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Comparative immuneendocrine responses to stressors in rainbow trout (Oncorhynchus mykiss) and gilthead sea bream (Sparus aurata).

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Comparative immunoendocrine responses to stressors in rainbow trout (*Oncorhynchus mykiss*) and gilthead sea bream (*Sparus aurata*).

Resposta immunoendocrina comparada a factors estressants en la truita de riu (Oncorhynchus mykiss) i l'orada (Sparus aurata).

Memòria presentada per
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Contents

1. Abstract

All animals may be exposed to stressors along their life. Fish under aquaculture conditions inhabit a potentially stressful medium that may impose extra challenges to the animal. In fish, the stress response and their consequences on immune system have been widely described. It involves the activation of the Hypothalamic-Pituitary-Interrenal (HPI) and Brain-Sympathetic-Chromaffin (BSC) axis as the neuroendocrine crosstalk that promotes the secretion of stress hormones by interrenal and chromaffin cells located at head kidney. In this ambit, the fish head kidney is equivalent of mammalian bone marrow and adrenal gland, functionality in one single multifunctional structure including haemato- and lymphopoiesis and also releasing the main stress hormones (glucocorticoids and catecholamines). Therefore, the stress response not only will activate the HPI axis to secrete hormones but also will modulate the fish immune response affecting the expression of cytokines and the whole immune reaction. Until recently, it was assumed that the effect of hormones involved in the physiological stress response (such as cortisol, ACTH, adrenaline) is species-independent and rather constant but, to our knowledge no study compared the effect of hormones or identical stress conditions in several species at the same time. Thus, our aim was to unveil the interaction between the neuroendocrine and the immune system in two different fish species (rainbow trout as a freshwater fish model and gilthead sea bream as a marine fish model) in response to stress hormones through evaluating the gene expression.

At first, it was intended to investigate the cytokine modulation of pro- (IL-1β, IL-6, TNF-α) and anti-inflammatory cytokines (IL-10, TGF-β) by stress hormones (cortisol, ACTH, adrenaline) and antagonist specific hormonal inhibition in rainbow trout (*Oncorhynchus mykiss*) and gilthead sea bream (*Sparus aurata*) head kidney primary cell culture (HKPCC). To this end, HKPCCs were incubated first with antagonist receptor for 30 min prior incubation with cortisol (100 ng ml^{-1}), adrenocorticotropic hormone (ACTH, 150 ng ml^{-1}), or adrenaline (1 μM) for two hours. Data showed that cortisol and ACTH decreased the expression of immune-related genes in sea bream but not in rainbow trout. On the other hand, while adrenaline was found to be suppressor of the pro-inflammatory cytokines (IL-1β, IL-6) in rainbow trout, the opposite effect was induced in sea bream increasing expression of (IL-1β, IL-6). Based on the results obtained, we also aimed to investigate the interaction between endocrine and immune system particularly the immune cell response in the presence of an immune stimulator (*Vibrio anguillarum* bacterin). HKPCC were treated with antagonist receptors and then with stress hormones as mentioned before. Then, *V. anguillarum* bacterin (dilution 1:40) was added at the same time than stress hormones and the effect was evaluated after two hours of incubation. Our results show an increase of pro- (IL-1β, IL-6, TNF-α) and anti-inflammatory cytokines (IL-10, TGFβ1) after *V. anguillarum* treatment in both species. In the presence of *V. anguillarum*, the stress hormones (cortisol, ACTH and adrenaline) did not modulate the expression of immune-related genes in rainbow trout, whereas in sea bream cortisol was able to reduce stimulated gene expression in all

cytokines. Overall, the studies performed in HKPCC confirm the close regional interaction between endocrine and cytokine messengers in the head kidney even in the presence of *V. anguillarum* bacterin, and also a clear difference between the two fish species in both the sensitivity to bacterin and to hormonal stimuli. Altogether, in contrary of classical view of stress hormone as an immune suppressor, we demonstrated distinct effect of hormones in different species.

Considering that the stress hormones modulated differentially the cytokine expression in trout and sea bream at regional level, we evaluated the effect of anoxia and *V. anguillarum* immersion (formalin killed bacteria) on the gene expression of rainbow trout and sea bream at physiological level, evaluating the expression pattern both at mucosal and systemic level. The response of fish to external stressors after 1, 6 and 24 hours post-stress was studied at first in mucosal tissue (skin, gills and intestine) of fish, which are in intimate contact with the immediate environment. Thus, external stimuli may produce local alterations in mucosal tissues when these potential threats are perceived by specific tissue receptors located at these regions. At such level, the stress and immune response to these stressors was evaluated in skin, intestine and gills surfaces. Our results showed a differential gene expression pattern between rainbow trout and gilthead sea bream, confirming that the response to a stressor at local level is tissue-dependent and species-specific. Thereby we have found gilthead seabream more stressed than rainbow trout.

The response to these stressors was also evaluated at systemic level based on most of data that to date has been reported on stress physiological response. Therefore, the effect of stress on HPI axis activation and gene expression (cytokines and stress genes) was evaluated in spleen and liver of rainbow trout and sea bream. In this particular part of the work, we included the expression analysis in zebrafish as another model organism, in this case a labratory model.

Importantly, at plasmatic level cortisol increased at 1 hours post anoxia (hpa) and then decreased at 6 hpa in trout and zebrafish but not in sea bream whose cortisol levels remained high compared to control, suggesting a greater degree of responsiveness to stress in gilthead seabream. Moreover, when fish were exposed to anoxia plus vaccination the cortisol level was also augmented at 1 and 6 h in rainbow trout and seabream and restored to the basal level at 24 h, whereas in zebrafish the response was higher indicating a longer response of HPI axis to the combination of both stressors. The gene expression pattern in spleen and liver was found to be differential and time dependent among species. Altogether, these results indicate that similar cortisol secretion kinetics takes place in the studied species. However, the modulation of genes appears to be decreased rather than the increment observed at mucosal level.

By taking into considering, the highest expression of cortisol at systemic level in sea bream, the expression response in hypothalamus and pituitary hormone stress genes after anoxia and vaccination was studied in the gilthead sea bream. The results show that stress by

anoxia is primarily stimulating both hypothalamic CRH and pituitary POMCB and PRL at short time whereas vaccine stimulates hypothalamic (CRHBP and TRH) and pituitary peptides (POMC, PRL, GH) at longer time points, as a possible result of feedback since GR expression in hypothalamus is also stimulated. Thus, the results confirm that at central nervous system different stressors show different stimulatory abilities as anoxia is perceived immediately and vaccine is not, which could be directly associated to the particular dynamics of the central stress response.

In summary, the present work supports the view that different species show a differential immune-related gene expression profile when subjected to several stressors. These differences may reside in the distinct sensitivity to the bacterin stimuli and to hormonal stimuli, and also to the dynamics of the central response to those stressors. We also conclude that fish from seawater may be more stressed than fresh water fish as this is confirmed with result obtained in cortisol and gene expression regulation.

Resum

Tots els animals poden estar exposats a factors estressants al llarg de la seva vida. Els peixos en condicions d'aqüicultura habiten un mitjà potencialment estressant que pot suposar un desafiament addicional a l'animal. En els peixos, la resposta a l'estrès i les seves conseqüències en el sistema immune han estat àmpliament descrits. Es tracta de l'activació de l'eix Hypothalamic-Hipofisari-Interrenal (HPI) i del Cervell-Simpatico-Cromafí (BSC) com a resposta neuroendocrina que promou la secreció de les hormones de l'estrès per cèl·lules interrenals i cromafins situades al ronyó anterior. En aquest àmbit, el ronyó anterior de peix equival a la medul·la òssia i la glàndula adrenal de mamífers, funcionant en una sola estructura multifuncional, incloent hemato- i limfopoiesi i també alliberant les hormones d'estrès principals (glucocorticoides i catecolamines). Per tant, la resposta a l'estrès no només activarà l'eix d'HPI per segregar hormones, sinó que també modularà la resposta immune dels peixos que afecta l'expressió de citoquines i tota la reacció immunitària. Fins fa poc, es va suposar que l'efecte de les hormones implicades en la resposta a l'estrès fisiològic (com el cortisol, l'ACTH, l'adrenalina) seria independent de les espècies i prou constant, però segons el nostre coneixement, cap estudi hauria comparat l'efecte de les hormones o de les condicions d'estrès idèntiques en diverses espècies. D'aquesta manera, l'objectiu era donar a conèixer la interacció entre el sistema neuroendocrí i el sistema immunitari en dues espècies de peixos diferents (truita arc-iris com a model de peix d'aigua dolça i orada (com a model de peix marí) en resposta a les hormones de l'estrès mitjançant l'avaluació de l'expressió gènica.

Primer es va intentar investigar la modulació de citoquines, pro-inflamatòries (IL-1β, IL-6, TNF-α) i antiinflamatòries (IL-10, TGF-β) per hormones d'estrès (cortisol, ACTH, adrenalina) i l'inhibició hormonal específica per antagonistes en la truita de arc-iris (Oncorhynchus mykiss) i l'orada (Sparus aurata) en cultiu de cèl·lules de ronyó anterior (HKPCC). A aquest efecte, el HKPCC es va incubar primer amb receptor antagonista durant 30 minuts abans de la incubació amb cortisol (100 ng ml-1), hormona adrenocorticotròpica (ACTH, 150 ng ml-1), o adrenalina (1 μM) durant dues hores. Les dades mostraren que el cortisol i l'ACTH disminueixen l'expressió de gens immunitaris en orada, però no en la truita arc-iris. D'altra banda, mentre que l'adrenalina era supressora de les citoquines proinflamatòries (IL-1β, IL-6) en la truita arc iris, es va induir l'efecte contrari (IL-1β, IL-6). Sobre la base dels resultats obtinguts, també es va tractar d'investigar la interacció entre el sistema endocrí i el sistema immune, en particular la resposta a la cèl·lula immune en presència d'un estimulador immune (el bacteri Vibrio anguillarum). El HKPCC es va tractar amb receptors antagonistes i després amb hormones d'estrès com s'ha esmentat anteriorment. Després, la bactèria V. anguillarum (dilució 1:40) es va afegir al mateix temps que les hormones de l'estrès i l'efecte es va avaluar després de dues hores d'incubació. Els nostres resultats mostren un augment de les citoquines pro-(IL-1β, IL-6, TNF-α) i antiinflamatòries (IL-10, TGFβ1) després del tractament de V. anguillarum en ambdues espècies. En presència de V. anguillarum, les hormones de l'estrès (cortisol, ACTH i adrenalina) no modulaven l'expressió de gens

immunes en la truita arc iris, mentre que en orada el cortisol va poder reduir l'expressió gènica en totes les citoquines. En general, els estudis realitzats al HKPCC confirmen l'estreta interacció regional entre missatgers endocrins i de citoquines al ronyó anterior, fins i tot en presència de bacteri V. anguillarum, i també una clara diferència entre les dues espècies de peixos tant en la sensibilitat a la bactèria com en els estímuls hormonals. Al contrari de la visió clàssica de l'hormona de l'estrès com un supressor immune general, es va demostrar un efecte diferenciat d'hormones en diferents espècies.

Tenint en compte que les hormones de l'estrès modulaven de manera diferenciada l'expressió de citocina en truites i orades a nivell regional, es va avaluar l'efecte de l'anoxia i la immersió amb V. anguillarum (bacteris en formol) sobre l'expressió gènica de la truita arc iris i orada a nivell fisiològic, avaluant el patró d'expressió tant a nivell mucosal com sistèmic. La resposta dels peixos als estressors externs després d'1, 6 i 24 hores després de l'estrès es va estudiar al principi en el teixit mucós (pell, intestí i brànquies) de peixos, ja que està en contacte íntim amb l'entorn immediat. Per tant, els estímuls externs poden produir alteracions locals en els teixits de la mucosa quan aquestes amenaces potencials són percebudes per receptors específics de teixits localitzats en aquestes regions. En aquest nivell, es va avaluar l'estrès i la resposta immune a aquests estressors en les superfícies de la pell, l'intestí i les brànquies. Els nostres resultats van mostrar un patró d'expressió gènica diferencial entre la truita arc iris i l'orada, confirmant que la resposta a un estressor a nivell local és dependent del teixit i específica de l'espècie. D'aquesta manera, hem trobat que l'espècie marina és més estressable que la truita d'aigua dolça.

La resposta a aquests estressors també es va avaluar a nivell sistèmic tenint en compte les dades que fins ara s'han informat sobre la resposta fisiològica a l'estrès. Per tant, es va avaluar l'efecte de l'estrès sobre l'activació de l'eix HPI i l'expressió gènica (citoquines i gens d'estrès) a la melsa i al fetge de la truita arc iris i l'orada. En aquesta part particular del treball, incloem l'anàlisi d'expressió en peix zebra com un altre organisme model, en aquest cas un model de laboratori.

És important destacar que, a nivell plasmàtic, el cortisol augmenta a 1 hora després de l'anòxia (hpa) i després es va reduir a les 6 hpa a la truita i el peix zebra, però no a l'orada, els nivells de cortisol es van mantenir elevats en comparació amb el control, suggerint un major grau de resposta a l'estrès. A més, quan els peixos van estar exposats a la vacuna, el nivell de cortisol també es va augmentar a les 1 i 6 hores a la truita de l'arc iris i a l'orada es va restaurar el nivell basal a les 24 h, mentre que en el peix zebra la resposta va ser més alta, la qual cosa que indica una resposta més àmplia de l'eix d'HPI a la combinació d'ambdós estressors. El patró d'expressió gènica en la melsa i el fetge va ser diferent i dependent del temps en cada espècie. En conjunt, aquests resultats indiquen que es produeix una cinètica de secreció de cortisol similar a l'espècie estudiada. Tanmateix, la modulació dels gens sembla disminuir a nivell sistèmic en lloc de l'increment observat a nivell de mucosa.

Prenent els resultats anteriors en consideració, es va estudiar l'expressió a nivell dels òrgans centrals, és a dir la resposta d'expressió en hipotàlem i hipofisari després de l'anoxia i la vacunació en l'orada. Els resultats mostren que l'estrès per anòxia és principalment estimulador tant en el CRH hipotàlem com en POMCB i PRL hipofisari, mentre que la vacuna estimula els pèptids de hipotàlem (CRHBP i TRH) i pituïtaris (POMC, PRL, GH) a temps més llargs, com a resultat possible de l'acció retroalimentadora del cortisol i el receptor GR en hipotàlem. D'aquesta manera, els resultats confirmen que, en el sistema nerviós central, diferents estressors mostren diferents habilitats estimulants, ja que es detecta immediatament l'anòxia i la vacuna més tard, que podria estar directament associada a la dinàmica particular de la resposta central a l'estrès.

En resum, el present treball dóna suport a la visió que diferents espècies mostren un perfil d'expressió gènica diferencial immunitària depenent dels diversos factors estressants. Aquestes diferències poden residir en la sensibilitat diferent als bacteris i als estímuls hormonals, i també a la dinàmica de la resposta central a aquells estressors. També es conclou que els peixos de l'aigua de mar podrien ser més sensiblel a l'estrès que els peixos d'aigua dolça segons confirmaria el resultat obtingut en cortisol i en la regulació de l'expressió gènica.

Chapter one

2.1 Introduction

2.1 Stressing the inflammatory network: immuno-endocrine responses to allostatic load in fish

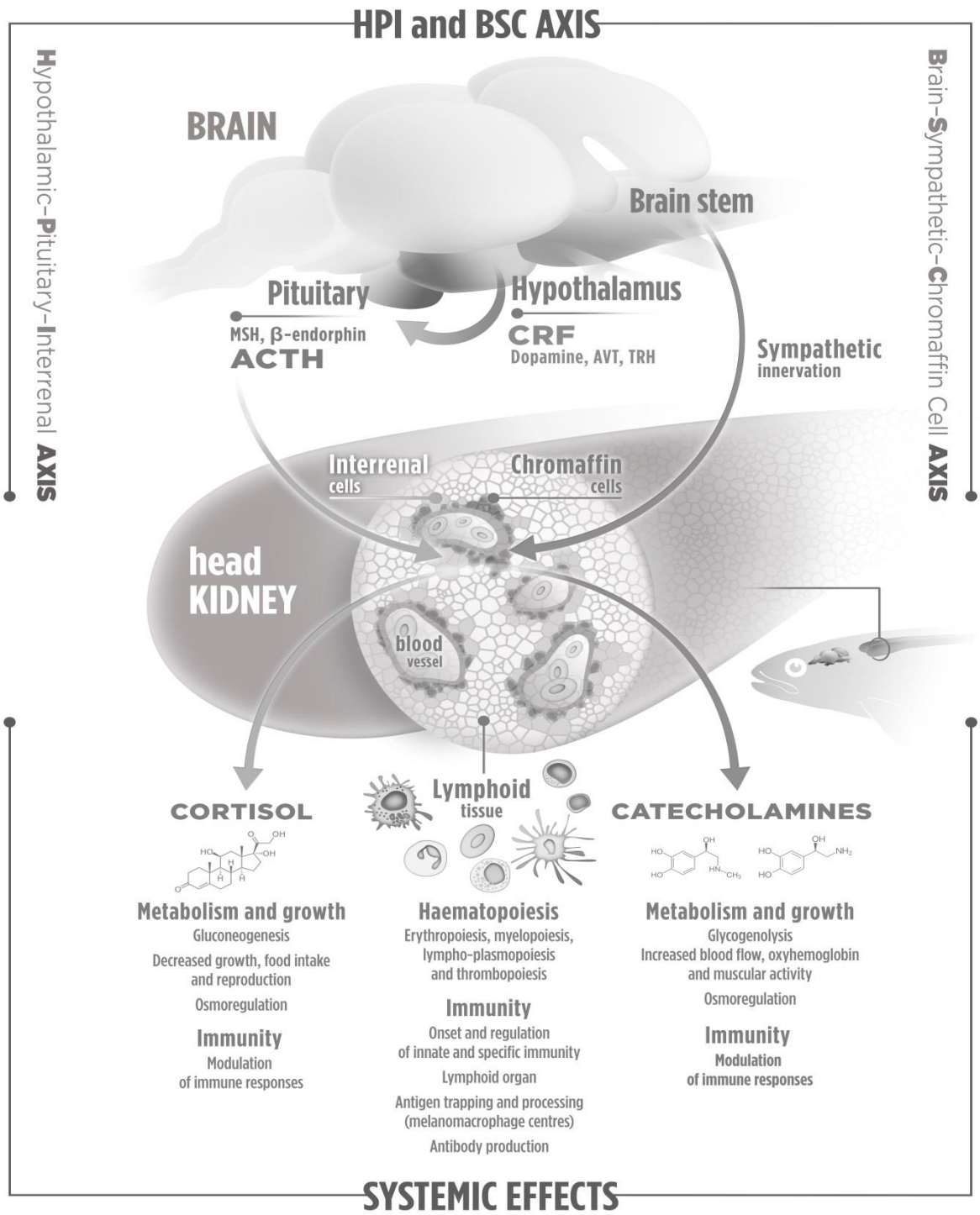
1. Networking the neuroimmunoendocrine responses in fish: the nodal head kindey

Fish inhabit an intrinsically stressful medium. The physical and colligative properties of water facilitate the dispersion and resilience of xenobiotics, opportunistic pathogens and endocrine disruptors among other potentially distressing agents, hence translating the viral/bacterial load to a harmful allostatic load. Fish are considered a paraphyletic group of vertebrates, therefore numerous individual and populational differences exist in how fish species respond to stress circumstances, in particular on how individuals interpret and perceive the external signals or stimuli as a threat, thus activating or maintaining an active stress response. Imbalance in the systems generated by the allostatic load and the stress response has to be rapidly compensated because this situation may damage the host when it becomes chronic. Thus, a number of mediators from the neural, endocrine and immune systems will be activated to regulate the overall response to this allostatic load. Lesions or parasite occurrence in skin or gills, or local environmental changes in the gut caused by diet composition or pathogens, may also initiate local changes triggering the cytokines- or neural peptides-mediated immune response (Parra et al., 2015).

The physiological effects of waterborne fish diseases remain species-specific, but a common pattern emerges: in fish, the activation of Hypothalamic-Pituitary-Interrenal (HPI) and Brain-Sympathetic-Chromaffin cell (BSC) axis directs the neuroimmunoendocrine crosstalk (Bernier and Peter, 2001), and the head kidney harbours the cellular scaffold that integrates the stress response. In the extensive systemic network of stress-activating immunitary defences and hormonal secretions, the proximal part of the kidney acts as a regulatory nodal organ, cradles the haematopoietic tissue and releases the main stress hormones, corticosteroids and catecholamines (Figure 1). In fish, the head kidney encompasses the mammalian bone marrow and adrenal gland functionality in one single multifunctional structure (Figure 2).

Cortisol (the main active corticosteroid in fish) is secreted by head kidney interrenal cells in a concatenated response after perception of stress by the CNS, involving release of hypothalamic corticotrophin releasing hormone (CRH) from hypothalamic paraventricular nucleus (PVN) and the adrenocorticotropic hormone (ACTH) by the pituitary gland. Released ACTH is recognized and bounded to melanocortin receptor 2 (MC2R) in the surface of interrenal cells and activates the steroidogenic signalling pathway leading to cortisol secretion as the final product of HPI axis activation (Aluru and Vijayan, 2008). The head

Figure 1:


HPI and BSC AXIS
Hypothalamic-Pituitary-Interrenal AXIS
Brain-Sympathetic-Chromaffin Cell AXIS
BRAIN
Brain stem
Pituitary
MSH, β-endorphin
ACTH
Hypothalamus
CRF
Dopamine, AVT, TRH
Sympathetic
innervation
Interrenal
cells
Chromaffin
cells
head
KIDNEY
blood
vessel
Lymphoid
tissue
CORTISOL
CATECHOLAMINES
Metabolism and growth
Gluconeogenesis
Decreased growth, food intake
and reproduction
Osmoregulation
Immunity
Modulation
of immune responses
Haematopoiesis
Erythropoiesis, myelopoiesis,
lympho-plasmopoiesis
and thrombopoiesis
Immunity
Onset and regulation
of innate and specific immunity
Lymphoid organ
Antigen trapping and processing
(melanomacrophage centres)
Antibody production
Metabolism and growth
Glycogenolysis
Increased blood flow, oxyhemoglobin
and muscular activity
Osmoregulation
Immunity
Modulation
of immune responses
SYSTEMIC EFFECTS

kidney also receives sympathetic innervation, involving release of catecholamines (adrenaline and noradrenaline) by chromaffin cells.

The degree of activation of the neuroendocrine stress axis in fish depend on the type of stressors, intensity and also the duration of stimuli (Tort, 2011). The stress response is characterised by its rapidity. Thus, the level of cortisol in zebrafish measured at frequent intervals over 1 hour, show initial increments already at 3 min post stress, peaking at 15 min (Ramsay et al., 2009). Results obtained by our group also showed both in rainbow trout and sea bream subjected to acute stress the secretion of cortisol at the first minutes, peaking at 1 h , though the level can be still high after the first hours returning to control level at 12-16 h (Castillo et al., 2008; Fierro-Castro et al., 2015). When the stressor is chronic or fish are subjected to a pathogenic stressor such as bath administration of *Vibrio anguillarum*, cortisol levels are maintained for several days or weeks after the initial exposure (Khansari et al., submitted).

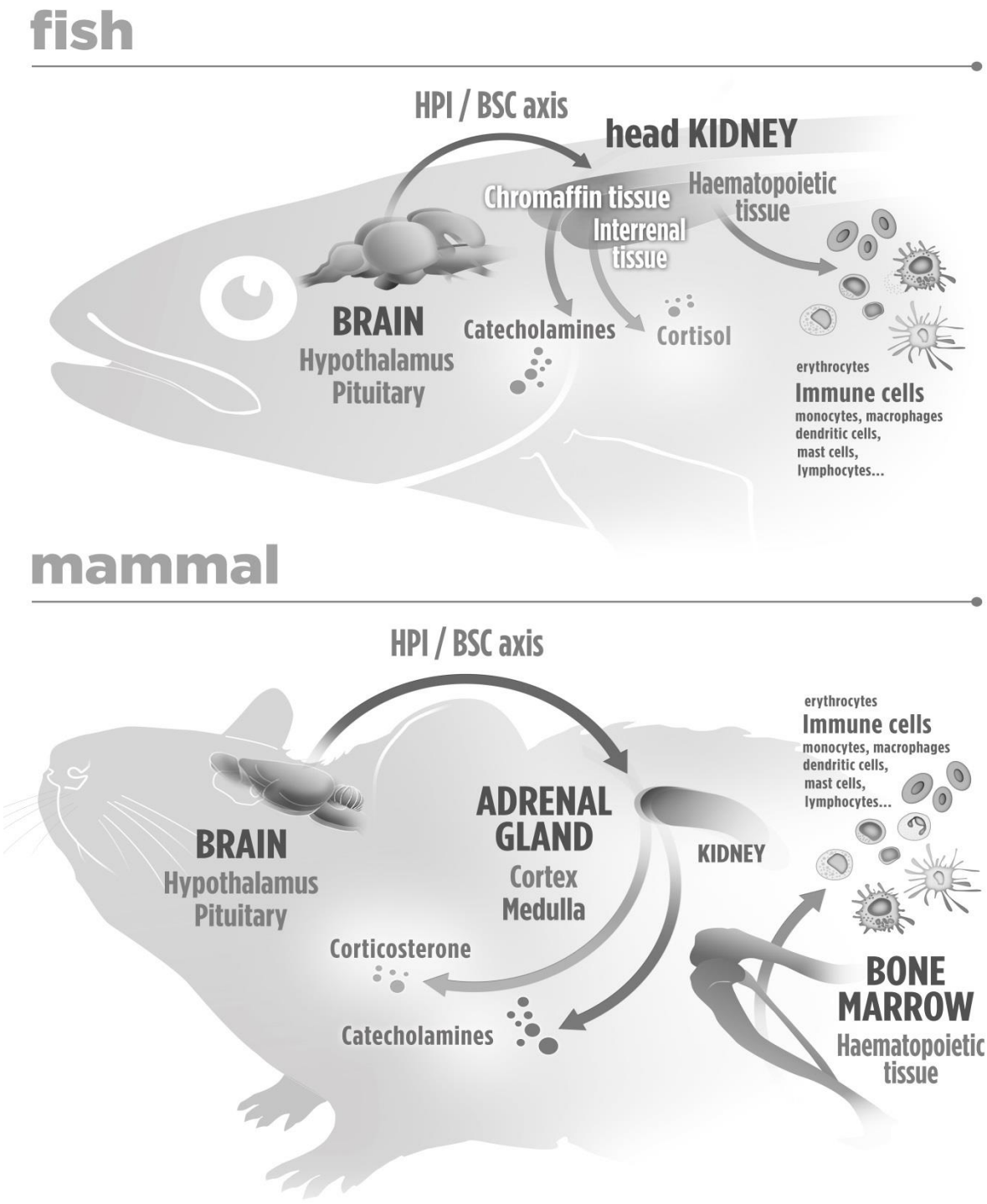
Both immune and endocrine cell types share common receptors (Baigent, 2001). Cortisol has receptors in different leukocyte cell types and it has been shown to modulate the immune response (Ader et al., 1995; Bury et al., 2003; Harris and Bird, 2000). B- and T-cells are affected by cortisol reducing lymphocyte proliferation and reduction of natural killer cell (NK) activity and also impairment of antibody secretion (McEwen et al., 1997). These effects have been shown either *in vitro* or *in vivo* studies (Castillo et al., 2009; Fast et al., 2008; Philip et al., 2012). ACTH receptors have been detected in spleen and thymus and furthermore, teleost fish show corticotropic-releasing-factor (CRF) immunoreactivity in both gills and skin macrophage-like cells (Mazon et al., 2006). Other hormones such as leptins have also been found in the thymus and spleen of carp (Huising et al., 2006), indicating that the acute stress response may have a direct effect on immunological tissues (Mola et al., 2005). Moreover, production of other hormones such as growth hormone and prolactin has been demonstrated to occur in fish immune cells (Yada, 2007; Yada and Tort, 2016).

The full account of the overall neuroimmunoendocrine interactions in fish are beyond the scope of this review. Here, we aim to briefly survey the main characteristics of fish immune system and describe the effects, characteristics and mediators of its stress-related endocrine regulation.

2. The facts and feats of fish immune system

Besides the head kidney, the organography of immune tissues in fish resembles that of the mammals (Figure 2), and, being vertebrates, both innate and adaptive branches of the immune system participate in defensive responses. The innate immunity is the primary and traditionally *non-specific* line of defence (Aderem and Ulevitch, 2000), and has been defined as the mechanism of recognition and response to pathogen-associated molecular patterns (PAMPs) such as polysaccharides, lipopolysaccharides (LPS), peptidoglycans, bacterial DNA or double strand viral RNA (Medzhitov and Janeway Jr, 2002; Sercarz, 1989). Tissue damage

Figure 2:


fish
HPI / BSC axis
head KIDNEY
Chromaffin tissue
Haematopoietic tissue
Interrenal tissue
BRAIN
Hypothalamus
Pituitary
Catecholamines
Cortisol
erythrocytes
Immune cells
monocytes, macrophages
dendritic cells,
mast cells,
lymphocytes...
mammal
HPI / BSC axis
erythrocytes
Immune cells
monocytes, macrophages
dendritic cells,
mast cells,
lymphocytes...
BRAIN
Hypothalamus
Pituitary
ADRENAL GLAND
Cortex
Medulla
KIDNEY
Corticosterone
Catecholamines
BONE MARROW
Haematopoietic tissue

also elicits a danger/damage-associated molecular patterns (DAMPs) inflammatory response when cellular contents or molecular immunity mediators are spilled in the extracellular matrix. The pathogenic signatures are recognized by pathogen recognition receptors (PRRs) found in immune cells, including lymphocytes, macrophages and neutrophils and soluble components on cellular compartments, endosomes, lysosomes and endolysosomes (Akira et al., 2001). Currently, more than 17 Toll-like receptors (TLRs), a particularly widespread PRR, have been described in fish (Tanekhy, 2014) with conserved TLR-signalling pathways (Purcell et al., 2006), although with different features and high diversity (Palti, 2011).

The adaptive responses are orchestrated by the recognition of specific antigens by B and Tcell specialized receptors (BCR and TCR, respectively) that triggers a clonal expansion of selected lymphocyte populations and initiates the production of specific tailored antibodies. In fish, the levels of antibodies produced in response to an infectious process are maintained for a longer time compared to the mammal response model. However, the dynamics of immune response is slower and smaller in magnitude (Roberts, 2012). Low temperatures may also have a higher impact on Tcell immune function(Figure 3), although this is a highly species-specific effect (Cheng et al., 2017; Raida and Buchmann, 2007; K Rakus et al., 2017; Krzysztof Rakus et al., 2017). The immune defence in fish can be considered focused on innate parameters because the activation is quick, roughly constant and temperature independent (Makesh et al., 2015), while the acquired immune response in fish takes at least couple of weeks to be fully active in comparison to innate immune response (Ye et al., 2013). In zebrafish, the level of specific antibody was gently increased and reached highest levels within 28 days post vaccination (Zhang et al., 2012). It has been suggested that the ectothermy of fish may have limited the progression of their adaptive responses, favouring a more robust innate immunity (Magnadóttir, 2006). Nevertheless, the adaptive immune response is of fundamental importance in fish defence since both T-cell responses and the synthesis of immunoglobulins are necessary to mount an efficient immune response against pathogens.

2.1. Immune sensing: mucosal portals of entry

The aquatic environment harbours a wide variety of potential threats involving biological, physical and chemical agents that interact with the immune cells and molecules nested in the mucosal surfaces. According to mammalian nomenclature, these Mucosal-Associated-Lymphoid-Tissues (MALT) include (Figure 3) the gut-associated-lymphoid tissue (GALT), skin-associated-lymphoid-tissue (SALT), gills-associated-lymphoid-tissue (GIALT) and the most recently proposed nose-associated-lymphoid-tissue (NALT) (Abelli et al., 2009; Salinas et al., 2011; Parra et al. 2015). The epithelium of these tissues is protected by a mucus layer which acts as a chemical and physical barrier, therefore becoming the immediate innate immune protection and acting as the first line of defence against pathogens (Gomez et al., 2013). In addition, mucosal tissues include a number of immune competent cells ready for an immediate response against immune threats. For instance, the gut mucosal immune system

is armed by different cell-type populations including lymphocytes, plasma cells, granulocytes and macrophages both in the epithelium or dispersed in the *lamina propia* (Rombout et al., 2011). The GALT component of mucosal immunity can be traced back in the evolution of jawed vertebrates and it is considered to have played a significant role in forming the variegation of the molecules present in gut. Skin is the largest mucosal surface in fish and it has been shown to be a metabolic, endocrine and immune active tissue (Parra et al., 2015). There are some molecules that confer antimicrobial ability to the skin mucus such as lysozyme, complement components, lectins, proteolytic enzymes and Igs (Nigam et al., 2012). Thus, it has been reported that infection with *Gyrodactylus derjavini* induced the expression of TNF-α, TGF-β and COX2 in rainbow trout skin (Lindenstrøm et al., 2004). Also husbandry stressors suppressed the activity of enzymes and genes involving cytokines such as IL-β1, TNF-α and IGF-1 in skin of *Scophthalmus maximus* after long-term stress (Jia et al., 2016). Recently, it was reported that teleost skin evokes gut-like immune responses with a predominant role of IgT, a teleost immunoglobulin specialized in gut immunity (Xu et al., 2013). We recently showed that acute handling stress and *Vibrio anguillarum* exposure promoted the expression of cytokines that are involved in inflammation response including IL-β1, TNF-α, IL-6, IL-10 in skin of rainbow trout and gilthead sea bream, therefore illustrating the importance of this barrier in inflammatory response. Together with skin, GIALT is the organ with most intimate contact with the aquatic environment, representing a crucial organ to deal with pathogens and also a portal of entrance for them. Thus, two common pathogens such as *Vibrio anguillarum* and *Aeromonas salmonicida* enter to fish via gills (Baudin-Laurencin and Germon 1987; Svendsen, Dalmo, and Bogwald 1999). Most immune cell-types are present in gills including lymphocytes, macrophages, eosinophils, granulocytes, neutrophils and antibacterial proteins (dos Santos et al., 2001). B-cells and the presence of IgT have been also reported in gills (XU et al., 2013). It has been described that bacterial challenges with *Vibrio anguillarum* and *Aeromonas salmonicida* induce changes in antibacterial proteins and cytokine expression in the gills of Atlantic cod (*Gadus morhua*) expressing IL-β1, IL-22, IFN-γ and IL-8. Therefore, it seems that the immune system implicated on fish mucosal barriers shows a rich repertoire of innate immune components and a clear interaction with the evironmental microbiota involved. Finally, the mucosal immune system also relies on NALT. The olfactory sensory system appears to be one of the most ancient organs and its mucosa has been shown to be essential for all animals acting as mucosal defence against either waterborne antigens in aquatic vertebrates or airborne antigens in terrestrial vertebrates. Rainbow trout has been shown to be armed by NALT immune features showing innate and adaptive immune capabilities (Tacchi et al., 2014). Trout olfactory tissue express immune features such as immune gene expression and immunoglobulin production, demonstrating IgT coating ability to the majority of bacteria (Tacchi et al., 2014). As in mammals, the existence of nose mucosal immune features in fish indicates a vital role in facing with waterborne antigens.

2.2. Humoral messengers: acute-phase response proteins (ARPs), cytokines and antibodies

ARPs

A number of proteins are important players of the first-line inflammatory responses in fish, amongst them complement, lysozyme, and a plethora of antimicrobial proteins (AMPs). Upon antigen exposure, ARPs convey the cellular crosstalk and help to regulate the onset on innate immune responses.

Complement is composed of at least 35 proteins that are devoted to attacking the bacterial membrane. It can be initiated and activated by three distinct pathways partially overlapped: the classical pathway (CP), the alternative pathway (AP) and the lectin pathway (LP). The CP activation is induced by binding of antibody to the complement C1 complex, made by C1q and two serine protease (C1r and C1s), or by sticking directly of the C1q component to the bacteria surface. After hydrolysing of C3 to C3(H2O), which obtains a C3b-like composition capable of binding to factor B(Bf) and factor D, leading in formation of the alternative C3 convertase (Holland and Lambris, 2002). The Alternative Pathway is initiated by the spontaneous hydrolysis of C3, an abundant protein of blood plasma that after sucesive reactions produce C3-convertase and after binding properdin, C5-convertase which binds sequentially a number of other complement proteins ending with a membrane attack complex that is able to lyse bacteria (Sunyer et al., 2003). The Lectin Pathway is initiated by microbial polysaccharides that interact with circulating lectins. In fish, C3 component participates as a central complement component in all three pathways. Therefore the presence of C3 even at early stage of the fish life has been shown to exist because of its pivotal role in innate immune system (Løvoll et al., 2007). A study performed in our group illustrated that C3 is suppressed in liver as a producer of complement after cortisol administration in rainbow trout, whereas the repression was not induced in spleen, thus C3 appears to be regulated after stress condition in tissue specific response (Cortés et al., 2013). One of the characteristics of the complement system in fish is the high variety of their proteins, thus offering a wide variety of isoforms, which contrasts with the lower variability found in mammals (Sunyer et al., 1997, 1998). This variety of isoforms has been associated to a greater possibility of fish to respond to a wider variety of antigens at innate immune level (Sunyer et al., 1998).

Lysozyme is known as a bactericidal agent playing a crucial role in the innate immune system against broad spectrum of invading microorganisms. Although primarily lysozyme is concerned with defence against Gram-positive bacteria, also Gram-negative bacteria can be lysed by this enzyme (Magnadóttir, 2006). Lysozyme can activate the complement system, therefore once the outer membrane of bacteria is destroyed by complement, then lysozyme appears to be effective to process the pathogen (Grinde, 1989). Lysozyme is present in lymphoid tissue, plasma and body fluids and it has also been found on fish skin mucus (Nigam et al., 2012; Paulsen et al., 2001).

Pentraxins (C-reactive protein, CRP and serum amyloid protein, SAP) are lectins, which are found in the body fluids of both vertebrates and in vertebrates and thus are involved in the so-called acute phase of response showing increments at serum level after tissue damage, trauma or infection. The pentraxins participate in the innate immune response through induction of complement proteins playing a role in the identification and elimination of apoptotic cells (Haas et al., 2000; Szalai et al., 1992).

Fish can also fight pathogens by producing a broad spectrum of antimicrobial peptides (AMPs). Overall, AMPs are secreted into the circulation that is the major target for antigens. A long list of antimicrobial peptides in fish has been proposed with immunomodulatory properties (Rajanbabu and Chen, 2011). Indeed, previous antecedents demonstrated that antimicrobial peptides are capable of modulating the cytokines response in mammals and fish (Bowdish et al., 2005; Chiou et al., 2006). Also, in Coho salmon was shown protection against *Vibrio anguillarum* after a previous administration of AMPs (Jia et al., 2000).

Cytokines

Cytokines are secreted proteins with growth, differentiation, and activation functions that regulate the nature of immune responses (Reyes-Cerpa et al., 2013). Cytokines are active at the portals of entry resulting in elimination of the antigen. IL-1β is an endogenous pyrogen produced and released at the early stage response to infections, lesions and stress (Duque and Descoteaux, 2014). It has been reported the expression of IL-1β in fish following infection of both bacterial (Rojo et al., 2007; Zhang et al., 2013) and also viral pathogens (Reyes-Cerpa et al., 2014, 2012). This is because in fish IL-1β is (as in mammals) one of the most important initiator of pro-inflammatory response with key roles on the stimulation of pro-inflammatory mediators such as other cytokines and prostaglandins in macrophages, and activation of lymphocytes (Alvarez-Pellitero, 2008; Dinarello, 2009). IL-1β can activate the expression of IL-6, a pleiotropic cytokine which has pro-inflammatory function, being induced by LPS, poly I:C and IL-1β both in the macrophage cell line RTS-11 and head kidney macrophages (Costa et al., 2011). IL-6 also promotes the differentiation of B-cells into plasma cells and activating cytotoxic T cells (Duque and Descoteaux, 2014). It has been also reported that IL-6 can significantly down-regulate the expression of pro-inflammatory cytokines, suggesting a potential role of trout IL-6 in limiting host damage during inflammation (Costa et al., 2011). Other relevant pro-inflammatory cytokine is TNFα, which plays an important role in a wide range of host responses, including cell proliferation and differentiation but also necrosis and apoptosis. This cytokine can stimulate the acute phase response and is one of the first to be released in response to pathogens as well as after stress episodes (Teles et al., 2011). Also, TNFα is able to exert its effect in many organs as well as induction of IL-6 together with IL-β (Baud and Karin, 2001). Beyond the systemic immune organs which are directly implicated in immune response, the expression of IL-β1 and TNF-α has been also observed in the brain of sea bream and sea bass after Nodavirus exposure (Poisa-Beiro et al., 2008) indicating that other organs may be involved in the

cytokine synthesis and, therefore, in the fish response against pathogens. The pro-inflammatory cascade is strictly regulated by anti-inflammatory mediators, TGFβ1 and IL-10, responsible to maintain the response under control to avoid tissue damage (Cools et al., 2007; Hu et al., 2008). TGFβ1 is often associated with IL-10 as an immunosuppressive cytokine (Cools et al., 2007) regulating the immune response by blocking the activation of lymphocytes and monocyte-derived phagocytes and promoting tissue repair after a local inflammatory response (Li et al., 2009). Importantly, the expression of TGFβ1 has also been associated to fish disease resistance, thus promoting a persistence phenotype in those fish infected with viral pathogens (Reyes-Cerpa et al., 2014; Reyes-López et al., 2015). On the other hand, IL-10 is produced by activated macrophages, B cells and T cells (Mosser and Zhang, 2008), thus playing a pivotal role on the resolution of infection and, therefore, in reducing tissue damage caused by inflammation (Moore et al., 2001; Ouyang et al., 2011). Taking together, the cytokine response is crucial for fish to induce an efficient and tightly regulated immune response.

