COMPARATIVE ENDOCRINE STRESS RESPONSES IN VERTEBRATES

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COMPARATIVE ENDOCRINE STRESS RESPONSES IN VERTEBRATES

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EDITORIAL
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Editorial: Comparative Endocrine Stress Responses in Vertebrates

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Keywords: editorial, stress, responses, endocrine, vertebrates

Editorial on the Research Topic

Comparative Endocrine Stress Responses in Vertebrates

The stress response in vertebrates is characterized by involving physiological regulatory systems and a number of organs, tissues, and effector pathways in order to both respond to the stressor effects and overcome the situation and recover homeostasis. Although differences in specific mechanisms are encountered in different animal groups and even between interspecies and intraspecies, the stress response involves an endocrine activation in all groups of vertebrates. Since an increasing number of scientific works are currently published in this field, the idea of collecting and reviewing such advances originated the initiative of a Topic Collection on the Comparative Stress responses in Vertebrates. In the following set of papers, the readers will find out an interesting update of the latest work in the field of the endocrine responses to stress in vertebrates, from general approaches to specific contributions and methodological updates.Of course, it is not intended that this *Topic* collection be exhaustive or complete, as many untreated aspects could be added, and some vertebrate groups are not well-represented into the collection, but this overview is anyway interesting to show what are some of the current working areas in this field.

Among the general approaches presented in this Topic collection, a novel contribution is the concept of *stressotope* by Balasch and Tort, linking the adaptive set of responses of the animal to particular biotopes associated with specific conditioning factors involving the maximum overall stress responses across immune-neuroendocrine relevant physiological levels and scenarios, including the characterization of behavioral response.

In relation with this stressotope concept, the work by Sánchez-Vázquez et al., deep into the relationship among factors regulating the circadian rhythms in animals particularly under stress situations, showing that not only a number of specific environmental factors are connected to circadian rhythms, but also that the proper oscillations of the environmental factors are significantly involved.

In another of the works in this *Topic*, Gómez-Boronat et al., investigating in daily cycles, demonstrate that the misalignment of external cues such as day-night photocycles and feeding time may temporarily alter fish homeostasis, thus involving a stress situation for the animals.

Other contributions make relevant insights in the comparative approach of endocrine stress regulation in vertebrates. In the first one, Narayan and Vanderneut provided invaluable insights into how wild koalas respond physiologically to environmental trauma and disease, a species not often represented in the scientific literature of comparative stress responses. In addition, this paper includes an applied aspect on how methods of care, husbandry, and treatment can be used to reduce the impacts of stressors with the ultimate aim of increasing the rehabilitation possibilities and future release of this species in the wild.

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In another paper, Höglund et al. show the role of Tryptophan and the associated metabolic pathways in the regulation of serotonergic activity in the fish brain, a key mechanism intimately associated to stress and behavioral responses of animals. Numerous studies have shown that elevated dietary Trp has a suppressive effect on aggressive behavior and post-stress plasma cortisol concentrations in vertebrates. These effects are believed to be mediated by the brain serotonergic system, even though mechanisms involved are not well-understood.

Also regarding key components in the diet, the work by Herrera et al., have looked at the studies on stress attenuation in animals through diet or supplement components. Other than the development of new technologies to monitor and improve environmental conditions of farmed animals, particularly fish, beneficial additives in the daily meal have been included in order to mitigate the effects of husbandry stressors. Immunological, nutritional, and metabolic changes have been assessed in these trials, always associated to endocrine regulation. The biochemical and physiological functionality of those feed additives may strongly affect the stress response and, even, such additives may act as neurotransmitters, hormone precursors, energy substrates, or cofactors implying multi-systematic and multi-organic responses that modify the response to stress.

Suarez-Bregua et al. focused their approach in a less studied area in lower vertebrates as the endocrine relationship between glucocorticoid metabolism and the parathyroid hormone family peptides. The paper deeps into the response driven by these hormones and other key regulators of mineral homeostasis in connection with bone remodeling processes, which involves important consequences in terms of harmonic growth and skeletal deformities.

A more specific comparative work on the stress and endocrine responses is presented by a group of researchers from Greece, Norway, and The Netherlands. Thus, Samaras et al. focus on the differential responses between two close warm water marine aquacultured species, sea bass, and sea bream. In this paper, they show how significant can be the species-specific molecular and neuro-regional differences between two similar species sharing many environmental and geographical conditions. This points out how important can be the variability of specific mechanisms between species, even from close-related groups, though sharing the basic patterns of molecular and endocrine molecules and pathways.

Finally, regarding key methodological contributions, the paper by Aerts rises the importance of the methodological aspects associated to the molecules currently chosen to define a stressed status. He demonstrates that it is pivotal to know the involved regulatory molecules and to understand how these molecules are synthesized, regulated, and excreted, together with how these molecules grasp their actions on a plethora of biological processes in many organs and tissues.

Collectively, the *Topic* highlights current research areas and future directions in the dynamic field of vertebrate stress endocrinology. Beyond theoretical knowledge, the field of research provides powerful tools to enable researchers to make objective assessments of the physiological state of animals, to understand how animals respond to environmental change and human interventions.

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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ORIGINAL RESEARCH
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Allostatic Load and Stress Physiology in European Seabass (*Dicentrarchus labrax* L.) and Gilthead Seabream (*Sparus aurata* L.)

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The present study aimed to compare effects of increasing chronic stress load on the stress response of European seabass (*Dicentrarchus labrax*) and gilthead seabream (*Sparus aurata*) to identify neuroendocrine functions that regulate this response. Fish were left undisturbed (controls) or exposed to three levels of chronic stress for 3 weeks and then subjected to an acute stress test (ACT). Chronic stress impeded growth and decreased feed consumption in seabass, not in seabream. In seabass basal cortisol levels are high and increase with stress load; the response to a subsequent ACT decreases with increasing (earlier) load. Basal cortisol levels in seabream increase with the stress load, whereas the ACT induced a similar response in all groups. In seabass and seabream plasma α-MSH levels and brain stem serotonergic activity and turnover were similar and not affected by chronic stress. Species-specific molecular neuro-regional differences were seen. *In-situ* hybridization analysis of the early immediate gene *cfos* in the preoptic area showed ACT-activation in seabream; in seabass the expression level was not affected by ACT and seems constitutively high. In seabream, expression levels of telencephalic *crf, crfbp*, *gr1*, and *mr* were downregulated; the seabass hypothalamic preoptic area showed increased expression of *crf* and *gr1*, and decreased expression of *mr*, and this increased the *gr1/mr* ratio considerably. We substantiate species-specific physiological differences to stress coping between seabream and seabass at an endocrine and neuroendocrine molecular level. Seabass appear less resilient to stress, which we conclude from high basal activities of stress-related parameters and poor, or absent, responses to ACT. This comparative study reveals important aquaculture, husbandry, and welfare implications for the rearing of these species.

Keywords: allostasis, aquaculture, cortisol, CRF, repeated stress, serotonin

INTRODUCTION

The concept of allostasis, which states that animals "achieve constancy through change" [adjusting set points of regulatory loops to prevailing needs; (1, 2)] is gaining popularity in fish stress physiology. Allostasis involves synthesis of prior knowledge with predicted current needs and resetting of one or more physiological set points accordingly. A successful stress response involves the reorganization of the organism's energy budget, their immune system, as well as neural and endocrine mechanisms to successfully cope with a given stressor. The stress response then results in a timely return to pre-stress conditions, and restoration of homeostasis so-called eustress (3). If the response fails, or is inadequate, allostatic overload will occur. This is usually seen under chronic stress conditions when individuals are no longer able to successfully cope with continued stress challenge (4). The term "allostatic load" is used to describe the capacity of an organism to cope with a certain challenge by acclimating its behavior and physiology. Stress responses are meant to be compensatory and adaptive, to allow the animal to overcome the threat; when the animal succeeds in this we refer to stress as eustress. However, when an animal is facing an intense or chronic stress, the stress response might lose its adaptive significance, become dysfunctional and ultimately result in adverse effects such as inhibition of growth, failure to reproduce, and impeded resistance to pathogens. This condition is called distress (3, 5–7).

The stress response in fish (in fact in any vertebrate) is initiated by activating the hypothalamic–sympathetic axis followed by the activation of the hypothalamus–pituitary gland–interrenal gland (HPI) axis. The former results in the release of adrenaline and noradrenaline to quickly induce hyperglycemia and fuel fight or flight (3, 8). However, due to the rapid release and clearing of catecholamines from the circulation [seconds to minutes; (9)] it is difficult to obtain accurate data on the resting levels of adrenaline and noradrenaline, and for that reason these parameters are not commonly assayed. The endocrine stress steroid axis (HPI-axis) will subsequently produce (hyperglycemic) cortisol to guarantee energy for coping with the new conditions and counteract changes in energy budgeting induced by the stressor. Indeed, corticotrophin-releasing factor (CRF) is secreted from the preoptic area [POA; (10–13)]. The axons of CRF-producing cells project directly to *pars distalis* ACTH cells (12). CRF is released there and will then bind the CRF-receptors (CRF_1R) located on the ACTH cells (12). This process is believed to be modulated by CRF-binding-protein (CRF-BP), which binds CRF and therefore reduces its bioavailability (12, 14, 15). Hypothalamic CRF neurons also project to the pituitary *pars intermedia* and induce release of α-melanophore-stimulating hormone (α-MSH) (16, 17); in particular, increased constitutive release under conditions of chronic stress (18, 19) may act as corticotrope, lipolytic or anorexigenic signal (3).

ACTH acts via a specific melanocortin receptor type 2 (MC2R), expressed exclusively on interrenal cells in the head kidney of fish (16, 20); this receptor acts as a dimer and is associated with four melanocortin receptor associated proteins [MRAPs; (21–23)]. MC2R activates pathways that result in synthesis of cortisol from cholesterol and subsequent secretion to the bloodstream (24). The mechanisms regulating its expression are not yet fully described, but in seabass it seems that exogenous cortisol administration can exert negative feedback on *mc2r* gene expression (20). Cortisol, the single steroid produced by interrenal cells in fish, signals in target tissues *via* either a mineralocorticoid or several glucocorticoid receptors (MR and GRs, respectively). Once cortisol is bound, these transcription factors bind specific DNA sequences (GR- and MR-responsive elements) in target-gene promoters and control mineralocorticoid and glucocorticoid activities as required to cope with imposed challenges (8, 25, 26).

The aim of the present study was to study the neuroendocrine regulation of European seabass (*Dicentrarchus labrax* L.) and gilthead seabream (*Sparus aurata* L.) upon exposure to different intensities and types of chronic stress. These species constitute the largest portion (approximately 90–95%) of the Mediterranean aquaculture production, and have high economic and societal value. They, however, show often enigmatic differences in their physiology (27), especially the responsiveness and susceptibility to stress (28) and react differently to an acute stressor, when previously exposed to chronic (crowding) stress (29–33). Moreover, seabream seems more resilient than seabass in terms of growth under stress (31, 32, 34). Based on that and to study the effects of different stress loads on the response and identify key neuroendocrine features that regulate these differences between these species, seabass and seabream were exposed to increasing levels of repeated stress episodes combining common aquaculture stressors, such as confinement, chasing and air-exposure (as a model for chronic stress) for 3 weeks and were then subjected to an acute stress test [ACT; (35)]. Fish were sampled for "baseline values" and 1-h post-stress to assess interrenal steroid production capacity. The general performance of fish (food intake and growth) was monitored over the experiment; levels of plasma cortisol and α-MSH were quantified at the end of the experiment. *In-situ* hybridization of the immediate early gene *cfos* was carried out to give anatomical resolution in gene activity; then expression of a set of key target genes in the telencephalon and preoptic area was analyzed.

MATERIALS AND METHODS

Animals

Hatchery produced seabass (14-months-old) and seabream (12-months-old) were provided by the Institute of Marine Biology, Biotechnology and Aquaculture of the Hellenic Centre from Marine Research (HCMR) and Forkys S.A. (Sitia, Greece), respectively. In total 160 seabass of 28.69 ± 4.04 cm (mean ± SD) fork length and 380 ± 83.1 g body mass and 160 seabream with 25.05 ± 1.14 cm fork length and 322 ± 54.8 g body mass were used. Fish were kept at HCMR in Gournes, Crete, Greece.

Abbreviations: 5-HIAA, 5-hydroxyindoleacetic acid; 5-HT, 5-hydroxytryptamin; α-MSH, *alpha*-melanocyte-stimulation hormone; *crf*, corticotropin-releasing factor; *crf-bp*, corticotropin-releasing factor binding protein; *gr1*, glucocorticoid receptor 1; HPI axis, Hypothalamus–Pituitary–Interrenal axis; *mc2r*, melanocortin receptor type 2; *mr*, mineralocorticoid receptor; MRAPs, melanocortin receptor associated proteins; POA, preoptic area; ACT, acute stress test.

Duplicate groups of fish were divided according to body weight over eight cylindrical 500-L tanks with flow-through filtered seawater at a final stocking density of 16.2 ± 0.2 kg m^{-3} for seabass and 14.8 ± 0.3 kg m^{-3} for seabream. The fish were then left to acclimatize for 3 weeks before the start of the experiment. The water temperature was kept at 19°C and the photoperiod was set at 12L:12D. Fish were fed *ad libitum* during the experiment and the quantity of the food consumed was measured daily per tank (by collecting uneaten pellets within 1 h after feeding). The feed used consisted of 44% protein and 19% lipids (Irida S.A., Greece).

Experimental Design

The experimental treatment consisted of exposing seabass and seabream groups to three different chronic stress regimes, varying in intensity, over a period of 21 (seabass) or 24 (seabream) days (**Table 1**). The experiments were conducted in July 2013 for seabass and October 2013 for seabream. The stressors used were chosen in a way that they reflect common aquaculture practices and have been previously shown to elicit stress responses in both species. Specifically, these stressors were confinement (30, 36, 37), confinement and chasing (38, 39) and a combination of confinement, chasing and air-exposure (28, 40) (**Table 1**). In detail, the low stress regime consisted of subjecting fish to a confinement stressor for 30 min every 2nd day; this was accomplished by lowering a net into the tank to decrease the available space to 50% (doubling the density) while keeping a constant water volume and similar water quality. The medium stress regime consisted of subjecting fish to both confinement (conducted as previously described) and chasing of the fish for 5 min with a net every 2nd day. The high stress regime consisted of confinement (to only 25% of the tank volume) for 30 min, chasing for 5 min every 2nd day, and air exposure for 1 min once per week. These stressors were applied to the fish between 10.00 and 12.30 h.

Two days after the end of the chronic stress treatments 10 out of 20 fish per tank were immediately sampled (referred to as T0 fish) after netting and deep anesthesia with 0.5% (v/v) 2-phenoxyethanol. Blood was drawn via heparinized syringes, centrifuged (2,000 × *g* for 10 min) and the plasma stored at −80°C until further analysis. The spinal cord was cut to kill the fish and telencephalic, preoptic area and brainstem samples were collected, snap-frozen in liquid N_2, and stored at −80°C. The 10 remaining fish were acutely stressed by subjecting them to a net chase for 5 min and then air-exposure for 1 min. The fish were then left undisturbed for 1 h [when the peak cortisol response after stress is observed; (28, 37, 40–42)] and deeply anesthetized before sampling (T1 fish), as explained above.

TABLE 1 | Stress applied to seabass and seabream for three different stress loads.

			Stress load		
Stressor	**Time (min)**	**Frequency**	**Low**	**Medium**	**High**
Confinement*	30	Every 2 days	√	√	√
Chasing	5	Every 2 days		√	√
Air exposure	1	Every 7 days			√

Confinement and chasing were performed once every 2 days; air-exposure was performed once a week.

**Confinement in the Low and Medium stress groups was performed by restraining the fish to 50% of the initial water volume, for the High stress group to 25% of the volume.*

The laboratories of the Hellenic Centre for Marine Research are certified and have obtained the codes for breeding and husbandry of animals for scientific purposes (EL 91-BIO-03, EL 91-BIO-04). All procedures involving the handling and treatment of fish were approved by the HCMR Institutional Animal care and use committee in accordance to Greek (PD 56/2013) and EU (Directive 63/2010) legislation on the care and use of experimental animals following the principles of refinement, replacement and reduction in animal experimentation.

Plasma Analysis

Plasma cortisol levels were determined by radioimmunoassay, according to Gorissen et al. (43). Plasma α-MSH levels were evaluated by radioimmunoassay using the L9 α-MSH antibody (44). The antiserum shows 100% cross-reactivity with des-, mono-, and di-acetyl α-MSH. Tracer α-MSH-peptide was labeled with ^{125}I through the iodogen method (45).

Brainstem 5-HT Neurochemistry

Frozen brain stems were homogenized in 4% (w/v) ice-cold perchloric acid (PCA) containing 0.2% EDTA and 40 ng ml^{-1} epinine (deoxyepinephrine as an internal standard) with a Potter–Elvehjem homogenizer. After centrifuging samples for 5 min at 15,493 rcf, the supernatant was analyzed by high-performance liquid chromatography (HPLC). The mobile phase was; 12 μmol L^{-1} EDTA, 86 mmol L^{-1} sodium phosphate and 1.4 mmol L^{-1} sodium octyl sulfate in deionized water (resistance 18.2 MΩ cm^{-1}), containing 7% acetonitrile; pH was set to 3.1 with phosphoric acid. The system consisted of a solvent delivery system (Shimadzu, LC-10AD, Kyoto, Japan), an auto-injector (Famos, Spark), a reverse phase column (4.6 × 100 mm, H0ichrom, C18, 3.5 mm) and an ESA Coulochem II detector (ESA, Bedford, MA, USA) with two electrodes at −40 and +320 mV. A conditioning electrode with a potential of +40 mV was used to oxidize possible contaminants before analysis. Brain stem concentrations of 5-HT and the 5-HT metabolite 5-Hydroxyindoleacetic acid (5-HIAA) were quantified by comparison with standard solutions of known concentrations and corrected for recovery of the internal standard using HPLC software (CSW, DataApex Ltd, Prague, the Czech Republic). The 5-HT turnover was quantified by ratio of 5-HIAA/5-HT.

RNA Isolation

Brain tissue was dissected into telencephalon and preoptic area using a stereo microscope, as *per* Madaro et al. (35, 46). Tissues were homogenized in TRIzol reagent (Gibco BRL) according to manufacturer's instructions. RNA concentration and purity were determined by measuring absorbance at 260 and 280 nm with Nanodrop® ND-1000 UV–Vis spectrophotometry (Peqlab, Erlangen, Germany).

Synthesis of cDNA

Synthesis of cDNA was performed as *per* Madaro et al. (35, 46). RNA (100–500 ng) was reverse-transcribed by a series of incubations: 10 min at 25°C, followed by 50 min at 42°C and 15 min at 70°C; cDNAs were then diluted five times and stored at −20°C until further analysis.

Real-Time Quantitative PCR

Oligonucleotides used in the qPCR analysis are shown in **Table 2**. To each diluted cDNA sample, 16 μl of a mix containing: 10 μl iQ™ SYBR® Green Supermix (2x) (Bio-Rad, Hercules, CA, USA), 0.7 μl (10 μM) primer forward, 0.7 μl (10 μM) primer reverse, 4.6 μl DEPC H2O was added. The amplification protocol was carried out on a CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA) and consisted of 3 min at 95°C, followed by 40 cycles of amplification (95°C for 15 s and 60°C for 1 min). A melting curve was generated for each sample to assess specificity of the PCR products.

In-situ Hybridization

For *in-situ* hybridization fish were sampled directly from their holding tank (at basal conditions, $n = 2$/species) and 1 h post-stress conditions (chasing for 5 min and air exposure for 1 min, $n = 2$/species). All fish were quickly and deeply anesthetized with 1% (v/v) phenoxyethanol and fixed by vascular perfusion with 4% PF in 0.1 M Sørensen's phosphate buffer (PB; 28 mM NaH_2PO_4, 71 mM Na_2HPO_4, pH 7.2). Dissected brains were post-fixed in the same fixative for 16 h at 4°C. The tissue was washed three times 20 min in PB, cryopreserved overnight in 25% sucrose in PB at 4°C, embedded in Tissue-Tek OCT-Compound (Sakura Fintek) and stored at −80°C until sectioning.

Adjacent transverse 12 μm sections were cut with a Leica CM 1850 cryostat (Leica Microsystems, Wetzlar, Germany), collected on SuperFrost Ultra Plus glasses (Menzel Glaser, Braunschweig, Germany) and dried at 65°C for 10 min. Digoxigenin-labeled riboprobes were prepared with a digoxigenin (DIG)-RNA labeling mix following the manufacturer's instructions (Roche Diagnostics, Mannheim, Germany). The *cfos* ISH probes for seabream and seabass were 542 and 467 nucleotides long, respectively. Forward GGCTCGAGTTCATTCTCGCT and reverse GTCGTTGCTGTTGCTTCCTC and forward TCTGGGATGGTGGTCTGTGA and reverse CCAGCCTTTGATCTCCTCGG primers were used to clone the *cfos* probe primers in seabream and seabass, respectively. The quality and quantity of the synthesized riboprobes were assessed by agarose gel electrophoresis. Pretreatment and treatment of sample for ISH was conducted as specified earlier (48). The reaction with chromogen substrate (3.4 μl of nitroblue-tetrazolium, 3.5 μl of 5-bromo-4-chloro-3-indoylphosphate (Roche Diagnostics, Indianapolis, IN, USA) and 0.24 mg/ml levamisole in visualization buffer) was carried out for 3–24 h in darkness at room temperature (samples were routinely checked to avoid overstaining). The reaction was terminated with stop solution (10 mM Tris-HCl, 1 mM EDTA, 150 mM NaCl, pH 8.0) and tissue was mounted in ProLong Gold (Invitrogen, Carlsbad, CA, USA). Photomicrographs were taken by a digital camera (Leica DFC 320, Leica 350 FX) attached to a Leica DM 6000B microscope using the LEICA APPLICATION SUITE, version 3.0.0 image acquisition and processing software.

Statistical Analysis

For plasma analyses and gene expression data, normal distribution of data was tested with the D'Agostino and Pearson omnibus normality test. Cortisol data were analyzed using linear regression. Other plasma analyses were assessed by two-way ANOVA, gene expression data and brain mono-amine data were tested using one-way ANOVA. Significance of effects were subsequently determined by Tukey's *post-hoc* tests or unpaired Student's *t*-testing, where appropriate (α-level was adjusted for multiple comparisons). For all statistical tests $P < 0.05$ was taken as the fiducial limit, unless otherwise stated (in case of multiple comparisons). All statistical analyses were

TABLE 2 | Primer sequences used in RT-qPCR for seabass and seabream.

	Gene	Forward primer 5′ to 3′	Reverse primer 5′ to 3′	Accession no
Seabass (*D. labrax*)	*crf*	CGCTACGAATGTCGGGCTAT	GGGAGTTTTGGGTTTGGGGA	JF274994
	gr1	TCAGTGGCTTGCTCAAGGAG	GGGCTTCTGCTGGTGAGAAT	AY549305
	mr	CCTGTCTCCTCTATGAATGG	AATCTGGTAATGGAATGAATGTC	JF824641
	elf1α	CAAGGAGGGCAATGCCAGT	GAGCGAAGGTGACGACCAT	AJ866727
	rpl17	TTGAAGACAACGCAGGAGTCA	CAGCGCATTCTTTTGCCACT	AF139590
	pomca	CAGAGACACCGATCATCCCG	TCTTCAGGGAAAACCTCGGC	AY691808
Seabream (*S. aurata*)	*crf*	CGCTACGAATGTCGGGCTAT	GGGAGTTTTGGGTTTGGGGA	KC195964
	crf-bp	GATTTCGTGCAGCTGTTGGG	CAGCCGATCTTCATGTGGGT	KC195965
	gr1	AGTGCTCCTGGCTCTTCCTA	GCTTCATCCGCTCCTCGTT	DQ486890
	mr	CGCCTGGCTGGAAAGCAGATG	GAGGTCAGGGGCAAAGTAGAGCAT	(47)
	elf1α	TGGTGATGCTGCCATTGTC	AGCCACTGTCTGCCTCAT	AF184170
	fau	AGCCCAACTCTGCCATCA	AATCCTGCCACCAGAACCT	(47)
	pomca1	CCGCTGCTCACGCTCTTC	GGCTGCTCGTCTTCTGTCTCT	(47)

The sequence for seabass crf-bp was not available at the time of experimentation.

performed with GraphPad Prism 7.0 (GraphPad Software Inc., La Jolla, CA, USA).

RESULTS

Chronic Stress, Growth, and Food Intake in Seabass and Seabream

In seabass growth decreased with increasing stress intensity, not in seabream (**Figures 1A,B**). For feed consumption, there was a significant interaction for seabass between stress and time [$F_{(6, 95)} = 2.36$; $P = 0.037$], with higher feed consumption in controls compared to stressed groups in the 2nd and 3rd week of the experiment (**Figure 1C**). In seabream, no differences in feed consumption were observed among any of the groups [$F_{(3, 143)} = 0.45$; $P = 0.717$] (**Figure 1D**).

Plasma Cortisol, Stress Load and Acute Stress Response

Regression analysis of seabass plasma cortisol showed a significant effect of stress load on basal cortisol levels [$F_{(1, 75)} = 27.03$; $P < 0.0001$; $R^2 = 0.2649$; **Figure 2A**], as well as a significant effect of the ACT [$F_{(1, 76)} = 44.61$; $P < 0.0001$; $R^2 = 0.3699$; **Figure 2B**]. Basal cortisol levels increased with increasing stress load, whereas plasma cortisol after the ACT decreased with increasing stress load. For seabream a significant regression between stress load and plasma cortisol was found for basal cortisol only [$F_{(1, 77)} = 8.86$; $P = 0.0039$; $R^2 = 0.1032$; **Figure 2C**], not for plasma cortisol after the ACT [$F_{(1, 76)} = 3.55$; $P = 0.0634$; $R^2 = 0.04463$; **Figure 2D**]. There were significant interactions between chronic and acute stress in both species [$F_{(3, 147)} = 29.27$; $P < 0.0001$ for seabass, and $F_{(3, 149)} = 3.37$; $P = 0.0178$ for seabream].

Plasma α-MSH Levels and Chronic Stress

In both species no effect of chronic stress treatments on basal plasma α-MSH was observed (data not shown), nor was any interaction effect found between chronic and acute stressors. Values varied around 270 pM for seabass and 250 pM for seabream.

Monoamines in the Brain

In both species no effect of chronic stress on brain stem monoamine content was observed (data not shown). 5-HT turnover (as quantified by 5-HIAA/5HT ratio's) ranged between 0.40 and 0.50 for seabream and 0.25 and 0.30 for seabass.

In-situ Hybridization of *cfos*

There were species-specific differences in the *cfos* mRNA abundance in the preoptic area, particularly at basal levels. That is, while no labeling of *cfos* mRNA was seen in seabream samples, in seabass high mRNA abundance was found in the preoptic area. This suggests activation of the POA at basal conditions in dependence of degree of stress load. Notably, *cfos* abundance increased in seabream and remained high in seabass post-stress (**Figures 3A,B**).

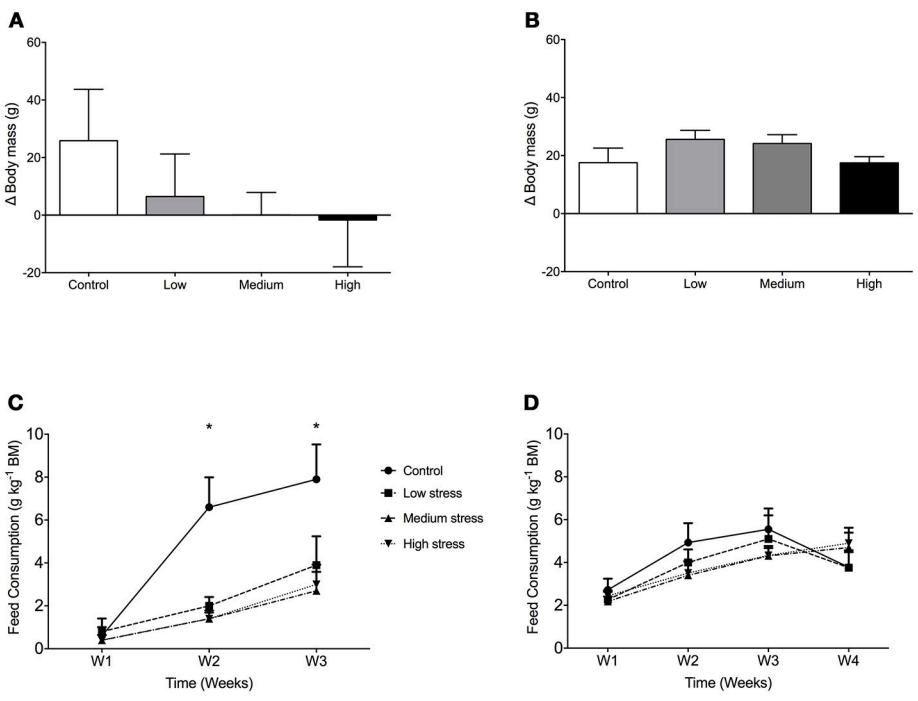

FIGURE 1 | Growth and daily food consumption in sea bass and seabream. Body mass gain in seabass **(A)** and seabream **(B)** in controls and after exposure to low, medium or high chronic stress (mean + 1 SD; $N = 2$; $n = 40$). Daily food consumption of seabass **(C)** and seabream **(D)**, expressed as gram dry food per kg of fish. Two-way ANOVA showed significant differences between control and the rest of the groups in seabass (*$P < 0.05$).

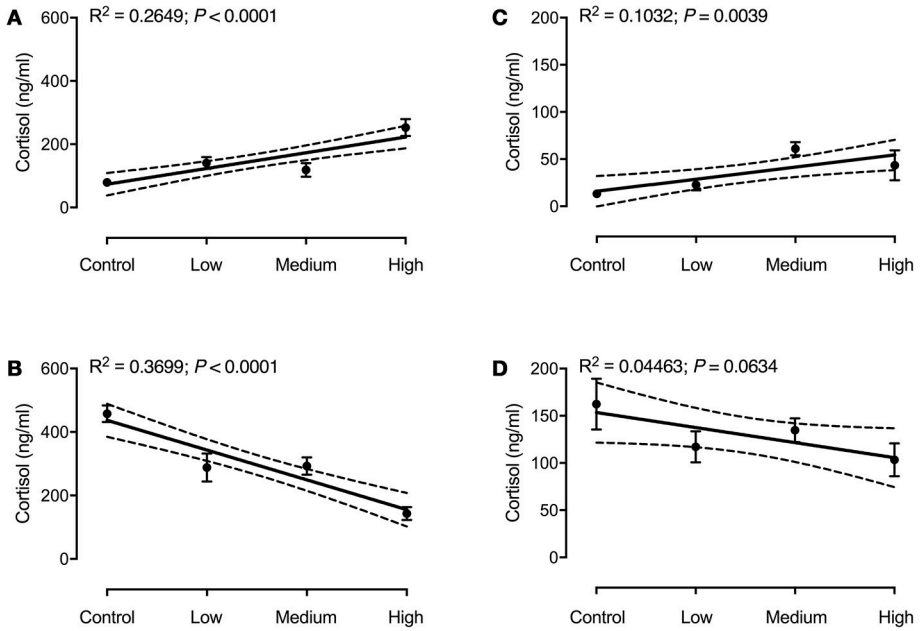

FIGURE 2 | Plasma cortisol in seabass **(A,B)** and seabream **(C,D)** in controls and after exposure to chronic stress, before **(A,C)** and after an acute stress test **(B,D)**. Data are expressed as mean ± SEM ($N = 2$; $n = 20$). Linear regression (solid line) results are shown in each panel. The 95% confidence interval is shown as dashed lines.

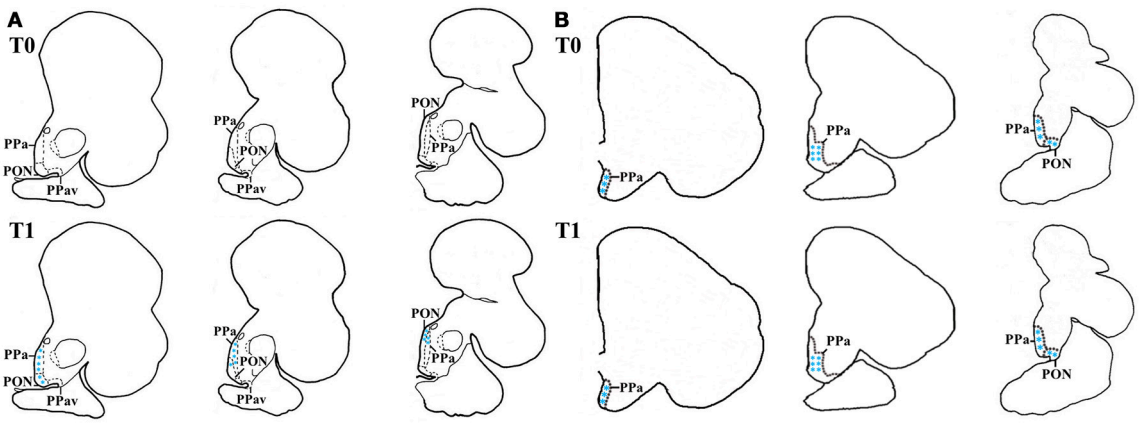

FIGURE 3 | *In-situ* hybridization of *cfos* in seabass and seabream brain before (T0) and after acute stress (T1). Schematic representation of transverse brain sections containing the POA in seabream **(A)** and seabass **(B)** illustrating *cfos* mRNA transcript abundance before (T0) and after acute stress (T1). The blue stars represent labeled cells within each area.

Gene Expression in POA

In seabass, the *gr1* and *mr* expressions had increased and decreased, respectively, in the high stress group compared to all other groups [*gr1*: $F_{(3,\ 68)} = 16.50$; $P < 0.0001$, *mr*: $F_{(3,\ 68)} = 25.94$; $P < 0.0001$; **Figures 4A,C**]. Consequently, the *gr1*/*mr* ratio was significantly higher in the high stress group compared to all other groups [$F_{(3,\ 68)} = 47.60$; $P < 0.0001$; **Figure 4E**]. In seabream no significant differences were found in the expression of *gr1* and *mr* (**Figures 4B,D**) or in the *gr1*/*mr* ratios (**Figure 4F**).

In seabass POA *crf* expression was affected by the intensity of chronic stress [$F_{(3,\ 68)} = 8.974$; $P < 0.0001$]. In this species the expression of *crf* was higher in the high stress compared to the control and medium stress groups (**Figure 5A**). In seabream, no significant differences in *crf* and *crf-bp* expression were evident between groups (**Figures 5B,C**). No primer sequence for *crf-bp* in seabass was available at the time of these studies.

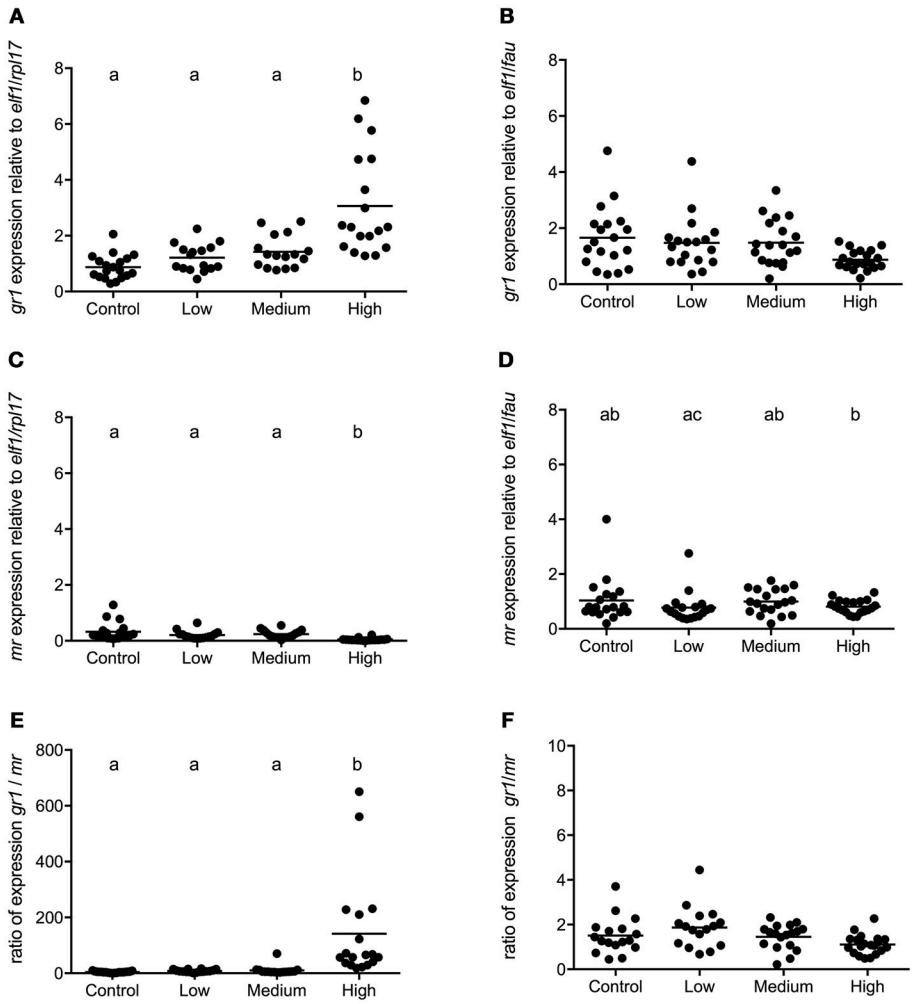

FIGURE 4 | *gr1* and *mr* gene expression in POA of seabass and seabream. Expression of *gr1*, *mr* and *gr1/mr* ratio in seabass **(A,C,E)** and in seabream **(B,D,F)** for control fish and for groups previously subjected to chronic stress. Data are shown for individual fish; the black lines indicate the mean ($N = 2$; $n = 20$). One-way ANOVA showed a significant effect of chronic stress; different letters indicate significant differences between groups ($P < 0.05$).

A significant correlation between *gr1* and *crf* (Spearman $r = 0.570$; $P < 0.0001$) was found for seabass, for all experimental groups. For seabream there was no significant correlation between these parameters (Spearman $r = -0.1076$; $P = 0.379$).

Gene Expression in Pituitary Gland

In seabass low, medium, and high levels of chronic stress decreased transcript abundance of *pomca* [$F_{(3, 57)} = 5.434$; $P = 0.002$; **Figure 6A**]. In seabream no significant effect of chronic stress on *pomca1* expression was observed [$F_{(3, 68)} = 1.574$; $P = 0.20$; **Figure 6B**].

Gene Expression in Telencephalon

In seabass a high degree of variation in telencephalic gene expression was observed and chronic stress further increased this variation. No statistical differences existed in the expression of *crf* and unlike the pattern in POA, the *gr1/mr* ratio was not affected by chronic stress load (**Figures 7A,C**).

In seabream, telencephalic *crf* mRNA levels were significantly lower in the highly stressed seabream compared to all others [$F_{(3, 72)} = 5.03$; $P = 0.0033$] while the *gr1/mr* ratio had decreased as stress load increased (**Figures 7B,D**).

DISCUSSION

Insight in fish stress handling is crucial to guarantee welfare and product quality in aquaculture and fisheries (49). In the present study, we compared two fish species with great relevance to Mediterranean aquaculture that differ widely in their life history and stress handling capacities.

It is well known that stress is energy consuming, leads to decreased food consumption and thus growth in fish (50–52). Indeed, the seabass decreased their feed consumption due to chronic stress. Moreover, body mass decreased with

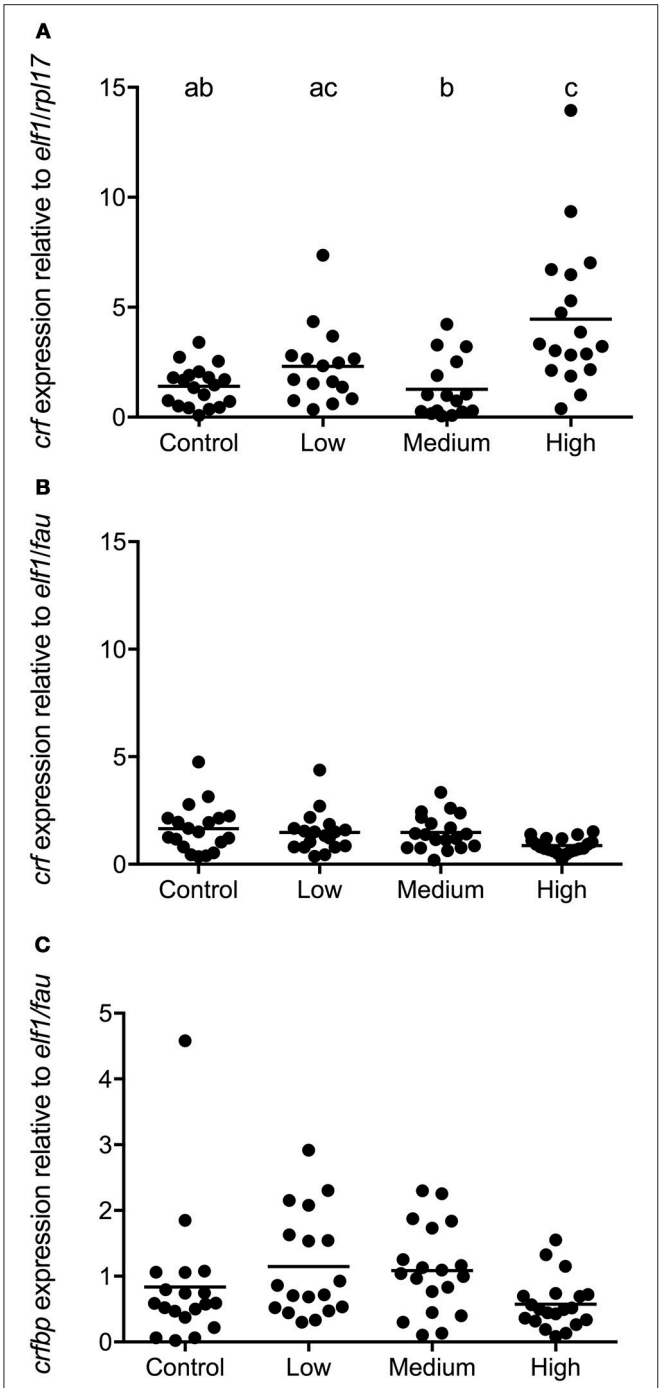

FIGURE 5 | *crf* and *crf-bp* gene expression in POA of seabass and seabream. Expression of *crf* in seabass **(A)** and in seabream **(B)**, as well as *crf-bp* in seabream **(C)**, for control fish and for groups previously subjected to chronic stress. Data are shown for individual fish; the black lines indicate the mean ($N = 2$; $n = 20$). One-way ANOVA showed a significant effect of chronic stress; different letters indicate significant differences between groups ($P < 0.05$).

increasing stress load. Both feed consumption and growth were unaffected by a similar stress imposed on seabream. From these observations, we conclude that the stress load in this study was significant but not extreme and that seabream apparently are more resilient.

In general, it is believed that reduced feeding intake induced by stress is regulated by a combination of behavioral and physiological adaptations to stress (53). These adaptations alter energy expenditure allocation (50, 51), which may in turn lead to growth reduction. Seabass individuals are sensitive to common aquaculture practices such as tank cleaning, which can lead to reduced feed intake for up to 3 days (54), and a significant reduction in growth (51, 55). On the contrary, seabream seems to be more resilient to stress, and did not show differences in growth between control and daily-stressed fish (32). Taken together our data confirm earlier reports showing lower resilience of seabass to stress compared to seabream.

Cortisol in fish combines glucocorticoid and mineralocorticoid actions, by redistributing energy away from growth and reproduction toward survival mechanisms including regulation of hydromineral balance (8, 56, 57). Therefore, high and persistent elevated concentrations of circulating cortisol can affect a wide range of metabolic, immune and reproductive functions (8, 25).

It is shown here that seabass subjected to increasing intensity of (chronic) stress mildly elevate basal plasma cortisol levels (range: 50–200 ng/ml; **Figure 2**) compared to controls; remarkably, basal levels of cortisol in seabass are remarkably high compared to the generally accepted "non-stress" level seen in most fish (up to 20 ng/ml). Seabass is in general characterized by high cortisol values and variation (27, 28, 39, 58, 59), and the current results point out that chronic stress can further increase these high (basal) cortisol levels.

The decreasing response in seabass to the ACT with increasing stress load history indicates that cortisol production capacity is impeded when the stressor persists, the interrenal tissue becomes exhausted (46, 60, 61). In other words, the stress intensity in severely stressed fish exceeded their coping ability (62, 63), the stress given presented an allostatic overload (3). Indeed, repetitive common handling stress on this species, such as tank cleaning (51) or exposure to high-density stress (30, 31) cause changes in circulating cortisol levels. It seems therefore that the intensity and type of the (chronic) stressor and the sum of stressors imposed (e.g., handling, suboptimal water quality and light conditions) need consideration in defining their effects on cortisol response and stress regulation in seabass. In this respect, small-scale laboratory experiments such as presented here are highly informative in aquaculture policy making.

Contrary to what was observed in seabass, in seabream no significant differences were observed in cortisol levels between chronically stressed groups at basal conditions, and all groups responded with increased cortisol to acute stress, and we take this to indicate a healthy physiological functioning of the HPI axis in this species and strong capability to handle stress. The outcome of this comparative study makes us confident that the stress imposed reflects (presumed) realistic conditions.

In our studies we did not bisect the pituitary gland into pars distalis and pars intermedia, we did not isolate ACTH- or MSH-cells, and therefore *pomc* expression levels shown could reflect both ACTH and MSH activities. Only, in seabass we found an

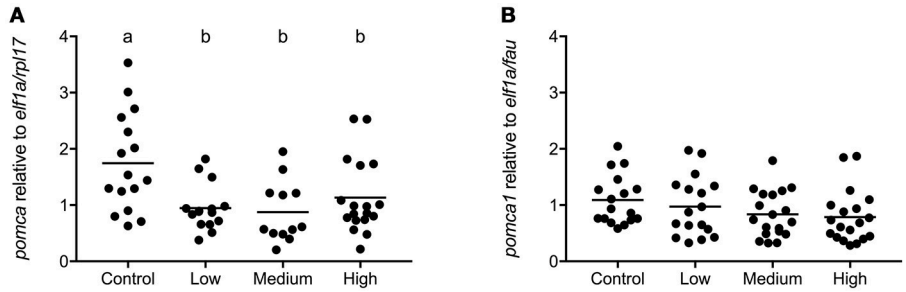

FIGURE 6 | *pomca* gene expression in pituitary gland of seabass and seabream. Expression of *pomca* in seabass **(A)** and in seabream **(B)**, for control fish and for groups previously subjected to chronic stress. Data are shown for individual fish; the black lines indicate the mean ($N = 2$; $n = 20$). One-way ANOVA showed a significant effect of chronic stress in seabass only; different letters indicate significant differences between groups ($P < 0.05$).

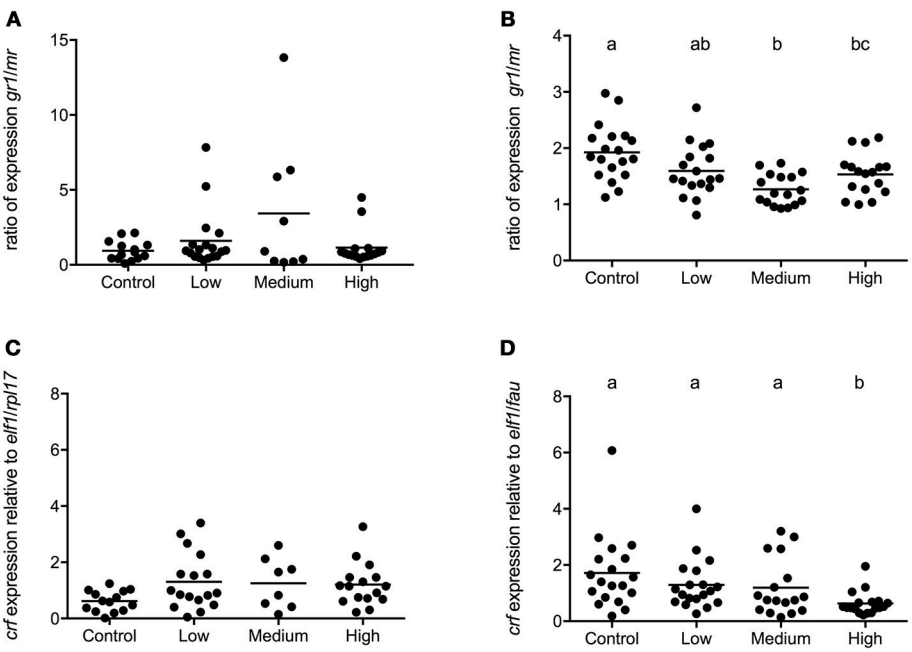

FIGURE 7 | Gene expression in telencephalon of seabass and seabream. Expression of *gr1/mr* **(A)** and *crf* **(C)** in seabass and *gr1/mr* **(B)** and *crf* **(D)** in seabream for controls and for groups previously subjected to chronic- low, medium, and high stress. Data are shown for individual fish; the black lines indicate the mean ($N = 2$; $n = 20$). One-way ANOVA showed a significant effect of chronic stress; different letters indicate significant differences between groups ($P < 0.05$).

inhibitory effect of stress on *pomc* expression, while preoptic *crf* expression was unaffected (low and medium stress) or up-regulated (high stress); so *pomc* expression had increased either to replenish POMC-derived protein stores or an as yet unknown short feedback loop affects the pituitary gland under stress in this fish.

Plasma α-MSH in some species may serve as modulator of the stress response (40, 64), and particularly under chronic stress conditions α-MSH may act as (mild) corticotrope (8, 18), lipolytic, or anorexigenic signal (3). At present little is known about *plasma* α-MSH actions on brain functioning in relation to feeding; The option of plasma MSH as signal to brain (stem) centers [α-MSH is a cyclic molecule which may easily and passively pass the blood brain barrier; (65)] involved in feeding control requires further studies. It has been reported for Mozambique tilapia (*Oreochromis mossambicus*) that plasma α-MSH is only regulated under chronic stress conditions, but not after an acute stressor (66). In their studies on seabream, Arends and colleagues air-exposed naïve fish for 3 min and reported a very high peak in cortisol level (1,400 vs. 414 nM in this study after an ACT). These high cortisol levels correlated with elevated MSH-levels (which we did not observe in the present study) from which then was concluded that air-exposure has a major effect on catecholaminergic pathways as ACTH was not into play (40). Major differences in experimental design (e.g., 1 vs. 3 min air-exposure, chasing before air-exposure, pre-conditioning to different stress levels) may make the difference in outcome between these two studies. Importantly, habituation

of the catecholaminergic response induced by the chronic stress application cannot be excluded. Indeed, in our experiment fish responded to acute stress with an increase in plasma cortisol, not in α-MSH levels. Possibly, acetylation of α-MSH (independent from total levels of α-MSH) is affected by chronic stress. The corticotropic activity of α-MSH in Mozambique tilapia increases with increasing degree of acetylation (des-, mono- di-acetyl α-MSH) (18) and a shift in α-MSH species (apart from total levels) could result in a differential contribution of α-MSH to cortisol production. However, these aspects were not analyzed in the present research. The consequence of acetylation of the POMC-derived peptides MSH and endorphin(s) is differential: MSH may become more biopotent, endorphins become inactivated by acetylation (18, 66). Is it the protection against the powerful actions of endorphins to consider in POMC-peptide stress regulation? More detailed studies are needed.

