05$). A positive or negative Z-score indicates activation or inhibition of the pathway.

their function, microarray expression results, and IPA pathway analysis results, as listed below.

Glutathione S-transferase A1 (GSTA1), Interferon Regulatory Factor 7 (IRF7), Radical S-adenosyl Methionine Domain Containing 2 (RSAD2) and Similar to Interferon-inducible Protein IFI56 (IFIT2) were selected because they were significantly regulated by all the treatments and also involved in detoxification and immune response.

Vitellogenin (Vtg), which is a widely used biomarker of exposure to estrogenic compounds, is an Apolipoprotein and involved in the transport of lipids. Our expression microarray results also indicated that many genes involved in lipid

metabolism and transport were regulated by the EDC exposure. Hence, we selected some regulated genes within this pathway such as Low Density Lipoprotein Receptor-Related Protein 8 (LRP8) and Retinol-binding protein 4 (RBP4) that were detected in the expression microarray data, as well as Low Density Lipoprotein Receptor (LDLR) which was not included in the set of microarray probes.

In order to confirm the predicted regulatory networks and biological functions that were disrupted by EDC exposure, we also quantified the expression levels of some genes involved in AhR, AR and IGF signaling as well as the PPAR/RXR activation pathway. These genes included the Insulin receptor (INSR) and cytochrome p450 27B1 (CYP27B), which were included in the expression microarray data, and cytochrome p450 1A1 (CYP1A1), insulin like growth factor1 (IGF1), gonad aromatase (CYP19a1), androgen receptor (AR), Doublesex and mab-3 related transcription factor1 (DMRT1) and LDLR (in testis) were also selected, but were not included in the set of microarray probes.

Vitellogenin and Lipid Metabolism

The expression microarray results demonstrated that Vtg1 is highly elevated by NP, BPA and the mixture compared to the untreated control (**Table 5**). These results were confirmed by qPCR (**Figure 4**). Microarray results also showed the same trend for Vtg3. DEHP did not have any significant effects on Vtg1 expression. The expression microarray data revealed that individual compounds and the mixture also disrupted a number of genes involved in lipid metabolism (**Table S1**). Some examples included various classes of Apolipoproteins and their binding proteins such as APOC1, APOA1, APOA4, LRP8, LRP1B, LDL-related protein, INSR, and HDLBP. LRP8 was significantly up-regulated by NP in the expression microarray data (**Table 5**) and qPCR (**Figure 4**). Our qPCR results also demonstrated that NP, BPA and DEHP significantly altered RBP4, but however, the expression microarray data showed that NP was the only treatment that up-regulated this gene. NP and DEHP significantly up-regulated LDLR in male liver but none of the treatment had a significant effect on LDLR in testis (**Figure 5B**). INSR showed down-regulation in response to DEHP and the mixture exposure from the expression microarray data (**Table 3**), but it was up-regulated by DEHP from qPCR analysis (**Figure 4**).

Immune Response and Detoxification Pathway

A number of key elements in the immune response such as IFIT2, IRF7, and SRAD were highly inhibited by all of the four treatments from the expression microarray data and were confirmed by qPCR (**Table 5**). CYP27B, another gene with known roles in immune response, was down-regulated by the mixture treatment in both microarray and qPCR analysis. In contrast, GSTA1, which is involved in the detoxification pathway, was significantly elevated by all of the experimental treatments as demonstrated by expression microarray analysis and qPCR data (**Figure 6**)

Represented Genes in Predicted Regulators and Canonical Pathways

IPA pathway analysis predicted the cascades of upstream transcriptional regulators that can explain the observed gene expression pattern. AhR and AR were among the top identified regulators. According to this analysis, the observed dysregulation in our dataset might be the result of the up-regulation of AhR and down-regulation of AR (**Table 4**). qPCR results consistent with the pathway analysis, demonstrated that CYP1A1, a marker for AhR activation, was significantly up-regulated by all four EDC exposures (**Table 6**) while AR expression was significantly down-regulated by BPA, DEHP and the mixture exposure (**Figure 6**). IGF1 signaling was one of the canonical pathways that were significantly affected by chemical exposure (**Table 3**). However, the expression level of IGF1, a key element in this pathway, was not influenced by any of the exposure groups (**Figure 5**). A possible explanation of this observation could be that other proteins and receptors in this pathway might be altered independently of IGF1 in response to EDC exposure as suggested in our expression microarray results.