Antibodies

Immunoglobulins are produced by B-cells (Parra et al., 2013). Immunoglobulins can be categorized into different classes according to the nature of the C domain of the heavy (H) chain. Until recently fish were thought possessing only IgM and IgD, but interestingly in 2005 new immunoglobulins heavy chain IgT and IgZ were reported in rainbow trout and zebrafish, respectively (Danilova et al., 2005; Hansen et al., 2005). IgT is chiefly polymeric and it is expressed in the gut mucus in much higher numbers than that observed in the serum, while IgM appears to be circulating in the serum. Although production of specific antibodies against pathogens is assumed to be one of the efficient routes to cause immunoprotection, there has been suggested that because of low affinity of IgM to pathogen, antibody induction cannot prevent pathogen infection at early stages (Raida et al., 2011). Therefore, IgT is proposed to play an essential role in the mucosal immune response (Zhang et al., 2012). Unpublished results of our group also show that the transcription of IgM can also be regulated after acute handling stress, though a previous study showed immunoglobulin M expression after temperature changes in gills, spleen, head kidney and intestine (Cui et al., 2010). Also it has been shown that different environmental factors such as salinity influenced on the circulating level of immunoglobulins (Dominguez et al., 2004).

2.3. Cellular immunity

The innate immune response is also characterized by a pivotal action of macrophages as one of the primitive players of the host response to tissue invasion, resulting in phagocytosis and pathogen killing. A study performed by Mosser and colleagues (Mosser and Edwards, 2008) suggested that distinct pathogen invasions usually determine macrophages activation patterns into classically activated or alternatively activated macrophages. The activation of these macrophages normally result in secretion of pro-inflammatory cytokines such as IL-β1,

TNF-α, IL-12 and IL-23, and also chemokines such as CXCL8-11. Thus, they take part in host defence against intracellular pathogens (Mosser and Edwards, 2008). Alternatively activated macrophages are also known as an anti-inflammatory mediators playing essential role in protection of the host. After presence of pathogen in the host and activation of macrophages, the triggered response has to be rapidly controlled through phagocytosis, tissue repair and resolution of the infection. These macrophages regulate the pro-inflammatory response by imposing the anti-inflammatory activities to suppress IL-12 and boosting the production of IL-10 to protect the host form damage (Mosser and Edwards, 2008).

The identification of T cell markers such as α and β T cell receptor genes (TCR), CD3, CD4, CD8, CD28, CD40L, and also the presence of cytokines and chemokines suggest the presence of T helper (Th)1, Th2, Th17 and subsets of regulatory T cells in teleosts (Reyes-Cerpa et al., 2013). Lymphocytes express three distinct functions including provision of appropriate microenvironment for development of immune effector cells, mediating quality control (positive and negative selection) and regulating memory and pathogen elimination through the synthesis of immunoglobulins (Scapigliati, 2013). An important feature of fish lymphocytes that has been poorly studied is the correlation between nervous and immune system. Most of the immune components, especially lymphocytes, express receptors for neuromediators, thus being another bilateral regulatory network through direct neuro-immune connections. In vertebrates, CD4 T lymphocytes recognize peptides presented by MHC class II molecules on antigen-presenting cells and proliferate in response to autocrine IL-2. The presence of CD8(+) dendritic-like cells provides evidence of the presence of specialized antigen-presenting cells (Granja et al., 2015). In fish, functional activities related to Th1 and Th2 response have been shown in rainbow trout and zebrafish observing expression of CD4 (Takizawa et al., 2011; Zhu et al., 2012). Contrary to tetrapods, teleosts contain two CD4 genes, called CD4-1 and CD4-2 (Castro et al., 2011; Laing and Hansen, 2011). The biotechnological advances have allowed the generation of specific antibodies anti-CD4 in salmonids. Thus, it has been described the existence of T lymphocytes CD4-1(+)CD3ε(+) (Maisey et al., 2016) but also lymphocytes CD4-1(+)CD4-2(+) and CD4-2(+) and, in addition, one CD4-1(+) myeloid subset (Takizawa et al., 2016). Thus, the utilization of antibodies will provide additional evidence to understand the role of different cell-type populations on the immune response in teleost fish.

Among the most striking features of lymphocytes of fish is their ability to perform phagocytosis, as compared to mammalian lymphocytes. Three major B cell lineages have been described in teleosts, those expressing either IgT or IgD, and the most common lineage which co-expresses IgD and IgM. Thus Li et al., (2006) demonstrated for the first time that about 40% of fish lymphocytes have phagocytic activity and intracellular bactericidal capacities. This finding represented a paradigm shift as professional phagocytosis was believed to be exclusively performed by some cells of the myeloid lineage This property of fish B cells suggest that lymphocytes have evolved to maintain early functional capacities of

cells that have been lost further in the evolution as both white and red blood cells become more specialized. This involves a less specialization but higher versatility of fish blood cells to fight against pathogens. This phagocytic capacity was also found as well in other groups of vertebrates, amphibians and reptiles, suggesting that this innate capacity was evolutionarily conserved in certain B cell subsets of vertebrates. Moreover, it appears that phagocytic B-1 B cells have a potent ability to present particulate antigen to CD4(+) T cells (Sunyer, 2012). At present, these studies that were carried out originally on fish B cells have led to the discovery of new innate and adaptive roles of B cells in mammals (Sunyer, 2012)

Erythrocytes are the most abundant cell in circulatory system. Fish erythrocytes half-life has been shown to be 80-500 days compared to 120 days in humans (Fischer et al., 1998). Nevertheless, the main differential characteristic is that fish red blood cells present nucleus, as in other lower vertebrates, contrary to mammals. It is assumed that during the evolution fish erythrocytes conserved the nucleus and therefore the ability to transcript, translate and produce proteins, whereas mammalian erythrocytes are not able to do it because of the lack of nucleus and they evolved in a deeper specialisation on oxygen carrying. Because fish erythrocytes express proteins it has been investigated whether these cells could also have a role in the immune system and therefore express specific immune-related proteins (receptors, effectors). Morera and Mackenzie (2011) demonstrated the existence of such direct role of erythrocytes in the immune response and thus it was demonstrated the presence of TLR receptors and a positive response to viral and bacterial molecular patterns (Morera and MacKenzie, 2011). Therefore, although the immune role of erythrocytes may be moderated or low, they show the potential of immune reactivity and therefore the advantage to be the most abundant cell-type in bloodstream. Although fish are protected with innate mechanisms and mucosal barriers as a first line of defence, once pathogens clear this first line and access to circulation, the erythrocytes could play a role in the circulatory bed. It has also been described that erythrocytes take part in facilitating the clearance of pathogen by macrophages (Passantino et al., 2002), providing more evidence in the role of nucleated erythrocytes as an immune-like cell type.

Not only erythrocytes have been shown to be involved in immune defence, but also thrombocytes have been reported to play a putative immune role. They can present round, oval, ellipsoidal or spiked forms (Meseguer et al., 2002) and their main function is a regulator of hemostasis and blood clotting (Semple et al., 2011). But also it has been evident that beyond hemostasis, they participate in modulating the innate and adaptive immune response initiating the inflammatory response through interaction with leukocytes in case of injury (Smyth et al., 2009; Tavares-Dias and Oliveira, 2009). The expression of TLRs on platelets has also been found on birds, mice and human (Shiraki et al., 2004), but the mechanisms behind the immune role of thrombocytes have not yet to be elucidated.

3. The fish response to stress and pathogens as examples of how regulatory systems interact

After sensing a stressor (physical, chemical, environmental) the fish responds to stress activating the CNS to release stress hormones which will be the responsible to promote the physiological mechanisms able to counteract these stimuli (Reid et al., 1998). The classical view of these stress hormones is that cortisol, adrenaline and ACTH are suppressors of the immune system, but recent work demonstrates that hormones are modulating rather than suppressing agents (Tort, 2011).

Although in mammals and fish adrenaline has been found to suppress the expression of some inflammatory cytokines as well as inhibiting the phagocytic activity of leukocytes and also decreasing plasma levels of IgM (Chen et al., 2002; Zinyama et al., 2001), an enhancing effect of adrenaline was reported in mammals showing up-regulation of IL-6 (Liao et al., 1995). In a recent study, we also observed the enhancing influence of adrenaline on IL-β and IL-6 in sea bream head kidney (Khansari et al., submitted). Thus, the immunomodulatory influence of adrenaline on immune system remains to be investigated.

The secretion of cytokines after pathogen recognition by the host to mount an efficient response, will also induce a regulatory effect of these cytokines on HPI axis. The pro-inflammatory cytokines IL-1, TNF and IL-6 induce glucocorticoid and ACTH secretion following infection and inflammation (Besedovsky and Del Rey, 1996). In the same way, recent results from our lab show that in rainbow trout and gilthead sea bream HPI axis is simulated to release cortisol after the exposure to *Vibrio anguillarum* bacterin (not published data).

Also, it has been reported that the stimulation with PAMPs, such as LPS, elicits a rapid and dose-dependent activation of HPI which result in the release of ACTH and cortisol (Beishuizen and Thijs, 2003). Therefore, the direct influence of immune system mediators, such as cytokines, could also lead to hormone secretion into bloodstream. However, the detailed mechanism by which the fish HPI axis is stimulated by cytokines has not been fully described to date.

In addition to immune cells, non-immune cells are able to secrete cytokines. Thus, a large number of cytokines are influencing the HPI axis such as IL-β1, TNF-α and IL-6 (Turnbull and Rivier, 1999). Regarding the nervous system, neurons of the hypothalamus and pituitary such are capable of producing cytokine such as TGF, IL10 and IL-18 (Calcagni and Elenkov, 2006).

3.1 Hormones modulating the immune system

Glucocorticoids (GCs) are known to modulate the immune system in vertebrates exerting a complex action on a number of immune components (Weyts et al., 1998).

Our group showed the effect of cortisol on induced-gene expression profile with LPS in rainbow trout macrophages. Thus, cortisol inhibits the expression of genes involved in cell adhesion, cell surface receptor linked signal transduction, humoral immune responses, apoptotic regulation and complement system (MacKenzie et al., 2006). In fact, it has been evident that roughly every cell type of the immune system is affected by glucocorticoids (Ehrchen et al., 2007).

There is less studies in which cortisol was not able to modulate gene transcription. In rainbow trout macrophages Castro et al., (2011) did not induce changes after different co-incubation times with cortisol and immune stimuli administration. Previously, both the suppressing and enhancing (immunomodulatory) effects of cortisol have been largely reported in fish hepatocytes and head kidney cells (Castillo et al., 2009; Fast et al., 2008; Philip et al., 2012). The cortisol administration has been shown the suppression of the immune response at cytokines level for IL-β1, IL-6, TNF-α and TGF-β1 in sea bream but not in rainbow trout (Khansari et al., submitted). Therefore, this suggests that GCs effect in fish could act in a tissue-specific and species-specific manner.

Catecholamines have not been studied as much as glucocorticoids in fish because of sampling difficulties related to the fact that catecholamines are released in seconds, therefore difficulting the obtention of basal values. (Schreck et al., 2016). In addition, a simple elevation of adrenaline in blood contributes to impairing the regulation of blood Na^+ and Cl^-, thereby modifying the ion concentration balance in plasma and causing osmotic disturbances. Physiological and pharmacological concentrations of adrenaline have been shown to increase HSP70 in hepatocytes of salmonid (Ackerman et al., 2000). There is evidence that suggests an effect of adrenaline also in the modulation of the immune response. Thus, previous studies have shown that nitric oxide (NO), TNF-α and IL-10 were simultaneously increased following LPS treatment *in vitro* and that adrenaline dampened both NO and TNF-α with concurrent enhancement of IL-10 (Zinyama et al., 2001). Also, Kepka and colleagues (Kepka et al., 2013) have shown that injection of zymosan plus adrenaline reduced the percentage of monocytes/macrophages 24 h after injection. Also, a significant increase of apoptosis and decrease of IL-β1 was observed in the monocyte/macrophages treated with zymosan and adrenalin, while at 24 h IL-β1 was increased by adrenaline (Kepka et al., 2013).

The role of ACTH on immune system has been poorly investigated in fish, though its implication in cortisol secretion pathway. ACTH was shown to be one of the first neuropeptides binding to immune component cells thus modulating the immune response in mammals, and also ACTH induced an increased a cytotoxic response in T-lymphocytes (Johnson et al.,2005). In fact numerous studies have illustrated immunological influence of ACTH suppressing the activation of both human granulocytes and invertebrate immunocytes (Smith et al., 1992). ACTH has been found to prevent antibody response to T-cells as well as repressing interferon gamma secretion (IFN-γ) in murine splenocytes (Johnson et al., 1982).

ACTH can also promote B-lymphocytes growth and differentiation, modulating the IgM secretion and proliferation of phytohemagglutinin (PHA)-stimulated peripheral blood mononuclear cells (Bost et al., 1990). Thereby the immune modulatory effect of ACTH after neuroendocrine activation can be also expected in fish. The presence of ACTH was detected by immunocytochemistry in all stages of *Dicentrarchus labrax* even 2 days post hatch, revealing that the MC2R receptor should be present at early stages (Mola et al., 2005, 2004). Immunoreactivity of ACTH has also been shown, suggesting an important role of this neuropeptide to preserve the alteration of body haemostasis within the first fish life developmental stage (Mola et al., 2005). Moreover, it has also been shown that ACTH can directly regulate catecholamine secretion in rainbow trout (Reid et al., 1996), thus it could be involved in the endocrine-immune interaction through both stress axes.

Aside from ACTH secretion by neuroendocrine system, ACTH concentration was also detected in immune cells presenting in mammalian white blood cells and invertebrate immune components that have an essential role in phagocytosis. In fact, a study performed by Csaba and Pallinger illustrated the autoimmune regulation of the ACTH in immune cells (Csaba and Pallinger, 2007). In other words, immune cells can be well innervated and express receptors for neurotransmitters and neurohormones.

It is noteworthy that the interpretation of the *in vivo* obtained results can be a challenge fundamentally because the interaction between different effectors of the endocrine and nervous systems and their differential influence on the immune response. Thus, the *in vitro* model can be useful to study the individual impact of a specific hormone on immune cells or pathways. Previous studies performed in our group have revealed the stimulatory effect of ACTH on cytokines expression, enhancing TNF-α, IL-6 and TGF-β expression after 1 h of incubation. However, ACTH has also an immunosuppressive effect on IL-β1, IL-6, TNF-α, IL-10 and TGF-β1 after 2 h of exposure in sea bream head kidney (Castillo et al., 2009). On the other hand, ACTH was not able to mediate any modulatory effect in sea bream after stimulation with LPS and *Vibrio anguillarum* in either sea bream or rainbow trout (Castillo et al., 2009; Khansari., submitted). Therefore, hormones would play a modulatory rather than suppressive role on the immune system, but the specific actions resulting from these interactions between systems are subjected to the specific experimental conditions such as the previous history of immune and endocrine inputs.

Overall, during activation of the immune system or after an infectious process, glucocorticoids function to modify inflammatory response which is normally considered as an energy saving mechanism during stress incidents in which more energy resources are required and so, an additional allostatic load (Sapolsky et al., 2000). Thereby, not only is immune activity dampening, but immune cells may be catabolized as a source for protein and glucose, hence the function of GCs and in particular the bidirectional interaction between neuroendocrine and immune system appear to be indispensable as well as crucial for the animal integrity and thus cope with the pathogens invasion.

3.2 Immune inducers modulating the endocrine system.

As the communication between neuroendocrine and immune systems is bidirectional, endocrine activation also appears to be mediated by the immune system. In fact, the challenge involved in the process of an infection results in an allostatic load for the animal, therefore triggering a stress response resulting in the release of stress hormones. Thus, the presence of the pathogen *Lepeophtheirus salmonis* in Atlantic salmon increased the level of cortisol as well as glucose (Bowers et al., 2000). Sea lice, one of the major pathogens for the farmed Atlantic salmon, induces cortisol and glucose elevations (Mustafa et al., 2000). An acute cortisol response was also observed in channel catfish following an *E. ictaluri* challenge (Bilodeau et al., 2003), similar than that observed in rainbow trout, *Oncorhynchus mykiss*, following *Vibrio anguillarum* infection (Ackerman and Iwama, 2001). The injection of *Pseudomonas anguilliseptica*, an opportunistic pathogen for sea bram, induced an increase of cortisol, but only after 3 days of injection (Acerete et al., 2007). Regarding the infection consequences, it has been shown that under infection and inflammation, expression of IL-1, TNF and IL-6 induced secretion of glucocorticoids and ACTH (Besedovsky and Del Rey, 1996; Calcagni and Elenkov, 2006). Experiments using bacterial lipopolysaccharides (LPS) also showed induction of the HPI axis response. Van Enckevort et al., treated tilapia with *Escherichia coli* LPS stimulated the HPI axis and CRH and cortisol release but produced inhibition of ACTH and stimulation of α-MSH release after in vitro pituitary treatment (Pepels et al., 2004; Van Enckevort et al., 2002), although there was no effect of LPS on plasma cortisol in channel catfish (Weber et al., 2005). In yellow perch, Haukenes and Barton showed that an injection of LPS (3 mg/kg) elicited an increase of cortisol secretion even stronger than under handling stress (Haukenes and Barton, 2004). In *Oncorhynchus mykiss* injections of trout recombinant interleukin-1 beta (rIL-1 beta) or *E. coli* lipopolysaccharide (LPS), at concentrations known to induce immune/inflammatory responses in vivo (0.1-0.6 nmol/kg and 1.3 mg/kg respectively), significantly elevated plasma cortisol levels in a dose- and/or time-dependent manner (Holland et al., 2002). Other studies also showed increase of cortisol and cortisol receptor (GR) in sea bream, although at much higher concentrations than for mammals (Acerete et al., 2007) which may indicate a higher tolerance of fish to microbial PAMPs. LPS treatment on head kidney primary culture also showed an increase of the expression of the StAR protein, the key step to activate the cortisol production in fish (Castillo et al., 2008). Moreover, recent results from our laboratory showed that inactivated *Vibrio anguillarum* affected the HPI axis in rainbow trout and gilthead sea bream by releasing cortisol together with the up-regulation of cytokines including IL-β1, TNF-α and IL-6. Altogether, these antecedents indicate that the PAMP-mediated PRR identification and activation of the immune response may also activate the neuroendocrine machinery, interacting each other to mount an efficient global physiological response against the pathogen to recover the homeostasis.

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3. Objectives

The main objective of the present thesis was to contribute to understand the interaction between the neuroendocrine and the immune system under the situations of stress through the expression of genes involved in this interaction. This investigation has been undertaken in trout and seabream as two model species directly associated to aquacultured fish.

This objective has been splitted in the following specific objectives:

-To study the direct interaction between hormones and cytokines under the conditions of primary culture of the head kidney tissue, one of the main regulatory centers of the stress response in fish. This response was also studied in the presence of a pathogen.

-To study both the systemic and the peripheral response at the level of mucosal tissues when fish (trout and seabream) were subjected to anoxia, vaccination or the combination of both stressors.

-To study the response of the brain and pituitary protein expression under the same stressors of vaccination and anoxia.

Experimental design:

To fulfil the objectives, the experiments were designed as follows

- First, it was intended to investigate the interactions at the level of head kidney tissue as the one of the main regulatory centres, thus focusing on the cellular interactions without systemic influences. In order to precise this interaction, the preparation included the administration of anti
- In addition the same experiment was replicated, this time with the presence of an immune stimulator, the bacterin of *Vibrio anguillarum*, a common pathogen of marine and freshwater fishes. This was done in order to simulate the situation of both stress and pathogen occurrence in the tissue.
- Second, it was intended to look at the response at peripheral level, i.e. in the mucosae of the fish (trout and seabream). As it has been shown in the last years, in the mucosa many processes are taking place, among them innate immune responses and stress responses. Therefore skin mucus, gut mucus and gill mucus were studied.
- Once we knew the tissue response and the peripheral response, we wanted, of course, to look at the overall systemic response, identifying physiological reactions, metabolic reactions and gene reactions. This could bring a wider overview of the gene response to stressor in the species studied
- Finally, we assessed the gene response at the highest regulatory level, i.e., the hypothalamic and pituitary centres, as the places in which the overall response is regulated.

4. Chapter second

4.1. Cytokine modulation by stress hormones and antagonist specific hormonal inhibition in rainbow trout (*Oncorhynchus mykiss*) and gilthead sea bream (*Sparus aurata*) head kidney primary cell culture

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Abstract

A tight interaction between endocrine and immune systems takes place mainly due to the key role of head kidney in both hormone and cytokine secretion, particularly under stress situations in which the physiological response promotes the synthesis and release of stress hormones which may lead into immunomodulation as side effect. Although such interaction has been previously investigated, this study evaluated for the first time the effect of stress-associated hormones together with their receptor antagonists on the expression of cytokine genes in head kidney primary cell culture (HKPCC) of the freshwater rainbow trout (*Oncorhynchus mykiss*) and the seawater gilthead sea bream (*Sparus aurata*). The results showed a striking difference when comparing the response obtained in trout and seabream. Cortisol and adrenocorticotropic hormone (ACTH) decreased the expression of immune-related genes in sea bream but not in rainbow trout and this cortisol effect was reverted by antagonists mifepristone but not spironolactone. On the other hand, while adrenaline reduced the expression of pro-inflammatory cytokines (IL-1β, IL-6) in rainbow trout, the opposite effect was observed in sea bream showing an increased expression (IL-1β, IL-6). Interestingly, this effect was reverted by antagonists propranolol but not phentolamine. Overall, our results confirm the regional interaction between endocrine and cytokine messengers and a clear difference in the sensitivity to the hormonal stimuli between the two species.

Keywords: stress hormones; antagonist receptors; immune response; rainbow trout (*Oncorhynchus mykiss*); gilthead sea bream (*Sparus aurata*); head kidney primary cell culture.

1. Introduction

Stressors may compromise the overall health status in fish including increased susceptiblity to pathogens and reduced disease resistance. The stress response, as a general, non-specific and widespread reaction, involves all physiological systems, and particularly the neuroendocrine and immune systems which are tightly connected (Engelsma et al., 2002). In fish, the head kidney plays a principal role in this network since, not only is crucial for the organization of the systemic stress response in fish, secreting corticosteroids and catecholamines, but also for its main role in the immune response as hematopoietic tissue, and in energetics as the producer and supplier of oxygen carrying red blood cells.

The Hypothalamus-Pituitary-Interrenal (HPI) and Sympathetic Adreno-Medullar (SAM) axis, as the two major pathways by which the endocrine response is organized, have been demonstrated that can modify the immune function in mammals and fish (MacKenzie et al., 2006; Padgett and Glaser, 2003). Cortisol is secreted by head kidney interrenal cells in a concatenated response involving the hypothalamic corticotrophin-releasing hormone (CRH) and the adrenocorticotropic hormone (ACTH) secretion. Thus, secreted ACTH is recognized by melanocrotin receptor 2 (MC2R) on the surface of the interrenal cells and activates a signalling cascade which mediates the secretion of cortisol. Cortisol is the major glucocorticoids (GCs) in teleost fish and the final product of HPI axis activation. It plays essential roles in energy homeostasis, including balance maintenance, modulation of the immune response, and regulating behaviour through genomic (slow) and non-genomic (fast) mechanisms in the central nervous system (Castro et al., 2011; Cortés et al., 2013; Mommsen et al., 1999). Cortisol activates glucocorticoid receptors (GRs) in responsive cells leading to modulation of target genes expression. Corticosteroids bind to GRs forming a complex that is transported to the nucleus where it binds to DNA at glucocorticoid response elements (GREs) present in the promoters of several genes. This interaction usually involves changes in gene transcription (trans-activation) by interacting with co-activator molecules. As the role of receptors is pivotal, two GR (GR1 and GR2) and also one mineralocorticoid receptors (MR) cloned and sequenced in teleost species (Bury et al., 2003; Greenwood et al., 2003; Stolte et al., 2008a) have been assessed. In zebrafish (*Danio rerio*) a splice variant for GR similar than that in mammals has been described (Alsop and Vijayan, 2009, 2008). Interestingly, the circulating level of aldosterone is extremely low in fish and cortisol palliate this situation binding to MRs and playing a principal role in, for example, the acclimation of teleost to seawater environment (Mancera et al., 2002; McCormick, 2001; Prunet et al., 2006; Takahashi and Sakamoto, 2013). Therefore, in teleost cortisol plays both glucocorticoid and mineralocorticoid functions through GRs and MRs, respectively (Mccormick and Bradshaw, 2006).

Adrenaline is the main catecholamine product of the activation of SAM axis after sympathetic innervation of the head kidney chromaffin cells. Adrenaline modulates cardiovascular and respiratory function in order to rapidly mobilize the available energy

reservoirs and to maintain an adequate oxygen level to satisfy the increased energy demand which the stress response implies (Reid et al., 1998). There is evidence of the β-adrenoreceptor existence which recognises adrenaline and mediates its effects (Chadzinska et al., 2012; Fabbri et al., 1998; Jozefowski and Plytycz, 1998). Thus, cortisol, ACTH and adrenaline are currently present in the head kidney after an integrated stress episode.

Cytokines are key regulators of the immune response which are produced mainly at the site of entry of pathogen and regulate the activation of resident immune cells. Although a powerful inflammatory response is crucial to overcome an infection, this might be a double edged sword bringing damage to the host tissue, hence the provoked pro-inflammatory response has to be rapidly regulated (Chadzinska et al., 2008; Wang and Secombes, 2013). Among cytokines, IL-1β is an endogenous pyrogen (very responsive because of the fast expression) produced and released at the early stage response to infections, lesions and stress (Duque and Descoteaux, 2014). In fact, this cytokine is an initiator of pro-inflammatory response with key roles on the stimulation of pro-inflammatory mediators, such as other cytokines and prostaglandins in macrophages, and activation of lymphocytes (Alvarez-Pellitero, 2008; Dinarello, 2009). IL-1β can activate the expression of IL-6, a pleiotropic cytokine which has both pro- and anti-inflammatory functions, promoting differentiation of B-cells into plasma cells and activating cytotoxic T cells (Duque and Descoteaux, 2014). Another relevant actor in the pro-inflammatory response is TNF-α, which is able to exert its effect in many organs and induces IL-6 together with IL-β (Baud and Karin, 2001). This cytokine can stimulate the acute phase of immune response and is one of the first to be released in response to pathogens as well as after stress episodes (Teles et al., 2011). The pro-inflammatory cascade is strictly regulated by anti-inflammatory mediators, IL-10 and TGF-β1, responsible of maintaining the response under control to avoid tissue damage (Cools et al., 2007; Hu et al., 2008). IL-10 is produced by activated macrophages, B cells and T cells (Mosser and Edwards, 2008), while TGF-β1 has been described mostly associated with IL-10 as an immunosuppressive cytokine (Cools et al., 2007) regulating the immune response by blocking the activation of lymphocytes and monocyte-derived phagocytes and promoting tissue repair after a local inflammatory response (Li et al., 2009). Thus, in this study, we have analysed the hormone effect over some of the main cytokines involved in pro- (IL-1β, IL-6, TNF-α) and anti-inflammatory (IL-10 and TGF-β1) response.

The relationship between immune and neuroendocrine system is bidirectional because hormones affect immune cells but cytokines can also modulate HPI function (Calcagni and Elenkov, 2006). Although neuroendocrine and immune systems were initially considered to act independently, it is now recognized that an extensive communication network controls an orchestrated neuroendocrine-immune interaction (Engelsma et al., 2002). Thus, it is not surprising that most of the immune cells such as lymphocytes, monocytes, macrophages and granulocytes express receptors for many neuroendocrine products and their expression is regulated after hormone secretion (Bury et al., 2003; Duque and Descoteaux, 2014). In previous results obtained in our group the effects of adrenaline, adrenocorticotropic

hormone (ACTH) and cortisol on the expression of pro- (IL-1β, IL-6, TNF-α) and anti-inflammatory (TGF-β1) cytokines was evaluated in sea bream head kidney cells, showing a down-regulation in the expression of these cytokines after 2 h of treatment (Castillo et al., 2009). However, there is no evidence on whether antagonists for specific stress hormones receptors may revert the cytokine gene expression regulation observed after hormone administration. Therefore, the aim of this study was to assess the *in vitro* effects of the main stress hormones alone or in combination with their antagonist receptor on the gene expression of key pro-inflammatory (IL-1β, IL-6, TNF-α) and anti-inflammatory cytokines (IL-10, TGF-β) in head kidney primary cell culture. We used spironolactone and mifepristone to antagonize GR and ACTH receptors (Alderman et al., 2012) and propranolol and phentolamine as β-adrenoreceptor and α-adrenoreceptor antagonists, respectively. Two fish species (rainbow trout and gilthead sea bream) were considered in order to determine whether fish from different characteristics such as water environment or genetic background, respond similarly to the same experimental conditions. This is the first study to assess the effect of antagonist receptors over stress hormones regulation of immune-related genes.

2. Material and methods

2.1. Animals

Oncorhynchus mykiss with body weight of 120-140 g were obtained from a local fish farm (Piscifactoria Andres, St Privat d'en Bas, Spain). *Sparus aurata* with body weight of 60-70 g were obtained from AQUACULTURA ELS ALFACS,S.L. (Tarragona). Fish were transferred to the Universitat Autonoma de Barcelona (UAB) fish facility (AQUAB) to acclimatize them to laboratory conditions. All experimental procedures involving fish were submitted and authorized by the Ethical Committee of the "Universitat Autonoma de Barcelona" that agrees with the international Guiding Principles for Biomedical Research Involving Animals (EU2010/63).

2.2. Head kidney primary cell culture (HKPCC) preparation

Rainbow trout (n=6) and gilthead sea bream (n=6) head kidneys were isolated from euthanized fish by overdose of MS-222 (Sigma). Head kidney was isolated and immediately placed in DMEM – high glucose (Sigma) and kept in ice. Tissue was homogenized through a 100 µm nylon cell strainer (Falcon). HKPCC was prepared in DMEM – high glucose (Sigma) at concentration of $2x10^6$ cells/ml. The head kidney cells (1ml) were left undisturbed in an incubator under optimal culture temperature conditions (16 °C for rainbow trout; 18°C for sea bream, and 5% CO_2) for 2 h to settle in a 24-well culture plate (Jet Biofil) before adding antagonist receptors and/or hormones. The HKPCC includes hematopoietic, immune

(macrophages and lymphocytes) and endocrine cells (chromaffin and corticosteroidogenic cells).

2.3. Hormones and pharmacological agents

Cortisol (Hydrocortisone; Sigma cat #H2882-1G), adrenocorticotropic hormone (ACTH; Sigma cat #A0423-1 MG) and adrenaline (Adrenaline; Sigma cat #Y0000882-5MG) were dissolved according to manufacturer's instruction. Mifepristone or RU-486 (Sigma cat #M8046-100MG) as antagonist receptor for cortisol and ACTH, spironolactone (Sigma cat#S3378-1G) as antagonist receptor for cortisol, and propranolol (Sigma cat #P8688-100MG) and phentolamine (Sigma cat #P7547-100MG) as antagonist receptor for adrenaline, were used to evaluate the hormones effect in rainbow trout and gilthead sea bream on the immune response at gene expression level. Mifepristone, propranolol and phentolamine were dissolved in absolute ethanol while spironolactone was dissolved in chloroform. The final concentration of each solvent was ≤ 0.1% in order to have no influence on cell culture preparation.

2.4. In vitro experimental design.

In order to evaluate the hormone-dependent immune gene expression pattern in rainbow trout and gilthead sea bream, HKPCCs were pre-treated with antagonist receptors for 30 min previous to the incubation with hormone. Thus, RU-486 and spironolactone were added to cell culture separately and together to evaluate the cortisol effect; RU-486 was added to evaluate the ACTH effect; and propranolol and phentolamine were added separately and together to evaluate the adrenaline effect. Antagonist receptors were added at a final concentration 10-fold higher than that of stress hormones. Then, HKPCCs were incubated for 2h with cortisol (100 ng ml^{-1}), ACTH (150 ng ml^{-1}), or adrenaline (1 µM) based on previous reports (Castillo et al., 2009; Kelly and Chasiotis, 2011). Non-treated cells were considered as negative control for both fish species. Six replicates were analysed for each condition and fish species.

2.5. RNA isolation and cDNA synthesis

Total RNA was isolated from stimulated and control HKPCC using TRI reagent (Sigma) according to manufacturer's instructions. The RNA pellet was dissolved in nuclease free-water and immediately stored at -80ºC until use. The RNA concentration was quantified by NanoDropND-2000 spectrophotometer (Thermo Scientific). RNA (500 ng) was used as template to synthesize complementary DNA (cDNA) using iScript cDNA kit (Bio-Rad Laboratories) according to manufacturer's instructions.

2.6. Gene expression analysis

The hormone-dependent immune gene expression pattern on HKPCC was determined by real-time PCR. The gene expression levels of immune-related genes (IL-1β, IL-6, TNF-α, IL-10,

TGF-β1) were analysed. We first tested several housekeepings candidates (18S, EF1α and RPL27 in seabream, and EF1α and β-actin in rainbow trout) to elucidate which one had less variation (data not shown). Thus, β-actin (for rainbow trout) and 18S (for sea bream) were selected because of the less variability presented. Specific primers used for rainbow trout (Table 1) and gilthead sea bream (Table 2) are indicated. The primers for gilthead sea bream IL-6 and TNF-α were previously designed (Boltana et al., 2014). The other primers are first used in this study and they were designed with Primer-Blast. The primer secondary structure and primer specificity were checked with OligoAnalyzer (version 3.1) and Primer-Blast software, respectively. Real-time PCR reactions were performed with iTaq universal sybr green supermix (Bio-Rad Laboratories) using 2 µl of 1:40 dilution of cDNA (for genes of interest in rainbow trout) and 2 µl of 1:6 dilution of cDNA (for genes of interest in gilthead sea bream) or 1:1000 dilution (for housekeeping gene in both species). Primers for all genes were used at final concentration of 500 nM. The thermal condition used were 3 min at 95ºC of pre-incubation followed by 40 cycles at 95ºC for 30 s and 60ºC for 30 s. All the reactions were performed in duplicate using CFX384 Touch Real-Time PCR Detection System (Bio-Rad Laboratories). The quantification was done according to Pfaffl method corrected for efficiency of each primer set (Pfaffl, 2001). Value for each experimental condition was expressed as normalized relative expression, calculating in relation to values of control group and normalized against those of the housekeeping gene β-actin and 18S for rainbow trout and sea bream, respectively.

2.7. Statistical analysis

Differences among treatments were analysed by one-way analysis of variance (ANOVA) followed by Student-Newman-Keuls (SNK) test, data homogeneity of variance was checked and where homogeneity was not significant nonparametric tests were performed. All data were analysed using the SPSS version 20.0 software package. Differences in gene expression were considered significant when p-value < 0.05 among groups.

3. Results

No significant differences were observed after evaluation of expression of the pro-inflammatory cytokines IL-1β, IL-6 and TNF-α after cortisol incubation. In the same way, the expression of the anti-inflammatory cytokines IL-10 and TGF-β1 did not significantly vary among treatments (Figure 1). The same effect was observed when HKPCC was incubated with cortisol and the antagonist receptors RU-486 or spironolactone, or in combination (Figure 1). These results indicate that cortisol did not significantly modulate the expression of neither pro- nor anti-inflammatory cytokines in rainbow trout HKPCC at the concentration and incubation time tested (Figure 1). Regarding gilthead sea bream the expression of IL-1β, IL-6 and TFN-α, and the anti-inflammatory cytokines IL-10 and TGF-β1 were down-regulated by cortisol on HKPCC (Figure 2). Importantly, this effect was reverted by mifepristone, thus

restoring basal expression level. However, the effect of the antagonist spironolactone was not able to recover the basal expression values. Interestingly, only the effect of RU-486 antagonist was able to reverse the cortisol effect for all genes (Figure 2).

ACTH administration was not able to significantly modulate the transcript abundance of IL-1β, IL-6 and TNF-α, nor IL-10 and TGF-β1 in rainbow trout HKPCC (Figure 2). No differences were neither observed in any evaluated gene when the HKPCC was previously incubated with RU-486 (Figure 3). The results show that ACTH, at dose and time point used in this study, did not significantly induce the expression of immune related genes in rainbow trout HKPCC. In contrast, the administration of ACTH on gilthead sea bream HKPCC significantly dropped the expression level of IL-6, TNF-α and IL-10 compared to control (Figure 4). This down-regulated expression induced by ACTH did not return to control level when the antagonist receptor RU-486 was administered. Interestingly, no significant differences were registered for IL-1β and TGF-β1 when HKPCC was incubated with ACTH alone nor in combination with the antagonist receptor.

Adrenaline down-regulated the expression of IL-1β and IL-6 in rainbow trout HKPCC, and the same trend was observed for IL-10, although in the last was not statistically significant (Figure 5). No differences were observed for TNF-α and TGF-β1. The effect of adrenaline was reverted by propranolol, resulting in recovery of the expression of IL-1β and IL-6 to basal level after treatment. Importantly, this effect was not observed when the HKPCC was treated with phentolamine, although only the administration of propranolol was able to reverse the adrenaline effect in co-administration treatments of both receptor antagonists restoring the expression observed in control treatment (Figure 5). No effect was observed in the gene expression of TNF-α and TGF-β1 when HKPCC was incubated with adrenaline and antagonist receptors propanolol or phentolamine, or in combination. An opposite effect was observed in gilthead sea bream HKPCC, where the incubation with adrenaline up-regulated the expression of IL-1β and IL-6 compared to control, and the same effect was apparent for TNF-α and TGF-β1 when HKPCC was incubated both with adrenaline although no significant (Figure 6). When sea bream HKPCC was pre-treated with propranolol this up-regulation was reverted, and expression remained at control level. By contrast, administration of phentolamine produced a similar increase in gene expression than adrenaline, therefore not antagonizing the adrenaline effect. The administration of both antagonists induced the same effect than propranolol alone. The same trend, although not significant, was also observed for TGF-β1, IL-10 and TNF-α (Figure 6). Altogether, these results indicate that adrenaline promoted the expression of pro-inflammatory cytokines and this effect was reverted by propranolol but not by phentolamine in sea bream HKPCC.

4. Discussion

Previous studies in several vertebrates have shown that stress, involving adrenaline and cortisol secretion, suppress immune system function under some circumstances while enhancing it under others (Dhabhar, 2009). As a consequence, a harmful or helpful effect

will occur depending on the type of regulation of the immune mechanisms and the species affected, because of the existence of several critical factors that can modulate the effect of the stress hormones and their degree of sensitivity.

Head kidney possess the unique characteristic of being a hematopoietic organ with endocrine and immune functions, homologous to mammalian adrenal glands (Kepka et al., 2013; Tort et al., 2003). Thereby, as head kidney is an intermingled organ, obtaining pure population of cells and determining the percentage of the different cell types is extremely tough, and all previous research concerning stress and immune response has been performed in whole head kidney tissue (Castillo et al., 2009; Gravel and Vijayan, 2006). Nevertheless, new techniques such as laser microdissection microscopy (LMD) has been recently used to isolate the interrenal cells of head kidney and may help to investigate the role of specific cells (Fierro-Castro et al., 2015). In this study the percentage of interrenal cells was determined to be around 2%, increasing 6 fold under chronic stress and not acute stress conditions, thus indicating that histological changes require time. In another work in carp an increment of size of interrenal cells was observed after stress (Ruane et al., 2005). Regarding immune cells, although the majority of cell types forming the head kidney are hemopoietic cells, there is a variety of immune cells such as stromal (reticular cells), macrophages and granulocytes, and several lymphoid cells (Fishelson, 2006). We also wanted to compare two species that are adapted to different environments including temperature, salinity or oxygen requirements, but also genetic diversity, since different fish species may show genetic changes which acquire particular relevance when some of them duplicated genomes during evolution (Nakatani et al., 2007). Although in many occasions the investigations on the endocrine or immune system of fish may assume that the responses to stimuli could be the same or similar, it should be remembered that many factors including internal and external are influencing such responses (Magnadottir, 2006)(see Bowden, 2009) and a number of conditions (temperature range, latitude, salinity, depth range, surrounding microbiota, size, activity, feed composition and regime) will be associated to one particular species and not to the other, thus generating differential responses.

All these factors may influence the response to physiological adaptations in terms of endocrine and immune responsiveness and the interaction between them.