No differences in the 5-HT turnover rate were observed between chronic stress groups in both species. Generally, mammalian studies show that chronic stress and increased allostatic load affect 5-HT neurochemistry [reviewed by Beauchaine et al. (67)]. Similarly, chronic stress, induced by high stocking densities, resulted in elevated basal levels of brain stem 5-HT turnover in rainbow trout (68). However, upon an acute stress, already chronically stressed trout showed blunted stress responses including telencephalic 5-HT responsiveness (69). However, the present results indicate that chronic stress does not affect basal 5-HT neurochemistry, which is somewhat in contrast to the aforementioned rainbow trout studies. However, it is important to point out that in the rainbow trout studies fish were exposed to a continuous stressor, while in the present study they were repeatedly exposed to a combination of high-intensity stressors (different densities, chasing and air-exposure). Of note, the experimental design of the present study did not include brain 5-HT responsiveness to an acute stressor.

Stress can significantly alter the expression profile of genes related to metabolic, immune and cell signaling functions (70–72). The expression of glucocorticoid receptors and heat shock proteins is altered when seabass are chronically stressed by high rearing density (71, 73). Changes in the expression of stress-related genes have also been reported in seabream exposed to different rearing densities (33) or to unpredictable chronic low intensity stress in the early stages of life (74).

In the present study there was a remarkable difference in basal *cfos* expression in POA of seabass and seabream. In seabream the gene was apparently and essentially silent in unstressed seabream, but *cfos* expression was clearly seen after acute stress. In seabass, *cfos* expression in the POA was found under basal as well as post-stress conditions. It has been suggested that *cfos* expression is up-regulated after acute exposure to (hypercapnia) stress in seabass (72). Moreover, in zebrafish *cfos* expression seems to be upregulated after exposure to chronic stress (75). Still, however, literature on this aspect is limited and we can only speculate that the high expression of *cfos* confirms and reflects high HPI-axis activity in seabass, in agreement with the endocrine pre- and post-acute stress concentrations of cortisol, glucose and lactate in seabass, compared to those of seabream (28).

The significant increase in POA *crf* expression in the highly stressed seabass indicates that their impaired cortisol response to acute stress is not related to a dysfunction of the POA, but must be sought rather in exhaustion of the interrenal tissue (as discussed above) or in the pituitary corticotropes (35). Indeed, the seabass interrenal gland appears to be the key tissue where regulation of cortisol responsiveness occurs (Samaras and Pavlidis, submitted). Meanwhile, seabream coped well with the stress imposed. These fish presented both low cortisol levels and unaltered *crf* expression in the POA. This is in agreement with results reported for Atlantic salmon subjected to a similar unpredictable chronic stress (35). Taken together, the present study shows a species-specific regulation of the HPI axis to chronic stress.

A profound difference in cortisol receptor profile was found between seabass and seabream. The *gr1*/*mr* ratio showed an over 100-fold increase in highly stressed seabass, compared to control groups, while in seabream the ratio remained unaffected by stress. The drastic ratio shift in seabass resulted from a combined increase in *gr1* expression and decrease in *mr*-expression; we speculate that this shift is best explained by differential feedforward and feedback mechanisms of cortisol on these targets, respectively. Shifts in *gr*/*mr* ratio are indicators of impaired appraisal, poor learning and fear avoidance in vertebrates (76–78). In zebrafish (79) and trout (80) chronic stress increased the brain *gr*/*mr* ratio and this was associated with diminished cognitive quality and inhibitory avoidance learning. In mammals, *gr*/*mr* ratio shifts make the brain prone to steroid-induced pathologies (81) and we suggest here that the same may hold for fish (82–84).

If we take the *gr*/*mr* ratio as indicator of allostatic load [as done in rodent studies; (77, 81, 85)], also in fish, then our chronic stress paradigm induces allostatic overload and thus the ratio may be considered an appropriate indicator of stress load. We propose that such a receptor profile is a trait common to vertebrates, and originally developed in fish, the earliest vertebrates.

Finally, the telencephalon is an important target for cortisol feedback, illustrated by changes in *gr*/*mr* ratio in e.g., zebrafish (82–84). Indeed, in seabream we observed both decreasing *gr*/*mr* ratio's and *crf* expression levels with increasing stress load. To appreciate a stress response it is important to recognize and appreciate the role of complex behavior in this response, memory, learning, appraisal and prediction are crucial in coping with a dynamic environment and requires brain structures that facilitate such behavior. Evidence is accruing that the fish telencephalon/forebrain contains structures homologous and partly analogous to the mammalian hippocampus, amygdala, pyriform cortex, and isocortex (3). For zebrafish we have shown via inhibitory fear avoidance learning that the amygdala equivalent (dorsomedial pallium) is involved in acquisition of memory, a likely process involving MR activity, while in hippocampal neuronal clusters (dorsolateral pallium) GR facilitates consolidation of memory (86). A surprising functional parallel seems to exist in fish and mammalian system (81) steering stress-related behavior. The absence of

this response in *gr*/*mr* ratio's and *crf* expression in seabass to chronic stress corroborates the notion that this species resides outside its allostatic comfort zone in the current experimental paradigm.

CONCLUSIONS

In this experiment seabass and seabream were found to react very differently to stress. Specifically, seabass appear to be more susceptible to stress in terms of reduced food intake and growth, as well as the regulation of plasma cortisol levels. Seabream compared to seabass appeared to have a strong resistance and lower sensibility to the stress regimes used in this experiment. This study substantiates species-specific differences in (endocrine and neuroendocrine) stress physiology from gene expression to growth performance and (learning) behavior. These considerations on species-specificity should draw attention of those involved in diversification programmes in aquaculture practices.

AUTHOR CONTRIBUTIONS

AS, NP, MP, LE, GF, and MG conceived and designed the experiments. AS, NP, NM, FS, LE, GF, and MG carried out the experiments. CE, EH, TP, JZ, and MV analyzed the samples. AS, CE, GF, and MG analyzed, interpreted the data, and drafted the manuscript. All authors have critically revised and approved the manuscript.

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Stress, Glucocorticoids and Bone: A Review From Mammals and Fish

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Glucocorticoids (GCs) are the final effector products of a neuroendocrine HPA/HPI axis governing energy balance and stress response in vertebrates. From a physiological point of view, basal GC levels are essential for intermediary metabolism and participate in the development and homeostasis of a wide range of body tissues, including the skeleton. Numerous mammalian studies have demonstrated that GC hormones exert a positive role during bone modeling and remodeling as they promote osteoblastogenesis to maintain the bone architecture. Although the pharmacological effect of the so-called stress hormones has been widely reported, the role of endogenous GCs on bone mineral metabolism as result of the endocrine stress response has been largely overlooked across vertebrates. In addition, stress responses are variable depending on the stressor (e.g., starvation, predation, and environmental change), life cycle events (e.g., migration and aging), and differ among vertebrate lineages, which react differently according to their biological, social and cognitive complexity (e.g., mineral demands, physical, and psychological stress). This review intends to summarize the endogenous GCs action on bone metabolism of mammals and fish under a variety of challenging circumstances. Particular emphasis will be given to the regulatory loop between GCs and the parathyroid hormone (PTH) family peptides, and other key regulators of mineral homeostasis and bone remodeling in vertebrates.

Keywords: glucocorticoids, stress, bone, vertebrates, PTH3, PTHLH

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INTRODUCTION

Glucocorticoids (GCs) are central steroid hormones on endocrine stress response modulation and whole-body homeostasis in vertebrates. Downstream of the hypothalamic-pituitary-adrenal/interrenal (HPA/HPI) axis, regulated by a negative feedback loop, circulating CGs exert diverse actions by binding to glucocorticoid receptor (GR) placed on nearly every tissue in the body (1). In addition to well-known effects on glucose metabolism, immune system, reproduction, feeding, circadian rhythm, behavior, and cognition, GCs also regulate bone metabolism (2–4). Bone is a metabolically active tissue, shaped at an early stage of development and continuously remodeled throughout an animals' lifetime. Bone remodeling regulated by systemic hormones, neural, and local factors, involves the coupled action of osteoclasts, osteoblasts, and osteocytes to replace old and damaged bone. This process preserves the mechanical strength and stiffness of the skeleton, maintains calcium-phosphorus homeostasis, acid/base balance, and releases growth factors as well as organic material embedded in bone (5, 6).

In vertebrates, the GCs action is complex. Despite stress hormones have long been considered as catabolic hormones, a dual metabolic effect has been found in the skeleton. Physiological levels of GCs are vital for normal skeletogenesis and bone mass accrual, which highlights an important anabolic role (7). However, an increase of GCs over the basal levels causes reduced bone growth, bone resorption and bone mineral loss as seen in Cushing's syndrome and GCs-induced osteoporosis (GIO), as well as other associated pathologies such as diabetes or sarcopenia (8–10). In humans, Cushing's syndrome (also named hypercortisolism) is characterized by an increased production of endogenous cortisol or GCs drugs resulting in detrimental effects on bone metabolism (11). Patients suffering from Cushing's disease exhibit a reduced bone mineral density, increased risk of fracture, suppression of osteoblastic differentiation and apoptosis of both osteoblasts and osteoclasts, among other symptoms (12, 13). Moreover, sustained exposure to exogenous GCs is also responsible for the so-called GIO as a consequence of long-term GC therapy (14). GIO has recently been investigated in fish, with zebrafish incubated in GCs showing reduced bone growth and impaired bone regeneration (15).

On the other hand, endogenous/exogenous GCs have been proposed to act as key regulators of osteocalcin expression in bone. Osteocalcin is a calcium-binding peptide synthesized by osteoblasts and osteocytes, involved in skeletal mineralization and, regulation of insulin production (16). Elevated GC levels suppress the osteoblast activity and inhibit the osteocalcin release in mammals (17). Therefore, GCs affecting bone formation also indirectly cause changes in whole-body energy metabolism (8). GCs are known to interact with parathyroid hormone (PTH) family members. Human PTH1 (PTH—the master regulator of bone mineral homeostasis) showed corticotropic activity in adrenocortical cell cultures (18). A feedback regulatory loop between cortisol and PTH3 (parathyroid hormone like hormone—PTHLH) has been described in vertebrates (18–20). In mammals, PTH3 participates in embryonic skeletal development (21), calcium mobilization during fetal-placental transport (22) and lactation (23, 24). While in fish, duplicated Pth3 factors are hormones involved in calcium uptake (25, 26), mineral release from scales (27), skeletogenesis and early mineralization (28).

To date, a substantial body of research has focused on the bone effects caused by a pathological increase of endogenous and exogenous GC levels, but few studies have reported the changes produced on bone metabolism due to the elevation of stress-induced GCs. As a natural mechanism, all organisms react to extrinsic and intrinsic stressors through the GC-mediated hormonal response to restore the equilibrium and preserve homeostasis. In this context, the skeleton is one of the target organs of the stress hormones and bone remodeling is an essential process that enables it to respond to changing conditions by modifying its structure and mineral composition. Stress responses are characterized by being variable across vertebrates and they are closely related to the type of stressor as well as the lineage-specific biology and ecology (29, 30). In this article, we review the action of stress-induce GCs on bone metabolism in vertebrates. Briefly, we define the current knowledge on the effect of endogenous GCs on bone under normal physiological conditions. Then, we describe how several stress factors affect bone mineral metabolism in two different vertebrate lineages: mammals (primarily human), which are endothermic terrestrial vertebrates, and fish, characterized as ectothermic aquatic vertebrates.

ENDOGENOUS GCs ON BONE DEVELOPMENT AND HOMEOSTASIS

Endogenous GC hormones regulate the expression of target genes through GR signaling within bone cells, affecting skeletal development and metabolism. The skeleton responsiveness to GCs and the subsequent activation or inhibition of the gene expression depends on the level of circulating stress hormones, the intracellular availability of active GCs and the GR activity (1). To date the study of GC actions on bone has focused on mammalian models. Initially, investigations were based on the global GR deletion which led to premature death in newborn mice by respiratory failure (31). This was followed by more advanced molecular approaches such as the bone cell-specific GR gene deletion or the osteoblasts-targeted transgenic expression of 11βHSD2 (enzyme that catalyzes the conversion of active to inactive GCs) to disrupt intracellular GC signaling. These studies contributed to better define the endogenous GCs effects under various physiological conditions. *In vivo* and *in vitro* studies carried out in cell cultures derived from 11βHSD2 overexpressing transgenic mice have reported the positive action of endogenous GCs during bone development (32, 33). GCs appeared to be essential for mice osteoblastogenesis as they control the lineage commitment of mesenchymal progenitor cells through osteoblasts by promoting the activation of Wnt signaling. In turn, Wnt proteins act on mesenchymal cells to increase the expression of β-catenin and RUNX2, the master regulator of osteoblast differentiation. Also, osteoblast GC activity disruption in 11βHSD2 transgenic mice revealed an important role for normal intramembranous ossification and proper cartilage removal during cranial development (34, 35). In addition to the GC actions during skeletogenesis in mammals, several studies have pointed out that endogenous GCs are also required to maintain the bone mass accrual and skeletal integrity across adulthood. Inactivation of osteoblast-specific GC signaling by using a GR knockout mouse model (36) or 11βHSD2 expressing transgenic mice (37, 38) resulted in a decrease of bone mineral density in adults, which was dependent on the skeletal site and sexual maturity (37). Moreover, a downregulation in the expression of osteoblasts differentiation markers (i.e., *Col1a1*, *Runx2*, bone sialoprotein, and osteocalcin) was found, suggesting failed osteoblastogenesis as well as mature osteoblast function (36, 38). Therefore, the major effects of endogenous GCs on bone development and homeostasis are probably due to its direct actions on osteoblasts. Nevertheless, due to a close and reciprocal interconnectivity between osteoblasts and osteoclasts for skeletal metabolism, *in vivo* studies involving endogenous GCs and osteoclasts are needed to specifically dissect the cellular actions on the skeleton.

STRESS-INDUCED GLUCOCORTICOID EFFECTS ON BONE MINERAL METABOLISM

GCs, including cortisol and/or corticosterone in mammals as well as cortisol in fish, are synthesized in the adrenal cortex of mammals, but in the interrenal tissue of the head kidneys in teleosts (39). In response to stress, the pituitary gland signals the adrenal gland/interrenal tissue to release GCs. These GCs are released into the blood and initiate numerous cellular events that promote changes in cells and tissues for adaptation to stressful stimuli (40) (**Figure 1**). In this context, it is important to distinguish between the degrees of stress that can ultimately affect bone homeostasis. Acute stress is sudden and transitory and it may trigger skeletal remodeling as an adaptive response, which confers survival advantage (41). After exposure to an acute stressor, GCs levels are rapidly increased in the blood before returning to basal levels via negative feedback mechanisms. However, chronic stress is a long-term stressor, sustained for a prolonged period of time or due to a frequently occurring stressor (41), through which GCs levels remain elevated which could lead to several pathological conditions including bone mineral loss (2). Stress-induced bone resorption can result in calcium and phosphate release and it can lead to irreversible damage of the bone architecture resulting in mechanical instability. In addition to intensity and duration of the stressor, the stress responses of vertebrates are highly variable depending on the type of stressor and the way it is perceived by each kind of species. Some key stress factors affecting bone mineral metabolism in mammals and fish are described in this section including mineral demands, environmental change, starvation, physical exercise, psychological stress, and aging (**Figure 1**).

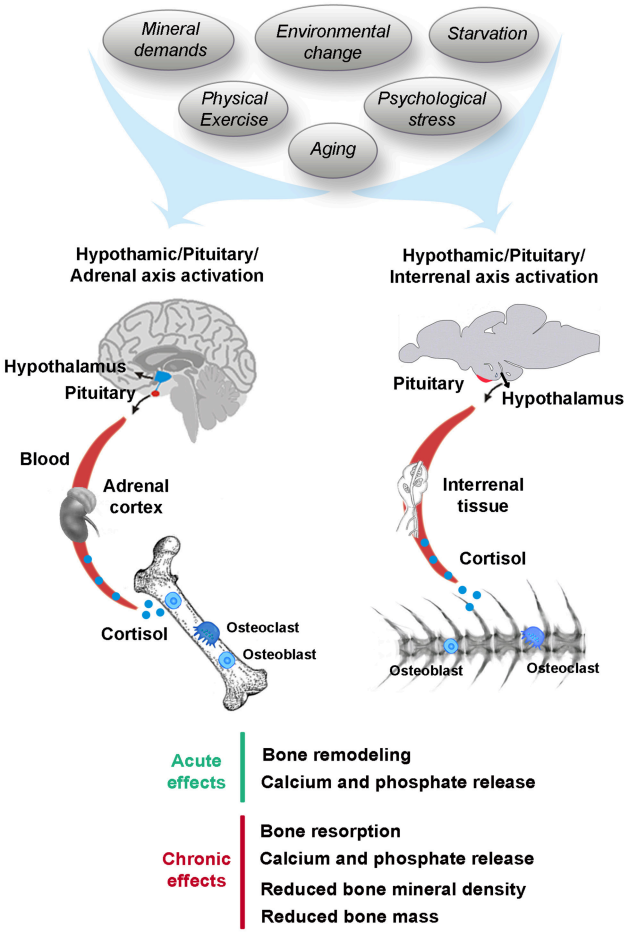

FIGURE 1 | Stress-induced cortisol affecting bone mineral metabolism in human and fish. Stressors including mineral demands, environmental change, starvation, physical exercise, psychological stress, or aging trigger the hypothalamic-pituitary-adrenal/interrenal (HPA/HPI) axis activation in human and fish, respectively, leading acute and chronic effects on the skeleton.

Mineral Demands

The skeleton is the major mineral storage organ in the vertebrate body and takes part in the regulation of calcium-phosphate metabolism. Thus, skeleton provides calcium and phosphate through bone resorption to compensate the inadequate availability of minerals in the environment and/or in the diet to maintain essential ionic levels in blood (5, 42). Unlike terrestrial vertebrates, fish can absorb minerals from surrounding water across the skin, oral and branchial epithelium, so stressors related to water and ion homeostasis have a greater physiological impact (29). In teleosts, the role of cortisol on osmoregulation has widely been reported (43) but, the contribution of cortisol on the ionic balance related to bone mineral homeostasis has received less attention (44). Previous studies showed that fish exposed to low calcium water levels give rise to high plasma cortisol levels in rainbow trout (45, 46), and stimulates the gene expression of steroid 11β-hydroxylase (final-step enzyme for cortisol synthesis) as well as glucocorticoid receptor (*gr*) in zebrafish (47). Moreover, cortisol treatment was shown to induce *in vitro* calcium transport in cultured rainbow trout gill epithelium, which supports its hypercalcemic role (48). Also, tilapia exposed to exogenous cortisol showed an increase in calcium uptake and upregulation of epithelial Ca^{2+} channel (*ecac*) gene expression (49). It would therefore appear that teleost fish regulate the calcium uptake to cope with a fluctuating water environment which is closely related to bone homeostasis. Alternatively, studies with juvenile seabream showed a plasma cortisol increase after prolonged exposure to low calcium availability in the water and/or diet, which resulted in reduced whole-body calcium and phosphorus contents (50). In the European eel, chronic cortisol treatment induced mineral loss in vertebral bone through osteoclastic resorption and osteocytic osteolysis (51). Interestingly, it has been suggested that cortisol mobilization of bone mineral stores in eel may be evidence of an ancestral stress-induced physiological process (51) related to the effects of stress events in mammals (e.g., starvation, physical exercise, psychological stress, or aging).

An interaction between hypercalcemic PTH factors regulating bone mineral metabolism and cortisol has been reported in mammals and fish (**Table 1**). Both PTH1 and PTH3, stimulated

TABLE 1 | Summary of some of the reported studies including PTH-cortisol regulatory interactions in mammals and fish.

Hormone	Species	Action	Tissue	References
PTH1	*Homo sapiens*	Cortisol release	Adrenocortical cells culture	18
PTH3	*Homo sapiens*	Cortisol release	Adrenocortical cells culture	18
Cortisol	*Mus musculus*	PTH3 expression increase	Kidney	19
Pth3	*Sparus aurata*	Cortisol release	Isolated interrenal glands	20
Cortisol	*Sparus aurata*	Pth3 expression decrease	Blood	52

cortisol release from human adrenocortical cells *in vitro* (18), although only the gene encoding PTH3 appears to be regulated by GCs (19). Similarly, piscine Pth3 showed *in vitro* corticotropic activity on isolated sea bream interrenal glands (20). In turn, sustained cortisol levels in sea bream as a consequence of a 24h confinement stressor or *in vivo* cortisol intraperitoneal injection resulted in a decrease in plasma Pth3 levels (52). Similar to cortisol, sea bream PTH3 is produced in interrenal tissue in fish (20, 53) and therefore an autocrine and/or paracrine regulatory mechanism between these two hormones was proposed (52). However the underlying molecular regulation remains unclear and it is possible that Pth3 acts indirectly at other levels of the HPI axis. Contradictory results regarding the cortisol-Pth3 reciprocal regulation were found in sea bream exposed to limited calcium availability in the long-term. Fish either under low calcium water along with a calcium-sufficient diet or under regular calcium water but calcium-deficient diet showed elevated plasma cortisol and Pth3 levels (50).

Environmental Change

Environmental stressors like temperature fluctuations are a critical feature of homeostasis in an organism. This is of particular relevance for ectothermic animals such as fish, where temperature directly influences their normal physiology. Sea bream exposed to water temperatures below 13°C develop winter syndrome, which is characterized by a multi-organ dysfunction together with a high but transient rise of plasma cortisol levels triggering a stress response (54, 55). A recent study in sea bream has revealed the impact of cold challenge, which increased the cortisol production and affected bone homeostasis in juveniles (55). Thus, fish exposed to low temperature during early development showed altered enzymatic activities of alkaline phosphatase (ALP) and tartrate-resistant acid phosphatase (TRAP) as well as calcium content changes on the vertebral bone (55). Interestingly, temperature is also a modulator of the expression of PTH family members. Zebrafish embryos exposed to cold (18°C) stress showed up-regulated mRNA levels of *pth1a*, *pth1b*, *pth3a, pth3b,* and *pth1rb*, while those exposed to a hot (38°C) stress down-regulated mRNA levels of these genes (56). Therefore, it is likely that such changes may impact mineral balance, altering bone development in embryos. However, to our knowledge, there are no studies showing a correlation between temperature-driven levels of cortisol and PTH family members affecting bone metabolism.

Starvation

A common stressor in the wild is food deprivation, which can be caused by adverse weather, decline in prey availability, increased predator pressure and migration or hibernation, among others. Under these conditions, it is well know that GCs are released into the blood to promote the mobilization and utilization of energy reserves and mineral stores in vertebrates (57). Regarding migratory teleost fish like salmonids, spawning migration is a very challenging situation since they undergo not only fasting but also exhausting exercise, changes in osmoregulation and sexual maturation (58). Thus, migratory salmonids, essentially as adults returning to spawning grounds, experience a strong activation of the neuroendocrine axis resulting in elevated plasma corticosteroid levels (59) as well as marked resorption of the skeleton. In particular, the anadromous Atlantic salmon was reported to experience a dramatic skeletal transformation caused by a decrease in the bone mineral content, halastic demineralization, osteoclastic resorption, and reduced vertebral bone mass (60–62). Nevertheless, a recent study in the migratory European eel showed that sexually mature fish via cortisol injection exhibited severe bone loss in the vertebrae and skull, while plasma cortisol levels were reduced (63). Therefore, a cortisol-independent bone resorption mechanism has been suggested in migratory eels (63). Some mammalian species also experience a situation of nutritional deprivation during hibernation similar to that observed in migratory fish. Small mammals such as little brown bats and hamsters lose a significant bone mineral volume during hibernation (64, 65), but only high plasma cortisol levels have been detected in bats (66). On the other hand, cortisol is increased in hibernating bears, however they maintain a typically balanced bone turnover which prevents bone reabsorption excess and osteoporosis (67, 68). Furthermore, fasting studies in humans have shown an increase in blood cortisol concentration (69) accompanied by a decrease of PTH secretion, which is suggested to have some positive effect on the bone health (70).

Physical Exercise

Physical exercise represents a stressful experience for all organisms. In mammals, physical activity promotes direct effects on bone metabolism via mechanical forces (i.e., weight-bearing activities), but also indirectly through hormonal factors (71). Hence, exercise causes HPA axis activation and the subsequent release of GCs into the blood. Although physical exercise has been reported to prevent bone mineral loss and to sustain bone health, long-term intense exercise is reported

to cause hypercortisolism, which can result in osteopenia and osteoporosis (71). Some studies have showed that over-trained runners exhibit elevated ACTH and cortisol basal concentrations compared with moderately trained runners and sedentary subjects (72). However, the HPA axis activation was attenuated in over-trained runners after exposure to an acute exercise, suggesting a certain adaptation to physical exercise (72). Other investigations have reported that highly trained male master cyclists (73) and competitive male cyclists show low bone mineral density in the hip and spine, however there is no clear association between bone mineral content and excess of GC secretion (74). Exercised fish show improved growth and increased bone remodeling (75). However, the most extreme examples of possible interactions between GCs and bone metabolism during exercise may arise from migratory fish such as the salmonids or eels (see also under *Aging*). In experiments that were aimed to simulate to some extent the skeletal-loss consequences of a 5,000 km migration to reproductive grounds (51, 63) demonstrated that cortisol induced a significant bone demineralization of Europen eel vertebrae, with significant decreases of the mineral ratio and the degree of mineralization of vertebral sections. Using histology and image analysis of ultrathin microradiographs they showed the induction by cortisol of different mechanisms of bone resorption, including periosteocytic osteolysis and osteoclastic resorption. These effects were further enhanced by sex steroids. Specificity of cortisol action was investigated by comparison with the effects of sex steroids, namely estradiol, related to the stimulated synthesis of vitellogenin (Vg), an oviparous specific phospho-calcio-lipoprotein. Such effects of estradiol have been profusely shown in salmonids (76). However, in above study, the ready-to-migrate eels were not actually exercised but simply injected with steroids and thus the evidence for the effects of exercise-related GCs.

Psychological Stress

It has recently been demonstrated that psychological stress affects bone metabolism in humans and some animal models (77–81). Although the psychological stress response is complex, as it depends on individual interpretation, it has been suggested that long-term psychological stress produces altered HPA axis activity and as a consequence, GC release affecting bone health (77). In rats, chronic psychological stress by anxiety neurosis results in the loss of mandibular bone matrix (78). Post-traumatic stress disorder, which is related to altered serum GCs, caused a decrease of bone mineral density and bone mineral contents in young mice (79). In humans, the relationship between depression and bone mineral density has also been associated with stress-induced cortisol effects. Post-menopausal women with depression showed loss of bone mineral density in the lumbar spine and femur compared to non-depressed subjects, as well as a higher cortisol production after an acute stress experience (80). Furthermore, pre-menopausal women suffering from chronic depression presented a negative correlation between cortisol levels and bone mineral density, as well as low osteocalcin levels suggesting a decrease in bone formation (81). Recently there has been increased attention to the impact of social or psychological stress in fish, in parallel with the recognition of an increased degree of sentience and multiple individual coping styles, to which some may even refer as "personalities" in fish. The way fish exhibiting those different coping styles address stressful events determines to some extent their rank, access to food, energy expenditure, growth rates and cortisol response levels (82, 83). However, to date, there is no information on the impact of psychological stress and induced GC levels on fish bone.

Aging

Aging is an imbalance between damage and repair that makes organisms undergo an increasing vulnerability to challenges during the post-maturational life, decreasing their ability to survive (84). Along these lines, aging disturbs the homeostatic system but perhaps it should not be considered as a stressor since it does not elicit *per se* a physiological stress response. However, aging is closely related to responsivity to stress and it seems to produce similar effects to those seen in the chronic stress response. In mammals, aging causes greater HPA axis activation and thereby an excess production of GCs that negatively affect bone metabolism (7). It has been proposed that HPA axis hyperactivity could be due to a decrease in the number of GC receptors in the brain, which in turn affects the negative feedback regulation, but can also be the result of repeated stress events (7). An age-related increase of corticosterone as well as upregulation of 11βHSD1 (enzyme that activates GCs) expression in bone, which led to reduced bone vasculature and skeletal fragility in mice (85). Studies in humans have provided evidence that elevated cortisol levels affect bone mineral density. Thus, elderly men and women with a high level of evening salivary cortisol had a reduced bone mineral density in the lumbar spine (86). Also, high plasma cortisol levels in older women contributed to bone loss in the femoral neck (87). Additionally, a positive correlation between cortisol concentration and bone loss rate was found in the lumbar spine in elderly men (88). Fish grow continuously throughout their lives and usually their skeleton maintains its integrity with aging. A few exceptions can be found in semelparous species, such as many salmonids and eels (51, 63, 76) in which sexual maturation, reproduction and related skeletal remodeling coincide with the end of life. Both GC and sex steroids increase along the migratory route and peak levels coincide with important organ and skeletal remodeling. In pink salmon specifically, cortisol levels rise over 20-fold in both males and females (89) being thus likely that GCs may have important effects over bone metabolism. Despite the fact that most fish do not appear to undergo important skeletal changes as they age, the use of fish as models for probing into aging-related health conditions with impacts on bone mineral metabolism in human offers ample possibilities, since they can be treated and selected to simulate such conditions, including those directly or indirectly related to disturbances in circulating GCs (90–93).

CONCLUSION

In response to a variety of stressful situations and/or stimuli that challenge the internal equilibrium in vertebrates, bone appears to be a target organ for stress-induced GCs produced by HPA/HPI axis activation. In mammals, as in fish, elevated GC levels sustained over time result in bone resorption, which alters the

mineral balance and damages the bone structure. Although this evidence suggests that stress-induced GCs may act in a similar fashion to that of therapeutic GCs, there is a gap in the knowledge about the cellular and molecular mechanisms involving the stress response, cortisol and bone mineral metabolism in vertebrates. Studies utilizing mammalian models based on the pathological increase of endogenous GCs and pharmacological GCs reported that the bone effect of these hormones could be due to its direct action on osteoblasts (34, 35). However, the actions of stress-induced GCs on bone cells as well as the interactions between GCs and other factors regulating bone homeostasis are currently unknown.

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Quantification of a Glucocorticoid Profile in Non-pooled Samples Is Pivotal in Stress Research Across Vertebrates

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Vertebrates are faced continuously with a variety of potential stressful stimuli and react by a highly conserved endocrine stress response. An immediate catecholamine mediated response increases plasma glucose levels in order to prepare the organism for the "fight or flight" reaction. In addition, in a matter of minutes after this (nor)adrenaline release, glucocorticoids, in particular cortisol or corticosterone depending on the species, are released through activation of the hypothalamic-pituitary-interrenal (HPI) axis in fish or hypothalamic-pituitary-adrenal (HPA) axis in other vertebrates. These plasma glucocorticoids are well documented and widely used as biomarker for stress across vertebrates. In order to study the role of glucocorticoids in acute and chronic stress and gain in-depth insight in the stress axis (re)activity across vertebrates, it is pivotal to pin-point the involved molecules, to understand the mechanisms of how the latter are synthesized, regulated and excreted, and to grasp their actions on a plethora of biological processes. Furthermore, in-depth knowledge on the characteristics of the tissues as well as on the analytical methodologies available for glucocorticoid quantification is needed. This manuscript is to be situated in the multi-disciplinary research topic of glucocorticoid action across vertebrates which is linked to a wide range of research domains including but not limited to biochemistry, ecology, endocrinology, ethology, histology, immunology, morphology, physiology, and toxicology, and provides a solid base for all interested in stress, in particular glucocorticoid, related research. In this framework, internationally validated confirmation methods for quantification of a glucocorticoid profile comprising: (i) the dominant hormone; (ii) its direct precursors; (iii) its endogenously present phase I metabolites; and (iv) the most abundant more polar excreted exogenous phase I metabolites in non-pooled samples are pivotal.

Keywords: vertebrate, stress, HPI, HPA, glucocorticoid, profile

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KEY CONCEPTS

Accurate Identification and Quantification of Stressors Experienced by an Individual

The sheer diversity in potential stressors, individual perception and subsequent reaction to these stressors, and the plethora of metabolic processes mediated by glucocorticoids render accurate identification and quantification of the stressors experienced by an individual pivotal.

Analysis of the Dominant Glucocorticoid Is Affected by Other Steroids

Glucocorticoid quantification can be biased by (i) the less dominant hormone; (ii) other steroids; (iii) direct precursors of the dominant hormone and the dominant hormone itself produced in extra-interrenal or extra-adrenal tissues; (iv) phase I metabolites present in the body; and (v) phase I metabolites present on the sample as contaminants.

Analysis of the Dominant Glucocorticoid Is Affected by the Sample Tissue

Results can be enhanced or suppressed by tissue specific compounds, and potential effects should be analytically validated.

Analysis of the Dominant Glucocorticoid Is Affected by the Analytical Methodology Used

Glucocorticoid analysis should best be performed using confirmation methods. Hereby, UPLC-MS/MS is considered the gold standard for quantitation of glucocorticoids in complex biological tissues as it has the needed sensitivity, selectivity and the advantage of having the capability to perform multi-analyte assays, even across compound classes.

Analysis of the Dominant Glucocorticoid Is Affected by the Lack of Analytical Validation

Methods should best be developed in an EN ISO/IEC 17025 regulated environment and analytically validated according the criteria of international standards to ensure full traceability and quality of the results in time.

INTRODUCTION

Moberg (1) defined stress as "a highly complex multi-dimensional phenomenon promoted by several noxious or unpredictable stimuli (stressors) that cause a physiological response (stress) aimed to maintain or recover the body homeostasis." Stressors are diverse and generally classified based on their: (i) type (i.e., chemical, physical, and psychological); (ii) duration [i.e., transitory (acute) or long-term (chronic)]; (iii) severity; (iv) (un)predictability; and (v) (un)controllability (2). Hereby, stress can be perceived as harmful or negative (distress), as well as a neutral or even as a positive condition (eustress) (3).

Organisms are faced continously with a variety of potential stressful stimuli and have developed over time a plethora of mechanisms to cope with changes and challenges in their environment (4). When faced with such stressful stimuli, vertebrates, ranging from fish to humans, react by a highly conserved endocrine stress response. An immediate catecholamine mediated response increases plasma glucose levels in order to prepare the organism for the "fight or flight" reaction (5). In addition, in a matter of minutes after this (nor)epinephrine [(nor)adrenaline] release, glucocorticoids, in particular cortisol (11β,17α,21-trihydroxypregn-4-ene-3,20-dione or $C_{21}H_{30}O_5$) or corticosterone (11β,21-dihydroxypregn-4-ene-3,20-dione or $C_{21}H_{30}O_4$) depending on the species, are released through activation of the hypothalamic-pituitary-interrenal (HPI) axis in fish (6) or hypothalamic-pituitary-adrenal (HPA) axis in other vertebrates (2). These plasma glucocorticoids are widely used as biomarker for stress across vertebrates (7, 8) and considered as adaptation hormones as they mediate a redistribution of energy (i.e., glucose) in order to restore pre-stress conditions. However, failure to regain homeostasis (maladaptation) will inevitably lead to chronic stress making the individual prone to the detrimental effects of glucocorticoid mediated actions (e.g., decreased growth, decreased reproduction, immune suppression, increased mortality). In the concept of "allostasis" [i.e., constancy through change by resetting the set-points for homeostasis in accordance to environmental cues (9, 10)], this situation can be described as: the transition from allostatic load (when the stress can be overcome, "eustress") to allostatic overload (when the stress cannot be overcome and becomes "distress") (5, 11). The dominant hormone, cortisol or corticosterone, respectively, is pleiotropic and affects all major homeostatic systems of the vertebrate's body. Besides modulating actions, which alter an organism's response to a stressor, also preparative actions, which alter the organism's response to a subsequent stressor or aid in adapting to a chronic stressor, are distinguished (2). Hereby, a plethora of physiological processes are modulated including central nervous system (CNS) and cardiovascular functions, the metabolic system [e.g., bone metabolism (12), stimulation of gluconeogenesis, proteolytic processes in the muscle and lipolysis in the adipose tissues to increase plasma glucose levels)], the immune system (inflammatory response and lymphocyte production), growth, reproduction, and behavior (13). Furthermore, physiological amounts of glucocorticoids are also essential for normal renal tubular function and thus for water and electrolyte homeostasis (14, 15).

The perception of potential stressors by an individual varies (16, 17) and depends on various factors including but not limited to the species, genetic background, previous experiences (18), gender (19), age, and types as well as duration of the stressors (20, 21). The stress response will vary accordingly between individuals and physiological and behavioral responses tend to be associated in distinct suites of correlated traits, called "stress coping styles" (22). Hereby, the proactive stress coping style (active coping or "fight-flight") is associated with low HPI or HPA axis responsiveness, but with high sympathetic reactivity, and is characterized by a high level of active avoidance, aggression and other actions indicating active attempts to counteract the stressful stimulus. The opposite is seen in reactive coping (passive coping or "conservation-withdrawal") (22).

In all, the sheer diversity in potential stressors, individual perception and subsequent response to these stressors, and the plethora of metabolic processes mediated by glucocorticoids render accurate identification and quantification of the stressors experienced by an individual pivotal.

ANALYSIS OF THE DOMINANT GLUCOCORTICOID IS AFFECTED BY OTHER STEROIDS

By the Less Dominant Glucocorticoid

The vertebrate stress response is mediated by the stress system which is activated when encountering environmental stressors but also when the body is at rest, hereby responding to various signals (e.g., circadian, neurosensory, blood-borne, and limbic) (23). The noradrenergic synthesizing neurons of the locus coeruleus/norepinephrine-central sympathetic system in the brainstem as well as the corticosteroid releasing hormone (CRH) and arginine vasopressin (AVP) synthesizing neurons of the hypothalamic paraventricular nuclei (PVN) comprise the central components, while the systemic sympathetic and adrenomedullary nervous systems and the HPI or HPA axis comprise the peripheral components of the stress system (24). Once triggered, CRH stimulates the release of adrenocorticotropic hormone (ACTH) from the pituitary, which results in glucocorticoid release, mainly cortisol, and corticosterone depending on the species, from the head kidney or adrenal gland, respectively. In ray-finned fish, cortisol predominates but corticosterone is also present; in the remaining fish species, the dominant or sole glucocorticoid varies. In this framework, 11-deoxycortisol in agnate fish (25); 1α-hydroxycorticosterone in sharks and rays (26); and 11-deoxycorticosterone in teleost fish (27, 28), were shown to be active glucocorticoids. In amphibians, reptiles and birds, the dominant glucocorticoid is corticosterone, while mammals, most placentals and marsupials secrete primarily cortisol. However, some rodents (e.g., rats and mice) secrete primarily or only corticosterone, whereas most other rodents secrete primarily or only cortisol (e.g., guinea pigs), while hamsters secrete both glucocorticoids in equal quantities. As a consequence, the less dominant glucocorticoid should be considered during analytical validation as it can cause cross-reactivity and subsequently bias glucocorticoid quantification.

By Other Steroids

Glucocorticoids have a typical steroid structure consisting of a cyclopentaphenanthrene nucleus comprising three fused cyclohexane rings in a non-linear arrangement and a terminal cyclopentane ring. Most glucocorticoids possess a Δ4-3-keto group, a carbon ketol side-chain at C_{17} and generally an oxygen function at C_{11}. The orientation of the groups attached to the steroid ring system is pivotal for the biological activity (29). As a consequence, other steroids including (i) androgens (C_{19}-steroids such as testosterone); (ii) estrogens (C_{18}-steroids such as estrone); (iii) mineralocorticoids (C_{21}-steroids such as aldosterone); and (iv) progestagens (C_{21}-steroids such as progesterone) (30), can be considered as physical-chemical similar molecules and should be taken into account during analytical validation as these compounds can cause cross-reactivity and subsequently bias glucocorticoid quantification.

By Direct Precursors of the Dominant Glucocorticoid and the Dominant Glucocorticoid Produced in "Extra-Interrenal" or "Extra-Adrenal" Tissues

All steroids are derivatives of cholesterol ($C_{27}H_{46}O$) (31). Though, glucocorticoids were initially thought to be exclusively synthetized by the interrenal or adrenocortical cells, respectively, numerous studies have shown that they are also synthesized locally in so called "extra-interrenal" or "extra-adrenal" tissues (32). At present, these tissues include but are not limited to: primary lymphoid organs (33), intestine (34), CNS (35), cardiovascular system (36), skin (37–39), hair follicle (40), lung (41), kidney (42), and retina (43).

As a consequence, quantification of the dominant glucocorticoid produced by the HPI or HPA axis can be biased by direct precursors of the dominant hormone and the dominant hormone itself produced in extra-interrenal or extra-adrenal tissues, making the quantification (or at least analytical validation) of these other glucocorticoids of importance.

By the Manner How Glucocorticoids Are Regulated

Systemically, glucocorticoid levels are influenced by distinct brain regions including structures of the limbic system (i.e., amygdala and hippocampus) and the midbrain (i.e., prefrontal cortex) (44) as well as by the hypothalamus, pituitary, and interrenal cells or adrenal cortex, respectively (45). In addition, the glucocorticoid pathway is controlled by the dominant glucocorticoid through a negative feedback loop. Besides this stress reactivity, glucocorticoid release is under control of a circadian clock (46). In humans the secretion of cortisol from the adrenal glands was shown to follow a diurnal cycle with a profound increase after awakening (47, 48).

Local regulation of glucocorticoid levels is mediated by access to target cells mediated by carrier proteins (49), by pre-receptor metabolism due to metabolic enzymes and by the availability of glucocorticoid (GR) and mineralocorticoid (MR) receptors.

By the Non-free Dominant Glucocorticoid in the Blood

Glucocorticoid levels vary rapidly due to the pulsatile nature of its secretion, rendering the dynamics of its binding critical determinants of tissue levels of free hormone and consequent hormone signaling. In most vertebrate species, the major proportion of circulating glucocorticoids are bound to a plasma glycoprotein called corticosteroid binding globulin (CBG) (50, 51). Subsequently, the free fraction is small (52). Since CBG is too large to leave the capillaries under normal circumstances, glucocorticoids bound to it remain in circulation. According to the "free hormone" hypothesis, it is the concentration of free,

unbound hormone that determines how much glucocorticoids diffuses out of the capillaries and reaches the tissues. However, as CBG-bound glucocorticoids were shown to be released by enzymatic cleaving of the CBG molecule (53) and cell surface receptors for the CBG-glucocorticoid complex were shown to be present in certain tissues (54), one could argue that the glucocorticoid dissociation from CBG is part of the mechanism that makes the hormone biologically active.

In all, when focusing on cortisol producing vertebrates, cortisol is transported in blood more than 90% protein bound, approximately 70% with high affinity to CBG and 20% with low affinity to albumin, but it dissociates so rapidly that it is generally thought to be free (55). However, evidence indicates a dichotomous pattern with respect to CBG in these vertebrates: (i) a dominant branch where high levels of CBG bind most of the glucocorticoid which applies to the majority of vertebrates; and (ii) a smaller branch where low levels of CBG bind almost none of the glucocorticoid which applies to the fish (56). As a consequence, glucocorticoid analysis should be analytically validated to ensure that solely the free fraction of cortisol is quantified.

By Phase I Metabolites of the Dominant Glucocorticoid Present in the Body

Intracellular cortisol within the endoplasmatic reticulum of cells is regulated by local enzymes in a tissue-specific way independently of its plasma concentration (57). The intracellular enzyme 11β-hydroxysteroid dehydrogenase (11β-HSD) is bidirectional (58): 11β-HSD type 1 is a reductase that converts the 11-keto metabolite cortisone to its active form 11-hydroxy cortisol, amplifying glucocorticoid action in liver and visceral adipose tissue, but also in brain, bone, gonad, muscle and other GR-expressing tissues including the eye, while 11β-HSD type 2 catalyzes the oxidation of cortisol to cortisone (a hydroxyl group at C_{11} becomes a carboxyl group) and is co-expressed with the MR in the kidney, colon and salivary gland and inactivates cortisol to cortisone, thereby enabling aldosterone to bind to the MR (59, 60). In addition, cortisone was found to be further reduced to 20β-hydroxycortisone by 20β-HSD type 2 (61). As a consequence, glucocorticoid analysis should include cortisone as the latter is rapidly interconverted to and from cortisol as well as 20β-dihydrocortisone.

Corticosteroids affect a variety of target tissues over a broad range of time scales, ranging from slow gene transcription dependent to rapid gene transcription independent actions. Following uptake from the circulation, binding can occur by the two major functional groups of vertebrate corticosteroid receptors: GR and MR distinguished by their amino acid sequences and ligand specificity (62, 63). Most studies were performed on human intracellular genomic receptors [gGR reviewed by (64) and gMR reviewed by (65) as well as by (66)] regulating transcriptional activity of steroid target genes. Far less is known regarding the non-genomic effects mediated by the extracellular membrane glucocorticoid (mGR) and mineralocorticoid (mMR) receptors [for review see (67)], which allow rapid modulation of synaptic transmission and membrane ion currents hereby playing a key role in signal transduction at the synapse, the key neuron-to-neuron interface involved in learning and memory and as such in traumatic memories during times of stress (68, 69). As a consequence, glucocorticoid analysis should take into account the effect of phase I metabolites present in the body (i.e., cortisone and 20β-dihydrocortisone) as both compounds could potentially bind to GR and MR and are also excreted in minor proportions to the environment (see further).

By Phase I Metabolites of the Dominant Glucocorticoid Present in the Environment

The dominant glucocorticoid, cortisol or corticosterone, respectively, is controlled by the ratio of *de novo* synthesis to catabolism by the action of the respective enzymes involved. In this framework, steroids undergo extensive bio-transformations which decrease their biological activity and increase their water solubility by converting them to hydrophilic compounds that can be excreted. In general, these bio-transformations are divided into: (i) phase I metabolism which usually includes oxidation (e.g., hydroxylation) and/or reduction (e.g., hydrogenation) reactions; and (ii) phase II metabolism which usually involves conjugation reactions with polar groups such as glucuronide or sulfate and resulting into a highly hydrophilic product, which facilitates excretion in the urine or feces.

Cortisol and cortisone are metabolized in the liver (70). The main pathways of phase I metabolic reaction include: (i) oxidation and reduction at C_{11}; (ii) reduction of the C_4-C_5 double bond; and (iii) reduction at C_{20} (30, 71, 72). In a next step, (allo)-tetrahydrocortisol (THF) and (allo)-tetrahydrocortisone (THE) is (i) conjugated at a hydroxy group rapidly with glucuronic acid or sulfate and excreted in the urine or (ii) cleaved to the C_{19} steroids 11-hydroxy or 11-oxo-androsterone or etiocholanolone. In humans, non-metabolized cortisol and cortisone were shown to comprise only about 0.1% of the total urinary cortisol metabolites. At least 90% of the tetrahydro-derivatives of cortisol and cortisone are excreted into the urine as glucuronide or sulfate conjugates (73). Alternatively, reduction of the 20-oxo group by 20α- or 20β-hydroxysteroid dehydrogenase yields α and β cortols and cortolones, respectively, with subsequent oxidation at the C_{21} position to form the extremely polar metabolites, cortolic, and cortolonic acids (71). In addition, hydroxylation at C_6 to form 6β-hydroxycortisol as well as reduction of the C_{20} position, which may occur without A ring reduction giving rise to 20α- and 20β-hydroxycortisol are described (74).

Overall, approximately 50% of secreted cortisol appears in the urine as THF/allo-THF/THE, 25% as cortols/cortolones, 10% as $C_{19}O_3$ steroids (androstanes), and 10% appears as cortolic/cortolonic acids. The remaining 5% metabolites are free, non-conjugated steroids (cortisol, cortisone and 6β- and 20α/20β-metabolites of cortisol and cortisone). As a consequence, glucocorticoid analysis should include the most abundant phase I metabolites such as THF and THE as they are indicative for possible contamination of the sample with glucocorticoids from urine, feces, water, as well as from anthropogenic contamination (e.g., from hands).

ANALYSIS OF THE DOMINANT GLUCOCORTICOID IS AFFECTED BY THE TISSUE USED FOR GLUCOCORTICOID QUANTIFICATION

The type of tissue used for glucocorticoid quantification is of utmost importance as each tissue incorporates glucocorticoids in accordance with the processes by which it is formed hereby defining the timeframe of interrenal or adrenocortical activity that the tissue represents. Subsequently, a proper tissue for chronic stress quantification should allow a retrospective (i.e., over a certain period of time) view of the stress axis activity, and subsequently should possess the capacity to incorporate glucocorticoids in a stress (i.e., in reaction to stress full stimuli eliciting a glucocorticoid mediated response) and time (i.e., over a certain period of time) dependent manner (75). The type of tissue also determines the structural changes of the dominant glucocorticoid that may occur via processes of conjugation to glucuronides and sulfates, metabolic conversion via enzymatic action and bacterial breakdown (8). As a consequence, the effect of the tissue on the analysis results, as the latter can be enhanced or suppressed by tissue specific compounds, should be analytically validated. In practice, the choice of tissue depends on various factors including but not limited to: (i) the species; (ii) the nature of the study; (iii) acute vs. chronic stress quantification; (iv) the tissues available for sampling; and (v) logistical feasibility. **Table 1** provides an overview of the temporal window of stress axis (re)activation that is being reported in tissues commonly used for glucocorticoid analysis across vertebrates. Hereby, it should be noticed that at present no tissue for chronic stress quantification exists for amphibians.

ANALYSIS OF THE DOMINANT GLUCOCORTICOID IS AFFECTED BY THE ANALYTICAL METHODOLOGY USED

Glucocorticoids are measured using a wide variety of analytical methods including radio- (RIA) and enzyme (EIA) immunoassay, gas chromatography (GC), high performance liquid chromatography coupled to ultraviolet or fluorescence detection (HPLC-UV or FL), gas or liquid chromatography coupled to tandem mass spectrometry (GC- or LC-MS/MS) as well as sensor based techniques. In practice, the technique of choice depends mainly on the availability of qualified operators and sophisticated equipment in the laboratory.

TABLE 1 | Tissues commonly used for glucocorticoid analysis across vertebrates.

Tissue	Temporal window on HPI/HPA (re)activity	References
Vertebrate egg	Maternal deposition	(76)
Vertebrate plasma/serum	Snapshot	(57)
Whole body of fish larva	Snapshot	(77)
Mammalian saliva	Minutes	(78)
Vertebrate urine	Minutes to hours	(72)
Vertebrate feces	Minutes to days	(79)
Vertebrate excreta	Minutes to days	(80)
Water	Minutes to days	(81)
Reptilian shed skin	Weeks to months	(82)
Avian feather	Weeks to months	(83)
Fish scale	Weeks to years	(75)
Mammalian hair	Weeks to years	(84)

By Screening Methods

Immunoassays are most often chosen because they are fast, cheap, easy to perform, and commercially available for the dominant glucocorticoid in widely used tissues such as plasma of well-studied vertebrate species. RIA and EIA are both competitive binding assays necessitating an antibody directed against certain parts of the dominant glucocorticoid. While RIAs rely on a radioactive isotope (e.g., tritium or iodine) to generate a radioactive signal, EIAs use enzymes to generate a colorimetric signal to quantify the dominant glucocorticoid. Though immunoassays are sensitive (i.e., sufficient low levels can be detected) for the glucocorticoid of interest, major disadvantages are the lack of specificity (i.e., as they show high cross-reactivity with precursors and phase I metabolites of the targeted glucocorticoid as well as with substances with similar physical-chemical properties such as other steroids due to the poly-reactive nature of antibodies), the high lot-to-lot variation of antibodies (85), and the necessity to measure hormones individually. For example, Rettenbacher et al. (86) stated that their results for egg corticosterone could be explained by cross-reactions of the antibody used in the corticosterone EIA with other steroids, probably of gonadal origin as Hackl et al. (87) found a similar distribution pattern for progesterone. Subsequently, immunoassays should always be analytically validated in-depth.