DISCUSSION

In this study, we investigated the transcriptome changes associated with exposure to BPA, NP, DEHP and their tertiary mixture in the male FHM hepatic tissue. Using expression microarrays enabled us to identify the genes that were differentially regulated by each EDC treatment relative to the untreated control. We used expression microarray data to generate several hypotheses of the molecular mechanisms in response to EDC exposure rather than solely on the quantitative measure of changes in gene expression. In this context, observed variations in the transcriptome following exposure to these EDC contaminants can be used to identify potential candidate biomarkers for toxicity screening and determination of molecular pathways that are affected by these contaminants. The identified biomarkers could be used in toxicity assessments when a population is exposed to a variety of contaminants with a broad or perhaps unknown mechanism of action. More than 50% of the significantly altered genes were unique for each treatment. There is a potential to use some of these genes as a tool for biomonitoring specific contaminants and their potential health impacts.

A number of commonly regulated genes in response to all four EDC treatments such as IRF7, IFIT2, and RSAD were important elements in the inflammatory and immune response. Therefore, dysregulation of these genes in addition to their capacity for use as a molecular sensor of EDC exposure could reflect the impact of contaminants on health by adversely affecting the immune system. In this context, IRF members are central mediators in the regulation of innate immune responses (87). Expression of IRF members including IRF7 elevates in response to pathogens (88). IFIT proteins and RSAD2 are among interferon –stimulating genes that are induced by viral infection to suppress the virus infectivity (89, 90). Several studies showed that knocking down of these immune response genes could result in increased virus

TABLE 5 | Comparison of microarray analysis and qPCR results.

Functional pathway	Gene name	qPCR results				Microarray analysis			
		NP	BPA	DEHP	Mixture	NP	BPA	DEHP	Mixture
Immune response and detoxification	IFIT2	↓	↓	↓	↓	↓	↓	↓	↓
	RSAD2	↓	↓	↓	↓	↓	↓	↓	↓
	IRF7	↓	↓	↓	↓	↓	↓	↓	↓
	GSTA1	↑	↑	↑	↑	↑	↑	↑	↑
	CYP27B1	-	↑	-	↓	-	-	↓	↓
Lipid metabolism	Vtg1	↑	↑	-	↑	↑	↑	-	↑
	LRP8	↑	-	↑	-	↑	-	-	-
	RBP4	↑	↑	↑	↑	↑	-	-	-
	INSR	-	-	↑	-	-	-	↓	↓

Relative changes in gene expression when compared to control group based on ANOVA followed by Tukey Test ($P < 0.05$, $n = 12$) for qPCR and $P < 0.0001$ and > 2 fold changes for microarray. Arrow direction indicates up or down regulation relative to control, bold and thin arrows represent significant and non-significant changes respectively and "-" indicates no significant changes.

