WELFARE AND STRESSORS IN FISH: CHALLENGES FACING AQUACULTURE

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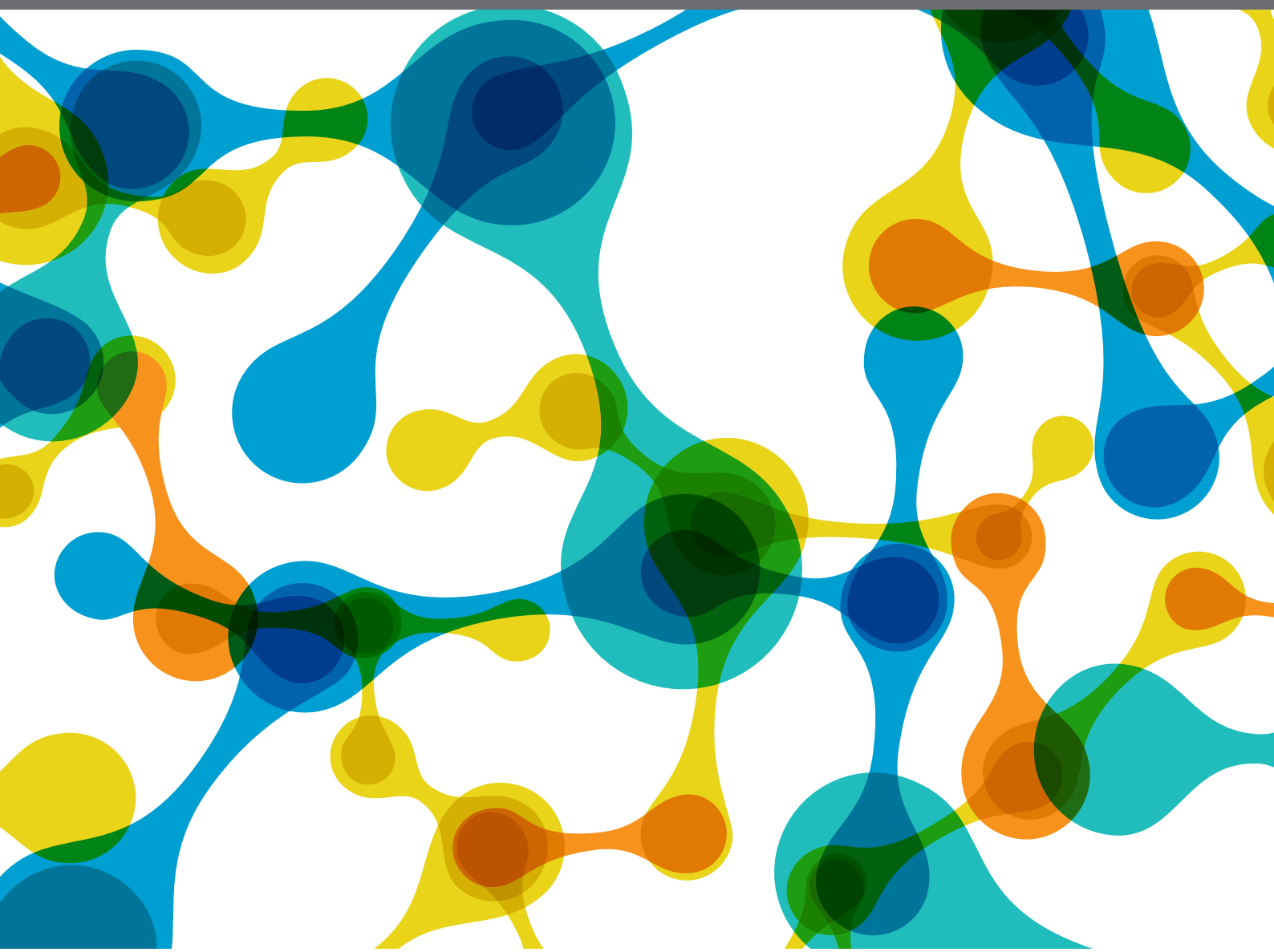

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WELFARE AND STRESSORS IN FISH: CHALLENGES FACING AQUACULTURE

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EDITORIAL
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Editorial: Welfare and Stressors in Fish: Challenges Facing Aquaculture

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Keywords: fish welfare, environmental stressors, dietary imbalance, handling, fish monitoring devices, stress-reducing agents, HPI axis, energy balance

Editorial on the Research Topic

Welfare and Stressors in Fish: Challenges Facing Aquaculture

Aquaculture production is in a process of expansion as demand increases globally. Nevertheless, the intensive production of fish requires carefully monitored and controlled environments that, if not adhered to, may lead to stressed animals, compromising their health and survival (Ashley, 2007). Efforts to improve welfare and to reduce mortality in fish aquaculture are reflected in European Directive 2010/63/EU, and its implementation has been evaluated by Toni et al. (2018). Improving the welfare of farmed fish (e.g., by reducing stress) can result in enhanced productivity. Therefore, applying correct management protocols is important for the economic success of this industry. In addition, there is now an increased awareness among the public and the scientific community of the importance of understanding the physiological and behavioral bases of stress responsiveness and welfare in fish (Conte, 2004; Huntingford et al., 2006; Braithwaite and Ebbesson, 2014; Castanheira et al., 2017). Ethical aspects of the use of fish for aquaculture, research as well as in fisheries have also been considered (Huntingford and Kadri, 2009; Bovenkerk and Meijboom, 2013).

Intensive production may involve decreased environmental quality, including increased fish density and the appearance of production-related diseases, which are challenges faced by aquaculture. Domestication could lead to lower stress levels of the fish population for certain aquaculture environment. However, domestication may impair stress coping when fish experience a change in that environment. Therefore, the effects of multiple concurrent stressors, the process of domestication and the mechanisms associated with stress responsiveness are aspects that still need to be investigated in cultured fish. For these reasons, this Research Topic was aimed at expanding general knowledge on the physiological responses of cultured aquatic animals to current practices as well as finding alternatives to improve conditions.

Over the past few decades we have seen an increased number of studies characterizing the stress response in fish, however, the information for other aquatic organisms, such as the cephalopods, is limited. Several Octopodidae species have great potential for aquaculture. Unfortunately, the lack of stress-related biomarkers in this taxon presents an obstacle to evaluate maintenance conditions. Barragán-Méndez et al. assessed physiological responses related to fishing capture of *Eledone moschata*, *E. cirrhosa,* and *Octopus vulgaris* as a first step toward studying the physiology of stress. The authors reported information on energy mobilization in response to capture, which returned to pre-stress values within the first 24 h.

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Cortisol is believed to be the main hormone mediating the physiological stress response in vertebrates. Sustained swimming activity at optimal speeds is associated with improved growth and lower secreted cortisol levels in various fish species. Palstra et al. demonstrated that best growth in zebrafish (*Danio rerio*) occurs at the optimal swimming speed and is associated with increased cortisol levels. However, the response was not directly mediated by the glucocorticoid receptor (Gr), suggesting that cortisol is not the main determinant of exercise-enhanced growth in this species. This study showed that 36 genes in white skeletal muscle, involved in transcriptional regulation and protein ubiquitination, play a major role in the growth-promoting effects of exercise in fish.

In addition to swimming, other factors impact fish homeostasis and may act as stressors. Among them water dissolved oxygen levels and stocking density are relevant factors under intensive aquaculture production. Therefore, physiological responses and the molecular mechanisms unchained by stressors need to be clarified to establish optimal animal welfare and maximize efficiency, especially when multiple factors may coexist. Magnoni et al. investigated the response of rainbow trout (*Oncorhynchus mykiss*) subjected to both long-term environmental hypoxia and dietary electrolyte-imbalance followed by an acute stressor. In spite of a decrease in feed intake, stress markers were not altered by hypoxia. The dietary challenge profoundly affected fish homeostasis, evoking oxidative stress and impaired immune status, which led to stress and potentiating the negative effect of the acute stressor, suggesting a synergistic effect. Furthermore, Hernández-Perez et al. showed that high stocking density in rainbow trout alters liver metabolism, a response also modulated by circadian rhythms. This study confirmed that high stocking density induces a stress response that is finely, differently and time-dependently regulated by glucocorticoid pathways. The circadian oscillator located in the liver was affected by the stressor, modulating the energy partitioning. Likewise, Martos-Sitcha, Simó-Mirabet et al. demonstrated by using metabolic and transcriptomic approaches that moderate hypoxia in gilthead seabream (*Sparus aurata*) produces a hypometabolic state with a negative impact on feed intake and growth rate, effects that were intensified with high stocking density. The response was accompanied by an improvement in the feed efficiency, an enhancement in the O_2-carrying capacity, as well as a differential regulation of metabolic and stress markers. This was shown by several physiological hallmarks responding to the stressor across several organs, suggesting a different contribution of each tissue to the allostatic load.

The fish skin is a multifunctional organ with highly relevant physiological roles. Kulczykowska summarizes the current knowledge of the skin function as a cutaneous stress response system, where cortisol, melatonin, and derived peptides act together to protect the organism against unfavorable conditions. The study of the various skin functions along with the impact of environmental and biotic factors is an exciting research area rapidly expanding due to its relevance in cultured fish. Sanahuja et al. investigated the response of the skin mucus proteome in the gilthead seabream exposed to changes in water temperature. Authors found that proteins associated with a stress response were up-regulated in fish exposed to low temperatures. However, proteins related to metabolic activity were down-regulated in response to cold, evidencing depressed skin metabolism. Results show a partial loss of mucus functionality under chronic cold exposure, which may affect fish welfare under farming conditions. Interestingly, parameters measured in the skin mucus can be used in a non-invasive approach to assess fish welfare.

Detrimental effects caused by adverse rearing conditions could be alleviated by therapeutic strategies, which is a promising area of research aimed at improving aquaculture production. The use of different amino acids in fish diets may be an interesting tool to mitigate stress responses, although the effect may be dependent on the species, the stressor and quantity supplemented. Azeredo et al. investigated the effects of tryptophan (Trp) dietary supplementation in Senegalese sole (*Solea senegalensis*) held at high stocking densities. The study showed that fish fed a Trp supplemented diet were better prepared to cope with a biotic challenge, showing a decreased cumulative mortality. The effects of dietary Trp supplementation have been investigated in the meagre (*Argyrosomus regius*) as well, a cultured species with increasing importance in Southern Europe. In this study Asencio-Alcudia et al. showed that the expression of several genes related to the immune response were up-regulated in fish fed the amino acid supplemented diet, suggesting its potential to improve tolerance and/or alleviating acute response to handling stressors of this species.

Anesthetics could be used to reduce the negative effects of stressors associated with aquaculture, including transport. Jerez-Cepa et al. and Teles et al. investigated the effects of a sedative dose of clove oil (CO) and MS-222 on hallmarks of the HPI axis regulation, energy management, and oxidative stress in gilthead seabream after simulated transport and further recovery. Jerez-Cepa et al. showed that HPI axis response was modified at plasma level, with differences depending on the anesthetic employed. Gene-expression related to cortisol production in the head kidney matched with the increased plasma cortisol levels immediately after transport in CO-sedated fish, but these levels remained constant in MS-222-sedated fish. Differential changes in the energy management of carbohydrates, lipids, and amino acids were dependent upon the anesthetic employed. In addition, Teles et al. reported that the use of both CO and MS-222 interferes with fish antioxidant status. The expression levels of genes related to antioxidant response and cell-tissue repair were altered in several tissues, confirming that both sedatives may have long-term effects on fish defenses, although minimizing the stress associated with transport. These results are highly relevant to aquaculture considering that oxidative stress may increase the fishes' susceptibility to other stressors and to pathogens.

Essential oils (EOs) could be a promising tool to reduce the stress associated with aquaculture procedures, improving welfare. EOs may be used as sedatives/anesthetics by reducing oxidative stress and/or boosting the immune response. Souza et al. reviewed the potential application of several EOs extracted from plants in fish subjected to several stressors. EOs have been reported to have a modulatory effect on the metabolic response of fish when included in the diet or added into the water.

EOs displayed reduced adverse effects as compared to synthetic compounds, although adequate concentrations and chemotypes to be used need to be further investigated.

Vaccination is a therapeutic strategy widely used in aquaculture to improve fish health although this may involve handling stress, implying a compromise in the fishes' welfare. Whether this vaccination elicits stress responses and enhances the crosstalk between the immune and endocrine systems in the brain or pituitary is unclear. Liu et al. investigated the stress and immune responses in gilthead seabream exposed to two different vaccine routes. The authors report that no stress response was induced for both routes of vaccination in the brain or pituitary using gene expression analysis, although plasma cortisol response was linked to both procedures. However, the authors showed an alteration of corticotropin-releasing hormone-binding protein (*crhbp*) and glucocorticoid receptor *(gr),* which suggests that the proteins coded by both genes could play a relevant role in the feedback regulation of HPI axis after vaccination.

Finally, technical solutions aiming to monitor fish welfare using less-invasive and non-lethal procedures by employing sentinel organisms are novel tools to be implemented in aquaculture. Martos-Sitcha, Sosa et al. described the design and validation of a reprogrammable and miniaturized device that can be attached to the operculum of gilthead seabream and European seabass (*Dicentrarchus labrax*). The authors reported how this device recorded and quantified the activity and respiratory frequency in fish kept in rearing tanks after its corresponding validation with swimming respirometry. Tagging the fish with this device was shown to have a minimal and transient impact, demonstrating that this miniaturized device could be a suitable tool when characterizing the physiological and behavioral responses of fish leading to improved performance and welfare in farmed species.

This Research Topic included several aspects related to the assessment of welfare in cultured fish, describing the stress response and the implementation of new procedures in order to decrease the negative effects of stressors existing in aquaculture practices. In addition, new devices to assess the wellbeing of relevant species are described. However, further studies will be required to better understand the mechanisms mediating stress responses and the possible strategies to reduce the impact of stressors associated with aquaculture.

AUTHOR CONTRIBUTIONS

JM-S, JM, PP, and LM contributed writing this editorial.

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Acute-Stress Biomarkers in Three Octopodidae Species After Bottom Trawling

*Cristina Barragán-Méndez[1], Ignacio Sobrino[2], Adrián Marín-Rincón[1], Sergio Fernández-Boo[3], Benjamin Costas[3,4], Juan Miguel Mancera[1] and Ignacio Ruiz-Jarabo[1]**

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Several Octopodidae species have a great potential for the diversification of worldwide aquaculture. Unfortunately, the lack of stress-related biomarkers in this taxon results an obstacle for its maintenance in conditions where animal welfare is of paramount relevance. In this study, we made a first approach to uncover physiological responses related to fishing capture in *Eledone moschata*, *Eledone cirrhosa*, and *Octopus vulgaris*. Captured octopus from all three species were individually maintained in an aquaculture system onboard of oceanographic vessel in south-western waters of Europe. Haemolymph plasma and muscle were collected in animals at the moment of capture, and recovery was evaluated along a time-course of 48 h in *Eledone* spp., and 24 h for *O. vulgaris*. Survival rates of these species captured in spring and autumn were evaluated. Physiological parameters such as plasma pH, total CO_2, peroxidase activity, lysozyme, hemocyanin, proteases, pro-phenoloxidase, anti-proteases, free amino acids, lactate and glucose levels, as well as muscle water percentage, free amino acids, lactate, glycogen and glucose values were analyzed. The immune system appears to be compromised in these species due to capture processes, while energy metabolites were mobilized to face the acute-stress situation, but recovery of all described parameters occurs within the first 24 h after capture. Moreover, this situation exerts hydric balance changes, as observed in the muscle water, being these responses depending on the species assessed. In conclusion, three Octopodidae species from south-western waters of Europe have been evaluated for stress-related biomarkers resulting in differentiated mechanisms between species. This study may pave the way to further study the physiology of stress in adult octopuses and develop new methodologies for their growth in aquaculture conditions.

Keywords: acute stress, bottom trawling, *Eledone*, Octopodidae, *Octopus*, physiological recovery

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INTRODUCTION

Cephalopods are of interest for human consumption and their fisheries constituted 6.4% of total world trade of fish products in 2016, and are amongst the most captured species (in tons) in marine fisheries (FAO, 2018). Wild octopus are caught every year, reaching 350,000 tons with a trade value of 1.5 billion dollars (Mouritsen and Styrbaek, 2018), being China and Morocco the largest

exporters of octopus, while Japan, the United States and Spain are the most important consumer markets (FAO, 2018).

Landings of octopus in Europe consist exclusively of three Octopodidae species: musky octopus (*Eledone moschata* Lamarck, 1798), horned octopus (*Eledone cirrhosa* Lamarck, 1798) and common octopus (*Octopus vulgaris* Cuvier, 1797). The latter species dominates European catches and landings, and is taken in greater numbers in southern waters (Pierce et al., 2010). In south Atlantic waters of Europe (Gulf of Cadiz, Spain) all three octopus species are captured by an artisanal and bottom trawling fleet (Sobrino et al., 2011). While *O. vulgaris* is the target species accounting for more than 87% of total Spanish octopus catches due to its high economically value in market, both *Eledone* spp. are routinely discarded after trawling due to their low market prices (Jereb et al., 2015). Bathymetric differences exist between these species, with *O. vulgaris* inhabiting depths from 0 to 200 m, *E. moschata* could be captured between 15 to 200 m depth, and *E. cirrhosa* occurs at deeper waters, between 50 and 300 m, or more (Pierce et al., 2010).

Octopus landings have declined fairly consistently since the mid-1980s (Jereb et al., 2015). Some pressure has been also generated on these species after the new Common Fisheries Policy in Europe, as it points out at a compulsory landing obligation for those species regulated by quotas or minimum sizes, including captured juvenile octopus (Regulation 1380/2013/UE). However, as reported in Article 13 of the regulation, captured species could be released back into the sea if robust scientific evidences indicated high survival rates and physiological recovery. Moreover, yearly variations on octopus captures due to environmental processes such as rainfalls, river discharges, and oceanographic currents are of great importance (Sobrino et al., 2002; Sánchez et al., 2004; Sobrino et al., 2011; Otero et al., 2016; Roura et al., 2019), highlighting aquaculture as a mandatory future to provide an increasing market supply of these species.

It is known that stress is a physiological response aimed to maintain the basal homeostatic levels of an organism (Chrousos, 2009). In vertebrates, these physiological responses have been broadly grouped as primary, secondary and tertiary (Barton, 2002), but knowledge on cephalopods is scarce. Primary stress responses include the release of neuroendocrine messengers such as catecholamines in cephalopods (Malham et al., 2002) and vertebrates (Reid et al., 1998), amongst other hormones (Schreck et al., 2016). Secondary stress responses are defined by the actions promoted by these hormones, including changes in the management of energy resources and the immune system (Costas et al., 2011; Schreck et al., 2016). If the stressful situation extends over time it can lead to exhaustion of the energy reserves, depression of the immune system, impairment of the behavior and reproduction, and eventually death of the animal (Wedemeyer et al., 1990; Schreck et al., 2016).

It was described that intermediary metabolism of cephalopods relies in amino acids and carbohydrates as a primary source of energy (Aguila et al., 2007; Lamarre et al., 2016; Speers-Roesch et al., 2016; Morales et al., 2017). In this sense, the muscle of molluscs is a source of proteins and carbohydrates that may be used as catabolic substrates when necessary (Lee et al., 2015). Thus, muscle glycogen seems to be an important source of glucose to be oxidized through glycolytic pathways (Storey and Storey, 1983), producing lactate under anaerobic circumstances (Gladden, 2004).

After acute-stress situations, sustained anaerobiosis results in a net accumulation of protons and metabolic CO_2 and hence, plasma acidosis (Hochachka et al., 1983). Changes in pH and temperature regulate oxygen transport in the haemolymph (Oellermann et al., 2015a,b). Hemocyanin (Hc), the respiratory pigment in cephalopods, is dissolved in the haemolymph and accounts for 98% of total proteins present in octopus blood (Aguila et al., 2007). It should be noticed that Hc exhibits phenoloxidase (PO) activity in cephalopods and crustaceans, which is involved in the innate defense mechanism (Adachi et al., 2003; Lacoue-Labarthe et al., 2009), and has shown to be affected by repeated sampling procedures in *E. cirrhosa* (Malham et al., 1998a) and infections in *O. vulgaris* (Castellanos-Martinez et al., 2014). The pro-PO activation is produced by proteases (Decker and Jaenicke, 2004). The immune system in cephalopods is poorly known to date, and lacks an adaptive immune response, but shows a good and efficient innate immune system composed of cellular and humoral (dissolved proteins in plasma) defense factors (Castellanos-Martinez et al., 2014). Thus, lysozyme activity appears to act non-specifically against a wide range of invaders in *E. cirrhosa* (Malham et al., 1998b), peroxidases possess antimicrobial activity that eliminates H_2O_2, maintaining the redox balance of the immune system (Rodríguez et al., 2003), antiproteases protect against bacterial proteases as well as endogenous proteases released by host cells (Malham and Runham, 1998; Malham et al., 1998b), while proteases like anti-trypsin show anti-inflammatory activity (Guttman et al., 2015).

The aim of this study was to evaluate acute-stress responses in three Octopodidae species (*E. moschata*, *E. cirrhosa*, and *O. vulgaris*). As fisheries processes were described as a source of acute-stress for the organisms (Lund et al., 2009; Gallagher et al., 2014; Veldhuizen et al., 2018), animals were captured by bottom trawling in the Gulf of Cadiz (south western waters of Europe) and allow to recover in onboard aquaria. Survival rates of these species were also evaluated altogether with physiological recovery responses. Results from this study may serve to improve the aquaculture of these species.

MATERIALS AND METHODS

Geographic Location, Vessel and Tows Characteristics

Octopus were captured by bottom trawling aboard the O/V "Miguel Oliver" (length: 70 m; engine power: 2x 1000 kw) during three different trawling surveys off the Gulf of Cadiz (south-western waters of Europe, Spain) in spring (March 2017 and 2018), and autumn (November 2017) (**Figure 1**). International standards were used during fishing processes, including 1 h of trawling at a constant depth (ANON, 2015). Temperature and salinity values were collected in each haul by a conductance-temperature-density probe (CTD) placed in the net.

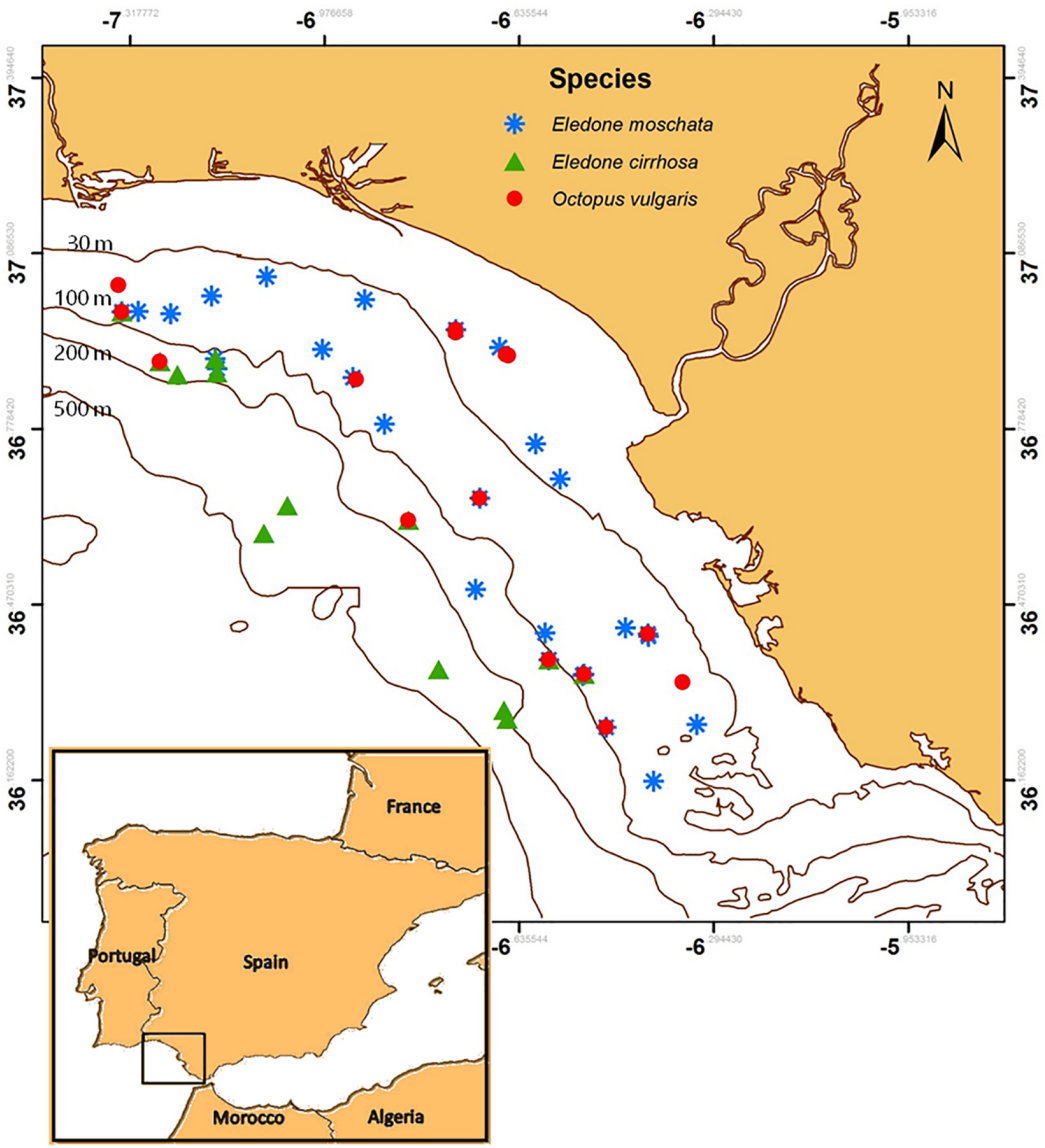

FIGURE 1 | Sampled area off the Gulf of Cadiz (south-western Atlantic waters of Europe, Spain). Blue asterisks, green triangles, and red circles indicate sampling points of *Eledone moschata*, *Eledone cirrhosa*, and *Octopus vulgaris*, respectively. Bathimetric lines and their depths (m) are indicated.

Animals Employed

Three Octopodidae species were studied to reinforce the results from an inter-specific point of view: *E. moschata*, *E. cirrhosa*, and *O. vulgaris*. Captures in this area depend on yearly variations (Sánchez et al., 2004; Sobrino et al., 2011) and were not constant along this study (**Table 1**). Depth of capture, as well as environmental temperature and salinity at the bottom, at the surface and in the recovery aquaria is shown in **Table 1**. Animals of both sexes were randomly selected aboard the vessel. *E. moschata* and *E. cirrhosa* weight was 197 ± 32 g and 117 ± 1 g, respectively. As *O. vulgaris* is an economically important species, with a minimum commercial size of 1 kg, this study was conducted with animals below that size, weighting 463 ± 26 g. Animals were kept and handled following the guidelines for experimental procedures in animal research from the Ethics and Animal Welfare Committee of the University of Cadiz, according to the Spanish (RD53/2013) and European Union (2010/63/UE) legislation. All experiments have been carried out under a special permit of scientific fishing granted to the Spanish Institute of Oceanography, and approved by the Spanish General Secretariat of Fisheries (project SUREDEPAR, Fundación Biodiversidad, Ministry for the Ecological Transition, Spain).

Recovery Aquaria Onboard

After bottom trawling, no more than 10 animals per species and trawl were introduced into onboard aquaria and allow to recover. A portable aquaculture system was specially designed for this purpose. This system consists of 30 independent

TABLE 1 | Environmental conditions of the experiments conducted in this study, including parameters of the fishing area (bottom), and temperature in the aquariums (tanks) and in the air of the fishing deck (air) with *E. moschata*, *Eledone Cirrhosa*, and *Octopus vulgaris* captured during bottom-trawling in the Gulf of Cadiz (Spain).

Species	Season year	Exp.	Depth (m)	Salinity (psu)	Temperature (°C)		
					Bottom	Tanks	Air
E. moschata	Spring 2017	R	76 ± 18	36.2 ± 0.1	14.0 ± 0.5	16.5 ± 0.1	18.6 ± 0.7
	Autumn 2017	S	78 ± 9	36.2 ± 0.0	16.6 ± 0.5	21.3 ± 0.3	22.6 ± 0.7
	Spring 2018	S	71 ± 10	36.3 ± 0.0	15.4 ± 0.1	17.4 ± 0.1	18.6 ± 0.1
E. cirrhosa	Spring 2017	R	290 ± 77	36.2 ± 0.1	13.9 ± 0.2	16.5 ± 0.1	18.6 ± 0.7
	Spring 2018	S	208 ± 49	36.3 ± 0.0	15.2 ± 0.2	17.4 ± 0.1	18.6 ± 0.1
O. vulgaris	Autumn 2017	R, S	21 ± 2	36.5 ± 0.0	21.0 ± 0.2	21.3 ± 0.3	22.6 ± 0.7
	Spring 2018	S	87 ± 12	36.3 ± 0.0	15.4 ± 0.1	17.4 ± 0.1	18.6 ± 0.1

"Exp." indicates the experiments during that season, including time-course recovery studies (R), and evaluation of the survival rates (S). Data are represented as mean ± SEM.

aquariums, painted in black with an upper light entrance, of 5 L each with a flow-throw system of seawater collected from the surface of the ocean during navigation. The system also has a charcoal filter and a protein skimmer to remove possible contaminants and/or dissolved nitrogenous molecules. Animals suffered around 65 min of air exposure (*E. moschata* 68 ± 2 min, *E. cirrhosa* 63 ± 7 min, and *O. vulgaris* 64 ± 2 min, without statistical differences between species), mimicking commercial fisheries in the Gulf of Cadiz, Spain (personal observation), in a lower fishing deck where environmental conditions include high environmental humidity, no sun radiation, low room irradiance, and constant temperatures below 23°C (see **Table 1**) before being introduced into the aquaria. Animals were fasted during experiments onboard. Dissolved oxygen was maintained above 80% saturation (>6.5 mg O_2 L^{-1}) by means of external aeration of the collected seawater.

Physiological Recovery Curve

Animals were sampled (see description in see section "Sampling") at times 0 h (immediately after capture and after air exposure) and after a few hours of being introduced into the aquaria. We aimed at the evaluation of the physiological recovery by conducting a time-course survey, assuming that acute-stress responses have been overcome when the physiological variables analyzed reach stable values over time, as described before in crustaceans (Barragán-Méndez et al., unpublished), elasmobranches (Barragán-Méndez et al., 2019), and teleosts (Skrzynska et al., 2018). In order to carry out this objective *E. moschata* (n = 55) and *E. cirrhosa* (n = 36) captured during 4 independent trawls for each species in March 2017 were randomly introduced into the recovery aquaria. A group of randomly selected animals was sacrificed before being introduced into the aquaria and samples were taken, constituting time 0 h (n = 18 *E. moschata* and n = 12 *E. cirrhosa*). Those animals introduced into the aquaria were maintained for 24 h (n = 18 *E. moschata* and n = 12 *E. cirrhosa*) and 48 h (n = 19 *E. moschata* and n = 12 *E. cirrhosa*) before being sampled. No *O. vulgaris* were captured in March 2017 but in November 2017 (n = 44 animals captured in 7 independent trawls). As both *Eledone* spp. appeared to be physiologically stable after 24 h (all analyzed parameters collected in March 2017 shown significantly similar values between times 24 and 48 h for each species, see section "Results"), and physiological recovery in crustaceans (Barragán-Méndez et al., unpublished) and teleost fish (Costas et al., 2011) occurs within the first 4 to 6 h, *O. vulgaris* were sampled at time 0 h (n = 15), 6 h (n = 15), and 24 h (n = 14) after recovery in the aquaria. A maximum of five animals per species and trawl were sampled at each time to reinforce independence of the results.

Survival Rates in Spring and Autumn

In order to study differences due to seasonality in the octopus' survival rates after capture, *E. moschata* were collected in autumn 2017 and in spring 2018 (from 10 and 13 hauls, respectively), *O. vulgaris* were collected in autumn 2017 and in spring 2018 (from 7 and 12 hauls, respectively), and *E. cirrhosa* were only captured in spring 2017 (11 hauls). After the triage process in the lower fishing deck aboard, a maximum of 5 octopus per species and trawl were introduced into the recovery aquaria and survival rates were evaluated 24 h later, as it was stated that giant Pacific octopus showed no retarded mortality after 24 h recovery (Conners and Levine, 2017). Animals were monitored every other hour (from 8 am to 1 am) during the experiments. Octopuses were considered alive if they show signs of breath, and responded to physical stimulation by moving their bodies when the observer opened their aquaria. Dead animals show a clear pale body colouration, a lack of body movements and rigidness. Dead animals were immediately removed from their aquaria.

Physiological Recovery in Spring and Autumn

In order to evaluate possible physiological differences between animals captured in spring and autumn, those animals employed in the survival rate evaluation were sampled. Haemolymph and muscle samples were collected at time 0 h (after circa 65 min of air exposure), and 24 h after recovery.

Sampling

Animals were anesthetized by decreasing water temperature to circa 4°C (by covering the aquariums with ice for 5 min, without physical disturbance of the animals) and addition of 2.5% ethanol for 2 min after that, conforming the principles of Directive 2010/63/EU, and following described procedures

(Malham et al., 2002; Estefanell et al., 2011; Sancho et al., 2015). Haemolymph samples were taken with sterile 1 mL 25 G syringes (circa 300 μL per animal) from the principal heart as described before (Aguila et al., 2007). Euthanasia was confirmed by severing the brain between the eyes. Muscle samples were collected from the third left arm. All samples were taken quickly (in less than 1 min per animal) to avoid an additional stress due to handing (Malham et al., 2002; Lawrence et al., 2018). As octopuses were maintained in individual aquaria, with darkened walls, the sampling process did not affect remaining animals in the aquarium (i.e., there were no disturbances due to visual contact, noise, or chemical distress). The soluble fraction of the haemolymph (referred to plasma hereafter, unless it could contain cytoplasmic components of the haemocytes) was obtained after centrifugation (10,000 × *g*, 4 min). All samples were immediately frozen at −20°C (by employing a refrigerated mixture that immediately freeze the samples). The samples were maintained at −20°C for less than 9 days, transferred to the Department of Biology (University of Cadiz, Spain) in dry ice and then maintained at −80°C.

Analysis of Plasma and Muscle Parameters

Plasma pH and TCO_2

Plasma pH was measured immediately after centrifugation with a mini-electrode (HI1083B, Hanna Instruments, Rhode Island, United States), and a 15 μL sample was collected for immediate analysis of total CO_2 (TCO_2) by means of an infra-red gas analyser (IRGA, S151, Qubit systems, Kingston, ON, Canada). The protocol for TCO_2 analysis was as follows: 15 μL of plasma were introduced into a 3.0 mL exetainer vial (Labco, United Kingdom) under a CO_2/water-free atmosphere (air pumped throw a 2 m length and a diameter of 18 mm tube filled with ½ silica gel and ½ soda lime) and hermetically closed. 100 μL 0.1 N HCl were injected into the vial and vigorously mixed with the sample. The needle of an empty 5 mL syringe was introduced into the vial followed by the introduction of the needle of a 5 mL syringe full of CO_2/water-free air. Air inside the vial was mixed by means of the two 5 mL syringes. Finally, 5 mL of that mixtured air was introduced into the IRGA and the concentration (in ppm) of CO_2 was analyzed. A standard curve was done with serial dilutions of $NaHCO_3$ diluted in distilled water (0 mM HCO_3^-).

Plasma Photographs *in vivo*

Haemolymph was photographed when circulating inside the blood system and while sampling as a proxy to estimate oxygen saturation rates between experimental groups. Due to the high amount of hemocyanin (Hc) in the plasma, haemolymph turns blue when Hc is oxidized (due to the cupper ions of the Hc molecule), and translucent when reduced. We aimed at grossly differentiate colored haemolymph between sampling groups.

Plasma Energy Metabolites

Plasma glucose and lactate levels were analyzed using commercial kits from Spinreact (St. Esteve de Bas, Girona, Spain) adapted for 96-well microplates. Total α-amino acid levels were assessed colorimetrically using the ninhydrin method of Moore (Moore, 1968) adapted for 96-well microplates. Plasma total proteins were determined using the Pierce BCA protein assay kit (Thermo Fisher Scientific) according to manufacturer recommendations.

Plasma Immune Status

Plasma hemocyanin concentration was measured spectrophotometry as previously described for octopuses (Aguila et al., 2007; Roumbedakis et al., 2017).

Plasma lysozyme activity was measured as described (Swain et al., 2007): 20 μL of sample and 180 μL of a solution of *Micrococcus lysodeikticus* (N3770, Sigma-Aldrich; 0.2 mg mL^{-1}, 0.04 M sodium phosphate buffer, pH 6.2) were added into a 96-well microplate. Blanks for each sample were done with 20 μL of the sample and 180 μL of sodium phosphate buffer. Reaction proceeds for 20 min at 37°C and afterward absorbance was measured at 450 nm. A standard curve was done with lyophilized hen egg white lysozyme (L6876, Sigma-Aldrich) serially diluted in Na_2HPO_4 buffer.

Peroxidase activity was measured as described (Quade and Roth, 1997), with some modifications: 15 μL plasma in duplicate were diluted in 135 μL of HBSS without Ca^{2+}/Mg^{2+} (H6648, Sigma-Aldrich) in a flat-bottomed 96-well plate, followed by the addition of 50 μL 10 mM TMB (T8768, Sigma-Aldrich) and 50 μL 5 mM H_2O_2. After 2 min the reaction was stopped with 50 μL 2 M H_2SO_4. Blank was done with 150 μL HBSS. Optical density was read at 450 nm. Peroxidase activity (U mL^{-1}) was determined defining 1 unit as that which produces an absorbance change of 1 OD.

Total PO-like activity activity was measured spectrophotometrically using L-DOPA (L-3,4-dihydroxyphenylalanine) as substrate, and trypsin (Sigma) as activator following described methodologies (Ji et al., 2009) with some modifications. Briefly, 15 μL plasma were incubated for 30 min at 25°C with 100 μL trypsin (1 mg mL^{-1}), with further addition of 100 μL L-DOPA (3 mg mL^{-1}) and absorbance measurements every 5 min at 490 nm in a SynergyHT microplate reader. Units of PO-like activity were calculated using Lambert–Beer law taking the gradient of slope of each sample and molar extinction coefficient of the L-DOPA (3700 M^{-1} cm^{-1}).

Protease activity was quantified using the azocasein hydrolysis assay according to the method of Ross et al. (2000) with some modifications. Briefly, protease activity was assayed in 10 μL plasma with 60 μL PBS and 125 μL 2% azocasein in 100 mM ammonium bicarbonate buffer. Samples were incubated for 24 h at 30°C. The reaction was stopped by adding 250 μL 10% trichloro acetic acid and the mixture centrifuged (10,000 × *g*, 10 min). 100 μL of the supernatant was mixed with 100 μL 1 N NaOH and optical density read at 450 nm. Trypsin was employed as standard.

Total antiprotease activity was determined by the ability of plasma to inhibit trypsin activity according to Ellis (1990) and modifications by Machado et al. (2015). Briefly, 10 μL of plasma were incubated with the same volume of a trypsin solution (5 mg mL^{-1} in $NaHCO_3$, 5 mg mL^{-1}, pH 8.3) for 10 min at 22°C in polystyrene microtubes. To the incubation mixture, 100 μL of phosphate buffer (NaH_2PO_4, 13.9 mg mL^{-1}, pH 7.0), and

125 μL of azocasein (20 mg mL^{-1} in $NaHCO_3$, 5 mg mL^{-1}, pH 8.3) were added and incubated for 1 h at 22°C. Finally, 250 μL of trichloroacetic acid were added to each microtube and incubated for 30 min at 22°C. The mixture was centrifuged at 10,000 × *g* for 5 min at room temperature. Afterward, 100 μL of the supernatant was transferred to a 96 well-plate that previously contained 100 μL of NaOH (40 mg mL^{-1}) per well. The OD was read at 450 nm in a SynergyHT microplate reader. Phosphate buffer in place of plasma and trypsin served as blank whereas the reference sample was phosphate buffer in place of plasma. The percentage inhibition of trypsin activity compared to the reference sample was calculated. All analyses were conducted in duplicates.

Muscle Energy Metabolites and Water Content

Frozen muscle was finely minced on an ice-cooled Petri dish and homogenized by ultrasonic disruption in 7.5 volumes ice-cold 0.6 N perchloric acid, neutralized using 1 M potassium bicarbonate, centrifuged (30 min, 3,220 × *g* and 4°C), and the supernatant used to determine tissue metabolites. Tissue lactate and amino acid levels were determined spectrophotometrically as described for plasma. Tissue glycogen concentration was assessed with amino glucosidase as described (Keppler and Decker, 1974). Glucose obtained after glycogen breakdown (after subtraction of free glucose levels) was determined with a commercial kit (Spinreact, see before). Muscle water content was analyzed by dehydrating pre-weighted muscle at 65°C until achieving constant weight (around 48 h). The percentage of water was calculated as the difference in weight between the fresh and the dry muscle divided by the fresh weight (Barragán-Méndez et al., 2018).

All assays were carried out using a PowerWave™ 340 microplate spectrophotometer (Bio-Tek Instruments, Winooski, VT, United States) using KCjunior™ data analysis software for Microsoft®.

Statistics

Normality and homogeneity of variances were analyzed using the Shapiro–Wilk's test and the Levene's test, respectively. Two-way nested ANOVA test was performed with trawl (as the nested factor for each season) and time as the factors of variance. No significant differences were described due to trawls in any of the dependant variables. Thus, differences between groups were tested using one-way ANOVA with recovery time (0, 24 and 48 h or 0, 6, and 24 h) as independent variables. When necessary, data was logarithmically transformed to fulfill the requirements for ANOVA. Tukey *post hoc* test was used to identify significant differences between groups. Differences between survival rates and concentration of the physiological parameters between the groups at 0 and 24 h were evaluated by Student's *t*-test. In addition, to establish the effect of the season, differences in plasma and muscle parameters between survivors at time 24 h after capture in spring and autumn, were also evaluated by Student's *t*-test. Statistical significance was accepted at $p < 0.05$. All the results are represented as mean ± SEM.

RESULTS

Survival Rates in Spring and Autumn

Eledone moschata and *O. vulgaris* did not show different survival rates between seasons despite the differences observed in the environmental variables described at the sea bottom, the aquariums and the air at the fishing deck (**Table 2**). *E. moschata* showed a survival rate of 100.0 ± 0.0% (91 animals captured in 13 hauls) and 92.7 ± 3.9% (83 animals captured in 10 hauls) in spring and autumn, respectively. *E. cirrhosa* was only captured in spring and its survival rate was 73.3 ± 14.1% (49 animals captured in 11 hauls). In the case of *O. vulgaris* its survival rate was 76.0 ± 11.0% in spring (72 animals captured in 12 hauls) and 75.7 ± 4.3% in autumn (44 animals captured in 7 hauls; mean ± SEM). All mortality occurred within the first 8 h after capture. We were unable to spot any major difference (external injuries or physical damage) between alive or dead animals.

Physiological Recovery Curve

Physiological changes in plasma glucose, muscle glycogen and plasma pH and TCO_2 after an acute-stress situation, and further recovery in onboard tanks in *E. moschata*, *E. cirrhosa*, and *O. vulgaris* are shown in **Figures 2–4**, respectively.

All three species shown the same responses for all four variables. Plasma pH and TCO_2 changes were shared by all three species, with minimum pH and maximum TCO_2 at time 0 h ($p < 0.05$), and maximum pH and minimum TCO_2 at all other times ($p < 0.05$, one-way ANOVA followed by a Tukey *post hoc* test). Maximum levels of plasma glucose and muscle glycogen are described at time 0 h, immediately before being introduced into the recovery aquariums. Both *Eledone* spp. showed statistically lower concentrations at times 24 h and 48 h after recovery (without differences between both groups, $p < 0.05$, one-way ANOVA followed by a Tukey *post hoc* test), in the same way as *O. vulgaris* showed statistically lower values at times 6 and 24 h after recovery (without differences between both groups, $p < 0.05$).

Other physiological parameters analyzed in plasma and muscle in a time-course following recovery in onboard aquariums after bottom-trawling are shown in **Table 3** (*E. moschata*), **Table 4** (*E. cirrhosa*), and **Table 5** (*O. vulgaris*).

TABLE 2 | Survival rates of *E. moschata*, *E. cirrhosa*, and *O. vulgaris* captured during bottom-trawling in the Gulf of Cadiz (Spain).

Species	Season	Survival (%)	Trawls (*n*)	Animals (*n*)
E. moschata	Spring	100.0 ± 0.0	13	91
	Autumn	92.7 ± 3.9	10	83
E. cirrhosa	Spring	73.3 ± 14.1	11	49
O. vulgaris	Spring	76.0 ± 11.0	12	72
	Autumn	75.7 ± 4.3	7	44

The table shows the number of trawls included and the total number of animals employed in every experiment. Data are represented as mean ± SEM. No statistical differences were described in the survival rates between spring and autumn for any species (Student's t-test, p < 0.05).

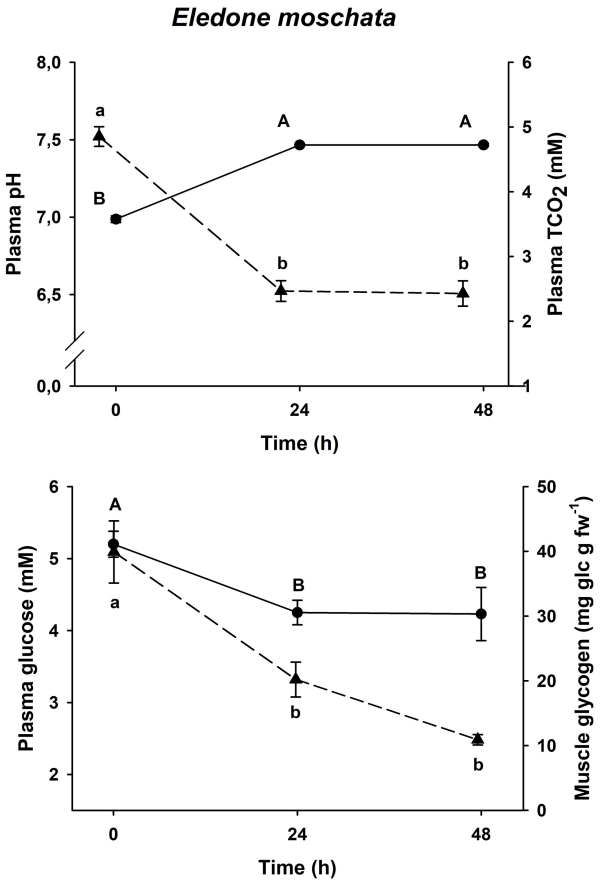

FIGURE 2 | Time course recovery of plasma pH (continuous line) and TCO_2 (dashed line) (upper figure) and plasma glucose (continuous line) and muscle glycogen (dashed line) (lower figure) in bottom trawled *E. moschata* in the Gulf of Cadiz (Spain). Plasma and muscle samples were taken just after bottom trawling (0 h) and at different recovery times (24 and 48 h). Data are expressed as mean ± SEM (n = 18–19 per group). Different capital letters indicate significant differences between groups for plasma pH and glucose, while different lowercase letters indicate significant differences between groups for plasma TCO_2 and muscle glycogen (one-way ANOVA followed by a *post hoc* Tukey test, $p < 0.05$). *fw* means fresh weight.

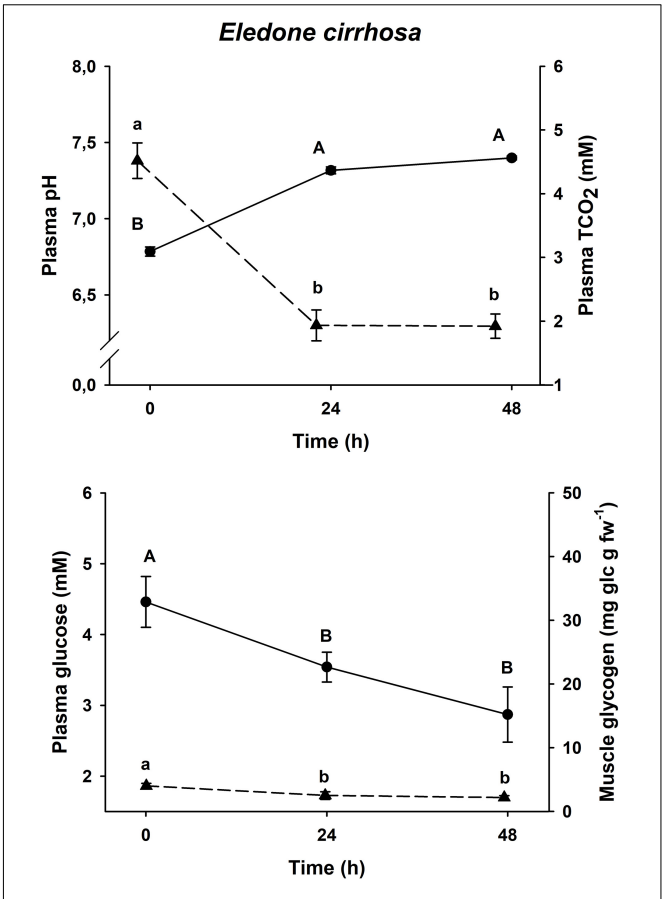

FIGURE 3 | Time course recovery of plasma pH (continuous line) and TCO2 (dashed line) (upper figure) and plasma glucose (continuous line) and muscle glycogen (dashed line) (lower figure) in bottom trawled *E. cirrhosa* in the Gulf of Cadiz (Spain). Plasma and muscle samples were taken just after bottom trawling (0 h) and at different recovery times (24 and 48 h). Data are expressed as mean ± SEM (n = 12 per group). Different capital or lowercase letters indicate significant differences between groups for the same variable (one-way ANOVA followed by a *post hoc* Tukey test, $p < 0.05$). More details are described in **Figure 2**.

Plasma and muscle lactate did not show statistical changes in any of the species tested ($p < 0.05$). Plasma amino acids show maximum concentration at time 0 h in all three species, and minimum concentrations at the end of the experiment, without statistical differences between the last two sampling times in all species ($p < 0.05$). Plasma Hc shows minimum concentrations at time 0 h in *E. moschata* and *O. vulgaris*, and maximum concentrations in all other times, without significant differences between the last two recovery times ($p < 0.05$). Hc in *E. cirrhosa* did not change along the experiment. After 24 h recovery, Hc values were 1.56 ± 0.06 mM in *E. moschata*, 0.95 ± 0.07 mM in *E. cirrhosa*, and 1.18 ± 0.06 mM in *O. vulgaris*. Plasma lysozyme activity shows the opposite profile than plasma Hc, with maximum concentrations at time 0 h in *E. moschata* and *O. vulgaris*, and minimum concentrations in all other times, without significant differences between the last two recovery times ($p < 0.05$). Lysozyme activity in *E. cirrhosa* did not change along the experiment.

Muscle glucose only showed statistical changes in *E. cirrhosa*, with maximum concentrations at time 0 h, and minimum at time 48 h ($p < 0.05$). Muscle amino acids only showed statistical changes in *E. moschata*, with maximum concentrations at time 0 h, and minimum at time 48 h ($p < 0.05$). Muscle water content only showed statistical changes in *E. cirrhosa*, with its minimum percentage at time 0 h, and maximum at time 48 h ($p < 0.05$). After 24 h recovery, muscle water content was 78.7 ± 0.3% in *E. moschata*, 78.2 ± 0.2% in *E. cirrhosa*, and 79.2 ± 0.2% in *O. vulgaris*.

Physiological Differences Between Spring and Autumn

Eledone moschata was sampled in spring and autumn and physiological recovery was analyzed by sampling plasma and

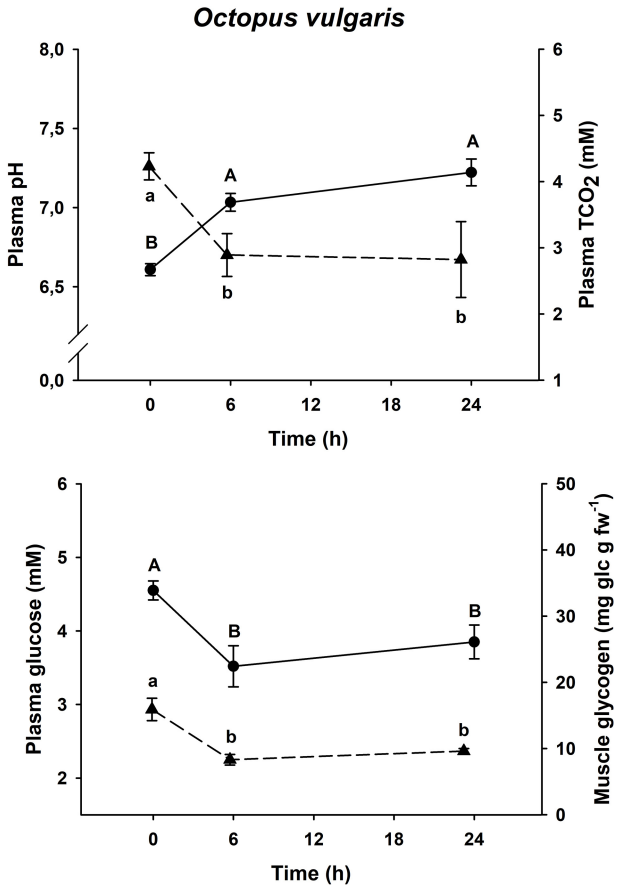

FIGURE 4 | Time course recovery of plasma pH (continuous line) and TCO2 (dashed line) and plasma glucose (continuous line) and muscle glycogen (dashed line) (upper figure) and (lower figure) in bottom trawled *O. vulgaris* in the Gulf of Cadiz (Spain). Plasma and muscle samples were taken just after bottom trawling (0 h) and at different recovery times (6 and 24 h). Data are expressed as mean ± SEM (*n* = 14–15 per group). Different capital or lowercase letters indicate significant differences between groups for the same variable (one-way ANOVA followed by a *post hoc* Tukey test, $p < 0.05$). More details are described in **Figure 2**.

muscle at times 0 and 24 h after recovery in onboard aquariums (**Table 6**); *E. cirrhosa* was only sampled in spring at times 0 and 24 h (**Table 7**); and *O. vulgaris* was sampled in spring (**Table 8**); and autumn (**Figure 4** and **Table 5**).

E. moschata (Table 6)

Plasma glucose was significantly lower at time 24 h in autumn when compared to spring (3.8 ± 1.6 mM vs. 4.5 ± 0.2 mM). Plasma lactate in autumn at both sampling times shown almost double concentration than in spring (above 0.60 mM in autumn, and circa 0.37–0.38 mM in spring). Plasma amino acids only show differences due to season at time 0 h, with lower concentration in autumn. Plasma proteins shown no changes due to sampling time in spring. Plasma hemocyanin and lysozyme activity displayed significant differences due to season only at time 24 h, with higher values in autumn than in spring. Other immune system parameters in plasma were

TABLE 3 | Physiological parameters in plasma and muscle of *E. moschata* after bottom trawling in spring 2017.

Parameter	Time post-recovery		
	0 h	24 h	48 h
Plasma lactate (mM)	0.35 ± 0.04	0.36 ± 0.02	0.35 ± 0.04
Plasma amino acids (mM)	21.8 ± 1.0 A	19.6 ± 0.9 AB	16.0 ± 1.7 B
Plasma hemocyanin (mM)	0.94 ± 0.05 B	1.56 ± 0.06 A	1.48 ± 0.08 A
Plasma lysozyme (μg mL^{-1})	0.38 ± 0.05 A	0.19 ± 0.03 B	0.20 ± 0.04 B
Muscle glucose (mg glc g fw^{-1})	23.3 ± 2.5	27.4 ± 2.2	24.1 ± 2.2
Muscle lactate (μg g fw^{-1})	31.3 ± 3.5	26.9 ± 2.9	29.6 ± 2.8
Muscle amino acids (μmol g fw^{-1})	202 ± 23 A	158 ± 9 AB	134 ± 12 B
Muscle water (%)	78.7 ± 0.2	78.7 ± 0.3	79.4 ± 0.5

Samplings were done during a time course after recovery in onboard tanks (0, 24 and 48 h). Data is shown as mean ± SEM (n = 18–19 per group). Differences between sampling times are indicated with different capital letters (p < 0.05, one-way ANOVA followed by a Tukey's post hoc test). fw means fresh weight.

TABLE 4 | Physiological parameters in plasma and muscle of *E. cirrhosa* after bottom trawling in spring 2017.

Parameter	Time post-recovery		
	0 h	24 h	48 h
Plasma lactate (mM)	0.45 ± 0.07	0.56 ± 0.06	0.48 ± 0.07
Plasma amino acids (mM)	18.4 ± 2.0 A	12.3 ± 1.7 B	11.9 ± 1.2 B
Plasma hemocyanin (mM)	0.81 ± 0.03	0.95 ± 0.07	0.98 ± 0.11
Plasma lysozyme (μg mL^{-1})	0.35 ± 0.03	0.35 ± 0.04	0.37 ± 0.04
Muscle glucose (mg glc g fw^{-1})	11.5 ± 1.6 A	7.7 ± 1.7 AB	5.2 ± 2.0 B
Muscle lactate (μg g fw^{-1})	13.0 ± 3.2	14.5 ± 4.3	14.6 ± 2.6
Muscle amino acids (μmol g fw^{-1})	123 ± 9	128 ± 7	129 ± 6
Muscle water (%)	78.1 ± 0.3 B	78.2 ± 0.2 AB	79.9 ± 1.2 A

Samplings were done during a time course after recovery in onboard tanks (0, 24, and 48 h). Data is shown as mean ± SEM (n = 12 per group). Differences between sampling times are indicated with different capital letters (p < 0.05, one-way ANOVA followed by a Tukey's post hoc test). fw means fresh weight.

TABLE 5 | Physiological parameters in plasma and muscle of *O. vulgaris* after bottom trawling in autumn 2017.

Parameter	Time post-recovery		
	0 h	6 h	24 h
Plasma lactate (mM)	0.22 ± 0.01	0.24 ± 0.03	0.28 ± 0.06
Plasma amino acids (mM)	16.7 ± 0.9 A	13.7 ± 2.9 AB	8.2 ± 1.8 B
Plasma hemocyanin (mM)	0.87 ± 0.01 B	0.83 ± 0.04 B	1.18 ± 0.06 A
Plasma lysozyme (μg mL^{-1})	0.17 ± 0.02 A	0.11 ± 0.02 B	0.05 ± 0.02 B
Muscle glucose (mg glc g fw^{-1})	26.3 ± 2.7	18.5 ± 3.9	26.9 ± 5.3
Muscle lactate (μg g fw^{-1})	24.1 ± 3.7	25.9 ± 3.1	18.7 ± 4.9
Muscle amino acids (μmol g fw^{-1})	81.8 ± 6.9	95.9 ± 7.2	99.6 ± 8.3
Muscle water (%)	79.8 ± 0.3	78.8 ± 0.6	79.2 ± 0.2

Samplings were done during a time course after recovery in onboard tanks (0, 6, and 24 h). Data is shown as mean ± SEM (n = 14–15 per group). Differences between sampling times are indicated with different capital letters (p < 0.05, one-way ANOVA followed by a Tukey's post hoc test). fw means fresh weight.

TABLE 6 | Plasma and muscle physiological parameters in *E. moschata* immediately after bottom trawling in the Gulf of Cadiz (0 h) and 24 h after recovery in water tanks in spring 2018 and autumn 2017.

Parameter	Spring		Autumn	
	0 h	24 h	0 h	24 h
Plasma glucose (mM)	5.6 ± 0.1	4.5 ± 0.2*	5.4 ± 0.6	3.8 ± 1.6*#
Plasma lactate (mM)	0.37 ± 0.03	0.38 ± 0.03	0.68 ± 0.26#	0.60 ± 0.24#
Plasma amino acids (μmol g fw^{-1})	15.8 ± 1.0	12.5 ± 1.0*	13.9 ± 0.7#	11.7 ± 0.7*
Plasma proteins (mg mL^{-1})	75.6 ± 2.8	75.2 ± 2.6	N/A	N/A
Plasma hemocyanin (mM)	0.84 ± 0.04	1.06 ± 0.06*	0.86 ± 0.01	1.34 ± 0.05*#
Plasma lysozyme (μg mL^{-1})	0.11 ± 0.02	0.04 ± 0.00*	0.15 ± 0.11	0.14 ± 0.09#
Plasma peroxidase (U mL^{-1})	6.5 ± 0.6	5.3 ± 0.5	N/A	N/A
Plasma protease (%)	0.86 ± 0.42	0.0 ± 0.0*	N/A	N/A
Plasma antiprotease (%)	0.94 ± 0.11	3.52 ± 0.81*	N/A	N/A
Plasma total PO-like activity (mU mL^{-1})	41.9 ± 3.0	57.3 ± 5.0*	N/A	N/A
Muscle glucose (mg glc g fw^{-1})	44.8 ± 3.6	47.3 ± 5.2	49.6 ± 3.3	56.7 ± 4.2
Muscle glycogen (mg glc g fw^{-1})	45.8 ± 6.4	22.0 ± 5.0*	60.9 ± 5.4	40.4 ± 3.5*#
Muscle lactate (μg g fw^{-1})	14.7 ± 3.7	9.9 ± 3.1	38.6 ± 1.9#	39.7 ± 2.2#
Muscle amino acids (μmol g fw^{-1})	67.9 ± 8.0	44.3 ± 6.6*	75.4 ± 2.4#	74.8 ± 3.1#
Muscle water (%)	84.1 ± 0.6	82.5 ± 0.5	78.9 ± 1.3	80.1 ± 1.5*#

Data is shown as mean ± SEM (n = 43 and 41 per group in spring and autumn, respectively). Asterisks (*) indicate significant differences between 0 h and 24 h for each season (p < 0.05, Student's t-test). Hashtags (#) indicate significant differences between spring and autumn for groups sampled at the same time (p < 0.05, Student's t-test). N/A, not available. fw means fresh weight.

only analyzed in spring and will be employed to compare inter-specific differences between all three octopus spp. No changes are also described for muscle glucose with either time or season. However, muscle glycogen shown significantly higher values at time 24 h in autumn that in spring (40.4 ± 3.5 and 22.0 ± 5.0 mg glc g^{-1} fw, respectively). Muscle lactate and amino acids were significantly higher at both sampling times in autumn than in spring. Water content in the muscle show differences due to season only at time 24 h, with 80.1 ± 1.5% in autumn, and 82.5 ± 0.6% in spring.

E. cirrhosa (Table 7)

As *E. cirrhosa* was only sampled in spring, no comparison was possible between spring and autumn. The analyzed physiological parameters from samples collected in spring 2017 and 2018 are similar. Plasma glucose, amino acids, proteins and peroxidase activity, as well as muscle glucose, glycogen and water content shown higher values at time 0 h than at time 24 h. No changes are observed due to sampling time in plasma lactate, hemocyanin lysozyme and muscle lactate and amino acids ($p < 0.05$, Student's *t*-test).

TABLE 7 | Plasma and muscle physiological parameters in *E. cirrhosa* immediately after bottom trawling in the Gulf of Cadiz (0 h) and 24 h after recovery in water tanks in spring 2018.

Parameter	Time post-recovery	
	0 h	24 h
Plasma glucose (mM)	3.9 ± 0.1	2.7 ± 0.2*
Plasma lactate (mM)	0.39 ± 0.03	0.32 ± 0.03
Plasma amino acids (μmol g fw^{-1})	42.2 ± 2.6	24.8 ± 2.7*
Plasma proteins (mg mL^{-1})	82.7 ± 1.7	56.7 ± 3.9*
Plasma hemocyanin (mM)	0.80 ± 0.06	0.74 ± 0.09
Plasma lysozyme (μg mL^{-1})	0.11 ± 0.03	0.10 ± 0.02
Plasma peroxidase (U mL^{-1})	9.4 ± 1.0	4.2 ± 0.5*
Plasma protease (%)	12.3 ± 2.4	0.0 ± 0.0*
Plasma antiprotease (%)	13.8 ± 0.16	2.42 ± 0.44*
Plasma total PO-like activity (mU mL^{-1})	53.7 ± 6.4	76.2 ± 11.2
Muscle glucose (mg glc g fw^{-1})	21.0 ± 2.2	14.9 ± 1.6*
Muscle glycogen (mg glc g fw^{-1})	3.5 ± 0.4	1.9 ± 0.4*
Muscle lactate (μg g fw^{-1})	10.6 ± 4.7	7.3 ± 5.0
Muscle amino acids (μmol g fw^{1})	123 ± 6	110 ± 6
Muscle water (%)	85.9 ± 1.0	83.0 ± 0.6*

Data is shown as mean ± SEM (n = 20–29 per group). Asterisks (*) indicate significant differences between 0 h and 24 h (p < 0.05, Student's t-test). fw means fresh weight.

TABLE 8 | Plasma and muscle physiological parameters in *O. vulgaris* immediately after bottom trawling in the Gulf of Cadiz (0 h) and 24 h after recovery in water tanks in spring 2018.

Parameter	Time post-recovery	
	0 h	24 h
Plasma glucose (mM)	5.4 ± 0.2#	3.6 ± 0.2*
Plasma lactate (mM)	0.27 ± 0.02	0.22 ± 0.01
Plasma amino acids (μmol g fw^{-1})	19.0 ± 2.0	13.4 ± 1.5*
Plasma proteins (mg mL^{-1})	92.9 ± 1.5	75.6 ± 5.5*
Plasma hemocyanin (mM)	0.80 ± 0.02	1.13 ± 0.08*
Plasma lysozyme (μg mL^{-1})	0.16 ± 0.02#	0.07 ± 0.01*
Plasma peroxidase (U mL^{-1})	5.4 ± 0.7	3.2 ± 0.3*
Plasma protease (%)	43.3 ± 2.9	22.2 ± 2.2*
Plasma antiprotease (%)	0.46 ± 0.04	0.66 ± 0.06*
Plasma total PO-like activity (mU mL^{-1})	12.2 ± 1.5	20.5 ± 3.4*
Muscle glucose (mg glc g fw^{-1})	34.0 ± 2.1	18.2 ± 2.7*
Muscle glycogen (mg glc g fw^{-1})	5.4 ± 0.7#	2.0 ± 0.3*#
Muscle lactate (μg g fw^{-1})	15.7 ± 3.5	8.8 ± 4.2
Muscle amino acids (μmol g fw^{-1})	115 ± 7#	95 ± 6 *
Muscle water (%)	81.5 ± 0.4#	83.0 ± 0.6#

Data is shown as mean ± SEM (n = 25–36 per group). Asterisks (*) indicate significant differences between 0 h and 24 h (p < 0.05, Student's t-test). Hashtags (#) indicate significant differences between spring and autumn for groups sampled at the same time (p < 0.05, Student's t-test). fw means fresh weight.

O. vulgaris (Table 8)

Plasma glucose at time 0 h in spring was significantly higher than in autumn (5.4 ± 0.2 mM and 4.5 ± 0.1mM, respectively), and no differences are described at time 24 h between seasons. Plasma lactate shown changes neither with sampling time nor due

to season. Plasma amino acids shown no statistical differences due to season but to sampling time, with higher concentrations at time 0 h. Plasma proteins, analyzed only in spring, shown higher values at time 0 h. Plasma hemocyanin was statistically similar between season for each sampling time. Plasma lysozyme show higher activity rate in autumn than in spring at time 0 h (0.17 ± 0.02 and 0.16 ± 0.02 μg mL^{-1}, respectively). Plasma peroxidase was only analyzed in spring, and its activity was higher at time 0 h than at time 24 h (5.4 ± 0.7 and 3.2 ± 0.3 U mL^{-1}, respectively). Muscle glucose did not show differences between seasons at any sampling time. Glycogen in muscle showed statistically differences at time 0 h between spring and autumn (5.4 ± 0.7 and 15.9 ± 1.7 mg glc g^{-1} wet weight, respectively) and at time 24 h (2.0 ± 0.3 and 9.6 ± 0.4 mg glc g^{-1} wet weight, respectively). Lactate levels did not present differences between time or season. In the case of amino acids in muscle a significant decrease was observed 24 h after recovery in spring (115 ± 7 μmol g^{-1} wet weight after fishing and 95 ± 6 μmol g^{-1} wet weight after 24 h). Differences at time 0 h between seasons were also showed, as muscle amino acids in autumn were 81.8 ± 6.9 μmol g^{-1} wet weight. Finally, muscle water content show differences only due to season, with values at time 0 h of 81.5 ± 0.4% in spring and 79.8 ± 0.3% in autumn; and values at time 24 h of 83.0 ± 0.6% in spring and 79.2 ± 0.2% in autumn.

Changes in the Color of Haemolymph

By taking pictures of sampled octopus, framing the ventral heart and the circulatory system, we have been able to clearly distinguish two hemocyanin states: oxidized and reduced (**Figure 5**). Those animals at time 0 h presented a translucent haemolymph, which correspond to a reduced hemocyanin under circulatory conditions. Moreover, in those animals that survived for 6 h or more, the haemolymph acquires a very striking turquoise blue color, as indicative of an oxidized state of the hemocyanin.

Immune System Differences in Plasma: Comparison Between Species

Several plasma parameters were analyzed in all three species at times 0 and 24 h in spring (**Tables 6–8**).

Plasma hemocyanin and total PO-like activity displayed circa 33% lower values at time 0 h in *E. moschata* and *O. vulgaris*. Lysozyme activity showed changes due to sampling time only in *E. moschata* and *O. vulgaris*, with higher values at time 0 h in both species. Statistically higher peroxidase activity and plasma concentration of proteins were described in *E. cirrhosa* and *O. vulgaris* but in *E. moschata* at time 0 h. Proteases showed higher activities in all three species at time 0 h, reaching values of 0.86 ± 0.42, 12.3 ± 2.4, and 43.3 ± 2.9% in *E. moschata*, *E. cirrhosa*, and *O. vulgaris*, respectively; and values at time 24 h of 0.0 ± 0.0% in both *Eledone* spp., and 22.2 ± 2.2% in *O. vulgaris*. Antiproteases (antitrypsin activity) in all three species, otherwise, showed statistically higher activity at time 24 h, with values around 0.46 to 1.38 mU mL^{-1} at time 0 h, and 2–4 mU mL^{-1} in both *Eledone* spp., and 0.66 ± 0.06 mU mL^{-1} in *O. vulgaris*.

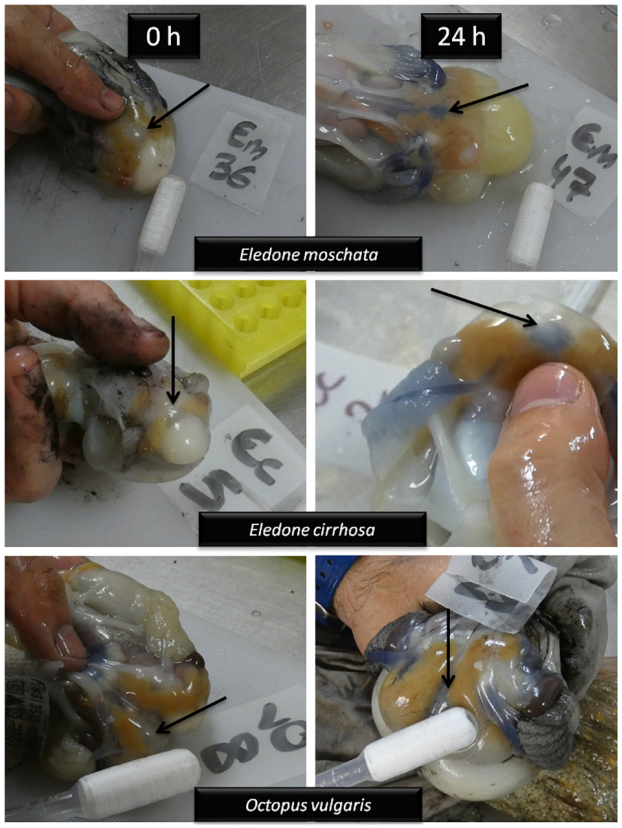

FIGURE 5 | Detailed photographs of the circulatory system of *E. moschata*, *E. cirrhosa*, and *O. vulgaris* captured by bottom trawling at times 0 and 24 h after recovery into onboard tanks. Immediately after capture (0 h) haemolymph is translucent, and turned into turquoise blue 24 h after recovery, evidencing reduced and oxidized hemocyanin, respectively. Arrows indicate ventral heart.

DISCUSSION

The present pilot study serves to unravel physiological consequences of an acute-stress situation in Octopodidae species. Taking advantage of animals captured by trawling, which is considered a source of acute-stress in marine organisms (Lund et al., 2009; Gallagher et al., 2014; Veldhuizen et al., 2018), we have managed to decipher some of these mechanisms through the physiological stability of octopus. Survival rates of three species (*E. moschata*, *E. cirrhosa*, and *O. vulgaris*) captured in spring and autumn in South Western Atlantic waters of Europe were evaluated, and physiological responses related to energy management and the immune system were assessed. This study described that octopus rely on amino acids and carbohydrates to face an acute-stress challenge, and that the immune system is compromised during the first hours after capture, but managed to recover in less than 24 h. However, to the best of our knowledge, this study is the first to describe, in juvenile and adult octopus, haemolymph and muscle physiological parameters after a dramatic event such as being trawled and exposed to air. These results can be useful to improve aquaculture

conditions through the recognition of stress situations that may affect welfare.

Survival Rates

Survival rates were evaluated in three Octopodidae species in spring and autumn after 1 h of being trawled followed by 65 min of air exposure (mimicking commercial procedures). After such a dramatic event, octopus were allowed to recover in onboard aquariums, showing differentiated survival rates 24 h later depending on the species: *E. moschata* (as high as 100% survival in spring, and 92% in autumn), *E. cirrhosa* (circa 73% in spring) and *O. vulgaris* (above 75% in both spring and autumn). In light of these results, there is a lack of differences in the survival rates for each species between spring and autumn. However, it has been described before that invertebrates such as crustaceans and bivalves presented higher mortality rates in months with higher water temperatures (Raicevich et al., 2014; Mehault et al., 2016; Clements et al., 2018). The fact that octopuses had no differences between survival rates due to environmental temperature changes (within the range evaluated in this study, and 1.0–4.5°C difference between the bottom of the ocean and the aquariums) could indicate a high capacity to acclimate to different environmental conditions, as suggested before for *O. vulgaris* embryo and paralarvae (Garrido et al., 2017; Nande et al., 2018).

The novelty of this study relies on the evaluation of the survival rates in discarded octopus under fisheries conditions. Previously, only one study evaluated survival rates of captured octopus (Conners and Levine, 2017). These authors studied the giant Pacific octopus (*Enteroctopus dofleini*) captured by pot-fishing and trawling, and evaluated its condition factor after capture. However, following their criteria for assessing condition of octopus immediately after capture, most of the animals collected in the present study should be considered dead (though survival rates 24 h later were above 73% in all three species), as they did not move in response to handling, they show color fading to gray or white, and show flaccid arms and suckers not adhering to surfaces (personal observations done during the experiments described herein). Thus, we considered necessary to conduct experiments where captured octopus are allowed to recover in appropriate water conditions for a few hours to evaluate survival rates. We hypothesize that bottom trawling under the circumstances described in the present study was such an intense process (lasting almost 2 h, including trawling and air exposure) that animals were exhausted when observed for the first time (prior to sampling and/or introducing into recovery aquariums). Moreover, Conners and Levine (2017) maintained a few animals in onboard tanks and observed no delayed mortality rates after 24 h recovery. In our study, all deceased octopus were considered dead in less than 8 h after being introduced into the aquariums. Coinciding with other taxa, survivors (after an acute-stress situation such as being captured and exposed to air) seem to be recovered in a few hours (Barragán-Méndez et al., unpublished) (Bergmann et al., 2001; Costas et al., 2011; Skrzynska et al., 2018; Barragán-Méndez et al., 2019). In this sense, the analysis of certain physiological parameters seems to be a good proxy to evaluate recovery. This study aims at evaluating the maximum survival rates of these species after capture ("maximum" as the animals were recovered in individual tanks with environmental conditions similar to those in the wild). We assumed that immediately discarded octopus would fall prey to predation rapidly after being returned to the ocean due to their catatonic state after capture. However, future advances should be focused on the way of diminishing dead of discards due to predators as, for example, releasing the animals through a tube submerged a few meters below the ocean surface to reduce bird's capture, or allowing captured octopus to be recovered in onboard tanks for a certain period of time before being discarded.

pH, TCO_2, and Hypoxia

In this study, blood pH and TCO_2 were altered by bottom-trawling and further recovery in onboard tanks, suggesting a metabolic acidosis after an acute-stress episode altogether with an anaerobic situation, as described before for other aquatic animals (Mandic and Regan, 2018). It was stated that maintaining constant levels of these parameters in intra- and extracellular fluids of marine organisms are of paramount importance for the correct maintenance of the acid-base balance (Tresguerres and Hamilton, 2017). Thus, in this study, survivors of all three Octopodidae species managed to presumably recover plasma basal pH and TCO_2 levels in less than 24 h after an acute-stress situation as seen by the lack of differences between the last sampling points in the described experiments.

It should be mention that pH of the haemolymph and environmental temperature contribute to the oxygen affinity of dissolved Hc (Oellermann et al., 2015a,b). It was stated that *E. moschata* shows 100% oxygen binding to Hc (at temperatures similar to those described in this study) at plasma pH 7.5, while the maximal pH-dependent release of oxygen by Hc occurs at pH 7.0 (Oellermann et al., 2015a). Thus, our results of plasma pH confirmed that photographs taken of *Eledone* spp. and *O. vulgaris* in this study after an acute-stress challenge evidenced a deep blood anaerobiosis, as translucent haemolymph could be thus associated to 0% oxygen being transported by Hc, while octopus maintained in water aquariums for more than 6 h show maximum oxygen linked to Hc as seen by the deep-turquoise color of haemolymph. Our results show lower plasma pH levels in *O. vulgaris* compared to *Eledone* spp. This fact may define tolerance to hypoxia in these species, and also be a reason for the higher mortality rates of *O. vulgaris* when compared to *E. moschata* captured in the same geographical area (similar depths and water temperatures). However, further studies should be conducted to locate the causes of death of these species after a stress like the one described here.

Another striking result is that plasma TCO_2, mostly in the form of HCO_3^- at circulating pH conditions in the blood (Boutilier et al., 1985) in all three Octopodidae species, is as low as 2–3 mM at pH 7.4–7.5; while marine crustaceans show plasma HCO_3^- levels around 10–20 mM (at plasma pH 7.5–7.8 and water temperature 15–20°C) (Howell et al., 1973); teleost fish show 5–10 mM HCO_3^- in plasma at a pH around 7.6–7.8 (Boutilier et al., 1985); and mammals evidenced higher plasma HCO_3^- concentrations around 20 mM at pH 7.4 (Pamenter et al., 2018). As maintenance of blood pH in vertebrates is mostly

controlled by plasma bicarbonate (Tresguerres and Hamilton, 2017), plasma of octopus may be buffered by proteins such as Hc, which constitutes 98% of all proteins in plasma of these species (Aguila et al., 2007). This low bicarbonate levels in cephalopods may be of interest to further investigate the formation of their hard structures and thus evaluate growth rates (Arkhipkin et al., 2018), and/or to describe their acid-base mechanisms.

Immune System

The immune system of all three studied Octopodidae was also altered due to an acute-stress situation, with species-specific differences. It was stated that the general health status of octopus is related to the activation of immune mechanisms facing harmful situations (Roumbedakis et al., 2017). Thus, lower Hc concentrations in plasma of *E. moschata* and *O. vulgaris* in this study may be associated to the conversion of Hc into a phenoloxidase (PO)-like enzyme, as was described for crustaceans and other invertebrates (Adachi et al., 2003; Decker and Jaenicke, 2004). Supporting this idea, some studies conducted in cuttlefish (*Sepia officinalis*) embryo shown increased PO activity after exposure to dissolved metals (Lacoue-Labarthe et al., 2009), while *O. vulgaris* infected by a protozoan parasite show changes in blood Hc (Castellanos-Martinez et al., 2014). In the present study, plasma Hc reached stable values circa 1–1.5 mM in all animals after 24 h recovery, similar to those levels described in several cephalopod species (Senozan et al., 1988; Aguila et al., 2007; Sancho et al., 2015; Roumbedakis et al., 2017). Our hypothesis that Hc acts as a total PO-like enzyme in octopus is based in the fact that after bottom trawling *E. moschata* and *O. vulgaris* had 60–80% less Hc and pro-PO activity than 24 h later, when recovered. Future studies, based in these results and actual knowledge in arthropods, may be considered to explore the Hc involvement in the immune system in cephalopods.

Plasma lysozyme in *E. moschata* and *O. vulgaris* showed higher activities immediately after the acute-stress situation, maintaining lower and stable values after 24 and 6 h, respectively. Lysozyme is an essential component of the innate immunity of octopus and part of their antibacterial mechanism (Malham et al., 1998b), and it was described not only in plasma but also in other tissues (Malham et al., 1998b; Martinez-Montaño et al., 2017). As for Hc, lysozyme activity shows no changes in *E. cirrhosa*. The reason for this lack of lysozyme response was unknown, though the activity of this enzyme in both *Eledone* spp. is similar. However, it was described in *E. cirrhosa* that injection of *Vibrio anguillarum* caused an increase in lysozyme activity in the branchial heart without changes in the activity of this enzyme in the haemolymph, while also reduced plasma antiprotease activity (Malham et al., 1998b), coinciding with our results in all three species. *E. cirrhosa* has a well develop immune system in the haemolymph, with bacteriostatic, lysozyme, protease, antiprotease and peroxidase activities, as seen by our results and as described by other authors (Malham and Runham, 1998). It was also described for this species that repeated sampling of haemolymph induced an increase in the haemocytes (Malham et al., 1998a). Thus, *E. cirrhosa* seemed to present a differentiated immune response compared to *E. moschata* and *O. vulgaris*, which may explain observed differences in the mortality rates between both *Eledone* spp. However, protease and antiprotease activities seemed to behave similarly in all three species, and may highlight shared immune responses in Octopodidae. According to these results, it can be speculated that antiproteases increased in response to the high protease activity observed at time 0 h, as an endogenous mechanism to protect host cellular damage. Interestingly, only *E. cirrhosa* specimens showed the opposite pattern, with lower antiprotease activity at 24 h, a fact that could be related to their lower survival rates. Such remarkable similarities and differences deserve future approaches to unravel the mechanisms of action in these interesting species.

Another relevant immune mechanism is the presence of peroxidase activity in plasma of the haemolymph. Peroxidase enzymes may act on the same substrates than PO (Lacoue-Labarthe et al., 2009). In this study, peroxidase activity reached similar values in plasma of all three species at time 24 h post-recovery. However, immediately after capture there is a substantial increase in this defense mechanism in *E. cirrhosa* and *O. vulgaris*. Due to the toxicity of peroxide (H_2O_2) molecules (substrates in the peroxidase reaction) and its precursor, the anion superoxide (O_2^-), these two species may face a greater oxidative stress than *E. moschata*, causing cellular injuries via protein and DNA oxidation and lipid peroxidation (Kowaltowski et al., 2009) and higher mortality rates, as described in this study. Moreover, those species evaluated in this study with the highest mortality rates (*E. cirrhosa* and *O. vulgaris*) also displayed changes in muscle water content, which is related to dehydration/overhydration processes after an acute-stress situation such as air-exposure (Barragán-Méndez et al., 2018), while *E. moschata* (with survival rates above 92%) shows no differences in muscle moisture depending on sampling time. Thus, similar stressful situations may induce differentiated responses depending on the species, and evidenced that aquaculture of octopus may require further studies focused on their physiological stress responses.

Carbohydrates Metabolism

Muscle of invertebrates acts as a reserve for carbohydrates, stored in the form of glycogen (Lee et al., 2015; Speers-Roesch et al., 2016). Glycogen is catabolized to form free glucose that, through glycolytic pathways, is employed to produce energy under stressful situations in cephalopods (Speers-Roesch et al., 2016). This situation is observed in Octopodidae species after capture by bottom-trawling, with muscle glycogen stores gradually being consumed to maintain circulating free glucose levels constant (presumably at homeostatic basal levels in these species). This carbohydrate consumption is evidenced during the first hours after stress, and reached metabolic equilibrium after 6–24 h recovery in onboard tanks. In vertebrates, lactate dehydrogenase (LDH) activity under anaerobic conditions like those described in this study produced lactate as a final end product of glycolysis (Gladden, 2004). However, our results highlighted a negligible amount of lactate in the arm's muscle of Octopodidae, and low and constant levels of circulating plasma lactate. This metabolic response may be related to the low LDH activity in *O. vulgaris* paralarvae (Morales et al., 2017), or the lack of LDH activity in *S. officinalis* (Speers-Roesch et al., 2016), resulting

in a striking result that deserve future evaluation of anaerobic catabolism in cephalopods.

Amino Acids Metabolism

It was stated that *O. vulgaris* and other cephalopods have a protein-dominated metabolism (Katsanevakis et al., 2005; Petza et al., 2006), which explains the great amount of plasma and muscle amino acids in all three species in this study after an acute-stress situation. Free amino acids catabolism in octopus indicates a well-adapted use of proteins as a source of energy (Roumbedakis et al., 2017), and are employed as gluconeogenic substrates that are incorporated into glycogen deposits (Hochachka and Fields, 1982) and plasma glucose (Linares et al., 2015). In other invertebrates such as the oyster *Crassostrea virginica*, relying on amino acids catabolism to face anoxia also requires some glycolytic energy production to meet overall tissue requirements of recovery, returning to control values by 24 h, as described in the present study in all three Octopodidae species (Eberlee et al., 1983). Further studies describing the consumption of specific essential and non-essential amino acids during acute-stress situations will be of help for the aquaculture industry, as the development of specific diets containing those amino acids may minimize negative consequences of stress.

CONCLUSION

This study described, for the first time, physiological acute-stress responses in three Octopodidae species. Recovery processes include maintaining the acid-base balance of the blood, and returning to basal levels of immune system parameters (such as hemocyanin, lysozyme, and peroxidase activities), as well as mobilization of energy molecules such as carbohydrates and amino acids to face the stressful process. Designed as a pilot study onboard a fishing vessel, further approaches should be conducted in controlled lab-conditions to better characterize secondary stress responses in cephalopods. Future studies could also measure physiological parameters *in vivo* at fixed time points, to reveal the exact time-course of plasma biomarkers after a stress situation. Aquaculture of these species may guarantee proper welfare conditions for their culture, but nowadays the lack of knowledge about the stress responses of these animals, among other issues, slows the development of this industry worldwide.

ETHICS STATEMENT

Animals were kept and handled following the guidelines for experimental procedures in animal research from the Ethics and Animal Welfare Committee of the University of Cadiz, according to the Spanish (RD53/2013) and European Union (2010/63/UE) legislation. All experiments have been carried out under a special permit of scientific fishing granted to the Spanish Institute of Oceanography, and approved by the Spanish General Secretariat of Fisheries (project SUREDEPAR, Fundación Biodiversidad, Ministry for the Ecological Transition, Spain).

AUTHOR CONTRIBUTIONS

CB-M, IS, BC, JM, and IR-J conceived and designed the study. CB-M, AM-R, SF-B, and IR-J carried out experimental procedures. CB-M, AM-R, SF-B, BC, and IR-J analyzed and interpreted the data. CB-M, IS, BC, JM, and IR-J wrote the original draft. All authors reviewed, edited, and approved the final version of the manuscript.

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Cortisol Acting Through the Glucocorticoid Receptor Is Not Involved in Exercise-Enhanced Growth, But Does Affect the White Skeletal Muscle Transcriptome in Zebrafish (*Danio rerio*)

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Forced sustained swimming exercise at optimal speed enhances growth in many fish species, particularly through hypertrophy of the white skeletal muscle. The exact mechanism of this effect has not been resolved yet. To explore the role of cortisol, we first subjected wild-type zebrafish to an exercise protocol validated for exercise-enhanced growth, and showed that exercised zebrafish, which indeed showed enhanced growth, had higher cortisol levels than the non-exercised controls. A central role was therefore hypothesized for the steroid hormone cortisol acting through the Glucocorticoid receptor (Gr). Second, we subjected wild-type zebrafish and zebrafish with a mutant Gr to exercise at optimal, suboptimal, and super-optimal speeds and compared them with non-exercised controls. Exercised zebrafish showed growth enhancement at all speeds, with highest growth at optimal speeds. In the Gr mutant fish, exercise resulted in growth enhancement similar to wild-type zebrafish, indicating that cortisol signaling through Gr cannot be considered as a main determinant of exercise-enhanced growth. Finally, the transcriptome of white skeletal muscle tissue was analyzed by RNA sequencing. The results of this analysis showed that in the muscle tissue of Gr mutant fish a lower number of genes is regulated by exercise than in wild-type fish (183 vs. 351). A cluster of 36 genes was regulated by exercise in both wild-type and mutant fish, and in this cluster genes involved in transcriptional regulation and protein ubiquitination were overrepresented. Because these two processes appear to be regulated in both wild type and mutant fish, which both display exercise-enhanced growth, we suggest that they play an important role in the growth of muscles upon exercise.

Keywords: swimming exercise physiology, hypothalamic-pituitary-interrrenal (HPI) axis, cortisol, glucocorticoid receptor, RNAseq

INTRODUCTION

Fish can be stimulated to exercise by inducing swimming behavior against a water flow. Interestingly, swimming exercise has been shown to improve the growth rate in a variety of fish species when the fish swim at speeds which can be maintained for long periods of time (Jobling et al., 1993; Davison, 1997; Palstra and Planas, 2011; Davison and Herbert, 2013). The molecular and physiological mechanisms behind this effect are still unclear. Recently, increased growth upon exercise has also been shown in the cyprinid zebrafish *Danio rerio* (Palstra et al., 2010), which has enabled research on the functional mechanisms behind exercise-enhanced growth. The advantages of this highly versatile experimental animal model will be exploited for this purpose in the present study.

This study will focus on the role of the stress hormone cortisol, since it has been shown that species such as rainbow trout (Postlethwaite and McDonald, 1995) and Atlantic salmon (Herbert et al., 2011) lower the secreted levels of cortisol under sustained exercise conditions. Cortisol is a steroid hormone that is secreted upon stress by the interrenal tissue in fish. This secretion is tightly regulated by the hypothalamic-pituitary-interrenal (HPI) axis. Upon acute stress, corticotrophin-releasing hormone (CRH) is secreted from the hypothalamus, which subsequently induces the secretion of adrenocorticotropic hormone (ACTH) from the pituitary gland, which in turn stimulates cortisol secretion from the interrenal tissue.

During stress, the effects of cortisol are mediated by an intracellular receptor, the glucocorticoid receptor (Gr). Upon activation by cortisol, the Gr acts as a transcription factor, regulating the expression of a wide variety of target genes. The zebrafish Gr is encoded by a single gene (Alsop and Vijayan, 2008; Schaaf et al., 2008) and one functional isoform is expressed (Vijayan et al., 2005; Chatzopoulou et al., 2017). Interestingly, most other fish species express two functional isoforms, Gr1 and Gr2, which are encoded by separate genes (Bury et al., 2003; Greenwood et al., 2003). The main result of the transcriptional changes induced by Gr-mediated cortisol signaling is a shift in priorities in the body by eliciting a mobilization of nutrients and oxygen to relevant organs like the heart and brain, while suppressing other systems like growth, reproduction and the immune response (Chrousos and Gold, 1992; Whirledge and Cidlowski, 2010). Considering the catabolic, growth-suppressing effects of Gr-mediated cortisol signaling on muscle tissue (reviewed by Sadoel and Vijayan, 2016), we hypothesized that the lowering of circulatory cortisol levels during exercise allows for the enhancement of muscle growth and hypertrophy.

To test this hypothesis, we have investigated in the present study whether exercise-enhanced growth originates from altered cortisol levels. First, the effects of sustained exercise on plasma cortisol levels were assessed. Second, we investigated the effect of cortisol signaling through Gr on exercise-enhanced growth at suboptimal, optimal, and super optimal swimming speeds. For this purpose, an available Gr mutant zebrafish line (Ziv et al., 2013) was used, which has a mutation in the Gr gene, effectively inhibiting the Gr-mediated effects of cortisol. Contrary to the wild-type zebrafish, Gr mutants should not experience exercise-dependent enhancement of muscle growth. Third, we have studied the transcriptional effects of cortisol during exercise-enhanced growth by RNA sequencing. Since exercise-induced growth mainly reflects hypertrophy of white skeletal muscle, we focused on this tissue. Recently we have performed transcriptome analyses (Palstra et al., 2014), which showed the activation of a series of complex transcriptional networks in white skeletal muscle of wild-type zebrafish in response to exercise. In this study, the cortisol/Gr-dependency of the exercise-induced transcriptional changes has been established.

MATERIALS AND METHODS

Experimental Fish and Conditions

The experimental protocols complied with the current laws of the Netherlands and were approved by the animal experimental committee (DEC Nos. 2012101, 2013091, and 2013169).

Wild-type zebrafish (*D. rerio)* of 3 months old were provided by the zebrafish facilities of the Institute of Biology Leiden (Leiden, Netherlands). A Gr mutant fish line (gr^{s357}; Ziv et al., 2013) was used which was made available by Dr. Herwig Baier (University of San Francisco). Fish of this line have a point mutation in the DNA-binding domain of the Gr gene, effectively prohibiting all Gr-mediated effects of cortisol.

Fish were housed for 2 weeks in aquaria with fresh water at 28°C at a photoperiod regime of 16L:8D and fed *ad libitum* with automatic feeders twice per day (DuplaRin pellets, Dupla, Gelsdrof, Germany). Water quality was continuously monitored and controlled.

Experimental Set-Up and Procedure for Exercise in Zebrafish

After 2 weeks in aquaria, fish were either sampled as start group or divided into an exercise and non-exercise group (group sizes shown below). The exercise and non-exercise groups were introduced in Blazka-type 127 L swim tunnels as described by Van den Thillart et al. (2004), one tunnel for each group. The exercise group was subjected to a long-term training protocol as described by Palstra et al. (2010) which involved forced sustained exercise at swimming speeds of 0.3 m s^{-1} or ∼10 BL s^{-1} (suboptimal speed), 0.4 m s^{-1} or ∼13 BL s^{-1} (optimal swimming speed) or 0.5 m s^{-1} or ∼16 BL s^{-1} (super-optimal swimming speed), for 6 h day^{-1} (09.00–15.00 h), for 5 days $week^{-1}$ and for 4 weeks long. The non-exercise group remained at 0.1 m s^{-1}.

After three days of acclimatization in the swim tunnels, the training protocol was started. The motor speed of the tunnels with fish of the exercise group was increased slowly from resting conditions (0.1 m s^{-1}) to the experimental swimming speeds. After swimming for 6 h, the speed was decreased slowly until the resting flow rate of 0.1 m s^{-1} was reached again. At the minimal flow rate of 0.1 m s^{-1} all fish could move freely in all directions, while at the flow rate of 0.4 m s^{-1} fish were forced to swim against the flow at their optimal speed. No mortality occurred among non-exercised and exercised fish due to experimental treatment.

The swim tunnels were placed in a climatized room and connected to a single storage tank holding a total fresh water

volume of 500 L, recirculating continuously over a bio filter keeping water quality parameters consistent. Conditions were maintained at their photoperiod and feeding regime and at a temperature of 28°C. After the 4 weeks experimental period, exercised, and non-exercised fish were released from the tunnels. Fish were anesthetized with clove oil (dissolved 1:10 in 100% ethanol and used at a 1000 times dilution in distilled water) and measured for body weight (BW) and body length (BL; total body length TL in exp. 1, standard body length SL in exp. 2 because some fish had veil tails).

Experimental Design

Two experiments were performed, and an overview of the design of these experiments is presented in **Figure 1**. In experiment 1, at the start of the 4 week experimental period fish were either sampled as start group ($N = 25$) or divided into an exercise and non-exercise group (each $N = 25$). The exercise group was forced to sustained exercise at an optimal swimming speed of 0.4 m s^{-1}.

In experiment 2, both Gr mutant and wild-type zebrafish were either measured as start group ($N = 10$ Gr mutant and $N = 10$ wild-type fish) or divided into three exercise groups and a non-exercise group (each $N = 15$ Gr mutant and $N = 15$ wild-type zebrafish). The three exercise groups were subjected to exercise at sub-optimal swimming speed (0.3 m s^{-1} or ∼10 BL s^{-1}), optimal swimming speed (0.4 m s^{-1} or ∼13 BL s^{-1}) or super-optimal swimming speed (0.5 m s^{-1} or ∼16 BL s^{-1}), while the non-exercise group remained at 0.1 m s^{-1}.

Cortisol Measurements Using ELISA

To determine plasma cortisol concentrations, the tails of the fish in experiment 1 were cut and blood was extracted with heparinized micro capillaries. Micro capillaries were then spun for 5 min at 11,000 rpm in a micro centrifuge. Sufficient blood could only be collected for larger fish: $N = 5$ fish of the start group; $N = 6$ non-exercised fish, and $N = 9$ exercised fish. After centrifugation, the plasma was collected in Eppendorf tubes and stored at –80°C for cortisol measurement. Cortisol plasma concentrations were determined by ELISA (Demeditec Diagnostics GmbH, Kiel-Wellsee, Germany), according to the manufacturer's instructions.

RNA Isolation From White Muscle Tissue

After the 4 weeks experimental period in experiment 2, the white muscle fillet was dissected dorsally from the lateral line in the epaxial quadrant along the whole length of the fish and stored in RNAlater (Ambion) at –20°C. Tissue was lysed in QIAzol Lysis Reagent. A Qiagen TissueRuptor was used to cut up the tissue samples and RNA was extracted using the Qiagen miRNeasy Mini Kit according to the manufacturer's description (Qiagen Benelux BV, Venlo, Netherlands). RNA was eluted in 50 µl and quantified by Nanodrop (Thermo Fisher Scientific, Amsterdam, Netherlands). Integrity of the RNA was confirmed using an Agilent Bioanalyzer 2100 total RNA Nanoseries II chip (Agilent, Amstelveen, Netherlands).

Genotyping

Fish were genotyped after experiment 2 so the SL and BW data could be assigned as belonging to Gr mutant or wild-type zebrafish. Extracted total RNA was reverse-transcribed using the iScript™ cDNA Synthesis Kit (Bio-Rad). The cDNA samples were used for genotyping using a custom-designed Kompetitive Allele Specific PCR (KASP) genotyping assay. Finally, $N = 48$ Gr mutant ($N = 13$ non-exercised; $N = 11$ optimal exercised; $N = 11$ suboptimal exercised; $N = 13$ super optimal exercised) and $N = 45$ wild-type ($N = 12$ non-exercised; $N = 11$ optimal exercised; $N = 10$ suboptimal exercised; $N = 12$ super optimal exercised) zebrafish were identified.

RNAseq: Library Preparation and Sequencing

RNAseq was performed on RNA of fish that rested ($N = 3$) or swam at Uopt ($N = 3$) for each of both Gr mutant and wild-type zebrafish groups to make all cross comparisons possible. Illumina RNAseq libraries were prepared from 1 µg total RNA using the Illumina TruSeq RNA Sample Prep Kit v2 according to the manufacturer's instructions (Illumina, San Diego CA, United States). All RNAseq libraries (150–750 bp inserts) were sequenced on an Illumina HiSeq2500 sequencer as 1 × 50 nucleotides single-end reads according to the manufacturer's protocol. Image analysis and base calling were done using the Illumina pipeline. Total yield varied from ∼9 to ∼16 M reads per sample.

RNAseq: Data Analysis

Illumina reads were aligned against the zebrafish genome sequence (GRCz10.80) using TopHat (version 2.0.5; Trapnell et al., 2009). Secondary alignments of reads were excluded by filtering the files using SAMtools (version 0.1.18; Li et al., 2009). Aligned fragments per predicted gene were counted from SAM alignment files using the Python package HTSeq (version 0.5.3p9; Anders et al., 2015). In order to make comparisons across samples possible, these fragment counts were corrected for the total amount of sequencing performed for each sample. As a correction scaling factor, library size estimates determined using the R/Bioconductor (release 2.11) package DESeq (Anders and Huber, 2010) were employed. Read counts were normalized by dividing the raw counts obtained from HTSeq by its scale factor. Detailed read coverage for individual genes was extracted from the TopHat alignments using SAMtools. Raw RNAseq data (reads) have been submitted to NCBI's Gene Expression Omnibus (GEO) as GSE120253 study with sample accession numbers GSM3396885 – GSM3396896[1].

Comparisons were made between Uopt and non-exercised groups for both Gr mutant and wild-type zebrafish. This way, the molecular regulation of physiological processes that characterize the effects of exercise in the white skeletal muscle could be determined. Then, these exercise effects were compared between Gr mutant and wild-type zebrafish in order to identify the communalities and differences between mutants and wild-type

[1]https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE120253

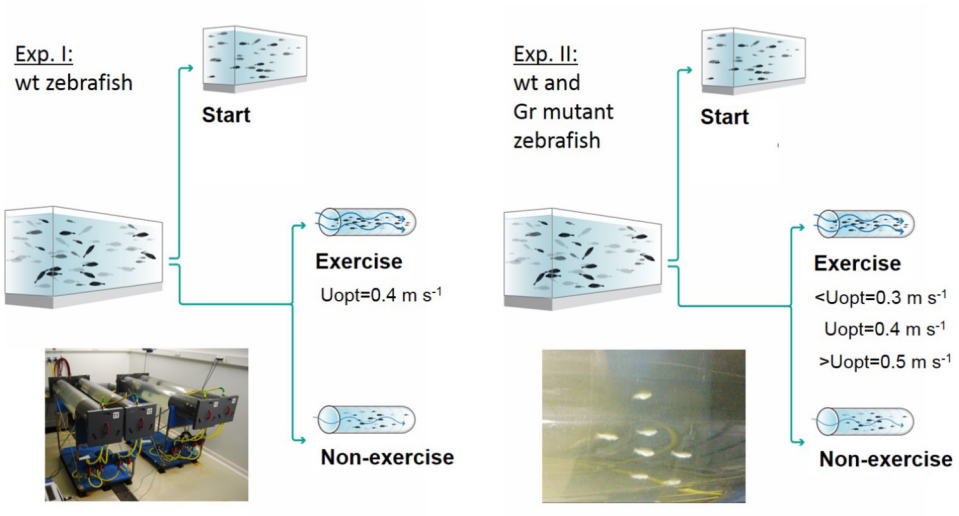


FIGURE 1 | Experimental set-ups. Experiment I was executed with wild-type (wt) zebrafish with a control group sampled at the start of the experiment, a non-exercised group and an exercised group which was subjected to forced sustained swimming at the optimal swimming speed (Uopt) of 0.4 m s^{-1} for a period of 4 weeks in a swim tunnel. Experiment II had a similar set-up but was executed with wild-type zebrafish and Gr mutants. Now there were three exercise groups swimming at the sub optimal (<Uopt), optimal, and super optimal (>Uopt) swimming speeds of 0.3, 0.4, and 0.5 m s^{-1}, respectively.

zebrafish in response to exercise. Particularly, it is expected that this will raise insight into the role of the Gr in the response to exercise. Gene expression of differentially expressed genes, both up- or downregulated, was functionally characterized and classified using DAVID 6.8 (The Database for Annotation, Visualization and Integrated Discovery, Huang et al., 2009a,b).