The drawbacks of immunoassays have stimulated the development of new screening methods. Electrochemical biosensors have shown potential for fast, accurate and sensitive analysis of glucocorticoids. However, a continuing challenge is the sensitivity and stability of the surface bound bio-recognition molecules, which depends on the matrix used for their immobilization on the sensor (88). Besides the use of antibodies, molecular imprinting, which involves the synthesis of polymers in the presence of a template to produce the complementary binding sites with specific recognition ability, is also used. During this formation, the functional monomers are polymerized in the presence of a template, which is subsequently removed by washing and/or extraction after polymerization, resulting in a molecularly imprinted polymer (MIP) (89). A library of cortisol-imprinted polymers was prepared by Baggiani et al. (90), while Moreno-Guzmán et al. (91) reviewed the existing immunosensors for human cortisol.

In all, the lack of or insufficient in-depth analytical validation is the main cause of inconsistent results generated by immunoassays in the pertinent literature.

By Confirmation Methods

For confirmatory purposes, chromatographic techniques such as GC and LC, especially when coupled to (tandem) MS, are preferred since they allow a high resolution as required for complex biological tissues (92). Major disadvantages are the need for qualified operators and sophisticated equipment, high costs and complex sample preparations.

Significant improvement in the specificity of glucocorticoid measurements was achieved with the introduction of GC-MS/MS, however, accurate quantification is limited to analytes which can be derivatized (93) in order to increase their volatility (94). Because of limited sensitivity, low throughput and labor-intensive sample preparation, GC-MS/MS is not optimal for measuring glucocorticoid profiles. HPLC is well suited for the separation of glucocorticoids, though when coupled to UV or FL it lacks the sensitivity and specificity to distinguish glucocorticoid traces from the biological matrix background (29). Because of its inherent sensitivity and selectivity, LC-MS/MS is considered the gold standard method for quantitation of glucocorticoids in complex biological tissues (92, 95, 96). It has the further advantage of having the capability to perform multi-compound assays, even across compound classes (97).

ANALYSIS OF THE DOMINANT GLUCOCORTICOID IS AFFECTED BY THE LACK OF ANALYTICAL VALIDATION

Overall, glucocorticoid levels to be quantified are considered "trace levels" as they are situated in the ppb ($\mu g\ kg^{-1}$ or $\mu g\ L^{-1}$) and ppt ($ng\ kg^{-1}$ or $ng\ L^{-1}$) range. Regardless the sample tissue and analytical methodology used, it is pivotal to demonstrate that results are accurate, precise, and not biased by interfering compounds rendering results highly reliable. Subsequently, every procedure [i.e., parameter(s)/tissue combination using a specific analytical methodology] should be analytically validated. In this framework, working according the criteria of international standards such as EN ISO/IEC 17025 (98) and Commission Decision No. 2002/657/EC (99, 100), whereby experiments are carried out by well trained and authorized personnel in a controlled environment are a must. Hereby, the use of calibrated equipment, products with a certificate of analysis as well as performing all tests in standardized conditions hereby registering all details in logbooks is of importance. In addition, determination of the performance characteristics such as accuracy, trueness, precision, sensitivity, specificity and cross-reactivity with structurally related compounds are of utmost importance as they can influence the interpretation of results between studies. In particular immunoassays are prone to be biased by this as the used antiserum differs between assays leading to differences in cross-reactivity (8). Subsequent, physiological (i.e., by pharmacologically induced physiological changes in circulating glucocorticoid levels and to evaluate whether these changes are reflected in measured concentrations afterwards) as well as biological (i.e., glucocorticoid measurements in relation to cortical activity and the experience of stress) validation is needed in order to state that the method is fit for purpose (7).

As a consequence, one should try to use methods developed in an EN ISO/IEC 17025 regulated environment and analytically validated according the criteria of international standards as this ensures full traceability and quality of the results in time.

CONCLUSION

At present, most studies in the pertinent literature have focused on the quantification of the dominant glucocorticoid, cortisol or corticosterone depending on the species, using immunoassays. Hereby, one should bare in mind that: (i) results are prone to bias by cross-reactivity from other glucocorticoids as well as substances with similar physical-chemical properties, making analytical validation a must; (ii) immunoassays are screening methods which do not allow quantification of multiple substances, making them not suited for quantification of a glucocorticoid profile needed to obtain a more accurate and complete view on the HPI or HPA axis (re)activity, respectively. However, in-depth validated immunoassays for the dominant glucocorticoid can be useful in cases when only an indication (i.e., qualitative) of stress is needed. In addition, the use of pooled samples (e.g., for whole body of fish larva) renders it impossible to take into account the coping style of a single individual.

As a consequence, internationally validated confirmation methods for quantification of a glucocorticoid profile comprising: (i) the dominant hormone (e.g., cortisol); (ii) its direct precursors (i.e., 17α-hydroxyprogesterone and 11-deoxycortisol; as both will certainly lead to cortisol production); (iii) its endogenously present phase I metabolites (i.e., cortisone and 20β-dihydrocortisone; as feedback regulation of cortisol at pre-receptor level is mediated by 11β-HSD and 20β-reductase, respectively); and (iv) the most abundant more polar excreted phase I metabolites (i.e., tetrahydrocortisol and tetrahydrocortisone; to establish if exogenous glucocorticoids present in the environment (e.g., from water) or anthropogenic derived glucocorticoids (e.g., from hands) may have influenced the results) in non-pooled samples are pivotal in stress research across vertebrates.

AUTHOR CONTRIBUTIONS

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

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Time-Lag in Feeding Schedule Acts as a Stressor That Alters Circadian Oscillators in Goldfish

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The circadian system controls temporal homeostasis in all vertebrates. The light-dark (LD) cycle is the most important *zeitgeber* ("time giver") of circadian system, but feeding time also acts as a potent synchronizer in the functional organization of the teleost circadian system. In mammals is well known that food intake during the rest phase promotes circadian desynchrony which has been associated with metabolic diseases. However, the impact of a misalignment of LD and feeding cycles in the entrainment of fish circadian oscillators is largely unknown. The objective of this work was to investigate how a time-lag feeding alters temporal homeostasis and if this could be considered a stressor. To this aim, goldfish maintained under a 12 h light-12 h darkness were fed at mid-photophase (SF6) or mid-scotophase (SF18). Daily rhythms of locomotor activity, clock genes expression in hypothalamus, liver, and head kidney, and circulating cortisol were studied. Results showed that SF6 fish showed daily rhythms of *bmal1a* and *clock1a* in all studied tissues, being in antiphase with rhythms of *per1* genes, as expected for proper functioning clocks. The 12 h shift in scheduled feeding induced a short phase advance (4–5-h) of the clock genes daily rhythms in the hypothalamus, while in the liver the shift for clock genes expression rhythms was the same that the feeding time shift (∼12 h). In head kidney, acrophases of *per* genes underwent a 12-h shift in SF18 animals, but only 6 h shift for *clock1a*. Plasma cortisol levels showed a significant daily rhythm in animals fed at SF6, but not in SF18 fish fed, which displayed higher cortisol values throughout the 24-h. Altogether, results indicate that hypothalamus, liver, and head kidney oscillate in phase in SF6 fish, but these clocks are desynchronized in SF18 fish, which could explain cortisol alterations. These data reinforce the hypothesis that the misalignment of external cues (daily photocycle and feeding time) alters fish temporal homeostasis and it might be considered a stressor for the animals.

Keywords: goldfish, hypothalamus, interrenal tissue, liver, circadian system, food intake, clock genes

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INTRODUCTION

The circadian system in vertebrates is formed by a widespread network of self-sustainable endogenous clocks located in central and peripheral tissues (Albrecht, 2012; Schibler et al., 2015; Costa et al., 2016; Isorna et al., 2017). These clocks generate circadian endogenous rhythms with a period close, but generally not equal, to 24 h, providing a temporal organization for

physiological and behavioral activities making it possible to predict environmental changes (i.e., *zeitgebers*; Albrecht, 2012; Tsang et al., 2014; Challet, 2015). The most important environmental factor that entrains circadian oscillators is the light-dark (LD) cycle, and clocks synchronized by this *zeitgeber* ("time giver" in German) are named Light-Entrainable Oscillators (LEOs; Reppert and Weaver, 2002; Mendoza and Challet, 2009). However, feeding time is also an important *zeitgeber*, especially for peripheral clocks, and clocks entrained by feeding-fasting cycles are known as Feeding-Entrainable Oscillators (FEOs; Damiola et al., 2000; Mendoza and Challet, 2009).

The circadian clocks machinery is well conserved in vertebrates and it is based on transcriptional-translational feedback loops. The positive limb of the main loop is represented by two transcription factors, CLOCK (Circadian Locomotor Output Cycles Kaput) and BMAL1 (Brain and Muscle ARNT-Like 1), whose heterodimer binds to an E-box rich region in the promoter of the negative limb genes *period* (*per*) and *cryptochrome* (*cry*) (Gekakis et al., 1998; Nakamura et al., 2008). This binding promotes the expression of these last two clock genes, whose products PER and CRY heterodimerize in the cytoplasm and translocate into the nucleus to repress CLOCK-BMAL1 transactivation (Hastings et al., 2007; Nader et al., 2010; Schibler et al., 2015). Moreover, the CLOCK-BMAL1 heterodimer also induces the expression of genes known as clock-controlled genes (CCG), which are considered the outputs of the clock by binding to the E-boxes in their promoters (Hastings et al., 2007; Vatine et al., 2011; Albrecht, 2012). The functioning of this molecular mechanism is conserved, although several copies of these clock genes have been reported in fishes (Vatine et al., 2011; Sánchez-Bretaño et al., 2015a).

In mammals, the master pacemaker is a LEO located in the suprachiasmatic nucleus of the hypothalamus (Reppert and Weaver, 2002; Welsh et al., 2010) that controls in an hierarchical manner the rest of pacemakers widely distributed over the organisms (Dibner et al., 2010). It is evident that the organization of the circadian system in fish is less hierarchical than in mammals, since a master clock has not been clearly identified yet (Moore and Whitmore, 2014; Sánchez-Bretaño et al., 2015a; Isorna et al., 2017). Despite the greater or lesser hierarchical role of central pacemakers, evidences of the physiological relevance of peripheral circadian clocks in vertebrates are emerging. It is suggested that the entrainment of peripheral clocks by feeding-fasting cycles allows peripheral tissues to anticipate food supply, and potentially optimizing processes required for food digestion, metabolism, and energy storage and utilization (Vera et al., 2007; Lamia et al., 2008). Indeed, food intake during the rest phase promotes circadian desynchrony, which has been associated with metabolic diseases in mammals (Ferrell and Chiang, 2015; Ramirez-Plascencia et al., 2017), thus a time-lag feeding schedule can be considered a stressor that alters temporal homeostasis. In fish, feeding time is a potent *zeitgeber* for peripheral oscillators of the gastrointestinal tract (Isorna et al., 2017). In fact, feeding time affects daily locomotor activity rhythms (Aranda et al., 2001; Cavallari et al., 2011; Feliciano et al., 2011); clock genes expression in liver, gut, and encephalic tissues (López-Olmeda et al., 2009, 2010; Feliciano et al., 2011; Nisembaum et al., 2012; Tinoco et al., 2014); and daily profile of circulating cortisol (Montoya et al., 2010; Cowan et al., 2017). But a variety of results are obtained depending on species and protocols employed (Cowan et al., 2017). Nevertheless, the effect of feeding time on the clock of the interrenal tissue has not been investigated in any fish species to date, and it is unknown if this oscillator behaves as a LEO or a FEO. In fact, the paradigm of a time-lag in feeding schedule and its consequences in locomotor activity, peripheral oscillators and cortisol production has not been studied all at once and in the same species.

Therefore, the aim of this work was to study, if a time-lag in scheduled feeding alters temporal homeostasis in fish and to test its possible role as a stressor. To this end, we have studied the effects of 12 h shifted feeding schedule on daily expression of clock genes in the hypothalamus and two peripheral oscillators, the liver and the head kidney in goldfish (*Carassius auratus*). We have also investigated if this paradigm affects circulating cortisol daily rhythms as stress indicator and hepatic leptin expression as a putative output of the liver clock. The interest to study such oscillators is based on several reasons. The hypothalamus plays a key role in the control of both, energy homeostasis and the hypothalamus-pituitary-interrenal (HPI) axis, acting as an integrative core of environmental and endogenous signals. The role of the liver as a nexus between metabolism and circadian system in mammals and fish has been outlined (Albrecht, 2012; Schmutz et al., 2012; Tsang et al., 2014; Schibler et al., 2015), emphasizing this tissue as a key food-sensitive clock. Finally, the interrenal tissue (contained in the head kidney) is the main source of cortisol, which initiates the stress response (Schreck and Tort, 2016), and its daily rhythm is considered as the most robust hormonal rhythmic output in vertebrates (Isorna et al., 2017; Spencer et al., 2018).

MATERIALS AND METHODS

Animals and Housing

Goldfish (*C. auratus*) with a body weight (bw) of 24 ± 5 g were obtained from a local commercial supplier (ICA, Madrid, Spain). Fish were housed in 60 l aquaria with filtered and aerated fresh water (21 ± 2°C) under a 12 h light and 12 h darkness (12L:12D) photoperiod (lights on at 8 am, considered as *Zeitgeber* Time 0, ZT 0). Fish were fed with automatic feeders that daily delivered food pellets (1% bw; Sera Pond Biogranulat, Heinsberg, Germany) at ZT 2. Animals were acclimated during 2 weeks under these conditions before the beginning of the experiments. The experiments comply with the Guidelines of the European Union Council (UE63/2010), and the Spanish Government (RD53/2013) for the use of animals in research and were approved by the Animal Experimentation Committee of Complutense University (O.H.-UCM-25-2014), and the Community of Madrid (PROEX 107/14).

Experimental Design

Two groups of fish maintained under the same 12L:12D photoperiod (lights on at 8 a.m.) were fed with different schedules with automatic feeders to avoid the negative effects of the human feeding activities. One group (n = 36, placed in six aquaria, six fish/tank) was daily fed at mid-photophase (ZT 6, named Scheduled Feeding 6, SF6), and the other one (n = 36, placed in six aquaria) was daily fed at mid-scotophase (ZT 18, named SF18). Three weeks later, goldfish were sampled each 4 h throughout a 24 h cycle (one tank (n = 6) per sampling time at ZT 5, ZT 9, ZT 13, ZT 17, ZT 21, and ZT 1). Blood was collected from the caudal vein of anesthetized animals (tricaine methanesulfonate, MS-222, 0.14 g/l; Sigma-Aldrich, Madrid, Spain), and plasma was obtained after blood centrifugation and stored at −80°C until assay. Fish were then sacrificed by anesthetic overdose (MS-222, 0.28 g/l), and hypothalamus, head kidney, and liver were quickly collected, frozen in liquid nitrogen and stored at −80°C until analysis.

Locomotor Activity Recordings

Daily locomotor activity was recorded during the experimental period by six infrared photocells (Omron Corporation, E3S-AD12, Japan) fixed on the walls of each aquarium wall. Two photocells were located below the automatic feeder (for recording feeding-related activity), while the remaining four photocells were placed at a height of 3–9 cm above the bottom in each aquaria wall (for recording general locomotor activity). With this arrangement of photocells, we obtained reproducible actograms, more photocells increase the total amount of activity but does not affect daily profiles. Each photocell continuously emitted an infrared light beam which was interrupted each time fish swam in that zone, generating an output signal. The number of light beam interruptions was automatically registered every 10 min by a computer with specific software (Micronec, Spain). The aquaria walls were covered with opaque paper to minimize external interferences during the experiment. Data were analyzed using the chronobiology software EL TEMPS®(Prof. Antoni Díez Noguera, University of Barcelona), and actograms and periodograms were performed.

Gene Expression Analysis

Total RNA from hypothalamus, head kidney, and liver were isolated using TRI®Reagent (Sigma-Aldrich) and treated with RQ1 RNase-Free DNase (Promega, Madison, United States) according to the manufacturer's instructions. Then, 0.3 μg of total RNA was reverse transcribed into cDNA in a 25 μl reaction volume using random primers (Invitrogen, Carlsbad, United States), RNase inhibitor (Promega), and SuperScript II Reverse Transcriptase (Invitrogen). The reverse transcription reaction conditions consisted of an initial step at 25°C for 10 min, an extension at 42°C for 50 min, and a denaturalization step at 70°C for 15 min. Real-Time quantitative PCRs (RT-qPCRs) were carried out by duplicate in a CFX96 Real^{TM}-Time System (Bio-Rad Laboratories, Hercules, United States), using iTaq^{TM} Universal SYBR®Green Supermix (Bio-Rad Laboratories) using a 96-well plate loaded with 1 μl of cDNA and a final concentration of 0.5 μM of each forward and reverse primers in a final volume of 10 μl. Each PCR run included also a four-points serial standard curve, non-retrotranscribed-RNA (as positive control) and water (as negative control). The RT-qPCR cycling conditions consisted of an initial denaturation at 95°C for 30 s and 40 cycles of a two-step amplification program (95°C for 5 s and 60°C for 30 s). A melting curve was systematically monitored (temperature gradient at 0.5 C/5 s from 70 to 90°C) at the end of each run to confirm the specificity of the amplification reaction. The Gene Data Bank reference numbers and the primers (Sigma-Aldrich) sequences employed for target genes (clock genes: *per1a*,

TABLE 1 | Accession numbers of the genes and primers sequences employed in quantitative RT-qPCR studies.

Gene	Accession number		Primer sequence 5′→3′	Product (bp)
per1a	EF690698	Forward	CAGTGGCTCGA ATGAGCACCA	155
		Reverse	TGAAGACCTG CTGTCCGTTGG	
per1b	KP663726	Forward	CTCGCAGCTC CACAAACCTA	235
		Reverse	TGATCGTGCA GAAGGAGCCG	
per2a	EF690697	Forward	TTTGTCAATC CCTGGAGCCGC	116
		Reverse	AAGGATTTGC CCTCAGCCACG	
per3	EF690699	Forward	GGCTATGGCAGT CTGGCTAGTAA	130
		Reverse	CAGCACAAAAC CGCTGCAATGTC	
bmal1a	KF840401	Forward	AGATTCTGTT CGTCTCGGAG	161
		Reverse	ATCGATGAGTC GTTCCCGTG	
clock1a	KJ574204	Forward	CGATGGCAGC ATCTCTTGTGT	187
		Reverse	TCCTGGATCTG CCGCAGTTCAT	
leptin al	FJ534535	Forward	AGCTCCTCA TAGGGGATC	192
		Reverse	TAGATGTCGTT CTTTCCTTA	
ef-1α	AB056104	Forward	CCCTGGCCA CAGAGATTTCA	101
		Reverse	CAGCCTCGAA CTCACCAACA	

per period; bmal1a, brain and muscle ARNT-like 1a; clock1a circadian locomotor output cycles kaput 1a; ef-1α, elongation factor-1α.

per1b, *per2a*, *per3*, *bmal1a,* and *clock1a*; and *leptin aI)* and the reference gene (*ef-1α*) are shown in **Table 1**. The $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001) was used to determine the relative mRNA expression (fold change). Data obtained were normalized to the group with the lowest expression in each gene.

Plasma Cortisol Assay

Plasma cortisol levels were determined by enzyme–linked immunosorbent assay (ELISA) using a commercial kit (Demeditec, Schleswig-Holstein, Germany), previously validated for goldfish plasma (Azpeleta et al., 2010). The lowest analytical detectable level of cortisol that can be distinguished from the zero calibrator was 3.79 ng/ml. Free cortisol values were expected to be within the range described by the manufacturer (10–800 ng/ml), therefore no dilution was necessary.

Data Analysis

The existence of significant periods in daily locomotor activity was analyzed by constructing chi-square periodograms with a significance level set at 0.05 (EL TEMPS®). A one-way ANOVA followed by the *post hoc* Student-Newman-Keuls (SNK) test was performed to compare data obtained for gene expression and cortisol levels at different sampling points (using SigmaPlot 12.0 statistics package). When necessary, data were transformed to logarithmic or square root scale to normalize and to obtain homoscedasticity. Statistical differences among groups were noted with different letters. In addition, we have performed a Mann-Whitney *U* Test for analyzing the differences between the mean of cortisol levels in fish fed at ZT 6 and ZT 18. A probability level of $p < 0.05$ was considered statistically significant in all tests. Daily (24 h) significant rhythms in gene expression and cortisol were determined by Cosinor analysis fitting the data to sinusoidal functions by the least squares method (Duggleby, 1981). The formula used was $f(t) = M + A\cos(t\pi/12 - \Phi)$, where $f(t)$ is the gene expression level at a given time, the mesor (M) is the mean value, A is the sinusoidal amplitude of oscillation, t is time in hours, and Φ is the acrophase (time of peak expression). Non-linear regression allows the estimation of M, A, Φ, and their standard errors (SE), which are calculated on the residual sum of squares in the least-squares fit (Duggleby, 1981; Delgado et al., 1993). Significance of Cosinor analysis was defined by the noise/signal of amplitude calculated from the ratio *SE(A)/A* (Nisembaum et al., 2012).

RESULTS

Effects of Feeding Time on Synchronization of Locomotor Activity Daily Rhythms

Daily locomotor activity was registered during 14 days before sampling. Representative double-plotted actograms with the general locomotor activity of fed fish at ZT 6 and ZT 18 are shown in **Figures 1A,B**, respectively, while the feeding-related activity is shown in **Figure 1C** (SF6) and **Figure 1D** (SF18). General activity of SF6 goldfish displayed a diurnal significant rhythm (evidenced by a significant 24 h period; **Figure 1A**), with higher general activity during the photophase (80% of total activity). As

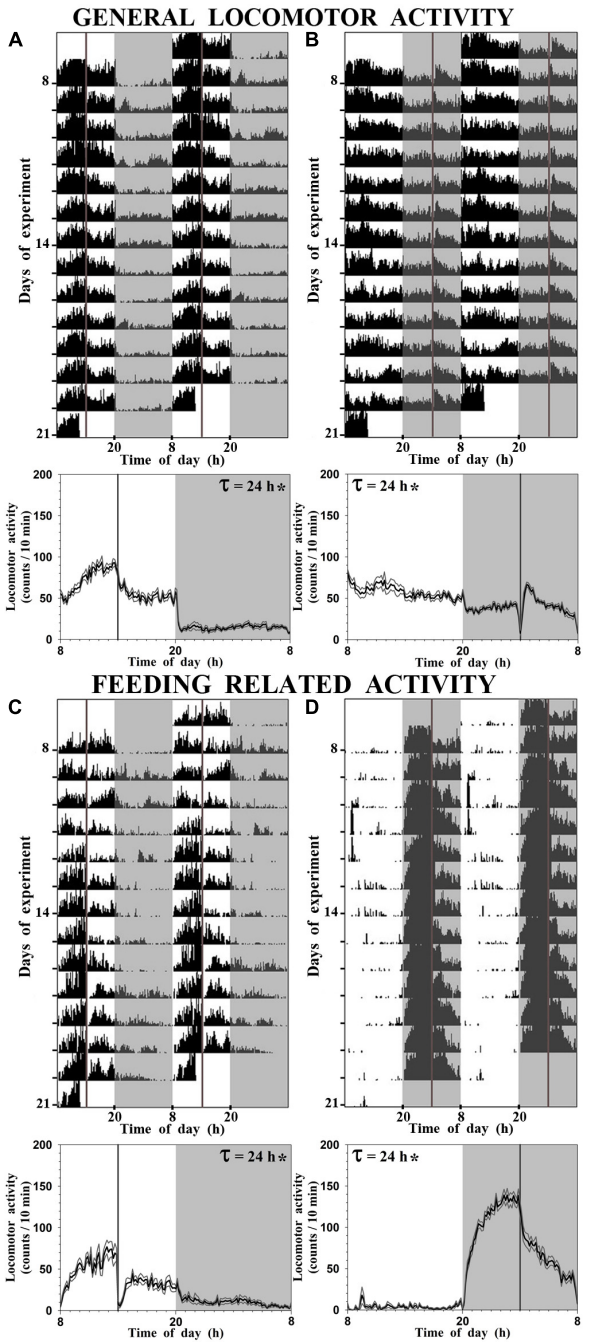

FIGURE 1 | Representative actograms and daily average waveform of goldfish maintained under a 12L:12D photoperiod and fed at ZT 6 [SF6, **(A**,**C)**] or at ZT 18 [SF18, **(B,D)**]. For convenient visualization, data in the actograms have been double-plotted in a time scale of 48 h. **(A,B)**, general locomotor activity; **(C,D)**, feeding-related locomotor activity. In the average waveform graphs, black line represents the mean, gray lines represent the SEM, and τ indicates the period of the rhythm when significant (*).

expected, the feeding-related activity was concentrated around 3–4 h before scheduled feeding, corresponding to the food anticipatory activity (FAA), with a significant daily rhythm with a period of 24 h (**Figure 1C**). When scheduled feeding time was shifted to the mid-scotophase, the general locomotor activity remained rhythmic (period of 24 h), but its 24 h profile was flattened (**Figure 1B**), and surprisingly general locomotor activity continued being higher during the photophase (60% of total activity). Nevertheless, fish fed at ZT 18 showed a robust FAA during the night with a significant daily rhythm (period of 24 h; **Figure 1D**).

Daily Rhythms of Clock Genes Expression in Goldfish

In the hypothalamus of SF6 animals, all studied genes exhibited significant 24 h rhythms (**Figure 2**), with acrophases of *per1* genes at the end of the dark phase (ZT 22.7 for *per1a*; **Figure 2A**) and at the light onset (ZT 1.2 for *per1b*; **Figure 2B**). These rhythmic profiles are in antiphase with those shown by *bmal1a* (ZT 11.3; **Figure 2E**) and *clock1a* (ZT 14.3; **Figure 2F**). Hypothalamic *per3* expression in the SF6 fish peaked around ZT 4 (**Figure 2D**), while the maximum expression of *per2a* occurred at midday (ZT 7.6; **Figure 2C**). The expression profiles of the clock genes in the scheduled-fed goldfish at ZT 18 also showed 24 h rhythms in the hypothalamus (**Figures 2A,B,D,E**), except for *per2a* and *clock1a*, whose rhythms were lost (**Figures 2C–F**). The shift in the scheduled feeding time from ZT6 to ZT18 advanced 4–5 h the acrophases in the case of *per1a, per1b,* and *bmal1a* genes, and 9 h for *per3* (**Figures 5A,B**) in hypothalamus.

In the head kidney, all examined clock genes showed significant daily variation in their expression in both groups of scheduled-fed goldfish (SF6 and SF18; **Figure 3**), with the exception of *per2a* and *bmal1a*, which lost their significant daily rhythmicity when scheduled feeding was shifted from midday to midnight (**Figures 3C–E**). The daily expression profiles in the head kidney of SF6 fish were broadly similar to the rhythms observed in the hypothalamus, with similar acrophases, as it can be observed in polar graphs (**Figures 5A–D**). However, a slight shift seems to exist for *per1b* and *per1a* in the head kidney of SF6 fishes compared to the hypothalamus of the same animals (**Figures 5A–C**). The amount of *per1* transcripts peaked at the early morning, which is in antiphase with the expression of *bmal1a* and *clock1a*, whose acrophases were located at the end of the light phase and beginning of the dark phase, as occurs in the hypothalamus. Thus, hypothalamic and head kidney oscillators seem to be in phase in SF6 fish. In contrast to the minor effect observed in the hypothalamus, the 12 h-shift in feeding schedule produced a complete shift (11–13 h) in *per1* and *per3* rhythms in the head kidney of goldfish, but only a 6 h advance for *clock1a*, suggesting that these negative and positive elements of the head kidney clock were not in antiphase. The expression of *per2a* showed a significant rhythm in the head kidney of SF6 but not in SF18 fish, as occurs in the hypothalamus, with similar acrophases in both tissues.

Clock genes expression in the goldfish liver displayed significant 24 h rhythms in both SF6 and SF18 fish (**Figure 4**), except for *per2a,* which did not show daily rhythmicity in any studied groups (**Figure 4C**). In SF6 animals, rhythmic profiles of clock genes expression were similar to those observed in the hypothalamus and the head kidney. The acrophases of *per1* rhythms are located at the light onset (ZT 0.7 and ZT 0.9 for *per1a* and *per1b,* respectively; **Figures 4A,B**) or the early morning (ZT 3.4 h for *per3*; **Figure 4D**), which is in antiphase with *bmal1a* (ZT 10.0) and *clock1a* genes (ZT 9.0; **Figures 4E,F**, **5E,F**). When feeding schedule was shifted from midday to midnight, all clock genes also underwent a 12 h shift in their acrophases, being moved to the LD transition in the case of *per* genes and to the light onset for *bmal1a* and *clock1a* genes (**Figures 4**, **5**). Thus, the hepatic oscillator seems to be in phase (i.e., positive elements *vs.* negative elements) in both SF6 and SF18 fishes, as in the hypothalamus, but not in the head kidney.

Comparing the clocks in the three analyzed tissues, in SF6 animals these clocks ticked at time (i.e., clock genes are in phase in the different tissues). However, acrophases of clock genes rhythms in the hypothalamus of SF18 animals were in antiphase with the hepatic ones, being the head kidney oscillator in an intermediate condition. Another different aspect of the liver oscillator, compared to the hypothalamus, and the head kidney, is referred to the amplitudes of the genes, which were much higher in the liver. In this sense, the amplitudes of *per* genes were more than 10 times higher than in the hypothalamus and about 3–5 times higher than in the head kidney in both SF6 and SF18 animals.

Daily Rhythms of Circulating Cortisol and Leptin Expression in the Liver

Circulating cortisol displayed a significant daily rhythm in goldfish fed at midday with a robust amplitude (143.8 ng/ml) and the acrophase during the scotophase (at ZT 18.9; **Figure 6A**) 6 h before lights on. By contrast, in the SF18 group this 24 h rhythmicity was fully abolished. Moreover, the SF18 fed fish showed significantly higher levels of cortisol (202.19 $\pm$ 22.78 ng/ml) than that observed in SF6 fed fish (126.95 $\pm$ 23.06 ng/ml) ($p < 0.05$, Mann-Whitney *U* Test). Hepatic *leptin aI* expression showed significant daily rhythms in both SF6 and SF18 fish (**Figure 6B**). The acrophase of *leptin aI* rhythm was found at the middle of the scotophase (ZT 17.6) in fish fed at ZT 6, while it was shifted at midday (ZT 5.8) in SF18 fish. Thus, the 12-h-shift in feeding schedule from midday to midnight induced a 12-h shift in the rhythmic expression of *leptin aI* in goldfish liver.

DISCUSSION

Results obtained clearly show that a shift in feeding schedule alters temporal homeostasis in goldfish, as it differently affects clocks (i.e., clock genes expression rhythms) in the hypothalamus, the liver, and the head kidney. In fish fed at midday, these three oscillators tick at time with similar acrophases for each gene

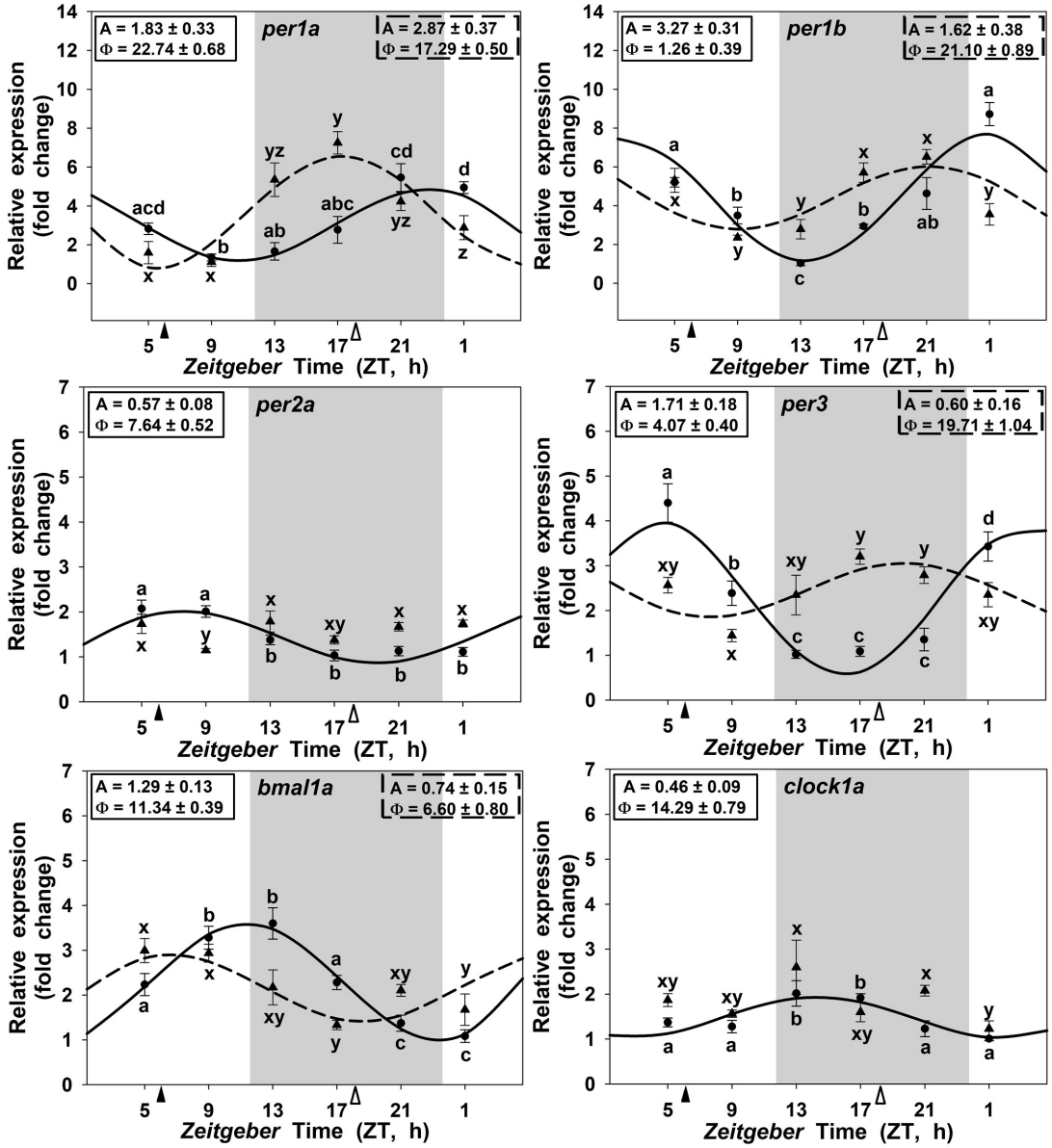

FIGURE 2 | Daily profile of clock genes expression in the hypothalamus of SF6 (●) and SF18 (▲) goldfish maintained under a 12L:12D photoperiod. Gray area indicates the dark period while feeding time is indicated by triangles in the x-axis (solid, ZT 6; white, ZT 18). Data obtained by RT-qPCR are shown as mean ± SEM (n = 6) in relative units ($2^{-\Delta\Delta Ct}$ method). Different letters (**a–d** in SF6 and **x–z** in SF18) indicate significant differences. When Cosinor [$SE(A)/A < 0.3$] was significant, periodic sinusoidal functions were represented as solid waves (SF6 fish) or dashed waves (SF18 fish), and amplitudes and acrophases (A and Φ, respectively) are shown at the top of the panels (SF6, left; SF18 right).

in the different tissues. However, in fish fed at mid-scotophase, daily expression rhythms of clock genes are not in phase in the different tissues, and *per1* and *clock-bmal* genes do not follow their characteristic profiles of expression in antiphase, particularly in the head kidney. Then, time-lag in feeding schedule seems to represent a stressor for the animals, since alters the temporal homeostasis, with increases in plasma cortisol and the disappearance of its daily rhythm in fish fed in the mid-scotophase.

It is widely known that food acts as a potent *zeitgeber* for circadian rhythms when restricted or provided on a periodic basis (Hara et al., 2001; Stephan, 2002). As expected, goldfish adapted their daily locomotor activity to feeding schedule; SF6 fish showed a robust FAA in the photophase while SF18 fish showed it during the scotophase. It is previously reported that a scheduled feeding under continuous light (Vera et al., 2007; Feliciano et al., 2011), at the start or the end of the photophase (Aranda et al., 2001), or at the beginning of the scotophase

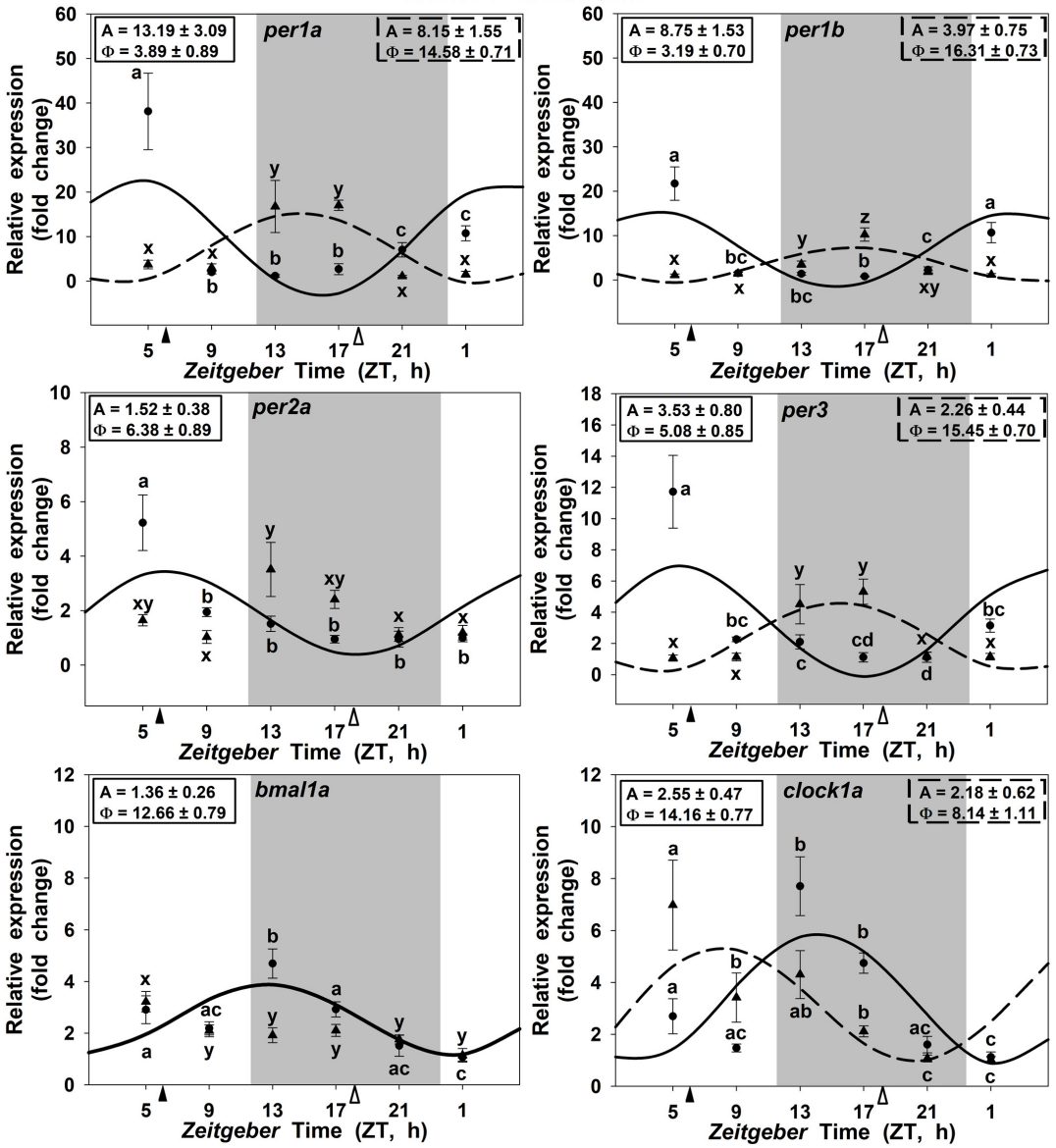

FIGURE 3 | Daily profile of clock genes expression in the head kidney of SF6 (●) and SF18 (▲) goldfish maintained under a 12L:12D photoperiod. Gray area indicates the dark period while feeding time is indicated by triangles in the x-axis (solid, ZT 6; white, ZT 18). Data obtained by RT-qPCR are shown as mean ± SEM (n = 6) in relative units ($2^{-\Delta\Delta Ct}$ method). Different letters (**a–c** in SF6 and **x–z** in SF18) indicate significant differences. When Cosinor [$SE(A)/A < 0.3$] was significant, periodic sinusoidal functions were represented as solid waves (SF6 fish) or dashed waves (SF18 fish), and amplitudes and acrophases (A and Φ, respectively) are shown at the top of the panels (SF6, left; SF18 right).

(Vivas et al., 2011) synchronizes daily activity to feeding time in goldfish. However, it has been also reported that if both *zeitgeber*s are present, both are important (Aranda et al., 2001). In this sense, our data revealed that SF6 goldfish are clearly diurnal (80% of the activity during the photophase), but SF18 fish has not became nocturnal, since they reduce their locomotor activity during daytime but remain active through the 24 h. In fact, they continue to move more during the photophase (60%) than during the scotophase. Thus, it seems that goldfish is not as flexible as previously suggested in terms of daily activity pattern (Isorna et al., 2017). Currently, it is not possible to discern if the alteration of locomotor activity rhythm in SF18 goldfish is related to the time-lag observed in clock genes expression, or if it is due to the loss of cortisol rhythm. Further studies are needed to assess such possibilities.

In fish fed at midday (ZT 6), the *per1a* and *per1b* genes in the hypothalamus, the head kidney and the liver displayed significant daily rhythms with their acrophases at the onset of the photophase or at the end of the scotophase, in accordance with previous reports in goldfish also maintained in 12L:12D and fed

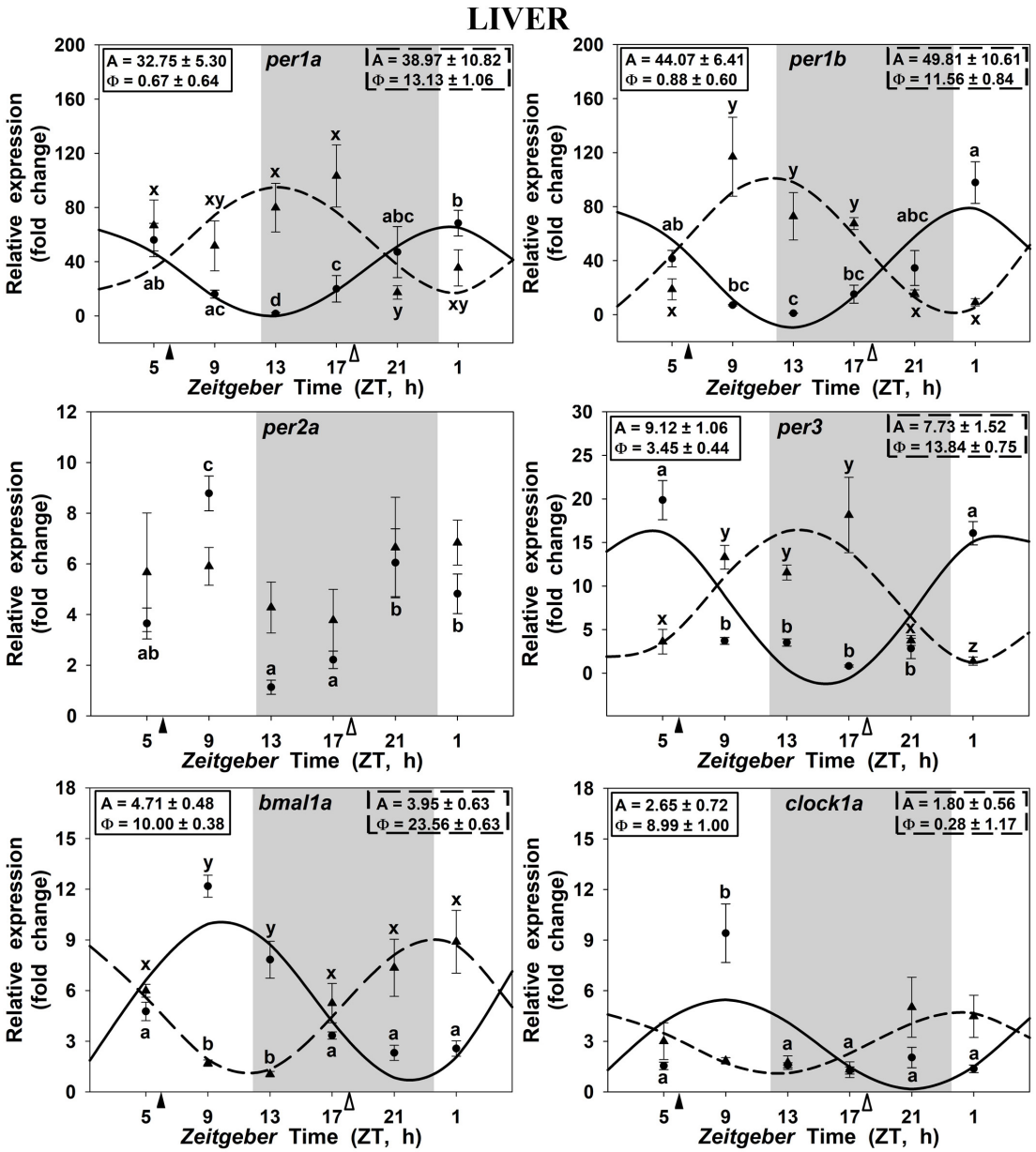

FIGURE 4 | Daily profile of clock genes expression in the liver of SF6 (•) and SF18 (▲) goldfish maintained under a 12L:12D photoperiod. Gray area indicates the dark period while feeding time is indicated by triangles in the x-axis (solid, ZT 6; white, ZT 18). Data obtained by RT-qPCR are shown as mean ± SEM (n = 6) in relative units ($2^{-\Delta\Delta Ct}$ method). Different letters (**a–c** in SF6 and **x–z** in SF18) indicate significant differences. When Cosinor [$SE(A)/A < 0.3$] was significant, periodic sinusoidal functions were represented as solid waves (SF6 fish) or dashed waves (SF18 fish), and amplitudes and acrophases (A and Φ, respectively) are shown at the top of the panels (SF6, left; SF18 right).

during the photophase at ZT 2 (Velarde et al., 2009; Nisembaum et al., 2012; Sánchez-Bretaño et al., 2015b). Similarly, a *per1* peak around the dark-light transition has been also reported in other teleosts, as zebrafish brain (*Danio rerio*; Sanchez and Sanchez-Vazquez, 2009; Vatine et al., 2011), European sea bass brain and liver (*Dicentrarchus labrax*; Sánchez et al., 2010), rainbow trout hypothalamus (*Oncorhynchus mykiss*; Patiño et al., 2011), Senegalese sole retina and optic tectum (*Solea senegalensis*; Martín-Robles et al., 2012), or Nile tilapia brain (*Oreochromis niloticus*; Costa et al., 2016). All these findings support the hypothesis that *per1* genes anticipate the light arrival in fish under these conditions (Isorna et al., 2017). Moreover, the clock genes of the positive limb of the loop (*bmal1a* and *clock1a*) were in antiphase with the negative limb genes (*per*) in these three tissues, showing their acrophases almost in the LD interphase, as previously reported in goldfish (Nisembaum et al., 2012), and other fish species under a LD photocycle (Patiño et al., 2011; Vatine et al., 2011; Martín-Robles et al., 2012; Costa et al., 2016).

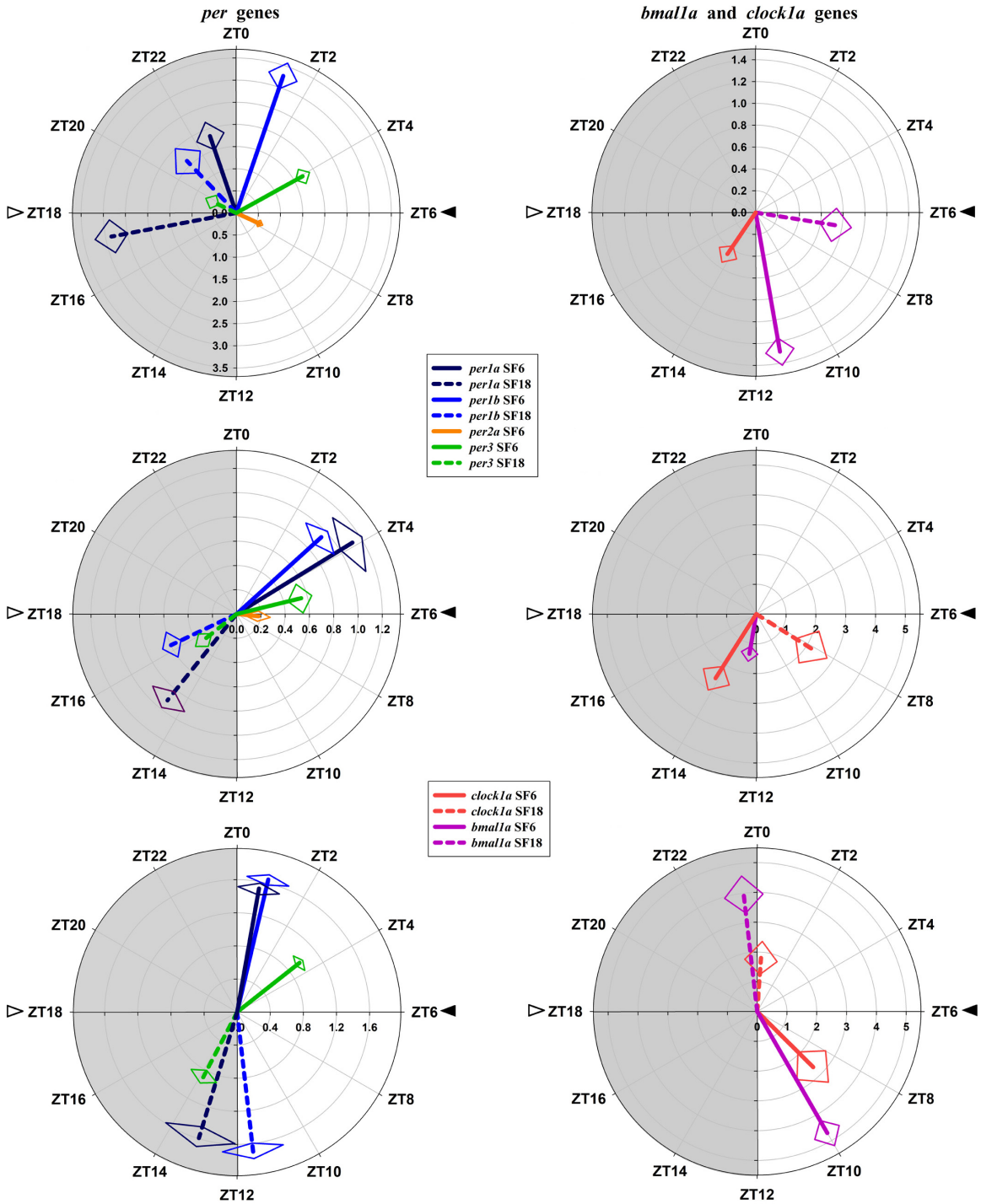

FIGURE 5 | Polar representations of parameters defining clock genes rhythms. **(A,B)** hypothalamus, **(C,D)** head kidney, **(E,F)** liver. The length of the vector (radial axis) indicates the value of the amplitude [fold change of relative expression, **C,E** in logarithmic scale]. The angular position indicates the acrophase (ZT, zeitgeber time). The SE of these two parameters is represented by the rhombus at the end of each vector.