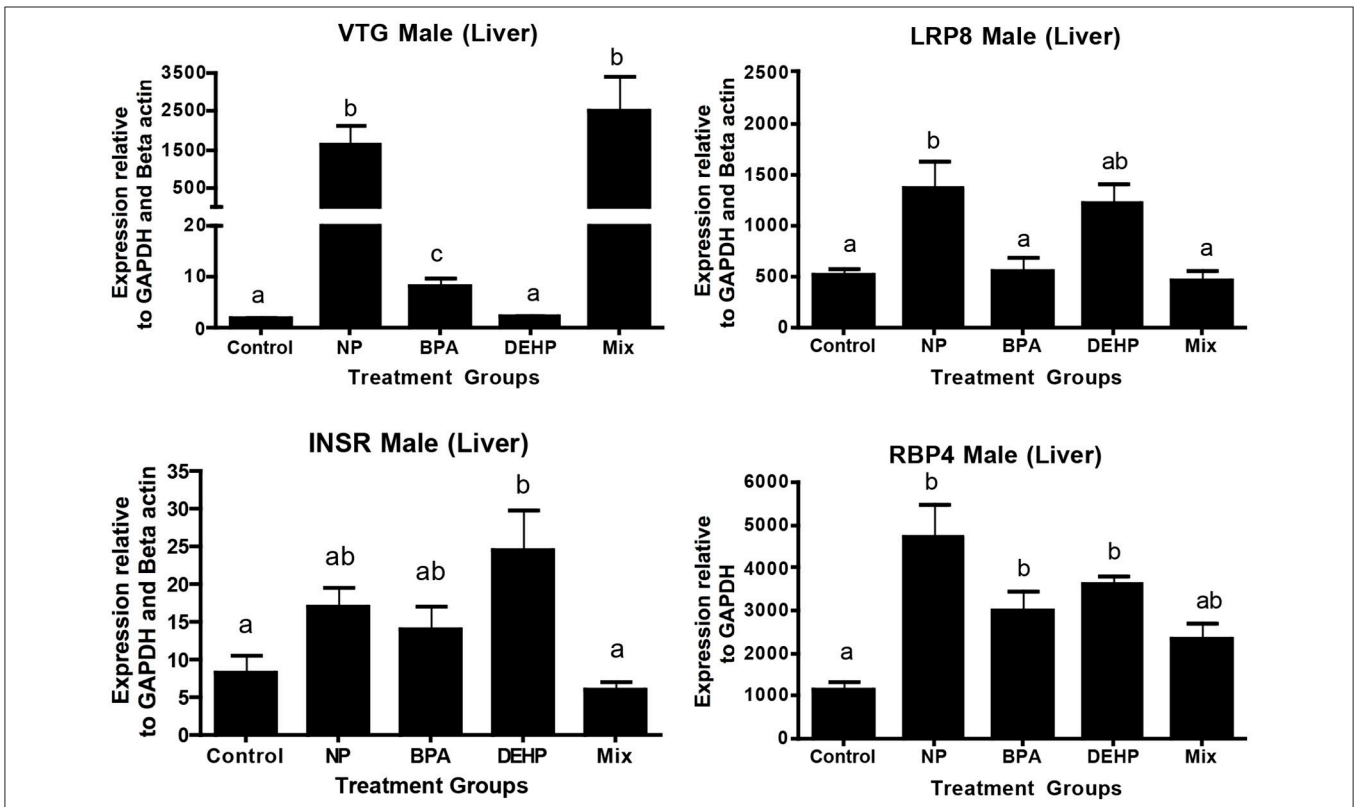

FIGURE 4 | Validation of expression microarray results by quantitative real-time PCR (qPCR). Effects of 100 μg/L of NP, BPA, DEHP and the mixture of three chemicals on genes involved in lipid metabolism and transportation following 21 days exposure. The expression levels were measured using qPCR and normalized against the mean of GAPDH and β-actin (mean ± SEM). Different letters indicate a significant difference (ANOVA followed by Tukey Test, $P < 0.05$, $n = 12$).

population in the body (89) or triggering breast cancer cell metastasis (91). Our findings in this study have demonstrated that IRF7, IFIT2, and RSAD are significantly down-regulated by all four EDC treatments. These results suggest that the EDC exposure may increase the fish susceptibility to pathogens and other stressors.