Statistics Biometric Data

Statistical analyses were carried out with IBM SPSS Statistics 22.0. Datasets were first tested for normal distribution with Kolmogorov–Smirnov tests and all data were normally distributed. As for experiment I, one-way ANOVA was performed with Tukey *post hoc* testing. As cortisol exponentially increased, log transformed data were tested. As for experiment II, treatments for each of the groups (wild-type or Gr mutant) were tested similarly. Responses to exercise were determined for each treatment vs. the controls at the start within each group, and then pairwise compared between groups with Student *t*-tests. Differences at $P \leq 0.05$ were considered significant. Data are presented as averages ± standard errors (AV ± SE).

RESULTS

Exercise-Enhanced Growth and Cortisol Levels in Wild-Type Zebrafish

In the first experiment, the effects of exercise at optimal swimming speed were tested on size and cortisol levels (**Figure 2**). Exercise effects were highly significant for length ($P = 0.007$), weight ($P = 0.001$) and cortisol levels ($P = 0.009$). Exercised fish were significantly longer than fish at the start of the experiment ($P = 0.006$; 7.3%). They were 5.6% longer than non-exercised fish but this difference was not significant ($P = 0.062$; **Figure 2A**). Exercised fish were significantly heavier (39.1%) than non-exercised fish: 0.32 ± 0.01 vs. 0.23 ± 0.02 g, respectively ($P < 0.001$; **Figure 2B**). Non-exercised fish tended to have a lower weight when compared to the fish of the start group ($P = 0.063$).

The average cortisol levels were 3.5-fold higher in exercised fish vs. non-exercised fish (0.173 ± 0.031 vs. 0.049 ± 0.016 μg ml^{-1}, respectively) but due to high individual variation this difference was not significant. Cortisol concentrations of exercised fish were significantly different ($P = 0.007$) from cortisol data of fish of the start group (0.011 ± 0.003 μg ml^{-1}).

Exercise-Enhanced Growth in Wild-Type and Gr Mutant Zebrafish at Different Swimming Speeds

In the second experiment, the effects of exercise at suboptimal, optimal, and super optimal swimming speed were tested on size of Gr mutant and wild-type zebrafish (**Figure 3**). In general, exercise enhanced growth similarly in the mutant and wild-type fish. Both groups increased in length and weight over time under exercise conditions (group and treatment effects were highly significant for length, $P < 0.001$ for both groups, and weight, $P = 0.016$ for wt and $P < 0.001$ for Gr mutants, without significant interaction). The growth patterns indicated most pronounced growth when swimming at Uopt ($P < 0.01$). Gr mutants swimming at Uopt were on average 0.12 g ($P < 0.001$; 40%) heavier than the controls at the start and 0.09 g ($P = 0.018$; 27%) heavier than the non-exercised fish. Despite the fact that Gr mutants are bigger than wt fish, comparison of treatment effects between both groups showed that groups similarly responded

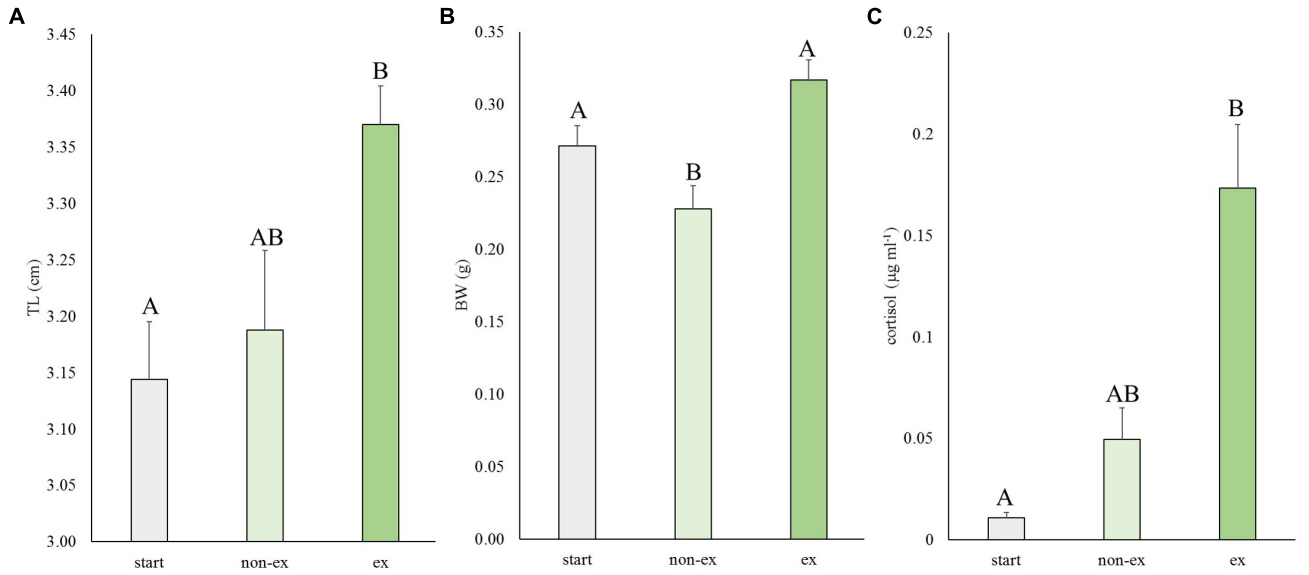


FIGURE 2 | Experiment I: Exercise effects on size and cortisol levels. **(A)** Total length (TL), **(B)** body weight (BW), and **(C)** cortisol levels in wild-type fish at the start of the experiment and after 4 weeks without or with subjection to the daily exercise protocol (non-ex/ex). Differences in letters mark significant differences ($P < 0.05$).

with growth to exercise. Only Gr mutants swimming at super optimal speed showed a significantly different response in weight than the wild type fish that were swimming at this speed ($P = 0.008$).

Transcriptome Analysis of Muscle Tissue in Wild-Type and Gr Mutant Zebrafish During Exercise-Enhanced Growth

The transcriptome of white skeletal muscle tissue was analyzed by using RNA sequencing. RNA was collected from wild type and Gr mutant fish, and for both groups non-exercised and exercised (Uopt) fish were used, yielding four experimental groups. In wild type fish, the comparison exercised vs. non-exercised showed significant differential expression of 351 genes at $P < 0.05$ (1.10%). Of these 351 differentially expressed genes (DEGs), expression of 192 genes was upregulated and expression of 159 genes was downregulated by exercising. In Gr mutants, this comparison showed significant differential expression of 183 genes at $P < 0.05$ (0.57%). Of these 183 DEGs, expression of 49 genes was upregulated and expression of 134 genes was downregulated upon exercise. Comparing exercised and non-exercised fish, the wild-type fish and the Gr mutants have only 36 DEGs in common (**Figure 4** and **Supplementary Material S1**). Of these DEGs, expression of 20 genes was in the same direction and of 16 genes in opposite direction. The wild-type fish have 315 specific DEGs (**Figure 4** and **Supplementary Material S2**) and the Gr mutants have 147 specific DEGs (**Figure 4** and **Supplementary Material S3**). Gene ontology was analyzed for the common (**Figure 4**) and Gr mutant and wild-type zebrafish specific DEGs. Gene ontology for the 36 common DEGs revealed three annotation clusters with genes involved in transcription factor activity and protein ubiquitination (6 DEGs), zinc ion binding (4 DEGs) and membrane transport (5 DEGs). As these DEGs are shared between both groups that show exercise-enhanced growth, they represent important functions in white muscle physiology during hypertrophy.

Gene ontology for the 315 specific DEGs for wild-type zebrafish revealed 12 annotation clusters with enrichment scores > 1, with DEGs involved in the zona pellucida; steroid biosynthesis; oxygen transport, heme, and iron; collagen, and others. Gene ontology for the 147 specific DEGs for Gr mutant zebrafish revealed three annotation clusters with enrichment scores > 1, with DEGs involved in hydrolase activity; GTP binding; transport, transporter activity and transmembrane transport.

Thus, after comparing exercised and non-exercised fish, 351 DEGS were found in the wild-type fish and 183 DEGs in the Gr mutants. When focusing on DEGs involved in muscle growth and structure (**Table 1**), a group of 22 appropriate DEGs could be identified which were associated with growth factors, hormones and receptors; myosin and myoglobin, and collagen. Interesting is the expression of the *oxidative stress induced growth inhibitor 1*, downregulated in both groups. By far most of these genes were, however, differentially regulated when comparing Gr mutant and wild-type zebrafish: genes which were up or down regulated in one group were not significantly regulated in the other group. The most extreme examples are the expressions of *growth arrest-specific 7a* (fc 12.26) and *opioid growth factor receptor-like 1* (fc 9.28) which were upregulated in wild-type zebrafish, and *insulin-like growth factor binding protein 6a* (fc 40.26) and *growth regulation by estrogen in breast cancer 1* (fc 10.61) which were upregulated in Gr mutant fish. Furthermore, expression of several myosin related genes is upregulated in wild-type zebrafish but not in Gr mutant zebrafish. Also nine collagen types were upregulated in wild-type zebrafish but expression of one other type was

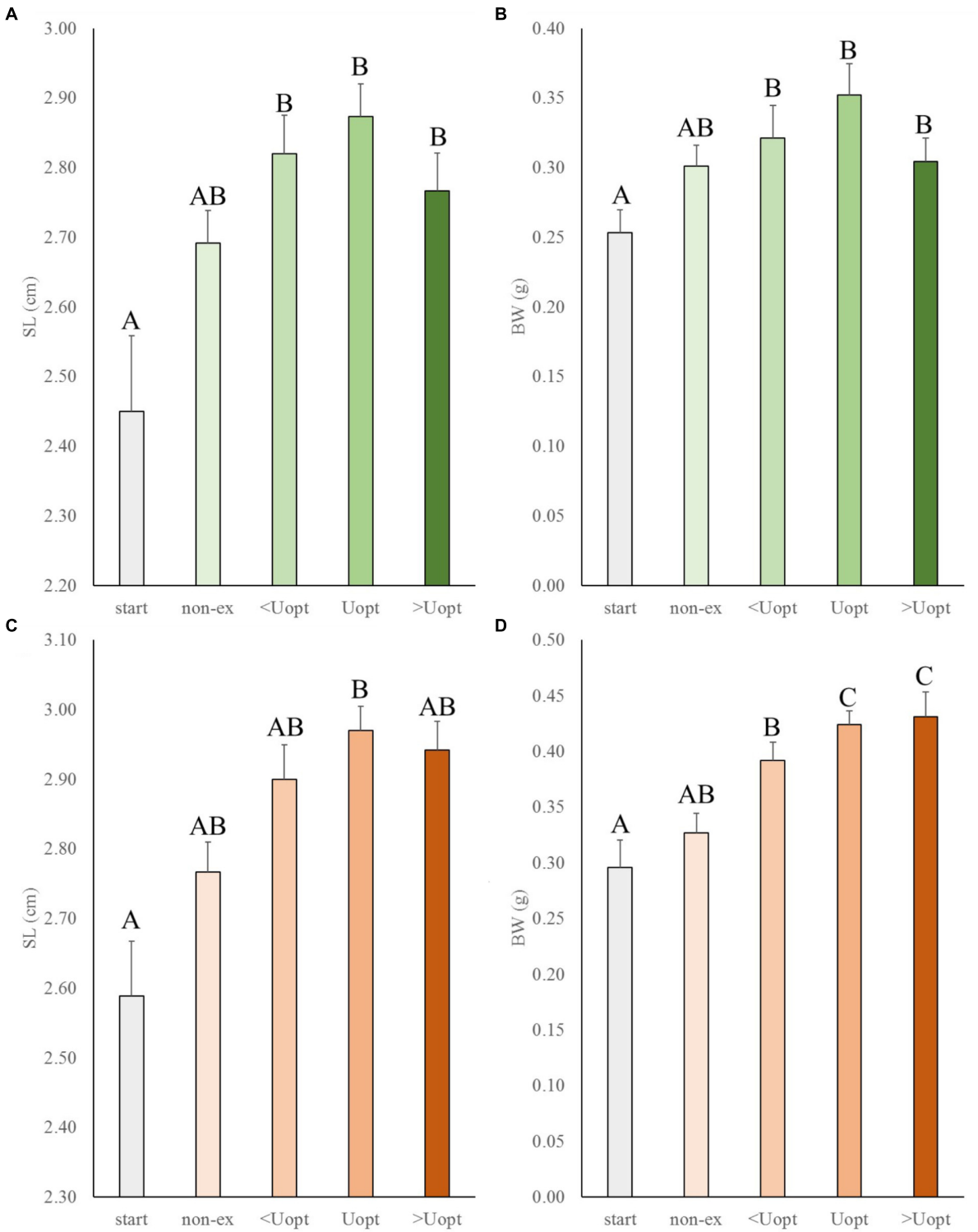
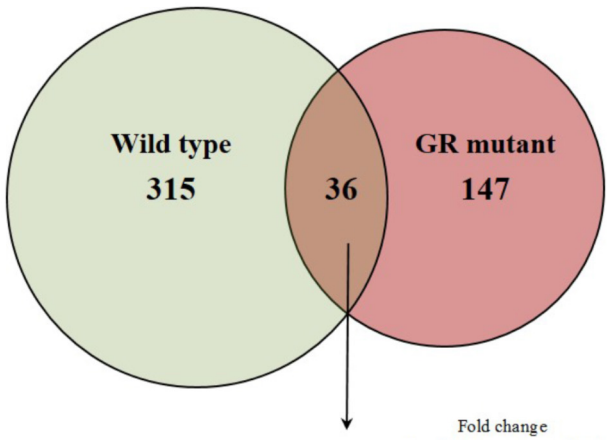


FIGURE 3 | Experiment II: Exercise effects on size in wild-type and Gr mutant zebrafish. **(A)** Standard length (SL) and **(B)** body weight (BW) in wild-type zebrafish (green), and **(C)** SL and **(D)** BW of Gr mutant zebrafish (orange), at the start of the experiment (start), after 4 weeks without (non-ex) and with subjection to the daily exercise protocol swimming at the suboptimal (<Uopt), optimal, and super optimal (>Uopt) swimming speeds of 0.3, 0.4, and 0.5 m s^{-1}, respectively. Differences in letters mark significant differences ($P < 0.05$).



	Fold change exercised vs. non-exercised wild-type	Fold change exercised vs. non-exercised Gr mutant
nucleus: transcription factor activity, ubiquitination		
ankyrin repeat and SOCS box containing 15b	14.99	11.53
nuclear factor, interleukin 3 regulated, member 5	8.11	5.42
F-box protein 32	4.54	4.65
cyclin-dependent kinase inhibitor 1D	2.92	4.36
tumor protein p53 inducible nuclear protein 1	2.93	3.87
D site albumin promoter binding protein b	0.30	0.22
zinc ion binding		
tripartite motif containing 63a	3.91	3.87
prostaglandin reductase 1	0.13	0.22
nuclear receptor subfamily 1, group d, member 1	0.05	0.09
APAF1 interacting protein	0.00	Inf
membrane		
si:dkey-184p9.7	25.99	17.28
polymeric immunoglobulin receptor-like 2.3	14.46	0.05
C-type lectin domain family 4, member A	2.99	0.13
major facilitator superfamily domain containing 2ab	2.67	0.14
zgc:152791	0.28	0.07

FIGURE 4 | Enriched functional categories of differentially expressed genes (DEGs) specific for wild-type zebrafish, for Gr mutants and the DEGs that both groups had in common. Also shown is the functional annotation clustering of the commonly expressed genes.

upregulated in mutants. Finally, it is worth mentioning that the *progesterone receptor* was upregulated at fc 13.68 in Gr mutant zebrafish while expression was not significantly differential in wild-type zebrafish.

DISCUSSION

In this study we have shown that exercise enhances growth and that optimal growth occurs at the optimal swimming speed. Moreover, we have shown that exercise increases cortisol levels in zebrafish. By applying swimming exercise physiology to a mutant zebrafish line with a functional knockout of the glucocorticoid receptor, we have investigated the role of cortisol signaling in exercise-enhanced growth. Our results show that cortisol signaling through the Gr receptor is not a main determinant of exercise-enhanced growth. However, RNAseq analysis revealed modulatory effects of cortisol, mediated by Gr,

TABLE 1 | Genes associated with muscle growth and structure which are differentially expressed in wild-type and/or Gr mutant zebrafish, exercised at Uopt in comparison with non-exercised zebrafish, as fold change.

	Fold change (exercised vs. non-exercised)	
Description	**Wild-type**	**Gr mutant**
fibroblast growth factor 13a	2.80	n.s.
oxidative stress induced growth inhibitor 1	0.21	0.16
growth arrest-specific 7a	12.26	n.s.
opioid growth factor receptor-like 1	9.28	n.s.
insulin-like growth factor binding protein 1a	3.04	n.s.
growth hormone receptor b	0.32	n.s.
insulin-like growth factor binding protein 6a	n.s.	40.26
growth regulation by estrogen in breast cancer 1	n.s.	6.05
myosin, heavy chain b	2.56	n.s.
myosin IIIA	4.32	n.s.
tropomyosin 1 (alpha)	2.70	n.s.
myoglobin	3.25	n.s.
collagen, type XI, alpha 2	4.33	n.s.
collagen, type VIII, alpha 1b	3.88	n.s.
collagen, type XI, alpha 1b	3.26	n.s.
collagen, type I, alpha 1a	3.24	n.s.
collagen, type I, alpha 2	3.19	n.s.
collagen, type I, alpha 1b	2.73	n.s.
procollagen, type V, alpha 1	2.57	n.s.
collagen, type VI, alpha 1	2.52	n.s.
collagen, type VI, alpha 2	2.45	n.s.
collagen, type X, alpha 1b	n.s.	10.61

at the transcriptional level in white skeletal muscle tissue during exercise-enhanced growth, which apparently do not affect growth but most likely alter the physiology of the muscle tissue.

Exercise Enhances Growth

The enhancement of growth by exercise in experiment 1 of this study was highly similar to previously published results (Palstra et al., 2010). Body weight was 39.1% higher in the exercised fish of this study vs. the non-exercised fish, which is very similar to the 41.1% as reported previously. Total length was 5.6% higher in the exercised fish of this study, which is identical to the length increase reported earlier. This validates this experimental procedure with the purpose to enhance growth by exercise and it confirms that zebrafish is a good model to study exercise-induced growth.

In the previous study and in experiment 1 of the present study, the body weight of the non-exercised fish had decreased after the 4 week experimental period. However, in experiment 2 of this study, the non-exercised fish in experiment 2 had increased in weight during the four-week exercise period. Apparently, growth conditions were better in the second experiment. A possible explanation for this difference could be that we briefly increased the flow in the tunnel after feeding in order to remove feed left overs and waste. As both exercised and non-exercised fish showed a better growth rate in the second experiment, the difference in growth between the two groups remained similar.

Optimal Growth at the Optimal Swimming Speed

Exercised fish showed growth enhancement which was highest at the optimal swimming speed and lower at both sub and super-optimal speeds. This implicates that the optimal swimming speed for growth can be predicted by the optimal swimming speed as determined on basis of respirometry. The optimal swimming speed is the speed at which the lowest oxygen uptake per unit distance, or lowest cost of transport (COT), occurs. The lowest COT can be calculated by equaling the first derivative of the polynomial function of COT vs. speed to zero (Palstra et al., 2008). Hereby we functionally link oxygen consumption to growth performance allowing for high throughput quick scan tests for growth prediction by oxygen consumption. This association was also suggested by Davison and Herbert (2013) on basis of data for several salmonids and yellowtail kingfish. Thus, zebrafish can be added to the list of species that show optimal exercise-enhanced growth at the optimal swimming speed, and this is probably true for more cyprinid species like common carp. In addition, this finding supports the hypothesis that fish reap the benefits of the exercise at a certain optimal swimming speed without wasting energy on aggressive behavior (at speeds which are too low) or using excessive energy for swimming (at speeds which are too high) (Davison, 1997; Palstra et al., 2010).

Exercise Increases Cortisol Levels

Exercise-enhanced growth is accompanied by higher cortisol levels in zebrafish than occurring at resting conditions. These results are in contrast with results in other fish species like rainbow trout (Postlethwaite and McDonald, 1995; Milligan et al., 2000) and Atlantic salmon (Boesgaard et al., 1993; Herbert et al., 2011), which secrete lower levels of cortisol under sustained exercise conditions than in non-exercised controls. Generally, high circulatory cortisol levels are associated with growth retardation (reviewed by Wendelaar Bonga, 1997). For example, fish subjected to stress-induced increases of cortisol levels show significantly decreased growth rates, even when food intake levels are maintained (Barnes, 2006). But this association may be context-dependent and restricted to situations of distress, while sustained exercise at optimal speeds may increase cortisol levels but still represent a eustress situation (Korte et al., 2005).

Gr-Mediated Cortisol Signaling Does Not Affect Skeletal Muscle Growth *per se* but Alters Its Transcriptional Regulation

As exercise-enhanced growth occurred similarly in wild-type fish and Gr mutants, we conclude that Gr-mediated cortisol signaling does not affect skeletal muscle growth *per se*. Cortisol signaling through Gr can therefore not be considered as a main determinant of exercise-enhanced growth. The conclusion is also supported by the fact that salmonids show exercise-enhanced growth in combination with decreased cortisol levels (Postlethwaite and McDonald, 1995; Herbert et al., 2011) and a cyprinid like zebrafish in combination with increased cortisol levels (this study). On the other hand, it could be argued that growth enhancement in the Gr mutant is due to residual receptor activity mediating some cortisol effects, but these effects appear to be very subtle (Facchinello et al., 2017).

However, even though exercise-enhanced growth at optimal swimming speed was similar between wild-type and Gr mutant zebrafish, the effects of exercise in wild-types and mutants on muscle gene expression were highly different. The differentially regulated functional gene categories between Gr mutant and wild-type zebrafish varied greatly and reflected the functional relevance of Gr-mediated cortisol effects (e.g., Ellis et al., 2012), among others as key controller of fish intermediary metabolism and physiological homeostasis (reviews by Wendelaar Bonga, 1997; Mommsen et al., 1999) under this situation of chronic exercise stress. We therefore suggest that, although muscle growth is not affected by cortisol signaling during exercise, Gr mediated effects of this hormone have great effects on the physiology of the muscle tissue.

When comparing exercised and non-exercised fish, wild-type and Gr mutant zebrafish had 36 DEGs in common. Gene ontology analysis showed that six of these genes belonged to the cellular component *nucleus* and the functional categories protein ubiquitination and transcription factor activity. Since wild-type and Gr mutant zebrafish show similar growth upon exercise, this common category probably represents processes that are crucial for muscle growth. Protein ubiquitination is a protein degradation pathway consisting of the strongly expressed and upregulated expression of genes such as *ankyrin repeat and SOCS box containing 15b* and *F-box protein 32*. The upregulation of these catabolic genes during muscle growth may be counterintuitive, but these findings are consistent with our earlier findings in zebrafish (Palstra et al., 2014), and data indicating activation of catabolic regulators in hypertrophied muscles in humans subjected to resistance training (Leger et al., 2006). These results suggest an involvement of genes involved in protein degradation and the ubiquitin proteasome pathway or other proteolytic systems in the growth of skeletal muscles upon exercise. The proteolytic processes that these genes moderate may be important in providing the muscle with amino acids under exercise. Increased protein turnover may therefore be a prerequisite for exercise-induced hypertrophy of fast muscle fibers in adult zebrafish, as previously suggested (Palstra et al., 2014). Probably even more important for the mechanism behind exercise-enhanced growth is the common differential expression of several transcription factors such as *nuclear factor, interleukin 3 regulated, member 5*; *tumor protein p53 inducible nuclear protein 1* and *D site albumin promoter binding protein b*. These transcription factors may represent the exercise fingerprint of enhanced growth and indicate the crucial pathways that are at the base of white skeletal hypertrophy as induced by exercise. The same may well be true for *oxidative stress induced growth inhibitor 1* of which the downregulated expression will lead to stimulated growth.

Remarkably, expressions of several genes which were considered as key marker genes in a recent study on exercised zebrafish by Rovira et al. (2017) were not differentially regulated

in our study. Expression of *mechanistic target of rapamycin* (*mtor*), key regulator in the PI3K/AKT/mTOR pathway toward protein biosynthesis in the muscle, was high but not differentially regulated in or between exercised Gr mutant and wild-type zebrafish. Neither were the expressions of myogenic transcription factors such as *paired box protein pax7* and *myocyte enhancer factor mef2* differentially regulated, nor the expression of any of the subunits for *AMP-activated protein kinase* (*prkaa1*, *prkaa2*, *prkab1a*, *prkab1b*, *prkab2*, *prkag1*, *prkag2a*, *prkag2b*, *prkag3a*, *prkag3b*) and *peroxisome proliferator-activated receptor gamma coactivator 1-alpha* (PGC-1α; *ppargc1a*). Therefore we cannot confirm the exercise-induced activation of the PI3K/AKT/mTOR, the mTOR-MEF2 and the AMPK-PGC1α signaling pathways toward muscle growth and proliferation, myogenesis and energy homeostasis, not in wild-type fish, nor in Gr mutants. As Rovira et al. (2017) did not report on growth of their experimental fish, and in our study we find high but no differential expression of these genes, we suggest that these pathways may play a role in muscle growth in general, but not specifically in exercise-enhanced growth.

As mentioned, when comparing exercised and non-exercised fish, wild-type and Gr mutant fish had only few DEGS in common (36) and had many specific DEGs (315 and 147, respectively). Among these specific DEGs were genes encoding growth factors, proteins involved in muscle contraction (e.g., myosins, troponin, and myoglobin) and in the extracellular matrix (e.g., collagens). Most pronounced differences when comparing genes which were differentially expressed between wild-type zebrafish and Gr mutants, were on the one hand the upregulated expressions of several genes in wild-type zebrafish such as *growth arrest-specific 7a* (*gas7a*) and *opioid growth factor receptor-like 1* (*ogfrl1*), while on the other hand the expressions of *insulin-like growth factor binding protein 6a* (*igfbp6a*) and *growth regulation by estrogen in breast cancer 1* (*greb1*) were upregulated in Gr mutants. Both *Growth arrest-specific 7a* as the *Opioid growth factor receptor-like 1* appear to have suppressive effects on growth. Expression of *growth arrest-specific 7a* occurs in cells that enter a quiescent state (Ju et al., 1998) and may have a function in neuronal development (Zhang et al., 2016). The *Opioid growth factor receptor-like 1* binds the opioid growth factor, an endogenous opioid peptide which plays an inhibitory role in cell proliferation during growth (Zagon et al., 2002). *Insulin-like growth factor binding protein 6a* is considered as a negative IGF I or II regulator and therewith functions as indirect muscle growth inhibitor (reviewed by Duan et al., 2010; Fuentes et al., 2013; Vélez et al., 2017). *Growth regulation by estrogen in breast cancer 1* (GREB1) promotes growth by estrogen-dependent proliferation of (breast cancer) cells (Camden et al., 2017). Besides estradiol, it may respond to other closely related steroid hormones and their receptors such as progesterone (Camden et al., 2017) which binds to the strongly upregulated progesterone receptor. As wild-type zebrafish and Gr mutants showed similar exercise-enhanced growth, these differentially regulated genes probably alter the physiology rather than the growth of the muscle tissue. Alternatively, they may indicate compensatory mechanisms in absence of cortisol signaling but leading to the same growth.

Other signaling pathways, such as cortisol signaling through the mineralocorticoid receptor (Vijayan et al., 2005; Prunet et al., 2006; Alsop and Vijayan, 2008) and activation of compensatory growth mechanisms, may be important for exercise-enhanced growth. For example, the GH/IGF axis and/or anabolic androgens moderating the hypertrophy of the white skeletal muscle may be involved. However, the androgen receptor was not differentially expressed in our study making a major role for anabolic androgens less plausible. We did find important differential regulation of growth factor expression which supports a major role for the GH/IGF axis in exercise-enhanced growth of zebrafish. Indeed, evidence has been provided for a pivotal role of the GH/IGF axis in exercise-enhanced growth of aquaculture fish such as coho salmon, rainbow trout, and Gilthead seabream (reviewed by Vélez et al., 2017).

CONCLUSION

In conclusion, cortisol signaling cannot be considered as a main mechanism behind exercise-enhanced growth. Moreover, as Gr mutants could fulfil the long term exercise protocol, even at the highest speeds, cortisol signaling cannot even be considered as crucial in the functioning of the white muscle under exercise. However, we demonstrate that cortisol has large effects on the transcriptional response of the muscle tissue to exercise, suggesting that the physiology of the muscle is altered. Finally, we identified several Gr-independent transcriptional responses to exercise, which may be at the base of exercise-enhanced growth.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this manuscript will be made available by the authors, without undue reservation, to any qualified researcher.

AUTHOR CONTRIBUTIONS

AP and MS conceived and designed the study. AP, SM, RD, and MS acquired the data. AP, RD, and MS analyzed and interpreted the data and drafted and reviewed the manuscript. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2018.01889/full#supplementary-material

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Acute Stress and an Electrolyte-Imbalanced Diet, but Not Chronic Hypoxia, Increase Oxidative Stress and Hamper Innate Immune Status in a Rainbow Trout (*Oncorhynchus mykiss*) Isogenic Line

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In aquaculture, fish may be exposed to sub-optimal rearing conditions, which generate a stress response if full adaptation is not displayed. However, our current knowledge of several coexisting factors that may give rise to a stress response is limited, in particular when both chronic and acute stressors are involved. This study investigated changes in metabolic parameters, oxidative stress and innate immune markers in a rainbow trout (*Oncorhynchus mykiss*) isogenic line exposed to a combination of dietary (electrolyte-imbalanced diet, DEB 700 mEq Kg^{-1}) and environmental (hypoxia, 4.5 mg O_2 L^{-1}) challenges and their respective controls (electrolyte-balanced diet, DEB 200 mEq Kg^{-1} and normoxia, 7.9 or mg O_2 L^{-1}) for 49 days. At the end of this period, fish were sampled or subjected to an acute stressor (2 min of handling/confinement) and then sampled. Feeding trout an electrolyte-imbalanced diet produced a reduction in blood pH, as well as increases in cortisol levels, hepato-somatic index (HSI) and total energy content in the liver. The ratio between the lactate dehydrogenase (LDH) and isocitrate dehydrogenase (IDH) activities decreased in the liver of trout fed the DEB 700 diet, but increased in the heart, suggesting a different modulation of metabolic capacity by the dietary challenge. Several markers of oxidative stress in the liver of trout, mainly related to the glutathione antioxidant system, were altered when fed the electrolyte-imbalanced diet. The dietary challenge was also associated with a decrease in the alternative complement pathway activity (ACH_{50}) in plasma, suggesting an impaired innate immune status in that group. Trout subjected to the acute stressor displayed reduced blood pH values, higher plasma cortisol levels as well as increased levels of metabolic markers associated with oxidative stress in the liver. An interaction between

diet and acute stressor was detected for oxidative stress markers in the liver of trout, showing that the chronic electrolyte-imbalance impairs the response of rainbow trout to handling/confinement. However, trout reared under chronic hypoxia only displayed changes in parameters related to energy use in both liver and heart. Taken together, these results suggest that trout displays an adaptative response to chronic hypoxia. Conversely, the dietary challenge profoundly affected fish homeostasis, resulting in an impaired physiological response leading to stress, which then placed constraints on a subsequent acute challenge.

Keywords: rainbow trout, dietary imbalance, metabolic capacity, fish homeostasis, chronic hypoxia, stressors

INTRODUCTION

Farming fish involves following certain practices and procedures which may include handling, low water levels, confinement, and crowding (Conte, 2004). These procedures can act as stressors when habituation is not present (Pickering, 1993; Wendelaar Bonga, 1997; Bratland et al., 2010; Nilsson et al., 2012). Additionally, chronic sub-optimal rearing conditions may also give rise to stress responses if full adaptation is not displayed. However, the information available on existing conditions that may act as stressors during rearing, along with their interactions, is very limited. This is an area of increasing interest, as stressors have been linked to a reduction in both growth performance and immune condition; hampering health and welfare in fish (Portz et al., 2006).

One of the known existing factors of stress in cultured fish species is the persistent low oxygen concentration (chronic hypoxia). Nevertheless, its consequences are still poorly documented. In addition, long-term dietary imbalances affect fish homeostasis and energy balance. The induction of stress by dietary imbalances have been described in fish, including responses linked to partial or total replacement of fish meal and fish oil by alternative sources (Montero et al., 2003, 2008, 2010; Gómez-Requeni et al., 2004). Previous studies have shown that growth performance, feed intake and nutrient digestibility are altered when fish are fed electrolyte imbalanced diets (Dersjant-Li et al., 1999, 2000; Saravanan et al., 2013b; Magnoni et al., 2016). However, the induction of stress by this type of dietary imbalance is yet to be investigated in fish.

The dietary electrolyte balance (DEB) is defined as the sum of the mineral cations minus the sum of mineral anions present in the diet. Differences in DEB may occur when feed ingredients containing different quantities of cations (Na, K, Ca, and Mg) and anions (Cl and P) are included in the diet formulation (Patience and Wolynetz, 1990). It was previously shown that rainbow trout (*Oncorhynchus mykiss*) fed an electrolyte imbalanced diet (DEB 700) were energetically less efficient than those fed an electrolyte balanced diet (DEB 200). In spite of this change in energy balance, feed intake and growth performance were not affected (Magnoni et al., 2018).

The objective of this study was to determine whether electrolyte imbalanced diet may affect the ability of trout to cope with chronic hypoxia conditions. The assessment of physiological impacts of combined stress factors (diet and hypoxia) was analyzed in fish before and after applying an acute stressor (handling/confinement). This acute stress condition is commonly present in aquaculture, and was used to determine the physiological coping ability of the fish.

A set of plasma parameters related to stress and innate immune response were determined, as they are commonly associated with fish welfare. Additionally, several parameters were analyzed in liver and heart, since their energy use are altered when subjected to stress conditions (Wendelaar Bonga, 1997; Hermes-Lima et al., 2001), including changes in oxidative stress and metabolic response. An isogenic heterozygous family of rainbow trout was used as fish model, due to its genetic uniformity providing low intra-specific variability and high reproducibility.

MATERIALS AND METHODS

Fish and Housing

The isogenic heterozygous family of rainbow trout (R23) obtained by crossing two homozygote isogenetic lines was produced by INRA/PEIMA (France) experimental fish facilities (Sadoul et al., 2015). Fish were housed in the Aquatic Metabolic Unit (AMU) tanks of Aquaculture and Fisheries group, Wageningen University, The Netherlands. Thirty rainbow trout (115.2 $\pm$ 2.0 g) were randomly assigned to each of the twelve experimental tanks (200 L). The tanks were connected to a water recirculation system consisting of a trickling filter, an oxygenation unit, a sump, a drum filter (Hydrotech 500®), and a cooling/heating system for maintaining uniform water quality throughout the study. The oxygenation unit maintained the DO levels by injecting oxygen into the water and was facilitated with separate automatic probes for the detection of water flow and oxygen consumption. Water temperature was set at 14 $\pm$ 1°C. Photoperiod was maintained at 12:12 (Light: Dark) with daybreak set at 07:00 h.

Experimental Diets and Feeding

Two isoproteic (45% DM) and isoenergetic (22 kJ gDM^{-1}) diets, floating pellets of 4 mm, were extruded by Research Diet Services (Wijk bij Duurstede, The Netherlands). Diets, upon arrival to the AMU at University of Wageningen, were stored in a room under controlled conditions during the trial. The two diets were formulated to provide a contrast in electrolyte content (DEB);

200 or 700 mEq Kg^{-1}. This difference was created by adding different amounts of Na_2CO_3 and diamol (inert filler) in the diets. Fish were fed the experimental diets to apparent satiation, twice a day for 49 days. Ingredients and proximate composition of the experimental diets are included in **Supplementary Table S1**.

Experimental Conditions

The difference in DO levels was induced by adjusting the rate of water flow into the tanks as described by Saravanan et al. (2013a). The water volume was kept constant at 200 L in all tanks. For the normoxia groups, the rate of water inflow into each tank was kept at 7.2 ± 0.0 L min^{-1} (mean ± SEM) with a mean water DO level of 10.2 ± 0.1 mg O_2 L^{-1}. The DO level in the outflowing water remained at 7.9 ± 0.1 mg O_2 L^{-1}. Hypoxia conditions were created by gradually reducing the rate of water inflow into the tank (2.2 ± 0.0 L min^{-1}) with a DO level of 10.2 ± 0.1 mg O_2 L^{-1} for the first 3 days after the start of the experiment. DO level in the outflowing water remained at 4.5 ± 0.1 mg O_2 L^{-1} and was kept equal in all the hypoxic tanks. The DO level in the water outlet was considered to be equivalent to ambient DO level of the tanks, as differences between both DO levels (outlet versus inside the tank) shown in several previous experiments were negligible (<0.2 mg O_2 L^{-1}, e.g., Tran-Duy et al., 2008). The force of the evenly distributed water supplied by the inlet in each tank together with the swimming activity prevented the occurrence of water stratification within the tanks. The DO level applied in the hypoxia treatment is recognized as an environmental challenge, with the value decided based on the reported incipient DO level of 6.0 mg O_2 L^{-1} for rainbow trout (Pedersen, 1987).

Experimental Design

Employing a 2 × 2 design (DEB diets and DO levels), the experimental tanks (12) were divided into three experimental blocks, with four tanks randomly assigned within each of three blocks (n = 3 tanks per treatment). Fish were not fed on the day prior to sampling.

Fish from each experimental group were divided into 2 sub-groups for the application of a standardized handling/confinement protocol (acute stress) and posterior sampling. A control sub-group in which three fish per tank (9 per treatment) were sampled by reducing the exposure to potential stressors. Subsequently, a group of 3 fish per tank were netted and exposed to a confinement stress (2 min at a density of 200 kg/m^3) and transferred back to their original tank (empty) for 1 h. Then fish from both experimental sub-groups were netted and euthanized for sampling. A sampling effect was prevented by sampling the tanks following the order tanks were installed in the room, starting with the control sub-group and with water from sampled tanks on flow through to prevent water re-entering the RAS circulation. A lethal dose of 2-phenoxy ethanol solution (1 mg/L) was used in all the sampling procedures.

Blood was drawn from the caudal region with a heparinized syringe. Blood pH was immediately measured (pH meter, WTW pH 320; pH electrode, WTW SenTix Sp). The duration of procedure (blood withdrawal and pH measurement) was strictly standardized to 1 min for all fish to minimize blood pH variation (Saravanan et al., 2013b). Blood was centrifuged at 3000 g for 20 min at 4°C, and plasma samples were frozen and stored for later analyses. Fish, liver, and heart were weighed and sampled. Samples were frozen in liquid nitrogen and stored at −80°C for further analyses.

Plasma Metabolite Content

Lactate concentration in plasma was quantified using a commercial kit (LO-POD, Spinreatc, Sant Esteve de Bas, Spain). Glucose concentration in plasma was quantified using a commercial kit (GOD-POD, Spinreatc, Sant Esteve de Bas, Spain). An ELISA kit based on the competitive link between cortisol and related monoclonal antibodies was used to quantify cortisol levels in plasma of rainbow trout (RE 52061, IBL International, Hamburg, Germany). Validation of the kit for determination of cortisol in plasma of trout was performed. The intra- and inter-assay coefficient of variation in plasma samples were <9% and <10%, respectively. The linearity showed an r^2-value of 0.96. Results from this validation indicated the suitability for the use of this kit to quantify changes in cortisol levels in rainbow trout. All measurements were performed in triplicates, following the recommendations provided by the manufacturers.

Energy Balance in Tissues

Total protein, glycogen and lipids contents were measured spectrophotometrically in the tissue homogenates according to De Coen and Janssen (1997, 2003). Liver and heart tissues were homogenized in phosphate buffer (1/10 vol, 0.1 M, pH = 7.4). Proteins in the homogenates were precipitated with the addition of cold 15% trichloroacetic acid and incubated at −20°C for 10 min. After a centrifugation at 1000 g for 10 min, the resulting supernatant was used for glycogen quantification by adding phenol (5%) and H_2SO_4 (conc.). After 30 min incubation at 20°C, glucose was quantified by measuring the absorbance at 490nm. The protein pellet obtained after trichloroacetic acid precipitation was resuspended in NaOH (1N), incubated at 60°C for 30 min and then neutralized with HCl (1.67 M). The resuspended pellet was used to quantify total protein content by the Bradford (1976) method measuring absorbance at 600 nm, using bovine serum albumin as standard.

As for lipids extraction, chloroform, methanol, and Mili-Q water were added to homogenates in a 2:2:1 proportion, respectively. The organic phase was separated after centrifugation, treated with H_2SO_4 at 200°C, and used to quantify total lipid content at 400 nm, using tripalmitin as standard. Total protein, glycogen, and lipid content of each sample was expressed as mg per g of wet weight tissue. Total energy content was calculated as the sum of the protein, glycogen and lipids contents in each tissue, transformed into energetic equivalents using values for the enthalpy of combustion (24 kJ/g proteins, 17.5 kJ/g carbohydrates, and 39.5 kJ/g lipids), as described by De Coen and Janssen (1997), and expressed as kJ g^{-1} wet weight wet tissue.

An aliquot of homogenate was centrifuged for 5 min, at 3000 g (4°C) and the supernatant was used for lactate dehydrogenase (LDH) and isocitrate dehydrogenase (IDH) activity measurements. LDH was measured following the method described by Vassault (1983) and adapted to microplate. IDH

was measured following the method described by Ellis and Goldberg (1971) with the adaptations of Lima et al. (2007). For the normalization by protein content, Bradford (1976) method was applied to quantify protein in the supernatant fraction, using bovine *G*-globulin as standard and reading absorbance at 600 nm. Energy consumption rate (Ec: measured through the electron transport system -ETS- activity) was performed according to the procedure described by De Coen and Janssen (1997, 2003). Measurements were performed at 25°C in triplicates using proper reaction blanks in a synergy H1 Hybrid Multi-Mode microplate reader (Biotek® Instrument, Vermont, United States).

Oxidative Stress Markers in Liver

Liver and heart samples were homogenized in phosphate buffer (1/10 vol., 0.1 M pH 7.4). Enzymatic analyses were all carried out with the reaction mixtures and homogenate dilution established in preliminary tests. Protein concentration was assayed in homogenates using bovine serum albumin as standard (Bradford, 1976). Lipid peroxidation (LPO) was determined by quantifying the presence of formed thiobarbituric acid reactive substances (TBARS) (Ohkawa et al., 1979). Glutathione reductase (GR) (EC1.8.1.7) and glutathione peroxidase (GPx) (EC 1.11.1.9.) were evaluated based on NADPH (Sigma, Portugal) oxidation at 340 nm (Mohandas et al., 1984; Cribb et al., 1989). Total glutathione (TG) and oxidized glutathione (GSSG) were evaluated by the formation of 5-thio-2-nitrobenzoic acid at 412 nm (Baker et al., 1990). Reduced glutathione (GSH) was calculated as the difference between TG and GSSG. Changes in absorbance were measured at 22°C in a PowerWave™ microplate spectrophotometer (BioTek Instruments), and reactions were performed in triplicates. Substrate was omitted in reaction blanks and background activity was subtracted from that measured in the presence of substrate.

Innate Immune Parameters in Plasma

Immune status was assessed through the determination of key parameters, namely lysozyme, peroxidase and the alternative complement pathway (ACH_{50}) activities. Lysozyme activity was evaluated according to Lie et al. (1986), employing hen egg white lysozyme (Sigma, Germany) as standard and *Micrococcus lysodeikticus* (0.5 mg mL^{-1}; 0.05 M sodium phosphate buffer; pH 6.2) as bacterial suspension. Peroxidase activity (U mL^{-1} plasma) was determined based on the methodology described by Quade and Roth (1997). ACH_{50} activity was analyzed according to Sunyer and Tort (1995) using a concentration of 2.8×10^8 cells mL^{-1} rabbit erythrocytes. The reciprocal of the plasma dilution giving 50% haemolysis of erythrocytes equals to one ACH_{50} unit.

Measurements and Calculations

Weight gain (WG) was calculated as:

$$WG\ (g) = BW_f - BW_i$$

where BW_i and BW_f are the initial and the final body weight of fish in the trial, respectively.

Feed intake (FI) was calculated as:

$$FI\ (g\ DM) = FI_{TOT}/\ n \times t,$$

where FI_{TOT} is the total FI per tank over the experimental period corrected for dead fish and uneaten feed, n is the number of fish per tank and t is the experimental period (days).

Uneaten feed (pellets) were collected on the surface at the end of each feeding period (1 h). Pellets remaining on the bottom of the tank were collected by a decantation unit. All the uneaten pellets were counted and the amount calculated by taking and average weight of pellets in each diet production lot. The amount of feed was registered daily and accounted for in the feed intake calculation. No mortalities were recorded during the trial, except one fish fed the DEB 200 diet in hypoxia (98.9% survival).

Feed conversion ratio (FCR) was calculated as:

$$FCR = FI/(BW_f - BW_i)$$

Specific growth rate (SGR) was calculated as:

$$SGR(\%BWday^{-1}) = (lnBW_f - lnBW_i)/time(days) \times 100$$

The hepato-somatic index was calculated as:

$$HSI(\%) = 100 \times [liverweight(g)/BW(g)]$$

The cardio-somatic-index was calculated as:

$$CSI(\%) = 100 \times [heartweight(g)/BW(g)]$$

The effects of DEB 200 and DEB 700 on growth performance and feed intake of this trout isogenic line subjected chronic hypoxia or normoxia has been published in Magnoni et al. (2018). However, to assist with the interpretation of the results presented in the current study, growth performance and feed intake parameters were included as **Supplementary Table S2**.

Statistical Analysis

Final body weight (BW_f), weight gain (WG), and specific growth rate (SGR) were analyzed for the effects of the diet and DO levels along with their interactions by a two-way ANOVA analysis (triplicate tanks). All the other parameters in fish were analyzed for the effects of diet, DO levels and acute stress, along with their interactions by a three-way ANOVA analysis ($n = 9$). When differences in mean values were detected by the ANOVA analysis, *post hoc* tests were applied (Holm-Sidak method). In all these analyses, data sets were tested for normality (Shapiro–Wilk) and for equal variance (Levene's test), and if these assumptions were not met, data transformation was applied (Natural logarithm). Statistical analysis was made using SigmaPlot 12.0 (Systat Software, Inc. 2011) and significant differences were considered for $P < 0.05$.

RESULTS

Metabolic Markers in Blood and Plasma

Blood pH remained similar in fish reared at different DO levels (**Table 1**). Feeding trout an electrolyte-imbalanced diet (DEB

TABLE 1 | Effect of dietary electrolyte balance (DEB), dissolved oxygen levels, and acute stress on blood parameters of rainbow trout.

	DEB 200				DEB 700				Factors			Interactions			
	Normoxia		Hypoxia		Normoxia		Hypoxia								
	C	S	C	S	C	S	C	S	D	O	S	DOS	DO	DS	OS
pH	7.06 ± 0.2	6.99 ± 0.02	7.08 ± 0.02	7.02 ± 0.02	7.0 ± 0.01	6.95 ± 0.03	7.07 ± 0.02	6.92 ± 0.03	**	ns	**	ns	ns	ns	ns
Cortisol	6.63 ± 1.95	28.00 ± 4.88	6.58 ± 1.84	34.88 ± 8.50	11.66 ± 1.80	41.62 ± 4.52	8.55 ± 1.68	31.84 ± 6.35	*	ns	**	ns	ns	ns	ns
Lactate	0.26 ± 0.07	0.45 ± 0.11	0.31 ± 0.03	0.65 ± 0.13	0.27 ± 0.06	0.60 ± 0.10	0.29 ± 0.07	0.63 ± 0.05	ns	ns	**	ns	ns	ns	ns
Glucose	5.40 ± 0.31	7.22 ± 0.60	5.39 ± 0.45	6.83 ± 0.47	5.34 ± 0.30	7.29 ± 0.16	5.31 ± 0.44	6.32 ± 0.47	ns	ns	**	ns	ns	ns	ns

*Plasma cortisol levels in ng mL^{-1}. Plasma lactate and glucose concentrations in mM. Values are mean of fish (n = 9) ± SE. C, Control; S, Acute stress. Three-way ANOVA analysis results. D, Diet; O, dissolved oxygen levels; DOS, DO, DS, and OS, Interactions; ns, not significant P > 0.1; *P < 0.05; **P < 0.01.*

700) or subjecting the fish to acute stress induced a reduction on blood pH ($P < 0.001$).

Plasma cortisol levels were increased in trout subjected to acute stress or when fed an electrolyte-imbalanced diet ($P < 0.001$ and $P < 0.005$, respectively). However, cortisol levels remained unaltered when fish were subjected to chronic hypoxia. Plasma lactate and glucose concentrations were increased in fish subjected to acute stress ($P < 0.001$) but remained similar when fed diets with varied DEB or when maintained at different DO levels.

Energy Use in Tissues

The hepato-somatic index (HSI) of trout was 16% higher in fish fed the DEB 700 diet ($P < 0.05$) (**Table 2**). All the parameters related to energy content measured in liver of trout remained similar at different DO levels. However, total energy content in the liver was increased by 6% in fish fed the electrolyte-imbalanced diet ($P < 0.05$).

The cardiac-somatic index (CSI) of trout was reduced by 6% in fish subjected to hypoxia ($P < 0.05$) (**Table 3**). The energy stored as glycogen in the heart of trout was increased by 27% in fish reared in hypoxia ($P < 0.01$) and decreased by 26% when subjected to acute stress ($P < 0.01$).

A decrease in IDH activity ($P < 0.01$), with an increase in LDH to IDH activity ratio ($P < 0.05$) was detected in the liver of trout reared in hypoxia (**Table 4**). On this tissue a decrease in LDH activity ($P < 0.05$), as well as in the LDH to IDH activity ratio ($P < 0.01$) were detected in trout fed the DEB 700 diet. However, all these metabolic parameters remained similar when acute stress was applied.

On the other hand, enzyme activities and parameters related to energy-use in the heart of trout remained similar in fish reared at different DO levels (**Table 5**). Both electrolyte-imbalanced diet and acute stress increased LDH to IDH activity ratio in the heart of trout ($P < 0.05$). In addition, acute stress decreased the IDH activity measured in the trout's heart ($P < 0.05$).

Oxidative Stress Markers in Liver

All the oxidative stress parameters analyzed in this tissue remained similar in fish reared at different DO levels (**Table 6**). However, in trout fed with the DEB 700 diet, a decrease in the GPX activity ($P < 0.05$), as well as an increase in the TG and GSSG levels ($P < 0.05$) were observed. In addition, acute stress induced increases in GR activity ($P < 0.05$) and GSH content ($P < 0.05$) in trout's liver.

Innate Immune Status

All the parameters analyzed in plasma remained similar in fish reared at different DO levels (**Table 7**). However, feeding trout an electrolyte-imbalanced diet produced a decrease in ACH_{50} levels ($P < 0.05$). Acute stress increased lysozyme activity ($P < 0.05$).

Interactions Between Experimental Factors

No interaction between dietary treatment and DO levels was observed in parameters related to fish performance (**Supplementary Table S2**). In addition, no interactions were observed between the factors investigated when analyzing markers related to stress, metabolism or innate immune status in plasma (**Tables 1**, **7**, respectively). Furthermore, the energy content of the heart of trout did not display any interaction between the experimental factors investigated (**Table 3**).

By contrast, the total energy content of the liver was affected by the diet and the DO levels ($P < 0.05$), as well as by the DO levels and the acute stressor ($P < 0.01$) (**Table 2**). The diet also influenced the energy consumption (Ec) value in the liver when the acute stressor was considered ($P < 0.05$). Additionally, the dietary treatment influenced DO levels ($P < 0.05$), acute stressor ($P < 0.01$), and all the three factors together ($P < 0.05$) when the marker of oxidative stress, GPx activity, was analyzed in liver (**Table 6**). The three factors together displayed significant interaction when several markers of energy use in heart of trout were investigated (LDH, IDH, LDH/IDH, Ec) (**Table 5**). DO levels and acute stress revealed significant interactions when glycogen content was analyzed in liver ($P < 0.05$) (**Table 2**).

DISCUSSION

This study analyzed the prolonged effects of dietary electrolyte-imbalance and environmentally low DO levels, along with an acute stressor (handling/confinement), on various functions including: (i) acute stress responses at the level of plasma or cellular stress, (ii) tissue metabolism (liver and heart), and (iii) innate immunity. By implementing such approach, the aim

TABLE 2 | Effect of dietary electrolyte balance (DEB), dissolved oxygen levels and acute stress on hepato-somatic index (HSI), protein, lipids, glycogen, and energy content in liver of rainbow trout.

	DEB 200				DEB 700				Factors			Interactions			
	Normoxia		Hypoxia		Normoxia		Hypoxia								
	C	S	C	S	C	S	C	S	D	O	S	DOS	DO	DS	OS
HSI	1.42 ± 0.22	1.31 ± 0.15	1.25 ± 0.17	1.39 ± 0.23	1.56 ± 0.18	1.73 ± 0.19	1.29 ± 0.13	1.81 ± 0.32	*	ns	ns	ns	ns	ns	ns
Protein	61.87 ± 5.65	61.50 ± 2.61	61.82 ± 7.63	54.51 ± 4.56	59.35 ± 3.12	58.09 ± 3.34	61.54 ± 3.92	53.68 ± 6.41	ns	ns	ns	ns	ns	ns	ns
Lipids	24.47 ± 1.95	22.10 ± 1.17	25.33 ± 1.86	28.06 ± 2.89	26.37 ± 2.80	26.86 ± 3.31	23.43 ± 0.73	28.83 ± 3.46	ns	ns	ns	ns	ns	ns	ns
Glycogen	95.18 ± 14.62	74.50 ± 8.59	88.15 ± 14.31	103.74 ± 10.19	109.81 ± 6.32	105.52 ± 10.08	83.47 ± 7.69	112.72 ± 10.72	ns	ns	ns	ns	ns	ns	*
Total energy	4.12 ± 0.13	3.65 ± 0.14	4.03 ± 0.16	4.23 ± 0.17	4.39 ± 0.13	4.30 ± 0.21	3.86 ± 0.82	4.40 ± 0.16	*	ns	ns	ns	*	ns	**

*Protein, lipids and glycogen expressed as mg g^{-1} weight wet tissue. Total energy content expressed as kJ g^{-1} weight wet tissue. Values are mean of fish (n = 9) ± SE. C, Control; S, Acute stress. Three-way ANOVA analysis results. D, Diet; O, dissolved oxygen levels; DOS, DO, DS, and OS, Interactions; ns, not significant P > 0.1; *P < 0.05; **P < 0.01.*

was to provide a refined tool to evaluate stress response in fish. The prolonged conditions implemented, DEB 700 diet and hypoxia, were designed to produce deep physiological challenges in trout. These conditions were chosen over other conditions, such as repeated physical handling, as this may increase the likelihood of skin, mucus or fin damages, and could make interpretation of chronic stress responses difficult, as this may increase the likelihood of skin, mucus or fin damages, and could make interpretation of chronic stress responses difficult due to co-factors that could be present (e.g., opportunistic pathogens), leading to erroneous conclusions.

The Electrolyte-Imbalanced Diet Acts as a Chronic Stressor in Rainbow Trout

Growth performance and FI remains unchanged when trout was fed an electrolyte-imbalanced diet at satiety for 49 days (**Supplementary Table S2**). In spite of the lack of changes in these parameters, markers of energy use, oxidative stress response, and innate immune status were all altered in trout. In particular, the DEB 700 diet induced increases in HSI and total energy content in the liver of the trout. These changes could be related to an energetic constrain imposed by electrolyte-imbalanced diet, as mechanisms managing acid-base homeostasis may involve an increased energy expenditure. In fact, feeding a DEB 700 diet on this isogenic trout line has shown to produce a 65% increase in the metabolizable energy requirements for maintenance, suggesting higher energy demand to maintain acid-base balance in that group (Magnoni et al., 2018). Such scenario is supported by a postprandial decrease in blood pH of trout fed the DEB 700 diet (**Table 1**). An alteration on the energy balance is shown by the increase in the total energy content stored in liver of trout fed the DEB 700 diet, possibly related as well to changes in the relative importance of aerobic and anaerobic metabolism to provide energy to this organ.

As any stress condition carries additional energetic requirements, biomarkers on anaerobic and aerobic pathways of energy production may provide important indications in terms of global energetic demands toward a stress condition. In particular, LDH activity has a key role in anaerobic pathways and changes on this enzyme activity are usually associated to increased energy demand, e.g., due to stressful conditions (Dahlhoff, 2004). On the other hand, IDH, an enzyme involved in energy production through the aerobic pathways, catalyzes a key step of the Krebs cycle, and is also involved in antioxidant defenses, where it is crucial for the regeneration of NADPH required for glutathione conjugation pathways (Jo et al., 2001; Lee et al., 2002). For example, in juvenile European seabass (*Dicentrarchus labrax*) an increased IDH activity was detected in liver when exposed to high temperature, which has been linked to a stress response (Almeida et al., 2015). An increased reliance in aerobic metabolism (low LDH/IDH activities ratio) usually enables a more efficient energy metabolism than the anaerobic pathways. In our study, LDH/IDH activities ratio was decreased in liver of trout by the electrolyte-imbalanced diet. In contrast, the heart of trout displayed an increase in LDH/IDH activities ratio when fed

TABLE 3 | Effect of dietary electrolyte balance (DEB), dissolved oxygen levels and acute stress on cardiac-somatic index (CSI), protein, lipids, glycogen, and energy content in heart of rainbow trout.

	DEB 200				DEB 700				Factors			Interactions			
	Normoxia		Hypoxia		Normoxia		Hypoxia								
	C	S	C	S	C	S	C	S	D	O	S	DOS	DO	DS	OS
CSI	0.15 ± 0.01	0.15 ± 0.01	0.13 ± 0.01	0.14 ± 0.01	0.14 ± 0.01	0.15 0.01	0.13 ± 0.01	0.14 ± 0.01	ns	*	ns	ns	ns	ns	ns
Protein	57.23 ± 4.17	51.06 ± 2.61	51.47 ± 2.24	53.57 ± 3.98	55.29 ± 3.61	52.16 ± 4.72	46.58 ± 3.76	51.51 ± 4.27	ns	ns	ns	ns	ns	ns	ns
Lipids	23.93 ± 3.31	17.83 ± 1.28	18.64 ± 2.02	21.04 ± 1.94	17.92 ± 1.24	19.63 ± 1.49	16.90 ± 1.66	18.59 ± 1.55	ns	ns	ns	ns	ns	ns	ns
Glycogen	4.35 ± 0.51	2.79 ± 0.27	4.80 ± 0.22	4.34 ± 0.40	4.12 ± 0.32	2.61 ± 0.34	4.57 ± 0.32	4.20 ± 0.56	ns	**	**	ns	ns	ns	ns
Total energy	2.40 ± 0.19	1.98 ± 0.92	2.25 ± 0.11	2.38 ± 0.14	2.11 ± 0.10	2.07 ± 0.16	1.87 ± 0.13	2.04 ± 0.16	ns	ns	ns	ns	ns	ns	ns

*Protein, lipids, and glycogen expressed as mg g^{-1} weight wet tissue. Total energy content expressed as kJ g^{-1} weight wet tissue. Values are mean of fish (n = 9) ± SE. C, Control; S, Acute stress. Three-way ANOVA analysis results. D, Diet; O, dissolved oxygen levels; DOS, DO, DS, and OS, Interactions; ns, not significant P > 0.1; *P < 0.05; **P < 0.01.*

the electrolyte-imbalanced diet, indicating that the metabolic capacity of liver and heart are modulated differently when subjected to the dietary challenge.

Key components of the glutathione system keeping redox balance, including GPX activity, TG, and GSSG levels, were all altered by diet in the liver of trout. In particular, GPx activity has been suggested as a reliable marker of stress in fish subjected to prolonged dietary or environmental challenges (Winston, 1991; Martínez-Álvarez et al., 2005; Sitjà-Bobadilla et al., 2005; Fontagné-Dicharry et al., 2018). Therefore, this study was able to confirm importance of this stress marker in the liver of trout subjected to a prolonged dietary challenge.

Haemolytic activity due to alternative complement pathway, measured as ACH_{50}, is interpreted by numerous authors as a sign of a prompter innate immune system, improving the resistance to pathogens (Chiu et al., 2008; Biller-Takahashi et al., 2012). It is known that ACH_{50} activity in fish changes with stress conditions (Boshra et al., 2006). Furthermore, decreased ACH_{50} activity has been linked to chronic stress produced by crowding in gilthead seabream (*Sparus aurata*) juveniles (Montero et al., 1999b). Trout fed the DEB 700 diet displayed a decreased ACH_{50} activity in plasma, which reinforces the conclusion that the disruption on fish homeostasis generated by the electrolyte-imbalanced diet could be evoking a stress response. Fish are able to mount successful and robust innate responses (Tort et al., 2003), such as the measured in this study. Previous studies have shown that the innate immune response in fish is altered by chronic crowding stress (Rotllant et al., 1997; Montero et al., 1999a) and by a combination of dietary supplementations and chronic crowding stress (Montero et al., 1999b). However, future studies should investigate the response of adaptive immune parameters to combined stressors such as those implemented in this study.

Rainbow Trout Shows a Full Adaptive Response to Chronic Hypoxia

Hypoxia tolerance appears to be variable in close phylogenetic related species of fish (Mandic et al., 2009), also displaying marked changes during development (Ishibashi et al., 2005). In this sense, the heterozygous isogenic line of rainbow trout used in this study, reared for many generations, provides a unique model with low intraspecific variability, adequate to test the effect of chronic hypoxia. This study is a follow-up of a previous one (Magnoni et al., 2018), in which it was shown that both feed intake (FI) and growth performance were decreased when this trout line was exposed to chronic hypoxia. In particular, rainbow trout maintained under hypoxia (4.5 ± 0.1 mg O_2 L^{-1}) reduced their feed intake and specific growth rate by 33 and 22%, respectively (**Supplementary Table S2**). In a previous study, Glencross (2009) also showed a decrease in FI in rainbow trout exposed to hypoxia (28 days at 4.29 mg O_2 L^{-1}). Therefore, the decrease in FI during chronic hypoxia detected in the present study is probably not linked to chronic stress, but more likely to a reduction in appetite triggered by the inhibition of food-anticipatory behavior, as has been suggested by previous studies (Glencross, 2009; Folkedal et al., 2012a,b). Corticotropin-releasing factor in the forebrain appears to mediate sustained anorexia during chronic hypoxia (Bernier and Craig, 2005), suggesting that this mechanism may be an essential energy-saving strategy due to the reduction in fish maintenance energy. Also suppression of appetite may save energy by reducing the postprandial increase in O_2 consumption associated with digestion, absorption, transformation, and storage of nutrients (Jobling, 1981, 1993). In this study, we cannot completely rule out the possibility that chronic hypoxia could act as a chronic stressor in trout, inhibiting feed intake by altering feeding behavior. However, as will be discussed in the next section, we propose that this may not be the case for this study, since the cortisol response was not altered after chronic low DO levels, suggesting that trout is adapted to these particular conditions.

There is increasing evidence that rearing fish under intensive aquaculture conditions may alter their cardiovascular physiology (Gamperl and Farrell, 2004). The DO level applied in the hypoxia treatment during this study (4.5 mg O_2 L^{-1}) is recognized as an environmental challenge, with the value decided based on the critical DO level for feed consumption and for both growth rate and feed conversion efficiency (6 and 7 mg O_2 L^{-1}, respectively) for rainbow trout (Pedersen, 1987). However, a previous study showed that rainbow trout reared at hatcheries, with sub-optimal water quality conditions, appear to have a remarkable degree of tolerance to short-term severe hypoxia, which has been

TABLE 4 | Effect of dietary electrolyte balance (DEB), dissolved oxygen levels and acute stress on energy use in liver of rainbow trout.

	DEB 200				DEB 700				Factors			Interactions			
	Normoxia		Hypoxia		Normoxia		Hypoxia								
	C	S	C	S	C	S	C	S	D	O	S	DOS	DO	DS	OS
LDH	2699.12 ± 274.64	3349.83 ± 437.29	2990.73 ± 298.11	3092.40 ± 315.63	2287.73 ± 281.52	2579.46 ± 204.78	2754.66 ± 114.60	2606.28 ± 297.18	*	ns	ns	ns	ns	ns	ns
IDH	84.64 ± 4.95	85.89 ± 8.59	70.13 ± 4.98	74.58 ± 4.99	94.09 ± 3.82	93.19 ± 4.51	80.05 ± 3.12	76.34 ± 4.20	ns	**	ns	ns	ns	ns	ns
LDH/IDH	32.43 ± 3.48	39.30 ± 3.26	43.01 ± 3.18	42.69 ± 4.69	25.08 ± 3.64	28.20 ± 2.58	34.83 ± 1.96	33.56 ± 2.89	**	*	ns	ns	ns	ns	ns
Ec	211.37 ± 23.23	175.51 ± 12.78	268.48 ± 28.93	182.61 ± 22.62	183.38 ± 13.03	205.08 ± 15.22	199.70 ± 18.66	177.93 ± 22.04	ns	ns	ns	ns	ns	*	ns

*Lactate dehydrogenase (LDH) and iso-citrate dehydrogenase (IDH) activities are expressed as nmol min^{-1} mg^{-1} tissue. Energy consumption value (Ec) is expressed as mJ mg^{-1} weight wet tissue. Values are mean of 9 fish ± SEM. C, Control; S, Acute stress. Three-way ANOVA analysis results; D, Diet; O, dissolved oxygen levels; DOS, DO, DS, and OS, Interactions; ns, not significant $P > 0.1$; *$P < 0.05$; **$P < 0.01$.*

TABLE 5 | Effect of dietary electrolyte balance (DEB), dissolved oxygen levels and acute stress on energy use in heart of rainbow trout.

	DEB 200				DEB 700				Factors			Interactions			
	Normoxia		Hypoxia		Normoxia		Hypoxia								
	C	S	C	S	C	S	C	S	D	O	S	DOS	DO	DS	OS
LDH	596.40 ± 55.20	506.88 ± 41.59	736.26 ± 79.99	580.91 ± 53.58	596.66 ± 37.47	627.02 ± 42.02	556.57 ± 42.57	676.77 ± 45.19	ns	ns	ns	ns	ns	*	ns
IDH	452.34 ± 28.98	380.32 ± 22.44	443.31 ± 23.85	427.57 ± 20.33	440.96 ± 16.12	364.53 ± 30.59	390.43 ± 19.87	407.13 ± 17.12	ns	ns	*	ns	ns	ns	*
LDH/IDH	1.32 ± 0.08	1.34 ± 0.08	1.52 ± 0.09	1.35 ± 0.08	1.35 ± 0.07	1.78 ± 0.12	1.42 ± 0.06	1.67 ± 0.10	*	ns	*	ns	ns	*	ns
Ec	192.29 ± 11.73	182.52 ± 10.64	190.61 ± 22.37	160.44 ± 11.72	159.85 ± 18.85	141.11 ± 9.69	148.62 ± 9.36	208.08 ± 21.60	ns	ns	ns	*	*	ns	ns

*Lactate dehydrogenase (LDH) and iso-citrate dehydrogenase (IDH) activities are expressed as nmol min^{-1} mg^{-1} tissue. Energy consumption value (Ec) is expressed as mJ mg^{-1} weight wet tissue. Values are mean of fish (n = 9) ± SE. C, Control; S, Acute stress. Three-way ANOVA analysis results. D, Diet; O, dissolved oxygen levels; DOS, DO, DS, and OS, Interactions; ns, not significant $P > 0.1$; *$P < 0.05$; **$P < 0.01$.*

TABLE 6 | Effect of dietary electrolyte balance (DEB), dissolved oxygen levels and acute stress on several markers of oxidative stress in liver of rainbow trout.

	DEB 200				DEB 700				Factors			Interactions			
	Normoxia		Hypoxia		Normoxia		Hypoxia								
	C	S	C	S	C	S	C	S	D	O	S	DOS	DO	DS	OS
GPx	0.64 ± 0.09	0.41 ± 0.03	0.55 ± 0.04	0.46 ± 0.03	0.34 ± 0.04	0.48 ± 0.07	0.50 ± 0.03	0.47 ± 0.08	*	ns	ns	*	*	**	ns
GR	3.27 ± 0.44	4.09 ± 0.33	2.56 ± 0.25	3.13 ± 0.25	3.34 ± 0.32	4.59 ± 0.57	4.11 ± 0.40	3.72 ± 0.53	ns	ns	*	ns	ns	ns	ns
TG	1.14 ± 0.18	1.24 ± 0.09	1.06 ± 0.07	1.20 ± 0.07	1.28 ± 0.07	1.45 ± 0.11	1.28 ± 0.12	1.34 ± 0.13	*	ns	ns	ns	ns	ns	ns
GSSG	0.53 ± 0.08	0.45 ± 0.05	0.57 ± 0.06	0.41 ± 0.07	0.55 ± 0.06	0.61 ± 0.04	0.64 ± 0.05	0.54 ± 0.04	*	ns	ns	ns	ns	ns	ns
GSH	0.61 ± 0.11	0.79 ± 0.08	0.56 ± 0.06	0.79 ± 0.10	0.73 ± 0.07	0.84 ± 0.08	0.64 ± 0.10	0.86 ± 0.13	ns	ns	*	ns	ns	ns	ns
LPO	5.62 ± 0.43	5.81 ± 0.52	6.38 ± 0.93	5.70 ± 0.41	5.48 ± 0.28	6.07 ± 0.79	5.28 ± 0.71	5.11 ± 0.46	ns	ns	ns	ns	ns	ns	ns

*Glutathione peroxidase (GPx) and Glutathione reductase (GR) activities in nmol min^{-1} mg prot^{-1}. Total glutathione (TG), oxidized glutathione (GSSH), reduced glutathione (GSH), and lipid peroxidase (LPO) in nmol mg prot^{-1}. Values are mean of fish (n = 9) ± SE. C, Control; S, Acute stress. Three-way ANOVA analysis results. D, Diet; O, dissolved oxygen levels; DOS, DO, DS, and OS, Interactions; ns, not significant P > 0.1; *P < 0.05; **P < 0.01.*

TABLE 7 | Effect of dietary electrolyte balance (DEB), dissolved oxygen levels and acute stress on innate immune status in plasma of rainbow trout.

	DEB 200				DEB 700				Factors			Interactions			
	Normoxia		Hypoxia		Normoxia		Hypoxia								
	C	S	C	S	C	S	C	S	D	O	S	DOS	DO	DS	OS
ACH50	13.64 ± 2.12	15.80 ± 2.28	16.25 ± 3.25	20.43 ± 2.16	10.90 ± 2.09	10.19 ± 2.20	8.59 ± 0.96	10.72 ± 1.59	*	ns	ns	ns	ns	ns	ns
Lysozyme	522.22 ± 24.26	645.37 ± 44.03	633.33 ± 64.67	596.30 ± 50.29	570.37 ± 60.26	815.74 ± 138.37	544.79 ± 63.27	576.85 ± 35.05	ns	ns	*	ns	ns	ns	ns
Peroxidase	116.53 ± 4.46	123.04 ± 4.87	120.26 ± 5.80	119.74 ± 4.90	126.42 ± 3.91	106.08 ± 14.32	128.59 ± 4.58	125.60 ± 2.25	ns	ns	ns	ns	ns	ns	ns

*Alternative complement pathway ACH50, Lysozyme and Peroxidase activities expressed in U mL^{-1}. Values are mean of fish (n = 9) ± SE. C, Control; S, Acute stress. Three-way ANOVA analysis results. D, Diet; O, dissolved oxygen levels; DOS, DO, DS, and OS, Interactions; ns, not significant P > 0.1; *P < 0.05; **P < 0.01.*

linked to advantageous cardiovascular adaptations to enhance the animal's function (Faust et al., 2004). Previous studies have shown that chronic hypoxia in both zebrafish (*Danio rerio*) and the cichlid *Haplochromis piceatus* lead to decreases in the ventricular space, suggesting a profound restructuration of this organ (Marques et al., 2008). Our results also suggest that the trout population used in the trial displayed a good capacity to adapt to the chronic hypoxic conditions. In particular, the rainbow trout subjected to 49 days of hypoxia displayed a reduction in heart to body mass index (CSI), but increased the glycogen content compared to the normoxic group. Therefore, the rainbow trout isogenic line appears to have the capacity to fully adapt to the applied hypoxic levels, which was confirmed by the lack of significant change in all the markers of oxidative stress (liver) and innate immune status (plasma) markers investigated on this study.

Reliability of Markers for Acute and Chronic Stress

Changes in plasma cortisol levels are commonly used to assess acute stress conditions in fish, as a peak in the release of this hormone is associated with stress response. As expected, in this study, the cortisol levels in plasma of trout subjected to acute stressor were significantly increased when compared to their controls. However, the utility of changes in the levels of this cortisol to evaluate chronic stress in fish is not always possible due to several mechanisms reducing cortisol levels, including negative feedback (Pickering and Stewart, 1984; Mommsen et al., 1999) and the increased metabolic clearance rate of cortisol, all mechanisms which overall result in suppression of the cortisol response (see reviews Wendelaar Bonga, 1997; Gorissen and Flik, 2016). Thus, in rainbow and brown trout, chronic confinement stress for 6 weeks resulted in an elevation of plasma cortisol levels up to 4 weeks before returning to basal level after 42 days (Pickering and Pottinger, 1989; Person-Le Ruyet et al., 2008). A similar response was observed in Atlantic salmon parr or smolt exposed to repeated chasing stress over 3 weeks, when a decline in plasma cortisol levels was observed after 1 week, suggesting a rapid habituation/desensibilization of the HPI axis (Madaro et al., 2016). However, cortisol response to chronic stressors may vary according to species and/or the type of stressor. For example, seabass exposed for 57 days to water quality deterioration did not show any significant changes in the basal cortisol levels (Santos et al., 2010), whereas in other study in seabream and seabass, the same parameter increased after 3 weeks when exposed to three levels of chronic stress (confinement/chasing/air exposure) (Samaras et al., 2018). Another approach to characterize chronic stress status is the analysis of cortisol response to acute confinement stress, which affects the HPI axis responsiveness. This approach has been implemented in rainbow trout and salmon by applying chronic crowding stress or repeated acute stress over several weeks, resulting in a less pronounced cortisol response to acute stress (Madaro et al., 2015, 2016; Moltesen et al., 2016). In contrast, seabass exposed to water quality deterioration showed an increased cortisol response to acute stress (Santos et al., 2010) and a similar response was also observed in trout exposed to chronic hypoxia (Leguen and Prunet, unpublished). Overall, this data indicates that chronic stress generally leads to dysregulation of cortisol production (basal and/or acute stress levels) which varies according to species and stressor. In the present study, exposure of rainbow trout to chronic low DO levels did not modify cortisol levels nor cortisol response, which fits with our previous conclusions that indicate fish were adapted to these hypoxic conditions.

In this study, the prolonged dietary challenge increased the cortisol levels in plasma of trout 1.23 times. However, such increase was modest compared to the spike of 4.08 times in plasma cortisol levels of trout when subjected to the acute stressor. In spite of these observed changes, cortisol levels were not significantly different in trout fed the diets with different DEB and then subjected to acute stress ($P < 0.057$), also indicated by the lack of interactions between both factors ($P = 0.284$). These results may suggest that the capacity of trout to mount a proper cortisol response when subjected to an acute stressor remained unaltered by the dietary treatment. These results contrast with studies presented in the previous paragraph where chronic stress leads to a clear dysregulation of cortisol responsiveness in fish. Therefore, additional studies are required to further appraise how the cortisol response and the allostatic load of trout may be altered under chronic stressors, for example when subjected to dietary challenges.

No detectable changes were observed in LPO, a marker of oxidative damage, in the liver of trout exposed to the experimental conditions, even when facing the acute stressor. However, components of the glutathione system (GR and GSH) were increased in liver of trout subjected to the acute stressor, indicating an alteration on the balance between prooxidants/antioxidants in this organ. In addition, another component of the glutathione system, the GPx activity in liver, displayed an interaction between dietary treatment and acute stress, as well as for all three factors together ($P < 0.01$), suggesting that the prolonged dietary challenge places a constraint in trout exposed to a subsequent acute stressor.

The innate immune system in fish is constitutive, reacting at short time scale to non-specific cues and induced by several external molecules (Tort et al., 2003; Tort, 2011). In particular, lysozyme activity is used as an indicator of non-specific immune response (Tort et al., 1996, 1998), rapidly induced in fish exposed to acute stressors (Demers and Bayne, 1997; Rotllant and Tort, 1997). Although lysozyme activity can vary considerably between fish species, this enzymatic activity in plasma appears to be modulated in response to both physical and nutritional cues (Möck and Peters, 1990; Montero et al., 1999b). The increased lysozyme activity in plasma of trout subjected to acute stress observed in this study suggest a link between stress and suppression of the innate immune response, which has important implications for aquaculture. As it was suggested earlier by Fevolden et al. (1999), it is possible to hypothesize that the enhanced lysozyme activity after exposure to the acute stressor may not be directly related to pathogen resistance, and may rather reflect disease-susceptible fish. In this study ACH_{50}

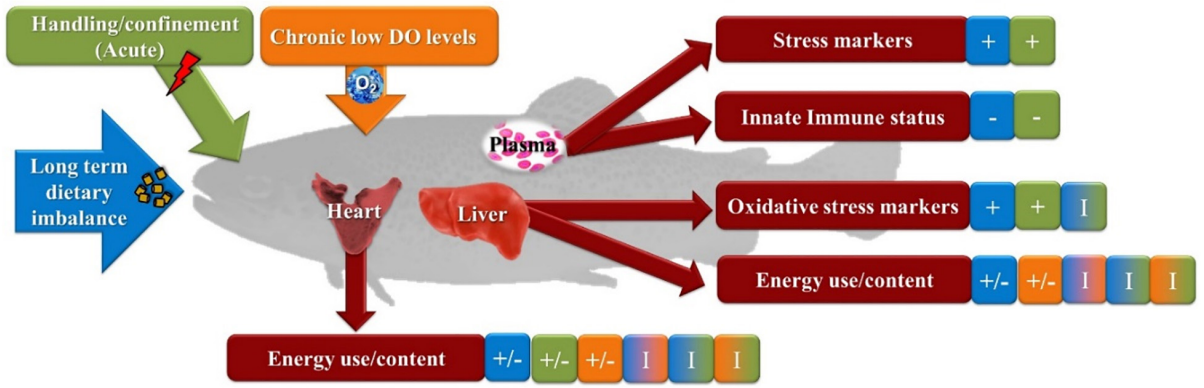

FIGURE 1 | Summarized output of biomarkers studied in rainbow trout exposed to a combination of chronic and acute conditions. Fish were fed a balanced- or an electrolyte-imbalanced diet (DEB 200 or DEB 700, respectively) and reared at different environmental oxygen levels (normoxia or hypoxia) for 49 days. Subsequently fish were subjected to a standard acute stress protocol (2 min of handling/confinement) and compared to undisturbed group (control). +, increased by the condition. −, decreased by the condition. I, Interactions.

activity was decreased by the dietary imbalance, where lysozyme activity was decreased by the acute stressor in plasma of rainbow trout. Surprisingly, no interaction between the two factors was detected for any of the innate immune status parameters analyzed. Therefore, it will be necessary to further investigate how chronic stressors may be affecting not only the innate but also the adaptive immune response of rainbow trout exposed to acute challenges.

A summarized description of the results obtained from this study is presented in **Figure 1**. This study suggest that the trout isogenic line displays a complete adaptive response to chronic hypoxia, whereas the challenge imposed by feeding an electrolyte-imbalanced diet profoundly affected fish homeostasis after 49 days. Such physiological condition in fish appear to lead to stress response as indicated by several markers of oxidative stress and decreased innate immune response. Although no interactions in the markers for innate immune response were detected between the different factors analyzed, the dietary challenge, by feeding trout the DEB 700 diet, suggests an impairment of the physiological response in this fish to a subsequent acute stressor. This is shown in this study by an increase in the levels of several oxidative stress markers detected in liver of trout exposed to the acute stressor in trout fed the electrolyte-imbalance diet. Nevertheless, the results presented here were obtained in an isogenic line and may not necessarily reflect the response from other rainbow trout used in aquaculture.

CONCLUSION

In conclusion, this study suggests that dietary imbalances present during rearing may act as chronic stressors, interacting with other well-established stressors such as the acute stressors applied on this study. These results are of great interest to the aquaculture industry as both of these factors may be present in cultured fish and could be linked to negative effects. Future studies should further investigate the physiological and behavioral aspects associated to such stress responses, and how to reduce their occurrence.

ETHICS STATEMENT

The fish trials were approved and carried out according to the Wageningen University Ethics Board for experimentation with animals (DEC, Registration protocol 2014056.a), under Dutch and EU legislation on the handling of experimental animals.

AUTHOR CONTRIBUTIONS

LM, IG, PP, and JS conceptualized the study. LM, SN, EE, ML, RO, IL, PP, IG, and JS were involved in the methodology and formal analysis. LM, SN, PP, and JS were involved in writing the original draft. All authors reviewed the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2019.00453/full#supplementary-material

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Influence of Stress on Liver Circadian Physiology. A Study in Rainbow Trout, *Oncorhynchus mykiss*, as Fish Model

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In vertebrates stress negatively affects body homeostasis and triggers a battery of metabolic responses, with liver playing a key role. This organ responds with altered metabolism, leading the animal to cope with the stress situation, which involves carbohydrate and lipid mobilization. However, metabolism among other physiological functions is under circadian control within the liver. Then, metabolic homeostasis at system level involves circadian timing systems within tissues and cells, and collaborate with each other. During chronic stress, cortisol maintains the liver metabolic response by modulating carbohydrate- and lipid-related metabolism. Stress also disrupts the circadian oscillator within the liver in mammals, whereas little information is available in other vertebrates, such as fish. To raise the complexity of this process, other candidates may mediate in such effect of stress. In fact, sirtuin1, a link between cellular sensing of energy status and circadian clocks, participates in the response to stress in mammals, but no information is available in fish. Considering the role played by liver in providing energy for the animal to deal with an adverse situation, and the existence of a circadian oscillator within this tissue, jeopardized liver circadian physiology during stress exposure might be expected. Whether the physiological response to stress is a well conserved process through the phylogeny and the mechanisms involved in such response is a question that remains to be elucidated. Then, we provide information at this respect in mammals and show comparable results in rainbow trout as fish animal model. Similar to that in mammals, stress triggers a series of responses in fish that leads the animal to cope with the adverse situation. Stress influences liver physiology in fish, affecting carbohydrate and lipid metabolism-related parameters, and the circadian oscillator as well. In a similar way than that of mammals different mediators participate in the response of liver circadian physiology to stress in fish. Among them, we confirm for the teleost rainbow trout a role of nuclear receptors (*rev-erb*β), cortisol, and sirt1. However, further research is needed to evaluate the independent effect of each one, or the existence of any interaction among them.

Keywords: stress, liver, metabolism, clock genes, mammals, fish, rainbow trout

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INTRODUCTION

When an animal is subjected to stress coordinated behavioral and physiological responses initiate in order to compensate and/or adapt to a new situation. However, the animal is enabled to overcome such threat. When the animal experiences intense stress, the response becomes dysfunctional, and can lose its adaptive value, thus probably resulting in inhibited growth, reproductive failure, and decreased resistance to pathogens, among other negative effects (Barton et al., 1987; Barton and Iwama, 1991). The physiological responses to a stressor are either specific for a single, or a group of related stressors (more typical), or non-specific, commonly due to different stressors. All the levels of animal organization are involved in a process known as integrated response to stress (Wendelaar-Bonga, 1997). By other hand, depending on the duration, stress can be considered as acute or chronic. Acute stressors are events that animal experiences for a short time period, for example, handling procedures. Chronic stress refers to a given situation that persists for longer time periods, thus with the physiological response of the animal being permanently activated (Tort and Teles, 2011). Among other negative effects, prolonged exposure to stress causes alterations in dendritic architecture, synapse density, and neurogenesis within the brain (Shonkoff et al., 2009). Also, gene expression can change, among other effects (for a review, see McEwen et al., 2015). Hypothalamic–pituitary–adrenal gland (HPA) activation persists during repeated stress, which leads to increased risk of depression and anxiety disorders (McEwen, 2008), as consequence of altered corticosterone system, the orexin system and others (Sargin, 2018). Whatever the case, stress may have severe negative effects on animal's welfare.

With respect to fish, this vertebrate group have also developed mechanisms to cope with stress-induced alterations of internal homeostasis, which is indicative of the physiological stress response to be a marked adaptive character (Wendelaar-Bonga, 1997) all over vertebrates. Although the most frequent stressors in fish are those related to changes in water quality (temperature, salinity, oxygenation, pH, contaminants) and interaction with individuals of the same or different species (competition, predation, etc.) (Wendelaar-Bonga, 1997; Gesto et al., 2013), intensive fish aquaculture brought new potential stressful situations, such as high stock density, confinement, low water renovation, the presence of sick individuals, transport, altered photoperiod and feeding schedule, etc. Then welfare might be adversely affected, which influences fish growth and plant productivity.

In vertebrates, it is well described that a stressful stimulus triggers a response of two neurohormonal pathways: (1) the hypothalamic–sympathetic–chromaffin (HSC), which once activated enhances plasma catecholamines level very quickly (even seconds) and their half-life is brief (minutes); (2) the HPA, which is known as hypothalamic–pituitary–interrenal cells (HPI) in fish, and once activated, cortisol synthesis and release tend to rise slowly and remaining high when stress persists over time (chronic stress). Regarding this HPA/HPI axis, stress is responsible of glucocorticoids production and release from the adrenal gland, which is fish locates at the head kidney. Then, the hypothalamic paraventricular nucleus receives stress signals from hippocampus, amygdala and prefrontal cortex, and stimulates CRH secretion, thus activating the HPA axis. Glucocorticoids need to be newly synthesized, then delaying the response. As a consequence, slower dynamic than that of HSC is observed.

In the same way than that of other vertebrates, these axes in fish are regulated by specific biochemical factors, including adrenocorticotropic hormone (ACTH), corticotropin-releasing factor (CRF), arginine vasotocin (AVT), and brain neurotransmitters, DA, NA, and 5-HT (Winberg and Nilsson, 1993; Wendelaar-Bonga, 1997; Balment et al., 2006). This vertebrate group is also characterized by hormonal dynamics being differentially affected the type of stress, intensity, species and their previous story (Barton et al., 1987; Barton, 2002; Aluru and Vijayan, 2009; Gesto et al., 2013), which makes difficult to fully understand how stress is acting.

Among many other effects all over the body, stress hormones induce metabolic reprogramming in a tissue-specific way, in order to deal with the increase of energy demand during stress exposure, thus affecting specific tissues such as the liver. In this way, cortisol effects on metabolism have been extensively studied in mammals, with the hormone increasing glucose availability in liver through activating the major gluconeogenic pathways and the synthesis of glycogen, together with the inhibitory effect of the ability of other tissues to capture glucose (Goldstein et al., 1992, 1993; Fujiwara et al., 1996). In fish it has been described a great variety of metabolic effects of cortisol depending on the species and situations in which high levels of cortisol are generated. However, there is a consensus on the strong increase in gluconeogenesis and lipolytic potential in liver as a consequence of the stress-induced increase of plasma cortisol levels (Vijayan et al., 1991, 1994; López-Patiño et al., 2014b). It is also well known that cortisol metabolic action in fish is mediated by glucocorticoid receptors (GRs), which in most teleost fish display two isoforms (GR1 and GR2) that locate in a large number of central and peripheral tissues (Ducouret et al., 1995; Teitsma et al., 1997, 1998; Jaillon et al., 2004).

By other hand, metabolism, together with many other physiological functions is under circadian control in all living organisms in such a way that metabolic homeostasis at system level needs the timed collaboration of cells and tissues all over the body. Such rhythms are driven by cell autonomous clocks at central and peripheral locations, which synchronize the organism to the environmental cycles, even in the absence of environmental cues (Hardin and Panda, 2013). Clocks molecular mechanism is highly conserved in phylogeny and although with some differences, it is easily identifiable in the different organisms. The basic mechanism involves a series of feedback loops between the transcription and transduction processes of certain genes (called "clock genes") and their protein products (Panda et al., 2002). The most known model determines that the system initiates with the accumulation of cytoplasmic CLOCK and BMAL1 proteins as the products of *clock* and *bmal1* transcription. These proteins form a heterodimer (CLOCK/BMAL1) that returns to the nucleus, joining E-Box promoters in the target genes, including the negative branch genes of the loop: *per* and *cry*. Their transcription results in increased cytoplasmic levels of their protein products,

PER and CRY, so that when the levels are high enough they dimerize, thus inhibiting the CLOCK/BMAL1 complex function (Kondratov, 2007).

Mammalian circadian system is the most studied so far, and the suprachiasmatic nucleus (SCN) is considered the main pacemaker. Then, the SCN hosts the master oscillator containing specific molecular elements (clock genes) that create/control circadian rhythms of most functions all over the organism. In support of this, behavioral, endocrinological, and physiological circadian variations disappear in lesioned SCN (Moore et al., 1995). SCN receives information mainly through three different pathways: retinohypothalamic tract (RHT), intergeniculate leaflet (IGL), and medial nucleus of the rafe (RM). RHT pathway originates at the retina and is the main photic-related input, thus playing a critical role in generating circadian rhythms (Weaver, 1998; Gooley et al., 2001), whereas the other pathways participate as inputs of photic information to the suprachiasmatic nucleus (IGL) or non-photic information (RM). In addition, rhythms of clock genes expression has been reported in tissues other than the SCN, such as liver, muscle, adipose tissue, pancreas, kidney, lung, and ovary (Balsalobre, 2002; Mühlbauer et al., 2004; Peirson et al., 2006; Zvonic et al., 2006), which is indicative of the existence of circadian oscillators in them all.

In non-mammalian vertebrate groups multiple coupled central circadian oscillators exist. These oscillators can locate within different tissues such as retina, pineal gland and hypothalamus, but their functioning appear to remain quite similar to that of mammals (Menaker et al., 1997; Falcón et al., 2010; López-Patiño et al., 2011). In addition to hypothalamus in fish, retina and pineal organ also host circadian oscillators, since both tissues are photosensitive, clock genes express rhythmically within them, and release an endocrine output, such as the melatonin (Falcón, 1999; Falcón et al., 2007). In the same way than that of mammals, clock genes rhythmically express in peripheral tissues of non-mammalian vertebrates, such as liver, heart, intestine, and muscle of birds (Chong et al., 2003), liver, heart, muscle, lung, and testis of reptiles (Della Ragione et al., 2005; Vallone et al., 2007), and liver, heart, spleen, and gall bladder of fish (Kaneko et al., 2006; Velarde et al., 2009; Betancor et al., 2014; Hernández-Pérez et al., 2017).

Specifically for fish, the circadian system is typically composed by multiple oscillators located throughout the body, and mainly entrain to external inputs, LD and feeding-fasting cycles, among others. Coordination among them all leads for successful control of different rhythmic functions (locomotor activity, hormonal rhythms. . .). The synthesis and release of specific outputs allow them to adjust all these functions.

One of the peripheral locations described for the circadian system is the liver. In fish, some reports describe rhythmic expression of clock genes within this tissue in different species such as goldfish (*per* and *cry,* Velarde et al., 2009), Atlantic salmon (*bmal1*, Betancor et al., 2014) and rainbow trout (*clock*, *bmal1*, *per* and *rev-erbβ*, Hernández-Pérez et al., 2017). Interestingly, such rhythms perfectly fit with those of metabolism-related parameters within this peripheral location (Polakof et al., 2007; Betancor et al., 2014; Paredes et al., 2014; Hernández-Pérez et al., 2015).

Independently of where the oscillatory machinery is located, its functioning can be disrupted by different factors. Among them, stress is the most studied so far. Accordingly, glucocorticoids and epinephrine synchronize circadian tissue clocks (Balsalobre et al., 2000; Akiyama et al., 2003; So et al., 2009), but stress affects the circadian system in many other ways. For example, the effectors of the stress response exert their action through specific receptors. Regarding cortisol as the main glucocorticoid, the hormone displays daily rhythms of plasma levels and binds to either GRs or mineralocorticoid receptors (MR), with the latest binding to cortisol even at the time of the day in which plasma glucocorticoid levels are in the minimum. This makes MR signaling pathway not to be effective in conveying time-of-day information (see rev. Koch et al., 2017). The other mediator, GR, is expressed all over the organism (Kino and Chrousos, 2004; Chrousos and Kino, 2005; Nader et al., 2010), but not at the SCN, which makes this tissue not to get any synchronizing feedback through GRs in vertebrates (Balsalobre et al., 2000), whereas no information is available at this respect in other groups such as fish.

Regarding GR signaling, classical and non-classical pathways are reported (Beato et al., 1987; Freedman, 1992; Groeneweg et al., 2012) and contribute to a high level of complexity. Among the classical ones, the interaction of GR dimers with glucocorticoid response elements within regulatory regions of GC target genes is the most studied so far, and such elements are identified in some clock genes (So et al., 2009). Transcription of target genes can be also activated by other transcription factors, nuclear factor-κB (NF-κB), activator protein-1 (AP-1), or STAT5 (Scheinman et al., 1995; Garside et al., 2004; Kino and Chrousos, 2004; Chrousos and Kino, 2005; De Bosscher and Haegeman, 2009). Additionally, GR binding to negative glucocorticoid response elements mediates the *trans*-repression of negatively regulated genes (Surjit et al., 2011). The interaction of GRs with DNA can influence surrounding DNA-bound transcription factors as well (Groeneweg et al., 2012; Samarasinghe et al., 2012). The activation of one of these pathways takes place in minutes to hours. On the contrary, the non-classical signaling is independent of transcription and gene expression (Groeneweg et al., 2012), thus resulting in a faster response (seconds to minutes). Altered activity of some kinases (phosphoinositide 3-kinase, PI3K; AKT, and mitogen-activated protein kinases, MAPKs) is responsible of this pathway.

The influence of stress on circadian rhythms has been addressed in rodents. Accordingly, clock genes phase advance in peripheral organs of acutely stressed mice during the early day (Tahara et al., 2015). On the contrary, stress exposure at different time of the day causes a phase delay or the loss of synchrony. This is indicative of the time of the day-dependence of the influence of stress on peripheral clocks. However, no changes were observed within the SCN, where GRs do not express in this vertebrate group, but chronically repeating a given procedure, such as social defeat results enhances the amplitude of *Per2* rhythm within the SCN (probably as consequence of activated indirect mechanisms), and downregulates *Per2* and *Cry1* expression within the adrenal gland of animals stressed

at the early dark phase, whereas stress the early day phase advances the adrenal oscillator but has no effect on the SCN clock (Bartlang et al., 2014).

Studies at this respect in other vertebrate groups such as fish are scarce. In this way, goldfish receiving a cortisol administration display similar results than those observed in mammals, with inhibited expression of some clock genes within the liver (Sánchez-Bretaño et al., 2015). However, no evidence exists relative to how stress affects rhythmic physiology within this tissue in this vertebrate group, but recent results indicate altered metabolism-related parameters within rainbow trout liver following acute stress (López-Patiño et al., 2014b), where a circadian oscillator has been reported to exist in the same species (Hernández-Pérez et al., 2015, 2017).

On the other side, chronic or repeated exposure to a stressor makes the body to adapt, resulting in altered functions such as energy metabolism, which may raise the incidence of metabolic disorders, as reported for humans and rodents (see rev. Koch et al., 2017). Even when it is of high clinical interest to address the impact of social stress on circadian functions, it is likely that available data are not sufficient, since many studies do not report the time of stress exposure, and just few compare the impact of the stressor all over the day. Also, stress response is dependent of the stressor (Gattermann and Weinandy, 1996), which makes difficult to compare results among studies. Depressive and anxiety-related behaviors are reported for rat subjected to chronic mild stress applied only during the light phase (Aslani et al., 2014). Also, other stressors (cat smell, tail shock, and immobilization) are more effective when applied when animals are in their phase of inactivity (Retana-Marquez et al., 2003; Cohen et al., 2015; Fonken et al., 2016).

Regarding fish, chronic stress might negatively affect the circadian system, followed by the alteration of rhythmic behavioral and physiological functions. In fact, endocrine rhythms are outputs of the circadian system, whereas some hormones may play a role as inputs to the circadian system in hypothalamic and peripheral oscillators (Challet, 2015; Coomans et al., 2015). Among them, glucocorticoids, as stress response mediators, display daily rhythms in fish (in the same way than other vertebrate groups), and cortisol rhythm in fish synchronizes to feeding fasting cycle and feeding time (see rev. Isorna et al., 2017). This is indicative of the hormone to be an output of the circadian system, thus being under circadian control. Accordingly, daily variations of plasma cortisol was reported for different fish species (Cerdá-Reverter et al., 1998; Pavlidis et al., 1999; Small, 2005; Ebbesson et al., 2008) and even related with feeding and metabolism, suggesting a possible synchronization of the cortisol secretion with feeding time (Spieler and Noeske, 1984). However, it is not defined whether cortisol is an input to the circadian system in fish or not. It is reported that cortisol stimulates the *per1a* and *per1b* expression, and inhibits that of *clock* and *bmal1* in liver of goldfish (Sánchez-Bretaño et al., 2016). Then, cortisol could mediate the effects of stress on liver circadian physiology in fish. Our preliminary results in rainbow trout point to such role, since liver of mild-stress animals shows decreased amplitude of *clock1a* and *bmal1* mRNA abundance daily rhythms, together with altered *per1* rhythm (unpublished data). Then, glucocorticoids may play a key role as mediators of the altered functioning of the circadian system observed in stressed fish.

Taking into account the interactions between cortisol, metabolism and circadian system, it is possible that the alteration of cortisol levels as a consequence of stress can trigger effects on liver metabolism and circadian system in fish, as in mammals. Accordingly, our aim was to elucidate how stress affects gene expression of clock- and metabolism-related genes, and to corroborate if metabolic response to chronic stress exposure results from altered circadian machinery within the liver of rainbow trout (*Oncorhynchus mykiss*), in the same way than that reported for other vertebrate groups.

MATERIALS AND METHODS

Animals

Rainbow trout (*Oncorhynchus mykiss* Walbaum) of 94 ± 8 g of body weight were transferred to our facilities (Faculty of Biology; Vigo, Spain) from a local hatchery (A Estrada, Spain). Animals adapted to our laboratory conditions for at least 15 days before any experiment was performed. Then, fish remained in 120 L tanks (10 kg of fish/m^3) with filtered and continuous water renovation (13.5 ± 1°C). Food consisted on a commercial (Dibaq Diproteg, Segovia, Spain) dry pellet diet (1% body weight), and was provided at zeitgeber time (ZT/CT) 2. Trout were kept in a 12:12 LD photoperiod, with lights on at ZT0. Light intensity was 500 lux at the water surface during photophase, and did not exceed 0.3 lux at scotophase. Experiments comply with European Union Council Guidelines (2010/63/EU), and Spanish Government (RD 53/2013) for the use of animals in research. Also, the Animal Care Committee at the Vigo University approved all the animal protocols, following the international ethical standards (Portaluppi et al., 2010).

Sampling

Fish were deeply anesthetized with MS-222 (50 mg L^{-1}) buffered to pH 7.4 with sodium bicarbonate, and weighed. Animals were sacrificed and sampled every 4 h during a 24-h light/dark cycle, starting at ZT0 (lights on). Accordingly, samplings time points were ZT0, ZT4, ZT8, ZT12, ZT16, ZT20, and ZT0′ on the following day. Time needed for sacrifice and sampling procedures was never longer than 15 min/time point. Once deeply anesthetized, caudal puncture with ammonium heparinized syringes was performed for individual blood collection. Fish were sacrificed immediately after, and liver from each animal was dissected and divided into two portions under sterile conditions. Each portion was placed in sterile RNase-free 1.5 mL Eppendorf tubes, and immediately frozen in liquid nitrogen, and stored at −80°C until assayed for metabolites content, enzyme activities, and gene expression quantification. Blood was centrifuged to obtain plasma samples that were immediately frozen on liquid nitrogen and stored at −80°C until assayed for cortisol, glucose and lactate levels.

Experimental Design

To evaluate the impact of stress on liver rhythmic physiology in rainbow trout, two experimental groups of fish (N = 56) were randomly distributed in seven tanks each, which were initially acclimated to standard lighting conditions (L:D 12:12) for at least 2 weeks. After that, one of the groups of fish remained in the same condition (Control, C), while the second one was submitted to high density stress (Stress, ST), by reducing water level of the tanks up to reach a density of 70 kg of fish/m^3 (Conde-Sieira et al., 2014). Fish remained 72 h in these experimental conditions, being fed at the same time as during the acclimatization phase (ZT2). On the last day fish were captured from each tank (control and stress groups) and sampled as mentioned above.

Assessment of Cortisol Levels and Liver Enzyme Activities

The Enzyme Immunoassay Kit (Cayman, Ann Arbor, MI, United States) was purchased for plasma cortisol assessments, following manufacturer's specifications. A portion of liver was assessed for enzyme activities. Then, homogenization by ultrasonic disruption was performed for each, in 10 vol. ice-cold buffer: 50 mmol L^{-1} Trizma (pH 7.6), 5 mmol L^{-1} EDTA, 2 mmol L^{-1} 1,4-dithiothreitol, and a protease inhibitor cocktail (Sigma P-2714). The homogenate was centrifuged (10 min at 10,000 × g) and the supernatant collected for enzyme assays. The INFINITE 200 PRO microplate reader (Tecan, Grödig, Austria) was used for enzyme activities determination. The reaction rate of each enzyme was determined by the increase/decrease of NAD(P)H absorbance at 340 nm. Reactions initiated after addition of homogenates (15 μL), at a pre-established protein concentration, omitting the substrate in control wells. Then, reactions did proceed at 20°C for 3–10 min. Enzyme activities were expressed as relative to mg of protein. Then, protein content in each homogenate was assayed in triplicate following the bicinchoninic acid method with BSA (Sigma, Saint Louis, MO, United States) as standard. Enzymatic analyses were performed at maximum rates, with the reaction mixtures set up in preliminary tests to render optimal activities. GK, PEPCK, G6Pase, PK, HOAD, and FAS activities were evaluated as described previously (Polakof et al., 2007, 2008; Librán-Pérez et al., 2012).