Is feeding time able to modify such clock genes rhythmicity? As previously mentioned, food acts as a potent *zeitgeber* nor only for circadian activity rhythms (Aranda et al., 2001; Stephan, 2002; López-Olmeda et al., 2009; Refinetti, 2015) but also for clock synchronization (Damiola et al., 2000; Feliciano et al., 2011; Nisembaum et al., 2012) in mammals and fish. Our findings revealed that feeding time exerts different effects on clock genes expression at central and peripheral levels. In the hypothalamus,

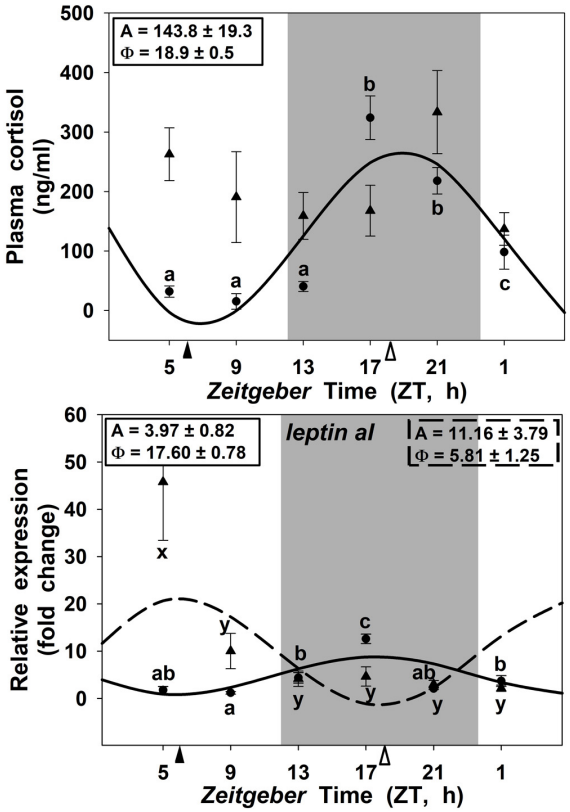

FIGURE 6 | Daily profiles of plasma cortisol **(A)** and *leptin-al* expression in the liver **(B)** of SF6 (•) and SF18 (▲) goldfish maintained under a 12L:12D photoperiod. Gray area indicates dark period and feeding time is indicated by triangles in the x-axis (solid, ZT 6; white, ZT 18). Data are shown as mean ± SEM (n = 6). Different letters (**a–c** in SF6, and **x–z** in SF18) indicate significant differences. When Cosinor [$SE(A)/A < 0.3$] was significant, periodic sinusoidal functions were represented as solid waves (SF6 fish) or dashed waves (SF18 fish), and amplitudes and acrophases (A and Φ, respectively) are shown at the top of the panels (SF6, left; SF18 right).

a 12 h shift in the feeding schedule (adjusting the feeding time at the mid-scotophase) induced a minor shifting of 4–5 h in the acrophases of the target genes (except *per2a* as expected and below discussed), in agreement with previous reports in the European sea bream (*Sparus aurata*; (Vera et al., 2013), and the Nile tilapia brain (Costa et al., 2016). These findings indicate that feeding time is able to induce a slight displacement of the acrophases, but the LD cycle seems to be the main synchronizer of the rhythmic expression of hypothalamic clock genes, as previously suggested (Hara et al., 2001; Sanchez and Sanchez-Vazquez, 2009; Feliciano et al., 2011; Nisembaum et al., 2012; Tinoco et al., 2014). Interestingly, the amplitudes of the central clock genes were diminished when the food was supplied at midnight (except for *per1a*), suggesting that feeding-fasting cycles enhance LD driven-daily rhythms, in agreement with previous reports (Sánchez-Bretaño et al., 2015a).

It is worthy to highlight the case of *per2a*, the only gene that did not change its expression pattern in any of the three studied tissues when feeding time was shifted. Previous reports have shown that *per2a* displayed a rhythmic expression in some central and peripheral tissues of goldfish, under a LD cycle with acrophases at midday (Velarde et al., 2009; Nisembaum et al., 2012), as in sea bass brain (Herrero and Lepesant, 2014). Such rhythms usually disappear in constant conditions, light or darkness (Feliciano et al., 2011; Nisembaum et al., 2012; Vera et al., 2013), showing that *per2a* rhythmicity is strongly dependent of the LD cycle. Indeed, it is well-known that *per2a* is a light-induced gene with a key role in the molecular mechanism that entrains the LEOs in zebrafish (Vatine et al., 2011; Moore and Whitmore, 2014; Ben-Moshe et al., 2014; Ceinos et al., 2018). Our results support this role of *per2a* as a light-dependent clock gene also in goldfish.

A substantial finding is the 12 h shifting in the acrophases of all hepatic clock genes when feeding time was shifted 12 h (from midday to midnight). Unlike in the hypothalamus, amplitudes of all rhythms shown by the different clock genes in the liver were not significantly affected by feeding time. Vera et al. (2013) obtained comparable results, reporting a 6–7 h shifting in the liver of sea bream fed at mid-photophase compared to fish fed at the mid-scotophase. All these data point out that feeding time is a synchronizer powerful than the LD cycle in the liver, as it is previously proposed in mammals (Damiola et al., 2000; Stokkan et al., 2001; Kornmann et al., 2007). This conclusion was also suggested by Feliciano et al. (2011), who demonstrate significant rhythms for clock gene expression driven by the last meal, independently of previous feeding approaches (random or scheduled feeding). Therefore, the hepatic clock might be a peripheral FEO in goldfish. In terms of adaptation to the new scheduled feeding, the shift in clock genes expression could be an advantage for the animal physiology. However, overt rhythms (i.e., outputs of the circadian system) are complex and usually dependent of more than one oscillator. Thus, although liver clock genes are synchronized to receive food at mid-scotophase, metabolic rhythms could not be adapted. In this sense, lipid metabolism rhythmicity is linked to the LD cycle, independently of feeding time in zebrafish and sea bream liver (Paredes et al., 2014, 2015), although feeding time drives clock genes oscillations in the last species (Vera et al., 2013). Surprisingly, our results show that hepatic leptin expression rhythms match with clock genes expression rhythms in liver, and the acrophase is 12 h shifted in SF6 compared to SF18 animals. This suggests that maybe not all of the metabolic outputs are driven by the same *zeitgebers* in the liver of goldfish.

Regarding the head kidney, fish fed at midday exhibit significant daily rhythms in the expression of all clock genes, with genes of the positive and negative limbs of the loop in antiphase (except *per2a*, as above discussed), confirming the existence of a functional clock in this tissue, as in the adrenal gland of mammals (Son et al., 2008; Kwon et al., 2011). Even though, the interrenal tissue of goldfish is not directly related to the gastrointestinal system, feeding time seems to play an important role on its synchronization, since the expression of *per1* genes had a peak just before the expected feeding time in both experimental groups (at ZT∼4 when food was provided at ZT 6, and at ZT∼15 when provided at ZT 18). Hence, the 12 h time-lag in the feeding time shifted the rhythmic expression pattern of *per1* genes, similarly

as the liver's response. This is not surprising, given that several peripheral clocks appear to be entrained by food in mammals (Albrecht, 2012) and in fish (López-Olmeda et al., 2010; Feliciano et al., 2011). For instance, food intake has been proven to be a potent synchronizer not only for the liver (Damiola et al., 2000; Stokkan et al., 2001; Kornmann et al., 2007), but also for the heart (Schibler et al., 2003; Mukherji et al., 2015) in mammals. In fish, meal time synchronizes the expression of clock genes in posterior intestine and liver of goldfish (Feliciano et al., 2011; Nisembaum et al., 2012; Tinoco et al., 2014), as well as in heart and fin of zebrafish (Cavallari et al., 2011). These evidences suggest that the feeding schedule has an essential role on the organization of the circadian system in vertebrates, beyond exclusively regulating digestive functions. Although it clearly seems that the interrenal tissue of midday-fed fish is a functional circadian clock, the fact that *clock1a* is not in antiphase with *per1* genes, and *bmal1a* lost its rhythmicity in goldfish fed at mid-scotophase, calls into question the functionality of the clock under this time-lag condition, and support that temporal homeostasis in SF18 animals is altered. Then, the time-lag in feeding schedule may be a stressor for goldfish.

The better adaptation of SF6 fish compared to SF18 is also supported by cortisol results. Our results demonstrate the existence of a daily cortisol rhythm in fish fed at midday, with a peak 5 h before the light onset, which correlates with the functional interrenal clock observed in this group. Conversely, animals fed at the mid-scotophase did not show a daily cortisol rhythm, owing to the fact that the basal levels of this hormone are constantly elevated, being 10 times higher than the basal levels found in midday-fed fish. Such cortisol increase in SF18 fish could be a response to a stressful situation, such as the conflict between environmental cues (light/dark cycle and meal time), that mismatches the phase of hypothalamic, hepatic, and interrenal oscillators. This alteration of circulating cortisol might be due to an altered functionality of the interrenal clock in fish fed at mid-scotophase, in agreement with the hypothesis (under debate) that a local functional clock in the interrenal tissue is necessary to maintain cortisol daily rhythms. In this sense, it is suggested that the adrenal clock could influence the circadian changes in circulating glucocorticoids in mammals (Oster et al., 2006). In fact, fish, and mammals are able to maintain daily cortisol rhythms after an hypophysectomy and in absence of cyclic ACTH levels (Srivastava and Meier, 1972; Meier, 1976), and adrenal clock genes maintain their cyclic expression in rats without a functional hypophysis (Fahrenkrug et al., 2008).

In summary, a time-lag in feeding schedule mismatches clock genes expression in the hypothalamus, the liver, and the interrenal tissue. The increment in cortisol values and the loss of its daily rhythmicity in goldfish fed at mid-scotophase could indicate that these fish are under a stressor. Thus, our results show that the loss of temporal homeostasis can negatively affect the physiology in goldfish and the underlying links between clocks and functional outputs deserve to be explored.

AUTHOR CONTRIBUTIONS

MG-B, NdP, and EI conceived and designed the experiments. MG-B, NS, and EI analyzed the samples. All authors participated in sampling animals, interpreted findings, drafted, and revised the manuscript.

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Physiological Stress in Rescued Wild Koalas Are Influenced by Habitat Demographics, Environmental Stressors, and Clinical Intervention

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Koalas are rescued from the wild often with incidence of burns from bushfire, injury from animal attacks, vehicle collision, and diseases. Exposure to environmental stressors (trauma and disease) could generate physiological stress and potentially impact the outcomes of clinical management intervention and rehabilitation of rescued wild koalas. It is important to quantify the stress physiology of wild koalas upon registering into clinical care. This study demonstrates the first report of physiological stress assessment in rescued wild koalas ($n = 22$) to determine the potential influences of habitat-specific demographics, stressor category, and clinical diagnosis. Fecal samples were collected from the koalas at rescue and routinely during hospitalization to provide a longitudinal assessment of the koala's stress response throughout clinical care. Fecal glucocorticoid metabolites (FCM) enzyme-immunoassay was used to index physiological stress non-invasively. Koalas were admitted with exposure to various categories of environmental trauma such as vehicle collision, dog attack, burns from forest fire (this also related to conditions such as copious drinking and flat demeanor), and other injury. The main disease diagnosed was chlamydial infections. In terms of environmental interactions, it was found that habitat-specific demographics, location where the rescued koala was found, especially the rural-urban fringe, influenced FCM levels. Furthermore, there was significant interaction between location, stressor category, and clinical diagnosis for mean FCM levels. However, these factors were not predictive of the clinical outcome (euthanized or released). Overall, the results provide invaluable insights into how wild koalas respond physiologically to environmental trauma and disease and how methods of care, husbandry, and treatment can be used to further reduce the impacts of stress with the ultimate aim of increasing the rehabilitation and future release of rescued koalas to revive the declining mainland populations.

Keywords: koala, rescue, rehabilitation, stress, environmental trauma, disease

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INTRODUCTION

Global biodiversity is in rapid decline with an increase in human use of Earth's natural resources (1). Australia is home to some of the world's most distinctive and unique fauna with 80 percent of its terrestrial mammalian species being endemic (1). However, worldwide mammalian biodiversity is showing rapid declines largely due to factors such as habitat degradation and hunting (1). It is estimated that over 50 percent of all mammal species extinctions worldwide over the past 200 years are from Australia (2). Since 1788, 28 Australian endemic land mammals have become extinct and this rate is increasing (1). These figures make Australia the worst record for mammal conservation with rates of extinction exceeding that of any continent (2, 3). There are a multitude of both environmental factors and species attributes being recognized as causations of this species decline (3). These are inclusive of anthropogenic induced environmental changes (4), shelter and foraging habitat, regional productivity, fecundity, longevity and phylogeny (3). Further factors include the introduction of predators such as cats and foxes as well as incidence of infectious diseases (3). The International Union for Conservation of Nature (IUCN) now lists 56 Australian land mammals as threatened and an additional 52 as near-threatened (1). One these threatened species is the koala (*Phascolarctos cinereus*), being recognized as threatened under both Commonwealth and State legislation (5).

Koala mortality is of increasing concern with multiple environmental and anthropogenic factors attributing to this species decline (6). Disease has been considered as one of the prevalent causes of losses (7). Both retrovirus and trypanosomes are some of the pathogens affecting koala losses however the most recognized is the incidence of Chlamydia (6). A review of historical records has recognized chlamydiosis symptoms to be present in cases as early as the 1800s (6). Symptoms associated with the disease are inclusive of kerato-conjunctivitis, pneumonia, urinary tract infections, and genital tract infections, especially in female koalas (6). These can cause adverse effects such as infertility in some koala populations (6). The spread of the disease is also Australia wide in both captive and wild populations, with little indication suggesting that it is location specific (6). Currently the diagnosis of chlamydia requires intense clinical examination including PCR detection and ultrasonography (6, 8). In general, the disease is usually presumed in koalas experiencing some of the symptoms such as sore eyes, chest infections, and "wet bottom" or "dirty tail" (6). In a wildlife hospital or clinical setting, the infection is treated through the use of antimicrobial drugs but the results thus far are mixed (6). There has been progress in the development of a chlamydial vaccine to control the disease in koala populations (6).

Further associated factors of mortality and injury to the wild koala is vehicle collision, bushfire and dog attacks (9, 10). Vehicle collision are of particular concern in heavily urbanized environments where there are small fragmented koala populations (9). In particular localities, such as Phillip Island in Victoria, vehicle collisions make up for 60% of the mortality for koala populations (9). The incidence of bushfire also threatens koala population survival causing burns and respiratory issues to individuals (10). Like the trends of road mortality, bushfire frequency is heightened in areas of habitat fragmentation (10).

The hypothalamo-pituitary adrenal (HPA) axis is active during stress, which causes release of corticotropin releasing-hormone (CRH), which travels through the hypophyseal portal system to release adrenocorticotropic hormone (ACTH) from the anterior pituitary and into the blood stream (11). ACTH then acts to release glucocorticoid (GC) steroid hormone from the cortex of the adrenal gland. GCs can either be in the form of cortisol or corticosterone and dependant on the species, either cortisol or corticosterone, or even both, are produced. Cortisol is the major GC in mammals (eutherian and metatherian species) while corticosterone is the major GC in fish, amphibians, reptiles, and birds. The effects of GCs can last from several minutes to hours. Depending on amount by which GCs are elevated can provide an insight into the severity of the stressor and how an animal reacts to it (12, 13). In koalas, cortisol has been identified as the major circulating GC (14), however both cortisol and corticosterone metabolites have been measured in excreta (15, 16). Levels of FCMs in adult healthy male and female koalas have been reported earlier in response to an ACTH stimulation test as follows; Pre-ACTH challenge; males (7.1 ± 1.29 ng/g dry feces, $n = 6$) and females (3.9 ± 0.51 ng/g dry feces, $n = 18$). Mean fecal cortisol metabolite concentrations in the males and females after the ACTH challenge were as follows: Males (8.9 ± 0.80 ng/g dry feces, $n = 19$) and females (6.7 ± 0.47 ng/g dry feces, $n = 12$).

The types of stressors and their duration can provoke an array of neuroendocrine responses and immunity capabilities of an individual (17). There is a proposed link between environmental factors affecting koala population declines such as the modification of landscapes and disease incidences, and the effect of physiological stress on immune capabilities (18). It is recognized that there is an influence of stress on disease susceptibility in wildlife species (18). Prolonged stressors or chronic stressors, result in reductions of basic immune processes (19). Short term stressors (acute stressors) however generally enhance immune responses (19). Baseline stress then describes the absolute basal levels of stress hormone secretion experienced by the individual in a state where there are no posed threats (20, 21). It is recognized that baseline GC levels have the ability to change as the organism encounters environmental fluctuations and therefore stress causes elevation of cortisol secretion (21). In wild koala populations there is no knowledge of cortisol levels in rescued koalas.

An understanding of the relationships between stress, incidence of disease and trauma and clinical outcomes is key for conservation management of wildlife populations (22). The measurement of GCs is key into investigating these relationships as they are able to indicate the stress response and physiological resilience of the animal (22). The use of non-invasive techniques such as fecal GC metabolite measurements is a significant tool to measure the stress responses whilst not increasing stress responses through invasive interactions (i.e., blood collection) (15). Being a folivore with a natural diet consisting of *Eucalyptus*

spp., which is extremely high in fibers, the koala requires a long gut system to be able to digest these products (23). In general, diets that are higher in fiber will cause a delay in GC release and gut transit time (24). It is currently approximated that digestion and GC transition to feces will take an average of at least 213 h (23). In koalas, fecal based hormone monitoring technique is highly suitable due to their long gut system and therefore a lengthy excretory lag-time of over 9 days. Therefore, the first fecal sample collected at rescue provides a window into quantifying the physiological stress responses of koalas to environmental stressors (15).

The success of wildlife rehabilitation is based on successful treatment as well as long term survival and ultimate release of the patient koalas (25). Fertility is also a leading driver in the success of rehabilitation (26). In general, there is a greater need for research in the rehabilitation process (26). Currently the success of chlamydial treatments such as topical ointment and antibiotics is lacking with high failure rates of recovery (26). Whilst there has been exploration surrounding infection treatments in clinical settings, there is no research that investigates if prior life experiences have impact on an animal's recovery and outcomes. In this study, we attempt to find out the effects of environmental stressors on the outcome of koalas in a clinical setting.

The measurement of fecal glucocorticoid metabolites and the koalas long gut system therefore allows us to have an understanding of the stress experienced by koalas several days before arrival to the clinic and also gives indication as to whether absolute baseline stress levels could affect clinical outcomes. It is hypothesized that those rescued koalas admitted to the veterinary clinic experiencing prior heightened stress levels (e.g., burn victims from bush fire) will have lowered success to recovery in the clinic and will be mainly euthanized.

MATERIALS AND METHODS

Study Koalas

This study was done through formal approval by the Charles Sturt University ACEC Committee (Protocol number: A16044). Koala health data was collected in partnership with Adelaide Koala and Wildlife Hospital (AKWH), South Australia. The hospital is dedicated to the emergency treatment, rehabilitation of injured or orphaned native wildlife. During the koala's admittance in clinic, they were housed individually in large cages and provided with fresh water and various assortments of Eucalyptus species. Sampled koalas were those in care at AKWH during the sampling period of 2015–2016.

Health Data, Habitat Demographics, and Stressor Categories

Health data provided was inclusive of hospital records for the koalas with matched fecal sampling done ($n = 22$). Hospital records contained details of health checks, age, sex, weight, stressor categories, treatments, and outcomes.

Using the AKWH records that were provided, a health summary was created for each koala which detailed their basic information (age, sex, location found, etc.) and then what treatment was used, treatments administered, how long they were in hospital for, and what their outcome was. Stressor category, location, and clinical outcomes were all categorized to allow for statistical analysis.

Habitat was categorized using Google Maps to identify the habitat demographics where the koala was found by rescuers. The habitat demographics categories included; "National Park" which indicated that the koala was picked up from within a national park, "Rural" which indicated an area that was sparsely populated and mainly included large lots of grass lands and open areas, "Semi-Urban" which was a location that was moderately populated and situated near or fringed by parkland, forest, or open grasslands, and "Urban" which were areas that is densely populated and a distance from any forests or parklands.

Stressor categories were as follows; Healthy koalas were identified as with good body condition score of >4.0 (27) and no physical signs of disease. Suspected infection cases showed physical signs such as red/swollen/sore eyes/conjunctiva, discharge, red cloaca, wet bottom, swollen genital however tested negative for chlamydia (PCR testing return negative). Injury included physical injuries sustained from any physical trauma apart from dog-attack or vehicle collision. Burn victims were koalas that were rescued from bush fire impact. Dehydrated patient identified as a koala that was found to be drinking an abnormal quantity of water for an abnormal length of time (e.g., some rescued koalas recorded drinking for over 40 min). Flat demeanor was noted when a rescued koala was found in a state of not exhibiting normal behaviors, seemed slow and depressed or was not responding to external stimulus appropriately.

Diagnosis was determined through veterinary testing and examinations. For example, a koala that had vehicle collision was found to have multiple fractures so this is what it was ultimately treated for. Another koala may have been found on the ground but ended up being treated for an infected pouch, so infected pouch was its diagnosis. Due to the nature of chlamydia and its intermittent shedding, the PCR tested negative or positive was used as a diagnosis as a –ve or +ve result could influence the FCM levels. Other common diagnosis included renal failure, arthritis (inability to climb), diabetes and respiratory illness.

Fecal Sample Collection

Fecal samples were collected from 22 koala patients admitted to the AKWH from the period of 2015–2016. During routine cage cleaning, 1–5 fresh pellets were collected from each koala daily at the same time period in the morning to avoid potential influence of circadian rhythms on FCMs. Sample size (days) ranged from $n = 2$ days−36 days depending on the length of time that each koala stayed in the clinic. Fresh pellets were initially identified by intensity of smell, mucous covering and lack of dehydration. Samples were placed into Ziplock© bags and labeled with the animal's name, date, identification number, and time of sample collection. Samples were stored at −20°C until they were sent on ice to the laboratory *via* overnight freight. Upon delivery, the fresh fecal samples were immediately frozen to minimize effects of sample age on FCM levels. All samples were analyzed within 1 month of collection.

Sample Preparation

Frozen fecal samples were dehydrated in a freeze dryer for a 24 h period (or until completely dried). Once dry, samples were ground into a fine powder up using a mortar and pestle. Each mortar and pestle was cleaned using 10% ethanol between samples. The ground up powder was sifted through a find mesh strainer to remove all course particles. A 0.2 grams (g) +/− 0.001 g sample of sifted product was weighed out into a labeled test tube and then stored in a −20°C freezer.

Fecal Cortisol Metabolite Extraction

Samples were removed from the −20°C freezer and 2 milliliters (mL) of 90% ethanol solution was added to the test tube. Tubes were vortexed at medium-high speed on an Eppendorf mini-spin centrifuge for a minimum of 30 s to thoroughly mix the solution. Tubes were then placed into a +80°C water bath for 10 min to allow hormones to dissolve in the solution. Whilst in the bath, tubes were gently shaken to ensure feces stayed submerged in ethanol and did not spill over the top of the tube. After 10 min, the contents of the tube were poured into an Eppendorf tube, closed and then centrifuged at 10,000 RPM for 5 min until the liquid residue separated from the hormones dissolved in ethanol. Following this, 0.6 mL was aliquoted into a new, clean, and labeled Eppendorf tube. Tubes were left open and stored in a laminar flow chamber for a minimum of 24 h until the ethanol has evaporated and the tube was completely dry. Once tubes were completely dried, 1 mL of assay buffer (39 mM NaH_2PO_4, 15 mM NaCl and 0.1% bovine albumin, pH 7.0) was added to the tube. Clean pipette tips were used to scrape off as much of the residue as possible. Tubes were vortexed at medium-high speed on an Eppendorf mini-spin centrifuge for a minimum of 30 s. Following this, they were centrifuged at 10,000 RPM for 10 min. After centrifugation, 850 microliters (μL) of supernatant was pipetted into a clean labeled Eppendorf tube avoiding any of the solid section of the solution when pipetting. If the sample appeared to still be cloudy, tubes were re-centrifuged for 10 min and then pipetted again into a new tube. Samples were then stored in a −20°C freezer until ready for use.

Hormone Analysis

Validation of the fecal cortisol metabolites (FCM) extraction method is described in (15) and follows the previously described extraction protocols of (28–30). FCM concentrations were determined using a polyclonal anti-cortisol antiserum (R4866) diluted to 1:15,000, horseradish peroxidase (HRP) conjugated cortisol 1: 80,000 and cortisol standards (1.56–400 pg $well^{-1}$). Sample extracts were then assayed in duplicate on Nunc Maxisorp™ plates (96 wells). Plates were coated with appropriately diluted cortisol antibody and left to stand and incubate for a minimum of 12 h in a fridge at 4°C. The plates were then washed using an automated plate washer (ELx50, BioTek™) with phosphate-buffered saline containing 0.05% Tween 20. The dilution factor for the FCMs in koala fecal extracts where based on the concentration of pooled samples that resulted in 50% binding on the parallelism curve [see (15)].

For each assay, 50 μL of cortisol standard, control, and diluted fecal extract was added to each well-based on the plate map, immediately following 50 μL of HRP was added. Plates were covered and incubated at room temperature for exactly 2 h. After 2 h of incubation, plates were washed and 50 μL of substrate buffer (0.01% tetramethylbenzidine and 0.004% H_2O_2 in 0.1 M acetate citrate buffer, pH 6) was added to each well to generate a color change. Color reaction was halted after 15 min using 50 μL of stop solution (0.5 $molL^{-1}$ H_2SO_4). To quantify the concentration of FCM in each sample the plates were read at 450 nm (with reference to 630 nm) on an ELx800 (BioTek™) microplate reader.

Statistical Analysis

Data was statistically analyzed using SYSTAT software version 13.0. All FCM data was first log transformed to meet the assumptions of normality. Graphs were plotted in GraphPad Prism software. All FCM data points (from rescue to end point of clinical recuperation) for each koala were used to calculate mean levels that provided absolute baseline levels of FCMs for each koala. A GLMM ANOVA was used to compare level of significant difference between mean FCM (variable) and factors included (sex, koala ID, length of stay, stressor category, habitat location, diagnosis, and clinical outcome). *Post-hoc* comparison for interaction between habitat location, stressor category, and clinical diagnosis as determinants of mean FCM levels was done using Dunn's multiple comparison test. $P < 0.05$ was used as the level of significance.

RESULTS

Mean FCM Levels Relative to Koala Habitat Demographics

GLMM Analysis of Variance results showed that mean FCM levels were significantly different between individual koalas ($F = 26.33$, df = 11, 220; $p < 0.001$). There was no significant difference in mean FCM levels between male and females, however the length of stay in the hospital was significant ($p < 0.05$).

Stressors experienced in rural localities included; vehicle collision, dog attacks, flat demeanor (associated with bushfire) and having a wet "dirty" bottom. Vehicle collision was the leading stressor in rural localities making up 33% of cases. All other stressors in rural habitats recorded occurrence of 17%.

Stressors experienced in semi-urban (rural-urban fringe) localities included; continuous drinking, eye discharge and flat demeanor, all of which were equally high occurrence at 27%.

Individuals in urban habitats experienced multiple stressors including; continuous drinking, dog attacks, eye discharge, flat demeanor and vehicle collision. Eye discharge had the highest occurrence (29%) followed by vehicle collisions and flat demeanor both at equal occurrence of 21%.

Analysis of Factors and Interactions With Mean FCM Levels

Mean Fecal Cortisol Metabolites (FCM) Levels by Locations

Mean levels of FCM were not significantly different between locations ($F = 1.31$, df = 3, 167, $p = 0.27$; **Figure 1**). The highest mean FCM levels were present in koalas found at rural-urban fringe or semi-urban localities followed by rural and urban locations (**Table 1**). Koalas rescued from national parks had lowest mean FCM levels (**Table 1**). *Post-hoc* comparisons showed significant difference ($p < 0.05$) between all location comparisons, except for comparisons between urban vs. national park and rural vs. rural-urban fringe ($p > 0.05$ for all comparisons; **Table 1**).

FCM Levels by Stressor Category

There was a significant difference between mean FCM levels for the different stressor categories ($F = 5.33$; df = 7, 240; $p < 0.001$; **Figure 2**). FCM levels were highest for koalas with chlamydia), followed by koalas impacted by bushfire (including burns and flat demeanor), vehicle collision, dog-attack, veterinary check, suspected infection, dehydration, and other injury (**Table 2**). *Post-hoc* comparisons showed that only level of significant difference in FCM levels were between bushfire vs. veterinary check and bushfire vs. other injury (**Table 2**).

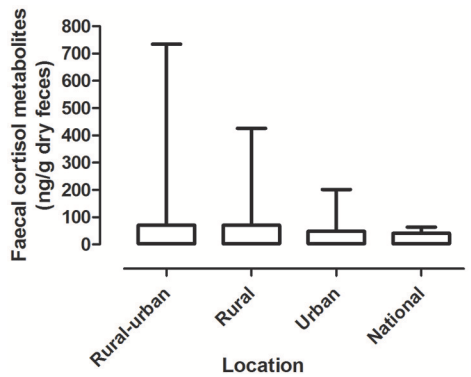

FIGURE 1 | Shows the mean + range of fecal cortisol metabolites (FCM) levels in koalas distributed by locations.

Mean FCM Levels by Diagnosis

There was a significant difference between the mean FCM levels for diagnosis ($F = 3.96$; df = 5, 50; $p = 0.0046$; **Figure 3**). Koalas that were diagnosed with respiratory illness had the highest mean FCM, followed by respiratory illness, other injury, infected pouch, burns, other infection, Chlamydia +, diabetes, Chlamydia –, renal failure, healthy koala (**Table 3**). *Post-hoc* comparison showed level of significant difference only between comparison of healthy koala vs. other injury. A caveat here is low sample sizes for some of the diagnosis (see **Table 1**). Thus, categories with $n = 1$ sample size were excluded from the statistical analysis.

In all cases of diagnosis for renal failure, arthritis (inability to climb) and diabetes the outcome was euthanasia. For koalas with diagnosis of burns, heat stress and respiratory illness, all cases ended with release. Diagnosis of chlamydia, other infections and injuries had cases of both release and euthanasia outcomes.

Mean FCM Levels by Multiple Factors

Significant interaction (*) was found between location, stressor, diagnosis, and outcome as predictors of FCMs levels in the koala patients (**Figure 4**). The test results were as follows:

$$\text{Location}^*\text{diagnosis}(F = 28.87, p = 0.00)$$
$$\text{Location}^*\text{stressor}^*\text{diagnosis}(F = 14.89, p = 0.00)$$
$$\text{Location}^*\text{stressor}^*\text{outcome}(F = 3.16, p = 0.044)$$
$$\text{Location}^*\text{Stressor}^*\text{diagnosis}^*\text{outcome}(F = 25.09, p = 0.00).$$

DISCUSSION

This study has provided new knowledge on the physiological stress responses of rescued wild koalas in relation to their habitat demographics, stressor category, and clinical intervention. The results showed that all of these factors interacted to influence levels of physiological stress (indexed using fecal GC metabolites) in the rescued koalas (**Figure 4**). Therefore, the clinical outcome (release or euthanasia) can be influenced by both the pre-rescue conditions as well as the clinical environment that is provided to the koalas in care.

Koalas that were rescued from rural-urban fringe locations had the highest FCM values while those in urban had the lowest (excluding national park and unknown). In rural locations, interestingly road collision was a leading stressor in 33% of rural cases. A study by Griffith et al. (31) on trends of koala admission

TABLE 1 | Shows the descriptive statistics and *post-hoc* comparisons of fecal cortisol metabolites (FCM) levels in koalas distributed by location.

Category			Fecal cortisol metabolites (ng/g dry weight)					Statistical comparisons	
Number	Location	Sample size	Min	Max	Median	Mean	S.E.M	*post-hoc* comparison (c.q.)	Significant ($p < 0.05$)
1	Urban	77	3.26	202	24	48.34	5.492	I c.f.2	yes
2	Rural-urban fringe	47	13.42	734.6	38.39	69.87	15.69	I c.f. 3	yes
3	Rural	27	2	426.4	31	68.48	17.32	I c.f.4	no
4	National Park	17	15.82	64.22	36.85	39.74	3.395	2. c.f.3	no
								2 c.f.4	yes
								3. c.f.4	yes

to wildlife hospitals found that male koalas to have increased risks of vehicle accidents during the summer period where tourism was high. Griffith et al. (31) further found that vehicle accidents coincided with periods of land clearance with those koalas experiencing these anthropogenic induced threats to be more likely to be admitted to the wildlife hospital. Furthermore, (32) in their study compared major and minor roads and found the incidence of road mortality to be much greater on minor (rural) roads. It was also found that minor roads caused greater habitat destruction than the major roads of urban environments (32). Of the koalas found in rural locations ($n = 6$), four of these ended with a final outcome of euthanasia. Koalas in rural locations will experience less exposure to human activities compared to those in urban environments (33). However, koalas in an urban environment are found to be more resourceful, using all trees in the area, being able to better exploit patchy areas and increased ability to find mates in fragmented landscapes due to a life history of adaption to these experiences (33).

In both semi-urban and urban environments, eye discharge was at the highest occurrence. In semi-urban environments, other factors such as excessive drinking and sitting on the ground had equal high occurrence. Eye discharge was generally diagnosed as kerato-conjuntivitis, which is a leading symptom of Chlamydia (34). Red cloaca, eye discharge, wet bottom, and swollen genitals were all regarded as chlamydial symptoms (urban; $n = 4$). This suggests that in both urban and semi-urban environments, Chlamydia is the leading environmental threat.

During our study period at the AKWH, 17 koalas were diagnosed with *C. percorum* (no PCR, PCR +ve and PCR –ve). Chlamydial infections were higher in females ($n = 12$) compared to males ($n = 5$). In female infections ($n = 12$), four resulted in a final outcome of euthanasia. Gonzalez-Astudillo et al. (35) found koala females to be at a higher risk of poor clinical outcomes when diagnosed with chlamydiosis. Females have been found

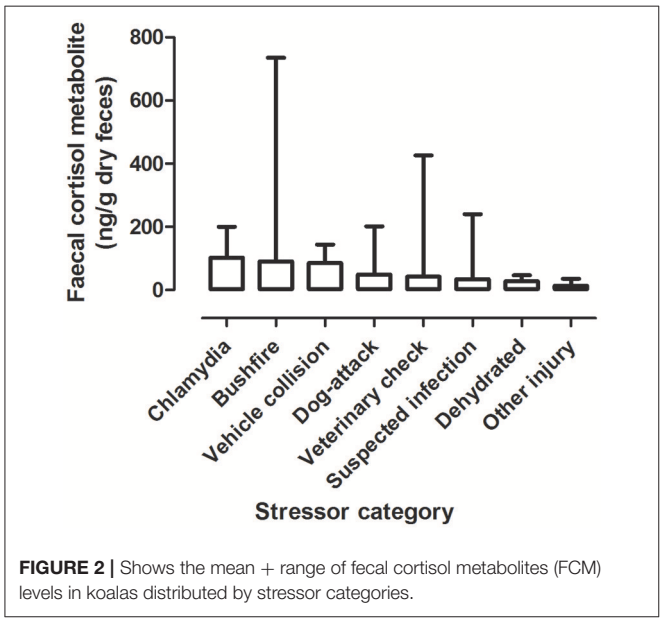

FIGURE 2 | Shows the mean + range of fecal cortisol metabolites (FCM) levels in koalas distributed by stressor categories.

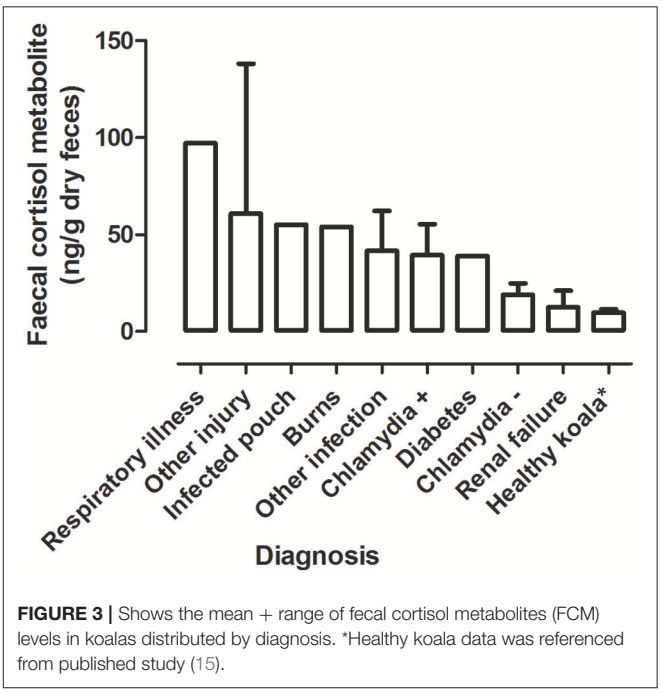

FIGURE 3 | Shows the mean + range of fecal cortisol metabolites (FCM) levels in koalas distributed by diagnosis. *Healthy koala data was referenced from published study (15).

TABLE 2 | Shows the descriptive statistics and *post-hoc* comparisons of fecal cortisol metabolites (FCM) levels in koalas distributed by stressor category.

Category			Fecal cortisol metabolites (ng/g dry weight)					Statistical Comparisons	
Number	**Stressors**	**Sample Size**	**Min**	**Max**	**Median**	**Mean**	**S.E.M.**	***post-hoc* comparison (c.f.)**	**Significant ($p < 0.05$)**
1	Other injury	20	6	36	9.5	13.55	2.067	4 c.f. 7 and l c.f. 7	yes
2	Dehydrated	7	14	47	26	27.71	4.96	All other pairwise comparisons	no
3	Suspected infection	14	7	240	16	34.14	16.13		
4	Veterinary Check	120	3	426	24.5	42.69	4.837		
5	Dog-attack	12	14	202	30.5	49.17	15.36		
6	Vehicle collision	10	22	144	83	85.5	15.2		
6	Bushfire	53	2	735	75	91.09	13.55		
7	Chlamydia	5	54	200	76	102.4	26.62		

Data for healthy koalas were referenced from earlier published work (15).

TABLE 3 | Shows the descriptive statistics and *post-hoc* comparisons of fecal cortisol metabolites (FCM) levels in koalas distributed by diagnosis.

Category			Fecal cortisol metabolites (ng/g dry weight)					Statistical comparisons	
Number	**Diagnosis**	**Sample Size**	**Min**	**Max**	**Median**	**Mean**	**S.E.M**	***post-hoc* comparison (c.f.)**	**Significant ($p < 0.05$)**
1	Chlamydia–	3	8	8	28	5.859		All pairwise comparisons except,	no
2	Chlamydia+	8	5	5	146	16.02		4 c.f. 7	yes
3	Other infection	5	9	9	118	20.59			
4	Other injury	4	21	21	138	26.22			
5	Burns	1	54	54	54				
6	Renal failure	2	4	4	21	8.5			
7	Healthy koala*	29	2.153	2.153	46.44	1.681			
8	Infected pouch	1	55	55	55				
9	Respiratory illness	1	97	97	97				
10	Diabetes	1	39	39	39				

**Healthy koala data was referenced from published study (15).*

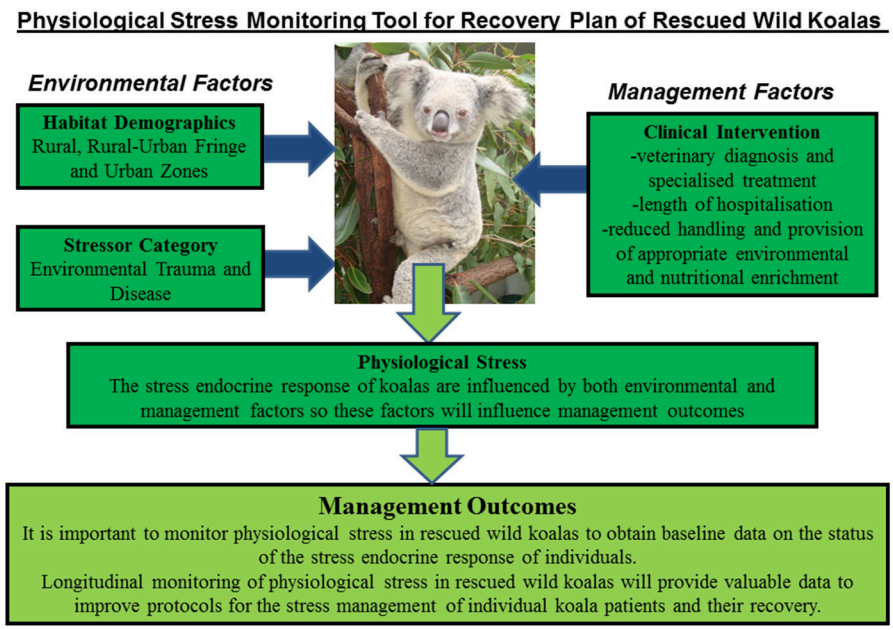

FIGURE 4 | Shows the conceptual diagram summarizing the main findings of this study. That is, physiological stress in rescued wild koalas has the influence of both environmental factors (pre-rescue) and management factors (post-rescue). The pre-rescue factors include anthropogenic induced environmental stressors that generate physiological stress in wild koalas such as habitat fragmentation, forest fires, vehicle collision, dog-attacks etc. The habitat-specific factors, especially rural-urban fringe zone create ecological problems for koalas associated with accessing habitat and food sources. The post-rescue factors are mainly associated with the clinical management of koalas which include their veterinary care and diagnosis, length of stay, treatment, and rehabilitation. It is important to carefully assess physiological stress in wild rescued koalas in order to obtain real-time data on their physiological status at the point of rescue and to apply fecal glucocorticoid monitoring in clinical care to better understand their physiological response to human interventions. The application of non-invasive hormone monitoring can assist us to better manage and reduce stress for koalas under human care.

to express more explicit signs of chlamydiosis and the disease often causes female infertility, resulting in higher euthanasia rates in clinical settings (35). Chlamydia has been recognized as a contributing factor to koala population declines due to high incidence, detrimental impacts of the disease and the symptoms involved (35).

Clinical interventions are crucial for the appropriate care and recuperation of rescued wild koalas. Increased handling during treatment as well as a decreased success in antibiotic treatment may influence stress levels (7). Other diagnosis, such as renal failure can often be indicative of oxalate nephrosis in koalas which can be a detrimental disease to koala populations (36).

In conclusion, it is evident from the outcomes of this research that the nature of environmental stressor (trauma and/or disease) and habitat-specific demographics (location of rescue) can have influence on the physiological stress responses of wild koalas and their eventual recovery in clinical care. It is therefore important to monitor the physiological stress responses of wild rescues koalas using non-invasive techniques such as fecal glucocorticoid metabolite enzyme-immunoassays to provide early index of stress levels in koala patients and apply the data to understand how koalas perceive environmental stress (37) and improve their responses to clinical care and management.

AUTHOR CONTRIBUTIONS

EN conceptualized this research and collaborated with the Adelaide Koala and Wildlife Hospital. EN supervised TV for an Honors research project. TV carried out part of the lab work under the supervision of EN. EN conducted the data analysis and interpretation.

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REVIEW
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Netting the Stress Responses in Fish

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In the last decade, the concept of animal stress has been stressed thin to accommodate the effects of short-term changes in cell and tissue physiology, major behavioral syndromes in individuals and ecological disturbances in populations. Seyle's definition of stress as "the nonspecific (common) result of any demand upon the body" now encompasses homeostasis in a broader sense, including all the hierarchical levels in a networked biological system. The heterogeneity of stress responses thus varies within individuals, and stressors become multimodal in terms of typology, source and effects, as well as the responses that each individual elicits to cope with the disturbance. In fish, the time course of changes after stress strongly depends on several factors, including the stressful experiences in early life, the vertical transmission of stressful-prone phenotypes, the degree of individual phenotypic plasticity, the robustness and variety of the epigenetic network related to environmentally induced changes, and the intrinsic behavioral responses (individuality/personality) of each individual. The hierarchical heterogeneity of stress responses demands a code that may decrypt and simplify the analysis of both proximate and evolutionary causes of a particular stress phenotype. We propose an analytical framework, the *stressotope*, defined as an adaptive scenario dominated by common environmental selective pressures that elicit common multilevel acute stress-induced responses and produce a measurable allostatic load in the organism. The stressotope may constitute a blueprint of embedded interactions between stress-related variations in cell states, molecular mediators and systemic networks, a map of circuits that reflect the inherited and acquired stress responses in an ever-changing, microorganismal-loaded medium. Several features of the proposed model are discussed as a starting point to pin down the maximum common stress responses across immune-neuroendocrine relevant physiological levels and scenarios, including the characterization of behavioral responses, in fish.

Keywords: stress, stressotope, fish, teleost, plasticity, transcriptomics, phenomics

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INTRODUCTION

When studying the adaptive ecophysiology of stress in teleosts, the largest group of fishes and therefore of vertebrates, their extremely diverse life stories appear. This diversity impedes a unified and common description of stress-related effects of environmental insults in fish, and, in consequence, is understandably overlooked in comparative interspecies analyses of stress physiology. Often, the physiological effects of stressors are treated as species-specific features of the chosen animal, but not always expressly acknowledged as such. Therefore, in the literature, the uncovered stress-related feature of a single or few species becomes, misleadingly, a prominent characteristic of *all* teleosts.

Reducing the exogenous and endogenous covariates that elicit stress-related responses undoubtedly helps to reproduce a more focused physiological process in the laboratory. However, this approach veils the adaptive and, more importantly, *content-rich* interactions between stress-related gene expression and phenotype turnover across the life stories of each species. Consequently, the high diversity of teleost lifestyles enriches the physiological analysis of stress effects in fish, but also flaws a unified description of common responses to stress. To overcome this dilemma, the analysis of pan-specific common predictors of stress-related responses should be entrusted to the accurate selection of more explanatory variables. For example, when analyzing the effects of high or low temperatures on physiological performance in ectothermic species, choosing species-specific optimal temperature limits (thermopreferundum) as baseline values allows for comparing the effects of common stressors (1, 2). This approach assumes that the thermic reference summarizes the adaptive pathway to temperature tolerance evolved in a particular biotope (and, implicitly, part of the adaptive life story of each species), and guarantees a more realistic description of the "natural" (or *eustressed*, see below) vs. maladaptive (*distressed*) pathways of stress responses. The same applies for the comparative inter-species analysis of immune responses to stressors in adult fish, where we should consider specifically the maturation of primary and secondary immune organs rather than the relative size of fishes. The microorganism load may substantially differ between marine and freshwater realms, but both environments share the deleterious effects of the communities of resilient low-abundance pathogens (3). Therefore, diverse stress-related physiological adaptations in teleost inhabiting aquatic biocenosis are to be expected, as well as the inter-species commonalities of biological signal transduction and physiological axes. Given that, the degree of functional maturation of immune-related organs and tissues becomes a proxy for adult/mature physiology and allows for the effective cross-species comparison of immune responses to stress in a microbial-rich environment. These examples suggest that when we analyze a particular stress-related phenotype we are not only describing the physiological outcome of specific gene networks, but also the recapitulation of the evolutionary life-stories of each individual (**Figure 1**).

Considering the complex influences between environmental stressors and pathogen communities, in this short review we propose a modified biotope concept (4) for analyzing stress-induced *abnormal* responses (i.e., capable of inducing an allostatic load that compromise the evolutionary conserved activation of regulatory stress-related physiological axis responsive to normal/adaptive stress, see below). This approach would reduce the complexity of species-specific stress analysis to a set of common descriptors, endogenous and exogenous, of such responses. Here, we define a teleost "stressotope" as an adaptive scenario dominated by common environmental selective pressures that elicit common multilevel severe stress-induced responses and produce a measurable allostatic load in the organism.

Defining the components and dynamics of a stressotope may help to reframe the variability of interspecific stress responses in teleosts in terms of the cross-linked interactions between niche characteristics, diverse genomic scaffoldings and phenotypic specificities that define a set of common, multilevel stress responses in fish. Several endogenous and exogenous features that may be relevant to modeling stressotopes are presented below as a starting point, by no means exhaustive, to discuss the value of this ecophysiological approach to analyze the commonalities to stress responses.

STRESSING THE STRESS RESPONSES

Although some definitions and general considerations on the stress concept involve the idea of an altered status and physiological exceptionality, it is also true that coping with stressors, the stress course, and the response of the organism are not only a common mechanism but also a very sound and conserved response among living species. Hence, the stress responses should be considered as one of the basic and important mechanisms that are key to maintain the physiological, cellular and molecular stability (*homeostasis*) of the organism. A myriad of mechanisms available to face the impact of stressors will be selected or modulated depending on many factors: the species itself, the environmental conditions, and chiefly, the intensity, duration and predictability of the stressor. Therefore, an important part of the machinery behind the stress response is the same that is engaged after other stimuli that are not considered stressors, such as reproductive changes, exercise, immune stimulants, feeding, light-dark transitions or the presence of conspecifics or enrichment objects. That is why it is also difficult to make a definition of the stress concept with precision.