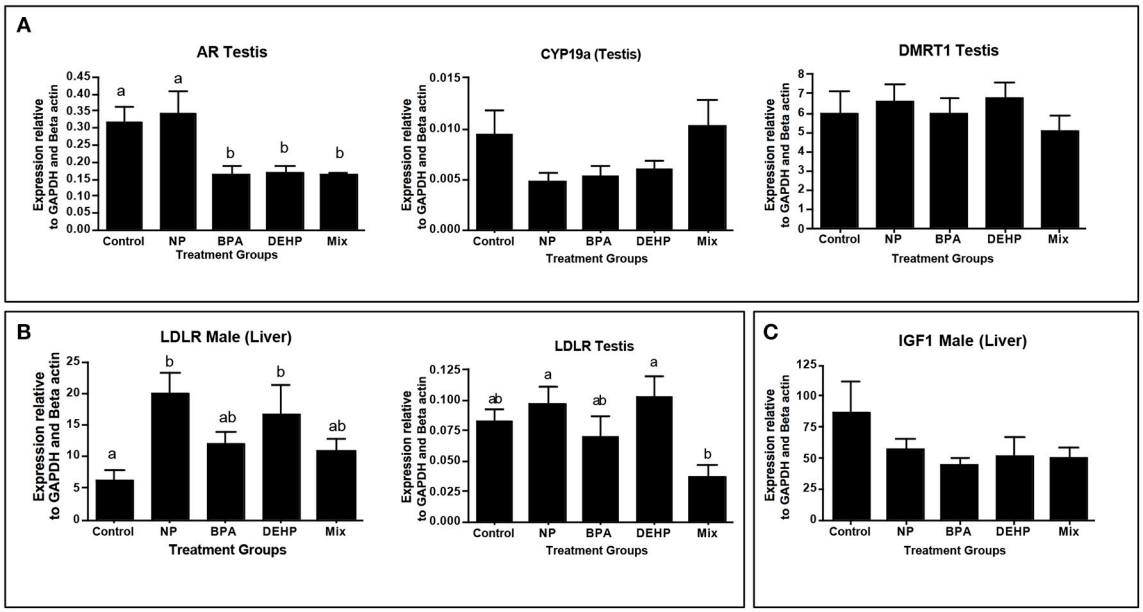

FIGURE 5 | Confirmation of top regulators predicted by ingenuity pathway analysis (IPA) software, using quantitative real-time PCR (qPCR). Top regulators were predicted based on significantly regulated genes relative to control in microarray analysis. Genes were selected based of their function in the altered pathways: Androgen signaling **(A)**, PPAR/RXR Activation **(B)** and IGF1 signaling **(C)**. The expression levels were measured using qPCR and normalized against the mean of GAPDH and β-actin (mean ± SEM). Different letters indicate a significant difference (ANOVA followed by Tukey Test, $P < 0.05$, $n = 12$).

TABLE 6 | Comparison of top regulators predicted by IPA (fisher exact test $p < 0.05$) and gene expression of representative genes of the pathway using qPCR (ANOVA followed by Tukey Test, $P < 0.05$, $n = 12$).

Predicted top regulators	Direction of pathway regulation (predicted by IPA)				Real-time PCR results				
	NP	BPA	DEHP	Mixture	Represented genes in the pathway	NP	BPA	DEHP	Mixture
AhR	↑	↑	↑	-	CYP1A1(liver)	↑	↑	↑	↑
AR	↓	-	↓	↓	AR (testis)		↓	↓	↓
					DMRT1 (testis)	-	-	-	-
					Cyp19a1 (testis)	-	-	-	-

Arrow direction indicates up or down regulation relative to control, bold and thin arrows represent significant and non-significant changes respectively and "-" indicates no significant changes.

There is increasing evidence for the impacts of EDCs on the immune response (3, 92, 93). Our results are consistent with the reports that various class of environmental contaminants including BPA cause acute inflammatory response in zebrafish larvae by modulation of neutrophils and gene transcription, suggesting immunotoxicity (94). High concentrations of BPA were found to increase proliferation of a subset of immune cells including splenocytes, lymphocytes, thymocytes and B cells in goldfish or mice [Reviewed in (3)]. BPA could also exert negative impacts on the regulation of immune-associated genes in medaka (95) and juvenile common carp (*Cyprinus carpio*) (93). NP regulates various elements of the innate immune response in the Pacific oyster that was induced by bacterial infection (96). Exposure to 17alpha-ethylinestadiol (EE2) affected a large number of pathways associated with immune system in fathead minnow (97). In addition, AhR controls the expression of a number of genes involved in the immune response [Reviewed in (98)]. Therefore, the EDCs tested in this study may also act on the immune system via AhR.

Both innate and adaptive immune systems play a critical role in modulation of metabolic health. Exposure to environmental contaminants such as EDCs has been associated with a variety of physiological alterations, including reproductive impairment, metabolism dysregulation and immune dysfunction [Reviewed in (99)]. In our study, pathway analysis identified at least 10 signaling pathways that were altered by these treatments. These included pathways implicated in the Acute Phase Response, PI3K/AKT, EIF2 and IGF-1 signaling, PPAR/RXR activation, LXR/RXR activation, and NRF2-mediated oxidative stress response.