Real-Time Quantitative RT-PCR (qPCR)

The TRIzol® (Life Technologies, Grand Island, NY, United States) method was used for total RNA extraction in individual rainbow trout liver. The extract was mixed with RQ1-DNAse (Promega, Madison, WI, United States). Then, 2 μg of RNA from each sample was reverse transcribed into cDNA, for which M-MLV reverse transcriptase (Promega) and Random Primers (Promega) were used. To discard genomic contamination of the RNA extract a negative control of each sample was assessed in the absence of reverse transcriptase.

To perform the qPCR, Maxima™ SYBR Green qPCR Master Mix (Thermo Scientific, Waltham, MA, United States) and the Bio-Rad MyIQ Real Time PCR system (BIO-RAD, Hercules, CA, United States) were used. All primers and probes (**Table 1**) were designed according to existing sequences for rainbow trout genes, and obtained from Sigma.

Relative quantification of each gene transcript was assessed and β-*actin* expression was selected as housekeeping, since it does homogeneously express through the 24-h cycle independently of the experimental condition. Thermal cycling initiated with 3 min incubation at 95°C; followed by 40 steps of PCR (heating for 10 s at 95°C for denaturing, and specific annealing for 30 s and extension at 50°C for 30 s). After the last PCR cycle, melting curves were monitored (50°C temperature gradient at 0.5°C/s from 50 to 95°C) to corroborate that only one fragment was amplified. Relative mRNA expression level was calculated using the standard comparative delta-Ct method. Relative quantification of each gene transcript with the β-*actin* reference gene transcript was evaluated according to the Pfaffl method (Pfaffl, 2001). For each gene, samples from the same time point were processed in parallel, and expression was assessed in triplicate within the same microplate. Only efficiency values ranging from 85% to 100% were accepted (R^2 for each gene was always higher than 0.985).

Statistical Analysis

To determine the existence of significant differences of gene expression between time points within a given experimental condition (C and ST) and gene, the one-way ANOVA analyses were carried out, followed by the Student–Newman–Keuls test for multiple comparisons. Also, the rhythm of expression for each gene was analyzed by fitting periodic sinusoidal functions to the gene expression levels across the sampling time points using the formula $f(t) = M + Acos(t\pi/12 - \varphi)$. Thus, $f(t)$ reflected gene expression level at a given time point, the mesor (M) was the mean value, A was the sinusoidal amplitude of the oscillation, t was time in hours, and φ was time of the peak (acrophase). Non-linear regression allowed to estimate M, A, and φ, and their standard error (SE) (Delgado et al., 1993). For the sinusoidal function, all parameters were expressed as the value ± standard error (SE). The SE based on the residual sum of squares in the least-squares fit. A rhythm of expression was consistent only if either $P < 0.05$ from the ANOVA test, and $SE(A)/A < 0.3$ provided by the cosinor analysis, according to the principle of a noise/signal ratio less than 0.3, with "signal" being the amplitude and "noise" its error (Halberg and Reinberg, 1967).

RESULTS

Plasma Metabolite Levels

Cortisol, glucose, and lactate plasma contents are represented in **Figure 1**. In Control, cortisol levels were highest at the early light period (ZT4) and basal levels were observed during the light-dark transition (ZT12), when started to rise slowly. Stress by high stocking density enhanced cortisol levels, which resulted in a significant raise of mean cortisol levels (58.53 ng/mL) relative to that of Control (29.01 ng/mL), and altered rhythmic profile of the hormone. Then stressed trout displayed peaking cortisol levels during the night (ZT16) and basal levels at the very end of the dark phase (ZT0′).

TABLE 1 | Primers' sequences, forward (F), and reverse (R) of different genes measured along the study, with the specific annealing temperature and reference for each gene.

Gene	Sequence	Annealing T[a]	References
gk	**F:** *GCACGGCTGAGATGCTCTTTG*	60	AF053331 (GenBank)
	R: *GCCTTGAACCCTTTGGTCCAG*		
pk	**F:** *CCATCGTCGCGGTAACAAGA*	59	AF246146 (GenBank)
	R: *ACATAGGAAAGGCCAGGGGC*		
pepck	**F:** *GTTGGTGCTAAAGGGCACAC*	59	AF246149 (GenBank)
	R: *CCCGTCTTCTGATAAGTCCAA*		
g6pasa	**F:** *CTCAGTGGCGACAGAAAGG*	55	cay0019b.d.18_3.1.s.om.8.1-1693 (Sigenae)
	R: *TACACAGCAGCATCCAGAGC*		
GLUT2	**F:** *GTGGAGAAGGAGGCGCAAGT*	59	AF321816 (GenBank)
	R: *GCCACCGACACCATGGTAAA*		
fas	**F:** *GAGACCTAGTGGAGGCTGTC*	59	tcab0001c.e.06 5.1.s.om.8 (Sigenae)
	R: *TCTTGTTGATGGTGAGCTGT*		
hoad	**F:** *GGACAAAGTGGCACCAGCAC*	59	Tcad0001A.l.15_3.1.om (Sigenae)
	R: *GGGACGGGGTTGAAGAAGTG*		
GR1	**F:** *AGAAGCCTGTTTTTGGCCTGTA*	59	NM_001124730.1 (GenBank)
	R: *AGATGAGCTCGACATCCCTGAT*		
GR2	**F:** *CATCGCAGACCAGTCTGAAC*	55	AY495372.1 (GenBank)
	R: *AGCAGCAGCAGAACCTTCAT*		
clock1a	**F:** *CTCAAGACGAAAAACCAGTTAGAA*	57	AF266745 (GenBank)
	R: *AGGCTCTTTGGGGTCGAT*		
bmal1	**F:** *TGGACATTTCCTCCACGATG*	55	GQ489026.1 (GenBank)
	R: *TCTTGTCCCTGCGTCTCTTC*		
per1	**F:** *AAGTCGTAGAGGAAGACCCA*	55	AF228695 (GenBank)
	R: *ATCTGTCTGCACATACCGCT*		
rev-erbβ	**F:** *AGCAGTGCCGCTTCAAGA*	56	AF342943.1 (GenBank)
	R: *CGGCCAAACCTAACAGAGTC*		
sirt1	**F:** *GCTACTTGGGGACTGTGACG*	55	EZ774344.1 (GenBank)
	R: CTCAAAGTCTCCGCCCAAC		
b-actin	**F:** *GATGGGCCAGAAAGACAGCTA*	55	NM_001124235.1 (GenBank)
	R: *TCGTCCCAGTTGGTGACGAT*		

Plasma glucose levels showed a daily oscillation in control animals, with higher values at day-time (ZT4) and basal levels at the night onset (ZT12). Stress caused an increase in the averaged levels (6.86 mM), compared to Control (4.60 mM), but also the daily oscillation to disappear.

Lactate levels displayed a similar daily oscillation that that of glucose, with higher values being observed in samples collected at ZT8, and basal levels during the late night period (ZT20). Stress exposure resulted in the increase of averaged lactate levels (2.14 mM) compared to that of control group (1.77 mM), but also the daily oscillation changed, thus with the highest levels occurring at the day–night transition (ZT12), i.e., with a 4-h delay.

Liver Metabolite Levels

Hepatic levels of glycogen, glucose and lactate in control and stressed trout are represented in **Figure 2**. Glycogen content in control fish daily oscillated, thus being higher during the day onset (ZT0) and being low during the second half of the night (ZT20). Averaged content was 156.4 ± 11.2 μmol/g tissue. Stress exposure resulted in a 20% decrease of averaged glycogen content up to 125.6 ± 7.6 μmol/g tissue, together with the loss of the daily fluctuation.

No significant daily oscillation was observed for glucose and lactate content in liver of control non-stressed trout. Averaged contents were 32.9 ± 0.7 μmol/g tissue, and 2.3 ± 0.1 μmol/g tissue, respectively. When trout were subjected to high stocking density the averaged content of each metabolite significantly decreased (glucose: 25.6 ± 0.3 μmol/g tissue; lactate: 0.8 ± 0.1 μmol/g tissue) relative to Control.

Carbohydrate Metabolism-Related Parameters

The daily profile of enzyme activity and mRNA abundance of carbohydrate metabolism enzymes, and glucose transporter expression (GLUT2) in trout liver of Control and Stress groups is shown in **Figure 3**. The *cosinor* analysis corroborated the presence of significant rhythms for most of them. Accordingly, GK activity in liver of control fish displayed a daily variation with significantly higher values during the day (ZT4) and basal levels at night (ZT20). Averaged activity along the 24-h cycle was of 2.71 mU/mg prot. Stress caused a drastic increase of averaged

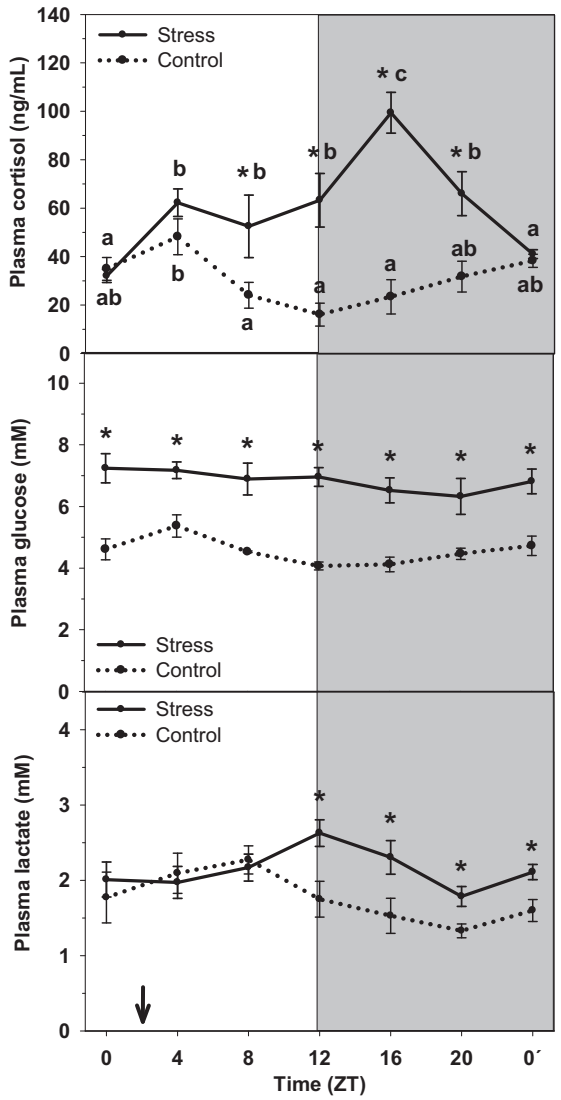

FIGURE 1 | Daily profile of plasma cortisol, glucose and lactate plasma levels in control (dashed line) and stress (continuous line) rainbow trout. Each value is the mean ± SEM (n = 8/group). *$P < 0.05$ relative to Control at the same time point. Different letters indicate significant differences ($P < 0.05$) between time points within the same experimental group. Gray band indicates the dark phase of the daily cycle. The arrow indicates feeding time (ZT2).

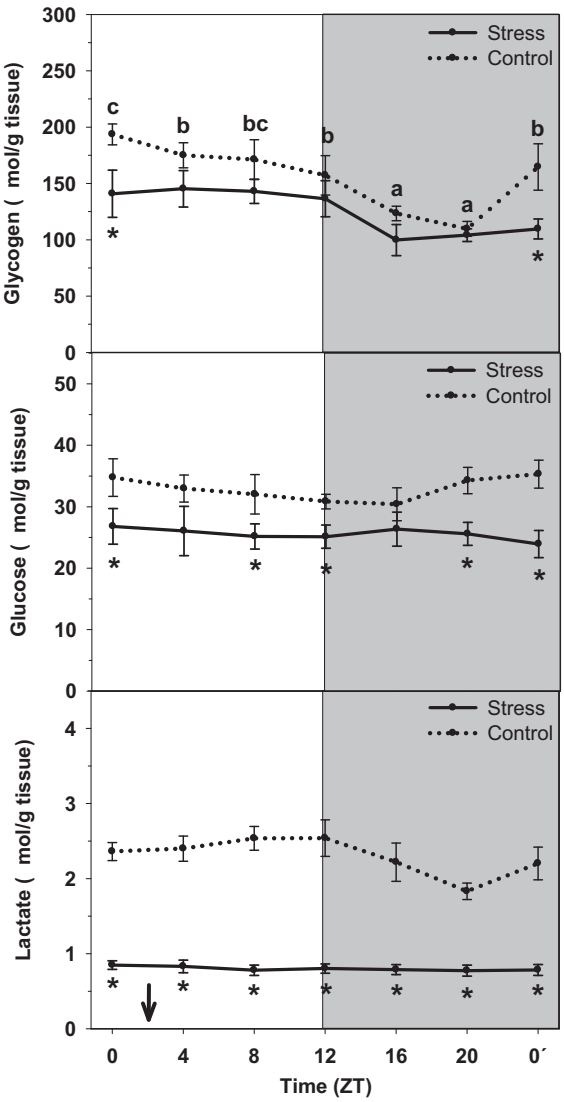

FIGURE 2 | Daily profile of glycogen, glucose and lactate liver levels in control (dashed line) and stressed (continuous line) rainbow trout. Each value is the mean ± SEM (n = 8/group). *$P < 0.05$ relative to Control at the same time point. Different letters indicate significant differences ($P < 0.05$) between time points within the same experimental group. Gray band indicates the dark phase of the daily cycle. The arrow indicates feeding time (ZT2).

activity, up to 25% (3.41 mU/mg prot) relative to that of Control. Such increase was mainly due to the increase of the enzyme activity observed in stressed trout at night, which also made the daily fluctuation not to reach significance level. In addition, a significant rhythm was observed for *gk* mRNA abundance in control fish, with peaking levels during the first half of the day (ZT4). Stress caused a 4-h shift of peaking levels, which advanced to the light onset (ZT0), but also a nearly 45% increase of averaged expression (38.6 ± 11.6 relative fold change units) compared to control group (26.8 ± 8.2 relative units).

A significant daily rhythm of PK activity was noted in non-stressed fish, with the highest values being observed during the second half of the day (ZT8), thus progressively decreasing to reach basal levels at the night–day transition (ZT0′). The magnitude of the oscillation was attenuated in stressed trout, which resulted in a slight decrease of mean activity levels (845.6 ± 24.1 mU/mg prot) relative to that of Control (913.1 ± 48.6 mU/mg prot). In the same way than the activity, *pk* rhythmically expressed in Control, with peaking values at the early night (ZT12) and basal levels during the end of the night. Stress by high stocking density affected such rhythm in such a way that it was almost blunted and averaged expression was inhibited (2.1 ± 0.1 relative fold change units) to almost half of that of Control (3.7 ± 0.5 relative units).

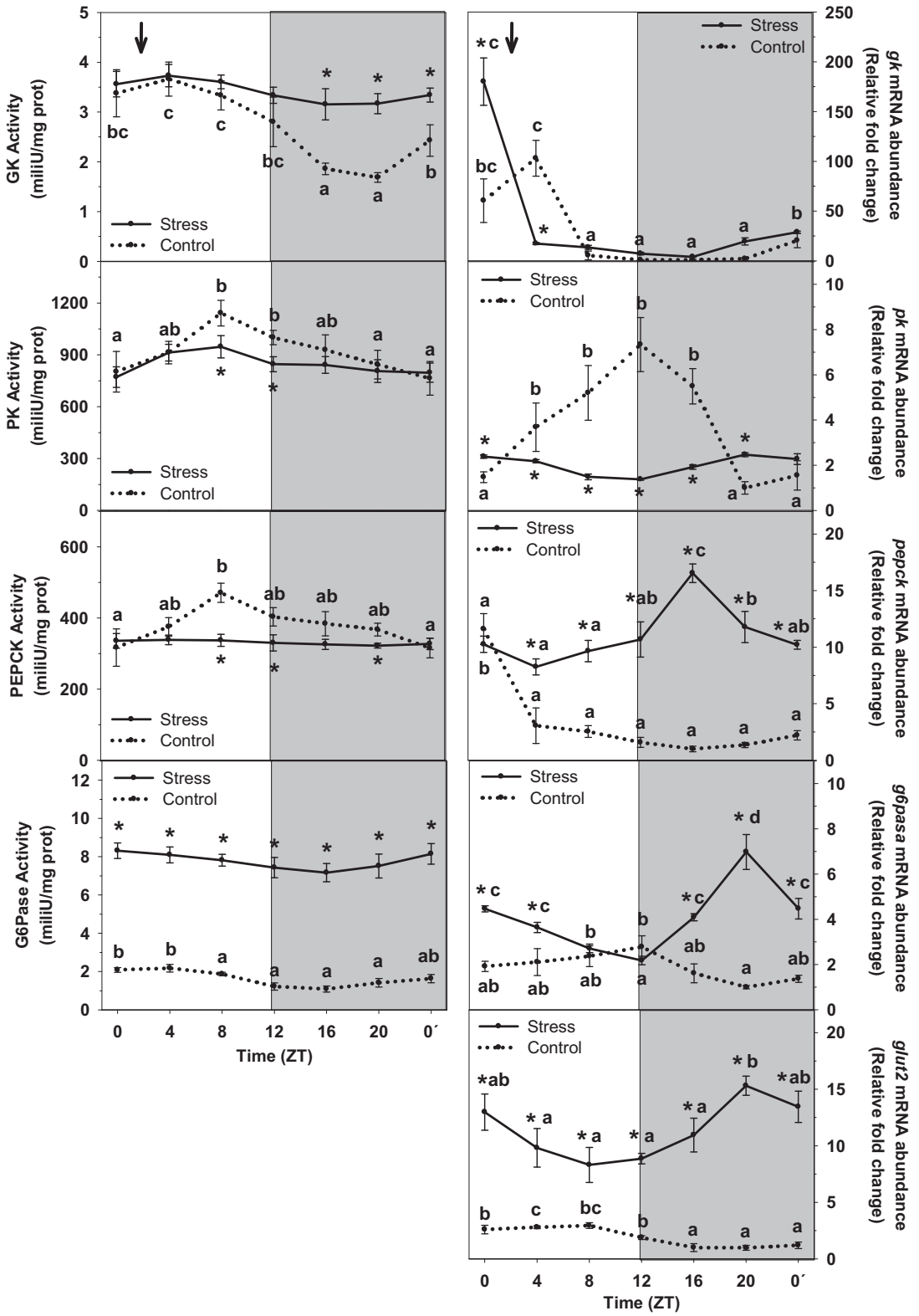

FIGURE 3 | Daily profile of enzyme activity and gene expression of different parameters of hepatic carbohydrate metabolism in fish subjected at control (dashed line) and stress (continuous line) conditions. Each value is the mean ± SEM (n = 8/group on enzyme activity and n = 4/group on gene expression). *$P < 0.05$ relative to Control at the same time point. Different letters indicate significant differences ($P < 0.05$) between time points within the same experimental group. Gray band indicates the dark phase of the daily cycle. The arrow indicates feeding time (ZT2).

PEPCK enzyme activity rhythmic in control group, with peaking levels occurring at ZT8, and basal levels being observed around the day onset (ZT0). Averaged activity levels were 376.2 ± 20.1 mU/mg prot. This rhythm disappeared in trout subjected to high stocking density, and a decrease of averaged enzyme activity was also noted (331.1 ± 2.4 mU/mg prot), relative to that of non-stressed fish. On the other hand, *pepck* displayed rhythmic expression in liver of control group, with peaking values during the day onset (ZT0) and basal levels at the first half of the night (ZT16). Stress exposure resulted in a dramatic alteration of this profile in such a way that averaged expression abruptly increased (11.1 ± 0.6 relative fold change units) relative to that of Control (2.5 ± 0.4 relative units), but also peaking values shifted to ZT16, thus with an 8-h advance.

G6Pase enzyme activity showed a slight daily variation in liver of control fish, with higher levels during the first half of the day (ZT4). Averaged levels were 1.7 ± 0.1 mU/mg protein. Stress exposure enhanced the enzyme activity thus making averaged levels to be higher (7.8 ± 0.2 mU/mg protein) than those of control group. However, the profile of the rhythm was not affected in stressed fish, in which peaking levels of G6Pase activity occurred during the day onset. The mRNA expression did also display a rhythmic profile in control fish, with peaking values during the early night period (ZT12) and basal levels at the late night (ZT20). Averaged expression levels were 1.8 ± 0.1 relative fold change units. Stress exposure enhanced *g6pase* expression in such a way that averaged levels raised to 4.1 ± 0.3 relative units, but also the phase of the rhythm was affected (ZT20) thus displaying an 8-h delay relative to that of Control.

Expression of *glut2* in non-stressed fish was rhythmic and peaking levels were found at day-time (ZT8). Averaged levels of mRNA abundance in this group were 2.1 ± 0.2 relative fold change units. Stress altered such profile by enhancing mRNA mean expression up to 11.4 ± 0.6 relative units, but also by phase-shifting the rhythm in such a way that it was in antiphase (peaking levels at ZT20) compared to control group.

Lipid Metabolism-Related Enzymes

Figure 4 shows daily variations of FAS and HOAD enzyme activities and mRNA abundance in liver of animals subjected or not to stress by high stocking density. FAS activity displayed a significant increase during the first half of the day (ZT4) and basal levels at night (ZT16), but the daily variation did not reach significance level. Averaged activity levels were 1.04 ± 0.13 mU/mg protein. Stress exposure blunted the daily variation, which resulted in a decrease of averaged enzyme activity to 0.37 ± 0.03 mU/mg prot, relative to control group. Control fish showed a significant rhythm of *fas* mRNA abundance. Peaking levels were noted in samples collected at the day onset (ZT0) and a minimum during the day–night transition. Averaged expression in this group was 6.5 ± 1.3 relative fold change units. Stress exposure enhanced *fas* expression, thus with averaged levels (14.6 ± 1.5 relative units) duplicating those of Control. The profile of the rhythm was also affected in such a way that two increases of *fas* abundance were observed, at ZT0, and during the night (ZT16), when basal were expected, as in Control.

HOAD enzyme activity did not show any daily variation at any experimental condition. However, stress inhibited HOAD activity, thus with averaged levels (136.7 ± 3.1 mU/mg prot) being lower than those of control group (482.7 ± 16.1 mU/mg prot). The daily rhythm of *hoad* mRNA abundance reached significance level in both experimental groups, in which peaking levels occurred during the second half of the dark period (ZT20) and basal levels at the night onset (ZT12). No differences were found between groups.

Clock Genes Expression

The rhythm of clock genes mRNA abundance in liver of rainbow trout and the effect of stress on such rhythms is represented on **Figure 5**. In control fish, the rhythm of *clock1a* was significant, and peaking values were observed during the day–night transition (ZT12), whereas basal values occurred at the day onset (ZT0). Averaged mRNA levels were 2.5 ± 0.2 relative fold change units. Stress inhibited gene expression but did not affect the profile of the rhythm, thus displaying peaking values (ZT12) in phase with that of Control. However, averaged expression decreased in stressed fish (1.5 ± 0.1 relative units) reaching a 60% of that of control group.

Similarly to that of *clock1a*, assessment of *bmal1* mRNA abundance revealed the existence of a significant rhythm with peaking values occurring at ZT12, and basal levels being observed at day-time (ZT4). Averaged expression in this group was 2.3 ± 0.2 relative fold change units. This profile was not affected when trout were subjected to stress. Then, peaking values were observed in samples collected at ZT12, as in control group. However, the inhibitory effect of stress in *bmal1* expression was noted and averaged mRNA levels decreased (1.7 ± 0.1 relative units) respect that of Control.

The daily profile of *per1* mRNA abundance in liver of control trout reached significance level, with peaking values occurring at day-time (ZT4) and basal levels during the end of the night (ZT20). Averaged mRNA levels were 1.6 ± 0.1 relative fold change units. Stress exposure affected such profile in a way that a shift of peaking levels was noted. Then, the time of the peak was observed during the second half of the night (ZT20), i.e., with an 8-h advance relative to that of Control. Averaged mRNA levels slightly increased in stressed fish, up to 1.9 ± 0.2 relative units.

By other hand, assessment of *rev-erb*β mRNA abundance revealed the existence of a significant daily rhythm in liver of control fish. Peaking levels were found at the end of the night (ZT20), and basal levels at the end of the day (ZT12). Averaged mRNA levels were 1.8 ± 0.1 relative fold change units in this group. Stress enhanced *rev-erb*β expression in such a way that averaged mRNA levels in stressed trout were near 33% higher (2.4 ± 0.1 relative units) than those of Control. No variation was observed for the profile of *rev-erb*β expression in stressed fish, thus with peaking mRNA levels occurring at ZT20, and basal levels at ZT12, as in control group.

Expression of sirt1, GR1, and GR2

To identify the role played by sirtuin1 as possible mediator of the effect of stress on liver rhythmic physiology and its interaction with cortisol as a well-known mediator of such effect, the daily

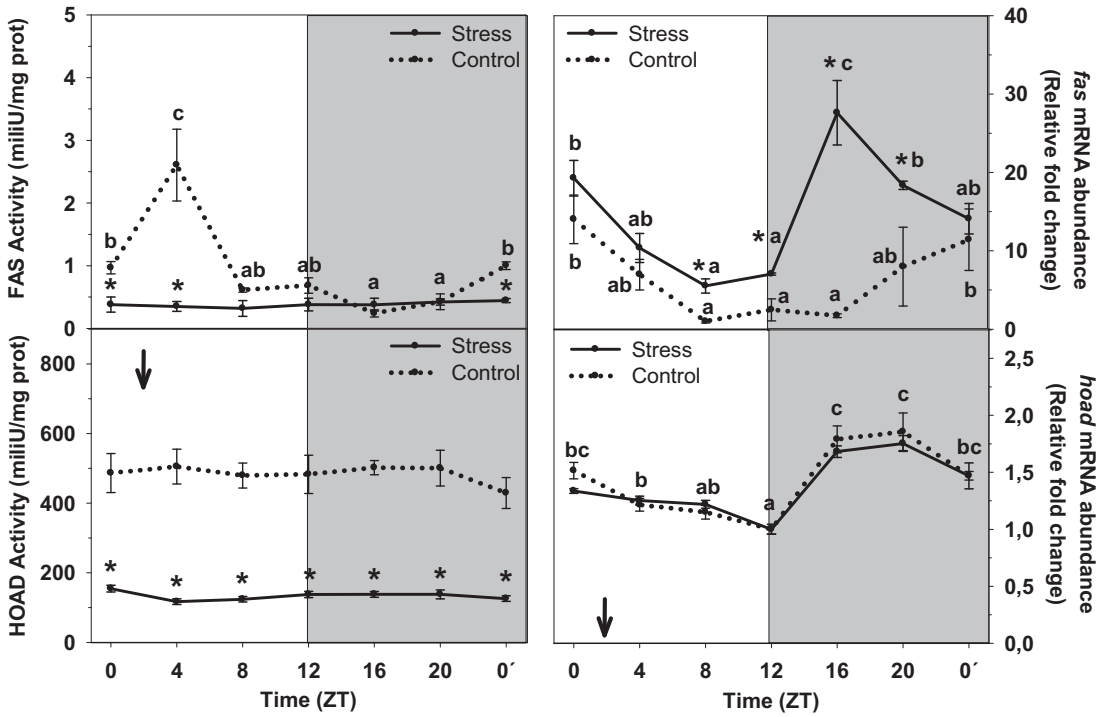

FIGURE 4 | Daily profile of enzyme activity and gene expression of different parameters of hepatic lipids metabolism in fish subjected at control (dashed line) and stress (continuous line) conditions. Each value is the mean ± SEM (n = 8/group on enzyme activity and n = 4/group on gene expression). *$P < 0.05$ relative to Control at the same time point. Different letters indicate significant differences ($P < 0.05$) between time points within the same experimental group. Gray band indicates the dark phase of the daily cycle. The arrow indicates feeding time (ZT2).

profile of mRNA abundance of *sirt1* and glucocorticoid receptors (*gr1* and *gr2*) was also evaluated in trout subjected or not to stress (**Figure 6**). Control group exhibited a significant rhythm of *sirt1* in liver, with the peak being observed at ZT4 and a minimum during the night onset (ZT12). The Averaged mRNA abundance was 1.9 ± 0.3 relative fold change units. This profile was not affected by stress exposure, but enhances expression was noted, thus averaged levels raised to 3.3 ± 0.2 relative units in stressed trout.

Both GRs (GR1 and GR2) did exhibit a significant rhythm of mRNA abundance with high nocturnal levels and basal levels occurring at day-time independently of the experimental condition. Averaged mRNA levels for control group were 2.2 ± 0.3 relative fold change units for *gr1*, and 2.5 ± 0.3 relative units for *gr2*. Stress exposure resulted in the increase gene expression for both *gr1* and *gr2*. Then, averaged levels of mRNA abundance were 6.9 ± 0.4 relative units for *gr1*, and 5.1 ± 0.4 relative units for *gr2*, thus triplicating (*gr1*) and duplicating (*gr2*) that of Control.

DISCUSSION

Stress triggers in the body a series of responses in order to obtain the energy needed to deal with an adverse situation. Liver is an important source of that energy. When exposed to a stressful situation, a cascade of hormonal signaling occurs in which cortisol participates in the maintenance of the physiological response.

Our results show daily rhythm of cortisol in non-stressed fish, with peaking levels being observed at the same temporal window than feeding time. Exposure to high stocking density results in enhanced cortisol averaged levels and a significant disturbance of its daily rhythm, with peaking levels occurring at night (ZT16). These results agree with that previously reported for the same species (Gesto et al., 2013), in which cortisol levels increased shortly after animals were subjected to stress and remained elevated for several days. The daily rhythms of plasma cortisol levels have been also described in humans and rodents, and display a robust oscillation with the peak occurring shortly before the active phase initiates, morning in humans and early evening in nocturnal rodents (see rev. Koch et al., 2017). In addition, disruption of the circadian clock machinery (responsible of the daily rhythm of the hormone) associates with altered glucocorticoid concentration and daily profile, but also metabolic impairments and depression (Turek et al., 2005; Albrecht, 2010; Mukherjee et al., 2010; Barclay et al., 2012; Leliavski et al., 2014).

Complementarily, plasma glucose and lactate levels displayed a daily oscillation in control fish. Stress exposure resulted in enhanced glucose and lactate levels and altered oscillation. Such results might be expected, since stress response results in the activation of those mechanisms involved in providing nutrients all over the time a stressor is present. On the contrary, a daily

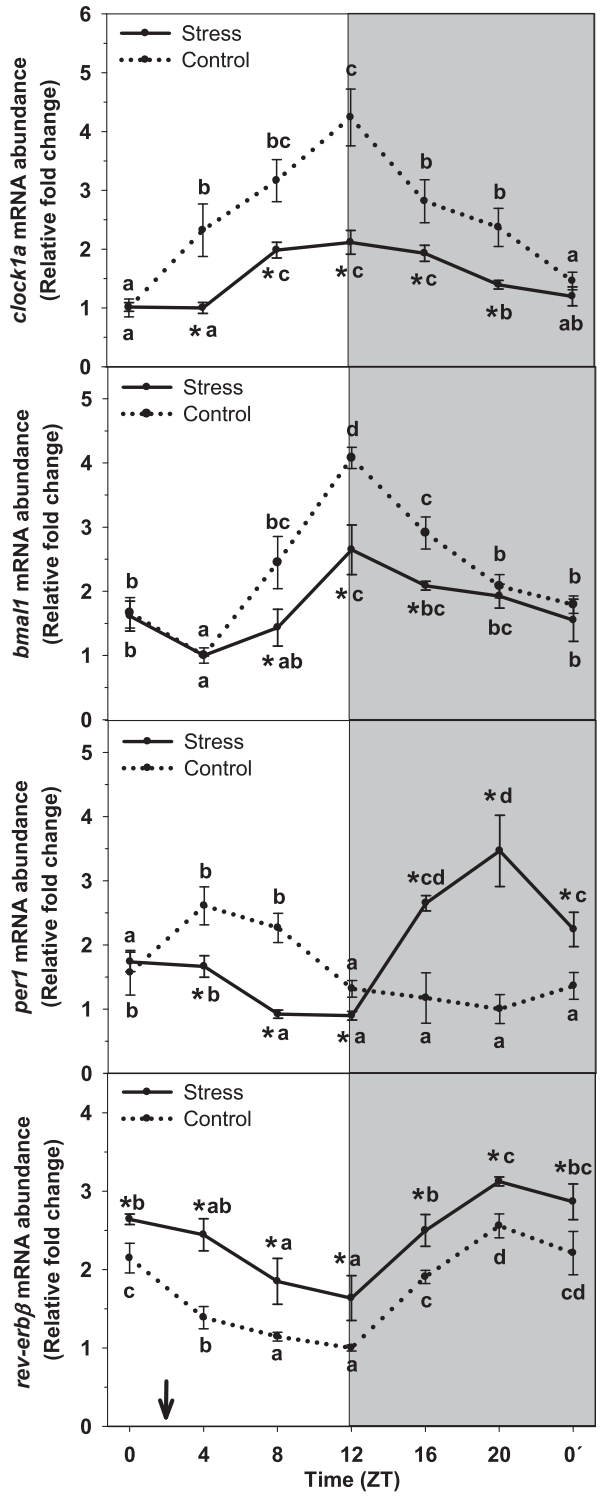

FIGURE 5 | Daily profile of hepatic clock gene expression (*clock1a*, *bmal1*, *per1* and *rev-erbβ*) in fish subjected at control (dashed line) and stress (continuous line) conditions. Each value is the mean ± SEM (n = 4). *$P < 0.05$ relative to Control at the same time point. Different letters indicate significant differences ($P < 0.05$) between time points within the same experimental group. Gray band indicates the dark phase of the daily cycle. The arrow indicates feeding time (ZT2).

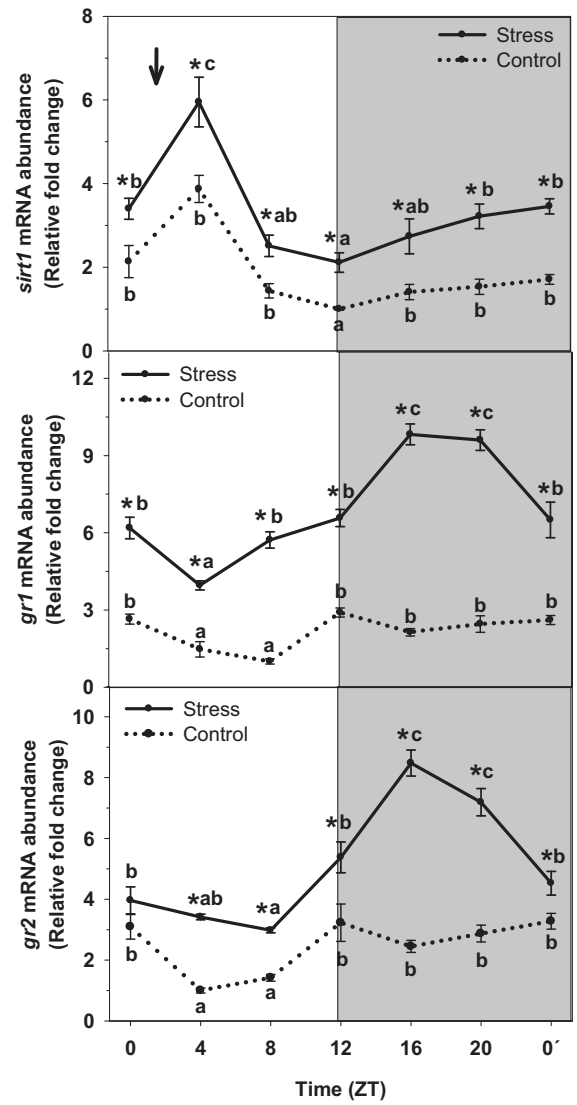

FIGURE 6 | Daily expression profile of *sirt* and glucocorticoids receptors (*GR1* and *GR2*) in the liver of rainbow trout subjected at control (dashed line) and stress (continuous line) conditions. Each value is the mean ± SEM (n = 4). *$P < 0.05$ relative to Control at the same time point. Different letters indicate significant differences ($P < 0.05$) between time points within the same experimental group. Gray band indicates the dark phase of the daily cycle. The arrow indicates feeding time (ZT2).

oscillation was also found for glycogen, glucose, and lactate content in liver of non-stressed fish. Stress exposure resulted in a decrease of them all, leading for the oscillation to even disappear (lactate). Such results are indicative of hepatic metabolism to change in order to mobilize resources in order for the animal to cope with, and overcome the adverse situation.

Changes in Parameters Related to Carbohydrate and Lipid Metabolism

Daily rhythms of enzyme activity and mRNA abundance are observed in most of the carbohydrate metabolism-related

parameters within the liver of non-stressed trout. These rhythms are reported to be under circadian control in trout liver (Hernández-Pérez et al., 2015, 2017). Stress affects the daily oscillation of enzyme activities in a way that the amplitude of most of them decreases (GK, PK) and even disappears (PEPCK). In addition, the daily rhythm of mRNA abundance for carbohydrate metabolism-related parameters is also affected, thus increasing averaged levels (*gk*, *pepck*, *g6pase*, and *glut2*), but also displaying a phase-shifting. All these changes are consistent with increased glycogenolytic capacity (enhanced G6Pase enzyme activity and mRNA abundance) and enabled glucose transport (increased *GLUT2* abundance) that occur during stress exposure, as previously reported for by us trout subjected to acute stress (López-Patiño et al., 2014b). These results are in contrast to those reported for gilthead seabream subjected to changes in water salinity, in which a decrease in glycogenolytic capacity during chronic stress (Laiz-Carrión et al., 2003). Such discrepancy may be indicative of a species- and stress nature-specific dependence of liver physiological response to stress in fish.

Stress can induce the increased cortisol-mediated glyconeogenic capacity (Mommsen et al., 1999; Gilmour et al., 2012). In support of that, our results reveal a significant increase of mRNA abundance for the key enzyme in glucose synthesis, *pepck*, but not in the enzyme activity. However, the daily profile of both, enzyme activity and mRNA abundance, is altered in liver of stressed trout relative to that observed in control fish. Then, during the response to stress, regulatory mechanisms may exist at both transcriptional and post-transcriptional levels during stress exposure. Similar results are described for cultured trout hepatocytes (Sathiyaa and Vijayan, 2003), but also for animals subjected to different stressors (Aluru and Vijayan, 2009).

On the other hand, increased hepatic glucose production under stress conditions makes this carbohydrate available for use in glycolytic processes. Then, during stress exposure, one might expect a decrease of liver glycolytic capacity, as herein reported with decreased PK activity and mRNA abundance, together with altered daily oscillation of both. Such results agree with that previously reported for trout subjected to 60-min manipulation (Wiseman et al., 2007), or gilthead sea bream exposed to a drop of temperature (Kyprianou et al., 2010), and brook trout (*Salvelinus fontinalis*) stressed by density (Vijayan and Leatherland, 1990). All these changes occur in order to ensure glucose supply to other tissues during prolonged stress. Then, liver is important during the response to stress, since this organ mobilizes and releases glucose reserves into the bloodstream according to that needed.

With respect to lipids metabolism within the liver of rainbow trout, daily variations of mRNA abundance are observed for *fas* and *hoad*, but the respective enzyme activities do not display such oscillation. This is in agreement with our previous data describing daily rhythms of lipid metabolism-related parameter in liver of the same species (Hernández-Pérez et al., 2015, 2017), thus with the control exerted by a circadian oscillator. Stress exposure results in altered enzyme activities, but also in a change of the rhythm of *fas* abundance. Studies carried out in other teleost species describe that acute stress leads to increased plasma fatty acids levels in Atlantic salmon (Waring et al., 1992) and carp (Ruane et al., 2001). Such results together with that herein reported point to a decrease of liver lipogenic activity during stress exposure. In addition, decreased HOAD activity is indicative of increased fatty acid release from the liver into the blood. Then, when trout is subjected to stress, the liver displays changes in enzyme activities and mRNA abundance that even affect to their daily oscillation. These changes occur in order for this organ to provide energy resources that allow the whole organism to cope with the adverse situation.

Hepatic Circadian System

Being able to anticipate daily changes in the environment is an evolutionary advantage for most species on earth. Then, organisms from plants to mammals have developed endogenous circadian oscillators that make possible for them to estimate the time of day. The alteration of the activity of such endogenous clocks results in an adverse situation for the animal, since rhythmic behavioral and physiological functions may decouple. Stress exposure is reported to negatively affect the activity of the circadian system (Takahashi et al., 2013). Glucocorticoids, as stress response mediators, play a main role in such effect in mammals, at least in peripheral locations hosting circadian oscillators such as heart, kidney or liver (Balsalobre et al., 2000). Our results in fish not only point to cortisol as mediator of the effect of stress on the circadian system at peripheral locations (herein reported), but also at the hypothalamus, where the central circadian oscillator is located (Naderi et al., 2018). In the same way, glucocorticoids administration in goldfish results in altered rhythmic functions (López-Patiño et al., 2014a; Sánchez-Bretaño et al., 2015).

With respect to our herein reported results, daily rhythms of clock genes exist in liver of rainbow trout. Those rhythms perfectly fit with those previously reported by us in the same tissue, for which the presence of a circadian oscillator was evidenced (Hernández-Pérez et al., 2017). Stressing trout by high stocking density results in decreased amplitude of *clock1a* and *bmal1* rhythms and averaged mRNA levels. However the phase of the rhythm remained similar to that of non-stressed animals. On the contrary, *per1* expression displayed both a phase-shift and a slight increase of averaged mRNA levels in stressed trout. These results agree with that previously reported for liver of mammals subjected to moderate stress, where *bmal* and *per* expression is altered (Takahashi et al., 2013). These coincidences are indicative of the circadian system to be influenced by stress, with the underlying mechanisms being apparently well conserved in vertebrates.

By other hand, *rev-erb*β displays significant rhythms of mRNA abundance in liver of rainbow trout, which is indicative of a regulatory role played by this nuclear receptor in fish liver circadian physiology. In this way, studies carried out in mammals indicate the modulatory action of *rev-erb*α in modulating different aspects of liver metabolism (Duez and Staels, 2009), but also the inhibitory effect exerted by the nuclear receptor on *bmal1* expression (Guillaumond

et al., 2005). Thus, when *rev-erb* expression increases, one may expect decreased *bmal1* mRNA abundance. This is exactly what we found in liver of trout stressed by high stocking density, where enhanced *rev-erb*β expression was noted all over the day, thus resulting in a significant increase of averaged mRNA levels, relative to non-stressed animals. However, stress did not affect the phase of such rhythm. Then, in a stressful situation, altered hepatic energy status leads for *rev-erb*β abundance to increase, thus inhibiting *bmal1* expression with the subsequent desynchronization of the hepatic oscillator. Such results confirm the role played by this nuclear receptor during the response of liver circadian system to stress in fish.

Cortisol appears to also mediate effects of stress on the circadian system, since the glucocorticoid enhances *per1a* and *per1b*, and inhibits *clock* and *bmal1* in liver of goldfish (Sánchez-Bretaño et al., 2016). Then, we did evaluate the rhythmic expression profile of the general glucocorticoid receptors (GR1 and GR2) and how can stress affect such profile, as long as the activation of these receptors results in altered profile of clock genes expression in mammals (see rev. Koch et al., 2017). Our results reveal significant rhythms of *gr1* and *gr2* abundance, which is indicative of the circadian system to control them in fish, as reported for mammals, in which the sensitivity of GRs is also under circadian regulation (Lamia et al., 2011). On the other side, the activation of GRs modulates the transcription of certain genes (Pratt, 1993; Jewell et al., 1995), with clock genes among them. Then, the inter-relationship between circadian system and glucocorticoids is plausible in fish. Stress by high stocking density not only enhances plasma cortisol levels (peaking at night), but also results in increased mRNA levels of *gr1* and *gr2*, with the peaks occurring at the same time (ZT16) than that of the glucocorticoid. Such enhanced expression may be responsible of the decrease of averaged *clock1a* and *bmal1* expression herein reported. At the same time, *per1* expression increases in stressed trout, and a phase-shift is also observed. Overall, a desynchronization of liver circadian physiology occur in stressed trout.

By other hand, our previous results reveal that cortisol may not be the only mediator of the effect of stress on trout circadian physiology, since treatments with the general GRs antagonist does not totally prevent such effect of stress on hypothalamic clock genes (Naderi et al., 2018). Accordingly, we evaluated the expression of *sirtuin1*, which may be considered as a link between circadian clocks and sensing of cell energy status (see rev. Delgado et al., 2017). Sirtuin1 relates to the circadian system through two main mechanisms, the first one consists on the dependence on the NAD^+ as cofactor, which has a rhythmic biosynthesis, thus driving the rhythm of sirtuin1 activity (Nakahata et al., 2009; Ramsey et al., 2009). In addition, SIRT1 modulates the cell circadian system at central level, thus influencing the daily rhythms of *bmal1*, *per2* and *cry1* (Asher et al., 2010), and in peripheral tissues, such as liver (Nogueiras et al., 2012). Our results in rainbow trout agree with that above mentioned, since a daily rhythm of *sirt1* mRNA abundance is observed in trout liver of non-stressed trout, thus pointing at the interaction between sirtuin1 and circadian system to also exist in fish. By other hand, no change of the daily profile but an increase of averaged *sirt1* mRNA levels was observed in liver of stressed trout. We previously reported similar results for hypothalamus of trout exposed to identical stressing conditions (Naderi et al., 2018). In addition, our preliminary results also confirm the key role of *sirt1* during the response to stress of the circadian system, since the sirt1 inhibitor (EX527) prevents the expected variation of *clock genes* and *sirt1* expression when trout are subjected to identical stressing conditions (unpublished). However, we cannot discard the interaction between sirt1 and other mediators such as cortisol during the response to stress in rainbow trout. Further research is needed to evaluate these interactions.

In summary, our data confirm in fish that stress triggers a series of responses in the body in order to obtain energy that bring the animal the chance to deal with such adverse situation. Since liver plays an important role as source of that energy, stress also influence the physiology of this organ. Different mediators are involved in the response of liver circadian physiology to stress specially that of carbohydrate and lipid metabolism-related parameters and the circadian oscillator that locates in this organ. Among them, we confirm in fish a role for nuclear receptors (*rev-erb*β), cortisol, and sirt1 as mediators. However, to evaluate the independent effect of each mediator or the existence and nature of any interaction among them deserve further research.

DATA AVAILABILITY

The datasets generated for this study can be found in GenBank, AF053331, AF246146, AF246149, AF321816, NM_001124730.1, AY495372.1, AF266745, GQ489026.1, AF228695, AF342943.1, EZ774344.1, and NM_001124235.1.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of Guidelines of he European Union Council (2010/63/UE) and of the Spanish Government (RD 55/2013) for the use of animals in research. Protocols were approved by the Ethics Committee at the University of Vigo.

AUTHOR CONTRIBUTIONS

JH-P and FN conducted the main experimental work and performed all the samples analysis. JH-P wrote the manuscript under the supervision of JM and ML-P. JS, JM, and ML-P conceived the experiments. JS and JM contributed with both reagents and goods. All the authors contributed to, and approved the manuscript.

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Tissue-Specific Orchestration of Gilthead Sea Bream Resilience to Hypoxia and High Stocking Density

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Two different O_2 levels (normoxia: 75–85% O_2 saturation; moderate hypoxia: 42–43% O_2 saturation) and stocking densities (LD: 9.5, and HD: 19 kg/m^3) were assessed on gilthead sea bream (*Sparus aurata*) in a 3-week feeding trial. Reduced O_2 availability had a negative impact on feed intake and growth rates, which was exacerbated by HD despite of the improvement in feed efficiency. Blood physiological hallmarks disclosed the enhancement in O_2-carrying capacity in fish maintained under moderate hypoxia. This feature was related to a hypo-metabolic state to cope with a chronic and widespread environmental O_2 reduction, which was accompanied by a differential regulation of circulating cortisol and growth hormone levels. Customized PCR-arrays were used for the simultaneous gene expression profiling of 34–44 selected stress and metabolic markers in liver, white skeletal muscle, heart, and blood cells. The number of differentially expressed genes ranged between 22 and 19 in liver, heart, and white skeletal muscle to 5 in total blood cells. Partial Least-Squares Discriminant Analysis (PLS-DA) explained [R2Y(cum)] and predicted [Q2Y(cum)] up to 95 and 65% of total variance, respectively. The first component (R2Y = 0.2889) gathered fish on the basis of O_2 availability, and liver and cardiac genes on the category of energy sensing and oxidative metabolism (*cs*, *hif-1*α, *pgc1*α, *pgc1*β, *sirt*s *1-2-4-5-6-7*), antioxidant defense and tissue repair (*prdx5*, *sod2*, *mortalin*, *gpx4*, *gr*, *grp-170*, and *prdx3*) and oxidative phosphorylation (*nd2*, *nd5*, and *coxi*) highly contributed to this separation. The second component (R2Y = 0.2927) differentiated normoxic fish at different stocking densities, and the white muscle clearly promoted this separation by a high over-representation of genes related to GH/IGF system (*ghr-i*, *igfbp6b*, *igfbp5b*, *insr*, *igfbp3*, and *igf-i*). The third component (R2Y = 0.2542) discriminated the effect of stocking density in fish exposed to moderate hypoxia by means of hepatic fatty acid desaturases (*fads2*, *scd1a*, and *scd1b)* and muscle markers of fatty acid oxidation (*cpt1a*). All these findings disclose the different contribution of analyzed tissues (liver ≥ heart > muscle > blood) and specific genes to the hypoxic- and crowding stress-mediated responses. This study will contribute to better explain and understand the different stress resilience of farmed fish across individuals and species.

Keywords: hematology, hypometabolism, hypoxia, limiting oxygen saturation, *Sparus aurata*, stocking density, tissue-specific transcriptomics

INTRODUCTION

Several attempts have been made over the course of last years to monitor the ecological and physiological impacts of a reduced O_2 availability in aquatic environments (Ekau et al., 2010; Richards, 2011; Zhu et al., 2013; Deutsch et al., 2015). The magnitude and orchestration of adaptive responses from a physiological point of view, including blood hematology and metabolic regulation, reflects the duration and intensity of hypoxic stimuli in different marine species (Martos-Sitcha et al., 2017; Cadiz et al., 2018), being defined the limiting O_2 saturation (LOS) as the threshold level where regulatory mechanisms are no longer sufficient to maintain O_2 consumption without compromising any physiological function (Remen et al., 2015, 2016). To minimize hypoxia impact, fish reduce feed intake and reorganize its metabolism to limit the tissue O_2 demand (Hopkins and Powell, 2001; Bermejo-Nogales et al., 2014b). This allows to preserve aerobic metabolism by means of a restricted mitochondrial respiration and a shift in substrate preferences, as it has been reported in humans and rodents during the metabolic adaption of skeletal muscle to high altitude hypoxia (Murray, 2009). Other adaptive responses include changes in the production and scavenging of reactive oxygen species (ROS) (Lushchak and Bagnyukova, 2006; Bermejo-Nogales et al., 2014a), gill surface functionality (Nilsson, 2007) and hemoglobin (Hb)-O_2 binding characteristics (Jensen and Weber, 1982; Nikinmaa, 2001). In most aquaculture scenarios, these adaptive features are commonly associated to increases in temperature and high stocking rearing densities (Person-Le Ruyet et al., 2008; Vikeså et al., 2017), which in turn can compromise water quality resulting in impaired fish growth and immunity (Pickering, 1993; Van Weerd and Komen, 1998; Montero et al., 1999; Ashley, 2007). Indeed, Arctic charr (*Salvelinus alpinus*) or meager (*Argyrosomus regius*) kept at high stocking densities evidenced a better growth performance than fish reared at low densities as long as water quality was preserved (Jørgensen et al., 1993; Millán-Cubillo et al., 2016). The impact of crowding stress can also be minimized when O_2 levels are not below LOS (Ruer et al., 1987; Araújo-Luna et al., 2018). Unraveling the combined effects of hypoxia and high rearing density are, thereby, crucial to warrant welfare during intensive fish farming.

Progress toward a more sustainable and environmentally friendly aquaculture requires important investments in both conventional and new methodologies for a less invasive and more refined phenotyping of individual farmed fish. Main achievements so far include the use of acoustic telemetry or stand-alone biosensors for the non-disturbing monitoring of feeding behavior or metabolic capabilities (Føre et al., 2017; Martos-Sitcha et al., 2019). In addition to that, major progress has been done with the advent of wide-holistic omics based on functional genomics, proteomics, metabolomics and metagenomics as powerful toolsets for the development of a highly technified aquaculture in different fish species (Yáñez et al., 2015; Martin and Król, 2017; Martínez-Porchas and Vargas-Albores, 2017; Alfaro and Young, 2018; Rodrigues et al., 2018). Such approaches are increasingly used in gilthead sea bream (*Sparus aurata*), a highly and economically important cultured fish species in the Mediterranean area. Thus, a first draft genome based on genetic-linkage maps (Pauletto et al., 2018) and other current genome initiatives will contribute to have major progress in selective breeding and epigenetic research in gilthead sea bream. Also, in this species, important research efforts have been conducted to define a reference pattern for skin/intestine mucus proteome (Estensoro et al., 2016; Pérez-Sánchez et al., 2017), gut microbiota (Piazzon et al., 2017), or serum metabolome (Gil-Solsona et al., 2019). Moreover, the use of high-density microarrays (Calduch-Giner et al., 2010, 2012, 2014), pathway-focused PCR-arrays (Benedito-Palos et al., 2014, 2016; Bermejo-Nogales et al., 2014b, 2015; Pérez-Sánchez et al., 2015; Magnoni et al., 2017; Martos-Sitcha et al., 2017) and more recently NGS (Piazzon et al., 2019) have contributed to define tissue-specific gene expression patterns in response to nutritional, environmental and parasite challenges. Likewise, the synchronization of the molecular clock of sea bream larvae, involving more than 2,500 genes with a clear circadian rhythmicity, has been proposed as certification of juvenile quality later in life (Yúfera et al., 2017). In the present study, we aim to go further on the definition of criteria of fish welfare and quality, regarding in depth the effect of two different initial stocking densities (9.5 kg/m^3, 19 kg/m^3) and O_2 saturation levels (85%, 42–43% O_2 saturation) in a 3-week trial with fast growing juveniles of gilthead sea bream. The analyzed parameters included the gene expression pattern of a set of growth and metabolic markers of liver, skeletal muscle, heart and blood cells in combination with data on growth performance, as well as blood hematology and biochemistry. The working hypothesis is that each tissue contributes differentially to the homeostatic load achievement, helping the generated knowledge to better exploit the plasticity and resilience of gilthead sea bream under stressful conditions.

MATERIALS AND METHODS

Animal Care

Gilthead sea bream juveniles of Atlantic origin (Ferme Marine du Douhet, Bordeaux, France) were reared from early life stages in the indoor experimental facilities of Institute of Aquaculture Torre de la Sal (IATS-CSIC, Spain) under natural photoperiod and temperature conditions (40°5′N; 0°10′E). Sea water was pumped ashore (open system) and filtered through a 10-μm filter. The O_2 content of water effluents in standard conditions was always higher than 85% saturation, and unionized ammonia remained below 0.02 mg/L.

Experimental Set-Up and Sampling

Juvenile fish (initial body weight 34.08 ± 0.31 g) were randomly distributed in 12 90 L tanks coupled to a recirculation system equipped with physical and biological filters, and programmable

temperature and O_2 devices (**Supplementary Figure 1**). Water temperature was daily monitored and maintained at 25−27°C. Fish were arbitrarily allocated to constitute two different initial stocking densities (six tanks per condition) fed once daily to visual satiety with a commercial diet (EFICO Forte 824, BioMar, Palencia, Spain): LD (low density, 25 fish/tank, 9.5 kg/m^3) and HD (high density, 50 fish/tank, 19 kg/m^3). Fish were allowed to acclimate to these conditions for 12 days before any manipulation. Fish behavior during acclimation was assessed by routine camera monitoring, and also by visual observation regarding to normal feeding performance. After the acclimation period, the water parameters of three tanks of each initial stocking density were kept unchanged, constituting the normoxic (>5.5 ppm O_2; >85% O_2 saturation) groups of each experimental condition (LDN, low density normoxia; HDN, high density normoxia). Fish maintained in the remaining six tanks experienced a gradual decrease in the water O_2 level until reaching 3.0 ppm (42–43% O_2 saturation), constituting the hypoxic groups of each experimental condition (LDH, low density hypoxia; HDH, high density hypoxia). The normal range of variation in O_2 levels was marked by a rapid drop (3.8–4 ppm normoxic groups; 2.3 ppm hypoxic groups) 15–30 min after feeding, with a rapid restoration of reference values in less than 1 h by the automatic entrance of clean water from the main reservoir tank. This system allowed maintaining unionized ammonia below toxic levels (<0.50 mg/L) in both HDN and HDH groups.

After 22 days under these experimental conditions and following overnight fasting (>20 h after last meal), 12 fish (4 per tank) per experimental condition (LDN, LDH, HDN, and HDH) were anesthetized with 3-aminobenzoic acid ethyl ester (100 mg/L), weighed and blood was taken from caudal vessels with EDTA-treated syringes (less than 5 min for all the fish sampled for each tank). All lethal samples were collected between 10.00 am and 12.00 am to reduce the biologic variability due to circadian rhythms and postprandial-mediated effects, since temperatures used in the present experimental approach (25−27°C) ensured that digestion processes have been completed (Gómez-Requeni et al., 2003). One blood aliquot (25 μL) was directly collected into a microtube containing 500 μL of stabilizing lysis solution (REAL total RNA spin blood kit, Durviz, Valencia, Spain) and stored at −80°C until total RNA extraction. Other aliquots were processed for haematocrit (Ht), Hb, and red blood cells (RBC) counting. The remaining blood was centrifuged at 3,000 × *g* for 20 min at 4°C, and plasma samples were frozen and stored at −20°C until biochemical and hormonal analyses were performed. Prior to tissue collection, fish were killed by cervical section. Liver and viscera were weighed, and representative biopsies of liver, dorsal skeletal muscle, and complete hearts were immediately snap-frozen in liquid nitrogen and stored at −80°C until extraction of total RNA.

Blood Biochemistry and Hormonal Parameters

Measures of Ht were conducted using heparinized capillary tubes centrifuged at 1,500 × *g* for 30 min in a Sigma 1-14 centrifuge (Sigma, Germany). Hb was assessed using a Hemocue Hb 201+ (Hemocue, Sweden). Counts of RBC were made in a Neubauer chamber, using an isotonic solution (1% NaCl). Plasma glucose was analyzed using the glucose oxidase method (Thermo Electron, Louisville, CO, United States). Lactate was measured in deproteinized samples (perchloric acid 8%) by an enzymatic method based on the use of lactate oxidase and peroxidase (SPINREACT S.A., Girona, Spain). Total antioxidant capacity in plasma samples was measured with a commercial kit (Cayman Chemical, Ann Arbor, MI, United States) adapted to 96-well microplates. This assay relies on the ability of antioxidants in the samples to inhibit the oxidation of ABTS [2,2′-azino-di-(3-ethylbenzthiazoline sulphonate)] to ABTS radical cation by metmyoglobin, a derivatized form of myoglobin. The capacity of the sample to prevent ABTS oxidation is compared with that of Trolox (water-soluble tocopherol analog) and is quantified as mM Trolox equivalents. Plasma cortisol levels were analyzed using an EIA kit (Kit RE52061m IBL, International GmbH, Germany). The limit of detection of the assay was 3.01 ng/mL with intra- and inter-assay coefficients of variation lower than 3 and 5%, respectively. Plasma growth hormone (Gh) was determined by a homologous gilthead sea bream RIA as reported elsewhere (Martínez-Barberá et al., 1995). The sensitivity and midrange (ED50) of the assay where 0.15 and 1.8 ng/mL, respectively. Plasma insulin-like growth factors (Igf) were extracted by acid–ethanol cryoprecipitation (Shimizu et al., 2000), and the concentration of Igf-I was measured by means of a generic fish Igf-I RIA validated for Mediterranean perciform fish (Vega-Rubín de Celis et al., 2004). The sensitivity and midrange of the assay were 0.05 and 0.7–0.8 ng/mL, respectively.

Gene Expression Analysis

Total RNA from liver, white muscle and heart was extracted using a MagMax-96 total RNA isolation kit (Life Technologies, Carlsbad, CA, United States), whereas total RNA from total blood cells was extracted using the REAL total RNA spin blood kit including a DNase step. The RNA yield in all tissues was >3.5 μg, with absorbance measures ($A_{260/280}$) of 1.9–2.1. Synthesis of cDNA was performed with the High-Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA, United States) using random decamers and 500 ng of total RNA in a final volume of 100 μL. Reverse transcription (RT) reactions were incubated 10 min at 25°C and 2 h at 37°C. Negative control reactions were run without RT.

The 96-well PCR-array layout was designed for the simultaneous profiling of a panel of 43 (liver), 44 (white muscle and total blood cells) or 34 (heart) genes, including markers of GH/IGF system (13), lipid metabolism (10), energy sensing and oxidative metabolism (12), antioxidant defense and tissue repair (10), muscle growth and cell differentiation (8), respiration uncoupling (3), xenobiotic metabolism (2), nuclear receptors (3), transmembrane translocation (8), mitochondrial dynamics and apoptosis (5), as well as OXPHOS (22) (**Table 1**). qPCR reactions were performed using an iCycler IQ Real-time Detection System (Bio-Rad, Hercules, CA, United States). Diluted RT reactions were conveniently used for qPCR assays in a 25 μL volume in combination with a SYBR Green Master

Mix (Bio-Rad, Hercules, CA, United States) and specific primers at a final concentration of 0.9 μM (**Supplementary Table 1**). The program used for PCR amplification included an initial denaturation step at 95°C for 3 min, followed by 40 cycles of denaturation for 15 s at 95°C and annealing/extension for 60 s at 60°C. All the pipetting operations were made by means of an EpMotion 5070 Liquid Handling Robot (Eppendorf, Hamburg, Germany) to improve data reproducibility. The efficiency of PCRs (>92%) was checked, and the specificity of reactions was verified by analysis of melting curves (ramping rates of 0.5°C/10 s over a temperature range of 55–95°C) and linearity of serial dilutions of RT reactions (>0.99). Fluorescence data acquired during the extension phase were normalized by the delta-delta C_T method (Livak and Schmittgen, 2001) using *actb* in the liver, white muscle and heart, or *cox4a* in total blood cells, as the housekeeping gene due to its stability among different experimental conditions (average C_T varied less than 0.5 in each tissue). For multi-gene analysis, data on gene expression were in reference to the expression level of *cs* in the liver, *igfr2* in the white muscle, *gcr* in the heart, and *tim8a* in total blood cells of LDN fish, for which a value of 1 was arbitrarily assigned (**Supplementary Tables 2–5**, respectively).

This manuscript follows the ZFIN Zebrafish Nomenclature Guidelines for gene and protein names and symbols[1].

Statistical Analysis

Data on growth performance, blood biochemistry, and gene expression were analyzed by two-way analysis of variance (ANOVA) with O_2 levels (normoxia and moderate hypoxia) and stocking conditions (low and high stocking densities) as main factors. These analyses were followed by the SNK *post hoc* test for comparisons among different groups. The significance level was set at $P < 0.05$. Analyses were performed using SigmaPlot v13 (Systat Software Inc., San Jose, CA, United States). Unsupervised multivariate analysis principal component analysis (PCA) was first performed on data as an unbiased method to observe trends within conditions at different experimental groups, using EZinfo v3.0 (Umetrics, Umeå, Sweden). To achieve the maximum separation among experimental groups, Partial Least-Squares Discriminant Analysis (PLS-DA) was sequentially applied with joint data from liver, heart and white muscle, excluding the results from total blood cells due to its low contribution to the total variance. The quality of the PLS-DA model was evaluated by R2Y(cum) and Q2Y(cum) parameters, which indicated the fitness and prediction ability, respectively. Additionally, 200 random permutation tests to avoid over-fitting of the supervised model were carried out by SIMCA-P+ v15.0 (Umetrics). Cross-validation analysis of variance (CV-ANOVA) was applied (*p*-value = 0.037). The contribution of differential genes along liver, white muscle and heart tissues was assessed by means of Variable Importance in Projection (VIP) measurements. A VIP score > 1.1 was considered to be an adequate threshold to determine discriminant variables in the PLS-DA model (Wold et al., 2001; Li et al., 2012; Kieffer et al., 2016).

[1]https://wiki.zfin.org/display/general/ZFIN+Zebrafish+Nomenclature+Conventions

RESULTS

Growth Performance

Data on feed intake, growth and somatic indexes (hepatosomatic index, HSI; viscerosomatic index, VSI) are shown in **Table 2**. Fish reared under moderate hypoxia evidenced lower feed intake, which resulted in reduced weight gain and SGR. This also affected liver and viscera weight as well as HSI. This general impairment of feed intake and growth was further evidenced in fish kept at the highest density, though FE was improved in moderate hypoxia and more especially in fish kept at HD (HDH group).

Blood Analysis

Data on blood hematology and biochemistry are shown in **Table 3**. The results show a significant effect of O_2 level, with a generalized increase in Hb, Ht, RBC content, MCH, cortisol and Gh plasma levels, as well as a widespread decrease in MCHC, MCV and plasma lactate levels. Overall this feature was more pronounced in fish maintained under LD conditions. In contrast, the rearing density effect was mostly evidenced in plasma cortisol levels, which showed a pronounced rise in HD fish that was exacerbated by hypoxic conditions. Noticeably, significant O_2 level and rearing density interactions were found for cortisol, but also for Ht, MCHC, MCH, and TAA.

Gene Expression Profiling

All genes selected for PCR-arrays were found at detectable levels in the four tissues analyzed. Results of gene expression profiling in hepatic selected genes are presented in **Supplementary Table 2**. Among them, 22 out of 43 genes were affected by at least one of the experimental factors or by its interaction (i.e., the combined effect of confinement stress and hypoxia exposure leading to expression changes that could not be attributed to a single parameter), being 11 differentially expressed (DE) in response to O_2 level. Relative expression of markers from GH/IGF system (*ghr-i*), oxidative metabolism (*nd2*), and antioxidant defense and tissue repair (*gpx4*, *prdx5*) was significantly down-regulated by moderate hypoxia in LDH and HDH groups. In addition, several genes of lipid metabolism (*elovl1*, *fads2*, and *scd1b*) were up-regulated in the LD group, whereas markers of oxidative metabolism (*nd5*), and antioxidant defense and tissue repair (*gr*, *sod2*, and *grp-75*) were down-regulated in fish kept at HD conditions. Stocking density also affected 11 genes related with the GH/IGF system (*ghr-i*, *ghr-ii*, and *igf-i*), lipid metabolism (*elovl6*, *fads2*, *scd1a*, s*cd1b*, and *lpl*), oxidative metabolism (*ucp1*, *pgc1α*) and antioxidant defense and tissue repair (*grp-75*). A statistically significant interaction of O_2 levels and rearing density was found for *igf-ii*, *fads2*, *scd1a*, *scd1b*, *pgc1ß*, *gr*, *prdx3*, and *grp-170* genes.

In white skeletal muscle, 20 out of 44 DE genes were affected at least by one of the experimental condition or by their interaction (**Supplementary Table 3**). Markers of the GH/IGF system were mostly affected by stocking density (*ghr-i*, *igf-ii*, *igfbp3*, *igfbp5b*, *igfbp6b*, *insr*, and *igfr1*) rather than by O_2 levels (*igfr2*). Moderate hypoxia up-regulated *myod2* expression as the sole effect on genes related to muscle growth and cell

TABLE 1 | Genes included in the liver (L), white muscle (M), heart (H), and total blood cells (B) pathway-focused PCR arrays.

Gene name/category	Symbol
GH/IGF system	
Growth hormone receptor I	*ghr-i* LMH
Growth hormone receptor II	*ghr-ii* LMH
Insulin-like growth factor-I	*igf-i* LMH
Insulin-like growth factor-II	*igf-ii* LMH
Insulin-like growth factor binding protein 1a	*igfbp1a* L
Insulin-like growth factor binding protein 2b	*igfbp2b* L
Insulin-like growth factor binding protein 3	*igfbp3* M
Insulin-like growth factor binding protein 4	*igfbp4* L
Insulin-like growth factor binding protein 5b	*igfbp5b* M
Insulin-like growth factor binding protein 6b	*igfbp6b* M
Insulin receptor	*insr* M
Insulin-like growth factor receptor I	*igfr1* M
Insulin-like growth factor receptor II	*igfr2* M
Energy sensing and oxidative metabolism	
Sirtuin 1	*sirt1* LMH
Sirtuin 2	*sirt2* LMH
Sirtuin 3	*sirt3* LMH
Sirtuin 4	*sirt4* LMH
Sirtuin 5	*sirt5* LMH
Sirtuin 6	*sirt6* LMH
Sirtuin 7	*sirt7* LMH
Carnitine palmitoyltransferase 1A	*cpt1a* LMHB
Citrate synthase	*cs* LMHB
Proliferator-activated receptor gamma coactivator 1 alpha	*pgc1α* LMH
Proliferator-activated receptor gamma coactivator 1 beta	*pgc1β* LMHB
Hypoxia inducible factor-1 alpha	*hif-1α* LMH
Respiration uncoupling	
Uncoupling protein 1	*ucp1* L
Uncoupling protein 2	*ucp2* BH
Uncoupling protein 3	*ucp3* M
Xenobiotic metabolism	
Aryl hydrocarbon receptor 1	*ahr1* H
Cytochrome P450 1A1	*cyp1a1* H
Outer and Inner transmembrane translocation (TOM and TIM complex)	
Mitochondrial import receptor subunit Tom70	*tom70* B
Mitochondrial import receptor subunit Tom34	*tom34* B
Mitochondrial import receptor subunit Tom22	*tom22* B
Mitochondrial import inner membrane translocase subunit 44	*tim44* B
Mitochondrial import inner membrane translocase subunit 23	*tim23* B
Mitochondrial import inner membrane translocase subunit Tim8A	*tim8a* B
Mitochondrial import inner membrane translocase subunit Tim10	*tim10* B
Mitochondrial import inner membrane translocase subunit Tim9	*tim9* B

Gene name/category	Symbol
Lipid metabolism	
Elongation of very long chain fatty acids 1	*elovl1* L
Elongation of very long chain fatty acids 4	*elovl4* L
Elongation of very long chain fatty acids 5	*elovl5* L
Elongation of very long chain fatty acids 6	*elovl6* L
Fatty acid desaturase 2	*fads2* L
Stearoyl-CoA desaturase 1a	*scd1a* L
Stearoyl-CoA desaturase 1b	*scd1b* L
Lipoprotein lipase	*lpl* L
Peroxisome proliferator-activated receptor α	*pparα* L
Peroxisomeproliferator-activated receptor γ	*pparγ* L
Antioxidant defense and tissue repair	
Catalase	*cat* LMH
Glutathione peroxidase 4	*gpx4* LMH
Glutathione reductase	*gr* LMH
Peroxiredoxin 3	*prdx3* LMHB
Peroxiredoxin 5	*prdx5* LMHB
Superoxide dismutase [Mn]	*Mn-sod/sod2* LMHB
Glucose-regulated protein, 170 kDa	*grp-170* LMH
Glucose-regulated protein, 94 kDa	*grp-94* LMH
70 kDa heat shock protein, mitochondrial	*mthsp70/grp-75/mortalin* LMH
Glutathione S-transferase 3	*gst3* B
Muscle growth and cell differentiation	
Myoblast determination protein 1	*myod1* M
Myogenic factor MYOD2	*myod2* M
Myogenic factor 5	*myf5* M
Myogenic factor 6	*myf6/mrf4/ herculin* M
Myostatin/Growth differentiation factor 8	*mstn/gdf-8* M
Myocyte-specific enhancer factor 2A	*mef2a* M
Myocyte-specific enhancer factor 2C	*mef2c* M
Follistatin	*fst* M
Nuclear receptors	
Glucocorticoid receptor	*gcr* H
Estrogen receptor alpha	*er-α* H
Nuclear respiratory factor 1	*nrf1* B
Mitochondrial dynamics and apoptosis	
Mitofusin 2	*mfn2* B
Mitochondrial fission factor homolog B	*miffb* B
Mitochondrial Rho GTPase 1	*miro1a* B
Mitochondrial Rho GTPase 2	*miro2* B
Apoptosis-related protein 1	*aifm1* B
OXPHOS (Complex IV)	
Cytochrome c oxidase subunit I	*coxi* LMHB
Cytochrome c oxidase subunit II	*coxii* LMHB

(Continued)

TABLE 1 | Continued

Gene name/category	Symbol	Gene name/category	Symbol
OXPHOS (Complex I)		Cytochrome c oxidase subunit III	*coxiii* B
NADH-ubiquinone oxidoreductase chain 2	*nd2* LMHB	Cytochrome c oxidase subunit 4 isoform 1	*cox4a* B
NADH-ubiquinone oxidoreductase chain 5	*nd5* LMHB	Cytochrome c oxidase subunit 5A, mitochondrial-like isoform 2	*cox5a2* B
NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 1	*ndufa1* B	Cytochrome c oxidase subunit 6A isoform 2	*cox6a2* B
NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 3	*ndufa3* B	Cytochrome c oxidase subunit 6C1	*cox6c1* B
NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 4	*ndufa4* B	Cytochrome c oxidase subunit 7B	*cox7b* B
NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 7	*ndufa7* B	Cytochrome c oxidase subunit 8B	*cox8b* B
NADH dehydrogenase [ubiquinone] 1 beta subcomplex subunit 5	*ndufb5* B	SCO1 protein homolog, mitochondrial	*sco1* B
NADH dehydrogenase iron-sulfur protein 2	*ndufs2* B	Surfeit locus protein 1	*surf1* B
NADH dehydrogenase iron-sulfur protein 7	*ndufs7* B	Cytochrome c oxidase assembly protein COX15 homolog	*cox15* B
NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, assembly factor 2	*ndufaf2* B		

differentiation. In contrast, many genes related to energy sensing, oxidative metabolism, and antioxidant defense and tissue repair were down-regulated by low O_2 levels (*sirt1*, *ucp3*, *hif-1α*, *prdx5*, and *sod2*) or up-regulated in HD conditions (*sirt4*, *sirt7*, *coxi*, *hif-1α*, and *gpx4*). Additionally, a significant interaction between O_2 levels and rearing density is reported for *cpt1a* and *grp-170*.

In heart, changes in O_2 saturation and stocking density triggered significant differences in 19 out of 34 genes presented in the array (**Supplementary Table 4**). Up to 13 genes, including markers of the GH/IGF system (*ghr-i*), energy sensing and oxidative metabolism (*sirt1*, *sirt5*, *sirt6*, *sirt7*, *cs*, *nd5*, *pgc1α*, *pgc1ß*, and *hif-1α*) and antioxidant defense and tissue repair (*cat*, *prdx5*, and *sod2*) were down-regulated under moderate hypoxia, especially in HD conditions. The xenobiotic metabolism marker *cyp1a1* was up-regulated by hypoxia in both LD and HD. Stocking density also down-regulated the expression of several genes involved in energy sensing and oxidative metabolism (*sirt3*, *sirt5*, *cs*, and *nd2*) as well as antioxidant defense and tissue repair (*gr*, *prdx3*, *prdx5*, *grp-170*, and *grp-75*), preferentially under low O_2 levels.

In total blood cells, only 5 out of 44 genes were DE mainly by the interaction among different experimental conditions (**Supplementary Table 5**), being responsive to the stress challenge enzyme subunits of Complex I (*ndufaf2*) and Complex IV (*coxi*, *coxii*, *cox6a2*, and *cox15*) of the mitochondrial respiratory chain.

In order to assess the differential contribution of the DE genes in the physiological response to moderate hypoxia and rearing density, the tissue (liver, white skeletal muscle, and heart) gene expression dataset was analyzed by PLS-DA. The discriminant model was based on six components, which explained [R2Y(cum)] 95% and predicted [Q2Y(cum)] 65% of total variance (**Figure 1A**). The validity of the PLS-DA model was validated using a permutation test (**Supplementary Figure 2**). The first three components showed cumulative values for R2Y and Q2Y of 0.836 and 0.493, respectively. A clear separation between normoxic (LDN, HDN) and hypoxic (LDH, HDH) groups was observed along the first component (R2Y = 0.2889) (**Figures 1B,C**). Component 2 (R2Y = 0.2927) clearly separated LDN and HDN normoxic groups (**Figure 1B**), whereas component 3 (R2Y = 0.2542) discriminated LDH and HDH hypoxic groups (**Figure 1C**).

Genes with a contribution to VIP > 1.1 in component 1 were a total of 39, with a main contribution of heart (19) and liver (14) genes involved in energy sensing and oxidative metabolism (14), antioxidant defense and tissue repair (12) and OXPHOS (**Figure 2**). When the second component was also considered, a total of 44 genes presented VIP values > 1.1 (**Figure 3**), and 11 out of the 21 new genes (highlighted in yellow) were from white skeletal muscle. Energy sensing and oxidative metabolism (12), antioxidant defense and tissue repair (11), GH/IGF system (11) and OXPHOS (6) were the main categories. Considering the VIP values from the three main components (**Figure 4**), most of the genes due to component three contribution (highlighted in purple) were related to lipid metabolism.

DISCUSSION

Hypoxia in aquatic habitats is a common disturbance that is predicted to occur in the future more extensively, more frequently and for longer periods of time (Intergovernmental Panel on Climate Change, 2014), becoming a major aquaculture stressor around the world. This is especially true in the case of intensive fish farming, and unraveling the adaptive hypoxic responses helps to better understand the nature of metabolic disturbances after short- and long-term exposures to challenging O_2 levels. Blood physiological landmarks remain mostly unaltered in juveniles of gilthead sea bream exposed over 24 h to moderate hypoxia (40% O_2 saturation), whereas changes in Ht and circulating levels of Hb, glucose and lactate are reported few hours after acute hypoxia (20% O_2 saturation) (Martos-Sitcha et al., 2017). In the same study, gene expression profiling of total blood cells evidenced a consistent transcriptional response after strong hypoxic challenges, which serve to ensure a reduced but more efficient aerobic ATP production during severe hypoxia. Herein, the combined effects of moderate hypoxia (42–43%

TABLE 2 | Effects of rearing density and dissolved oxygen level on gilthead sea bream growth performance on a 21-days feeding trial.

	LD		HD		*P*-value		
	Normoxia	**Hypoxia**	**Normoxia**	**Hypoxia**	**[O_2]**	**Density**	**Interaction**
Initial body weight (g)	34.54 ± 1.11	34.22 ± 0.27	34.32 ± 0.34	33.25 ± 0.45	n.s.	n.s.	n.s.
Final body weight (g)	56.04 ± 1.89	51.65 ± 0.71	54.02 ± 0.50	48.54 ± 1.05**	0.003	n.s.	n.s.
Feed intake (g DM/fish)	23.78 ± 1.63	18.52 ± 0.7*	24.57 ± 1.06	17.54 ± 0.47**	< 0.001	n.s.	n.s.
Weight gain (%)[1]	62.21 ± 0.31	50.94 ± 1.34**	57.43 ± 1.42	45.97 ± 1.31**	< 0.001	0.003	n.s.
SGR (%)[2]	2.30 ± 0.01	1.96 ± 0.04**	2.16 ± 0.04	1.80 ± 0.04**	< 0.001	0.004	n.s.
FE (%)[3]	0.91 ± 0.03	0.94 ± 0.02	0.80 ± 0.02	0.87 ± 0.01*	0.039	0.003	n.s.
Liver weight (g)	0.94 ± 0.07	0.67 ± 0.03**	0.90 ± 0.06	0.63 ± 0.03***	< 0.001	n.s.	n.s.
Viscera weight (g)	4.41 ± 0.28	3.84 ± 0.18	4.42 ± 0.19	3.68 ± 0.10**	0.002	n.s.	n.s.
HSI (%)[4]	1.64 ± 0.07	1.33 ± 0.06**	1.58 ± 0.07	1.25 ± 0.06**	< 0.001	n.s.	n.s.
VSI (%)[5]	7.78 ± 0.29	7.65 ± 0.25	7.87 ± 0.24	7.38 ± 0.22	n.s.	n.s.	n.s.

Values on body weight, feed intake, growth, and feed efficiency are the mean ± SEM of triplicate tanks. Values on tissue biometric indexes are the mean ± SEM of 12 fish (4 fish per replicate tank). P-values are the result of two-way analysis of variance. Non-significance (P > 0.05) is stated by "n.s.". Asterisks in each row indicate significant differences with oxygen level for a given rearing density (SNK test, P < 0.05). [1]Weight gain (%) = (100 × body weigh increase)/initial body weight. [2]Specific growth rate = 100 × (ln final body weight – ln initial body weight)/days. [3]Feed efficiency = wet weight gain/dry feed intake. [4]Hepatosomatic index = (100 × liver weight)/fish weight. [5]Viscerosomatix index = (100 × viscera weight)/fish weight.

TABLE 3 | Effects of rearing density and dissolved oxygen level on blood hematology and plasma levels of metabolites, hormones, and total antioxidant capacity.

	LD		HD		*P*-value		
	Normoxia	**Hypoxia**	**Normoxia**	**Hypoxia**	**[O_2]**	**Density**	**Interaction**
Hemoglobin (g/dl)	7.18 ± 0.24	7.73 ± 0.21	7.38 ± 0.14	7.77 ± 0.26	0.041	n.s.	n.s.
Haematocrit (%)	22.18 ± 1.10	32.91 ± 1.65***	28.27 ± 1.77	29.90 ± 1.39	< 0.001	n.s.	0.004
RBC× 10^{-6} (cells/μl)[1]	2.45 ± 0.07	2.74 ± 0.07**	2.38 ± 0.06	2.82 ± 0.08***	< 0.001	n.s.	n.s.
MCHC (pg/10 μm^3)[2]	34.07 ± 1.12	24.00 ± 1.18***	26.62 ± 1.73	26.46 ± 1.10	< 0.001	n.s.	< 0.001
MCH (pg/cell)[3]	89.79 ± 4.21	116.6 ± 4.46**	116.5 ± 8.28	109.5 ± 7.21	n.s.	n.s.	0.010
MCV (μm^3)[4]	29.50 ± 1.02	28.33 ± 0.76	31.36 ± 0.93	27.73 ± 0.96*	0.014	n.s.	n.s.
Glucose (mg/dl)	54.39 ± 1.58	52.17 ± 2.44	58.04 ± 1.78	52.73 ± 2.79	n.s.	n.s.	n.s.
Lactate (mg/dl)	16.30 ± 2.78	4.81 ± 1.41**	10.22 ± 3.06	4.99 ± 0.84	0.001	n.s.	n.s.
TAA (mM Trolox)[5]	1.34 ± 0.04	1.45 ± 0.04	1.48 ± 0.03	1.43 ± 0.03	n.s.	n.s.	0.026
Cortisol (ng/ml)	23.40 ± 5.67	21.08 ± 5.32	35.69 ± 11.15	79.25 ± 9.05**	0.036	< 0.001	0.027
Growth hormone (ng/ml)	2.34 ± 0.83	6.71 ± 1.17*	5.39 ± 1.29	8.33 ± 4.20	n.s.	n.s.	n.s.
Insulin-like growth factor-I (ng/ml)	46.06 ± 4.76	46.59 ± 4.77	45.78 ± 2.27	41.03 ± 6.29	n.s.	n.s.	n.s.

Values are the mean ± SEM of 10–12 fish (4 fish per replicate tank). Non-significance (P > 0.05) is stated by "n.s.". P-values are the result of two-way analysis of variance. Asterisks in each row indicate significant differences with oxygen level for a given rearing density (SNK test, P < 0.05). [1]Red blood cells [2]Mean corpuscular hemoglobin concentration. [3]Mean corpuscular hemoglobin. [4]Mean corpuscular volume. [5]Total antioxidant activity.

O_2 saturation) and rearing density (initial density 19 kg/m^3, leading up to 30 kg/m^3 at the end of experiment) in a 3-week trial highlighted reduced growth and a different contribution of target tissues to the homeostatic load in challenged fish. As discussed below, the ultimate mechanisms for this adaptive stress response remain far to be established, though probably they have a major impact in mitochondrial respiration uncoupling, which varies across life, tissues, individuals and species (Rolfe and Brand, 1996; Hulbert et al., 2002, 2006). Indeed, improved energy efficiency and reduced mitochondrial respiration uncoupling becomes a priority with low food availability (Auer et al., 2015), and the expression of mitochondrial uncoupling proteins (UCP2/UCP3) is differentially regulated by feed restriction in glycolytic (white skeletal muscle) and highly oxidative (heart and skeletal red muscle) tissues of gilthead sea bream (Bermejo-Nogales et al., 2014a).

Growth impairments due to long-term hypoxia exposure have been noticed in a wide-range of farmed fish, including turbot (*Scophthalmus maximus*), European sea bass (*Dicentrarchus labrax*), and Atlantic salmon (*Salmo salar*) (Pichavant et al., 2001; Remen et al., 2016; Cadiz et al., 2017; Vikeså et al., 2017). As reported herein in gilthead sea bream, a primary response is the inhibition of feed intake which would favor a hypo-metabolic state with a reduced ROS production and risk of oxidative stress. This is supported by lowered plasma levels of lactate, which would reflect in hypoxic fish, and in a lower extent in HDN, a low basal metabolism rather than a shift of aerobic to anaerobic metabolism. This metabolic re-adjustment has also been reported in gilthead sea bream juveniles facing multiple sensorial stressors in a model of chronic stress that mimic daily aquaculture operations (Bermejo-Nogales et al., 2014b). Thus, according with the oxystatic theory (Dam and Pauly, 1995;

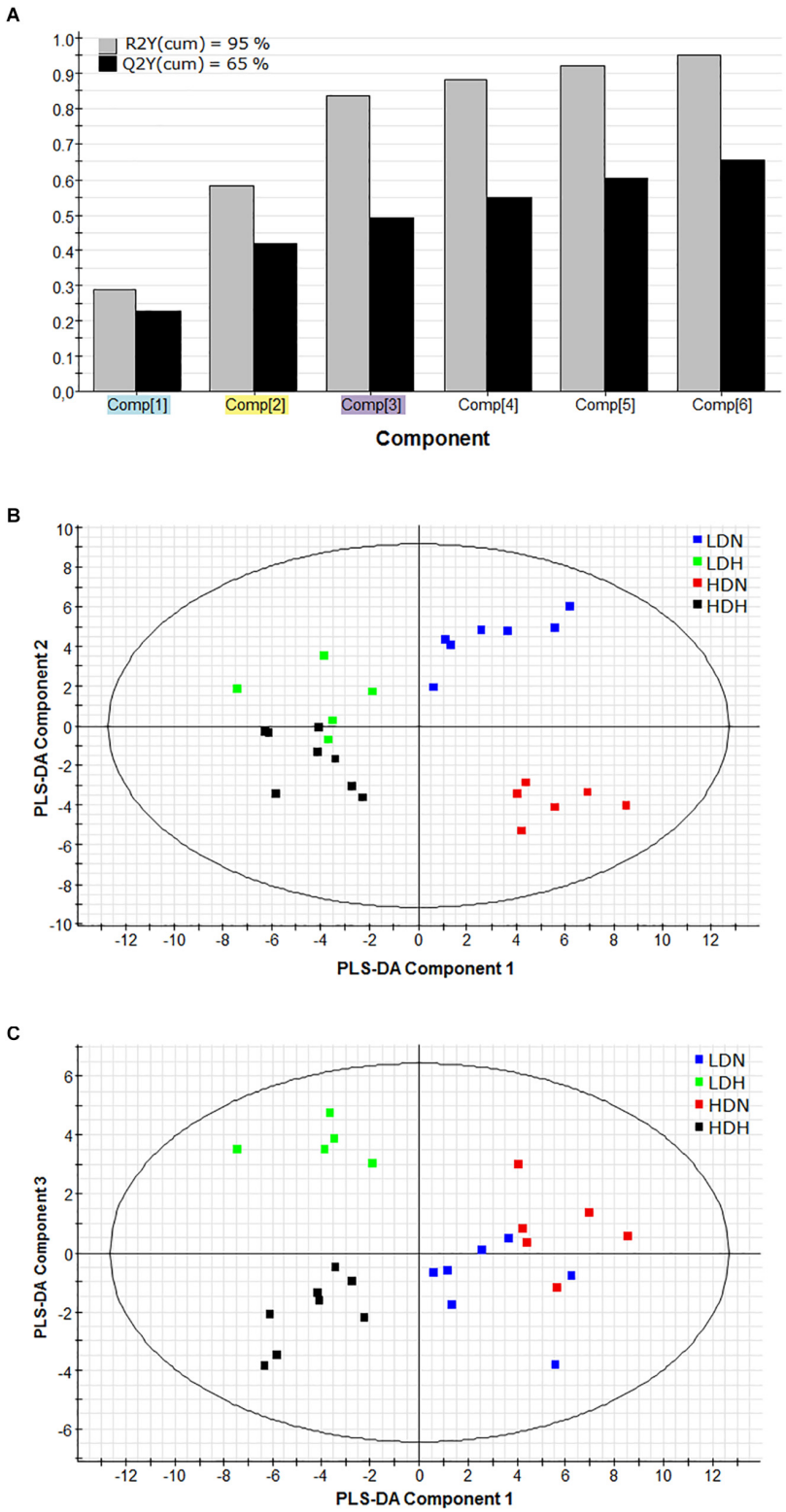
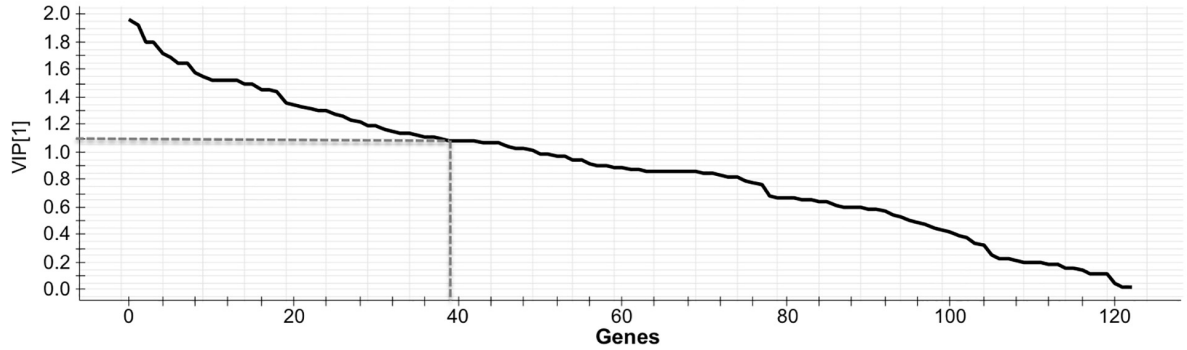


FIGURE 1 | (A) Graphical representation of the goodness-of-fit of the PLS-DA model. **(B)** Two-dimensional PLS-DA score plot representing the distribution of the samples between the first two components in the model. **(C)** Two-dimensional PLS-DA score plot representing the distribution of the samples between the first and third components in the model. R^2(cum), explained variance; Q^2(cum), predicted variance; LDN, low density normoxia; LDH, low density hypoxia; HDN, high density normoxia; HDH, high density hypoxia.

A



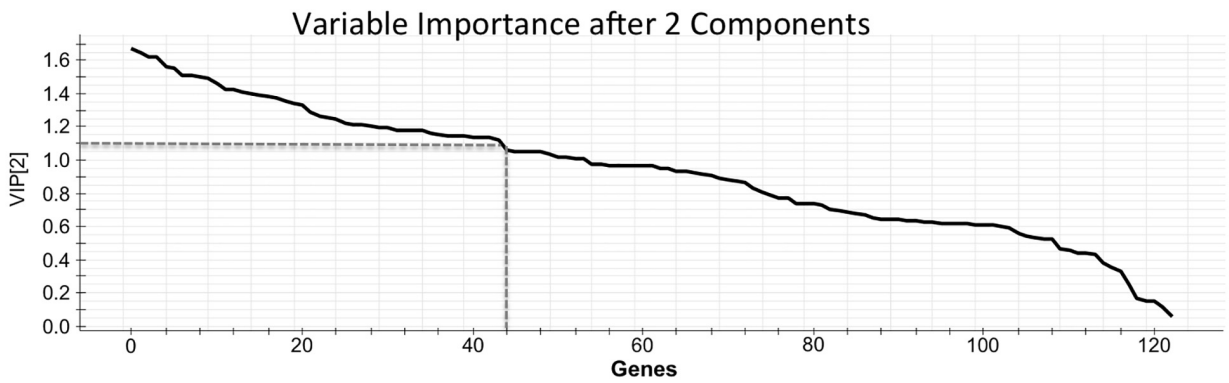
B

Order	Gene (tissue)	VIP (Component 1)	LD		HD		P-value		
			Normoxia	Hypoxia	Normoxia	Hypoxia	[O2]	Density	Interaction
1	*prdx5* (Liv)	1.964	0.59 ± 0.03	0.44 ± 0.03**	0.60 ± 0.06	0.43 ± 0.02*	<0.001	0.935	0.833
2	*nd2* (Liv)	1.926	37.29 ± 2.44	29.65 ± 2.15*	38.21 ± 3.14	26.85 ± 2.26*	<0.001	0.713	0.468
3	*nd5* (Heart)	1.801	45.09 ± 3.28	40.14 ± 2.49	44.19 ± 3.70	31.03 ± 0.90**	0.003	0.085	0.154
4	*Mn-sod / sod2* (Heart)	1.799	2.52 ± 0.15	1.93 ± 0.16*	2.19 ± 0.12	1.63 ± 0.07**	<0.001	0.024	0.902
5	*mthsp70/grp-75/mortalin* (Heart)	1.718	1.58 ± 0.08	1.58 ± 0.13	1.52 ± 0.08	1.20 ± 0.03**	0.082	0.021	0.084
6	*Mn-sod / sod2* (Liv)	1.691	1.20 ± 0.05	1.02 ± 0.08	1.30 ± 0.09	0.94 ± 0.08*	0.001	0.955	0.280
7	*cs* (Heart)	1.653	9.29 ± 0.54	7.79 ± 0.47*	7.95 ± 0.25	6.80 ± 0.25**	0.002	0.007	0.666
8	*hif-1α* (Heart)	1.651	6.67 ± 0.57	5.35 ± 0.36*	6.15 ± 0.31	4.52 ± 0.28*	0.001	0.100	0.692
9	*gpx4* (Liv)	1.584	21.34 ± 2.51	14.41 ± 1.48*	18.20 ± 2.14	12.28 ± 1.03*	0.002	0.171	0.788
10	*pgc1α* (Heart)	1.553	2.55 ± 0.15	2.28 ± 0.15	2.74 ± 0.26	2.08 ± 0.10*	0.012	0.966	0.274
11	*nd2* (Heart)	1.529	68.14 ± 4.68	70.41 ± 5.69	64.19 ± 5.02	52.61 ± 2.44*	0.322	0.026	0.145
12	*nd5* (Liv)	1.525	16.51 ± 0.83	14.41 ± 0.73	18.11 ± 1.54	14.20 ± 1.11*	0.010	0.533	0.416
13	*igfr2* (WM)	1.521	0.93 ± 0.08	0.89 ± 0.06	1.15 ± 0.08	0.81 ± 0.03*	0.005	0.298	0.029
14	*sirt1* (Heart)	1.518	0.22 ± 0.01	0.20 ± 0.01	0.21 ± 0.01	0.18 ± 0.01*	0.023	0.219	0.572
15	*ucp3* (WM)	1.498	11.65 ± 1.78	6.43 ± 0.93*	16.89 ± 4.46	6.91 ± 0.74*	0.006	0.272	0.359
16	*sirt5* (Heart)	1.496	0.99 ± 0.08	0.94 ± 0.05	0.96 ± 0.05	0.75 ± 0.04**	0.023	0.051	0.160
17	*prdx5* (WM)	1.460	28.69 ± 1.98	20.68 ± 1.10**	27.18 ± 2.14	22.48 ± 1.14*	<0.001	0.933	0.329
18	*prdx5* (Heart)	1.449	6.26 ± 0.52	4.34 ± 0.40*	4.95 ± 0.30	3.95 ± 0.16*	<0.001	0.029	0.231
19	*gr* (Liv)	1.441	0.36 ± 0.02	0.34 ± 0.02	0.42 ± 0.04	0.28 ± 0.02**	0.006	0.929	0.050
20	*pgc1β* (Heart)	1.362	0.97 ± 0.04	0.85 ± 0.06	0.96 ± 0.05	0.84 ± 0.05	0.026	0.834	0.983
21	*sirt7* (Heart)	1.350	0.07 ± 0.01	0.06 ± 0.00	0.07 ± 0.01	0.05 ± 0.00*	0.003	0.608	0.395
22	*grp-170* (Heart)	1.337	0.91 ± 0.06	0.92 ± 0.04	0.90 ± 0.05	0.77 ± 0.03*	0.191	0.070	0.103
23	*sirt2* (Liv)	1.316	0.30 ± 0.01	0.31 ± 0.02	0.34 ± 0.03	0.28 ± 0.01	0.347	0.870	0.062
24	*sirt6* (Heart)	1.309	0.05 ± 0.00	0.05 ± 0.00	0.05 ± 0.00	0.04 ± 0.00*	0.042	0.211	0.099
25	*cyp1a1* (Heart)	1.297	2.58 ± 0.25	3.63 ± 0.32*	2.71 ± 0.23	3.79 ± 0.32*	<0.001	0.601	0.960
26	*coxi* (Heart)	1.268	329.3 ± 18.4	310.7 ± 14.8	332.4 ± 27.5	278.2 ± 19.3	0.086	0.480	0.393
27	*prdx3* (Liv)	1.258	0.82 ± 0.05	0.87 ± 0.05	0.94 ± 0.07	0.72 ± 0.07*	0.178	0.787	0.030
28	*pgc1β* (Liv)	1.239	0.42 ± 0.06	0.50 ± 0.07	0.71 ± 0.08	0.39 ± 0.06**	0.103	0.220	0.008
29	*sirt2* (Heart)	1.215	0.34 ± 0.02	0.31 ± 0.02	0.33 ± 0.03	0.28 ± 0.01	0.081	0.454	0.644
30	*elovl1* (Liv)	1.194	10.63 ± 0.46	13.41 ± 0.79*	10.53 ± 0.66	12.69 ± 1.01	0.003	0.593	0.682
31	*sirt4* (Heart)	1.190	0.06 ± 0.00	0.05 ± 0.00	0.05 ± 0.00	0.05 ± 0.00	0.074	0.312	0.542
32	*ghr-i* (Liv)	1.161	3.06 ± 0.27	2.18 ± 0.26*	3.70 ± 0.36	2.79 ± 0.35*	0.007	0.049	0.977
33	*mthsp70/grp-75/mortalin* (Liv)	1.146	0.56 ± 0.04	0.55 ± 0.04	0.80 ± 0.10	0.55 ± 0.05*	0.033	0.044	0.053
34	*sirt2* (WM)	1.141	1.45 ± 0.06	1.49 ± 0.08	1.61 ± 0.14	1.43 ± 0.06	0.475	0.605	0.259
35	*elovl4* (Liv)	1.132	0.44 ± 0.05	0.49 ± 0.03	0.52 ± 0.05	0.41 ± 0.03	0.480	0.948	0.057
36	*sirt1* (Liv)	1.127	0.10 ± 0.01	0.09 ± 0.01	0.12 ± 0.01	0.10 ± 0.01	0.067	0.089	0.467
37	*grp-170* (WM)	1.111	1.80 ± 0.08	1.93 ± 0.12	2.09 ± 0.12	1.72 ± 0.11*	0.270	0.705	0.025
38	*igfr1* (WM)	1.107	1.76 ± 0.09	1.81 ± 0.09	2.36 ± 0.21	1.93 ± 0.12	0.183	0.014	0.083
39	*ghr-i* (Heart)	1.102	0.39 ± 0.01	0.35 ± 0.02	0.38 ± 0.02	0.30 ± 0.03	0.021	0.257	0.507

FIGURE 2 | (A) Graphical representation of the variable importance (VIP) scores after component 1. **(B)** Ranking list of genes showing VIP score values above 1.1 and their relative gene expression. Liv, liver; WM, white muscle. Values on relative expression are the mean ± SEM of eight fish (2–3 fish per replicate tank). *P*-values are the result of two-way analysis of variance. Asterisks in each row indicate significant differences with oxygen level for a given rearing density (SNK test, $P < 0.05$).