Along the years and among the authors that have dealt with the concept of stress (5), several definitions have been provided following the initial definition, "the non-specific response of the body to any demand placed upon it" that was proposed by Hans Selye in 1951 (6). Several concepts have been proposed that agree with the current consensus that stress responses emerge when the stimulatory demand exceeds the natural regulatory capacity of an organism (7). For instance, Selye's *eustress* and *distress* (8) responses differentiate between a "normal" state, in which no significant alterations are recorded and the homeostasis is not impaired (although some hormonal, metabolic or molecular stress-related mechanisms can work), and an "abnormal" state in which significant alterations are regarded, an overall perception of alarm occurs and the stress-related mechanisms are highly engaged. *Hormesis* has been defined as any process in which a cell or an organism exhibits a biphasic response to exposure to increasing amounts of a specific condition (9). It is currently applied to chemical stimuli but it has been applied to amounts of sensory stimulus, metabolic alterations and stressors. Thus, low-dose exposures would elicit a stimulatory, beneficial or compensatory response (eustress), whereas high doses elicit inhibition, alteration or suppression (*distress*). Likewise, the term *allostasis* (10), refers to a concept linked to the energetics or the "economy management" of the body resources. Any stressor may lead to an allostatic load that first, compromises the

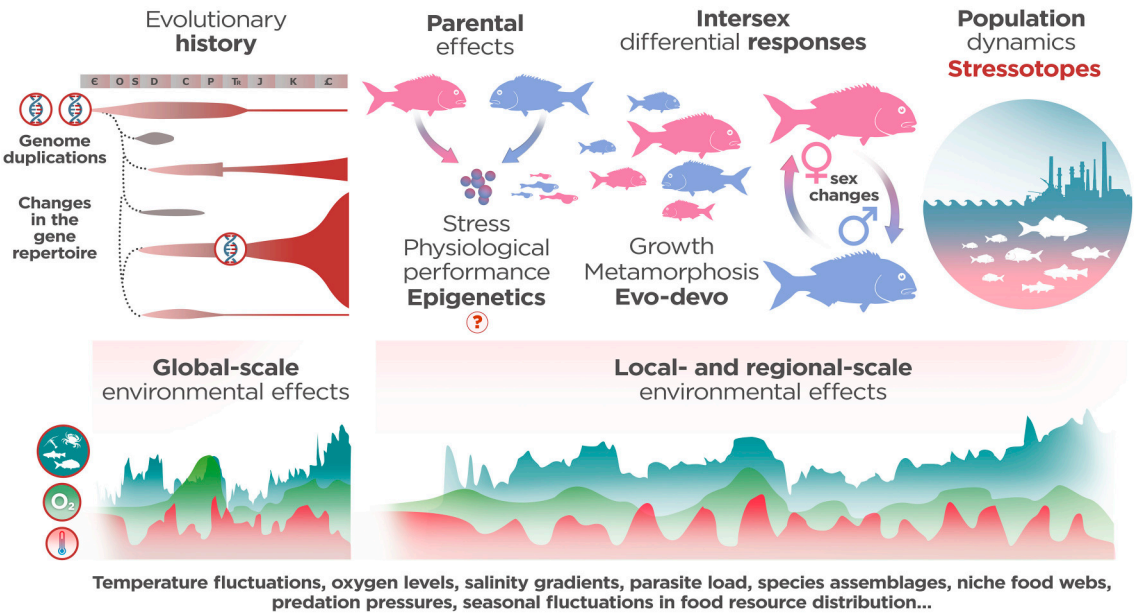

FIGURE 1 | Several influences that shape distress-related phenotypes in fish. The analysis of common responses to stress relies on (1) the evolutionary life-stories endured by each species (i.e., genome duplications, changes in stress-related gene pools, changes in oxygen and temperature levels over geologic time and dynamics of extinction/speciation throughout Earth's history) that constraint the evolvability of biological systems and (2) the pattern and scale of environmental effects in a particular biotope. When analyzing a particular stress-related phenotype the physiological outcomes of specific gene networks during the twin processes of development and growth/metamorphosis have been taken into account, but also the effects of epigenetic transmission of cortisol sensitivity, the differential responses to stressors between sexes and the behavioral interactions within populations as starting points. In this sense, a stressotope defines the boundaries of common pan-specific maladaptive stress responses in a particular/local biotope not only from the perspective of abnormal changes in environmental resources, but also, from the global-scale changes recapitulated in the life story of each individual, i.e., the functional genomics and phenomics of stress intertwined with functional ecology across spatial scales.

overall balance of the organism, and second, involves a higher demand of resources that either leads to a higher acquisition of food/energy or induces a number of physiological and metabolic internal compensations in order to retain the lost balance. This results in maladaptation, which indicates that the regulatory mechanisms have not been able to compensate the effects of the stressor. Maladaptation is often associated to chronic stress since heavy acute stressors may result in death, and mild ones in recovery. These chronic stressors leading to maladaptation are very relevant in farmed animals, including fish subjected to artificial conditions.

The *perception* of stress involves the receptor-mediated sensing of the stressor, either physiologically at neuro-endocrine or cellular levels. The perception mechanisms are important, not only to act as transducers of alarm signals but also to discriminate the intensity of the stress stimuli and therefore the threshold required to trigger the response mechanisms. In fish, neuroendocrine signaling affects and becomes regulated by the onset of immune responses, due to the peculiar organization of the head kidney, a hematopoietic tissue made from a mixture of endocrine, hematopoietic and immune cell populations, akin to the mammalian adrenal gland and bone marrow. As in the rest of vertebrates, those responses are mainly mediated by the activation of two hormonal axes in fish, the sympatho-chromaffin (SC) axis and the hypothalamic-pituitary-interrenal (HPI) axis (11). The SC axis activates a fast stress response, involving the cardio-respiratory system by increasing ventilatory and heart rates, heart stroke volume, and blood perfusion in gills and muscle, providing glucose supply to critical tissues, with adrenaline being one of the major mediator hormones. An activated HPI axis contribute to the re-organization of resources by increasing the catabolic pathways, supplying glucidic sources, processing fatty acids for energy, and suppressing other high-cost energy and longer-term processes such as those of immune responses, being plasmatic cortisol levels one of the major mediators (12).

By binding to glucocorticoid (GR) or mineralocorticoid (MR) receptors, cortisol regulates neuroimmunoendocrine circuitries elicits stress-induced immunosuppression and contributes to allostatic imbalances. That is why is particularly suited for stress-related surveys in natural and artificial environments and the focus of the search for common global markers of stress states in fish. However, the levels of cortisol in distressed fish and, consequently, the individual perception and physiological effects of the intensity of the stressors, are usually strongly biased for neuroendocrine and immune systems in a highly species-specific manner, which makes the prognosis of stress recovery both apparently simple and dauntingly complex (13). Moreover, within-species diversity in cortisol levels also differs between behavioral phenotypes. As discussed below, selecting for "bold"

(proactive) and "shy" (reactive) individuals in a population also segregate animals as low- or high-cortisol responders, masking the common cortisol-related responses to stress. A side effect of this behavioral phenotyping can be seen in experiments with paired trout, in which agonistic competition for food resources leads to cortisol-based hierarchical social labeling, with animals ranging from dominant (proactive, usually with low plasmatic cortisol levels) to subordinate (reactive, usually with high plasmatic cortisol levels). When the social status is reversed, cortisol levels in former subordinates are recovered quickly, rendering useless the measure of cortisol levels as a global long-term common marker of social stress (14). The direct effects of social status on plasmatic cortisol levels should also be balanced out by analyzing the food control exerted by the dominant conspecifics that may indirectly elevate cortisol levels in food deprived stressed subordinates.

Cortisol implants may fail to act as a proxy of behavioral patterns in teleosts (15, 16), and the repeatability of cortisol profiles is higher in reared as opposed to free-living fish due to the artificial control of environmental variables (17). Circadian and seasonal cycles of cortisol secretion must also be considered for assessing the sensitivity and adaptability to stressors (18), considering that cortisol are involved in the synchronization of circadian systems in fish (19). This clearly indicates that a more complex multiscale approach (i.e., from cellular activation to organism and population dynamics in specific stressotopes) will be desirable to describe the effects of stressors.

Besides cortisol, other mediators of stress responses, namely major regulatory axis components (ACTH, CRH, proopiomelanocortin –POMC- peptides, β-endorphin, α-MSH), opioids and a myriad of immune cytokines have been extensively used to define commonalities in altered stress states, but the species bias remain. In the last decade the quest for commonalities of stress responses in fish has focused in peripheral structures such as the mucosae, that sense and distribute alarm signals from pathogens, parasites, bacteria, injuries, sudden changes of salinity or oxygen or the presence of chemicals in the water (20, 21). Skin, gills or intestine may often be the first structures that sense the stressors, but they do so again in a marked species-specify manner (20, 22, 23). The reorganization of the overall metabolism to cope with the stressors also involve an alteration of thyroidal axis (24) related to the energetics and mobilization of fat resources, especially in fish undergoing severe metamorphosis regulated by environmental shortages, such as in smolting salmons (25, 26). Under stress, growth is arrested, the reproductive processes are suppressed or depressed and chronic stressors induce immune suppression, in particular in expensive processes such as white cell production and antibody production, whereas other responses such as phagocytosis may be maintained (27, 28). However, as seen in whole organism physiological responses, at the cellular level the delicate equilibrium between adaptive and maladaptive stress seems to be the norm. Reactive oxygen species (ROS), for example, signal oxidative stress as an evolutionary conserved phagocyte response to infection or xenobiotics (29). However, as part of the environmental stress response, the expression of ROS-related genes vary in hermetic fashion: mild oxidative stress promote the expression of antioxidant defenses that, if defeated, lead to enhanced gene expression that may have distressed outcomes (30). The effects of stress-essential (responsive to specific stressors) and stress-induced (involved in metabolic and high order neuroendocrine axis activation) genes (31) reach from cellular disturbances all the way up to systemic processes, and demand a multilevel approach to determine stress sensing and resolution in a stressotope context.

Notwithstanding the intensity of the stressor, in fish as in other vertebrates, the onset of short-term stress mechanisms usually correlates with genome-fixed and protective adaptive responses to seasonal and predictable environmental perturbations and health insults, whereas long-term responses to stressors tend to be considered as harmful expressions of allostatic imbalances in an unpredictable or pathogen-ridden environment (32). This brings the necessity for a broad multilevel framework that may define more precisely the effect of stressors in cellular, physiological, pathological/clinical and (eco)systemic scenarios.

OVERCOMING THE SCENIC FEAR

Ancient and extant biotic and abiotic dynamics of aquatic environments shaped the adaptive/essential stress responses of fish in a species-specific fashion and should be considered when defining a stressotope. Here we discuss the effects of environmental stressors from a dual perspective, including the physical heterogeneity (natural and man-made) and the reeducation of genomic landscapes in populations placed under explicitly perceived predation risk.

The term "fishes" continue to be a phylogenetic trap that encompass a loosely grouping of more than 28,600 species of ray-finned fish (Actinopterygii) and elasmobranchs, unequally distributed in freshwater (12,740 species) and marine (15,886 species) environments (33). The distribution and diversity of life story patterns in extant fish reflect the differential characteristics of both realms that helped to shape the organization and expression of stress-related genome structures. Teleosts comprise a monophyletic group that accounts for roughly 98% of species of ray-finned fishes. Both marine and freshwater environments seem to be dominated by percomorphs and ostariophysians (34), but marine fishes show an unexplained low diversity in a realm that covers 70% of the Earth's surface (35). Several competing hypothesis have been suggested unsuccessfully to explain such differences, ranging from ecological constrictions, homogeneity-heterogeneity of water biotopes or ocean's net primary productivity and spatial heterogeneity [see (34–36) for a comprehensive review]. Freshwater fishes inhabit a 0.01% of available planetary water volume, usually more fragmented, prone to isolation and barred to dispersal of organisms than oceanic environments (37). This favors intense selective pressures that quite frequently lead to niche-specific diversification, adaptive radiations and increasing speciation, the many phenotypes of African cichlids being the most cited example of such processes (38). It has also been described a greater resilience to extinction in these freshwater low-density, high-diversity specialized fish populations compared to their

marine counterparts (39), probably due to the differential exploitation of resources (detritivores seem to be more abundant in freshwater environments) and large-scale geological perturbations. In this sense, freshwater taxa seem to be more affected and selected for temperature and climatic variations (33).

Anoxia and osmotic changes affect teleost performance, but, being fish ectothermic and oxygen levels and saline content strongly dependent of temperature, thermal conditions largely define the boundaries of stressotopes. In fish, a sudden drop in temperature diminishes the production of immune cellular and molecular resources, impairs T cell-dependent immune responses and may led to cellular inactivation or anergy (40–42). High temperatures correlate with enhanced parasite transmission and resilience within hosts' bodies (43, 44), even when the onset of behavioral fever may stimulate phagocytic activation and modulate innate humoral responses (45). In fish, shifting too far away from thermopreferendum wakes up distress-induced genes and alters the responsiveness of HPI and immune axis (46), but the overall effect may be modulated by acclimation to temperature changes (42). In this sense, the plasticity of phenotypic responses to thermic-related stressors dictates the type and relevance of physiologic variables to be included in a stressotope.

From those observations it is clear that the number and distribution of fish species and, consequently, their physiological strategies to cope with stress result from, and are influenced by the different rates of speciation and extinction (i.e., net diversification) in each environment. Several model species, such as trout, zebrafish or carps inhabit freshwater niches and may endure unexpected selective pressures due to the limitations of toxic drainages, xenobiotic clearance or dissolved oxygen-consuming autotrophic blooms, common to lentic environments. Under these conditions a high turnover of species richness, together with accelerated evolution of stress-related homeostatic mechanisms is to be expected. For example, in fast-growing short-lived killifish species, the exposome, defined as an adding-up response to a lifetime expositions to environmental insults (47) correlates with a fast paced adaptation to Human Induced Rapid Environmental Changes, HIREC (48). Complexity, severity and pace of HIREC changes have been proposed to explain the rapidly acquired tolerance to stress of different populations of killifish (*Fundulus heteroclitus*) in polluted estuaries (49). In this species, a maladaptive stress scenario forced the emergence of genetic polymorphisms related to xenobiotic clearance and stress responses such as the aryl hydrocarbon receptor (*ahr*) signaling pathways, cytochrome P450 1A (*cyp1a*), heat shock proteins (*hsp70*), multidrug resistance transport proteins (*mrp*) and estrogen receptors (*esr2b*). In this model of distress modulation, the environmental trade-offs defined a pattern of gene expression and the emergence of low-responders stress-tolerant populations, but the fitness costs depended on specific particularities of newly adapted phenotypes. This suggests that the physiological costs of evolving tolerances to specific stressors strongly depend on the population and individual fitness in a particular niche. In other words, in diversified population assemblages, well-characterized and common stress phenotypes expressed from stress-related genetic markers may quickly reverse in a population-specific manner, hindering the definition of a set of common stress genes. Moreover, the expression of gene regulatory networks observed in different populations of killifish was complex enough to preclude a one-to-one relationship between clusters of expressed genes and adaptive features of observed fish phenotypes (50), probably due to the heterogeneity of xenobiotic stressors. Even so, under strong selective pressures, convergent evolution may favor the expression of a handful of stress-induced genes (51, 52), shared among populations and, possibly, species. This may be useful for the purposes of establishing a common set of pan-specific responses to different stressors in fish.

The effects of chronic stressors are context-dependent and involve a long-term activation of HPI, SC, and other physiological axis (reproductive, immunological, thyroidal/metabolic) influenced by stress. In the quest for rationalize and simplify stress responses across species, an even more applied definition of stress may help (53): *perceived* anticipatory stress, acute or not, resulting from continuous predation risk. Laundré's "Landscape of Fear" (LoF) defines this perceived stress considering the risks of foraging in unsafety habitats (54). Predation risk, parasite load, metabolic trade-offs associated to seasonal resource shortages, living in high density populations, or artificial habitats, HIREC influences and evolved life story traits have been used to frame the stress related to a particular biotope, usually measuring behavioral patterns and glucocorticoid levels as distress indicators (55, 56). However, despite the content-rich description of these analyses, few studies have approached the effects of LoF in fish. Behavioral cascades and patterns of risk aversion have been documented in coral reef fishes (57–59) and juvenile salmonids (60). In a highly simplified model of predator-prey relationship between trout (*Oncorhynchus. mykiss*) and its prey, (*Daphnia pulex*) in a salinized environment coupled with alarm kairomones, osmotic stress diminished the predatory pressure and favored prey abundance, whereas alarm cues reduced trout aggression (61). The effects of combined stressors, however, did not affect trout growth, probably due to the limitations of the model.

The individual's perception of stress may also collide with the maladaptive effects of HIREC-related ecological traps. Albeit scarcely studied in fish, man-made changes in an otherwise low-quality habitat may attract fishes unable to properly evaluate the amount of resources available. As a result, a behavioral glitch may lead to a struggle to survive in an "evolutionary trap" (62). For example, drifting fish aggregation devices act as supernormal stimuli (63) and may lure tuna species to misinterpret habitat resources (64); coho salmon (*O. kisutch*) prefer spawning habitats that greatly reduce their survival (65); and increased water acidification confounds visual cues in damselfish (*Pomacentrus amboinensis*) reducing their antipredator responses (66).

Taken together, those studies confirm not only that the complexity of the stressotope should be assessed against a minimum common number of informative variables (**Figure 2**), not restricted to binary food webs, but also the relevance of *eco*physiological approaches to describe a unified response to stress in teleosts. Both net diversification and the effects

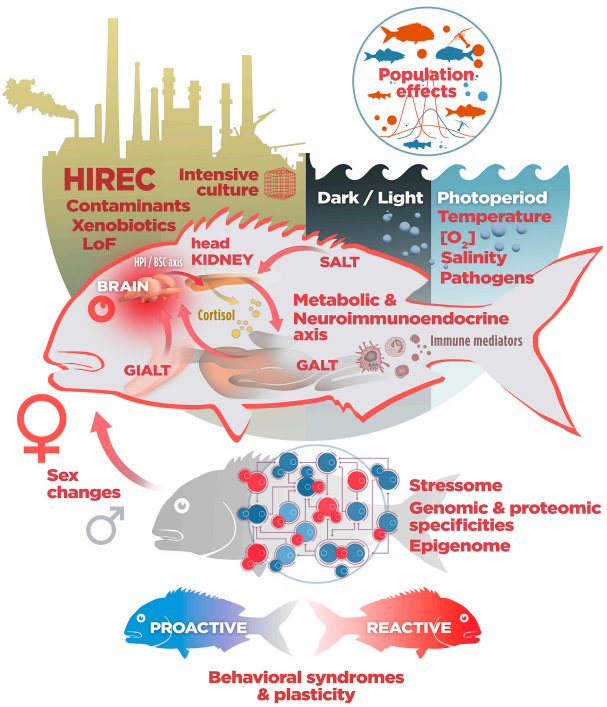

FIGURE 2 | A non-exhaustive list of stressotope components. In fish, the fate of stress responses to natural (temperature and oxygen variations, changes in salinity and photoperiod, abundance of pathogens in freshwater and marine realms...), artificial (cultured) and Human Induced Rapid Environmental Changes (HIREC) depends mainly, but not only, on environmental insults, perceived stressful scenarios influenced by continuous predation risk (Landscape of Fear, LoF) and species-specific intersex differential activation of stress, immune and metabolic axes. To what extent phenotypic plasticity helps to cope with maladaptive stressors in turn depends on evolutionary conserved life stories and behavioral repertoires (see the text for details and abbreviations).

of perceived risk of depredation and foraging in natural and artificial habitats provide a coarse-grained description of environmentally-related impacts on stress physiology in teleosts and may help to discriminate shared mechanisms common to stress responses in fish, but the historical genomic remodeling must also be considered.

ROLLING GENOMES

To delineate a stressotope, a set of pan-specific genes involved in maladaptive responses to stress must be defined. In the seascape of fish phenomes, genomes are being continuously tested and polished against the evolutionary coupling between environmental and endogenous selective pressures. This affects specifically the recent omics interpretations of adaptive physiology of stress in fish. In less than a decade, stress studies have evolved from moleculocentric analysis to genocentric approaches, and lately, to genome-wide association studies, proteomic analysis and high throughput genomic interpretations of genetic and epigenetic networks' cross-talking with environmentally-induced phenotypes that have been thoroughly reviewed elsewhere (67–70). Dissecting genome-based responses to severe stressors implies an extensive analysis of gene regulatory networks and interactions in cellular and tissue environments. To make the analysis of genome-phenome interactions more manageable, we can define a "stressome," or catalog of genes and its products expressed when the organism suffers a maladaptive stress, a concept borrowed from studies of microbial resistance to stressful insults (71) that has been coined to characterize the roadmap to stress-related changes in genomic, proteomic, and metabolomic arenas (72, 73). Stressomes pave the way to a precise definition of stressotopes, but several methodological and conceptual issues have arisen in the course of the genocentric turn of fish stress physiology, mainly the scarcity of model species and the peculiarities of fish genomes that affect their expression, plasticity and evolvability under maladaptive scenarios.

Several species of teleosts are considered the gold standard for developmental, evo-devo, stress-related, and toxicogenomic studies (20, 74–80). However, to date <0.5% of those species have a detailed, but still far from being systematic, coverage of genomic data (81). From the vantage point of comparative studies, teleost genomes differ from those of other vertebrates in terms of divergence and redundancy. In addition to the two events of whole genome duplication common to early vertebrates, teleost endured another round of teleost-specific genome duplication 320 million years ago (Mya) (82). Some lineages widely used as model species, such as Salmonidae and Cypriniformes have experienced yet another process of tetraploidization, ~80 and 8 Mya, respectively (83, 84). To what extent this diversification leads *per se* to increased phenotypic plasticity and adaptability to environmental stressors by means of neofuncionalization of duplicated genes is still controversial (85, 86), being the subfuncionalization (the functional division of ancestral genes among the duplicated ones), loss of genes or slow evolution of duplicate genes three major outcomes of genome duplication (87, 88). For example, the recent (<10 Mya) independent evolution of anadromy in salmonid clades has been correlated to cooler temperatures that opened new estuarine and freshwater habitats, and also redefined previous stressotopes, favoring speciation (85). As described for extremely diversified non-tetraploid cichlids, several ecophysiological factors may influence a successful radiation to stressful environments without specific genome duplications. Instead, genome-wide diversifying selection on key genes, gene duplication and regulation by microRNAs and transposable elements may have allowed their adaptive radiation (89). Additionally, the teleost genomes analyzed to date seem to have suffered accelerated rates of nucleotide divergence, high rate of intron turnover and dramatic loss of conserved noncoding sequences and *cis*-regulatory elements [see (90) for a comprehensive review] that may contribute to their great phenotypic diversity in response to stressful ever-changing environments. However, this may impair the inclusion of a set of common stress-related genes as required when defining a stressotope.

This implies that the species-specificity biases the comparative genomics of teleosts, but a stressome made of a set of common predictors of distress still can be assembled from genome-wide

analysis. This is the case for annual killifish genomes that contain several *hsp* transcripts and genes associated with mitochondrial function that confer resistance to severe (and more importantly, predictable) environmental anoxia stress during development and diapausa stages (91). Atlantic cod (*Gadus morhua*) also has a surprisingly high number of major histocompatibility complex (MHC) I genes that supply the absence of MHC II components, thus maintaining functional antigen trapping and processing pathways during the onset of immune responses (92, 93) in microbial-rich environments. Despite their disparate life stories, metabolism, longevity and genome scaffolding, both species can still act as genomic models and source of candidate predictors for distress-related markers because the processes evaluated (the extreme stress tolerance and the alternate antigen processing) recruit enough identical or very similar categories of predictors for an effective description of a common stressome. Gene expression profile outcomes may differ between stressors and species, and the methodology is certainly not without pitfalls [see (94, 95) for a detailed discussion], but including the adaptive life stories and the environmental biotope may normalize the analysis of physiological responses to distress. For instance, uncovering the seasonal oscillations of stress-related regulatory networks may help to define stressotopes in a more realistic way. Cortisol has been shown to induce the expression of *per1a* and *per1b* and repress *bma11a* and *clock* genes that control circadian rhythms in fish, and it has been proposed to act as a modulator of molecular oscillators (19, 96). Molecular clocks that respond to environmental factors such as light and dark cycles, food availability and thermal conditions vary both in natural and in HIREC environments and may contribute to the ticking of stressomes in a set of defined stressotopes involving migration and breeding scenarios (97).

Epigenetic modification of xenobiotic and temperature stress-related gene expression should also be considered to define a teleost stressome. Fish genomes differ from those of mammals in the number of methylated sites retained early in development and contain exclusive DNA methyltransferase genes that may help in the vertical transmission of the epigenome (98, 99), but the overall modulation of gene expression follows the vertebrate pattern (100). Epigenetic analyses have been used to test the effects of captive rearing in salmons, suggesting that hatchery-induced epigenetic changes impair the osmoregulatory seawater acclimation and swimming performance during smoltification (101). In zebrafish (*Danio rerio*), xenobiotic exposure modified methylation patterns during embryogenesis (102). Diversification of cortisol-responder phenotypes in stickleback (*Gasterosteus aculeatus*) offspring of stressed mothers has been ascribed also to epigenetic changes (103) signaled by glucocorticoid receptors. Little is known about the long-term effects of vertical transmission of stressed phenotypes in fish, but higher responses to cortisol may reduce the fitness of hatchlings and contribute to allostatic load in stressful environments (104). In addition, adaptive epigenetic modifications of gene expression strongly depend upon the degree, intensity and predictability of environmental changes that may propitiate maladaptive outcomes of epigenetic modifications, such as the epigenetic traps discussed below.

Teleost inhabit a stress-prone scenario that favors the evolution of highly reactive immunological surfaces, such as fish mucosal skin, gills, or gut, infiltrated by mucosa-associated lymphoid tissues (MALT), exquisitely sensitive to pathogenic or xenobiotic insults (21), and that's why the analysis of interfacial tissues can be so rewarding to define a stressome. Fish skin scaffolding consists of a highly secretory non-queratinized living tissue that harbors stress-sensing cells, skin associated lymphoid tissues (SALT) packed with B and T cells, resident or errand myeloid phagocytes and cells that produce microbicidal molecules and protective mucus. Teleost SALT induce and regulate local adaptive immune responses that may communicate with other mucosal tissues (branchial, GIALT, and intestinal, GALT) and influence the immune reactivity of systemic lymphoid (head kidney, spleen, thymus) and metabolic (liver) organs. In addition to immunological sensing and regulation, fish gills and gut are also involved in osmoexcretory/acid-base balance and energetic metabolism (105, 106). In fish, such multipurpose organs serve both as probes to distressful environmental changes and as effectors of allostatic rearrangements of stress-related hormonal axis, and may be specially suited to define minimum common molecular markers of distress across species. In a recent study (20), the short-term effects of hypoxia and vaccination against *Vibrio anguillarum* elicited a strongly interspecific differential response of pro-inflammatory and stress-related genes in MALT of gilthead seabream (*Sparus aurata*), a marine species, and rainbow trout (*Oncorhynchus mykiss*), a freshwater teleost, being the former more responsive to stressors. The stress- and immune-related transcripts tested (*lysozyme*, *c3*, *igm*, *hsp70*, *cox2*, *Il1β*, *tnfα*, *il6*, *il10*, and *tgfβ1*), together with the analysis of mucosal- and plasmatic-derived cortisol levels constitute a typical set of markers of distressed states that may help to define a minimum common set of gene-driven responses to stressors in teleosts.

JANIAN PHENOMES

Nested in the archaic roman pantheon, a two-headed figure, Janus, represent, among other things, the transition from one state to another, or from the past to the future. In both vertebrates and invertebrates, behavioral phenotypes may change during the lifetime of an individual, following a reaction norm defined by environmental changes that enhance or suppress the expression of key behavioral mediators, and constrained by the adaptability of the genome (107). A stressotope should consequently be defined by the ontogenic variations and changing phenotypes that the organism endure in diverse environments. In teleosts, the study of relevant stressful-prone "janian" phenotypes has come to focus in recent years in the grounds of fish welfare, and include among others the ecological distribution of differentiated behavioral syndromes or individualities ("personalities") ruled by environmental stressors [extensively reviewed in (108) and not to be discussed here], the pathogen effects on physiological modifications underlying sequential sex changes and the

physiological changes linked to transition from freshwater to marine realms in diadromous species.

The majority of fish follow the usual vertebrate gonochorism, with both sexes being determined genetically or environmentally (109). Several teleosts also indulge in a plethora of rare vertebrate reproductive modes ranging from simultaneous and sequential hermaphroditism to parthenogenesis (110, 111) that have been ascribed to differential ecological selective pressures (111), diversification of reproductive mediators by means of whole genome duplication events (86) and fish-specific idiosyncrasies of gonadal axis. Males and females usually inhabit the same environment, but the selective pressures faced by both sexes may differ owing to variations in size, competition for resources, diet, microhabitat use aggressiveness and metabolic trade-offs between gamete production/fecundity and immune resistance to parasitic load (112), even in sex-role-reversed species (113).

Several sex-biased effects of parasitism and facultative infections have been described in natural and artificial populations of teleosts. Poeciliids have been used as a model to highlight the relevance of sex-specific evolution of physiological responses to environmental changes on a macroevolutioanry basis (114). Polygynous guppies (*Poecilia reticulata*) parasitized by *Gyrodactylus* spp., showed an increased responsiveness to infection in females that lead to differential evolution of resistance phenotypes (115). Male guppies also differ from females in the navigational abilities associated to increased dispersion and mobility in complex environments (116) and seems to be more prone to parasite infection than females (117). Unpredictable chronic stress (social isolation, crowding, tank changes, thermal variations, and chasing) affect zebrafish males but not females (118), highlighting the double effect of species-specificity and sex-biased covariation in stress studies. The offspring of largemouth bass females (*Micropterus salmoides*) treated with cortisol showed lower responsiveness to stress and exhibit less exploratory behavior and aggression than those of non-treated females (119), adding to the stressotope equation the still imprecisely described mechanism of vertical transmission of stress-related phenotypes.

Parasitic load and unexpected environmental changes may also contribute to the stressful effects of sex-biased physiologies. Parasite burden accounts for a large portion of stressors in aquatic habitats, and in vertebrates immunocompetence depends largely on male and female sex hormones, being testosterone generally immunosuppresive and estrogens enhancers of immune system in a broad sense (120). Vertebrate males also tend to rely more than females in Th1-mediated immune responses (linked to defensive responses against intracellular bacterial and viral parasites) whereas females display generally higher Th2-mediated extracellular responses against parasites (121). Both T-cell related immune responses have been described in fish, albeit with species-specific kinetics that may interfere or potentiate with the resistance to severe infection (122) and the intensity of distress responses. However, sex-specific responses to reproductive hormones may be altered by HIREC changes in water composition, as demonstrated by the effects of endocrine disrupting chemicals such as 17β-oestradiol in host-pathogen interaction between males and females of three-spined sticklebacks (*Gasterosteus aculeatus*) and the cestode parasite *Schistocephalus solidus* (123). When exposed to high doses of estradiol, parasitized stickleback males were found to be greatly affected, more than females by parasite growth.

A reduction of fitness in one sex has also been suggested as the trigger of selective vulnerabilities in species with environmentally-directed sex determination (ESD). Unexpected temperature changes may influence epigenetic regulation of breeding strategies in teleosts with ESD as described for mangrove killifishes (124). Similar to the "ecological traps" discussed above, severe environmental or HIREC variations could skew the sex ratio by inducing short term epigenetic changes that favor accelerated adaptation to novel environments but can become "epigenetic traps" in the long term, benefiting one sex and decreasing the fitness of the other (125). The same holds true for sequential hermaphroditic species (126), such as the extensively farmed Sparidae. Several species of this family practice protandrous (changing sex from males to females) and protogynous (the opposite) hermaphroditism (127). In protandrous gilthead sea bream (*Sparus aurata*) populations, the few large fertile females surrounded by many smaller males skew the sex ratio and have greater fitness measured by the number of offspring (128). In this species, reproductive success may be linked to the high rates of evolution of female-biased genes compared to male-biased genes (129), probably due to differential selective pressures for both sexes at each stage. This suggests that the effect of environmental stressors may affect the sex-biased expression of genes in hermaphrodites in a different way from what has been described in gonochoristic teleosts.

In diadromous species, the still poorly understood and complex influence of glucocorticoids as mediators of stress responses modulates stressome structure and function. In teleosts, crossing continental and oceanic aquatic environments stresses the physiology of osmoregulation and metabolism in a complex combination of enhancing and suppressive expression of HPI, growth and thyroidal axes. A recent study embraced the joint analysis of ontogenetic stages, sexual, and parasitic effects in hypoxia-stressed European eels (*Anguilla anguilla*), defining a limited stressotope to modulate the causes and consequences of the stepped decline in eel populations (130). Parasitized eels showed stronger levels of plasmatic cortisol and higher gill Na+/K+—ATPase activity that added up to physical constraints (salinity, temperature) to mark female eels in the last stage of silvering to be more prone to be stressed by the combined effects of several stressors. The synergistic effects of parasitism, hypoxia and biotic factors included in the analysis of eel physiology signal the way by which a comprehensive and realistic study of stress responses should be performed. In anadromous salmonids, for instance, long-lasting migrations subdue the cortisol resistance and chronically stress semelparous species. To date, the crosstalk between immune and hormonal components remains unsolvable due to the complexity of the activation/suppression interplay between cortisol, thyroid, growth and sex hormones, B cell lymphopoiesis, inflammation, antibody responses and the development of immunological memory at different stages of their life cycle (131). In this case, the stressotope demands a pronounced level of multiscale complexity to integrate the

adaptive vs. maladaptive effects of stress in such migratory species.

As discussed above, fish stressotopes harbor several opportunistic and obligate parasitic, fungal, viral, and bacterial pathogens that may transmit stress-prone phenotypes vertically, by parasite colonization of gonadal tissues, and direct cortisol effects into eggs (119, 132) and affect not only broodstock and natural populations but both sexes differentially as well. Therefore, the puzzling diversity of teleost reproductive strategies may be also partially explained assuming compensatory genetic changes that overcome maladaptive responses to distressful environments. This leads to plastic reproductive adaptations between sexes to predatory and pathogenic pressures by virtue of sex-specific differences in the reproductive hormonal axis.

Overall, these and other studies imply that to accurately define a stressotope, the range of abnormal values in distress physiological adjustments, the scope of stressome components to be included in the analysis of allostatic load and the intersex differential responses to severe stressors, should necessarily be taken into account. Considering that in teleosts, as in the rest of vertebrates, steroids regulate reproductive outcomes but also metabolism, stress responses, behavior and immune function, usually in a seasonal way (133, 134), the differential effect of estrogens- and testosterone-derived mediators must be included in the stressome catalog.

CONCLUSION

We have outlined some of the key processes and influences required to properly define a stressotope, ranging from the molecular to the ecological ones. Stress is a foreground concept defined against a background of interactions between network genome expression and phenome consolidation in a particular ecological niche. A stressotope approach that could help to elucidate common responses to diverse stressful scenarios is not only informative but also necessary to reduce the diversity of fish lifestyles to a minimum common set of telltales and indicators of allostatic loads originating from multiple and recurrent stressors. There is a growing shift in the literature of stress responses in fish toward a more integrate view of allostatic description. However, this approach is still hampered by the lack of analytical tools, peculiarities of fish genomes and the fuzzy definition of common inter-specific endpoints of distress-related physiological changes across behavioral phenotypes. Moreover, fish are considered more labile and diverse in their physiology than other vertebrates. We can describe teleosts as animals that indulge in sex changes, inhabit environments hostile to ectothermic metabolisms, grow indefinitely, modify their coping styles, or individualities in response to environmental and parasitic insults (135, 136), have higher rates of cell proliferation in the adult brain compared to mammals, and that are strongly dependent on the social interactions and physical environments (137, 138). Therefore, a roadmap for minimum common descriptors of stress responses, a stressotope, must be drawn considering the behavioral plasticity of teleosts, an integrative concept that harbors the cross-linked effects of neuroimmunoendocrine cross-talks that integrate in a variable set of phenotypes from specific activation of pan-specific stressomes.

AUTHOR CONTRIBUTIONS

JB and LT conceived and wrote the review and JB crafted the figures. Both authors contributed to manuscript revision, read, and approved the submitted version.

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Tryptophan Metabolic Pathways and Brain Serotonergic Activity: A Comparative Review

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The essential amino acid L-tryptophan (Trp) is the precursor of the monoaminergic neurotransmitter serotonin (5-hydroxytryptamine, 5-HT). Numerous studies have shown that elevated dietary Trp has a suppressive effect on aggressive behavior and post-stress plasma cortisol concentrations in vertebrates, including teleosts. These effects are believed to be mediated by the brain serotonergic system, even though all mechanisms involved are not well understood. The rate of 5-HT biosynthesis is limited by Trp availability, but only in neurons of the hindbrain raphe area predominantly expressing the isoform TPH2 of the enzyme tryptophan hydroxylase (TPH). In the periphery as well as in brain areas expressing TPH1, 5-HT synthesis is probably not restricted by Trp availability. Moreover, there are factors affecting Trp influx to the brain. Among those are acute stress, which, in contrast to long-term stress, may result in an increase in brain Trp availability. The mechanisms behind this stress induced increase in brain Trp concentration are not fully understood but sympathetic activation is likely to play an important role. Studies in mammals show that only a minor fraction of Trp is utilized for 5-HT synthesis whereas a larger fraction of the Trp pool enters the kynurenic pathway. The first stage of this pathway is catalyzed by the hepatic enzyme tryptophan 2,3-dioxygenase (TDO) and the extrahepatic enzyme indoleamine 2,3-dioxygenase (IDO), enzymes that are induced by glucocorticoids and pro-inflammatory cytokines, respectively. Thus, chronic stress and infections can shunt available Trp toward the kynurenic pathway and thereby lower 5-HT synthesis. In accordance with this, dietary fatty acids affecting the pro-inflammatory cytokines has been suggested to affect metabolic fate of Trp. While TDO seems to be conserved by evolution in the vertebrate linage, earlier studies suggested that IDO was only present mammals. However, recent phylogenic studies show that IDO paralogues are present within the whole vertebrate linage, however, their involvement in the immune and stress reaction in teleost fishes remains to be investigated. In this review we summarize the results from previous studies on the effects of dietary Trp supplementation on behavior and neuroendocrinology, focusing on possible mechanisms involved in mediating these effects.

Keywords: serotonin, stress, aggression, immune response, fatty acids, dietary supplementation

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INTRODUCTION

Tryptophan (Trp) is an essential amino acid in all animals, which is synthesized and provided to higher trophic levels by bacteria, fungi and plants. In addition to being a component for protein synthesis, Trp is also the obligatory substrate for the production of several important bioactive substances. For example, tryptophan is a substrate for the synthesis of serotonin (5-hydroxytryptpamine, 5-HT) in the brain and gut, and melatonin in the pineal gland. In vertebrates, central 5-HT plays an integrative role in the behavioral and neuroendocrine stress response (1–3). Accordingly, effects of dietary Trp on the neuroendocrine stress response have been reported in a variety of species, spanning from teleosts to humans (4–10). However, the mechanisms underlying this link between Trp metabolism and the stress response are not fully understood.

In mammals, the majority of Trp is catabolized and transformed through the kynurenic pathway to bioactive substances which potentially can interact with the stress response (11). Moreover, infections, stress, and changes in the gut microbiome have all been shown to shunt Trp metabolism from 5-HT production toward this pathway (12, 13). Consequently, pathological changes in stress responsiveness, as in depression, have been related to nutritional factors, stress and immune function in humans (14, 15). However, in non-mammals, information on the kynurenic pathway and its interactions with central 5-HT signaling and the stress response is scattered and/or limited.

Dietary manipulations affecting Trp availability to the brain have been used as a tool to investigate involvement of the 5-HT system in behavior, mood and cognition in humans (16–18). Likewise, the dietary Trp content have been shown to affect endocrine and behavioral responses to stress in teleost fishes (10, 19, 20). This review summarizes the results from previous studies on the effects of dietary Trp supplementation on the behavioral and neuroendocrine stress response, focusing on possible mechanisms involved in mediating these effects. We also present a hypothesis on how the diet could be used to improve fish stress tolerance through interactions with the Trp metabolic pathways.

L-TRYPTOPHAN AVAILABILITY AND BRAIN SEROTONERGIC ACTIVITY

In serotonergic neurons Trp serves as the precursor for 5-HT. The 5-HT metabolic pathway is initiated by Trp being hydroxylated to the intermediate 5-hydroxytryptophan (5-HTP), which is subsequently decarboxylated to become 5-HT. Tissue levels of 5-HTP are usually low since this substance is rapidly decaroxylated by the enzyme aromatic amino acid decarboxylase [for review see (21)]. Thus, the rate limiting step in the biosynthesis of 5-HT is the hydroxylation of Trp which is catalyzed by the enzyme tryptophan hydroxylase (TPH) (**Figure 1**). This enzyme is specific for 5-HT producing cells, however, it is present in two different isoforms, TPH1 and TPH2 [reviewed in (22, 23)].

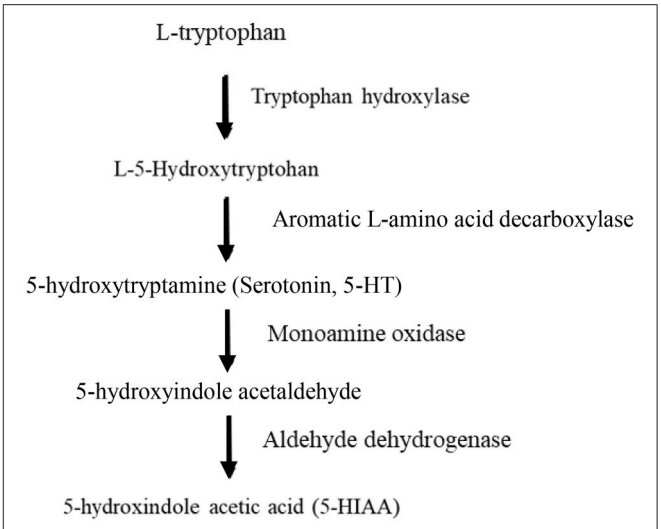

FIGURE 1 | Biosynthetic pathway of serotonin.

In amniotes 5-HT neurons are only present in the raphe area of the hind brain whereas in anamniotes, including teleosts, 5-HT cell bodies are also located in pretectal areas and basal forebrain. In zebrafish (*Danio rerio*) raphe and pretectal 5-HT cells express TPH2, whereas diencephalic and hypothalamic 5-HT cells express TPH1 (TPH1a and TPH1b) and TPH3, respectively (23). Interestingly, TPH2 show a Km for its substrate which is in the range of *in vivo* brain levels of Trp (24). Consequently, the rate of 5-HT synthesis in cells expressing TPH2 is drastically affected by changes in Trp availability, an effect which is probably not seen in 5-HTergic cells expressing other TPH isoforms (22). Moreover, the rate of 5-HT synthesis is believed to be reflected in the release of 5-HT, often quantified as the concentration of the catabolite 5-hydroxyindole acetic acid (5-HIAA), or the 5-HIAA/5-HT ratio. Thus, changes in Trp availability may have direct effects on 5-HTergic tone. Coherent to this, Russo et al. (25) made the interesting suggestion that Trp may act as signal to the brain, transferring information on peripheral homeostasic challenges to the 5-HT system which in turn could act to defend homeostasis. Dietary composition as well as stress, physical activity and immune system activation will all have effects on plasma Trp concentrations, and thus on brain Trp availability and raphe 5-HTergic activity (25). Such Trp related changes in 5-HTergic activity could have direct effects on behavior as well as endocrine status through 5-HT projections to telencephalic and hypothalamic areas. It could be argued that such effects may be less important in teleost fish since they have extra-raphe located 5-HT cell populations expressing the TPH1 isoform, making them less responsive to changes in Trp availability. However, in teleosts, as well as in other vertebrates, the raphe 5-HTergic cells have a wide projection pattern innervating most brain regions (23). Still, it has to be acknowledged that very little is known about the role of telesost forebrain 5-HT cell population in the control of behavior and endocrine functions (23).

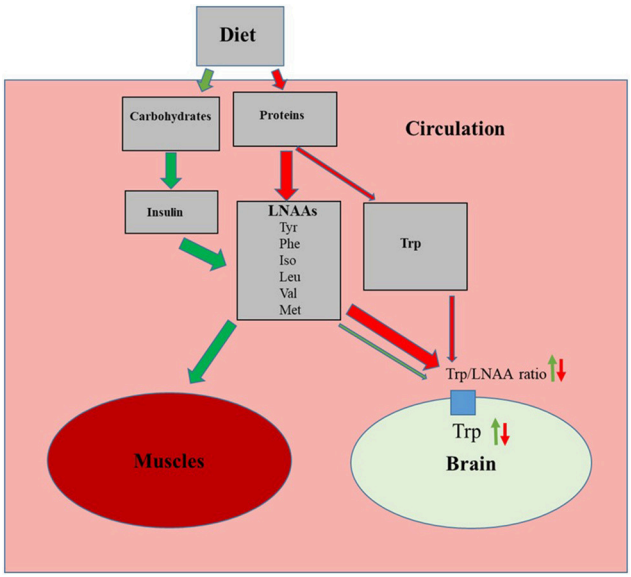

FIGURE 2 | Effects of the proteins and carbohydrates on influx of tryptophan (Trp) to the brain. Green arrows indicate activation of carbohydrate induced pathway, resulting increased muscle uptake of large neutral amino acids (LNAAs; Tyr, tyrosine; Phe, phenylalanine; Iso, isoleucine; Leu, leucine; Val, valine and Met, methionine) which in turn increases plasma Trp/LNAA ratio and brain Trp levels. Red arrows indicate how a normal dietary protein source, with relatively low Trp content, decreases the plasma Trp/LNAA ratio and brain Trp levels.

FACTORS AFFECTING TRP UPTAKE TO THE BRAIN

Dietary Effects on Trp Availability

The essential amino acid Trp enters the brain in competition with other large neutral amino acids (LNNAs; i.e., valine, isoleucine, leucine, tyrosine, phenylalanine and methionine) through a common transporter protein. Thus, the amount of Trp entering the brain depends on the plasma concentrations of Trp in relation to the other LNAAs [for references see reviews (26, 27)]. Hence, ingestion of a normal protein source, usually containing 0.5–1% Trp, results in a relatively small increase in Trp but a larger elevation of plasma concentrations of other LNNAs (28). This results in a decrease in the plasma Trp/LNAA ratio and thus reduced Trp influx to the brain (**Figure 2**). Dietary carbohydrates, on the contrary, increase brain Trp levels. This is due to elevated insulin which in turn promote uptake of LNAAs except Trp to the skeletal muscles, thereby increasing plasma Trp/LNAA ratio and Trp influx to the brain (**Figure 2**) (26, 27). This differential amino acid uptake to skeletal muscles is caused by the fact that Trp in blood plasma is bound to albumin whereas other LNAA are not. Trp influx to the brain is then promoted by the common LNAA transporter protein in the blood brain barrier having a much higher affinity for Trp compared to albumin (27).

Studies in rainbow trout (*Oncorhynchus myliss*) show that the amino acid composition of trout albumin differs from that of mammalians and lacks the binding site for indoles (29, 30). Thus, in rainbow trout, the majority of plasma Trp is in its free non-protein bound state (31, 32). This assumption is further strengthened by a study by Ruibal et al. (33) showing that hyperglycemia induced elevation of plasma insulin levels did not affect brain 5-HT activity in rainbow trout. It is not known if the lack of Trp binding by albumin is specific for rainbow trout or if it represents a more general trait of teleost albumin. However, it is possible that in teleost fishes brain influx of Trp could be more dependent of the dietary amino acid composition than on carbohydrates.

The Kynurenic Pathway

In fact, only a minor fraction of the Trp pool is utilized for 5-HT biosynthesis. In mammals, the majority of Trp enters the kynurenic pathway and is converted to other bioactive substances than 5-HT, such as kynurenic acid and quinolinic acid (**Figure 3**) [for references see review (11)]. The first stage of this pathway is catalyzed by the hepatic enzyme tryptophan 2,3-dioxygenase (TDO) and the extrahepatic enzyme indoleamine 2,3-dioxygenase (IDO), enzymes that are induced by glucocorticoids and pro-inflammatory cytokines, respectively (34). Thus, chronic stress and infections can shunt available Trp toward the kynurenic pathway and thereby lowering brain 5-HT synthesis while simultaneously increasing the production of other Trp based bioactive substances. Moreover, since a majority of Trp follows the kynurenic pathway (<95%, **Figure 3**) relative small changes in the activity of this pathway can have rather big impact on the Trp influx to the brain (35). Accordingly, decreased Trp influx to the brain as a result of stress or inflammation/infection induced activation of the kynurenic pathway have been suggested to be an underlying factor for mental illnesses and dysregulation of the neuroendocrine stress axis (12, 14, 15).

Generally, IDO is more nonspecific than TDO, and catabolizes other indoleamines than Trp. Moreover, two distinct IDO genes, IDO1 and IDO2, have been identified in vertebrates. Earlier studies suggested that IDO1 arose by a gene duplication in mammals (36). However, recent phylogenetic analyses show that IDO1 are present in reptiles and in teleosts, indicating that the gene duplication occurred in the common ancestor of vertebrates (37). In mammals, the activation of dendritic cells results in IDO1 induction with the depletion of Trp levels locally or systemically, a mechanism by which interferons inhibit the growth of certain bacteria, intracellular parasites, and viruses (34). Moreover, an elevation of the activity of the kynurenic pathway also inhibits T lymphocyte replication which results in immunosuppression and tolerogenicity. In line with this, IDO1 have been suggested to play an important role in preventing fetal rejection and in facilitating immune escape of tumor cells (34). In addition, some products of the kynrunic pathway may act anti-inflammatory (38, 39). However, to which extent these anti-inflammatory Trp catabolites acts back on the activity kynurenic pathway and thereby affecting Trp influx to the brain and/or central 5-HT signaling is to our knowledge unknown.

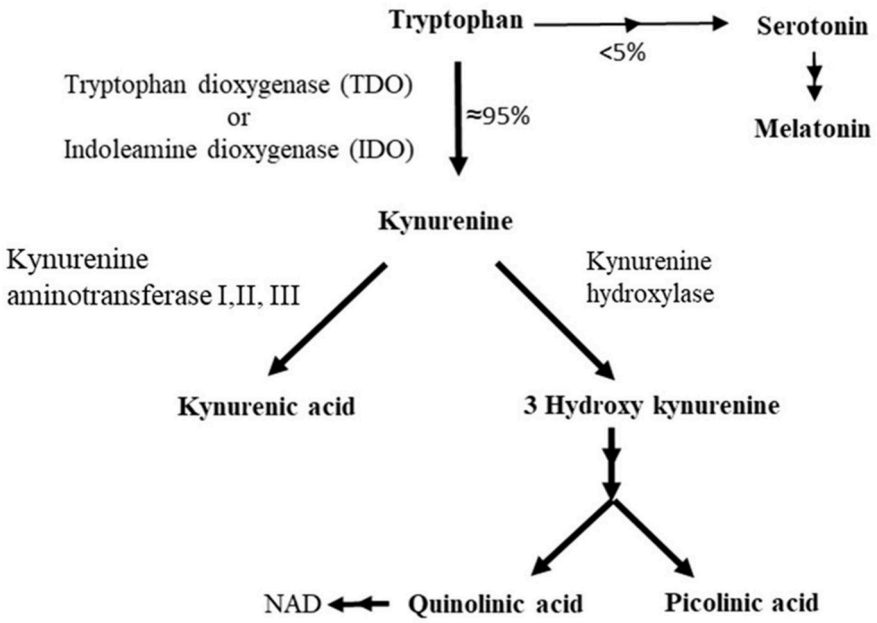

FIGURE 3 | Major metabolic pathway of tryptophan in mammals.

The Trp catabolizing efficiency of IDO2 and non-mammalian IDO1 seems to be lower than mammalian IDO1, and their function and involvement in the immune response in comparative model species is far less understood (37). However, recently, it has been demonstrated that treatment with bacterial lipopolysaccharide (LPS) induces and upregulation of IDO expression in rainbow trout, suggesting that this enzyme is involved in the immune response in non-mammalian vertebrates (40). Moreover, in the aforementioned study, expression of IDO was induced by the pro-inflammatory cytokine interferon gamma (IFNγ) in an *in vitro* cell model, indicating similar induction mechanisms as those in mammalian IDO1 (40). This suggests that systemic infection may decrease Trp influx to the brain of teleost fishes in the same way as in mammals, and result in behavioral and physiological changes (see section Kynurenine pathway).

Acute Stress

As discussed above chronic stress may result in lowered brain Trp availability as a consequence of a stress-induced activation of the kynurenine pathway. However, acute stress has been reported to have the opposite effect elevating brain Trp levels in both mammals (41, 42) and teleost fish (3, 10). This stress-induced increase in brain Trp concentrations appears at least in part related to a sympathetic activation and elevated levels of circulating plasma catecholamines (43). Plasma catecholamines stimulate lipolysis, resulting in elevated plasma levels of non-esterified fatty acids, which in turn could compete with Trp for binding to albumin and thus elevate the plasma pool of free Trp available for uptake into the brain [reviewed by (44)]. However, as discussed above, rainbow trout albumin appears to lack the Trp binding site, suggesting that mechanisms based on competition between Trp and non-esterified fatty acids are not involved in stress-induced increase in brain Trp in teleosts, at least not in rainbow trout. It has also been suggested that sympathetic activation results in increased permeability of the blood-brain barrier, another mechanism that could increase brain Trp influx (44).