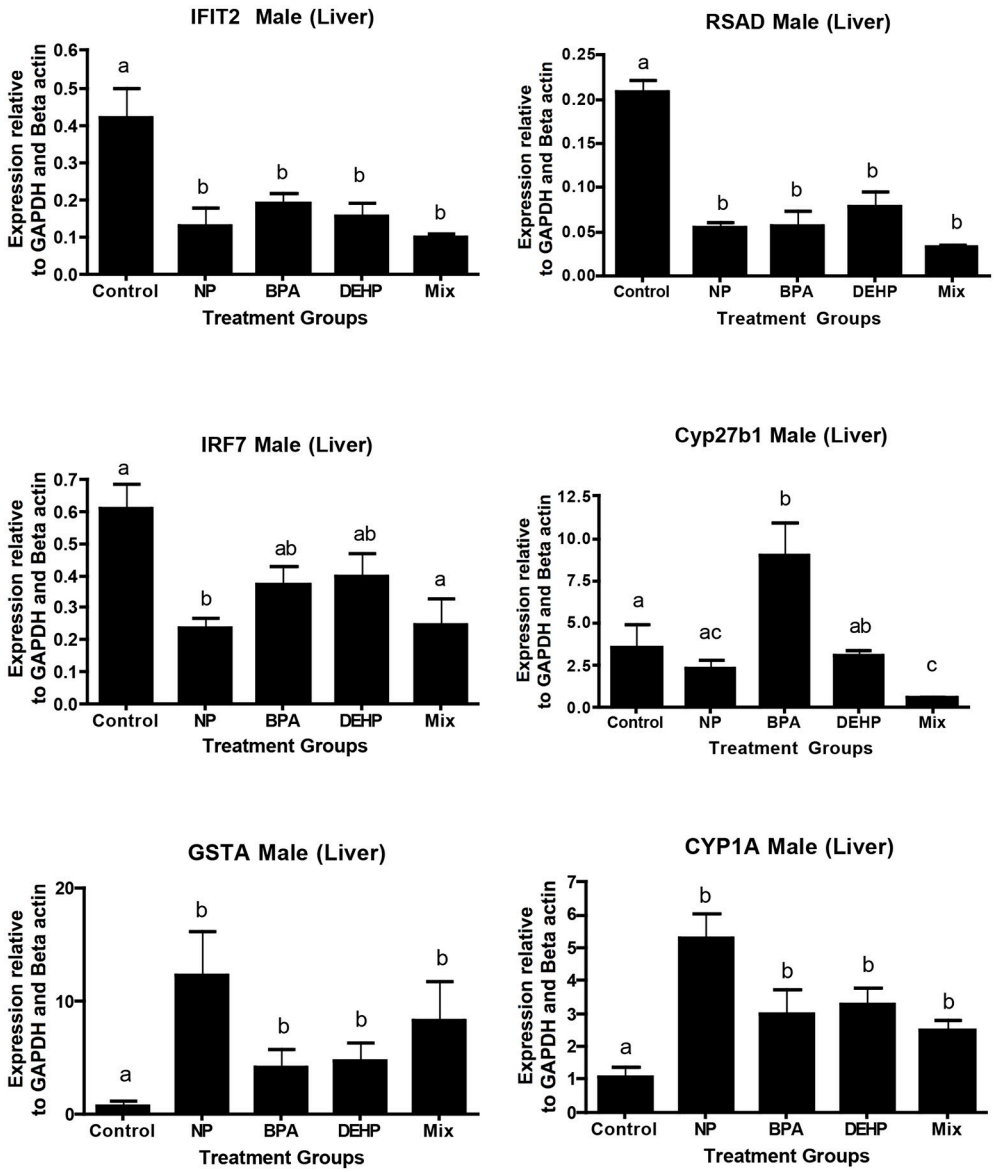

FIGURE 6 | Validation of microarray results by quantitative real-time PCR (qPCR). Effects of 100 μg/L of NP, BPA, DEHP and the mixture of three chemicals on genes involved in the immune response and detoxification following 21 days exposure. The expression levels were measured using qPCR and normalized against the mean of GAPDH and β-actin (mean ± SEM). Different letters indicate a significant difference (ANOVA followed by Tukey Test, $P < 0.05$, $n = 12$).

Fathead minnows that were treated to municipal wastewater effluent (MWWE) for 21 days showed disruption in the pathways correlated with a variety of physiological alterations, including reproductive and immune dysfunction, cell adhesion, inflammation, estrogen receptor signaling and WNT signaling (100). In our study, GSTA1 was also altered by all four EDC treatments. It has been demonstrated that GSTs are upregulated in many fish species exposed to oil and hydrocarbons (101, 102).