Saravanan et al., 2012), fish finely adjust feed intake and basal metabolism to available O_2, prioritizing feed efficiency at the expenses of maximum growth under restricted mitochondrial respiration. As a prove of this, the best FE and hormonal signatures for fast and efficient growth generally occurs before the achievement of maximum growth at the greater ration size (Brett, 1979; Pérez-Sánchez et al., 1995), pointing out a high metabolic plasticity in this euryhaline, eurytherm and euryoxic

A

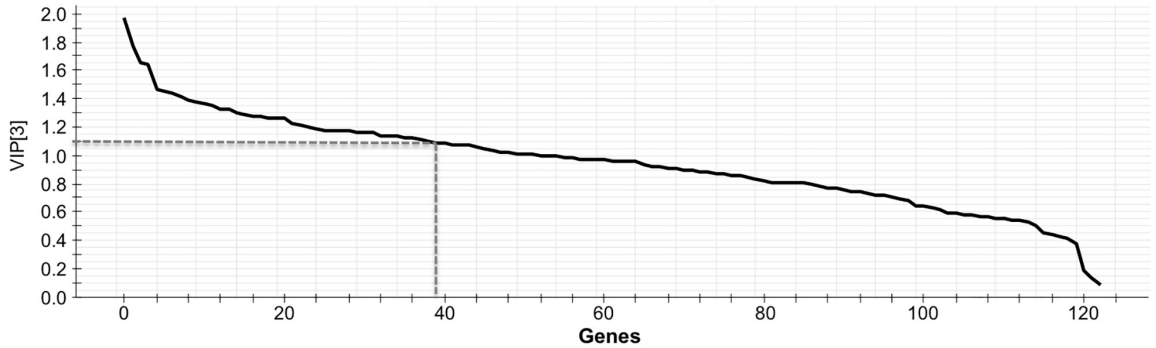


B

Order	Gene (tissue)	VIP (Component 1+2)	LD		HD		P-value		
			Normoxia	Hypoxia	Normoxia	Hypoxia	[O2]	Density	Interaction
1	*cs* (Heart)	1.675	9.29 ± 0.54	7.79 ± 0.47*	7.95 ± 0.25	6.80 ± 0.25**	0.002	0.007	0.666
2	*ghr-i* (WM)	1.644	6.42 ± 0.57	5.63 ± 0.20	4.14 ± 0.40	4.84 ± 0.51	0.915	0.002	0.101
3	*ucp1* (Liv)	1.618	23.04 ± 2.14	19.01 ± 1.99	17.55 ± 2.17	15.98 ± 1.46	0.218	0.002	0.527
4	*igfbp6b* (WM)	1.617	0.53 ± 0.05	0.59 ± 0.06	1.01 ± 0.10	0.65 ± 0.05*	0.056	0.001	0.009
5	*gpx4* (WM)	1.565	1.78 ± 0.48	1.01 ± 0.39	0.78 ± 0.21	0.43 ± 0.08	0.135	0.038	0.559
6	*Mn-sod / sod2* (Heart)	1.552	2.52 ± 0.15	1.93 ± 0.16*	2.19 ± 0.12	1.63 ± 0.07**	<0.001	0.024	0.902
7	*igfr1* (WM)	1.512	1.76 ± 0.09	1.81 ± 0.09	2.36 ± 0.21	1.93 ± 0.12	0.183	0.014	0.083
8	*igfbp5b* (WM)	1.510	5.16 ± 0.39	6.21 ± 0.35	7.08 ± 0.47	6.18 ± 0.29	0.846	0.019	0.016
9	*pgc1β* (Liv)	1.505	0.42 ± 0.06	0.50 ± 0.07	0.71 ± 0.08	0.39 ± 0.06**	0.103	0.220	0.008
10	*ghr-ii* (Liv)	1.491	2.39 ± 0.25	2.62 ± 0.35	4.00 ± 0.59	2.70 ± 0.26	0.182	0.032	0.059
11	*prdx5* (Heart)	1.460	6.26 ± 0.52	4.34 ± 0.40*	4.95 ± 0.30	3.95 ± 0.16*	<0.001	0.029	0.231
12	*insr* (WM)	1.423	3.17 ± 0.15	3.26 ± 0.29	4.30 ± 0.43	3.28 ± 0.17*	0.105	0.050	0.057
13	*lpl* (Liv)	1.421	5.77 ± 0.78	6.57 ± 0.56	8.08 ± 0.99	8.75 ± 0.82	0.361	0.010	0.949
14	*elovl6* (Liv)	1.409	0.89 ± 0.12	0.89 ± 0.14	0.52 ± 0.06	0.60 ± 0.07	0.683	0.004	0.713
15	*gpx4* (Liv)	1.402	21.34 ± 2.51	14.41 ± 1.48*	18.20 ± 2.14	12.28 ± 1.03*	0.002	0.171	0.788
16	*prdx5* (Liv)	1.389	0.59 ± 0.03	0.44 ± 0.03**	0.60 ± 0.06	0.43 ± 0.02*	<0.001	0.935	0.833
17	*nd5* (Heart)	1.385	45.09 ± 3.28	40.14 ± 2.49	44.19 ± 3.70	31.03 ± 0.90**	0.003	0.085	0.154
18	*pgc1α* (Liv)	1.372	0.06 ± 0.01	0.07 ± 0.01	0.10 ± 0.02	0.12 ± 0.02	0.304	0.010	0.558
19	*nd2* (Liv)	1.358	37.29 ± 2.44	29.65 ± 2.15*	38.21 ± 3.14	26.85 ± 2.26*	<0.001	0.713	0.468
20	*mthsp70/grp-75/mortalin* (Heart)	1.338	1.58 ± 0.08	1.58 ± 0.13	1.52 ± 0.08	1.20 ± 0.03**	0.082	0.021	0.084
21	*hif-1α* (Heart)	1.332	6.67 ± 0.57	5.35 ± 0.36*	6.15 ± 0.31	4.52 ± 0.28*	0.001	0.100	0.692
22	*igf-ii* (Liv)	1.287	2.85 ± 0.44	3.73 ± 0.53	5.37 ± 0.57	3.31 ± 0.76*	0.336	0.094	0.022
23	*pgc1α* (WM)	1.264	0.67 ± 0.21	0.30 ± 0.09	0.35 ± 0.07	0.27 ± 0.07	0.096	0.183	0.273
24	*sirt1* (Liv)	1.252	0.10 ± 0.01	0.09 ± 0.01	0.12 ± 0.01	0.10 ± 0.01	0.067	0.089	0.467
25	*sirt2* (Liv)	1.245	0.30 ± 0.01	0.31 ± 0.02	0.34 ± 0.03	0.28 ± 0.01	0.347	0.870	0.062
26	*mthsp70/grp-75/mortalin* (Liv)	1.223	0.56 ± 0.04	0.55 ± 0.04	0.80 ± 0.10	0.55 ± 0.05*	0.033	0.044	0.053
27	*igfr2* (WM)	1.209	0.93 ± 0.08	0.89 ± 0.06	1.15 ± 0.08	0.81 ± 0.03*	0.005	0.298	0.029
28	*nd2* (Heart)	1.209	68.14 ± 4.68	70.41 ± 5.69	64.19 ± 5.02	52.61 ± 2.44*	0.322	0.026	0.145
29	*ucp3* (WM)	1.204	11.65 ± 1.78	6.43 ± 0.93*	16.89 ± 4.46	6.91 ± 0.74*	0.006	0.272	0.359
30	*prdx3* (Heart)	1.199	2.14 ± 0.15	2.08 ± 0.17	1.97 ± 0.13	1.61 ± 0.14	0.168	0.041	0.305
31	*Mn-sod / sod2* (Liv)	1.192	1.20 ± 0.05	1.02 ± 0.08	1.30 ± 0.09	0.94 ± 0.08*	0.001	0.955	0.280
32	*sirt5* (Heart)	1.182	0.99 ± 0.08	0.94 ± 0.05	0.96 ± 0.05	0.75 ± 0.04**	0.023	0.051	0.160
33	*igf-i* (Liv)	1.180	9.51 ± 0.58	9.26 ± 1.20	13.29 ± 1.86	11.94 ± 0.79	0.514	0.013	0.654
34	*hif-1α* (WM)	1.178	5.45 ± 0.35	4.55 ± 0.30	6.67 ± 0.69	5.51 ± 0.31	0.028	0.020	0.773
35	*sirt1* (WM)	1.177	0.73 ± 0.04	0.70 ± 0.04	0.84 ± 0.04	0.72 ± 0.03*	0.050	0.104	0.263
36	*mthsp70/grp-75/mortalin* (WM)	1.158	5.58 ± 0.27	5.86 ± 0.43	6.96 ± 0.65	5.83 ± 0.47	0.380	0.163	0.147
37	*igfbp3* (WM)	1.156	9.33 ± 0.89	11.68 ± 1.03	13.17 ± 0.99	14.97 ± 2.18	0.143	0.015	0.845
38	*nd5* (Liv)	1.149	16.51 ± 0.83	14.41 ± 0.73	18.11 ± 1.54	14.20 ± 1.11*	0.010	0.533	0.416
39	*sirt4* (Heart)	1.145	0.06 ± 0.00	0.05 ± 0.00	0.05 ± 0.00	0.05 ± 0.00	0.074	0.312	0.542
40	*igf-i* (WM)	1.144	0.76 ± 0.09	0.67 ± 0.06	0.65 ± 0.11	0.63 ± 0.08	0.533	0.387	0.657
41	*gr* (Heart)	1.140	0.42 ± 0.03	0.40 ± 0.03	0.37 ± 0.01	0.33 ± 0.01*	0.136	0.012	0.574
42	*sirt1* (Heart)	1.138	0.22 ± 0.01	0.20 ± 0.01	0.21 ± 0.01	0.18 ± 0.01*	0.023	0.219	0.572
43	*cpt1a* (Heart)	1.138	1.63 ± 0.15	1.28 ± 0.12	1.23 ± 0.12	1.21 ± 0.10	0.142	0.071	0.189
44	*pgc1α* (Heart)	1.119	2.55 ± 0.15	2.28 ± 0.15	2.74 ± 0.26	2.08 ± 0.10*	0.012	0.966	0.274

FIGURE 3 | (A) Graphical representation of the variable importance (VIP) scores after component 2. **(B)** Ranking list of genes showing VIP score values above 1.1 and their relative gene expression. Cells shaded in blue highlight genes detected as VIP after component 1; cells shaded in yellow highlight genes detected as VIP after component 2. For further details, see legend on **Figure 2**.

A



B

Order	Gene (tissue)	VIP (Component 1+2+3)	LD		HD		P-value		
			Normoxia	Hypoxia	Normoxia	Hypoxia	[O2]	Density	Interaction
1	*fads2* (Liv)	1.966	3.82 ± 0.42	9.19 ± 0.97***	5.10 ± 0.60	4.98 ± 0.66	<0.001	0.048	<0.001
2	*cpt1a* (WM)	1.764	9.05 ± 1.02	6.72 ± 0.43*	7.44 ± 0.46	10.74 ± 1.25*	0.564	0.155	0.002
3	*scd1b* (Liv)	1.657	0.22 ± 0.03	0.52 ± 0.13*	0.22 ± 0.05	0.19 ± 0.02	0.076	0.024	0.026
4	*scd1a* (Liv)	1.639	0.43 ± 0.09	1.15 ± 0.19**	0.53 ± 0.23	0.56 ± 0.14	0.042	0.018	0.050
5	*pgc1β* (Liv)	1.462	0.42 ± 0.06	0.50 ± 0.07	0.71 ± 0.08	0.39 ± 0.06**	0.103	0.220	0.008
6	*pgc1α* (Liv)	1.456	0.06 ± 0.01	0.07 ± 0.01	0.10 ± 0.02	0.12 ± 0.02	0.304	0.010	0.558
7	*cs* (Heart)	1.435	9.29 ± 0.54	7.79 ± 0.47*	7.95 ± 0.25	6.80 ± 0.25**	0.002	0.007	0.666
8	*coxi* (WM)	1.414	740.2 ± 58.4	732.6 ± 26.9	834.2 ± 61.6	957.9 ± 57.1	0.271	0.005	0.214
9	*ghr-i* (WM)	1.385	6.42 ± 0.57	5.63 ± 0.20	4.14 ± 0.40	4.84 ± 0.51	0.915	0.002	0.101
10	*elovl6* (Liv)	1.376	0.89 ± 0.12	0.89 ± 0.14	0.52 ± 0.06	0.60 ± 0.07	0.683	0.004	0.713
11	*ucp1* (Liv)	1.362	23.04 ± 2.14	19.01 ± 1.99	17.55 ± 2.17	15.98 ± 1.46	0.218	0.002	0.527
12	*igfbp6b* (WM)	1.349	0.53 ± 0.05	0.59 ± 0.06	1.01 ± 0.10	0.65 ± 0.05*	0.056	0.001	0.009
13	*Mn-sod / sod2* (Heart)	1.326	2.52 ± 0.15	1.93 ± 0.16*	2.19 ± 0.12	1.63 ± 0.07**	<0.001	0.024	0.902
14	*ghr-ii* (Liv)	1.324	2.39 ± 0.25	2.62 ± 0.35	4.00 ± 0.59	2.70 ± 0.26	0.182	0.032	0.059
15	*gpx4* (WM)	1.306	1.78 ± 0.48	1.01 ± 0.39	0.78 ± 0.21	0.43 ± 0.08	0.135	0.038	0.559
16	*prdx5* (Heart)	1.286	6.26 ± 0.52	4.34 ± 0.40*	4.95 ± 0.30	3.95 ± 0.16*	<0.001	0.029	0.231
17	*igfbp5b* (WM)	1.273	5.16 ± 0.39	6.21 ± 0.35	7.08 ± 0.47	6.18 ± 0.29	0.846	0.019	0.016
18	*igfr2* (WM)	1.273	0.93 ± 0.08	0.89 ± 0.06	1.15 ± 0.08	0.81 ± 0.03*	0.005	0.298	0.029
19	*igfr1* (WM)	1.266	1.76 ± 0.09	1.81 ± 0.09	2.36 ± 0.21	1.93 ± 0.12	0.183	0.014	0.083
20	*igf-ii* (Liv)	1.265	2.85 ± 0.44	3.73 ± 0.53	5.37 ± 0.57	3.31 ± 0.76*	0.336	0.094	0.022
21	*lpl* (Liv)	1.260	5.77 ± 0.78	6.57 ± 0.56	8.08 ± 0.99	8.75 ± 0.82	0.361	0.010	0.949
22	*grp-170* (Liv)	1.224	1.03 ± 0.08	1.41 ± 0.17	1.37 ± 0.19	1.11 ± 0.08	0.635	0.878	0.023
23	*mthsp70/grp-75/mortalin* (Heart)	1.209	1.58 ± 0.08	1.58 ± 0.13	1.52 ± 0.08	1.20 ± 0.03**	0.082	0.021	0.084
24	*insr* (WM)	1.194	3.17 ± 0.15	3.26 ± 0.29	4.30 ± 0.43	3.28 ± 0.17*	0.105	0.050	0.057
25	*cpt1a* (Heart)	1.185	1.63 ± 0.15	1.28 ± 0.12	1.23 ± 0.12	1.21 ± 0.10	0.142	0.071	0.189
26	*prdx5* (Liv)	1.179	0.59 ± 0.03	0.44 ± 0.03**	0.60 ± 0.06	0.43 ± 0.02*	<0.001	0.935	0.833
27	*nd5* (Heart)	1.178	45.09 ± 3.28	40.14 ± 2.49	44.19 ± 3.70	31.03 ± 0.90**	0.003	0.085	0.154
28	*gpx4* (Liv)	1.176	21.34 ± 2.51	14.41 ± 1.48*	18.20 ± 2.14	12.28 ± 1.03*	0.002	0.171	0.788
29	*sirt4* (WM)	1.170	0.15 ± 0.01	0.14 ± 0.01	0.17 ± 0.02	0.18 ± 0.01	0.672	0.025	0.343
30	*sirt2* (Liv)	1.166	0.30 ± 0.01	0.31 ± 0.02	0.34 ± 0.03	0.28 ± 0.01	0.347	0.870	0.062
31	*sirt5* (Heart)	1.159	0.99 ± 0.08	0.94 ± 0.05	0.96 ± 0.05	0.75 ± 0.04**	0.023	0.051	0.160
32	*pgc1α* (WM)	1.157	0.67 ± 0.21	0.30 ± 0.09	0.35 ± 0.07	0.27 ± 0.07	0.096	0.183	0.273
33	*nd2* (Liv)	1.139	37.29 ± 2.44	29.65 ± 2.15*	38.21 ± 3.14	26.85 ± 2.26*	<0.001	0.713	0.468
34	*sirt4* (Liv)	1.132	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	0.02 ± 0.00	0.520	1.000	0.608
35	*sirt4* (Heart)	1.131	0.06 ± 0.00	0.05 ± 0.00	0.05 ± 0.00	0.05 ± 0.00	0.074	0.312	0.542
36	*coxii* (Liv)	1.125	44.07 ± 2.34	37.08 ± 1.90*	39.36 ± 3.29	39.94 ± 2.30	0.205	0.711	0.137
37	*sirt7* (WM)	1.117	0.24 ± 0.01	0.25 ± 0.02	0.27 ± 0.01	0.28 ± 0.02	0.801	0.047	0.898
38	*hif-1α* (Heart)	1.112	6.67 ± 0.57	5.35 ± 0.36*	6.15 ± 0.31	4.52 ± 0.28*	0.001	0.100	0.692
39	*hif-1α* (WM)	1.101	5.45 ± 0.35	4.55 ± 0.30	6.67 ± 0.69	5.51 ± 0.31	0.028	0.020	0.773

FIGURE 4 | (A) Graphical representation of the variable importance (VIP) scores after component 3. **(B)** Ranking list of genes showing VIP score values above 1.1 and their relative gene expression. Cells shaded in blue highlight genes detected as VIP after component 1; cells shaded in yellow highlight genes detected as VIP after component 2; and cells shaded in purple highlight genes detected as VIP after component 3. For further details, see legend on **Figure 2**.

fish species due to a permissive regulation of feed intake which allows to cope an efficient energy metabolism at slow growth rates. This also applies at the cellular level, where the maximum ATP yield per molecule of O_2 (P/O ratio) is highly dependent on ration size, as evidenced the increased P/O ratio of king penguins during periods of food shortage (Monternier et al., 2014) or liver mitochondria of brown trout (*Salmo trutta*) starved for 2 weeks (Salin et al., 2016).

Most of the hypoxia-mediated effects are accompanied by an enhanced O_2-carrying capacity denoted by a swelling, formation and/or release of new erythrocytes together with plasma volume reduction (Gallaugher and Farrell, 1998). A set of different mechanisms operate this complex response in fish. It must be considered that fish RBC are nucleated and have the ability to produce Hb during most of their life span (Speckner et al., 1989). As a general response to stress, erythrocytes from fish spleen reservoirs can be released into the blood (Pearson and Stevens, 1991). In the other hand, the affinity of fish Hb to O_2 can be modulated by allosteric regulation (variations in phosphate levels) (Val, 2000). This ability to increase blood O_2 affinity as a response to hypoxia is present even in fish living in well-oxygenated environments, as it is the case of the Antarctic fish bald rockcod (*Pathogenia borchgrevinki*) (Wells et al., 1989), a finding that highlights the general phenotypic plasticity of fish. Hypoxia studies on rainbow trout (*Oncorhynchus mykiss*) showed an initial increase in Hb concentration mediated by the release of spleen erythrocytes, but under persistent hypoxia conditions the increase of the O_2-carrying capacity arose from synthesis of new erythrocytes (Lai et al., 2006). These results suggest a complex and dynamic adaptation of fish to hypoxic conditions, a feature that could be species-specific. From our hematological data in the present work, hypoxia induced a slight increase of Hb content at both rearing densities, although the most evident and significant effects were the increase of the measured Ht, RBC count and corpuscular concentrations of Hb, which were secondly affected by rearing density. A similar enhancement of O_2-carrying capacity by means of Ht increase was observed not only in previous short-term acute hypoxia challenges in gilthead sea bream (Magnoni et al., 2017; Martos-Sitcha et al., 2017), but also in eels and rainbow trout (Wood and Johansen, 1972; Soivio et al., 1980). Conversely, changes in HSI, reflecting the amount of lipid and glycogen depots, are more informative of feed intake rather that hypoxic condition, though it is difficult to disclose the main factor. At the hormonal level, this is also inferred from the measurements of circulating levels of cortisol and Gh, which are well-known regulators of metabolic rates by their involvement on mitochondria function (see Mommsen et al., 1999; Bergan-Roller and Sheridan, 2018 for review).

In fact, cortisol is an established marker of crowding stress in gilthead sea bream (Arends et al., 1999; Skrzynska et al., 2018), as well as under other challenging conditions, and the reduced feed intake as a consequence of the stress challenge also enhanced the responsiveness of the hypothalamic-pituitary-adrenal axis (Sangiao-Alvarellos et al., 2005). Thus, cortisol is the main corticosteroid in teleost fish and its plasma levels can increase dramatically during unfavorable situations activating specific intracellular responses through glucocorticoid receptors present in many fish organs, which in turn alleviate the great energy demand in a systemic way by increasing blood metabolite concentrations and redistributing the energy balance in the organism (reviewed by Mosconi et al., 2006; Wendelaar Bonga, 2011). In fish, high plasma cortisol levels modulate the metabolism of carbohydrates by stimulating gluconeogenesis in liver, and also increase the availability of substrates derived from proteins and fats, although the role of cortisol in fish lipid metabolism has not been clearly established (Vijayan et al., 2010). All this agrees with the observation that the greater circulating concentration of cortisol was achieved herein in the HDH group, which also experienced a higher feed intake inhibition. However, this system cannot be continuously refed, and evidence in rodents points out toward a translocation of cortisol into mitochondria mediated by glucocorticoid receptors to reduce mitochondrial activity and the risk of oxidative stress (Du et al., 2009), a mechanism that could be possibly extended to other animal models including fish, although this fact has still not been documented. Thus, in the absence of a cortisol response, chronic cold-thermal stress up-regulates OXPHOS in gilthead sea bream, whereas the cortisol rise in fish facing multiple aquaculture sensorial stressors is accompanied by a pronounced transcriptional repression of all the hepatic complex units of the mitochondrial respiratory chain (Bermejo-Nogales et al., 2014b). A similar response has been reported after acute hypoxia exposure, though in this case the catalytic and regulatory enzyme subunits of Complex IV (the last electron acceptor of respiratory chain involved in the O_2 reduction) were up-regulated, maximizing the use of available O_2 for aerobic ATP generation (Martos-Sitcha et al., 2017). The aerobic scope and gene expression profiling of mitochondria is also highly regulated at the nutritional level by synthetic and natural dietary oils (Pérez-Sánchez et al., 2013; Martos-Sitcha et al., 2018), and the suppression of heptanoate effects upon exercise endurance is viewed as a protective measure to counteract disproportionate oxidative metabolic rates in fish fed fast energy-delivery nutrients (short/medium chain fatty acids). In other words, the interplay between stimulatory and inhibitory effects must be envisaged as a response to the energy needs, even if metabolic fuels were available. Accordingly, in the present study, the increased circulating levels of Gh in hypoxic/crowded fish will reflect a reduced feed intake and energy demand rather than a minor capacity to combat oxidative stress, as it is generally referenced in fish and other animal models overexpressing GH (Brown-Borg et al., 1999; Brown-Borg and Rakoczy, 2000; McKenzie et al., 2003; Almeida et al., 2013).

The gene expression profiling of key metabolic biomarkers also contributes to better understand the search of allostatic load in a challenging environment. Thus, the two-way ANOVA revealed the different involvement of tissues and gene categories into the stress-mediated responses. This observation is reinforced by the use of multivariate analysis, which offers the possibility to identify, at a high level of confidence, the most responsive tissues and biomarkers for a given stress stimuli in a factorial stress design. Using such approach, we are able to explain and to predict a high percentage of total variance, being noteworthy that liver, white skeletal muscle and heart remained responsive at long-term to changing O_2 and rearing density, whereas the expression pattern of blood cells became mostly unaltered with the imposed stress stimuli of medium intensity, in contrast with the previously reported wide expression change of mitochondrial-related genes in total blood cells in response to a more severe challenge (Martos-Sitcha et al., 2017). For this reason, the transcriptomic analysis of total blood cells during moderate hypoxia challenges

was not included in our PLS-DA model to avoid the background noise detected when introduced. In previous studies in gilthead sea bream and other animal models, liver and cardiac muscle are highly responsive to hypoxia (Everett et al., 2012; Hermes-Lima et al., 2015; Magnoni et al., 2017), and genes of these two tissues highly contributed herein to separate normoxic and hypoxic fish along the first component of our PLS-DA (R2Y = 0.2889). One of the most relevant genes participating in this discriminant feature is the *hif-1α*, a well-documented regulator of O_2 homeostasis. This transcriptional factor acts at a multi-regulatory level, managing the hypoxic responsiveness of a vast array of transcribed proteins including antioxidant enzymes (Nikinmaa and Rees, 2005; Lushchak and Bagnyukova, 2006). Concretely, herein, we show a clear down-regulation of *hif-1α* that was coincident with the repressed expression of other down-stream markers of antioxidant defense and tissue repair (*prdx5*, *sod2*, *mortalin*, *gpx4*, *gr*, *grp-170*, and *prdx3*). This intriguing result can be cautiously interpreted since Hif-1 is mostly regulated at the post-translational level (Ke and Costa, 2006), though this finding should be understood as a steady-state in which O_2 availability is reduced but maintained high enough to preserve aerobic metabolism at a relatively high level. This fact is supported by a reduced expression of *cs* and associated enzyme subunits of Complex I (*nd2*, *nd5*), used successfully in several studies as markers of mitochondria abundance and Krebs cycle activity (Larsen et al., 2012; Magnoni et al., 2017). In addition to that, several *sirts* (*sirt1, 2, 5, 6*, and *7*) of liver or cardiac muscle were overall down-regulated in hypoxic fish, especially in the case of HDH fish. These NAD^+-dependent deacetylases are energy sensors that act in gilthead sea bream as a link between nutrition and energy metabolism in different growth models with nutrients and genetic variables as source of variation (Simó-Mirabet et al., 2017a,b, 2018). This was extended herein to hypoxia/crowding stress, which indicates that most of the envisaged adaptive responses should include changes in the acetylation status of both nuclear histones, and cytoplasmic and mitochondrial metabolic enzymes.

The second component of our PLS-DA (R2Y = 0.2927) differentiates normoxic fish held at different stocking densities. In this case, the white skeletal muscle clearly promotes this separation mainly by the expression pattern of genes related to GH/IGF system (*ghr-i*, *igfbp6b*, *igfbp5b*, *insr*, *igfbp3*, *igf-i*). Components of liver and muscle GH/IGF system are differentially regulated by nutrients and seasonal environmental cues (reviewed by Pérez-Sánchez et al., 2018), but herein this observation becomes specially relevant for muscle *ghr-i* that highly contributes to discriminate the detrimental growth effects of crowding stress from those more related to hypoxia or water quality. Likewise, genes of *igfbp* repertoire highly contribute to this differentiation, though the discriminant role of Igfbp counterparts (*igfbp6b* > *igfpb5b* > *igfpb3)* was mostly reduced to skeletal muscle and *Igfbp3/5/6* clade. Functional divergence regarding the growth-inhibitory or growth-promoting action of *igfbp*s have been reported across species and physiological context (Garcia de la Serrana and Macqueen, 2018), but herein the overall depressed expression of the muscle *Igfbp* clade in HD fish is consistent with inhibitory rather than stimulatory growth-promoting effects, which also involves the regulation of insulin and Igfbp receptors with important implications on the final arrangements of carbohydrate, growth, and energy metabolism (reviewed by Reindl and Sheridan, 2012; Vélez et al., 2017). Indeed, fish are the first group in the vertebrate tree in which there is evidence of distinct insulin and Igf receptors, though certain cross-reactivity between ligand and receptors of insulin and Ifgs occurs and the specific-mediated effects are sometimes confounding. However, it is well-recognized that insulin stimulates Hif-1, whereas intermittent hypoxia induces insulin resistance in mice (Treins et al., 2002; Poulain et al., 2017). Likewise, *Igfbp1* knock-down alleviates the hypoxia-induced growth retardation in zebrafish (Kajimura et al., 2005), whereas the IGFBP4 expression is induced by hypoxia in U87 glioma cells (Minchenko et al., 2016). From our results it is also conclusive that the muscle expression of *igfr1 and igfr2* are specially responsive to hypoxia, but importantly *insr* in gilthead sea bream seems to be more receptive to crowding stress rather than hypoxic stress stimuli, though it remains to be established the functional relevance of this differential responsiveness to environmental stressors.

Finally, the third component of our multivariate approach (R2Y = 0.2542) discriminates the effect of stocking density in fish exposed to moderate hypoxia, with a marked contribution of hepatic fatty desaturases with Δ6 (*fads2*) or Δ9 (*scd1a*, *scd1b*) activities due to its strong and specific induction in LDH fish. A muscle marker of FA oxidation (*cpt1a*) was also consistently up-regulated in this group, but this response was opposite to that found in HDH group, which is indicative of the different regulation of muscle lipid catabolism by hypoxia in fish stocked at standard or high densities. Likewise, the major discriminant capacity of other factors related to lipid metabolism (*elovl6*) was achieved between normoxic fish held at LD and HD. Meanwhile, other elongases (*elovl5*) with a well-recognized role in the control of hepatic triglyceride storage did not take part of the group separation in the present study, though *elovl5* highly contributes to differentiate two gilthead sea bream strains with differences in growth performance and metabolic capacities (Simó-Mirabet et al., 2018). Previous studies, in gilthead sea bream (Benedito-Palos et al., 2013, 2014) and European sea bass (Rimoldi et al., 2016) have also evidenced an important effect of ration size on the hepatic and muscle regulation of most of the lipid biomarkers assessed in the present study, but again it is difficult to disclose what is the main factor (feed intake or the imposed stress condition) due to the logistic limitations of our experiment design that did not include pair-fed groups. However, as a general rule, stressors enhance the demand of specific nutrients and hypoxia in particular promote the cellular uptake of extracellular unsaturated fatty acids in mice cell lines (Ackerman et al., 2018). Moreover, in hypoxic stress, cancer cells enhance lipid synthesis that is important for membrane biosynthesis and energy storage for cell survival and proliferation (Huang et al., 2014), being induced this hypoxia lipogenic phenotype via dependent- and HIF1α-independent pathways (Valli et al., 2015). All this together supports the pronounced stimulation of *fads2* and *scd* desaturases in our stress model, which will promote the increase of the unsaturation

index of structural lipids as previously reported during feed restriction in gilthead sea bream (Benedito-Palos et al., 2013). In agreement with this, hypoxia stress on HeLa cells leads to significant changes in their membrane lipid profiles, and polyunsaturated phospholipid species are becoming stronger biomarkers for discriminating the effect of hypoxia treatment on membrane fluidity and further membrane-dependent functions (Yu et al., 2014).

A growing effort is devoted in fish to define a "stressome," or a catalog of genes expressed when an organism is challenged with a given stress, particularly those that comprise a common response to diverse stressful scenarios (as reviewed in Balasch and Tort, 2019). Our work follows a similar approach in order to determine not only the most consistent and reliable biomarkers for welfare assessment, but also the most (or least) convenient tissues for these analyses.

CONCLUDING REMARKS

The findings described herein evidence the re-adjustment of several biological functions in a factorial model of chronic stress, where most of the hypoxia-mediated effects on growth performance and energy metabolism were exacerbated in fish held at HD. The integrated data on blood hematology, biochemistry and hormonal profiling highlights a hypo-metabolic state with the enhancement of O_2-carrying capacity, being this metabolic feature accompanied by a reduction in voluntary feed intake and a more efficient energy metabolism at the expenses of slow growth rates. This notion was supported at the transcriptional level by global changes of tissue-gene expression profiles, which also evidenced tissue-specific orchestration of stress response reflecting the nature and intensity of stress stimuli, but also the different metabolic capacities of targeted tissues. Thus, the number of DE in response to a given stress stimuli varies across the targeted tissues (liver $\geq$ heart $>$ muscle $>$ blood), but importantly PLS-DA analysis also informs of the different tissue contribution to the allostatic load. Thus, liver and heart mostly contribute to cope with a global hypoxic response involving changes in energy sensing and production as well as antioxidant defense and tissue repair. In contrast, metabolic markers of skeletal muscle with a high over-representation of GH/IGF system mostly contribute to disclose the effects of rearing density not necessarily mediated by low O_2 levels. Likewise, lipid metabolism and hepatic fatty acid desaturases are becoming strong biomarkers of crowding stress in hypoxic fish, which reveals the complexity and metabolic plasticity of gilthead sea bream to cope with stress resilience under intensive fish farming. These results evidence the potential of the identified biomarkers for a reliable assessment of fish welfare, although for some tissues such as blood cells, responsiveness is highly dependent on the intensity of the challenge. Overall, this new knowledge will contribute to better explain and understand the different stress resilience of farmed fish across individuals and species.

ETHICS STATEMENT

All procedures decribed here were approved by the Ethics and Animal Welfare Committee of Institute of Aquaculture Torre de la Sal and carried out according to national (Royal Decree RD53/2013), and the EU legislation (2010/63/EU) on the handling of animals for experiments.

AUTHOR CONTRIBUTIONS

JM-S, JC-G, and JP-S conceived and designed the study. JM-S, PS-M, and VdlH carried out the experimental procedures. JM-S and JP-S wrote the original draft. All authors analyzed and interpreted the data, reviewed, edited, and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

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Stress Response System in the Fish Skin—Welfare Measures Revisited

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The skin of vertebrates acts as a biological barrier defending the organism against many harmful environmental factors. It is well established that the main stress hormone cortisol, together with antioxidants such as melatonin (Mel) and its biologically active metabolites set up a local stress response system in the mammalian skin. Recently, our research group has shown that in fish there are basic conditions for the functioning of a cutaneous stress response system (CSRS) similar to that in mammals, where Mel with its biologically active metabolite AFMK (N1-acetyl-N2-formyl-5-methoxykynuramine) and cortisol act together to protect organism against unfavorable environment. Since aquaculture is making an increasing contribution to the global economy and new laws are demanding people to respect the welfare requirements of animals there has been increasing interest in indicators of fish well-being in aquaculture. This article addresses the problem of on-farm assessment of fish welfare and proposes the CSRS as a new source of information on the welfare status of farmed fish.

Keywords: fish, welfare, skin, stress, melatonin, mucus, aquaculture, AFMK

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There has been growing public concern for the well-being of farmed fish since aquaculture is making an increasing contribution to the global economy and, at the same time, new laws are demanding people to respect the welfare requirements of animals to ensure their right treatment (for review, see Browman et al., 2018). This has resulted in considerable research on the impact of various aspects of aquaculture practice on fish welfare and in a growing body of literature identifying potentially harmful aspects thereof and proposing different approaches to assessing animal well-being (for review, see Huntingford et al., 2006; Ashley, 2007; Martins et al., 2012). The issue of the welfare of aquatic animals was revisited quite recently by Browman et al. (2018). Nowadays, there are many physiological, biochemical, and behavioral indicators of fish welfare, many of them linked with the systemic response to stress (see Reviews cited above). However, appropriate combinations of welfare measures feasible in a fish farm are still much sought-after. In this context, our research group of marine physiologists at the Institute of Oceanology of the Polish Academy of Sciences has recently begun to investigate the cutaneous system of response to stress (CSRS) as a new source of information on the welfare status of farmed fish.

The skin of vertebrates is more than just a protective barrier between the external and internal environments. This largest body organ, which is directly exposed to multiple external stressors, can initiate appropriate responses to preserve body homeostasis by generating signals for the systemic defense system, but not only. In, Slominski et al. (1995) proposed that there is a local system of response to stress in mammalian skin which is an equivalent to the hypothalamic-pituitary-adrenal axis (HPA). Since then, there has been growing evidence supporting that view and many biologically active molecules engaged in the system have been identified (for review, see Slominski et al., 2008, 2012). It turns out that there are not only stress hormones related to the HPA, such as corticotropin-releasing hormone, adrenocorticotropic hormone, and cortisol, but

also antioxidants, e.g., melatonin (Mel), with its biologically active metabolites, the kuramines N1-acetyl-N2-formyl-5-methoxykynuramine (AFMK) and N1-acetyl-5-methoxykynuramine (AMK) (Slominski et al., 2002, 2005, 2012), all engaged in the inactivation of free radicals in the cells (Hardeland, 2008; Galano et al., 2013). However, the epidermis that constitutes the outer layer of skin in vertebrates is diverse. The formation of a stratum corneum in the process whereby living keratinocytes are transformed into non-living corneocytes making a barrier against water-loss, mechanical and chemical stress, and infection is essential for all terrestrial vertebrates (Alibardi, 2009). The process of keratinization occurs, though to a lesser extent, also in fish (Mittal and Banerjee, 1980). But in fish, the epithelial mucus layer constitutes a first line of defense (Shephard, 1994), and therefore we consider the mucus a part of fish CSRS (see below).

Since it has been demonstrated that the exposure of mammalian skin to numerous pathological agents and harmful environmental factors induces production of biologically active compounds acting at both the cutaneous and systemic level to protect homeostasis (Slominski et al., 2005, 2012), one might expect to find a similar mechanism in fish. Recently, our research group has shown that in fish there are basic conditions for the functioning of a CSRS similar to that in mammals. Indeed, the main stress hormone cortisol, and antioxidants Mel and AFMK are present in fish skin (Kulczykowska et al., 2018). Moreover, *in vitro* experiments with European flounder skin explants have demonstrated that cortisol added to the incubation medium can stimulate Mel and AFMK release in a dose-dependent manner (Kulczykowska et al., 2018). Furthermore, the concentrations of cortisol used in the experiments mimicked plasma cortisol levels in fish exposed to different types of stress, such as handling, confinement, high stock density or food-deprivation (Mommsen et al., 1999; Barton, 2002). A question arises as to whether Mel and AFMK are formed in fish skin. One can expect the answer to be "yes" because a constitutive expression of genes encoding aralkylamine N-acetyltransferase (AANAT; EC2.3.1.87; the enzyme in Mel biosynthesis), albeit low, has been found in the skin of both the rainbow trout (*Oncorhynchus mykiss*) (Fernández-Durán et al., 2007) and the three-spined stickleback (*Gasterosteus aculeatus*) (Kulczykowska et al., 2017). Moreover, specifically *cutaneous* synthesis of Mel would seem to be confirmed by studies in various fish species where higher Mel concentrations were found in their skin than in plasma or muscles (Kulczykowska et al., 2018; our unpublished data). It can thus be presumed that, in fish subjected to unfavorable conditions, when their circulating cortisol concentration is high, the cutaneous synthesis of Mel/AFMK will increase, even though AANAT gene expression and Mel/AFMK production in the skin of non-stressed individuals is normally low (discussed by Kulczykowska et al., 2018). A stimulation of Mel production in organs such as the gut and skin when exposed directly to environmental pollutants is considered a protective action in any vertebrate organism (Hardeland, 2005; Tan et al., 2007). For instance, in white stork nestlings (*Ciconia ciconia*) living near a copper smelter, toxic compounds in the diet can stimulate Mel synthesis on-the-spot in the gut, to serve as a first-line defensive step in the protective mechanism of the organism (Kulczykowska et al., 2007). Various oxidative stress measures are commonly used in fish biology (Birnie-Gauvin et al., 2017), but not yet Mel and its metabolites.

Certainly, one might expect to find some dissimilarity between the stress response systems in fish skin and in terrestrial vertebrates' skin. The aquatic environment and the specific structure/function of fish skin make a difference! The skin in fish is covered by mucus and this outermost layer has been considered a first line of defense against a wide range of detrimental environmental conditions (Shephard, 1994) and pathogens (Benhamed et al., 2014). Analysis of skin mucus, which is continuously secreted by cells, contains many components, and is easily collected, can be a valuable source of information on the welfare status of farmed fish. Metabolites such as glucose, lactate, protein, and cortisol in skin mucus have already been studied to judge their suitability for determination of physiological response to different stressors (De Mercado et al., 2018; Fernández-Alacid et al., 2018). A positive relation between stress markers in plasma and skin mucus was recently demonstrated by Fernández-Alacid et al. (2019). This is good news from the point of view of elaboration of a new non-invasive, quick, and simple assay to detect stress responses in fish, especially in aquaculture. The current research of our group at the Institute of Oceanology focuses on the CSRS and its components, Mel and AFMK, synthesized and released in response to stress in fish. Analysis of cortisol together with Mel and AFMK in the mucus seems to be a promising approach to on-farm assessment of fish welfare.

AUTHOR CONTRIBUTIONS

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

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Chronic Cold Stress Alters the Skin Mucus Interactome in a Temperate Fish Model

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Temperate fish are particularly sensitive to low temperatures, especially in the northern Mediterranean area, where the cold season decreases fish-farm production and affects fish health. Recent studies have suggested that the skin mucus participates in overall fish defense and welfare, and therefore propose it as a target for non-invasive studies of fish status. Here, we determine the mucus interactome of differentially expressed proteins in a temperate fish model, gilthead sea bream (*Sparus aurata*), after chronic exposure to low temperatures (7 weeks at 14°C). The differentially expressed proteins were obtained by 2D-PAGE of mucus soluble proteins and further assessed by STRING analyses of the functional interactome based on protein-protein interactions. Complementarily, we determined mucus metabolites, glucose, and protein, as well as enzymes involved in innate defense mechanisms, such as total protease and esterase. The cold mucus interactome revealed the presence of several subsets of proteins corresponding to Gene Ontology groups. "Response to stress" formed the central core of the cold interactome, with up-regulation of proteins, such as heat shock proteins (HSPs) and transferrin; and down-regulation of proteins with metabolic activity. In accordance with the low temperatures, all proteins clustered in the "Single-organism metabolic process" group were down-regulated in response to cold, evidencing depressed skin metabolism. An interactome subset of "Interspecies interaction between species" grouped together several up-regulated mucus proteins that participate in bacterial adhesion, colonization, and entry, such as HSP70, lectin-2, ribosomal proteins, and cytokeratin-8, septin, and plakins. Furthermore, cold mucus showed lower levels of soluble glucose and no adaptation response in total protease or esterase activity. Using zymography, we detected the up-regulation of metalloprotease-like activity, together with a number of fragments or cleaved keratin forms which may present antimicrobial activity. All these results evidence a partial loss of mucus functionality under chronic exposure to low temperatures which would affect fish welfare during the natural cold season under farm conditions.

Keywords: gilthead sea bream, low temperatures, mucus interactome, string analysis, zymography

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INTRODUCTION

Fish from temperate latitudes are typically exposed to broad fluctuations of water temperature. In nature, fish may use behavioral responses to overcome the threat that such fluctuations pose, through migration or by descending in the water column to take advantage of more stable temperatures. However, fish under aquaculture conditions cannot enact this natural behavior. When temperature variations approach certain upper or lower limits, according to the thermal tolerance range of the species, the consequences can be highly deleterious or even fatal. Both acute and chronic exposure to suboptimal temperatures generally have suppressive effects, particularly on adaptive immunity [reviewed in Abram et al. (2017)]. This has traditionally been assumed to be responsible for winter mortality in a large number of wild fish populations (Hurst, 2007). Furthermore, evidence has accumulated which suggests that diseases and handling disturbances in cultured species are also related to low water temperatures (Toranzo et al., 2005; Ibarz et al., 2010a). Gilthead sea bream have been cultured successfully for several decades and are an important species for the European aquaculture industry. However, they are particularly sensitive to low temperature, especially in the northern Mediterranean area, where cold affects fish health and decreases fish-farm production. A drop in temperature causes cold-induced fasting, thermal stress, and metabolic depression, resulting in a lower immune capacity and the fish being more susceptible to infection (Ibarz et al., 2010a). Moreover, in this species, there is no significant thermal compensation under sustained cold conditions and in such a situation any additional stress factors can cause fish to suffer metabolic collapse, even during cold recovery (Sánchez-Nuño et al., 2018a,b).

Management of fish farms is crucial to ensure fish health and welfare. Although potential stressors can be found at all stages of the production cycle, they are likely to be of greatest importance during the particularly sensitive period at low temperatures, during which fish are immunodepressed and suffer metabolic alterations (Tort et al., 1998a,b; Ibarz et al., 2010a; Silva et al., 2014). For this reason, analysis of the epidermal mucus has recently been proposed as a putative non-invasive and reliable method by which to study the response of fish physiology to environmental challenges (Benhamed et al., 2014; Sanahuja and Ibarz, 2015; Cordero et al., 2017; De Mercado et al., 2018; Fernández-Alacid et al., 2018, 2019). This method could replace other more invasive and deleterious diagnosis methods, such as hematological or histological analysis. In teleosts, the skin mucus is the first barrier against physical and chemical attacks. In addition to the structural mucin matrix, it contains components related to defense, metabolism, environmental influences and nutritional status (Esteban, 2012; Sanahuja and Ibarz, 2015). The skin mucus represents an important portal of pathogen entry, since it induces the development of biofilms and represents a favorable microenvironment for bacteria; the main disease agents in fish [reviewed in Benhamed et al. (2014)]. Skin mucus can trap and immobilize pathogens before they come into contact with epithelial surfaces, because it is impermeable to most bacteria and many pathogens (Mayer, 2003; Cone, 2009). Mucus is secreted by epidermal cells, mainly goblet cells, in a continuous effort to ensure its composition is adequate to prevent stable colonization by potentially infectious microorganisms as well as invasion by metazoan parasites (Ingram, 1980; Ellis, 2001; Nagashima et al., 2003). Thus, alterations in skin mucus due to low temperature conditions would modify this surface barrier and may facilitate bacterial adhesion, colonization, and entrance.

Therefore, the composition and characteristics of skin mucus are very important for the maintenance of its immune functions (Cone, 2009), as well as for other biological roles attributed to it: locomotion, respiration, ion regulation, excretion, and thermal regulation (Esteban, 2012). To extend the characterization of fish skin mucus, several studies have addressed the general mucosa proteome (Rajan et al., 2011; Guardiola et al., 2015; Sanahuja and Ibarz, 2015) and changes in skin mucus proteome in response to infections (Easy and Ross, 2009; Provan et al., 2013; Rajan et al., 2013). Fish mucus also serves as a repository of numerous innate immune factors; specific activities of enzymes, such as lysozyme, phosphatase, esterase, and protease also play an important role in mucosal immunity, which includes inhibitory or lytic activity against pathogens (Guardiola et al., 2014a). An interesting variety of protease families play important roles in mucus, such as serine and cysteine proteases, which are involved in organism defenses against bacteria and protozoa by lysing the parasite; or metalloproteases, which are involved in the activation of pro-cathepsin D, an enzyme that hydrolyses proteins for peptide production (Aranishi and Nakane, 1997; Cho et al., 2002b; Rakers et al., 2013). However, there is little information, at the level of skin mucus, on the role, and relevance of the activities of these proteases in cultured marine species, or their relationship with temperature fluctuations.

All this indicates the need to study the importance of mucus for overall fish defenses and welfare status during the problematic low-temperatures period of fish culture. Thus, the aim of the present work was to determine the main changes in the gilthead sea bream mucus interactome, based on protein–protein interactions, after chronic exposure to low temperatures (7 weeks at 14°C). The differentially expressed proteins were obtained by 2D-PAGE of soluble mucus proteins and further studied by STRING analysis of the functional interactome. The protease activities of skin mucus were also characterized by zymography, to identify different digestion bands. Our results therefore provide better understanding of mucus functionality at low temperatures in temperate marine species.

MATERIALS AND METHODS

Animal Conditions

Gilthead sea bream, with an average body weight of 145 g, were obtained from a local fish farm and acclimated indoors at the facilities of the Faculty of Biology of the University of Barcelona (Barcelona, Spain) at 22°C for 2 weeks, using standard commercial fish feed (Skretting ARC). Following this period, the fish were randomly distributed into two groups in a water-recirculating system. The system was composed of 400 L tanks with solid and biological filters. Water temperature and oxygen concentration were monitored, while nitrite, nitrate,

and ammonia concentrations were maintained at initial levels throughout the experimental period. For the experiment, the fish were initially maintained at 22°C for 4 weeks, after which time mucus samples were obtained non-invasively from 12 animals (Warm), and thereafter the water temperature was cooled to 14°C over 5 days (at 1.5°C per day) and maintained at this temperature the remained of a total 7 weeks period (including the 5 days cooling down period). At the end of this period, mucus samples were obtained from 12 animals (Cold). For both samplings, warm and cold, fish were 24 h-fasted. All animal-handling procedures were conducted following the European Union Council (86/609/EU) and Spanish and Catalan government-established norms and procedures and with Ethics and Animal Care Committee of the University of Barcelona approval (permit no. DAAM 9383).

To collect mucus samples, fish were lightly anesthetized with 2-phenoxyethanol (100 ppm, Sigma-Aldrich) to avoid stress of the manipulation. Sterile glass slides were used to carefully remove mucus from the over-lateral line from the front in the caudal direction, as explained in Fernández-Alacid et al. (2018). The sterile glass was gently slid along both sides of the animal and the epidermal mucus was carefully pushed into a sterile tube (2 mL). Non-desirable areas of the operculum, and ventral-anal and caudal fins were avoided. The mucus collected was immediately frozen with liquid nitrogen and stored at −80°C until analysis.

Two-Dimensional Electrophoresis of Mucus Samples

Protein Extraction

Mucus samples for two-dimensional electrophoresis (2D-PAGE) protocols were solubilized in an equal volume of ice-cold lysis buffer (4 mL · g^{-1} tissue; 7 M urea, 2 M thiourea, 2% w/v CHAPS; and 1% protease inhibitor mixture) and centrifuged at 20,000 g for 15 s at 4°C, with the resultant supernatant aliquoted, avoiding pellet resuspension, and surface lipid layer. The supernatants obtained were subjected to a clean-up procedure (ReadyPrep 2-D clean-up kit, BioRad, Alcobendas, Spain) to enhance protein extraction, as previously described in Sanahuja and Ibarz (2015), and the proteome map of soluble skin mucus proteins was obtained by 2D-PAGE. The significantly expressed proteins were further analyzed by LC-MS/MS and identified using database retrieval. Protein concentration was determined by the Bradford assay with bovine serum albumin (BSA) as standard (BioRad).

Dimensional Electrophoresis Separation

Two mucus samples were polled in order to obtain 450 μg of protein dissolved in 450 μL of rehydration buffer containing 7 M urea, 2 M thiourea, 2% w/v CHAPS, 0.5% v/v IPG buffer, 80 mM DTT, and 0.002% bromophenol blue. Five such samples of skin mucus protein extract from each condition (Warm and Cold) were loaded onto 24 cm, pH 3-10 NL IPG strips (GE Healthcare, Madrid, Spain). Isoelectric focusing was performed using an IPGhor instrument (Amersham Biosciences, Stockholm, Sweden), following the manufacturer's instructions (active rehydration at 50 V for 12 h followed by a linear gradient from 500 to 8,000 V, at 48,000 V · h^{-1}). The focused strips were equilibrated in two steps as follows: 15 min with equilibration buffer I (65 mM DTT, 50 mM Tris-HCl, 6 M urea, 30% glycerol, 2% SDS, and bromophenol blue) and then 15 min with equilibration buffer II (135 mM iodoacetamide, 50 mM Tris-HCl, 6 M urea, 30% glycerol, 2% SDS, and bromophenol blue). Equilibrated strips were set directly onto 12.5% polyacrylamide gels, sealed with 0.5% w/v agarose, and separated at a constant voltage of 50 V for 30 min followed by 200 V for about 6 h, until the blue dye reached the bottom of an Ettan DALT II system (Amersham Biosciences). Proteins were fixed for 1 h in methanol: acetic acid, 40:10, and stained overnight using colloidal Coomassie Brilliant Blue G-250. Gel staining was removed by consecutive washing steps with distilled water until the best visualization was achieved.

Gel Image Analysis

Gels stained with Coomassie Brilliant Blue were scanned in a calibrated Imagescanner (BioRad) and digital images captured using Quantity-One software (BioRad). The images were saved as uncompressed TIFF files. Gel images were analyzed using the software package ImageMaster 2D, version 6.01 (GE Healthcare). Proteins were detected using the automated routine of the ImageMaster 2.0 software, combined with manual editing when necessary to remove artifacts. The background was removed, and normalized volumes were calculated as follows: the volume of each protein spot was divided by the total volume of all the protein spots included in the analysis. Normalized protein spot values were used to select the 300 most abundant proteins in each condition to be further analyzed for their differential expression.

Protein Digestion

Protein in-gel trypsin was digested manually (sequencing grade modified, Promega). Selected spots with differential expression were manually cut out from reference gels and were washed sequentially with 25 mM ammonium bicarbonate (NH_4HCO_3) and acetonitrile (ACN). The proteins were reduced with 20 mM DTT solution for 60 min at 60°C and alkylated with a 50 mM solution of iodine acetamide for 30 min at room temperature. After sequential washings with buffer and acetronitrile, the proteins were digested overnight at 37°C with 80 ng of trypsin. Peptides were extracted from the gel matrix with 10% formic acid (FA) and can, pooled and dried in a vacuum centrifuge. The trypsin-digested peptide samples were analyzed by LC-MS/MS.

LC-MS/MS Analysis

Dry-down peptide mixtures were analyzed in a nanoAcquity liquid chromatographer (Waters, Cerdanyola del Vallés, Spain) coupled to an LTQ-Orbitrap Velos (Thermo Scientific, Barcelona, Spain) mass spectrometer. Trypsin digests were resuspended in 1% FA solution and an aliquot was injected into chromatographic separation equipment. The peptides were trapped in a Symmetry C18TM trap column (5, 180 μm × 20 mm, Waters), and were separated using a C18 reverse-phase capillary column (ACQUITY UPLC M-Class Peptide BEH column; 130 Å, 1.7, 75 μm × 250 mm, Waters). The gradient used for the elution of the peptides was 1 to 40% B in 20 min, followed by 40 to 60% in 5 min (A: 0.1% FA; B: 100% CAN,

0.1% FA), with a 250 nL · min^{-1} flow rate. Eluted peptides were subjected to electrospray ionization in an emitter needle (PicoTipTM, New Objective, Woburn. MA, USA) with an applied voltage of 2,000 V. Peptide masses (m/z 300–1,700) were analyzed in data dependent mode where a full Scan MS was acquired in the Orbitrap with a resolution of 60,000 FWHM at 400 m/z. Up to the 10th most abundant (minimum intensity of 500 counts) peptides were selected from each MS scan and then fragmented in the linear ion trap using CID (38% normalized collision energy) with helium as the collision gas. The scan time settings were: Full MS: 250 ms (1 microscan) and MSn: 120 ms. The.raw data files generated were collected with Thermo Xcalibur (v.2.2).

Database Search

The.raw files obtained in the mass spectrometry analysis were used to search the public database Uniprot Actinopterygii (v.23/3/17). A database containing common laboratory contaminant proteins was added to this database. The software used was Thermo Proteome Discoverer (v1.4.1.14) with Sequest HT as the search engine. The following search parameters were applied: 2 missed cleavage sites as well as fixed and variable modifications; carbamidomethyl of cysteine and oxidation of methionine, respectively. Peptide tolerance was 10 ppm and 0.6 Da for MS and MS/MS spectra, respectively. Both a target and a decoy database were searched in order to obtain a false discovery rate (FDR), and thus estimate the number of incorrect peptide–spectrum matches that would exceed a given threshold. The results were filtered so only proteins identified with at least 2 high confidence (FDR > 1%) peptides were included in the lists.

Interactome Analysis

Gene Ontology (GO) enrichment analysis was performed with the UniProt-IDs of identified proteins retrieved from UniProt knowledgebase (UniProtKB). The UniProt-IDs were submitted to PANTHER (www.pantherdb.org) to cluster the proteins into different groups related to their biological process, according to GO annotation (GO terms). Only results with $p < 0.05$ were accepted. The interactome was derived from confidence analysis of the protein–protein interaction network by the STRING Program v10.5.

Biochemical Parameters

Before mechanical homogenization, the scales collected in mucus samples were individually removed. Mucus samples were diluted (v/v) with Milli-Q water to extract the mucus adhered to the scales. The mechanical homogenization was performed by a sterile Teflon stick to desegregate the mucus mesh before centrifugation at 14,000 g. The resultant mucus supernatants were collected avoiding the surface lipid layer, aliquoted, and stored at −80°C.

Glucose concentration was determined by an enzymatic colorimetric test (LO-POD glucose, SPINREACT®, Girona, Spain). Briefly, glucose oxidase (GOD) catalyzes the oxidation of glucose to gluconic acid. The hydrogen peroxide (H_2O_2) formed is detected by a chromogenic oxygen acceptor phenol, 4–aminophenazone (4-AP), in the presence of peroxidase (POD). Following the manufacturer's instructions for plasma determination, but with slight modifications, 10 μL of mucus extracts or standard solutions (from 0 to 100 mg · dL^{-1}) were mixed in triplicate with 200 μL of working reagent and incubated for 10 min at 37°C. The OD was determined at $\lambda = 505$ nm with a microplate reader (Infinity Pro200 spectrophotometer, Tecan, Barcelona, Spain). The glucose values were expressed as μg glucose · mL^{-1} of skin mucus.

The protein concentration of the homogenized mucus was determined using the Bradford assay (Bradford, 1976) with BSA as standard (Sigma). Mucus samples or standard solutions (from 0 to 1.41 mg · mL^{-1}) were mixed in triplicated with 250 μL of the Bradford reagent and incubated for 5 min at room temperature. The OD was determined at $\lambda = 596$ nm with a microplate reader (Infinity Pro200 spectrophotometer, Tecan). The protein values were expressed as mg protein · mL^{-1} of skin mucus.

Esterase activity was determined according to the method of Ross et al. (2000). Equal volumes of skin mucus and 0.4 mM p-nitrophenyl myristate substrate in 100 mM ammonium bicarbonate buffer containing 0.5% Triton X-100 (pH 7.8, 30°C) were incubated. The OD was continuously measured at 1 min intervals over 3 h at 405 nm in a plate reader. The initial rate of the reaction was used to calculate the activity. One unit of activity was defined as the amount of enzyme required to release 1 mmol of p-nitrophenol product in 1 min. Enzyme activity was measured as mIU · mg^{-1} of protein.

Total alkaline protease activity (TPA) was spectrophotometrically measured in the homogenates following Moyano et al. (1996). Thus, the samples first reacted in 50 mM Tris-HCl pH 9.0 buffer containing 1% casein. After 30 min, the reaction was stopped by adding trichloroacetic acid (TCA, 12%). The samples were then maintained for 1 h at 4°C and centrifuged (7500 g, 5 min, 4°C). Supernatant absorbance was measured at 280 nm. Each sample was analyzed in triplicate and individual blanks were established by adding TCA solution before the homogenate. Bovine trypsin was used as the standard. Enzyme activity was measured as IU · mg^{-1} of protein.

Lysozyme activity was measured according to the turbidimetric method described by Parry et al. (1965) with some modifications. One hundred ml of skin mucus diluted 1/2 with 10 mM PBS, pH 6.2, was placed in flat-bottomed 96-well plates in triplicate. To each well, 100 ml of freeze-dried *Micrococcus lysodeikticus* (0.3 mg · ml^{-1}, Sigma) was added as a lysozyme substrate. The reduction in absorbance at 450 nm was measured after 0 and 15 min at 22°C in a plate reader. One unit of lysozyme activity was defined as a reduction in absorbance of 0.001 min^{-1}. The units of lysozyme present in skin mucus were obtained from a standard curve made with hen egg white lysozyme (HEWL, Sigma). Enzyme activity was measured as mIU · mg^{-1} of protein.

Zymography

Individual alkaline protease activities were also studied using zymograms according to the method established in fish by Santigosa et al. (2008) and modified by García-Meilán et al. (2013). Briefly, 30 μg of mucus protein was diluted and loaded on 12% polyacrylamide gel. Electrophoresis was performed at

a constant current of 15 mA per gel for 90 min (Bio Rad Mini PROTEAN Tetra Cell, 4°C). Protease-active fractions were visualized using the method described by García-Carreño et al. (1993) where the gels were incubated at 4°C under agitation in Tris-HCl 50 mM pH 8.2 solution containing 2% casein. After 30 min, the temperature was raised to room temperature for 90 min with shaking. The gels were washed and stained in a methanol:acetic:water solution (40:10:40) with 0.1% of Coomassie Brilliant Blue R-250 (Bio-Rad). Destaining was carried out using the same solution without colorant until the right visualization of the digested bands was achieved. Pure trypsin was used as a positive control. To determine the molecular weight of protease fractions, a commercial weight marker was used (RPN 800E, GE Healthcare). The gels were further scanned in an ImageScanner III (Epson J181A) and caseinolytic bands were identified. Total protein was normalized using the Quantity One software (Bio-Rad) including total lane intensity. Negative images from each sample were captured to show the intensity for the corresponding caseinolytic band. The relative digestion units for each band were obtained by the relation between the band quantification (from the negative image) and the total lane intensity (previously removing the background). Digestion band intensity was calculated as arbitrary units of casein digestion capacity: the area intensity of each specific digested band, via the negative image, was related to the total intensity of the respective undigested lane, see **Supplementary Material** for detailed information.

Western Blot

Mucus samples were centrifuged at 12,000 g for 10 min and the protein concentration in the supernatant measured. Supernatants were treated with Laemmli loading buffer and 30 μg of proteins resolved on SDS-polyacrylamide (10%) gels and transferred to nitrocellulose. Membranes were then blocked overnight (depending on the antibody affinity) with 4% Non-Fat Dry Milk (BioRad) in Tris-buffered saline (TBS) (pH 7.4) containing 0.05% (w/v) Tween 20 (TTBS). Membranes were washed three times in TTBS and probed for 1 h with the following primary antibodies: anti-cytokeratin-8 (Thermo-Scientific) and anti-actin (Sigma-Aldrich). Detection was performed with an adequate HRP-conjugated IgG (Santa Cruz Biotechnology, Heidelberg, Germany). The blots were visualized with enhanced chemiluminescence (Clarity from Bio-Rad) and detected and scanned on a Fujifilm LAS-3000 Imager (Fujifilm Corporation, Tokyo, Japan). Digital images were quantified using Quantity One software (BioRad) and normalized by the total amount of protein detected by Ponceau S staining (Sigma-Aldrich).

Statistical Analysis

Metabolite amounts, enzyme activities, zymography, and Western blot comparison between Warm and Cold were analyzed by Student's *t*-test. Proteins (spots) that were found to vary in abundance between the Warm and Cold samples were analyzed for significance using Student's *t*-test. The Shapiro-Wilk test was first used to ensure the normal distribution of the data, while the uniformity of the variances was determined by Levene's test. All statistical analysis was undertaken with commercial software (PASW version 21.0, SPSS Inc., Chicago, IL, USA). The STRING databases were used to obtain direct protein–protein interactions (PPI), the interactome, by the search tool for the retrieval of interacting genes/proteins STRING Program v10.5 (Szklarczyk et al., 2017). The selected stat indicators were the "clustering coefficients" and "PPI enrichment *p*-value," which correspond to a measure of how connected the nodes in the network are, and the "count in gene set" which indicates the number of proteins included and their "False discovery rate." The enrichment tests, from STRING software, are done for a variety of classification systems (Gene Ontology, KEGG, Pfam and InterPro), and employ a Fisher's exact test followed by a correction for multiple testing (Benjamini and Hochberg, 1995; Rivals et al., 2007).

RESULTS

Mucus Proteome

The aim of our mucus proteome analysis was to determine the differentially expressed proteins in skin mucus by comparing the "Warm" mucus proteome and "Cold" mucus proteome at the end of the extended period at 14°C. More than 1,200 protein spots were detected in the mucus proteome of all the samples. Of these spots, 20 were down-regulated and 32 were up-regulated due to the chronic cold (master gel with labeled spots is shown in **Supplementary Figure 1**). **Table 1** shows the mass spectrometry characterization of the differentially expressed spots, followed by MASCOT database searches which yielded their theoretical pI and molecular weight, and established probable protein identity. The table also shows the observed molecular weight and pI, in accordance with its location in the 2D gel. Most of the proteins identified correspond to protein sequences that have already been reported in teleost species, except for three spots which correspond to structural proteins that show the greatest homologies to distinct species of mammals.

The proteins identified were clustered, firstly according their main function as: structural-, metabolic- or protective-related proteins. Accordingly, **Table 2** summarizes the name of the proteins belonging to each GO group; only six proteins could not be directly grouped. The proteins were also analyzed using the "cellular component GO," for their specific location. Forty-six of the fifty-two proteins (**Table 2**) belong to the "extracellular vesicular exosome" (GO: 0070062, $p = 1.43e\text{-}37$) indicating that all these proteins could be released into the extracellular region directly via exosomal vesicles. Moreover, the STRING databases were used to obtain direct protein–protein interactions (**Figure 1A**).

The resulting Cold-mucus interactome has a central core of differentially expressed proteins (18 different proteins) related to the biological process "Response to stress" (GO:0006950, $p = 7.05e\text{-}06$, **Figure 1B**; **Table 2**). This group clustered together 11 over-expressed proteins. Some were associated with a protective role, such as four spots identified as transferrin (TF, spots 1, 2, 133, 236), three different heat shock proteins (HSP8, spot 6; and HSPA1, spots 44, and 154) and a lectin-type form (MBL-2, spot 181). Others were associated with matrix structure functions, such as β-actin (ACTB, spots 184, 192) and keratins (KRT8, spots

TABLE 1 | Identification of the 52 differentially expressed proteins by cold in gilthead sea bream epidermal mucus grouped by Structural, Metabolic, or Protective functions.

Spot[a]	INT[b]	SEM[b]	INT[c]	SEM[c]	T-Stu[d]	INT[d]	Protein identity[e]	Accession n^0[e]	Gene[f]	Theoretical[e]		Observed[e]		Peptides[e]	SQ[e]	Species[e]	Gene[f]	UniprotKB[f]
ID	(%)		(%)			FOLD		(gI)	symbol	MW	pI	MW	pI	matched	(%)		number	
1	0.38	0.003	0.53	0.048	0.026	1.39	Transferrin	327243044	TF	74.23	6.30	75.00	7.11	38/(52)	79	*Sparus aurata*	7018	P02787
2	0.31	0.010	0.43	0.043	0.044	1.37	Transferrin	327243042	TF	76.10	5.90	72.00	6.10	17/(35)	30	*Sparus aurata*	7018	Q90YH6
5	0.35	0.009	0.17	0.021	0.001	0.49	Deoxycytidylate deaminase	FG590567	DCTD	13.80	6.80	11.00	4.40	1/(1)	12	*Sparus aurata*	1635	P32321
6	0.28	0.014	0.37	0.026	0.014	1.33	Stress protein HSC70-1	212274295	HSPA8	71.50	5.20	66.00	4.40	14/(23)	26	*Seriola quinqueradiata*	3312	P11142
15	0.03	0.004	0.25	0.071	0.026	7.56	40S ribosomal protein like 12	47224253	RPS12	14.40	7.24	12.00	7.25	10/(10)	64	*Tetraodon nigroviridis*	6206	P25398
26	0.15	0.007	0.12	0.011	0.048	0.76	Coactosin-like	85719983	COTL1	10.00	5.50	11.00	4.60	1/(1)	10	*Ictalurus punctatus*	23406	Q14019
38	0.26	0.011	0.21	0.013	0.021	0.81	Coactosin-like	47221902	COTL1	16.20	4.90	11.00	4.00	4/(8)	22	*Tetraodon nigroviridis*	23406	Q14019
44	0.15	0.019	0.23	0.010	0.005	1.50	Heat shock protein A1	47223819	HSPA1A	71.40	5.20	66.00	4.30	11/(15)	19	*Tetraodon nigroviridis*	3303	P08107
47	0.05	0.003	0.19	0.036	0.028	4.02	Hnrpa01 protein	323649982	HNRNPA1	13.71	6.39	18.00	6.34	2/(8)	39	*Perca flavescens*	3178	P09651
56	0.13	0.004	0.08	0.004	0.000	0.66	Proliferating cell nuclear antigen	430721599	PCNA	28.66	4.72	26.00	4.07	9/(9)	58	*Dicentrarchus labrax*	5111	P12004
71	0.04	0.005	0.13	0.026	0.033	2.99	Periplakin-like	551527179	PPL	205.96	6.18	76.00	6.02	1/(9)	5	*Xiphophorus maculatus*	5493	O60437
82	0.03	0.008	0.09	0.008	0.001	2.91	Keratin. type II cytoskeletal 8-like	551498795	KRT8	53.45	5.01	54.00	4.49	5/(15)	30	*Xiphophorus maculatus*	3856	P05787
94	0.05	0.005	0.13	0.012	0.000	2.53	Epiplakin	221047999	EPPK1	30.75	4.84	39.00	6.40	3/(5)	25	*Epinephelus coioides*	83481	P58107
97	0.02	0.003	0.16	0.031	0.006	7.13	Peptidyl-tRNA hydrolase	317418901	PTRHD1	20.20	4.63	16.00	3.97	3/(3)	26	*Dicentrarchus labrax*	391356	Q6GMV3
98	0.03	0.003	0.13	0.023	0.008	4.14	Keratin 12	528509044	KRT12	49.81	5.35	15.00	3.97	3/(11)	17	*Danio rerio*	3859	Q99456
111	0.02	0.002	0.05	0.010	0.040	2.45	60S ribosomal protein	11095761	RPL23A	10.91	9.70	15.00	8.09	3/(3)	29	*Oncorhynchus mykiss*	6147	P62750
119	0.11	0.008	0.13	0.007	0.037	1.24	Protein disulfide-isomerase-like	475653184	PDIA3	55.87	5.60	54.00	4.89	13/(18)	31	*Dicentrarchus labrax*	2923	P30101
121	0.10	0.018	0.05	0.006	0.048	0.46	Alpha 2 globin	99122203	HBA2	15.83	8.72	12.00	4.11	8/(8)	52	*Sparus aurata*	3040	P69905
124	0.16	0.006	0.12	0.014	0.037	0.72	Myosin light polypeptide 6	229366002	MYL6	17.00	4.50	11.00	3.80	5/(7)	36	*Anoplopoma fimbria*	4637	P60660
126	0.04	0.014	0.11	0.016	0.023	2.42	Keratin. type II cytoskeletal 5-like	P13647	KRT5	62.34	7.74	76.00	6.67	16/(27)	43	Mammal sps.	3852	P13647
133	0.06	0.008	0.11	0.015	0.026	1.75	Transferrin	327243044	TF	74.23	6.30	75.00	4.74	27/(27)	45	*Sparus aurata*	7018	P02787

(Continued)

TABLE 1 | Continued

Spot[a] ID	INT[b] (%)	SEM[b]	INT[c] (%)	SEM[c]	T-Stu[d]	INT[d] FOLD	Protein identity[e]	Accession n[0e] (gI)	Gene[f] symbol	Theoretical[e] MW	Theoretical[e] pI	Observed[e] MW	Observed[e] pI	Peptides[e] matched	SQ[e] (%)	Species[e]	Gene[f] number	UniprotKB[f]
134	0.18	0.011	0.11	0.020	0.026	0.63	Malate dehydrogenase mitochondrial	410905057	MDH2	35.80	8.60	32.00	8.10	10/(16)	37	*Takifugu rubripes*	4191	P40926
140	0.18	0.023	0.12	0.011	0.032	0.64	Esterase D	348524078	ESD	31.60	5.90	31.00	5.20	3/(6)	14	*Oreochromis niloticus*	2098	P10768
144	0.11	0.005	0.15	0.011	0.042	1.36	Betaine homocysteine M-transferase	388260758	BHMT	44.07	6.71	43.00	8.39	13/(18)	64	*Sparus aurata*	635	Q93088
152	0.17	0.024	0.09	0.017	0.018	0.51	Translation initiation factor 5A	47209413	EIF5A	17.50	5.20	14.00	4.40	3/(15)	13	*Tetraodon nigroviridis*	1984	P63241
154	0.11	0.008	0.21	0.036	0.029	1.99	Heat shock 70 kDa protein 1-like	410933029	HSPA1L	52.50	5.24	16.00	6.93	2/(4)	11	*Takifugu rubripes*	3305	P34931
155	0.24	0.029	0.14	0.015	0.016	0.59	Keratin type I cytoskeletal 13	229366514	KRT13	49.72	5.36	17.00	4.14	3/(17)	27	*Anoplopoma fimbria*	3860	P13646
159	0.18	0.006	0.10	0.012	0.002	0.59	Inorganic pyrophosphatase-like	432903493	PPA1	33.40	5.10	33.00	4.50	6/(9)	23	*Oryzias latipes*	5464	Q15181
160	0.07	0.006	0.15	0.021	0.016	2.24	Inositol monophosphatase 1-like	583999941	IMPA1	27.26	5.30	27.00	5.12	11/(12)	57	*Neolamprologus brichardi*	3612	P29218
163	0.20	0.009	0.09	0.013	0.000	0.43	14-3-3 protein zeta/delta	10719663	YWHAZ	28.10	4.70	24.00	4.10	2/(9)	7	*Fundulus heteroclitus*	7534	P63104
167	0.04	0.009	0.13	0.021	0.008	3.45	Keratin. type I cytoskeletal 13	229366514	KRT13	48.48	5.33	20.00	3.94	3/(15)	22	*Anoplopoma fimbria*	3860	P13646
169	0.03	0.004	0.16	0.034	0.014	4.74	Keratin. type II cytoskeletal 5-like	573882490	KRT5	61.05	5.41	13.00	4.50	4/(20)	25	*Lepisosteus oculatus*	3852	P13647
170	0.03	0.002	0.10	0.015	0.006	3.98	Intermediate filament protein ON3	551498797	ION3	58.55	5.48	12.00	4.75	2/(15)	22	*Xiphophorus maculatus*	N/A	P18520
176	0.13	0.014	0.08	0.011	0.023	0.64	Proteasome subunit alpha type-6-like	410916067	PSMA6	27.40	6.35	23.00	6.96	23/(23)	63	*Takifugu rubripes*	5687	P60900
177	0.14	0.018	0.08	0.012	0.027	0.60	UBQ-like modifier-activating enzyme	432865628	UBA1	117.97	5.76	96.00	4.51	3/(8)	11	*Oryzias latipes*	7317	P22314
181	0.02	0.004	0.12	0.029	0.023	5.77	F-type lectin 2	334883514	Rb-FTL2	34.53	6.34	27.00	6.34	2/(2)	9	*Oplegnathus fasciatus*	N/A	F7J049
184	0.04	0.001	0.10	0.023	0.034	2.72	β-actin	6693629	ACTB	41.81	5.48	42.00	7.49	3/(24)	65	*Pagrus major*	60	P60709
189	0.03	0.003	0.14	0.019	0.002	5.11	Keratin. type II cytoskeletal 5-like	18858425	KRT5	58.55	5.41	13.00	4.74	4/(17)	19	*Danio rerio*	3852	P13647
190	0.25	0.022	0.12	0.021	0.002	0.48	Gelsolin	FM026536	GSN	85.90	5.90	77.00	6.90	2/(3)	6	*Dicentrarchus labrax*	2934	P06396

(Continued)

TABLE 1 | Continued

Spot[a] ID	INT[b] (%)	SEM[b]	INT[c] (%)	SEM[c]	T-Stu[d]	INT[d] FOLD	Protein identity[e]	Accession n^{0}[e] (gi)	Gene[f] symbol	Theoretical[e] MW	Theoretical[e] pI	Observed[e] MW	Observed[e] pI	Peptides[e] matched	SQ[e] (%)	Species[e]	Gene[f] number	UniprotKB[f]
192	0.06	0.002	0.10	0.008	0.006	1.82	β-actin	261286856	ACTB	40.83	5.83	12.00	7.00	3/(3)	10	*Anguilla japonica*	60	P60709
193	0.05	0.007	0.09	0.012	0.025	1.84	Keratin. type II E3-like protein	48476437	N/A	38.60	4.96	13.00	4.28	11/(20)	46	*Sparus aurata*	N/A	Q4QY72
197	0.02	0.004	0.08	0.019	0.035	3.36	Peptidyl-prolyl cis-trans isomerase F	348508637	PPIF	21.02	8.94	13.00	9.04	2/(3)	17	*Oreochromis niloticus*	10105	P30405
199	0.16	0.009	0.09	0.008	0.001	0.58	Protein disulfide-isomerase-like	498926878	PDIA3	57.40	4.60	52.00	3.60	6/(11)	11	*Maylandia zebra*	2923	P30101
201	0.03	0.006	0.08	0.015	0.021	2.53	Septin-2-like	551486665	SEPT2	40.03	6.28	36.00	6.26	4/(16)	66	*Xiphophorus maculatus*	4735	Q15019
205	0.12	0.004	0.08	0.016	0.039	0.65	UMP-CMP kinase-like	348500565	CMPK1	24.90	8.60	20.00	6.80	2/(7)	11	*Oreochromis niloticus*	51727	P30085
206	0.05	0.009	0.09	0.011	0.018	1.91	Keratin. Type II cytoskeletal 1	P04264	KRT1	65.98	8.12	14.00	9.32	18/(21)	38	Mammal sps.	3848	P04264
207	0.18	0.036	0.08	0.013	0.040	0.42	Periplakin-like	499048295	PPL	184.00	5.90	98.00	7.00	7/(5)	4	*Maylandia zebra*	5493	O60437
213	0.03	0.006	0.10	0.019	0.011	3.68	Keratin. Type II cytoskeletal 1	P04264	KRT1	65.98	8.12	16.00	8.72	30/(34)	49	Mammal sps.	3848	P04264
234	0.14	0.014	0.08	0.014	0.030	0.61	Aldo-keto reductase family	432941989	AKR1B10	35.62	6.46	31.00	7.54	1/(7)	18	*Oryzias latipes*	57016	O60218
236	0.04	0.003	0.07	0.010	0.012	2.00	Transferrin	327243044	TF	74.23	6.30	74.00	4.73	19/(19)	35	*Sparus aurata*	7018	P02787
247	0.15	0.020	0.08	0.006	0.008	0.52	Malate dehydrogenase	551491925	MDH1	38.40	7.60	34.00	7.10	6/(15)	18	*X. maculatus*	4190	P40925
251	0.03	0.003	0.07	0.013	0.020	2.55	Keratin. type I cytoskeletal 13	212995	KRT13	49.72	5.36	14.00	4.76	2/(11)	16	*Carassius auratus*	3860	P13646

[a] *Spot number from* ***Supplementary Figure 1*** *and the corresponding spot ID in* ***Tables 1***, ***2***.

[b] *Mean and standard error of the mean (SEM) for each individual spot from 5 replicate Warm condition gels (pools of soluble protein extract from 2 or 3 fish).*

[c] *Mean and standard error of the mean (SEM) for each individual spot from 5 replicate Cold condition gels (pools of soluble protein extract from 2 or 3 fish).*

[d] *Statistic Student T-test ($p < 0.05$) and intensity fold for each individual spot from 5 replicate gels.*

[e] *Protein identities, accession number, theoretical, and observed MW and pI, peptides matched (unique peptides), percentage sequence coverage (SQ) and species identification were supplied by the Mascot Search Results (Matrix science). Further details of search conditions in Material and Methods section.*

[f] *Gene symbol, gene number (Entrez gene database from NCBI, http://www.ncbi.nlm.nih.gov/), and UniprotKB (http://www.uniprot.org) of each protein were obtained from the Genecards database search process (http://www.genecards.org). The UniprotKB number was used for further Gene Ontology enrichment analysis in* ***Table 2***.

TABLE 2 | Regulation and biological process aggrupation of differentially expressed proteins sorted by Structural, Metabolic, or Protective functions.

Spot ID[a]	Protein identity[b]	Gene[c] symbol	Regulation[d]		Biological process groups				Exosome[e]
			UP-	DOWN-	Response[e] to stress	Metabolic[e] process	Transport[e]	Interspecies[e] interaction	
PROTECTIVE PROTEINS									
1, 2, 133, 236	Transferrin	TF	*		X		X		X
6	Stress protein HSC70-1	HSPA8	*		X		X	X	X
44	Heat shock protein A1	HSPA1A	*		X		X	X	X
154	Heat shock 70 kDa protein 1-like	HSPA1L	*		X		X		X
181	F-type lectin 2	MBL-2	*					X	-
119, 199	Protein disulfide-isomerase-like	PDIA3	*	*	X	X	X		X
121	Alpha 2 globin	HBA2		*	X	X			X
177	Ubiquitin-like modifier-activating enzyme 1-like	UBA1		*	X				X
163	14-3-3 protein zeta/delta	YWHAZ		*	X				X
140	Esterase D	ESD		*		X			X
METABOLIC PROTEINS									
15	40S ribosomal protein like 12	RPS12	*				X	X	-
111	60S ribosomal protein	RPL23A	*				X	X	X
47	Heterogeneous nuclear ribonucleoprotein A1	HNRNPA1	*				X	X	X
197	Peptidyl-prolyl cis-trans isomerase F	PPIF	*		X		X		-
160	Inositol monophosphatase 1-like	IMPA1	*		O				X
144	Betaine homocysteine M-transferase	BHMT	*		O				X
97	Peptidyl-tRNA hydrolase	PTRHD1	*						X
56	Proliferating cell nuclear antigen	PCNA		*	X	X			X
176	Proteasome subunit alpha type-6-like	PSMA6		*	X	X		X	X
152	Translation initiation factor 5A	EIF5A		*		X			X
5	Deoxycytidylate deaminase	DCTD		*		X			X
247	Malate dehydrogenase	MDH1		*		X			X
134	Malate dehydrogenase mitochondrial	MDH2		*		X			X
159	Inorganic pyrophosphatase-like	PPA1		*		X			X
205	UMP-CMP kinase-like	CMPK1		*		X			X
234	Aldo-keto reductase family 1 member B10-like	AKR1B10		*		X			X
STRUCTURAL PROTEINS									
170	Intermediate filament protein ON3-like	ION3	*				X		-
201	Septin-2-like isoform X2	SEPT2	*				X	X	X
94	Epiplakin-like protein	EPPK1	*					O	-
193	Keratin, type II E3-like protein	N/A	*						-
206, 213	Keratin, Type II cytoskeletal 1	KRT1	*		X				X
82, 251	Keratin, type II cytoskeletal 8-like	KRT8	*		X			X	X

(Continued)

TABLE 2 | Continued

Spot ID[a]	Protein identity[b]	Gene[c] symbol	Regulation[d]		Biological process groups				Exosome[e]
			UP-	DOWN-	Response[e] to stress	Metabolic[e] process	Transport[e]	Interspecies[e] interaction	
184, 192	β-actin	ACTB	*		X		X		X
126, 169, 189	Keratin, type II cytoskeletal 5-like	KRT5	*						X
98	Keratin 12 isoform X1	KRT12	*						X
155, 167	Keratin, type I cytoskeletal 13	KRT13	*	*					X
71, 207	Periplakin-like	PPL	*	*				O	X
124	Myosin light polypeptide 6	MYL6		*					X
26,38	Coactosin-like	COTL1		*	X				X
190	Gelsolin-S1/S2-like	GSN		*	X	X		X	X

[a] *Spot number from **Supplementary Figure 1** (where green spots are over-expressed and pink spots were under-expressed) and the corresponding spot ID in **Tables 1**, **2**.*
[b] *Protein identities were supplied by the Mascot Search Results (Matrix science). Further details of search conditions in Material and Methods section.*
[c] *Gene symbol of each protein were obtained from the Genecards database search process (http://www.genecards.org).*
[d] *Up- or Down- protein regulation in cold condition. The intensities of each protein and statistical analysis Student T-test ($p < 0.05$) are shown in **Table 1**.*
[e] *Classification of proteins into different categories based on Gene Ontology enrichment analysis (GO) using UniprotKB number (shown in **Table 1**). Related to Biological process GO: Response to stress (GO:0006950, p = 7.05e-06); Single-organism metabolic process (GO:0044710, p = 3.85e-02); Transport (GO:0006810, p = 2.39e-02); Interspecies interaction between organisms (GO:0044419, p = 2.22e-05). An additional cluster of Cellular component categories has been added: Extracellular vesicular exosome (GO:0070062, p = 1.43e-37).*

82 and 251; and KRT1, spots 206 and 213). While yet others were associated with other stress-related proteins and enzymatic activity (PDIA3, spot 118; PPFI, spot 197; IMPA 1, spot 160; and BHMT, spot 144). This group also included seven under-expressed proteins: three with a protective role (HBA2, spot 121; UBA1, spot 177; and YWHAZ, spot 163), two with metabolic activity (PCNA, spot 56; and PSMA6, spot 176), and two actin-related proteins, gelsolin (GSN, spot 190) with actin-assembly regulatory function and coactosin (COTL1, spots 26, 38) with actin filament-stabilizer function.

The second most significant group of protein interactions, namely "Single-organism metabolic process" (GO:0044710, $p =$ 3.85e-02), included thirteen proteins that are under-expressed at low temperatures (**Figure 1C**). Most of these proteins showed enzymatic activities: related to lipid metabolism, such as an esterase (ESD, spot 140) and an inorganic pyrophosphatase (PPA1, spot 159); enzymes required for cellular nucleic acid biosynthesis, a deaminase (DCTD, spot 5) and a kinase (CMPK1, spot 206); and other activities, such as proteasomal (PSMA6, spot 176), malate dehydrogenases (MDH1, spot 247; and MDH2, spot 134), and an oxidoreductase (AKR1, spot 234). The "Transport" group (GO:0006810, $p =$ 2.39e-02, **Figure 1D**) represents biological processes related to the directed movement of substances into, out of or within a cell, or between cells. This group included 11 proteins modified in the mucus interactome; all over-expressed, indicating a putative increased response at low water temperatures of skin mucus exudation. They belong to protective functions (HSPs and PDIA3), to structural functions of intermediate filaments (ION3, spot 170; and SEPT2, spot 201), to ribosomal activity (RPS12, spot 15; and RPL23A, spot 111), and to protein folding (PTRHD1, spot 97; and HNRNPA1, spot 47). Finally, a number of proteins was grouped within the biological process "Interspecies interaction between organisms" (GO:0044419, $p =$ 2.22e-05, **Figure 1E**). This GO group clustered together seven over-expressed proteins (HSPA8, HSPA1A, KRT8, RPS12, RPL23A, HNRNPA1, and SEPT2) and two under-expressed proteins (GSN and PSMA6). Moreover, other proteins that were over-expressed were also related to this process of species interaction at the extracellular matrix level, such as lectin (MBL2, spot 181), a carbohydrate-binding protein, and two proteins in the plakin structures of the skin barrier function: epiplakin (EPPK1, spot 94) and periplakin (PPL, spots 71 and 207).

Biochemical Parameters and Mucus Zymography

Levels of soluble glucose and soluble protein in skin mucus were also obtained before and after the 7 weeks cold challenge at 14°C. We then calculated the glucose/protein ratio individually to normalize putative mucus dilution during the sampling process (data in **Table 3**). The present study revealed that skin mucus glucose exudation was greatly affected by the cold challenge: a 5-fold reduction from 15.9 ± 2.0 to 3.4 ± 0.4 $\mu g \cdot mL^{-1}$ of mucus extract ($p < 0.05$). However, the amount of soluble mucus protein was not modified by the cold challenge. As a result, the glucose/protein ratio was reduced by 6-fold, evidencing different affectation of glucose and protein exudation capacity. As regards the enzymatic activities of total protease (TPA), esterase and lysozyme, all related to the immune response, they showed no cold compensation via increased presence in mucus at the end of cold period: values of TPA were around 1.6-1.7 (IU · mg $protein^{-1}$) and esterase activity was around 0.6 (IU · mg $protein^{-1}$); whereas lysozyme activity was not detectable under the current analytical conditions.

To characterize the alkaline protease activity pattern of sea bream skin mucus, zymographic analysis was performed using casein digestion activity for the first time in skin mucus of this species. The resulting zymograms (**Figure 2A**) show the presence of two digested bands with caseinolytic activity at the molecular weights of 12–15 kDa (low MW-band or L-band) and 76–80

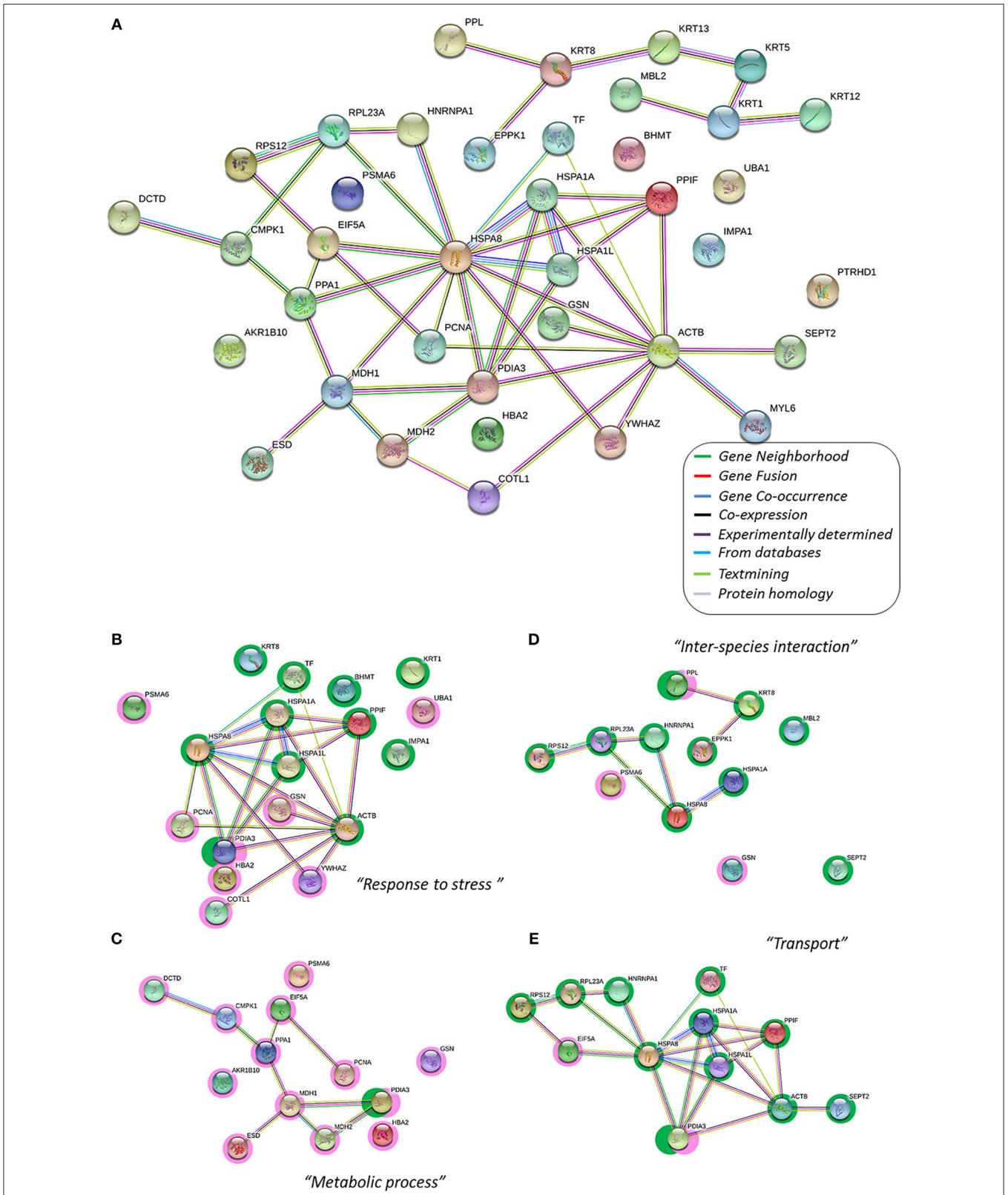

FIGURE 1 | The protein–protein interaction network, the interactome, of gilthead skin mucus proteins differentially expressed by chronic low temperatures. In this network, nodes are proteins, lines represent the predicted functional associations, and the color of the lines represents the strength of the predicted functional interactions between the proteins, according to the STRING databases (Szklarczyk et al., 2017). **(A)** Total protein interactome; all protein listed in **Table 2** have been

(Continued)

FIGURE 1 | included to obtain the network. Relevant data from the network stats (such as the clustering coefficient and the PPI enrichment *p*-value) are provided in **Supplementary Table 1**. **(B–E)** Main Gene Ontology clusters obtained by GO-enrichment groups with significance (see **Table 2**), where green shaded nodes correspond to proteins that are up-regulated by chronic cold stress and pink shaded nodes corresponded to down-regulated proteins due to chronic cold stress. Each sub-cluster have been performed using the protein groups from **Table 2**. Relevant data from the network stats and the functional enrichment process are also provided in **Supplementary Table 1**.

TABLE 3 | Metabolites and enzymatic parameters of epidermal mucus after a cold challenge.

	Warm	Cold
Glucose (μg/mL)	14.1 ± 0.8	3.4 ± 0.4*
Protein (mg/mL)	14.4 ± 0.5	15.4 ± 1.5
Glucose/Protein ratio (μg/mg)	0.97 ± 0.1	0.22 ± 0.0*
Total protease activity (IU/mg pr)	1.6 ± 0.3	61.6 ± 0.9
Esterase (mIU/mg pr)	0.56 ± 0.04	0.60 ± 0.01
Lysozyme (IU/mg pr)	n.d	n.d

Values are mean ± SEM from pools of 2 fish (n = 6). Asterisks indicate significant differences between Warm and Cold conditions (p < 0.05; Student's T-test). N.d, no detected.

kDa (intermediate MW-band or I-band). Enlarged gel images are provided as **Supplementary Figure 2**. Individual activities were calculated for both the I-band and the L-band (**Figures 2B,C**, respectively) measuring the intensity of each specific digested band, via a negative image, and then normalizing by the total intensity of the respective undigested lane (details of negative image evaluation are provided in **Supplementary Figure 2**). Although total protease measured spectrophotometrically was not affected by cold challenge, the zymography study revealed that the caseinolytic activity of the specific I-band increased 5-fold in response to the chronic exposure to low temperature.

Identification of Protein Fragments With Putative Antimicrobial Activity

The study of proteins that were significantly expressed by 2D-PAGE revealed a number of proteins located at a lower molecular weight (Observed MW) than expected (Theoretical MW); they are plotted in **Figure 3A**. Ten of these proteins correspond to different keratin fragments, so-called "KDAMPs" (keratin-derived antimicrobial peptides), all of which were significantly over-expressed (**Figure 3A**). Two spots identified as KRT1 had observed MWs of 14 and 16 kDa, instead of the theoretical 66 kDa (data in **Table 1**); two spots identified as KRT5 had observed MWs of 13 kDa, instead of the theoretical 61 kDa; one spot identified as KRT8, spot 251, had an observed MW of 14 kDa, instead of the theoretical 50 kDa; one spot identified as KRT12, spot 98, had an observed MW of 15 kDa, instead of the theoretical 50 kDa; one spot identified as KRT13, spot 167, had an observed MW of 20 kDa, instead of the theoretical 49 kDa; and one spot identified as KRT-E3, spot 193, had an observed MW of 13 kDa instead of the theoretical 39 kDa. Besides keratin fragments, two additional structural proteins were identified as protein fragments with lower MWs: ION3, spot 170, and ACTB, spot 192, with observed MWs of around 12 kDa. **Figure 3A** also includes the relative abundance of two ribosomal proteins, related to putative antimicrobial activity (see the Discussion section): 40S ribosomal protein (RPS12, spot 15) and 60S ribosomal protein (RPL23A, spot 111) increased 7.5- and 2.5-fold in sea bream mucus at low temperatures.

Finally, **Figure 3B** shows the Western blot analysis of cytokeratin-8 and β-actin, to compare with the proteome data. At least two clear bands appeared for cytokeratin-8: at 40 kDa, with no coincidence with significantly increased spots of KRT8; and at 14 kDa, coinciding with the KRT8 fragment (spot 251) reported above, with a possible extra band at 20 kDa. However, neither Western blot band was significantly over-expressed. For β-actin, a single band appeared at around 45–48 kDa, corresponding to the expected MW; however, no lower MW bands were observed which could have matched with the actin fragment (ACTB, spot 192) observed in the proteome.

DISCUSSION

Monitoring and reporting the general health status and welfare of fish is an important issue for fish farms. With the aim of combining the search for biomarkers with a non-invasive method, here for the first time we studied the skin mucus proteome of gilthead sea bream subjected to low temperatures for an extended period. We combined the valuable screening of differentially expressed proteins in the mucus proteome with the evaluation of some innate defenses, such as TPA, and esterase and lysozyme activity. In addition, skin mucus metabolites, glucose, and protein were analyzed as new indicators of fish welfare (in accordance with Fernández-Alacid et al., 2018, 2019) and mucus zymography was characterized, as it is classically performed on gut mucosa (Alarcón et al., 1998; Santigosa et al., 2008).

The amounts of soluble glucose and protein in skin mucus have recently been proposed as non-invasive markers of fish responses to stress challenges, together with mucus lactate and cortisol levels (Cordero et al., 2017; De Mercado et al., 2018; Fernández-Alacid et al., 2018, 2019). The drastic reduction in soluble glucose exuded after 50 days of low temperature exposure would seem to indicate a chronic condition of low-energy availability, as is also true for glucose plasma values during cold-associated reduced ingesta (Ibarz et al., 2010b; Sánchez-Nuño et al., 2018a). Whereas, soluble mucus glucose was reduced by a half in response to 2 weeks of deprivation at warm temperatures (Fernández-Alacid et al., 2018), here, the sustained low-temperature condition reduced mucus glucose 5-fold. The lower levels of glucose exudation not only indicated energy sparing but would seem to be associated with a compromised state at low temperatures. The importance of maintaining soluble carbohydrates in fish mucus has been reported, because bacteria adhesion correlates negatively with carbohydrate-rich mucus

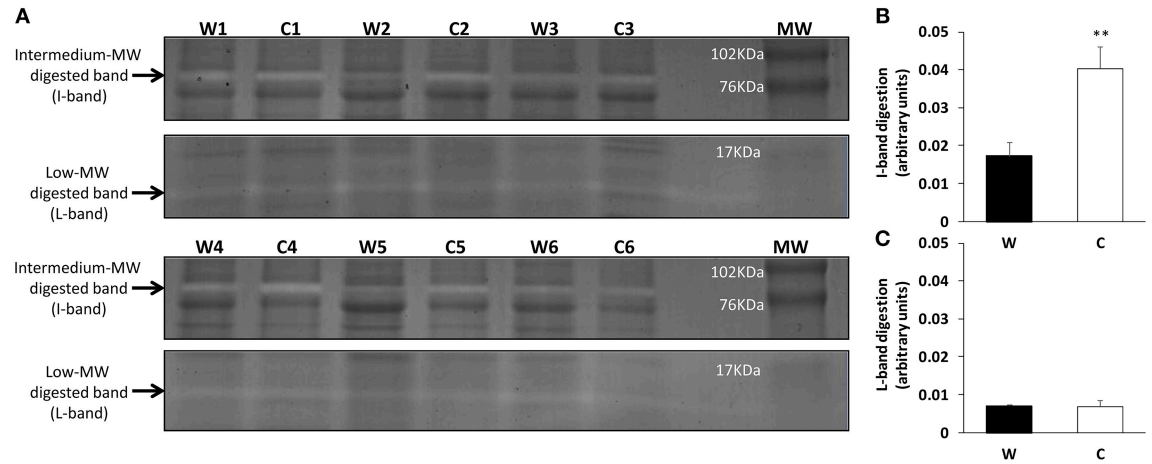

FIGURE 2 | Zymograms of skin mucus protease activities of warm (W) and cold challenged (C) gilthead sea bream. **(A)** Gel zymography: electrophoresis was performed on polyacrylamide (12% acrylamide) gels. Two clear digested bands were appreciated and quantified. To determine the molecular weight of the protease fractions, a commercial weight marker was used (MW-lane). The gels were cut to simplify interpretation (intact gels are provided as **Supplementary Figure 2**). **(B)** Intermediate band relative intensity **(C)** Low band relative intensity. Both the I-band and L-band intensity were calculated as arbitrary units of trypsin digestion capacity (see **Supplementary Figure 2** for detailed information). ** indicates significant differences ($p < 0.01$; Student's *t*-test).

constituents and positively with lipid- and protein-rich mucus constituents (Tkachenko et al., 2013).

Fish epidermal mucus serves as a repository of numerous innate immune response protein components, playing roles in inhibitory or lytic activity against different types of pathogens, such as glycoproteins, lysozyme, complement proteins, C-reactive protein, flavoenzymes, proteolytic enzymes, and antimicrobial peptides (Guardiola et al., 2014a,b; Sanahuja and Ibarz, 2015). Among these, the most commonly characterized have been proteases, lysozyme and esterases. In response to low temperatures, neither TPA nor esterase activity changed. This is in contrast to reported increased activities when fish are exposed to pathogens, stress or environmental factors, such as salinity (Easy and Ross, 2009; Caruso et al., 2011; Jung et al., 2012; Loganathan et al., 2013). In addition, we can expect the functionality of these enzymes to be temperature dependent, with activity reduced at 14°C compared to 22°C. Thus, the same amount of enzyme at lower temperatures would mean weakened defenses during the cold season, due to a lack of cold adaptation, as has repeatedly been reported for sea bream metabolism during the cold season (Vargas-Chacoff et al., 2009; Ibarz et al., 2010b; Silva et al., 2014; Sánchez-Nuño et al., 2018a,b). With regard to lysozyme activity, we detected no mucus activity, in spite of it having been reported in several species including sea bream (Guardiola et al., 2014b).

The release of proteases into skin mucus may act directly on a pathogen or may prevent pathogen invasion indirectly by modifying mucus consistency to increase the sloughing of mucus and thereby the removal of pathogens from the body surface (Aranishi et al., 1998). The zymographic evaluation in the current study, comparing warm and cold caseinolytic activity, showed two well-defined bands at MWs of ∼12–15 kDa (L-band) and 76–80 kDa (I-band). This demonstrates for the first time the presence of different protease activities in sea bream skin mucus. The L-band in the zymography matched trypsin-like activity: a low-molecular-weight serine protease with strong bactericidal activity against Gram positive bacteria, which has been observed in the skin mucus of rainbow trout (Hjelmeland et al., 1983), Atlantic salmon (Braun et al., 1990; Ross et al., 2000), and olive flounder (Jung et al., 2012). Meanwhile, the I-band matched reported activity of metalloproteases in the skin mucus of Atlantic salmon (Firth et al., 2000) and several freshwater species (Nigam et al., 2012). In higher vertebrates, metalloprotease production has been associated with response to injury, disease or inflammation (Woessner, 1991), activating various immune factors, such as cytokines, chemokines, receptors (McCawley and Matrisian, 2001), other proteases like cathepsines, and antimicrobial peptides (Cho et al., 2002a,b). Interestingly, the cold challenge increased those particular activities 5-fold in gilthead sea bream, reflecting differences between mucus protease properties according to stressor. The existence of trypsin-like serine proteases has been considered to play an important role in innate immunity, on top of its digestive function [reviewed in Esteban (2012)]. However, low temperatures did not alter the L-band activity of sea bream mucus, indicating, as with TPA, the lack of cold adaptation of trypsin-like activities. Further studies are needed of the specific role of skin mucus proteases and environmental challenges in fish.