TRP AND THE NEUROENDOCRINE STRESS RESPONSE

Stress Responses Are Modified by Trp Availability and Brain 5-HT Functions

As mentioned earlier in this review, the positive relationship between Trp availability and brain 5-HT production is well conserved within the vertebrate linage. Coherent to this, the involvement of 5-HT in the neuroendocrine regulation of the stress response seems to be similar within this linage. 5-HT plays a central role in control of the hypothalamus–pituitary–adrenal axis (HPA axis) in mammals, and the hypothalamic–pituitary–interrenal axis (HPI axis) in fish. This, mainly through its effects on the release of corticotropin-releasing factor (CRF) from the hypothalamus (45, 46). In addition, extra hypothalamic 5-HT appears be involved in appraisal and stress coping mechanisms, modulating behavioral and neuroendocrine responses to stressors (47, 48). Furthermore, as mentioned in section The Kynurenic pathway and Acute stress, stress by itself can influence the Trp influx to the brain, and thereby affect 5-HT signaling and the stress response. Moreover, the HPA/HPI axis are under feedback control on serval levels, including central 5-HT signaling. Thus, the link between Trp and the 5-HT system

and how they control behavioral and neuroendocrine stress responses appears complex with 5-HT having context dependent effects (19, 22, 49).

Effects of Elevated Dietary Trp

Long-term effects of Trp dietary manipulations on the neuroendocrine stress response have been observed in both mammals and teleost fishes [for a review see (49)]. For instance, in pigs, elevated dietary Trp had stress suppressive effects, including elevated hypothalamic 5-HT and lowered post stress plasma cortisol levels, effects that peaked after 5 days of dietary Trp enrichment (50). Similarly, (51) showed that post-stress plasma cortisol levels returned to baseline earlier after social stress in pigs fed Trp enriched feed for 7 days. Interestingly, a similar time frame for the suppressive effects of dietary Trp supplementation on glucocorticoid release has also been demonstrated in fish (for references see **Table 1**). For instance, studies in rainbow trout show that suppression of the neuroendocrine stress response is present after 7, but not after 3 or 28 days of treatment with dietary Trp supplementation (52). Furthermore, in the earlier studies showing a suppressive effect of elevated dietary Trp on the neuroendocrine response to an acute stressor the effects were investigated during or directly following a period of dietary Trp supplementation (10, 52). However, in recent studies in sea water reared Atlantic salmon (*Salmo salar*), the suppressive effect on post-stress plasma cortisol seems to appear between 2 and 8 days after terminating the Trp supplementation. Moreover, in Atlantic salmon, this suppressive effect was still present at 21 days post Trp supplementation (7, 53). Basic et al. (53) suggested that such slow acting Trp-induced alterations of HPI-axis reactivity could be related to smoltification, a process where salmonid fish adapt to sea water. Moreover, these long-term alternations of HPI axis reactivity was not related to changes in hypothalamic 5-HT neurochemistry. Instead they coincided with changes in dopaminergic neurochemistry in this brain part, effects which may be related to elevated activity of the kynruneric pathway, as discussed in section The Kynurenic pathway. Similar results were shown in the study performed by Höglund et al. (7), where 5-HTergic activity in hypothalamus did not follow the long term Trp induced suppressive effect on post stress cortisol levels. The latter study also included telencephalon and 5-HT activity followed the same general pattern as cortisol in this brain part. Höglund et al. (7) suggested that such region specific differences could be related to 5-HT signaling in telencephalon being more dependent on projections from the hindbrain raphe, a nucleus where 5-HT neurons are highly sensitive to available Trp, see section L-tryptophan availability and brain serotonergic activity.

Generally, teleost fishes have a remarkable neurogenic and regenerative capacity throughout ontogeny, and it has been suggested that structural changes may underlie long-lasting effects on telencephalic neurochemistry induced by elevated dietary Trp in teleost fishes (7). This type of brain architectural changes is supported by mammalian studies, showing that the 5-HT system is involved in the organization and development of its own neural projection pattern (65). In addition, a positive relationship between dietary Trp content and neural proliferation markers, such as (exogenous) 5-bromo-2-deoxyuridine and brain derived neurotrophic factor (BDNF) has been demonstrated in rats (66), which lends further support for the suggestion that dietary Trp can induce structural changes in the brain.

There are studies in teleost fishes showing effect of longer Trp treatment periods than 7 days (**Table 1**). For example, Tejpal et al. (60) showed that a 60 days of dietary Trp supplementation decreased baseline plasma cortisol values as well as the cortisol response to 60 days of crowding stress. Moreover, longer Trp treatment periods have also been shown to act stimulatory on plasma cortisol responses. For example, an immune challenge by i.p. injection of inactivated *Photobacterium damselae* suspension resulted in elevated cortisol values in seabass fed Trp supplemented feed for 2 weeks as compared to fish given standard feed fish (67). Furthermore, there is a rather high variability in the effect of elevated dietary Trp on baseline cortisol values (**Table 1**). This variability could reflect interspecific differences in Trp metabolism and neuroendocrine mechanisms (49). Moreover, Höglund et al. (19) suggested that such variation could be related to differences in HPI axis activation due to divergent rearing environments. For example, in the studies performed by Lepage et al. (10, 52, 62), fish were kept socially isolated while in other studies they were group reared (4, 7, 53, 54). Considering the fact that the 5-HT system is affected by social interaction (3, 22, 68), this type of rearing differences may explain some of the variability in the response to elevated dietary Trp. Moreover, studies in humans and rats suggest that individual variation in 5-HT neurotransmission underlies differences in the response to dietary Trp manipulation (27). It has become increasingly clear that individual variation in HPA/I axis reactivity is as widespread phenomena in the vertebrate linage (69). Still, if such individual variation is related to sensibility to dietary manipulations of dietary Trp content in non-mammalian vertebrates remains to be investigated.

Kynurenine Pathway

As mentioned above, in the section about factors affecting Trp uptake to the brain. Trp influx to the brain and brain 5-HT signaling can be modulated by the activation of the kynurenic pathway. In addition, metabolites of this pathway may affect neuronal signaling involved in stress coping processes [reviwed by (14)]. The metabolite in the first step of this pathway, kynurenine, readily passes the blood brain barrier (70). In the brain it is further degraded to kynurenic acid or quinolinic acid. Further down this pathway quinolinic acid produces neurotoxic compounds such as NMDA receptor agonists and oxidative radicals (71) while kynurenic acid is neuroprotective by being an NMDA receptor antagonist [for references see (14)]. In mammals, the neuroprotective kynurenic acid is mainly produced in astrocytes, while neurotoxic compounds are produced in macrophages and microglia (34). It has been suggested that an imbalance between these neurodegenerative and neuroprotective factors are involved in brain dysfunctions, including poor stress coping ability, in depression (72). In

TABLE 1 | Effects of dietary tryptophan supplementation on the behavioral and endocrine stress response in teleost fishes.

Species	Dose (x std feed)	Treatment (days)	Behavior	Plasma cortisol		Stressor		References
				Baseline	Stress	Type	Post Trp terat. (days)	
Oncorhuncus mykiss	2	7	N. i.	–	–	Confinement 2 h	1	(10)
	4	7	N. i.	↑	↓	Confinement 2 h	1	
	8	7	N. i.	↑	↓	Confinement 2 h	1	
Oncorhuncus mykiss	8	3	N. i.	↑	-	Confinement 2 h	1	(52)
	8	7	N. i.	–	↓	Confinement 2 h	1	
	8	28	N. i.	–	–	Confinement 2 h	1	
Gadus morhua	2	7	N. i.	–	–	Confinement (0.5 h)	1	(4)
	2	7	N. i.	–	–	Confinement (0.5 h)	2	
	2	7	N. i.	–	–	Confinement (0.5 h)	4	
	3	7	N. i.	–	–	Confinement (0.5 h)	1	
	3	7	N. i.	–	–	Confinement (0.5 h)	2	
	3	7	N. i.	–	–	Confinement (0.5 h)	4	
	4	7	N. i.	–	↓	Confinement (0.5 h)	1	
	4	7	N. i.	–	–	Confinement (0.5 h)	2	
	4	7	N. i.	–	–	Confinement (0.5 h)	4	
Salmo salar	2	7	N. i.	–	–	Confinement (0.5 h)	1	(53)
	2	7	N. i.	–	–	Confinement (0.5 h)	2	
	2	7	N. i.	↓	↓	Confinement (0.5 h)	10	
	3	7	N. i.	↓	–	Confinement (0.5 h)	1	
	3	7	N. i.	–	–	Confinement (0.5 h)	2	
	3	7	N. i.	↓	↓	Confinement (0.5 h)	10	
	4	7	N. i.	–	–	Confinement (0.5 h)	1	
	4	7	N. i.	–	↑	Confinement (0.5 h)	2	
	4	7	N. i.	↓	↓	Confinement (0.5 h)	10	
Oreochromis niloticus	4	7	N. i.	–	–	Chasing (0.3 h)	0	(54)
	10	7	N. i.	↓	–	Chasing (0.3 h)	0	
Salmo salar	2	7	N. i.	–	–	Crowding (1 h)	8	(7)
	2	7	N. i.	–	↓	Crowding (1 h)	21	
	3	7	N. i.	–	–	Crowding (1 h)	8	
	3	7	N. i.	–	↓	Crowding (1 h)	21	
Dicentrarchus labrax	2	14	N.i	–	↑	24 h post immune challenge	0	
Cyprinus carpio	5	15	N. i.	N. i.	↓	Saltwater (6 h)	0	(55)
Cyprinus carpio		21	N. i.	↓	↓	Cu+ exsposure (7 days)	0	(56)
Cichlasoma dimerus	8	28	N. i.	↓	N. i.			(57)
Labeo rohita	1.7[a]	45	N. i.	↓	N. i.			(58)

(Continued)

TABLE 1 | Continued

Species	Dose (x std feed)	Treatment (days)	Behavior	Plasma cortisol		Stressor		References
				Baseline	Stress	Type	Post Trp terat. (days)	
	2.4[a]	45	N. i.	↓	N. i.			
	2.9[a]	45	N. i.	↓	N. i.			
Labeo rohita	2.8	60	N. i.	N. i.	↓	Temp and/or salt (30 days)	0	(59)
	4.8			N. i.	↓	Temp and/or salt (30 days)	0	
Cirrhinus mrigala	∼3[a]	60	N. i.	↓	↓	High rearing density (60 days)	0	(60)
Sander lucioperca	3	7-60	N. i.	N. i.	↓	Emersion	0	(61)
	6	7-60	N. i.	N. i.	↓	Emersion	0	
AGGRESSION								
Oncorhyncus mykiss	36	0	–				0	(20)
		3	–				0	
		7	↓				0	
	360	0	–				0	
		3	–				0	
		7	↓				0	
Oncorhuncus mykiss	8	7	↓	N. i	↓	A smaller conspefic (1 h)	1	(62)
Gadus morhua	6[a]	4–10	↓	N. i	N. i	3 × social interact. (0.15 h/day)	0	(63)
Matrinxã Brycon amaz.	2	7	↓	N. i.	↓	Social interaction (0.3 h)	0	(64)
	4	7	↓ Anneroxia	N. i.	↓	Social interaction (0.3 h)	0	
Salmo trutta	3.6	7	↓	N. i.	N. i.	Novel environment (3 days)	1–3	(19)

↑, ↓, *and* – *refers to stimulating, suppressive or no effect t compared to standard feed.*

N.i. Not investigated.

[a]*Estimated from similar feed recipe.*

addition, studies in rats show that dietary Trp can affect brain levels of kynurenic acid (73), which in turn effects other neurotransmitters, such as dopamine and glutamine through activation of NMDA and/or a7 nicotinic acetylcholine receptor (74, 75). Central effects of Trp metabolites produced by the kynurenic pathway in teleost fishes are, to our knowledge, largely unknown. Still, effects of dietary Trp supplementation on dopaminergic neurochemistry in Atlanitic salmon (53) and Atlanitic cod (*Gadus morhua*) (4) have been suggested to be related to elevated levels of kynurenic acid (53).

BEHAVIORAL EFFECTS OF ELEVATED DIETARY TRP

There is a general consensus that low levels of central 5-HT are associated with high levels of aggression within the vertebrate subphylum (3, 69). In line with this, human studies show that alterations of the dietary Trp content changes irritability and aggressive behavior [for references see review by Young and Leyton (76)]. For example, human lab studies show that dietary Trp induces a dose dependent effect on aggressive responses, where Trp supplementation and depletion induced the lowest highest aggression, respectively (77, 78). This negative relationship between dietary Trp content and aggression is further supported by studies on rats and birds, showing that Trp loading can attenuate aggressiveness (79, 80). Similarly, there are studies in teleost fishes showing a general suppressive effect on aggressive behavior by dietary Trp supplementation (20, 63, 64). Furthermore, in the study performed by Winberg et al. (20) the attenuating effects of dietary Trp on aggressive responses during territorial defense followed the same time-coarse as the effects on the neuroendocrine stress response in rainbow trout (52), with a peak after 7 days of treatment. This together with a study performed by Höglund et al. (19), showing that the same treatment time attenuated the anorexic response to a novel environment, strongly suggest that Trp affects 5-HT signaling and the integrating role of this neurotransmitter in behavioral and neuroendocrine stress responses.

Dietary Trp supplementation have also been shown to reduce cannibalism in juvenile grouper (*Epinephelus coioides*) (81) and pike perch (*Sander lucioperca*) (82). However, the behavioral components of this response were not studied. Differences in body size is a main factor underlying cannibalism in piscivorous fish (83), and one possible explanation to the reduced cannibalism could be a more homogeneous growth due to reduced competition for food in fish given Trp supplemented food. The behavioral effect of dietary Trp manipulations in teleost fishes are summarized in **Table 1**.

CONCLUSIONS AND SUGGESTION FOR DIRECTION OF FURTHER STUDIES

A positive relationship between dietary Trp and brain 5-HT activity seems to be present across the vertebrate linage. However, there appear to be differences between teleost fishes and mammals when it comes to plasma Trp transport since teleost albumin lacks the indole binding site (29, 30). This makes Trp influx to the brain less sensitive to carbohydrates in fish compared to mammals. On the other hand, behavioral and neuroendocrine effects of elevated dietary Trp are similar in all vertebrates. Studies in mammals and teleost fishes show that these effects, including suppression of aggressive behavior, attenuation of stress induced anorexia and lower post stress plasma cortisol, appear after 3–7 days of elevated dietary Trp intake. It has been suggested this slow time-course reflects 5-HT induced structural changes in the brain (7). However, further studies are needed to verify this assumption.

In mammals the majority of Trp enters the kynurenic pathway. The first stage of this pathway is catalyzed by the enzymes TDO and IDO that are induced by glucocorticoids and pro-inflammatory cytokines, respectively. Thus, chronic stress and infections can shunt available Trp toward the kynurenic pathway and thereby lowering the rate of brain 5-HT synthesis while simultaneously increasing the production of other Trp metabolites [for references see (14)], which potentially can affect behavioral and endocrine responses to stress. So far, the kynurenic pathway have been neglected when investigating effects of dietary Trp supplementation in teleost fishes. It has previously been pointed out that effects of dietary Trp is context dependent, where especially the stress status of the animals can affect the outcome of dietary Trp manipulation (19). A recent study demonstrates that the expression of IDO mRNA is upregulated by LPS in rainbow trout (40), suggesting that bacterial infection can affect the catabolic faith of Trp also in fish. Previously dietary Trp supplementation have been suggested as a strategy for reducing unavoidable stress, such as stress related to transport, size grading and vaccination, in aquaculture (84). However, considering that inflammatory processes might affect the catabolic faith of Trp in teleost fish, anti-inflammatory treatments should also be considered.

In humans, low circulating levels of the ω3 fatty acids, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), and a decreased ratio of EPA to the ω6 fatty acid arachidonic acid (ARA) have been associated with psychiatric ailments and poor stress coping ability (15). Moreover, a diet with high DHA and EPA have been shown to affect serotonergic transmission and to prevent such psychiatric ailments [for references see (15)]. The mechanisms for this anti-depressive action of ω3 fatty acids are currently not fully understood. However, it is possible that a diet with high ω3 content results in a suppression of pro-inflammatory eicosanoids, which in turn may reduce the activity of the kynurenic pathway, increasing Trp influx to the brain, and subsequently stimulate brain 5-HT synthesis.

The relative amount of marine ω3 fatty acids has decreased in commercial fish feed. Potentially, this may result in poorer stress coping ability trough dietary effects on central 5-HT signaling. Thus, we hypothesize that it is not only the relative amount of Trp to other LNAAs in the diet that is important for producing stress resilient robust fish. Rather, there is an interplay between dietary amino and fatty acids that decides the effects of Trp supplementation, where ratio ω3 to ω6 fatty acids in the diet influences the catabolic faith of Trp. Studies demonstrating a

negative relationship between HPI-axis reactivity and the ration of ω3 to ω6 fatty acids in the diet (85, 86) lends support to this hypothesis. However, if such effects of dietary fatty acid composition are related to changes in the activity of the kynurenic pathway is currently not known.

AUTHOR CONTRIBUTIONS

EH and SW drafted the manuscript. EH, ØØ, and SW finalized the manuscript.

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frontiers in Endocrinology

REVIEW
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Environmental Cycles, Melatonin, and Circadian Control of Stress Response in Fish

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Fish have evolved a biological clock to cope with environmental cycles, so they display circadian rhythms in most physiological functions including stress response. Photoperiodic information is transduced by the pineal organ into a rhythmic secretion of melatonin, which is released into the blood circulation with high concentrations at night and low during the day. The melatonin rhythmic profile is under the control of circadian clocks in most fish (except salmonids), and it is considered as an important output of the circadian system, thus modulating most daily behavioral and physiological rhythms. Lighting conditions (intensity and spectrum) change in the underwater environment and affect fish embryo and larvae development: constant light/darkness or red lights can lead to increased malformations and mortality, whereas blue light usually results in best hatching rates and growth performance in marine fish. Many factors display daily rhythms along the hypothalamus-pituitary-interrenal (HPI) axis that controls stress response in fish, including corticotropin-releasing hormone (Crh) and its binding protein (Crhbp), proopiomelanocortin A and B (Pomca and Pomcb), and plasma cortisol, glucose, and lactate. Many of these circadian rhythms are under the control of endogenous molecular clocks, which consist of self-sustained transcriptional-translational feedback loops involving the cyclic expression of circadian clock genes (*clock*, *bmal*, *per,* and *cry*) which persists under constant light or darkness. Exposing fish to a stressor can result in altered rhythms of most stress indicators, such as cortisol, glucose, and lactate among others, as well as daily rhythms of most behavioral and physiological functions. In addition, *crh* and *pomca* expression profiles can be affected by other factors such as light spectrum, which strongly influence the expression profile of growth-related (*igf1a*, *igf2a*) genes. Additionally, the daily cycle of water temperature (warmer at day and cooler at night) is another factor that has to be considered. The response to any acute stressor is not only species dependent, but also depends on the time of the day when the stress occurs: nocturnal species show higher responses when stressed during day time, whereas diurnal fish respond stronger at night. Melatonin administration in fish has sedative effects with a reduction in locomotor activity and cortisol levels,

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as well as reduced liver glycogen and dopaminergic and serotonergic activities within the hypothalamus. In this paper, we are reviewing the role of environmental cycles and biological clocks on the entrainment of daily rhythms in the HPI axis and stress responses in fish.

Keywords: daily rhythm, light, temperature, HPI axis, wavelength, thermocycles, fish welfare

ENVIRONMENTAL CYCLES AND BIOLOGICAL CLOCKS IN FISH

The environment is rarely constant and fluctuates most of the time. Although some environmental changes are unpredictable (e.g., metereological phenomena such as rain or wind), other cyclic fluctuations such as tides, day length, moon phases and seasons are highly predictable. These environmental cycles are governed by geophysical cycles originating from the rotation of the Earth and the Moon around the Sun. Time-keeping systems (i.e., circadian clocks) have evolved since the most primitive forms of life to cope with natural cycles and anticipate periodic events (1). In fish, as in other vertebrates, most behavioral and physiological processes exhibit rhythms, which are driven by molecular clocks made up of transcriptional/translational loops of several clock genes (*per*, *clock*, *bmal*, *cry*, *ror,* and *reverb*) (2, 3).

Light and temperature cycles are the two main synchronizing signals (so called "zeitgebers" or time-givers) to entrain biological clocks. Light information is transduced into a nocturnal rhythm of melatonin that acts as an internal zeitgeber setting up the phase of individual pacemakers. Daylength, the basis for photoperiodism and seasonality, is coded by the duration (longer/shorter) of the nocturnal melatonin rhythm (4). In addition, light characteristics should be considered, since the underwater photo-environment is peculiar as light is absorbed differently by the water column, so that only blue light ($\lambda \sim$450 nm) reaches deep marine waters (up to 200 m in clear oceanic waters -euphotic zone), while red light ($\lambda > 600$ nm) is quickly absorbed within the first 20 m. Thus, melatonin synthesis is suppressed by light differently depending on the wavelength: shorter (blue) being more effective than longer (red) wavelengths (5). Artificial lights differ greatly from the natural solar light, because classic light bulbs (incandescent filaments) produce a reddish inefficient light underwater, while fluorescent tubes produce sharp peaks at specific wavelengths far from natural daylight. Modern light-emitting diode (LED) technology, however, provides better cost-effective lighting systems which can be used for different purposes in aquatic research (6). Using such technology, light spectrum has been found to affect the ontogeny of the molecular clock, as *clock*, *per,* and *bmal* gene expression was affected by lighting conditions during early larval development. Furthermore, larvae reared under constant darkness became arrhythmic, while under light/dark cycles of different wavelengths their daily activity rhythms appeared earlier under blue than under white or red lights (7).

The daily day/night alternation not only imposes a light cycle but also a temperature cycle, as the water warms up during the day following sunrise, and cools down at night after sunset. Such a daily thermo-cycle (TC, 12 h cold:12 h warm) synchronizes the circadian clock, which periodicity (tau) is temperature-compensated and remains constant in a wide range of temperatures, with a Q10 value for tau around 1 (8). Actually, clock transcriptional regulatory elements are entrained by TC in embryos and primary cell lines of zebrafish (*Danio rerio*) (9), although light controlled elements (p*er2* and c*ry1a*) do not show rhythmic expression under TC (10). Regarding melatonin, as early reported by Underwood and Calaban (11) in lizards, its rhythmic secretion can be synchronized in constant dark (DD) and constant light (LL) by daily temperature cycles as low as 2°C in amplitude (melatonin peaking during the cold phase). In pike *in vitro* pineal culture, rhythmic melatonin production persisted in TC (10°C:20°C) and DD, which peaked during the hight temperature (12). Nevertheless, TC cycles synchronized with good strength a melatonin rhythm under DD, providing the high temperature coincided with the subjective dark. Synchronization persited, but the rhythm was of lower amplitude when the high temperature was given during the subjective day. In all cases, the TC rhythm didn't entrain the melatonin rhythm as a release into constant temperature resulted in a rapid damping of the melatonin rhythm. As to locomotor activity rhythms, however, under TC and ahemeral light-dark (LD) cycles (conflicting zeitgebers), zebrafish displayed relative coordination, while in constant dim light they synchronized to TC, and they also free-run in constant temperature. These findings indicate that TC alone can entrain zebrafish rhythms, suggesting the participation of both light- and temperature-entrainable oscillators which are weakly coupled (13, 14).

PHOTOTRANSDUCTION AND MELATONIN RHYTHMS IN FISH

Melatonin is a key hormone acting in the circadian system of vertebrates, and it is mainly produced by the pineal gland. In fish, the pineal is a complex structure located in an evagination of the roof of the diencephalon, which exhibits photoreceptive characteristics (15, 16). The pineal epithelium contains photoreceptor cells that resemble the retinal cones of the retina, both on a structural and functional point of view (17–19). These cells elaborate an electrical message at night when they are depolarized, which results in the release of an excitatory neurotransmitter. Meanwhile, light induces hyperpolarization of the photoreceptor cells and inhibits the discharge of the pineal neuronal units (20–22). In addition, as early reported by Falcon et al. (23), photoreceptor cells contains the amino

acid (tryptophan) and all the indole compounds (serotonin, N-acetylserotonin, melatonin) and enzymes (see later) to produce melatonin (24–29). The pineal hormone displays daily and seasonal patterns of secretion with elevated levels at night and basal levels during the day, regardless of the fish species studied. Therefore, robust and predictable rhythms of melatonin secreted from the pineal to the blood and likely to the CSF, with which the pineal epithelium communicates in its apical part (30) are expected. The rhythmic melatonin output, which reflects the prevailing photoperiod, is an efficient signal to entrain a wide number of processes that occur at daily and seasonal levels (4).

The synthesis of melatonin also occurs in the retina, which in teleost has been usually, but not exclusively, associated with photoreceptor cells (31–33). Although rhythmic on a daily basis, the pattern of retinal melatonin is substantially different from that in the pineal organ, with melatonin content peaking during the night, or at different times during the day or modifying the phase of the rhythm throughout seasons depending on the species (34–37). Moreover, retinal melatonin is thought to act as a local neuromodulator within the eye (32, 38, 39) and it could be metabolized *in situ* (40), which prevents retinal melatonin to be released to the blood. More doubt arises from a synthesis of the hormone in other body tissues of fish, the intestine being reported to hold relevant amounts of melatonin (41–43). In addition, the presence of mRNA transcripts of melatonin synthesis enzymes has been reported in the digestive tract of several teleost species such as goldfish (44), carp (45), and rainbow trout (43), with daily rhythms that adjust to the prevalent photoperiod. Although a more formal demonstration of melatonin synthesis in fish intestine is needed, it seems like its contribution to plasma melatonin rhythms should be very poor in comparison with the pineal melatonin source, as low night levels or lack of plasma melatonin rhythms are found in pinealectomized fish (43, 46).

Studies in several teleost provide well-founded data about the distribution of melatonin binding sites in wide range of body tissues (47–50). Therefore, this hormone can be involved in multiple physiological processes, most of them displaying daily and/or seasonal rhythms, such as those of locomotor activity, skin pigmentation, food intake, osmoregulation, growth and reproduction [for reviews (3, 4, 51, 52)]. Thus, the melatoninergic output is part of the time-keeping system and enable the fish to synchronize with the closest environment (51). The characteristics of its daily rhythm are well conserved independently on the organization of the system that controls such rhythm. The LD cycle is the prevalent cue that directly or indirectly through the circadian clock system, controls pineal melatonin synthesis and adjust its daily profile in blood (29, 51, 53, 54). The nocturnal rise in melatonin observed in all vertebrates is the consequence of two enzymatic steps that transform serotonin into melatonin: arylalkylamine N-acetyltransferase (AANAT) catalyses serotonin synthesis, whereas hydroxyindol-*O*-methyl transferase (HIOMT) transforms N-acetylserotonin in melatonin (55). In vertebrates, AANAT enzyme is the rate-limiting step for clock-dependent light influence on melatonin synthesis, since this enzymatic activity displays daily oscillations with light inhibiting it during daytime (56). Interestingly, teleost fish, unlike other vertebrates, possess two AANAT subfamilies, namely AANAT1 and AANAT2, which is likely to derive from the whole genome duplication that occurred close the origin of fish (57–59). Whereas, AANAT1, which is homologous with the AANAT found in tetrapods, is expressed preferentially in the retina and discrete brain areas of fish, AANAT2 is more specifically expressed in the pineal gland and has no equivalent in other vertebrates (22, 60).

In contrast to that of mammals, fish pineal photoreceptors cells contain the whole machinery of a light entrained circadian system: photoreceptor unit, clock machinery and melatonin production system (25, 29, 61, 62). Indeed, melatonin synthesis in most teleost species continues to be rhythmic in pineal explants and this rhythm adjusts to a 24-h cycle when they are exposed to a fluctuating light environment (25, 31, 63–65). The connection between pineal clock system and rhythmic melatonin synthesis occurs through a CLOCK-BMAL dimer binding to an *E-box* in the *aanat2* promoter (66–68). Thus, accumulation of *aanat2* mRNA as a result of increased gene transcription during the second half of the day allows AANAT2 protein to be high soon after night onset. Light at the following day resets the clock, which makes AANAT2 enzyme activity and melatonin synthesis to drop (69). The salmonidae lineage, which includes the rainbow trout (*Oncorhynchus mykiss*) and Atlantic salmon (*Salmo salar*), breaks this rule since it lacks an intra-pineal oscillatory mechanism (70). Because of that, rhythmic melatonin synthesis occurs only under an LD cycle both *in vivo* and *in vitro* (71–74). Additionally, melatonin synthesis from fish pineal varies between seasons, which is interpreted by the clock machinery, then modulating annual rhythms (36, 75, 76). Light properties such as intensity and spectrum impact on the amplitude of the melatonin peak, therefore melatonin secretion varies in fish as a result of water depth, time of the day (dawn and dusk), weather conditions, moon phase or latitude (4). Moreover, water temperature is another external factor that acts on the pineal organ to influence melatonin rhythm, through the regulation of AANAT2 activity. A good correlation of AANAT2 activity at night exists for some teleost such as rainbow trout, pike (*Esox lucius*), sea bream (*Sparus aurata*), and zebrafish, with optimal physiological temperatures (12, 29, 60, 72). This strongly supports that both light and temperature act together to provide accurate tuning to daily and annual cycles of melatonin in fish (4, 77).

RHYTHMS IN THE HPI STRESS AXIS

A wide variety of physiological variables display rhythmicity in fish, among them many factors of the endocrine system such as those produced at all levels of the hypothalamus-pituitary-interrenal (HPI) axis (78, 79), which is the main neuroendocrine circuit involved in the primary response to stress in fish, together with the catecholamine-producing chromaffin cells from the hypothalamic sympathetic nervous system (80, 81). The hypothalamus synthesizes corticotropin-releasing hormone (Crh) which in turn stimulates the synthesis

and release of adrenocorticotropic hormone (Acth) from the pituitary (82). Acth is generated from the cleavage of the Proopiomelanocortin (Pomc) and stimulates the production and release of glucocorticoids in the cells of the fish interrenal tissue (82) (**Figure 1**). The main glucocorticoid produced by fish is cortisol which, besides its main role in the stress response and stress-related homeostasis, influences many other processes such as behavior, growth, reproduction, and osmoregulation (80, 82, 84, 85).

Studies on the rhythmicity of factors from the HPI axis have mainly focused on cortisol, whose daily rhythms have been described in a wide variety of species (78, 86). In addition, daily rhythms have also been reported in other factors from the HPI axis such as the hypothalamic *crh* and pituitary *pomc* gene expression (83, 87, 88). Regarding cortisol, the characteristics of the rhythm such as mesor (similar to the median), amplitude (difference between mesor and highest or lowest point), and acrophase (the time of day when the highest values can be found) are species-dependent. Cortisol rhythms persist under environmental constant conditions, i.e., constant light (LL) or darkness (DD), in some species such as gilthead sea bream, Senegalese sole and rainbow trout (89–91). This persistence in the absence of external cues (free-running) indicates that the rhythm is controlled by circadian oscillators located within the organism (79).

Moreover, besides the daily rhythms that seem to be mainly controlled by variations in the LD cycle, cortisol is also influenced by seasonal variations in photoperiod and water temperature and

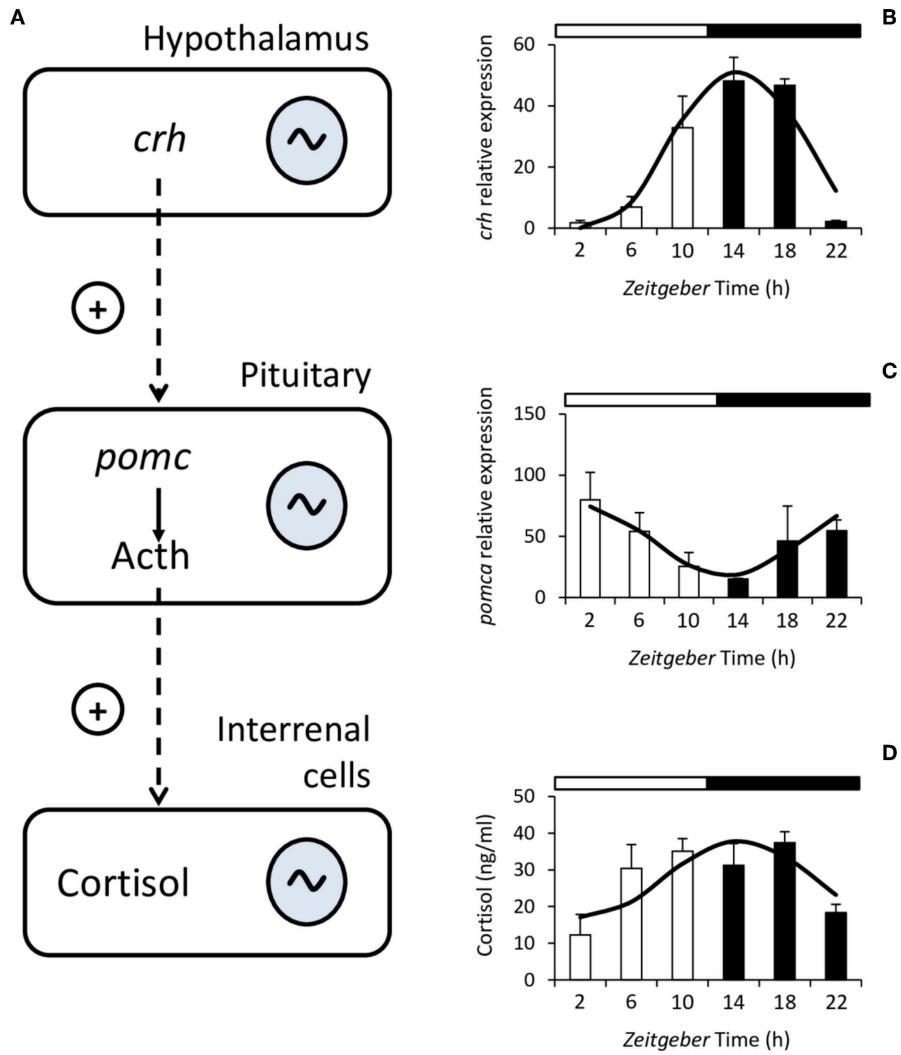

FIGURE 1 | Schematic diagram of the hypothalamus-pituitary-interrenal (HPI) axis **(A)**. Corticotropin-releasing hormone (Crh) is synthesized in the hypothalamus and stimulates, at the pituitary, the synthesis and release of adrenocorticotropic hormone (Acth), which is formed from Proopiomelanocortin (Pomc). Acth stimulates the production and release of cortisol in the interrenal cells. In fish, the HPI axis presents daily rhythms at all of its levels. To the right of the figure, representative examples of the rhythms of *crh* expression **(B)**, *pomca* expression **(C)**, and plasma cortisol **(D)** from Senegalese sole are shown. Mean ± S.E.M. are represented by the bars and errors, the continuous curve represents the cosine function calculated from a significant Cosinor analysis ($p < 0.05$). White and black bars above the graphs represent the light and dark period, respectively. Modified with the permission of authors from López-Olmeda et al. (83).

by feeding time. Annual cortisol variations have been described in several fish species and they seem to correlate mainly with the seasonal reproduction, with the highest annual cortisol levels being located around the spawning season (78). On the other hand, a fixed feeding time can act as the entraining signal of cortisol rhythms in the absence of other external signals such as the LD cycle (92, 93), and different fixed feeding times are able to shift the cortisol rhythm (94). Therefore, the season of the year and the feeding strategy are factors that should be considered when studying cortisol rhythms.

STRESS AND MELATONIN INTERPLAY IN A RHYTHMIC ENVIRONMENT

Light disturbance either in natural environment, i.e., artificial nighttime lighting, or during farming is another critical factor that could induce stress in animals, including fish (95–97). In this context, studies on environmental stress effects on vertebrate circadian systems are still scarce. In mammals, constant light exposure or food intake out of circadian phase potentially alter the diurnal level of secreted glucocorticoids (GC) and stress-induced GC response (98). Additionally, GC and catecholamines can act as synchronizers of circadian clocks (99, 100). The glucocorticoid receptors (GR) are expressed ubiquitously in nearly all tissues and organs, with the exception of SCN, where no GR expression was noted (99). However, several genomic and non-genomic pathways exist, through which GC can influence circadian core clock genes. In this context, stress at the photophase onset causes a phase-advance of mRNA expression of several core clock genes in peripheral organs (101). Meanwhile, when applied at different times during the photophase, it causes delay or even loss of synchrony, indicating that influence of stress on peripheral clocks depends on the time of day.

In fish, environmental stressors are increasingly related to changes in water conditions including elevated temperature (e.g., global warming or proximity to nuclear plants or cities), presence of pollutants, and oxygen deficits. Routine husbandry in aquaculture also involves further factors, such as stocking conditions, handling, feeding and social interactions, among others (102–104), several of which are also influenced by human intervention. In fish, the effect of stress induced by high density stocking on the daily profile of hypothalamic mRNA abundance of circadian clock genes (*clock1a*, *bmal1*, *per1,* and *rev-erbβ-like*) was recently studied. Decreased amplitude and mean expression levels for most of these genes appeared in stressed trout, except for *rev-erbβ-like* whose expression increased (105). Furthermore, treatment of trout with the GR antagonist, mifepristone, previously exposed to a stressor failed to prevent these stress-induced changes, suggesting that cortisol is not directly modulating clock gene expression within the hypothalamus in trout. Additionally, this study provides evidence for the involvement of Sirtuin1 (Sirt1), a member of the histone deacetylases family which links cellular metabolism and circadian clocks in mammals (106) and fish (91). Sirt1 deacetylates *bmal1* and *per2* in the liver (107) and activates hypothalamic SCN pacemaker in mice (108). Moreover, *sirt1* mRNA accumulates rhythmically under normal LD conditions and increases sharply in the hypothalamus of stressed trout (105). Therefore, Sirt1 is a good candidate to mediate the effects of stress on the circadian clock genes, not only in peripheral metabolic tissues (liver), but also centrally at the hypothalamic level, where a neuronal network integrates the effects of stress to modulate nutrient sensing information and regulate feeding behavior (109, 110). It is also involved in the regulation of the rhythmic profile of clock genes at the brain level (105), suggesting a role of Sirt1 in the crosstalk between stress response and central circadian system in fish.

The pineal melatoninergic system in vertebrates has been also reported to be influenced by stress and GC treatment in early studies in the 70s [e.g., (111)], and later both *in vitro* (112, 113) and *in vivo* (114–117). In rodents, forced physical activity every 2 h for the 24 h around the clock, results in lower melatonin levels at night, thus flatting normal daily melatonin rhythm (118). Additionally, chronic stress alters the expression of sympathetic markers in rodent pineal gland and increases plasma melatonin concentrations (119). Increased melatonin levels during daytime after immobilization alone or together with dexamethasone treatment were reported in the avian ring dove (*Streptopelia capicola*) (114). A prolonged, but not acute, treatment with dexamethasone also suppressed melatonin production in chick pineal gland and retina, with Aanat activity being significantly lower than that of controls (115). Regarding fish, it seems that pineal melatonin is very sensitive to different environmental stressors, although differences were observed depending on the species and stress type. Rainbow trout initially adapted to freshwater conditions (6 ppt) that were later transferred to isosmotic (12 ppt) and hyperosmotic conditions (18 ppt) showed an increased melatonin content at night in pineal gland and plasma, as compared to the initial status, both in a short-term (6 h) and long-term (5 days) exposure (120). A stimulatory effect of salinity on pineal *aanat2* mRNA abundance and enzyme activity was identified at day- and night-time, with melatonin synthesis enzymes under the regulation of cortisol. This suggests that increased blood osmolality and plasma cortisol levels induced by the hypersaline environment promotes melatonin synthesis in the pineal organ of rainbow trout by increasing Aanat activity independently of the regulatory action exerted by light. In cocho salmon, however, plasma melatonin remain constant during parr to smolt transformation, but increased upon seawater entry (121). Other stressors, like chasing and high-stocking density inhibit melatonin synthesis at night, thus disrupting melatonin rhythms and the capacity of fish to translate environmental information (122). A drop in pineal serotonin content, *aanat2* gene expression, and Aanat enzyme activity was also reported at night. This fits with a diminished N-acetylation pathway as a consequence of lower substrate availability and enzyme activity. In this context, cortisol is likely to have a key role in mediating stress-effects on melatonin synthesis in the pineal organ of trout. In fact, intraperitoneal (IP) cortisol implants reduced melatonin synthesis at night in a similar way than exposure to stressors, and incubation of cultured pineal organs with cortisol reduced

melatonin synthesis during the dark phase of the 24-h cycle, with this effect prevented when a GR antagonist was added (113, 122).

Several published studies also support a modulatory role of GC in teleost pineal organ. High cortisol concentrations (100 ng/ml) mimicking stressed conditions were shown to reduce melatonin secretion from cultured pineal organs of tilapia (*Oreochromis mossambicus*) (123), although a similar effect was not observed at night, when cortisol was physiologically elevated in stressed fish. In contrast, socially subordinated rainbow trout displayed concomitant increases in cortisol and melatonin levels in blood, suggesting that social status of the animals may modify the circadian cycles of these hormones. In the North African catfish (*Clarias garieinus)*, treatment with corticosteroid hormones in a μM to mM range inhibited pineal AANAT activity in a dose-dependent way during different phases of the breeding cycle (124). Meanwhile, rainbow trout pineal organs incubated with the GC analog, dexamethasone, at nM concentrations also exhibited inhibition of AANAT2 activity, without affecting HIOMT activity (113). Since a daily variation of *gr* mRNA has been reported in the pineal organ (123) it is plausible that GC effects on melatonin synthesis are modulated by oscillation of GR signaling, which involves the activation of glucocorticoid-responsive elements at the Aanat promoter (113). Alternatively, GC actions are also likely mediated by cell surface receptors that modify Ca2+ and cAMP levels (82), therefore being potentially able to modulate rhythmic melatonin synthesis by the photosensitive pineal cells (4).

In fish, the stress response involves a series of physiological components organized in two neuroendocrine axes, the brain-sympathetic-chromaffin (HSC), and the HPI tissues, whose activation by stressors lead to increased catecholamines and cortisol blood levels, respectively (125). Several studies showed that melatonin might play a role in alleviating stress effects in teleosts, which in many cases relates to the modulation of neuroendocrine responses within the HPI axis. For instance, Munro (126) showed that intracerebroventricular (i.c.v.) injections of melatonin (10 μg) reduced aggressive behavior in the cichlid *Aequidens pulcher* to a mirror presented 20 min later, whereas Larson et al. (127) reported that socially subordinated fish have higher night melatonin levels and no elevation of cortisol levels compared to non-stressed fish. On the other hand, several studies report that treatments with melatonin at doses mimicking nocturnal increase of the hormonal levels were able to reduce stress effects in fish. Thus, melatonin given orally (40–200 mg/g food) or dissolved in water (10 μM) attenuated several effects of chronic stress in rainbow trout (128), and Senegalese sole (*Solea senegalensis*) (129), such as elevated plasma cortisol, inhibited food intake, altered activity of some digestive enzymes, and increased plasma lactate levels and liver glycogenolitic potential (128). Accordingly, Gesto et al. (130) showed that adding melatonin at doses as low as 10 nM into the fish tank was effective in reducing the intensity of stress response induced at short-term by chasing. Thus, a simple treatment with melatonin attenuated the response to cortisol, including the increase of hypothalamic *crh* mRNA content and that of enzymes involved in the steroidogenesis pathways at the head kidney, which normally allow cortisol secretion to increase soon after fish is stressed. Also, intraperitoneal (i.p.) administered melatonin at doses as low as 10 μg/g body weight for 7 days resulted in reduced plasma cortisol levels and locomotor activity of goldfish (*Carassius auratus*) (131), thus suggesting that peripheral melatonin inhibits the stress response and displays additional sedative effects in teleost.

The mechanisms through which melatonin mitigates stress is currently unknown, although both central and peripheral actions of melatonin are suspected to be involved. In fish, the brain serotonergic system is believed to play a role in the activation of the neuroendocrine responses to both acute and chronic stress, including social stress (132–134). An increased serotonergic function starts immediately after exposure to the stressor, particularly affecting the hypothalamus and telencephalon, two regions that receive serotonergic neuronal endings (132, 133). At the level of the hypothalamus-preoptic area, serotonin stimulates the HPI axis by increasing Crh release, which boosts the downstream GC stress response (125, 134). Studies have revealed that melatonin can interact with serotonin to modulate its function (109, 130, 135). Moreover, melatonin ability to reduce stress in teleosts has been usually associated with simultaneous changes in brain serotonergic activity (109, 130, 133). Indeed, melatonin treatment decreased *crh* mRNA in sole which was upregulated by environmental stressors (130), pointing to a melatonin interplay with serotonin- and Crh-containing neurons in the hypothalamic-preoptic area. Specific studies are lacking to demonstrate the underlying mechanisms of the actions of melatonin on brain serotonin at the cellular level, as well as those that activate the endocrine response to stress. For instance, 5-HT1A-like receptors were involved in mediating increases in *crh* mRNA and Acth hormone secretion in the Gulf toadfish to crowding stress (136) and to modulate HPI axis response in Arctic charr (*Salvelinus alpinus*) (137). This suggests these receptors are potential candidates for serotonin-mediated effects of melatonin to reduce stress response in teleosts, and this hypothesis should be further tested.

Additionally, the possibility that melatonin acts directly on adrenal tissue to modulate GC secretion exists, as reported in mammals (138), and also suggested in fish where i.p., but not i.c.v., melatonin treatment was able to reduce cortisol secretion (131). The presence of melatonin binding sites and mRNA expression of melatonin receptors has been demonstrated in several teleost species (47, 48). Finally, besides applying pharmacological and molecular tools to gain knowledge on the melatonin-cortisol interaction, it is intriguing to know whether the endogenous high levels of melatonin at night are involved in modulating cortisol secretion, either through the HPI axis and interrenal cells or by tuning the daily rhythmic cortisol profile, through the circadian system.

LIGHT AND TEMPERATURE STRESSORS DURING EARLY DEVELOPMENT AND ADULTHOOD

The environment during early life stages permanently alters behavior and physiology by "programming" the expression of selected genes. Actually, environmental stress in early life

can impair normal development, predisposing to disease in adulthood (139). Light characteristics (intensity and spectrum) change underwater and affect fish embryo and larvae development (140). In fact, constant light, constant darkness or LD cycles of red lights lead to increased malformations and mortality, whereas LD cycles of blue light produced best hatching rates and growth performance in European sea bass and Senegalese sole (141, 142). In zebrafish, LD cycles of different light wavelengths (violet, blue, green, yellow, red, and white) led also to differences in development, growth, malformations and ultimately survival, upregulating the expression of key genes of the somatotropic (*igf1a* and *igf2a*) and stress axis in fish (crh and pomca) (143). On one hand, growth was enhanced in larvae exposed to LD cycles of violet and blue lights, which showed also significantly higher expression of *igf1* and *igf2*. On the other hand, the LD cycles of violet light produced the highest malformation rates and increased expression of crh, while the best survival rate and feed intake was achieved in fish exposed to LD cycles of blue light (**Figure 2A**).

Light spectral responses may differ depending on the fish species. In tench, locomotor activity and cortisol levels were influenced by light spectrum, since juvenile tench kept under white and blue lights were less active at night, and cortisol levels were higher in fish kept under white light than in those under constant darkness (144). Fish under red light behaved in a similar fashion as those in darkness. In fact, in some fish species red light may stimulate feeding activity, although such an increase in feeding does not necessarily elicit higher growth. That is the case of Nile tilapia, which showed higher feed intake under red light than under white, blue, green and yellow lights, but failed to show differences in growth rates of feed conversion efficiencies (145). This lack of growth differences despite the increase in food intake maybe related to changes in metabolism, which made food energy being channeled to stress or swimming. In this species, however, blue light prevented confinement stress responses and produced lowest cortisol levels compared to fish under green or white lights (146, 147). In Atlantic cod and turbot (*Scophthalmus maximus*), larvae reared under shorter wavelengths (blue and green lights) showed significantly enhanced growth in comparison to larvae reared under longer wavelengths (red light) (148). Reproduction was also affected by light color, nest construction in Nile tilapia being enhanced under blue light as well (149).

Background color and light contrast are further relevant issues to be considered. In Jundiá (*Rhamdia quelen*), a south american aquacultured fish, the combination of tank color and shelter availability reduced stress responses as cortisol levels decreased in fish kept in talks with blue walls and shelter (150). In the Caspian kutum (*Rutilus frisii*), the color of the tanks (black, blue, red, yellow or white) appeared also to influence food intake and lipid content without changing growth or feed conversion rates (151). Eurasian perch (*Perca fluviatilis*) larvae also showed better growth and prey intake when raised in black tanks compared to gray tanks (152). The combination of different light and wall tank colors affected also the welfare of beluga (*Huso huso*), since red light had a negative impact in growth, while blue light reduced plasma cortisol and glucose (153, 154). In summary, there seems to be a general consensus in different species pointing at shorter wavelengths (blue and green -the ones matching the natural marine underwater photoenvironment) having a positive effect on fish welfare, regardless of their life stage.

The role of temperature regulating fish metabolism, reproduction, development and other adaptive responses has been widely reported (155). Temperature tolerance in fish has been linked with global warming issues (156) and nutritional factors such as dietary lipids (157). As to the effects of daily thermo-cycles (TC) on fish welfare, however, little is known. An early paper by Spieler et al. (158) reported in goldfish that increasing water temperature from 14 to 23°C for 4 h at different times (7, 11, 15, 19, 23, or 3 h) every day resulted in different body weight and gonadalsomatic index. In Senegalese sole, larvae exposed to TC (22°C-day:19°C-night) grew better, showing fastest development and lowest malformation rates, than those raised under constant temperature (20.5°C) or a reversed daily thermocyle (CT, 19°C-day:22°C-night) (141) (**Figure 2B**). Moreover, in juvenile sole, daily thermocycles proved to affect sex steroid concentrations (higher estradiol in TC fish), sex determination (which occurred earlier in fish under TC) and sex differentiation: fish exposed to TC showing a higher female proportion (71%) than those under CT (18%) or constant temperature (38%) (141). Similar results were obtained in zebrafish larvae kept under two constant (24°C and 28°C) and two daily thermocycles: 28°C-day:24°C-night (TC) and 24°C-day:28°C-night (CT), embryo development and larval growth being fastest under 28°C and TC, which also showed the highest survival and lowest malformation rates (159). Moreover, in that report sex ratio was also strongly affected by the temperature regime, so that CT and TC produced more females (around 80%), and highest expression of ovarian aromatase (*cyp19a*), which converts androgens into estrogens and thus led to female differentiation.

Acclimation to a cyclic thermal environment can increase thermal tolerance, particularly during early development since the thermal history of larvae induces irreversible changes. As reported by Schaefer and Ryan (160), fish zebrafish larvae reared under daily thermocycles (28 ± 6°C) showed greater tolerance than those reared under constant (28°C) or stochastic (random variations, mean 28°C) temperature regimes. Ongoing research further support these observations as zebrafish larvae challenged to cold/heat shocks (16°C/36°C, respectively) showed reduced mortality rates and enhanced expression of heat shock protein (*hsp70*) when reared under a daily thermocycle as compared to a constant rearing temperature (de Alba et al. unpublished).

TIME-DEPENDENT STRESS RESPONSES AND DETOXIFICATION RHYTHMS

The endocrine system of fish responds differently depending on the time of the day. For instance, daily differences have been reported in the response to exogenous treatments that affect endocrine pathways controlled by the hypothalamus-pituitary (HP) system such as the administration of exogenous Gh or Gnrh agonists (Gnrha) (161–163).