The PPAR family of nuclear receptors has been associated with a number of inflammatory disease states. PPARγ agonists have been shown to suppress the expression of proinflammatory cytokines in the spinal cord and BPA may alter the regulatory/anti-inflammatory axis through interaction with PPARs [Reviewed in (3)]. Exposure to weakly estrogenic effluent reduced immune system activity such as reductions in the number of circulating lymphocytes, but increased nonspecific immune activation in fathead minnow (103).

Lipid molecules through interactions with a large family of nuclear receptors regulate signaling cascades involved in development, cellular differentiation, and metabolism. PPARs exert the majority of their effects as heterodimers with the retinoid x receptor (RXR) and are some of the key regulators of lipid and carbohydrate metabolism that also

regulate inflammatory responses [Reviewed in (104)]. In African sharptooth catfish (*Clarias gariepinus*), exposure to DEHP led to dysregulation of PPAR gene expression and protein levels which consequently affects lipid homeostasis (105). Expression of genes related to lipid accumulation, oxidative stress and proteolysis were altered in female rare minnow (*Gobiocypris rarus*) following exposed to different doses of BPA in a non-monotonic pattern (38).

Exposure to BPA in zebrafish resulted in dysregulation of fatty acid synthesis pathway, leading to the production of cholesterol esters and conversion to lipid droplets (106). Dietary administration of NP, *t*-OP or BPA individually and in a mixture altered the expression of genes involved in lipid metabolism resulting in hepatic steatosis in juvenile gilt-head sea bream (*Sparus aurata*) (107, 108). In adult male gilt-head sea bream dietary exposure to BPA elevated the amounts of lipids and triglycerides in the liver and dysregulated the levels of endocannabinoids (EC) (109). Male burbot fish (Lota lota) from the lakes contaminated with POP (persistent organic pollutant) showed that FXR/RXR and LXR/RXR activation, and the NRF2-mediated oxidative stress response were the most significantly affected pathways. The authors also discovered that differentially-regulated genes were significantly involved in lipid and vitamin A metabolism (110). PPAR, a nuclear receptor involved in the regulation of lipid homeostasis, was also identified as upstream key elements involved in the response to POP. Thus, exposure to EDC contaminants can potentially exert adverse health impact by producing harmful compounds in the body through activation of inflammation, (oxidative) stress, lipid peroxidation, and other natural processes (111).

The presence of complex chemical mixtures in the environment and our limited knowledge on mechanisms of toxicity and the interaction of chemicals in a mixture are the main challenges for design methodology guidelines to evaluate the potential ecological and human health risk assessment (64, 112). Several mathematical based models have been designed to predict the toxicity effects of EDC mixtures [Reviewed in (52, 113)]. However, the toxicokinetic interactions of mixture components, and their physiological and metabolism pathways that are altered by each compound in a mixture and also their adverse outcome at the level of the biological organization often have been neglected in these studies (113). In the present study, the gene expression pattern of the mixture-exposed FHM was found to be different from individually exposed chemicals. Exposure to a mixture of BPA, DEHP and NP led to an integrated stress response by inhibiting the Acute Phase Response Signaling and activating the EIF2 Signaling, which are common adaptive mechanisms (114). This stress response was detected in a greater extent in the EDC mixture compared to the individual exposures. While we acknowledge that the mixture in this study is not completely representative of the environmental mixture, the information obtained in this study is important for the overall understanding of toxicokinetic interactions of contaminants when they are mixed.

With an increase in emerging chemicals to the environment, there is a growing demand for ecological risk assessors to evaluate each chemical and in combination more accurately in a shorter time while using less resources and animals (115). Advances in biological science and the applications of Omics technologies along with advances in computational analytical tools may provide valuable information on mechanistic pathways that link molecular events to adverse outcomes at the ecological level. Transcriptomics combined with computational pathway analysis could provide some useful information on the key events at the cellular or organ level which facilitates predictive outcome effects in organisms or population (59, 68, 115–117). The Omics approach could characterize the biochemical pathways targeted by the contaminants and the common regulators perturbed by the chemical exposure which can be subsequently applied to ecotoxicology research (66).