4.1. The effect of cortisol on rainbow trout and sea bream head kidney primary cell culture

It has been shown that GR and MR receptors in salmonid fish bind selectively to the antagonist mifepristone and spironolactone respectively (McCormick et al., 2008). Here we provide evidence of cytokines gene expression modulation by GR and MR cortisol receptors after using mifepristone and spironolactone, respectively. These cortisol receptors are expressed in the central nervous system among other tissues of the teleost fish (Greenwood et al., 2003). We have administered both antagonists since it is not clear that upon addition

of cortisol as well as one GR blocker the effect could be fully blocked at basal expression level. Moreover, the hormone may exert its effect through MR in addition to GR. Previous investigations provided evidence that teleosts express a MR-like gene (with high homology to a mammalian MR gene, (Stolte et al., 2008b) that controls the corticosteroid second function as regulator of ions and water transportation and chloride cell proliferation (Mccormick and Bradshaw, 2006; Takahashi and Sakamoto, 2013). More importantly, it has been shown that MR is also expressed under stress circumstances (Sloman et al., 2001). These receptors of the neuroendocrine system, together with other neuroendocrine products of HPA and SAM axes (Padgett and Glaser, 2003) would be shared by lymphocytes, monocyte-macrophages or granulocytes which may end-up with changes in cellular trafficking, proliferation, cytokine secretion and antibody production (Madden et al., 1995).

In the present work, cortisol did not show any significant modulatory effect on cytokine expression in rainbow trout, while, in sea bream cortisol produced an inhibitory effect on both pro-inflammatory (IL-1β, IL-6, TNF-α) and anti-inflammatory cytokines (IL-10, TGF-β1). Glucocorticoids (GCs) are well known immunosuppressive molecules, but their effects on particular immune fish cell types are not clear yet, with few available studies describing direct GCs actions on innate cell responses (Tort, 2011; Verburg-van Kemenade et al., 2011; Yada and Nakanishi, 2002). The suppression of the immune response agrees with previous studies illustrating that glucocorticoids down-regulate and modulate cytokine mRNA levels (Castillo et al., 2009; Flory and Bayne, 1991) and confirms the immunosuppressive effect in other fish species (MacKenzie et al., 2006; Saeij et al., 2003). In fact, the immunosuppressive effect of cortisol has been already reported in fish head kidney tissue and hepatocytes (Philip et al., 2012; Sathiyaa and Vijayan, 2003; Tort, 2011). Furthermore, cortisol administration has also been shown to modulate the immune response by reducing the number of circulating T- and B-like lymphocytes (Pazirandeh et al., 2005; Pulsford et al., 1993; Purton et al., 2004) and inducing apoptosis of B-cells (Weyts et al., 1998).

Differences in cytokine regulation after cortisol treatment on different fish species have also been previously reported. In trout hepatocytes, cortisol up-regulated the expression of IL-1β (Philip et al., 2012). Also, repeated handling stress and the consequent high plasma cortisol, produced an elevation of IL-1β in Atlantic salmon head kidney macrophages (Fast et al., 2008). On the other hand, down-regulation of IL-1β has been observed in gilthead sea bream head kidney cells incubated with cortisol (Castillo et al., 2009) and even no alteration for IL-1β was shown in a trout macrophage cell line after cortisol administration (Castro et al., 2011).

Concentration is clearly of relevant importance, since it has been shown that glucocorticoid receptors GR1 and GR2 in fish may be responsive to different cortisol concentrations (Stolte et al., 2006; Sturm et al., 2016), which may explain differences in the response observed in our study between rainbow trout and gilthead sea bream not only at cytokine but also at GR gene expression patterns. The circulating cortisol concentration in non-stressed fish is

generally lower than 20 ng ml^{-1} for many species, while after induced stress, cortisol may range from 50 ng ml^{-1} to several hundreds of nanograms. These levels may change substantially depending on the species or the intensity and duration of the stressor (Mauri et al., 2011; Tort, 2011). Though, it should also be noted that differences have also been observed in reports in which the same cortisol concentration (100 ng ml^{-1}) has been used (Castillo et al., 2009; Philip et al., 2012).

The genes that encode for many pro-inflammatory proteins are controlled by pro-inflammatory transcription factors, mainly NF-κB and AP-1 which become activated during inflammatory events (Adcock and Caramori, 2001; Barnes, 2006). GCs inhibit the effect of these transcription factors by several mechanisms (Refojo et al., 2001). The first trans-repressive mechanism involves physical interaction of the GR with the transcription factor or with its co-activator NF-κB that is present in almost all cell types in the immune system and the majority of inflammatory cytokines genes are under positive regulation by these proteins (Wissink et al., 1998). Cross-coupling of GR with NF-κB was shown to repress promoters associated to pro-inflammatory cytokines genes (such as IL-1, IL-6 and TNF-α) through down-regulation of NF-κB transcriptional activity. Another mechanism is the induction of inhibitory proteins by GCs affecting the inflammatory signal transduction pathway of corticosteroids and decreasing protein synthesis by reducing the mRNA stability after activation of inflammatory genes (Clark, 2003; Scheinman et al., 1995).

Altogether, these antecedents support the down-regulation of pro-inflammatory cytokines gene expression observed in this study. Thus, a different effect of cortisol would occur depending on the species affected and/or a differential effect when administered to a single cell type or to a complex mixture of cells such as the head kidney tissue.

4.2. The effect of ACTH on rainbow trout and sea bream head kidney primary cell culture

ACTH circulates through the bloodstream and stimulates head kidney interrenal cells to produce cortisol (Barton, 2002; Barton and Iwama, 1991). We observed that ACTH administered at the concentration of 150 ng mL^{-1} did not exert any significant effect on the cytokine gene expression measured in trout head kidney cells. Interestingly, ACTH was effective in sea bream head kidney cells suppressing both pro-inflammatory and anti-inflammatory cytokines excluding IL-1β and TGF-β1. ACTH not only activates interrenal cells to release cortisol in head kidney, it has also been demonstrated that other tissues, such as thymus and spleen, express ACTH receptors even at early stage of development (Mola et al., 2005). Therefore the direct influence of ACTH on immune system can be expected after induction of acute stress. On the other hand, the effect of this hormone on immune system following application of an antagonist drug has not been studied in sea bream so far.

The obtained findings did not show any inhibition effect of mifepristone administered with ACTH on cytokines gene expression. Although there is no evidence that confirms that mifepristone can block StAR or MC2R of the cortisol secretion pathway, previous in vivo

study in head kidney tissue from fish fed with mifepristone produced significantly less cortisol in response to ACTH stimulation compared to control-fed fish (Alderman et al., 2012). Although more studies are necessary in this area, the reduced capacity to synthesize cortisol could be attributed to a reduction of StAR mRNA levels. Again as the effect of ACTH on interrenal cells and immune cells is mediated by MC2R, thus our obtained result illustrated the elevation of the receptors after 2 h ACTH incubation. The findings is in agreement with the previous study that accomplished in our research group and other research showing elevation of the receptor after acute handling stress (Aluru and Vijayan, 2008; Fierro-castro et al., 2015).

4.3. The effect of adrenaline on rainbow trout and sea bream head kidney primary cell culture

Adrenaline, as the main catecholamine product of the SAM axis in head kidney chromaffin cells, has not been studied as much as other stress hormones such as glucocorticoids. Since head kidney is an organ directly involved in endocrine and immune functions, it may facilitate a direct paracrine interaction between the endocrine and hematopoietic tissues (Verburg-van Kemenade et al., 2011). Likewise the study carried out by Chadzinska and the colleagues demonstrated the paracrine effect of adrenaline on both pro-inflammatory and anti-inflammatory mediators in head kidney of carp (Chadzinska et al., 2012). Three main adrenergic receptors α1, α2 and β have been found that could play essential role in this interaction. Although little attention has focused on adrenergic receptors in fish, β-adrenoreceptors appear to be present in leukocytes of all vertebrate classes including fish (Chadzinska et al., 2012; Nance and Sanders, 2007). Radioligand binding experiments confirmed the expression of β-adrenoreceptors on goldfish head kidney, spleen and peritoneal leukocytes (Jozefowski and Plytycz, 1998) and some works highlighted that the effect of adrenaline would be mediated mainly by the β-adrenergic receptor (Fabbri et al., 1998) and that activation of this receptor may be responsible of many stress-related immunological changes (Nance and Sanders, 2007). In fact, in our study the β-adrenergic receptor but not α-adrenergic receptor was reduced in rainbow trout after incubation with adrenaline. Accordingly, Jetschmann et al. (1997) observed that the administration of adrenaline on human natural killer cells decreased β-adrenergic receptor. The same effect of adrenaline has also been reported in fish, in rainbow trout, a strong trend towards decrease was observed in hepatocyte β-adrenoceptor after agonist exposure as well as decreased the numbers of red blood cells surface β-ARs (Dugan et al., 2003; Nickerson, 2003). Thereby the effect of adrenaline on β-adrenoceptor in sea bream remains to be elucidated.

Here we illustrate for the first time that short-time incubation of head kidney cells with adrenaline increases the mRNA levels of pro-inflammatory cytokines IL-1β and IL-6 in seabream, but not the expression of anti-inflammatory cytokines. Importantly, the induction

significantly took place in the opposite direction, as pro-inflammatory cytokines (IL-1β and IL-6) were suppressed in rainbow trout while increased in sea bream.

In mammals, adrenaline has been found to suppress the expression of some inflammatory cytokines (Li et al., 2003; Zinyama et al., 2001). Similarly, in tilapia (*Oreochromis aureus*), it has been observed that catecholamines secretion induced by cold stress inhibited the phagocytic activity of leukocytes and decreased plasma levels of immunoglobulin M (IgM). Moreover other studies in carp revealed regulation of the innate immune indicators by catecholamines both *in vivo* and *in vitro*, also affecting cell apoptosis, and interestingly indicating time dependent regulation (Chadzinska et al., 2012; Chen et al., 2002; Kepka et al., 2013). Conversely, the enhancing effect of adrenaline on pro-inflammatory cytokines observed in sea bream has been also reported in isolated perfused rat liver preparations showing up-regulation of IL-6 (Liao et al., 1995). In addition to regulation of immune function by GCs, it is known that catecholamines also modulate immune function including cell proliferation, cytokine and antibody production, cytolytic activity and cell trafficking (Madden, 2003; Rabin, 1999; Watzke et al., 2007). The adrenergic stimulation can be differently regulated depending on which cell type or tissue is stimulated, suggesting local effects of stress hormones, as is already known for glucocorticoids. To date, most of the data on catecholamine studies have demonstrated an immunomodulatory effect of adrenaline, though the stimulatory effect of adrenaline in head kidney appears to be species and condition-dependent.

In this study we also aimed to assess the influence of adrenaline administration in combination with propranolol (β-antagonist), its major receptor antagonist. A study in zebrafish showed much higher expression of β2a-adrenoreceptor gene in lymphoid organs such as spleen and head kidney (Wang et al., 2009). Expression of β2-adrenoreceptors in spleen as well as head kidney of rainbow trout has been also recorded, highlighting the role of β-adrenoreceptors on the modulatory effect of both the catecholamine and its receptors under stress situations (Nickerson et al., 2001; Reid et al., 1998). Thus, it has been proposed that receptors would play an important role as modulators of the effects of catecholamines on immune cells since this interaction is crucial to avoid tissue damage after activation of pro-inflammatory responses (Smith and Vale, 2006). Accordingly, our results would support the role of this modulatory effect by β-adrenoreceptors, since propranolol prevented the suppressive/enhancing effect of catecholamines on rainbow trout and sea bream head kidney cytokines. The adrenaline-induced inhibition by propranolol is consistent with its demonstrated suppressive effect in immune system in other fish species such as the air-breathing fish *Channa punctatus* (Roy and Rai, 2008), and in mammals (Li et al., 2003). Our results also show that phentolamine did not exert the same action of reverting adrenaline effects, and thus no significant differences were found between adrenaline and adrenaline plus phentolamine treated groups. Therefore modulation of the immune response after adrenergic stimulation would be carried out through β-adrenoreceptors.

Conclusion

The present study shows that stress hormones have a significant effect on the expression of the main inflammatory cytokines, both pro-inflammatory and anti-inflammatory, in the head kidney, and that this effect is highly dependent on the species. Whereas cortisol and ACTH did not induce relevant effects in rainbow trout at the concentrations tested, adrenaline induced significant suppression. In sea bream, cortisol and ACTH concentrations exerted significant suppressive effects on cytokines while adrenaline showed a significant up-regulation. Overall, after hormone administration a clear difference between the two species in both direction of the effect (enhancement or suppression) and in the sensitivity to the different hormones was observed. In addition our results with hormone antagonists showed that mifepristone but not spironolactone could revert the suppressive effect of cortisol and that propranolol but not phentolamine reverted the effects of adrenaline.

Abbreviations

HPI: hypothalamus-pituitary-interrenal; SAM: sympathetic-adreno-medullar; CRH: corticotrophin-releasing hormone; ACTH: adrenocorticotropic hormone; MC2R: melanocrotin receptor 2; GCs: glucocorticoids; GREs: glucocorticoid response elements; GR: glucocorticoid receptor; MR: mineralocorticoid receptor; AR: adrenergic receptor; HKPCC: head kidney primary cell cluture.

Competing interests

The authors declare that they have no competing interests.

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Legends

Figure 1. qRT-PCR quantification of specific mRNA abundance in rainbow HKPCC after antagonist receptors and cortisol exposure. Data is shown as mean ± SEM. CTRL: control; CO: cortisol; CO+RU: cortisol + mifepristone; CO + SP: cortisol + spironolactone; CO+RU+SP: cortisol+mifepristone+spironolactone.

Figure 2. qRT-PCR quantification of specific mRNA abundance in seabream HKPCC after antagonist receptors and cortisol exposure. Data is shown as mean ± SEM. Significant differences (one-way ANOVA) are shown by different letters as follows: A (p-value < 0.01), and a (p-value < 0.05). CTRL: control; CO: cortisol; CO+RU: cortisol + mifepristone; CO + SP: cortisol + spironolactone; CO+RU+SP: cortisol+mifepristone+spironolactone.

Figure 3. qRT-PCR quantification of specific mRNA abundance in rainbow trout HKPCC after antagonist receptor and ACTH exposure. Data is shown as mean ± SEM. CTRL: control; ACTH: Adrenocorticotropic hormone; ACTH+RU: ACTH + mifepristone

Figure 4. qRT-PCR quantification of specific mRNA abundance in seabream HKPCC after antagonist receptor and ACTH exposure. Data is shown as mean ± SEM. Significant differences (one-way ANOVA) are shown by different letters as follows: α (p-value < 0.001), A (p-value < 0.01), and a (p-value < 0.05). CTRL: control; ACTH: Adrenocorticotropic hormone; ACTH+RU: ACTH + mifepristone.

Figure 5. qRT-PCR quantification of specific mRNA abundance in rainbow trout HKPCC after antagonist receptors and adrenaline exposure. Data is shown as mean ± SEM. Significant differences (one-way ANOVA) are shown by different letters as follows: A (p-value < 0.01), and a (p-value < 0.05 CTRL: control; AD: adrenaline; AD+PR: adrenaline+propranolol; AD+PHE: adrenaline+phentolamine; AD+PR+PHE: adrenaline+propranolol+phentolamine

Figure 6. qRT-PCR quantification of specific mRNA abundance in seabream HKPCC after antagonist receptors and adrenaline exposure. Data is shown as mean ± SEM. Significant differences (one-way ANOVA) are shown by different letters as follows: A (p-value < 0.01), and a (p-value < 0.05 CTRL: control; AD: adrenaline; AD+PR: adrenaline+propranolol; AD+PHE: adrenaline+phentolamine; AD+PR+PHE: adrenaline+propranolol+phentolamine.

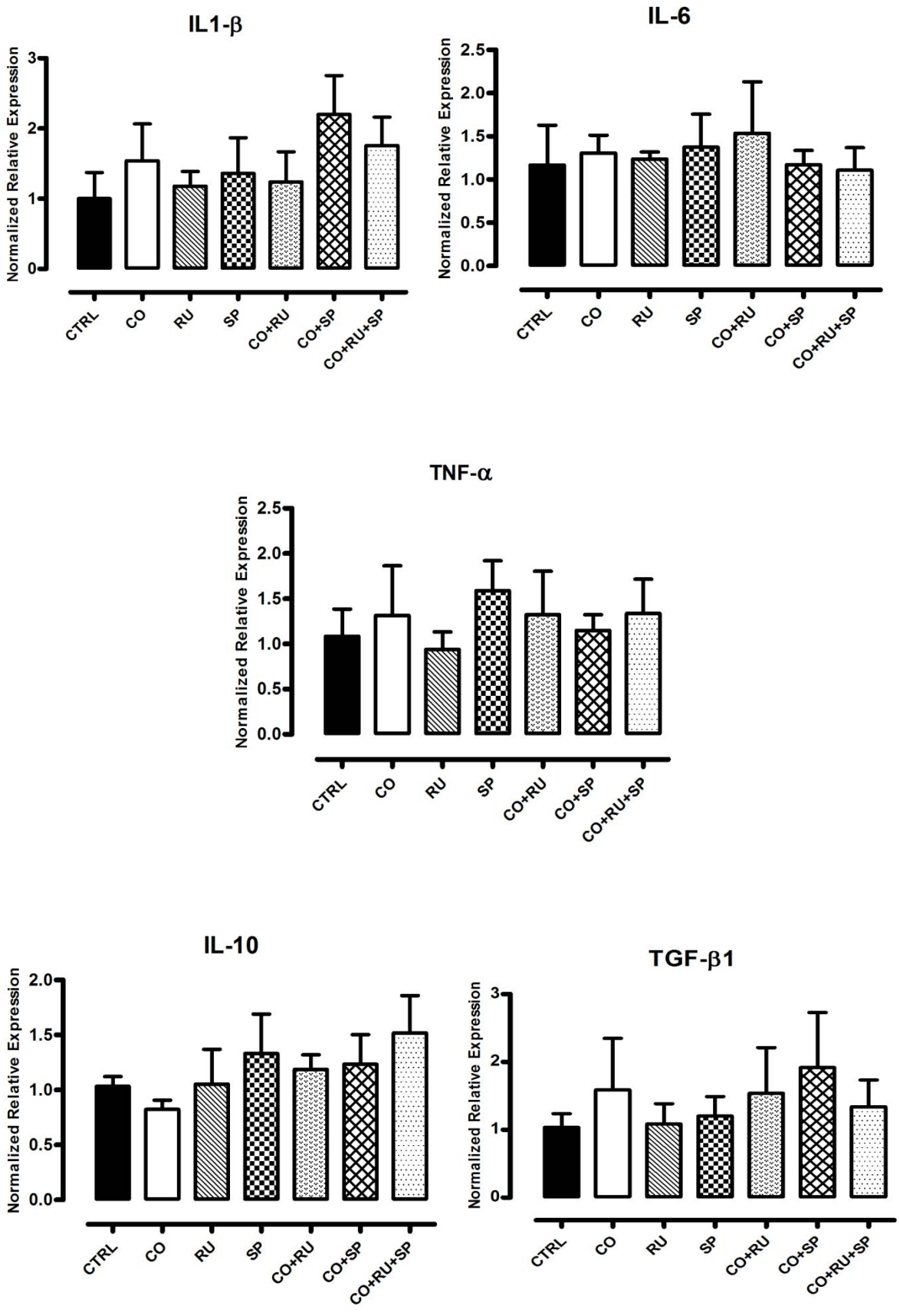
Table 1: Primers used in real-time PCR for gene expression analysis for rainbow trout

Gene	GenBank Accession number	Sequence 5′–3′	Product size
β-actin	NM_001124235.1	**FW:**GGACTTTGAGCAGGAGATGG	186
		RV:ATGATGGAGTTGTAGGTGGTCT	
IL-1β	NM_001124347.2	**FW:**TGAGAACAAGTGCTGGGTCC	148
		RV:GGCTACAGGTCTGGCTTCAG	
IL-6	NM_001124657.1	**FW:**GAGTTTCAGAAGCCCGTGGA	149
		RV:AGCTGGTACACTTGCAGACC	
TNF-α	NM_001124357.1	**FW:**CACACTGGGCTCTTCTTCGT	155
		RV:CAAACTGACCTTACCCCGCT	
IL-10	NM_001245099.1	**FW:**CCGCCATGAACAACAGAACA	105
		RV:TCCTGCATTGGACGATCTCT	
TGF-β1	NM_001281366.1	**FW:**GCCAAGGAGGTCCACAAGTT	146
		RV:GTGGTTTTGATGAGCAGGCG	
GR1	NM_001124730.1	**FW:**TTCCTTTCCTCCCTGTCAGT	171
		RV:ATCCTCCTCCGTCTTGATGA	
GR2	AY495372.1	**FW:** ACTTTGAGTTGACAGGCTCC	177
		RV: TGGTGTTGGAACCGCTAAAA	
α-AR	EF667964.1	**FW:** GTCAACTCCACAAACGCCAC	144
		RV: GGTCATCACCGCCAGAATGA	
β-AR	AY044093.1	**FW:** CTCCTCAACACCTGGCACTT	160
		RV: GCTTGGTCAGTAGGGATGGG	

Table 2: Primers used in real-time PCR for gene expression analysis for gilthead sea bream

Gene	GenBank Accession number	Sequence 5′–3′	Product size
18S	AY587263.1	**FW:**ACCAGACAAATCGCTCCACC	172
		RV:AGGAATTGACGGAAGGGCAC	
IL-1β	AJ277166.2	**FW:**TCAGCACCGCAGAAGAAAAC	115
		RV:TAACACTCTCCACCCTCCAC	
IL-6	EU244588.1	**FW:**ATCCCCTCACTTCCAGCAGA	129
		RV:GCTCTTCGGCTCCTCTTTCT	
TNF-α	AJ413189.2	**FW:**TCGTTCAGAGTCTCCTGCAG	320
		RV:AAGAATTCTTAAAGTGCAAACACACCAAA	
TGF-β1	AF424703.1	**FW:**AGACCCTTCAGAACTGGCTC	145
		RV:ACTGCTTTGTCTCCCCTACC	
IL-10	JX976621.1	**FW:** GAGCGTGGAGGAATCTTTCAA	154
		RV: GATCTGCTGGATGGACTGC	
GR	DQ486890.1	**FW:** ACTGAGGAGGGAGGTCTATT	195
		RV: GGACTCTGGGACTTCTAACA	

Figure 1:


IL1-β
Normalized Relative Expression
0
1
2
3
CTRL
CO
RU
SP
CO+RU
CO+SP
CO+RU+SP
IL-6
Normalized Relative Expression
0.0
0.5
1.0
1.5
2.0
2.5
CTRL
CO
RU
SP
CO+RU
CO+SP
CO+RU+SP
TNF-α
Normalized Relative Expression
0.0
0.5
1.0
1.5
2.0
2.5
CTRL
CO
RU
SP
CO+RU
CO+SP
CO+RU+SP
IL-10
Normalized Relative Expression
0.0
0.5
1.0
1.5
2.0
CTRL
CO
RU
SP
CO+RU
CO+SP
CO+RU+SP
TGF-β1
Normalized Relative Expression
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1
2
3
CTRL
CO
RU
SP
CO+RU
CO+SP
CO+RU+SP

Figure 2:

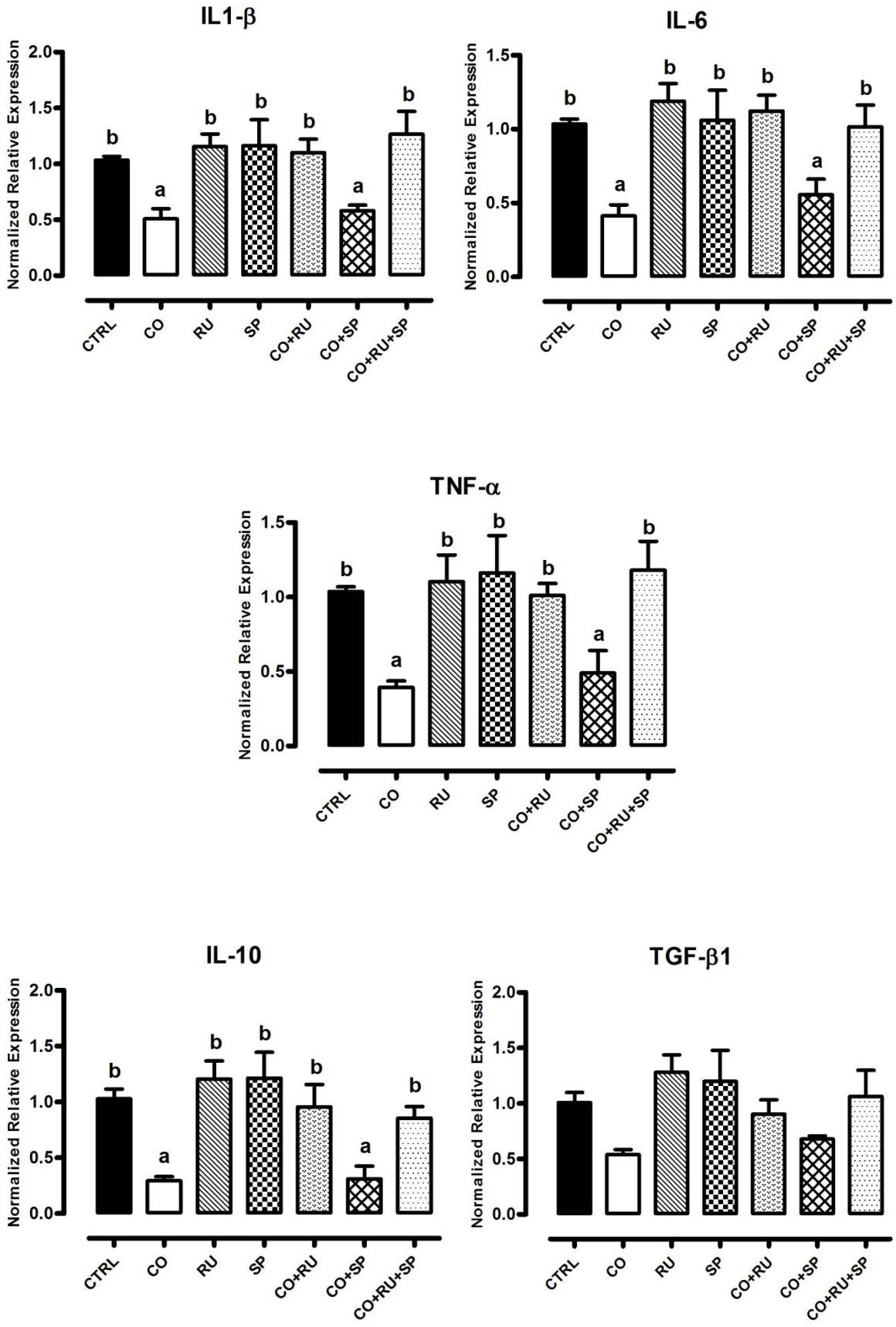
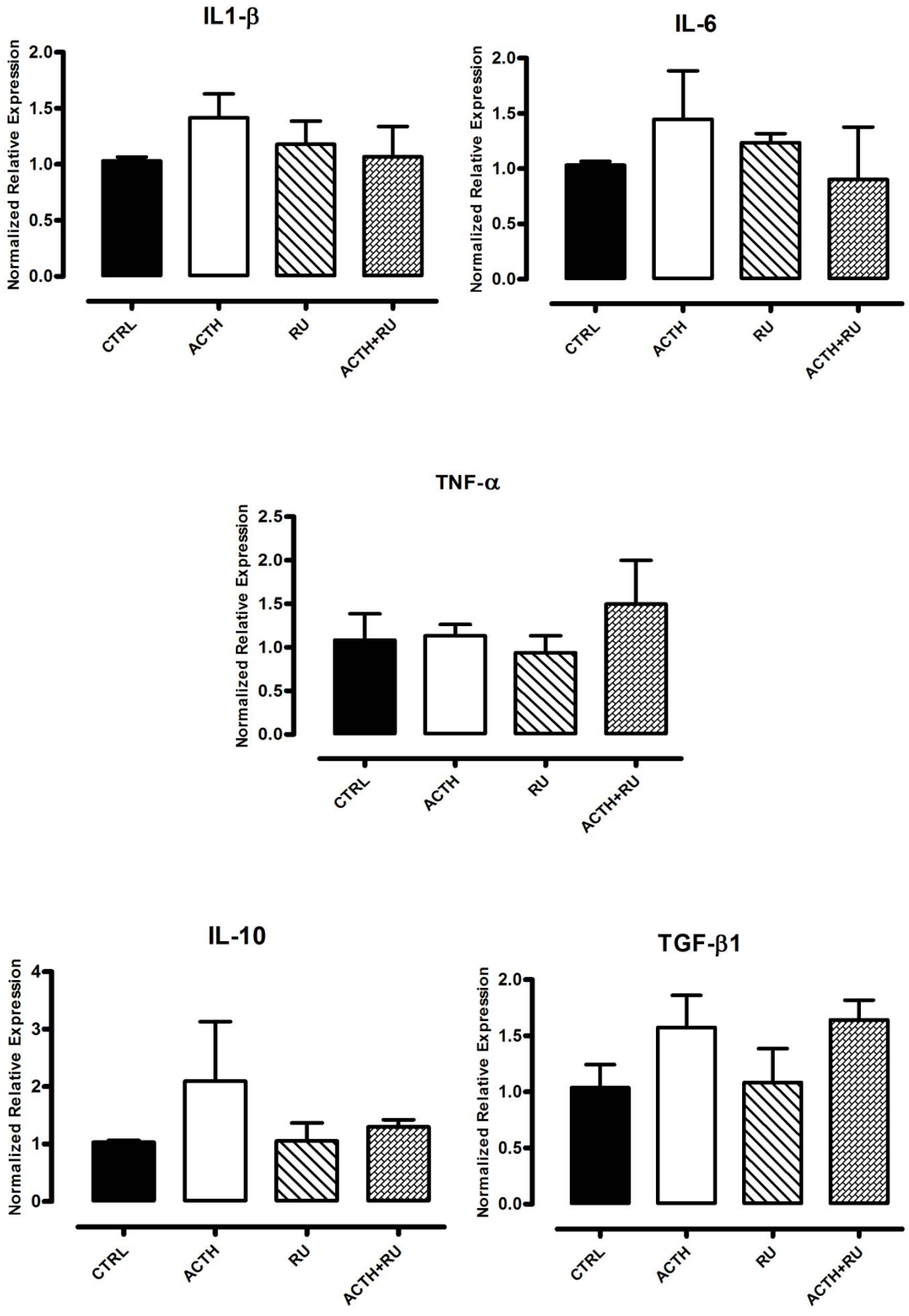

IL1-β
IL-6
TNF-α
IL-10
TGF-β1
Normalized Relative Expression
CTRL
CO
RU
SP
CO+RU
CO+SP
CO+RU+SP

Figure 3:


IL1-β
Normalized Relative Expression
2.0
1.5
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CTRL
ACTH
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IL-6
Normalized Relative Expression
2.0
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0.5
0.0
CTRL
ACTH
RU
ACTH+RU
TNF-α
Normalized Relative Expression
2.5
2.0
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0.0
CTRL
ACTH
RU
ACTH+RU
IL-10
Normalized Relative Expression
4
3
2
1
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CTRL
ACTH
RU
ACTH+RU
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CTRL
ACTH
RU
ACTH+RU

Figure 4:

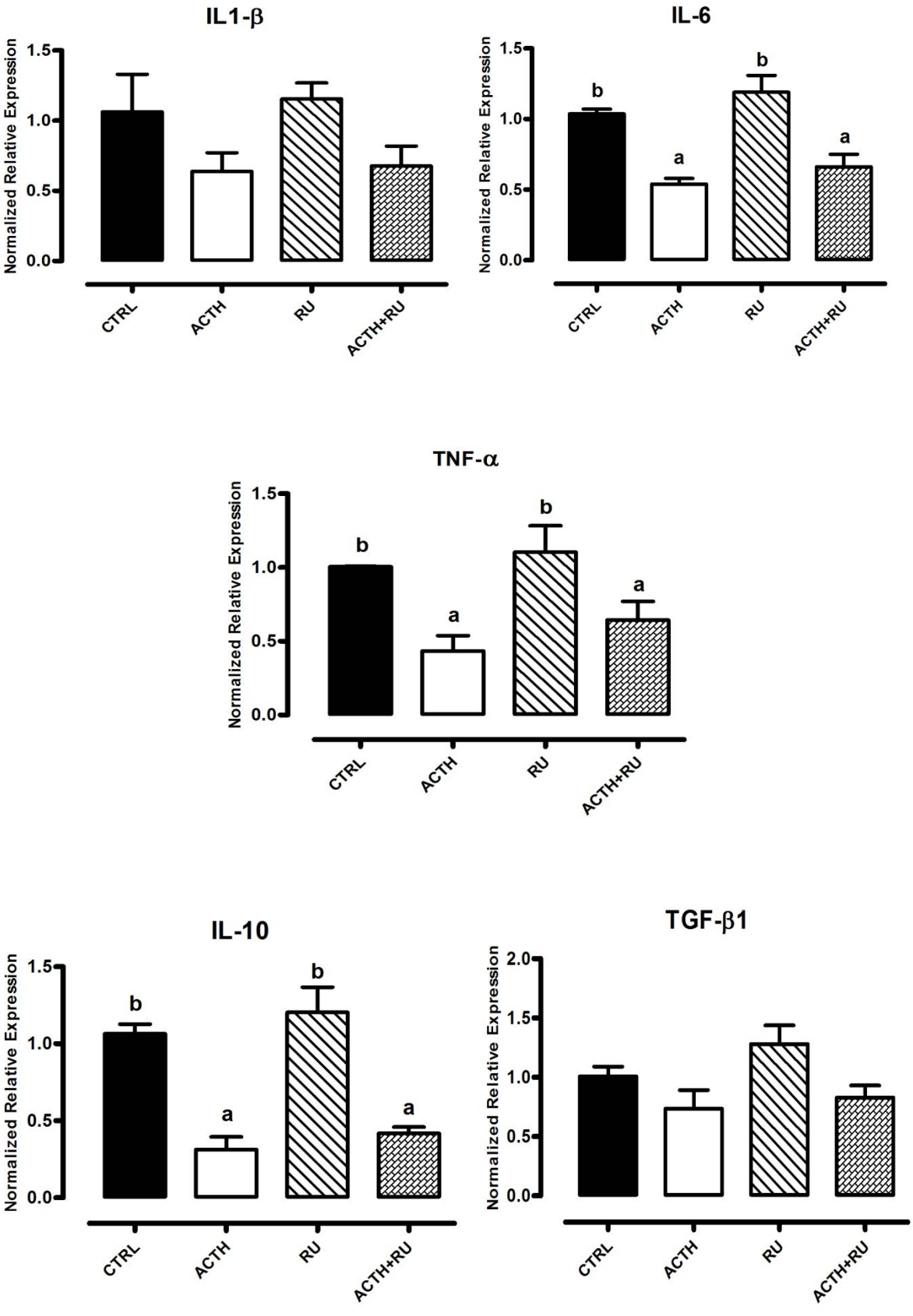
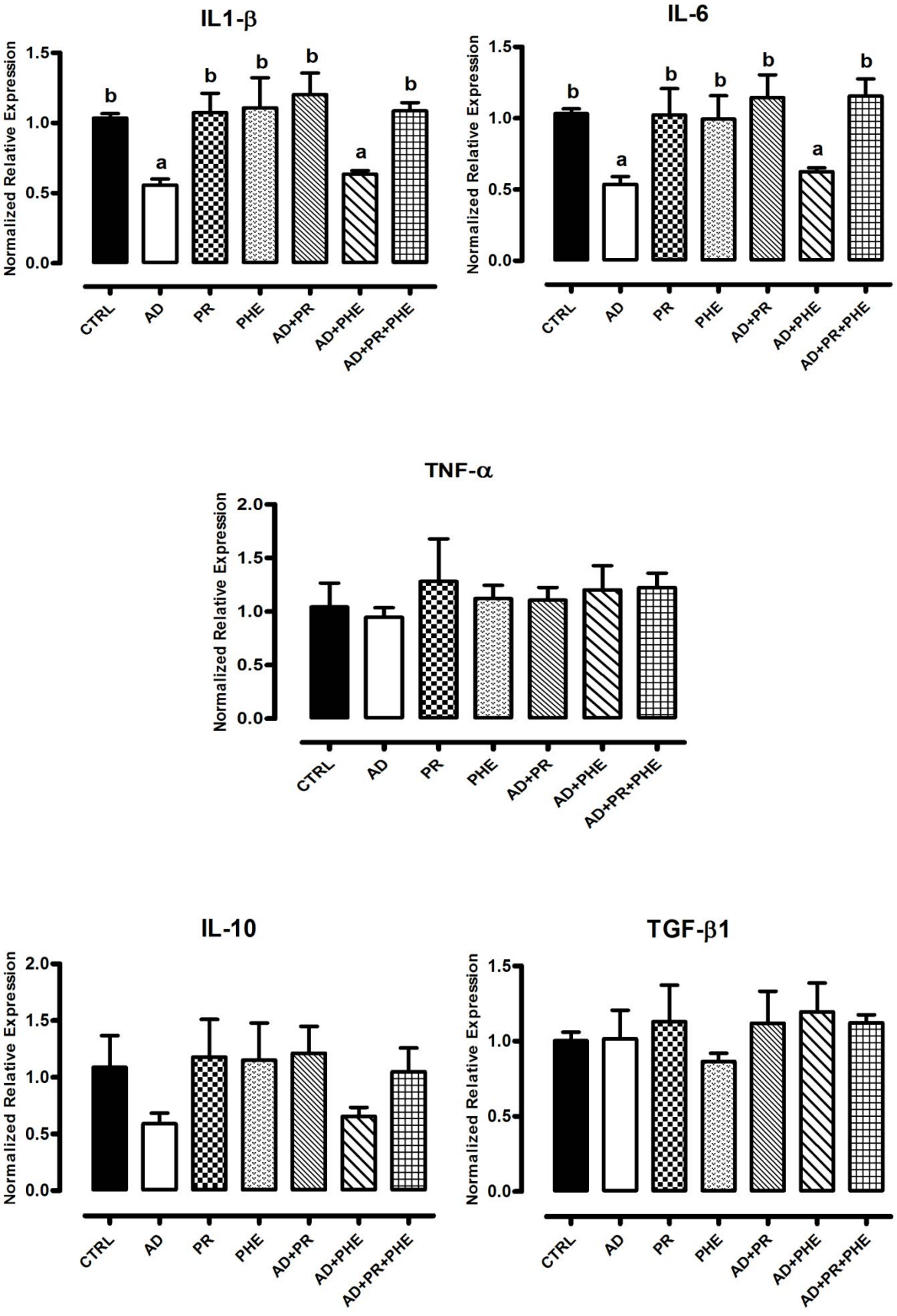

IL1-β
Normalized Relative Expression
1.5
1.0
0.5
0.0
CTRL
ACTH
RU
ACTH+RU
IL-6
b
a
b
a
TNF-α
b
a
b
a
IL-10
b
a
b
a
TGF-β1
2.0
1.5

Figure 5: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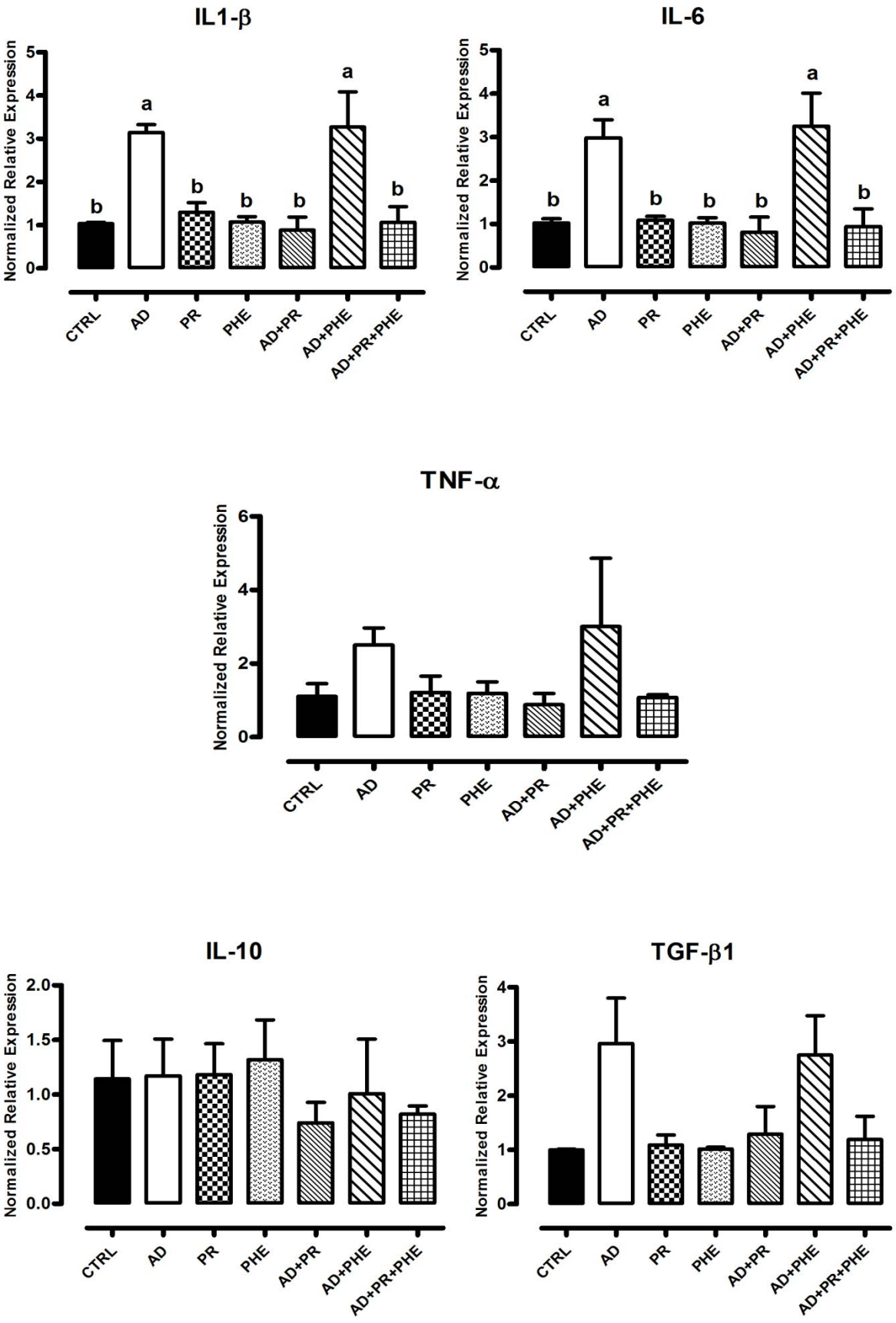
IL-6
TNF-α
IL-10
TGF-β1
Normalized Relative Expression
CTRL
AD
PR
PHE
AD+PR
AD+PHE
AD+PR+PHE
a
b

Figure 6:


IL1-β
IL-6
TNF-α
IL-10
TGF-β1
Normalized Relative Expression
CTRL
AD
PR
PHE
AD+PR
AD+PHE
AD+PR+PHE
a
b

5. Chapter third

5.1. Modulatory effect of stress hormones on the cytokine response of rainbow trout and gilthead sea bream head kidney tissue stimulated with *Vibrio anguillarum**

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Abstract

The effect of stress hormones cortisol, adrenocorticotropic hormone (ACTH) and adrenaline on inflammatory cytokine expression has been investigated in head kidney tissue of trout and sea bream previously exposed to *Vibrio anguillarum*. Since there is no evidence of whether antagonists for stress hormone receptors may influence the interaction between hormones and cytokines after bacterial administration, our study evaluated such an interaction in presence of stress hormones (cortisol, ACTH, adrenaline), hormone receptor blockers and inactivated *Vibrio anguillarum* in head kidney primary cell culture (HKPCC). Mifepristone, spironolactone, propranolol and phentolamine were used to block GR, MR, MC2R, and β-/α-adrenoreceptors.