The mucus proteome has been shown to be a powerful tool to devise putative bioindicators of fish welfare and physiological status via non-invasive methods in several fish species, such as Atlantic cod (Rajan et al., 2011), lumpsucker (Patel and Brinchmann, 2017), discus (Chong et al., 2005), European sea bass (Cordero et al., 2015), and gilthead sea bream (Jurado et al., 2015; Sanahuja and Ibarz, 2015). Differentially expressed

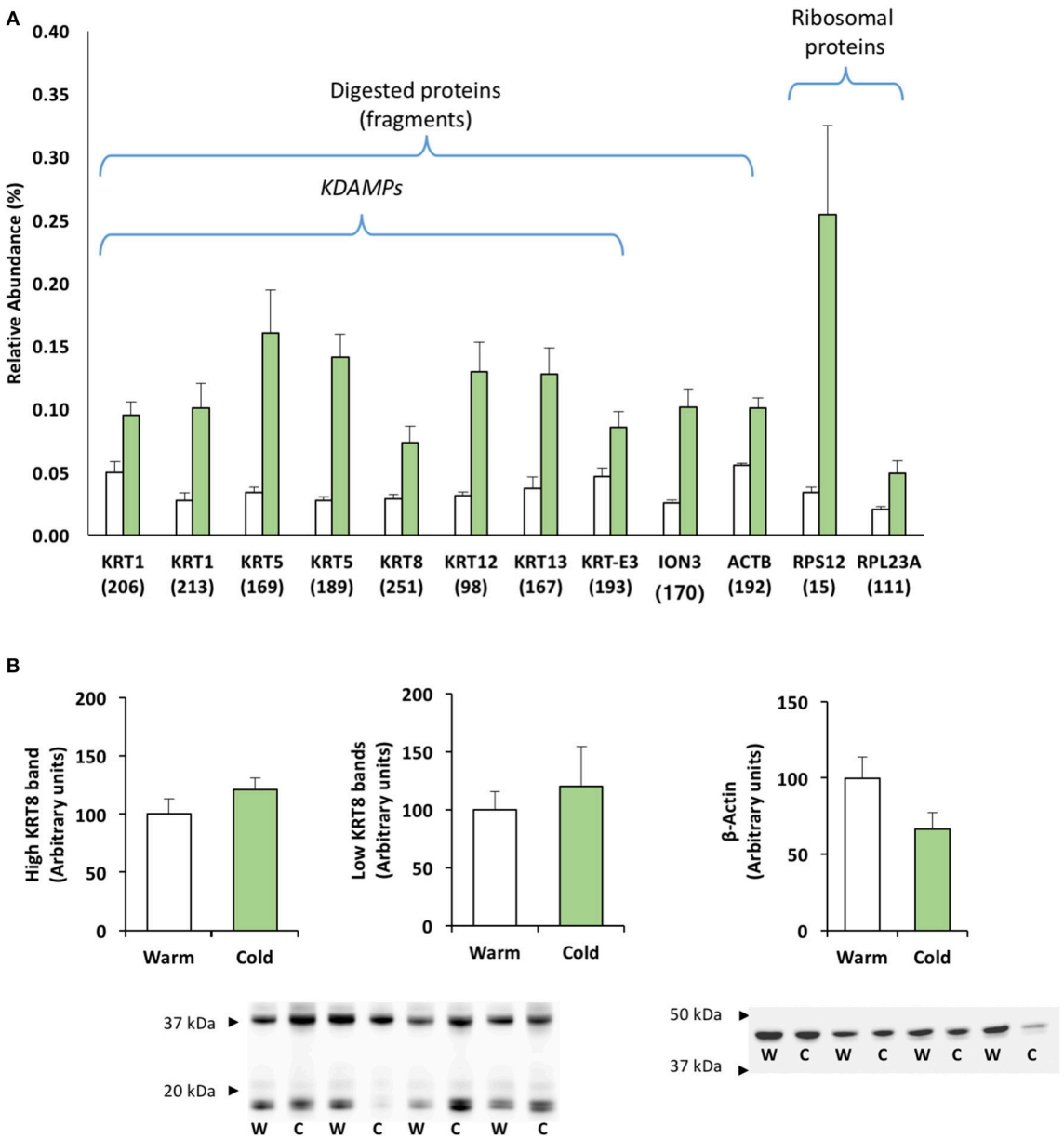

FIGURE 3 | Relative expression of identified protein fragments with putative antimicrobial activity. **(A)** Histogram of protein abundance. Values corresponded to mean ± S.E.M. of the relative abundance of differentially expressed proteins. The digested proteins corresponded to proteins identified with observed MW lower than theoretical MW (see details in **Table 1**). Over-expressed ribosomal proteins are shown due to their antimicrobial activity. **(B)** Cytokeratin-8 (KRT8) and β-actin relative abundances by Western blot analysis.

proteins in skin mucus have been studied in response to aquaculture stressors, such as infection (Provan et al., 2013; Rajan et al., 2013; Valdenegro-Vega et al., 2014), handling or crowding (Easy and Ross, 2009, 2010; Pérez-Sánchez et al., 2017), and nutritional challenges (Micallef et al., 2017). Here, for the first time, we study how the mucus proteome responds to the environmental challenge of low temperatures, as in the cold season: one of the main concerns for gilthead sea bream aquaculture, reviewed in Ibarz et al. (2010a). Our study goes beyond a list of individual proteins with expressions that are modified by low temperatures, and attempts to elucidate the relationship of the modified proteins by building the interactome, or protein–protein interactions, using STRING tools (Szklarczyk et al., 2017). Despite initially proposed protein classification as structure, metabolism or protection related, the resulting interactome showed a central core strongly linking most of the differentially expressed proteins under cold conditions, and a satellite subset network including all the keratin forms detected

together with periplakin and epiplakin proteins. From that central core of the cold interactome, four main subsets were obtained via enrichment analysis corresponding to GO groups with significance.

Within the "Response to stress" GO group (GO:0006950), consistent protein–protein interactions were reported for 12 proteins, indicating that defensive proteins, such as HSPs, TF, and PDIA3; metabolic proteins, such as PCNA, PPIF, and PSMA6; and structural proteins, such as GSN and COTL1, work together, also in skin mucus. Furthermore, whereas proteins with enzymatic activities (PDIA3, UBA1, PCNA or PSMA6) were down-regulated, the defensive proteins HSPs and TF were up-regulated. HSP forms and TF have been proposed as welfare biomarkers in mucus (Sanahuja and Ibarz, 2015), since the presence of chaperones has been related with mucus protein stability (Iq and Shu-Chien, 2011; Rajan et al., 2011) and the TF withholds iron and makes bacterial survival difficult, playing a role as an activator of fish macrophages (Stafford et al., 2001). Their up-regulation at low temperatures can probably be attributed to an increase of these unspecific and innate responses. All the proteins clustered as "Single-organism metabolic process" (GO:0044710) were under-expressed at low temperatures. In the skin mucus of sea bream, several proteins related to metabolism, and mainly with carbohydrate metabolism, were previously reported (Jurado et al., 2015; Sanahuja and Ibarz, 2015; Pérez-Sánchez et al., 2017). Once again, studies of challenges to different fish species have reported the increased presence of metabolic proteins in the skin mucus proteome (Provan et al., 2013; Rajan et al., 2013). For instance, a number of proteasome subunits and ubiquitin were up-regulated in fish mucus in response to infections (Bricknell et al., 2006; Rajan et al., 2013). In contrast, we attributed the current down-regulation of detected activities in mucus under cold conditions to overall metabolic depression (Ibarz et al., 2010b; Sánchez-Nuño et al., 2018a,b), which also affects exudation of these enzymes from epidermal cells. Thus, a lower presence of metabolic proteins exuded at low temperatures is also an indicator of lower metabolism in skin, and a putative lower capacity to cope with further challenges, such as infections.

Another interactome subset was linked to "Interspecies interaction between organisms" (GO:0044419), which included mainly up-regulated mucus proteins. This interactome subset evidenced a favorable condition for bacteria adhesion at low temperatures due to changes in the proteome. *Hsp70* may be a stress-induced surface adhesin, mediating sulfatide recognition, that could be used by bacteria to facilitate surface adhesion (Valizadeh et al., 2017), just as lectin types are used by infectious organisms to bind with complementary host structures (Acord et al., 2005). Septins, together with actin, are increasingly recognized as playing important roles in bacterial entry into host cells (Mostowy et al., 2009) including those of fish (Willis et al., 2016). Meanwhile, 40S ribosomal protein is required for an adhesion process that depends upon both cell–cell and cell–substrate adherence of several fungal pathogens (Kim et al., 2010); although in fish, greater amounts of ribosomal proteins in skin mucus were reported in response to infection (Esteban, 2012). Epiplakin and periplakin, as desmosome components, and keratin-8, seem to work together in maintaining tissue integrity, mainly in keratinocyte layers (Long et al., 2006). Their up-regulation was observed in the present study, which is a signal of a putative response to block bacterial entry or to regulate epithelial cell turnover in chronic low temperature conditions.

Interestingly, the interactome approach resulted in a group of proteins being clustered in the "Transport" GO-group (GO:0006810), and all were over-expressed. It is well-known that mucus cells in fish epidermis package their products in secreting vesicles and release the contents through exocytosis processes (Long et al., 2013), similarly to the mucus-secreting cells of mammals (Verdugo, 1990). However, the molecular mechanisms underlying the synthesis and release of bioactive mucus products, and the responses of mucus cells to environmental stressors or pathogens, remain largely unknown. Our results would indicate that, in spite of overall depression under cold conditions, fish made efforts to maintain the rate of mucus secretion at low temperatures, because mucus turnover (the balance between continuous secretion and replacement) is crucial to prevent potential infections (Esteban, 2012). However, further studies should focus on mucus turnover and renewal under natural and challenged conditions, considering both epidermal cell activities, and mucus properties, and composition.

Finally, the proteome map of gilthead sea bream skin mucus at low temperatures showed a number of fragments or cleaved proteins, mainly keratin forms. Recently, interest in the presence of cleaved keratins has increased due to their putative antimicrobial function as membrane pore-forming peptides in mammals (Tam et al., 2012). The so-called KDAMPs are produced by proteolysis via extracellular proteases. In fish, little information on the roles of keratin as antimicrobial peptides is available. Different reports have shown that keratins from skin mucus also possess anti-bacterial activity, owing to their pore-forming properties (Molle et al., 2008; Rajan et al., 2011). For gilthead sea bream, Sanahuja and Ibarz (2015) noted the presence of keratin fragments in the skin mucus proteome and Pérez-Sánchez et al. (2017) also revealed by Western blot the presence of several forms, with different MWs, of cytokeratin-8 as a product of proteolytic activity. In accordance with that, in the current study we identified two bands for cytokeratin-8, which corresponded to the proteome presence of a small fragment (around 14 kDa). An increasing number of antimicrobial peptides in fish mucus are found to be derived by proteolysis from larger proteins with other known functions, such as ribosomal proteins (Cho et al., 2002b). It seems that matrix metalloproteinase 2 is involved in the regulation of that proteolysis in mucus, activating cathepsin forms. Thus, up-regulation of the specific metalloprotease activity detected by zymography together with higher concentrations of ribosomal and keratin fragments in skin mucus suggest an increased innate defense via new antimicrobial peptides during chronic cold in sea bream. This is the first approach using 2D-SDS-PAGE coupled to LC-MS/MS analysis to report a number of differentially expressed protein fragments in skin mucus. As it would be difficult to identify fragments of native proteins by the respective antibodies, as occurred here with the different spots corresponding to actin, further approaches will be necessary to focus on those fragments, the

sequence to be identified and the antimicrobial role attributed to them.

CONCLUSION

Skin mucus studies have been shown to be a powerful tool to devise putative bioindicators of fish welfare and physiological status via non-invasive methods. Here, we demonstrate that the skin mucus proteome also reflects the reported overall depression of gilthead sea bream metabolism and immune response at low temperatures. Under a chronic cold challenge, the capacity of fish to exude protective components to the main external fish barrier was altered, reducing mainly proteins related to enzymatic activity. However, alternative innate defenses appeared, such as HSPs, transferrin or lower-molecular-weight antimicrobial peptides. Additionally, some mucus proteins related to pathogen adhesion were increased at low temperatures, which would favor infection processes. In view of present results, further studies are necessary to enhance understanding of the impact of low environmental temperatures on the acute or short-term performance of host–pathogen systems, as well as during temperature recovery. Specifically, it would be advantageous to elucidate the underlying mucosal defense mechanisms that result in host mortality when fish suffer cold stress under farm conditions.

AUTHOR CONTRIBUTIONS

IS, LF-A, SS-N, BO-G, and AI performed the experiments. IS and AI designed the trial. All authors revised the manuscript, agreed to be accountable for the content of the work, and agreed to be listed and approved the submitted version of the manuscript.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2018.01916/full#supplementary-material

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Dietary Tryptophan Induces Opposite Health-Related Responses in the Senegalese Sole (*Solea senegalensis*) Reared at Low or High Stocking Densities With Implications in Disease Resistance

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High rearing densities are typical conditions of both inland and onshore intensive aquaculture units. Despite obvious drawbacks, this strategy is nonetheless used to increase production profits. Such conditions inflict stress on fish, reducing their ability to cope with disease, bringing producers to adopt therapeutic strategies. In an attempt to overcome deleterious effects of chronic stress, Senegalese sole, *Solea senegalensis*, held at low (LD) or high density (HD) were fed tryptophan-supplemented diets with final tryptophan content at two (TRP2) or four times (TRP4) the requirement level, as well as a control and non-supplemented diet (CTRL) for 38 days. Fish were sampled at the end of the feeding trial for evaluation of their immune status, and mortalities were recorded following intra-peritoneal infection with *Photobacterium damselae* subsp. *piscicida*. Blood was collected for analysis of the hematological profile and innate immune parameters in plasma. Pituitary and hypothalamus were sampled for the assessment of neuro-endocrine-related gene expression. During the feeding trial, fish fed TRP4 and held at LD conditions presented higher mortalities, whereas fish kept at HD seemed to benefit from this dietary treatment, as disease resistance increased over that of CTRL-fed fish. In accordance, cortisol level tended to be higher in fish fed both supplemented diets at LD compared to fish fed CTRL, but was lower in fish fed TRP4 than in those fed TRP2 under HD condition. Together with lower mRNA levels of *proopiomelanocortin* observed with both supplementation levels, these results suggest that higher levels of tryptophan might counteract stress-induced cortisol production, thereby rendering fish better prepared to cope with disease. Data regarding sole immune status showed no clear effects of tryptophan on leucocyte numbers, but TRP4-fed fish displayed inhibited alternative complement activity (ACH50) when held at LD, as

opposed to their HD counterparts whose ACH50 was higher than that of CTRL-fed fish. In conclusion, while dietary tryptophan supplementation might have harmful effects in control fish, it might prove to be a promising strategy to overcome chronic stress-induced disease susceptibility in farmed Senegalese sole.

Keywords: amino acid, neuro-endocrine response, functional feed, cortisol, crowding stress

INTRODUCTION

Every physiological response (e.g., metabolism, mineral balance, reproduction, growth, and immune function) is at least partly regulated by neuroendocrine mechanisms, which, in teleosts, are orchestrated by the hypothalamus-pituitary-interrenal (HPI) axis. Neuroendocrine signals are released upon stimulation of HPI axis and responses are highly different within each context. In fish, the stress response, triggered by both internal and external signals, consists of the production and secretion of different molecules, such as the corticotropin-releasing hormone (Crh) and Crh-binding protein (Crhbp) in the hypothalamus, adrenocorticotropic hormone (ACTH) in the pituitary, and cortisol in the head-kidney interrenal tissue (Bonga, 1997; Flik et al., 2006). The latter has been generally recognized as the most important stress indicator in fish (Tort, 2011) and, amongst other intermediates, ACTH is the ultimate cortisol secretion-inducer. ACTH is a polypeptide hormone encoded in the medium region of the proopiomelanocortin gene (*pomc*) which post-translational processing yields not only ACTH, but γ, α and β-melanocyte-stimulating hormone (MSH) and β-endorphin (Takahashi et al., 2013; Navarro et al., 2016). In most teleost species, given the earlier genome duplication, two forms of proopiomelanocortins are present, named *pomca* and *pomcb*. In Senegalese sole (*Solea senegalensis*) the two isoforms have been shown to depict subfunctionalization and therefore are expected to be differently modulated by stress (Wunderink et al., 2012). HPI axis activity is also indirectly modulated by serotonin, which receptors activity seem to both stimulate and inhibit ACTH secretion in fish, hence, cortisol release (Höglund et al., 2002).

Being a precursor of the monoamine serotonin, tryptophan availability dictates serotonin production and so can indirectly (through adrenocorticotropic hormone action) affect stress response in fish, as already reviewed in several fish species (Hoseini et al., 2017). In fact, an increased tryptophan availability in the diet was already reported to affect endocrine and behavioral responses to stress in fish (Winberg et al., 2001; Lepage et al., 2002; Höglund et al., 2007), including the Senegalese sole (Costas et al., 2012). The later study also highlighted the importance of feeding time, a fact already reported previously for the rainbow trout (*Oncorhynchus mykiss*) (Lepage et al., 2003) and also reviewed by Hoseini et al. (2017). Nevertheless, the nature of stress factors that effectively activates an HPI response is wide-ranging and it eventually plays a role in dictating response development (Martos-Sitcha et al., 2014). While dietary supplementation of tryptophan lowered cortisol levels in acute crowding stressed rainbow trout (Lepage et al., 2002), this effect seems to not so clear during longer periods of time (Hoseini et al., 2017). Having this in mind, it remains to be elucidated whether a long-term tryptophan feeding presents a clear role on the modulation of the HPI axis response under chronic stressful conditions in fish.

The importance of neuroendocrine-immune interactions is not unidirectional. Not only immune stimulation has the ability to trigger a neuroendocrine response. Indeed, the presence of nerve fibers innervating teleosts head-kidney, and cortisol receptors in leucocytes, show the importance of neuroendocrine mechanisms in regulating other physiological processes such as the immune response (Tort, 2011). Cortisol well-known immunosuppressive effects in fish (Saeij et al., 2003; Varsamos et al., 2006), and the immune-mediated cortisol release (Haukenes and Barton, 2004) clearly illustrate the abovementioned relationship, particularly when stressful situations last through time and turn into chronic stress conditions.

Chronic stress imposed by high rearing densities in farmed fish and daily husbandry routines may impair immune defenses and thereby increase fish susceptibility to disease (Tort, 2011). It is therefore of critical importance to find and adopt strategies to minimize the impact of such aquaculture-related practices on fish health. A significant effort is being made to find compounds that display immune-enhancing properties, such as pro- and prebiotics, vitamins, minerals, fatty acids, etc. (Manning and Gibson, 2004; Li et al., 2009; Nayak, 2010). Given their versatility in terms of biological roles they play, amino acids dietary supplementation is becoming an important research topic in fish immunonutrition. Within amino acids, the potential benefit of tryptophan in the diet was investigated in different fish species (reviewed by Hoseini et al., 2017; Höglund et al., 2019), although the results are not yet conclusive. Therefore, this study was conceived to evaluate the potential benefits of tryptophan dietary surplus at different levels on Senegalese sole neuroendocrine and metabolic response in a factorial design (3×2) in fish held at low or high stocking densities as a chronic stress source. Furthermore, the present work aims to unravel possible effects of dietary tryptophan supplement on Senegalese sole immune condition and disease resistance to a bacterial infection.

MATERIALS AND METHODS

Experimental Diets

A practical diet was formulated to be isonitrogenous and isolipidic (45% crude protein and 16% crude fat) and to meet the estimated Senegalese sole amino acids profile, and served as control (CTRL). Two other diets were formulated similar to CTRL and supplemented with 0.86 and 1.72% tryptophan (diets

TRP2 and TRP4, respectively). Lowest supplementation level was selected based on preliminary studies in which cortisol-mediated immunosuppression was reduced (Costas et al., 2012, 2013b). Lepage et al. (2002) on rainbow trout demonstrated tryptophan role in counteracting cortisol elevation when dietary inclusion levels were as high as eightfold the tryptophan requirement level. This study served as a reference to the use of a higher (4×) tryptophan supplementation level. The essential amino acids profile from the control diet was formulated considering the requirement previously determined for Senegalese sole juveniles (Silva et al., 2010). In the absence of specific data on tryptophan requirement of Senegalese sole, requirement data for other species were applied (Kaushik, 1998).

Detailed information on diet composition and proximate analysis is given in **Table 1**. All ingredients were ground, mixed together and dry-pelleted in a laboratory pellet mill (CPM, California Pellet Mill, Crawfordsville, IN, United States). Proximate analysis of the diets was performed according to the Association of Official Analytical Chemists methods (AOAC, 2000) and amino acids analysis was carried out according to Machado et al. (2015) and tryptophan was measured by a spectrophotometric method as described by De Vries et al. (1980). Amino acids composition of the diets is presented in **Table 2**.

TABLE 1 | Ingredients and proximate composition of the experimental diets.

	Experimental diets		
Ingredients (% dry weight)	**CTRL**	**TRP2**	**TRP4**
Fish meal[a]	25	25	25
CPSP[b]	5.0	5.0	5.0
Corn gluten[c]	20	20	20
Soybean meal[d]	14.4	12.9	11.3
Wheat meal[e]	18.2	18.8	19.5
Cod liver oil	11.7	11.7	11.7
Vitamin mix[f]	1.0	1.0	1.0
Mineral mix[g]	1.0	1.0	1.0
Choline chloride (50%)	0.50	0.50	0.50
Binder (Aquacube)[h]	1.0	1.0	1.0
Agar	1.0	1.0	1.0
Tryptophan		0.86	1.72
Proximate analysis (% dry weight)			
Dry matter (%)	89.3	89.2	89.6
Crude protein	47.1	47.1	47.0
Crude fat	16.4	16.3	16.4
Ash	10.2	10.9	10.1

[a]Steam dried LT fish meal, Pesquera Diamante, Perú (CP: 70.7% DM; CF: 9.1% DM). [b]Soluble fish protein concentrate; Sopropêche, France (CP: 80.4% DM; CF: 19.7% DM). [c]Sorgal, Portugal (CP: 68.3% DM; CF: 2.9% DM). [d]Sorgal, Portugal (CP: 53.5% DM; CF: 1.0% DM). [e]Sorgal, Portugal (CP: 14.1% DM; CF: 2.2% DM). [f]Vitamins (mg kg^{-1} diet): retinol acetate, 18000 (IU kg^{-1} diet); cholecalciferol, 2000 (IU kg^{-1} diet); alpha tocopherol acetate, 35; sodium menadione bisulfate, 10; thiamin-HCl, 15; riboflavin, 25; calcium pantothenate, 50; nicotinic acid, 200; pyridoxine HCl, 5; folic acid 10; cyanocobalamin, 0.02; biotin, 1.5; ascorbic acid, 50; inositol, 400. [g]Minerals (mg kg^{-1} diet): cobalt sulfate, 1.91; copper sulfate, 19.6; iron sulfate, 200; sodium fluoride, 2.21; potassium iodide, 0.78; magnesium oxide, 830; manganese oxide, 26; sodium selenite, 0.66; zinc oxide, 37.5; dibasic calcium phosphate, 5.93 (g kg^{-1} diet); potassium chloride, 1.15 (g kg^{-1} diet); sodium chloride, 0.40 (g kg^{-1} diet). [h]Agil, England (guar gum, polymethyl carbamide, manioc starch blend, hydrate calcium sulfate).

Fish and Experimental Design

This study was carried out at the Interdisciplinary Centre of Marine and Environmental Research facilities, Porto, Portugal. Healthy, non-vaccinated Senegalese sole (45.3 ± 0.3 g) juveniles were obtained from a Portuguese commercial fish farm with no history of photobacteriosis. After 2 weeks of acclimatization, fish were weighed, measured and distributed to 18 flat-bottomed, shaded tanks (0.05 m^2; 6.5 L) of two separate seawater recirculation systems [temperature: 20 ± 1°C; salinity: 24 ppt; natural light-dark cycle (June, 2013, 41°09′07.0″N 8°36′56.1″W)]. Fish were distributed to establish two different stocking densities, one per system: in one of the systems, each of 9 tanks held 14 fish (12.5 kg m^{-2}, LD group) and was considered the control group, while in the other system, each of 9 tanks held 34 fish (31 kg m^{-2}, HD group) and fish were regarded as chronically stressed. It is noteworthy to mention that LD cannot be considered as unstressed animals but fish undergoing a lower degree of stress. Density levels were chosen in accordance to that currently used in super-intensive production systems for Senegalese sole. Dietary treatments were randomly assigned to triplicate tanks of each density group. Fish were fed twice a day (9 am and 4 pm) until apparent satiety for 38 days, and water parameters, including nitrogenous compounds, were monitored daily.

Fish were fasted 24 h prior to sampling to avoid any influence of feeding on cortisol and glucose levels (Arends et al., 1999). At day 39, 4 fish per tank (12 fish per dietary treatment, and per density) were killed by immersion in 2-phenoxyethanol (1 mL

TABLE 2 | Determined amino acid composition (g 16 g^{-1} N) of the experimental diets.

	Experimental diets		
Amino acid analysis (g 16 g^{-1} protein)	**CTRL**	**TRP2**	**TRP4**
Lysine	5.54	5.57	5.41
Arginine	5.22	5.14	5.17
Histidine	2.99	2.91	2.86
Isoleucine	4.67	4.54	4.47
Leucine	7.62	7.50	7.37
Valine	7.11	6.88	6.89
Methionine	2.08	2.06	2.08
Phenylalanine	3.66	3.59	3.55
Threonine	6.07	6.04	6.00
Tyrosine	2.57	2.62	2.61
Aspartic Acid	8.18	8.15	8.22
Glutamic acid	16.4	16.1	16.1
Serine	5.62	5.64	5.27
Glycine	6.64	6.49	6.25
Alanine	7.54	7.36	7.13
Proline	7.12	7.14	7.09
Tryptophan	0.97	2.28	4.36

L^{-1}; Sigma). Blood was collected from the caudal vessel with heparinized syringes and processed for hematological analyses as described below. The remaining blood was centrifuged for 10 min at 10,000 × *g* and 4°C and then stored at −80°C until further analyses. Pituitary and hypothalamus were also collected and kept in a 1/10-relation w/v of RNAlater® stabilization solution (Ambion Inc., Austin, TX, United States) at 4°C for 24 h and then stored at −80°C until assayed. For gene expression purposes, two fish per tank were used ($n = 6$).

The experiments were approved by the Animal Welfare Committee of the Interdisciplinary Centre of Marine and Environmental Research and carried out in a registered installation (N16091.UDER). Experiments were performed by trained scientists in full compliance with national rules and following the European Directive 2010/63/EU of the European Parliament and the European Union Council on the protection of animals used for scientific purposes.

Bacteria Inoculum Preparation

Photobacterium damselae subsp. *piscicida* (*Phdp*) strain PC566.1 was isolated from Senegalese sole and kindly provided by Professor Alicia E. Toranzo (Departamento de Microbiología y Parasitología, Facultad de Biología, University of Santiago de Compostela, Spain). Stocked bacteria were cultured for 48 h at 22°C on tryptic soy agar supplemented with NaCl at 1% (TSA-1) and then inoculated into tryptic soy broth similarly supplemented with NaCl at 1% (TSB-1) (both media from Difco Laboratories) and cultured overnight at the same temperature, with continuous shaking (100 rpm, Rotator DSR2100V). Exponentially growing bacteria were collected by centrifugation at 3,500 × *g* for 30 min, resuspended in sterile phosphate-buffered saline (PBS) and adjusted to a LD_{50} [5×10^3 colony forming units (CFU) mL^{-1}] by reading absorbance against a growth curve, according to Costas et al. (2013a). Final bacterial concentration was confirmed by plating serial dilutions onto TSA-1 plates and counting the number of CFU after incubation at 22°C for 48 h.

Bacterial Challenge

At the end of the feeding trial, 20 fish per treatment (i.e., density and diet) were intraperitoneally injected with either 100 μL *Phdp* (5×10^3 CFU mL^{-1}) or 100 μL PBS and redistributed to duplicate tanks (five fish per tank) of four independent seawater recirculation systems under the same conditions described above. Feeding protocol and daily maintenance were kept as for the pre-challenge trial except for water temperature that was increased to 22°C to simulate a possible scenario during natural outbreaks (Arijo et al., 2005). Fish mortality was recorded for 3 weeks. Moribund or dead fish were removed, weighed, and sampled for bacteria isolation by plating head-kidney samples onto TSA-1 plates.

Analytical Procedures With Blood and Peripheral Leucocytes

An aliquot of gently homogenized blood was used to perform total white blood cells (WBC) and red blood cells (RBC) counts, hematocrit (Ht), and hemoglobin (Hb; SPINREACT kit, ref. 1001230, Spain) determinations. Mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC) were also calculated as follows:

- MCV (mm^3) = (Ht/RBC) × 10
- MCH (pg $cell^{-1}$) = (Hb/RBC) × 10
- MCHC (g 100 mL^{-1}) = (Hb/Ht) × 100

Blood smears were also prepared from fresh homogenized blood, air dried and stained with Wright's stain (Hemacolor; Merck) after fixation with formaldehyde-ethanol (10% of 37% formaldehyde in absolute ethanol). The slides were examined (1000×) and at least 200 leucocytes were counted per smear and classified as thrombocytes, lymphocytes, monocytes, and neutrophils. Peroxidase activity was carried out as described by Afonso et al. (1998) in order to facilitate identification of neutrophils. Absolute value ($\times 10^4$ mL^{-1}) of each cell type was subsequently calculated.

Plasma Cortisol

Plasma cortisol levels were measured using enzyme-linked immunosorbent assays (ELISAs) performed in microtitre plates (MaxiSorp™, Nunc, Roskilde, Denmark), as previously described by Martos-Sitcha et al. (2014) for other teleost species. Steroids were extracted from 5 μL plasma in 100 μL RB [10% v/v potassium phosphate buffer (PPB) 1 M, 0.01% w/v NaN_3, 2.34% w/v NaCl, 0.037% w/v EDTA, and 0.1% w/v bovine serum albumin (BSA)] and 1.2 mL methanol (Panreac), which was then allowed to evaporate over 48–72 h at 37°C. Cortisol ELISA standard (Cat. #10005273), goat anti-mouse IgG monoclonal antibody (Cat. #400002), specific cortisol express ELISA monoclonal antibody (Cat. #400372), and specific cortisol express AChE tracer (Cat. #400370) were obtained from Cayman Chemical Company (MI, United States). Standards and extracted plasma samples were analyzed in duplicate. The standard curve was constructed through 1:1 serial dilutions from 2.5 ng mL^{-1} to 9.77 pg mL^{-1} ($R^2 = 0.992$). The lower limit of detection (95.85% of binding, ED98.75) was 14.6 pg mL^{-1}. The percentage of recovery was 95%. Inter- and intra-assay coefficients of variation (calculated from sample duplicates) were 3.67 ± 1.18% and 2.89 ± 0.63%, respectively. Cross-reactivity of the specific antibody toward intermediate steroid synthesis or metabolism products was determined by the supplier (Cayman Chemical Company, MI, United States).

Plasma Metabolites

Plasma glucose, lactate and triglycerides were assessed using commercially available Spinreact kits (Glucose HK Ref. 1001200; Lactate Ref. 1001330; Triglycerides Ref. 1001311; Sant Esteve d'en Bas, Girona, Spain), adapted for 96-well microplates. All assays were carried out on a microplate reader (BioTek Instruments, Winooski, VT, United States) using the KCjunior Data Analysis Software for Microsoft Windows XP.

Innate Humoral Parameters

Plasma bactericidal activity was measured according to Graham et al. (1988) with some modifications (Machado et al.,

2015). Total bactericidal activity is expressed as percentage, calculated from the difference between the dissolved formazan in samples and the one formed in the positive controls (100%). The bactericidal activity was calculated as the percentage of non-viable bacteria.

Alternative complement pathway activity (ACH50) was evaluated as described by Sunyer and Tort (1995). The ACH50 units were defined as the concentration of plasma inducing 50% hemolysis of rabbit red blood cells.

A turbidimetric assay was used to evaluate lysozyme activity following the method as described by Costas et al. (2011).

Total peroxidase activity in plasma was measured following the procedure described by Quade and Roth (1997). Peroxidase activity was determined by defining one unit of peroxidase as that which produces an absorbance change of 1 OD (units mL^{-1} of plasma).

Gene Expression Analysis

Total RNA was isolated from individual pituitaries using NucleoSpin®RNA XS kit (Macherey-Nagel), and from hypothalamus using NucleoSpin®RNA II kit (Macherey-Nagel). The on-column RNase-free DNase digestion was used for gDNA elimination. The manufacturer's instructions were followed in this procedure. Additionally, the amount of RNA was fluorimetrically measured with the Qubit® 2.0 Fluorometer (Invitrogen™, Life Technologies) and its quality was determined in a 2100 Bioanalyzer using the RNA 6000 Nano Kit (Agilent Technologies). Only samples with a RNA Integrity Number higher than 8.5, which is indicative of clean and intact RNA, were used for real-time PCR (qPCR).

Primer oligonucleotide sequences and final concentrations used in the qPCR reactions are shown in **Table 3** and were as described by Wunderink et al. (2011, 2012), except for *gr1* and *gr2*, which were designed with NCBI Primer Blast Tool and the oligo analyzer tool of IDT®. The efficiency of *gr1* and *gr2* primer pairs was analyzed in serial fivefold dilutions of cDNA by calculating the slope of the regression line of the cycle thresholds versus the relative concentration of cDNA. Total RNA was used to synthesize the first strand cDNA by reverse transcription (RT) reaction using qSCRIPT™ cDNA Synthesis Kit (Quanta BioSciences). The qPCR was carried out with Fluorescent Quantitative Detection System (Eppendorf Mastercycler ep realplex 2 S). Each reaction mixture (10 μL) contained 0.5 μL each specific forward and reverse primer, and 5 μL PerfeCTa SYBR® Green FastMix™ (Quanta Biosciences). Reactions were conducted in semi-skirted twin-tec 96-well real-time PCR plates (Eppendorf) covered with adhesive Masterclear real-time PCR Film (Eppendorf). The thermocycling procedures were as previously described by Wunderink et al. (2011, 2012). Melting curves were used to ensure that only a single PCR product was amplified and to verify the absence of primer-dimer artifacts. Each sample was run in triplicate. The results were normalized to β-actin levels due to its low variability (less than 0.10 C_T in each tissue) under our experimental conditions. Relative gene quantification was performed using the $\Delta\Delta C_T$ method (Livak and Schmittgen, 2001).

Statistical Analysis

Statistical analyses were performed with STATISTICA (StatSoft, Inc., 2013, version 12) for WINDOWS. Results are expressed as means ± SD. Data were analyzed for normality and homogeneity of variance and, when necessary, outliers were removed using the STATISTICA tool for outliers and extremes removal. Data were analyzed by Multifactorial ANOVA with dietary treatment and rearing density as variables. Whenever significant differences were found among groups, a multiple-comparisons Tukey HSD test was performed to identify significantly different groups. For every test, the level of significance chosen was $p \leq 0.05$.

RESULTS

Diets and Growth Performance

Experimental diets were well accepted and survival was 100% for all treatments at the end of the experimental period. FBW, SGR, VFI, K, and HSI were not affected by diet composition. K was also not affected by rearing density, whereas FBW, SGR, and HSI were higher in fish reared at LD in contrast to the increased VFI observed in HD fish (**Table 4**).

Cumulative Mortality in Challenged Fish

Mortalities in LD fish started 5 days after infection in the CTRL group, while fish fed TRP4 started to die at day 6 post-injection; no mortalities were registered in fish fed TRP2 (**Figure 1A**). At the end of the challenge trial, cumulative mortality in fish fed CTRL was 30% reaching its maximum value at day 6, while fish fed TRP4 reached 80% of mortalities which were extended from the 14th day after injection until the end of the experiment.

At HD, mortality in fish fed CTRL presented a similar response to that observed in fish fed the same diet at LD (30% cumulative mortality, **Figure 1B**). In contrast, mortality increased to 10% in fish fed TRP2 and decreased also to 10% in fish fed TRP4, although a delay over time (5 days) was observed in TRP2 with respect to the TRP4 group.

Hematology

Regardless of rearing density, fish fed TRP4 showed higher Hb content than fish fed TRP2, but no differences were observed compared to fish fed CTRL (**Table 5**). At Fish maintained at LD did not show differences in MCV between treatments, while at HD fish fed TRP4 presented higher MCV than fish fed CTRL and TRP2 (**Table 5**). MCV of fish fed TRP4 was also higher at HD than that of their counterparts under LD condition. At LD there were also no differences in MCH between groups, while at HD it was also higher in fish fed TRP4 than TRP2. In fish held at HD, MCH was higher in fish fed TRP4 and the CTRL than their LD counterparts. No differences were observed regarding Ht and MCHC (**Table 5**).

In fish fed TRP4, peripheral RBC were lower in fish held at HD than at LD, no other differences being observed between diets or rearing densities (**Figure 2A**). No effects of diet or rearing density were detected on total WBC (**Figure 2B**). In contrast, peripheral neutrophils concentration decreased in TRP4-fed fish

TABLE 3 | Specifications of real-time PCR assays including forward (F) and reverse (R) primers, GenBank ID (NCBI), efficiencies (Eff) of PCR reactions, annealing temperature (Ta), and length of amplicon.

Gene	Acronym	GenBank ID	Eff (%)[1]	Ta (°C)	Amplicon length (bp)	Primer sequence (5′–3′)
Corticotropin releasing hormone	*crh*	FR745427.1	98.1	60	135	F: CCTGACCTTCCACCTGCTAC R: GAGATCTTTGGCGGAGTGAA
Corticotropin releasing hormone-binding protein	*crhbp*	FR745428.1	101.3	60	118	F: GGCAATGGCATAGACACCTC R: CACTGGACACCAGCCTCAC
Glucocorticoid receptor 1	*gr1*	AB614369.1	105.6	60	154	F: CATGACGACCCTGAACCGAT R: CCAGCCCAGACTGAAAGACA
Glucocorticoid receptor 2	*gr2*	AB614370.1	100.0	60	150	F: ACCATGCTGTCTGTGCTCAA R: AGACTTGGCCCACTTGACTG
Proopiomelanocortin A	*pomca*	FR851915.1	102.2	60	127	F: AAGGCAAAGAGGCGTTGTAT R: TTCTTGAACAGCGTGAGCAG
Proopiomelanocortin B	*pomcb*	FR851916.1	103.9	60	110	F: GTCGAGCAACACAAGTTCCA R: GTCAGCTCGTCGTAGCGTTT
β-actin	*actb*	DQ485686.1	99.6	60	108	F: TCTTCCAGCCATCCTTCCTCG R: TGTTGGCATACAGGTCCTTACGG

[1] *Efficiency of PCR reactions (represented in percentage) were calculated from serial dilutions of tissue RT reactions in the validation procedure.*

TABLE 4 | Initial (IBW) and final (FBW) body weight, specific growth rate (SGR), voluntary feed intake (VFI), condition factor (K), and hepatosomatic index (HSI) in Senegalese sole after 38 days held at different treatments.

Parameters	Treatments					
	LD			HD		
	CTRL	TRP2	TRP4	CTRL	TRP2	TRP4
IBW (g)	45.24 ± 0.08	44.98 ± 0.37	45.07 ± 0.47	45.47 ± 0.10	45.49 ± 0.06	45.50 ± 0.21
FBW (g)	51.78 ± 0.78	50.86 ± 0.85	51.47 ± 0.36	48.60 ± 0.15	48.89 ± 0.80	48.67 ± 0.48
SGR (% day^{-1})	0.36 ± 0.05	0.36 ± 0.06	0.39 ± 0.03	0.20 ± 0.01	0.21 ± 0.04	0.20 ± 0.02
VFI	0.19 ± 0.01	0.19 ± 0.01	0.10 ± 0.01	0.30 ± 0.01	0.29 ± 0.01	0.30 ± 0.01
K (g cm^{-3})	1.21 ± 0.01	1.21 ± 0.01	1.21 ± 0.07	1.20 ± 0.06	1.21 ± 0.04	1.21 ± 0.03
HSI (%)	1.31 ± 0.17	1.33 ± 0.32	1.46 ± 0.43	1.09 ± 0.21	1.21 ± 0.32	1.04 ± 0.15

Parameters	Density	Diet	Density × diet	LD	HD
FBW	<0.001	ns	ns	B	A
SGR	<0.001	ns	ns	B	A
VFI	<0.001	ns	ns	A	B
K	ns	ns	ns		
HSI	<0.001	ns	ns	B	A

Values are expressed as means ± SD (n = 3; except for HSI n = 12). A and B represent significant differences between density treatments regardless of dietary treatment, whereas ns means no significant differences. Two-way ANOVA; Tukey post hoc test; $p \leq 0.05$. Voluntary feed intake (VFI) = Crude feed intake/average body weight [(initial body weight+final body weight)/2]/days.

under HD compared to their counterparts held at LD, but no other differences were observed among groups (**Figure 3A**). Regarding circulating monocytes, numbers were higher in fish fed TRP2 than in fish fed CTRL and TRP4, irrespective of rearing density (**Figure 3B**). Finally, lymphocytes and thrombocyte concentrations were not significantly affected by experimental treatments (**Figures 3C,D**, respectively).

Plasma Cortisol and Metabolites

Plasma cortisol levels were lower in fish held at HD than at LD, regardless of dietary treatment. Regarding dietary treatments, fish fed TRP4 showed lower plasma cortisol concentration compared to fish fed TRP2, but no significant differences were observed compared to CTRL-fed fish due to high variability in fish fed TRP2 (**Figure 4**).

Plasma glucose and lactate levels were lower in fish fed TRP4 than TRP2, regardless rearing density (**Figures 5A,C**, respectively). Differently, dietary treatment did not affect plasma triglycerides, while levels were lower in fish held at HD (**Figure 5B**).

Innate Humoral Parameters

Plasma bactericidal activity increased in fish fed TRP2 and held at LD compared to fish fed CTRL or TRP4 under the same density conditions, whereas dietary treatments did not affect the bactericidal activity of Senegalese sole held at HD (**Figure 6A**).

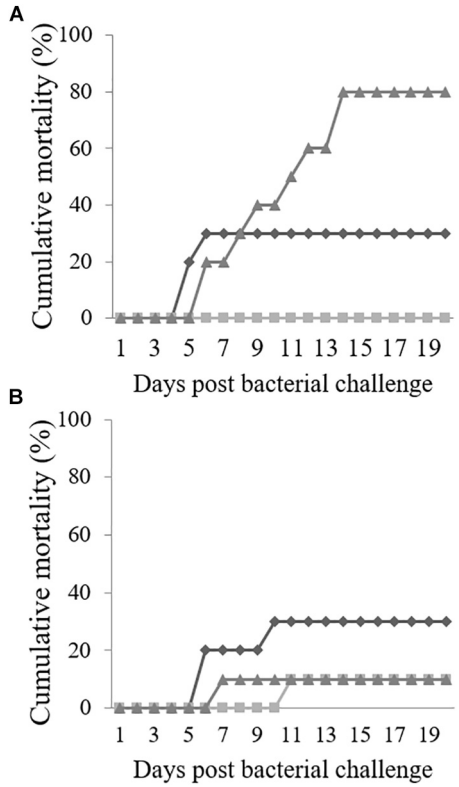

FIGURE 1 | Cumulative mortality (%) of Senegalese sole reared at low **(A)** or high **(B)** stocking densities, fed CTRL (♦), TRP2 (■) or TRP4 (▲), and intraperitoneally injected with *Phdp* (n = 10).

Still, a significant interaction was observed in fish fed the CTRL diet which translated in an enhanced bactericidal activity in fish held at HD compared to their counterparts reared at LD (**Figure 6A**). On the other hand, neither dietary treatments, nor rearing density significantly affected plasma peroxidase levels (**Figure 6B**). Regarding plasma ACH50 levels, interactive effects between density and dietary treatments were observed, in which fish fed CTRL and TRP2 held at LD presented higher ACH50 activity than those maintained at HD (**Figure 6C**). Differently, ACH50 was higher in fish fed TRP4 held at HD compared to those at LD. Moreover, TRP2-fed fish showed higher ACH50 activity than those fed TRP4 under LD condition, while at HD, both supplemented diets enhanced ACH50 activity compared to those fed CTRL.

Gene Expression

Expression for *crh* was not affected by diet composition, but increased in fish held at HD regardless dietary treatments (**Figure 7A**), whereas *crhbp* expression level was higher in TRP2-fed fish than in CTRL-fed fish regardless stocking density conditions (**Figure 7B**). Expression for *pomca* was down-regulated in fish fed TRP2 and TRP4 under HD condition compared to CTRL-fed animals, whereas fish held at HD and fed CTRL also presented higher *pomca* transcripts respect to their counterparts at LD (**Figure 7C**). Moreover, HD conditions increased *pomcb* gene expression relatively to LD, while no diet-mediated effects were observed (**Figure 7D**). Dietary treatments did not affect *gr1* transcript levels (**Figure 7E**), which contrasted a down-regulation of *gr2* gene expression in TRP2-fed fish at HD compared to their LD counterparts (**Figure 7F**).

DISCUSSION

High stocking density conditions are used in intensive fish farms and though hardly unavoidable, they are regarded as one of intensive aquaculture production biggest bottlenecks, as it represents an important stress-inducing factor (Saeij et al., 2003; Varsamos et al., 2006; Aedo et al., 2015). Amongst other negative effects, growth, immunity, and disease resistance are known to be severely compromised in fish undergoing chronic stress (Tort, 2011). In the present study, fish held at HD decreased growth performance (i.e., FBW and SGR) even though VFI was enhanced, in line to that described for other flatfish species such as common sole *Solea solea* (Schram et al., 2006), turbot *Scophthalmus maximus* (Irwin et al., 1999), or California halibut *Paralichthys californicus* (Merino et al., 2007). This tertiary stress response has already been observed in many other chronically stressed fish (Bonga, 1997), including the Senegalese sole (Arjona et al., 2009). However, results from this study contrast previous studies, which suggested that Senegalese sole cope well with high stocking densities with no implications for growth under both experimental and farming conditions (Salas-Leiton et al., 2008; Costas et al., 2013b; Andrade et al., 2015). This apparent discrepancy could be related to differences among developmental stages, rearing conditions or genetic factors (Barton, 2002), which may have interfered in the typical coping style described for Senegalese sole (Costas et al., 2008; Andrade et al., 2015). In fact, rearing tanks (i.e., shape and size) from the present study are likely to have negatively influenced fish growth performance. This is an important issue to have into account in future studies with a clear impact/knowledge transfer to the industry.

Cortisol, considered the most direct evidence of stress in fish (Flik et al., 2006), was relatively high in fish fed CTRL and kept under LD condition compared to levels measured in Senegalese sole in control conditions in previous studies (Costas et al., 2008, 2013b; Salas-Leiton et al., 2010; Wunderink et al., 2011). Still, we should bear in mind that both cortisol levels and density conditions from fish fed CTRL at LD from the present study are comparable to those held at high density by most of the mentioned studies. Moreover, the fact that CTRL-fed fish held at HD did not show higher plasma cortisol than their LD counterparts might be related to negative feedback mechanisms established in the HPI axis, as a strategy of chronically stressed animals to attenuate an exacerbated stress response (Bonga, 1997; Mommsen et al., 1999).

Though not significantly different from CTRL-fed fish, plasma cortisol concentrations were comparatively higher in TRP2-fed fish compared to TRP4. These cortisol patterns might be related to the tryptophan role in the central nervous system as an exclusive precursor of serotonin. Neuronal serotonin concentration is expected to be higher upon increased availability

TABLE 5 | Hematocrit (Ht), Hb, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC) in Senegalese sole after 38 days held at different treatments.

Parameters	Treatments					
	LD			HD		
	CTRL	TRP2	TRP4	CTRL	TRP2	TRP4
Ht (%)	13.6 ± 4.3	11.7 ± 1.3	12.5 ± 2.0	11.8 ± 1.9	12.7 ± 2.2	13.2 ± 1.9
Hb (g dL^{-1})	2.7 ± 0.5	2.5 ± 0.5	2.9 ± 0.6	2.5 ± 0.8	2.4 ± 0.6	2.9 ± 0.6
MCV (μm^3)	94.4 ± 34.9	105.3 ± 25.5	110.4 ± 23.8*	130.0 ± 32.6 a	122.7 ± 13.6 a	180.7 ± 16.7 b#
MCH (pg $cell^{-1}$)	22.2 ± 8.7*	25.8 ± 4.8	25.0 ± 3.4*	34.2 ± 4.1 ab#	22.3 ± 8.3 a	39.4 ± 8.6 b#
MCHC (g 100 mL^{-1})	22.4 ± 3.0	22.5 ± 2.6	24.1 ± 3.9	21.8 ± 8.8	18.6 ± 5.7	22.7 ± 4.1

Parameters	Density	Diet	Density × diet	CTRL	TRP2	TRP4
Ht	ns	ns	ns			
Hb	ns	0.022	ns	ab	a	b
MCV	<0.001	0.002	0.035			
MCH	<0.001	0.012	0.003			
MCHC	ns	ns	ns			

Values are expressed as means ± SD (n = 12). Different letters mean significant differences between dietary treatments. Different symbols denote significant differences between densities ($p < 0.05$), whereas ns means non-significant differences. Two-way ANOVA; Tukey post hoc test; $p \leq 0.05$.

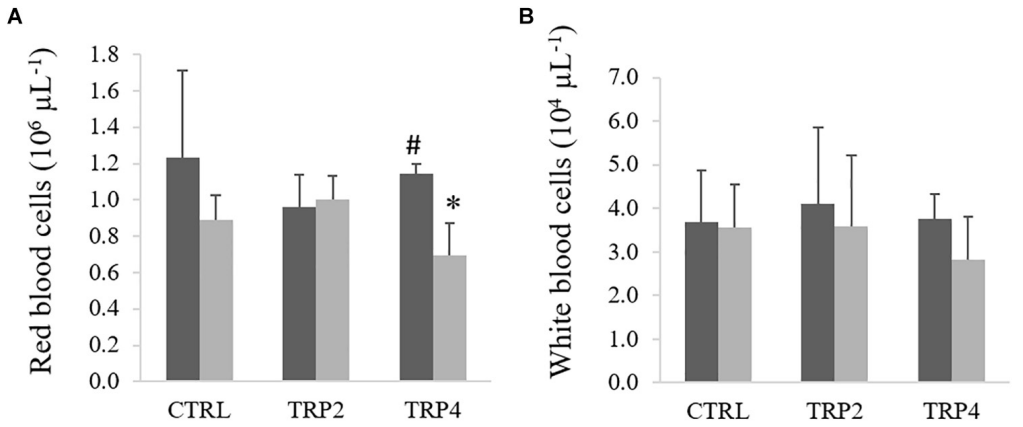

FIGURE 2 | Red blood cells **(A)** and total white blood cells **(B)** in Senegalese sole held at low (■) or high (■) stocking densities and fed dietary treatments for 38 days (mean ± SD, *n* = 9). Different symbols indicate significant differences attributed to stocking density. Two-way ANOVA; Tukey *post hoc* test; $p \leq 0.05$.

of its precursor (tryptophan), as tryptophan hydroxylase is not saturated at tryptophan physiological levels (Herrero et al., 2007; Azeredo et al., 2017; Hoseini et al., 2017). Furthermore, serotonin is partly responsible for either inhibiting or stimulating pathways in central nervous system (Spinedi and Negrovilar, 1983) as previously observed in rainbow trout (Winberg and Lepage, 1998; Winberg et al., 2001; Lepage et al., 2002). This might therefore be the mechanism explaining the increasing trend in cortisol secretion in TRP2-fed fish, even though fish were in LD stocking conditions. In providing a tryptophan surplus, serotonin production was enhanced inducing an endocrine response in these fish. These results are in accordance with those obtained in rainbow trout, which cortisol levels increased with graded inclusion of dietary tryptophan in non-stressed fish while decreased in stressed animals (Lepage et al., 2002, 2003). However, in the present study, such gradual increase of tryptophan did not similarly induce cortisol production, as TRP4-fed fish did not present higher cortisol concentration than those fed TRP2. This impairment of cortisol response, together with the observed increased disease susceptibility might be pointing a possible toxic effect of the highest supplementation level of tryptophan, which in turn could produce a clear misbalance for the correct functioning of the HPI axis, masking or desensitizing the stress response at peripheral level. At HD stocking conditions, it is expected that a neuroendocrine response is developed in which increased cortisol production is usually observed. Present results corroborate once more the aforementioned studies, as Senegalese sole reared at HD and fed tryptophan supplemented diets produced cortisol in lower (TRP2-fed fish) or similar (TRP4-fed fish) amounts

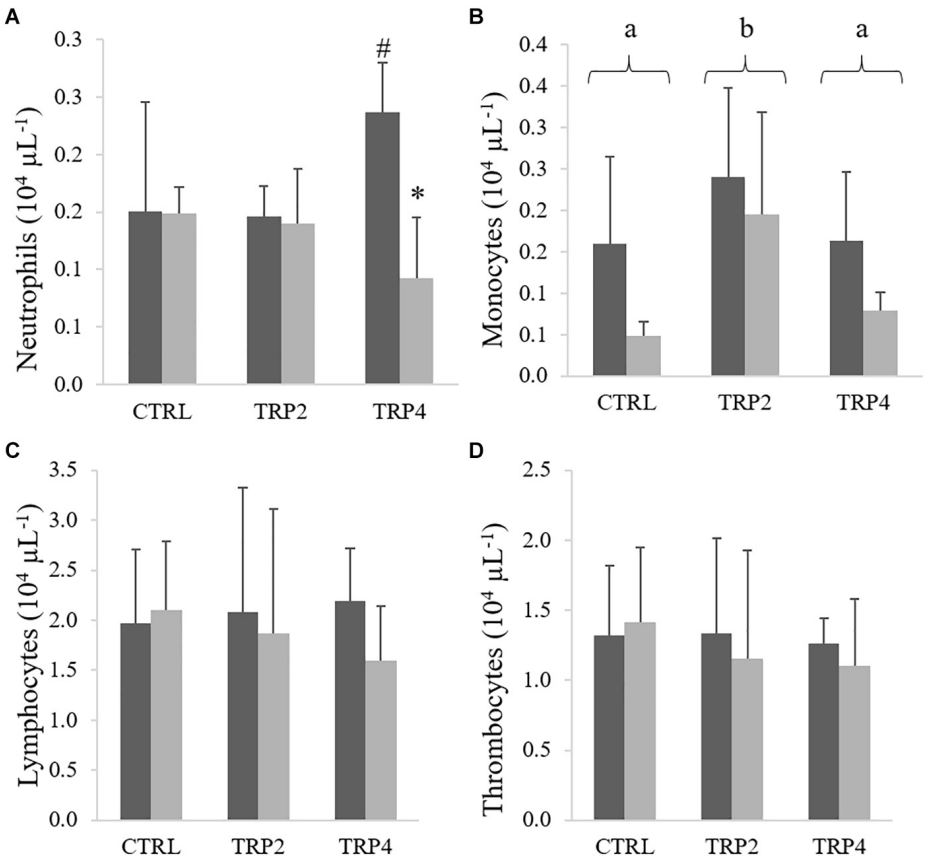

FIGURE 3 | Neutrophils **(A)**, monocytes **(B)**, lymphocytes **(C)**, and thrombocytes **(D)** in Senegalese sole held at low (■) or high (■) stocking densities and fed dietary treatments for 38 days (mean ± SD, $n = 9$). Different symbols indicate significant differences attributed to stocking density. Different letters stand for significant differences between dietary treatments. Two-way ANOVA; Tukey *post hoc* test; $p \leq 0.05$.

than their LD counterparts. This apparent adaptation to a stressful situation might again have been mediated by serotonin neuronal intervention, which is known to inhibit pituitary ACTH secretion during an ongoing neuroendocrine response (Höglund et al., 2002).

Since tryptophan – through its metabolite serotonin – has a direct impact on the central nervous system (Höglund et al., 2005, 2007; Gesto et al., 2013), brain mediators of stress response are expected to be modulated as well. The involvement of serotonin in the neuroendocrine regulation of the stress response seems to be similar within the vertebrate linage sine serotonin plays a role in the control of the HPI axis in fish, mainly through its effects on the release of corticotropin-releasing factor from the hypothalamus (Winberg et al., 1997). In the present study, stressful conditions potentiated an increase of *crh* expression that was transversal to all dietary treatments, while tryptophan dietary supplementation did not seem to affect Crh production, at least at a molecular level. Yet, *crhbp* gene expression did not follow the same response and was unaffected by stocking conditions in fish held in HD. Moreover, up-regulation of *crhbp* in fish fed TRP2 relatively to CTRL fish suggests that nutritional modulation of the HPI axis might start as high as at the hypothalamus. The mRNA from *crhbp* is translated to a protein known to regulate Crh availability and activity. Regulatory mechanisms might both prolong Crh half-life, as by binding to Crh prevents it from being degraded, and reduce Crh activity by sequestering ligand from receptor (Seasholtz et al., 2002; Moltesen et al., 2016). In spite of *crh* not differing between dietary treatments, the increased expression of *crhbp* in TRP2-fed fish points at tryptophan higher availability as a key modulator of the HPI axis activity.

An even clearer aspect of tryptophan neuroendocrine modulation is the *pomca* down-regulation in fish held at HD and fed TRP2 or TRP4, relative to CTRL. Both *pomca* and *pomcb* are structurally mostly identical, encoding several bioactive peptide hormones such as ACTH, β-MSH, and β-endorphin (Navarro et al., 2016). However, the two paralogs have distinct cleavage sites, thereby each yielding different hormones; *pomca* seems to translate to functional ACTH, while *pomcb* gives rise to β-MSH (Wunderink et al., 2012). Accordingly, present results show that *pomca*, and not *pomcb*, is modulated by stress, as fish fed CTRL showed a rise in *pomca* mRNA levels when held at HD stocking conditions. In contrast, dietary tryptophan prevented such increase in *pomca* transcripts in fish held at HD, possibly denoting tryptophan indirect modulation of the HPI axis activity. Indeed, *pomca* mRNA levels in HD fish fed TRP2 and TRP4 seem correlated ($R^2 = 0.67$, $P = 0.018$) with a decreasing trend in plasma

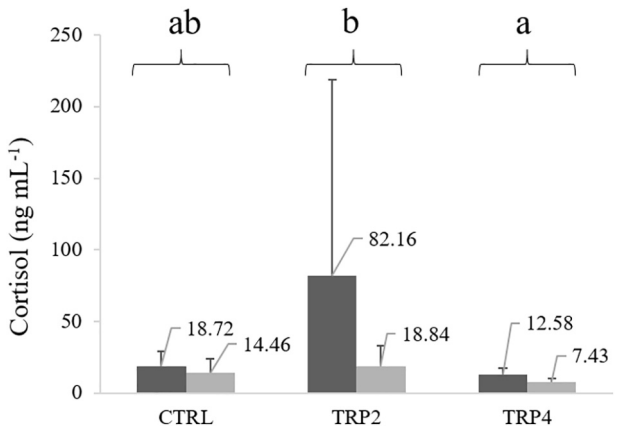

FIGURE 4 | Plasma cortisol levels in Senegalese sole held at low (■) or high (■) stocking densities and fed dietary treatments for 38 days (mean ± SD, n = 12). Values above each column are cortisol mean concentrations. Different letters stand for significant differences between dietary treatments. Two-way ANOVA; Tukey *post hoc* test; $p \leq 0.05$.

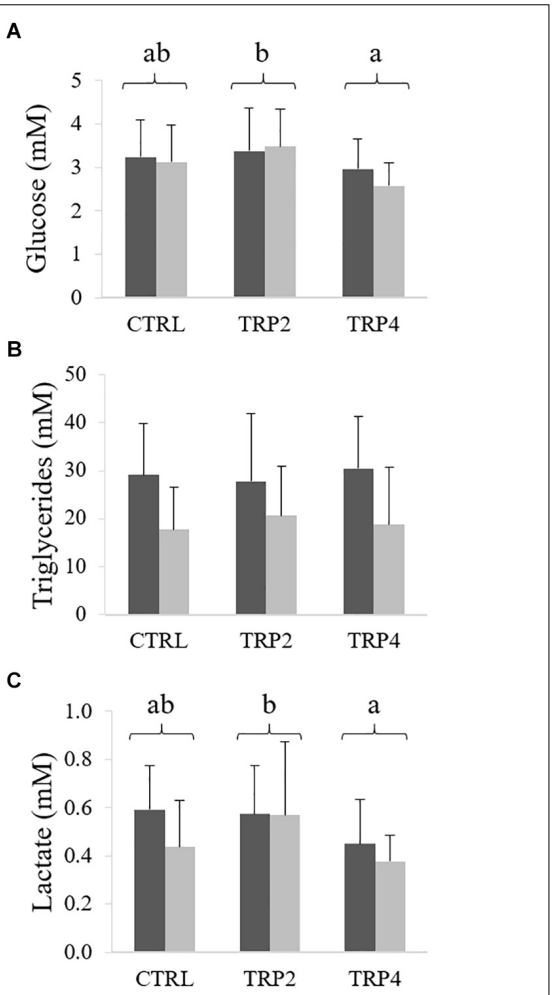

FIGURE 5 | Plasma glucose **(A)**, lactate **(B)**, and triglycerides **(C)** levels in Senegalese sole held at low (■) or high (■) stocking densities and fed dietary treatments for 38 days (mean ± SD, n = 12). Different letters stand for significant differences between dietary treatments. Two-way ANOVA; Tukey *post hoc* test; $p \leq 0.05$.

cortisol levels in fish fed dietary tryptophan supplements. Despite not as elucidating, results on the expression of cortisol receptor *gr2* seem to corroborate those of cortisol itself and of *pomca* as its expression is down-regulated in TRP2-fed fish held at HD compared to their LD counterparts.

In the present study, available data may suggest a possible relationship between increased serotonin and *pomca* mRNA levels in fish reared in HD in which the tryptophan supplementation was effective in reducing the stress response. For instance, it is known that pharmacological blockade of serotonin receptors increases the expression of POMC in rainbow trout (Pérez-Maceira et al., 2016). It is tempting to speculate that the study pointed to an implication of brain serotonin in some of the effects previously described in stressed fish and those fish supplemented with tryptophan, and reinforces the need to further explore the links between tryptophan nutrition, serotonin synthesis and the implication of the HPI axis in stressed fish.

Glucose and triglycerides stores mobilization and glucose anaerobic metabolism are activated whenever a stress response is triggered and, therefore, are considered part of the secondary stress response (Bonga, 1997). Accordingly, glucose plasma levels were observed to increase along with cortisol as a response to chronic stress (Costas et al., 2008, 2013b). Differently, in the present study, glucose concentration was not affected by high stocking density and a similar pattern was observed in both great and Siberian sturgeon, *Huso huso* and *Acipenser baerii*, respectively, exposed to similar chronic stress conditions (Rafatnezhad et al., 2008; Hasanalipour et al., 2013). Triglycerides levels were lower in plasma of fish held at HD than in those in LD conditions, as previously observed by Yarahmadi et al. (2015) in rainbow trout held at high density for 30 days. Lower triglycerides could possibly be a direct consequence of lower feed intake known to be related to stressful holding conditions as previously observed in the Atlantic cod, *Gadus morhua* (Lambert and Dutil, 2001). This, together with the observed lower HSI in HD groups and the fact that no such differences were observed either in glucose nor in lactate levels, might be a signal of total energy substrate exhaustion in fish undergoing a challenging, chronic stress response.

Cortisol is known to modulate several physiological responses, including immune responses. Thus, if an acute stress might be a trigger of immunological mechanisms either directly or via the HPI axis (Tort, 2011), chronic stress conditions have long been associated with immunosuppression and general poor welfare in fish (Tort, 2011; Gadan et al., 2012; Philip et al., 2012). Neither dietary treatments nor stocking density seemed to affect total WBC. However, fish reared at HD and fed TRP4 had decreased number of RBC and increased erythrocyte volume (MCV index) and hemoglobin content (MCH) compared to their LD counterparts, which might be a compensatory strategy to ensure oxygen transport (Robb and Abrahams, 2003). As no

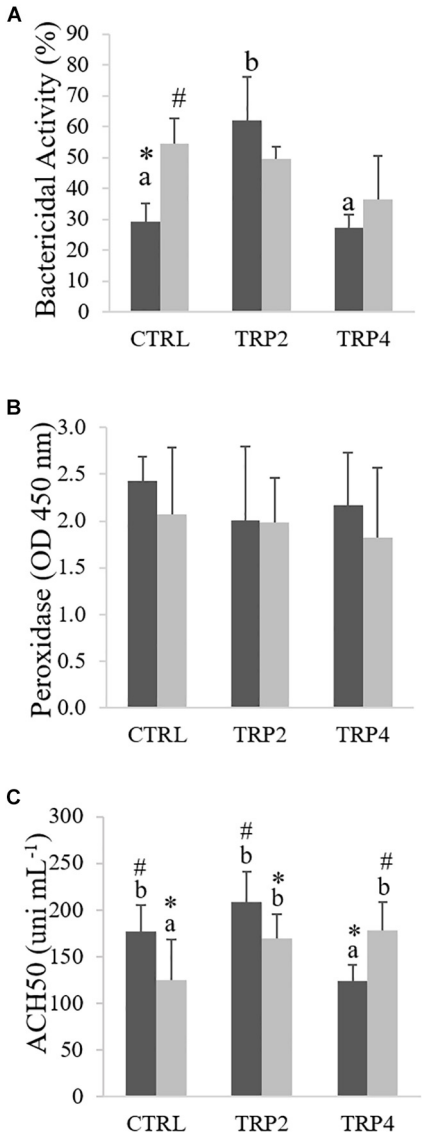

FIGURE 6 | Plasma total bactericidal activity **(A)**, peroxidase **(B)**, and ACH50 activity **(C)** in Senegalese sole held at low (■) or high (■) stocking densities and fed dietary treatments for 38 days (mean ± SD, $n = 12$). Different symbols indicate significant differences attributed to stocking density. Different letters stand for significant differences between dietary treatments. Two-way ANOVA; Tukey *post hoc* test; $p \leq 0.05$.

direct connection is known to exist between tryptophan and erythrocyte oxygen transport, further research is required to unveil the biological mechanisms associated to stress response that are triggering these hematological changes.

Indoleamine 2,3-dioxygenase-mediated tryptophan catabolism is known to modulate T-cell proliferation and impair other immune cells function (Frumento et al., 2001; Moffett and Namboodiri, 2003). Yet, its activity is only triggered with immune stimulation in both teleosts and mammals (Munn and Mellor, 2013; Cortes et al., 2016). Although present results are only related to general immune status (i.e., in the absence of immune stimulation), stressful conditions and the inherent HPI axis activation are likely to activate other immune mechanisms, in light of what is known about regulatory pathways between immune and neuroendocrine systems (Verburg-Van Kemenade et al., 2011). This bi-directional communication between immune and neuroendocrine systems was perhaps what led total bactericidal activity to increase in fish fed CTRL held at HD. Hence, indoleamine 2,3-dioxygenase cannot be excluded as possible mediator of changes in cell counts. Though not statistically significant, neutrophil concentration in TRP4-fed fish held at LD tended to be higher than in CTRL-fed fish, which could give cues about the immune system activation produced during the days previous to the end of the trial (with the subsequent stabilization of mortality rates), or even about the mediated orchestration to further induce a refractory answer during infection in a longer response. Interestingly, this apparent neutrophilia, together with higher monocyte concentration in TRP2-fed fish, is in opposite direction from what is expected of indoleamine 2,3-dioxygenase action.

Peroxidase activity is a signal of cell activation and it was not significantly affected in this study, suggesting that increased tryptophan availability did not enhance cell activation, even though improved cell recruitment. Moreover, effects of tryptophan supplementation on cell response are quite different from those on plasma ACH50 levels and on disease resistance. ACH50 plays an important role in the innate immune machinery, being responsible for pathogen lysis and opsonization (Nonaka and Smith, 2000), and in the present study it varied in line with the ability of fish to overcome infection. ACH50 was inhibited in fish fed TRP4 and held at LD. However, while ACH50 levels decreased in fish fed CTRL and under HD condition, dietary tryptophan supplementation not only counteracted this stress-induced decrease, but enhanced plasma ACH50 activity in fish held at HD. Similar results were previously reported by Hoseini et al. (2016), in Persian sturgeon, *Acipenser persicus*, fed tryptophan-supplemented diets and subjected to an acute stress.

Dietary tryptophan supplementation seemed to translate into a double-edged sword in Senegalese sole reared at LD since it induced opposite effects in terms of immune responses and disease resistance. On the one hand, fish fed TRP2 showed increased plasma bactericidal and ACH50 activities as well as circulating monocyte numbers, which translated in no mortalities after a bacterial challenge. On the other hand, despite it did not significantly affect other immune parameters, TRP4 decreased plasma ACH50 activity and led to the highest cumulative mortalities among experimental treatments. These data are indeed intriguing and suggest further experiments to unravel tryptophan role during a disease challenge. Although the contrasting responses appear not to be mediated by the HPI axis, since fish reared at LD and fed both tryptophan treatments showed similar responses, more genes linked to synthesis and release would be of assistance. Whether the observed increased disease susceptibility in fish fed TRP4 and held at LD is mediated by indoleamine 2,3-dioxygenase-related mechanisms needs to be further explored.

In contrast, it has been reported that tryptophan needs to increase in Senegalese sole reared under high density conditions (Costas et al., 2008). In the present study, both dietary tryptophan treatments seemed to compensate this increase demand, which

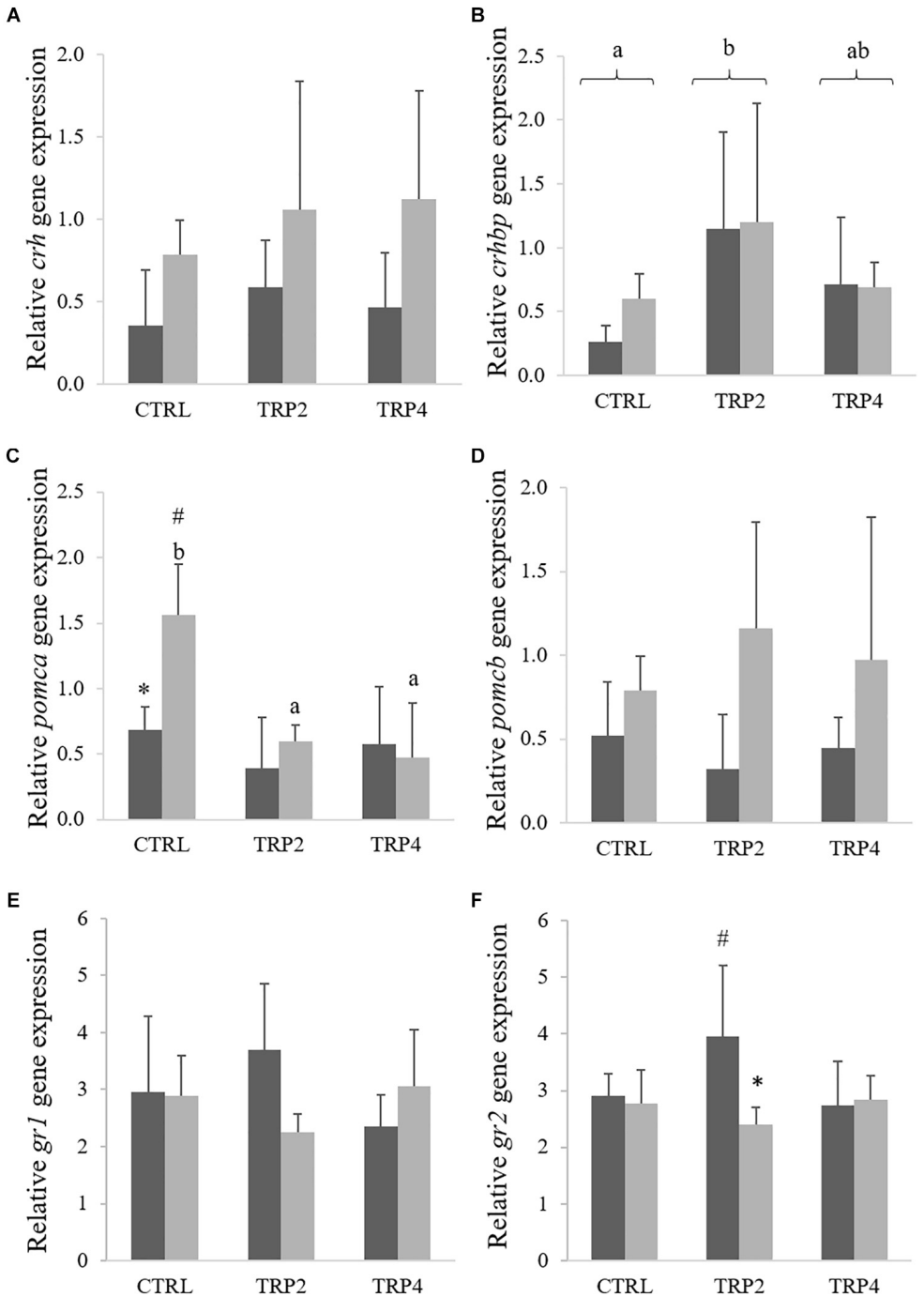

FIGURE 7 | Corticotropin-releasing hormone (*crh*, **A**), corticotropin-releasing hormone-binding protein (*crhbp*, **B**), proopiomelanocortin A (*pomca*, **C**) and B (*pomcb*, **D**), and glucocorticoid receptor 1 (*gr1*, **E**) and 2 (*gr2*, **F**) in Senegalese sole held at low (■) or high (■) stocking densities and fed dietary treatments for 38 days (mean ± SD, $n = 6$). Different symbols indicate significant differences attributed to stocking density. Different letters stand for significant differences between dietary treatments. Two-way ANOVA; Tukey *post hoc* test; $p \leq 0.05$.

translated in lower *pomca* transcripts, plasma cortisol levels, enhanced immune status and disease resistance. In this context, dietary tryptophan surplus as treatment to counteract the negative effects of chronic stress seems to be realistic for Senegalese sole.

In summary, this study suggested that tryptophan dietary treatment could be a promising strategy to counteract chronic stress-induced immunosuppression in Senegalese sole reared at high stocking density. In fish reared at low density, dietary tryptophan supplementation above requirements seems

to be both beneficial and harmful, depending on the level of supplementation, an effect that remains to be unraveled. Furthermore, both nature and duration of stress inflicted in fish might be determinant while evaluating tryptophan potential in modulating the neuroendocrine response.

ETHICS STATEMENT

This study was carried out by trained scientists in full compliance with national rules and following the European Directive 2010/63/EU of the European Parliament and the European Union Council on the protection of animals used for scientific purposes. The protocol was approved by the Animal Welfare Committee of the Interdisciplinary Centre of Marine and Environmental Research and carried out in a registered installation (N16091.UDER).

AUTHOR CONTRIBUTIONS

RA, MM, and JM conducted the main experimental work and performed all humoral and cellular analysis. RA wrote the manuscript under the supervision of BC. JM-S and GM-R analyzed the plasma cortisol and metabolites, and performed the brain and hypophyseal gene expression analyses. HP was responsible for dietary formulation and determined amino acid composition of the experimental diets. AO-T, AA, JMM, and BC conceived the experiments and contributed with both reagents and goods. All authors contributed to and approved the manuscript.

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Stressors Due to Handling Impair Gut Immunity in Meagre (*Argyrosomus regius*): The Compensatory Role of Dietary L-Tryptophan

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In the context of intensive aquaculture, meagre (*Argyrosomus regius*) is one of the most important new aquaculture species in Southern Europe and several studies are focused on the optimization of its culture. Nevertheless, stressors such as handling during transport or culture maintenance may affect the immune system, thereby impairing some immune responses or provoking cellular damage. One strategy that has been used to avert this type of negative stress response is the supplementation of amino acids to improve resistance to stress. In this experiment, meagre (105.0 ± 2.6 g, mean ± standard deviation) juveniles were fed two diets for a period of 7 days, the first a commercial diet supplemented with 1% tryptophan (Trp) and second, the same commercial diet without tryptophan supplementation (control group). The effects of two types of handling stressors (air exposure and confinement/netting) on fish fed both diets was evaluated in terms of gene expression of the selected gut immunity markers, such as (1) innate immune response processes: *c3 complement* (*c3*), *lysozyme* (*lys*), and *cyclooxygenase* (*cox2*); (2) humoral immune response processes: *interferon type 1* (*ifn1*), *mx protein* (*mxp*), *interleukin 1b* (*il-1b*), *tumor necrosis factor 1a* (*tnf1a*), and *interleukin 10* (*il-10*); (3) antimicrobial peptides: *defensin* (*def*), *hepcidin* (*hep*), *piscidin* (*pis*), and a marker for mitochondrial respiration: *glyceraldehyde 3-phosphate dehydrogenase* (*gapdh*). Samples of the anterior intestine were collected at 1 and 6 h post-stress (hps). Results showed that in fish fed 1% Trp, the air exposure resulted in an upregulation of gene expression at 6 hps for *c3*, *lys*, *cox2*, *ifn1*, *mxp*, *il-10* and *gapdh*, and *il-1b* and *pis*. The confinement/netting test for fish fed 1% Trp resulted in an upregulation of *c3* and *mxp* and a downregulation of *cox2*, *ifn1*, *il-1b*, *tnf1a*, *il-10*, *def*, *hep*, and *gapdh* at both post-stress times (1 and 6 hps). According to the present study, dietary supplementation with 1% Trp may be considered as a proper nutritional strategy for improving tolerance and/or alleviating acute response to handling stressors.

Keywords: *Argyrosomus regius*, amino acid, stress, immune response, gene expression, aquaculture, diet supplement

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INTRODUCTION

Interest in animal welfare has increased in the last decade because of ethical or legal concerns, as well as its effect on growth performance and product quality (Ashley, 2007; Noble et al., 2012; Toni et al., 2019). In this context, routine aquaculture procedures often involve fish manipulation outside water for a period of time, thereby causing stress, immunosuppression, general discomfort, or even mortality in the case of extended manipulation and exposure times. Under stressful conditions, the immune system can become depressed accentuating the risk for infectious diseases (Barton, 2002; Kumar et al., 2012; Gisbert et al., 2018; Khansari et al., 2018). Consequently, recent advances in the field of aquafeed formulation have led to the development of new feed strategies, using so-called "functional feeds," to enhance the sustainability of fish production, concerning the growth, survival, and health of the animal by reducing the stress imposed during different handling processes and/or modulating immune functions (Olmos-Soto et al., 2015). In this sense, different nutritional studies have demonstrated that dietary supplementation with essential amino acids, which are precursors to other significant biomolecules, i.e., biogenic amines, heme, or purine and pyrimidine groups (Nelson and Cox, 2013; Andersen et al., 2016), can improve several physiological functions that regulate key metabolic pathways in the organism thus improving survival, development, growth, health, welfare, and reproduction in different vertebrates, including fish species (Li et al., 2009).

Among them, several studies have shown that tryptophan (Trp) enhanced survival and growth by decreasing aggressive behavior, mitigating crowding stress, and improving post-stress recovery in different fish species (Hseu et al., 2003; Höglund et al., 2005; Hoseini et al., 2012; Herrera et al., 2017; Azeredo et al., 2017a). As Trp is a substrate for serotonin and melatonin, it can be expected to have a significant role in establishing behavioral states; furthermore, it is also a substrate for nicotinamide adenine dinucleotide (NAD) synthesis; so, there are major implications for electron transport and energy generation in the cell (Richard et al., 2009). In addition, this essential α-amino acid has also been shown to have significant impacts on immune functions controlled by specific pathways, *via* its role as a structural component of specific transcription factors (Mamane et al., 1999). More recently, meagre (*Argyrosomus regius*) fed with a diet supplemented with Trp modulated its serological immune parameters after stress caused by air exposure (González-Silvera et al., 2018).

With regard to the abovementioned considerations of diet supplementation, the impact of animal handling on stress and systemic immunity has been widely studied in livestock production and aquaculture practices (Barton, 2002; Kumar et al., 2012), whereas limited information is available about the impact of routine animal handling on the immunity of mucosal tissues, and more specifically the intestine (Schokker et al., 2015). The mucosal immunology of the gut is key in not only maintaining a balanced response toward the commensal bacteria that are a normal part of the microbiota but also the prevention of growth of pathogens. Many immune genes are involved in this complex process (Gómez et al., 2013; Ostaff et al., 2013). Moreover, the physiological response to stress is similar between vertebrates and teleost fish where two pathways of response to stress stimulation are activated: the brain-sympathetic-chromaffin cell axis, responsible for a quick release of circulating catecholamine and the hypothalamic-pituitary-interrenal axis (HPI) in charge of the release of an endocrine cascade, which leads to secondary responses that induce changes in cellular metabolites that ultimately alter immune gene expression in fish (Wendelaar-Bonga, 1997; Barton, 2002). However, the gut-associated lymphoid tissue (GALT) in fish is quite different from that of mammals (Foey and Picchietti, 2014). While some general features of the mucosal immunity of the gut is shared with other better studied vertebrates (Gómez et al., 2013), there are unique features such as a class of immunoglobulins, Ig T that replaces the Ig E normally associated to mucosal immunity in higher vertebrates (Zhang et al., 2010; Mutoloki et al., 2015). In addition, chronic stress increases cortisol levels to a point at which the stress impacts negatively on the overall immune responses (Richard et al., 2009). It is well known that the immune system of the gut is responsive to oral vaccination (Mutoloki et al., 2015), but the responsiveness of gut immunity to different stressors is of special relevance. Vaccination protocols require significant handling, and negative consequences from the handling stressors may also influence the effectiveness of the administered vaccine. It is toward this end that we evaluated inclusion of Trp in the diet of fish groups receiving different types of stress and evaluate the concomitant immune response at different levels: (1) innate immune response and (2) endocrine signaling of effector cells. It is in this context that it is interesting to reduce the negative impacts of stress through dietary supplements.

Therefore, the objective of the present study was to evaluate the impact of two common aquaculture handling stressors like air exposition and confinement/netting on local gut immunity in fish fed a diet supplemented with 1% Trp compared to another group of fish fed the same diet, but not supplemented with this essential amino acid. To achieve the abovementioned goal, the following gut immunity markers were used to evaluate the potential stressor-alleviating effects of Trp on local gut immunity: (1) innate immune response processes: *c3 complement* (*c3*), *lysozyme* (*lys*), and *cyclooxygenase* (*cox2*); (2) humoral immune response processes: *interferon type 1* (*ifn1*), *mx protein* (*mxp*), *interleukin 1b* (*il-1b*), *tumor necrosis factor 1a* (*tnf1a*), and *interleukin 10* (*il-10*); (3) antimicrobial peptides: *defensin* (*def*), *hepcidin* (*hep*), *piscidin* (*pis*), and a marker for mitochondrial respiration: *glyceraldehyde 3-phosphate dehydrogenase* (*gapdh*).

MATERIALS AND METHODS

Fish Maintenance and Experimental Diets

Fish used in this trial (n = 150) were obtained from the Olhão Fish Farming Pilot Station (EPPO) of the Portuguese Institute of Atmosphere and the Sea (EPPO-IPMA, Olhão, Portugal) and kept in four re-circulating seawater tanks (600 L; 25 fish each) in the IFAPA Centro Agua del Pino (Cartaya, Spain).

Fish body weight (BW) was 105.0 ± 2.6 g (mean ± standard deviation). Water temperature was maintained at 19.0 ± 1.0°C with a flow rate of 2 m^3 h^{-1} and 33 PSU. The photoperiod was natural (N37°13′4.31″), and fish were fed a commercial pelleted diet (L4-Alterna, Skretting, Burgos, Spain; 47% protein, 20% lipid, 7% ash, 4% cellulose, 1% total phosphorus, and 18 MJ kg^{-1} energy) at a feeding rate of 1% BW day^{-1}. Fish were allowed to acclimatize to experimental conditions for 21 days before the start of the trial.

For the study, the same commercial diet that was used during the acclimation process was also used for the nutritional phase of the study. In particular, pellets were finely ground and then mixed with water (400 ml kg^{-1} dry feed) and Trp (Ref. T0254; Sigma-Aldrich, St. Louis, USA) was added in order to achieve a final concentration of 1% dry weight. Then, the mixture was pelleted into 2 mm diameter and 20–25 cm length strips that were dried at 60°C for 24 h, and manually cut into 2–3 mm size pellets. At the same time, half of the commercial feed was re-pelleted without Trp supplementation, and this batch of feed was used as a control diet. Both control (C) and experimental (T) diets were stored at 4°C until use.

Experimental Design

Fish were fed both diets for 7 days (two tanks per treatment) prior to evaluating the impact of handling stress on local gut immunity. Two different types of stressors were used as previously described by González-Silvera et al. (2018). An air exposition stress treatment (A), in which all fish from each experimental tank were netted and kept out of the water for 3 min, and then returned to their respective experimental tanks; and a confinement/netting stress treatment (N), in which water levels in experimental tanks were reduced (until 20 cm) and then, fish were chased with a net (without air exposure) for 3 min. This procedure was repeated every 10 min for 1 h. In both treatments (A and N), gut samples were collected at 1 and 6 h post-stress (1H and 6H). In order to evaluate whether stressors had an impact on local gut immune function in meagre, and to set up a baseline for comparative studies, samples from each experimental group (C and T = control and tryptophan diets, respectively) were taken prior to stress episodes (B = baseline).

For tissue sampling purposes, fish from both treatments were sacrificed at different sampling points (B, 1H, and 6H) with an overdose of anesthetic (>1 ml L^{-1} 2-phenoxyethanol, Sigma, USA), their digestive system dissected and a portion of the anterior intestine (*ca.* 1 cm in length) was obtained from each fish (n = 6 per sampling point and experimental condition). This portion of the gut included the intestinal mucosa, submucosa, and *muscularis*, since the thin layer of smooth musculature could not be removed from gut samples. Then, tissue samples were fixed at 4°C for 24 h in RNAlater® (Invitrogen, Waltham, USA) and frozen at −80°C until analysis (RNA extraction).

The experiment complied with the Guidelines of the European Union Council (2010/63/EU) and the Spanish Government (RD1201/2005; RD53/2013 and law 32/2007) for the use of laboratory animals. All experimental protocols were approved by the Ethical Committee of the IFAPA (Seville, Spain).

Total RNA Extraction and cDNA Synthesis

Total RNA from the anterior intestine was extracted using the RNeasy Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. RNA concentration and purity were determined by spectrophotometry (NanoDrop2000, Thermo Fisher Scientific, Madrid, Spain) measuring the absorbance at λ = 260 and 280 nm. The integrity of extracted RNA was verified with visualization of the 28S and 18S ribosomal RNA bands by electrophoretic separation on a 1.2% agarose gel. For cDNA synthesis, RNA was reverse transcribed in a 20 µl reaction volume containing 2 µg total RNA using SuperScript™ First-Strand Synthesis System for RT-PCR (Invitrogen, Waltham, USA) with oligo (dT) (0.5 µg $µl^{-1}$), random hexamer primers (50 ng $µl^{-1}$) and 10 mmol L^{-1} dNTP mix, 10× RT buffer [200 mmol L^{-1} Tris-HCl (pH = 8.4), 500 mmol L^{-1} KCl], 25 mmol L^{-1} $MgCl_2$, 0.1 mol L^{-1} DTT, RNaseOUT (40 U $µl^{-1}$), and SuperScript™ II RT, followed by RNAse H treatment. The resulting cDNA was diluted 1:20 in DEPC-treated water, and the prepared dilutions were used for gene expression analysis.

Real-Time qPCR

The qPCR reactions were performed for the six individuals per treatment in duplicate on a CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad, Madrid, Spain). Each reaction (20 µl) contained 10 µl SsoAdvanced™ Universal SYBR® Green Supermix (Life Technologies, Carlsbad, California, USA), 0.50 µl forward primer, 0.50 µl reverse primer, 2 µl of cDNA sample, and 7 µl of RNase/DNase-free water. The real-time qPCR protocol started with 95°C for 3 min, followed by 40 cycles of 15 s at 95°C followed by 30 s at the specific annealing temperature for each primer pair followed by 30 s at 72°C with a final melt curve step to establish standard melt curve profiles for each amplicon. Relative expression was calculated using CFX Manager 3.0 (Bio-Rad, Madrid, Spain) software, with elongation factor 1 alpha (EF1α) and hypoxanthine-guanine phosphoribosyltransferase (HPRT) as endogenous controls, while basal (pre-stress) samples from control and Trp groups were used for normalizing the relative quantification within each experimental diet group. All samples were run in duplicate with a negative control (no RT enzyme) included to confirm absence of genomic DNA contamination and an additional negative control on each plate containing no template cDNA.

Statistical Analysis

To determine differences between gene expression of experimental diets and the time of exposure to stress, all data were checked for normality (Kolmogorov test) and homoscedasticity (Levene test), and analyzed by two-way ANOVA using Tukey as a *post hoc* test, with a significance level of $p < 0.05$ using the statistical software Statistica 7 (StatSoft, Inc., USA). These results were plotted using a surface response analysis for clearer visualization. A heatmap for visualizing different patterns in gene expression was also conducted by means of GraphPad Prism version 7 (GraphPad Software, La Jolla, California, USA). In order to evaluate whether both tested stressors had an impact on local gut immunity in fish fed the C diet, gene expression levels in gut samples from fish prior to stress (CB) and samples

taken 1 h after both stress treatments (CA1H and CN1H) were compared by means of the relative expression software tool (REST) analysis 2009 (v2.0.13). Finally, a principal component analysis (PCA) was used to discriminate gene expression, to identify the treatments associated with 1% Trp supplementation and stressors and to minimize the influence of inter-individual variations.

RESULTS

In the present study, gene expression of several key genes of the innate and humoral immune system was assessed in *A. regius* juveniles fed C and T diets before and after air exposure (A) and confinement/netting stressors (N). Regarding the air exposure stress, an upregulation in gene expression (asterisks) was found between gut samples taken before the stress episode and at 1 hps for *c3*, *lys*, *cox2*, *il-10*, *def*, and *hep* genes in fish fed the C diet (REST analysis, $p < 0.05$). Regarding the results from fish fed the T diet, several genes (*lys*, *cox2*, *def*, and *hep*) did not show statistical differences ($p > 0.05$) in gene expression patterns when fish were stressed by air exposure. However, *gapdh*, *c3 complement*, *ifn1*, *mxp*, *tnf1a*, and *il-10* showed the highest expression due to air exposure ($p < 0.05$) at 1 hps (TA1H) and the lowest expression at 6 hps (TA6H) in fish fed with the 1% Trp supplementation, while *il-1b* and *pis* showed the highest expression due to air exposure ($p < 0.05$) at 6 hps (TA6H) when fish were fed the Trp diet (**Figures 1, 2**).

Netting stress resulted in a high level of gene expression (asterisks) in *lys*, *ifn1*, *tnf1a*, *il-10*, *pis*, *def*, and *hep* genes in fish fed the C diet (**Figure 3**; REST analysis, $p < 0.05$). When considering the results of gene expression from fish fed the T diet, gene expression results for confinement/netting stress showed statistical differences at 1 hps ($p < 0.05$), with the highest and lowest expression values, at TN1H and TN6H, respectively, for *gapdh*, *c3 complement*, *cox2*, and *mxp*. On the other hand, the gene expression of *lys*, *ifn1*, *tnf1a*, *il-10*, *pis*, and *hep* was elevated when meagre was exposed to the confinement/netting stress,

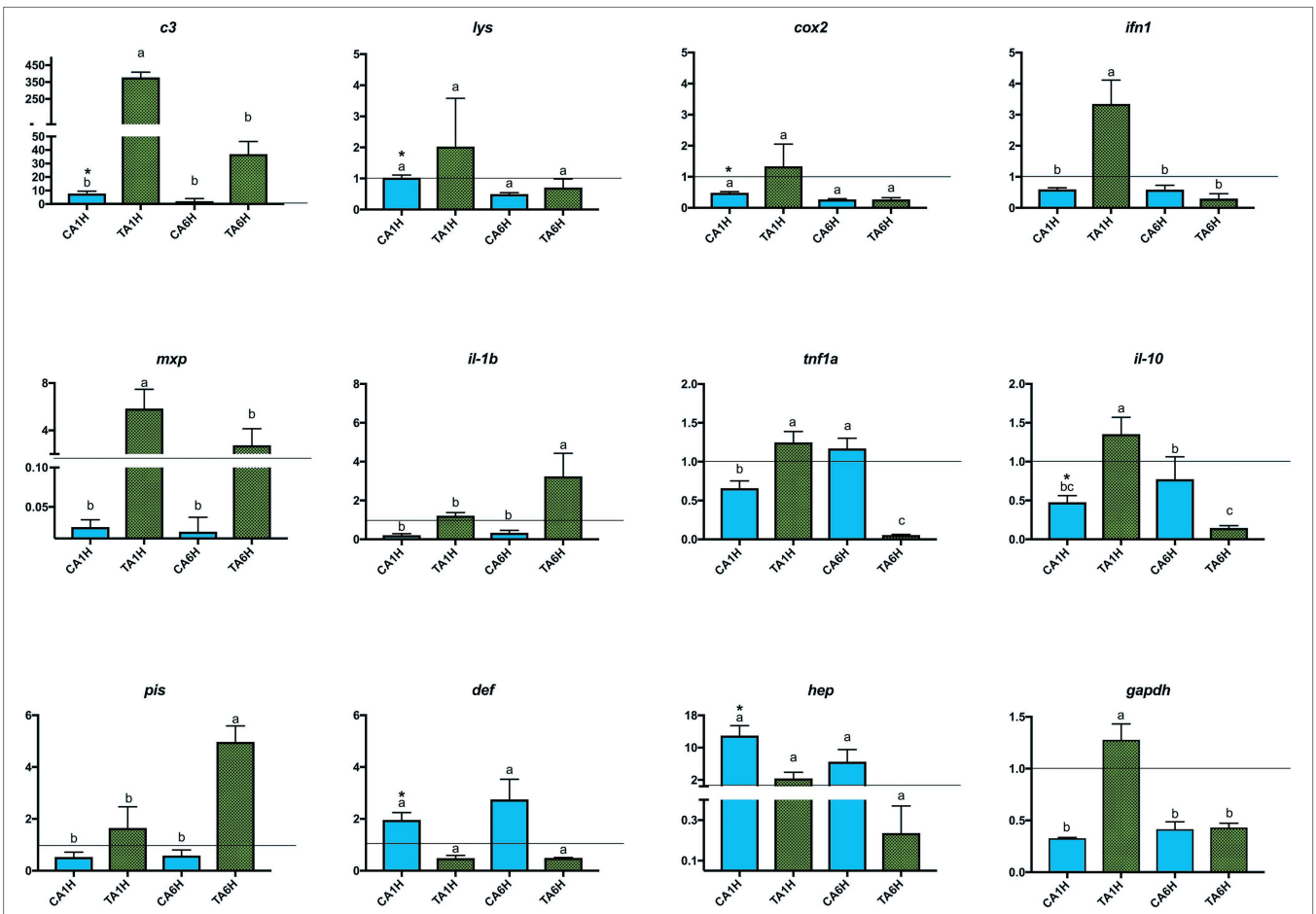

FIGURE 1 | Gene expression levels in gut-associated lymphoid tissue (GALT) after air exposure stressor at 1 and 6 h post-stress. Quantitative PCR of specific mRNA markers of innate immune response processes (*c3 complement protein*, *lysozyme*, *cox2*, *mxp*, *piscidin*, *defensin,* and *hepcidin*), immune cytokines (*ifn*, *il-1b*, *tnf1a*, and *il-10*) and *gapdh,* normalized with elongation factor 1a and hypoxanthine-guanine phosphoribosyltransferase ($n = 6$ per experimental group). Asterisk shows differences between control without stress (resting fish) and control samples after 1 h of air exposure stress test without 1% Trp supplementation (REST analysis), whereas different letters denote statistically significant differences between experimental groups (two-way ANOVA). C, control feed; T, 1% Trp feed; A, air exposure stressor; 1H/6H, 1/6 h post-stress.

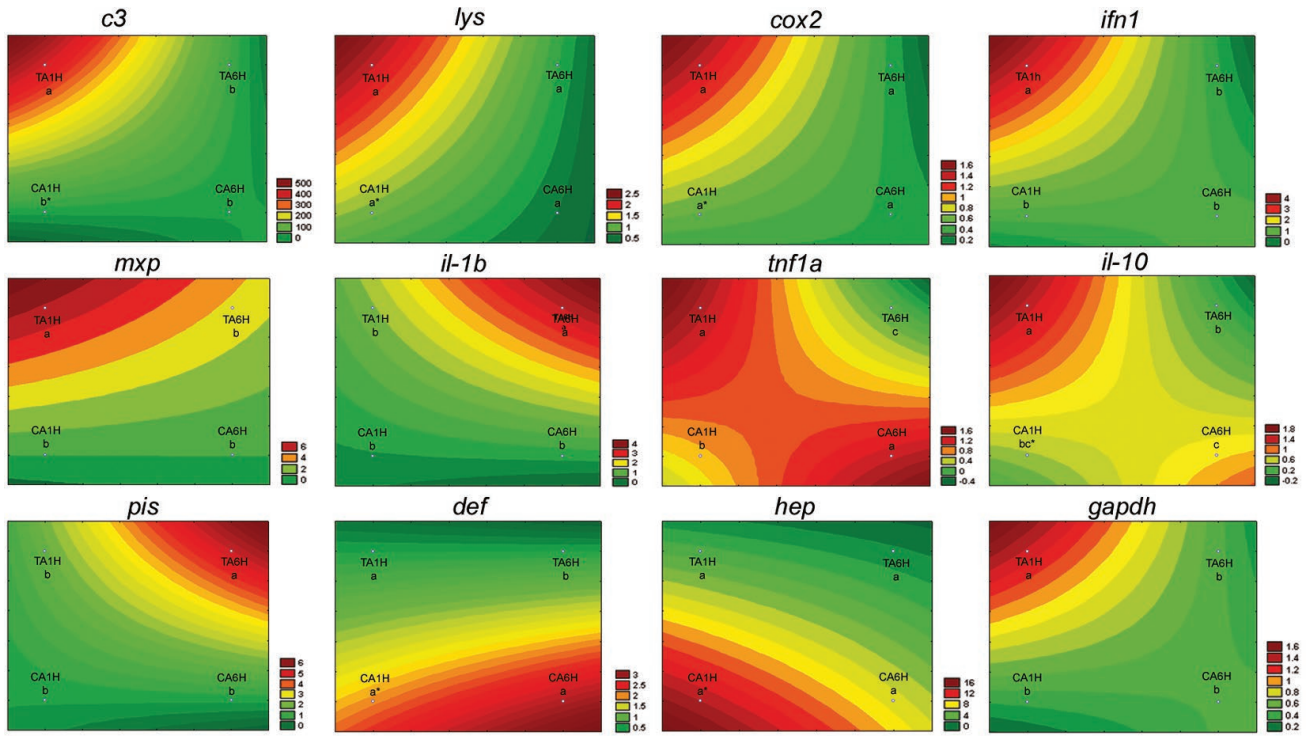

FIGURE 2 | Visualization of gene expression results in gut-associated lymphoid tissue (GALT) after air exposure stressor at 1 and 6 h post-stress by means of response surface analysis. Quantitative PCR of specific mRNA markers of innate immune response processes (*c3 complement protein*, *lysozyme*, *cox2*, *mxp*, *piscidin*, *defensin*, and *hepcidin*), immune cytokines (*ifn*, *il-1b*, *tnf1a*, and *il-10*) and *gapdh,* normalized with elongation factor 1a and hypoxanthine-guanine phosphoribosyltransferase (n = 6 per experimental group). Asterisk shows differences between control without stress (resting fish) and control samples after 1 h of air exposure stress test without 1% Trp supplementation (REST analysis), whereas different letters denote statistically significant differences between experimental groups (two-way ANOVA). C, control feed; T, 1% Trp feed; A, air exposure stressor; 1H/6H, 1/6 h post-stress.

but only with the control diet at 1 hps (CN1H), whereas for the CN6H group (6 hps), *il-1b* showed a reduced expression and *def* a higher expression (**Figure 4**).

Results from the PCA (**Figure 5A**) showed two different groups of genes (*cox2*, *c3 complement*, *mxp*, and *gadph*) that were completely separated from the second one (*tnf1a*, *il-10*, *pis*, *hep*, and *lys*), whereas other genes like *ifn1*, *il-1b*, and *def* did not explain the relations between any dietary groups for either treatment. Results from PCA indicated that the first five components explained 99.5% of the cumulative variance; thus, the genes that better explained the association with the treatments were *gadph* (44.6%), *cox2* (28.7%), *ifn1* (15.4%), *il-1b* (8.6%), and *tnf1a* (2.0%). On the other hand, PCA between treatments also showed two groups: the first group included CA1H, TA6H, TN6H, CA6H, and CN6H, which was separated from the second group (TN1H and TA1H). Finally, fish from the treatment CN1H were totally separated from both groups (**Figure 5B**). According to this analysis, treatments that included 1% Trp (diet T) had a higher number of overexpressed genes from the second group (*tnf1a*, *il-10*, *pis*, *hep*, and *lys*) and they did not show any relation with those genes from the first group (*gadph*, *cox2*, *c3 complement*, and *mxp*). Additionally, fish fed the C diet and exposed to confinement/netting stress (CN1H) had a higher expression of genes from the second group (*tnf1a*, *il-10*, *pis*, *hep*, and *lys*).

Finally, the heatmap analysis clearly showed a higher gene expression pattern in meagre fed the Trp diet for air exposure at 1hps; by contrast, gene expression decreased under netting/confinement exposure after 1 and 6 hps (**Figure 6**).