Some molecular initiating events caused by particular classes of chemicals and their adverse outcomes are well-documented. For instant, the chemicals that activate the xenobiotic-metabolizing enzymes through binding to AhR, can result in reproductive impairment, cancer, perturbation in immune response, and early-life-stage mortality [Reviewed in (118)]. Estrogen receptor (ER) agonists are another example where binding of an estrogen-mimicking chemical to the ER resulted in changes in expression of estrogen-responsive genes, alterations in plasma sex steroid and vitellogenin concentrations at the cellular level, and gonadal abnormalities (such as intersex) at the organ level. These key events lead to changes in secondary sex characteristics and reproductive behavior and consequently may influence exposed populations (115). However, further study on time and dose dependent effects of EDC's is required to fill the gap between traditional toxicity biomarkers (reproduction and growth) and transcriptome data. It is necessary to obtain basic knowledge about biological systems and the molecular mechanisms by which chemicals disturb them, to improve the ability to predict the potential adverse effects of compounds and to identify the compounds that caused observed effects in the environment (115, 119). Network analysis makes it possible to identify the adverse outcome pathways (AOPs) and to predict the mechanisms underlying chemical toxicity and its link to human diseases and endocrine disorders (116).

Useful molecular biomarkers to evaluate biological responses to environmental contaminants need to be robust, reliable, and repeatable in various laboratories. Using transcriptome datasets generated from six independent laboratories, which exposed male fathead minnows to 17alpha-ethylinestadiol (EE2), it was demonstrated that EE2 regulates processes related to lipid metabolism and peroxidation, innate immune response, and IGF signaling in the liver of male fathead minnow (120). Results of our study also revealed these pathways were altered in response to chemical exposure. However, the 18 commonly regulated transcripts shown in our study are not among the identified estrogen-responsive transcripts that were found in the EE2 exposed fish. This is not surprising considering the chemical property of our experimental compounds. BPA and NP are traditionally considered as estrogen mimicking compounds and DEHP is known as an androgen receptor antagonist. It should be noted that these compounds are not only estrogenic and are known to interact with different types of hormone receptors and consequently alter distinct signaling pathways, when compared

to EE2. In this regard, overlap of specificity for BPA and DEHP were shown in a number of previous studies (23, 49).

Using GSEA (gene set enrichment analysis)-based tools makes it possible to use non-human model organisms to make a potential connection between environmental conditions that lead to differentially-expressed gene sets and human disorders. This approach evokes hypotheses regarding circumstances that lead to potential onset of human diseases and dysregulation of biological processes. Also, comparing the gene expression profile of the model organism after exposure to a contaminant to a human gene set involved in a particular pathway and function, can make it possible to predict the adverse impacts of a given treatment to humans (69).

In summary, our results demonstrate that exposure to BPA, DEHP, NP and a mixture of all these EDCs can trigger distinct gene expression patterns in fathead minnow. Despite the differences, all these treatments regulate many key elements in the immune response pathway that might result in increasing susceptibility of fish to pathogens and other stressors. Novel canonical pathways and causal networks were also identified in response to each of the contaminants as well as the mixture. Our results provide information about the molecular mechanisms of the chemicals in a mixture compared to the individual exposure. The EDC mixture inhibited Acute Phase Response Signaling and activated the EIF2 Signaling, which are common adaptive pathways functioning in the integrated stress response (114). This stress response was not observed in the individual treatments. We also investigated a number of relevant genes affected by all the EDC treatments, and the expression of most of these genes detected by expression microarrays were validated by qPCR. We propose that these genes could potentially act as biomarker candidates in screening for the presence and biological effects of these contaminants.

Using gene set enrichment analysis of transcriptome data allowed the use of a non-human model organisms to make a potential connection between environmental conditions that lead to differentially-expressed gene expression and human disorders. Network analysis made it possible to predict the potential mechanisms underlying chemical toxicity and its potential link to human diseases and disorders caused by contaminants.

AUTHOR CONTRIBUTIONS

AZ and HH designed research. AZ performed research. AZ, DH, and PG analyzed data. GC provided intellectual input on microarray analysis. AZ and HH wrote the paper. HH provided funding, oversight, intellectual input on experimental design and data analysis.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2018.00749/full#supplementary-material

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