Our results show an increase of the pro-inflammatory and anti-inflammatory response after *Vibrio anguillarum* treatment in both species. Cortisol, ACTH and adrenaline did not modulate the expression of immune-related genes in rainbow trout, while in sea bream cortisol was able to reduce stimulated gene expression in all cytokines. This effect was only restored to basal expression level in IL-1β and TNF-α by mifepristone alone or in combination with spironolactone. ACTH reduced both pro-inflammatory and anti-inflammatory cytokine expression excluding IL-1β in sea bream. Adrenaline enhanced the expression of IL-1β and TGF-β1 stimulated by *Vibrio* in sea bream, and the effect was diminished by propranolol alone or in combination with phentolamine. Overall, other than an expected effect of the tested antagonists, our results confirm the close regional interaction between endocrine and cytokine messengers in the head kidney against the *Vibrio anguillarum* treatment, and a clear difference between the two fish species in both the sensitivity to *Vibrio anguillarum* and to hormonal stimuli.

1. Introduction

The stress response in animals is a general, non-specific and widespread reaction involving all physiological systems [1], and particularly the immune and neuroendocrine systems are tightly connected [2,3]. The activation of the Hypothalamus-Pituitary-Interrenal (HPI) and Sympathetic-Adreno-Medullar (SAM) axis, as the two major pathways by which the endocrine response is organized, can modulate the immune function [4,5]. On the other hand, it has also been demonstrated that several cytokines are able to stimulate hormone secretion in HPA [6]. Cortisol, the major glucocorticoid (GCs) in teleost fish and the final product of HPI axis activation, is secreted by head kidney interrenal cells in a concatenated response involving the hypothalamic corticotrophin-releasing hormone (CRH) and the adrenocorticotropic hormone (ACTH). Thus, secreted ACTH is recognized by melanocortin receptor 2 (MC2R) on the surface of the interrenal cells and activates a signalling cascade which mediates the secretion of cortisol. Cortisol plays an essential role in several biological functions such as energy homeostasis, hydromineral balance, modulation of the immune response, and behaviour [7–9]. These actions are initiated by genomic (slow) and non-genomic (fast) mechanisms in the central nervous system [7,10,11]. Cortisol binds to glucocorticoid receptors (GRs) forming a complex that is transported to the nucleus where it binds to DNA at glucocorticoid response elements (GREs) present in the promoters of several genes. This interaction usually involves changes in gene transcription (trans-activation) by interacting with co-activator molecules. Two GRs (GR1 and GR2) and also one mineralocorticoid receptor (MR) have been cloned and sequenced in several teleost species [12,13], except in zebrafish (*Danio rerio*) where a splice variant for GR, similar than in humans, has been described [14,15]. Since the circulating level of aldosterone is extremely low in fish, cortisol palliates this situation by binding to MR and playing a principal role in osmoregulation [8,16,17]. Therefore, in teleosts cortisol plays both glucocorticoid and mineralocorticoid functions through GRs and MRs, respectively [18]. Adrenaline is the main catecholamine product following the activation of SAM axis after sympathetic innervation of the head kidney chromaffin cells. Adrenaline modulates cardiovascular and respiratory functions in order to rapidly mobilize the available energy reservoirs and to maintain an adequate oxygen level to satisfy the increased energy demands involved in the stress response [19]. There is evidence of β-adrenoreceptor existence in fish, which recognizes adrenaline and mediates its effects [20,21].

It has been demonstrated that lymphocytes, monocytes, macrophages and granulocytes have receptors for many neuroendocrine products and also that their expression is regulated after hormone secretion [22]. Indeed, it has been shown that similar or even identical hormone or cytokine signalling molecules are synthesized by cells and tissues from both systems [23], hence allowing bi-directional communication. Thus, specific receptors for stress hormones have been found in immune cells [3,24].

The teleost head kidney is both an endocrine and immune organ: the hematopoietic tissue, reponsible for the synthesis of blood cells, is situated adjacent to the endocrine tissue, creating a unique location for bilateral endocrine-immune interactions [2,25,26]. Although these two systems were initially considered to act independently, an extensive and orchestrated network controls such immune and neuroendocrine system interaction [27,28].

Vibriosis is one of the bacterial diseases that can bring huge losses to aquaculture. Its etiological agent, *Vibrio anguillarum*, causes a widespread lethal haemorrhagic septicaemia affecting both marine and freshwater fish [29,30]. This bacterium has several serogroups including O1, O2 and O3, which are considered the most pathogenic [31]. In fact, it has been reported that 80% of O2 serogroup cannot be killed by the complement system in trout [30,32]. Hence, the *in vitro* administration of a *Vibrio anguillarum* contributed to first, validate the cytokine response under immune activation and second, to check the immune-endocrine interaction under these circumstances.

In previous results of our group, the effects of stress hormones on the expression of pro- and anti-inflammatory cytokines have been evaluated in sea bream head kidney cells showing down-regulation of the expression of LPS induced-cytokines after 2 h of treatment [33]. It has also been reported that inflammatory cytokines were quickly increased by both alive or inactive *Vibrio anguillarum* in seabass [33,34]. However, few studies have been performed looking at the interaction between stress-associated hormones and the immune cell response in the presence of *Vibrio anguillarum*. Moreover, there is no evidence on whether antagonists for specific stress hormone receptors may restore the putative cytokine modulation observed after hormone administration. Therefore, we evaluated both pro-inflammatory (IL-1β, IL-6, TNF-α) and anti-inflammatory cytokine (IL-10, TGF-β) expression of head kidney tissue treated with stress hormones in presence of inactivated *Vibrio anguillarum*. We also included receptor antagonists to better precise the interaction hormones-cytokines. We have used mifepristone (to antagonize both GR and ACTH receptors), spironolactone (to antagonize MR effect), phentolamine (α-adrenoreceptor antagonist), and propranolol (β-adrenoreceptor antagonists).

This assessment has been performed in two species (rainbow trout and gilthead sea bream) that are adapted to different environments including range of temperature, salinity or oxygen requirements, and also showing genetic differences [35]. All these factors may influence the response in terms of endocrine and immune responsiveness and the interaction between them. This is the first study to assess the effect of antagonist receptors and stress hormones in immune related genes after an inactivated pathogen exposure.

2. Material and methods

2.1. Animals

Rainbow trout (*Oncorhynchus mykiss*), body weight of 120-140 g were obtained from a local fish farm (Piscifactoria Andres, St Privat d'en Bas, Spain). Gilthead sea bream (*Sparus aurata*) body weight of 60-70 g were obtained from AQUACULTURA ELS ALFACS,S.L. (Tarragona). Fish were transferred to the UAB fish facility (AQUAB) to acclimatize them to laboratory conditions. All experimental procedures involving fish were submitted and authorized by the Ethical Committee of the "Universitat Autonoma de Barcelona" that agrees with the international Guiding Principles for Biomedical Research Involving Animals (EU2010/63).

2.2. Head kidney primary cell culture (HKPCC) preparation

Rainbow trout (n=6) and gilthead sea bream (n=6) head kidneys were isolated from euthanized fish by an overdose of MS-222 (Sigma). Head kidney was isolated and immediately placed in 24-well cell culture plate with 1 ml DMEM – high glucose (Sigma) and kept in ice. Tissue was homogenized and passed through a 100 µm nylon cell strainer (Falcon). HKPCC was prepared in DMEM – high glucose (Sigma) at a concentration of $2x10^6$ cells/ml. The head kidney cells (1ml) were left undisturbed in an incubator under optimal culture temperature conditions (16 °C for rainbow trout; 18°C for sea bream, and 5% CO_2) for 2 h to settle in a 24-well culture plate (Jet Biofil). The HKPCC includes hematopoietic, immune (macrophages and lymphocytes) and endocrine cells (chromaffin and corticosteroidogenic cells).

2.3. Hormones and pharmacological agents

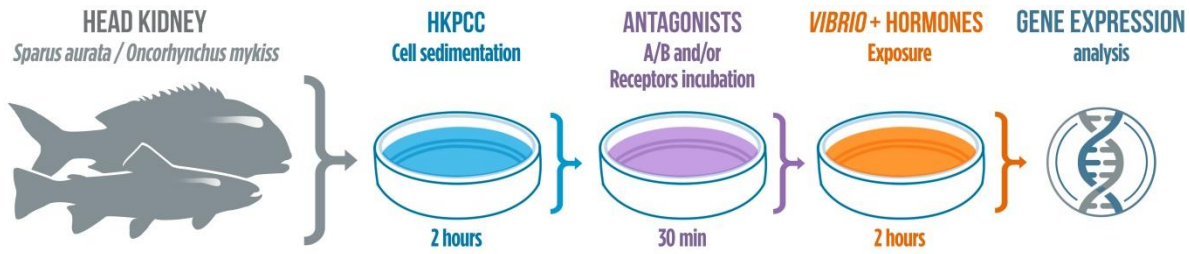
Cortisol (Hydrocortisone; Sigma cat #H2882-1G), adrenocorticotropic hormone (ACTH; Sigma cat #A0423-1 MG) and adrenaline (Adrenaline; Sigma cat #Y0000882-5MG) were dissolved according to manufacturer's instruction. Mifepristone (or RU-486, Sigma cat. #M8046-100MG) as antagonist receptor for cortisol and ACTH, spironolactone (Sigma cat#S3378-1G) as antagonist receptor for cortisol, and propranolol (Sigma cat #P8688-100MG) and phentolamine (Sigma cat #P7547-100MG) as antagonist receptors for adrenaline, were used to evaluate the hormones effect on the immune response at gene expression level. Mifepristone, propranolol and phentolamine were dissolved in absolute ethanol while spironolactone was dissolved in chloroform. Thus, the final concentration of each solvent was ≤ 0.1% in order to have no influence on cell culture preparation.

2.4. Inactivated *Vibrio anguillarum*

ICTHIOVAC ^{R} VR (HIPRA) was the source for the inactivated *Vibrio anguillarum*. The composition consists of inactivated *Vibrio anguillarum*, serotypes O1, O2α (the most pathogenic) and O2β with relative percent survival (RPS) ≥ 60%. The inactivated *Vibrio anguillarum* isolation was carried out by centrifugation for 10 min 10000*g* at 4°C. The pellet was washed twice with DMEM. Finally, the inactivated bacteria stock solution was prepared by resuspending the pellet in 1 ml (of DMEM). Different dilutions were then tested in order to determine the bacteria working solution. The 1:40 dilution was chosen based on the proinflammatory cytokine expression effect.

2.5. In vitro experimental design.

In order to evaluate the hormone-dependent immune gene expression pattern, the already settled HKPCC were treated with antagonist receptors for 30 min. Thus, RU-486 (GR antagonist) and spironolactone (MR antagonist) were added to the cell culture separately and together to evaluate the cortisol effect; RU-486 was added to evaluate the ACTH effect; propranolol (nonselective beta blocker) and phentolamine (nonselective α-adrenergic antagonist) were added separately and together to evaluate the adrenaline effect. Then, HKPCCs were incubated for 2h with cortisol (100 ng ml^{-1}), ACTH (150 ng ml^{-1}), or adrenaline (1 µM). The addition of inactivated *Vibrio anguillarum* (dilution 1:40) was performed at the same time than stress hormones. Antagonist receptors were added at a final concentration of 10-fold higher than that of stress hormones. HKPCC alone (as non-treated group) and HKPCC treated with *Vibrio anguillarum* only (as bacteria treated group) were included in the study. Six replicates were analysed for each condition and fish species.



2.6. RNA isolation and cDNA synthesis

Total RNA was isolated from stimulated and control HKPCC using TRI reagent (Sigma) according to manufacturer's instructions. The RNA pellet was dissolved in nuclease free-water and immediately stored at -80°C until use. The RNA concentration was quantified by

NanoDropND-2000 spectrophotometer (Thermo Scientific). RNA (500 ng) was used as template to synthesize complementary DNA (cDNA) using iScript cDNA kit (Bio-Rad Laboratories) according to manufacturer's instructions.

2.7. Gene expression analysis

The gene expression levels of immune-related genes (IL-1β, IL-6, TNF-α, IL-10, TGF-β1) were analysed by real-time qPCR. We first tested several housekeepings candidates (18S, EF1α and RPL27 in sea bream, and EF1α and β-actin in rainbow trout) to elucidate those with less variation. Thus, β-actin (for rainbow trout) and 18S (for sea bream) were selected based on their lowest variability (data not shown). Specific primers for rainbow trout (Table 1) and gilthead sea bream (Table 2) were designed with Primer-Blast. The potential primer secondary structure and also primer specificity were checked with OligoAnalyzer (version 3.1) and Primer-Blast software, respectively. Real-time PCR reactions were performed with iTaq universal sybr green supermix (Bio-Rad Laboratories) using 2 µl of 1:40 dilution of cDNA (for genes of interest in rainbow trout) and 2 µl of 1:6 dilution of cDNA (for genes of interest in gilthead sea bream) or 1:1000 dilution (for housekeeping gene in both species). Primers for all genes were used at a final concentration of 500 nM. The thermal conditions used were 3 min at 95°C of pre-incubation followed by 40 cycles at 95°C for 30 s and 60°C for 30 s. All reactions were performed in duplicate using CFX384 Touch Real-Time PCR Detection System (Bio-Rad Laboratories). The quantification was done according to Pfaffl method corrected for efficiency of each primer set (Pfaffl, 2001). Values for each experimental condition are expressed as normalized relative expression, calculated in relation to values of control group (non-treated control) and normalized against those of the housekeeping gene β-actin (rainbow trout) or 18S (sea bream).

2.8. Statistical analysis

Gene expression values are represented as mean value ±SEM. Differences were considered significant when p-value < 0.05 among groups. Significant differences on gene expression among all groups treated with stress hormones and *Vibrio anguillarum* were analysed by one-way analysis of variance (ANOVA) followed by Student-Newman-Keuls (SNK) test. All data was analysed using the SPSS version 20.0 software package.

3. Results

In order to determine the efficient dilution of *Vibrio anguillarum* to induce gene expression, head primary cell culture was treated with different dilutions of bacteria including 1:20, 1:100, 1:500 and 1:2500. The 1:20 and 1:100 dilution of bacteria were able to induce a

robust 73.928 and 18.217 fold expression of IL-1β respectively, whereas 1:500 and 1:2500 dilutions did not show IL-1β stimulation (Figure 1).

In order to examine whether the inactivated *Vibrio anguillarum*-induced cytokine expression is modulated by hormones, the mRNA abundance of genes related with pro-inflammatory (IL-β, IL-6, TNFα) and anti-inflammatory (TGF-β1 and IL-10) response compared to controls, RT-qPCR analysis was carried out. The results showed that *Vibrio* clearly modulated HKPCC cytokine expression. Cortisol did not induce a differential effect on the expression of pro-inflammatory cytokine IL-1β in rainbow trout together with IL-6 and TNF-α. The same response was observed for IL-10 and TGF-β1 (Figure 2). As expected, when HKPCC was co-incubated with the antagonists, no effects were observed.

A different pattern was obtained in cortisol treated sea bream showing a down-regulated expression of all cytokines assessed. This cortisol down-regulation effect was only reverted and restored to basal expression level for IL-1β and TNF-α by the antagonist receptor Mifepristone (RU-486) or in combination with spironolactone (Figure 3), but not with spironolactone alone. Overall, these results indicate that cortisol does not modulate the *Vibrio anguillarum*-induced cytokine expression in rainbow trout but does modulate it in sea bream at the same concentrations and times tested.

ACTH administration did not significantly alter the transcript abundance of any of the cytokines evaluated in rainbow trout (Figure 4). Consequently, it was not surprising that the treatment with the antagonist receptor RU-486 produced no effect on gene expression modulation. Unlike the results observed in rainbow trout, the expression of IL-6, TNF-α, IL-10 and TGF-β1 was significantly diminished by ACTH in sea bream HKPCC (Figure 5). The administration of RU-486 was not able to restore their expression values to control level (Figure 5).

Administration of adrenaline did not significantly alter the mRNA levels of both pro-inflammatory (IL1β and IL6, TNF-α) and anti-inflammatory cytokines (TGF-β1 and IL10) in rainbow trout HKPCC compared to samples treated with *Vibrio anguillarum* (Figure 6). Accordingly, when HKPCC was treated with propranolol or co-administered with propranolol and phentolamine, no changes were observed in the gene expression among the different groups treated with antagonists in rainbow trout HKPCC (Figure 6).

When sea bream HKPCC was treated with adrenaline, an up-regulation of IL1-β and TGF-β1 transcriptional level was observed. The same trend, although no significant, was also observed on IL-6 and TNF-α (Figure 7). Once sea bream HKPCC was pre-treated with propranolol alone or in combination with phentolamine, the expression of IL-1β and TGF-β1 returned to control level (Figure 7). However, the administration of phentolamine alone did not block the adrenaline induced reduction of IL-1β and TGF-β1 expression in sea bream. No changes were registered on other genes (Figure 7). Therefore, these results suggest that

adrenaline induced IL-1β and TGF-β1 increment and the effect was restored to control level mainly by the action of propranolol but not phentolamine in sea bream.

Figure 1:

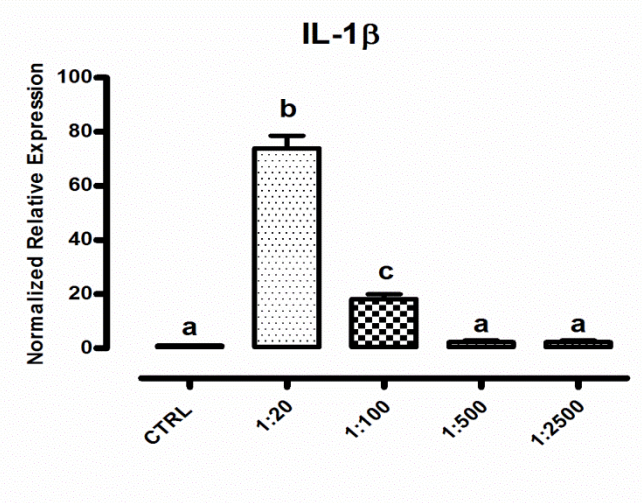


Figure 2:

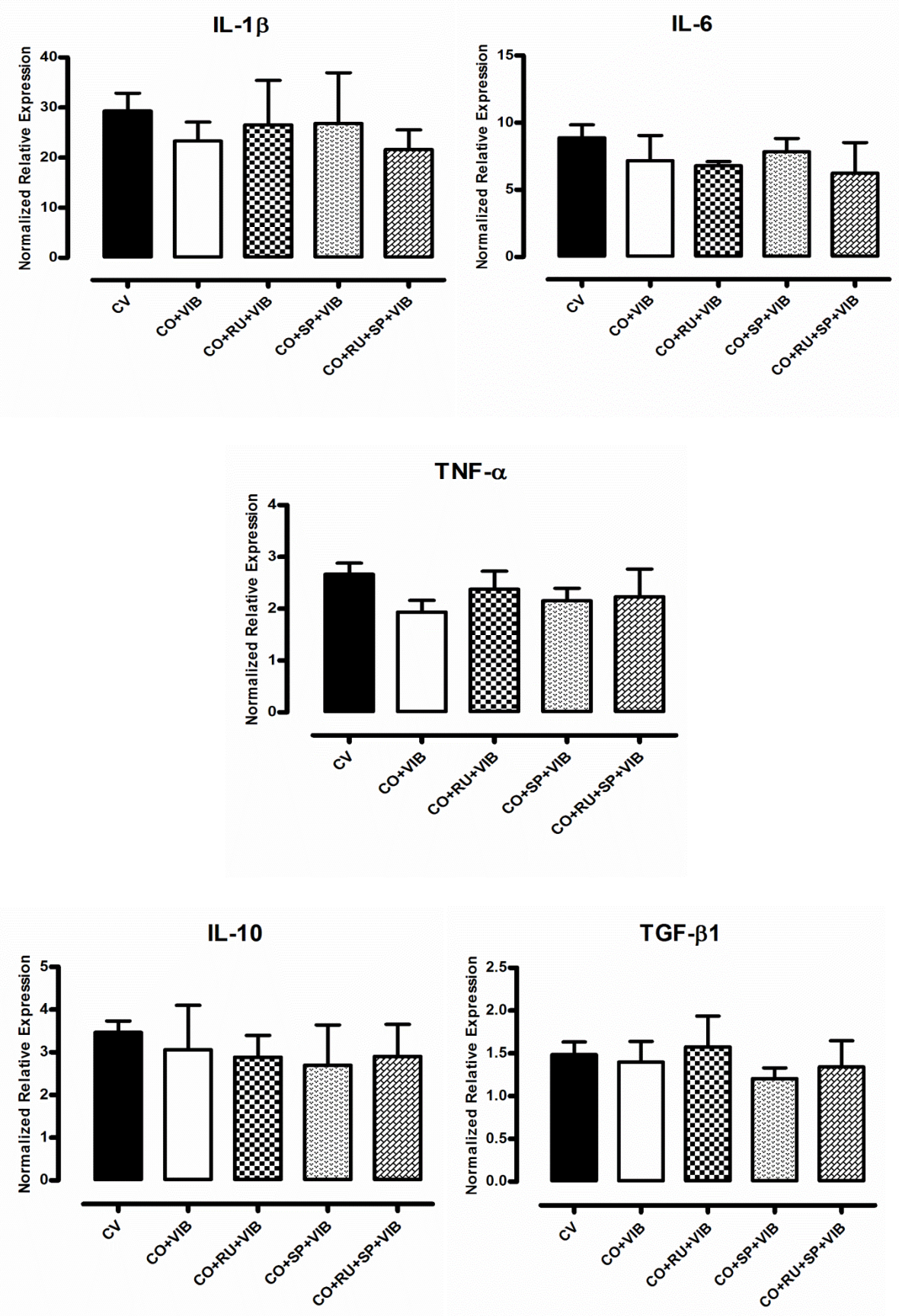

IL-1β
Normalized Relative Expression
0
10
20
30
40
CV
CO+VIB
CO+RU+VIB
CO+SP+VIB
CO+RU+SP+VIB
IL-6
Normalized Relative Expression
0
5
10
15
CV
CO+VIB
CO+RU+VIB
CO+SP+VIB
CO+RU+SP+VIB
TNF-α
Normalized Relative Expression
0
1
2
3
4
CV
CO+VIB
CO+RU+VIB
CO+SP+VIB
CO+RU+SP+VIB
IL-10
Normalized Relative Expression
0
1
2
3
4
5
CV
CO+VIB
CO+RU+VIB
CO+SP+VIB
CO+RU+SP+VIB
TGF-β1
Normalized Relative Expression
0.0
0.5
1.0
1.5
2.0
2.5
CV
CO+VIB
CO+RU+VIB
CO+SP+VIB
CO+RU+SP+VIB

Figure 3:

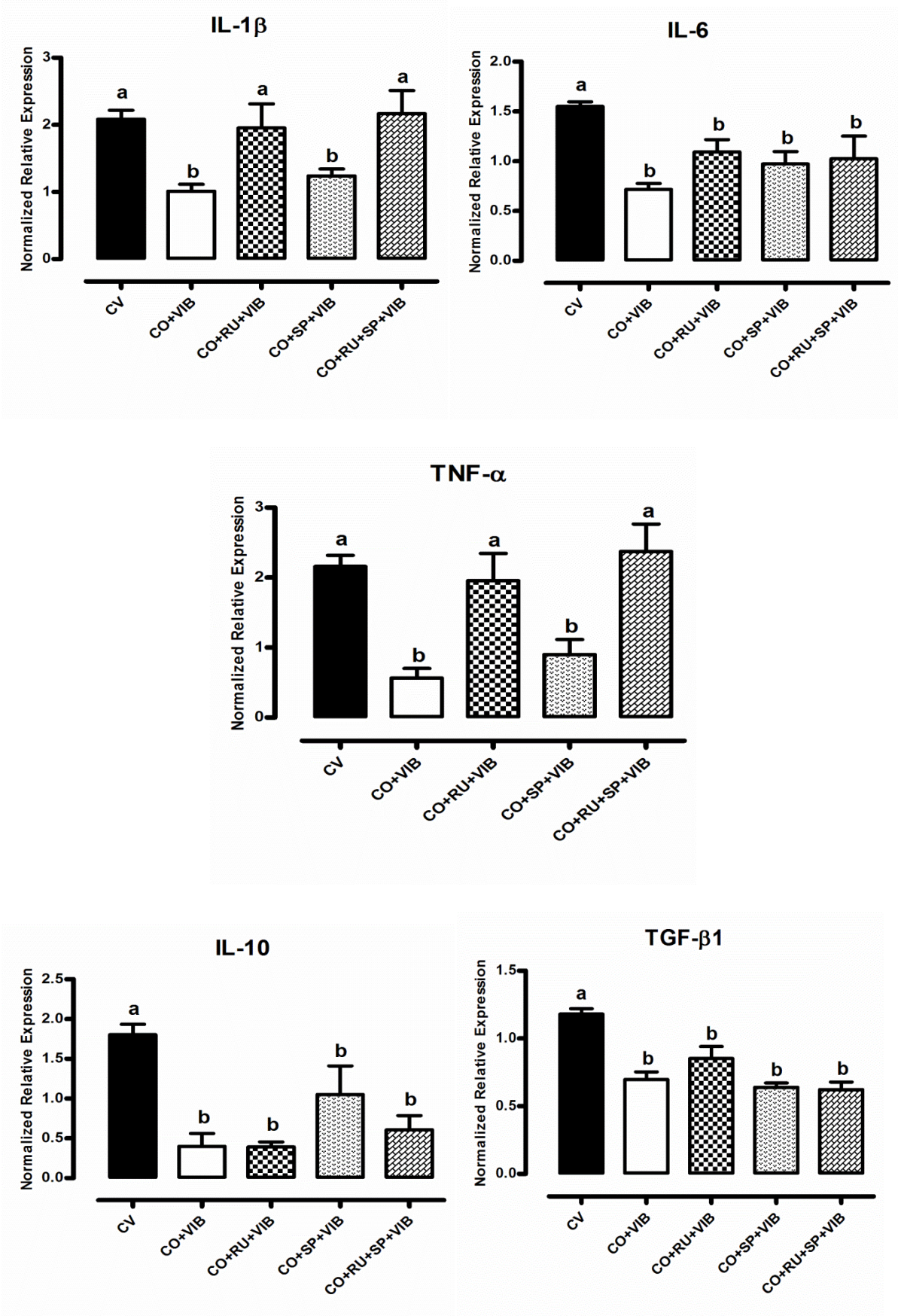

IL-1β
Normalized Relative Expression
0
1
2
3
a
b
a
b
a
CV
CO+VIB
CO+RU+VIB
CO+SP+VIB
CO+RU+SP+VIB
IL-6
Normalized Relative Expression
0.0
0.5
1.0
1.5
2.0
a
b
b
b
b
CV
CO+VIB
CO+RU+VIB
CO+SP+VIB
CO+RU+SP+VIB
TNF-α
Normalized Relative Expression
0
1
2
3
a
b
a
b
a
CV
CO+VIB
CO+RU+VIB
CO+SP+VIB
CO+RU+SP+VIB
IL-10
Normalized Relative Expression
0.0
0.5
1.0
1.5
2.0
2.5
a
b
b
b
b
CV
CO+VIB
CO+RU+VIB
CO+SP+VIB
CO+RU+SP+VIB
TGF-β1
Normalized Relative Expression
0.0
0.5
1.0
1.5
a
b
b
b
b
CV
CO+VIB
CO+RU+VIB
CO+SP+VIB
CO+RU+SP+VIB

Figure 4:

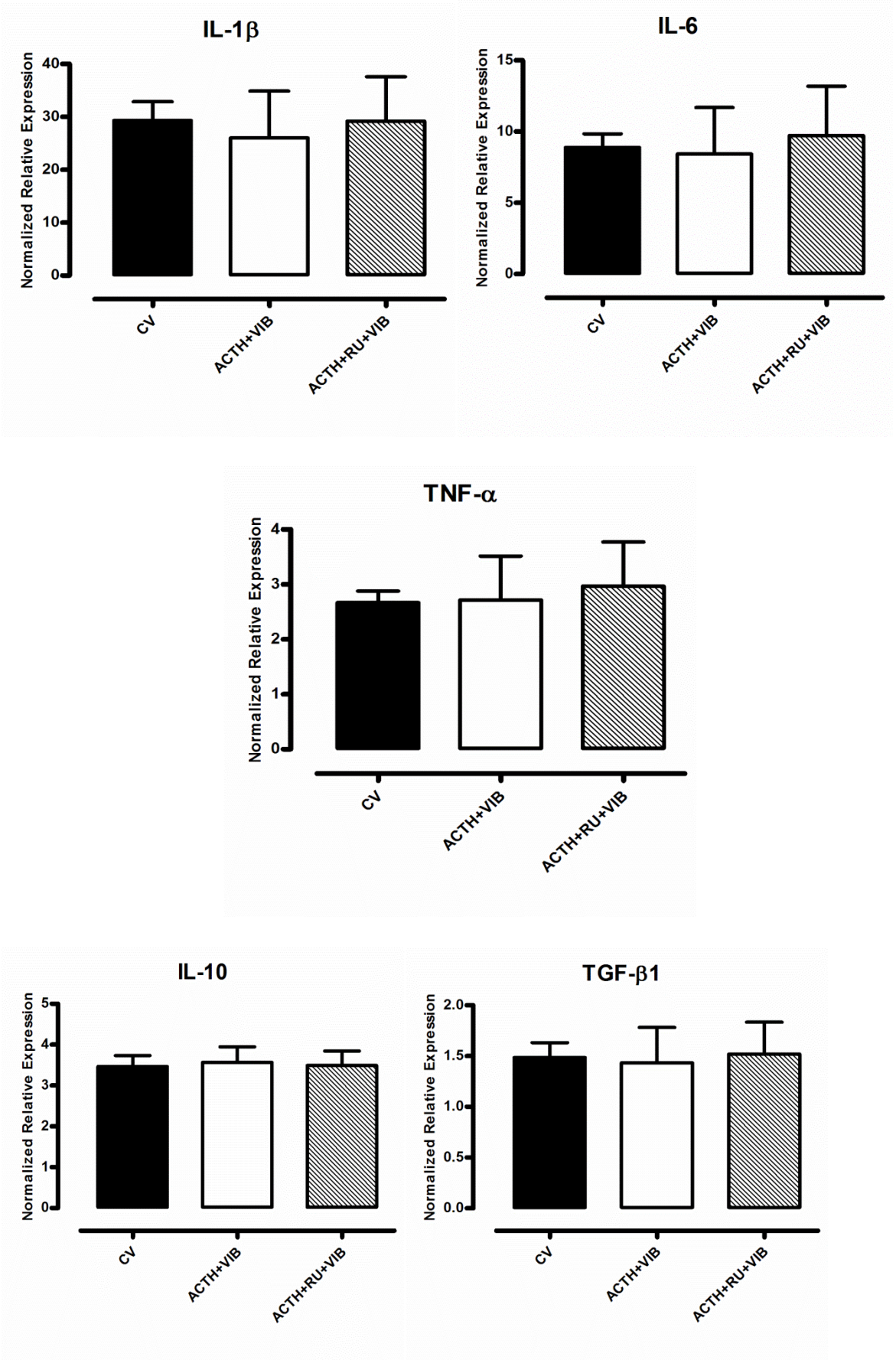

IL-1β
Normalized Relative Expression
40
30
20
10
0
CV
ACTH+VIB
ACTH+RU+VIB
IL-6
Normalized Relative Expression
15
10
5
0
CV
ACTH+VIB
ACTH+RU+VIB
TNF-α
Normalized Relative Expression
4
3
2
1
0
CV
ACTH+VIB
ACTH+RU+VIB
IL-10
Normalized Relative Expression
5
4
3
2
1
0
CV
ACTH+VIB
ACTH+RU+VIB
TGF-β1
Normalized Relative Expression
2.0
1.5
1.0
0.5
0.0
CV
ACTH+VIB
ACTH+RU+VIB

Figure 5:

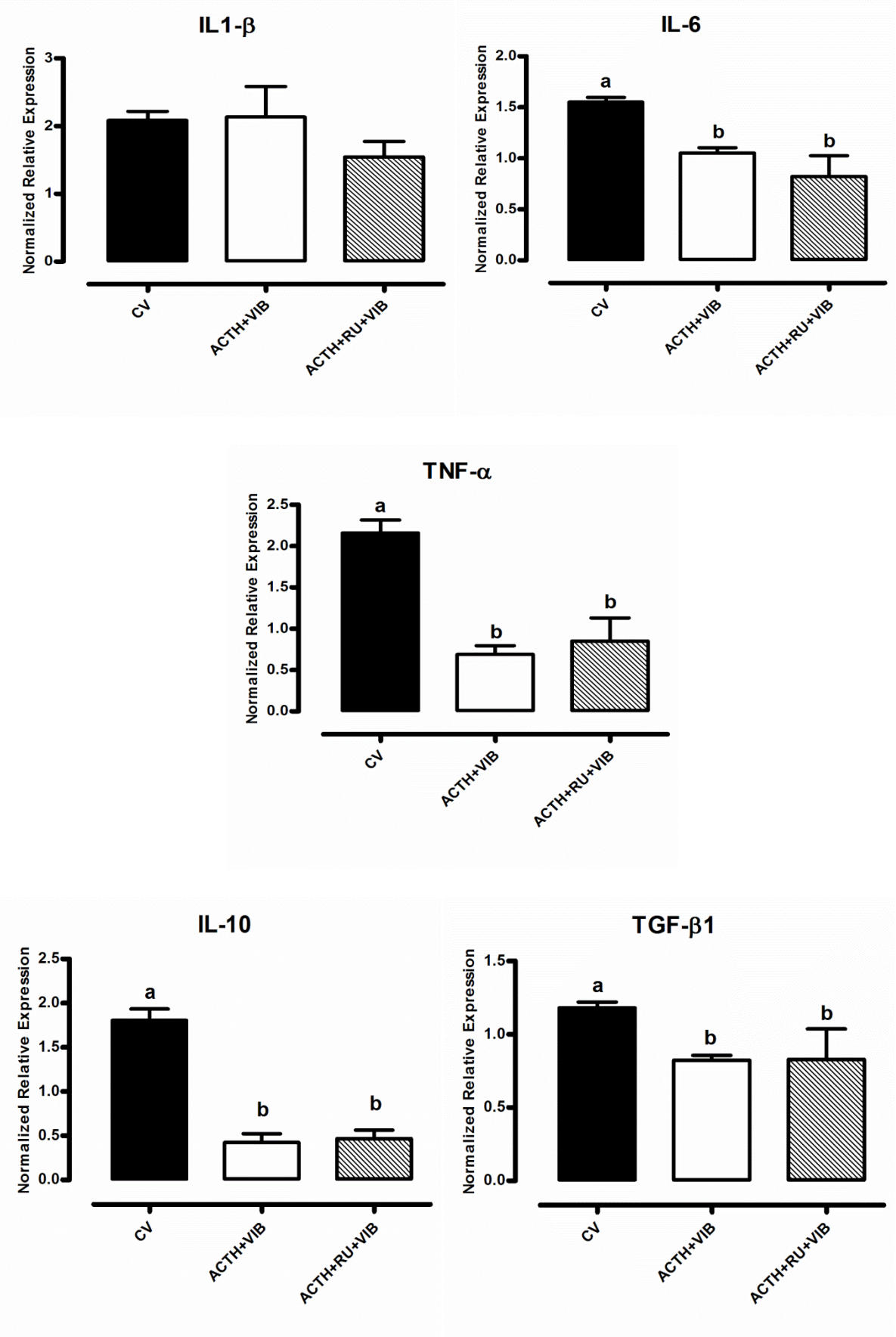

IL1-β
Normalized Relative Expression
3
2
1
0
CV
ACTH+VIB
ACTH+RU+VIB
IL-6
Normalized Relative Expression
2.0
1.5
1.0
0.5
0.0
a
b
b
CV
ACTH+VIB
ACTH+RU+VIB
TNF-α
Normalized Relative Expression
2.5
2.0
1.5
1.0
0.5
0.0
a
b
b
CV
ACTH+VIB
ACTH+RU+VIB
IL-10
Normalized Relative Expression
2.5
2.0
1.5
1.0
0.5
0.0
a
b
b
CV
ACTH+VIB
ACTH+RU+VIB
TGF-β1
Normalized Relative Expression
1.5
1.0
0.5
0.0
a
b
b
CV
ACTH+VIB
ACTH+RU+VIB

Figure 6:

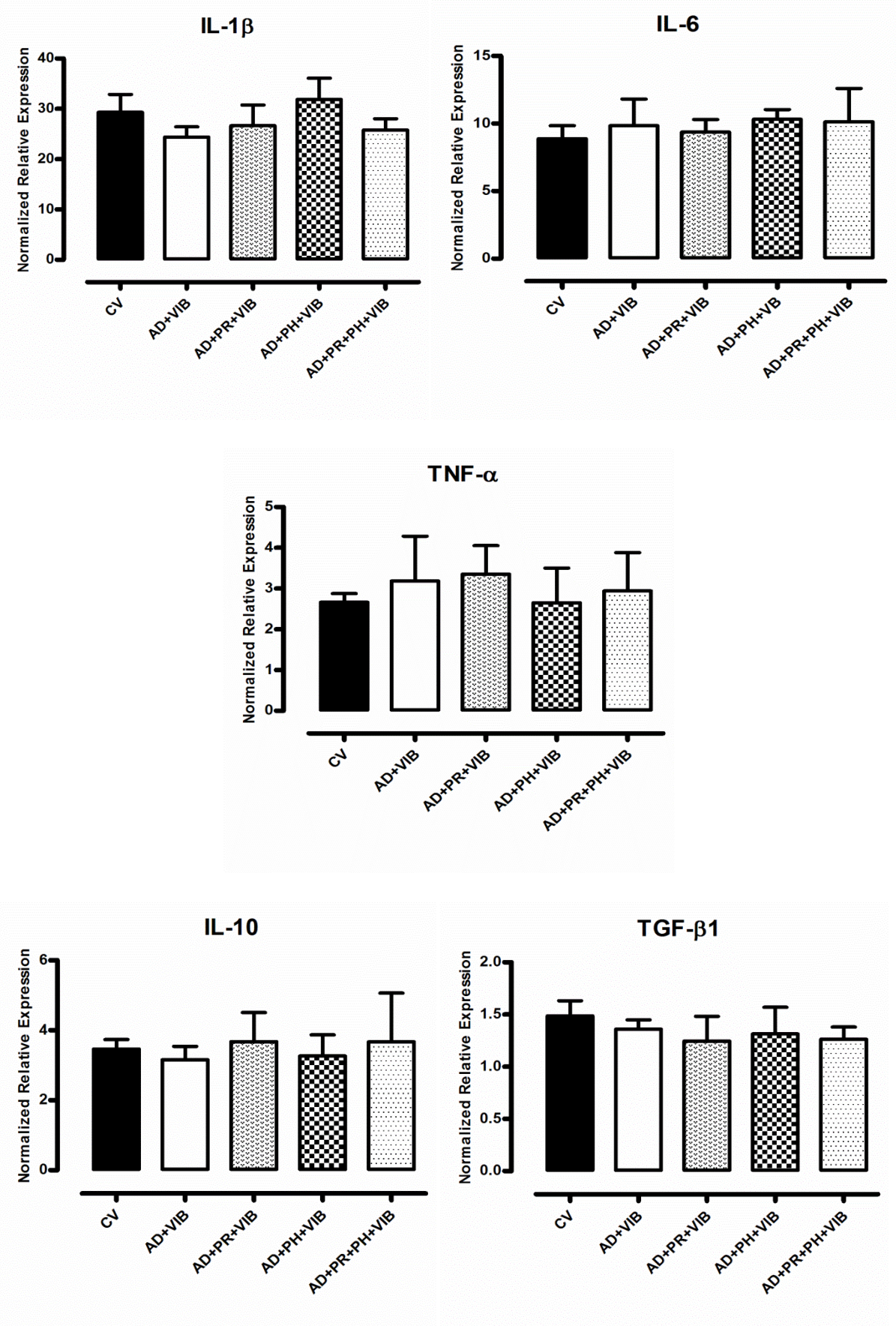
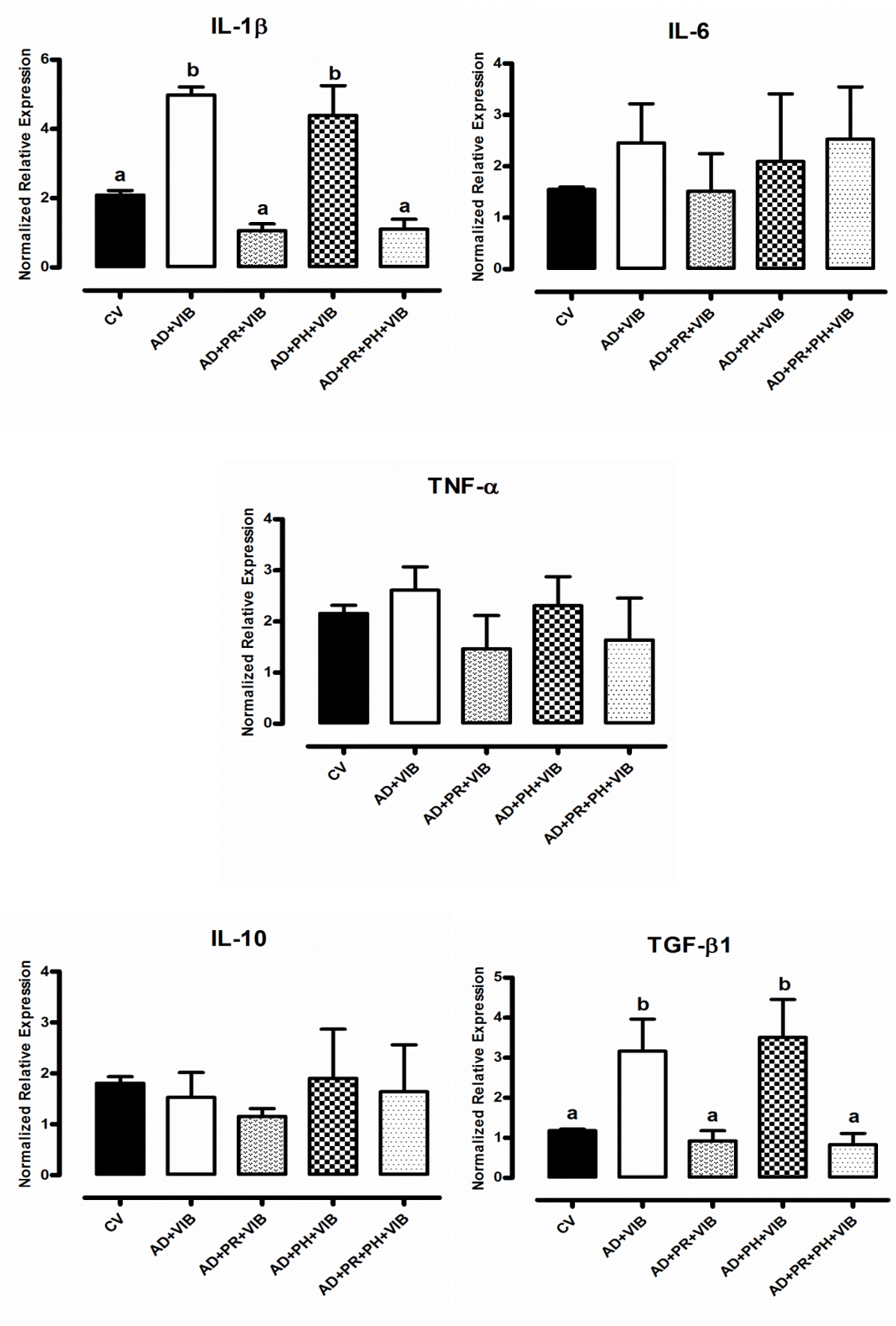

IL-1β
Normalized Relative Expression
40
30
20
10
0
CV
AD+VIB
AD+PR+VIB
AD+PH+VIB
AD+PR+PH+VIB
IL-6
Normalized Relative Expression
15
10
5
0
CV
AD+VIB
AD+PR+VIB
AD+PH+VB
AD+PR+PH+VIB
TNF-α
Normalized Relative Expression
5
4
3
2
1
0
CV
AD+VIB
AD+PR+VIB
AD+PH+VIB
AD+PR+PH+VIB
IL-10
Normalized Relative Expression
6
4
2
0
CV
AD+VIB
AD+PR+VIB
AD+PH+VIB
AD+PR+PH+VIB
TGF-β1
Normalized Relative Expression
2.0
1.5
1.0
0.5
0.0
CV
AD+VIB
AD+PR+VIB
AD+PH+VIB
AD+PR+PH+VIB

Figure 7:


IL-1β
Normalized Relative Expression
a
b
a
b
a
CV
AD+VIB
AD+PR+VIB
AD+PH+VIB
AD+PR+PH+VIB
IL-6
Normalized Relative Expression
CV
AD+VIB
AD+PR+VIB
AD+PH+VIB
AD+PR+PH+VIB
TNF-α
Normalized Relative Expression
CV
AD+VIB
AD+PR+VIB
AD+PH+VIB
AD+PR+PH+VIB
IL-10
Normalized Relative Expression
CV
AD+VIB
AD+PR+VIB
AD+PH+VIB
AD+PR+PH+VIB
TGF-β1
Normalized Relative Expression
a
b
a
b
a
CV
AD+VIB
AD+PR+VIB
AD+PH+VIB
AD+PR+PH+VIB

Legends

Figure 1. qRT-PCR quantification of IL-1β mRNA abundance in rainbow trout HKPCC after *Vibrio anguillarum* incubation. HKPCC was exposed to *Vibrio* for 2 hours at different dilutions 1:20, 1:100, 1:500, 1:2500, and the expression was evaluated. Data is shown as mean ± SEM. Significant differences (one-way ANOVA) are shown with different letters.

Figure 2. qRT-PCR quantification of specific mRNA abundance in rainbow trout HKPCC after cortisol and *Vibrio anguillarum* incubation. HKPCC was exposed to antagonist receptors mifepristone (RU) and/or spironolactone (SP) for 30 minutes first and second cortisol (100 ng ml^{-1}) and *Vibrio* for 2 hours. The expression of IL-1β, IL-6, TNF-α, IL-10 and TGF-β1 was evaluated. Data is shown as mean ± SEM. All cytokines were significantly elevated in control treated with *Vibrio anguillarum* versus untreated control. No significant differences (one-way ANOVA) are observed. CV: control Vibrio; CO+VIB: cortisol+Vibrio; CO+RU+VIB: cortisol + mifepristone+Vibrio; CO + SP+VIB: cortisol + spironolactone+Vibrio; CO+RU+SP+VIB: cortisol+mifepristone+spironolactone+Vibrio

Figure 3. qRT-PCR quantification of specific mRNA abundance in gilthead sea bream HKPCC after cortisol and Vibrio anguillarum incubation. HKPCC was exposed to antagonist receptors mifepristone (RU) and/or spironolactone (SP) for 30 minutes first and second cortisol (100 ng ml-1) and Vibrio for 2 hours. The expression of IL-1β, IL-6, TNF-α, TGF-β1, and IL-10 was evaluated. Data is shown as mean ± SEM. All cytokines were significantly elevated in control treated with *Vibrio anguillarum* versus untreated control except TGF-β1. Significant differences (one-way ANOVA) are shown by different letters ($p< 0.05$). CV: control Vibrio; CO+VIB: cortisol+Vibrio; CO+RU+VIB: cortisol + mifepristone+Vibrio; CO + SP+VIB: cortisol + spironolactone+Vibrio; CO+RU+SP+VIB: cortisol+mifepristone+spironolactone+Vibrio

Figure 4. qRT-PCR quantification of specific mRNA abundance in rainbow trout HKPCC after ACTH and *Vibrio anguillarum* incubation. HKPCC was exposed to first antagonist receptor mifepristone (RU) for 30 minutes and second ACTH (150 ng ml^{-1}) and *Vibrio* for 2 hours. The expression of IL-1β, IL-6, TNF-α, IL-10 and TGF-β1 was evaluated. Data is shown as mean ± SEM. All cytokines were significantly elevated in control treated with *Vibrio anguillarum* versus untreated control. No significant differences (one-way ANOVA) are observed. CV: control Vibrio; ACTH+VIB: Adrenocorticotropic hormone+Vibrio; ACTH+RU+VIB: ACTH + mifepristone+Vibrio

Figure 5. qRT-PCR quantification of specific mRNA abundance in seabream HKPCC after ACTH and Vibrio anguillarum incubation. HKPCC was exposed to first antagonist receptor mifepristone (RU) for 30 minutes and second ACTH (150 ng ml-1) and Vibrio for 2 hours. The expression of IL-1β, IL-6, TNF-α, IL-10 and TGF-β1 was evaluated. Data is shown as mean ± SEM. All cytokines were significantly elevated in control treated with *Vibrio anguillarum* versus untreated control except TGF-β1. Significant differences (one-way ANOVA) are shown by different letters ($p< 0.05$). CV: control Vibrio; ACTH+VIB: Adrenocorticotropic hormone+Vibrio; ACTH+RU+VIB: ACTH + mifepristone+Vibrio

Figure 6. qRT-PCR quantification of specific mRNA abundance in rainbow trout HKPCC after adrenaline and Vibrio anguillarum incubation. HKPCC was exposed to first antagonist

receptors propanolol (PR) and/or phentolamine (PH) for 30 minutes and second adrenaline (1 μM) and Vibrio for 2 hours. The expression of IL-1β, IL-6, IL-10 and TGF-β1 was evaluated. Data is shown as mean ± SEM. All cytokines were significantly elevated in control treated with *Vibrio anguillarum* versus untreated control. Significant differences (one-way ANOVA) are shown by different letters ($p < 0.05$). CV: control Vibrio; AD+VIB: adrenaline+Vibrio; AD+PR+VIB: adrenaline+propranolol+Vibrio; AD+PH+VIB: adrenaline+phentolamine+Vibrio; AD+PR+PH+VIB: adrenaline+propranolol+phentolamine+Vibrio

Figure 7. qRT-PCR quantification of specific mRNA abundance in seabream HKPCC after adrenaline and Vibrio anguillarum incubation. HKPCC was exposed to first antagonist receptors propanolol (PR) and/or phentolamine (PH) for 30 minutes and second adrenaline (1 μM) and Vibrio for 2 hours. The expression of IL-1β, IL-6, IL-10 and TGF-β1 was evaluated. Data is shown as mean ± SEM. All cytokines were significantly elevated in control treated with *Vibrio anguillarum* versus untreated control except TGF-β1. Significant differences (one-way ANOVA) are shown by different letters ($p < 0.05$). CV: control Vibrio; AD+VIB: adrenaline+Vibrio; AD+PR+VIB: adrenaline+propranolol+Vibrio; AD+PH+VIB: adrenaline+phentolamine+Vibrio; AD+PR+PH+VIB: adrenaline+propranolol+phentolamine+Vibrio

4. Discussion

Fish head kidney plays an important role in the immuno-endocrine interaction because of its hematopoietic, lymphoid and endocrine functions [36]. The *in vivo* immunological response to stressors is dependent on the actions of various hormones, their interaction with each other, the action on immunocompetent cells and the interaction with other endogenous factors such as cytokines. To further investigate the effect of the stress hormones without all these interactions, we have selected an *in vitro* model which has been demonstrated to be useful in different fish species [33,37–39]. Several works have previously looked at the effect of LPS on fish immune system and have elucidated its potential for mediating pro-inflammatory cytokines mRNA abundance [40,41]. In our study we show the effect of stress hormones on cytokine-induced expression by inactivated *Vibrio anguillarum* exposure.

Vaccination or administration of inactivated pathogens induces immune protection in fish. Previous studies indicate that attenuated *V. anguillarum* and lipopolysaccharide (LPS) from *V.anguillarum* induce immune protection in fish, expressing a less intense pro-inflammatory response in zebrafish [42] and giving protective immunity in salmonids [43]. However, there is still much to learn about the mechanism of such a protection and which bacterial components of the *Vibrio anguillarum* induce the protection. Thus, in this study we have focused on cellular response, proposing cell-mediated immunity may be more important for protection as previous evidences have shown in mammals and also in fish [32,44].

4.1. The cytokine response provoked by inactivated *Vibrio anguillarum*

Antigen stimulation and the specific cytokine modulation result in distinct macrophage activation pattern upon infection [45]. There are few studies on the anti-bacterial ability of fish macrophages against *Vibrio anguillarum*, though both O1 and O2 strains have been reported to be eradicated by both rainbow trout and gilthead sea bream macrophages [32,46]. In the present study we evaluated the effect of inactivated *Vibrio anguillarum* on the HKPCC cytokines, observing the expected expression increase in both species although less intense in sea bream. Previous research demonstrated cytokine induction by LPS in different fish species [47–49], at very low (picomolar) concentrations [50,51]. The prominent message which has been also made evident in fish is the orchestration between pro-inflammatory and anti-inflammatory responses first allowing robust inflammatory cytokine response but also a rapid down-regulation in the later phase of inflammation [52], thus preventing damage to the host [27]. This protective process has been previously related to local activation of endocrine cells which may result in GC and catecholamine secretion [53]. In summary, these evidences point out to similar induction mechanisms but a difference in cytokine regulation among the two fish species during immune activation [32].

4.2. The effect of cortisol on rainbow trout and sea bream head kidney primary cell culture

During infectious processes glucocorticoids generally function to dampen inflammatory response which is normally understood as an energy saving procedure during stress episodes in which the allostatic load or the need for resources is exacerbated (Sapolsky et al., 2000). Thus, not only is immune activity suppressed, but immune cells may even be catabolized as a source of proteins and glucose [55]. Hence, glucocorticoids (GC) are well known immunosuppressive molecules, as confirmed as well by the results obtained by our group [4,7,33], but their effects on particular immune fish cell types are not yet completely clear [27,56]. In the present work, cortisol did not show significant modulatory effects on cytokine expression-induced by *Vibrio anguillarum* in trout head kidney, while in sea bream cortisol did produce a clear inhibitory effect on both pro-inflammatory (IL-1β, IL-6, TNF-α) and anti-inflammatory cytokines (IL-10, TGF-β1) and also on the constitutive levels of immune gene expression. Again in sea bream, cortisol modulated the Vibrio-stimulated gene expression, by suppressing both Vibrio-induced pro-inflammatory (IL-1β, IL-6, TNF-α) and anti-inflammatory cytokines (IL-10, TGF-β1), thus supporting the suppressive effect of this steroid on fish immune cells [4,10,33,57,58]. Differences in cytokine regulation in different fish species after cortisol treatment have also been previously reported. In trout hepatocytes and spleen, cortisol up-regulated the expression of IL-1β and TNF-α [7,59]. Also, repeated handling stress and the consequent high plasma cortisol produced an elevation of IL-1β in Atlantic salmon head kidney macrophages [7,60]. On the other hand, down-regulation of IL-1β has been observed in gilthead sea bream head kidney cells incubated with cortisol [33] and even no alteration for IL-1β and TNF-α in a trout macrophage cell line

and liver after cortisol and rIL-1β incubation [7,10]. It should be noted that similar results have been observed in reports in which the same cortisol concentration (100 ng ml^{-1}) has been used [33,59]. Therefore, the effect of cortisol on cytokine expression shows variable results depending on the treatment, *in vivo* vs. *in vitro*. For instance, IL-1β was enhanced in vitro in rainbow trout liver, while no changes were recorded at systemic level [7,59]. As a matter of fact, the cortisol concentration is also of relevant importance since it has been shown that glucocorticoid receptors GR1 and GR2 in fish may be responsive to different cortisol concentration levels [16,61,62].

We also administered both GR and MR antagonists since there are not previous studies that assessed the effectivity of either antagonists. Cortisol may exert its effect through MR in addition to GRs since it has been shown that MR is also expressed under stress circumstances [63]. Previous investigations provided evidence that teleosts express a MR-like gene (with high homology to a mammalian MR gene [13,57] that controls the corticosteroid function for adaptation to saline environments [17,18]. These corticoid receptors, together with other neuroendocrine products of the HPA and SAM axes [5] may be shared by lymphocytes, monocyte-macrophages or granulocytes which may end-up with changes in cellular trafficking, proliferation, cytokine secretion and antibody production [64]. Our results show that cortisol effects on *Vibrio*-stimulated immune related gene indicators (IL-1β and TNF-α) were abolished by mifepristone in sea bream head kidney, while the preventing effect of mifepristone was not observed for IL-6, IL-10 and TGF-β1. Our laboratory recently demonstrated the ability of Mifepristone to recover cytokine basal values in head kidney treated with cortisol (Khansari et al., submitted). Altogether, these findings suggest a different effect of cortisol depending on the species affected and/or a differential effect when administered to a single cell type or to a complex mixture of cells such as the head kidney tissue.

4.3. The effect of ACTH on rainbow trout and sea bream head kidney primary cell culture

ACTH not only activates interrenal cells to release cortisol, but it has also been demonstrated that other tissues, such as thymus and spleen, express ACTH receptors even at early stages of development [65]. Therefore a direct influence of ACTH on fish immune system can be expected after induction of acute stress. However, we observed that ACTH administered at a concentration of 150 ng mL^{-1} did not exert any significant effect on cytokine gene expression measured in trout head kidney cells whereas in sea bream ACTH suppressed both pro-inflammatory and anti-inflammatory cytokines excluding IL-1β, confirming previous results obtained in our group that showed down-regulating ACTH on sea bream HKPCC cytokine expression [33]. According to those results, when ACTH and LPS were co-incubated in sea bream head kidney cells, no effect was observed for IL-1 except for the increased of TNF-α [33]. This differential response on ACTH co-incubated with LPS or *V. anguillarum* may reside in the nature of the pathogenic stimuli. On the other hand, our

findings did not show inhibition effects of mifepristone administered with ACTH on cytokine gene expression. Although there is no evidence that confirms that mifepristone can block StAR or MC2R, previous in vivo study in head kidney tissue from fish fed with mifepristone produced significantly less cortisol in response to ACTH stimulation compared to control fish [66].

4.3. The effect of adrenaline on rainbow trout and sea bream head kidney primary cell culture

Adrenaline, as the main catecholamine product of the SAM axis in head kidney chromaffin cells, has not been studied as much as other stress hormones such as glucocorticoids. So far only a limited number of studies concerning the effect of catecholamines on the expression of inflammatory mediators have been published [33,67]. It is also noteworthy that the rapidity of catecholamine release into bloodstream (within seconds) make it difficult their study, in particular because of the difficulty of collecting blood samples without any excess stress [1]. Thus, the *in vitro* model appears as an important contribution to better understand functional interactions. In mammals, it is known that catecholamines modulate immune function including cell proliferation, cytokine and antibody production, cytolytic activity and cell trafficking [68–70]. Previous studies in several vertebrate groups have also shown that stress, involving adrenaline and cortisol secretion, suppress immune system function under some circumstances while enhancing it under others [71]. Nevertheless, to date, most of the data on catecholamine studies have demonstrated an immunomostimulatory effect of adrenaline, though most likely this effect appears to be species and condition-dependent [3] . Here we illustrate for the first time that short-time incubation of rainbow trout head kidney cells with adrenaline was not able to alter the mRNA levels of neither pro-inflammatory cytokines (IL-1β, IL-6 and TNF-α) nor anti-inflammatory cytokines (TGF-β1, IL-10). The induction of adrenaline was effective for both seabream pro- (IL-1β) and anti-inflammatory cytokines (TGF-β1). The enhancing effect of adrenaline on pro-inflammatory cytokines observed in sea bream has been also reported in isolated perfused rat liver preparations showing up-regulation of IL-6 [72], and also in common carp adrenaline injected together with zymosan elevated IL-1β expression [67]. Our findings confirm the immunomodulatory effect of catecholamines on fish immune system.

The tree main adrenergic receptors α1, α2 and β play an essential role in this interaction. Although little attention has focused on adrenergic receptors in fish, β-adrenoreceptors appear to be present in leukocytes of all vertebrate classes [73,74]. On the other hand, radioligand binding experiments confirmed the expression of β-adrenoreceptors on goldfish head kidney, spleen and peritoneal leukocytes [21] and some studies highlighted that the effect of adrenaline would be mediated mainly by the β-adrenergic receptor [20] and that activation of this receptor may be responsible for some stress-related immunological

changes [74]. Our findings show that adrenaline enhanced the *Vibrio*-stimulated gene expression (IL-1β and TGF-β1) and the effect was abolished by the antagonist propranolol alone, and also in combination with phentolamine. Therefore, adrenergic stimulation can be differently regulated depending on the cell type, tissue and also the fish condition. It should also be reminded that head kidney, as an organ that includes immune and endocrine cells, may facilitate a direct paracrine interaction between them [27].

5. Conclusion

The present results indicate a different stress hormone effect on Vibrio-induced cytokine expression in HKPCC. Whereas cortisol, ACTH and adrenaline did not induce relevant effects in rainbow trout head kidney at the concentrations tested, the same hormones exerted a significant cytokine gene expression modulation in sea bream. Altogether the results indicate a species-specific hormone effect between the two species evaluated at the same concentrations against *Vibrio anguillarum*,Meanwhile cellular immunity is also required for a protective response against *Vibrio anguillarum*.

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Table 1: Primers used for gene expression analysis in rainbow trout.

Gene	GenBank Accession number	Sequence 5′–3′	Product size
β-actin	NM_001124235.1	**F:**GGACTTTGAGCAGGAGATGG	186
		R:ATGATGGAGTTGTAGGTGGTCT	
IL-1β	NM_001124347.2	**F:**TGAGAACAAGTGCTGGGTCC	148
		R:GGCTACAGGTCTGGCTTCAG	
GR1	NM_001124730.1	**F:**TTCCTTTCCTCCCTGTCAGT	171
		R:ATCCTCCTCCGTCTTGATGA	
IL-6	NM_001124657.1	**F:**GAGTTTCAGAAGCCCGTGGA	149
		R:AGCTGGTACACTTGCAGACC	
TNF-α	NM_001124357.1	**F:**CACACTGGGCTCTTCTTCGT	155
		R:CAAACTGACCTTACCCCGCT	
TGF-β1	NM_001281366.1	**F:**GCCAAGGAGGTCCACAAGTT	146
		R:GTGGTTTTGATGAGCAGGCG	
IL-10	NM_001245099.1	**F:**CCGCCATGAACAACAGAACA	105
		R:TCCTGCATTGGACGATCTCT	

Table 2: Primers used for gene expression analysis in gilthead sea bream.

Gene	GenBank Accession number	Sequence 5′–3′	Product size
18S	AY587263.1	**F:**ACCAGACAAATCGCTCCACC	172
		R:AGGAATTGACGGAAGGGCAC	
IL-1β	AJ277166.2	**F:**TCAGCACCGCAGAAGAAAAC	115
		R:TAACACTCTCCACCCTCCAC	
GR	DQ486890.1	**F:**ACTGAGGAGGGAGGTCTATT	195
		R:GGACTCTGGGACTTCTAACA	
IL-6	EU244588.1	**F:**ATCCCCTCACTTCCAGCAGA	129
		R:GCTCTTCGGCTCCTCTTTCT	
TNF-α	AJ413189.2	**F:**TCGTTCAGAGTCTCCTGCAG	320
		R:AAGAATTCTTAAAGTGCAAACACACCAAA	
TGF-β1	AF424703.1	**F:**AGACCCTTCAGAACTGGCTC	145
		R:ACTGCTTTGTCTCCCCTACC	
IL-10	JX976621.1	**F:**GAGCGTGGAGGAATCTTTCAA	154
		R:GATCTGCTGGATGGACTGC	

6. Chapter forth

6.1 Fish mucosal immune system: Study of immune stimulation outcomes induced by acute stress and *Vibrio anguillarum* exposure in rainbow trout (*Oncorhynchus mykiss*) and gilthead seabream (*Sparus aurata*) mucosal surfaces.

Abstract

The response of fish to external stressors after 1, 6 and 24 hours post-stress was studied at first in mucosal tissue (skin, gills and intestine) of fish, which are in intimate contact with the immediate environment. Thus, external stimuli may produce local alterations in mucosal tissues when these potential threats are perceived by specific tissue receptors located at these regions. At such level, the stress and immune response to these stressors was evaluated in skin, gills and gut surfaces. Our results showed a differential gene expression pattern between rainbow trout and gilthead sea bream, confirming that the response to a stressor at local level is tissue-dependent and species-specific. And also anoxia and vaccine+anoxia were found to be an inducer of the cortisol secretion into skin mucus. Thereby we have found gilthead seabream more stressed than rainbow trout

1. Introduction

Fish (fresh water and sea water) are continuously exposed to aquatic microbial environment that are in close contact with epithelial barrier of their body. It is a suitable medium for microorganisms growth compare to air. Thus, fish mucosal immune system is imposed by additional challenge versus their terrestrial counterpart dealing with multiple of microorganisms. In addition to be physical barrier, fish is armed by mucosal tissues for maintaining the homeostasis (Hiroi et al., 2005; McCormick, 2001), and also play an important role in mucosal immune system fighting against pathogen and commensals (Dongarrà et al., 2013) and, thus considered as a very active and alive immunological arm (Salinas et al., 2011).. Recently fish mucosal immune system studies have been attracted more attention since it is accepted that the mucosal immune system is as complex as its systemic counterpart because of the complex relationship between mucosa and microorganisms in the aquatic environment in several and different surfaces, such as skin, gills and gut. Vertebrate lymphoid organ is classified to primary and secondary, that mucosa-associated lymphoid tissue (MALT) is one of the important secondary organs (Salinas et al., 2011) which is sub-divided into three distinct tissue according to mammalian nomenclature including skin-associated lymphoid tissue (SALT), gill-associated lymphoid tissue (GIALT) and eventually gut-associated lymphoid tissue (GALT). These mucosal surfaces are covered with a protective mucus layer which acts as a first line of defense against pathogens (Shephard, 1993). Mucus is permeable to some macromolecules, but also acts as an active barrier (Inami et al., 2009). Its compounds is slightly variable amongst species but it is mainly consist of water (95%), some glycoproteins and innate and adaptive factors, such as immunoglobulins (Igs) (Salinas et al., 2011). The majority of the investigations that have been published on fish mucosal immune system, are mainly devoted to the role and importance of antibodies in mucosal surfaces (Dongarrà et al., 2013; Gomez et al., 2013; Parra et al., 2015; Salinas et al., 2011; Xu et al., 2013), therefore, the cellular and molecular response, particularly cytokines response needs to be more investigated. The skin-associated lymphoid tissue of fish is the largest and also the metabolically most active tissue (Austin, 2006), possessing a unique histological diversity which plays a crucial role in mucosal immune system and also serves for communication, sensory perception, locomotion, respiration and ion regulation (Fast et al., 2002; Marshall and Bellamy, 2010). The skin being intimately and constantly immersed in the aquatic environment makes it not only highly susceptible as immune organ but also sensitive to stressors (physical and chemical). Similar to mammals, teleost skin is composed of an epidermis and dermis, although two important differences exist compared to mammals: the outer layer of fish skin is alive and secondly the presence of mucus secreting cells that confer antimicrobial features to skin including bacteriolytic molecules such as lysozyme, complement components, lectin, proteolytic enzyme and more importantly Igs (Nigam et al., 2012). Therefore the importance of fish skin in protecting the animal against pathogen compare to

mammals is more pivotal and complex because of existence of bacterial population which is around 10^2 to 10^4 cm^{-2} (Austin, 2006).

Herbivores, detritivores, omnivorous and carnivorous fish species differ from each other in terms of the presence or absence of stomach, the length of the intestine and the presence and number of pyloric caeca (Evans, 1998), but regarding gut mucosa, all fish show immune cells such as lymphocytes, plasma cells, granulocytes and macrophages situated in the epithelium or distributed in the lamina propia of the gut (Brandtzaeg et al., 2008; Rombout Jan et al., 2011), and intraepithelial lymphocytes (IELs) which include T cells and some B cells located among epithelial cells. These immune cells together with epithelial cells, goblet cells and neuroendocrine cells are responsible for producing and regulating the immune response in gut.

After gut, the gill-associated lymphoid tissue is the mucosal organ that also has a close interaction with the outer environment and it serves as an entry for numerous microorganisms. For instance, one of the entry portal of two common fish pathogens *Vibrio anguillarum* and *Aeromonas salmonicida* is through the gills (Baudin Laurencin and Germon, 1987). The gills of bony fish are made up four paired arches, each containing two rows posteriolaterally oriented filaments with lamellas covered by respiratory epithelium (Wilson and Laurent, 2002). It is also noteworthy that, the stress response is initiated through activation of the Hypothalamus-Pituitary-Interrenal axis (HPI) and Sympathetic-Adreno-Medullar (SAM) axis, first via perception of stimuli by central nervous system, producing catecholamines and cortisol which are released into circulation (Barton, 2002). Moreover cortisol as a final production of HPI axis, is the well-known indicators of stress situation experienced by fish (Tort, 2011). It has also been proven that the bidirectional interaction exist between HPI and immune system in fish (Engelsma et al., 2002), raising the question about the occurrence of such interaction in mucosal surfaces. Little is known about cortisol level in the mucus secreted in skin, gills and gut. Thus, the response of fish to stressors has been shown to be effective at different levels, from genes to tissue and from central to periphery. In spite of those investigations that have been performed at systemic level, up until know the mechanisms by which cortisol is secreted into skin or gills mucus in fish are not clear but the presence of hormones at this local surfaces can modulate specific tissue receptors and cytokine expression in mucosae. This study may also contribute to disclose of the occurrence of the interaction between endocrine and mucosal immune system, thus we used different time point 1, 6 and 24 h, since it has been demonstrated that in fish cortisol within several minutes up to 1 hour is secreted into circulation modulating immune function (Ramsay et al., 2009).

A side from that, the consequences of stress response activation on the immune response have been described mainly in systemic compartments including head kidney, liver and spleen and thus at fish mucosal level have been poorly investigated. Hence, the current work is devoted to study the mucosal immune response looking at the cytokines regulation

which is crucial in maintaining the fish integrity under immune challenges. We also determine how stress induces a mucosal endocrine response in fish. The present research also compares among skin, gills and gut mucosal surfaces and in two species, rainbow trout and gilthead seabream, living in different aquatic environments. Hence, fish were subjected to two different stressors, anoxia as a physical stressor and inactivated *Vibrio anguillarum* bath as an immune stressor (source of antigen).

2. Materials and methods

2.1. Fish and rearing condition

Rainbow trout (*Oncorhynchus mykiss*) with body weight of 120-140 g were obtained from a local fish farm (TroutFactory, Peramola, Spain). Gilthead sea bream (*Sparus aurata*) with body weight of 60-70 g were obtained from AQUACULTURA ELS ALFACS,S.L. (Tarragona). Fish were maintained in recirculating stock tanks (300 L) at the Aquarium facilities on Universitat Autònoma de Barcelona (AQUAB) in order to acclimatize them to laboratory conditions. Fish were kept at 13°C (rainbow trout) or 19°C (gilthead sea bream), photoperiod cycle 12 h light/ 12 h dark, and fed with commercial pellet at 1.5% of total body weight per day. Water quality indicators (dissolved oxygen, ammonia, nitrite, and pH) were analyzed periodically. All experimental procedures involving fish were submitted and authorized by the Ethical Committee of the "Universitat Autonoma de Barcelona" that agrees with the international Guiding Principles for Biomedical Research Involving Animals (EU2010/63).

2.2 *Vibrio anguillarum* bacterin

The inactivated vaccine ICTHIOVAC ^{R} VR (HIPRA), a formalin-killed *V. anguillarum* bacterin, was utilized as a source of antigen. The composition consist of inactivated *Vibrio anguillarum*, serotype O1, O2α (most pathogenic serogroup) and O2β, all with relative percentage survival (RPS) ≥ 60%.

2.3. Experimental design.

After three weeks of acclimatization, fish contained in tank stock (n=88 trouts; n=88 sea bream) were included in the study. A total of 44 fish were captured from tank stock and vaccinated by short immersion with a formalin-killed *V. anguillarum* bacterin according to manufacturer's instructions (Hipra) and then equally were distributed in two separated 300 l tanks (n=22 each tank). This group was the vaccinated group (henceforth *v group*). After 24 hours, one of the vaccinated-group tank (n=22) were subjected to anoxia stress by 1 minute

subsequently maintained out of water and immediately returned to separated tanks. Thus, these fish were called the vaccinated and stressed group (henceforth *v+s group*). On the other hand, 22 fish from tank stock were captured with net and also subjected to anoxia stress by 1 minute air exposure and immediately returned to other separated tanks. This group was identified as the stressed group (henceforth *s group*). As handled untreated control (control group), 22 fish were removed from the tank and mock-vaccinated in the same conditions than vaccinated group. Then, fish were returned to other tank. A total of six fish were randomly sampled at 1, 6, and 24 hours post-stress (hps) from each experimental group (control, s, v+s, v) and sacrificed by over-anesthetization in MS222 (1 g/L).

2.4 Sampling.

Trout skin mucus was samples according to Xu et al. (2013) with some modifications. Briefly, skin mucus was collected by scrapping carefully the skin surface in order to avoid blood contamination provoked by any potential injury during the procedure. Samples were transferred into a 1.5 tube and immediately kept on ice. Samples were suspended gently with help of 1.5 ml syringe and 25G needles to completely disaggregate them, and centrifuged at 400 × *g* for 10 min at 4°C. The obtaining supernatant was re-centrifuged at 10,000 × *g* for 10 min at 4°C. The supernatant (skin mucus) was recovered and immediately stored at −80°C. Skin tissue (immediately upper lateral line area and behind the dorsal fin, left side) sample was carefully taken to avoid any muscle trace contamination. Gills (third lamella from both sides) and gut (midgut)) were also samples from all fish. The spleen, brain, head kidney, heart, liver, muscle, gills, and intestine were carefully dissected out and immediately frozen in liquid nitrogen. Samples were stored at −80 °C for further assays.

2.5. Quantification of cortisol in skin mucus

Cortisol level in skin mucus was measured by radioimmunoassay (Rotllant et al., 2006). The antibody used for the assay was purchased from MO bio-medical LLC (OH, USA) and used in the final dilution of 1:4500. Antibody cross-activity with cortisol is 100%. The radio activity was quantified using a liquid scintillation counter. The lower detection limit of the cortisol assay is 0.16 ng/mL.

2.6. Specific antibody detection in skin mucus

The level of IgM in rainbow trout and gilthead seabream skin mucus at 1, 6, and 24 hours post-stress (hps) was determined by ELISA according to Reyes-López et al. (submitted) with some modifications. Rainbow trout (but not sea bream) skin mucus samples were 1/4 diluted in PBS+ 10 mM EDTA. Then, 50 µl $well^{-1}$ of sample was added and incubated at 4°C

onto Maxisorp microplates (Thermo Fisher Scientific) in duplicate. The unbound antigen was removed washing twice with 200 µl $well^{-1}$ of PBS. The nonspecific binding was then blocked with 100 µl $well^{-1}$ non-fat milk 5% in PBS for 1 hour at room temperature (RT) and then washed twice with PBS. The antibody mouse anti-trout IgM 4C10 monoclonal antibody (1/100 dilution in PBS) and anti-sea bream IgM monoclonal antibody (1/100 dilution in PBS determined by Western blot (Supplementary Figure 1)) (Aquatic Diagnostics Ltd, UK)) were used as primary antibody to detect the presence of IgM on skin mucus. Samples were incubated with 50 µl $well^{-1}$ of primary antibody for 1 h at RT, followed by three times washing with 200 µl washing buffer (PBS + 0.15% Tween 20). Samples were incubated with 50 µl $well^{-1}$ of goat-anti-mouse IgG conjugated with HRP (1/4000 dilution in PBS). The microplate was washed five times with 200 µl $well^{-1}$ of washing buffer and 50 µl $well^{-1}$ of Ultra-TMB (3,3',5,5'-tetrametilbenzidina; Thermo Fisher Scientific) was added as substrate. After incubation for 7 min at RT, 50 µl $well^{-1}$ of H_2SO_4 (2M) was added as stop solution and absorbance was determined at 450 (0.1s) nm with a microplate reader (Victor3, Perkin Elmer). All samples were evaluated in duplicated.

2.7. Isolation of RNA and cDNA synthesis

Total RNA was isolated from individual fish using TRI reagent (Sigma) according to manufacturer's instructions. The RNA pellet was dissolved in autoclaved miliQ-water and immediately stored at -80°C until use. The RNA concentration was quantified by NanoDropND-2000 spectrophotometer (Thermo Scientific). Total RNA (1 µg) was used as template to synthesize cDNA using iScript cDNA kit (Bio-Rad Laboratories) according to manufacturer's instructions and immediately stored at -20°C until use.

2.8. Quantitative real time PCR (qPCR)

Fish mucosal surfaces including skin, gills and gut were analysed using real-time PCR. The analysis included the evaluation of innate (Lysozyme, C3, IgM), pro- (IL-1β, IL-6, TNF-α) and anti-inflammatory (IL-10, TGF-β1), and stress- (COX2, HSP70) related gene expression profiles. We first tested several housekeeping candidate genes in rainbow trout (EF1α and β-actin) and also in sea bream (18S, EF1α and RPL27) to elucidate which one had less variation. Thus, β-actin (for rainbow trout) and 18S (for sea bream) were included on gene expression analysis. Specific primers used for rainbow trout (Table 1) and gilthead sea bream (Table 2) are indicated. Primers were designed with Primer-Blast. The primer secondary structure and annealing specificity were checked with OligoAnalyzer (version 3.1) and Primer-Blast software, respectively. The undesirable PCR products appearance was previously verified by single peak in the melting curve for each primer set. The primer amplification efficiency was determined in all mucosal surfaces included in our study. Real-time PCR reactions were performed with iTaq universal sybr green supermix (Bio-Rad Laboratories) using 1:40 cDNA dilution made for genes of interest in rainbow trout and

gilthead sea bream. Primers for all genes were used at final concentration of 500 nM. The thermal condition used were 3 min at 95°C of pre-incubation followed by 40 cycles at 95°C for 30 s and 60°C for 30 s. All the reactions were performed in duplicate using CFX384 Touch Real-Time PCR Detection System (Bio-Rad Laboratories). The quantification was done according to Pfaffl method (Pfaffl, 2001) corrected for efficiency of each primer set obtained for each mucosal surface evaluated. Value for each experimental condition was expressed as normalized relative expression, calculating in relation to values of control group and normalized against those of the housekeeping gene β-actin and 18S for rainbow trout and sea bream, respectively. The results are expressed as average of values obtained for the same treatment and time-points evaluated.

2.9. Statistical analysis

The statistical Package for Social Science Software (SPSS version 20) was used for statistical analysis, utilizing the generalized linear model (GzLM: http://refhub.elsevier.com/S0306-4530(15)00909-9/sbref0135). Stressors and dynamism of time were considered as a two factors. This statistical method is flexible since no homogeneity of variance is required. Where a statistical significant interaction was found between stressors and dynamic, we only fulfilled the correlation between time and IgM at protein level, cortisol and gene expression modulation, meanwhile comparing all groups versus control. It means that no data was analyzed without significant interaction between stressors as well as time. Differences in all data were considered significant when $p\text{-value} < 0.05$ among groups.