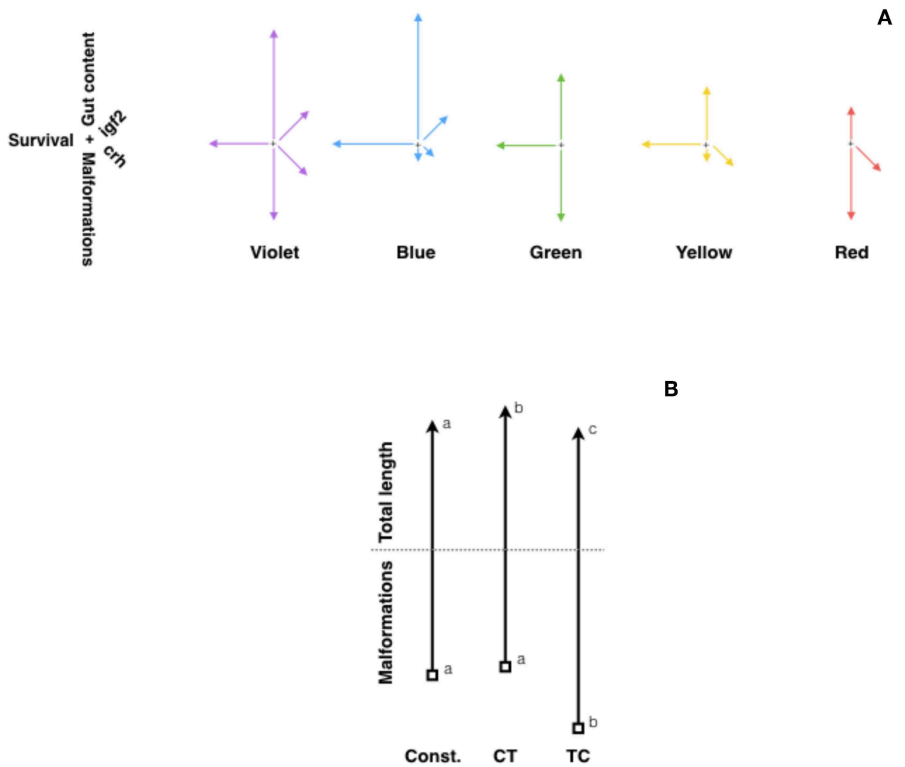

FIGURE 2 | Fitness diagrams of **(A)** zebrafish exposed to different light spectrum (violet, blue, green, yellow, and red), and **(B)** Senegalese sole larvae at 30 DPH raised under constant temperature (21.5°C), or two daily thermocycles: TC (22°C-day:19°C-night) or CT (19°C-day:22°C-night). In **(A)**, lines represent relative values for malformations (vertical, downwards arrow), survival rate (horizontal, left arrow), gut content (vertical, upwards arrow), and expression of *igf2* (rigth-up) and *crh* (right-down) genes. Modified from Villamizar et al. (143). In **(B)**, vertical upwards arrows represent relative values for total length, while downwards arrows represent malformation rates. Different letters indicate significant differences. Modified with the permission of authors from Blanco-Vives et al. (141).

This different response depending on the time of the day has been reported for the stress response in several fish species such as the green sturgeon (*Acipenser medirostris*), Senegalese sole, gilthead sea bream and African sharptooth catfish (*Clarias gariepinus*) (83, 88, 164–166). Senegalese sole subjected to an acute stress (air exposure) showed a greater cortisol production when the stress was applied at the beginning of the light phase as opposed to beginning of the dark phase (83) (**Figure 3**). Likewise, a similar stress applied to gilthead seabream at several time points throughout the 24-h cycle elicited greater cortisol responses during darkness compared with the light phase (88) (**Figure 3**). The daily patterns of locomotor behavior could be partially responsible for the species-dependent differences. Actually, a greater stress response was associated with the resting phase of the species: nocturnal sole presented higher stress during the day, while diurnal gilthead sea bream were more stressed during the night. This hypothesis should be further tested in different fish species, particularly in fish with dual phasing behavior (changing from diurnal to nocturnal) such as sea bass.

The effectiveness of drug absorption, administration, metabolism and elimination are also subjected to rhythmicity, which affects the final concentration of xenobiotics in the animals' blood and their bioavailability (167). In mammals, the existence of toxicity rhythms is widely accepted but in fish species, data remains scarce with only a few studies recently published. In particular, the time-dependent effect of several substances frequently used in aquaculture has been assessed, including anesthetics and veterinary medicines.

Anesthetics are administered to fish to immobilize them and minimize their stress response during research and routine procedures in fish farms (168). However, anesthetics need to fulfill a number of criteria before being approved for their use in aquatic animals and consequently, toxicology tests have to be performed to determine any toxic effects as well as the optimal concentration required to induce anesthesia, which will be species and temperature specific (169). In this context, it is also important to determine whether the time of administration can have an impact on the effect of these substances. In the case of tricaine methanesulfonate (MS-222), a licensed anesthetic for use in food sources, a daily rhythm of toxicity and effectiveness has been reported in gilthead sea bream (170) and zebrafish (171). In both species, a strong effect of the time of administration was found, with higher toxicity and effectiveness of MS-222 when fish were exposed during the day than at night. In the case of sea bream, the median lethal concentration (LC50) at mid-darkness (MD) was 25.7% higher than at mid-light (ML). In order

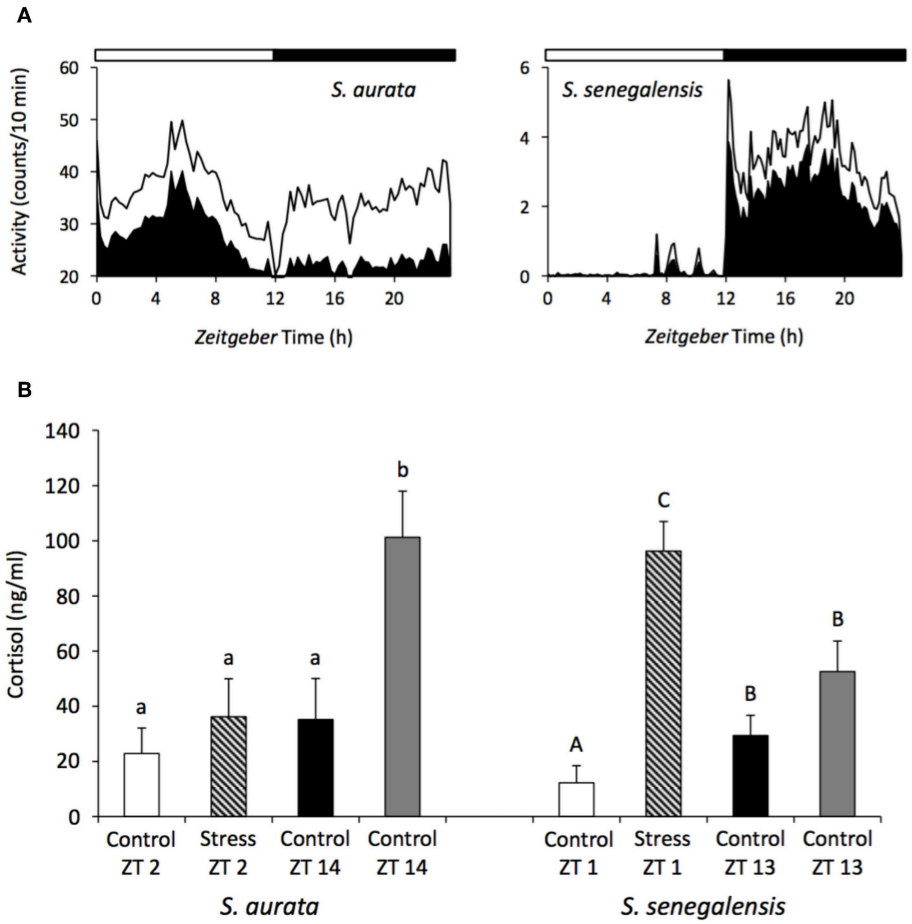

FIGURE 3 | Daily rhythms of locomotor activity **(A)** and differences in the cortisol response depending on the time of the day **(B)** in the gilthead sea bream and Senegalese sole. The black area in the waveforms represents the mean values of activity and the continuous line the S.D. White and black bars above the waveforms represent the light and dark period, respectively. A stress challenge was applied to both species, consisting of air exposure during 30 s, at different time points of the LD cycle: ZT2 and 14 h for sea bream, and ZT1 and 13 h for sole. Fish were sampled 1 h after the stress and cortisol was evaluated. Unstressed control groups were sampled at all-time points. Different letters indicated significant differences between groups (ANOVA, $p < 0.05$) (small case letter for sea bream and upper case letters for sole). Modified with the permission of authors from López-Olmeda et al. (83) and Vera et al. (88).

to determine the induction time of anesthesia at ML and MD, fish were also exposed to sublethal concentrations of MS-222, which revealed that during the day the activity of fish significantly decreased after 7 min of exposure whereas at night no effect was observed until fish had been exposed for 9 min. In addition, the recovery time was longer during the day (10 min) than at night (6 min) (170). These differences in the toxicological response of sea bream were correlated to higher plasma concentrations of MS-222, measured post-exposure, during the day than at night, suggesting a link between the plasma anesthetic levels and the degree of toxicity (172). In zebrafish, similar day-night differences in the effect of anesthetics (MS-222 and eugenol) were found. When fish were exposed to 190 mg/L of MS-222, the mortality rate was 82% at ML whereas at MD this rate descended to 14%. In the case of eugenol, a concentration of 80 mg/L also resulted in a higher mortality rate at ML than at MD (68 and 22%, respectively) which correlated with a shorter induction time of anesthesia during the day (171) (**Figure 4**). The authors of these studies concluded that toxicity rhythms may be related to the animal's daily pattern of activity. Higher toxicity/effectiveness of anesthetics was observed during the active phase of fish, possibly due to an increase of the ventilatory frequency and as a result, increased uptake of the xenobiotic from the water (170–172).

In Atlantic salmon, the time-dependent effects of hydrogen peroxide have also been investigated. Hydrogen peroxide is a veterinary medicine commonly used to treat ectoparasites such as sea lice (*Lepeophtheirus salmonis*) and amoebic gill disease (AGD) caused by *Neoparamoeba perurans*, but these treatments can have side effects on fish and trigger a stress response following exposure leading to increased mortalities in some cases (173). However, the stress response showed daily rhythmicity in salmon, with cortisol, glucose and lactate levels showing higher levels when the fish were treated during the day than at night (174). In addition, these authors also

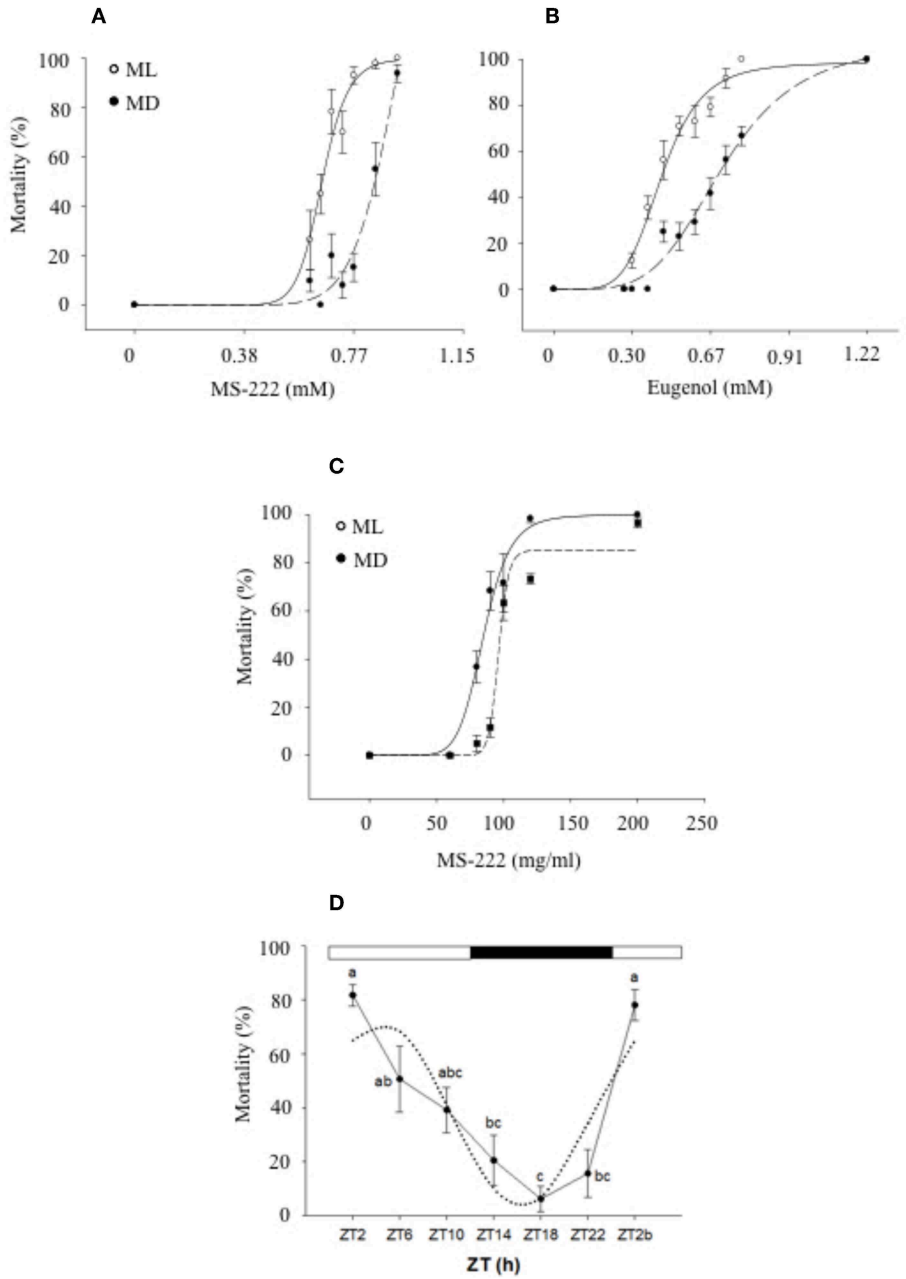

FIGURE 4 | Daily variations of mortality of zebrafish exposed to different MS-222 **(A)** and eugenol **(B)** concentrations after 15 min exposure at mid-light (ML; white circles) or mid-dark (MD; black circles) [with the permission of authors from Sánchez-Vázquez et al. (171)]. Sea bream mortality after 15 min exposure to different MS-222 **(C)** concentrations at ML or MD [with the permission of authors from Vera et al. (172)]. A logistic curve (dotted lines) was fitted to mortality rate (six independent replicates with $n = 8$). **(D)** Daily rhythm of mortality of zebrafish larvae exposed to 5% ethanol for 1 h. Different letters indicate significant differences (ANOVA I, $p < 0.05$), while the dotted black line represents the sinusoidal function fit (Cosinor analysis, $p < 0.05$).

investigated the effect of hydrogen peroxide on the oxidative stress response in liver, reporting that gene expression of key antioxidant enzymes (*gpx1*, *cat*, *hsp70,* and *mn-sod*) was up-regulated when fish were treated during the first half of the day, and in particular around 6 h after the lights onset (175).

In vertebrates, the liver is the main organ involved in detoxification, a process that includes multiple biochemical steps that convert lipophilic toxins into water-soluble metabolites that can then be eliminated from the organism via the urine (176). This system relies on a number of biotransformation enzymes and transporter proteins (177), some of which are

regulated by the circadian clock in mammals (178). In zebrafish, recent investigations have revealed that both detoxification genes and key transcription factors regulating their expression are also subjected to circadian control. In particular, the expression of hepatic PAR bZIP proteins (*tefa*, *tefb*, *dbpa,* and *dbpb*) and nuclear receptors (*ahr2*) showed daily and circadian rhythmicity, in tune with clock genes expression. These transcription factors and nuclear receptors regulate the expression of many detoxifying enzymes and ABC transporters, some of them also displaying rhythmicity in this species (*cyp1a*, *gstr1*, *mgst3a*, *sult2_st2*, *abcg2*, *abcb4*, *smtb*) (179). Altogether, this study provided evidence about the molecular mechanisms underlying the toxicity rhythms described before in fish species and suggested the existence of clock-control in their toxicological response.

The application of this field of research is evident when designing health strategies in the aquaculture industry. However, it is also important to highlight that zebrafish has become an animal model widely used in biomedical research, to assess the psychoactive and toxic effects of many drugs (180, 181), including the neurobehavioural effects of ethanol (182). Therefore, it is crucial to understand the effect of time of administration when designing these tests. In this context, recent research has revealed a daily rhythm in the effects of ethanol in zebrafish, characterized by higher mortality rates in larvae exposed to 5% ethanol at the beginning of the day (80%) than in the middle of the night (6%). In addition, behavioral effects in adults exposed to 1% ethanol were also more severe during the day, with key genes involved in ethanol detoxification in the liver showing circadian rhythmicity in continuous darkness (DD) (183).

In conclusion, fish chronotoxicity is a novel area of research that is showing promising prospects for the application of chronobiology concepts to optimize the administration of medicines in fish farms, which can lead to improve welfare of animals in commercial settings. Furthermore, increasing our knowledge about toxicity rhythms of drugs used in biomedical research will also have an impact on the application of therapies in humans.

PHOTODAMAGE IN THE RETINA

Although light is essential for vision, the trade-off is the production of reactive oxygen species (ROS) that can cause damage within the eye (184). In vertebrates, the negative effect of abnormal light conditions on the retina has been well reported, including studies in fish species. The existence of LD cycles is the most important environmental factor acting as a synchroniser of biological rhythms in vertebrates. For this reason, lighting conditions and photoperiod have been frequently used and manipulated in aquaculture to control the timing of reproduction, overcoming the problems associated with early maturation, such as reduced growth and feed efficiency (185, 186). In particular, continuous light (LL) conditions are commonly used during the production cycle of commercially relevant fish species to control the onset of puberty, increase growth rates, manipulate smoltification in salmonids and improve larvae performances (187–190). However, the use of artificial light sources and regimes can also have a negative impact on fish physiology at different levels, triggering the stress response through activation of the HPI axis, affecting the immune function and inducing retinal damage (191).

The effect of artificial light regimes during early development can be particularly detrimental to fish and have negative effects later during their life cycle. In zebrafish larvae, exposure to abnormal light-rearing conditions (LL or DD) affects their visual behavior and adversely influence the physiological development of the retina, as measured with electroretinogram (ERG) (192). However, artificial lighting systems are used throughout the production cycle in the aquaculture industry. Therefore, lights effects need to be evaluated at different stages of the fish life cycle, especially in those species showing phototactic behavior, as these fish would be exposed to high levels of irradiance when swimming close to the light source (193).

The use of LED technology has increased considerably in the last few years. LEDs have low electrical running costs, a long-life span and can be manufactured to yield specific wavelengths that can be modified according to a species' environmental requirements (194–196). However, the potential adverse effects of these light systems need to be assessed before implementing their use in aquaculture settings. To this end, several studies have focused on these effects in different fish species. In Atlantic salmon, Migaud et al. (191) exposed post-smolt fish to high intensity white and blue LED lights (LL) and investigated their effect on retinal morphology. The study found that high intensity LEDs did not cause retinal damage although the blue lights triggered a stress response in salmon. Similarly, when Atlantic cod were exposed to metal halide (LL, 16.58 ± 8.77 W/m^2), high green cathode lights (LL, 0.82 ± 0.15 W/m^2) or low green cathode lights (LL, 0.47 ± 0.18 W/m^2), no differences in the outer nuclear layer (ONL) thickness or ONL nuclei number were found between groups or in comparison to the control fish under simulated natural photoperiod (SNP, 0.08 ± 0.03 W/m^2) (197). However, when halogen lights were used, the exposure to continuous high intensity illumination resulted in the induction of retinal damage in Atlantic salmon (*Salmo salar*), Atlantic cod and European sea bass (198). This damage was characterized by morphological alterations that included higher melanin density, forming granules around the photoreceptor cells, photoreceptor necrosis and clear disorganization within the ONL. Interestingly, inter-species differences were found, with cod being the most sensitive species and sea bass the least (cod > salmon > sea bass). Regional variations in the effect of light on the ONL thickness and nuclei were also observed, with the central region of the retina presenting more acute damage. When fish were returned to a LD cycle, retinal regeneration occurred in the three species although the recovery time was also species-specific. Thus, cod showed retinal regeneration after 15 days in LD whereas at least 30 days were needed to observe the same effect in salmon and sea bass (198). In albino zebrafish, exposure to constant intense light also resulted in photoreceptor cell death in the central and dorsal retina, whereas many rods and cods were not affected in the ventral area. In addition, high levels of cell proliferation in both the ONL and inner nuclear layer (INL) were observed, suggesting

a potential compensation for the photoreceptors loss, with large numbers of PCNA (Proliferating Cell Nuclear Antigen)-positive cells localized in these layers, indicating a correlation between the magnitude of retinal damage and cell proliferation response (199). In normally pigmented individuals, similar results were found, with high light intensity causing extensive photoreceptor apoptosis and progenitor cell degeneration, mainly in the dorsal and central retinas. In particular, retinal damage triggered Müller glial dedifferentiation and proliferation response of progenitor cells that then migrated to the ONL (200).

Melatonin is also synthesized in the retina of teleost fish, showing marked daily rhythmicity. However, an inverse melatonin profile has been observed in plasma and eye in some fish species, which could be explained by the existence of two different AANAT isoforms and suggests a local function for ocular melatonin (201). One of these roles may be related to the antioxidant properties of this molecule, which can act as a free radical scavenger and also as an anti-apoptotic compound in the retina (202). Actually, recent studies in mammals have concluded that melatonin reduces and even inhibits retinal damage associated to oxidative stress. This anti-apoptotic function could be linked to the inducing effect of melatonin on antioxidant enzymes, as well as its suppressing effect on pro-oxidant compounds (203). In fish, the neuroprotective effect of melatonin against oxidative stress in the retina has not been evaluated yet. However, the antioxidant properties of this indolamine and the fact that its production in the eye of some fish species is higher during the day [reviewed by (204)] suggests that melatonin may play a role in protecting cells against retinal photodamage. Further investigations will be needed to prove this hypothesis.

In summary, there is ample scientific evidence that the use of artificial lights and protocols can induce retinal damage in fish, although important differences between light sources and species have been reported. For this reason, it is crucial to develop and test novel illumination technologies before their implementation in aquaculture systems, to ensure that animal welfare is not compromised. In addition, further studies on melatonin effects in the fish retina will be important to enable us to better understand the cellular mechanisms of retinal photodamage and elucidate whether this hormone play a role as a neuroprotector against light-induced oxidative stress in fish.

CONCLUDING REMARKS AND PRACTICAL ISSUES

Fish physiology is mainly rhythmic, governed by biological clocks which synchronizes to the (cyclic) environment in order to improve fitness and ultimately survival. Thus, stress responses in fish are not always straight forward, as they may respond differently on a time-dependent basis. Fish in captivity are challenged by many stressors and the chronobiological approach depicted here should be considered to improve their welfare. For instance, in farming conditions fish should be manipulated at the times when stress is better tolerated, whereas anesthetics and medicines should be used at the optimal times to enhance their efficacy while minimizing toxicity and side effects. Finally, keeping conditions regarding light spectrum and temperature cycles, should be also considered with care, particularly during early embryo and larval development as they may have long lasting irreversible effects. Light contamination at night should be particularly avoided, providing fish with a "melatonin friendly" environment.

AUTHOR CONTRIBUTIONS

FS-V, JL-O, and LV provided the figures. All authors contributed equally in the writing and revision of the manuscript.

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REVIEW
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The Use of Dietary Additives in Fish Stress Mitigation: Comparative Endocrine and Physiological Responses

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In the last years, studies on stress attenuation in fish have progressively grown. This is mainly due to the interest of institutions, producers, aquarists and consumers in improving the welfare of farmed fish. In addition to the development of new technologies to improve environmental conditions of cultured fish, the inclusion of beneficial additives in the daily meal in order to mitigate the stress response to typical stressors (netting, overcrowding, handling, etc.) has been an important research topic. Fish are a highly diverse paraphyletic group (over 27,000 species) though teleost infraclass include around 96% of fish species. Since those species are distributed world-wide, a high number of different habitats and vital requirements exist, including a wide range of environmental conditions determining specifically the stress response. Although the generalized endocrine response to stress (based on the release of catecholamines and corticosteroids) is detectable and therefore provides essential information, a high diversity of physiological effects have been described depending on species. Moreover, recent omics techniques have provided a powerful tool for detecting specific differences regarding the stress response. For instance, for transcriptomic approaches, the gene expression of neuropeptides and other proteins acting as hormonal precursors during stress has been assessed in some fish species. The use of different additives in fish diets to mitigate stress responses has been deeply studied. Besides the species factor, the additive type also plays a pivotal role in the differentiation of the stress response. In the literature, several types of feed supplements in different species have been assayed, deriving in a series of physiological responses which have not focused exclusively on the stress system. Immunological, nutritional and metabolic changes have been reported in these experiments, always associated to endocrine processes. The biochemical nature and physiological functionality of those feed additives strongly affect the stress response and, in fact, these can act

as neurotransmitters or hormone precursors, energy substrates, cofactors and other essential elements, implying multi-systematic and multi-organic responses. In this review, the different physiological responses among fish species fed stress-attenuating diets based on biomolecules and minerals have been assessed, focusing on the endocrine regulation and its physiological effects.

Keywords: fish, stress mitigation, additive, welfare, cortisol

INTRODUCTION

The study of stress in fish has significantly increased in the last years, mainly due to its close connection to animal welfare. It is widely accepted that a good fish welfare ensures a successful culture in fish farms, as in superior animal facilities. In this way, fish farmers are progressively recognizing it, since survival and growth, among other factors, are known to decrease under poor welfare conditions (1).

In spite of the negative perception of stress, it has been reported that, at low levels, it leads to a necessary and suitable response for adapting organisms to new environment/conditions; which is called eustress (2, 3). In contrast, distress is referred to a more severe and continuous stressful condition having suppressor effects on immune system and impairing physiological functions (4).

In fish farming, several zootechnical systems and variables are adjusted to achieve the maximum animal welfare without affecting the productive yield, though sometimes the right balance is very difficult to find. Besides the technological and infrastructural adaptations, the use of new feeding strategies is an easy and practical procedure to improve the fish welfare. In this context, the concept of functional food (providing beneficial effects on the organism besides the nutritional ones) has arisen as a new method to improve the general healthy status, including welfare (5). By this reason, several works on fish farming are based on the addition of specific substances with biological activity to conventional commercial fish feed in order to modulate or attenuate the stress response and, hence, improve the welfare (6–11). Those works focus on the stress response in fish fed experimental feeds, after submitting them to stressful procedures as netting, air exposure, high stocking density, chasing, and others. The diversity is very high, reporting many types of stressors and additives, and species, and, despite the methodological approach is similar, a wide range of stress markers (e.g., hormones, enzyme activity, immune parameters, gene expression, etc.) have been reported (12–15). The final goal is to find the most suitable additive and feeding strategy (i.e., time, concentration) to prevent fish from suffering, especially for typical stress-related processes in fish farming (e.g., grading, vaccination, fishing, etc.).

The stress response as a complementary study to nutritional issues has been carried out in many works, especially those on different protein, lipid, or carbohydrate concentrations and ratios in the diet (16–18). In this sense, those papers were, probably, the first evidences of dietary effects on the fish stress response (19). At the same time, vitamins (mainly ascorbic acid) were also target substances in that type of studies (20, 21). Lastly, thanks to new biotechnological protocols developing new substances, isolating/extracting biomolecules more efficiently, or including any additive in commercial feeds, many works have also described the effects of specific molecules (e.g., amino acids, nucleotides, polysaccharides, etc.) on the stress response (22–24).

For the last decades, studies dealing with proteins and amino acids have been the most abundant (**Figure 1**). The versatility of amino acids may justify their first place in this ranking, since some of them are directly involved in the neuroendocrine response. Fatty acids have also been frequently studied, especially those related to nutritional requirements (docosahexanoic, arachidonic, and eicosapentanoic acids). Some nucleotides, including trademarks, are progressively being assayed in fish; in spite of being stress alleviators, its interaction with the stress axis still remains unclear (25).

In this review, the literature on fish stress mitigation through feed additives based on biomolecules and minerals has been revised and analyzed, aiming at comparing the endocrine and physiological responses along farmed fish species.

THE ENDOCRINE STRESS RESPONSE IN FISH

Stress responses have been deeply studied in fish, showing the key role of the endocrine system in the process. The primary stress response is based on hormonal cascades; in fact, the stress response was initially referred as the general adaptative syndrome (GAS), consisting of a hormonal cascade which promotes the other responses to stressors (3). HPI (i.e., hypothalamus-pituitary-interrenal) and HSC (i.e., hypothalamic-sympathetic-chromaffin) axes are activated during this primary response, releasing corticosteroids and catecholamines (e.g., adrenaline, nor-adrenaline and dopamine) into the blood stream. Following several energy metabolic pathways are enhanced (secondary response) and, if stress stands, severe failures at organism level (e.g., pathologies, decreasing growth, dead) may appear (tertiary response) (26).

The hormonal cascade starts at the hypothalamus level, which secretes the corticotropin releasing hormone (CRH) to stimulate the pituitary for releasing ACTH (i.e., adrenocorticotropic hormone) and MSH (i.e., melanophore stimulating hormone) into the blood stream. As a result, chromaffin cells, and interrenal cells from the head-kidney release catecholamines, and cortisol, respectively. Therefore, plasma cortisol and catecholamines are considered good acute stress markers. In fact, adrenaline is considered to be the stress hormone, and cortisol the adaptive

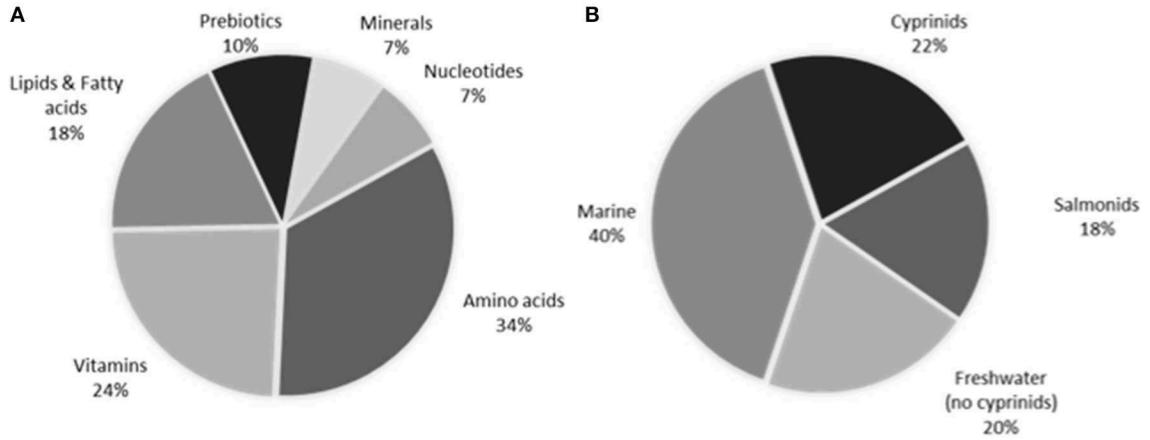

FIGURE 1 | Percentage of works for **(A)** every additive type, and **(B)** every fish species group type in the literature dealing with stress attenuation through feed additives in fish.

hormone (27). The effects of cortisol on energy metabolism and other physiological functions is already known in fish, indeed it is the responsible of the releasing of energy substrates to the blood stream (secondary response), stimulating glycolysis, and other metabolic pathways (28). The catecholamines role in the stress metabolic response is poorly known in fish, meanwhile it is known that affect carbohydrate and lipids metabolism in mammals (29).

Thanks to the development of powerful tools on molecular biology, the knowledge of the HPI signaling in teleosts has progressed significantly. Many corticosteroid precursors and receptors have already been characterized in several species, providing valuable data in the field (30–32). Therefore, the classical stress markers (plasma hormones, immune parameters, metabolic rates) are currently studied together specific molecular biomarkers. Eissa and Huang (33) have revised thoroughly all genes involved in the fish stress response depending on stressor type, and stated that the use of genomic tools to study the candidate genes associated with stress responses are often unique signatures or imprints of specific stressors and could determine early signs of stressors. Having this in mind, Kiilerich et al. (34) have recently studied the expression of glucocorticoid and mineralocorticoid receptors (i.e., GR1, GR2, and MR) at different levels, concluding that the control and release of cortisol after stress is regulated through a negative cortisol feedback occurring at pituitary level; to the date, it was thought that this feedback occurred at every level of the HPI axis. Other authors have concluded that cortisol regulation is also dependent on circulating glucose concentration under acute stress, reporting a stimulatory effect of increasing glucose levels on the cortisol release (35). Despite the latest progress in the subject, the regulation of stress axis, and mechanisms of cortisol action in fish still remains unclear. In this sense, Faught et al. (36) suggested that future studies should be focused on the rapid non-genomic effects of cortisol, since that pathway could be crucial in the transcriptional activation of non-GR target genes during stress.

In the study of other endocrine factors and hormones, beyond the "classical" cortisol and catecholamines, involved in the fish stress response, the leptins have been objective for years (37–40). It seems clear that leptin interacts with the HPI axis at both head-kidney and pituitary gland levels, though contradictory results have been published on ACTH stimulation (37, 41). Gorissen and Flik (41) have stated that this hormone may convey information on energy status and serve to downplay the stress response, contributing to the coordination of the balance between eustress and distress.

Continuing on new hormones and endocrine responses, Skrzynska et al. (42) have recently studied the involvement of the vasotocinergic and isotocinergic systems in the stress response. These authors have stated that changes in *avt* (arginine vasotocin) and *it* (isotocin) gene expression, and in their specific receptors (*avtrv1*, *avtrv2*, and *itr*) at central (hypothalamus and pituitary) and peripheral (liver and head-kidney) locations, demonstrate that vasotocinergic and isotocinergic systems could have a role in several physiological changes induced by air exposure, including metabolic and energy repartitioning processes as well as the control of synthesis and release of several hormones as the final product of different endocrine pathways.

Lastly, a very innovative and recent study has revealed the cytoprotective importance of the CRH in the stress-induced apoptosis during the ontogeny (43). These authors have demonstrated the relation between CRH and caspase-3 activity (an effector caspase that execute apoptosis) during zebrafish (*Danio rerio*) ontogeny. They also highlighted that it can be a novel function for CRH during a period of embryonic development when the HPI axis is not yet matured, and proposed that it may help mediating the impacts of early life stress on offspring phenotype.

Summarizing, the literature on endocrine responses to stress in fish is extensive, and significant advances have been achieved for the last years. A consensus exist on the HPI (and HSC) response after stress and the roles of the main factors, including tissues where they act. Nevertheless, the interaction of the

axis with other endocrine or metabolic processes is poorly understood. In most of cases, it has been stated that interaction exists (thanks to powerful bioindicators) though the intrinsic biochemical, physiological and endocrine processes involved in it have not been described yet.

PHYSIOLOGICAL ROLES OF DIETARY ADDITIVES

Additives are added in food to both improve the physiological effects on the consumer (probiotics, prebiotics, etc.) and provide/modify some physical food properties (texture, taste, color, etc.). The first group includes the stress attenuation, and diverse works on fish welfare have focused on it. The general biological functions and physiological roles of those additives on the fish stress response are summarized in **Table 1**. For the last 20 years, over 30 biomolecules and minerals, and around 38 fish species have been assayed in this subject. Below a more detailed revision depending on every additive group and it main physiological effects are shown.

Amino Acids

It has been described that stressful husbandry conditions affect amino acid metabolism in fish (45, 91) and under some stress situations an increase in the requirement of certain essential amino acids occurs, which is probably related with the synthesis of proteins, and other compounds related with the stress response (92). The role of specific amino acids and their metabolites on key metabolic pathways that are necessary for growth, immunity or resistance to environmental stressors and pathogens have been already reviewed in fish (92–94). Thus, amino acids not only serve as constituents of proteins and energy sources, but also can be converted into important biochemically active substances *in vivo*.

Arginine is the precursor for the synthesis of nitric oxide (NO) and polyamines in higher vertebrates. In fish, NO production plays an important role in cellular defense mechanisms and has been demonstrated in stimulated macrophages in fish (56). Moreover, dietary arginine can increase some innate immune mechanisms and disease resistance of fish following challenge with Phdp (*Photobacterium damselae piscicida*) (56).

Branched-chain amino acids (BCAA: leucine, isoleucine and valine) have an important role in regulating protein synthesis in skeletal muscle, being leucine the most effective in the regulation of this process (95). An increased proteolysis activity is usually observed in fish under stressful situations, together with a decrease in plasma levels of BCAA (91, 96). Therefore, dietary supplementation with BCAA, especially leucine, appears to be a promising tool to mitigate negative effects of stress in fish.

Tryptophan (Trp) is an essential amino acid with important roles in the regulation of the stress response. It can be converted to serotonin (5-hydroxytryptamine, 5-HT) and melatonin (97). Nevertheless, over 95% of the ingested Trp is catabolized primarily in the liver via kynurenine pathway and produces niacin, pyruvate and acetyl-CoA as the final products (98). Brain 5-HT is involved in the control of the HPI axis in fish and a correlation between brain 5-HT activity and plasma cortisol levels has been observed (99). Indeed, tryptophan directly or indirectly participates in a wide array of physiological pathways, as recently reviewed by Hoseini et al. (94). In fact, fish under stressful husbandry conditions dropped free tryptophan levels in the plasma compared to control specimens (45, 91). Therefore, dietary tryptophan supplementation seems to be a promising nutritional strategy for health management in aquaculture.

Tyrosine is a common precursor for important hormones and neurotransmitters, including thyroxine, triiodothyronine, epinephrine, norepinephrine, dopamine, and melanin. These molecules have important important roles during stress response in fish, and thus tyrosine could profoundly influence pigmentation development, feed intake, growth performance, immunity, and survival of fish (93). It has reported that plasma free tyrosine concentrations increase during acute stress responses, suggesting tyrosine importance during stress response (96, 100).

Methionine also plays an important role in the antioxidant and immune status of animals as the precursor of cysteine, which in turn is required for the synthesis of glutathione and taurine (101). Some studies have reported changes in plasma levels of methionine in stressed fish compared to control specimens after both acute and chronic stressful conditions (45, 96, 100). Methionine metabolism can be directed to three pathways with health implications: (i) it provides s-adenosylmethionine that is then decarboxylated and turned into an aminopropane donor that fuels polyamine turnover (102), (ii) s-adenosylmethionine is directly involved in methylation of several cell constituents such as DNA, adrenergic, dopaminergic and serotonergic molecules (93); (iii) it leads to the transsulfuration pathway that ends up in the formation of glutathione from homocysteine (103). Therefore, an eventual increase in the requirement of methionine in fish under stressful conditions should be carefully considered.

Although the dietary protein is not a dietary additive, that is a key source for obtaining amino acids with relevant role in the stress response. In this sense, the effects of dietary protein (with no details on amino acid composition) concentration and its relation to lipid/carbohydrate content in fish have been widely studied, focusing on the nutritional issues (see Introduction). Regarding stress response, some of them have included stress markers, searching the optimum protein content to improve fish health and welfare (14, 16, 17, 47). The endocrine processes are not described in these works in detail (focused on nutrition), though it is supposed that the effects on stress response are based on amino acid content of those experimental diets.

Vitamins

Vitamin C has been the object of the first works on stress attenuation through vitamin supplements, in both fish and superior animals (60, 104, 105). Moreover, from a nutritional perspective, the vitamin C content in fish feed is crucial since they are not able to synthesize it due to the lack of the enzyme L-gulonolactone oxidase, which is necessary to convert L-gulonic acid into vitamin C (106).

Its physiological role related to stress is based on the steroidogenesis inhibition through peroxidation of

TABLE 1 | Main additives and its physiological effects assayed in fish in experiments based on the study and reduction of the stress response.

Substance	General biological functions	Biological function related to stress system (described in fish)
Amino acids[1]	Enzymes, antibodies, hormones, pH regulation, cell signaling, muscle structure	Neurotransmitter and hormone precursors, anti-oxidative enzymes, enhancer of fatty acid oxidation
Vitamins[2]	Enzyme cofactor, antioxidants	Enzyme cofactor, antioxidant, immunostimulant
Lipids and fatty acids[3]	Building biological membranes, storing energy	Energy reserves, eicosanoid precursors
Prebiotics[4]	Storing and providing energy, building macromoecules	Energy source, prebiotic
Nucleotides[5]	Nucleic acids building, cell signaling	Immune system enhancer
Minerals[6]	Bone and tooth building, energy production, muscle function, enzyme cofactor, antioxidant	Enzyme cofactor

[1]*Morrow et al. (17), Hoğlund et al. (44), Aragão et al. (45), Tejpal et al. (46), Abdel-Tawwab (47), Wolkers et al. (48), Conde-Sieira et al. (35), Hooley et al. (16), Kumar et al. (49), Morandini et al. (50), Chen et al. (51), Tian et al. (52), Liu et al. (24), Habte-Tsion et al. (14), Babaei et al. (12), Azeredo et al. (7), Herrera et al. (8), Cabanillas-Gámez et al. (6), Harpaz (53), Papoutsoglou et al. (54), Lepage et al. (55), Costas et al. (56), Costas et al. (57), Martins et al. (58), Hoseini et al. (59).*

[2]*Thompson et al. (60), Montero et al. (61), Chen et al. (62), Belo et al. (63), Trenzado et al. (64), Liu et al. (20), Liu et al. (13), Falahatkar et al. (65), Miao et al. (66), Guimarães et al. (67), Imanpoor et al. (21), Jia et al. (10), Cheng et al. (68), Jakab Sándor et al. (69), Alves Martins et al. (70), Hwang et al. (71), Davis et al. (72).*

[3]*Lochmann et al. (73), Van Anholt et al. (74), Van Anholt et al. (75), Bransden et al. (76), Alves Martins et al. (77), Trushenski et al. (78), Araújo and Rosa (79), Xu et al. (80), Rezek et al. (81), Martins et al. (82).*

[4]*Xie et al. (83), Torrecillas et al. (84), Chen et al. (18), Forsatkar et al. (22).*

[5]*Tahmasebi-Kohyani et al. (85), Kenari et al. (23), Palermo et al. (86), Fu et al. (25), Fuchs et al. (87).*

[6]*Küçükbay et al. (88), Betancor et al. (89), Long et al. (90), Izquierdo et al. (11), Kumar et al. (9).*

polyunsaturated lipids and the enhancement of the immune system (107–110). However, the effect of this supplement on the cortisol biosynthesis could not be demonstrated in fish (60, 111). Over 10 years later, Trenzado et al. (64) kept supporting this lack of connection between cortisol secretion and vitamin C. Nevertheless, Liu et al. (20) reported the beneficial immunomodulatory and antioxidant effects of vitamin C in stressed fish, stating that dietary ascorbic acid supplements alleviate chronic stress effects. In this sense, Imanpoor et al. (21) have recently demonstrated that vitamin C is a beneficial dietary supplement for improving the growth performance, survival, skeletal development and resistance to salinity stress of common carp fry. In spite of being object in many studies, there is not a general statement on the beneficial effects on vitamin C on the stress resistance, though no study indicates negative consequences of this feed supplement.

Vitamin E is required to maintain flesh quality, immunity, the normal resistance of red blood corpuscles to hemolysis, the maintenance of normal permeability of capillaries, and heart muscle (112, 113) Similarly to vitamin C, vitamin E effects on cultured fish welfare are based in its role as immunostimulant and antioxidant (61, 114, 115). This vitamin has been assayed successfully as inhibitor of cortisol secretion; in fact the most of works highlight this role, besides its stimulating effects on the immune system (13, 61, 63). Therefore, it seems that vitamin E could be a better stress alleviator than vitamin C, though the interaction of both vitamins with the stress system and cortisol and catecholamines secretion (endocrine and primary response) would not be clear yet.

Few works have studied the effects on other vitamins on the stress response, with no clear results regards stress alleviation. For instance, vitamin A is involved in metabolism, acting as a steroid hormone regulating growth through glycoprotein and glycosaminoglycan synthesis, as well as by modulating cell differentiation (67). In spite of those key physiological roles, Guimarães et al. (67) have reported that vitamin A does not provide any protection against cold-induced stress in fish. In this sense, Miao et al. (66) have demonstrated that, contrarily to the objective of the above works, long-term high doses of vitamin D_3 lead to chronic stress and weaken the disease resistance. Therefore, the role and/or effects of vitamins different to C and E on the fish stress response are still unknown.

Lipids and Fatty Acids

The study of the effects of dietary lipids on stress response, based on endocrine markers is relatively recent. Although some previous works dealt with the stress response in fish fed different lipid content, these used other markers as mortality, and oxygen consumption (116–118) One of the first trials including endocrine effects did not report promising results since no evidence on the relation between dietary lipid content and stress response was found (73). However, several successful works in this subject were published later (74, 76).

The importance of lipids in stress response is based on the formation of eicosanoids, particularly prostaglandins. Concretely, the Arachidonic Acid (ArA) can transform into eicosanoids, acting as endocrine, paracrine and/or autocrine modulators of secretory mechanisms in various organs (74). It has been stated that prostaglandins can modulate the sensitivity of the hypothalamus–pituitary–adrenal (HPA) axis in mammals and alter the release of cortisol and corticosterone in the stress response (119–121). In fish the interaction between HPI (hypothalamus–pituitary–interrenal) axis (equivalent to mammal HPA axis) response and dietary ArA has also demonstrated (122, 123). That is the reason which the most of

studies on lipids and stress have focused in the dietary ArA as stress-attenuating biomolecule.

Mainly due to its key nutritional role, other fatty acids like docosahexanoic and eicosapentanoic acids (DHA and EPA) have been studied. Similarly, it has stated that several HUFAs (highly unsaturated fatty acids), for instance EPA, are also eicosanoid precursors. Besides eicosanoids, more fundamental processes like alterations in membrane properties and cellular signal transduction are supposed to contribute to the consistent effects of dietary DHA/EPA on growth, stress resistance and certain immune responses (80). Nevertheless, the knowledge of the interaction between HUFAs and HPI axis and cortisol secretion is very limited. Ganga et al. (124) have suggested that the oxygenated products of cyclooxygenase (COX) and lipoxygenase (LOX) derived from ArA, EPA, and DHA, respectively, may be major players in this regulation.

Besides HUFAs studies, the effects of dietary marine lecithine (mainly phospholipids) on stress response in fish have also reported (78). Phospholipids are known to facilitate digestion and absorption of lipids and other nutrients, form the structure of cellular membranes and support hyperplastic growth and may serve critical roles as the prevailing carriers of bioactive long-chain polyunsaturated fatty acids (LC-PUFA) and precursors to other physiologically active molecules (125). In fact, Trushenski et al. (78) stated that amending feed formulations with marine-origin phospholipid appears to be a practical approach to improve growth and stress tolerance in fish.

Astaxanthin (carotenoid) has also assayed as fish stress modulator and it has been reported that improves the acute overcrowding stress resistance though reduces the weight gain, CAT (catalase), and lysozyme activities (24). The anti-oxidative capacities of this compound are already known (126), though its relation to cortisol secretion decrease was not elucidated in that work.

Prebiotics

The use of dietary carbohydrates to mitigate stress in fish has not been studied in deep. In fact, these biomolecules has been studied in a few works since some prebiotics are composed of them (22, 84, 127). Mannan-oligosaccharides (MOS) are one of the most studied prebiotics in fish, stating that improves growth, feed conversion, stress resistance, and immune function (128–130). The way which MOS act on the HPI axis has not been studied, though it is probable that the stress reduction is a consequence of the general fish welfare improvement. Therefore, probably the stress attenuation is not related directly to the consumption of these additives or their derived biomolecules.

Nucleotides

Nucleotides refer to a group of biochemical substances (a purine or a pyrimidine base, a ribose or 2-deoxyribose sugar and one or more phosphate groups) with different physiological roles and biochemical functions since they are involved, for instance, in the vital cell function and metabolism, biosynthetic pathways, or mediating energy metabolism and cell signaling (131, 132). Dietary nucleotides are considered non-essential since neither prevailing biochemical malfunctions nor classical signs of deficiency are developed in endothermic animal models, and also due to the high rates of their *de novo* synthesis (e.g., RNA and DNA) that takes place in the human body, compared to the actual intake (133). The modulatory effects of dietary nucleotides on lymphocyte maturation, activation and proliferation, macrophage phagocytosis, immunoglobulin responses, gut microbiota as well as genetic expression of certain cytokines have been reported in endothermic animals (134).

The roles of nucleotides and metabolites in fish diets have been studied for almost 20 years, and most research has shown rather consistent and encouraging beneficial results in health management of both marine and freshwater fish. Li and Gatlin (132) reviewed the influence of dietary nucleotides on innate and adaptive immunity in fish and also suggested that dietary nucleotides would support lymphoid tissues that have limited "*de novo*" synthesizing capacity. Ringø et al. (135) recently pointed out that exogenous nucleotides have shown great potential as dietary supplements to enhance immunity and disease resistance of fish produced in aquaculture. Research on dietary nucleotides in fish has shown they may improve growth in early stages of development, alter intestinal structure, increase stress tolerance as well as modulate innate and adaptive immune responses (135). Despite occasional inconsistency in physiological responses, dietary supplementation of nucleotides has shown rather consistent beneficial influences on various fish species. In fact, fish fed nucleotide supplemented diets generally have shown enhanced resistance to viral, bacterial and parasitic infection (135, 136). However, little attention has been paid to the role of dietary nucleotides as stress-attenuating additives from an endocrine perspective.

Minerals

The importance of mineral nutrition in relation to skeletal metabolism and health in fish have been described by Lall and Lewis-McCrea (137). Most available literature on mineral nutrition have aimed at determining optimum levels in diets for fish, and particular emphasis have been paid in early nutrition (11). Therefore, much effort needs to be taken to look at specific mineral requirements during adverse farming conditions to optimize aquaculture profitability. It seems clear that organic and inorganic selenium are the most frequent minerals assayed in order to reduce stress in fish (11, 88–90, 138). Selenium is cofactor in the antioxidant enzyme glutathione peroxidase (GPx), playing a crucial role in the oxidative stress (139). Therefore, the studies are focused on the oxidative stress response, instead of the endocrine one. All works have stated the beneficial effects of Se supplements on stress resistance due to its antioxidant action, and only Long et al. (90) have demonstrated, in addition, their effects on the inhibition of cortisol secretion. Manganese and zinc also have been tested (11). Similar to selenium, their roles as cofactors in several essential enzymes have been related to stress parameters attenuation, mainly those related to oxidative stress.

ENDOCRINE AND NEUROENDOCRINE EFFECTS ALONG SPECIES

The most of endocrine responses in the literature are based on plasma cortisol analysis, though the use of molecular markers and other hormones is progressively growing (see previous sections). The wide diversity of fish species (over 38), and additive type used make very difficult to analyse the effects of an only additive along the species. By that reason, a previous classification according to taxonomy or other features is appropriate to compare the effects of additives along species (**Figure 1**).

Marine Species

The **Table 2** shows an overview on the works on stress attenuation with dietary additives in marine species. The intensively cultured species have been used in the most of experiments, such as gilthead seabream (*Sparus aurata*), European seabass (*Dicentrarchus labrax*), Senegal sole (*Solea senegalensis*), and turbot (*Scophthalmus maximus*). Sometimes there are contradictory results for the same species and additive (74, 150), although the clear different responses are usually derived from distinct species, hence those responses are probably species-specific.