DISCUSSION

Through commercial aquaculture activities (management protocols, vaccination protocols, crowding, and transportation, among others), fish are exposed to several stressors that threaten their welfare, survival, and the quality of the finished product(s). For these reasons, producers wish to alleviate stress during production to the extent that is possible (Galt et al., 2018). Barring elimination of stress, it would be beneficial to reduce the physiological response in fish to those stressors imposed upon them. As a consequence of external stressors, whether they are abiotic (hypoxia, temperature, osmotic shock, etc.) or biotic (predation, pathogens, handling, and transport by aquaculturists, among others), the stress manifests as a two-stage physiological response. The primary and secondary stress responses are well understood, and it is the release of endocrine factors during the primary response that leads to the secondary stress responses. The neuroendocrine pathways encompassed by the HPI and chromaffin sympathetic axes alter the blood chemistry in profound ways

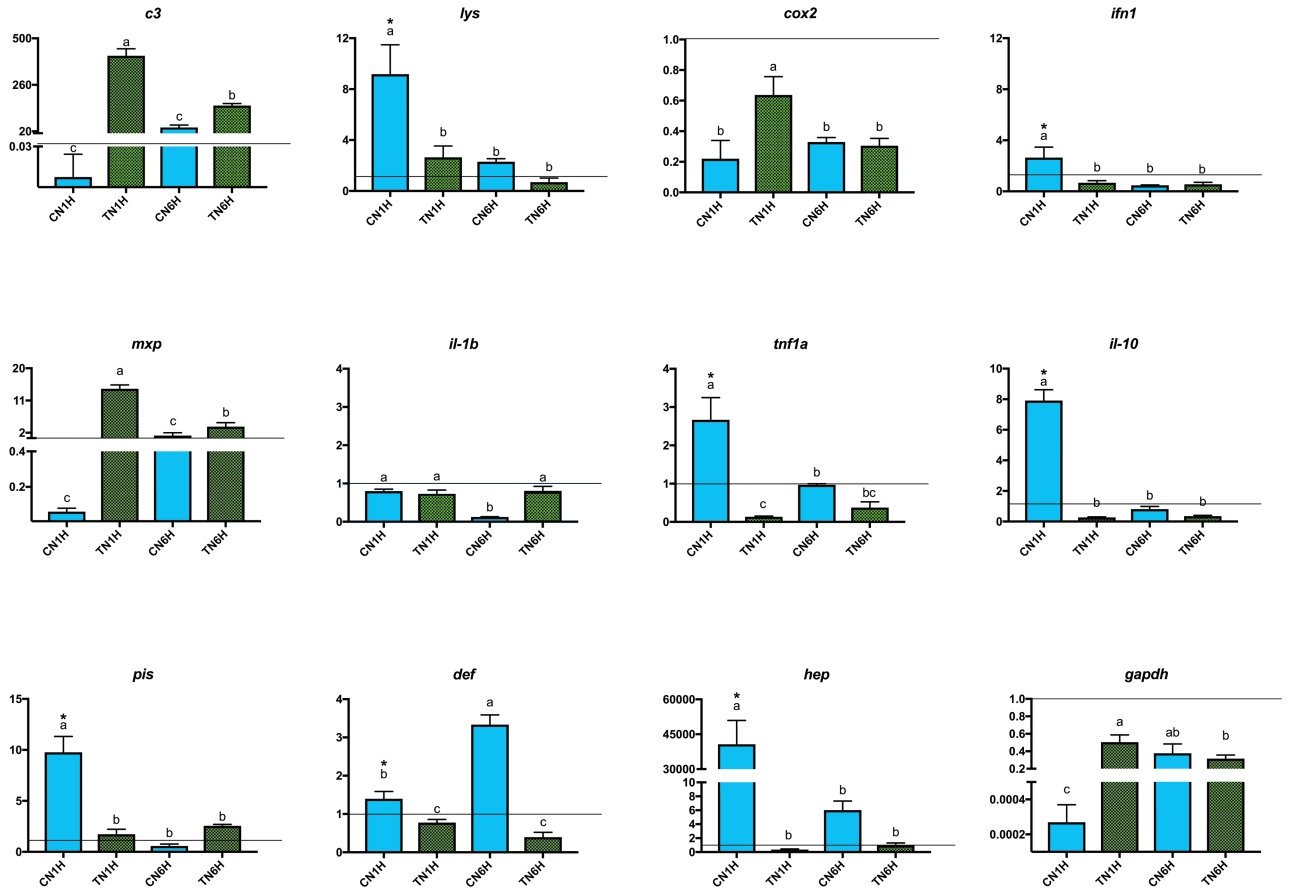

FIGURE 3 | Gene expression levels in gut-associated lymphoid tissue (GALT) after netting-handling stress at 1 and 6 h post-stress. Quantitative PCR of specific mRNA markers of innate immune response processes (*c3 complement protein*, *lysozyme*, *cox2*, *mxp*, *piscidin*, *defensin*, and *hepcidin*), immune cytokines (*ifn*, *il-1b*, *tnf1a*, and *il-10*) and *gapdh*, normalized with elongation factor 1a and hypoxanthine-guanine phosphoribosyltransferase (n = 6 per experimental group). Asterisk shows differences between control without stress (resting fish) and control samples after 1 h of air confinement/netting stress test without 1% Trp supplementation (REST analysis), whereas different letters denote statistically significant differences between experimental groups (two-way ANOVA). C, control feed; T, 1% Trp feed; N, netting stress; 1H/6H, 1h/6 h post-stress.

by the release of corticosteroids (Barton, 2002). This has immediate effects on all aspects of organ functioning *via* hormone receptors and secondary messenger systems, some of which involve specific gene transcription factors. In this study, we focused on the response of the anterior intestine to two different types of abiotic stressors: air exposure and netting/confinement. Since it has already been demonstrated that the stressors tested in the current study resulted in an increase in plasma cortisol levels in the same group of animals (Fernández-Alacid et al., 2019), herein we investigate the cascade of gene expression that follows the primary stress response in the intestine to understand whether the addition of L-tryptophan in the diet could alleviate the impact of handling stressors on the local gut immunity. Thus, the maintenance of a healthy gut, by alleviating some of the stress response, should have concomitant benefits for the overall health of the fish.

Results from the present study revealed that handling stressors affected local gut immunity as seen by changes in the gene expression patterns. In particular, meagre fed the C diet and stressed by air exposure for 3 min showed significant changes in the expression of several genes involved in innate immune responses (*c3*, *lys*, and *cox2*), humoral immune response processes (*il10*), and antimicrobial peptides (*def* and *hep*). In addition, stressing meagre fed the C diet by confinement and netting for 1 h resulted in significant changes in *lys*, *ifn1*, *tnf1a*, *pis*, *def*, and *hep*. These differences in the type and number of differentially expressed genes between both handling stressors may be due to their time of exposure (3 vs. 60 min with regard to air exposition and confinement/netting, respectively), as well as to the type of stressor. For instance, several studies have shown that the transcription factor hypoxia-inducible factor 1, which plays a major role in adaptive responses to hypoxia (Semeza, 1998), is also a mediator of immune functions (Hellwig-Bürgel et al., 2005; Palazón et al., 2014). This hypothesis may be supported by the fact that *cox2* was differentially expressed in fish stressed by air exposure, which agrees with some studies in higher vertebrates (Hellwig-Bürgel et al., 2005). However, the mechanisms by which different handling stressors

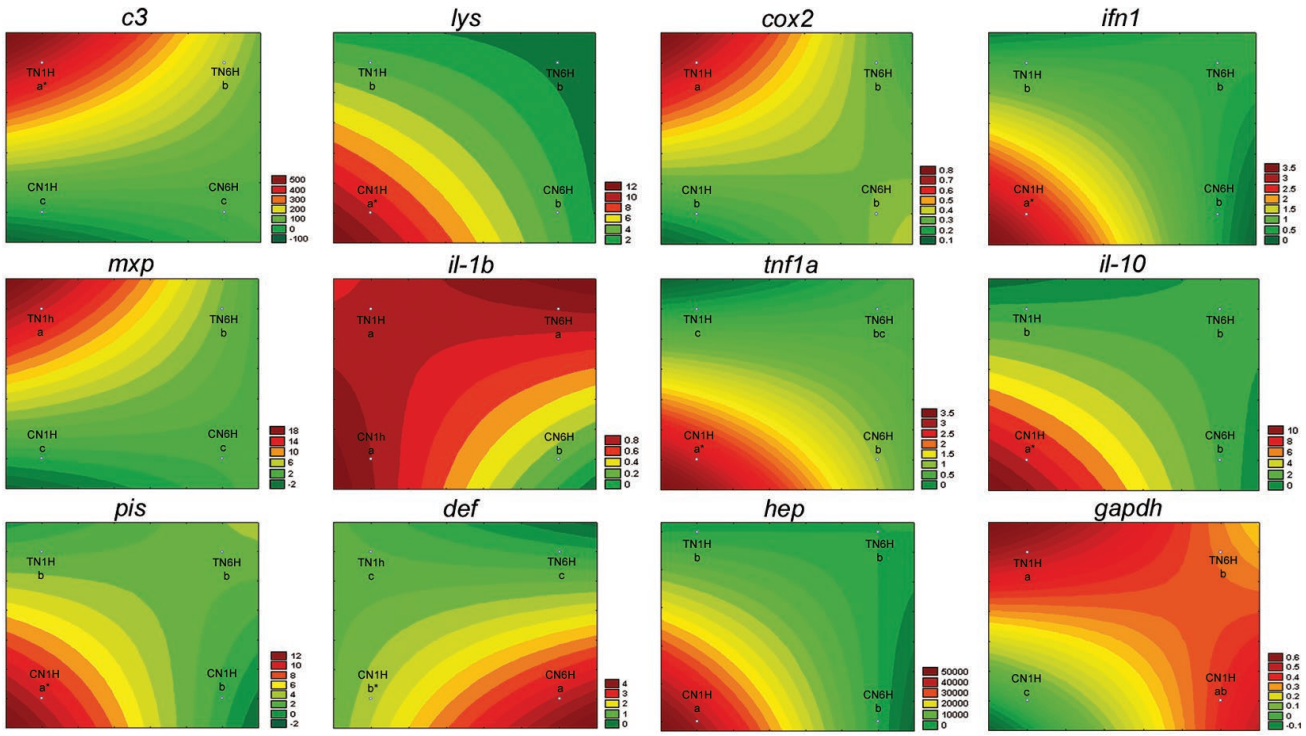

FIGURE 4 | Visualization of gene expression results in gut-associated lymphoid tissue (GALT) after netting-handling stress at 1 and 6 h post-stress by means of response surface analysis. Quantitative PCR of specific mRNA markers of innate immune response processes (*c3 complement protein*, *lysozyme*, *cox2*, *mxp*, *piscidin*, *defensin*, and *hepcidin*), immune cytokines (*ifn*, *il-1b*, *tnf1a*, and *il-10*) and *gapdh,* normalized with elongation factor 1a and hypoxanthine-guanine phosphoribosyltransferase (n = 6 per experimental group). Asterisk shows differences between control without stress (resting fish) and control samples after 1 h of air exposure stress test without 1% Trp supplementation (REST analysis), whereas different letters denote statistically significant differences between experimental groups (two-way ANOVA). C, control feed; T, 1% Trp feed; N, netting stress; 1H/6H, 1/6 h post-stress.

effect the expression of local gut immune markers was not the objective of the present study; thus, this study does not provide conclusive results on this issue and the authors suggest that further research which is more specifically designed to address this issue is warranted.

Tryptophan is the only precursor of serotonin, whose function as a neurotransmitter plays a key role in reducing stress and aggression, acting through the HPI axis to control osmoregulatory, hematological, immunological, and behavioral responses (Wen et al., 2014). Several studies have shown that supplementation of feed with small amounts of Trp reduces stress in various cultivated species and improves their immune response (Andersen et al., 2016). The results obtained in the present study showed that feeding meagre with a diet supplemented with 1% Trp for 7 days resulted in an upregulation of gene expression at 1 hps in the air exposure stress group for most of the gut immune genes evaluated, such as *c3*, *lys*, and *cox2* (innate immune system response), *ifn1*, *mxp*, and *il-10* (humoral immune system), *pis* (antimicrobial peptides), and *gapdh* (mitochondrial respiratory). On the other hand, the confinement/netting stress test exhibited its primary effect in fish fed the T diet only in expression of *c3* and *mxp* genes. As previously suggested, these results indicate that different stressors may have different effects on local gut immunity (Hellwig-Bürgel et al., 2005; Palazón et al., 2014), as well as demonstrating the positive effects of Trp dietary supplementation. These results from supplementation of Trp are due in part to it being provided as a free-tryptophan where, after its absorption, it is converted to serotonin by the enzyme tryptophan hydroxylase. Serotonin is involved in the modulation of the endocrine system and serum cortisol levels (Lim et al., 2013; O'Mahony et al., 2015). It has been estimated that in higher vertebrates, *ca.* 95% of serotonin is found in the gastrointestinal tract (Kim and Camilleri, 2000). Although serotonin was not measured in the brain nor in the gut of meagre in the current study, higher levels of cortisol in fish exposed to both handling stressors (Fernández-Alacid et al., 2019) seemed to validate the former postulate and confirmed our results in meagre fed diets supplemented with Trp. In our study, fish fed the 1% Trp supplementation and treated with the air exposure stress test showed an upregulation of genes involved in innate (*c3*, *lys*, and *cox2*) and humoral (*ifn1*, *mxp*, and *il-10)* responses. However, the gene expression response was not so clear for fish exposed to the confinement/netting test, which may be due to different types of physiological responses to brief hypoxia versus chasing with a net (Hellwig-Bürgel et al., 2005; Palazón et al., 2014). In this context, the importance of the abovementioned genes (*cox2*, *ifn1*, *il-1b*, *tnf1a*, and *gadph*) in the stress response in meagre fed the 1% Trp

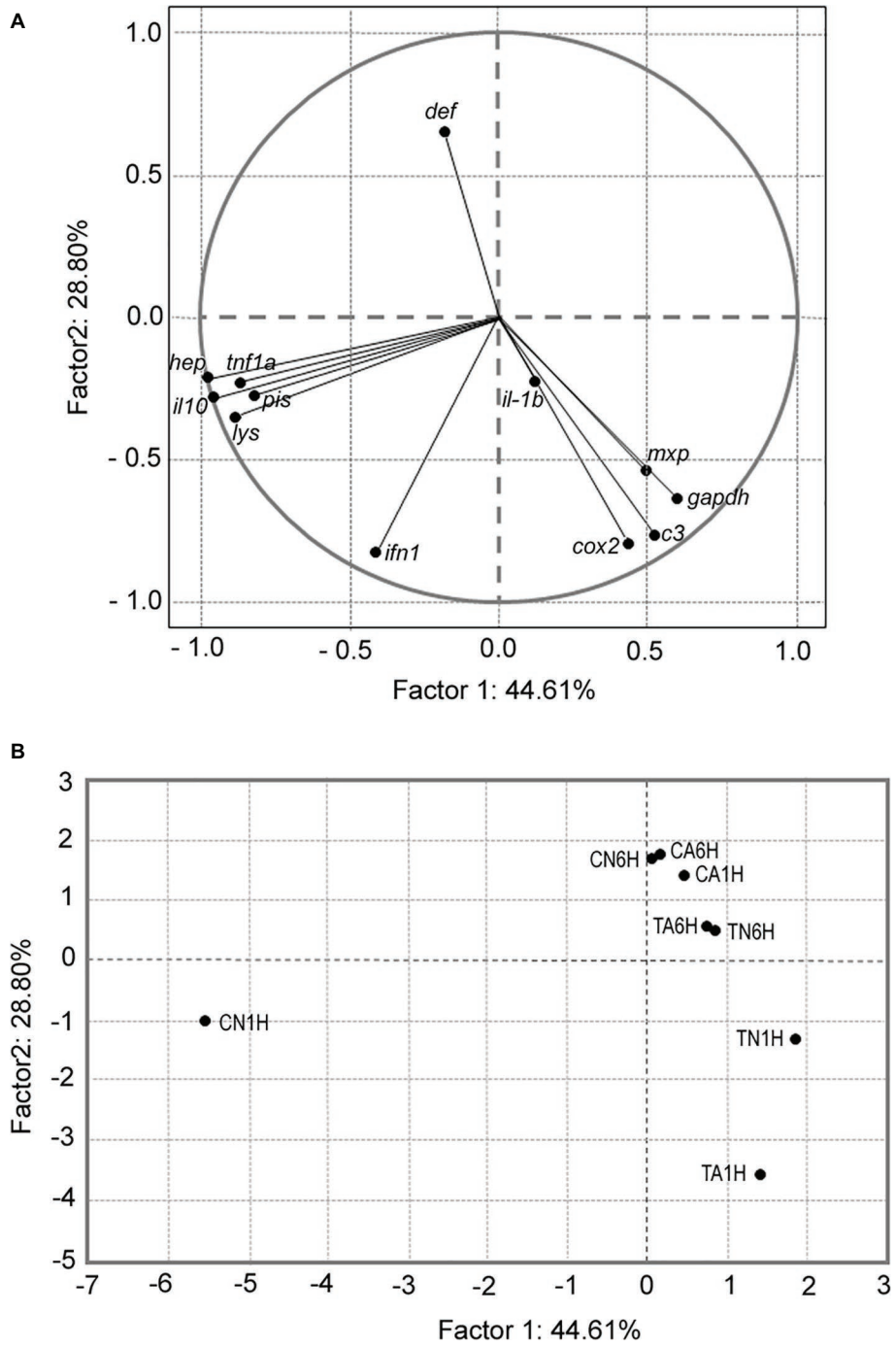

FIGURE 5 | Principal components analysis of **(A)** gene expression according to the treatments set; *gadph*, *cox2*, *ifn1*, *il-1b*, *tnf1a*, *il-10*, *pis*, *def*, *hep, c3 complement*, *lys*, and *mxp*. **(B)** Correlation between treatment combination (tryptophan, time, and stress). Each dot represents the average of three biological replicate analyses of samples in the plot. Factors F1 and F2 used in this plot explain 73.40% of the total variance, which allows confident interpretation of the variation.

diet was corroborated by the PCA results, as those genes explained more than 73% of the total variance observed. Considering the abovementioned results, air exposure and netting/confinement stressors may affect the biological homeostasis of serum Trp due to its involvement in serotonin synthesis (Lim et al., 2013), and consequently, these stressors can induce changes in metabolic, hematological, hydromineral, and structural responses (Barton and Iwama, 1991). Under present experimental condition, fish fed the C diet had elevated levels of circulating corticosteroids (Fernández-Alacid et al., 2019), which may reduce

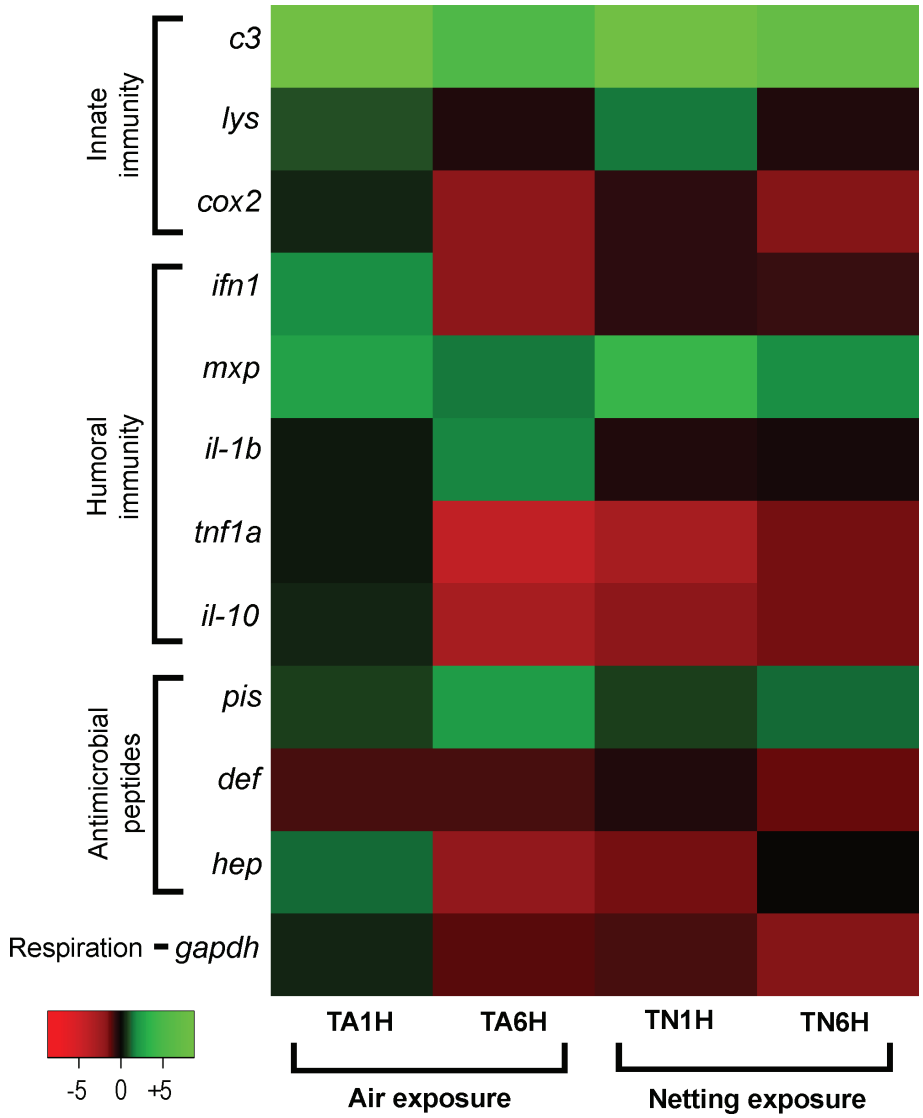

FIGURE 6 | Heatmap image portraying expression patterns in gut-associated lymphoid tissue (GALT) after the air exposure and confinement/netting stress tests. Samples represented are from supplementation with 1% Trp and treated with air exposure (TA1H and TA6H) or confinement/netting exposure (TN1H and TN6H), and Control diet groups exposed to each stress (CA1H and CA6H for air exposure; CN1H and CN6H for netting). Changes in expression of innate immune response genes (*c3 complement protein*, *lysozyme*, *cox2*, *mxp*, *piscidin*, *defensin*, and *hepcidin*), immune cytokines (*ifn*, *il-1b*, *tnf1a*, and *il-10*) and *gapdh*, normalized with elongation factor 1a and hypoxanthine-guanine phosphoribosyltransferase, are represented in color: the green scale indicates downregulated genes and the red scale indicates induced genes.

immuno-competence by influencing lymphocyte numbers and antibody-production capacity, which function in adaptive immune memory against pathogens, and changes in the external environment (Hoseini et al., 2012). Present results revealed that an adequate dietary supplementation of Trp generated an upregulation of genes from the innate and humoral immune systems, which would stimulate the gut-associated lymphoid tissue (GALT) and activate the immune system, providing protection to the host against potential pathogen invasions, as has been widely documented in other fish species (Dinan and Cryan, 2012; Lazado and Caipang, 2014; O'Mahony et al., 2015). In agreement with present results, a study conducted in European sea bass (*Dicentrarchus labrax*) fed diets supplemented with 0.5% of Trp and challenged with an intraperitoneal injection of *Photobacterium damselae* subsp. *Piscicida* demonstrated that immune-related genes were upregulated in the gut (Azeredo et al., 2017b). The abovementioned effect could not be corroborated in *A. regius* because no bacterial challenge was conducted in this study; however, our data on local gut immunity supported the hypothesis of a positive effect of this amino acid on the overall condition and immunity of fish.

In the present study, meagre fed the commercial diet supplemented with 1% Trp showed an upregulation of *c3* in both stress tests. Moreover, innate immune functions performed

by *lys* expression only increased in fish exposed to the air stress after 1 hps (TA1H), while for confinement/netting stress, *lys* expression was not modified by the addition of Trp to the diet. The expression of c3 complement protein functions toward removal of foreign bodies such as viral particles and bacteria by opsonization and the induction of bacterial lysis, thereby enabling targeted foreign organisms to be destroyed by phagocytes (Rooijakkers and van Strijp, 2007). C3 complement also participates in inflammatory reactions (Raftos et al., 2003) and functions as a modifier of acquired immunity (Sunyer et al., 1998; Claire et al., 2002; Lange and Magnadóttir, 2003; Ricklin et al., 2010). While lysozyme plays an important role functioning as a lytic enzyme by hydrolyzing the peptidoglycan component of bacterial cell walls (Davis and Weiser, 2011), thereby limiting bacterial infections and further activating the complement system and phagocytes (Paulsen et al., 2003); thus, these two innate effectors work together to clear infections. Since lysozyme is less specific in its action as it is not influenced by acquired immunity in the same way as the complement system (Claire et al., 2002), it would make sense that this molecular effector is more tightly controlled for preventing unwanted damage to the commensal microbiota of the gut (Gao et al., 2018).

In the air exposure stress test, fish fed with 1% Trp supplementation demonstrated an upregulation of gene expression at 1 hps (TA1H) of *c3*, *lys*, and *cox2*, although for the confinement/netting stress test, there was downregulation of expression for *cox2*. Prostaglandin endoperoxide H synthase, or *cox2*, functions in oxygenating a wide range of fatty acid and fatty esters (Vecchio et al., 2010), leading to the induction of inflammation *via* prostaglandin production (Dyall, 2017). The contrasting response in *cox2* expression with the two different stress exposure treatments may also be related to alternate pathways of gene signaling *via* transcription factors such as hypoxia-inducible factor 1alpha (HIF1a) and HIF1B (Pelster and Egg, 2018).

Regarding humoral immune response processes, our study showed that the supplementation of a commercial diet with 1% Trp increased the expression of *ifn1*, *mxp*, *tnf1a*, and *il-10* at 1 hps when fish were subjected to an air exposure stress test, while their expression decreased at 6 hps. By contrast, the confinement/netting stress test only resulted in an upregulation of *mxp* at 1hps. These results agree with a study in barramundi (*Lates calcarifer*) fed a diet supplemented with 1% Trp where there was high lysozyme activity and an upregulation of *mxp* expression after a challenge with nervous necrosis virus (NNV) (Chi et al., 2018). These results may be explained by the participation of interferon regulatory factors (IRF) in the regulation of the abovementioned proteins. IRFs are a group of transcription factors whose synthesis requires Trp (Mamane et al., 1999), as they contain a characteristic repeat of five tryptophan residues in a DNA-binding domain. Through this DNA-binding domain, IRF family members bind to a similar DNA motif, termed IFN stimulated response element (ISRE). It follows that when the free serum Trp is increased by a dietary supplement, there can be expected an increase in synthesis of IRF with down-stream effects on expression of immune genes such as *ifn1* and *mxp*. Among the molecular effectors examined herein, type I interferon (*ifn1*) can stimulate cellular effectors like natural killer cells and macrophages (Kvamme et al., 2013). In addition, *ifn1* positively regulates the *mxp* antiviral protein, whose coding sequence may be regulated by the presence of ISREs (Das et al., 2008). The increase in *ifn1* expression in meagre subjected to air exposure stress test at 1 hps (TA1H) could be explained by the abovementioned results. However, the confinement/netting stress test did not evidence this same expression pattern, as in the case of *cox2*, and it may be related to differences in transcriptional signaling.

The expression of *il-1b* increased at 6 hps for fish fed 1% Trp under air exposure stress (TA6H); however, when considering the confinement/netting stress test this gene had the lowest expression at 1 and 6 hps (TN1H and TN6H respectively) together with *ifn1*, *il-1b*, *tnf1a*, and *il-10*. Given these results, it seems to be that confinement/netting test is not so aggressive a stressor as the air exposure test. Among the cytokines studied herein, interleukin-1B (*il-1b*) influences the function of the HPI axis by stimulating the secretion of cortisol (Engelsma et al., 2002; Holland and Lambris, 2002). Additionally, *tnfα* is a significant pro-inflammatory cytokine produced by a variety of lymphocytes that promotes apoptosis and macrophage respiratory burst activity and can promote antiviral response *via il-1b* stimulation (Qin et al., 2001). It could be possible that in our study these pro-inflammatory cytokines responded in different ways to each stressor due to feedback or influence of other cytokines not presented in this study. In general, gene expression of the pro-inflammatory cytokines examined seemed to have a tendency to be influenced by the addition of 1% Trp in the diet; however, more research is necessary to elucidate this aspect.

With regard to cytokines, Trp can function as a first step in signaling T cell proliferation and this may be functioning in part due to the ISREs already mentioned (Jenkins et al., 2016). Once activated, different classes of T cells produce additional cytokines that have a variety of effects on a broad range of immune functions, some of which are more anti-inflammatory than pro-inflammatory. Interleukin 10 functions to modulate inflammatory responses. In this study under the influence of air exposure, *il-10* expression was upregulated at 1 hps (TA1H) in fish fed the 1% Trp supplementation, whereas for the same dietary treatment in the confinement/netting stress groups TN1H and TN6H, *il-10* showed a downregulation in expression, which could indicate a potential positive effect of Trp on gut condition. Ordinarily, *il-10* is associated with an anti-inflammatory response. Under a dietary supplement of 1% Trp and confinement/netting stress, depression of *il-10* expression would enable a more robust pro-inflammatory response, potentially benefitting the host. Although under such conditions where *il-10* is downregulated, there may be over-expression of inflammatory cytokines and activation of natural killer cells that could be a source of unwanted tissue damage, which has been detected in *D. labrax* fed 0.52% Trp supplementation (Azeredo et al., 2017c).

In the gut, antimicrobial peptides are expressed somewhat continuously to maintain a balance among commensal versus potentially pathogenic bacteria (Ostaff et al., 2013). This is achieved through what are likely tight controls over their expression to prevent unwanted damage to the host microbiota.

In this study, *pis* was upregulated at 6 hps in meagre fed the Trp diet and subjected to air exposure stress (TA6H), and this tendency was already seen at 1 hps (TA1H), even though it was not statistically significant. By contrast, *pis* expression was not affected by the same dietary treatment when fish were exposed to the confinement/netting stress test. Furthermore, *hep* and *def* expression in fish fed the 1% Trp diet and subjected to the air exposure test had a tendency toward reduced expression values at both sampled times (1 and 6 hps), even though no statistical differences were found due to high inter-individual variability. For fish exposed to the confinement/netting stress and fed the 1% Trp diet, we found *def* to be downregulated at both sampling times (1 and 6 hps). The alterations of expression of AMPs may be a cause of imbalance to the host microbiota (Ostaff et al., 2013), although since the expression of AMPs was different depending on the gene in question and the handling stressor applied no specific conclusions can be drawn about a general effect. Different stressor signals are involved since the response was markedly different for *pis* as compared to *hep* and *def* at 1 hps depending on the type of stressor (air exposure versus confinement/netting). Antimicrobial peptides, such as defensin, piscidin, and hepcidin proteins, are important effector molecules of the innate immune system (Campoverde et al., 2017) that are thought to function by lytic mechanisms (Campagna et al., 2007; Noga et al., 2009; Smith et al., 2010). Depending on the class of AMP considered, they can be effective against viral, fungal, or bacterial pathogens (Lauth et al., 2005; Falco et al., 2008; Casadei et al., 2009; Masso-Silva and Diamond, 2014; Campoverde et al., 2017). Stimulation using pathogen-associated molecular patterns (PAMPs) has been shown to specifically increase their expression under experimental trials (Casadei et al., 2009; Campoverde et al., 2017). Piscidin also has the capacity to modulate gene expression of pro-inflammatory and immune-associated genes (Lin et al., 2016; Yang et al., 2016). The capacity for *pis* to modulate expression of pro-inflammatory immune genes may explain the different patterns of expression seen between *pis* and the other two AMPs analyzed in the current study, or the downregulation of AMPs observed under the air exposure stress conditions applied in this study may be related to the upregulation of *il-10*. Keeping the AMP expression under multiple regulatory controls likely aids in preventing dysbiosis of the gut due to lysis of beneficial members of the host microbiota (Ostaff et al., 2013).

Finally, it is worth mentioning that under confinement/netting stress, the addition of Trp augmented the expression of *gapdh*. This increase in *gapdh* expression could provide a benefit for energy production, and therefore, improvements in all other metabolic events related to the animal's energy balance, which has been demonstrated *in vitro* using intestine cell cultures from European sea bass *D. labrax* supplemented with Trp (Azeredo et al., 2017c). This increment in *gapdh* gene expression was observed in meagre as a response of both stressors after 1 hps in fish fed the Trp diet in comparison to the control group. This gene was initially considered as a housekeeping gene in our analyses, but when checking its level of expression, the authors found that it was differentially regulated depending on the stressor considered, which may be attributed to this enzyme's function in cellular respiration. The transfer of electrons during respiration requires NAD^+ as an electron carrier; Trp is a metabolic precursor and requirement for NAD^+ synthesis. Therefore, Trp availability may be a rate-limiting point in the transfer of electrons and the functioning of respiration. With more availability of Trp, there is the possibility of an increase in the synthesis of NAD^+ (Richard et al., 2009), relieving this rate-limiting step in respiration. The increase in respiration would be made evident by an increase in the production of the key enzymes in this pathway, such as *gapdh*. Considering these results, special attention should be paid when using *gadph* as housekeeping genes in studies were fish may be exposed to potential stressing conditions.

CONCLUSIONS

The two handling stressors tested in this study, which are quite common under standard aquaculture practices, resulted in some distinctly different gene expression responses depending on the type of stress, the dietary supplementation of Trp, and the recovery time (time post-stress). Some of the differences observed in gene expression may be related to positive or negative feedback mechanisms, such as *Il-10* signaling of AMPs. Air exposure may have an important effect distinguishing the two responses. The differences between the two stress responses may be related to specific transcriptional regulatory pathways of local gut immune genes, such as HIFs, that will require further study to confirm. The inclusion of 1% Trp in diets for *A. regius* had clear pronounced effects on physiological functions, as would be expected since this particular supplement implicates the HPI and chromaffin sympathetic axes, which influence vital physiological functions. The stresses imposed in this study were meant to mimic those generated during fish aquaculture practices that are known to increase serum cortisol levels. The overall effect of the Trp supplemented diet on the mucosal immunity of the gut seemed to allow a more balanced response, alleviating some of the more damaging aspects of the stress response, such as elevated AMP expression that could change the microbiota and have long-term negative effects such as outbreaks of opportunistic bacterial pathogens, although this needs to be studied further using specific challenge experiments. Moreover, there was seen a significant increase in the nonspecific innate immune molecules *ifn*, *mxp*, and *c3* that should help to prime the immune system in a nonspecific manner against infections. Lastly, the energy balance would seem likely to benefit from an increase in the expression in *gapdh* that is related to the requirement for tryptophan as a substrate for NAD^+ synthesis. Further work is needed to understand the additional functions of Trp on gut health for the benefit of cultured fish species and confirm the potential mechanisms observed herein.

AUTHOR CONTRIBUTIONS

EG and MH were the scientific leaders and project supervisors, and designed the experiments presented in this paper.

KA designed the gene expression assays, directed the molecular analyses, and revised the English language. IG performed the *in vivo* experiment, and GA-A conducted the molecular analyses. Finally, all authors read and contributed in the preparation and revision of the final manuscript.

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Transport and Recovery of Gilthead Seabream (*Sparus aurata* L.) Sedated With Clove Oil and MS-222: Effects on Stress Axis Regulation and Intermediary Metabolism

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Transport processes between aquaculture facilities activate the stress response in fish. To deal with these situations, the hypothalamic-pituitary-interrenal (HPI) axis releases cortisol, leading to an increase in circulating energy resources to restore homeostasis. However, if the allostatic load generated exceeds fish tolerance limits, stress-related responses will compromise health and welfare of the animals. In this context, anesthetics have arisen as potential agents aiming to reduce negative effects of stress response. Here we assessed the effects of a sedative dose of clove oil (CO) and MS-222 on hallmarks involved in HPI axis regulation and energy management after simulated transport, and further recovery, in gilthead seabream (*Sparus aurata* L.) juveniles. Fish were placed in a mobile setup of water tanks where transport conditions were simulated for 6 h. Sedation doses of either CO (2.5 mg L^{-1}) or MS-222 (5 mg L^{-1}) were added in the water tanks. A control group without anesthetics was also included in the setup. Half of the animals ($n = 12$ per group) were sampled immediately after transport, while remaining animals were allowed to recover for 18 h in clean water tanks and then sampled. Our results showed that the HPI axis response was modified at peripheral level, with differences depending on the anesthetic employed. Head kidney gene-expressions related to cortisol production (*star* and *cyp11b1*) matched concomitantly with increased plasma cortisol levels immediately after transport in CO-sedated fish, but these levels remained constant in MS-222-sedated fish. Differential changes in the energy management of carbohydrates, lipids and amino acids, depending on the anesthetic employed, were also observed. The use of CO stimulated amino acids catabolism, while MS-222-sedated fish tended to consume liver glycogen and mobilize triglycerides. Further studies, including alternative doses of both anestethics, as well as the assessment of time-course HPI activation and longer recovery periods, are necessary to better understand if the use of clove oil and MS-222 is beneficial for *S. aurata* under these circumstances.

Keywords: welfare, *Sparus aurata*, anesthetics, transport, clove oil, MS-222, stress, intermediary metabolism

INTRODUCTION

Welfare of farmed fish is of concern for aquaculture industry due to its effects on production efficiency and related economic benefits (Sneddon et al., 2016). The routine husbandry and management processes in aquaculture facilities, e.g., handling, stocking density or transport, can result in an activation of the stress system and thus compromise animal welfare (Ashley, 2007; Sneddon et al., 2016). Primary stress responses in teleost fish are mediated by the activation of the hypothalamic-sympathetic-chromaffin (HSC) axis and the hypothalamic-pituitary-interrenal (HPI) axis, with the consequent release of catecholamines and cortisol into the blood, respectively (Wendelaar Bonga, 2011; Schreck and Tort, 2016). HSC axis is actually considered the proper stress axis, whereas HPI axis regulation and all the physiological responses derived from cortisol actions aimed at the acclimation of the fish (Koolhaas et al., 2011). Cortisol release starts by up-regulation of the neurohypothalamic factor corticotrophin-releasing hormone (CRH), whose levels are regulated by the CRH-binding protein (CRHBP) (Flik et al., 2006), and also by the thyrotropin-releasing hormone (TRH) (Ruiz-Jarabo et al., 2018). These factors control the release of proopiomelanocortin-derived hormones (POMCs) at hypophyseal level, like the adrenocorticotropic hormone (ACTH), into the bloodstream (Flik et al., 2006). Finally, ACTH stimulates cortisol synthesis in the interrenal cells of the head kidney through the activation of key enzymes such as the steroidogenic acute regulatory protein (StAR) and the 11β-hydroxylase (Cyp11b1) (Montero et al., 2015; Skrzynska et al., 2018).

In teleost fish, cortisol has a dual adaptive role as glucocorticoid and mineralocorticoid hormone, mediated by its interaction with gluco- and mineralo-corticoid receptors (Faught et al., 2016). However, their interactions and the physiological responses derived from different stress situations are still largely unknown (Kiilerich et al., 2018; Tsalafouta et al., 2018). Classically, the mineralocorticoid action of cortisol improves osmoregulatory performance of gills, the main tissue involved in ion-balance in fish, by regulating the activity of several ion pumps such as the Na^+/K^+-ATPase (NKA) (Mancera et al., 2002; Takei and McCormick, 2013) and the permeability of the epithelia (Kelly and Chasiotis, 2011). As a glucocorticoid, cortisol stimulates the intermediary metabolism and mobilizes energy to deal with the stress situation (Mommsen et al., 1999; Vijayan et al., 2010). Cortisol induces plasma hyperglycemia through breakdown of stored hepatic glycogen and increases gluconeogenesis from alternative non-glycolic sources (Barton, 2002), as triglycerides (TAG), free fatty acids (FFA), and amino acids to provide energetic substrates (Faught and Vijayan, 2016). Thus, the metabolic effects of cortisol are key players involved in fish welfare.

In this context, different anesthetics have arisen as a solution to improve animals' welfare during aquaculture procedures. Some of them, including benzocaine, MS-222 (tricaine methanesulfonate), clove oil, metomidate, isoeugenol, 2-phenoxyethanol, quinaldine, or ketamine are widely employed for different fish species (Ross and Ross, 2008; Priborsky and Velisek, 2018). These anesthetics have been proved useful for practical procedures in aquaculture processes such as class-size sorting, weighting, sampling, surgeries, or breeders manipulation (Weber et al., 2009; Toni et al., 2015). Their employment at sedation doses was also proposed during transport to minimize stress and improve welfare (Ashley, 2007; Sneddon et al., 2016). Nevertheless, fish responses to anesthetics are species-specific and dependant on compounds' chemical properties (Readman et al., 2017).

In Europe, the use of anesthetics in fish for human consumption is restrictive, and only MS-222, benzocaine and isoeugenol are approved (European Commission, 2010, 2011), while in the USA only MS-222 (ANADA 200-226) is permitted (FDA, 1997). However, the use of anesthetics during fish transport is still limited (European Council, 2005). In these terms, transport standards of the World Organization for Animal Health are focused on management and water quality, while sedation is not considered necessary to guarantee fish welfare (OIE, 2018). Despite this, a slight sedation during transport has proved to reduce the metabolic rate of fish, and consequently oxygen consumption and generation of waste products, improving water quality management (Zahl et al., 2012; Vanderzwalmen et al., 2018). Although some of these compounds can generate additional effects on the stress response of fish, such as increased cortisol levels, altered oxidative stress status and immune system, or even decreased food intake (Ortuño et al., 2002; Pirhonen and Schreck, 2003; Azambuja et al., 2011), further studies are necessary to evaluate their putative benefits during fish transport processes, as well as after a recovery period.

The purpose of this study was to determine the physiological effects of a sedation dose of MS-222 or clove oil in transported gilthead seabream (*Sparus aurata*) juveniles. Changes in mRNA levels of the main HPI factors in brain, pituitary and head kidney, and plasma cortisol were analyzed. Furthermore, intermediary metabolism in liver was assessed to determine changes in the management of carbohydrates, lipids and amino acids due to anesthetics' addition. MS-222 and clove oil are probably the most widely used anesthetics in the world, but their physiological effects may show differences depending on many factors (reviewed in Priborsky and Velisek, 2018). The results from this study will contribute to elucidate if these compounds improve fish welfare in transport processes related to aquaculture of *S. aurata*.

Abbreviations: MS-222, Tricaine Methanesulfonate; CO, Clove oil; HPI: Hypothalamic-Pituitary-Interrenal axis; CRH, Corticotrophin Releasing Hormone; CRHBP, Corticotrophin Releasing Hormone Binding Protein; TRH, Thyrotropin Releasing Hormone; POMC, Proopiomelanocortin; ACTH, Adenocorticotropic Hormone; StAR, Steroidogenic Acute Regulatory protein; Cyp11b1, 11β-hydroxylase; Nr3c1, glucocorticoid receptor; TAG, Triglycerides; GP, glycogen phosphorylase (EC 2.4.1.1); PK, pyruvate kinase (EC 2.7.1.40); GLDH, glutamate dehydrogenase (EC 1.4.1.2); AST, aspartate aminotransferase (EC 2.6.1.1); ALT, alanine aminotransferase (EC 2.6.1.2); G6PDH, glucose-6-phosphate dehydrogenase (EC 1.1.1.49); HADH, 3-hydroxyacyl-CoA dehydrogenase (EC 1.1.1.35); GPDH, glycerol-3-phosphate dehydrogenase (EC 1.1.1.8); FBP, fructose 1,6-bisphosphatase (EC 3.1.3.11); LDH-o, lactate dehydrogenase-oxidase (EC 1.1.1.27); NKA, Na^+/K^+-ATPase.

MATERIALS AND METHODS

Animal Maintenance

Fish were provided by *Servicios Centrales de Investigación en Cultivos Marinos* (SCI-CM, CASEM, University of Cadiz, Puerto Real, Cádiz, Spain; Spanish Operational Code REGA ES11028000312). Immature gilthead seabream (*S. aurata*) juveniles ($n = 160$, 42.7 ± 6.8 g body mass, mean ± SD) were transferred to the facilities of the Department of Biology at the Faculty of Marine and Environmental Sciences (CASEM, University of Cadiz, Puerto Real, Cádiz, Spain) and acclimated to laboratory conditions for 7 days in four flow-through 500 L tanks (approximate stocking density: 3.5 kg m^{-3}), with seawater in controlled conditions of salinity (38 ppt) and temperature (19 °C), and under natural photoperiod (May 2015; 13:11 h, light:dark; 36°31′45″ N, 6°11′31″ W). Animals were fed twice daily (1 % of tank biomass per day) with commercial pellets for *S. aurata* (Skretting España S.A., Spain). Fish were kept and handled following the guidelines for experimental procedures in animal research from the Ethics and Animal Welfare Committee of the University of Cadiz, according to the Spanish (RD53/2013) and European Union (2010/63/UE) legislation. The Ethical Committee from the Autonomous Andalusian Government approved the experiments (Junta de Andalucía reference number 28-04-15-241).

Characterization of Exposure to Anesthetics

S. aurata juveniles were exposed to different concentrations of clove oil (CO, extracted from cloves of *Eugenia* spp., Sigma-Aldrich C8392) and buffered MS-222 (tricaine methanesulfonate, Sigma-Aldrich E10521) to determine the optimal sedation dose for the simulated transport assay. Fish were transferred individually into 5 L glass-aquaria within different concentrations of CO: 5, 10, 20, 40, 60 mg L^{-1}; or MS-222: 10, 25, 50, 70, 80, 100 mg L^{-1} ($n = 8$). Doses selected were in accordance to other authors for the same species, fish size and water temperature (Mylonas et al., 2005; Vera et al., 2010). Biochemical conditions of the water employed were the same as those in the acclimation tanks. All aquaria were oxygen-saturated (> 90% O_2 saturation) with fine bubbles from an air stone to ensure maximum gas exchange efficiency. To avoid increased metabolic rates and O_2 consumption, animals were fasted for 24 h before anesthetics exposure. Water and anesthetic were freshly renewed for each fish to avoid metabolic waste accumulation in the aquaria and ensure concentration, respectively. Once each fish was placed into the aquarium, induction times to sedation and anesthesia were recorded for each stage. Two different grades, light and deep, were defined also for both sedation and anesthesia stages. The exposure was finished when fish either reached all the stages or after 30 min without changes in the induction progress. Then, fish were transferred to a clean-water aquarium to determine the recovery time. The progressive stages of the induction and recovery, defined in **Supplementary File 1**, were adapted from Ross and Ross (2008).

Simulated Transport and Sampling Procedure

Fish were randomly placed into a mobile setup of nine 15 L aquaria, and distributed in three different experimental groups in triplicate ($n = 72$). Then, *S. aurata* juveniles were transported for 6 h with either 2.5 mg of CO L^{-1} or 5 mg of MS-222 L^{-1}, plus a control group without anesthetics. The selected doses of anesthetics were half of the lowest concentration that induced a light sedation in the previous described characterization protocol (section Characterization of Exposure to Anesthetics in this manuscript; detailed results and explanations are provided in the results section). All aquaria were oxygen-saturated (> 90% O_2 saturation) with air stones. Animals were fasted for 24 h before the assay. To simulate transport conditions, every 20 min the mobile setup of aquaria was displaced for 5 min (mimicking noise and vibrational disturbances due to shaking) followed by 15 min of resting. After 6 h half of the animals were euthanized and sampled ($n = 12$ per experimental group), whereas the remaining fish were transferred to similar clean-water aquaria, allowed to recover for 18 h, and then euthanized and sampled as well. No food was supplied during recovery period (in total, animals were fasted for 48 h maximum). Sampling times were selected according to virtual industrial conditions. Thus, the transport started at 9 a.m. and finished at 5 p.m., when animals were sampled. The final end point (18 h after start of the recovery period) was selected as in this species, complete physiological recovery after an acute stress challenge occurs 6 h later (Skrzynska et al., 2018). Thus, the last sampling point was conducted at the same time of the day as the transport started, aiming at the emulation of a regular workday in aquaculture facilities.

Sampled animals were netted and deeply anesthetized with 2-phenoxyethanol (1 mL L^{-1}, Sigma-Aldrich 77699), to standardize the stunning protocol in all experimental groups. 2-phenoxyethanol was selected due to its low time to induce deep anesthesia (<1 min) without significant effects on the physiological parameters assessed (Toni et al., 2015; Priborsky and Velisek, 2018). Then fork length and body mass were recorded, and blood collected from caudal vessels with ammonium-heparinized syringes (Sigma-Aldrich H6279, 25000 units in 3 mL of saline 0.9 % NaCl). Plasma was separated from cells by centrifugation of blood (3 min, 10000 × *g*, 4°C) and snap frozen in liquid nitrogen. Fish were subsequently euthanized by spinal cord sectioning. Whole brain, pituitary and representative portions from head kidney were collected, placed into tubes with 10-volumes (v/w) of RNA*later*™ (Invitrogen by Thermo Fisher Scientific), held for 24 h at 4°C and then stored at −20°C until total RNA isolation. Liver was also excised, and the portions collected in microtubes were snap frozen in liquid nitrogen and stored at −80°C until metabolites quantification and enzymatic activities assessment. Additionally, the second gill arch on the left side was also excised, adherent blood was removed by blotting with absorbent paper and few branchial filaments collected and placed into microtubes with 100 μL of ice-cold sucrose-EDTA-imidazole (SEI) buffer (150 mM sucrose, 10 mM EDTA, 50 mM imidazole, pH 7.3) for analysis of Na^+/K^+-ATPase (NKA) activity.

Total RNA Isolation and mRNA Levels

Total RNA from brain and head kidney portions were isolated with NucleoSpin® RNA kits (Macherey-Nagel), whereas pituitaries were processed with NucleoSpin® RNA XS kits (Macherey-Nagel). To reduce gDNA contamination, an on-column rDNase digestion was carried out according to kits' specifications (Macherey-Nagel) on each sample. RNA quality was determined using a 2100 Bioanalyzer (Agilent Technologies), and total RNA quantified in a Qubit® 2.0 Fluorometer with Qubit™ RNA BR Assay Kit (Invitrogen by Thermo Fisher Scientific). Only samples with best RNA Integrity Number (RIN $>$ 8.0) were used for real-time PCR (qPCR). Before mRNA expression levels determination, RNA was reverse transcribed using a qSCRIPT™ cDNA Synthesis Kit (Quanta BioSciences™). Samples from brain and head kidney were equaled to 500 ng of RNA in a final volume of 20 μL for the cDNA synthesis, whereas for pituitary 50 ng of RNA were used.

The qPCR was performed by semi-quantitative fluorescence with a CFX Connect™ Real-Time PCR System (Bio-Rad Laboratories) in 96 white wells Hard-Shell® PCR plates covered with Microseal® "B" Seals (Bio-Rad). On each well, the total reaction mixture of 10 μL contained 0.5 μL of each specific reverse and forward primers, 5 μL of PerfeCTa SYBR® Green FastMix™ 2x (Quanta BioSciences™), and 4 μL of cDNA from each sample. The amount of cDNA template was 1 ng for pituitaries, and 10 ng for brain and head kidney samples. Primers for *crh* (GenBank acc. no. KC195964), *crhbp* (acc. no. KC195965), *trh* (acc. no. KC196277), *pomcα1* (acc. no. HM584909), *pomcα2* (acc. no. HM584910), and *star* (acc. no. EF640987) were used as described other works, from our research group, for *S. aurata* (Martos-Sitcha et al., 2014; Toni et al., 2015; Ruiz-Jarabo et al., 2018; Skrzynska et al., 2018). For *11β-hydroxylase* (*cyp11b1*, acc. no. FP332145), and *glucocorticoid receptor* (*nr3c1*, acc. no. DQ486890) genes, primers annealing temperature (50–60 °C), primers concentration (100, 200 and 400 nM), and template concentration (1:10 serial dilutions of cDNA, from 10 ng to 100 fg) were tested to optimize qPCR conditions. Two negative controls: NRT (no reverse transcriptase, 10 ng RNA/reaction), and NTC (no template control, only Tris-HCl 10 mM [pH 8.0], 0.1 mM EDTA) were also added to detect, respectively, possible gDNA contamination or primer-dimer by-products of PCR. Curves with 1:10 serial dilutions of template concentration (from 10 ng of cDNA to 100 fg for brain and head kidney; and from 1 ng to 10 fg for pituitary samples) were performed to test linearity and efficiency of each pair of primers. The reaction protocol for qPCR was conducted in the detection system as follows: 95°C, 10 min; [95°C, 15 s; 60°C, 30 s] × 40 cycles; plus melting curve ([from 60 to 95°C, 0.5°C per read, 70 reads], 95°C, 15 s) to ensure the amplification of a single product and the non-appearance of primer-dimers. Results were normalized to two reference genes, *β-actin* (*actb*, acc. no. X89920) and *elongation factor 1α* (*ef1a*, acc. no. AF184170), due to their low variability in our experimental conditions (M-value $<$ 0.5). Stability M-values of reference genes were 0.1491, 0.1490, and 0.2391 for brain, pituitary and head kidney, respectively. Relative gene expression was performed by ΔΔCq Normalize Expression Gene Study with Bio-Rad CFX Manager™ 3.1 software. Nucleotide primers designs and amplicon sizes, as well as efficiencies and R^2 from serial dilution curves are summarized in **Supplementary File 2**.

Plasma Parameters

Plasma cortisol levels were measured with a commercial Cortisol Enzyme Immunoassay Kit from Arbor Assays™ (NCal™ International Standard Kit, DetectX®, K003). Glucose, lactate and triglyceride levels in plasma were measured using commercial kits from Spinreact (St. Esteve de Bas, Girona, Spain) adapted to 96-well microplates. Plasma total protein concentration was determined with a BCA Protein Assay Kit (Pierce™, Thermo Fisher Scientific, USA, #23225) using BSA as a standard. Total α-amino acid levels were assessed colorimetrically using the ninhydrin method from Moore (1968) adapted to 96-well microplates. Plasma ammonium was measured following the method from Bower and Holm-Hansen (1980) adapted to 96-well microplates, wherein NH_4^+ reacts with salicylate and hypochlorite to form a spectrophotometrically measurable adduct at 650 nm. The method was validated through serial dilutions of *S. aurata* plasma samples with 0.6 % (w/v) NaCl solution at pH 7.2, to ensure plasma ammonia balance. The method resulted in a confident linearity up to 60 μM for 1:500 diluted plasma of *S. aurata*. All assays were performed using a PowerWave™ 340 microplate spectrophotometer (Bio-Tek Instruments, Winooski, VT, USA) using KCjunior™ data analysis software for Microsoft®.

Liver Parameters

Frozen liver samples were finely minced on an ice-cooled Petri dish and divided into two aliquots to assess enzyme activities and metabolite levels. The frozen tissue used for the assay of metabolites was homogenized by ultrasonic disruption in 7.5 volumes ice-cold 0.6 N perchloric acid, neutralized using 1 M KCO_3, centrifuged (30 min, 3220 × *g* and 4°C), and then the supernatant isolated to determine tissue metabolites. Tissue triglycerides levels were determined spectrophotometrically with commercial kits (Spinreact, see before). Tissue glycogen concentration was quantified using the method from Keppler and Decker (1974). Glucose obtained after glycogen breakdown with amyloglucosidase (Sigma-Aldrich A7420) was determined with a commercial kit (Spinreact, see before). Total α-amino acid levels were assessed colorimetrically with the ninhydrin method as described above for plasma samples.

Frozen liver portions for enzymatic activities assays were homogenized by ultrasonic disruption in 10 volumes of ice-cold homogenization buffer (50 mM imidazole, 1 mM 2-mercaptoethanol, 50 mM NaF, 4 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride (PMSF) and 250 mM sucrose; pH 7.5). The homogenate was centrifuged for 30 min at 3220 × *g* and 4°C, and the supernatant stored at −80°C for further analysis. The assays of GP (glycogen phosphorylase, EC 2.4.1.1), PK (pyruvate kinase, EC 2.7.1.40), GLDH (glutamate dehydrogenase, EC 1.4.1.2), AST (aspartate aminotransferase, EC 2.6.1.1), ALT (alanine aminotransferase, EC 2.6.1.2), G6PDH (glucose-6-phosphate dehydrogenase, EC. 1.1.1.49), HADH (3-hydroxyacyl-CoA dehydrogenase, EC 1.1.1.35),

GPDH (glycerol-3-phosphate dehydrogenase, EC 1.1.1.8), FBP (fructose 1,6-bisphosphatase, EC 3.1.3.11), and LDH-o (lactate dehydrogenase-oxidase, EC 1.1.1.27), were performed as previously described for *S. aurata* (Laiz-Carrión et al., 2003; Sangiao-Alvarellos et al., 2005, 2006; Polakof et al., 2006; Vargas-Chacoff et al., 2016). Enzyme activities were determined using a PowerWave™ 340 microplate spectrophotometer (Bio-Tek Instruments, Winooski, VT, USA) using KCjunior™ data analysis software for Microsoft®. Reaction rates of enzymes were determined by changes in absorbance from the reduction of $NAD(P)^+$ to NAD(P)H, measured at 340 nm and 37 °C, during pre-established times (10-15 min). Activities were referenced to protein content of homogenate (U mg $prot^{-1}$). Proteins were assayed in duplicate, as described for plasma samples.

Osmoregulation Parameters

Plasma osmolality was measured with a vapor pressure osmometer (Fiske One-Ten, Fiske, VT, USA) and expressed as mOsm kg^{-1}. Na^+/K^+-ATPase (NKA) activity in gills was determined in 96-well microplates as Mancera et al. (2002) performed for *S. aurata* based on the method described my McCormick (1993). The reactions were allowed to proceed at 25°C and changes in absorbance were monitored during pre-established times (5–10 min). Proteins were assayed in duplicate, as described for plasma samples.

Statistics

One-way ANOVA was performed for each anesthetic and stage during the induction progress (8 fish per treatment). The other variables were evaluated in 12 animals per experimental group and time (four animals per aquarium, in triplicate). Two-way nested ANOVA was performed to evaluate inter-tank variability of replicates for all parameters at each sampling point. Since no significant variability was determined due to triplicates in any of the dependent variables ($p \geq 0.25$), tanks were subsequently treated as a single group. When necessary, data were logarithmically transformed to fulfill the requirements for parametric statistical analysis. Gaussian distribution was confirmed using the Kolmogorov-Smirnov's test. The homogeneity of variances was analyzed by Levene's test. The effect of the anesthetics doses (none, clove oil or MS-222), and sampling times (after stress or after recovery), as well as its possible interaction in parameters assessed was tested using two-way ANOVA, with "anesthetic" and "time" as main factors. Tukey's *post hoc* test was used to identify significantly different groups. A Linear Regression Model was performed to determine correlation between *cyp11b1* and *star* mRNA levels. Statistical significance was accepted at $p < 0.05$. All the results are given as mean ± standard error of the mean (SEM).

RESULTS

Characterization of Exposure to Anesthetics

Induction times to sedation and anesthesia stages in *S. aurata* juveniles showed a dose-dependent variation for both clove oil (CO) and MS-222 (**Table 1**). Animals exposed to CO reached deep anesthesia above 20 mg L^{-1}, requiring almost 9 min at this concentration. However, this stage is reached in <3 min at 40 mg L^{-1} (159 ± 18 s), while taking significantly less time at 60 mg L^{-1} (80 ± 5 s, $p = 0.0001$). Recovery times at both highest concentrations of CO were similar, 359 ± 57 s and 403 ± 26 s respectively ($p = 0.9046$). MS-222 induced deep anesthesia above 70 mg L^{-1}, requiring more than 9 min at this concentration, and < 3 min at 80 mg L^{-1} (214 ± 9 s) and 100 mg L^{-1} (202 ± 15 s), without significant differences between the two highest concentrations ($p = 0.8032$). However, recovery time at 100 mg L^{-1} (525 ± 22 s) was significantly higher ($p = 0.0186$) than at 80 mg L^{-1} (290 ± 19 s). The lowest concentration of CO (5 mg L^{-1}) only induced light sedation after 12 min of exposure, and that of MS-222 (10 mg L^{-1}) only induced light sedation after 14 min of exposure. Consequently, half of those concentrations were selected for the simulated transport, 2.5 mg L^{-1} for CO and 5 mg L^{-1} for MS-222, to ensure that animals did not get a deeper sedation stage during the whole 6 h transport process. No mortality occurred due to anesthetics exposure.

Simulated Transport With Sedation Doses of Anesthetics

S. aurata juveniles transported under sedative conditions, with CO (2.5 mg L^{-1}) or MS-222 (5 mg L^{-1}), presented modifications in the HPI axis regulation as well as in metabolic responses derived from stress system activation. *P-values* determined by two-way ANOVA analysis for each factor of variation, and also the interaction between factors, are showed in **Supplementary File 3**. No mortality occurred, and no changes in behavior or clinical effects due to anesthetics

TABLE 1 | Characterization of the induction progress in *S. aurata* juveniles exposed to different concentrations of clove oil and MS-222.

Anesthetic (mg L^{-1})	Sedation stage		Anesthesia stage		Recovery
	Light	Deep	Light	Deep	
CLOVE OIL					
5	730 ± 159 A	*n.r.*	*n.r.*	*n.r.*	325 ± 31 *B*
10	79 ± 10 B	146 ± 15 A	*n.r.*	*n.r.*	1040 ± 26 A
20	47 ± 3 BC	83 ± 14 B	109 ± 6 A	528 ± 44 A	427 ± 30 B
40	34 ± 5 C	55 ± 9 BC	60 ± 8 B	159 ± 18 B	359 ± 57 B
60	17 ± 1 D	27 ± 2 C	36 ± 2 C	80 ± 5 C	404 ± 26 B
MS-222					
10	854 ± 126 A	*n.r.*	*n.r.*	*n.r.*	173 ± 28 D
25	281 ± 11 B	*n.r.*	*n.r.*	*n.r.*	354 ± 67 BC
50	110 ± 5 C	234 ± 6 A	1014 ± 109 A	*n.r.*	455 ± 52 AB
70	72 ± 2 D	112 ± 4 B	268 ± 19 B	547 ± 56 A	273 ± 20 CD
80	40 ± 4 E	57 ± 4 C	116 ± 15 C	214 ± 9 B	289 ± 19 CD
100	28 ± 4 F	44 ± 4 C	75 ± 17 D	202 ± 15 B	525 ± 22 A

Times (s) of induction to light and deep sedation and anesthesia, as well as recovery are represented as mean ± SEM (n = 8). n.r.: not reached stage. Different sets of capital letters represent statistical differences among doses for each stage of the induction progress (one-way ANOVA followed by a post-hoc Tukey test, p < 0.05).

administration were determined in fish during transport or recovery period.

HPI Axis Regulation

The simulated transport did not affect HPI axis gene expression in brain or pituitary for both tested anesthetics. Brain *crh*, *crhbp* and *trh* as well as pituitary *pomcα1* and *pomcα2* mRNA levels remained constant both after transport and recovery (**Table 2**). In head kidney, *cyp11b1* mRNA levels were reduced in CO-sedated fish ($p = 0.0099$) after transport, while the decrease observed in MS-222-sedated animals was not significant ($p = 0.0847$). On the contrary, after recovery, *cyp11b1* mRNA levels in CO-sedated fish increased significantly ($p = 0.0005$) respect to control animals ($p = 0.0154$); but in MS-222-sedated fish remained constant (**Table 2**). A similar response was determined for *star* (**Table 2**), as shown by a positive linear correlation ($r^2 = 0.9507$; $p < 0.0001$) with *cyp11b1* mRNA levels in head kidney. No changes were determined in the glucocorticoid receptor (*nr3c1*) after transport; but after recovery, contrary to MS-222-sedated animals, in CO-sedated fish its levels were significantly lower ($p = 0.0066$) in comparison to control animals ($p = 0.0183$) (**Table 2**). In addition, *S. aurata* juveniles transported with a sedation dose of CO enhanced plasma cortisol levels respect to control group ($p = 0.0018$); but in fish transported with MS-222 no changes were observed ($p = 0.7291$). After recovery, plasma cortisol levels in CO-sedated fish decreased significantly ($p = 0.0017$), and no differences were determined between treatments (**Figure 1**).

Plasma Parameters

Sedation doses of CO and MS-222 did not alter plasma glycaemia after 6 h of simulated transport. Although plasma levels in non-sedated fish were reduced after the recovery period ($p = 0.0078$), no differences between treatments were determined (**Table 3**). Plasma lactate levels were not affected by anesthetics addition; but after recovery, lactate levels strongly decreased in all sedation conditions ($p < 0.0001$ for all treatments) (**Table 3**). *S. aurata* juveniles sedated with CO did not present significant variations in plasma free amino acids after transport; but levels decreased after recovery period ($p = 0.0172$), and in comparison to non-sedated fish ($p = 0.0009$). In MS-222-sedated fish, no changes in plasma amino acids were determined (**Table 3**). Plasma total proteins and ammonia levels remained

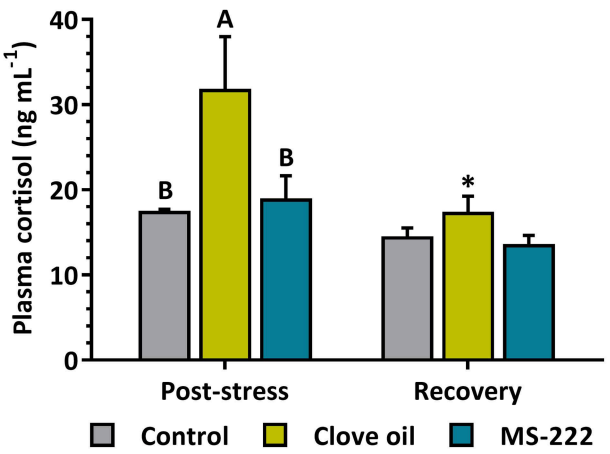

FIGURE 1 | Plasma cortisol (ng mL^{-1}) in *S. aurata* juveniles after 6 h of simulated transport (post-stress), under sedation doses of either: (i) control (none); (ii) clove oil (2.5 mg L^{-1}); or MS-222 (5 mg L^{-1}), and after 18 h of maintenance in clean and quite recovery tanks (recovery). Data are shown as mean ± SEM ($n = 12$). Different capital letters represent statistical differences among sedating conditions after simulated transport; and asterisks represent statistical differences between sampling times for each treatment (two-way ANOVA followed by *post hoc* Tukey test). Statistical significance was accepted at $p < 0.05$.

TABLE 2 | Gene expression levels (relative units) in brain (*crh, crhbp, trh*), pituitary (*pomcα1, pomcα2*) and head kidney (*star, cyp11b1, nr3c1*) in *S. aurata* juveniles after 6 h of simulated transport (post-stress), under sedation doses of either: (i) control (none); (ii) clove oil (2.5 mg L^{-1}); or MS-222 (5 mg L^{-1}), and after 18 h of maintenance in clean and quite recovery tanks (recovery).

	Post-stress			Recovery		
	Control	**Clove oil**	**MS-222**	**Control**	**Clove oil**	**MS-222**
BRAIN						
crh	1.20 ± 0.10	1.05 ± 0.05	1.08 ± 0.10	1.03 ± 0.05	1.20 ± 0.11	0.99 ± 0.07
crhbp	0.81 ± 0.09	0.95 ± 0.01	0.96 ± 0.05	1.09 ± 0.09	1.09 ± 0.07	0.91 ± 0.08
trh	1.11 ± 0.06	0.89 ± 0.07	0.90 ± 0.06	1.08 ± 0.11	0.98 ± 0.05	0.94 ± 0.13
PITUITARY						
pomcα1	0.86 ± 0.13	1.07 ± 0.11	0.94 ± 0.09	1.17 ± 0.13	1.08 ± 0.08	0.95 ± 0.12
pomcα2	0.75 ± 0.29	1.22 ± 0.33	0.90 ± 0.17	1.60 ± 0.32	1.22 ± 0.16	1.38 ± 0.33
HEAD KIDNEY						
star	1.94 ± 0.54 A	0.41 ± 0.09 B	0.78 ± 0.26 B	1.04 ± 0.24 b*	1.94 ± 0.22 a*	0.83 ± 0.15 b
cyp11b1	1.43 ± 0.34 A	0.35 ± 0.09 B	0.73 ± 0.24 AB	0.93 ± 0.18 b	1.81 ± 0.36 a*	0.77 ± 0.18 b
nr3c1	1.05 ± 0.04	1.01 ± 0.06	0.99 ± 0.05	0.98 ± 0.03 a	0.81 ± 0.06 b*	0.91 ± 0.04 ab

Data are shown as mean ± SEM (n = 12). Different capital letters represent statistical differences among sedating conditions after simulated transport; different lowercase letters represent statistical differences among treatments after recovery; and asterisks represent statistical differences between sampling times for each treatment (two-way ANOVA followed by post hoc Tukey test). Statistical significance was accepted at p < 0.05.

TABLE 3 | Plasma glucose (mg dL^{-1}), lactate (mg dL^{-1}), triglycerides (TAG, mg dL^{-1}), total proteins (mg mL^{-1}), amino acids (μmol mL^{-1}), ammonia levels (μmol mL^{-1}), and osmolality (mOsm kg^{-1}) in *S. aurata* juveniles after 6 h of simulated transport (post-stress), under sedation doses of either: (i) control (none); (ii) clove oil (2.5 mg L^{-1}); or MS-222 (5 mg L^{-1}), and after 18 h of maintenance in clean and quite recovery tanks (recovery).

	Post-stress			Recovery		
	Control	Clove oil	MS-222	Control	Clove oil	MS-222
Glucose	71.52 ± 5.44	68.8 ± 4.40	63.09 ± 4.36	55.57 ± 3.44*	57.88 ± 3.36	55.24 ± 1.34
Lactate	12.92 ± 1.61	10.09 ± 1.02	10.35 ± 1.05	2.38 ± 0.27*	3.12 ± 0.69*	3.59 ± 0.28*
TAG	185.0 ± 9.2 A	116.6 ± 12.5 B	131.8 ± 10.4 B	186.8 ± 19.6 b	200.7 ± 21.3 ab*	236.9 ± 16.9 a*
Proteins	30.76 ± 0.72	30.29 ± 0.81	29.92 ± 0.72	30.00 ± 0.34	30.76 ± 0.65	31.69 ± 0.48
Amino acids	11.66 ± 0.68	9.94 ± 0.86	11.25 ± 0.26	11.27 ± 1.21 a	7.10 ± 0.98 b*	9.35 ± 0.87 ab
Ammonia	13.82 ± 2.15	16.98 ± 2.71	19.55 ± 2.97	13.78 ± 2.45	14.05 ± 2.35	21.02 ± 3.22

Data are shown as mean ± SEM (n = 12). Different capital letters represent statistical differences among sedating conditions after simulated transport; different lowercase letters represent statistical differences among treatments after recovery; and asterisks represent statistical differences between sampling times for each treatment (two-way ANOVA followed by post hoc Tukey test). Statistical significance was accepted at $p < 0.05$.

without significant variations in all groups after transport and recovery period (**Table 3**). However, amino acids metabolism was modified by anesthetics addition, showing lower concentrations in both treatments after transport (CO: $p = 0.00456$; MS-222: $p = 0.0273$). The simulated transport also altered intermediary metabolism of lipids. As a result, plasma TAG levels decreased in both sedated groups after transport (CO: $p = 0.0050$; MS-222: $p = 0.0254$) (**Table 3**). After recovery, plasma TAG raised in both treated groups (CO: $p = 0.0013$; MS-222: $p < 0.0001$), but only in MS-222-sedated animals were higher compared to control fish ($p = 0.0231$) (**Table 3**).

Liver Parameters

In liver, glycogen content determined in CO-sedated fish was not significantly reduced after simulated transport, and remained constant after recovery. In MS-222-sedated fish, glycogen content decreased ($p = 0.0474$), but significantly raised up similar to control levels after recovery ($p = 0.0058$) (**Table 4**). On the contrary, no significant variations were determined in hepatic free glucose levels (**Table 4**). After recovery, hepatic amino acids content decreased in non-sedated fish ($p = 0.0383$) to similar levels of CO and MS-222 treatments (**Table 4**). No changes were determined in liver TAG content after the transport (**Table 4**), but higher hepatic TAG were determined in MS-222-sedated fish after recovery ($p = 0.0164$) (**Table 4**).

Liver metabolic enzymes were also modified due to anesthetics addition during transport. GP activity after simulated transport was lower in CO-sedated animals ($p = 0.0023$), but significantly enhanced after recovery ($p < 0.0001$) compared to control animals ($p < 0.0001$). However, no changes were determined for this enzymatic activity in MS-222-sedated fish (**Table 4**). Moreover, hepatic PK activity similarly enhanced in both sedated groups (CO: $p = 0.0013$; MS-222: $p = 0.0101$); but after recovery period, activities were restored as in control animals (CO: $p = 0.0021$; MS-222: $p = 0.0304$) (**Table 4**). LDH-o activity remained constant in all groups after the transport and the recovery period (**Table 4**). Hepatic enzyme activities related to amino acids metabolism were also modified. Thus, although AST activity was similar for all treatments after the stress, in non-sedated fish increased after the recovery period ($p = 0.0459$), being higher compared to CO-sedated animals ($p = 0.0118$). No changes were determined for this enzymatic activity in MS-222-sedated fish (**Table 4**). In the same way, ALT was not altered by transport in any treatment; but after recovery enhanced in all groups (control: $p = 0.0445$; CO: $p < 0.0001$; MS-222: $p = 0.0190$), being higher in CO-sedated fish ($p = 0.0375$) (**Table 4**). GLDH activity in CO-sedated animals was also reduced by transport ($p = 0.0081$); but after recovery, activity in non-sedated fish was reduced ($p = 0.0042$), and resulted similar for both groups ($p = 0.1670$). No changes were determined in GLDH activity in MS-222-sedated fish (**Table 4**). FBP activity remained constant after transport in all treatments; but CO-sedated fish presented higher activity of this enzyme after recovery ($p = 0.0167$) (**Table 4**). Hepatic enzymes from lipid metabolism were also modified due to transport with CO, as shown by the G6PDH decreased activity ($p = 0.0063$), with no changes in those animals sedated with MS-222. Alternatively, after recovery, G6PDH activity in MS-222-sedated fish increased significantly ($p = 0.0217$); being higher compared to non-sedated animals ($p = 0.0006$), in which a significant decrease of this activity was determined ($p = 0.0205$) (**Table 4**). On the other hand, any significant variations were determined in HADH or GPDH activities, neither after transport nor after recovery (**Table 4**).

Osmoregulatory Parameters

Plasma osmolality after 6 h of transport was not affected by anesthetics addition; but after recovery decreased in all the treatments (control: $p = 0.0008$; CO: $p < 0.0001$; MS-222: $p = 0.0079$) (**Figure 2**). On the other hand, gill Na^+/K^+-ATPase activity (NKA) after stress was reduced in animals with the sedation dose of MS-222 ($p = 0.0108$), but not in CO-sedated fish ($p = 0.1973$). After the recovery period, MS-222-sedated animals recovered similar values ($p = 0.0179$) as to control and CO groups (**Figure 2**).

DISCUSSION

Exposure of gilthead seabream (*Sparus aurata*) juveniles to clove oil (CO) and MS-222 evoked a differential response on the induction to anesthesia progress. In consequence, the

TABLE 4 | Glycogen (mg glc g tissue^{-1}), free glucose (mg glc g tissue^{-1}), TAG (mg g tissue^{-1}), amino acids (μmol g tissue^{-1}) and enzymatic activities (GP, PK, LDH-o, FBP, ALT, AST, GLDH, G6PDH, GPDH, HADH; U mg prot^{-1}), in liver of *S. aurata* juveniles after 6 h of simulated transport (post-stress), under sedation doses of either: (i) control (none); (ii) clove oil (2.5 mg L^{-1}); or MS-222 (5 mg L^{-1}), and after 18 h of maintenance in clean and quite recovery tanks (recovery).

	Post-stress			Recovery		
	Control	Clove oil	MS-222	Control	Clove oil	MS-222
METABOLITES						
Glycogen	14.31 ± 1.03 A	12.35 ± 1.72 AB	10.24 ± 0.72 B	13.7 ± 1.62	15.62 ± 1.90	16.06 ± 1.22*
Free glucose	6.77 ± 0.26	6.54 ± 0.49	5.88 ± 0.50	7.15 ± 0.54	7.03 ± 0.42	6.42 ± 0.82
TAG	38.98 ± 3.37	40.31 ± 7.02	39.32 ± 2.86	34.12 ± 3.32 b	35.62 ± 3.54 ab	45.95 ± 3.42 a
Amino acids	166.4 ± 8.5 A	141.3 ± 7.7 AB	137.4 ± 10.8 B	139.3 ± 7.7*	137.7 ± 7.3	134.6 ± 10.6
ENZYMES						
GP	1.66 ± 0.09 A	1.00 ± 0.20 B	1.55 ± 0.16 A	1.46 ± 0.11 b	3.58 ± 0.16 *a**	1.89 ± 0.26 b
PK	1.71 ± 0.12 B	2.52 ± 0.29 A	2.32 ± 0.12 A	1.84 ± 0.11	1.75 ± 0.14*	1.82 ± 0.15*
LDH-o	0.15 ± 0.01	0.17 ± 0.01	0.14 ± 0.02	0.14 ± 0.02	0.17 ± 0.02	0.16 ± 0.02
FBP	1.56 ± 0.08	1.58 ± 0.09	1.72 ± 0.09	1.53 ± 0.07 b	1.78 ± 0.06 a	1.67 ± 0.06 ab
ALT	15.00 ± 0.33	14.46 ± 1.12	14.36 ± 0.26	16.63 ± 0.55 b*	18.39 ± 0.53 *a**	16.44 ± 0.30 b*
AST	14.07 ± 0.72	14.25 ± 0.51	15.58 ± 0.87	16.20 ± 0.47 a*	13.20 ± 1.50 b	15.42 ± 0.30 ab
GLDH	12.37 ± 0.38 A	9.56 ± 1.18 B	12.51 ± 0.40 A	9.30 ± 0.54 ab*	7.89 ± 0.68 b	10.63 ± 0.75 a
G6PDH	5.03 ± 0.56 A	3.71 ± 0.35 B	4.57 ± 0.19 AB	3.89 ± 0.13 b*	4.04 ± 0.28 b	5.66 ± 0.18 a*
GPDH	0.75 ± 0.09	0.87 ± 0.11	0.60 ± 0.04	1.07 ± 0.09*	0.92 ± 0.16	0.99 ± 0.14*
HADH	3.92 ± 0.14 AB	4.33 ± 0.11 A	3.69 ± 0.26 B	4.26 ± 0.10	4.52 ± 0.28	4.21 ± 0.12

Different capital letters represent statistical differences among sedating conditions after simulated transport; different lowercase letters represent statistical differences among treatments after recovery; and asterisks represent statistical differences between sampling times for each treatment (two-way ANOVA followed by post hoc Tukey test). Statistical significance was accepted at $p < 0.05$.

use of these agents during a simulated transport process differentially regulated the HPI axis at peripheral level. Our results also showed that the metabolic rearrangement of carbohydrates, amino acids, and lipids depends on the anesthetic employed.

Prior to transport, fish were exposed to different concentrations of CO and MS-222 to characterize timing and doses to induce deep anesthesia, and also to define the sedative concentration for fish transportation. In teleost fish, the suggested maximum time to induce deep anesthesia by immersion should be <3 min, while the recovery time should take no more than 5 min (Ross and Ross, 2008). The optimal dose of CO determined for *S. aurata* juveniles was 60 mg L^{-1}, in accordance to what was described before for this anesthetic on this species (Mylonas et al., 2005). By employing MS-222, *S. aurata* reached the optimal deep anesthesia at 80 mg L^{-1}, similar to previous results reported (Vera et al., 2010).

Effects on HPI Axis

The simulated transport of *S. aurata* juveniles did not induce changes in HPI axis regulation at central level. Neither CO nor MS-222 induced significant changes in expression of the main neuroendocrine factors in the brain (*crh*, *crhbp*, and *trh*). In the pituitary, a similar lack of response was registered, since no significant changes induced by transport or anesthetics addition were determined in *pomcα1* or *pomcα2* levels. This suggests that, after 6 h of transport, fish were acclimated to stress conditions and primary responses of HPI-axis were not reflected at mRNA levels (Robertson et al., 1988). In the same way, anesthetic-doses selected seemed to be not enough to evoke a decrease in HPI-axis central factors after 6 h of stress. A similar response was obtained in silver catfish (*Rhamdia quelen*) transported for 6 h with sedation doses of an essential oil of *Myrcia silvatica* (Saccol et al., 2018). However, peripherally changes were found in HPI axis. So, the decreased *star* expression for both sedated groups after transport observed in head kidney, suggests a depression of HPI response; but the *star* expression enhancement for CO-sedated fish after the recovery time points out at a differential response of stress axis depending on the anesthetic, which can be assumed by different effects of the main compounds of each anesthetic in the central nervous system (Priborsky and Velisek, 2018). Interestingly, the high correlation found ($r^2 = 0.9507$) between *star* and *cyp11b1* in the experiment highlights the readiness of the interrenal cells, in terms of gene expression, for cortisol synthesis after cholesterol intake into the mitochondria (Hagen et al., 2006; Vijayan et al., 2010). It is described in *S. aurata* that changes in hypothalamic and pituitary factors of the HPI, altogether with plasma cortisol increase, occurs within the first 4 h after an acute stress situation (Skrzynska et al., 2018). This may explain the lack of changes in the present study, as fish had undergone a stressful challenge during transport, while recovered their basal levels of these parameters after 6 h. As plasma cortisol increased in the CO-group after transport (6 h after the start of the experiment), coinciding with lower expression of *cyp11b1* in the head kidney, we can postulate that CO may induce differential time responses to stress. After 18 h of recovery, time enough for this species to recover from an acute-stress situation (Skrzynska et al., 2018), fish shown similar cortisol levels in all treatments. However, CO-sedated

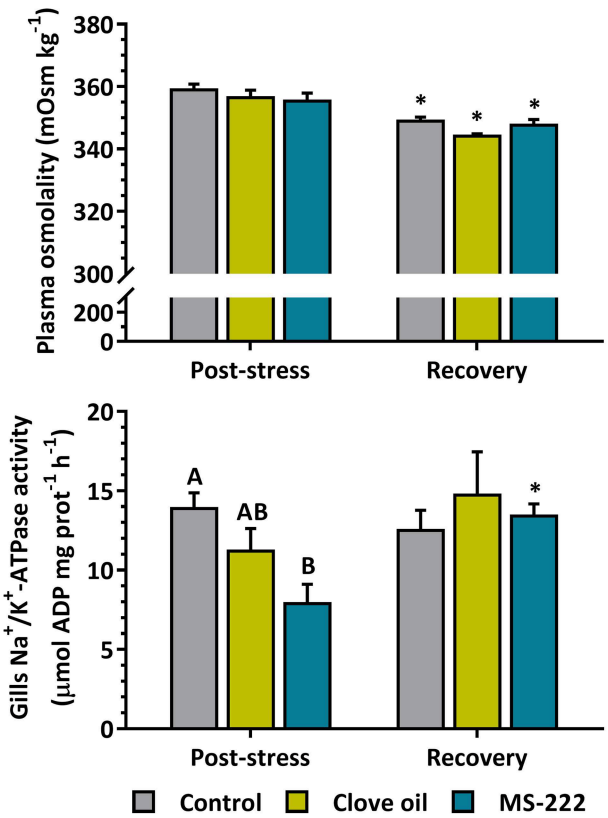

FIGURE 2 | Plasma osmolality (mOsm kg^{-1}) and gill NKA activity (µmol ADP mg prot^{-1} h^{-1}) in *S. aurata* juveniles after 6 h of simulated transport (post-stress), under sedation doses of either: (i) control (none); (ii) clove oil (2.5 mg L^{-1}); or MS-222 (5 mg L^{-1}), and after 18 h of maintenance in clean and quite recovery tanks (recovery). Different capital letters represent statistical differences among treatments after recovery; and asterisks represent statistical differences between sampling times for each treatment (two-way ANOVA followed by *post hoc* Tukey test). Statistical significance was accepted at $p < 0.05$.

fish shown that *cyp11b1* expression was stimulated and *nr3c1* decreased. These results could be associated to an additional stress originated by CO, related to increased cortisol levels as other authors reported for this species (Tort et al., 2002). In our experiment, MS-222-sedated fish did not show variations in neither plasma cortisol nor *cyp11b1* levels, but there were modifications in the intermediary metabolism as Molinero and Gonzalez (1995) reported before.

Effects on Intermediary Metabolism

Metabolic responses derived from the HPI axis activation have been described in other studies related to sedated fish transport (Toni et al., 2013; Zeppenfeld et al., 2014; Vanderzwalmen et al., 2018). In our study, the addition of CO and MS-222 has originated a differential rearrangement of energy sources in *S. aurata* juveniles. Plasma and liver metabolites, as well as hepatic enzyme activities, were modified due to anesthetics after a transport simulation and recovery period. These changes depend not only on the anesthetic employed but also on the metabolic pathways assessed.

Carbohydrates Related Metabolism

Plasma glucose and lactate are considered primary stress biomarkers, and increased levels are associated to HPI axis activation in fish (Faught et al., 2016). Glucose and lactate decrease in control group after recovery supports the effectiveness of transport simulation on the stress axis activation with a clearance of these metabolites from the blood for fuel supply in several energy-demanding tissues, but no differences were associated to the use of anesthetics. Nevertheless, described changes in hepatic carbohydrates reinforce the idea of energy expenditure imposed by transport and anesthetics. Thus, glycogen levels in the control group after transport and recovery are comparatively lower than those described in this species (Sangiao-Alvarellos et al., 2005; Skrzynska et al., 2018). These low levels could be associated to the acute stress and starving conditions in this experiment (Sangiao-Alvarellos et al., 2005; Skrzynska et al., 2018), or even to seasonal variations in hepatic energy stores (Vargas-Chacoff et al., 2009). In this study, PK activity enhancement in both sedated groups after transport is clearly associated to an increase in glycolysis, and thus energy production. Additionally, MS-222-sedated fish presented lower hepatic glycogen levels after transport, related to glucose demand increase due to HPI axis response (Vijayan et al., 2010; López-Patiño et al., 2014). It should be highlighted that hepatic free glucose values did not vary significantly due to transport and remained stable after the recovery. This remarks the potential to maintain glucose homeostasis despite the increase in energy requirements. Similarly, CO-sedated fish restored glycogen and free glucose levels after recovery. Even so, GP activity enhancement determined in recovered CO-treated fish points out at a tissue preparation to further energy demand, and the additional stress evoked by CO addition (Tort et al., 2002).

Amino Acids Related Metabolism

Proteins and amino acids are important sources of non-carbohydrate substrates for gluconeogenesis, and have been described as hepatic energy fuels in fish under different stress situations (Polakof et al., 2006; Vijayan et al., 2010). In this case, the addition of both anesthetics did not change plasma amino acids values after the stress. In the same way, neither AST and ALT activities (key enzymes in amino acids degradation), nor FBP (involved in gluconeogenic pathways) were modified after transport due to anesthetics addition. Despite this, hepatic amino acids content in both sedated groups was reduced; thus, it is difficult to elucidate the effectiveness of the sedation doses in amino acid catabolism. However, CO addition evoked a differential response in recovered fish, probably related to GLDH activity reduction shown after transport. The decrease in plasma amino acids and liver AST activity in CO-sedated fish, concomitantly to liver ALT and FBP activities increases suggest an enhancement of gluconeogenic pathways during recovery (Polakof et al., 2006). Contrarily, use of MS-222 did not affect amino acid metabolism in recovered fish.

Lipids Related Metabolism

Lipid mobilization is also related to HPI activation, mostly due to triglycerides (TAG) allocation as energy substrates for

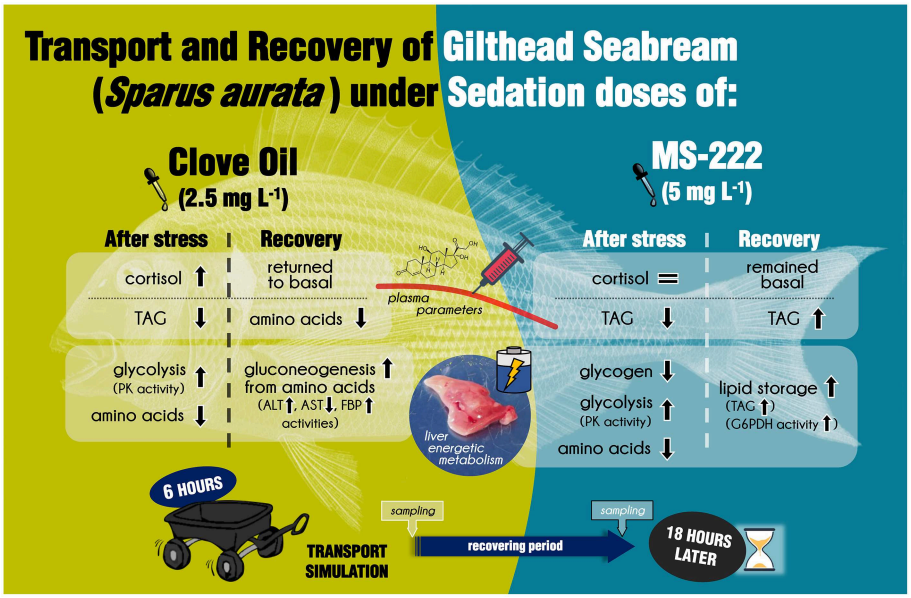

FIGURE 3 | Graphical summary of the main physiological effects determined in *S. aurata* juveniles after simulated transport, and 18 h after its recovery, under sedation doses of either clove oil or MS-222.

gluconeogenesis pathways in fish (Sheridan, 1988; Faught et al., 2016). In this case, CO and MS-222 addition stimulated TAG consumption in *S. aurata* juveniles, as the reduced plasma levels suggested. For both sedated groups, additional lipid requirement is suggested, but no changes in hepatic TAG content were determined. Therefore, exportation to muscle is suggested as energy substrates for this tissue (Vijayan et al., 1991). Additionally, MS-222-sedated fish presented a compensatory accumulation of TAG after the recovery. That is reflected by the increase in plasma and liver content, and also in liver G6PDH activity, which provides reducing equivalents for fatty acid synthesis (Tocher, 2003).

Effects on Osmoregulation

Transport processes have been demonstrated to alter osmoregulation in fish by the increase of ion fluxes through the gills, originating a net loss of ions (mainly sodium and chloride), and also accumulation of total ammonia in plasma (Azambuja et al., 2011; Becker et al., 2016). Slight sedation with essential oil of *Aloysia triphylla* in transported *R. quelen* has been reported to reduce these effects (Zeppenfeld et al., 2014). In this case, neither CO nor MS-222 induced changes in plasma osmolality after transport, but *S. aurata* juveniles presented lower levels in all groups after the recovery. This is in accordance to ion loss described in freshwater species as a consequence to stress exposure (Wendelaar Bonga, 2011), but anesthetics addition did not improve this response. Furthermore, the strong reduction in gill Na^+/K^+-ATPase (NKA) activity determined in MS-222-sedated fish after the transport could be related to increased plasma ammonia levels, but no significant differences were found. Ammonia cations are expelled mainly through the gills in teleost fish by, among others, ATP-consuming ion transporters such as the Na^+/H^+-exchanger (NHE) that can exchange $NH4^+$ instead of H^+ (Quijada-Rodriguez et al., 2017). Altogether, this information suggests that other osmoregulatory mechanisms such as the V-type H^+-ATPase or the co-transporter $Na^+/K^+/2Cl^-$ (NKCC) located not only in the gills, but also in other tissues such the intestine (Gregorio et al., 2013; Ruiz-Jarabo et al., 2017), are in co-operation with the branchial NKA to maintain plasma osmolality levels during transport.

Summary and Future Implications

Literature is enriched with studies focused on the characterization of anesthesia induction with clove oil and MS-222 in fish. Likewise, many physiological changes related to both anesthetics exposure, have been also described for *S. aurata* and other important aquaculture species (reviewed in Priborsky and Velisek, 2018). In addition, the stress response associated to transport is closely linked to duration of the process and water quality, and also to the anesthetic dose if sedation is implemented (Sampaio and Freire, 2016; Vanderzwalmen et al., 2018). In this way, the simulation performed in this work, with the doses selected of CO (2.5 mg L^{-1}) and MS-222 (5 mg L^{-1}), has resulted in a useful tool to address the use of these anesthetics in aquaculture practices. To summarize, the decrease of *star* and *cyp11b1* mRNA levels detected in head kidney for both sedated groups could be associated to a stress response inhibition. Nevertheless, the increase in plasma cortisol of CO-sedated fish, and the rebound in the expression of head kidney factors after recovery, shows a negative effect of this anesthetic. In conclusion, regarding HPI regulation, the dose of MS-222 employed could be enough to avoid and additional increase of cortisol levels; but for CO, the sedation dose evokes and additional response of interrenal cells that remained after recovery. This could be related to the faster excretion of MS-222 in comparison to eugenol (main compound of CO) (Priborsky and Velisek, 2018).

Additionally, side-effects of anesthetics were described in the intermediary metabolism of *S. aurata* juveniles (summarized in **Figure 3**). Both anesthetics, in the doses employed herein, increased liver glycolysis and also reduced hepatic amino acids content and plasma TAG, suggesting energy requirements enhancement during transport procedure. Furthermore, liver gluconeogenesis through amino acids catabolism was enhanced in CO-sedated fish, while in MS-222-sedated animals' recovery was associated to lipids consumption. Again, this could be related to the different metabolic dynamics of both chemicals in the organism. To conclude, neither clove oil nor MS-222 improved the stress response in *S. aurata* juveniles during transport with the information derived from this study. Moreover, side-effects on intermediary metabolism were evoked and reflected even 18 h later. Although the use of these agents is not dismissed, further studies should consider other doses of sedation, and analyze HPI modifications throughout the transportation process. Recovery of fish should also be addressed for prolonged periods, in order to evaluate how side-effects alter feed intake or growth rates of reared species.

ETHICS STATEMENT

Fish were kept and handled following the guidelines for experimental procedures in animal research from the Ethics and Animal Welfare Committee of the University of Cadiz, according to the Spanish (RD53/2013) and European Union (2010/63/UE) legislation. The Ethical Committee from the Autonomous Andalusian Government approved the experiments (Junta de Andalucía reference number 28-04-15-241).

AUTHOR CONTRIBUTIONS

JM, IR-J, and IJ-C conceived and designed the study. MF-C, TDSO, and IJ-C carried out the experimental procedures. IJ-C, MF-C and IR-J analyzed and interpreted the data. JM-S and GM-R supported molecular biology analysis. IJ-C, IR-J, and JM wrote the original draft. All authors have critically reviewed, edited and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2019.00612/full#supplementary-material

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Transport and Recovery of Gilthead Sea Bream (*Sparus aurata* L.) Sedated With Clove Oil and MS222: Effects on Oxidative Stress Status

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The use of anesthesia is a common practice in aquaculture to sedate fish and mitigate handling stress. Although the employ of anesthesia is considered beneficial for fish, as it reduces stress and improves welfare, at the same time it may induce hazardous side-effects. The aim of the present study was to investigate the effects of clove oil (CO) and tricaine methanesulfonate (MS222), two of the most used anesthetics, on several oxidative stress related parameters in gilthead sea bream (*Sparus aurata*), as these types of effects of anesthetics have been seldom investigated. To assess these effects, *S. aurata* juveniles were placed in a setup of mobile water tanks and were transported during 6 h with either 2.5 mg/L CO or 5 mg/L MS222. After transport, half of the fish were sampled, whereas the remaining fish were transferred to tanks without anesthetics where they were allowed to recover for 18 h before sampling. Changes in the expression levels of several target genes related with the antioxidant response and cell-tissue repair were evaluated in the gills, liver and brain. Those transcripts included glutathione peroxidase 1 (*gpx1*), catalase (*cat*), glutathione S-transferase 3 (*gst3*), glutathione reductase (*gr*), superoxide dismutase [Zn] (*sod2*), heat shock protein-70 (*hsp70*), and metallothionein (*mt*). Antioxidant enzymatic activities glutathione S-transferase, GST; catalase, CAT; and glutathione reductase, GR, levels of non-enzymatic antioxidants (non-protein thiols – NPT), and pro-oxidative damage, assessed as lipid peroxidation (LPO), were determined in gills, liver and brain. Acetylcholinesterase activity (AChE) was determined in plasma, gills, brain, muscle and heart as an indicator of neuro-muscular alterations. In plasma, the total antioxidant capacity (TAC) and total oxidative status (TOS) were also measured. Results showed that the use of both anesthetic agents, CO and MS222, interferes with fish antioxidant status. All tested biological matrices displayed alterations in antioxidant endpoints, confirming that these

substances, although minimizing the effects of transport stress, may have long term effects on fish defenses. This result is of high relevance to aquaculture considering that the oxidative stress, may increase the susceptibility to different environmental or biotic stress and different types of pathologies.

Keywords: anesthesia, clove oil, MS222, marine fish, antioxidants

INTRODUCTION

The use of light anesthesia or sedation on fish is a common practice in aquaculture to reduce the stress due to handling, transport, vaccination and blood sampling by depressing fish central and peripheral nervous systems. Tricaine methanesulfonate (MS222) is one of the most widely used anesthetics in fish and it is licensed for use in food fish in the United States and the European Union (FDA, 1997; European Commission, 2010; Velisek et al., 2011; Priborsky and Velisek, 2018). Clove oil (CO) has been proposed as a natural alternative and safer anesthetic, easily available and less expensive than MS222 with demonstrated efficiency in several fish species, including gilthead sea bream, *Sparus aurata* (Tort et al., 2002; Vera et al., 2010; Bodur et al., 2018). This anesthetic is a natural essential oil obtained from the clove tree (*Syzygium aromaticum*) and has as active ingredients eugenol and iso-eugenol (Javahery et al., 2012). This anesthetic has been used in several species, including *S. aurata* (Mylonas et al., 2005; Priborsky and Velisek, 2018). The iso-eugenol is also licensed for use in food fish in the European Union (European Commission, 2011), but not clove oil. The two compounds, MS222 and CO, are administrated by immersion of fish in water with the dissolved anesthetic, and thus mainly absorbed through the gills (Sneddon, 2012), biotransformed by the liver and probably the kidneys and mainly excreted through the gills and, to a lesser extent, through the urine and bile (Carter et al., 2011; Javahery et al., 2012).

The use of anesthesia is considered beneficial for fish as it reduces stress in handling procedures; however, it may induce hazardous side-effects (Ross and Ross, 2008; Priborsky and Velisek, 2018). It was previously demonstrated that MS222 induced an avoidance behavior in *Danio rerio* and *Oryzias latipes* (Readman et al., 2013, 2017), affected the natural cytotoxicity activity of head kidney leucocytes in *S. aurata* (Cuesta et al., 2003), and upregulated gill osmoregulatory genes in *Salmo salar* (Chance et al., 2018). On the other hand, CO has been shown to induce changes in some hematological parameters in *Carassius auratus* (Abdolazizi et al., 2011), enhance several skin mucosal immune parameters in *Oncorhynchus mykiss* (Soltanian et al., 2018) and induce the activity of serum and skin mucus natural hemolytic complement in *S. aurata* (Guardiola et al., 2018). However, the potential side effects of these anesthetic compounds on fish are not completely clear. These effects are species-specific and also dependent on compounds' pharmacodynamics in the organism, which are related to biological factors and water physico-chemical parameters (Ross and Ross, 2008).

The toxicity of xenobiotics often depends on their capacity to increase cellular levels of reactive oxygen species (ROS), which can happen by activation of their synthesis or by an imbalance in the antioxidant defenses (Oliveira et al., 2013). Among antioxidant defenses, non-enzymatic (e.g., non-protein thiols – NPT), as well as enzymatic responses (e.g., glutathione reductase – GR; glutathione *S*-transferases – GST and catalase, CAT) are frequently assessed as biomarkers of xenobiotic mediated oxidative stress (Oliveira et al., 2013). Overwhelmed antioxidant defenses may result in oxidative stress, which may be responsible for pernicious effects like inactivation of enzymes and peroxidative damage (e.g., lipid peroxidation – LPO). Metallothioneins (MT) are known to be over-expressed in organisms from environments with high metal concentrations (Viarengo et al., 1999). Nevertheless, they may also be used as biomarkers of a general stress response to environmental contaminants (Oliveira et al., 2009). It has been demonstrated that the MT gene promoter region contains not only genetic elements responsive to metals, but also sequences thought to be involved in oxidative stress response (Oliveira et al., 2009). Heat shock proteins (HSP) are also markers of cellular stress. Thus, both MT and HSP can also be used as biomarkers of a general cellular stress response to drugs/xenobiotics. In blood plasma, the total antioxidant capacity (TAC), that reflects the combined action of different antioxidants and the total oxidant status (TOS), that measures the different oxidant species present, have been proposed as valuable endpoints to assess the oxidative status in fishes after exposure to chemicals (Teles et al., 2016).

The aim of the present study was to investigate the potential side effects of MS222 and CO on the oxidative status (in gills, plasma, liver, and brain), neurotransmission (in gills, plasma, liver, muscle, and heart), immune function (in plasma) and cellular stress (in gills, liver, and brain) in gilthead sea bream (*S. aurata*) after a simulated transportation and a recovery period. Sedation doses of anesthetics are proposed to reduce stress during transport (Zahl et al., 2012; Vanderzwalmen et al., 2018), and *S. aurata* is an important aquaculture species with a high commercial importance. The selection of tissues was based on functions and anatomic locations. Gills were selected as they are a multifunctional organ with essential osmoregulatory, respiratory and immunologic roles (Evans et al., 2005) in close contact with the external medium and waterborne environmental substances (Oliveira et al., 2009); the liver is a major organ in terms of body weight, with relevant functions in energetics, intermediary metabolism, and storage of substances. The brain is the target organ of anesthetic agent's action in fish, and thus a potential target of side effects that may include oxidative stress; and, finally, blood has an important role in the transfer of substances absorbed through the gills, skin and gut, and provides information on the overall health status of the animal. In this regard, these biological

matrices were considered candidates for the detection of early warning signs of possible harmful effects of MS222 and CO used for the transport of *S. aurata* individuals.

MATERIALS AND METHODS

Fish Husbandry and Experimental Design

Juveniles of *S. aurata* were obtained from Servicios Centrales de Investigación en Cultivos Marinos (SCI-CM, CASEM, University of Cádiz, Puerto Real, Cádiz, Spain; Spanish Operational Code REGA ES11028000312). Immature gilthead sea bream (*S. aurata*) juveniles (n = 72, 42.7 ± 6.8 g body mass, mean ± SD) were transferred to the facilities from the Department of Biology at the Faculty of Marine and Environmental Sciences (CASEM, University of Cádiz, Puerto Real, Cádiz, Spain) and acclimated to laboratory conditions for 7 days in four flow-through 500 L seawater tanks (approximate stocking density: 3.5 kg m^{-3}), under stable conditions of salinity (38), temperature (19°C), and natural photoperiod for this season and latitude (May 2015; 13:11 h, light:dark; 36°31′45″ N, 6°11′31″ W). Animals were fed two times daily (1% of tank biomass per day) with commercial pellets for *S. aurata* (Skretting España S.A., Spain).

For the transport simulation, fish were randomly placed into a mobile setup of nine 15 L aquaria, and distributed in three different experimental groups in triplicate. Then, *S. aurata* juveniles were transported for 6 h with either 2.5 mg of CO L-1 (extracted from cloves of Eugenia spp., Sigma-Aldrich C8392) or 5 mg of MS222 L-1 (Sigma-Aldrich E10521), plus a control group without anesthetics.

Tested concentrations are related to anesthesia induction characterization previously done in gilthead sea bream with both anesthetics. The selected doses of the anesthetics were half of the lowest concentration that induced a light sedation in a previous characterization performed (data not shown). Higher concentrations were not considered in this experimental design to avoid the induction of deeper sedation or anesthesia stages during the transport of fish.