3. Results

3.1. Cortisol measurement in skin mucus

The level of cortisol in rainbow trout skin mucus after acute handling stress significantly mounted at 1 h post stress, when compare to the control group (Figure 1). At this time point cortisol in mucus reached to its maximum level (2.14 ng/mL) and it dropped at 24 h were at control level (0.266 ng/mL). Meanwhile cortisol was still higher compare to control at 6 h (1.154 ng/mL). Interestingly in vaccinated group cortisol significantly increased (1.43 ng/mL) at 1 h post stress versus control (25 h after vaccination) and after which cortisol declined was near control at 6 and 24 h post stress. In the treatment in which vaccine and acute handling stress was applied cortisol reached significantly to maximum level (1.913 ng/mL) at 6 h post stress (30 h after vaccination) versus control and drops at 24 h around control level. While in seabream the trend observed was not the same trend than that in rainbow trout because cortisol significantly increased at 1 h and 6 h compare to control but it taught the peak (3.411 ng/mL) at 6 h post stress and was shown around control at 24 h. In vaccinated group statistical analysis did not shown any difference neither in time course nor versus

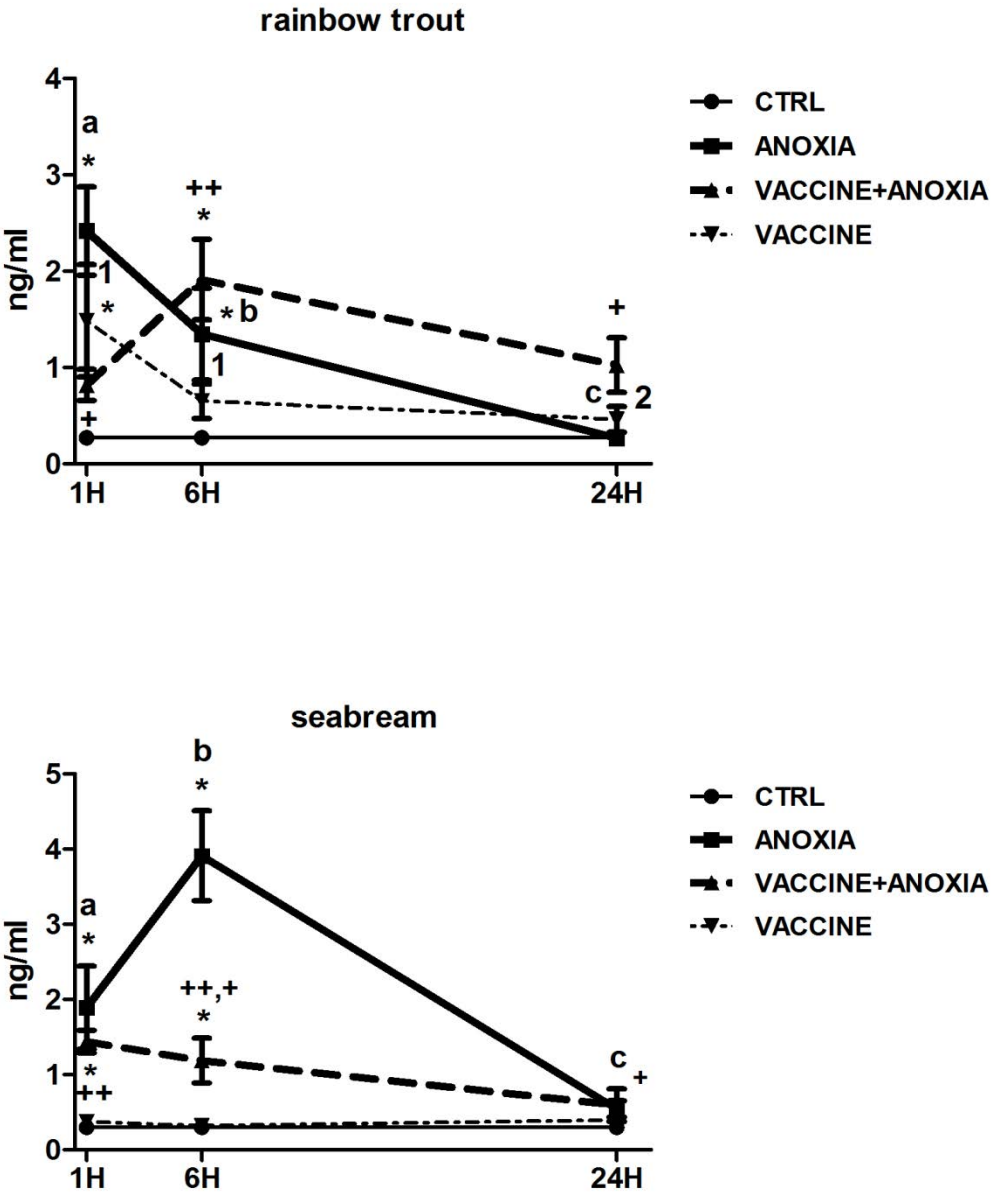
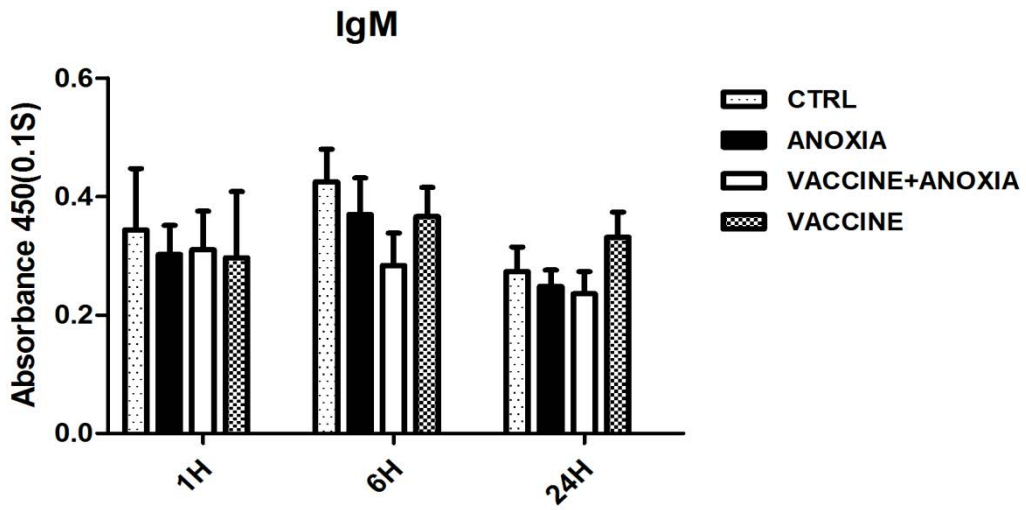


Fig.1. Changes in skin mucus cortisol level in rainbow trout and gilthead seabream in response to anoxia and *Vibrio anguillarum* exposure 1, 6 and 24 h post stress. Skin mucus cortisol was measured by radioimmunoassay. Date are presented as mean ±SE. significant differences are indicated by letters in stress group, vaccine+stress by ++ and vaccine group by number. * 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)

control. Interestingly in vaccine plus acute stress group cortisol was decreased at 1 h comparing stress and vaccine group, and also at 6 h was elevated, finally at 24 returned at control level in rainbow trout, whereas in gilthead seabream cortisol shown increment at 1 h only versus vaccine, at 6 h was decreased versus stress while increased comparing to vaccine, and lastly stayed around basal level.

3.2. Specific antibody detection in skin mucus

The titer of specific IgM at protein level was determined using ELISA with skin mucus of rainbow trout and seabream via proper preparation at different time point after acute handling stress and in-activated *Vibrio anguillarum* exposure. The quantity of specific antibody did not alter in rainbow trout in different treatment, while in seabream the level of IgM enhanced at 24 h comparing to control, in addition IgM level was shown to be altered reducing versus stress, whereas enhancing versus vaccine group at 24 h.



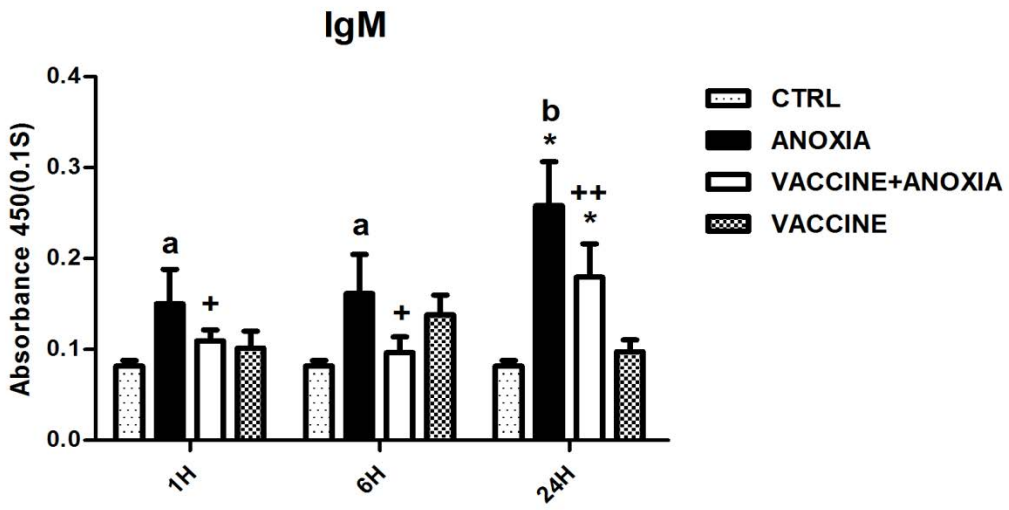


Fig.2. Changes in IgM skin mucus measured by ELISA (absorbance 450(0.1 S)) in rainbow trout and gilthead seabream in response to anoxia and *Vibrio anguillarum* exposure 1, 6 and 24 h post stress. Skin mucus cortisol was measured by radioimmunoassay. Date are presented as mean ±SE. significant differences are indicated by letters in stress group, vaccine+stress by ++ and vaccine group by number. * 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)

3.3. Cytokines regulation in rainbow trout and gilthead seabream GALT after stressors exposure

RT-qPCR was used to examine whether the anoxia as well as *Vibrio anguillarim* were able to drive differences in the mRNA abundance of a genes-cassette related with inflammation (IL1β, IL6, TNFα, IL10 and TGFβ1), stress HSP70 and COX2 or innate immune response including Lys, C3 and IgM in three mucosal-associated lymphoid tissues (MALT): skin, gut and gills. The result obtained in rainbow trout and gilthead seabream skin, gut and gills show overall significant interaction between treatment and time point at 1,6 and 24 hour, thus data without showing significant interaction between treatment and time were analysed.

3.3.1 Skin-associated lymphoid tissue (SALT)

In skin, a different gene expression pattern was observed comparing rainbow trout and gilthead sea bream. In rainbow trout, the anoxia + vaccine treatment promoted the upregulation at 1 hour post-stress (hps) of genes associated to the humoral arm of the innate response (IgM, C3) and stress response (HSP70). The expression of pro-inflammatory molecules (IL-1β, COX2) was also upregulated and their expression seem to be related to the upregulation also observed in the anoxia group. The same effect was also observed at 6 hps for IL-1β but not COX2. No modulation was observed at 1 hps in genes associated to anti-

inflammatory response (IL-10, TGFβ). However, the upregulation of TGFβ only was observed at 6 and 24 hps in the vaccinated group, probably linked with the upregulation also observed for lysozyme, C3, COX2 and HSP70. By contrast, all genes showed a marked down-regulation in a treatment- and time-independent manner.

In gilthead sea bream, the upregulation of genes in the anoxia+vaccine group was also observed. However, the magnitude and time-course of this modulation was different compared to rainbow trout. A high and decreasing expression from 1hps to 24 hpi in lysozyme and HSP70 was reported. The same expression pattern was also observed for TGFβ1. An increased gene expression peaked at 6 hps was noted for IgM and also pro-inflammatory (IL-1β, IL-6, TNFα, COX2) associated genes. The upregulation of IL-10 was modulated in the same manner. An increase in a time-dependent manner was registered only for C3. Importantly, this upregulation in the anoxia+vaccine group seems to be influenced by anoxia and vaccinated stimuli separately. Only in the cases of IL-1β and IL-6 the effect observed in the anoxia+vaccine group can be markedly associated to the expression registered in the anoxia group and vaccinated group, respectively. Importantly, the down-regulation of all genes evaluated was observed in the vaccinated group.

In summary, a lower gene expression magnitude was observed in rainbow trout than sea bream. While the expression observed in the anoxia+vaccine group was related to the expression of the anoxia group in rainbow trout, in gilthead sea bream was associated to a combination of both stimuli. Also, a similar expression of lysozyme, HSP70 and TGFβ1

Fig.3. qPCR quantification of specific mRNA accumulation in skin of rainbow trout and gilthead seabream subjected at 1, 6 and 24 h post challenge with acute handling stress and *Vibrio anguillarum* exposure. IL1β, IL6, TNFα, IL10, TGFβ1, HSP70, COX2, Lys, C3 and IgM shown are mRNA relative abundance to β-actin and 18S housekeeping gene in rainbow trout and gilthead seabream respectively. Date are presented as mean ±SE. significant differences are indicated by letters in stress group, vaccine+stress by ++ and vaccine group by number. * 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)

suggest that the anti-inflammatory cytokine could modulate the expression of these immune-related genes in both species subjected to stressors. A downregulation gene expression was particularly observed in the sea bream vaccinated group.

3.3.2 Gill-associated lymphoid tissue.

The gills showed a different gene expression pattern comparing both fish species. In rainbow trout, the anoxia and vaccine alone had a stronger effect on gene expression. In the vaccinated group the expression humoral innate genes (lysozyme, IgM) at 1 hps was modulated. The expression of pro-inflammatory genes (IL-1β, COX-2) was observed at 1 hps but at 6 hps the expression of IL-6 and HSP70 was also modulated. Importantly, IL-10 was also upregulated at 6 hps, suggesting that its modulation could be related to the control of the pro-inflammatory gene expression profile. On the other hand, the gene modulation in the anoxia group was observed at 6 hps on the profile of pro- (IL-1β, IL-6, TNFα, COX2) and anti-inflammatory genes (IL-10, TGFβ). Particularly, a downregulatory gene expression effect was observed in the anoxia group and anoxia+vaccinated group at 1 hps, suggesting that the stress by anoxia could influence the expression of vaccinated trout at early times post-stress.

On sea bream, a marked upregulation on the expression of innate immunity (lysozyme, C3) and pro-inflammatory cytokines (IL-1β, TNFα, COX2) was registered both at 1 and 6 hps. The upregulation of COX2 (1 hps) and IL-1β (6 hps) suggest a specific-gene expression effect of anoxia in sea bream. Also, the downregulation of several genes in the anoxia and vaccinated groups along the experiment propose that the stress stimuli alone may suppress the sea bream immune system.

Altogether, the results indicate that the upregulation of several immune-related genes after an acute stress is not a general response in teleost.

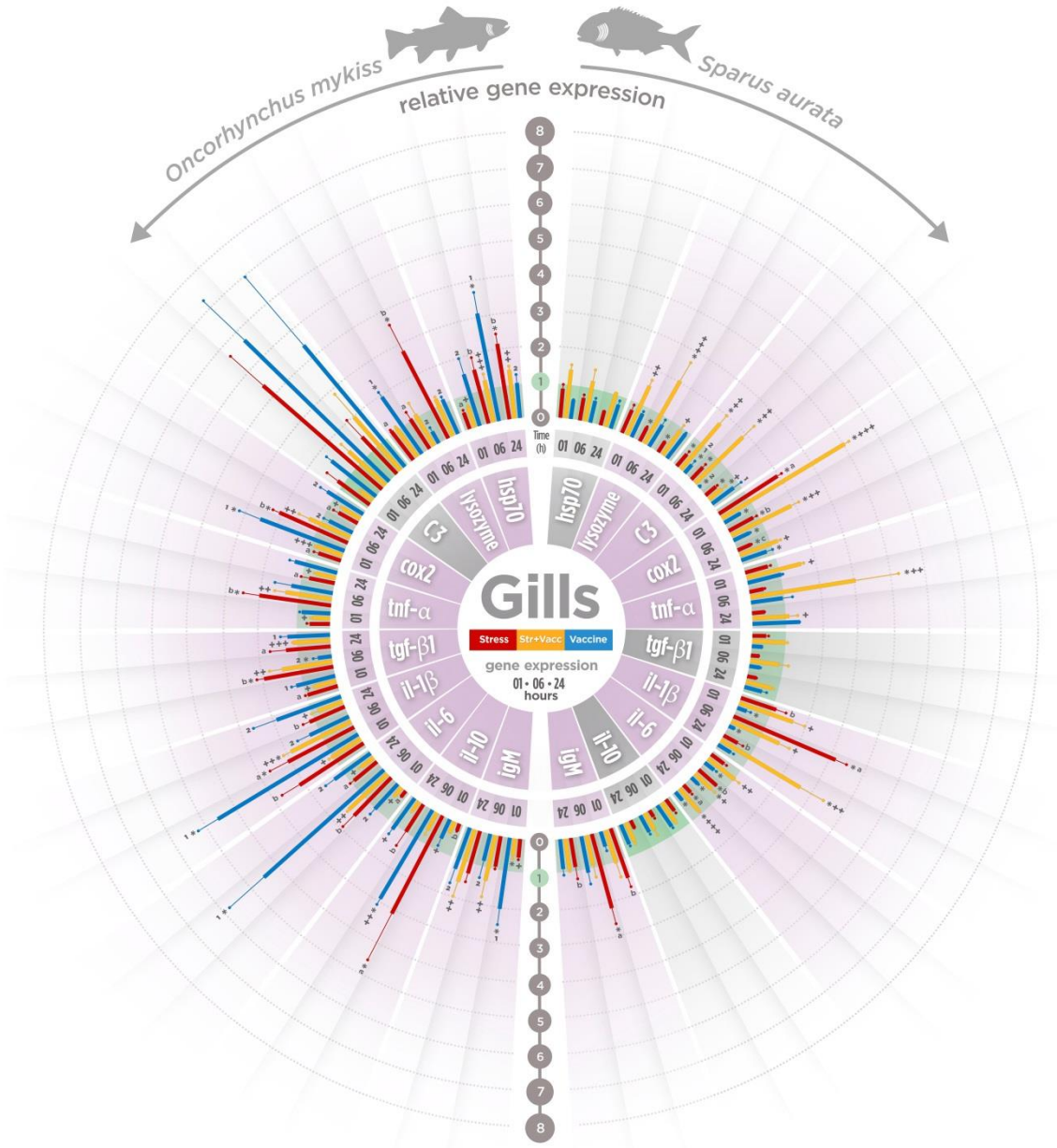


Fig.4. qPCR quantification of specific mRNA accumulation in gills of rainbow trout and gilthead seabream subjected at 1, 6 and 24 h post challenge with acute handling stress and *Vibrio anguillarum* exposure. IL1β, IL6, TNFα, IL10, TGFβ1, HSP70, COX2, Lys, C3 and IgM shown are mRNA relative abundance to β-actin and 18S housekeeping gene in rainbow trout and gilthead seabream respectively. Date are presented as mean ±SE. significant differences are indicated by letters in stress group, vaccine+stress by ++ and vaccine group by number. * 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).

3.3.3 Gut-associated lymphoid tissue.

The profile in gut showed that anoxia modulated the gene expression in rainbow trout and gilthead sea bream. While in trouts subjected to anoxia the upregulation of genes involved innate immunity (lysozyme, C3, IgM), pro- (IL-1β, TNFα, COX2) and anti-inflammatory response (TGF-β1) was observed at 1 hps, the same genes and also IL-6, IL-10 and HSP70 were upregulated at 6 hps. Thus, the anoxia induces the upregulation of immune-related genes in trout before than sea bream in gut.

Importantly, the same modulation of all the pro-inflammatory genes was also observed in trout at 1 hps in the anoxia+vaccinated group, indicating that this response could be directly influenced by the anoxia stress upregulation at the same time-point evaluated. On the other hand, the upregulation of the same genes (except IgM) was also upregulated in the vaccinated group after 6 hps. These results suggest that in gut the anoxia stress promote a similar immune-related gene expression modulation in both species although in a different time-dependent manner.

Taking altogether, the results obtained in the MALT evaluated suggest that the response to a stressor at local level is tissue-dependent and specie-specific.

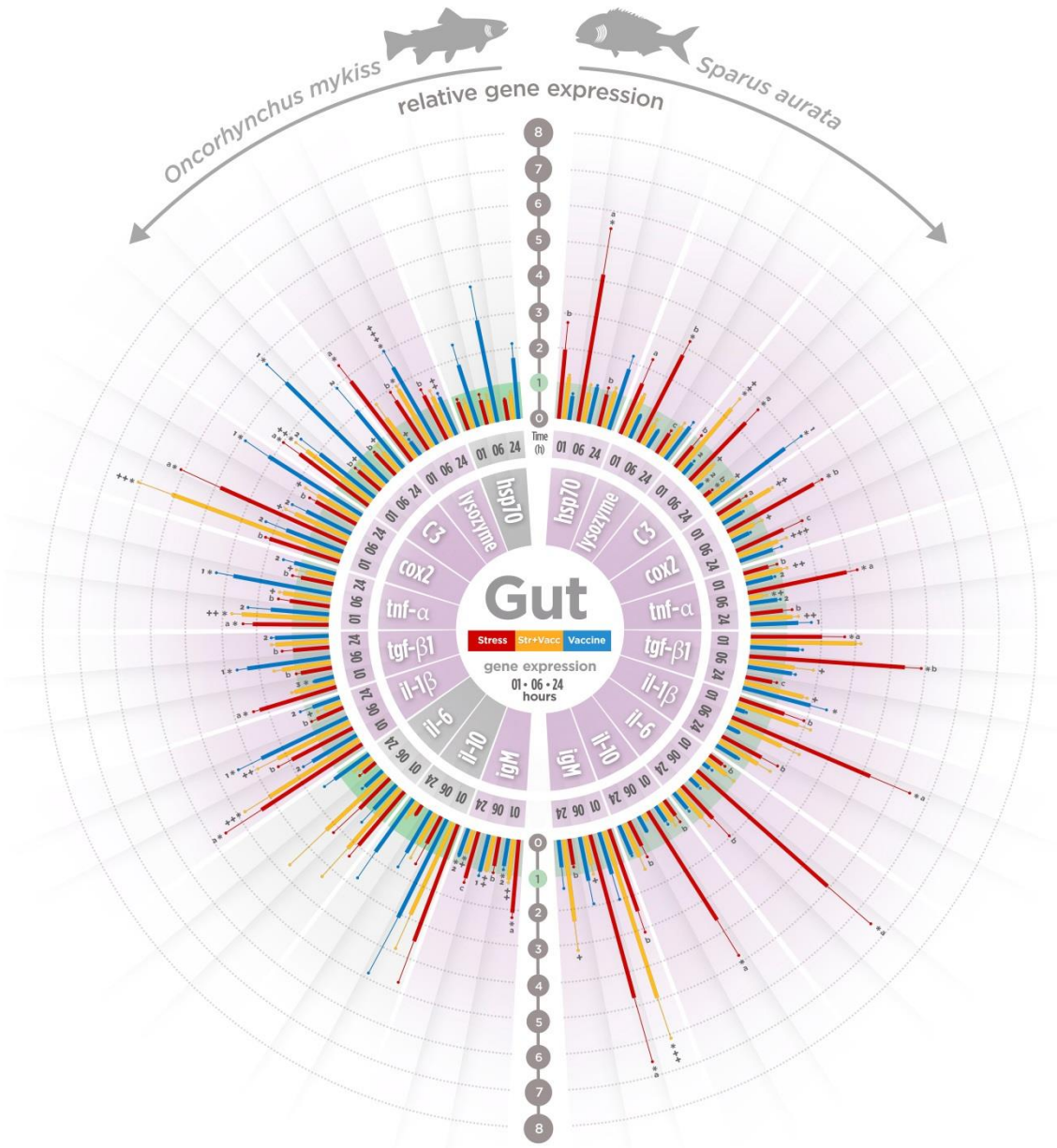


Fig.5. qPCR quantification of specific mRNA accumulation in gut of rainbow trout and gilthead seabream subjected at 1, 6 and 24 h post challenge with acute handling stress and *Vibrio anguillarum* exposure. IL1β, IL6, TNFα, IL10, TGFβ1, HSP70, COX2, Lys, C3 and IgM shown are mRNA relative abundance to β-actin and 18S housekeeping gene in rainbow trout and gilthead seabream respectively. Date are presented as mean ±SE. significant differences are indicated by letters in stress group, vaccine+stress by ++ and vaccine group by number. * 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).

4. Discussion

In this study, we investigated the effect of acute handling stress and vaccination on mucosal immune response of rainbow trout and gilthead seabream. We show for the first time the level of cortisol, and immunoglobulin M (IgM) at protein level in skin mucus and gene expression pattern in these two species subjected to two stressors. Further, we illustrated that short-term stress affected the cortisol level in skin mucus and the level of gene expression in both species at mucosal surfaces. As a matter of fact, unfavorable conditions often occurred within rearing period in fish farm by different stressors. It has been shown that stressors can be classified following different scale and source (Tort, 2011). Such factors are able to be stressful to the fish and to impose long-term disturbances of the mucosal surfaces (Jia et al., 2016; Sundh et al., 2010). The mucosal immune system has a key role in the defense mechanism against pathogens and thus it is considered as a very active immunological site (Salinas et al., 2011).The present study clearly illustrated that the mucosal barrier of the rainbow trout and gilthead seabream are absolutely affected by acute handling stress as well as *Vibrio anguillarum* as a source of antigen. Until recently, previous study have contributed to elucidate the significant role of immunoglobulins and B cells in producing protection in fish mucosal tissues (Salinas et al., 2011; Xu et al., 2013), since fish have direct interaction with environment makes the study of teleost mucosal immunity of particular interest. The present results suggest that stress induces disturbance in physical barrier, stimulating mucosal immune system in fish. It has also been shown that existence of reverse regulatory connection between cytokines and neuro-endocrine activation (Tort, 2011) may have played a key role for cortisol release into skin mucus by cytokines production.

4.1. Cortisol level in skin mucus of rainbow trout and gilthead seabream

The stress response has been defined as a non-specific and general reaction of an animal to stimuli result in cortisol secretion as a final production of the HPI axis (Barton and Iwama, 1991). It has been widely shown that the activation of HPI axis may suppress immune function because of the tight interaction between neuro-endocrine and immune system (Engelsma et al., 2002). Thus in this research we also assessed this plausible connection in mucosal barrier, measuring cortisol level in the skin mucus of both rainbow trout and seabream, though the mechanism of how cortisol is secretion need to be more investigated. Moreover there is no evidence that confirm that cortisol in mucosal surfaces is produced by specific cells especially in the skin. Our result shown that in rainbow trout cortisol reached to a maximum at 1 h after stress, gradually decreases at 6, thought the level was still high comparing to control and stayed at control level at 24h. Interestingly we observed an inverse pattern in seabream reaching to high level at 6 h post-acute handling stress showing a delay in cortisol rising in seabream. Similar result obtained by our group and other researcher confirm the elevation of cortisol in fish after subjecting to acute stress (Fast et al., 2008; Fierro-castro et al., 2015; Teles et al., 2013). The administration of in-activated

Vibrio anguillarum also elevated cortisol level in rainbow trout at 1 h and thus returned to the basal level at 6 and 24 h. Although, it is not clear that the mechanism of HPI axis activation or cortisol secretion by pathogen, but also it has been reported that there is an inverse interaction between neuro-endocrine and cytokines expression regulating cortisol secretion (Tort, 2011). In particular, it has been demonstrated that cytokine stimulation such as IL-β1, TNF-α and IL-6 are able to triggered stress response (Calcagni and Elenkov, 2006). Also in mammals IL-6 receptor has been detected in the neuro system, thus acting and demonstrating its role on HPA axis activation (Žarković et al., 2008). Also the result obtained is in agreement with previous study illustrating *Aeromonas salmonicida* infection induced cortisol elevation in fish (Magnadóttir et al., 2010). We have found a fascination result after vaccination following acute handling stress, reducing cortisol comparing to both stress and vaccine group at 1 h, interestingly at 6 h cortisol was enhanced versus both group and returned at basal level at 24 h. While in seabream cortisol was shown to be elevated at 1 h only versus vaccine, at 6 h was decreased compare to stress, whereas increased versus vaccine group. Therefor the results indicate the disordered pattern in both species after subjecting acute stress plus *Vibrio anguillarum*. It is also noteworthy that usage of two stressors did result in secretion of less cortisol at 1 h comparing to stress and vaccine group in rainbow trout and also the maximum level of cortisol in seabream after stress was 3.41 ng/ml, whereas in vaccine plus stress group the maximum was 1.43 ng/ml, showing double amount demonstrating two stressors produced less cortisol, thus this finding is in agreement with previous work performed in our group (Barton et al., 2005).

4.2. IgM at protein level in skin mucus of rainbow trout and seabream

Vaccination is considered to be one of the effective approaches to reinforce the fish immune system because of its easiness in aquaculture. Several studies have look at the correlation between infection and level of IgM since immunoglobulins is a crucial factor in humoral immunity (Zhang et al., 2013). It has also been reported that IgM secretion was induced in Channel catfish skin mucus after bacterial infection (Zilberg and Klesius, 1997). In fact IgM is one of the major immunoglobulins in skin mucus coating microbiota to provide protection against pathogen which are in close contact with skin (Parra et al., 2015). Measuring IgM by ELISA in the recent study showed no changes in trout skin mucus neither by acute handling stress nor *Vibrio anguillarum* and the obtained result is in agreement with previous work (Ye et al., 2013). Interestingly acute stress and combination of two stressors was able to increase the level of IgM at protein level in seabream skin mucus at 24 h. On the other hand, it is not surprise no alteration observed in vaccine group, since it has been shown that after infection the level of IgM needs time to be produced in the serum taking couple of weeks , though the rout of vaccination may play a crucial role in antibody secretion (Makesh et al., 2015).

4.3. Cellular response at gene expression level in rainbow trout and gilthead seabream SALT

Fish skin is one of the key barriers in protecting in the aquatic environment possessing similar basic feature in all fish species. In this section we tried to confirm the obtained results with those evidences which have already been published in mucosal tissue, otherwise they are already addressed with systemic as well as intestine. The teleost skin is metabolically active tissue and largest mucosal tissue which is constantly exposed to the external environment, thus makes it the most susceptible organ to different kind of pathogens and stressors (Buchmann, 1999). The current study illustrated that acute stress induced expression of IL-1β1 and TGF-β1 in trout skin, while in seabream skin IL-β1 and IL-6 were up-regulated at 24 h after stress, meanwhile *Vibrio* also stimulated TNF-α, IL6 and TGF-β1 at 24 h after time 0. As it was mentioned before the activation of pro-inflammatory and anti-inflammatory is an attempt of animal to protect the animal from tissue damage. Meanwhile the expression pattern observed suggests the sensitivity of seabream skin to vaccination. Recent report showed that long-term overcrowding stress suppressed the expression of IL-1β and TNF-α in skin of turbot rearing in high density for 120 days, thus the contradiction might be related to elevated cortisol after long-term crowding (Jia et al., 2016). Cox2 mRNA level was elevated in rainbow trout skin at 1 h and the inverse pattern observed for HSP70 decreased at 24 h, while stress induced COX2 expression at 24 h. administration of vaccine stimulated the level of COX2 and HSP70 at 24 h in skin of both species. The recent findings in turbot and salmon showed the increase of COX2 and HSP70 by long-term and acute stress in the skin and intestine (Jia et al., 2016; Oxley et al., 2010). Meanwhile the expression of inflammatory immune response genes TNF-α, COX2, inducible NOS2 in rainbow trout skin was shown to be induced by *Gyrodactylus derjavini* infection (Lindenstrøm et al., 2004). The expression of Lys was affected in the skin of rainbow trout and seabream in different time point earlier at 6 h in trout and later at 24 h in seabream with acute stress. Vaccination stimulated C3 and Lys in trout skin at 24 h, whereas C3 expression was elevated in seabream skin at 6 h by vaccination. Lysozyme and C3 is an important defense molecule of the innate immune system and it is established that the immune system of fish can be severely affected by various stress condition, leading to immunomodulatory in fish. Lysozyme is also present in mucus and is also expressed in wide variety of organ (Saurabh and Sahoo, 2008). Thus, skin as the largest barrier in fish with antimicrobial function such as lysozyme and complement can be responsive to environmental stressors. It has been also shown that Lys expression was increased by *Listonella anguillarum* inoculation in zebrafish (Rojo et al., 2007). IgM expression was not induced either by acute handing stress nor vaccination in the skin of both species. In fact, fish is also capable of producing antimicrobial molecules in skin mucus such as lysozyme, complement components, and Igs, considering that IgM is the most abundant immunoglobulin in the skin mucus since microbiota is covered by Igs (Nigam et al., 2012; Xu et al., 2013).

4.4. Cellular response at gene expression level in rainbow trout and gilthead seabream GIALT

In the current investigation we studied the expression of immune genes in the gills of rainbow trout and gilthead seabream after subjecting to acute handling stress and immunization. Acute handling stress induced expression of IL-1β1, TNF-α, IL-10 and TGF-β1 in rainbow trout at 6 h after stress, while in seabream IL-β1 was elevated at 6 h and interestingly IL-6 was declined at three time point by stress, no changes were observed for anti-inflammatory cytokines. Administrating Vibrio anguillarum in rainbow trout gills stimulated (IL-β1 and IL-10) and IL-6 transcriptional level at 1 and 6 h post time 0 respectively, while TGF-β1 was down-regulated at 6 h. whereas IL-β1 was declined at 1 and 6h restoring to the basal level at 24, and IL-6 also down-regulated at 3 time point. In fact *Vibrio anguillarum* causes serious diseases in fish, resulting in severe mortality and economic losses (Samuelsen et al., 2006). In fact gills is the mucosal organ after skin that has close contact with aquatic environment increasing the susceptibility of this organ to infection since it serves as a portal of entry. Up until now, the effect of acute stress on fish mucosal immunity especially on cytokines has not been studied, thus this is the first report about. The triggered inflammatory response by stress in both species showed that rainbow trout gills are more responsive comparing to seabream, once confronting to stress situations. It has been reported that one of the portals of entry of two commons fish pathogens *Vibrio anguillarum* and *Aeromonase salmonicida* is through the gills (Baudin Laurencin and Germon, 1987; dos Santos et al., 2001). The increase transcription of pro-inflammatory cytokines IL-β1 and IL-6, simultaneously with IL-10 increment in the gills of rainbow trout following exposure with vaccination indicate that inflammation could be the predominant response of mucosal surfaces to the pathogen invasion. It has been preciously demonstrated that IL-β1, IL-8, IL-22 and IFN-γ significantly were elevated in fish blood and gills with both in activated *Vibrio anguillarum* and *Aeromonase salmonicida* (Caipang et al., 2010, 2008). In contrary, the pro-inflammatory cytokines IL-β1 and IL-6 expression were down-regulated in seabream gills. Thus, the difference in expression pattern of genes in the seabream gills using Vibrio anguillarum could be related to specific-species and concentration of vaccine that is used in the current study. Transcriptional level of COX2 and HSP70 was induced by stress increased at 6 h and 24 h after stress respectively in gills trout. Interestingly seabream COX2 in gills was shown to be up-regulated at 1 h and declined at 6 and 24 h after stress. Vibrio anguillarum exposer in gills of rainbow trout stimulated COX2 and HSP70 at 1 and 6 h respectively, whereas vaccination was able to decrease COX2 expression at all time point in seabream gills. It has been already illustrated that acute stress and bacteria challenge are a inducer of COX2 expression, though there is no evidence on whether COX2 is modulated by acute and vaccination in gills it seems that a general increase of this gene is a response of animal to stress, although the pattern in seabream was different than that in rainbow trout seems to be species response to stimuli. HSP70 is one of the most studied genes in response to cellular stress since one of the crucial role that HSP70 plays is acting as a chaperon for folding of protein specially it is involved in GR signaling (Liberek et al., 2008). It also implicated in immune response in mammals since it has been demonstrated that like glucocorticoid effect, HSP70 can reinforce the anti-inflammatory

production and reduce the induction of pro-inflammatory response, thus it seems that it can also serves as a danger signal in fish (Shi et al., 2006; Wang et al., 2001). It is noteworthy that that seabream is shown to be less responsive to environmental stressors than trout as HSP70 expression was not induced by both acute handling stress and vaccination.

Lys at transcriptional level was increased at 24 h post stress in trout, while seabream gills for C3 and Lys have been declined at all time and 6 and 24 h respectively. Also IgM was expressed at 6 h after stress in seabream gills with acute stress exposure. While fish, in particular mucosal organs living in harmony with pathogens and commensals in water they are potentially exposed to stressors, in addition environmental stressors can affect on these surfaces in order to induce mucosal immunity activation. Thus, the innate components of innate and adaptive immune system are present in these barriers. Despite of existing some structural differences in intestine, skin and gills, It has been described that all of these surfaces shares immune function features to cope with stress situation (Gómez and Balcázar, 2008; Krasnov et al., 2012). Complement components and lysozyme are abundant in mucosal surfaces and their implications are crucial for the elimination of apoptotic cells and more importantly distraction of pathogen in fish and in mammals (Nakao et al., 2011; Pekkarinen et al., 2013). In the recent study C3 was not shown to be modulated neither by stress nor *Vibrio anguillarum*, it has been evident that C3 Up-regulation was observed in rainbow trout gills with Ich infection in the gills of rainbow trout (Olsen et al., 2011), thus indicating that either type of pathogen may also be important to trigger immune response or concentration of bacterin. In seabream gills both C3 and lysozyme were decreased illustrating inverse response to both stressors which also can be considered as a specific-species response. The suppression of seabream C3 and Lys can also be attributed to elevation of cortisol, as it has been demonstrated by our group through cortisol administration on rainbow trout innate immune response showing reduction of C3 and Lys in liver and head kidney (Cortés et al., 2013).

4.5. Cellular response at gene expression level in rainbow trout and gilthead seabream GALT

An inflammatory reaction can be provoked either by invasion of pathogen or external stressors. The primitive phase of an inflammation response is described by an increment of cytokines which are produced in response to disturbances (Chadzinska et al., 2008). Meanwhile intestine harbor a vast population of microbes which serves as a biological barrier against invading pathogen, making intestine as crucial surface in fish to maintain hemostasis since it has also been shown that stress increased the intestine permeability (Hara and Shanahan, 2006). Thus, it is shown that changes in dissolve oxygen and also pathogen induced gene expression modulation in fish gastrointestinal tract (Boltana et al., 2014; Niklasson et al., 2011).

On the other hand, Rojo and his colleague reported that the localization of *Vibrio anguilarum* occurred in the mouth and gastrointestinal tract after 2 hours of exposure and 6 h later in zebrafish intestine, hence, as intestine is one of the target tissue for pathogen this investigations could answer the questions about the involvement of this mucosal barrier in immune response (Rojo et al., 2007). In the current study, the obtained result shown that acute handling stress induced the expression of pro-inflammatory cytokines IL-1β, TNF-α, and also anti-inflammatory cytokine TGF-β1 in rainbow trout intestine at 1 hour post stress, while IL-1β, TNF-α, IL-6 and anti-inflammatory IL-10 and TGF-β1 were expressed with delay at 6 hour post stress in gilthead seabream intestine. Interestingly, *Vibrio anguillarum* expressing IL-1β and TNF-α at 6 h post time 0, and TGF-β1 was declined at 1 h but increased at 6 h post time 0 in rainbow trout. Whereas in gilthead seabream the effect of vaccine was not able to induce pro-inflammatory response except TNF-α was slightly increased and TGF-β1 increment was observed at 24 h after time 0.

Like in mammals, the fish inflammatory response is characterized by a first wave of expression of the pro-inflammatory cytokines IL-1β and TNF-α, followed by the expression of chemokines (Chadzinska et al., 2008). Also it is not surprising that within the activation of pro-inflammatory cytokines, anti-inflammatory expression is also derived in both species. It is worthy to note that during the last stage of inflammation, the anti-inflammatory cytokines including IL-10 and transforming growth factor-β (TGF-β1) are induced by macrophages, preventing excessive activation of the immune response and initiating the process of recovery, which is pivotal to reduce the inflammation (Verburg-van Kemenade et al., 2011). Although, a robust inflammatory response is expected for pathogen eradication because of microbicidal proteins, reactive oxygen species production which are potentially detrimental for host, hence the triggered-response should be rapidly suppressed. This result is in agreement with previous work demonstrating elevation of IL-1β after subjecting to acute handling stress (15s out of water) in Atlantic salmon head kidney macrophages, in contrast the expression level of IL-1β in Atlantic salmon intestine has been shown to decreased after 7 weeks exposing to 50% dissolved oxygen, as it is clear the influence of short-term and long-term stress may have induced different regulation on cytokines (Fast et al., 2008; Niklasson et al., 2011). Also vaccination of zebrafish and salmon by *Vibrio anguillarum* expressed IL-β, IL-8, IFN-γ and IL-10 in head kidney, spleen and liver (Kvamme et al., 2013; Zhang et al., 2012), thus vaccination can activate cytokines expression response to produce cellular protection in intestine. Meanwhile in the case of *Vibrio anguillarum*, gut could be the primary site of infection as demonstrated in turbot and zebrafish (Grisez et al., 1996; O'Toole et al., 2004).