Amino Acids

Fish present additional amino acid requirements when submitted to stressful rearing conditions, due to either increased energy demands or for the synthesis of stress-related proteins and other compounds related with the stress response (92). In this context, increasing evidence suggests the possibility of mitigating the negative physiological effects attributed to stress (see previous sections) by altering dietary amino acid levels.

Studies with flatfish species gathered some knowledge regarding the role of dietary arginine during chronic stressful conditions. It was observed that duration (e.g., 14/15 or 60 days) of handling procedures induced different responses in some innate immune parameters of Senegal sole and turbot (56, 140). While repeated acute stress reduced NO levels in turbot at both sampling times, a positive synergistic effect between dietary arginine and stress was observed in sole. Handling stress also decreased cellular ROS in both flatfish species, a fact that seems to be counteracted by dietary arginine after 60 days of feeding in turbot. Depending on the duration and severity of the stressor, increased glucocorticoid levels may enhance innate and adaptive immune responses while similar hormone levels may suppress immune function. Therefore, the suppressive effect of stress on the innate immune system is highly disputable and does not necessarily translate in decrease resistance to infection, as already suggested elsewhere (2, 159).

Tryptophan has been the central character in many stress mitigation studies in marine fish. A recent review has covered the involvement of tryptophan in 5HT and melatonin-mediated functions, along with its participation in the regulation of the immune system and its role as an antioxidant and antitoxic agent in fish (94). In general, a positive effect is usually attributed to tryptophan nutrition in stressed animals. In marine fish, a number of studies have already tested the effects of dietary tryptophan under both acute and chronic stressful conditions. In those works, feeding strategies varied from 7 to 39 days, being shorter times more frequently used prior to an acute stress event. Indeed, 7 and 10 days of tryptophan treatment decreased aggressive behavior and cannibalism rate in juvenile Atlantic cod (*Gadus morhua*) and grouper (*Epinephelus coioides*), respectively (141, 142). However, fish fed tryptophan supplemented diets and reared under non-stressful conditions seem to cope differently with the stress imposed depending on feeding time. For instance, Atlantic cod fed tryptophan supplemented diets for 7 days decreased plasma cortisol and glucose levels immediately after air exposure, whereas totoaba (*Totoaba macdonaldi*) and European seabass fed tryptophan surplus increased plasma cortisol levels after handling (chasing with a net for 45 min) and hypoxia (1 mg oxygen /L during 45 min) or an inflammatory insult, respectively, (6–8). In contrast, Senegalese sole juveniles fed tryptophan supplemented diets showed a trend to decrease plasma cortisol levels when reared at high stocking densities (i.e., 31 kg/m^2), which translated in enhanced disease resistance after 39 days of feeding.

Methionine also seems to play a role in the stress response probably due to its important role in the transsulfuration pathway. In a study with gilthead seabream, fish fed dietary methionine surplus for 30 days decreased plasma lactate levels and the superoxide dismutase (SOD) isoenzymatic profile (Mn-SOD and CuZn-SOD) in liver after hypoxia treatment (i.e., 2.8 mg oxygen /L during 5 h) (146). However, European seabass fed a methionine supplemented diet for 14 days showed the opposite trend with increased plasma cortisol levels at 24 h after an inflammatory insult (7).

While most research focused on the effects of individual dietary amino acids supplementation in fish submitted to stressful conditions, some other works increased the amount of digestible protein and therefore the availability of certain amino acids (AA). For instance, Costas et al. (147) observed that a slight increase in the availability of some dietary amino acids (arginine, phenylalanine, and tryptophan) may have a significant impact on amino acid metabolism, as indicated by changes in plasma amino acid levels compared to chronically stressed treatments. Therefore, providing those key AA in the diet may represent a metabolic advantage during predictable stressful events (e.g., handling and overcrowding associated to grading procedures), which may have a significant effect on growth and welfare in the longer term. Those effects on metabolism appear to be stronger after 14 days compared to 28 days of feeding, as indicated by the reduction of plasma glucose and lactate levels. Still, 28 days of feeding appear to have some effect on other processes related to the stress response. In a similar study, Senegalese sole exposed to a high density for 18 days and fed a diet with an increase in some key AA, counteracted the negative effects of chronic stress and increased plasma complement, lysozyme and peroxidase activities compared to their counterparts fed the control diet (57).

Vitamins

Vitamins have been demonstrated to improve immune responses to infection by affecting the proliferation and migration of immune cells such as phagocytic cells, equipping the fish with

TABLE 2 | General overview on the effects of dietary additives in marine fish submitted to stressful conditions.

Additive	Fish species	Stress condition/treatment	Feeding time, days	Test doses	Main effects on physiology and productivity
Arginine	*Solea senegalensis*[1]	Repeated daily handling (air exposure)	14	4.4–6.9 g 16 g^{-1} N	↑ ROS; ↑ NO
	Scophthalmus maximus[2]	Repeated handling (air exposure) every other day	15; 60	6–11 g 16 g^{-1} N	↓ Cortisol after 15 days ↑ ROS, plasma NO and ACH50 after 60 days ↑ Lysozyme after 15 and 60 days No effect on growth
Tryptophan	*Epinephelus coioides*[3]	Cohabitation for 10 days	10	0–1%	↓ Cannibalism rate ↑ Brain 5-HT contents ↓ Final weight
	Gadus morhua [4,5,6]	Cohabitation for 7 days	7	2,8%	↓ Aggressive behavior
		Air exposure (3 min) Thermal shock (from 10 to 15°C in 30 min)	7	0.26–1.62%	↓ Cortisol and glucose in plasma of air exposed fish
		Confinement stress (i.e., lowering of water level) for 30 min	7	0.4–1.58%	↓ Cortisol in plasma in a dose dependent manner
	Totoaba macdonaldi[7]	Handling (chasing with a net for 45 min) Hypoxia (1 mg oxygen /L during 45 min)	21	0.5–2.3%	↑ Cortisol levels in fish submitted to handling and hypoxia ↓ Telencephalic 5-HT content in stressed specimens
	Dicentrarchus labrax[8]	Inflammatory insult (intraperitoneal injection with an inactivated pathogen)	14	1.12–2.24 g 16 g^{-1} N	↑ Cortisol levels at 24 h after injection
	Argyrosomus regius[9]	Air exposure (3 min) Confinement and netting (3 min)	7	0.07–0.11%	↓ Plasma protease activity in fish submitted to air exposure (after 6 h) or confinement and netting (after 1 h) ↑ Plasma bactericidal activity in air exposed fish after 1 h
	Solea senegalensis[10]	High density (31 kg/m^2)	39	0.44–2.05%	↑ ACH50 in plasma ↑ Disease resistance
Methionine	*Sparus aurata*[11]	Hypoxia (2.8 mg oxygen /L during 5 h)	30	control; control + 0.3%	↓ Lactate in plasma ↓ SOD isoforms (Mn-SOD and CuZn-SOD) in liver
	Dicentrarchus labrax[8]	Inflammatory insult (intraperitoneal injection with an inactivated pathogen)	14	2.57–4.95 g 16 g^{-1} N	↑ Cortisol levels at 24 h after injection
Synergistic effects of amino acids	*Solea senegalensis*[12]	Repeated weekly handling (air exposure)	14; 28	Different amino acid mix	↓ Glucose and lactate after 14 days ↑ Lysozyme activity after 14 days ↑ Brain dopamine levels after 28 days
	Solea senegalensis[13]	High density (12 kg/m^2)	18	Different amino acid mix	↓ Cortisol, glucose and lactate ↑ ACH50, lysozyme and peroxidase levels in plasma
Vitamin C	*Sparus aurata*[14]	High density (12 Kg/m^3)	63	control; control + 0.025%	↓ Plasma lysozyme levels No effect on growth
	Sebastes schlegelii[15,16]	Exposure to hexavalent chromium (i.e., 120 and 240 mg/L)	14; 28	0.01–0.0 4%	↓ Plasma cortisol levels only at 14 days ↓ Chromium accumulation in blood, kidney, liver, gut, gills and muscle ↑ Haematocrit
Vitamin E	*Sparus aurata*[14]	High density (12 Kg/m^3)	63	control; control + 0.025%	↓ Plasma lysozyme levels ↑ ACH50 levels in plasma No effect on growth
	Huso huso[17]	Netting and air exposure (i.e., 1.5 min)	48	0.1–0.14%	↓ Plasma glucose levels ↑ WG
	Takifugu obscurus[18]	Exposure to ammonia-nitrogen for 48 h (i.e., 100 mg/L)	60	0.00023–0.03116%	↑ Expression levels of HSP, Mn-SOD, CAT and GR ↓ ROS in blood ↑ WG, SGR
ArA	*Sparus aurata*[19,20,21,22,23]	Daily salinity stress (fluctuating salinity over 24 h, from 25 to 40% and back to 25%)	20; 32	0.059–0.586% live prey DW	↑ Whole-body cortisol levels

(Continued)

TABLE 2 | Continued

Additive	Fish species	Stress condition/treatment	Feeding time, days	Test doses	Main effects on physiology and productivity
		Air exposure for 90 s	28; 50	0.15–0.75% *Artemia* DW	↓ Whole-body cortisol levels ↑ Growth
		Confinement: 5·min of submersion in dip-net	18	0.9–2.4%	↓ Plasmacortisol levels
		Crowding stress (43–49 kg/m^3)	240	0.2–1.11% FA	↓ Plasma cortisol levels
		Crowding stress (90–100 kg/m^3)	72	0.13–0.31% TFA	↓ Plasma cortisol and glucose levels ↓ Gene expression in cell- and tissue-repairing markers, antioxidant enzymes, nuclear receptors and transcription factors
	Solea senegalensis[24,25,26]	Air exposure (2 min)	14	0.1–2.3% *Artemia* DW	↑ expression levels of PPARα and PEPCK
		Chasing stress test consisting of 5 min net chasing	84	0.5–0.8% TFA	↑ Expression levels of glucocorticoid receptor 1 and 2 in liver
				0.5–0.8% TFA	↑ Expression level of genes related to defensive response against virus, antigen differentiation and cytokines ↑ Final weight
	Dicentrarchus labrax[27]	Handling 20 larvae per tank out of the water in a scoop net for 1 min	14	0.3- 1.2%	↓ Gene expression of StAR and CYP11β ↑ Expression level of genes related to glucocorticoid receptor complex
EPA	*Solea senegalensis*[25,26]	Chasing stress test consisting of 5 min net chasing	84	5.6–12%TFA	↑ Expression levels of glucocorticoid receptor 1 and 2 in liver
				5.6–12%TFA	↑ Expression level of genes related to defensive response against virus, antigen differentiation and cytokines ↑ Final weight
DHA	*Solea senegalensis*[25,26]	Chasing stress test consisting of 5 min net chasing	84	4.9–11.1%TFA	↑ Expression levels of glucocorticoid receptor 1 and 2 in liver
				4.9–11.1%TFA	↑ Expression level of genes related to defensive response against virus, antigen differentiation and cytokines ↑ Final weight
MOS (prebiotic)	*Dicentrarchus labrax*[28]	Confinement stressor (25 kg/m^3) Infection (intraperitoneal injection with (10^7 cfu *Vibrio anguillarum*/ml)	60	0–0.4%	↓ Plasma cortisol levels in infected and stressed and infected groups ↑ Plasma cortisol levels in stressed groups ↓ Side-effects of stress on microflora profiles
	Scophthalmus maximus[29]	Handling procedure (combination of capture, netting/ transfer, and crowding)	112	0–0.6%	↓ Plasma cortisol and glucose levels at 1 h following acute stress
Nucleotide (Optimum)*	*Sciaenops ocellatus*[30]	Confinement stress (transfer of 3 fish from 110 L aquaria to 0.4 L for 15 min)	42	0–0.2%	No changes in plasma cortisol levels No effect on growth
Commercial nucleotides	*Gadus morhua*[31]	Acute stress: Salinity: increase from 35 to 50% during 30 min Temperature: increase from 12 to 15°C for 1h Air exposure for 45 s	38	0.5–1 g/L (live prey enrichment)	↓ Survival after air exposure No changes in cortisol levels ↑ HIF-2α in whole larvae ↑ Growth
Nucleotide (Vannagen)*	*Solea solea*[32]	Catching, netting and hand-sorting for 1 min	56	0–0.04%	↓ Plasma cortisol and glucose levels at 1 and 4 h following acute stress ↓ Brain cannabinoid receptor 1A and 1B mRNA expression at 4 h following acute stress

(Continued)

TABLE 2 | Continued

Additive	Fish species	Stress condition/treatment	Feeding time, days	Test doses	Main effects on physiology and productivity
	Scophthalmus maximus[29]	Handling procedure (combination of capture, netting/ transfer, and crowding)	112	0–0.2%	↓ Plasma cortisol and glucose levels at 1 h following acute stress
Selenium (inorganic source–NaSe)	*Sparus aurata*[33]	Multiple stressful situations: persecution, handling and confinement for 2 h.	63	0.00002%	↓ Plasma cortisol levels at 2 h following acute stress
Selenium (organic source–SeMet)					

5-HT, Serotonin; ACH50, Alternative Complement Pathway; ArA, Arachidonic Acid (20,4n-6); CAT, Catalase; CYP11β, 11β-hydroxylase; DHA, Docosahexaenoic Acid (22,6n-3); DPH, Days Post-Hatch; DW, Dry Weight; EPA, Eicosapentaenoic Acid (20:5n-3); GR, Glutathione Reductase; HIF, Hypoxia Inducible Factor; HSP, Heat-Shock Proteins; MOS, Mannan Oligosaccharides; NO, Nitric Oxide; PEPCK, Phosphoenolpyruvate Carboxykinase; PPARα, Peroxisome Proliferator-Activated Receptor Alpha; ROS, Reactive Oxygen Species; SGR, Specific Growth Rate; SOD, Superoxide Dismutase; StAR, Steroidogenic Acute Regulatory Protein; TFA, Total Fatty Acids; WG, Weight Gain.

[1]Costas et al. (56); [2]Costas et al. (140); [3]Hseu et al. (141); [4]Höglund et al. (142); [5]Herrera et al. (8); [6]Basic et al. (143); [7]Cabanillas-Gámez et al. (6); [8]Azeredo et al. (7); [9]Gonzalez-Silvera et al. (144); [10]Azeredo et al. (145); [11]Pérez-Jiménez et al. (146); [12]Costas et al. (147); [13]Costas et al. (57); [14]Montero et al. (114); [15]Kim et al. (148); [16]Kim and Kang (149); [17]Falahatkar et al. (65); [18]Cheng et al. (68); [19]Koven et al. (150); [20]Van Anholt et al. (74); [21]Van Anholt et al. (75); [22]Ganga et al. (151); [23]Pérez-Sánchez et al. (152); [24]Alves Martins et al. (77); [25]Benítez-Dorta et al. (153); [26]Montero et al. (154); [27]Montero et al. (155); [28]Torrecillas et al. (84); [29]Fuchs et al. (87); [30]Li et al. (156); [31]Lanes et al. (157); [32]Palermo et al. (86); [33]Mechlaoui et al. (158).

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an improved resistance to diseases (160). Although vitamin levels required for fish are influenced by several factors such as environmental factors, few studies have gathered deep knowledge on the modulatory role of vitamins during stressful rearing conditions. Low levels of vitamin E in the diet depleted alternative complement pathway activity and non-specific haemaglutination whereas plasma cortisol basal levels were enhanced without a stressor influence (61). Moreover, this study concluded that fish fed a vitamin E-deficient diet presented lower stress resistance.

Positive effects of dietary vitamin E supplementation have observed in several marine fish species submitted to stressful conditions. For instance, pufferfish (*Takifugu obscurus*) fed vitamin E supplemented diets increased relative expression levels of HSP, Mn-SOD, CAT, and GR whereas ROS levels in blood decreased after acute exposure to ammonia nitrogen (100 mg/L) for 48 h (68). Moreover, beluga (*Huso huso*) submitted to netting and exposed to air for 1.5 min decreased post-stress plasma glucose levels when fed diets supplemented with vitamin E (65). In general, the stress response of the belugas observed in this study was relatively low, and the authors hypothesized that it could be related to greater resistance and/or weaker physiological responses to handling stress in that species. Montero et al. (114) observed that gilthead seabream reared at an initial stocking density of 12 Kg/m^3 (final density: 40 Kg/m^3) increased plasma cortisol and serum lysozyme levels whereas serum ACH50 values decreased. Those fish fed on Vitamin C or a Vitamin E supplemented diets did not change cortisol levels but a decrease in lysozyme was observed, in contrast to the augmentation in serum ACH50 from fish fed the vitamin E supplemented diet.

Lipids and Fatty Acids

It has been reported that dietary lipids can affect the fish stress response, measured as the ability to cope with different stressful situations (74, 75, 151, 152). However, the specific effect of individual fatty acids on the physiological response to stress is still poorly understood, particularly in terms of the modulatory role of fatty acids in the activation of the HPI axis. Arachidonic acid has played a central role in recent studies concerning research on the modulatory roles of dietary fatty acids in the fish stress response. The regulatory role of ArA on the ACTH-induced release of cortisol has been described *in vitro* for gilthead seabream by Ganga et al. (122) and for European seabass by Montero et al. (123). Seabream juveniles fed diets with a high inclusion of vegetable oils (e.g., linseed, rapeseed and palm oils), which translated in a drop in dietary ArA content, increased plasma cortisol levels following an acute overcrowding stress (124, 152). Similarly, feeding an ArA-supplemented diet to gilthead seabream juveniles for 18 days was effective to substantially diminish the cortisol response after net confinement, compared to fish fed a diet containing a low ArA level (74). Benítez-Dorta et al. (153) observed an increase in the level of mRNA expression in glucocorticoid receptor genes after a chasing stress in Senegalese sole juveniles fed a fish oil-based diet (i.e., with high ArA levels) compared to counterpart fed a vegetable oil-based diet (i.e., with low ArA levels). This decreased response to stress was in line to what was found in gilthead seabream larvae submitted to air exposure which showed a considerable drop in peak cortisol levels 28 or 50 days after hatching when they were fed ArA-enriched *Artemia* nauplii (75). In this sense, European seabass fed dietary ArA supplementation decreased the level of expression of P450 11β-hydroxylase (enzyme related cortisol-synthesis), which translated in an increased survival after an activity test consisting of handling procedures and transfer to a new tank (155). In contrast, pre-metarmophosing gilthead seabream larvae daily exposed to fluctuations in salinity increased whole-body cortisol levels when fed ArA-enriched *Artemia* metanauplii for 12 days, which translated in a decreased in survival at 32 days after hatching (150). These findings contrast with the survival-promoting effect of high dietary ArA in larvae exposed only to handling and having relatively low basal cortisol levels. These authors hypothesized that a clue for

those physiological mechanisms could be found in mammalian studies where not only prostaglandin E2 synthesized from the cyclooxygenase enzymes but other ArA metabolites, such as leukotrienes produced from the lipoxygenase enzyme system, also play an important role in ACTH secretion and adrenal steroidogenesis (121, 161).

The fish stress response is therefore nutritionally regulated, and in fact a study with gilthead seabream highlights that the magnitude and persistence of high plasma cortisol levels after overcrowding exposure are dependent on the source of dietary oils (124). Indeed, dietary oils source and, hence, dietary essential fatty acids clearly affected resting levels of glucocorticoid receptor genes expression in Senegalese sole juveniles and larvae and European seabass larvae (77, 153, 155). Moreover, Benítez-Dorta et al. (153) observed and increase in the level of mRNA expression in glucocorticoid receptor genes after a chasing stress in Senegalese sole juveniles fed a fish oil-based diet (i.e., with high ArA levels) compared to specimens fed a vegetable oil-based diet (i.e., with low ArA levels). Those experimental conditions also seemed to affect the Senegalese sole immune response to chasing stress (154).

ArA effects on the stress resistance seem to depend on ArA doses, species or type of stress, but these effects are also dependent on the abundance of n-3 LC-PUFA such as EPA and DHA, since these fatty acids are also essential for stress resistance (162, 163). For instance, ArA and particularly EPA promoted cortisol production in gilthead seabream interrenal cells (122). Moreover, Alves Martins et al. (164) hypothesized that the abundance of ArA relative to EPA (or their oxidized derivatives) in Senegalese sole fed a high ArA/EPA diet could influence StAR (Steroidogenic Acute Regulatory) protein, increase cortisol production and ultimately imply higher energy expenditure to cope with stress.

Prebiotics

The effects of prebiotics supplementation in relation to stress response have scarcely been studied in marine fish. For instance, Torrecillas et al. (84) observed that European seabass fed Bio-Mos® (Alltech, Inc., Nicholasville, KY, USA) dietary supplementation at 0.4% for 60 days reduced plasma cortisol levels in response to a challenge with *Vibrio anguillarum* (i.e., 107 cfu/ml) or to a combination of infection and confinement stress (25 kg/m^3). In contrast, European seabass submitted to confinement stress alone and fed Bio-Mos® increased plasma cortisol levels following acute stress whereas a lower effect of stress on gut microbiota was found in those fish fed 0.4% Bio-Mos® during 60 days compared to stressed fish fed a control diet. Indeed, it has been already reported that mannan oligosaccharides (MOS) supplementation reinforces epithelial barrier, stimulates the immune system, promotes growth and feed efficiency and effectively enhances disease resistance in fish (130). In another study, Fuchs et al. (87) studied the effects of a 6% yeast (*Saccharomyces cerevisiae*) product consisting of 20% beta-1,3/1,6 glucan and 17% MOS (ProEnMune, ProEn Protein, and Energie GmbH, Soltau, Germany) in turbot juveniles. In contrast to that observed by Torrecillas et al. (84), it was observed a decrease in plasma cortisol and glucose levels at 1 h after acute stress. However, this decrease in both primary and secondary stress responses observed in stressed turbot could be attributed to a synergistic effect of both beta-1,3/1,6 glucan and MOS from yeast, thus making difficult a direct comparison on the effects of dietary MOS within marine fish species submitted to stressful conditions.

Nucleotides

Studies on different fish species reported that dietary nucleotide supplementation enhanced their resistance to parasites, bacteria and virus (136), while the effects of those particular additives on the marine fish stress response still remain to be studied in detail. For instance, a study on Atlantic cod larvae suggested that a nucleotide-enriched *Artemia* can benefit growth whereas those larvae appeared to be more susceptible to acute stress as evidenced by the lower survival rates and higher *hif-2α* transcript levels in whole larvae, although cortisol levels were not affected (157). Likewise, red drum (*Sciaenops ocellatus*) juveniles fed a nucleotide product (i.e., Optimun, Chemoforma, Basel, Switzerland), which contained cytidine-50-monophosphate, disodiumuridine-50-monophosphate, adenosine-50-monophosphate, disodium inosine-50-monophosphate, disodium guanidine-50-monophosphate, and RNA, did not change plasma cortisol levels in after a 15 min confinement stress test, a fact that could be linked to a high individual variation among fish (156). In contrast, turbot juveniles submitted to an acute stress (i.e., handling procedure consisting of a combination of capture, netting/transfer, and overcrowding from 13.3 to 32.4 kg m^{-2}) and fed a product of purified yeast nucleotides for 112 days decreased both plasma cortisol and glucose levels at 1 h after acute stress. According to Palermo et al. (86), Senegalese sole fed a commercial source of nucleotides derived from yeast (Vannagen™, Chemoforma) for 8 weeks coped well with an acute stress challenge (i.e., catching, netting and hand-sorting for 1 min) and presented lower plasma cortisol and glucose levels than control fish. Those authors also reported a decrease in the mRNA expression level of brain cannabinoid receptors 1A and 1B in fish fed the nucleotides supplemented diet after acute stress, and suggested a putative nucleotides effect on the functional interaction between endocannabinoid signaling system and stress axis in fish, a fact that deserves further attention.

Minerals

Indeed, information regarding mineral nutrition in marine fish is still scarce, a lack of knowledge that seems to increase when assessing the stress response in fish. Selenium in particular is an essential trace element for fish (139), and therefore it plays an important role for growth and conservation of biological compounds, exerting protection against free radicals resulting from normal metabolism (165). An increase in dietary selenium supplementation (i.e., organic and inorganic forms) appeared to increase stress tolerance in gilthead seabream juveniles, as shown by the decreased plasma cortisol levels during the stress challenge in specimens submitted to acute stress (158). The later study reinforced the importance of dietary selenium supplementation

on health and welfare in gilthead seabream, similarly to that reported for salmonid species (see section Minerals below).

Salmonids

Atlantic salmon (*Salmo salmo*) and rainbow trout (*Onchorhynchus mykiss*) are the most studied salmonid species in the literature (**Table 3**). Contrarily to marine species, here it seems that stress responses are more consistent since, for the same species and additive, the results on stress parameters are not different among every work (55, 97, 167, 168).

Amino Acids

Research with salmonid species mainly studied the modulatory role of dietary tryptophan on the fish stress response, including aggressive behavior, to an acute stressful condition. Moreover, those studies particularly emphasized on the short-term effect of tryptophan treatment (e.g., 7 days). For instance, some recent findings showed that tryptophan administration can increase serotonergic activity by means of increased 5HT and/or 5HIAA (97, 167, 169); while others suggested a suppression in aggressive behavior and stress-induced anorexia (44, 166). In rainbow trout, a 7-day tryptophan treatment suppressed post-acute stress cortisol increase, a fact that appears to be modulated by serotonergic activity and ACTH release (97, 167).

In contrast, other researchers investigated if dietary tryptophan treatment may result in long-lasting effects on stress responsiveness. For instance, Atlantic salmon decreased post-acute stress cortisol levels at days 8, 10, and 21 following a 7-day period tryptophan administration (169, 170). The importance of tryptophan administration time on serotonergic activity and cortisol response has also been suggested for the rainbow trout (97). Still, there are no evidences for the effects of long-term dietary tryptophan administration on the stress response in salmonids, a fact that deserves further attention.

Vitamins

Few studies with salmonid species have focused on the modulatory role of vitamins during stressful rearing conditions. Thompson et al. (60) did not observe any evidence that dietary vitamin C (3.17 g/kg diet) can ameliorate the down regulation of the immune system that occurs following confinement stress in the Atlantic salmon, suggesting that vitamin C does not play a fundamental role in regulating the primary stress response in salmonids. In contrast, dietary supplementation of vitamin E (275.6 mg/kg diet) appears to enhance the MCV (Mean Corpuscular Volume) of rainbow trout reared at high density (i.e., 100 kg/m^3) for 42 days (171). 138 also reported a positive effect of vitamin E supplementation (500 mg/kg diet) in chronically stressed rainbow trout for 60 days. In this study, dietary vitamin E reverted the negative effects of high density (i.e., 80 kg/m^3) by decreasing plasma cortisol and lactate levels. Moreover, those fish also presented and enhanced SOD activity as well as a decrease in MDA (Malondialdehyde) in liver. A synergistic effect of dietary vitamin E supplementation with HUFA was also observed in chronically stressed rainbow trout with an increase of plasma cortisol after 42 days reared at high density (64). Those fish also showed an enhanced catalase activity in liver compared to their low density counterparts, a fact that could be related to the lipid-soluble character of vitamin E.

Nucleotides

Most studies concerning nucleotides nutrition in salmonids as a strategy to mitigate the negative effects of stress were performed with the same commercial additive (Optimun, Chemoforma, Augst, Switzerland). Rainbow trout fed diets containing 0.15–0.2% nucleotides from Optimun improved growth performance and several hematological and biochemical parameters, which translated in a significant reduction of plasma cortisol and glucose after exposure to acute handling and overcrowding stress (85). Leonardi et al. (174) also observed positive health-related effects in rainbow trout fed the same dietary additive at 0.03%, since those fish decreased plasma cortisol levels following challenge with infectious pancreatic necrosis virus. Furthermore, Caspian brown trout (*Salmo trutta caspius*) fed an Optimun supplemented diet (i.e., 0.25%) for 56 days decreased plasma cortisol and glucose levels after acute confinement and salinity stress (23). In contrast, rainbow trout fed an Optimun supplemented diet (i.e., 0.2%) for 45 days did not improve growth performance nor stressful condition in high density groups, which decreased serum ACH50 levels (173). Fu et al. (25) assayed diets supplemented with graded levels of Maxi-Gen^{TM} Plus (Canadian Bio-Systems Inc., Calgary, AB, Canada) with Atlantic salmon during smoltification, showing that the hypo-osmoregulatory ability was gradually enhanced when the dietary inclusion level of Maxi-Gen^{TM} Plus augmented from 0.05 to 0.20%, and from 0.20 to 0.60%. Moreover, an inclusion of 0.60% Maxi-Gen^{TM} Plus in the diet resulted in lower plasma cortisol levels of smolting Atlantic salmon compared to fish fed the control diet, suggesting reduced stress levels in fish during smoltification and desmoltification.

Minerals

Depending on its chemical form, selenium is a trace element with a narrow range between requirement and toxicity for most vertebrates, and thus some studies were undertaken to assess and recommend safe limits regarding selenium nutrition in salmonids (175, 176). However, few studies have been conducted with salmonid species submitted to stressful conditions. Rainbow trout submitted to acute stressful situations for 7 days or to crowding conditions (100 kg/m^3) for 86 days seem to increase selenium requirement for an optimal oxidative status (88, 165). In fact, Naderi et al. (172) reported a drop in serum lactate, alanine aminotransferase and alkaline phosphatase levels together with enhanced glutathione peroxidase activity in liver in rainbow trouts fed Se supplements under high density. Interestingly, in that study a positive synergistic effect between dietary organic selenium and vitamin E was observed, which translated in decreased serum cortisol levels as well as improved superoxide dismutase activity and low MDA levels in liver.

Cyprinids

In this order more than 10 additives and seven species have been assayed (**Table 4**). The most of works have been focused

TABLE 3 | General overview on the effects of dietary additives in salmonids submitted to stressful conditions.

Additive	Fish species	Stress condition/treatment	Feeding time, days	Test doses	Main effects on physiology and productivity
Tryptophan	*Salmo trutta*[1]	Transfer to a new environment	7	0.22–0.06 Trp/LNNA	↓ Stress-induced anorexia
	Oncorhynchus mykiss[2,3,4,5,6]	Resident/intruder test	3; 7	0.15–1.5%	↓ Aggressive behavior in fish fed for 7 days
		Lowering the water level for 2 h	7	0.44–3.57%	↓ Adrenocorticotropic hormone and cortisol levels in plasma ↑ Brain serotonergic activity
		Lowering the water level for 2 h	3; 7; 28	0.044–0.357%	↓ Adrenocorticotropic hormone and cortisol levels in plasma after 7 days of feeding
		Daily social interaction for 1 h followed by a resident/intruder test after 1 week	7	0.044–0.357%	↓ Aggressive behavior ↓ Cortisol levels in plasma
		Lowering the water level for 2 h	7	0.044–0.357%	↓ Cortisol and melatonin levels in plasma
	Salmo salar[7,8]	Confinement for 30 min at days 1, 2, and 10 after tryptophan treatment	7	0.4–1.58%	↓ Plasma cortisol levels at day 10 after tryptophan treatment
		Acute crowding stress for 1 h at days 8 and 21 after tryptophan treatment	7	0.44–1.2%	↓ Plasma cortisol levels at days 8 and 21 after tryptophan treatment
Vitamin C	*Salmo salar*[9]	Confinement for 2 h	161	0.0082–0.317%	↓ Plasma antibody titers at 43 days post-immunization
Vitamin E	*Oncorhynchus mykiss*[10,11]	High density (100 kg/m^3)	42	0.00256–0.02756%	↑ MCV
		High density (80 kg/m^3)	60	0.010475–0.060075%	↓ Cortisol and lactate levels in plasma ↑ SOD in liver ↓ MDA in liver ↑ SGR, WG, FI
Nucleotide (Optimum)*	*Oncorhynchus mykiss*[12,13]	Netting, air exposure for 30 s, and crowding at 100 kg/m^3 for 3 h	56	0–0.2%	↓ Plasma cortisol levels in infected and stressed and infected groups ↑ Plasma cortisol levels in stressed groups ↓ Side-effects of stress on microflora profiles ↑ WG, FE
		High density (30 kg/m^3)	45	0.2%	↓ Serum urea and ACH50 levels No effect on growth
	Salmo trutta caspius[14]	Netting, air exposure for 30 s, and crowding at 100 kg/m^3 for 3 h	56	0.15–0.5%	↓ Plasma cortisol and glucose levels at 8 h following acute stress ↑ Final weight
		Transfer to salt water (18 g/L)		0.15–0.5%	↓ Plasma cortisol levels at 120 h following acute stress ↑ Final weight
Nucleotide (Maxi-Gen Plus)#	*Salmo salar*[15]	Smoltification process	122	0.05–0.60%	↓ Plasma cortisol levels ↑ WG, FI
Selenium	*Oncorhynchus mykiss*[11,16,17]	High density (80 kg/m^3)	60	0.000035–0.000135%	↓ Serum lactate, ALP and ALT levels ↑ Hepatic GPx activity ↓ SOD activity in liver No effects on growth
		Acute stress for 7 days consisting of a combination of daily crowding and handling (i.e., netting and air exposure for 30 s) twice a day	70	0.00073–0.00074%	↑ ROS in blood ↑ Hepatic MDA ↑ Whole body copper
		High density (100 kg/m^3)	84	0.00008–0.00011%	↓ MDA levels in serum and muscle ↓ Serum GPx activity ↓ HSP70 expression in muscle ↑ Final weight, FI

ACH50, Alternative Complement Pathway; ALT, Alanine Aminotransferase; ALP, Alkaline Phosphatase; FE, Feed Efficiency; FI, Feed Intake; GPx, Glutathione Peroxidase; HSP70, Heat Shock Protein 70; LNNA, Large Neutral Amino Acids; MCV, Mean Corpuscular Volume; MDA, Malondialdehyde; ROS, Reactive Oxygen Species; SGR, Specific Growth Rate; SOD, Superoxide Dismutase; Trp, Tryptophan; WG, Weight Gain.

[1]Höglund et al. (44); [2]Winberg et al. (166); [3]Lepage et al. (167); [4]Lepage et al. (97); [5]Lepage et al. (168); [6]Lepage et al. (55); [7]Basic et al. (169); [8]Höglund et al. (170); [9]Thompson et al. (60); [10]Trenzado et al. (171); [11]Naderi et al. (172); [12]Tahmasebi-Kohyani et al. (85); [13]Yousefi et al. (173); [14]Kenari et al. (23); [15]Fu et al. (25); [16] Rider et al. (165); [17]Küçükbay et al. (88).

**Optimum® supplied by Chemoforma (Augst, Switzerland).*

#Maxi-Gen Plus® supplied by Canadian Bio-Systems Inc. (Calgary, AB, Canada).

TABLE 4 | General overview on the effects of dietary additives in cyprinids submitted to stressful conditions.

Additive	Fish species	Stress condition/treatment	Feeding time, days	Test doses	Main effects on physiology and productivity
Alanine and glutamine	*Cyprinus carpio*[1]	High density (80 g/L)	56	0–1%	↑ Serum IGF-I and insulin ↓ Serum glucagon ↑ GR gene expression ↑ WG
Tryptophan	*Labeo rohita*[2]	Thermal stress (34 and 38°C)	45	0–1.42%	↓ Blood glucose and serum cortisol ↓ AST and ALT activities ↓ LDH and MDH activities ↓ AchE, CAT, and SOD activities ↑ RGR, PER
	Cirrhinus mrigala[3]	Crowding stress (30 fish/75 L, 3-fold control group)	60	0–2.72%	↓ Blood glucose and plasma cortisol ↓ AST and ALT activities ↓ MDH activity ↑ AchE activity ↑ SGR, PER
Taurine	*Mylopharyngodon piceus*[4]	Crowding stress (100 g/L) for 24 h after experimental feeding	56	0–0.4%	↓ Serum glucose and cortisol ↑ Serum complement C3,lysozyme, SOD and glutathione ↑ WG
Vitamin C	*Cyprinus carpio*[5]	Salinity stress (0, 6 and 2 ppt)	48	0–0.1%	↓ Blood cortisol ↓ Skeletal malformations
Vitamins C + E	(*Notemigonus crysoleucas*)[6]	Vitamins C + E combinations and thermal stress (37°C)	119	0–0.000038% vit E 0–0.000222% vit C	Different interactive effects ↑ACH50 No effect on growth
Vitamin E	(*Megalobrama amblycephala*)[7]	Crowding stress for 48 h (100 g/L)	60	0.1–0.6%	↓ Serum glucose and cortisol ↓ Serum ALT and lysozyme activities ↑ Serum proteins ↓ Hepatic MDA content ↑ HSP70 expression ↑ SGR
Vitamins mix (C, B1, B6, and E)	*Cyprinus carpio*[8]	Handling (confinement) stress: 2 cm water depth for 2 h	14	Different mixes	↓ Mucus immunoglobulins No effect on growth
Lipids	(*Notemigonus crysoleucas*)[9]	Crowding stress (4 cm water depth for 2 h)	42	4–13% different oils	No changes in cortisol response
MOS (prebiotic)	(*Danio rerio)*[10]	Starvation, live transport and tank cleaning	56	0–0.4%	↓ Cortisol and CRH gene expression
Selenium	(*Megalobrama amblycephala*)[11]	Nitrite exposure (15 mg/L for 96 h)	60	0–0.00005%	↓ Serum cortisol ↓ Hepatic MDA content ↑ SOD, CAT and GPx activities and transcriptions

ACH50, Alternative Complement Activity; AchE, Acetylcholine Esterase; ALT, Alanine Aminotransferase; AST, Aspartate Aminotransferase; CAT, Catalase;; CRH, Corticotropin Releasing Hormone; GR, Glucocorticoid Receptor; HSP70, Heat Shock Protein 70 KDa; IGF-I, Insuline Growth Factor I; LDH, Lactate Dehydrogenase; MDA, Malondialdehyde; MDH, Malate Dehydrogenase; MOS, mannan-oligosaccharide; PER, Protein Efficiency Ratio; RGR, Relative Growth Rate; SGR, Specific Growth Rate; SOD, Superoxide Dismutase; WG, Weight Gain.
[1]Chen et al. (51); [2]Kumar et al. (49); [3]Tejpal et al. (46); [4]Tian et al. (52); [5]Imanpoor et al. (21); [6]Chen et al. (62); [7]Liu et al. (13); [8]Sándor et al. (69); [9]Lochmann et al. (73); [10]Forsatkar et al. (22); [11]Long et al. (90).

on amino acids and vitamins. Only two works have dealt with minerals and carbohydrates (22, 90).

Amino Acids

It seems clear that amino acid effects, concretely tryptophan (Trp) supplements, are consistent along cyprinid species. In this sense Kumar et al. (49) and Tejpal et al. (46) have reported significant cortisol secretion decreases after stress in rohu (*Labeo rohita*) and mrigal (*Cirrhinus mrigala*), respectively. In addition, abovementioned two studies papers have stated a growth enhancement after feeding Trp-enriched diets for 45–60 days. The amount of Trp in diet have been very similar in both papers, hence 1–1.5% Trp on dry matter basis is effective to attenuate the stress response in cyprinids. In addition, the stressors were different in both works, hence it seems that the stress response in cyprinids fed Trp supplements is enough consistent along species. Tejpal et al. (46) have also established a linear relation between Trp content and plasma cortisol for both

stressed (overcrowding) and non-stressed rohus, and have used that mathematical equation to define the optimum Trp content (1.36%) for the highest stress attenuation.

Other amino acids like alanine (Ala) and glutamine (Gln) did not affect cortisol response in carp (*Cyprinus carpio*) though growth performance was significantly improved (51). Spite of the lack of cortisol response in this work, other hormones variations reflected the addition of dietary amino acids. In fact, IGF-I (Insulin-like Growth Factor I) and insulin significantly increased with dietary Ala-Gln supplementation under overcrowding stress. Therefore, the authors concluded that Ala-Gln supplements enhance the ability of fish resistance to overcrowding stress, which may contribute to the better regulation ability for hormone secretion on fish.

Regards dietary total protein, Habte-Tsion et al. (14) have studied the effects of different protein ratios (28–36%) in feed on the stress response in the blunt snout bream (*Megalobrama amblycephala*). Under thermal stress, the cortisol secretion was minimum in fish fed diet containing 32% dietary protein. This treatment also showed positive results in other immune and stress oxidative parameters. Additionally, the authors reported that the specific molecular mechanisms by which the optimum dietary protein level reduced the level of cortisol in high temperature stressed blunt snout breams need to be researched.

The relation between dietary lipid and protein contents, and stress response have also tested in cyprinids. In those cases, the role of dietary proteins seems to more decisive than lipids since golden shiners did not show significant differences in the endocrine stress response depending on dietary lipid level, meanwhile Habte-Tsion et al. (14) stated that the optimum protein content for decreasing the cortisol response significantly in blunt snout bream was 32%.

Vitamins

Vitamins C and E have been assayed in some Cyprinid species. The beneficial antioxidant properties and the reduction of cortisol response after stress are common results in recent studies (13, 21, 62, 66, 69). Moreover, these have reported other positive effects like immune system and growth enhancement, higher survival and lower skeleton abnormalities. However, several differences have been detected among species. It seems that the vitamin C requirements to improve stress resistance in carp is around 50 mg/Kg diet, while golden shiners (*Notemigonus crysoleucas*) need more than 98 mg/Kg (21, 62). Similarly, 600 mg/Kg diet of vitamin E are reported to be enough to reduce the post-stress cortisol secretion in blunt snout bream (*Megalobrama amblycephala*) (13), and Chen et al. (62) point that 38 mg/Kg diet is a suitable vitamin E concentration to improve stress resistance in the golden shiner. In those cases, the vitamin requirements for improving the stress response are clearly different along species, which could be expectable since those requirements are in that way from a nutritional perspective.

Prebiotics

The inclusion of prebiotics, particularly MOS (mannan-oligosaccharides), in the diet have also demonstrated to have stress-attenuating effects at endocrine level in cyprinids (22). Both cortisol secretion and CRH expression level were significantly reduced after feed deprivation stress in zebrafish fed MOS. In addition, the inclusion of MOS in the diet of zebrafish reduced some anxiety-like behaviors in fish submitted to feed deprivation. Those authors stated that all the physiological alterations were the results of alteration in intestinal microbiota, and the modulation of gut microbiota by MOS play a role in the stress reactivity of zebrafish.

Other Freshwater Species

As in the other groups, amino acids and proteins are the most frequent substances assayed in these 11 different freshwater (excluding cyprinids) fish species (**Table 5**). This is the most heterogeneous group regards both species and stress response. Opposite endocrine stress responses have been described for every additive type in these species.

Amino Acids

In this group, the works have based on two different biomolecules content in diet: protein/lipid/carbohydrate ratios or tryptophan (Trp), and tilapia (*Oreochromis niloticus*) being the most frequent species. In the former, the study of stress response was a secondary objective beyond the nutritional aspects, meanwhile that response was the main objective in the latter.

Generally, the dietary protein level does not seem to have a significant effect on the stress response in these freshwater species. Concretely, Hooley et al. (16) did not report any plasma cortisol and glucose variations during hauling stress in tilapia; however, these authors pointed that it could be due to a limited ability to detect differences due to the limited number of fish examined at each time point and the high variability in responses between fish within a treatment. Neither Abdel-Tawwab (47) detected differences in plasma cortisol due to overcrowding stress in tilapias fed several protein levels. Lastly, Siberian sturgeon (*Acipenser bareii*) fed different protein, lipids, and carbohydrates levels only showed lower values of cortisol for low carbohydrate diets, regardless protein levels (12).

The effects of Trp-enriched diets on stress and other physiological parameters have been studied in freshwater species. Interestingly, three species have showed a similar stress response, presenting lower cortisol levels in Trp treatments for non-stressed fish, and no variation between those treatments when comparing pre- and post-stress cortisol. Concretely, *Brycon amazonicus* fed Trp supplements reduced their aggressiveness though the plasma cortisol did not vary (48). Contrarily, Martins et al. (58) found differences in plasma cortisol for undisturbed tilapias fed Trp supplements although, curiously, it increased significantly after stress for all treatments (control and Trp added). These authors indicate that despite altering the serotonergic activity, Trp-enriched diets do not always affect the HPI reactivity, as reported by Wolkers et al. (48). Despite Hoseini et al. (59) reported similar responses in Persian sturgeon (*A. persicus*), they went deeper in the study of the endocrine stress response and assessed the variations of serum thyroid hormones. In this sense, these authors have stated that exogenous tryptophan decreases serum levels of thyroid hormones probably via increase in serotonergic activity and elevated cortisol levels.

TABLE 5 | General overview on the effects of dietary additives in other freshwater species submitted to stressful conditions.

Additive	Fish species	Stress condition/treatment	Feeding time, days	Test doses	Main effects on physiology and productivity
Tryptophan	*Brycon amazonicus*[1]	Aggressiveness test (resident-intruder)	7	0.94–3.76%	↓ Aggressiveness No effect on physiological stress markers
	Cichlasoma dimerus[2]	Normal experimental conditions	28	control; control + 2.1%	↓ Plasma cortisol ↓ Brain serotonergic activity No effect on growth
	Acipenser persicus[3]	Confinement (0.5 h)	5; 10; 15	0.28–0.78%	↓ Serum thyroid hormones ↑ Serum cortisol
	Oreochromis niloticus[4]	Crowding (50% water volume)+chasing (20 min)	7	0.48–4.45%	↑ Brain serotonin metabolites No effect on the HPI axis
Protein levels	*Oreochromis niloticus*[5,6]	Experimental conditions (different protein levels)	70	25–45%	↑ Serum glucose, proteins and lipids ↑ ALT and AST activities ↑ SGR, PER, FI
		Simulated haul	84	28–36%	No effect on physiological stress markers No differences for WG, FI ↓ FCR
	Acipenser baerii[7]	Experimental conditions (different protein levels)	70	38–44%	↓ Amylase, SOD and CAT activities ↓ Plasma glucose ↑ SGR, WG
Vitamin C	*Leiocassis longirostris*[8]	Ammonia (1.03 and 9.6 mg/L total ammonia nitrogen)	60	0.0038–0.63%	Keeping of serum lysozyme and hepatic SOD activities ↑ SGR, FR
Vitamin E	*Piaractus mesopotamicus*[9]	High stocking density (20 Kg/m^3)	140	0–0.045%	↓ Plasma cortisol ↑ Kinetic activity of macrophage recruitment ↑ Giant cell formation
Commercial vitamin premix	*Ictalurus punctatus*[10]	Confinement (1 and 6 h)	540	Different mixes	No effect on plasma cortisol
DHA	*Prochilodus lineatus*[11]	Air exposure (60 s)	17	0.13–6.64% TFA (in *Artemia*)	↓ Whole-body cortisol No effect on growth
Astaxanthin	*Pelteobagrus fulvidraco*[12]	Crowding stress (2 days at 150 g/L)	60	0–0.008%g	↓ Serum cortisol and glucose ↓ ALT, AST, ALP and MDA activities ↓ Serum lysozyme activity ↓ SGR, WG
Zn	*Pangasius hypophthalmus*[13]	High lead (Pb) concentration (4 ppm)	75	0–0.002%	↓ Serum cortisol and HSP70 expression ↓ Blood glucose ↑ AChE activity ↓ CAT, SOD, GST, LPO activities

AchE, Acetylcholine Esterase; ALT, Alanine Aminotransferase; ALP, alkaline phosphatase AST, Aspartate Aminotransferase; CAT, Catalase; FCR, Factor Conversion Rate; FI, Feed Intake; FR, Feeding Rate; GST, Glutathione Transferase HPI, hypothalamus-pituitary-interrenal; LPO, Lipid Peroxydase; MDA, Malondialdehyde;; PER, Protein Efficiency Ratio; SGR, Specific Growth Rate; SOD, Superoxide Dismutase; TFA, Total Fatty Acids; WG, Weight Gain.
[1]Wolkers et al. (48); [2]Morandini et al. (50); [3]Hoseini et al. (59); [4]Martins et al. (58); [5]Abdel-Tawwab (47); [6]Hooley et al. (16); [7]Babaei et al. (12); [8]Liu et al. (20); [9]Belo et al. (63); [10]Davis et al. (72); [11]Araújo and Rosa (79); [12]Liu et al. (24); [13]Kumar et al. (9).

Only Morandini et al. (50) have reported a post-stress cortisol decrease in chanchita (*Cichlasoma dimerus*) fed Trp supplements. They also described an enhancement of the serotonergic activity hence it seems to be a common physiological reaction derived from this type of diets in studied freshwater species (see above). Those authors also analyzed the plasma sex steroid variations depending on the diet and did not find any differences in those hormones.

Vitamins

Commercial vitamin premix did not seem to affect the stress response (cortisol levels) in the Channel catfish (*Ictalurus punctatus*) (72). However, Belo et al. (63) reported that plasma cortisol did not vary in pacu (*Piaractus mesopotamicus*) submitted to overcrowding stress when fed vitamin E supplement (450 mg/Kg). These authors concluded that Vitamin E would seem to act on the stress response of pacus by preventing a stress-related immunosuppression. Contrarily, the serum cortisol levels in *Leiocassis longirostris* submitted to ammonia stress were not affected by the vitamin C supplements, and it was reported that chronic high-ammonia stress showed a tendency to inhibit the cortisol response (20).

Lipids and Fatty Acids

In this topic, Araújo and Rosa (79) researched on the effects of the docosaheanoic acid (DHA) in the feeding of *Prochilodus lineatus* larvae. The supplements were provided to the live prey (*Artemia*) during 16 h prior feeding. They stated that supplementation of

DHA-rich live feed to *P. lineatus* larvae can attenuate cortisol response to an acute stressor such as air exposure during metamorphosis, when higher mortalities are expected, and the physiological mechanisms underlying the effect of DHA on the larval stress response still need to be elucidated.

Finally, astaxanthin has also been used to reduce stress in yellow catfish (*Pelteobagrus fulvidraco*), stating that this supplement (80 mg/Kg) can improve the anti-oxidative capabilities, hepatic HSP70 levels, and acute overcrowding stress resistance of yellow catfish (24).

Minerals

Kumar et al. (9) performed a comprehensive work on the effects of zinc (Zn) supplements on several stressors in the catfish (*Pangasius hypophthalmus*). They studied the integrative stress response to high lead (Pb) concentration, assessing immune, endocrine, metabolic, and oxidative stress parameters. Both plasma stress markers (cortisol and glucose) and oxidative stress enzyme activities improved in fish fed Zn supplements. In addition, immune parameters were enhanced and survival was higher in the experimental diets. Concluding, Zn supplements (10–20 mg/Kg) improved the integrative stress response (endocrine and oxidative) to lead toxicity.

CONCLUSIONS

Overall, the possibility of mitigating the negative effects of stress and disease susceptibility of fish through dietary additives supplementation seems realistic, in particular concerning functional amino acids, fatty acids and minerals. Nevertheless, these nutritional strategies need to take into account several extrinsic (e.g., rearing systems, temperature, salinity, etc.) and intrinsic (e.g., age, genetic background, etc.) factors which in some cases could require tailor-made formulations. The link among the catabolism of those biomolecules and the HPI axis still remains unclear. For instance, the mechanism which serotonin coming from Trp supplements interact with the cortisol/corticosteroid secretion is poorly known.

Further studies are required for validating this nutritional strategy in order to improve welfare and survival in chronically stressed fish. It was observed that both stress response and immune function vary with type of stressors and stress duration. Therefore, once an optimal level of supplementation is achieved for a certain nutrient/additive and for a given species, its beneficial effects should be validated during different stressful conditions commonly found in aquaculture.

AUTHOR CONTRIBUTIONS

MH has coordinated the making of the manuscript, collected the most of papers for reviewing, and been the responsible for five main sections. JM has been responsible for three main sections. BC has been responsible for four main sections. All the authors have participated in the final revision of the manuscript.

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