No changes in behavioral or clinical effects due to anesthetics administration were determined during the transport or recovery period. All aquaria were fully oxygen-saturated with air stones. Animals were fasted for 24 h before the assay. To simulate transport conditions, every 20 min the mobile setup of aquaria was displaced for 5 min (mimicking noise and vibrational disturbances due to shaking) followed by 15 min of resting. After 6 h half of the animals were euthanized and sampled (n = 12 per experimental group, n = 4 animals per tank), whereas the remaining fish were transferred to similar clean-water aquaria to allow them to recover for 18 h, and then euthanized and sampled as well. Sampled animals were netted and deeply anesthetized with 2-phenoxyethanol (1 mL L^{-1}, Sigma-Aldrich 77699) for blood collection. 2-phenoxyethanol was selected due to its low time to induce deep anesthesia (less than 1 min) and to standardize the possible side-effects among groups (control, MS222 and CO). After blood collection (in less than 3 min since capture), fish were euthanized by spinal cord sectioning. Plasma was isolated by centrifugation (3 min, 10000 × g, 4°C), and frozen in liquid nitrogen. Gills, liver and brain were excised, flash frozen in liquid nitrogen and posteriorly used for gene expression analysis and biochemical endpoints assessment. The experiment complied with the Guiding Principles for Biomedical Research Involving Animals (EU2010/63), the guidelines of the Spanish laws (law 32/2007 and RD 53/2013), and authorized by the Ethical Committee of the Universidad de Cádiz (Spain) for the use of laboratory animals and the Ethical Committee from the Andalusian Government (Junta de Andalucía reference number 28-04-15-241).

RNA Isolation, Retrotranscription, and Real-Time Quantitative PCR

Total RNA was extracted from the selected tissues of control and exposed fish using Tri Reagent® (Sigma-Aldrich) and following manufacturer's recommendations. RNA quantification was done using a NanoDrop Spectrophotometer (Thermo Fisher Scientific, United States) and RNA quality checked with Experion, using the Experion Standard Sens RNA chip (Bio-Rad Laboratories, United States). Reverse transcription was performed using 1 μg of the total RNA using the iScript™ cDNA synthesis kit (Bio-Rad, United States) according to the manufacturer's instructions. Efficiency of the amplification was determined for each primer pair using serial fivefold dilutions of pooled cDNA and calculated as $E = 10^{(-1/s)}$, where s is the slope generated from the serial dilutions. RT-qPCR was run in a Bio-Rad CFX384 Real-Time PCR Detection System (Bio-Rad, United States). Reactions were done using iTaqTM Universal SYBR® Green Supermix (Bio-Rad, United States) according to the manufacturer's instructions. Briefly, 1 cycle at 95°C for 5 min, 40 cycles at 95°C for 30 s, 60°C for 30 s, and 72°C for 30 s were run; samples were performed in triplicates. Expression data, obtained from three independent biological replicates, were used to calculate the threshold cycle (Ct) value. After checking for primers efficiency, RT-qPCR analysis of all the individual samples was determined following the same protocol described above. NormFinder was used to identify the most appropriate housekeeping gene among the tested 3: elongation factor-1α (*ef1α*), α-tubulin (*tub*), and β-actin (*act*). Stability values of the individual candidate housekeeping genes were: 0.018 for ef1α, 0.022 for act, and 0.028 for tub in the gills; 0.017 for *ef1α* and *act*, and 0.018 for tub in the liver; 0.022 for tub, 0.038 for act, and 0.052 for ef1α in the brain. The best combination of two-genes was act + tub with a stability value of 0.017 and 0.010 for the gills ant the liver, respectively. For the brain, the best combination of two-genes was ef1α + act with a stability value of 0.029. The best combination of two genes for each organ was used for normalization. Normalized gene expression calculated with the ΔΔCt method (Livak and Schmittgen, 2001). Primers information is given in **Table 1**.

Sample Preparation for Biochemical Analyses

The samples of tissues for biochemical analysis were homogenized in potassium phosphate buffer (0.1 M, pH 7.4). Aliquots of the homogenate were separated for non-protein thiols (NPT), lipid peroxidation (LPO) assessment and

TABLE 1 | Sequences and efficiencies of primers used for quantitative real-time PCR analysis.

Gene name	Acronym	GenBank ID	Forward	Reverse
Elongation factor-1α	*ef1α*	AF184170	CCCGCCTCTGTTGCCTTCG	CAGCAGTGTGGTTCCGTTAGC
α-Tubulin	*tub*	AY326430	AAGATGTGAACTCCGCCATC	CTGGTAGTTGATGCCCACCT
β-Actin	*actin*	X89920	TCCTGCGGAATCCATGAGA	GACGTCGCACTTCATGATGCT
Glyceraldehyde 3-phosphate dehydrogenase	*gapdh*	DQ641630	TGCCCAGTACGTTGTTGAGTCCAC	CAGACCCTCAATGATGCCGAAGTT
Catalase	*cat*	JQ308823	TGGTCGAGAACTTGAAGGCTGTC	AGGACGCAGAAATGGCAGAGG
Superoxide dismutase (Mn)	*sod2*	JQ308833	CCTGACCTGACCTACGACTATGG	AGTGCCTCCTGATAT TTCTCCTCTG
Glutathione peroxidase 1	*gpx1*	DQ524992	GAAGGTGGATGTGAATGGAAAAGATG	CTGACGGGACTCCAAATGATGG
Glutathione reductase	*gr*	AJ937873	TGTTCAGCCACCCACCCATCGG	GCGTGATACATCGGAGTGAATGAAGTCTTG
Glutathione-S-transferase 3	*gst3*	JQ308828	CCAGATGATCAGTACGTGAAGACCGTC	CTGCTGATGTGAGGAATGTACCGTAAC
Metallothionein	*mt*	U93206	CTCTAAGACTGGAACCTG	GGGCAGCATGAGCAGCAG
Heat shock protein 70	*hsp70*	EU805481	AATGTTCTGCGCATCATCAA	GCCTCCACCAAGATCAAAGA

post-mitochondrial fraction (PMS) isolation (30 min at 15,000 *g*, 4°C).

Biochemical Analyses

The protein content of the homogenates and PMS fractions was determined. Protein content was determined by the Bradford (1976) method adapted to microplate. Prior to enzymatic analysis, the content of protein in the samples was normalized to 0.9 mg/mL. After enzymatic determinations, the protein concentration in each sample was quantified again and the measured value used to express the enzymatic activities per protein (Oliveira et al., 2012). Cholinesterase activity was determined in the gills, brain and heart was determined according to Ellman et al.'s (1961) method adapted to microplate, at 25°C. In the blood, AChE was determined according to Tecles and Cerón (2001) adapted for an automated analyzer (Olympus AU600; Olympus Diagnostica GmbH, Hamburg, Germany). The results were expressed as nmol of thiocholine formed per minute per mg of protein. Non-protein thiols content was estimated generally following the procedure described by Oliveira et al. (2010). Results were expressed as millimoles per milligram of protein. Catalase activity was determined by the method of Claiborne (1985) adapted to microplate. Absorbance was recorded spectrophotometrically at 240 nm (25°C) and enzyme activity expressed as micromoles H_2O_2 consumed per minute per milligram protein. Glutathione *S*-transferase activity was measured according to Habig et al. (1974) adapted to microplate. Absorbance was recorded at 340 nm (25°C) and expressed as nmol CDNB conjugate formed per minute per mg of protein. Glutathione reductase activity was assayed by the method of Carlberg and Mannervik (1975) adapted to microplate. Enzyme activity was quantified by measuring NADPH decrease at 340 nm (25°C) and expressed as nmol NADPH oxidized/min/mg protein. TAC was assessed in the plasma as described by Erel (2004), by measuring the 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonate) decolorization by antioxidants at 660 nm. The activity was expressed as mmol/L. TOS was measured in plasma as described by Erel (2005). The method is based on the reaction that the ferric ion makes a colored complex with xylenol orange in an acidic medium. The color intensity, which was measured spectrophotometrically at 560 nm (Olympus Diagnostica, GmbH) using 800 nm as the reference, is related to the total amount of oxidant molecules present in the sample. The assay was calibrated with hydrogen peroxide and the results were expressed in terms of micromole hydrogen peroxide equivalent per liter ($\mu molH_2O_2Equiv/L$). Lipid peroxidation levels were determined according to the protocols described by Oliveira et al. (2009). Absorbance was measured at 535 nm and LPO was expressed as nmol of thiobarbituric acid reactive substances (TBARS) formed per mg of protein.

Data and Statistical Analysis

Statistical analysis was done using the IBM SPSS Statistics 22 software package. One-way ANOVA was performed followed by the Dunnett's test to signal significant differences between treated and control groups. Statistically significant differences between treated groups and their respective controls groups are denoted with asterisks. One asterisk indicates $p < 0.05$, two asterisks indicate $p < 0.001$.

RESULTS

Molecular Responses

In gills, organisms treated with MS222 increased *gpx1* and *gst3* mRNA levels significantly after 6 h transportation, with mRNA levels returning to control values after 18 h recovery (**Figure 1**). However, *gr*, *cat*, and *sod2* expression levels significantly decreased after transportation and recovery period. Gill *mt* and *hsp70* mRNA levels were unaltered (data not shown). For CO treated fish, the gill's mRNA levels of *gst3* enhanced after transportation, returning to control values after recovery. However, the rest of transcripts assessed (*gpx1*, *cat*, *gr*, *mt*, and *hsp70*) didn't present any change.

Fish exposed to MS222 enhanced hepatic *sod2* and *gr* mRNA levels after 6 h of transportation, returning to control levels after 18 h of recovery; while no alterations were observed in mRNA levels of *gpx1* (**Figure 1**) as well as *cat* and *gst3* (data not shown). Respect to the assessed cellular stress indicators, although no significant effects were detected after transportation,

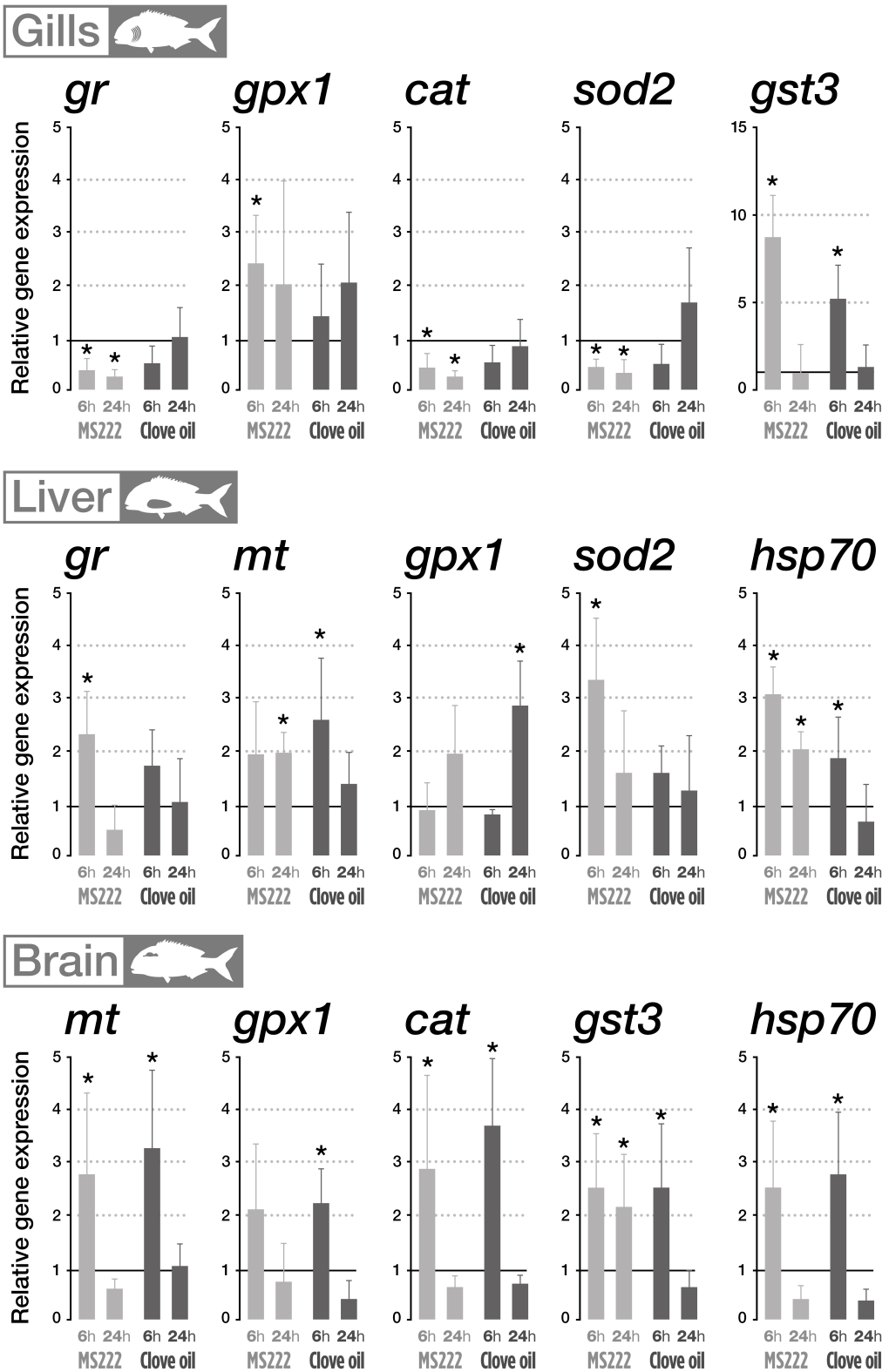

FIGURE 1 | Relative mRNA levels of target genes measured on gills, liver and brain of *S. aurata* sedated with tricaine methanesulfonate (MS222) or clove oil (CO) after 6 h transportation and 18 h recovery. The horizontal line originating at $y = 1$ denote the control group against which the expression was normalized. Bars represent the mean value of six fish with standard error. An asterisk indicates statistical significances compared to the control group ($p < 0.05$). Only parameters that denote statistically significant changes are presented.

MS222 stimulated significantly *mt* mRNA levels after recovery period. Finally, *hsp70* mRNA values were significantly higher than controls at both sampling periods. Concerning CO treated animals, a significant increase in *gpx1* mRNA levels was found after recovery period despite the lack of effects after transportation. All the other antioxidant related transcripts presented no significant differences with respect to control at both sampling points. However, for the cellular stress indicators (*mt* and *hsp70*), CO enhanced significantly hepatic mRNA expression after transportation but expression returned to control levels after recovery period.

In the brain, the use of MS222 during transportation increased mRNA levels of *mt*, *cat*, and *hsp70* after 6 h but returned to control levels after recovery period (**Figure 1**). Expression levels for *gst3* were significantly higher than controls after 6 and 24 h; while the expression of *gpx1* and *gr* displayed no significant differences from control throughout the experiment. CO, induced a similar pattern of change in expression levels of mt, *gpx1*, *cat*, gst3, and *hsp70*, with a significantly enhancement after transportation and returning to control levels after recovery period (**Figure 1**). The expression of *gr* in the brain was unaltered throughout the experimental assay (data not shown).

Biochemical Responses

The effects of MS222 and CO on neurotransmission indicators were assessed through the measurement of AChE activity on different biological matrices (gills, plasma, brain, muscle, and heart). Results showed that fish treated with MS222 significantly increased AChE activity in plasma both after 6 h transportation and after recovery period (**Figure 2**). However, in the heart, AChE was significantly inhibited after transportation, returning to control levels after 24 h recovery. Fish treated with CO only displayed significant differences to control in the heart, with a decreased activity after transportation (**Figure 2**).

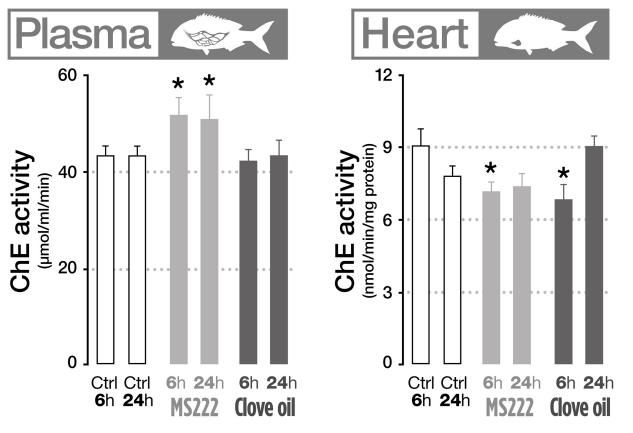

FIGURE 2 | Cholinesterase activity in plasma and heart of *S. aurata* sedated with tricaine methanesulfonate (MS222) or clove oil (CO) after 6 h transportation and 18 h recovery. Bars represent the mean value of six fish with standard error. An asterisk indicates statistical significances compared to the control group ($p < 0.05$). Only parameters that denote statistically significant changes are presented.

In terms of oxidative stress related parameters, plasma TAC and TOS levels in fish exposed to MS222 and CO were not significantly affected, when compared to their respective controls (data not shown). Gills enzymatic antioxidant defenses were not responsive to MS222 treatment, whereas CO treated organisms significantly increased CAT activity and decreased GST, at both sampling periods; while GR was significantly enhanced after transportation (**Figure 3**).

Considering hepatic enzymatic defenses, CAT and GST activities in fish exposed to MS222 displayed a significant decrease after 6 h transportation returning to control levels after recovery. No significant differences were found between controls and CO treated fish in terms of hepatic enzymatic antioxidant defenses (**Figure 3**). The assessed antioxidant defenses were not responsive to the tested anesthetics in the brain (data not shown). No significant differences between control and treated groups were found in the tested organs in terms of peroxidative damage (data not shown). Respect to non-enzymatic antioxidant defenses, assessed in this study through the quantification of NPT levels, differences to control situation were only found in the liver of fish treated with MS222 after 6 h transportation, returning to basal levels after recovery (**Figure 3**).

DISCUSSION

This study aimed to evaluate potential oxidative side effects of the use of MS222 and CO anesthetics in a simulated transportation of fish. These substances are being used in practice to reduce risk of stress-induced problems such as injuries, feeding and immune depression that may induce relevant physiological alterations in fish and compromise its development, overall health and welfare (Ross and Ross, 2008; Priborsky and Velisek, 2018). In addition, slight sedation during transport has been proved to reduce the metabolic rate of fish, and consequently oxygen consumption and generation of waste products, improving water quality management (Zahl et al., 2012; Vanderzwalmen et al., 2018). Despite the recognized applications of anesthetics, the side effects of anesthesia are unknown in many fish species (Bowker et al., 2015), being responses to these chemicals species- and anesthetic-specific (Readman et al., 2017). Thus, it becomes highly relevant to characterize the potential side effects of MS222 and CO to aquaculture fish-species, like *S. aurata*, where the use of anesthetics is a possible solution to improve animal welfare (Ashley, 2007). Our study confirmed that the use of anesthetics may modulate fish antioxidant responses. This is particularly relevant considering that here may be energetic costs to re-establish the oxidative status and that oxidative stress has been associated with an increased susceptibility to different abiotic and biotic stressors, development of different pathologies and constraint on growth.

The susceptibility of a given organ to a substance is modulated by (i) the different predisposition to accumulate the substance, (ii) its characteristic antioxidants basal levels, (iii) its adaptation capacity and consequent antioxidant activation, and (iv) the metabolic rate, thus increasing the potential to produce ROS and challenge the respective defenses (Oliveira et al., 2008).

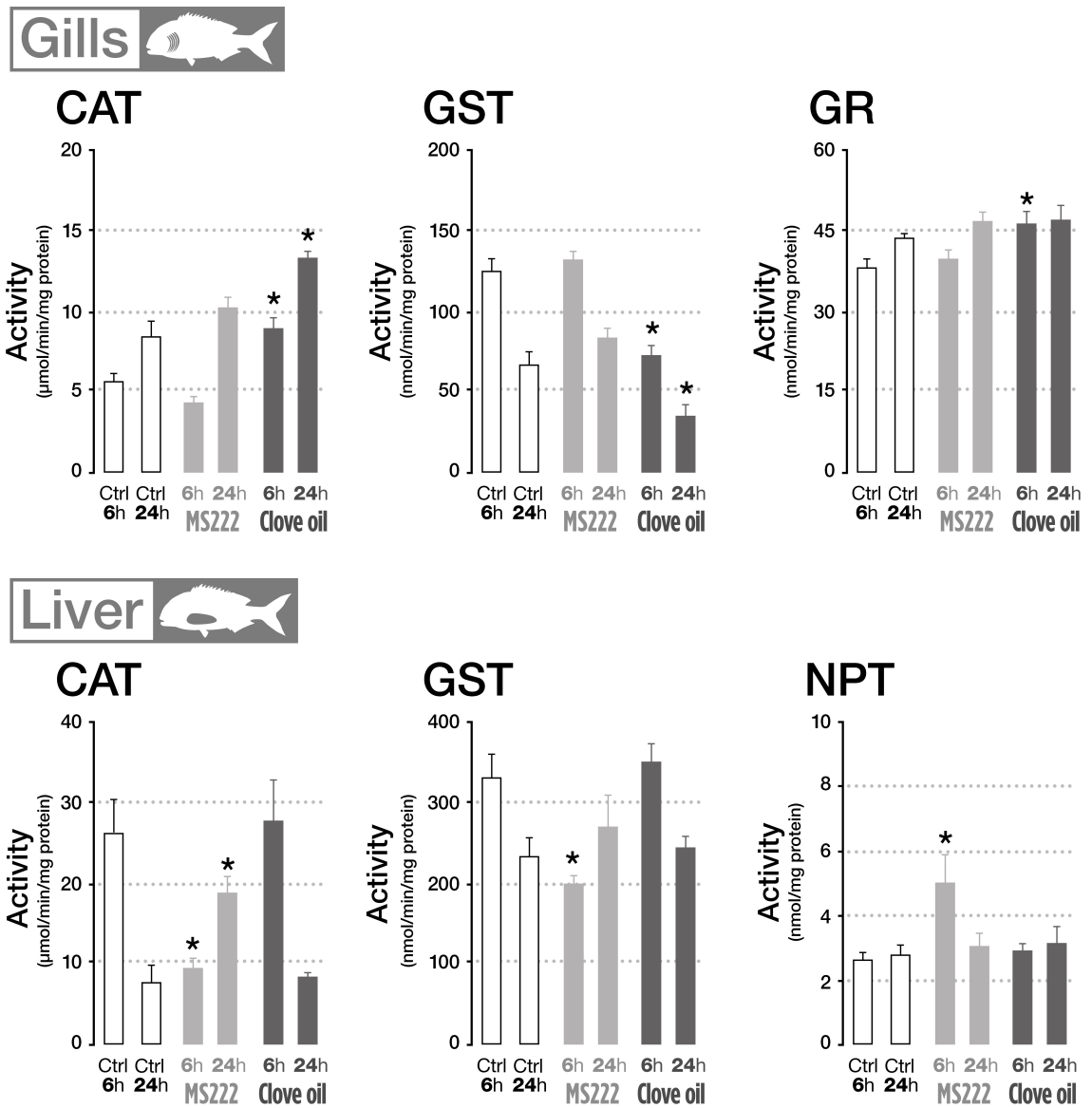

FIGURE 3 | Oxidative stress related parameters in the gills and liver of *Sparus aurata* sedated with tricaine methanesulfonate (MS222) or clove oil (CO) after 6 h transportation and 18 h recovery. An asterisk indicates statistical significances compared to the control group ($p < 0.05$). Only parameters that denote statistically significant changes are presented.

The assessed mRNA levels revealed that gills were more responsive to MS222 than to CO.

The observed increased expression of *gpx1* and *gst3* suggests production of reactive species (e.g., hydroperoxides) and activation of phase II biotransformation as well as an oxidative status that could impair gills' *sod2*, *cat*, and *gr* mRNA levels. However, hepatic expression levels of the assessed target genes suggest that liver was less responsive to MS222 than gills.

Only *sod2*, *cat*, and *hsp70* were responsive after 6 h transportation, increasing mRNA levels. However, after the recovery period, only *hsp70* remained different from controls. Despite not being responsive during transportation, *mt* mRNA levels appeared/were enhanced after recovery period. These responses support the idea that the liver presents basal antioxidant levels capable of protecting the cells from MS222 induced damage, despite the occurrence of cellular stress, as suggested by the *mt* and *hsp70* altered levels after recovery. Supporting the idea of increased ROS levels is the induction of *mt* mRNA levels, which have been reported to respond to non-metal compounds able to cause oxidative stress in fish cells, despite its upregulation by metals (Oliveira et al., 2009). Despite the lack of inhibition of the assessed antioxidants' mRNA levels, *hsp70* mRNA expression after 6 h transportation suggests the presence of stress. Contrary to the observed in the gills,

brain *cat* expression levels increased after transportation that could indicate lower susceptibility, likely associated with higher basal levels of antioxidants and higher responsiveness than gills. The mRNA levels of *gst3* in the brain were, like in the gills, significantly increased after 6 h transportation but, for this organ, mRNA levels remained higher than controls even after the recovery period, suggesting the formation of hydroperoxides. The *mt* and *hsp70* levels in the brain were also responsive to MS222 associated cellular stress. Concerning CO, gills' *gst3* expression values displayed a pattern of response similar to MS222. However, the mRNA levels of the other assessed endpoints were not altered when compared to controls suggesting a lower ability of CO to induce ROS. Nonetheless, changes in hepatic *mt* and *hsp70* expression suggest the existence of cellular stress that, after 24 h, disappears. However, *gpx1* was increased in the recovery period, probably due to an increased in ROS production. The brain appears more sensitive to CO than to MS222, with increased expression of *cat*, *gst3*, *mt*, and *hsp70* after 6 h that, after recovery return to control levels.

Considering the molecular responses, two of the most commonly assessed enzymatic activities, GST and CAT, were determined in the three organs, to understand the molecular responses. MS222 displayed no significant effects on gills' GST activity, despite the increased expression of *gst3* and, in the liver, the 6 h exposure caused a decreased significantly enzymatic activity. The brain GST activity was also not affected by MS222 despite the observed increased expression of *gst3*. CAT activity in the gills and brain was not significantly altered by MSS222, unlike hepatic GST activity that was inhibited after 6 h exposure despite the non-significant decrease in *gst3* mRNA expression. Concerning CAT activity, only the liver was sensitive to the treatment, with a significant decrease after 6 h and an increase after the recovery period. Previous studies have reported the ability of MS222 to decrease CAT activity in the erythrocytes of the marine fish *Dicentrarchus labrax* (Gabryelak et al., 1989). The non-enzymatic antioxidants in the liver and brain, show that liver was responsive to the MS222 6 h treatment, increasing the antioxidant defenses. With respect to CO, the biochemical endpoints suggest an ability to inhibit GST activity and induce the activity of CAT in the gills, effects that remain even after the recovery period and were not associated with effects on non-enzymatic antioxidants. In terms of peroxidative damage, the tested treatments revealed no effects. The TOS and TAC levels in the plasma, that have been proposed as a markers of the oxidative status in fish, showed that the anesthetics displayed no significant differences to control.

Overall, the data show that, under the present experimental design, anesthetics are able to affect biochemical endpoints in gills, liver and brain without significant consequences to the health of the tested organisms. Based on the molecular endpoints, CO appears to induce less effects or effects from which the fish are able to more easily recover than MS222. This could be expected based on the reports in different *in vitro* assays that show that CO can be an antioxidant, having reducing power, superoxide anion radical scavenging, hydrogen peroxide scavenging and metal chelating activities (Gülçin et al., 2012). The ability of MSS22 to affect antioxidant defenses such as peroxide metabolism enzymes (Gabryelak et al., 1989) and thus to compromise their use as biomarkers has been previously reported. Considering the effects on neurotransmission, data suggest that exposure to MS222 and CO may affect neurotransmission as significant decreases were found in the heart, despite the lack of effects observed for the other tissues. Furthermore, the increased levels of AChE found in the plasma of fish treated with MS222 may suggest pernicious effects of this anesthetic as previous studies have associated increases in AChE activity with ongoing apoptotic processes in the organism (Zhang et al., 2002, 2012) and production of free radicals and oxidative stress (Ferreira et al., 2012).

In conclusion, our results indicate that the use of both anesthetic agents, CO and MS222, interfere with fish antioxidant status. All tested biological matrices displayed alterations in antioxidant endpoints, confirming that these substances, although minimizing the effects of transport stress, may have long term effects on fish defenses. These results are of high relevance to aquaculture considering that an altered oxidative stress, induced by use of these anesthetics as stress-reducing agent during transport, may increase the susceptibility to different environmental or biotic stress and promote different types of pathologies.

ETHICS STATEMENT

The experiment complied with the Guiding Principles for Biomedical Research Involving Animals (EU2010/63), the guidelines of the Spanish laws (law 32/2007 and RD 53/2013), and authorized by the Ethical Committee of the Universidad de Cádiz (Spain) for the use of laboratory animals and the Ethical Committee from the Andalusian Government (Junta de Andalucía reference number 28-04-15-241).

AUTHOR CONTRIBUTIONS

JM and IJ-C designed the experiments and performed the experimental setup. IJ-C, MT, and MO conducted the experimental procedures. MT, MO, LF-M, and AT did the laboratorial analysis and analyzed the results. MT and MO wrote the manuscript. LT and JM revised the manuscript. All authors read and approved the manuscript for publication.

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frontiers in Physiology

REVIEW
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Essential Oils as Stress-Reducing Agents for Fish Aquaculture: A Review

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In fish, stressful events initiate a hormone cascade along the hypothalamus-pituitary-interrenal and hypothalamus-sympathetic-chromaffin (HSC) axis to evoke several physiological reactions in order to orchestrate and maintain homeostasis. Several biotic and abiotic factors, as well as aquaculture procedures (handling, transport, or stocking density), activated stress system inducing negative effects on different physiological processes in fish (growth, reproduction, and immunity). In order to reduce these consequences, the use of essential oils (EOs) derived from plants has been the focus of aquaculture studies due to their diverse properties (e.g., anesthetic, antioxidant, and antimicrobial), which have been shown to reduce biochemical and endocrine alterations and, consequently, to improve the welfare status. Recently, several studies have shown that biogenic compounds isolated from different EOs present excellent biological activities, as well as the nanoencapsulated form of these EOs may potentiate their effects. Overall, EOs presented less side effects than synthetic compounds, but their stress-reducing efficacy is related to their chemical composition, concentration or chemotype used. In addition, their species-specific actions must be clearly established since they can act as stressors by themselves if their concentrations and chemotypes used are not suitable. For this reason, it is necessary to assess the effect of these natural compound mixtures in different fish species, from marine to freshwater, in order to find the ideal concentration range and the way for their administration to obtain the desired biological activity, without any undesired side effects. In this review, the main findings regarding the use of different EOs as stress reducers will be presented to highlight the most important issues related to their use to improve fish welfare in aquaculture.

Keywords: aquaculture, natural compounds, fish stress, fish health, fish immundogy

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INTRODUCTION

The aquaculture industry deals with several stressful situations that can compromise the target species well-being, including handling, confinement, fertilization, transport, and other operations, from the hatchery to the final commercial stage (Ashley, 2007; Sampaio and Freire, 2016; Sneddon et al., 2016; Sánchez-Muros et al., 2017). Stress induced by such practices has long been suspected to

cause mortality, affecting the success in fish production with the consequent economic loss for this sector. In addition, the impact of aquaculture-related stressors can also predispose fish to disease (Segner et al., 2012).

Stress response is usually triggered by a wide range of physiological mechanisms in order to compensate the imbalances produced by the stressor and recover the homeostatic status of fish. The stress response is initiated and controlled by two hormonal systems, which lead to the production of catecholamines (such as adrenaline and noradrenaline, and their precursor dopamine) by the hypothalamus-sympathetic-chromaffin (HSC) axis, and corticosteroids (mainly cortisol) by the hypothalamus-pituitary-interrenal (HPI) axis (Wendelaar Bonga, 1997, 2011; Flik et al., 2006; Martos-Sitcha et al., 2014). Behavioral changes are used by the organism to overcome this situation, subsequently generating several responses to the stressor, including gene, metabolic, energetic, immune, endocrine, and neural changes (Schreck and Tort, 2016; **Figure 1**). Long-term consequences of repeated or prolonged stressful exposures are maladaptive by negatively affecting other necessary life functions, such as growth, development, disease resistance, behavior or reproduction and may even culminate in fish death (Wendelaar Bonga, 1997; Schreck and Tort, 2016). Therefore, several studies assessed the use of sedatives and anesthetics in order to find alternatives to minimize stress effects caused by the intense management practices in aquaculture (Ross and Ross, 2008; Neiffer and Stamper, 2009; Sneddon, 2012; Zahl et al., 2012). Recent excellent reviews have focussed on this topic (Hoseini et al., 2018; Priborsky and Velisek, 2018; Vanderzwalmen et al., 2018).

In addition, essential oils (EOs) derived from plants have been used in aquaculture studies because of their diverse properties that can improve health, growth and welfare of animals (Azambuja et al., 2011; Zeppenfeld et al., 2014; Souza et al., 2018a), as well as reduce stress processes (Saccol et al., 2016, 2018a; Souza et al., 2017b). There are reviews of the effects of EOs as sedatives, anesthetics, antioxidants, and antimicrobials (Cunha et al., 2018; Hoseini et al., 2018; Sutili et al., 2018). Hoseini et al. (2018) focused on stress-reducing effects, but only related to anesthetic effects of the EOs, while the other two reviews did not deal with this subject. Therefore, the aim of the present review is to discuss the possible mechanisms by which the different ways of applying EOs (waterborne and dietary exposure) may minimize stress responses induced by aquaculture procedures, as well as a future perspective of their use.

ESSENTIAL OILS

Essential oils extracted from plants contain compounds produced during plant secondary metabolism. They constitute one of the most important groups of raw materials for the food, hygiene, and cleaning products and pharmaceutical industries, perfumery, and others. They are complex mixtures of low molecular weight substances (Morais, 2009), with a wide variation in their chemical properties (Hussain et al., 2008). Several studies demonstrate that certain EOs, used as anesthetics and/or sedatives, reduced plasma cortisol levels and attenuated stress response (Cunha et al., 2010; Zeppenfeld et al., 2014; Souza et al., 2017b). Moreover, some EOs when added to therapeutic baths (concentrations lower than those that induce sedation) are able to prevent oxidative stress. The EO of *Melaleuca alternifolia*, for example, was also able to prevent the inhibition of splenic creatine kinase and pyruvate kinase activities caused by diseases (Baldissera et al., 2017c, 2018), thus demonstrating the possibility to be used as a stress-reducing agent in aquaculture practices. For example, anesthesia with the EO of *Lippia alba* (citral and linalool chemotypes) prevented plasma cortisol increase in silver catfish (*Rhamdia quelen*) exposed to air for biometry (Cunha et al., 2010; Souza et al., 2018a), and altered the expression of hormones and enzymes of the HPI axis, proving that it can directly influence this cascade (Souza et al., 2019). Furthermore, it is important to note that EOs have lipophilic properties and liposolubility, contributing to rapid dispersion through biological membranes, including the blood-brain barrier in the central nervous system (CNS), modulating brain function (Zahl et al., 2012; Manayi et al., 2016). It is known that several EOs exert their anesthetic effects by regulating the gamma-aminobutyric acid receptor complex (GABA), the main inhibitory neurotransmitter in the CNS (Bakkali et al., 2008; Chioca et al., 2013; Heldwein et al., 2014; Moreira et al., 2014; Raut and Karuppayil, 2015). Some EOs act in the benzodiazepine site of the $GABA_A$ receptor, but others do not (Heldwein et al., 2012; Silva et al., 2012; Garlet et al., 2016; Bianchini et al., 2017; Santos et al., 2017a). In addition, deep anesthesia with *Cymbopogon nardus* EO promotes a conspicuous depression on muscle contraction power with loss of muscle tonus and transient cardiorespiratory depression (Barbas et al., 2017b).

Definition

EOs are natural multicomponent systems of volatile, lipophilic, odoriferous and liquid substances, obtained from plant raw materials (Edris, 2007). The number of components of an EO generally ranges from 20 to 200, and they are named according to their concentration in the mixture, as (i) major constituents (from 20 to 95%), (ii) secondary constituents (1–20%), and (iii) trace components (below 1%). More than 3,000 distinct chemicals have been detected in EOs, with great variety of chemical structures (Sticher, 2015). Overall, they are classified as terpene hydrocarbons, simple and terpene alcohols, aldehydes, ketones, phenols, esters, ethers, oxides, peroxides, furans, organic acids, lactones, coumarins, or even sulfur compounds (Swamy et al., 2016).

Chemical Composition, Extraction Methods, and Stability of Essential Oils

The genetic characteristics of the producing plant are the most important factors determining the chemical composition of an EO and, consequently, interfere with their biological/pharmacological activities. In addition, the chemical composition is generally specific for a given organ

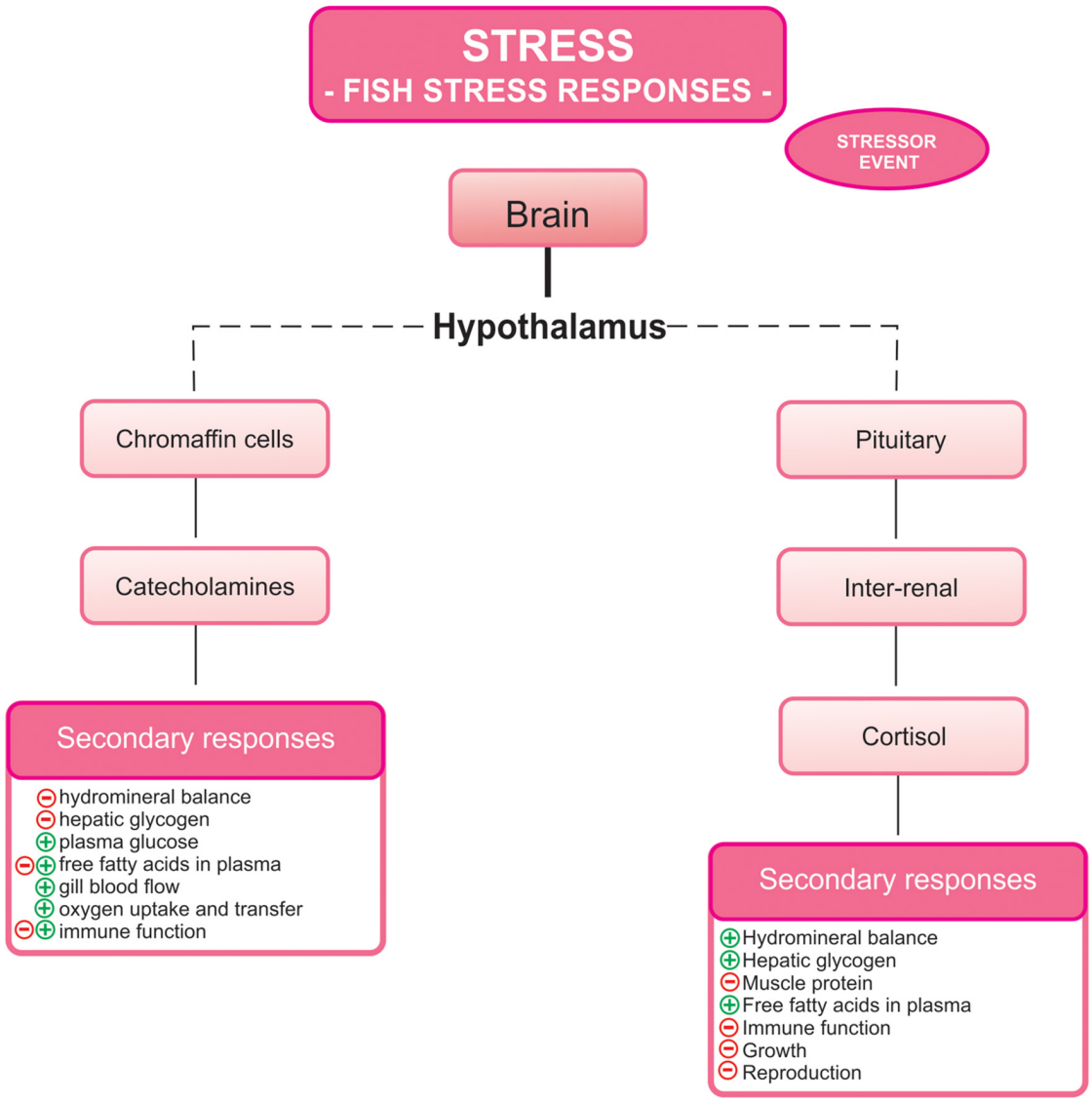

FIGURE 1 | Responses to stress in fish. The activation of the hypothalamus-sympathetic-chromaffin and hypothalamus-pituitary-interrenal axes culminates in the release of catecholamines and cortisol, respectively. These hormones induced several stress secondary responses (modified from Wendelaar Bonga, 1997). (+) indicates activation and/or increase and (–) indicates inhibition and/or reduction.

and characteristic of its stage of development, but edaphic and environmental conditions, as well as the extraction method used may cause significant variations (Figueiredo et al., 2008; Reyes-Jurado et al., 2015). Since chemical variability is often high among plants from their natural habitat, whenever possible, EOs should be obtained from cultivated plants that have genetic homogeneity, to ensure the consistency of the composition. However, it is also necessary to observe other aspects that influence the composition of an oil (Sticher, 2015), already cited above, trying to keep them constant.

The EO extraction methods are among the aspects that most affect the chemical characteristics. The main methods of EO extraction are steam distillation and its variants, cold pressing, supercritical fluid extraction, and solvent extraction (Luque de Castro et al., 1999; Capuzzo et al., 2013; Reyes-Jurado et al., 2015). Another variability factor is the occurrence of chemotypes or chemical races, which is often related to geographical variations (Figueiredo et al., 2008). This phenomenon is characterized by botanically identical plants which differ chemically (Aprotosoaie et al., 2017). Consideration should also be given to the relative low stability of EOs, which may undergo chemical changes mainly due to loss of volatile compounds, oxidation reactions and/or polymerization. However, various strategies can be observed to prevent their deterioration, including storage in small volume glass containers, maintaining them under low temperatures and protected from light. Further information on the stability of EOs can be obtained in Turek and Stintzing (2013).

EFFECTS OF EOS DURING DIFFERENT STRESS EVENTS

The interpretation of the general effects of EOs is somewhat complicated because only a few were tested in more than one fish species during different stress events. Furthermore, type and characteristics of stressors used may differ complicating the comparison between EOs. In addition, most studies were performed in freshwater species but not in marine species. Finally, other factors that hinder the comparisons are the existence of different chemotypes for the same plant and the variability of the composition of EOs even from the same chemotype.

Stress-Preventing Effects During Handling Procedures

Exhaustive swimming during attempts to escape from the capture induces changes in physiological parameters, such as increase in glucose and lactate values, when compared to resting animals (Brown et al., 2008; Olsen et al., 2013). Furthermore, previous studies showed that intense swimming activity during chase and capture can be sufficient to compromise flesh quality (Cole et al., 2003) and reproduction (Pankhurst and Van der Kraak, 1997). The handling procedure may also include exposure of fish to air, a situation that requires a short-term orchestration of different endocrine players from both HSC and HPI axes (Wendelaar Bonga, 2011; Skrzynska et al., 2018).

Despite the purpose of using an anesthetic being to mitigate stress, a common observation is that the substance itself may pose as a stressor, thus activating the stress response mechanism (Thomas and Robertson, 1991; Sladky et al., 2001; Bolasina, 2006) and these situations have been observed in fish exposed to MS-222, metomidate, quinaldine sulfate, benzocaine, and phenoxyethanol (Thomas and Robertson, 1991; Tort et al., 2002; Wagner et al., 2002). In addition, several synthetic anesthetics are aversive to fish even at low concentrations (Readman et al., 2013). In this sense, an increasing attention has been focused on the use of plant extracts in fish anesthesia due to a wide range of beneficial health benefits, such as antioxidant, antimicrobial, stress-relieving and immune-promoting effects (Zahl et al., 2010; Hoseini, 2011; Zeppenfeld et al., 2014; Souza et al., 2018b). Moreover, the EOs of *Lippia alba* (chemotype linalool) and *Aloysia triphylla* are not aversive to fish (Bandeira Junior et al., 2018), indicating another advantage compared to synthetic anesthetics.

The use of EOs as sedatives or anesthetics to minimize possible damage to fish resulting from handling was recently demonstrated in several species (Cunha et al., 2010; Benovit et al., 2012; Tondolo et al., 2013; Toni et al., 2015; Hashimoto et al., 2016; Pedrazzani and Ostrenski Neto, 2016; Ribeiro et al., 2016; Barbas et al., 2017a,b; Fogliarini et al., 2017; Bodur et al., 2018a,b; Hoseini et al., 2018; Souza et al., 2018a,b). For minor procedures such as biometry and collection of blood samples, lower concentrations of EOs can be used that induce tranquilization and light sedation, in order to minimize stress and reduce plasma cortisol levels (**Table 1**). The recommended concentration of clove oil (extracted from *Syzygium aromaticum* or *Eugenia aromatica*) in handling processes is 10–30 $mg{\cdot}L^{-1}$ (Javahery et al., 2012). However, exposure seawater gilthead seabream (*Sparus aurata*) at concentration of 44.5 $\mu L{\cdot}L^{-1}$ before air exposure for blood collection increased plasma cortisol and/or glucose values compared to control fish (handled as the anesthetized fish) (Bressler and Ron, 2004). Previous anesthesia with 10 $mg{\cdot}L^{-1}$ clove oil did not change plasma cortisol and glucose levels of the seawater meager manipulated for weighing compared to control fish (handled as the anesthetized fish) (Barata et al., 2016). On the other hand, for seawater Senegal sole (*Solea senegalensis*) anesthesia with 1000 $mg{\cdot}L^{-1}$ clove oil demonstrated a good ability of clove oil to prevent cortisol, lactate and glucose increases pre-mortem (Ribas et al., 2007). In addition, studies demonstrated that the faster anesthesia induction with eugenol and clove oil, the lower the stress response provoked by these anesthetics (Hoseini and Nodeh, 2013; Mirghaed et al., 2018) thus, low concentrations of eugenol besides causing slow anesthetic induction may cause disturbances in plasma glucose levels and plasma cortisol.

The effect of the EOs may also change according to the species and the type of application. For example, Nile tilapia anesthetized with 300 $\mu L{\cdot}L^{-1}$ of the EO of *A. triphylla* and then exposed to air for 1 min decreased plasma cortisol levels respect to control fish (handled as the anesthetized fish) (Teixeira et al., 2017). However, silver catfish anesthetized with 135 and 180 $mg{\cdot}L^{-1}$ and submitted to the same procedure did not change plasma cortisol values compared to control fish (Gressler et al., 2014). Interestingly, silver catfish fed diet supplemented with 2.0 mL per kg of this EO for 21 days and handled for blood collection presented lower plasma cortisol and lactate levels, but not glucose, than fish that received control feed and were submitted to the same procedure (Zeppenfeld et al., 2017; **Table 1**). However, the same dietary dose of this EO did not change whole body cortisol content in zebrafish handled for biometry (Zago et al., 2018).

The EO of *Ocimum americanum* used as anesthetic prevented plasma cortisol increase and Na^+ loss induced by aerial exposure (1 min) in silver catfish compared to control fish (handled as the anesthetized fish) (Silva et al., 2015). However, the EO of a plant from the same genus, but different species (*O. gratissimum*, with eugenol and 1,8-cineole as the main compounds), did not prevent this cortisol enhancement from air exposure. In addition, hyperglycemia was verified in fish exposed to 70 and 300 $mg{\cdot}L^{-1}$ at 1 and 4 h after handling, indicating no effect on attenuation of stress axis activation (Silva et al., 2015). Matrinxãs (*Brycon amazonicus*) transferred from cage nets to 20 L buckets with 60 $mg{\cdot}L^{-1}$ of the EO of *O. gratissimum* for 10 min also increased plasma glucose levels and did not prevent plasma lactate enhancement compared to control fish (handled as the anesthetized fish) (Ribeiro et al., 2016; **Table 1**). Consequently, even having eugenol as one of the main compounds, the efficacy of these two EOs to reduce stress is species-specific.

Anesthesia of silver catfish with the EO *L. alba* (300 $\mu L{\cdot}L^{-1}$) before handling and air exposure (1 min) prevented plasma cortisol increase observed 1 and 4 h later in control fish (handled

TABLE 1 | The use of essential oils (EOs) as stress-reducing agents in fish exposed to handling and air exposure.

Essential oil (EO)	Major compounds (%)	Fish species	Stress/ duration	Purpose/ concentration	Effect on fish physiology	References
Aloysia triphylla	Geranial (28.97)	*O. niloticus*	Air exposure/1 min	Anesthesia/300 $\mu L \cdot L^{-1}$	Prevents increase in plasma cortisol levels	Teixeira et al., 2017
	β-citral (20.78)	*R. quelen*	Handling	Diet supplemented with 2.0 mL per kg	Reduces plasmatic cortisol and lactate levels	Zeppenfeld et al., 2017
Eucalyptus sp.	1.8 cineole (80.84%)	*D. labrax*	Handling	Anesthesia/300 $\mu L \cdot L^{-1}$	Induces a stress response after 24 h of exposure, inducing plasma cortisol enhancement and up regulation of *hsp90* and *gr* gene expression in liver	Bodur et al., 2018b
Lippia alba	Linalool (55.26%)	*R. quelen*	Air exposure/1 min	Anesthesia and stress-reducing agent/100–500 $mg \cdot L^{-1}$	Reduces plasma cortisol levels	Cunha et al., 2010; Souza et al., 2018a
	β-linalool (50.56%)			Anesthesia/100–300 $\mu L \cdot L^{-1}$	Decreases TBARS and protein carbonyl levels in liver and kidney	Souza et al., 2018a
	Linalool (54.38%)		Handling and air exposure/1 min	Anesthesia/300 and 450 $\mu L \cdot L^{-1}$	Prevents Na^+-K^+-ATPase activity reduction due to handling.	Toni et al., 2014
	β-linalool (50.56%)			Anesthesia/100–300 $\mu L \cdot L^{-1}$	Decreases *slc6a2* and *crh* gene expression in brain.	Souza et al., 2019
	β-linalool (87.6%)	*S. aurata*	Persecution/1 min	Stress-reducing agent/ 35 $\mu L \cdot L^{-1}$	Increases *pomcb* expression in pituitary	Toni et al., 2015
Lippia alba	E-citral (29.84%)	*R. quelen*	Air exposure/1 min	Anesthesia/300 $\mu L \cdot L^{-1}$	Enhances protein carbonyl levels in liver and kidney	Souza et al., 2018a
					Increases *slc6a2*, *crh, hsd20b, hspa12a, hsp90* gene expression in brain	Souza et al., 2019
	Geranial (25.4%)	*C. macropomum*	Rapid air exposure for biometry	Sedation and stress-reducing agent/50 and 100 $\mu L \cdot L^{-1}$	Increases plasma glucose and ammonia values.	Batista et al., 2018
Hesperozygis ringens	Pulegone (95.17%)	*R. quelen*	Handling and air exposure/1 min	Anesthesia/300 and 450 $\mu L \cdot L^{-1}$	Prevents Na^+-K^+-ATPase activity reduction caused by handling.	Toni et al., 2014
Mentha spicata	Carvone (28.4%)	*C. carpio*	Handling	Anesthesia/5 mL L^{-1}	Reduces opercular rate and decreases plasma glucose levels after recovery	Roohi and Imanpoor, 2015
Ocimum gratissimum	Eugenol (43.3%)	*B. amazonicus*	Handling/fish transfer from cage to buckets and then back to cage	Anesthesia and stress-reducing agent/20–60 $mg \cdot L^{-1}$	Increases plasma glucose and lactate/ammonia values only at 60 $mg \cdot L^{-1}$	Ribeiro et al., 2016
	Eugenol (73.6%)	*R. quelen*	Handling	Anesthesia/70 and 300 $mg \cdot L^{-1}$	Enhances plasma glucose levels	Silva et al., 2012
Ocimum americanum	1,8-cineole (21.0%)	*R. quelen*	Air exposure/1 min	Anesthesia/300 and 500 $mg \cdot L^{-1}$	Prevents plasma cortisol increase and Na^+ loss	Silva et al., 2015
Origanum sp.	Carvacrol (78.16%)	*D. labrax*	Handling	Anesthesia/50 $\mu L \cdot L^{-1}$	Decreases stress-related genes (*cyb11b1* and *star*) expression in head kidney	Bodur et al., 2018b
Syzygium aromaticum (clove oil)	Eugenol	*C. carpio*	Handling	Anesthesia/700 $mg \cdot L^{-1}$	Increased plasma cortisol and glucose levels	Hoseini and Nodeh, 2013
		R. quelen	Handling	Anesthesia/50 $mg \cdot L^{-1}$	Decrease plasma cortisol and glucose levels	Cunha et al., 2010
		S. aurata and *O. mykiss*	Handling	Anesthesia/50–200 $\mu L \cdot L^{-1}$	Does not prevent plasma cortisol increase caused by handling	Tort et al., 2002

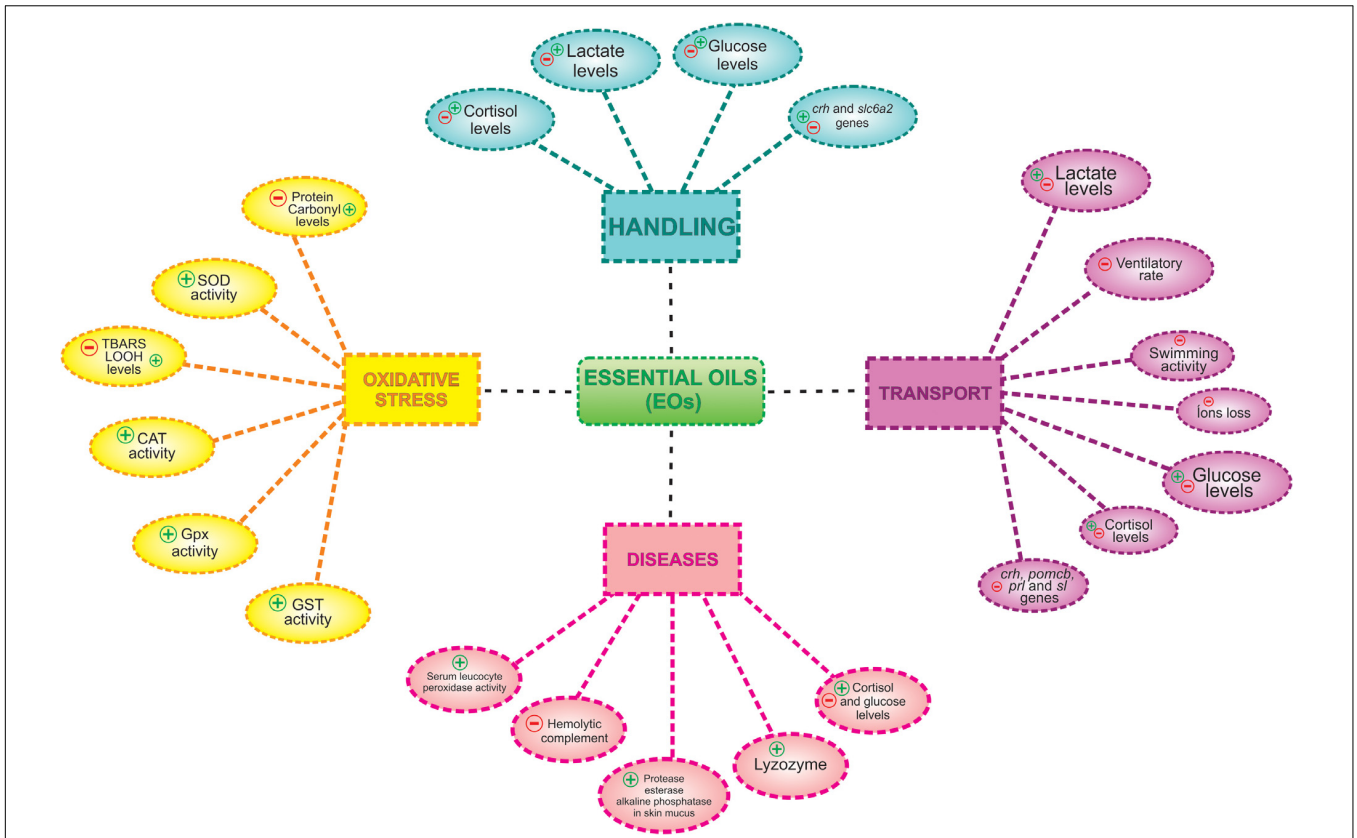

FIGURE 2 | Effects of essential oils (EOs) on physiological changes induced by aquaculture procedures, handling, transport, and diseases. (+) indicates activation and/or increase and (–) indicates inhibition and/or reduction.

as the anesthetized fish) (Cunha et al., 2010). In addition, both linalool and citral chemotypes of this EO (300 $\mu L \cdot L^{-1}$) reduced plasma cortisol through anesthesia and the first 10 min of recovery compared to control fish (handled as the anesthetized fish) (Souza et al., 2017b). Finally, individuals of this species anesthetized with linalool chemotype of this EO (100 and 300 $\mu L \cdot L^{-1}$) decreased, at 4 h after anesthesia and air exposure for biometry and compared to control fish, expression of genes directly related to stress: corticotropin releasing hormone (*crh*), and solute carrier family 6 (neurotransmitter transporter, noradrenalin) member 2 (*slc6a2*) (Souza et al., 2019). However, the citral chemotype is stressful for silver catfish because there was an up regulation of *slc6a2*, *crh*, 20β-hydroxysteroid dehydrogenase (*hsd20b*) and heat shock proteins 70 member 12 (*hspa12a*) and heat shock protein 90 (*hsp90*) (Souza et al., 2019; **Table 1**).

Similarly to other EOs, the efficacy of the linalool chemotype of the *L. alba* EO as a stress reducing agent can change with the concentration and/or species. So, 200 $\mu L \cdot L^{-1}$ did not change plasma cortisol levels in the hybrid tambacu (*Piaractus mesopotamicus* × *Colossoma macropomum*) subjected to handling compared to control fish (handled as the anesthetized fish) (Sena et al., 2016). However, anesthesia of tambaqui (*Colossoma macropomum*) with 50 and 100 $\mu L \cdot L^{-1}$ EO of this plant, but of the chemotype citral, did not prevent plasma glucose and ammonia enhancement due to rapid air exposure for biometry (Batista et al., 2018; **Table 1**).

Others EOs from different plants showed variable effects depending on the species tested. Anesthesia with the EOs of *Hesperozygis ringens* (137–277 $\mu L \cdot L^{-1}$) and *Ocotea acutifolia* (300 $\mu L \cdot L^{-1}$) did not change blood glucose levels in silver catfish (Silva et al., 2013). The EOs of *Myrcia sylvatica* and *Curcuma longa* anesthetized matrinxã and reduced plasma cortisol levels compared to control fish (handled as the fish exposed to EOs) (Saccol et al., 2017). Finally, the use of 50 $\mu L \cdot L^{-1}$ of oregano EO (*Origanum* sp.) as anesthetic decreased the expression of steroidogenic genes at 2 and 24 h compared to clove oil, demonstrating the efficiency of oregano EO in reducing secondary stress in European sea bass (Bodur et al., 2018a,b; **Table 1**).

In the marine dusky grouper the use of 50 and 300 $\mu L \cdot L^{-1}$ of the EO of *Aloysia polystachya* for, respectively, sedation and anesthesia, did not affect plasma glucose, Na^+, and Cl^- levels compared to control fish (handled as the anesthetized fish) (Fogliarini et al., 2017; **Figure 2**).

Usually, EOs are applied in water prior to handling. However, it would be interesting to test the efficacy of EOs if other means of administration were used. In this sense, dietary addition (0.5–2.0 $mL \cdot kg^{-1}$) of cinnamon (*Cinnamomun* sp.) EO did not alter plasma cortisol and glucose levels of Nile tilapia exposed to air for

3 min compared to control fish, which was fed diet without this EO and subjected to the same procedure) (Santos et al., 2016).

Stress-Preventing Effects During Transport Procedures

Live transportation of larvae, juvenile or broodstock is one of the major causes of stress in fish due to the capture, packing, high loading density, changes in water quality, the transport itself, unloading, and the final storage (Harmon, 2009; Sampaio and Freire, 2016). In all transport systems, including simple closed systems, such as sealed plastic bags partly filled with water and oxygen, homeostasis of the transported fish is mainly challenged by the build-up of ammonia (NH_3 and NH_4^+) due to excretion, and carbon dioxide (CO_2) from respiration (Lim et al., 2003; Paterson et al., 2003; Becker et al., 2016). These events cause changes in pH (Golombieski et al., 2003), which may alter fish metabolism and induce activation of the stress system. So, transportation can lead to a number of physiological responses such as catecholamines and corticosteroids release as well as blood glucose levels enhancement (Pottinger, 2008; Zeppenfeld et al., 2014). In addition, transport significantly decreased corticotrophin-releasing hormone (*crh*) and urotensin I (*ui*) expression levels in the brain of *Coilia nasus* and induced oxidative stress (Du et al., 2015). Furthermore, stress by transport induced an osmotic imbalance with ion loss in freshwater fish, showing a good correlation with transport stress (Becker et al., 2013, 2016; Parodi et al., 2014; Zeppenfeld et al., 2014; Garcia et al., 2015; Ostrensky et al., 2016; Teixeira et al., 2018; **Table 2**). Thus, during or prior to transport, sedation of the fish is desirable to reduce or prevent stress.

The EO of *L. alba* (linalool chemotype) at 10 or 20 $\mu L \cdot L^{-1}$ added to the transport water of silver catfish reduced net Na^+, Cl^-, and K^+ loss compared to control fish subjected to the same procedure (Becker et al., 2012). The same chemotype of this EO (10 $\mu L \cdot L^{-1}$) prevented plasma cortisol (but not glucose) increase provoked by transport in tambacu (Sena et al., 2016; **Table 2**). A similar concentration of this EO (15 $\mu L \cdot L^{-1}$) inhibited plasma glucose enhancement in slender seahorses (*Hippocampus reidi*) transported in plastic bags (Cunha et al., 2011). However, meager submitted to simulated transport with 12 $mg \cdot L^{-1}$ of the EO of *L. alba* (linalool chemotype) presented higher plasma cortisol levels compared to control fish subjected to the same procedure (Cárdenas et al., 2016; **Table 2**). The transport of common carp in freshwater with 50–200 $mg \cdot L^{-1}$ linalool decreased ammonia excretion, but increased serum cortisol, glucose and urea values and did not prevent the decrease of serum Na^+ and Cl^- provoked by transport compared to control fish submitted to the same procedure (Mazandarani et al., 2017). Recent studies showed that EO of *L. alba* (chemotype linalool) at 5–10 $\mu L \cdot L^{-1}$ reduced swimming activity of the black piranha, *Serrasalmus rhombeus*, compared to control fish subjected to the same procedure and may also be considered as options for the transport of this species (Almeida et al., 2018). These results obtained so far with the use of the EO of *L. alba* (linalool chemotype) and linalool for the transport of fish do not lead to the conclusion whether the differences are related to species, concentrations, or water quality (fresh and seawater).

The EO of *A. triphylla* has also been tested for transport procedures in freshwater and marine species. The use of this EO (40 and 50 $\mu L \cdot L^{-1}$) during a 6 h transport reduced net ion loss (Parodi et al., 2014), ammonia excretion and plasma cortisol levels in silver catfish compared to control fish submitted to the same procedure (Zeppenfeld et al., 2014). Plasma cortisol enhancement in fat snook due to transport was reduced by adding 20 $\mu L \cdot L^{-1}$ of this EO to the water (Parodi et al., 2016), whereas 30 $\mu L \cdot L^{-1}$ decreased ventilatory rate, ion loss and plasma glucose levels, with no effects preventing cortisol increase in Nile tilapia (Teixeira et al., 2018), both compared to their respective control fish subjected to the same procedure. In addition, this EO (25 $\mu L \cdot L^{-1}$) also decreased ventilatory rate of pacamã, *Lophiosilurus alexandri*, as well as ammonia excretion during 4 h transport (Becker et al., 2017; **Table 2**), also reducing the swimming activity of black piranha at concentration of 10 $\mu L \cdot L^{-1}$ (Almeida et al., 2018) both compared to their respective control fish subjected to the same procedure. Overall, this EO seems to be effective in reducing stress of transport in fish, irrespective of whether they are fresh- or seawater-adapted.

Transport of the Brazilian flounder for 7 h with the EOs of *Aloysia gratissima* (90 $mg \cdot L^{-1}$) and *Ocimum gratissimum* (10–20 $mg \cdot L^{-1}$) increased blood glucose levels compared to control fish subjected to the same procedure. In addition, the EO of *A. gratissima* induced mortality. Consequently, both EOs are not effective in transport of this species (Benovit et al., 2012). Moreover, the EO of *Nectandra megapotamica* at 15 or 30 $\mu L \cdot L^{-1}$ was not able to prevent the stress of transport in fat snook because it did not prevent deterioration in water quality and the post-transport mortality compared to control fish subjected to the same procedure (Tondolo et al., 2013; **Table 2**). Studies of the effect of these EOs in freshwater species are still lacking. Simulated transport (6 h) of gilthead seabream with clove oil at 2.5 $mg \cdot L^{-1}$ increased plasma cortisol levels and expression of head kidney gene-expressions related to cortisol production (*star* and *cyp11b1*) and stimulated amino acids catabolism (Jerez-Cepa et al., 2019). The gill's mRNA levels of *gst3*, a target gene related with the antioxidant response and cell-tissue repair, enhanced after transportation with clove oil, returning to control values after recovery. However, the rest of transcripts assessed (*gpx1*, *cat*, *gr*, *mt*, and *hsp70*) related to these responses did not present any alteration (Teles et al., 2019; **Table 2**). Authors proposed that alternative concentrations of this EO should be tested for the transport of gilthead seabream.

Transport of silver catfish with the EO of *Myrcia sylvatica* (25 or 35 $\mu L \cdot L^{-1}$) reduced plasma cortisol and lactate levels, concomitantly with the decrease in the gene expression of *crh* and proopiomelanocortin b (*pomcb*), *prolactin* and *somatolactin* mRNAs compared to control fish without EO administration and subjected to the same procedure (Saccol et al., 2018b). Exposure of silver catfish to the EOs of *Citrus* × *aurantium*, *Citrus* × *latifolia* (50–100 $\mu L \cdot L^{-1}$), increased ventilatory frequency, but reduced ion loss and ammonia excretion compared to control

TABLE 2 | The use of essential oils (EOs) as stress-reducing agents in fish transport.

Essential oil (EO)	Major compounds (%)	Fish species	Stress/ duration	Purpose/ concentration	Effect on fish physiology	References
Aloysia gratissima	NA	*P. orbignyanus*	Transport in plastic bags/7 h	Anesthetic/90 mg·L^{-1}	Increases plasma glucose values and mortality	Benovit et al., 2012
A. triphylla	E-citral (42.30)	*R. quelen*	Transport/4 h	Sedation/40 and 50 μL·L^{-1}	Reduces ammonia excretion and plasma cortisol levels	Zeppenfeld et al., 2014
	α-citral (20.41)	*L. alexandri*	Transport/4 h	Sedative and stress-reducing agent/25 μL·L^{-1}	Decreases total ammonia nitrogen levels and ventilatory frequency.	Becker et al., 2017
Curcuma longa	β-selinene (9.96)	*B. amazonicus*	Transport simulation/360 min	Sedation/40 μL·L^{-1}	Reduces plasma cortisol levels and preventing a stress response as well as excess of reactive oxygen species formation.	Saccol et al., 2017
L. alba	Linalool*	*R. quelen*	Transport/5, 6, 7 h	Slight sedation/10–20 μ·L^{-1}	Improves redox state	Azambuja et al., 2011
			Transport/4 h	Stress- reducing agent/1.5 and 3.0 μL·L^{-1}	Reduces net Na^+, Cl^-, and K^+ loss	Becker et al., 2012
			Transport/6 h	Sedation/30–40 μL^{-1}	Induces oxidative stress	Salbego et al., 2014
	Linalool (47.66)	Tambacu (*P. mesopotamicus* × *C. macropomum*)	Transport (8 h) and handling (0, 1 and 4 h)	Stress- reducing agent and anesthesia/10–200 μL·L^{-1}	Prevents plasma cortisol levels enhancement	Sena et al., 2016
		A. regius	Simulated transport/4 h	Anesthetic/12 mg·L^{-1}	Increases plasma cortisol values	Cárdenas et al., 2016
Myrcia sylvatica	β-phellandrene (31.48)	*B. amazonicus*	Transport simulation/6 h	Sedation/10 μL·L^{-1}	Decreases plasma cortisol levels as well as prevents stress response and excess of reactive oxygen species formation	Saccol et al., 2017
	β-phellandrene (31.48)	*R. quelen*	Pre-transport handling/transport (6 h)	Stress-reducing agent	Reduces plasma cortisol and lactate levels as well as increases *crh*, *pomcb*, *prolactin,* and *somatolactin* gene expression in brain	Saccol et al., 2018b
Nectandra grandiflora	NA	*C. macropomum*	Transport/2, 6, and 10 h	Sedation and stress-reducing agent/30 μL·L^{-1}	Enhances protection against oxidative damage mainly in muscle and gills	Barbas et al., 2017b
N. megapotamica	bicyclogermacrene (34.6)	*C. parallelus*	Transport/10 h	Stress-reducing agent/30 and 300 μL·L^{-1}	Increases plasma glucose and lactate levels.	Tondolo et al., 2013
Syzygium aromaticum (clove oil)	Eugenol	*O. niloticus*	Transport simulation/3.5 h	Sedation/20 μL·L^{-1}	Decrease plasma cortisol and glucose levels	Navarro et al., 2016
		O. aureus		3 mg·L^{-1}	Increase in plasmatic cortisol levels	Akar, 2011
				and 1 mg·L^{-1}	Decrease plasmatic cortisol levels	
		S. aurata	Transport simulation/6 h	Sedation/2.5 mg·L^{-1}	Increase in plasmatic cortisol levels and expression of *star* and *cyp11b1* genes in head kidney, interferes with fish antioxidant status	Jerez-Cepa et al., 2019; Teles et al., 2019

*NA, data not available, * percentage not stated.*

fish subjected to the same procedure (Lopes et al., 2018). The EOs of *Cunila galioides* (25 and 50 $\mu L \cdot L^{-1}$) and *Origanum majorana* (100 $\mu L \cdot L^{-1}$) also decreased ion loss (Cunha et al., 2017) and *Lippia origanoides* (5–10 $\mu L \cdot L^{-1}$) reduced ventilatory frequency in silver catfish after 6 and/or 8 h of exposure (Becker et al., 2018) both compared to their respective control fish subjected to the same procedure. For instance, these EOs apparently reduced stress and may also be useful for fish transport (**Figure 2**).

Stress-Preventing Effects During High Stocking Density

Recent studies demonstrated that EOs are capable of mitigating or preventing stress caused by different stocking densities. Dietary addition of 0.50 mL per kg EO *L. alba* (linalool chemotype) prevented the increase in cortisol levels in silver catfish submitted to a stressful condition of high stocking density (Souza et al., 2015). Similarly, a diet supplemented with *M. sylvatica* EO (2.0 mL per kg) for 90 days reduced cortisol levels in gilthead seabream after 22 days held at high stocking density (40 kg m^{-3}) (Saccol et al., 2018a). These results may stimulate new studies on the influence of EOs on stress reduction caused by the different stocking densities of fish in culture systems.

Regulation of Oxidative Stress

Pro-oxidant compounds are the oxygen reactive species (ROS) that can damage DNA, proteins and lipids (Evans and Halliwell, 1999; Morel and Barouki, 1999), whereas the antioxidants are any substance that can prevent or reduce the oxidation of a target molecule (Halliwell and Gutteridge, 2007). To maintain homeostasis, fish eliminate ROS to counteract oxidative stress and prevent or repair oxidative damage by antioxidant defense system, which includes antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione-S-transferase (GST), and also some non-enzymatic antioxidants such as non-protein thiol (NPSH) (Tu et al., 2012; Birnie-Gauvin et al., 2017), in order to avoid lipid peroxidation, and carbonylation of proteins, usually evaluated by the biomarkers thiobarbituric acid-reactive substances (TBARS) and carbonyl protein (PC), respectively. In this sense, the presence of ROS in the cells triggers biochemical reactions that will culminate in decreased cellular function due to oxidative damage caused in proteins, carbohydrates and lipids, which can lead to apoptosis and accumulation of oxidized molecular aggregates. Normally, an oxidative stress frame is triggered due to an imbalance between the oxidant and the antioxidant production, detected by low concentrations of antioxidant enzymes and higher prooxidant levels, culminating with a lower immune system response (Biller and Takahashi, 2018).

Several EOs, when used as anesthetics or sedative for manipulation and/or transport, exhibit antioxidant capacity (**Tables 1**, **2**). This antioxidant activity is also observed when the EOs are used at concentrations lower than those that induce sedation and as dietary supplements (**Tables 1**–**3**). Thus, the antioxidant effects of several EOs are not only due to the possibility of lower ROS production when fish are sedated or anesthetized.

We highlight the EO of *L. alba* because it presents different responses to oxidative stress, which varies according to its concentration, chemotype, and fish species. For example, the addition of this EO (10–20 $\mu \cdot L^{-1}$, linalool chemotype) during transport suppressed oxidative stress in silver catfish (Azambuja et al., 2011). However, this EO (linalool chemotype) at higher concentrations (30–40 $\mu \cdot L^{-1}$) induced physiological and oxidative stress in silver catfish and gilthead sea bream (Salbego et al., 2014; Toni et al., 2015). On the one hand, silver catfish anesthetized with the chemotypes linalool and citral did not increase renal and hepatic thiobarbituric acid reactive species levels after anesthesia, avoiding lipid damage. On the other hand, fish anesthetized with the citral chemotype showed higher protein carbonylation levels, superoxide dismutase, catalase, and glutathione S-transferase activities as well as non-protein thiol group values in both tissues compared to controls. Therefore, the EO of both chemotypes present antioxidant capacity, but anesthesia with higher concentrations of the linalool chemotype does not cause damage to lipids or proteins, being more effective in anesthetizing silver catfish (Souza et al., 2018a).

Interestingly, anesthesia of silver catfish with the EO of *A. triphylla* (135 and 180 $mg \cdot L^{-1}$), whose main compound is citral, was capable of preventing the formation of lipid peroxides in the liver, possibly due to the increase of catalase and glutathione-S-transferase activities (Gressler et al., 2014). Similarly, anesthesia and sedation of tambaqui with the EOs of *M. sylvatica* and *C. longa* resulted in lower levels of lipid peroxidation and higher activity of antioxidant enzymes (superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase, and glutathione-S-transferase), the content of non-protein thiols and total reactive antioxidant potential in several tissues (brain, liver, gills, and kidney) compared to control (Saccol et al., 2016). Anesthesia of common carp with 30 $mg \cdot L^{-1}$ clove oil did not alter TBARS or carbonyl protein levels in several tissues, but reduced superoxide dismutase in the brain as well as glutathione reductase and glutathione peroxidase in the brain and gills (Velisek et al., 2011).

In addition, the use of EOs in water during transport is also useful in order to activate the antioxidant defense system. Silver catfish transported with the EOs of *L. alba* (chemotype linalool) (10 and 20 $\mu L \cdot L^{-1}$) and *A. triphylla* (30 and 40 $\mu L \cdot L^{-1}$) in the transport water showed improvement in the redox state (Azambuja et al., 2011; Zeppenfeld et al., 2014; Salbego et al., 2017). However, this effect is dependent on the concentrations used. So, specimens treated with the EO of *L. alba* (chemotype linalool) prior to transport (200 $\mu L \cdot L^{-1}$ for 3 min) and transported for 6 h (with 30 or 40 $\mu L \cdot L^{-1}$) decreased significantly hepatic catalase, glutathione-S-transferase, glutathione peroxidase, non-protein thiol groups, and ascorbic acid levels compared to the control group (Salbego et al., 2014). This study also revealed that hepatic TBARS, protein oxidation levels, and the lipid peroxidation/catalase+glutathione peroxidase ratio were significantly higher in fish transported with both concentrations of this EO, indicating the existence of oxidative stress at hepatic level.

TABLE 3 | The use of essential oils (EOs) as stress-reducing agents in fish diseases.

Essential oil (EO)	Major compounds (%)	Fish species	Stress/ duration	Purpose/ concentration	Effect on fish physiology	References
A. triphylla	β-citral (20.78)	*R. quelen*	Infection by *A. hydrophyla*	Diet supplemented/2.0 mL per kg diet	Improves survival and decreases the total count of leukocytes, lymphocytes, and neutrophils	Santos et al., 2017b
Citrus limon	Limonene (54.4)	*O. mossambicus*	Infection by *E. tarda*	Diet supplemented/0.5, 0.75, and 1%	Enhances non-specific immune parameters and decrease mortality rate	Baba et al., 2016
H. ringens	Pulegone (96.63)	*R. quelen*	Infection by *A. hydrophyla*	Preventive baths/20 $mg{\cdot}L^{-1}$	Increases survival	Sutili et al., 2015
Melaleuca alternifolia	Terpinen-4-ol (27.15)	*R. quelen*	Infection by *A. hydrophyla*	Preventive baths/50 $\mu L{\cdot}L^{-1}$	Increases the non-specific immune system and prevents oxidative damage	Baldissera et al., 2017a
				Preventive baths/50 $\mu L{\cdot}L^{-1}$	Prevents alterations to purinergic enzymes and ameliorates the innate immune response.	Baldissera et al., 2017b
	Terpinen-4-ol (41.98)		Infection by *P. aeruginosas*	Preventive baths/50 $\mu L{\cdot}L^{-1}$	Ameliorates the hepatic antioxidant/oxidant status	Souza et al., 2017a
Ocimum gratissimum	1,8-cineole (40.4)	*O. niloticus*	Infection by *S. agalactiae*	Diet supplemented during 55 days/0.5, 1.0, and 1.5%	Improves growth, immune responses and disease resistance	Brum et al., 2017
O. americanum	β-linalool (46.6)	*R. quelen*	Infection by *A. hydrophyla*	Preventive baths/20 $mg{\cdot}L^{-1}$	Increases survival	Sutili et al., 2015
Zataria multiflora	NA	*C. carpio*	Low temperature and challenge by *A. hydrophyla*	Diet supplemented/30 and 60 $mg{\cdot}L^{-1}$	Enhances immune system	Soltani et al., 2010
Zingiber officinale	Geranial (24.0)	*O. niloticus*	Infection by *S. agalactiae*	Diet supplemented during 55 days/0.5 and 1.0%	Improves immune responses and disease resistance	Brum et al., 2017
Syzygium aromaticum (clove oil)	Eugenol	*R. quelen*	Infection by *A. hydrophyla*	Baths (5 and 10 $mg{\cdot}L^{-1}$)	Promoted the survival but did not change phagocytic activity, production of superoxide anion, serum hemolytic activity, and hematology.	Sutili et al., 2014

NA, data not available.

Exposure to EO of *Melaleuca alternifolia* (25 $\mu L \cdot L^{-1}$ – light sedation) for 6 h decreased hepatic TBARS levels followed by an increase in glutathione-S-transferase activity (Souza et al., 2018b). Finally, a sedative concentration (30 $\mu L \cdot L^{-1}$) of *N. grandiflora* EO enhanced protection against oxidative damage mainly in muscle and gills of tambaqui transported for up to 10 h (Barbas et al., 2017b; **Figure 2**).

The dietary supplementation for 20–60 days with different EOs usually improved oxidative status of freshwater fish. Channel catfish (*Ictalurus punctatus*) fed with 0.5 mL per kg EO of *Origanum vulgare* increased plasma lysozyme, catalase, and superoxide dismutase compared to fish fed a control diet (Zheng et al., 2009). The dietary addition of the EOs of *Cymbopogon citratus* (0.2 g per kg) and *Pelargonium graveolens* (0.4 g per kg) enhanced catalase and lysozyme activities and glutathione reductase content and reduced malondialdehyde from the whole body of the Nile tilapia compared to the basal diet (Al-Sagheer et al., 2018). Silver catfish fed 2.0 mL per kg EO of *A. triphylla* presented lower TBARS, lipid hydroperoxide, superoxide dismutase, catalase, and non-protein thiols in the brain, liver, and muscle of fish fed a control diet (Zeppenfeld et al., 2017). The same species fed diets supplemented with EO of *L. alba* (linalool chemotype, around 0.5–2.0 mL per kg) stimulated superoxide dismutase, catalase and glutathione peroxidase activities and non-protein thiols content, whereas reduced lipoperoxidation and TBARS in several organs compared to those fed a control diet (Saccol et al., 2013). Finally, hepatic superoxide dismutase and glutathione peroxidase activities enhanced while catalase, glutathione reductase, glutathione-S-transferase and malondialdehyde decreased in rainbow trout fed 0.5–1.0 g per kg diet of EOs of *Salvia officinalis*, *Mentha spicata*, and *Thymus vulgaris* compared to that of the control-diet fed group (Sönmez et al., 2015).

Regulation of Immune System and/or Bactericidal Effects

The negative influence of stress on the immune system is well documented (Tort et al., 2003; Tort, 2011). In this regard, there has been a trend in the use of EOs for improving immune responses and disease resistance in fish (Bulfon et al., 2015). Often, the immunostimulants are administered as immersion or food additives, usually improving the innate (non-specific) defense mechanisms, increasing resistance to specific pathogens and promoting a recovery from immunosuppression states caused by stress (Sakai, 1999; Barman et al., 2013).

Some EOs used as anesthetics were also related as good immunostimulants for fish (**Table 3**). Anesthesia of gilthead seabream with 44.5 $mg \cdot L^{-1}$ clove oil did not change serum lysozyme activity, respiratory burst, and pinocytosis activity of head kidney (Bressler and Ron, 2004), but a concentration of 55 $mg \cdot L^{-1}$ increased serum leucocyte peroxidase activity and decreased hemolytic complement activity, which led to the conclusion that this EO did not change immune response in this species (Bahi et al., 2018). However, rainbow trout anesthetized with 25 $mg \cdot L^{-1}$ clove oil enhanced lysozyme and bactericidal activity, as well as protease, esterase, and alkaline phosphatase activities in the skin mucus, indicating potentiated skin mucosal immunity (Soltanian et al., 2018). Similarly, preventive baths with the EOs of *H. ringens* and *O. americanum* (20 $mg \cdot L^{-1}$) for 5 days increased survival in silver catfish infected with *A. hydrophila* (Sutili et al., 2015). Some EOs can have antimicrobial effects and, at the same time, act as immunostimulants. This is supported by the effects of the EO of *Melaleuca alternifolia*, which showed an antimicrobial effect against *A. hydrophila,* increased the non-specific immune system and prevented oxidative damage in silver catfish in fish exposed to 50 $\mu L \cdot L^{-1}$ for 7 days prior to infection (Baldissera et al., 2017a; **Table 3**).

The EOs may also have an immunostimulatory effect when added to the diet. For example, diet supplemented with the EO of *Zataria multiflora* enhanced common carp immunity to some extent even though fish could not express their potential immunity during stress caused by low temperature (Soltani et al., 2010). After 35 days of supplementation with EO *O. gratissimum* (0.5%), the Nile tilapia presented the lowest value of hematocrit and the highest numbers of neutrophils at 35 days. It can be inferred that this reduction is due to an energy offset for production of defense cells – in this case, neutrophils (Brum et al., 2017; **Table 3**). Adel et al. (2015) also observed a significant increase in neutrophils in the cyprinid kutum (*Rutilus frisii*) after dietary supplementation with the EO of *Mentha piperita*, asserting that there was stimulation of the innate immune response. However in spite of improving survival of silver catfish after *A. hydrophila* infection, the addition of 2.0 mL per kg diet of EO of *A. triphylla* showed an immunosuppressive activity because the total count of leukocytes, lymphocytes and neutrophils decreased (Santos et al., 2017b). In addition, supplementing the diet with EO of *Citrus limon* peels (0.5, 0.75, and 1%) improve non-specific immune parameters and decreased mortality rates in the Mozambique tilapia *Oreochromis mossambicus* challenged with *Edwardsiella tarda* (Baba et al., 2016; **Table 3**). These results indicate that diets enriched with EOs often present promising results for fish, and some EOs may even aid in improving the immune system as well as helping to prevent disease outbreaks in aquaculture systems.

CONCLUSION AND FUTURE PERSPECTIVES ON THE USE OF EOS IN AQUACULTURE

Some EOs demonstrated advantages when used as sedatives/anesthetics compared to synthetic compounds because they are not aversive to fish, and reduce the stress responses related to handling and transport. On the other hand, their effects may vary according to plant variables such as chemotype, place of collection, climate, anatomical part from which the EO was extracted, altering significantly their composition and consequently their effects. Consequently, EOs should be obtained from cultivated plants that have genetic homogeneity, to ensure the consistency of their composition. Another perspective to be followed by future studies and by the pharmaceutical industry would be isolating/purifying the active compounds present in

the EOs to obtain more refined products that combine their beneficial effects with a better control of composition and maybe avoid undesired effects of uncontrolled compounds. Some isolated compounds, as linalool, have proportional anesthetic effect to the EO of *L. alba* chemotype linalool in silver catfish, but eugenol, which is the main compound of *O. gratissimum*, presents a narrower safety range that this EO. Additional studies comparing the effects and side effects of EOs and their isolated compounds are necessary to evaluate the advantages of using a given EO or its main compound(s). In addition, it is important to emphasize that the use of different concentrations of the EOs and the different fish species used can alter behavioral and physiological responses, as observed in synthetic anesthetics. As explained in a previous section, the stress-reducing effect of eugenol is better the faster the anesthesia induction. Is this relationship concentration/time to induce anesthesia/stress-reducing effect similar for EOs and their isolated compounds? Could this possible relationship explain, at least partially, the species-specific effects of the EOs studied so far?

Most studies show that EO also have promising potential for maintaining and promoting health, as well as preventing and potentially treating some diseases, ameliorating growth and antioxidant status. However, besides the high volatility, EOs can easily decompose, owing to direct exposure to heat, humidity, light, or oxygen, which would imply the loss of their effectiveness (Turek and Stintzing, 2013). Thus, it is interesting to develop technology to protect EOs when exposed to temperature variations and even to protect from low pH during gastric absorption (Zhang et al., 2016). Studies regarding the use of diets supplemented with several EOs to minimize the stress caused by handling or air exposure remain scarce. In addition, due to the species-specific effects of EOs, it would be interesting to check the efficacy of these EOs on more fresh- and seawater species.

The use of nanotechnology can potentialize the EO effects. Lecithin-based nanoemulsion of clove oil showed faster anesthesia induction and recovery in goldfish, *Carassius auratus*. Serum glucose was not different, but serum urea was higher in goldfish anesthetized with the nanoemulsion than with free clove oil (Gholipourkanani et al., 2015). The nanoencapsulated EO of *M. alternifolia* exert potent bactericidal action, presenting 100% of curative efficacy in silver catfish infected with *Pseudomonas aeruginosa*, while free EO only showed 70% of curative efficacy (Souza et al., 2017c). Consequently, nanotechnology shows promising results that deserve further analysis.

Medicinal plants have broad antimicrobial properties against important fish pathogens. Further studies on chronic and acute toxicity and on the deleterious effects of herbal medicines on treated organisms, non-target organisms, and the environment are encouraged. Most studies on effectiveness have been based on *in vitro* testing or even conducted under laboratory conditions. Therefore, further practical and economic studies are needed to enable replacement of the current treatments. Joint work between the supply chain, industry, and researchers are paramount to studying medicinal plants that may present antimicrobial effects against important fish pathogens.

AUTHOR CONTRIBUTIONS

CS wrote the manuscript and drew the figures. BH wrote from the section "Essential Oils." BB, JM-S, JM, and MB carried out the corrections on the manuscript.

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Brain and Pituitary Response to Vaccination in Gilthead Seabream (*Sparus aurata* L.)

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Vaccination is a widely used therapeutical strategy in aquaculture, but whether vaccination elicits stress responses in the central neuroendocrine system and enhances the crosstalk between the immune and endocrine systems in the brain or pituitary after vaccination is unclear. To answer this question two experiments using two different vaccine exposure routes, i.e., bath or intraperitoneal (i.p.) injection, were carried out on gilthead seabream (*Sparus aurata* L.). In the first one, the stress responses of fish subjected to waterborne *Vibrio anguillarum* bacterin were compared with responses after air exposure or their combination. In the second experiment, fish were subjected to an intraperitoneal injection of *Lactococcus garvieae* bacterin and we assessed the central stress response and also whether or not a significant immune response was induced in brain and pituitary. In both experiments, blood, brain and pituitary tissues were collected at 1, 6, and 24 h post stress for plasma hormone determination and gene expression analysis, respectively. Results indicated that bath vaccination induced a decreased central stress response compared to air exposure which stimulated both brain and pituitary stress genes. In the second experiment, injection vaccination kept unchanged plasma stress hormones except cortisol that raised at 6 and 24 h. In agreement, non-significant or slight changes on the transcription of stress-related genes were recorded, including the hormone genes of the hypothalamic pituitary interrenal (HPI) axis and other stress markers such as *hsp70*, *hsp90,* and *mt* genes in either brain or pituitary. Significant changes were observed, however, in *crhbp* and *gr*. In this second experiment the immune genes *il1*β, *cox2,* and *lys*, showed a strong expression in both brain and pituitary after vaccination, notably *il1*β which showed more than 10 fold raise. Overall, vaccination procedures, although showing a cortisol response, did not induce other major stress response in brain or pituitary, regardless the administration route. Other than main changes, the alteration of *crhbp* and *gr* suggests that these genes could play a relevant role in the feedback regulation of HPI axis after vaccination. In addition, from the results obtained in this work, it is also demonstrated that the immune system maintains a high activity in both brain and pituitary after vaccine injection.

Keywords: brain, pituitary, vaccination, immune response, stress response

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INTRODUCTION

Stress is defined as a state of real or perceived challenge for homeostasis that induces a response consisting in an array of biological reactions to compensate for the consequences of the threat created by the stressor (Tort and Teles, 2011; Schreck and Tort, 2016). After the stressor is perceived, the neuroendocrine cells of the ventral parvocellular section of the nucleus preopticus, secrete different neuroendocrine players: Corticotropin Releasing Hormone (CRH), CRH Binding Peptide (CRHBP), Arginin Vasotoccin (AVT) and Thyroid Releasing Hormone (TRH) that control the production of adrenocorticotropic hormone (ACTH) in corticotropic cells of the anterior pituitary gland (Flik et al., 2006). The release of ACTH into the bloodstream and interaction with the receptors of interrenal tissue, will subsequently induce cortisol release (Gorissen and Flik, 2016). Cortisol acts as a multifunctional hormone via binding to its receptors, the mineralocorticoid receptor (MR) and glucocorticoid receptors (GR), which are ubiquitously expressed in almost all tissues (Sapolsky et al., 2000; Teles et al., 2013). During the stress response, cortisol will redirect energy utilization among various organs in order to overcome the increased metabolic demand imposed by the stressor challenge. As a consequence, some processes such as immune response mechanisms may be affected or delayed (Kaattari and Tripp, 1987; Padgett and Glaser, 2003; Kudielka and Kirschbaum, 2007). For instance, hypothalamic CRH may act as an anti-inflammatory via stimulation of glucocorticoids and catecholamines; peripheral CRH acts as pro-inflammatory through direct action on immune cells (Karalis et al., 1997; Quintanar and Guzman-Soto, 2013) and cortisol acts generally as immunosuppressor or immunomodulator (Tort, 2011). Besides, ACTH has been reported to present immunoreactive activity in the thymus of goldfish (*Carassius auratus*) (Ottaviani et al., 1995). In addition, the expression of some immune genes in the central nervous system has been reported, and this suggests a potential cross-interaction between brain immune and neuroendocrine systems (Metz et al., 2006). Assuming that brain and pituitary are the hierarchical onset organs of the stress reaction (Cerdá-Reverter and Canosa, 2009), other central interactions have been shown to occur at brain and pituitary level, particularly the cortisol feed-back interaction via GR (Gorissen and Flik, 2016).

Vaccination is the most effective method used nowadays in aquaculture to prevent diseases caused by pathogens (Plant and LaPatra, 2011). Available data indicates that 2 h after *Vibrio anguillarum* bacterin exposure, the expression of both pro- and anti-inflammatory genes increase in gilthead seabream (*Sparus aurata*) head kidney primary cell culture (Khansari et al., 2017). Moreover, vaccination by immersion leads to alteration of some immune genes including complement *c3*, tumor necrosis factor alpha (*tnfα*), lysozyme (*lys*) *or* transforming-growth factor beta (*tgfβ*) in seabream mucosal tissues such as skin and gut (Khansari et al., 2018). Therefore, these previous results demonstrate that a non-specific immune response is elicited in immune tissues of fish shortly after vaccination. Also, serum or tissue antibodies such as immunoglobulin M and immunoglobulin T will increase at long-term after vaccination (Lamers et al., 1985; Mutoloki et al., 2015), together with some specific immune responses, thus contributing to the increased survival rate when fish are challenged a second time with a pathogen (Rodgers, 1990; Figueras et al., 1998). Similarly, the phagocytic activity of head kidney leucocytes isolated from turbot (*Scophthalmus maximus* L.) enhanced at 7 days post vaccination, and such increase lasted as long as 42 days (Figueras et al., 1998).

There are several vaccine delivery methods, including oral, immersion and injection, of which injection often shows better protection (Plant and LaPatra, 2011). However, the injection procedure can produce adverse reactions due to stress (Hastein et al., 2005), and this unavoidable stress is associated with short-term increase of plasma cortisol (Funk et al., 2004; Skinner et al., 2010). Work on stress or immune effects of vaccine delivered by intraperitoneal injection has been previously reported and shown to be dose and temperature dependent (Martínez et al., 2018; Oyarzún et al., 2019). Nevertheless, few data is available regarding the effects of vaccine on the Hypothalamus-Pituitary-Interrenal (HPI) axis at the brain and pituitary level. In a previous study of our research group, it has been shown that bacterin could elicit immune responses in cultured pituitary cells of rainbow trout (*Oncorhynchus mykiss*) (Liu et al., 2019), and so did when adding medium from cultured spleen cells to pituitary tissue preparations (Liu et al., 2019).

Taking all the above into consideration, the goal of the present study was to investigate the effect of vaccine in both brain and pituitary through different vaccination routes. We hypothesized that: (1) bath vaccination might evoke a significant stress response of the central neuroendocrine organs of fish; and (2) the bacterin vaccines can induce both a stress and immune response in brain and pituitary. To test these hypotheses, two experiments were performed: In the first experiment, *S. aurata* individuals were vaccinated by bath vaccine, subjected to air exposure stress or subjected to both (vaccine and air exposure). Plasma cortisol content as well as gene transcripts relevant to stress responses, specifically, *crh*, *crhbp*, *pomca*, *pomcb, gr, trh, gh, prl, sl1, and sl2* were tested in the pituitary and/or brain at 1, 6, and 24 h post treatments. In the second experiment, we tested whether a vaccine administered through intraperitoneal injection was able to elicit a central stress response. It was also evaluated whether brain and pituitary showed a significant immune response. As fish were taken out of the water for the injection, the responses to vaccination were tested against the air-exposed mock group, thus allowing consistent comparisons with the air exposure group from the first experiment. The air exposure stressor was selected for two reasons. One, because this is a previously used and validated type of stressor related to hypoxia or anoxia experiments (Skrzynska et al., 2018). Second, because we wanted to differentiate the response of the vaccine itself compared to the response induced by a non-biotic physical stressor.

MATERIALS AND METHODS

Fish Husbandry and Experimental Design

Two batches of gilthead seabream (110.8 ± 13.4 g and 285.6 ± 30.2 g) were transported in March and September 2017, respectively, from *Aquacultura Els Alfacs* (Tarragona, north-east Spain). Fish were stocked in the indoor water circular tanks (2000 L) 20 days at the Universitat Autonoma de Barcelona fish facility (AQUAB), under a 12L: 12D photoperiod, 21.4 ± 0.6°C temperature, and they were fed with a commercial diet (Skretting) once per day at a maintenance ration (1.5% body weight). During this period, no clinical signals of disease, malformation or injuries were observed, nor altered behavior. Water parameters including pH, NO_2, NO_3, NH_4/NH_3, temperature, and salinity were monitored every day. All experimental procedures were submitted by the Ethical Committee of the Universitat Autonoma de Barcelona (CEEAH), in accordance with the international European Guiding Principles for Biomedical Research Involving Animals (EU2010/63) and authorized by the regional authority (Generalitat de Catalunya Procedure Ref. 10208).

Vaccines and Sample Collection

Experiment 1

Seabream were vaccinated with ICTHIOVAC ^{R}VR by immersion according to guidelines recommended by the company (HIPRA). ICTHIOVAC ^{R}VR (HIPRA) is an inactivated commercial vaccine which is suitable for immersion delivery. The composition consists of inactivated *V. anguillarum*, serotype O1, O2α, and O2β with relative percent survival RPS ≥ 60%, presenting all pathogenic serotypes of the bacterium, including the serogroup O2α that is the most pathogenic serogroup of the bacterium (Frans et al., 2011). The second stressor, air exposure, consisted in 3 min out of the water. To this end, four groups of fish ($n = 18$ fish per group) were used for the experiment: (i) control group, fish treated with water free-vaccine in bucket, (ii) group treated with the vaccine, (iii) group subjected to air exposure during 3 min, and (iv) group exposed to both air exposure and vaccine. There were two replicate tanks in each group. It is worth to mention that vaccination was performed 24 h before air-exposure stress since the preliminary result in systemic immune organs did not show any significant alteration by vaccine at early time of vaccination (data not shown). Fish were sampled after 1, 6 and 24 h. Fish were anesthetized by an overdose of tricaine methanosulphonate (MS222) and the blood from each fish was quickly collected from the caudal vein by using a heparinized 2 mL syringe. After fast blood collection, the pituitary gland and brain of each fish were excised, immediately frozen in liquid nitrogen and stored under −80°C until use.

Experiment 2

Before the start of the experiment, a total of 36 fish were randomly divided into 2 groups (with two replicate tanks per group) as for mock injection and vaccination, and these fish were acclimatized for another 5 days in 200 L water circular tanks. During this period, water parameters and rearing conditions were kept the same as mentioned above. After 24 h fasting, all fish were slightly anesthetized by MS222 (0,1 g/L) Sigma-Aldrich, United States), and then they were quickly intraperitoneally injected with 1 mL sterilized PBS or 1 mL ICHTHIO-LG for the mock injection or vaccination groups, respectively. After the injection, fish were immediately returned to the corresponding experimental tanks. The whole operation lasted less than 3 min. Fish from both mock injection and vaccination groups were sampled at 1, 6, and 24 h post injection, and blood, pituitary and brain of 6 fish from each group at each sampling time point were collected. In brief, fish was anesthetized by an overdose of MS222, the blood of each fish was quickly collected from the caudal vein by using a 5 mL syringe, which was pre-rinsed with lithium heparin (Deltalab, Spain), and then transferred to a clean tube with one drop of lithium heparin. After fast blood collection, the pituitary gland and brain of each fish were excised, immediately frozen in liquid nitrogen and stored under -80^{o}C until use. ICTHIO-LG *Lactococosis* (HIPRA, Spain) is a vaccine obtained from inactivated *Lactococcus garvieae*, a pathogenic agent for both cultured freshwater and marine fish at water temperature above 15°C. The composition consists of inactivated *L. garvieae* with RPS > 75%.

Plasma Isolation and Test of Biochemical Parameters

Plasma was separated by blood centrifugation at 1500 × *g* for 10 min at 4°C. Then the isolated plasma of each fish was transferred to a clean tube and stored at −20°C until biochemical analyses. Plasma CRH and ACTH concentration were detected by using Fish CRH ELISA Kit (Cat: CK-E93386F, Yuan Ye Biotechnology, Shanghai, China) and Fish ACTH ELISA Kit (Cat: CK-E 93337F, Yuan Ye Biotechnology, Shanghai, China) according to manufacturer instructions, respectively. These Elisa kits used in the present study showed sensitivities of about 1.0 pg/mL. The intraassay coefficients of variation were <15% for both two kits. Seabream plasma cortisol levels were measured by radioimmunoassay (RIA) as described by Rotllant et al., 2006 (antibody from MO bio-medical LLC, United States, final dilution 1:4500, lower detection limit of the cortisol assay: 0.16 ng/mL, 100% antibody cross-reactivity with cortisol).

Total RNA Extraction and Reverse Transcription Quantitative Real-Time PCR (RT-qPCR)

Total RNA of each tissue was extracted according to the manufacturer's instructions with TRI reagent (Sigma-Aldrich, United States). The RNA concentration of RNA (260 nm) and the purity ratio (A260/A280) was measured with NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific Inc., United States). First-strand cDNA of each sample was synthesized from 1 μg total RNA by using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, United States) according to the user's manual.