Acute handling stress induced COX2 expression at 6 h in rainbow trout gut, while in seabream the high level expression were observed in COX2 and HSP70 at the same time. Bath vaccination by *Vibrio anguillarum* also altering COX2 mRNA abundance level interestingly 24 h post time 0, whereas HSP70 was up-regulated at three time point. In seabream vaccination was not able to alter COX2 and HSP70 among others. The

cyclooxygenase enzyme (COX) catalyze the conversion of arachidonic acid to prostaglandin H2 which is the main pathway leading to release of PGs, and also playing a key role in the induction of the inflammatory response, that their biosynthesis is significantly increased in inflamed tissue (Ricciotti and Fitzgerald, 2011; Smith, 2008). It has been shown that inducible COX2 is induced by inflammatory stimuli in mammals and also in fish (Griffin et al., 2007; Smyth et al., 2009). The result found, is in consistent with the previous work in Atlantic salmon showing increment of COX2a in midgut 1 h after stress (Olsen et al., 2012; Oxley et al., 2010). In fact is has been documented that stress response, in particular cortisol secretion may lead in altering the intestinal permeability and thus changing fish hemostasis. The general response to stress is decreasing arachidonic in intestine, therefore this decrease in eicosanoid content could be an attempt by fish to prevent the increment of intestinal permeability. At cellular level, the stress response is also characterized by the heat shock proteins (HSPs) which are highly conserved and present in all cells. In fact the 70 kDa protein family has been shown as a biomarker due to its rapid increment in response to stress situation (Jonsson et al., 2006), thus rainbow trout and seabream HSP70 appears to be responsive to stressors which is also in agreement with previous studies in fish (Bertotto et al., 2011; Nakano et al., 2004). The expression level of C3 in both species was shown to be altered by acute handling stress, so that C3 was increased at 1 h post stress, whereas it was expressed at 6 h in seabream, meanwhile C3 was down-regulated at 24 h after stress in seabream. Bath vaccination by *Vibrio anguillarum* up-regulated rainbow trout C3 at 6 h post time 0, while seabream C3 was first declined at 6 h and reached to maximum level at 24 h post stress. The same expression pattern than that observed for C3 were observed for Lys, up-regulating at 1 h post stress in rainbow trout by acute stress, while increasing in seabream at 6 h post stress. Stimulation of innate immune system through *Vibrio anguillarum* exposure was provoked only in rainbow trout Lys and increased at 6 h after time 0. IgM mRNA abundance was stimulated in both species by use of stress, interestingly at different time point, showing increment in rainbow trout at 1 h, whereas increasing in seabream at 6 h post stress. IgM was down-regulated in rainbow trout by vaccine at 1 h and 24 h after time 0, but no alteration were observed in seabream. The complement system and lysozyme are recognized as ones of the first lines of defense against pathogen through destroying it (Wang and Zhang, 2010). C3 is a central complement component which has also been shown to be expressed in fish (Huttenhuis et al., 2006; L??voll et al., 2006). The stimulation of C3 by acute handling stress is apparent; interestingly it has been occurred in few hours late in seabream. In fact, the extent to which husbandry stressors induce immune response is often ambiguous and remains to be more investigated because of the complexity in stress phenomenon (Davis et al., 2002). A study performed in our group demonstrated that C3 expression was suppressed in liver, whereas no changes observed in spleen, indicating the specific-tissue response after stress situation. On the other hand, increment of C3 in both species can be also attributed to the features of stressors which has already been reported as a significant factors (Tort, 2011). However, it should be noted that mucosal surfaces such as intestine, gills and skin could be more responsive as well as the

response is tissue and species dependent. Lipopolysaccharide challenge stimulated the expression of C3 in larva of zebrafish, thus C3 expression by *Vibrio anguillarum* can be supported by previous work (Wang et al., 2008). Lys was shown to be increased in both species by acute stress, it has been illustrated that lys expression was diminished in *Perca fluviatilis* spleen by acute stress, while was not altered in rainbow trout spleen (Cortés et al., 2013; Milla et al., 2010), therefore, the modulation of Lys by stress seems to be either tissue or species dependent. In 30 h after vaccination, Lys gene expression was only induced in rainbow trout intestine to eliminate invading *Vibrio anguillarum*, and thus this result is in agreement with previous result obtained vaccinated zebrafish by *Vibrio anguillarum*, showing induction 1 day after (Zhang et al., 2012).

These results suggest that vaccination by Vibrio was not inducer of Lys in gilthead seabream intestine; the induction may be either species-specific or bacterial species-dependent. The immune system is also armed for immunoglobulin M (IgM) secretion which is the first antibody to provide a pivotal defense to the immune system (Parra et al., 2013), surprisingly IgM mRNA was expressed in both species by acute handling stress. In spite of the studies focused on IgM induction particularly at protein level, the current study illustrated that IgM can be modulated by husbandry stress in mucosal surfaces, as it has already been reported that IgM can be also altered by environmental stressors (Dominguez et al., 2004), thereby the increment observed in stress fish can be supported by previous investigation looking at IgM expression induced by heat shock increasing at 3 h post stress in *Epinephelus coioides* in several tissues including gills, head kidney, intestine, spleen (Cui et al., 2010).

On the other hand, no changes observed in IgM expression in both species after vaccination cannot be a surprise since it has been demonstrated that in fish specifically in rainbow trout, the expression of IgM would takes a while (at least couple of weeks) to be induced after pathogen stimulation (Raida and Buchmann, 2007; Zilberg and Klesius, 1997), suggesting that Ig molecules need to recognize the bacterial pathogen within this stage. Concerning suppression of IgM at the early stage of vaccination in rainbow trout, it has been reported that an infection with *Aeromonas salmonicida* induced IgM repression in Atlantic cod (Magnadóttir et al., 2010).

4.6. Cellular response at gene expression level induced by both stressors in mucosal surfaces of trout and seabream

Aquaculture has been characterized by existence of different stressors. Once fish mucosal surfaces are in intimate contact with immediate environment these stressors can induce immune modulation, and thus affecting the health status of the fish (Aranguren et al., 2002). Again in the current study we tried to show that how fish response to different stressors which may induce short and long term effect (Varsamos et al., 2006). In this study looking at cellular response at gene expression level after usage of two distinct stressors,

revealed that the function and activation of immune system may have been influenced by several factors such as species, type and duration of stressors, thus the immune activation pattern can be totally different. Hence, the extent to which environmental stressors stimulate immune system have to be yet investigated (Davis et al., 2002). From our result, as an example rainbow trout gills IL-β was not altered in neither by stress nor stress+Vibrio whereas the expression level of IL-β1 was up-regulated by *Vibrio anguillarum*, in addition in the gills of seabream IL-β1 was stimulated at 6 h by acute handling stress, whereas was declined by *Vibrio anguillarum* exposure at this time, meanwhile the level of IL-β1 by administration of both stressors stayed at control level. Therefore the induction of cytokines response has been shown to be ambiguous. Previous study shown that crowding following bacterial challenge did not have considerable effects on innate immune parameters in European seabass and gilthead seabream (Mauri et al., 2011). Also similar result was observed by other investigator after applying chronic stress following lipopolysaccharide challenge in Yellow perch, illustrating that the cortisol level in those fish which were subjected to two stressors was less comparing to the rest (Haukenes and Barton, 2004). Our obtained result shown that both stressors individually were able to alter immune cytokines responses in rainbow trout and mucosal surfaces, whereas the combination did not behave as same as being individual. Therefore it may be suggested that their combination may could induced impairment on immune system function resulting in reduction of immune response efficiency.

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7. Chapter fifth

7.1. Comparative stress and immune response in rainbow trout (*Oncurhynvhus mykiss*), gilthead seabream (*Sparus aurata*) and zebrafish (*Danio rerio*), triggered by acute stress (anoxia) and *Vibrio anguillarum* immersion

Abstract:

The effect of stress on HPI axis activation and gene expression (cytokines and stress genes) was evaluated in spleen and liver of rainbow trout and sea bream. In this current work, we aimed to compare the stress and immune response of rainbow trout (as fresh water model), gilthead seabream (marine water model) and zebrafish (laboratory model). At plasmatic level cortisol increased at 1 hours post anoxia (hpa) and then decreased at 6 hpa in trout and zebrafish but not in sea bream whose cortisol levels remained high compared to control, suggesting a greater degree of responsiveness to stress in gilthead seabream. Moreover, when fish were exposed to anoxia plus vaccination the cortisol level was also augmented at 1 and 6 h in rainbow trout and seabream and restored to the basal level at 24 h, whereas in zebrafish the response was higher indicating a longer response of HPI axis to the combination of both stressors. The gene expression pattern in spleen and liver was found to be differential and time dependent among species. Altogether, these results indicate that similar cortisol secretion kinetics takes place in the studied species. However, the modulation of genes appears to be decreased, indicating the communication between neuro-endocrine and immune system result in genes suppression.

1. Introduction

Immune system has been categorized into innate (non-specific) and acquired (specific) system. However, an increasing number of findings demonstrate the intermingled function of both systems. In fact innate immune response has been shown to activate the adaptive response and determine the nature of the adaptive response resulting in maintenance of the haemostasis (Fearon and Locksley, 1996). As fish live in aquatic environment, a very high concentration of bacteria and commensals interact with its immune system, enabling the animal to fight with these potential invaders (Ellis, 2001). The innate mechanisms have been shown to be constitutive and readily responsive, thus producing protection via impairment of attachment, invasion or proliferation of the pathogen in the target tissue. Inflammation is one of the consequences of the immune activation which has been defined as a an adaptive response that is triggered by pathogens but also by other detrimental stimuli and conditions such as tissue injury (Barton, 2008). Inflammation is mediated by cytokines, polypeptides or glycoproteins regulating the immune system (Duque and Descoteaux, 2014). Thus, by induction of pro-inflammatory cytokines and pathogen recognition receptors (PPRs) will signal the neuro-endocrine system to cope systemically with these sutuations (Purcell et al., 2006). For instance, a viral infection leads to production of type I interferon-α and β, whereas the expression of pro-inflammatory IL-β1, TNF-α and IL-6 would be the hallmark of the infection caused by bacteria (Delves et al., 2006). Similar to mammals, fish inflammatory response is initiated by induction of a wave of pro-inflammatory cytokines such as IL-β1, TNF-α following by chemokines and IL-12, (Chadzinska et al., 2008). This induction is crucial for pathogen eradication but rapid regulation and termination of pro-inflammatory response appear to be also essential because permanent stimulation can produce damage to the host. Hence, expression of the anti-inflammatory indicators such as IL-10 and TGF-β1 within the last phase of the inflammation protect animal from injury (Pinto et al., 2007).

As it is mentioned already, other than activating cellular and humoral immune responses pathogens also activate Hypothalamic-pituitary-axis (HPI) resulting in hormone release into circulation (Bowers et al., 2000). In fact, hypothalamus sensors are in charge of receiving and monitoring information from the immediate environment of fish and secreting corticotrophin-realising-hormone (CRF) and then signalling to pituitary to secrete adrenocorticotropic hormone (ACTH) into bloodstream. Secreted ACTH is recognized by melanocortin receptor 2 (MC2R) on the surface of the interrenal cells of head kidney, eventually releasing cortisol as the main glucocorticoid (GCs) that can be measured at humoral and mucosal levels (Cortés et al., 2013; Rotllant et al., 2000). On the other hand, these two systems neuro-endocrine and immune system have been shown to function dependently, so that preserving the homeostasis (Engelsma et al., 2002; Selye, 1936). Moreover, it has been proven that this communication is effectively controlled by a complex network of bi-directional communication even sharing receptors from both systems that are capable of producing similar hormones and molecules (Baigent, 2001). Thereby this communication in fish needs to be more investigated to clarify how interaction is occurred.

Indeed, it has been evident that as in mammals, fish immune response is modulated by the final products of the neuro-endocrine activation such as cortisol, ACTH and adrenaline (Chadzinska et al., 2012; Smith et al., 1992; Zinyama et al., 2001), while other hormones such as growth hormone (GH), prolactin (PRL), reproductive hormones, melanin concentrating hormone (MCH), proopiomelanocortin (POMC) have also been effective in modulating the immune function (Harris and Bird, 2000). Hence, the response of the neuro-immune-endocrine to stressors appear to be integrated (Tort, 2011). Likewise, aside from modulating immune system via stress hormones, the secretion of hormones is considered to contribute in preparing and signalling to immune system for potential challenges as a danger signal.

Regarding immune organs, it has been demonstrated that most of the secondary lymphoid organs present in mammals are also recognized in fish excluding lymphatic nodules and bone marrow (Press and Evensen, 1999). Spleen has been found to function as a main secondary immune organ, as found in mammalian counterpart with abundant IgM^+ mature B cells (Zwollo et al., 2008). Spleen also functions in the clearance of blood-borne antigens and immune complex in in splenic ellipsoids, and presentation of the antigens and induction of the adaptive immune response (Zabotkina, 2005). In fish, spleen appears to act in haematopoiesis, antigen degradation and antibody production, though it serves as a secondary lymphatic and scavenging organ. It also acts as one of the potent weapon of the immune arms against parasite infection (Lefebvre et al., 2004). Meanwhile the spleen size of fish is frequently used as one of the simple immune parameters. The liver is not considered to be an immune organ. However, some relevant functions are produced in the liver that involves both immune and energetic roles, thus becoming important in front of a stress response.

The complement system is a major mediator of the innate immune system against pathogens. It is produced in liver, an organ also playing a role in stress response providing extra energy to protect proteins and immune cells from catabolism (Boshra et al., 2006),

As a matter of fact, a frequent practice in aquaculture is to vaccinate fish against common pathogens utilizing formalin-killed bacteria or bacterin as the source of antigen. The current work is devoted to study the effect of stressors on systemic tissue including spleen and liver looking at cortisol levels as the major indicator of the HPI axis activation, and cellular response including cytokines and gene regulation of specific tissues, which are implicated in the immune response. In other words, we tried to investigate how different stressors can affect on activation of the neuro-endocrine and immune system and thereby figuring out whether the interaction between them can take place in an early phase or later on. Therefore, the experimental design relies on three time points 1 h, 6 h and 24 h of HPI axis activation. Moreover, in the current work we also tried to determine how stress (anoxia) affects the systemic immune and endocrine response in fish after immersion in *Vibrio* bacterin. Ultimately, this is the first investigation dedicated to compare the stress and

immune response of three fish species including rainbow trout as a freshwater cultured fish model, gilthead seabream as marine cultured species and zebrafish as a laboratory model. Fish were subjected to the identical duration and intensity of acute handling stress and also to a dilution of vaccine *Vibrio anguillarum*.

2. Materials and methods

2.1. Fish and rearing condition

Rainbow trout (*Oncorhynchus mykiss*) with body weight of 120-140 g were obtained from a local fish farm (Piscifactoria Andres, St Privat d'en Bas, Spain). Gilthead sea bream (*Sparus aurata*) with body weight of 60-70 g were obtained from AQUACULTURA ELS ALFACS,S.L. (Tarragona) and zebrafish (*Danio rerio*) from were purchased from FLORAQUA (Barcelona) with body weight less than 1 gr. Fish were transferred to the UAB fish facility (AQUAB) to acclimatize them to laboratory conditions. All experimental procedures involving fish were submitted and authorized by the Ethical Committee of the "Universitat Autonoma de Barcelona" that agrees with the international Guiding Principles for Biomedical Research Involving Animals (EU2010/63).

2.2. Stressors (vaccination and anoxia)

In the current work two types of stressors have been applied including anoxia and *Vibrio* bacterin. Acute anoxia stress was carried out by catching fish with a net and subsequently maintaining them 1 minute out of water. For Vibrio treatment, fish were immersed in formalin-killed bacteria as a source of antigen. The gram-negative bacterium *Vibrio anguillarum* is an important pathogen that causes vibriosis in both marine and freshwater fish. ICTHIOVAC ^{R} VR (HIPRA) is inactivated commercial vaccine which is suitable for immersion used. The composition consist of inactivated *Vibrio anguillarum*, serotype O1, O2α and O2β with RPS ≥ 60%, presenting all pathogenic serotype of the bacterium. As it is demonstrated already the serogroup O2α is the most pathogenic serogroup of the bacterium. Vaccination performed by immersion making 1 to 10 dilution of vaccine according to guidelines recommended by company (HIPRA, Amer, Spain). To this end, four groups of fish (n=18 fish per group) were used for the experiment. The first group was used as a control (moved to the same bucket which was used for vaccination and immersed in water); the second was only subjected to anoxia, and third group was treated with vaccine. Fish were sampled 1h, 6h and 24h after anoxia stress. For the vaccine plus anoxia group, fish were first vaccinated and anoxia was applied 24h after vaccination.

2.3. Quantification of cortisol in plasma.

Cortisol level in plasma was measured by radioimmunoassay (Rotllant et al., 2006). The antibody used for the assay was purchased from MO bio-medical LLC (OH, USA) and used in the final dilution of 1:4500. Antibody cross-activity with cortisol is 100%. The radio activity was quantified using a liquid scintillation counter. The lower detection limit of the cortisol assay is 0.16 ng/mL.

2.4. Isolation of RNA and cDNA synthesis

Total RNA was isolated from individual fish using TRI reagent (Sigma) according to manufacturer's instructions. The RNA pellet was dissolved in nuclease free-water and immediately stored at -80ºC until use. The RNA concentration was quantified by NanoDropND-2000 spectrophotometer (Thermo Scientific). RNA (500 ng) was used as template to synthesize complementary DNA (cDNA) using iScript cDNA kit (Bio-Rad Laboratories) according to manufacturer's instructions.

2.5. Quantitative real time PCR (qPCR)

Fish mucosal surfaces including gut, gills and skin samples were analysed using real-time qPCR. The gene expression patterns of immune and stress-related genes (IL-1β, IL-6, TNF-α, IL-10, TGF-β1, IgM, COX2, Lys, HSP70 and C3) were analysed. We first tested several housekeepings candidates (18S, EF1α and RPL27 in sea bream, and EF1α and β-actin in rainbow trout) to elucidate which one had less variation (data not shown). Thus, β-actin (for rainbow trout) and 18S (for sea bream) were selected because of the less variability presented. Specific primers used for rainbow trout (Table 1) and gilthead sea bream (Table 2) are indicated. The primers for gilthead sea bream IL-6 and TNF-α were previously designed (Boltana et al., 2014). The other primers are first used in this study and they were designed with Primer-Blast. The primer secondary structure and primer specificity were checked with OligoAnalyzer (version 3.1) and Primer-Blast software, respectively. Meanwhile, the primers used gave a pure PCR product which is verified by single peak in the melting curve and the amplification efficiency were determined individually in all mucosal surfaces by qPCR and all were around 100%. Real-time PCR reactions were performed with iTaq universal sybr green supermix (Bio-Rad Laboratories) using cDNA dilution made for genes of interest in rainbow trout and gilthead sea bream. Primers for all genes were used at final concentration of 500 nM. The thermal condition used were 3 min at 95ºC of pre-incubation followed by 40 cycles at 95ºC for 30 s and 60ºC for 30 s. All the reactions were performed in duplicate using CFX384 Touch Real-Time PCR Detection System (Bio-Rad Laboratories). The quantification was done according to Pfaffl method corrected for efficiency of each primer set (Pfaffl, 2001). Value for each experimental condition was

expressed as normalized relative expression, calculating in relation to values of control group and normalized against those of the housekeeping gene β-actin and 18S for rainbow trout and sea bream, respectively.

2.6. Statistical analysis

The statistical Package for Social Science Software (SPSS version 20) was used for statistical analysis, utilizing the generalized linear model (GzLM: http://refhub.elsevier.com/S0306-4530(15)00909-9/sbref0135). Stressors and dynamism of time were considered as a two factors. This statistical method is flexible since no homogeneity of variance is required. Where a statistical significant interaction was found between stressors and dynamic, we only fulfilled the correlation between time and cortisol and gene expression modulation, meanwhile comparing all groups versus control. It means that no data was analyzed without significant interaction between stressors as well as time. Differences in all data were considered significant when p-value < 0.05 among groups.

3. Results

3.1 Cortisol measurement in the rainbow trout, gilthead seabream and zebrafish

After subjecting rainbow trout, gilthead seabream and zebrafish to acute handling stress, modulation of cortisol level nearly followed the same pattern, mounting at 1 h and dropping at 24 h after stress. In fact cortisol reached to the high level 1 h after stress (126.32 ng/ml) and the difference was statistically significant versus control, at 6 h dropped at control level. Plasma cortisol level in seabream showed greater magnitude (228.18 ng/ml) 1 h after stress, while the cortisol level gradually reduced at 6 h but still significantly elevated versus control (96.4 ng/ml). In zebrafish cortisol increased at 1 h (3.25 ng/g) comparing to control and decreased 6 h after stress. Immersion in *Vibrio anguillarum* was not capable of inducing any significant changes in plasma cortisol level in rainbow trout and seabream, though in rainbow trout at 6 h a slight tendency to increase was observed. Administration of acute anoxia stress together with *Vibrio* showed greater magnitude (159.85 ng/ml) in rainbow trout comparing to anoxia only, gradually decreasing at 6 h (135.4 ng/ml) also with significant difference compared to basal level and restoring the basal level at 24 h. Interestingly, gilthead seabream also showed the same pattern elevating at 1 h (144.08 ng/ml), slightly decrease at 6 h in time course (113.3 ng/ml) while still elevated versus control but with different magnitude. In zebrafish the level of cortisol was also affected by stressors increasing at 1 h, at 6 h with a greater difference between 1 and 24 h. At all levels at each time point differences were significant compared to control level.

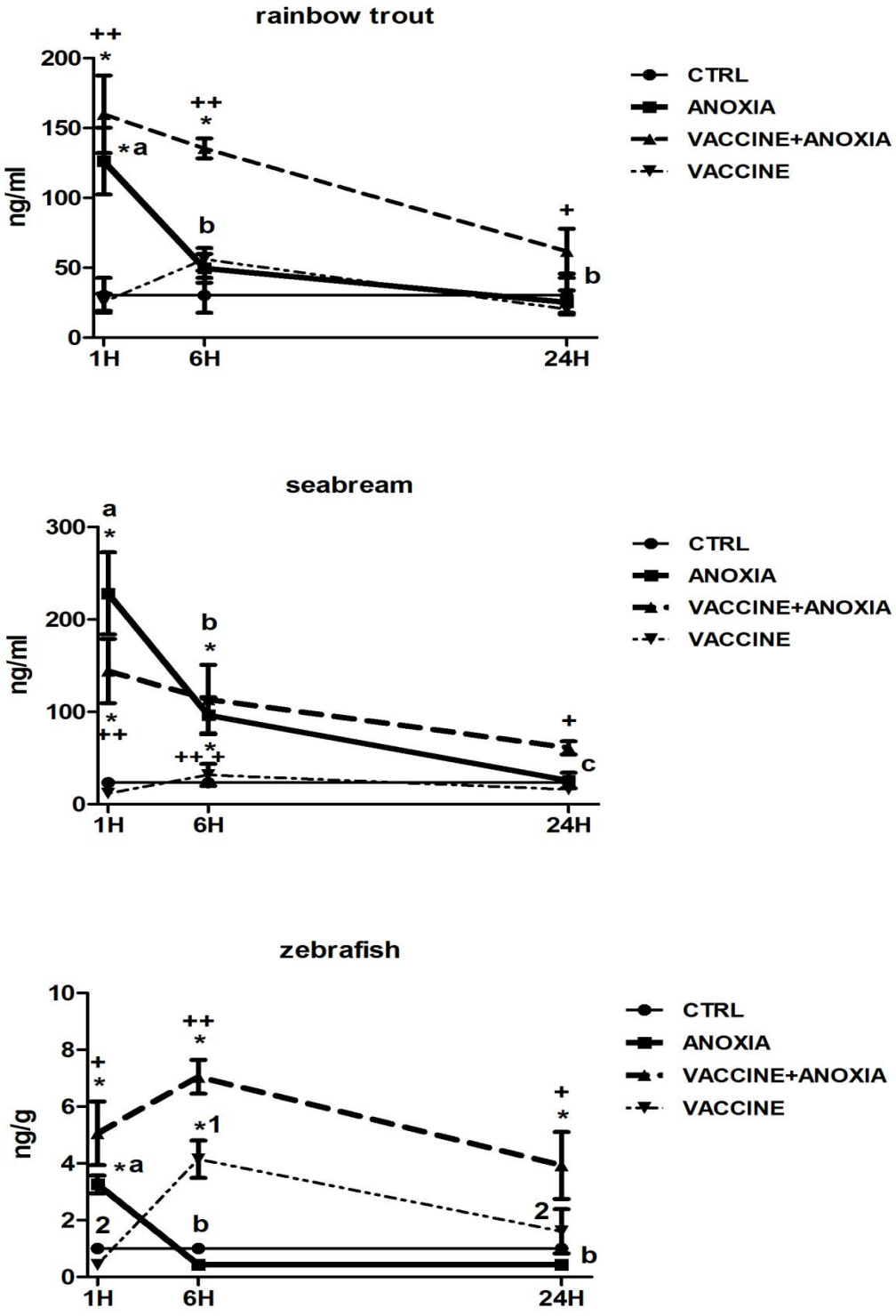

Fig.1. Changes in skin mucus cortisol level in rainbow trout and gilthead seabream in response to anoxia and *Vibrio anguillarum* exposure 1, 6 and 24 h post stress. Skin mucus cortisol was measured by radioimmunoassay. Date are presented as mean ±SE. significant differences are indicated by letters in stress group, vaccine+stress by ++ and vaccine group by number. * 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)

3.2 Gene expression modulation in rainbow trout, gilthead seabream and zebrafish liver

By using RT-qPCR we looked at gene modulation of genes-cassette related with inflammation (IL1β, TNFα, IL10 and TGFβ1), stress (HSP70, enolase and GR) or immune response including C3, Lys and IgM. The result obtained showed overall significant interaction between treatment and time at 1,6 and 24 hour and depending on the significant statistical interactions between treatment and times results will be discussed. Changes in gene expression are considered to be significant from 1 fold changes increase or reduction.

In rainbow trout acute handling stress was able to up-regulate the mRNA level of IL-β1 and TNF-α at 1 h post stress, and reaching to the basal level at 24 h, although in seabream the statistical analysis did not show any difference at 1 h versus 6 and 24 h in time course but IL1β1 increased at 1 and 6 h versus control and recovered at basal level at 24 h. Meanwhile seabream TNF-α was reduced within time course, though TNF-α expression showed an increment at 24 h versus 6 h but still down-regulated. In zebrafish no major changes were observed. Concerning anti-inflammatory cytokines, some alteration was observed for rainbow trout IL-10 but because of no existence of interaction between treatment and time, rainbow trout IL-10 did not show significant changes. Seabream IL-10 reached to the maximum transcriptional level at 24 h after stress, meanwhile was diminished at 1 h after anoxia and seabream TGF-β1 was down-regulated at 6 h after anoxia and then recovered to the basal level at 24 h. Zebrafish IL-10 was not detected in liver but TGF-β1 was decreased at 1 h after stress and then stayed at basal level at 6 and 24 h. *Vibrio* was able to induce major changes in seabream liver for IL-β1, increased at 24 h after anoxia and this increment was significant versus control. Regarding seabream, although TNF-α did tend to recover the basal level it was down-regulated within three time points after Vibrio treatment. TGF-β1 in seabream reached the control level after being decreased by *Vibrio anguillarum* at 1 and 6 h. In zebrafish the gene expression pattern for TGF-β1 did show reduction at each time point versus control.

Administration of the acute handling stress and vaccination in the liver of three species reduced the mRNA level of IL-β1 as a pro-inflammatory indicator in rainbow trout. In seabream stimulation of IL-β1 expression was observed after both stressors; no changes at 1 h and reaching to the maximum at 6 and decreased at 24 h, whereas the increase of IL-β1 compared to control was still significant at 6 and 24 h after stress. The expression of seabream TNF-α tended to reduce at 1 h and continually reduced at 6 and 24 h post anoxia, thought there was no significant change at 6h versus 24h and TNF-α was down-regulated respect to control at each time point. The transcriptional level seabream TGF-β1 begun to slightly decrease towards 24 h in time course, while the down regulation was shown to be significant at 6 h as well as 24 h compared to control. Zebrafish TGF-β1 reached the basal level at 6 h but the expression pattern was suppressed at 1 and 24 h versus control.

Regarding genes which are implicated in stress response the major stimulation was induced in rainbow trout GR, reaching the maximum at 1 h and recovering at 6 and 24 h, meanwhile the induction of GR was also significant compared to control, while GR was down-regulated in seabream at 1 and 6 h and stayed unaltered 24 h after stress. Acute handling stress slightly diminished HSP70 expression in seabream liver, so that the difference was observed between 1 and 24 h, meanwhile the expression was reduced at 6 and 24 h versus control. Interestingly, the major alteration for enolase in liver was induced by acute handling stress in zebrafish. Immersion of fish in *Vibrio anguillarum* vaccine was able to express enolase in rainbow trout at 24 h with a significant increment versus control, and the same pattern than that observed in rainbow trout was registered in seabream attaining the highest level at 24 as well as compared to control, whereas in zebrafish vaccination did not result in any modulation of enolase in liver. Interestingly, three different patterns for GR expression were observed in the liver of rainbow trout, gilthead seabream and zebrafish, so that vaccination did not induce any change in rainbow trout, but it was up-regulated at 24 h, whereas in zebrafish GR mRNA was suppressed after immersion in Vibrio at each time point.

Acute anoxia stress together with *Vibrio anguillarum* immersion roughly was not able to drive any changes for enolase in three species. HSP70 mRNA level showed a decrease tendency from 1 h towards 24 h, so that in both rainbow trout and seabream HSP70 expression was down-regulated at 6 and 24 h. Also combination of both stressors induced distinct patterns for GR in the three species including up-regulating at 6 h (also comparing to control) in rainbow trout and staying at about control level at 1 and 24 h. In gilthead seabream GR decreased over time course especially at 24 h after stress, whereas GR zebrafish was suppressed at 1 h, recovered to basal level at 6 and eventually decreased again at 24 h after stress.

The stimulation of the immune genes by acute stress showed that C3 was expressed in the liver of three species following different patterns, so that the temporal C3 expression reached the maximum at 1 h (compared to controls) in rainbow trout and returned to control level at 6 and 24 h after stress, whereas C3 in gilthead seabream and zebrafish was expressed with 24 h delay, up-regulating at 24 h and also versus control. The major changes were recorded after anoxia stress for Lysozyme in the liver of rainbow trout and zebrafish at 24 h after stress. Although IgM was detected in the liver of rainbow trout, no alteration was observed following treatments, so it seems that acute handling stress was not able to stimulate IgM expression in the liver of seabream, whereas IgM reached to the maximum in zebrafish 24 h after stress and the difference was significant compared to control. The C3 expression was induced with *Vibrio anguillarum* immersion in the liver of rainbow trout and gilthead seabream but not in zebrafish liver, so that C3 was mounted at 24 h in both species in time course, despite the pattern was slightly different in seabream increasing at 1 h as well, restoring to basal level at 6 h and eventually increasing at 24 h. Lysozyme expression was not stimulated neither in rainbow trout nor in gilthead seabream by vaccination,

whereas it was diminished at 1 h as well as 24 h comparing to control in zebrafish liver. No changes were found for the expression of IgM with Vibrio.

Acute handling stress plus *Vibrio anguillarum* stimulation did not show any modulation for C3 and Lys in the three species, while Lys was down-regulated in the liver of zebrafish 24 h after stress. Regarding IgM, although it was detectable in the liver of rainbow trout and zebrafish it was not shown to be modulated by stressors in these species. IgM was not stimulated neither by both stressors at short time after stimulation.

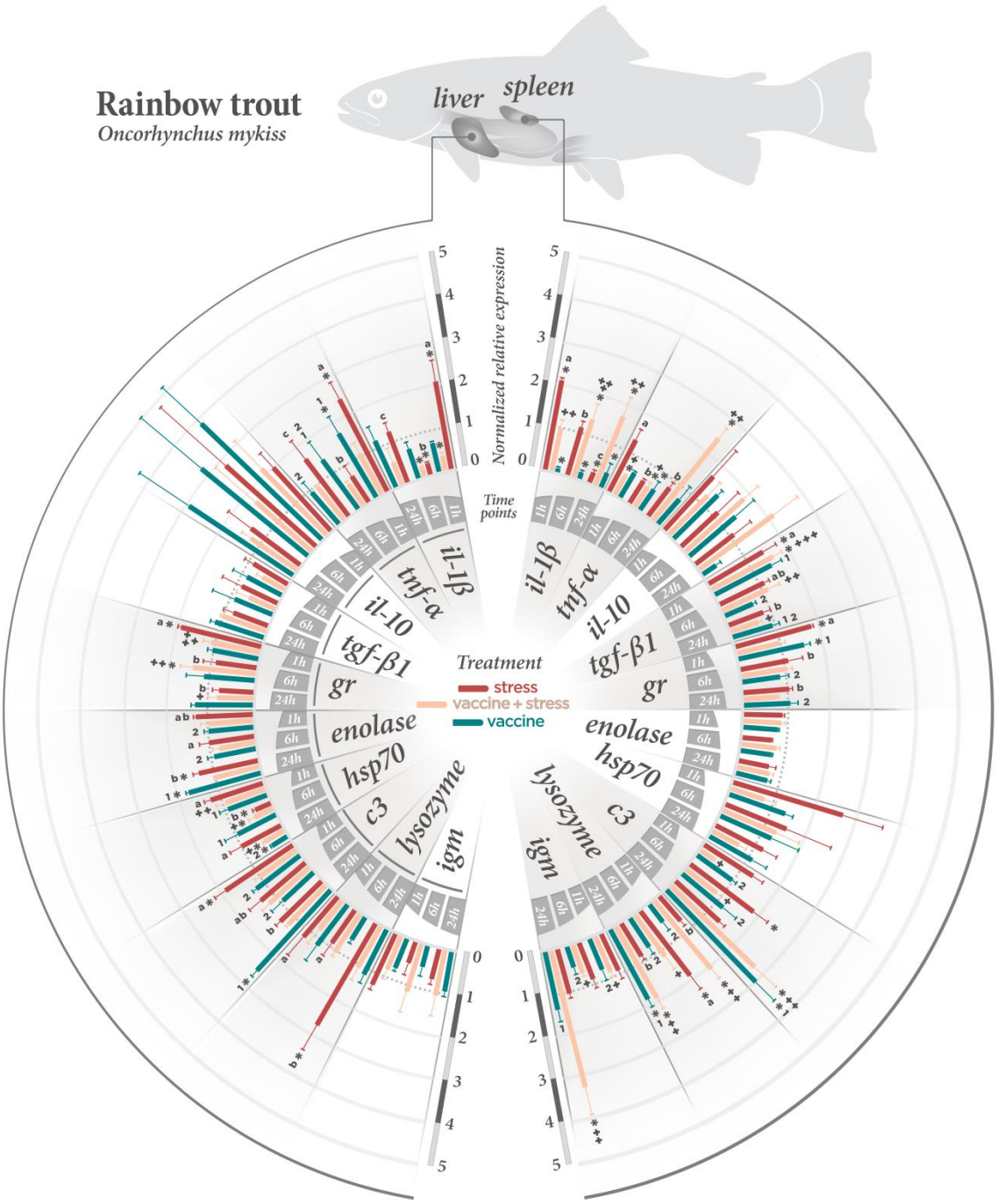

Fig.2. qPCR quantification of specific mRNA accumulation in rainbow trout liver and spleen subjected at 1, 6 and 24 h post challenge with acute handling stress and *Vibrio anguillarum* exposure. IL1β, TNFα, IL10, TGFβ1, enolase, GR, HSP70, Lys, C3 and IgM shown are mRNA relative abundance to β-actin and 18S housekeeping gene in rainbow trout and gilthead seabream respectively. Date are presented as mean ±SE. significant differences are indicated by letters in stress group, vaccine+stress by ++ and vaccine group by number. * 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).

3.3 Gene expression modulation in rainbow trout, gilthead seabream and zebrafish spleen

The modulation effect of acute anoxia stress and vaccination was also checked in spleen of rainbow trout, gilthead seabream and zebrafish. Acute stress was able to express IL-β1 at 1h and continuously reduce at 6h and 24 h although significant versus control in seabream. In zebrafish no major changes were observed. Concerning TNF-α, although a small modulation was observed it seems that acute stress was not able to drive any significant modulation at transcriptional level. The expression of IL-10 was only influenced by anoxia in gilthead seabream reaching the top at 6 h and dropping to basal level at 24 h. No major alteration was found for TGF-β1 in the three species. Immersion of fish in Vibrio did suppressed IL-β1 at each time point in rainbow trout without any significant modulation in time course; no changes were observed in seabream, while a significant reduction was observed at 1 h in zebrafish. IL-10 was stimulated by Vibrio only in seabream showing an increment versus control at 1 h. No change was found in three species for TGF-β1. Administration of acute handling stress and *Vaibrio anguillarum* in the spleen of three species showed a distinct pattern which was observed after the two stressors, thus the increment was statistically significant between 1 h and 24 h, beginning at 1 h and significantly increasing at 6h compared to control and gradually mounting at 24 h with. In seabream IL-β1 was shown to be up-regulated at each time point compared to control but the difference was significant between 24 and 1h and 6 h. Interestingly in zebrafish stressors suppressed IL-β1 in the spleen along time course. The expression of TNF-α in rainbow trout and zebrafish showed roughly the same pattern reaching to the maximum level at 24 h after anoxia and the increase was also significant compared to control, meanwhile in rainbow trout TNF-α was down-regulated at 1 and 6 h versus control. The administration of both stressors induced IL-10 expression only in seabream, so that IL-10 was increased at 6 h and reduced at 24 h, meanwhile the increase was significant versus control at 6 h.

Regarding genes which are implicated in stress response, acute anoxia stress was not able to stimulate the expression of enolase and HSP70 in the spleen of the three species. GR expression in rainbow trout and zebrafish showed distinct pattern, so that GR mRNA level was up-regulated at 1 h and recovered basal levels at 6 and 24 h, whereas in the spleen of zebrafish GR was expressed at 1 h, dropping at 6 h and mounting again at 24 h, with the increment at 1 h and 24 h statistically significant respect to control. The immersion of fish in Vibrio did not stimulate the expression of enolase and HSP70 in any of the three species. The mRNA level of GR was affected by *Vibrio* in rainbow trout, gilthead seabream and zebrafish, so that in rainbow trout GR was upregulated at 1 h recovering the basal level at 6 and 24 h, although in gilthead seabream no difference was found along time course, but GR increased at 1 and 6 h only compared to control. In zebrafish no alteration was found along time course but GR was increased at 6 and 24 h versus control after stress.

The application of anoxia and Vibrio immersion did not stimulate the expression of enolase as well as HSP70 in the spleen of the three species. The expression of GR was not affected

by both stressors in the spleen of rainbow trout and zebrafish, while GR transcriptional level showed a temporal expression reaching the maximum level at 6 h and recovering control levels at 24 h, while the increment observed at 6 h was also significant versus control. Regarding the expression of immune genes, it seems that acute anoxia stress did only stimulate the C3 mRNA level in rainbow trout at 6 h respect to control. Lysozyme was shown to follow the same expression pattern in three species reaching to the maximum at 6 h and dropped to basal level at 24h. IgM in the spleen of the rainbow trout was not stimulated after anoxia, not detected in gilthead seabream, whereas in zebrafish the transcriptional level was increased at 24 h and being significant versus control. *Vibrio angillarum* bath vaccination provoked the C3 response at 24 h (also versus control), while in seabream spleen a remarkable expression was observed at 6 h recovering control level at 24 h, and no changes were found in zebrafish. Distinct behavior of the Lysozyme gene was induced by *Vibrio*, so that rainbow trout Lysozyme was stimulated up-regulating at 24 h and also versus control. In seabream Lysozyme was not affected in the spleen, while in zebrafish Lysozyme was suppressed at 1 h and recovered basal level at 6 and again slightly reduced at 24 h. The expression of IgM was suppressed with immersion in Vibrio at all time points in zebrafish.

Anoxia plus vaccination showed C3 significant response in the species; rainbow trout and gilthead seabream C3 expression increased at 24 h, whereas zebrafish C3 increased at 6 h. Stressors were able to induce temporal alteration in Lysozyme mRNA level in rainbow trout up-regulating at 1 h, dropping at 6h and again increasing at 24 h. In seabream the increment was observed at 6 h, while in zebrafish Lysozyme decreased at 6 h, being these changes statistically significant compared to control. Both stressors induced IgM in the spleen of rainbow trout at 24 h (also comparing to control), while IgM was diminished at 6 h and 24 h only respect to control.