RT-qPCR was performed using iTaq^{TM} Universal SYBR®Green Supermix (Bio-Rad, United States) in a CFX Touch^{TM} Real-Time PCR Detection System (Bio-Rad, United States). In brief, a volume of 10 μL containing 0.4 μM of each upstream and downstream primer (**Table 1**), 2 μL of cDNA

TABLE 1 | Primer information used in the present study.

genes	Primer sequence (5′-3′)	Accession number	Product size (bp)	Efficiency
Il1β	F: TCAGCACCGCAGAAGAAAAC R: TAACACTCTCCACCCTCCAC	AJ277166.2	115	1.97
cox2	F: GAGTACTGGAAGCCGAGCAC R: GATATCACTGCCGCCTGAGT	AM296029.1	192	1.89
tnfα	F: TCGTTCAGAGTCTCCTGCAG R:AAGAATTCTTAAAGTGCAAACACACCAAA	AJ413189.2	320	2.24
c3	F: GTTCCACAACAACCCACAGC R:ACATACGCCATCCCATCCAC	HM543456.1	183	1.91
lys	F: TCATCGCTGCCATCATCTCC R:TGTTCCTCACTGTCCCATGC	AM749959.1	154	2.08
tgfβ1	F: AGACCCTTCAGAACTGGCTC R:ACTGCTTTGTCTCCCCTACC	AF424703.1	145	1.9
il10	F: GATCTGCTGGATGGACTGC R: GAGCGTGGAGGAATCTTTCAA	JX976621.1	154	2.02
il6	F: ATCCCCTCACTTCCAGCAGA R: GCTCTTCGGCTCCTCTTTCT	EU244588.1	129	2.04
hsp70	F: AGGTTGGGTCTGAAAGGAAC R: TGAACTCTGCGATGAAGTGG	EU805481.1	174	1.96
hsp90	F: GTGGATTCTGAGGACCTGCC R: GAGAGTCTTCGTGGATGCCC	DQ524994.1	196	1.96
mt	F: CTCTAAGACTGGAACCTG R: GGGCAGCATGAGCAGGAG	U93206.1	93	2.07
crh	F: ATGGAGAGGGGAAGGAGGT R: ATCTTTGGCGGACTGGAAA	KC195964.1	176	1.86
trh	F: GAAACGCTTTTGGGATAACTCC R: CGGCGTGACTCTTGTTTATGTT	KC196277.1	131	2.24
gh	F: CGTCTCTTCTCAGCCGAT R: GCTGGTCCTCCGTCTGC	U01301.1	131	1.79
prl	F: TGACATCGGCGAGGACAACATT R: CGGCAGCGGAGGACTTTCAG	AJ509807.1	111	1.84
crhbp	F: GCAGCTTCTCCATCATCTACC R: ACGTGTCGATACCGCTTCC	KC195965.1	147	1.95
pomca	F: AGCCAGAAGAGAGAGCAGTGAT R: ATCGGGTCAGAAAACACTCA	HM584909.1	120	1.92
pomcb	F: AGCTCGCCAGTGAGCTGT R: CCTCCTGCATCACTTCCTG	HM584910.1	81	2.07
gr	F: TGCTGGCGGAGATCATCACCA R:GCAGGCCAAGCGAAGGCTTA	DQ486890.1	182	2.01
18s	F: ACCAGACAAATCGCTCCACC R: AGGAATTGACGGGAAGGGCAC	AY587263.1	172	2.02
rpl27	F: AAGAGGAACACAACTCACTGCCCCAC	AY188520.1	160	2.01
	R: GCTTGCCTTTGCCCAGAACTTTGTAG			

product, 2.6 μl of MQ water, and 5 μL of 2 × iTaq Universal SYBR green Supermix were used for the RT-qPCR reaction. The cycling condition consisted of an initial denaturation cycle for 5 min at 95°C, 40 cycles of 15 s at 95°C, 30 s at 60°C. A melting curve analysis was carried out after the completion of RT-qPCR to verify no non-specific amplification. The reference genes 18S and RPL27 were used for normalization. The quantification was performed according to Pfaffl method (Pfaffl, 2001) and corrected for the efficiency of each primer set. Value for each experimental condition was expressed as normalized relative expression, calculated in relation to the values of control group and normalized against those of the reference gene 18S. The amplification efficiency and product size are listed in **Table 1**. Six biological replicates with two technical replicates were performed for the qPCR analysis.

Statistics

For the first experiment the statistical package for social science (SPSS, v20) software was used for the analysis. The Generalized Linear Model (GzLM) was utilized considering the stressors and time dynamics as a two between-subjects factor. This model is a more flexible statistical tool than the standard general linear model (GLM) in terms of types of distribution and different covariance structure of the repeated measures does not require homogeneity of variance and it admits missing values. After the main analysis, appropriate pairwise comparisons were carried out. In the second experiment, we used either one-way ANOVA followed by Fisher's LSD post-hot test, or unpaired student's *t*-test if the equal variances were not assumed. Differences among groups were considered significant when $P < 0.05$. All results were expressed as mean ± SEM.

RESULTS

Brain and Pituitary Stress Response to Bath Vaccine

Figure 1 shows the levels of plasma cortisol after both stressors and its combination at the respective time points. Cortisol did

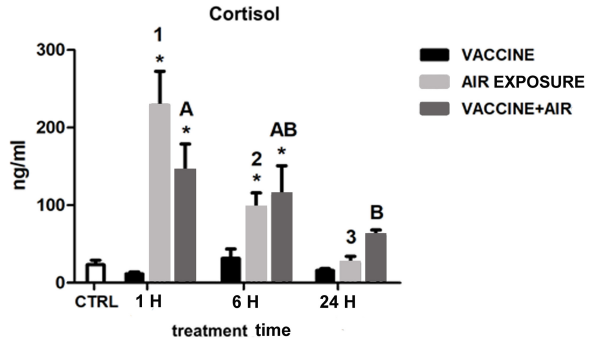

FIGURE 1 | Plasma cortisol concentration in gilthead seabream (*Sparus aurata* L.) at 1, 6, and 24 h post challenge after air exposure and *Vibrio* vaccine. Data are presented as mean ± SEM. Significant differences are indicated by numbers in air exposure group and by capital letters in vaccine plus air exposure group. Asterisk (*) indicates significant difference vs. control and the absence of a symbol indicates no difference ($P < 0.05$; General-linear-Model test was performed for multiple comparison).

not show any significant response after bath vaccine treatment compared to air exposure stress, which showed a classical acute response dynamics with a peak at 1 h, still significantly higher at 6 h, followed by further recovery of basal values at 24 h. The dynamics of the vaccine plus air exposure was similar to that observed after air exposure, although the recovery took place later on, indicating that the air exposure stressor was predominant in the cortisol response.

Regarding the response of the analyzed stress-related genes in the brain, no relevant changes were observed after bath vaccine treatment, except for a decrease of *crhbp* at 1 h, whereas air exposure showed significant increases in *crh, crhbp,* and *gr* and a significant down-regulation of *trh*. When both stressors were applied, *trh* was maintained down-regulated at 6 h and only *crhbp* increased significantly (**Figure 2**).

In the pituitary, bath vaccination showed a differential induction of *pomc* genes at short time (1 h) whereas at 6 h *prl* and *gh* showed significant increases. Air exposure increased the expression of *pomcb, gr* and *sl1* at 1 h, and *gr* at 6 h. After applying both stressors only slight changes were detected as for the reductions of *pomcb* at 6 h and *sl1* at 1 h, and the increase of *sl1* at 6 h (**Figure 3**).

Brain and Pituitary Stress and Immune Responses to Injected Vaccine

Plasma cortisol values significantly raised by 2.2- and 6.4-fold compared to the corresponding mock groups at 6 and 24 h post injection, respectively ($P < 0.05$). The differential cortisol increase of the vaccine-injected fish compared to the mock-injected fish was apparent at all time points. Regarding time course, both injected vaccine and mock groups presented the same cortisol dynamics, i.e., increases at 1 and 6 h and recovery at 24 h. However, the vaccinated group showed higher levels at either time compared to mock-injected group. The vaccine injected group also showed higher resistance to recovery at 24 h. Regarding CRH or ACTH, no alteration of plasma content in either group (mock or vaccination) was observed in none of different time points assessed 1, 6, and 24 h post injection (**Figure 4**).

In the second experiment, the expression of *crh* was almost unchanged at 1, 6, and 24 h post vaccination. Transcript of *gr* was not altered at 1 or 24 h, while there was a slight but significant up-regulation at 6 h post vaccination in the mock group. A similar trend can be observed in the heat shock proteins (HSP) *hsp70* and *hsp90* in which a significant increase was also observed at the same time point (6 h). As a whole, few changes were observed in brain genes, and the changes were higher in mock-injected fish than in vaccine-injected fish (**Figure 5**).

Similar than with the bath vaccine, the expression of stress genes in the pituitary showed a different pattern in which one gene, CRH binding protein (*crhbp*), substantially increased its expression (up to 15 fold at 6 h or up to 7 fold after 24 h) after vaccine injection. These increases contrast with the mock injected

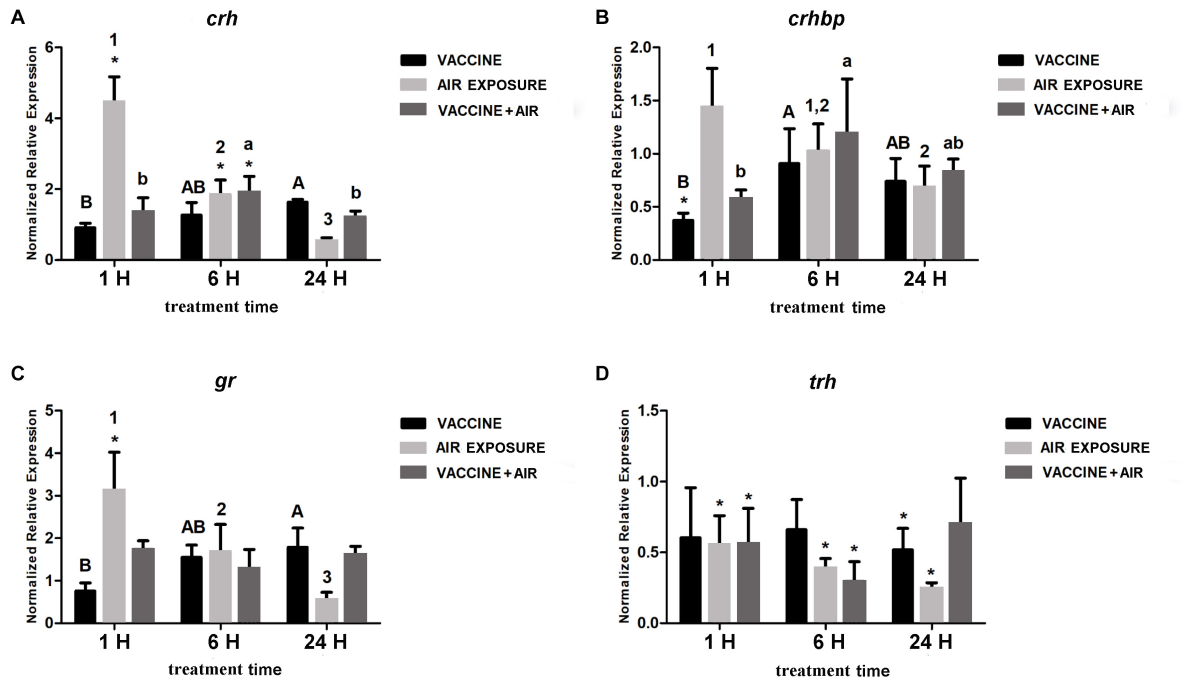

FIGURE 2 | qPCR quantification of specific mRNA accumulation in gilthead seabream (*Sparus aurata* L.) brain at 1, 6, and 24 h post challenge with *Vibrio anguillarum* exposure and air exposure. **(A)** *crh*; **(B)** *crhbp*; **(C)** *gr*; and **(D)** *trh* were shown as mRNA relative abundance. Data are presented as mean ± SEM. Significant differences are indicated by capital letters in vaccine group, by numbers in air exposure group, and by lowercase letters vaccine plus air exposure. Asterisk (*) indicates significant difference versus control and the absence of a symbol indicates no difference ($P < 0.05$; General-linear-Model test was performed for multiple comparison).

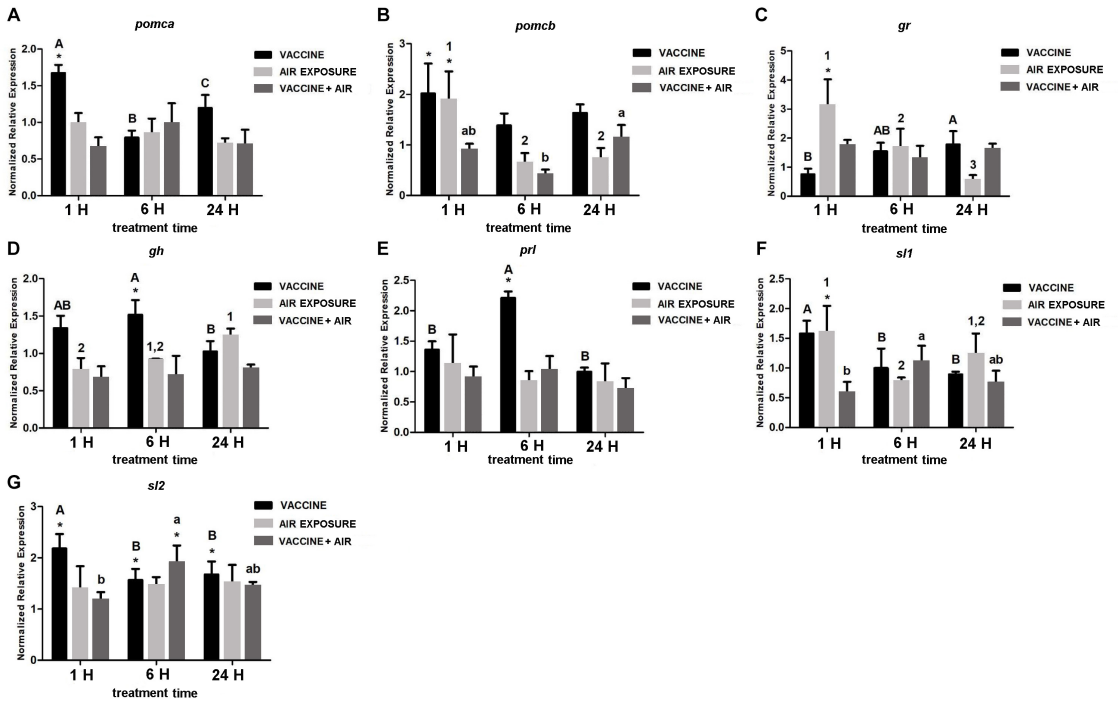

FIGURE 3 | qPCR quantification of specific mRNA accumulation in gilthead seabream (*Sparus aurata* L.) pituitary at 1, 6, and 24 h post challenge with air exposure and *Vibrio anguillarum* exposure. **(A)** *pomca*; **(B)** *pomcb*; **(C)** *gr*; **(D)** *gh*; **(E)** *prl*; **(F)** *sl1*; and **(G)** *sl2* were shown as mRNA relative abundance. Data are presented as mean ± SEM. Significant differences are indicated by capital letters in vaccine group, by numbers in air exposure group, and by lowercase letters vaccine plus air exposure. Asterisk (*) indicates significant difference versus control and the absence of a symbol indicates no difference ($P < 0.05$; General-linear-Model test was performed for multiple comparison).

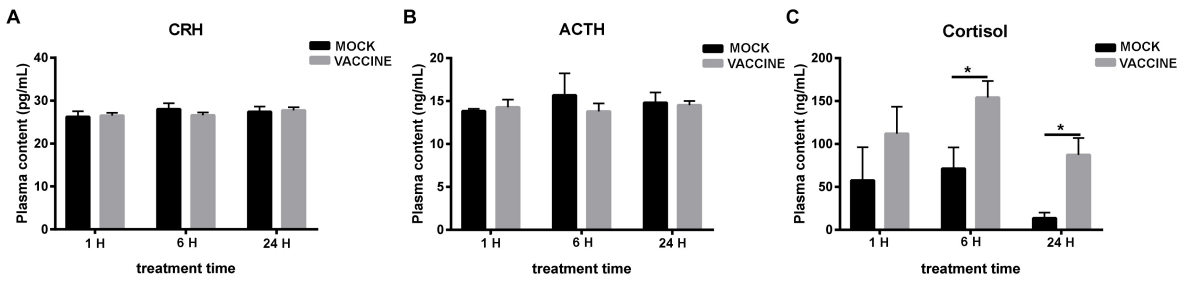

FIGURE 4 | Plasma CRH **(A)**, ACTH **(B)**, and cortisol **(C)** concentration in gilthead seabream (*Sparus aurata* L.) after *Lactococcus* bacterin or mock injection. Asterisk (*) indicates significant difference between mock and vaccination groups of each time point ($P < 0.05$).

fish in which the increase was moderate (between 3 and 5 fold), though showing the same dynamics. A similar significant trend, but more moderate (over two fold increases), was observed for *hsp70* but not for *hsp90*. The rest of the genes assessed, although showing some variations, did not change significantly their expression except for *gr* in which a significant down-regulation was observed at 6 h (**Figure 6**).

mRNA Expression of Immune Genes in Brain and Pituitary

In the second experiment it was intended to determine whether an injected vaccine induced immune gene expression changes in brain and pituitary other than in stress-related genes. The results showed a very clear picture as not only some cytokines increased their expression but the level of induction was very strong. Thus, the main pro-inflammatory cytokine *il1*β dramatically raised in the vaccination group at all three time points: 1, 6, and 24 h, by 12.0, 9.36, and 7.44 fold, respectively. Similarly, *cox2* was significantly up-regulated by 5.25, 7.77, and 3.46 fold in the vaccination group at 1, 6, and 24 h post injection, respectively. The expression level of both *il1*β and *cox2* peaked at 6 h post vaccination group. Other pro-inflammatory gene transcripts such as *il6* raised significantly only 1.7 fold at 1 h post vaccination ($P < 0.01$), and *tnf*α was kept almost unchanged.

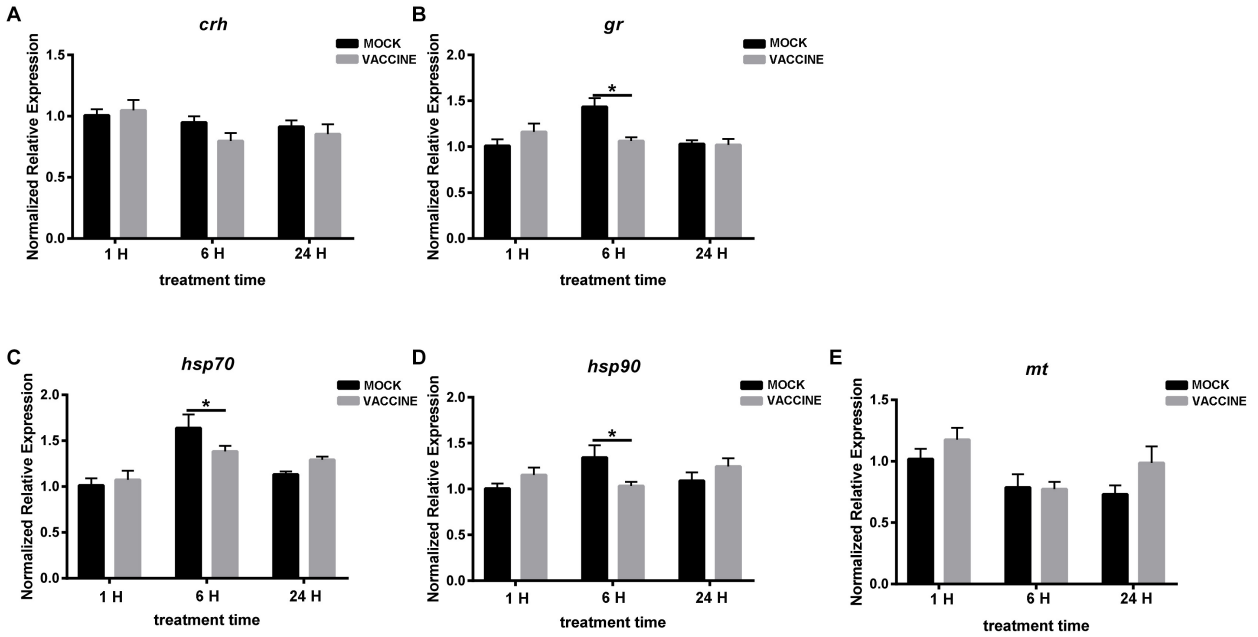

FIGURE 5 | mRNA expression of genes involved in the HPI axis in the brain of gilthead seabream (*Sparus aurata* L.) after *Lactococcus* bacterin or mock injection. **(A)** *crh*; **(B)** *gr*; **(C)** *hsp70*; **(D)** *hsp90*; and **(E)** *mt*. Asterisk (*) indicates significant difference between mock and vaccination groups of each time point ($P < 0.05$).

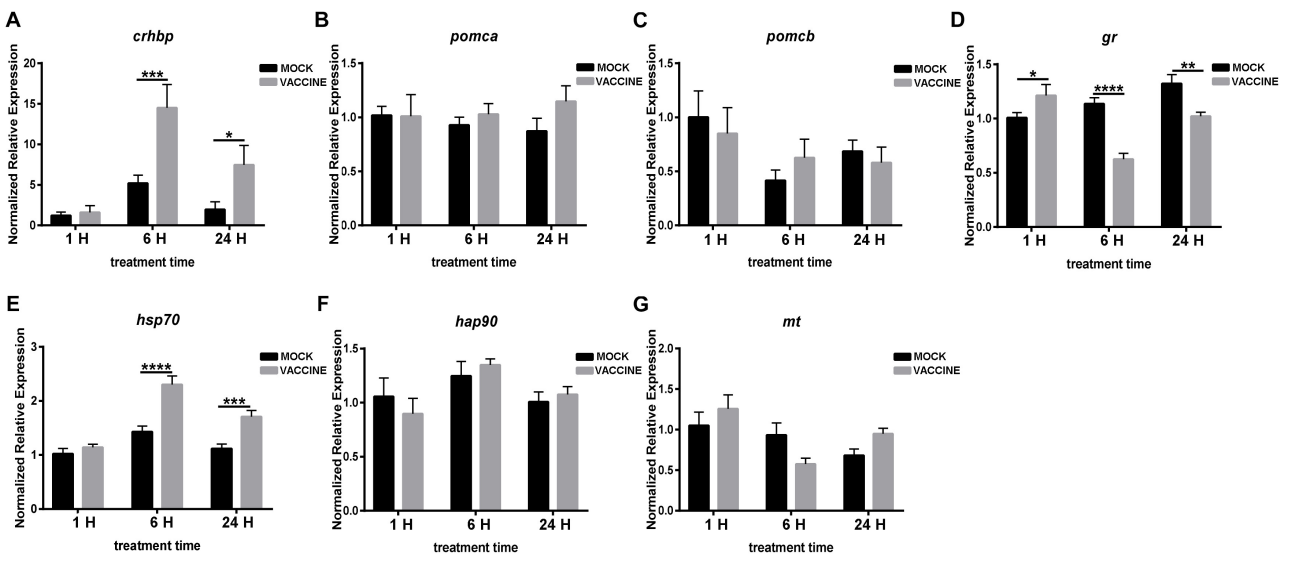

FIGURE 6 | mRNA expression of genes involved in the HPI axis in the pituitary of gilthead seabream (*Sparus aurata* L.) after *Lactococcus* bacterin or mock injection. **(A)** *crhbp*, **(B)** *pomca*, **(C)** *pomcb*; **(D)** *gr*; **(E)** *hsp70*; **(F)** *hsp90*; and **(G)** *mt*. Asterisk (*) indicates significant difference between mock and vaccination groups of each time point ($P < 0.05$).

Differently from the pro-inflammatory genes, the classical anti-inflammatory genes *tgf*β and *il10* showed no significant alteration in the vaccinated groups when compared with the corresponding mock injection groups. Nevertheless, the mean values showed a non-significant but apparent increasing trend (**Figure 7**). The expression of *lysozyme* gene (*lys*) significantly increased in the brain of seabream at 6 h post vaccination, although with just 1.61 fold, and a moderate raise was also observed at 24 h post injection. The transcript for the complement C3 component gene (c3) showed a significant increase at 1 h post vaccination, by 2.35 fold; however, it decreased at 6 and 24 h after vaccination when compared with the corresponding mock injection groups (**Figure 7**).

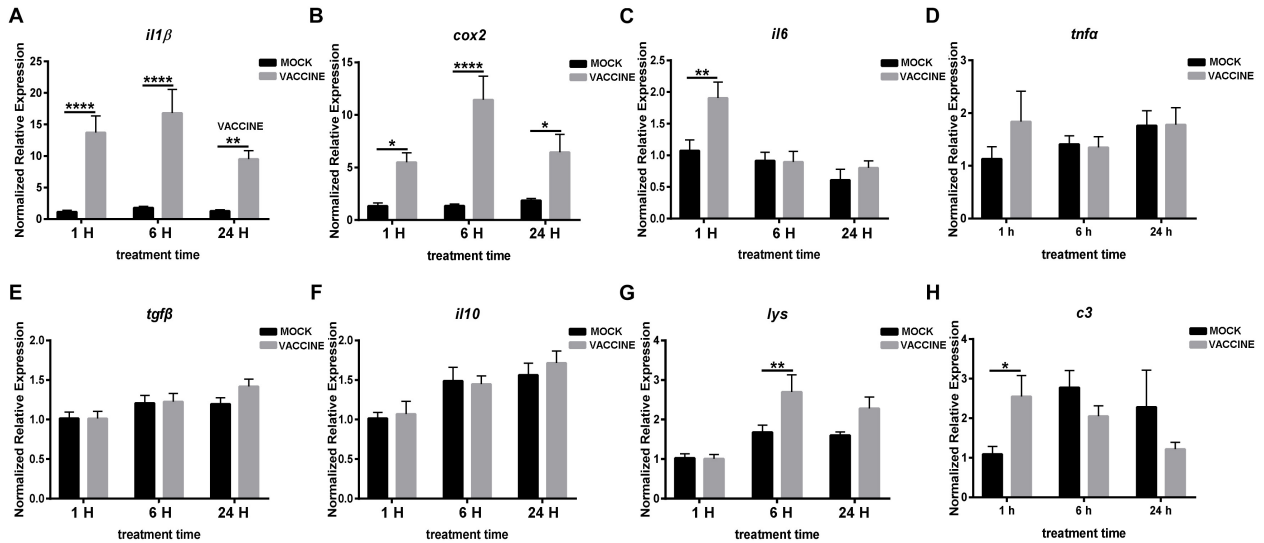

FIGURE 7 | RNA expression of pro-inflammatory genes *il1*β **(A)**, *cox2* **(B)**, *il6* **(C)**, *tnf*α **(D)**, anti-inflammatory genes *tgf*β **(E)**, *il10* **(F)** and some other immune components *lys* **(G)** and *c3* **(H)** in the brain of gilthead seabream (*Sparus aurata* L.) after *Lactococcus* bacterin or mock injection. Asterisk (*) indicates significant difference between mock and vaccination groups of each time point ($P < 0.05$).

Regarding pituitary, the expression of genes related to the immune responses are shown in **Figure 8**. The expression of pro-inflammatory gene *il1*β sharply and strongly increased at the three time points post vaccination, and it was significantly up-regulated by 20.65 and 2.94 fold at 1 and 24 h, respectively. A similar alteration trend was observed for the expression of *cox2* after vaccination, however, with less intensity. The *cox2* transcript raised by 4.37, 3.16, and 2.52 fold in 1, 6, and 24 h vaccination groups, respectively, but significance was observed only at 1 and 6 h time points. The *tnf*α transcript was up-regulated at 1 and 6 h post vaccination, and the significance was only observed in the early phase of vaccination (1 h), by 2.74 fold. The anti-inflammatory gene *tgf*β showed no alteration after vaccination, and *il10* presented a raising trend, although significant induction was only observed at 6 h. Compared to the mock injection, *lys* showed comparable levels at 1 h post injection, while it was distinctly up-regulated by 10.59 and 3.25 folds at 6 and 24 h, respectively.

DISCUSSION

Brain and Pituitary Stress Response to Vaccines

The combined results from the two vaccination experiments indicate that fish perceives the vaccine as a stressor but at a limited extent. Thus, bath vaccine did not induce plasma cortisol rise, while injection vaccine did produce a differential cortisol response compared to mock injection. In addition, neither plasma CRH nor ACTH values showed important alterations after vaccine injection. Therefore, in terms of plasma hormones, it seems that fish would not perceive vaccines as primary stressors stimulating the hierarchical activation of HPI axis, although it would indirectly activate cortisol release in the case of vaccine injection linked to the air exposition period during the injection procedure. In both experiments cortisol presented an acute response dynamics, peaking at 1 or 6 h and recovering at 24 h, in agreement with the studies previously reported for this species after subjecting seabream to acute stressors such as air exposure (Arends et al., 1999; Skrzynska et al., 2018). Similar increases of cortisol concentration in rainbow trout treated by vaccine injection have been previously reported as well (Funk et al., 2004; Skinner et al., 2010). However, in other works in which higher doses of bacteria were administered to *Eleginops maclovinus* or *S. maximus*, increases of cortisol lasted for 7 days or even longer after injection (Rodríguez-Quiroga et al., 2017; Oyarzún et al., 2019).

Our results also suggest that vaccines do not clearly activate the response of brain stress genes during the first hours. Thus, neither *crh* nor *crhbp* or *gr* showed relevant modulation after bath vaccine and only slight changes were observed in *hsp* and *gr* after injection. Therefore, this suggests that vaccine did not activate the central stress gene response unless a physical stressor (air exposure) was included, as observed in the vaccine plus air exposure groups. This agrees with the previously reported response of *S. aurata* to different stressors (Skrzynska et al., 2018).

On the contrary, in the pituitary, vaccine did induce the gene expression of stress-related hormones like *prl, gh* at 6 h and *pomca* and *pomcb* peptides at 24 h. Therefore, it seems that the pituitary was more sensitive than brain to immune stimulation, although at later time points (6 and 24 h). This may indicate, other than a higher sensitivity, that the pituitary stimulation could be not a direct effect, but resulting from the interaction through

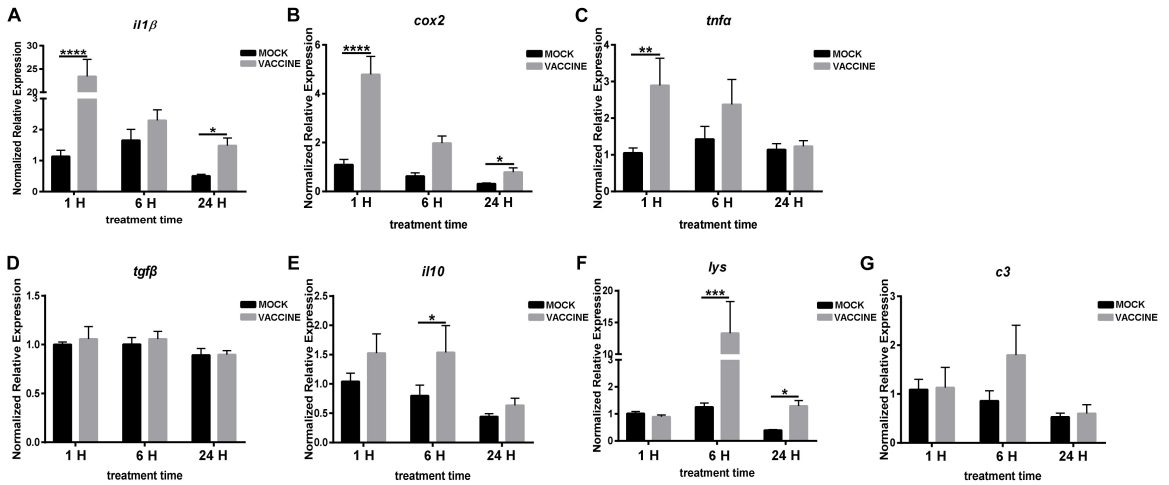

FIGURE 8 | mRNA expression of pro-inflammatory genes *il1*β **(A)**, *cox2* **(B)**, *tnf*α **(C)**, anti-inflammatory genes *tgf*β **(D)**, *il10* **(E)** and other soluble immune regulators *lys* **(F)**, *c3* **(G)** in the pituitary of gilthead seabream (*Sparus aurata* L.) after *Lactococcus* bacterin or mock injection. Asterisk (*) indicates significant difference between mock and vaccination groups of each time point ($P < 0.05$).

biological messengers such as cytokines. This would be supported by the fact that both brain and pituitary showed a robust pro-inflammatory cytokine response to vaccine (see **Figures 7**, **8**). At this point, the research has not gone further as receptors for *pomc* have not yet been cloned in seabream, although attempts have been made by several laboratories. The results for *trh* seem to follow a similar trend than *crh*, i.e., reduced activation of the stress axis, showing a small variation as a response to bath vaccine. At 1 h, levels were higher than at 6 h which matches with previous results of Ruiz-Jarabo et al. (2017) and Skrzynska et al. (2018). This response could also be related to an inhibition of the thyroid hormone axis modulating energetic responses, thus contributing to save energy resources. This moderate response is also linked to the expression of both *pomc* genes. Thus, *pomcb* showed a decrease of its expression at 6 h as *trh*, whereas *pomca* and *crh* maintained unaltered levels.

It is worth to note that vaccination caused a raise of cortisol together with some alteration of both *hsp* and *mt* in brain and pituitary. Metallothioneins, similarly than HSP are involved in stress response, and their expression can be induced by cortisol in fish (Hyllner et al., 1989) as a result of both abiotic and biotic stressors (Iwama et al., 1999; Roberts et al., 2010; Yamashita et al., 2010). In addition, our recent data in skin mucus showed both an increment of cortisol induced by *V. anguillarum* vaccine and also a significant rise of *hsp70* (Khansari et al., 2018). During stress, global RNA translation is supposed to be reduced to save energy, while a selective translation is up-regulated, which facilitates coping with challenges (Holcik and Sonenberg, 2005; Tort and Teles, 2011). Thus, after vaccination, a decreased expression of some stress genes together with the enhancement of other key response genes might be associated to energy savings, and thus protective immunity responses could be maintained (Pulendran and Ahmed, 2011).

Besides the role of GR in mediating the glucocorticoid effect of cortisol in target tissues, some other factors such as CRH and CRHBP could serve as potential feedback agents in the HPI axis. CRH is an ancient stress neuropeptide which is essential for facilitating the adaptive response to environmental stressors (Denver, 1999). Binding of CRH with its receptors in pituitary cells stimulates ACTH production. With a high affinity to CRH, CRHBP can sequester CRH in the circulation and thus modulate its bioavailability (Huising et al., 2004). Normally, a large proportion of total circulating CRH is complexed with CRHBP, and therefore the availability for receptor activation is low (Behan et al., 1997). Thus, CRHBP would act as another potential negative feedback agent of HPI axis as suggested by the up-regulation of *crhbp* in the pituitary of seabream after vaccination. This agrees with such a proposed role of CRHBP during acute stress in *S. aurata* in previous works (Martos-Sitcha et al., 2014).

Combining the results of *gr* expression and *crhbp* with the plasma cortisol content, we could speculate that after cortisol increase, up-regulation of *crhbp* and down-regulation of *gr* constitute two feedback factors of HPI axis. They would indirectly inhibit the bioavailability of circulating CRH and then suppress the new production of cortisol, reducing the binding of cortisol with GR and finally leading to the descent bioactivity of cortisol. Besides, induction of inflammation may help to eliminate potential invading pathogens and dead cells. On the other hand, prolonged hyperactivation of the immune response may be detrimental and therefore anti-inflammatory cytokines would help to regulate this activation process, which matches with the increase of *il10* observed both in brain and pituitary. Thus, the simultaneous alteration of plasma cortisol, decreased expression of *gr*, up-regulated expression of *crhbp* and pro-inflammatory genes, and the down-regulation of anti-inflammatory genes

could constitute a beneficial picture for homeostasis and recovery of fish.

Overall, in terms of the effect of the vaccine (route of vaccination or the bacterial species) the comparison between both experiments indicates that the stress response to vaccines focuses more in pituitary or head kidney (associated to the increase of cortisol) than in the brain. As mentioned before, while slight changes are recorded in either plasma hormones or genes in brain, in pituitary bath vaccine did modify *pomca, pomcb* expression at 1 h and *pomcb* at 6 h. In addition, *gr* increases at 6 h after bath and decreases after injection vaccination.

Immune Gene Response in Brain and Pituitary After Vaccine Injection

Immune responses in fish such as inflammatory and antibody response elicited by bacteria or pathogen-associated molecular patterns (PAMPs) including LPS, poly (I:C), can be detected notably in immune organs like spleen and head kidney (Cvitanich et al., 1991; Reyes-Cerpa et al., 2012; Martínez et al., 2018; Sheng et al., 2019). A relevant result of the present study is the strong immune response occurring both in brain and pituitary after vaccination regardless the overall stress response. Thus, very significant alterations of *il1*β*, cox2, lys, and c3* were observed in the brain and pituitary of vaccinated seabream. IL1β is one of the first cytokines produced at the inflammation site that contributes to induce the expression of other cytokines including TNFα, IL1α, IL6, IL8, COX2, MCP1 (Weber et al., 2010; Zou and Secombes, 2016). Cox2 is a potent mediator of inflammation encoding a prostaglandin-endoperoxide synthase 2, which is a rate limiting enzyme for formation of prostaglandins (PG) functioning under a wide variety of challenging conditions (Smith et al., 1996; Ricciotti and FitzGerald, 2011). The significant raise of pro-inflammatory signaling genes observed in brain and pituitary suggests that vaccines induced inflammation in these two tissues. It can also be speculated that stimulation of cortisol by the vaccine may be associated with an interaction of *il1*β expression at the pituitary, as previously proposed (see Tort and Teles, 2011). Moreover, the increased expression of *c3,* responsible for the complement protein C3, and even more the dramatic raise of *lys*, responsible for the bacteriolytic protein lysozyme (Sunyer et al., 1997; Hernández and Tort, 2003; Saurabh and Sahoo, 2008), indicates an effective activation of innate immune responses in the central neuroendocrine tissues after the intraperitoneal vaccination. The combination of the alteration of inflammatory genes plus the increase of the immune innate genes supports the occurrence of a significant immune response in the pituitary.

There is not precisely known what are the precise mechanisms of interaction between hormone elements and immune agents. Normally, due to the protection of the blood brain barrier, pathogens can hardly access to the brain or pituitary. However, some mediators such as cytokines can play a role of connecting antigens and response (Banks et al., 1995). In our previous works we observed that both the medium from the *in vitro* cultured spleen and recombinant IL1β presented a significant effect on the *in vitro* immune response of trout pituitary (Liu et al., 2019). Thus, the immune response in the central neuroendocrine system might be regulated by some mediators produced and released into the bloodstream by lymphoid organs as a response to the bacterin delivered by intraperitoneal injection. Further studies will be necessary to precise the mechanisms that can confirm this hypothesis.

Although both brain and pituitary present a robust immune reaction, it is worth noting that the expression trends of immune genes are different between these organs, and this may be related to the respective tissue architecture. Brain is constituted by neurons and also glia which account for an abundant portion of the brain cell population, acting as the primary resident macrophages to elicit both innate and adaptive immune responses (Yang et al., 2010; Schwartz et al., 2013; Lenz and Nelson, 2018). Thus, while pituitary have endocrine cells as the predominant population, just some stellate cells are hypothetically the functional immune cells (Glennon et al., 2015). In brain, the participation of glial cells in immune response could be quantitatively rather higher. Therefore, we can hypothesize that alterations in the transcription levels of immune genes in response to intraperitoneal vaccine injection might result from the different architecture and cell composition of these two organs, thus leading to different signaling elements involved in the immune response. In the case of *L. garvieae*, it is also possible that the strong induction of the pro-inflammatory response could be associated to the pathogenic neurodegenerative effect of the *L. garvieae* that has been shown to produce brain damage in fish (Vendrell et al., 2006). Moreover, although these hypotheses need further investigation, our findings support the fact that fish brain is capable of inducing a strong inflammatory response.

CONCLUSION

Vaccination, either via bath or injection did not involve a significant induction of brain-pituitary stress response, although cortisol showed a moderate increase. Other than assuming that such antigen stimulus does not involve a direct and high central perception response, the observed reaction could be also associated to the altered feedback genes of the HPI axis *gr* and *crhbp* that may have played a relevant role in preventing the maintenance of higher cortisol levels in brain and pituitary and therefore also preventing cortisol immunosuppressive consequences. Such a mechanism could modulate the initial stress response and the pleiotropic cortisol action, thus helping to prevent the putative suppression of an active immune response in the neuroendocrine centers. Thus, the raise of cortisol caused by the vaccination would not be achieved through the initial activation of the central brain-pituitary axis elements. Besides, a robust immune response was elicited both in brain and pituitary regardless the route of administration, bath or injection, as shown by the up-regulation of cytokines and innate response genes. Thus, results suggest an active and direct immune action of the vaccine components in brain and pituitary tissues uncoupled from the initial stress HPI axis response.

DATA AVAILABILITY

All datasets for this study are included in the manuscript and the supplementary files.

ETHICS STATEMENT

All experimental procedures were submitted by the Ethical Committee of the Universitat Autonoma de Barcelona (CEEAH), in accordance with the international European Guiding Principles for Biomedical Research Involving Animals (EU2010/63) and authorized by the regional authority (Generalitat de Catalunya Procedure Ref. 10208).

AUTHOR CONTRIBUTIONS

LT, JM, and FR-L conceived and designed the experiments. XL, AK, MT, and FR-L performed the experiments. XL, AK, GM-R, LT, and FR-L analyzed the data. YZ, GM-R, JM, and FR-L contributed the reagents and materials. XL, AK, MT, YZ, JM, GM-R, FR-L, and LT contributed to the writing of the manuscript. All authors read, corrected and approved the final manuscripts.

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Ultra-Low Power Sensor Devices for Monitoring Physical Activity and Respiratory Frequency in Farmed Fish

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Integration of technological solutions aims to improve accuracy, precision and repeatability in farming operations, and biosensor devices are increasingly used for understanding basic biology during livestock production. The aim of this study was to design and validate a miniaturized tri-axial accelerometer for non-invasive monitoring of farmed fish with re-programmable schedule protocols. The current device (AE-FishBIT v.1s) is a small (14 mm × 7 mm × 7 mm), stand-alone system with a total mass of 600 mg, which allows monitoring animals from 30 to 35 g onwards. The device was attached to the operculum of gilthead sea bream (*Sparus aurata*) and European sea bass (*Dicentrarchus labrax*) juveniles for monitoring their physical activity by measurements of movement accelerations in *x*- and *y*-axes, while records of operculum beats (*z*-axis) served as a measurement of respiratory frequency. Data post-processing of exercised fish in swimming test chambers revealed an exponential increase of fish accelerations with the increase of fish speed from 1 body-length to 4 body-lengths per second, while a close relationship between oxygen consumption (MO_2) and opercular frequency was consistently found. Preliminary tests in free-swimming fish kept in rearing tanks also showed that device data recording was able to detect changes in daily fish activity. The usefulness of low computational load for data pre-processing with on-board algorithms was verified from low to submaximal exercise, increasing this procedure the autonomy of the system up to 6 h of data recording with different programmable schedules. Visual observations regarding tissue damage, feeding behavior and circulating levels of stress markers (cortisol, glucose, and lactate) did not reveal at short term a negative impact of device tagging. Reduced plasma levels

of triglycerides revealed a transient inhibition of feed intake in small fish (sea bream 50–90 g, sea bass 100–200 g), but this disturbance was not detected in larger fish. All this considered together is the proof of concept that miniaturized devices are suitable for non-invasive and reliable metabolic phenotyping of farmed fish to improve their overall performance and welfare. Further work is underway for improving the attachment procedure and the full device packaging.

Keywords: aquaculture, sensor, swimming tests, fish welfare, physical activity, respiratory frequency, oxygen consumption

INTRODUCTION

Biosensor technology is increasingly used for a non-invasive measurement during farming and experimental studies of a range of variables that are directly or indirectly relevant for animal health and welfare (Ivanov et al., 2015; Neethirajan et al., 2017). Thus, cameras, sonars, acoustic telemetry and environmental biosensors of chemical and biological safety risk factors are currently used to improve feed conversion, product quality and disease prevention, reducing at the same time the environmental impact of farming operations (Saberioon et al., 2017; Føre et al., 2018; Enviguard EU project[1]). Such technological solutions contribute to the estimation of fish biomass for ration size assignment and medicinal dosages, or to the design of automated feeding strategies based on fish behavior in order to avoid unnecessary feed waste. Furthermore, the concept of sentinel animals fitted with biosensors, initially developed for the dairy industry (Simbeye et al., 2014), has been extended to the culture of oysters (Andrewartha et al., 2016) and fish (Staaks et al., 2010) with abundant literature on measurements of body temperature, animal position and depth in both wild and farmed fish (e.g., Clark et al., 2008; Payne et al., 2014; Metcalfe et al., 2016, among others). This approach is essential for the optimization of aquaculture operations, and the assessment of welfare and stress in farmed fish becomes a major challenge for a more efficient and ethical animal production.

With the advent of the MEMS (MicroElectroMechanical System) technology, high-precision and low-cost accelerometers are available as components of different electronic gadgets of physical activity (activity trackers, fitness band and heart rate monitors, sport-watches, smart pedometers and wellness monitors, among others). Acceleration data-loggers alone or in combination with pressure, temperature and heart-rate biosensors have also been used for tracing movement and estimating activity-specific energy expenditure or feeding behavior in a number of fish species, including juvenile hammerhead sharks (*Sphyrna lewini*, Gleiss et al., 2010), sockeye salmon (*Oncorhynchus nerka*, Clark et al., 2010), European sea bass (*Dicentrarchus labrax*, Wright et al., 2014), Atlantic cod (*Gadus morhua*, Claireaux et al., 1995), Atlantic salmon (*Salmo salar*, Kolarevic et al., 2016) and red-spotted groupers (*Epinephelus akaara*, Horie et al., 2017). These sensors operate as acoustic transmitter tags that contain a tri-axial accelerometer, which registers gravity forces and acceleration in the *x*-, *y*-, and *z*-directions. Currently, the smallest market size for these tags is 7 mm diameter and 20 mm length with a weight of 2.6 g in air and a typical battery life of 1–3 months, depending on measurement periods and transmission intervals. However, as pointed out by Kolarevic et al. (2016), the interference between transmitted signals limits the use of a large number of acceleration tags in a single rearing unit or in two neighboring units. Otherwise, in the aquatic environment, the use of low radiofrequency transmission is limited to 1–2 m of maximum communication range without repeaters (Palmeiro et al., 2011). Thus, with the double aim to produce small sensors and to combine measurements of physical activity and energy demand, the design and production of ultra-low power stand-alone devices using available technology was conducted within the AQUAEXCEL[2020] EU project. The resulting device (AE-FishBIT) was designed to be implanted on fish operculum for measurements of physical activity and respiratory frequency. To the best of our knowledge, there is not in the market any device designed to provide simultaneously these two types of measurements. The present work was envisaged as the proof of concept that data acquired with the constructed prototype (AE-FishBIT v.1s) can be used in a reliable manner at different tank scales for the assessment of physical activity and energy expenditure status of farmed fish. This functional validation of the device has been conducted in European sea bass and gilthead sea bream (*Sparus aurata*) as the two most significant farmed fish of the Mediterranean aquaculture (FAO, 2018).

MATERIALS AND METHODS

Hardware Architecture

The proposed device is a programmable and reconfigurable tri-axial accelerometer for measuring accelerations in *x*-, *y*-, and *z*-axes in the range of ± 8 *g* and sampling frequencies up to 800 Hz. The basic components disposed on a 0.8 mm 4-layer printed circuit board (pcb) are: (i) one accelerometer (MMA8451Q; NXP Semiconductors Research, Eindhoven, Netherlands), (ii) one high performance Li-ion battery (UMAC040130A003TA01; muRata Electronics, Kyoto, Japan) of low charge time (120 s), (iii) one microprocessor (KL17; NXP Semiconductors Research) with 256 kb flash memory and 32 kb RAM memory and (iv) one RFID tagging device (nano-transponder ID-100A-1.25; TROVAN, Madrid, Spain) for rapid identification. System integration includes water-proof packaging

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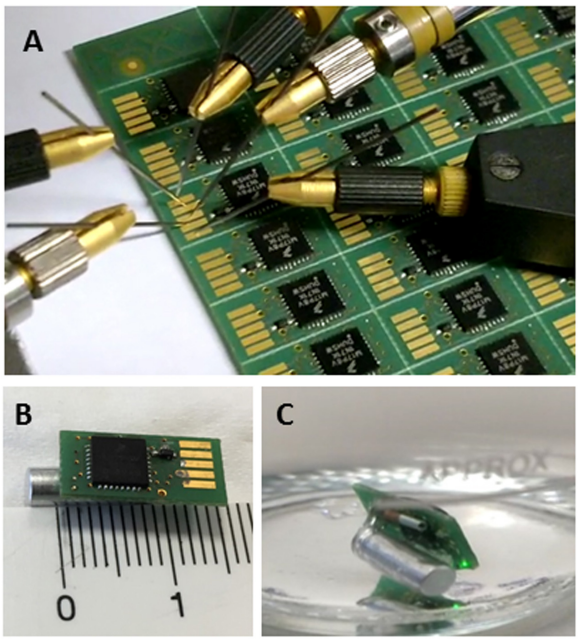

FIGURE 1 | (A) Electrical probing of pcb-mounted devices. **(B)** Photograph of AE-FishBIT v.1s before encapsulation. **(C)** Encapsulated AE-FishBIT v.1s in underwater operation. Flashing led light indicates the end of data acquisition program for user reference.

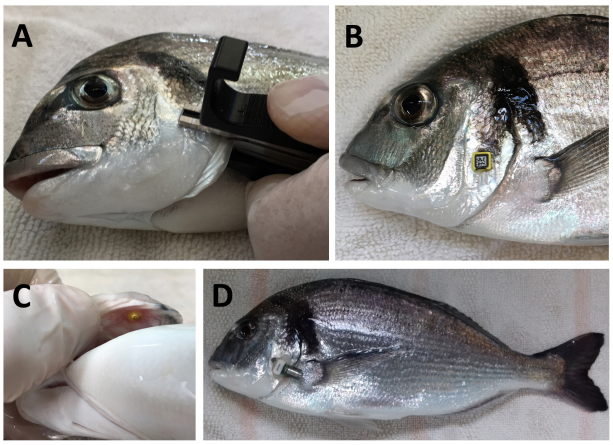

FIGURE 2 | Device attachment to sea bream operculum. **(A)** RapID tag is attached to fish operculum with the tag piercing tool. The external side of the tag **(B)** allows fixation with adhesive of a 3D-printed pocket. **(C)** Internal side of attached sea bream operculum. **(D)** Sea bream with an attachment with the RapiID tag and 3D pocket procedure.

(tested up to 6 bars of pressure in air and seawater environments) with isolated connector pins. The complete package size of the AE-FishBIT v.1s measures 14 mm × 7.2 mm × 7.0 mm with a total mass of 600 mg in air (**Figure 1**).

Sensor Location and Attachment Procedure

The operculum was chosen as the target location for the sensor as it allowed monitoring physical activity by measurements of accelerations in *x*- and *y*-axes, while records of operculum breathing (*z*-axis) served as a direct measurement of respiratory frequency. The device attachment was accomplished using small and light laboratory tags for identification of experimentation animals (RapID tags from RapID Lab, Inc., San Francisco, CA, United States) that were rapidly pierced to opercula of anesthetized fish (100 μg/mL 3-aminobenzoic acid ethyl ester MS-222, Sigma, St. Louis, MO, United States). In a second step, a rigid polyamide PA 2200 3D-printed pocket was fixed with an innocuous and quick drying aquarium adhesive (cyanoacrylate) to the exterior side of the RapID tag. Such approach allowed easy application and removal of the device in the pocket (**Figure 2**).

Data Acquisition and Software Processing

Software data processing aimed to assess respiratory frequency and fish activity. The first estimation was carried out with the signal of the *z*-axis of the accelerometer, and the second one was derived from the *x*- and *y*-axis signals of the accelerometer.

The recorded *z*-axis accelerations generated positive and negative signals, related to operculum opening and closing, respectively. Other movements superimposed to this *z*-axis signal were angular accelerations that informed of fish trajectory or side head movement, though the dominant periodic component was always operculum movement. To calculate the respiratory frequency, the *z*-axis signal of the accelerometer was band pass filtered between 0.5 and 8 Hz to reduce the noise (measurements were expected below 5 Hz), highlighting the periodic properties of the signal. The numbers of maxima or minima in the recorded signals were registered at a sampling rate of $f_s = 100$ samples per second. Then, the signal was derived and the number of crosses through zero was divided by two to obtain the signal period.

The signal of the accelerometer was noisy, and the low pass filter was not able to eliminate some spurious crosses through zero. To alleviate this noise drawback and to obtain a more accurate estimation of the number of peaks, they were estimated in N consecutive frames of T seconds. Experimentally, it was calculated that around half of the frames included spurious crosses through zero, increasing the value of the respiratory frequency. As trade-off between complex signal denoising processing algorithms and the limited energy and computational capability of the microprocessor, the final number of peaks (N_p) was defined as the 25% percentile of the N estimations. This 25% percentile was a conservative value below the 50% of usually noisy frames. Anyway, values between 20 and 40% provided similar results. Hence, the respiratory frequency was issued every $N \cdot T$ seconds and it was calculated as:

$$F_{resp} = N_p / T$$

Values of N, T and f_s were heuristically chosen to assure a good statistical representation. The value of f_s was established as 100 Hz. A value of $T_s = 10$ seconds was chosen as frame length. This value implied 5 peaks when the respiratory frequency was

2 Hz. N was established equal to 12 (respiratory frequency was calculated every 120 s), which allowed a reasonable estimation of the 25% percentile.

To describe the physical activity index of the fish spatio-temporal movement along the x- and y-axes, we relied on the simple optimization principle that the human movements strives to achieve maximally smooth movements by minimizing the first temporal derivative of acceleration, or "jerk," while constraining all higher derivatives to zero (Flash and Hogan, 1985). This fact has been also applied to measure the intensity of the movement of terrestrial livestock (Hamäläinen et al., 2011). In this way, the energy of the jerk can be seen as a measure of the intensity of fish movement. To ease microprocessor calculations, the physical activity index was defined as the averaged energy of the jerk along the frame. Similarly to the respiratory rate, the energy of the jerk was estimated on N consecutive frames of T seconds and the 25% percentile provided the activity index measurement. The values of N, T, and f_s were the same than in the respiratory frequency algorithm.

The procedure was as follows. The accelerations in the x- and y-axis, named $a_x(n)$ and $a_y(n)$, respectively, were derived as:

$$d_x(n) = a_x(n) - a_x(n-1)$$
$$d_y(n) = a_y(n) - a_y(n-1)$$

The standard deviations of $d_x(n)$ and $d_y(n)$ were calculated:

$$\sigma_x = \sqrt{\frac{\sum_{n=1}^{T\cdot f_s}(d_x(n)-\mu_x)^2}{T\cdot f_s}} \quad \text{and} \quad \sigma_y = \sqrt{\frac{\sum_{i=1}^{T\cdot f_s}(d_y(n)-\mu_y)^2}{T\cdot f_s}}$$

where μ_x and μ_y were the average of $d_x(n)$ and $d_y(n)$, respectively. The energy of the jerk was then obtained as:

$$E_{jerk} = \sqrt{\sigma_x^2(n) + \sigma_y^2(n)}$$

The physical activity index was the 25% percentile of the energies of the jerk estimated on N consecutive frames. A summary of the procedure for the calculation of respiratory frequency and physical activity index is shown in **Figure 3**.

Processing of the raw data generated by the tri-axial accelerometer and stored in the memory of the device required data transfer in a computer after tests. Using this procedure, the autonomy of the device was limited to the amount of memory, which was able to store a total of 6 min of raw data measured at a rate of 100 samplings per second. It was established that 2 min was a reliable time window for statistical calculations, and the device was then programmed to acquire three sets of 2 min of raw data for a given experimental schedule.

Software development also offered the possibility to process on-board recorded data by means of mathematical approximations to ease microprocessor function. This approach is not a high memory consuming process, and the autonomy of the system could be consequently increased up to 6 h of continuous data recording, allowing longer schedules as long as the battery of the device is operative. Algorithms for direct on-board calculation of respiratory frequency and physical activity index by the sensor were programmed through the FRMD-KL25Z algorithm platform and uploaded to the device. This step involved some changes on the algorithm to estimate the physical activity index due to the limited computational power of the sensor. For this purpose, the standard deviations of $d_x(n)$ and $d_y(n)$ were approximated by:

$$\sigma_x = \frac{\sum_{n=1}^{T\cdot f_s}|d_x(n)-\mu_x|}{T\cdot f_s} \quad \text{and} \quad \sigma_y = \frac{\sum_{n=1}^{T\cdot f_s}|d_y(n)-\mu_y|}{T\cdot f_s}$$

The energy of the jerk was estimated as:

$$E_{jerk} = \sigma_x + \sigma_y.$$

The value of T was changed from 10 to 10.24 s. It means a frame of 1024 data, which is a power of 2, more convenient for the sensor programming.

Functional Testing

Initial tests of the device were conducted in overnight fasted sea bream and sea bass juveniles (30–150 g body weight) reared in the indoor experimental facilities of Institute of Aquaculture Torre de la Sal (IATS-CSIC, Castellón, Spain) under natural photoperiod and temperature conditions (40°5′N; 0°10′E). Fish with the implanted device were exercised in an intermittent-closed swim tunnel respirometer of 10 L water volume (Loligo® Systems, Viborg, Denmark). The swim tunnel was submerged into a water bath that served as a water reservoir for flushing the respirometer after each closed respirometer run (flush pump: Eheim 1048, 10 L/min; Deizisau, Germany). To ensure constant high water quality, the water bath was connected by a second flush pump (Eheim 1250, 20 L/min; Deizisau, Germany) to a 100 L reservoir tank coupled to a re-circulatory system equipped with physical and biological filters and a programmable temperature system fixed at 24–25°C. Sea water (80–95% O_2 saturation) flowed back into the re-circulatory system by means of gravity, remaining unionized ammonia, nitrites and nitrates almost undetectable along the whole experiment. A thruster within the respirometer was used to generate a swimming current. Water velocities were calibrated with a hand-held digital flow meter that was ordered by the controller Movitrac® LTE 0.37kW/0.5HP (SEW Eurodrive, Normanton, United Kingdom). Respirometry runs and chamber flushing were automatically controlled with the DAQ-M instrument (Loligo® Systems) connected to a PC equipped with AutoResp™ software (Loligo® Systems). Water temperature and O_2 saturation within the respirometer were measured using a Witrox 1 single channel O_2 meter (Loligo® Systems, Viborg, Denmark), equipped with a needle-type fiber optical micro-sensor (NTH, PreSens Precision Sensing GmbH, Regensburg, Germany) and a temperature probe suspended into the water current within the respirometer.

For testing procedures, anesthetized fish with the AE-FishBIT v.1s prototype were transferred into the swim tunnel and allowed to recover and acclimate at a swimming speed of 0.5–1.0 body-lengths per second (BL/s), until their measurements of O_2 consumption rates (MO_2) reached a constant low plateau. This was achieved when the regression line between the O_2

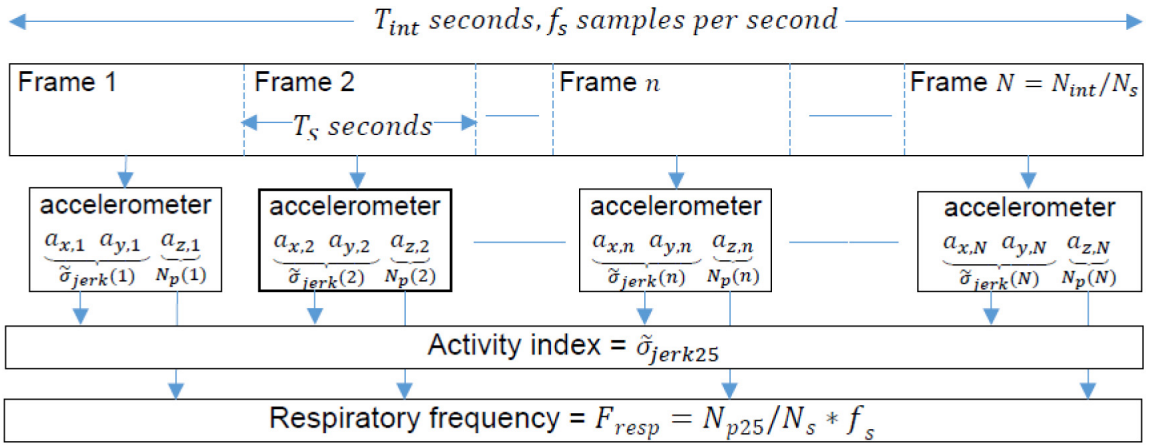

FIGURE 3 | Procedure diagram for the calculation of respiratory frequency and physical activity index.

consumption and time after transfer was not significantly different from zero during four consecutive 5 min intervals, and it typically happened after 30–45 min with MO_2 around 220–240 $mgO_2/kg/h$ (Martos-Sitcha et al., 2018). After this acclimation period, water velocity was increased in 0.5 BL/s steps, and specimens were submitted to controlled speeds between 1 and 4 BL/s during the first testing approaches, or from 1 BL/s until exhaustion in the final on-board validation of data recording. The range between 1 and 4 BL/s for the first tests was chosen to assure that AE-FishBIT measurements were obtained in the linear increase of metabolic rates according to O_2 consumption (Martos-Sitcha et al., 2018). Each swimming interval at a given velocity lasted 5 min, consisting in "flush-wait-measurement" cycles (60 s flush interval to exchange the respirometer water = "flush"; 30 s mixing phase in closed mode = "wait"; and a 210 s MO_2 measuring period in closed mode = "measurement"). During the measurement interval, O_2 saturation of the swim tunnel water was recorded every second. MO_2 was automatically calculated by the AutoResp^{TM} software from linear decreases (r^2 = 0.98–1.0) in chamber O_2 saturation during the measurement period at each discrete and specific speed using the appropriate constants for O_2 solubility in seawater (salinity, temperature, and barometric pressure). After the swim tunnel test, fish were anesthetized again to recover the AE-FishBIT for data download. For each fish, three data sets of 2 min raw data were acquired by the device at different speed steps. Additional tests were conducted in order to check the correlation of raw data calculated parameters with those coming from the on-board algorithms. To achieve this validation, devices were programmed to simultaneously store raw data (3 steps of 2 min data sets) and algorithm processed results.

For tests conducted with free-swimming fish (sea bream 292.5 ± 20.5 body weight, sea bass 161.6 ± 14.9 body weight) in 500 L tanks (stocking density ranging between 6 and 8 kg m^{-3} for both species), six devices were programmed to acquire 2 min raw data sets at three discrete times 1 day after device implantation (10:00 a.m., 2:00 p.m., and 6:00 p.m.). This schedule covered a wide range of possible circadian variations in activity and respiration indexes. Fish remained fasted during the day of data recording to avoid the possible distortion produced by feeding.

To check how blood markers of stress and welfare were affected by prototype implantation, polyamide PA 2200 dummy devices (same size and weight than the functional prototype) were implanted in active feeding sea bass (100–200 g) and sea bream of two different class of size (50–90 g; 300–500 g), reared at 22–24°C. After 1 week, tagged and non-tagged fish (n = 5–7 fish per group) were anesthetized and blood was quickly taken from caudal vessels with heparinized syringes. A blood aliquot was centrifuged at 3,000 × g for 20 min at 4°C, and the plasma was stored at −80°C until the subsequent biochemical assays. Plasma glucose levels were measured by the glucose oxidase method (Thermo Fisher Scientific, Waltham, MA, United States) adapted to 96-well microplates. Blood lactate was measured in deproteinized samples (perchloric acid 8%) using an enzymatic method based on the use of lactate dehydrogenase (Instruchemie, Delfzijl, Netherlands). Plasma triglycerides (TG) were determined using lipase/glycerol kinase/glycerol−3−phosphate oxidase reagent (Thermo Fisher Scientific). Plasma cortisol levels were analyzed using an EIA kit (Kit RE52061 m IBL, International GmbH, Germany). The limit of detection of the assay was 2.46 ng/mL with intra- and inter-assay coefficients of variation lower that 3 and 5%, respectively.

Ethics Statement

No mortalities were observed during fish manipulation and data recording, and all the described procedures were approved by the Ethics and Animal Welfare Committee of Institute of Aquaculture Torre de la Sal and carried out according to the National (Royal Decree RD53/2013) and the current EU legislation (2010/63/EU) on the handling of experimental fish.

Statistical Analysis

Daily variation of respiratory frequency and physical activity index in free-swimming sea bream and sea bass was analyzed by one-way ANOVA. Blood parameters of tagged and non-tagged (control) groups were compared by means of Student's t-test.

Analyses were performed using the SigmaPlot Version 13 for Windows (Systat Software Inc., Chicago, IL, United States). Multivariate partial least-squares discriminant analysis (PLS-DA) of data on respiratory frequency and physical activity index of exercised sea bream in the 1–6 BL/s water speed range was conducted with the EZ-info software (Umetrics, Sweden). The quality of the PLS-DA model was evaluated by R2Y and Q2Y parameters, which indicated the fitness and prediction ability, respectively.

RESULTS

Initials tests for the assessment of the functional significance of calculated outputs relied on PC post-processing of raw data. Challenges in the swim tunnel respirometer showed for both sea bream (**Figure 4A**) and sea bass (**Figure 4B**) a linear increase of MO_2 in the 1–4 BL/s speed range. The calculated respiratory frequency by data post-processing paralleled MO_2. At the same swimming speed, MO_2 and the calculated respiratory frequency were consistently higher in sea bream than in sea bass. However, for a given MO_2, the respiratory frequency was similar for both species as shown by the correlation plot of these two variables when all data were put together (**Figure 4C**). Regarding physical activity index, it increased exponentially rather than linearly with the increase of swimming speed, as the total jerk magnitude only reflects fish-related accelerations (**Figure 5**). This pattern was observed both in sea bream and sea bass, though it became more evident in sea bream at swimming speeds over 3 BL/s.

Registered values in free-swimming fish were in the measurable range by the PC-tested algorithms in the swim tunnel. In both fish species, the calculated respiratory frequency in holding tanks did not show significant daily variations from 10:00 a.m. to 6:00 p.m., ranging the respiratory frequency between 2.4–2.3 breaths/second in sea bream and 2.2–1.8 breaths/second in sea bass (**Figure 6A**). For the measurements of physical activity, the range of variation was also higher in sea bass, being statistically significant the decrease of physical activity from 0.150 ± 0.004 to 0.033 ± 0.001 relative units ($P < 0.05$). The observed decrease in sea bream varied from 0.147 ± 0.003 to 0.087 ± 0.005 relative units ($P = 0.12$) (**Figure 6B**).

For validation of on-board algorithms, a second step of swim tunnel tests was conducted. Sea bream juveniles of 80–100 g were exercised over 1–6 BL/s until exhaustion (fish were considered exhausted when they rested at the back grid for at least 5 s). Simultaneous raw data storage and algorithm on-board calculations allowed the comparison of both approaches for the calculation of respiratory frequency and physical activity indexes over the same 2 min period, and close linear correlations near to 1 between on-board and PC-calculated values were found (**Figure 7**). The different dynamics of on-board calculated parameters from low to submaximal exercise were observed. Respirometer measurements of MO_2 increased linearly up to a swim speed of 4.5 BL/s, paralleling the increase in the respiratory frequency, with a maximum respiratory frequency (MRF) close to 4 BL/s (**Figures 8A,B**). This short delay in the achievement of the maximum metabolic rate (MMR), defined as the maximum O_2 consumption in exercised fish, might be due, at least in part, to the instantaneous nature of sensor measurements (gill breathing) compared to the buffered

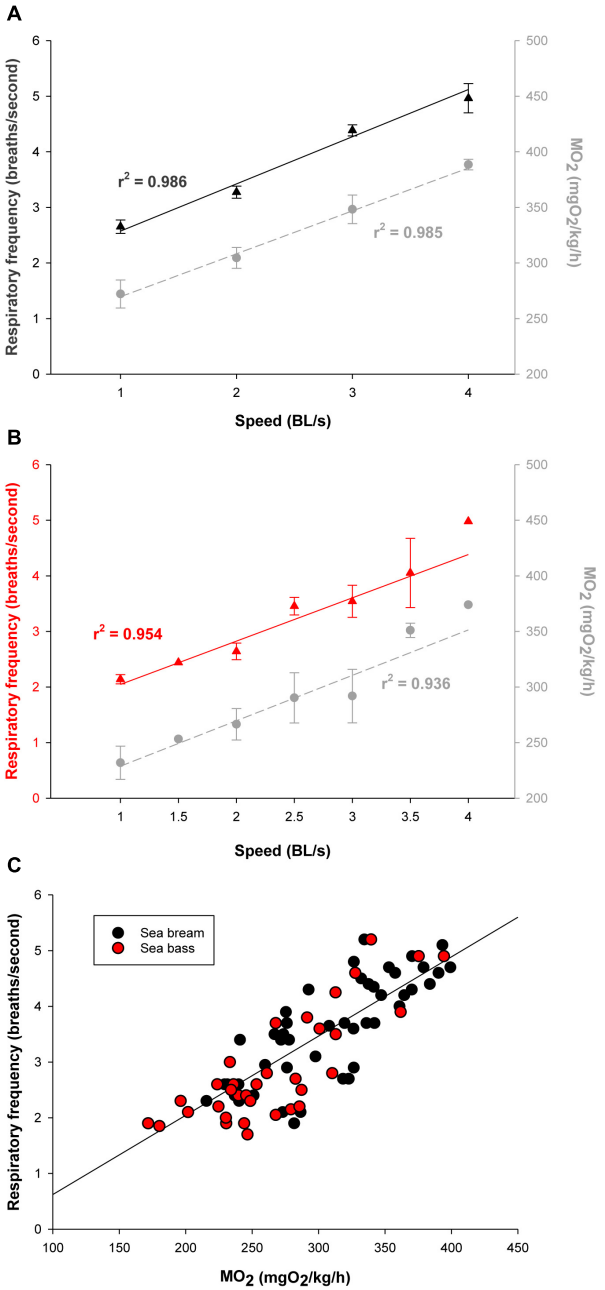

FIGURE 4 | (A) Measurements of MO_2 ($mgO_2/kg/h$; gray circles) and respiratory frequency (breaths/s; black triangles) of sea bream (35–60 g body weight range) with increasing speed in the swim tunnel respirometer. Each point represents mean ± SEM of 6–10 measurements from a total of 16 fish. **(B)** Measurements of MO_2 ($mgO_2/kg/h$; gray circles) and respiratory frequency (breaths/s; red triangles) of sea bass (90–150 g body weight range) with increasing speed in the swim tunnel respirometer. Each point represents mean ± SEM of 6 measurements. **(C)** Correlation plot of MO_2 and respiratory frequency values for each sea bream (black circles) and sea bass (red circles) individual.

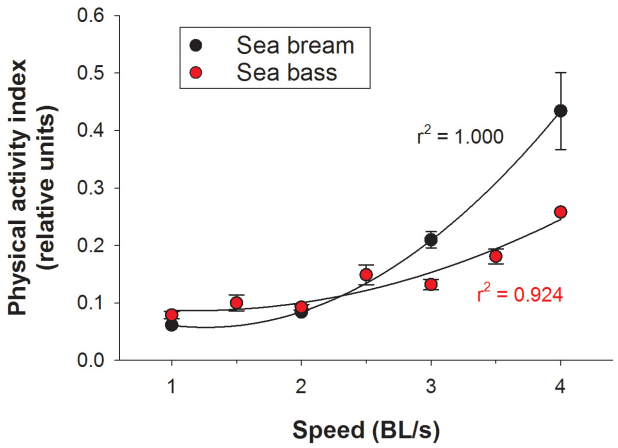

FIGURE 5 | Output of physical activity index with increasing speed in the swim tunnel respirometer for sea bream (black circles) and sea bass (red circles). Each point represents mean ± SEM of 6–10 measurements.

changes in the measurements of O_2 concentrations in the 10-L chamber of respirometer. In any case, these two types of respiration measurements decreased progressively and markedly with the increased contribution of anaerobic metabolism close to submaximal exercise. Likewise, measurements of physical activity achieved a maximum activity at 5 BL/s. This was preceded by a slight decrease of slope at 4.5 BL/s that became clearly negative with the enhancement of the unsustainable anaerobic metabolism close to submaximal exercise (**Figure 8C**). Any of the analyzed variables was informative enough to ascertain the aerobic/anaerobic scope when considered individually. However, given their different dynamic patterns in response to exercise, multivariate analyses with on-board processed data resulted in a fairly good differentiation of aerobic/anaerobic fish condition along the first component, with a 59% of total variance explained (R2Y) and 57% predicted (Q2Y) by the two components of the discriminant model (**Figure 9**).

The impact of device attachment in fish physiology was assessed by comparisons of circulating levels of markers of stress and welfare in tagged and non-tagged (control) free-swimming fish 1 week after implantation of dummy devices. No differences were found in circulating levels of cortisol (**Figure 10A**), glucose (**Figure 10B**) or lactate (**Figure 10C**) in 100–200 g sea bass nor sea bream for the two class of size analyzed. By contrast, TG levels in 50–90 g sea bream and 100–200 sea bass were significantly lowered ($P < 0.01$) in tagged fish in comparison with non-tagged ones (**Figure 10D**). This disturbance was not detected in larger fish (>300 g sea bream).

DISCUSSION

The present study is the proof of concept of the work conducted within the AQUAEXCEL^{2020} EU project for the design, programming and testing of a miniaturized device (AE-FishBIT v.1s) intended for individual fish phenotyping of metabolic condition and welfare. To achieve this end, different technological solutions already available as research tools or commercial products were initially considered (Tamayo et al., 2013; Bandodkar and Wang, 2014; Tamsin, 2015), but given the cost and methodological limitations when operating underwater, it was decided that the use of mechanical devices was more feasible than other options based on electric and/or optical sensors. The AE-FishBIT tri-axial accelerometer is able to register physical activity and operculum beats (two in one) as a direct measure of respiratory frequency. This sensor device was designed to work in stand-alone mode (no wireless data transmission) due to the constraints in size, weight, battery consumption and signal transmission in aquatic environments (Lloret et al., 2012; Climent et al., 2014). The AE-FishBIT also included a tag RFID system for an easier operability and data processing during fish data recording from resting to moderate or very active behavior under aerobic and/or anaerobic conditions.

When the system was tested in exercised juveniles, close correlations were found between O_2 consumption and calculated respiratory frequency in both sea bream and sea bass. For measurements of physical activity, the jerk of accelerations increased exponentially rather that linearly with the increase of swimming speed (**Figures 4**, **5**). Indeed, the jerk magnitude is independent of orientation and it only reflects accelerations, which are theoretically zero at the constant speeds imposed in the swim tunnel controlled conditions. It should then be assumed that when fish are induced to swim against the water current, the physical activity index will reflect the resistance of fish to match current water speed. Then, the steady increase of this index could be related with the number of intermittent (burst-and-coast) swimming bursts (Marras et al., 2010). Further increase in water speed should force a transition from burst-and-coast to continuous swimming that would decrease the number of burst and consequently decrease the physical activity index under forced swimming conditions (**Figure 8C**). Thus, the vigorous and irregular tail movements of fish resulted in a non-linear increase of the jerk of accelerations. This activity feature was more evident in the case of sea bream, which probably reflects a lower capability for fast swimming in comparison to sea bass with the spindle-shaped body characteristic of an active predator (Spitz et al., 2013), as indicates its common name in France "le loup de mer". Likewise, for a given MO_2, measurements of jerk accelerations were higher in free-swimming fish than in forced exercised fish (swim tunnel), where a more static position limits changes of trajectory and accelerations (**Figures 4–6**). From tests conducted across the light-phase in free-swimming animals, it is also conclusive that the range of variation was higher for measurements of physical activity rather than respiratory frequency, especially in the case of sea bass (**Figure 6**). However, this issue needs to be corroborated with a more continuous circadian data recording with the advent of new AE-FishBIT versions.

The usefulness of behavior monitoring remains yet to be fully exploited in aquaculture practice, though it is well known that swimming performance provides a complete measurement of animal fitness and perhaps stress behavior in fish kept under specific environmental conditions (Nelson, 1989; Plaut, 2001;

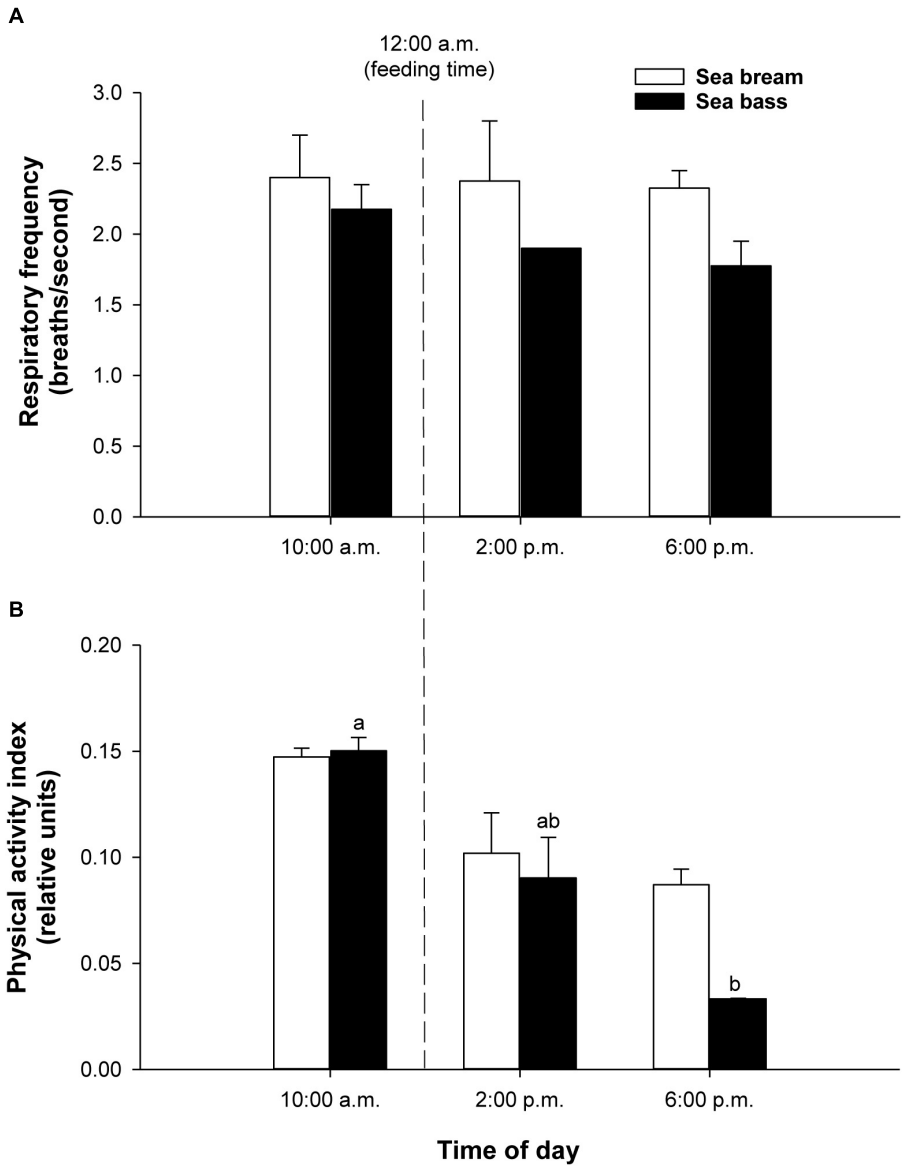

FIGURE 6 | (A) Daily variation of respiratory frequency values in free-swimming sea bream (292.5 ± 20.5 g mean body weight; white bars) and sea bass (161.6 ± 14.9 g mean body weight; black bars) in 500-L tanks. Each bar represents mean ± SEM of 6 measurements. **(B)** Daily variation of physical activity values in free-swimming sea bream (white bars) and sea bass (black bars) in 500-L tanks. Each bar represents mean ± SEM of 6 measurements from the same 6 individuals. Different superscript letters indicate significant differences ($P < 0.05$; one-way ANOVA).

Remen et al., 2016). Thus, measurements of MO_2, and secondly respiratory frequency, can be considered a good measurement of the energy expended by fish to integrate a wide-range of physiological processes influenced by both endogenous (e.g., size, age, and fish strain) and exogenous (light, temperature, O_2 concentration, time of day, etc.) factors. In our experimental conditions, MO_2 increased in sea bream and sea bass from 230–270 to 370–400 mgO_2/kg/h with the increase of swimming speed from 1 to 4 BL/s (**Figures 4A,B**). These reported values are in line with those found in previous studies in sea bream (Martos-Sitcha et al., 2018), European sea bass (Claireaux et al., 2006) or other farmed fish, such as meager (*Argyrosomus regius*) (Peixoto et al., 2017), challenged at water temperatures close to those set in our experimental approach. Thus, given the close parallelism between measurements of MO_2 and respiratory frequency, we can conclude that the AE-FishBIT provided reliable results of O_2 consumption in a wide-physiological range not only when the energy requirements did not exceed the O_2 demand for a sustainable aerobic metabolism, but also during the anaerobic phase that was largely increased at submaximal exercise (Ejbye-Ernst et al., 2016). This was demonstrated by additional swimming tests (**Figures 7–9**), prolonged until fish exhaustion at 6 BL/s, which also served to corroborate the suitability of on-board algorithms

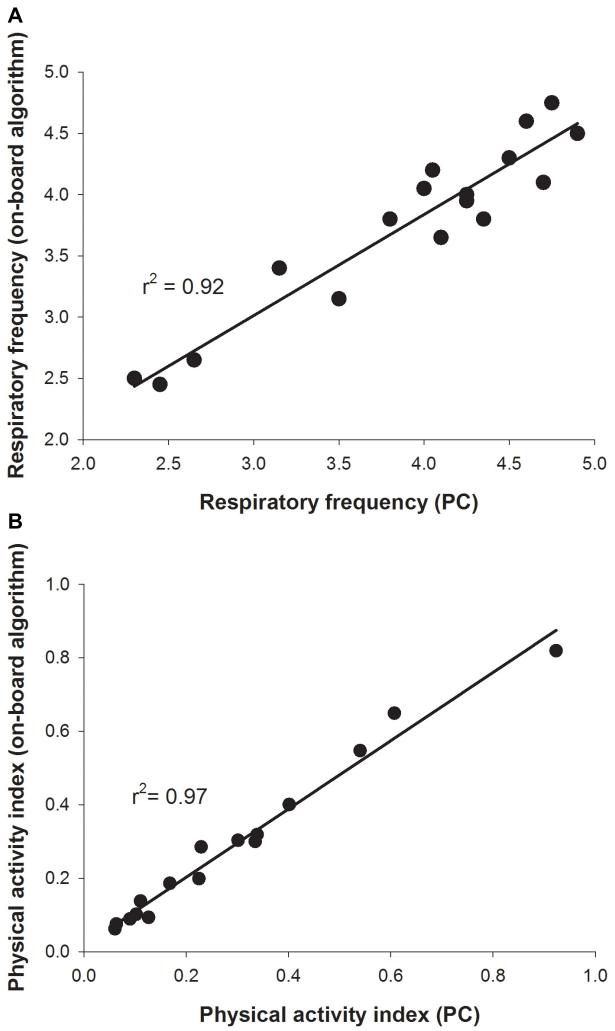

FIGURE 7 | Validation of on-board algorithms from low to submaximal exercise. Values are mean of six sea bream juveniles (141.3 ± 7.4 g mean body weight). **(A)** Correlation plot for a given swimming speed between respiratory frequency values calculated for 2 min raw data post-processed on a PC (*x*-axis) or on-board (*y*-axis). **(B)** Correlation plot for a given swimming speed between physical activity index values calculated for 2 min raw data post-processed on a PC (*x*-axis) or on-board (*y*-axis).

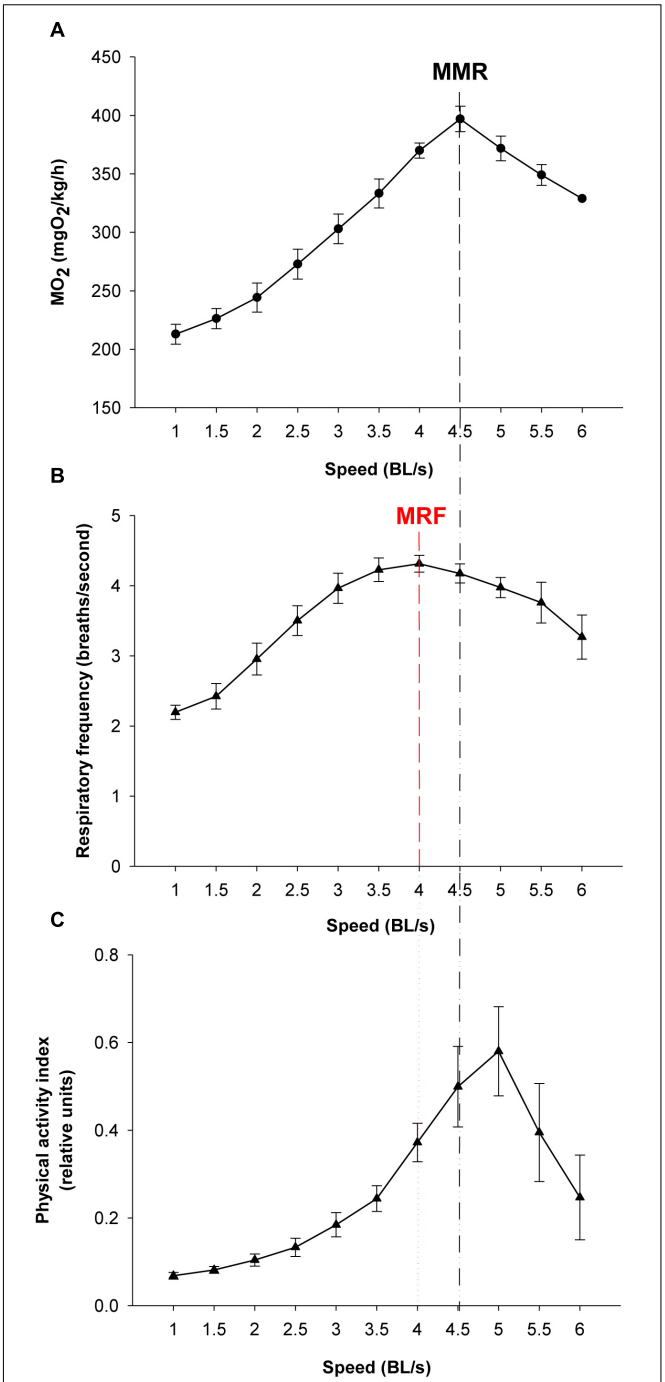

FIGURE 8 | Respirometer and on-board output from low to submaximal exercise. Values are the mean ± SEM of six sea bream juveniles (95.2 ± 3.7 g mean body weight) **(A)** Respirometer measurements of MO_2 (mgO_2/kg/h) with increasing swimming speed. Maximum metabolic rate (MMR) is marked. **(B)** Respiratory frequency (breaths/s) with increasing swimming speed. The maximum respiratory frequency (MRF) is marked. **(C)** Physical activity index with increasing swimming speed.

of respiratory frequency and physical activity through aerobic and anaerobic conditions.

The weight of the first AE-FishBIT prototype is 600 mg in air with an estimated 50% of buoyancy. These features make it very convenient for its use in fish of 30–35 g onwards, according to the empirical "2% tag/body mass" rule for implanted devices (Winter, 1996; Jepsen et al., 2004). Recent works claim that this rule can be even extended for some fish species up to 7% of body mass without detrimental effects on performance or survival (Smircich and Kelly, 2014; Makiguchi and Kojima, 2017). In the present study, we assessed the influence of device implantation on fish welfare by measurements of blood biochemical parameters in both sea bream and sea bass, and no differences were found between tagged and non-tagged fish in classical stress markers (cortisol, glucose, and lactate) 1 week after device attachment regardless of body weight (50->300 g). During this time, the observed feeding behavior was also considered to be quite similar

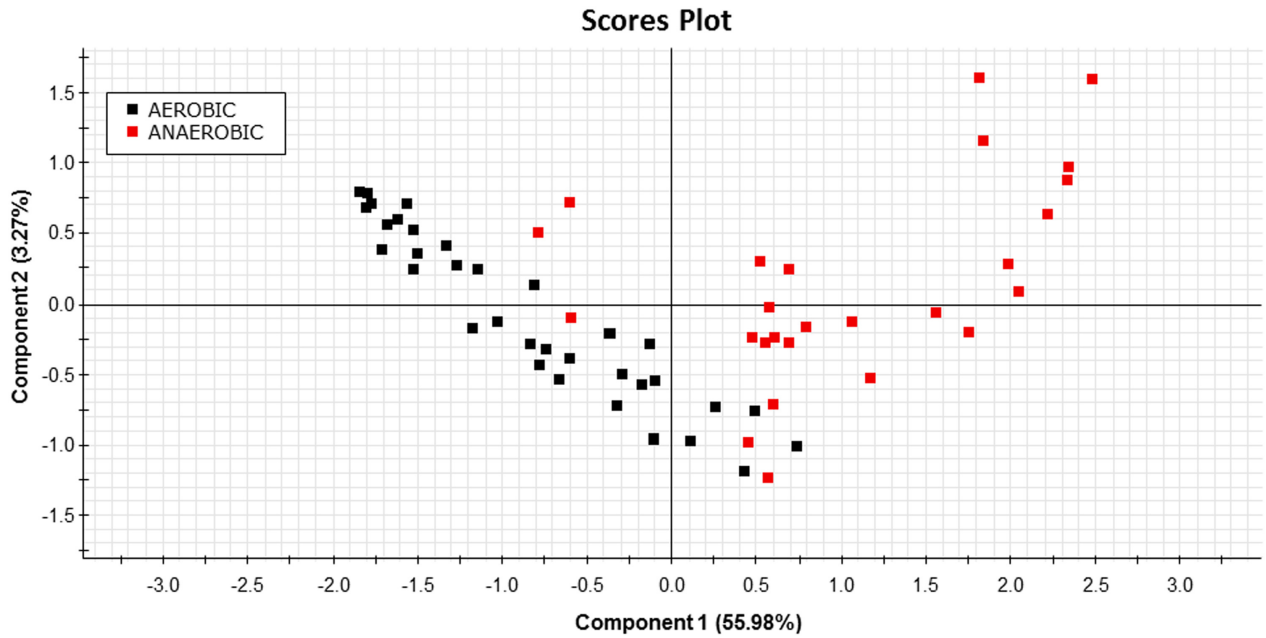

FIGURE 9 | PLS-DA score plot of on-board calculated parameters at different swimming speeds before (black squares) and after (red squares) the achievement of the MMR in sea bream juveniles from low to submaximal exercise.

in tagged and non-tagged fish. However, the observed decrease in plasma TG levels in tagged 100–200 g sea bass and 50–90 g sea bream would be indicative of, at least, a transient impairment of feed intake. This finding was not observed in the largest sea bream (>300 g), being this fact indicative of the importance of defining the critical fish size to minimize the impact of tag burden in welfare and behavior in different aquaculture scenarios (**Figure 10**). In parallel, important research efforts continue to be made to reduce the dimensions and weight of upcoming AE-FishBIT prototypes, keeping or even increasing the functional features of the device. At short-medium term, the main envisaged hardware strategies for this miniaturization will consider the use of: (i) flexible kapton instead of the current pcb rigid substrate, (ii) lighter and smaller long-life batteries and (iii) improved circuit packaging architecture based on Flip Chip (Lau, 2016) or embedded wafer level ball grid array (eWLB) technologies (Meyer et al., 2008). These solutions are based on currently available commercial components, but as a part of semi-industrialization packaging and production we cannot exclude the design of custom integrated circuits as the most ambitious and technologically challenging procedure for the final product miniaturization.

Data processing and software improvements with on-board algorithms are also key steps on the prototype development due to its major impact on data recording autonomy, which makes possible different short- and long-time schedules, adjusted for instance to 2 days (2 min recording each 15 min), 8 days (2 min recording each 60 min) or 24 days (2 min recording each 180 min). This relatively high autonomy also requires a low computational load, which did not compromise the usefulness of the microprocessor mathematical approximations, as demonstrated by the correlations of PC and on-board outputs in sea bream juveniles, exercised from low to submaximal exercise (**Figure 7**). This long coverage of exercise activity also highlighted that the system remained highly informative of metabolic condition with the shift of aerobic to unsustainable anaerobic metabolism, which reduces the efficiency of ATP production and promotes the accumulation of deleterious by-products (e.g., H^+, lactate) (Richards, 2009; Seibel, 2011). Indeed, aerobic locomotor activity is powered by red oxidative muscle fibers, but when approaching their maximum power capacity, a gait transition to anaerobic fueling is assisted by the activation of fast white muscle fibers that results in fatigue and depletion of muscle glycogen depots (Kieffer, 2000; Sänger and Stoiber, 2001). Some degree of activation of anaerobic metabolism occurs before reaching MMR, as it has been determined in different experimental models (Burgetz et al., 1998; Lee et al., 2003; Hinch et al., 2006; Teulier et al., 2013), including sea bream and guppy (*Poecilia reticulata*) (Ejbye-Ernst et al., 2016). However, as pointed out before, the closely related MRF and MMR will mark the start of a burst-assisted swimming, resulting in a change of metabolic scope and swimming energy partitioning (Peake and Farrell, 2004; Peake, 2008; Marras et al., 2013; Svendsen et al., 2015). Importantly, this energy transitioning can be monitored by the AE-FishBIT device, as discriminant multivariate analysis distinguished two main groups of fish behavior when the achieved MRF-MMR was taken as main criteria of aerobic/anaerobic classification (**Figure 9**). This complex trade-off is mediated by O_2 sensors (Norin and Clark, 2016), being defined the O_2 limiting saturation (LOS) as the O_2 threshold level that is no longer sufficient to maintain MO_2 at a given temperature and voluntary swimming activity (Remen et al., 2013, 2015). Acute hypoxia drives to

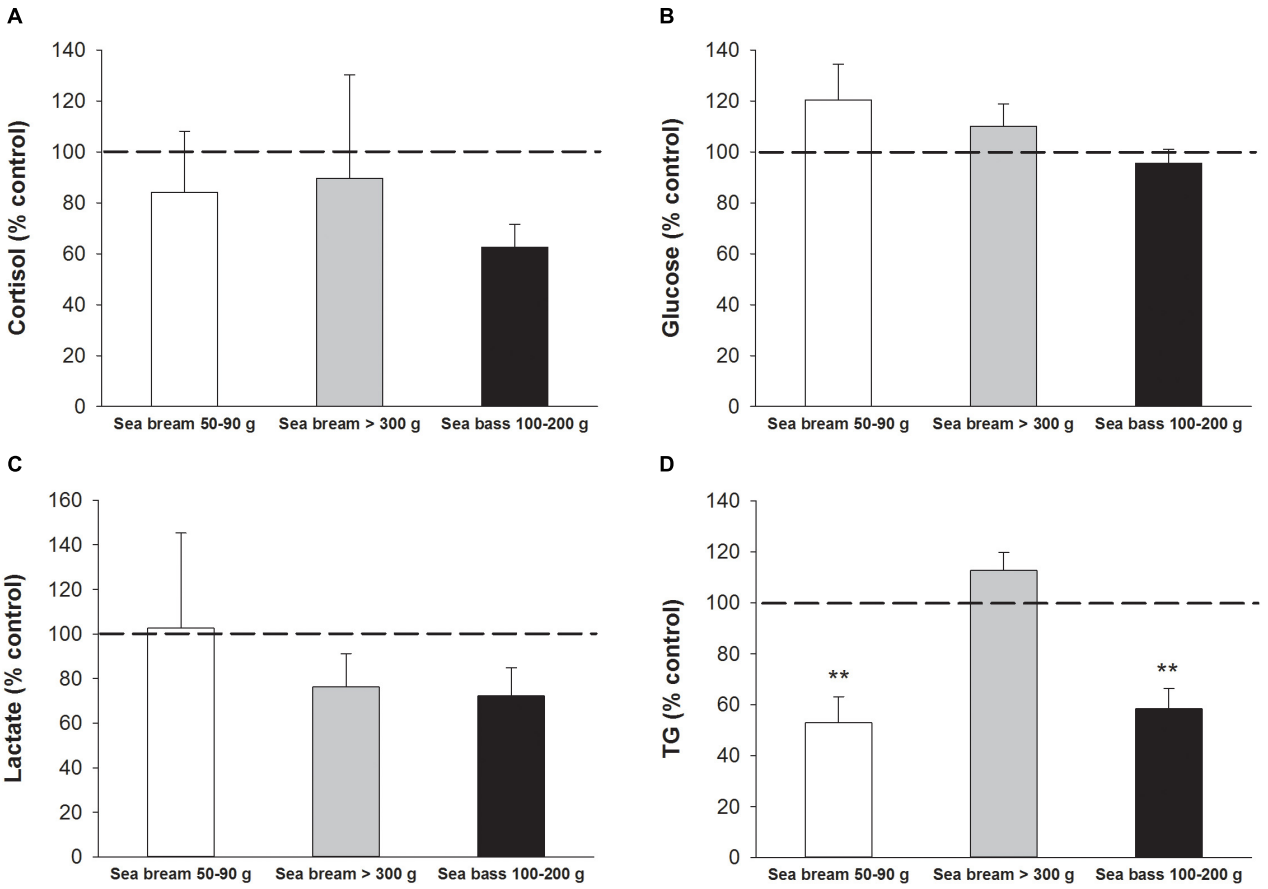

FIGURE 10 | Plasma levels of **(A)** cortisol, **(B)** glucose, **(C)** lactate, and **(D)** triglycerides represented as a percentage of non-tagged control values. Each bar represents mean ± SEM of $n = 5–7$ for each fish species (sea bream, sea bass) and class of size (405.3 ± 13.9 g; 69.2 ± 2.6; 172.9 ± 14.9 g). Asterisks indicate statistically significant differences with control ($P < 0.01$, Student's *t*-test).

the re-adjustment of sea bream mitochondrial machinery at transcriptional level, leading to a more efficient O_2-carrying capacity in blood cells (Martos-Sitcha et al., 2017). Different types of adaptive responses also occur under moderate hypoxia (above LOS), which reflects the tissue-specific responsiveness of liver, heart, skeletal muscle and blood cells, according to their metabolic capabilities and O_2 availability (Martos-Sitcha et al., unpublished). Indeed, fish transcriptomic meta-analysis in response to a vast array of challenged conditions highlights the key role of mitochondria in front of different cellular stresses, including hypoxia, hypercortisolism and malnutrition (Calduch-Giner et al., 2014). In particular, sea bream juveniles exposed to thermal stress or multiple sensory perception stressors (shaking, sound, light flashes, and water flow reversal) show adaptive responses of glycolytic pathways and mitochondrial respiratory chain (Bermejo-Nogales et al., 2014). How these adjustments at cellular level can be correlated with the monitored AE-FishBIT parameters is the upcoming envisaged challenge in a scenario of global change with increases of temperature, ocean acidification and reduced O_2 concentrations (Gruber, 2011).

Another important challenge is the improvement of the device attachment procedure to the operculum. External tagging procedures in fish are usually related to telemetry devices in large fish, with application near the dorsal or anal fins (Jepsen et al., 2015). At the present stage, the RapID tag and 3D pocket approach has served for the short-term validation experiences, but this system procedure was not operative for more than 1–2 weeks due to the appearance of macroscopic signals of damage and necrosis caused by loosening at the piercing location. This problem has been currently solved with the use of corrosion-resistant self-piercing fish tags (National Band & Tag Company, Newport, KY, United States) as the AE-FishBIT support. In our hands, the complete attachment implantation procedure takes less than 1 min, but automatization procedures like those used for massive fish vaccination (Skala Maskon, Stjørdal, Norway) need to be implemented for a more practical and routine use of this device in fish farming. In any case, specific attachment procedures need to be validated for each fish species, size and physiological condition in different aquaculture scenarios through production cycles.

Further improvements envisaged in the AE-FishBIT device include a more compact design (**Figure 11**) with the reallocation of the connection pins for charge and data transmission in the bottom side of pcb. The enclosure of the device is a

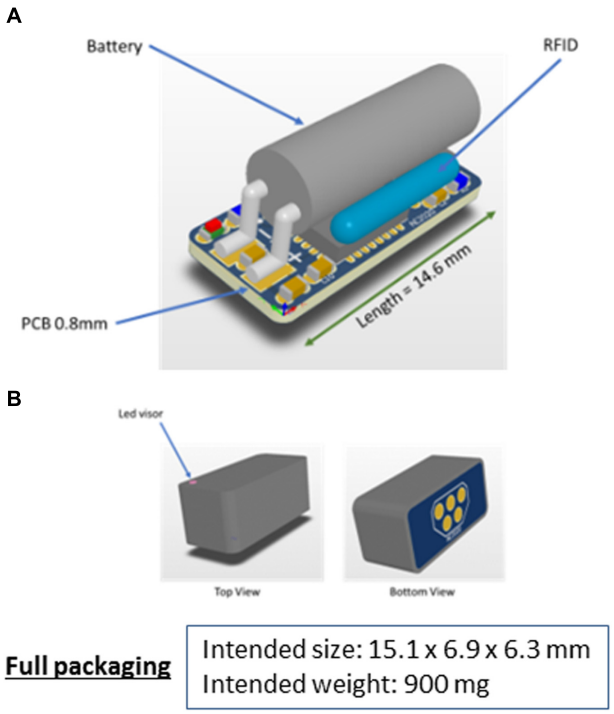

FIGURE 11 | Schematic 3D view of the intended AE-FishBIT v.2 prototype before **(A)** and after packaging **(B)**.

protective packaging that completely covers the edge of the printed circuit, which will improve the insulation of the sensor components. Additionally, the new printed circuit is manufactured in multilayer technology without through holes. The combination of the compact pcb and the protective enclosure results in a fully sealed device with a minimum increase of the total weight (around 900 mg). The design can stand water contact with the connector pins and the supply voltage of the battery is protected by a diode that prevents its discharge. During the experiments, data transmission signals are always in the high impedance state, which prevents the leakage of electrical current. The new intended prototype (AE-FishBIT v.2) has been tested underwater for more than 24 h without any additional protection in the electrical contacts. For longer experimental periods, the electrical contacts will be further protected with an adhesive waterproof lid. The removable cap will be adapted to be combined with the best attachment procedure for each fish species and experiment protocols.

CONCLUSION

A miniaturized device to register at the same time fish operculum beats (respiratory frequency) and physical activity has been designed, produced and tested in sea bream and sea bass as the proof of concept of the functional significance of the calculated data. The basic operating mode is stand-alone with autonomy of 6 h of continuous data recording with different programmable schedules. Validity and functional significance of tri-axial accelerations has been assessed under forced and voluntary exercise, and further work is underway to improve semi-industrial packaging and production. From a functional point of view, data analysis depicts the potential use of the proposed prototype for assessing the gait transition of metabolic scope and energy partitioning. The expected impact is the improvement of metabolic phenotyping in a slight invasive manner for the implementation of fish management and selective breeding. The prototype can also contribute to establish more strict and reliable welfare standards, and a better perception of quality controls in aquaculture production.

The prototype is protected by a registered patent (P201830305).

ETHICS STATEMENT

All procedures were approved by the Ethics and Animal Welfare Committee of Institute of Aquaculture Torre de la Sal and carried out according to the National (Royal Decree RD53/2013) and the current EU legislation (2010/63/EU) on the handling of experimental fish.

AUTHOR CONTRIBUTIONS

JP-S coordinated the different research teams. JS, DR-V and JM-N assembled the device components and implemented software programming. CC-D and MF established mathematical parameters for data outputs and on-board algorithm approximations. FB, EC, AV, and ML worked on device packaging and insulation procedures. JP-S, JM-S, and JA conducted functional tests. JM-S, JC-G, and JP-S wrote the manuscript. All authors edited the manuscript and read and approved the final manuscript.

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