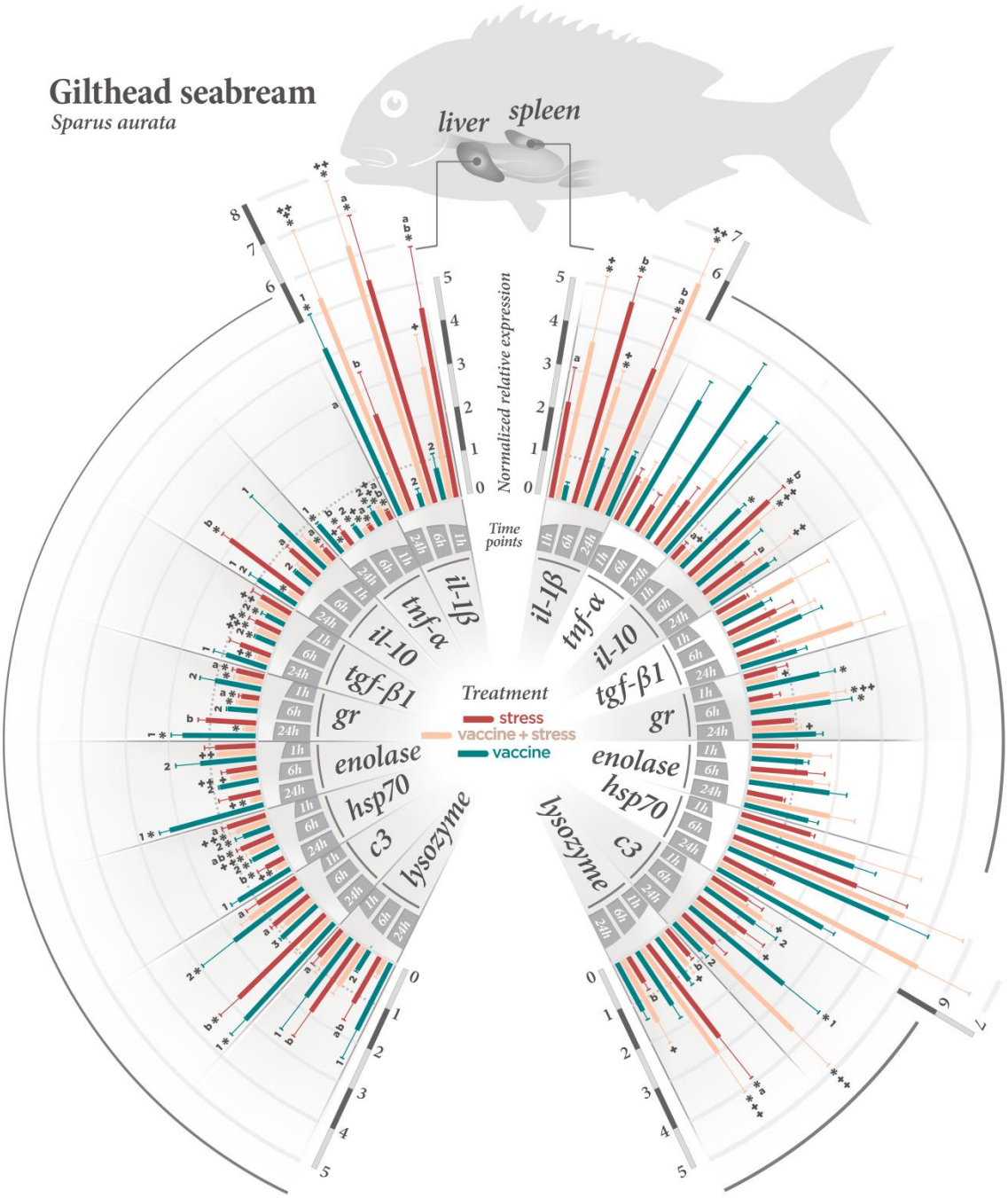

Fig.3. qPCR quantification of specific mRNA accumulation in gilthead seabream liver and spleen subjected at 1, 6 and 24 h post challenge with acute handling stress and *Vibrio anguillarum* exposure. IL1β, TNFα, IL10, TGFβ1, enolase, GR, HSP70, Lys, C3 and IgM shown are mRNA relative abundance to β-actin and 18S housekeeping gene in rainbow trout and gilthead seabream respectively. Date are presented as mean ±SE. significant differences are indicated by letters in stress group, vaccine+stress by ++ and vaccine group by number. * 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).

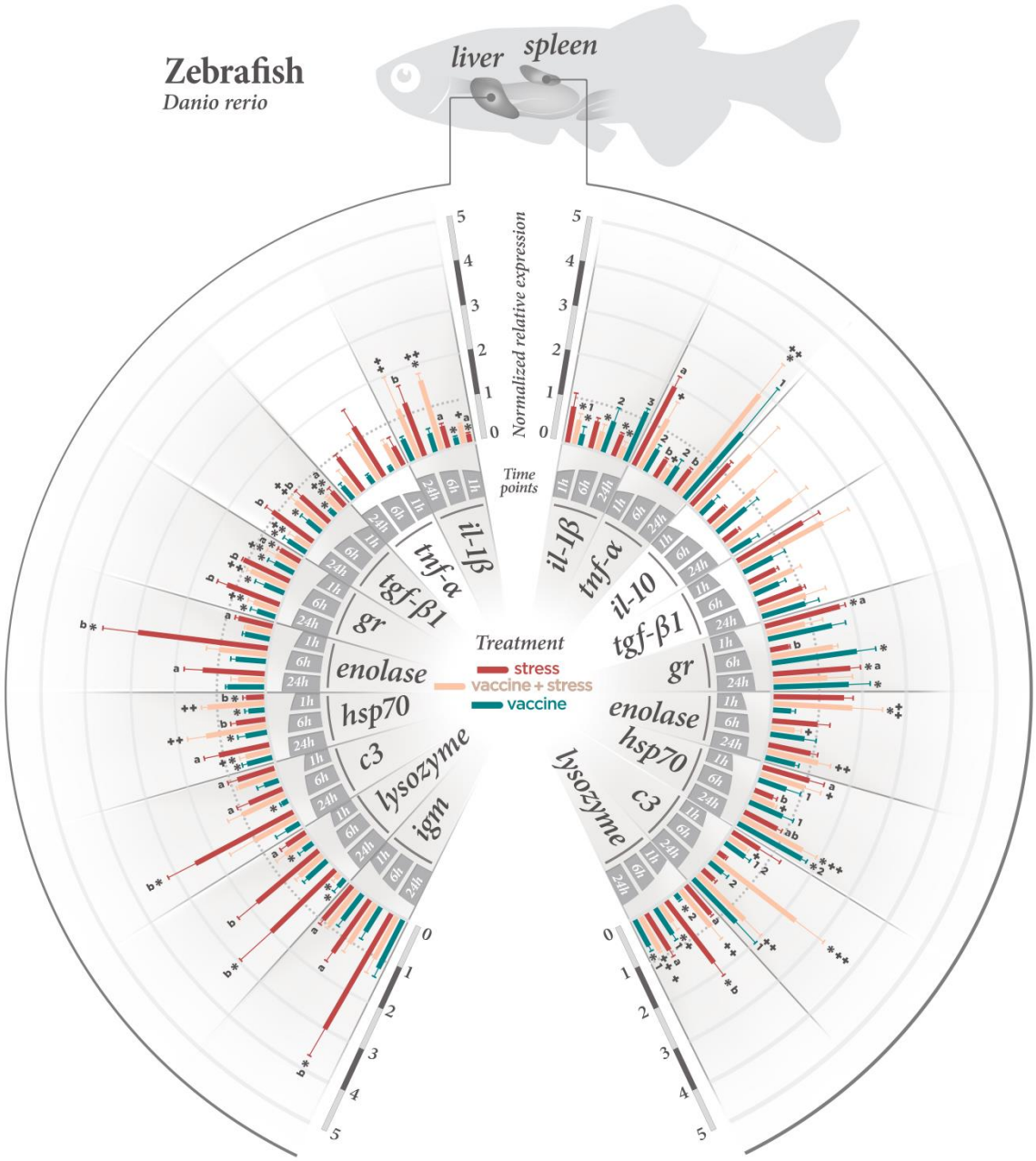

Fig.4. qPCR quantification of specific mRNA accumulation in zebrafish liver and spleen subjected at 1, 6 and 24 h post challenge with acute handling stress and *Vibrio anguillarum* exposure. IL1β, TNFα, IL10, TGFβ1, enolase, GR, HSP70, Lys, C3 and IgM shown are mRNA relative abundance to β-actin and 18S housekeeping gene in rainbow trout and gilthead seabream respectively. Date are presented as mean ±SE. significant differences are indicated by letters in stress group, vaccine+stress by ++ and vaccine group by number. * 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).

4. Discussion

4.1 Plasma cortisol level in rainbow trout, gilthead seabream and zebrafish

Plasma cortisol has been frequently used as an indicator of stress response in fish and other vertebrates (Barton and Peter, 1982; Ellis et al., 2012; Möstl and Palme, 2002) since stress and cortisol secretion are most often correlated. The present work describes the cortisol response in rainbow trout, gilthead seabream and zebrafish after acute handling stress and *Vibrio anguillarim* immersion; this can complete our previous results in mucosae and in vitro, and enable us to compare the pattern and also magnitude of stress response in different fish species after acute handling stress (Barton et al., 2005; Flos et al., 1988). As a matter of fact in this study we aimed to clarify the distinct response of HPI axis to stressors with identical intensity in three different species. Better comprehension how different fish respond to stressor will help the future of aquaculture. The obtained results illustrates that cortisol raised 1 h after stress in rainbow trout, gilthead seabream and zebrafish and returned to the basal level at 24 h as the normal pattern after acute stressors. Interestingly in seabream cortisol was still elevated at 6 h in comparison with other species indicating slight decrease of cortisol within time course. Our findings correspond with previous work in rainbow trout, *Perca fluviatilis* and zebrafish (Acerete et al., 2004; Aluru and Vijayan, 2006; Fierro-castro et al., 2015; Ramsay et al., 2009). A distinct range of cortisol compared to other studies confirms the role of nature of the stressor (air exposure, brief handling, transferring and net handling) and it has also been demonstrated that the intensity as well as duration of the stress greatly influence the stress response (Tort, 2011). It is also noteworthy that cortisol peacked in rainbow trout at 126.3 ng/ml, while in seabream was 228.18 ng/ml and in much less quantity in zebrafish (3.258 ng/gr), thus indicating the different degree of responsiveness to this stressors among species. Bath vaccination with Vibrio was not able to induce any cortisol secretion in rainbow trout and seabream, though a slight tendency towards increase was observed at 6 h in rainbow trout, while bath vaccination induced cortisol release at 6 h in zebrafish. In other fishes it has been reported that the presence of pathogen *Lepeophtheirus salmonis* in Atlantic salmon increased the level of cortisol as well as glucose (Bowers et al., 2000). Also sea lice, one of the major pathogen for Atlantic salmon, induced cortisol release (Mustafa et al., 2000). An acute cortisol response was also observed in channel catfish and rainbow trout,(Bilodeau et al., 2003) *Oncorhynchus mykiss*, following *E. ictaluri* and *Vibrio anguillarum* infection respectively (Ackerman and Iwama, 2001; Bilodeau et al., 2003). It should be also noted that the difference found in rainbow trout with the study of Ackerman and Iwama may have related to the injection of alive *Vibrio anguillarum* since the high concentration of the pathogen was observed in circulating blood. Comparing acute anoxia stress with Vibrio exposure, both stressors did produce cortisol elevation at 1h and 6 h in rainbow trout and seabream restoring the basal levels at 24 h, whereas in zebrafish the level was significantly higher over time course, thus illustrating the longer influence of stressors on HPI activation among species. Therefore zebrafish appear to be more responsive to combination of two

stressors. As with the study of Small and Bilodeau et al., (2005) in which exposure of *Edwardsiella ictaluri* in stressed *Ictalurus punctatus*, showed persistent plasma cortisol elevation in stressed catfish even several days after exposure, our results suggest longer activation of HPI axis after combination of the stressors in zebrafish.

4.2 Stress and immune gene expression in the liver of rainbow trout, gilthead seabream and zebrafish

Regarding the liver tissue, it has been evident that in mammals, liver does not cease to be involved in hematopoietic and immune roles after birth (Seki et al., 2000). Furthermore in fish, liver appears to be the major site for complement production and playing crucial role in pathogen eradication (Ellingsen et al., 2005). On the other hand liver is an important organ for metabolic regulation after stress in fish (Mommsen et al., 1999). Thus we tought that comparing stress and immune gene expression in rainbow trout, gilthead sea bream and zebrafish may help to have better understanding of how stress and immune system respond to the same intensity and duration of a stressor in three different species which belong to different environments as well as possessing different genetic background. IL-10 and TGF-β1 were not stimulated with anoxia in rainbow trout, and it is not surprising that IL-10 was up-regulated 24 h after stress in seabream, since apparently acute stress was not enough intense to induce IL-10 expression in the liver of zebrafish, while TGF-β1 was reduced 1 after stress in zebrafish and recovered to the basal level at 6 and 24 h after stress. As a matter of fact, IL-β1 is a major initiator of the inflammatory and immune response, and playing crucial role in induction of secretion of pro-inflammatory mediators by macrophages such as cytokines (IL-6) and prostaglandins (Dinarello, 2009). IL-β1 promotes differentiation of B-cells into plasma cells and activating cytotoxic T cells through activation of cytokines (Duque and Descoteaux, 2014). A pro-inflammatory reaction seems to be produced with the expression of TNF-α which is able to impose its effect in many organs and induce IL-β1 expression (Baud and Karin, 2001). This finding corresponds with the previous results, demonstrating the elevation of the IL-β1 after repeated handling stress and elevated plasma cortisol level and inducing the transcriptional level of this cytokine in head kidney macrophages in Atlantic salmon. Meanwhile IL-β1 induction was observed after cortisol incubation in rainbow trout hepatocyte *in vitro* (Fast et al., 2008; Philip et al., 2012). Previous work has illustrated that acute stress was capable of increasing the number of leukocyte in the head kidney of juvenile coho salmon (*Oncorhynchus kisutch Walbaum*) during 24 h of stress (Maule and Schreck, 1990). The constitutive expression of IL-β1 is higher than that observed in the liver of rainbow trout, as the level of cortisol secreted by acute stress is higher in seabream. Thus we suggest that the magnitude of the cortisol response may trigger a higher expression of IL-β1 as shown in previous studies in fish (Maule and Scherek, 1990). It has also been reported that central and peripheral IL-β1 was expressed in several organs including hypothalamus, pituitary and head kidney of *Cyprinus*

carpio after acute handling stress (Metz et al., 2006), whereas in zebrafish this cytokine was down-regulated, thus suggesting species-specific response in fish after acute handling stress. Concerning *Vibrio anguillarum* immersion, it has been evident that the innate mechanism of defense plays an indispensable role in protecting the animal against environmental biotic challenges. The Vibrio immersion provoked IL-β1 expression only in seabream at 24 h, in zebrafish it was not able to stimulate these cytokines after vaccination. Zhang, et al (2012) showed that vaccination of zebrafish with alive attenuated *Vibrio anguillarum* induced expression of IL-β1 in liver 7 days post vaccination. On the other hand, up-regulation of IL-β1 in seabream liver indicates that this member of the cytokines family is stimulated with vaccination at short term but not in rainbow trout and zebrafish. These findings are in agreement with other studies in which the effect of vaccination was investigated demonstrating enhanced expression of IL-β1 in blood (Caipang et al., 2008; Zhang et al., 2012), thus indicating less implication of the rainbow trout and zebrafish liver in innate immune response against Vibrio. Nevertheless, cytokines regulation has been reported in fish head kidney after bacterial exposure (Sepulcre et al., 2007) and no significant difference was obtained between alive and killed *Vibrio anguillarum*. In rainbow trout liver the effect of Ergosan as an immunomodulatory agent has been evaluated demonstrating an effect of Ergosan on TNF-α expression in agreement with our result obtained in rainbow trout after vaccination (Gioacchini et al., 2008). Meanwhile, isolated head kidney leukocytes can be stimulated with LPS, up-regulating the expression of TNF-α by LPS in rainbow trout (Laing et al., 2001). In contrary Cortes, et al (2013) sfter administration of the cortisol through implants in rainbow trout showed no alteration of cytokines expression including IL-β1, IL-6, TNF-α and TGF-β1. The response of the immune system to stress and bacterial infection included induction of the pro-inflammatory cytokines such as IL-β, IL-6 and TNFα, although a potent pro-inflammatory reaction is required for clearance of the pathogen but this may result in tissue damage and immune dysfunction. Thus the provoked pro-inflammatory reaction has to be terminated through anti-inflammatory components such as IL-10 and transforming growth factor-β (Delves et al., 2006; Verburg-van Kemenade et al., 2011). The expression of IL-10 occurred 24 h after stress only in seabream and is not a surprise since production of IL-10 both in fish and mammals take places at the late phase of the inflammation, thus induction of IL-10 prevents more damage to the stressed animal (Pinto et al., 2007). Regarding TGF-β1, the suppression of this cytokine might correspond to the suppressive effect of cortisol in both stressed seabream and zebrafish which is in agreement with our previous results obtained *in vitro* (Castillo et al., 2009; Khansari et al., 2017). The effect of glucocorticoids (GCs) is predominantly mediated by glucocorticoid receptors (GRs) in target tissues (Greenwood et al., 2003), inducing reduction or up-regulation at the promotors of the target genes. As liver is particularly involved in stress and immune response, thereby regulation of GR after stress may help to better appreciate the role of GR signaling. In rainbow trout the expression of GR was shown to be up-regulated simultaneously with elevation of cortisol in stressed and vaccine plus stress group, no changes in vaccinated group, whereas in gilthead seabream GR

was reduced in stresses group and V+S fish, whereas in zebrafish down-regulated in stress and V+S and also vaccinated group. The regulation of GR in rainbow trout is consistent with previous work demonstrating increment of GR mRNA abundance with cortisol treatment in rainbow trout hepatocytes (Aluru and Vijayan, 2007), whereas decreasing GR expression by cortisol in gilthead seabream and zebrafish appear to be in contrary with previous results obtained in fish. On the other hand decrease of GR transcription has been shown in mammals by GCs, thus the difference in regulation of GR in stress circumstances may be species-specific (Yudt and Cidlowski, 2002). Meanwhile our research group has already revealed the reduction of GR in seabream gut through cortisol implant (Teles et al., 2013). Thereby, this leads us to propose that distinct regulation of GR takes place in different species.

The enolase enzyme super family acts as a key role in glycolysis and it appears to be ubiquitous in all organisms (Babbitt et al., 1996). The expression of enolase in liver of rainbow trout was increased by both anoxia and vaccination. In seabream enolase was elevated only after vaccine, while anoxia stress induced enolase expression in zebrafish. In fact aside from its function for glucose catabolism, it has been documented that enolase acts as a cell associated stress protein (HAP), protecting cells within hypoxia (Roland et al., 2000). Also Gracy et al, (2001) illustrated that enolase increased in liver in longjaw mudsucker (*Gillicthys mirabilis*) by hypoxia. This result is consistent with our previous finding showing up-regulation of enolase in seabream brain after in vivo LPS challenge but not under high density challenge (Ribas et al., 2004), thus indicating that enolase in rainbow trout responds to anoxia and *Vibrio anguillarum* stressors suggesting the participation of enolase in a general stress response particularly in rainbow trout. The expression of C3 in the liver of rainbow trout, gilthead seabream and zebrafish was found to be upregulated at different time points in stressed fish, meanwhile the increment of this gene was increased in rainbow trout and seabream after vaccination by *Vibrio anguillarum*. It should be noted that complement system is present both in vertebrates and invertebrates acting as one of the potent weapons of innate immune system. Also it has been reported that complement bridges innate and adaptive immune response (Eikenberg, 1994; Gomez et al., 2013; Price and Boettcher, 1979). Thus, our finding suggest that complement could play a role in response to acute stress and *Vibrio anguillarum* immersion which has been used in the current study as a source of antigen. This is in agreement with (Sigh et al., 2004) showing upregulation of C3 in head kidney by parasites, in contrary the previous result obtained in our research group and other works that did not show any significant changes of C3 through cortisol implantation and immunization with live vaccine during 24 h (Jørgensen et al., 2008). Therefore, we reveal that in response to acute stress C3 is also responsive, though the regulation in seabream and zebrafish occurred 24 h later than rainbow trout. Lysozyme mRNA level was increased in rainbow trout and zebrafish 24 h after stress, was not altered in seabream and Vibrio vaccination reduced Lysozyme at 1 and 24 h. Lysozyme is synthesized both in liver and extra-hepatic sitse, however it has been demonstrated that its

function paly an essential role in producing protection against pathogens (Saurabh and Sahoo, 2008). Lysozyme may be on one of the first responses to be induced after immune and stress stimulation. The modulation found for lysozyme in rainbow trout and zebrafish is in agreement with the work of Caruso et al, (2002) in which a clear increase of lysozyme was observed in sheatfish *Silurus glanis* following stress and infection with *Edwardsiella tarda* (Caruso et al., 2002), though less responsivity was observed in seabream after applying two stressors.

4.3 Stress and immune gene expression in the spleen of rainbow trout, gilthead seabream and zebrafish

The response of stress and immune system to the identical duration and intensity of stressors in fish has not been evaluated yet, and in fact this is the first study comparing the response of neuro-endocrine and immune system in three fish species which possessing different genetic background and living in different environment, thus it may enable us to figure out the distinct ability of immune system to such stressors. However, while the genetic difference may be small and the molecular and cellular agents similar, then the function of the immune system might be different from one to other specie. The importance of the spleen and its morphology and function is already described in fish being as one of the most implicated lymphoid organ in response to immune activated situation (Press and Evensen, 1999). In the spleen we found significant expression of IL-β1 in rainbow trout and also gilthead seabream with higher magnitude and longer, while in zebrafish was decreased at 24 h. Inversely IL-β1 was down-regulated in rainbow trout, no changes in seabream and zebrafish induced by vaccine. The previous results illustrated the expression of this pro-inflammatory agent 5 days after cortisol injection mimicking chronic stress situation, while in the current study because of utilizing acute stress the immune response may be provoked earlier at the day first of stress administration (Cortés et al., 2013), meanwhile in Atlantic salmon head kidney short term stress did elevate IL-β1 transcription level at 1 and 3 h (Fast et al., 2008) and also Vibrio anguillarum increased IL-β1 expression in blood (Caipang et al., 2008). It also noteworthy that a side from different regulation of magnitude of IL-β1 derived in spleen of rainbow trout and seabream which may also be attributed to higher level of cortisol; nevertheless we revealed different regulation of this cytokine in three species in stress condition. IL-β1 is believed to function as the major actor of the early inflammation. The result has clearly showed different pattern induced by Vibrio in rainbow trout comparing seabream and zebrafish. Investigation of immune response in zebrafish induced by bath vaccination with Vibrio anguillarum did show IL-β1 increased 3 days after vaccination and no alteration at first day, thus supporting the current result in seabream and zebrafish stimulated by vaccine (Zhang et al., 2012), on the other hand indicating down-regulation of this cytokine at early stage of Vibrio exposure in rainbow trout.

The increase of seabream IL-10 in spleen which is regarded as an anti-inflammatory cytokines following exposure with the anoxia and *Vibrio anguillarum* indicates that induction of IL-10 in seabream could be the response of immune system to stressors utilized in this study using the same intensity and duration of stressors, thus anoxia and Vibrio were not potent enough to derive IL-10 response in rainbow trout and zebrafish. The expression of IL-10 in seabream is supported by regulation of inflammation in mammals in which pro-inflammatory cytokines such as IL-β1 and TNFα is regulated by IL-10 as its central role (Mege et al., 2006) and also fish head kidney, spleen, heart, liver and gills has been shown to express IL-10 with injection of poly I:C in Atlantic cod (Seppola et al., 2008), while infection with Vibrio anguillarum and Aeromonas salmonicida was not effective to modulate any changes in IL-10 in gills of Atlantic cod (Caipang et al., 2010). Interestingly TGF-β1 transcription was increased only in rainbow trout spleen after acute stress acting as the only anti-inflammatory agents induced in rainbow trout after stress. In contrary our recent result revealed that administration of cortisol on head kidney of rain bow trout *in vitro* did not stimulated the cytokines induction particularly TGF-β1, on the other hand the Castro et al, (2011) also showed no alteration for TGF-β1 in rain bow trout cell line after cortisol treatment (Castro et al., 2011; Khansari et al., 2017), thus we may attribute expression of this cytokine in rainbow trout to either some difference between *in vivo* and *in vitro* related to stress hormone administration or tissue-specific response. Overall, it seems that acute stress for one minute appears to be inducer of TGF-β and IL-10 in rainbow trout and gilthead seabream protecting them from tissue damage respectively.

In the current study glucocorticoid receptor (GR) has been shown to be up-regulated in rainbow trout and zebrafish with anoxia and also vaccine, while in seabream was increased only by vaccine. In fish cortisol is described as the predominant glucocorticoids regulating metabolism and immune modulation mediated through GR (Aluru and Vijayan, 2007; Sapolsky et al., 2000) and mineralocorticoid effect is mediated via mineralocorticoid receptor (MR) adjusting osmolality. In fact regulation of GR in mammals by cortisol is illustrated by reducing of the receptor mRNA, while in fish the findings demonstrated different regulation of GR by different concentration of cortisol (Alderman et al., 2012; Terova et al., 2005; Yudt and Cidlowski, 2002). Thus, our obtained result elevating GR mRNA by anoxia in agreement with GR increased by cortisol in rainbow trout hepatocyte (Sathiyaa and Vijayan, 2003). Regarding temporal elevation of GR in rainbow trout, gilthead seabream and zebrafish, GR induction in *Cyprinus carpio* head kidney and peritoneal leukocyte has been investigated demonstrating the expression of GR1a and GR1b. A rapid transient induction was seen for GR1(a,b) induced by LPS and also with Zymosan supporting the current increment of GR by Vibrio anguillarum exposure (Stolte et al., 2009), altogether it seems that anoxia was not able to derive any GR alteration at transcriptional level in seabream. Anoxia was found to be effective only on expression of C3 in rainbow trout not in seabream and zebrafish, meanwhile the usage of killed-Vibrio anguillarum induce C3 expression in rainbow trout and seabream in time dependent manner. The effect of stress in

animal has been described by inducing a variety of immune changes modulating the immune components. Thus expression of C3 in rainbow trout indicate immediate response of this specie to acute stress which belongs to acute phase protein and whose synthesis is enhanced upon an inflammation, however the elevation of C3 protein has been evident to be increase after acute stress (Sunyer et al., 1997). On the other hand, this experiment has clearly demonstrated that C3 which play a central role in complement system protecting rainbow trout and seabream against infection with no alteration in zebrafish. Infection of rainbow trout showed an increase of C3 in head kidney, spleen and skin (Jørgensen et al., 2008; Sigh et al., 2004). On the other hand, the effect of LPS and β-glucan was assessed in rainbow trout stimulating complement in vivo, while the transcription level of C3 in head kidney macrophages in vitro was not stimulated even by LPS stimuli, thus indicating the crucial role of C3 at systemic level (Løvoll et al., 2007). Altogether, zebrafish C3 was found to be unaltered during 48 hours post vaccination, thus Vibrio may modulate either expression of C3 later or othe subtype of C3 within vaccination. Rainbow trout, gilthead seabream and zebrafish spleen did respond to anoxia stress up-regulating lysozyme concomitantly at 6 h after stress, thus indicating the reasonable enhancement of innate immune parameter after facing to stress situation in order to cope with potentially invasive pathogen or regaining the integrity by animal. However in the current work enhancement of lysozyme was illustrated as the immediate effect of acute stress on immune system via altering the immune components including cytokines and other immune components. Air exposure of rainbow trout for 30 s showed increment of lysozyme activity after stress (Demers and Bayne, 1997). Therefore the result point out this is the first parameter of innate immune system that was found to be induced simultaneously in three different species to the same severity and length of stress. Regulation of lysozyme gene demonstrated different pattern after immersion of rainbow trout in Vibrio eliciting lysozyme expression, whereas no changes mediated in seabream and significant reduction in zebrafish. As lysozyme is of the chief antimicrobial peptide in humoral (innate) immunity, it has been documented that in gastrointestinal of human high secreted level of lysozyme mainly enable Peyer's patches to internalize and clear the bacteria (Lelouard et al., 2010). Similarly the expression of Lys showed significant rise in Indian carp, *Labeo rohita* head kidney after infection with *Edwardsiella tarda*, also infection of Japanese flounder with the same bacterial specie increased lysozyme mRNA in head kidney, spleen and ovary (Hikima et al., 1997). On the other hand, the suppression of lysozyme in zebrafish may be specie-specific response at the early stage of infection or vaccination since the result of Lyz expression after bath vaccination with attenuated *Vibrio anguillarum* showed tendency to decrease in zebrafish 1 day post vaccination (Zhang et al., 2012). Our results illustrated that acute handling stress and vaccination could not alter IgM expression in rainbow trout and seabream, whereas IgM was found to be down-regulated at 6 h post stress and increased at 24 h after stress. IgM also was reduced by *Vibrio anguillarum* exposure at early stage of exposure. However no regulation by Vibrio is not a surprise and is supported by previous investigation immunizing rainbow trout with alive vaccine against *Ichthyophthirius multifiliis* and no regulation was

seen even after 4 weeks, on the hand in the study performed by Cui et al., (2010) IgM expression was intensively increased in the orange-spotted grouper head kidney, spleen, thymus gland and blood cells from two weeks until 4 weeks after infection with *Vibrio alginolyticus* and rainbow trout following Yersinia ruckeri infection, thus indicating fish-species and bacterial-species dependent for regulation of IgM (Cui et al., 2010; Jørgensen et al., 2008; Raida and Buchmann, 2007). IgM is the only acquired immune component that has been reported to be modulated by environmental factor such as changing in temperature. Therefore we demonstrated that IgM at transcriptional level is only affected by anoxia in zebrafish spleen. This finding is in agreement with previous study showing heat stress enhanced IgM in head kidney spleen intestine of orange-spotted grouper at 3 h and also increment of IgM in Nile tilapia, *Oreochromis niloticus* (Cui et al., 2010; Dominguez et al., 2004).

In daily aquaculture practice fish are subjected to combination of stressors which belongs to distinct classification of stressors affecting immune system (Varsamos et al., 2006). Tort (2011) has classified stressors into different groups. In the nature stressors manly are considered as a cute since they are derived from challenge. On the other hand, the question that has to be clearly answered is how stress and immune system respond to different stressors which are described as an abiotic an biotic. Thus in the current study we aimed to investigate the occurrence of these two situation in rainbow trout, gilthead seabream and zebrafish. The extent to which different stressors modulate stress and immune response remain to be elucidated. It should be noted that for avoiding any repetition, in here we tried to explain the main message observed after stressors challenge. Overall, although the synergetic effect of two stressors was expected by immune system, the gene expression pattern rarely found to be up-regulated via effect of anoxia and Vibrio, thus it can be suggest that after confronting to combination of different stressors, provoking greater response with regard to magnitude can enhance the risk of tissue damage and also energy demanding which might bring more injury to the animal. This is supported by previous study since bacteriolytic activity under bacterial and viral challenge in gilthead seabream, *Sparus aurata* and European seabass, Dicentrarchus labrax was shown to be enhanced interestingly at low density condition (Mauri et al., 2011).

Conclusion:

Our findings suggest that the well-stablished short term effect of stress on immune function may be described by enhacemnet of glucocorticoids as well as stress and immune gene expression

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8. Chapter sixth

8.4. Expression response of the hypothalamus and pituitary hormone stress genes after vaccination and anoxia in the gilthead sea bream *Sparus aurata*

Abstract

Fish under aquaculture conditions may be subjected to husbandry stressors like transient anoxia or vacination procedures, but it is not known whether fish responds the same way if the stressors are perceived by different routes, and thus, in the present work we subjected sea bream to anoxia and vaccine administration. The results show that stress by anoxia is primarily stimulating both hypothalamic CRH and pituitary POMCB and PRL at short time whereas vaccine stimulates hypothalamic (CRHBP and TRH) and pituitary peptides (POMC, PRL, GH) at longer time points, as a possible result of feedback since GR expression in hypothalamus is also stimulated. Overall the results indicate that first, different stressors show different stimulatory abilities as anoxia is perceived immediately and vaccine is not, and second, that the dynamics of an overall central stress response may be a result of feed back activity as the results suggest after the vaccine treatment.

1. Introduction

Fish under aquaculture conditions may be subjected to different stressors, chemical, physical or biological such as transient anoxia, crowding, handling, ammonia exposure or pathogens or immunogens (Schreck & Tort, 2016; Tort, Pavlidis, & Woo, 2011). While physical stressors are currently perceived by the sensors of the nervous system, pathogens and immunogens such as vaccines enter by contact with skin or external mucosae (Parra et al., 2015) and therefore the physiological stress response takes place only when the immune mediators have interacted with hormone receptors thereby starting the endocrine cascade leading to the release of adrenaline and cortisol (Yada & Tort, 2016). Thus, in this work we have selected two stressors working through different routes such as anoxia and vaccine delivery in order to study the brain response at short term (1 to 48 hours) through gene expression and cortisol levels (Gornati, 2004; Momoda et al., 2007).

The initiation of the systemic stress response in fish and the regulation of the physiological response takes place in the hypothalamus and the pituitary. After the stressor is perceived, four peptides are secreted by the neuroendocrine cells of the ventral parvocellular section of the nucleus preopticus, i.e., Corticotropin Releasing Hormone (CRH)(Flik, Klaren, Burg, Metz, & Huising, 2006), CRH binding peptide (CRHBP), Arginin Vasotoccin (AVT) and Thyroid Releasing Hormone (TRH). CRH, CRHBP and AVT will contact with the receptors of the pars distalis of the pituitary gland to produce ACTH, whereas interaction in the pars intermedia will induce the secretion of MSH and b-endorphin (Bernier, 2009; Cerdá-Reverter & Canosa, 2009). Another part of the nucleus preopticus, the magnoecellular section projects CRH, CRHBP and AVT neuroendocrine cells in the pars nervosa of the pituitary (Gorissen & Flik, 2016). In this work we have assessed the gene expression response of the peptides involved in this process (CRH, CRHBP, TRH, POMCA, POMCB, GH and Prolactin). The sequences for these peptides are available for the seawater fish *Sparus aurata*, an important aquacultured species in the Mediterranean, as a result of previous work from our laboratories (Acerete et al., 2009; (Martos-Sitcha et al., 2014))

Assuming that this organization of the hypothalamus and pituitary is the hierarchical view of the onset of the brain stress reaction (Cerdá-Reverter & Canosa, 2009), other interactions are taking place in both hypothalamus and pituitary, and particularly the feed-back interaction with cortisol via Glucocorticoid Receptor (GR). Therefore stressors may influence peptide activity directly through brain perception or indirectly through glucocorticoid feed-back.

Hence, the present work focus in to ascertain the differential response of the genes encoding for these main peptides of the hypothalamic-pituitary axis in the seawater gilthead seabream to the above mentioned stressors: Transient anoxia, that may be the result of a pumping or oxygen supply failure in recirculation systems in aquaculture facilities or vaccine administration as vaccination of fish against common diseases using formalin-killed bacteria or bacterin as a source of antigen, is a frequent handling practice in aquaculture. In a previous work, it was shown that the stress response of sea bream changed depending on the type of stressor, i.e., salinity changes or food deprivation (Martos-Sitcha et al., 2014). However, no studies have assessed the central hypothalamic-pituitary gene response after vaccination.

2. Material and Methods.

2.1 Animal rearing

Gilthead sea bream (*Sparus aurata*) with body weight of 60-70 g were obtained from AQUACULTURA ELS ALFACS,S.L. (Tarragona). Fish were transferred to the UAB fish facility (AQUAB) to acclimatize to laboratory conditions. All experimental procedures involving fish were submitted and authorized by the Ethical Committee of the "Universitat Autonoma de Barcelona" that agrees with the international Guiding Principles for Biomedical Research Involving Animals (EU2010/63).

2.2 Stressors (anoxia and vaccination)

The gram-negative bacterium *Vibrio anguillarum* is an important pathogen that causes vibriosis in both marine and freshwater fish. ICTHIOVAC ^{R} VR (HIPRA) is an inactivated commercial vaccine which is suitable for immersion delivery. The composition consists of inactivated *Vibrio anguillarum*, serotype O1, O2α and O2β with RPS ≥ 60%, presenting all pathogenic serotypes of the bacterium. As it was already demonstrated the serogroup O2α is the most pathogenic serogroup of the bacterium. Vaccination was performed by immersion making 1 to 10 dilution of vaccine according to guidelines recommended by the company (HIPRA). The second stressor, anoxia, consisted in 1 minute out of the water. To this end, four groups of fish (n=24 fish per group) were used for the experiment. The first group was untreated and used as a control; another one was subjected to anoxia stress and finally the last one was treated with vaccine. Moreover in the vaccinated group, fish were exposed to an acute handling stress (1 minute of anoxia) 24 hours after vaccination and fish were sampled after 1h, 6h,24h and 48h.

2.3 Isolation of RNA and cDNA synthesis

Total RNA was isolated from individual fish using TRI reagent (Sigma) according to manufacturer's instructions. The RNA pellet was dissolved in nuclease free-water and immediately stored at -80ºC until use. The RNA concentration was quantified by NanoDropND-2000 spectrophotometer (Thermo Scientific). RNA (500 ng) was used as template to synthesize complementary DNA (cDNA) using iScript cDNA kit (Bio-Rad Laboratories) according to manufacturer's instructions.

2.4 Quantitative real time PCR (qPCR)

Fish mucosal surfaces including gut, gills and skin samples were analysed using real-time qPCR. The gene expression patterns of immune and stress-related genes (IL-1β, IL-6, TNF-α, IL-10, TGF-β1, IgM, COX2, Lys, HSP70 and C3) were analysed. We first tested several housekeepings candidates (18S, β-actin and RPL27) to elucidate which one had less variation (data not shown). Thus, 18S and RPL27 were selected because of the less variability presented. Specific primers used for gilthead sea bream (Table 1) are indicated. The primers for gilthead sea bream IL-6 and TNF-α were previously designed (Boltana, Tridico, Teles, Mackenzie, & Tort, 2014). The other primers are first used in this study and they were designed with Primer-Blast. The primer secondary structure and primer specificity were checked with OligoAnalyzer (version 3.1) and Primer-Blast software, respectively. Meanwhile, the primers used gave a pure PCR product which is verified by single peak in the melting curve and the amplification efficiency were determined individually in all mucosal surfaces by qPCR and all were around 100%. Real-time PCR reactions were performed with iTaq universal sybr green supermix (Bio-Rad Laboratories) using cDNA dilution made for genes of interest in rainbow trout and gilthead sea bream. Primers for all genes were used at final concentration of 500 nM. The thermal condition used were 3 min at 95ºC of pre-incubation followed by 40 cycles at 95ºC for 30 s and 60ºC for 30 s. All the reactions were performed in duplicate using CFX384 Touch Real-Time PCR Detection System (Bio-Rad Laboratories). The quantification was done according to Pfaffl method corrected for efficiency of each primer set (Pfaffl, 2001). Value for each experimental condition was expressed as normalized relative expression, calculating in relation to values of control group and normalized against those of the housekeeping gene β-actin and 18S for rainbow trout and sea bream, respectively.

2.5. Statistical analysis

The statistical Package for Social Science Software (SPSS version 20) was used for statistical analysis, utilizing the generalized linear model (GzLM: http://refhub.elsevier.com/S0306-4530(15)00909-9/sbref0135). Stressors and dynamism of time were considered as a two factors. This statistical method is flexible since no homogeneity of variance is required. Where a statistical significant interaction was found between stressors and dynamic, we only fulfilled the correlation between time and cortisol and gene expression modulation, meanwhile comparing all groups versus control. It means that no data was analyzed without significant interaction between stressors as well as time. Differences in all data were considered significant when $p\text{-value} < 0.05$ among groups.

3. Results

Figure 1 shows the level of plasma cortisol (in ng/ml) after both stressors and sampling times. As it can be seen, the levels of cortisol indicate an expected response curve after anoxia, with a peak after 1 hour, still significantly higher at 6 hours and followed by a further gradual recovery of basal values at 24 and 48 hours. The dynamics for vaccine plus anoxia is similar although the recovery takes place later on. It is noteworthy that the cortisol response after vaccine does not follow this pattern but not showing sensitivity to such treatment.

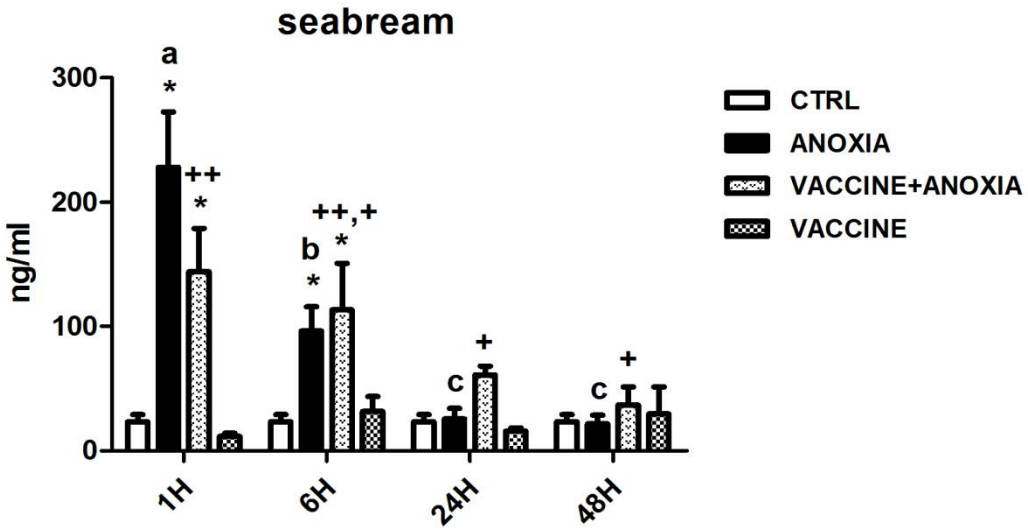

Fig.1. Changes in gilthead seabream plasma cortisol level in response to anoxia and *Vibrio anguillarum* exposure 1, 6, 24 and 48 h post stress. Skin mucus cortisol was measured by radioimmunoassay. Date are presented as mean ±SE. significant differences are indicated by letters in stress group, vaccine+stress by ++ and vaccine group by number. * 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)

The hypothalamic CRH gene expression shows an increase after anoxia at earlier times and a significant increase is also observed for the glucocorticoid receptor gene (GR), and CRHBP and TRH show an increase only after 48 hours of vaccine treatment. TRH gene shows a decrease at 1,6 and 24 hours. In the pituitary the vaccine treatment shows more effects

with an increase of POMCA at 24h and POMCB at 1 and 24h, PRL at 1h and GH at 6 hours. Anoxia induces increases in POMCB and PRL at earlier times and GH and POMCB also at later times.

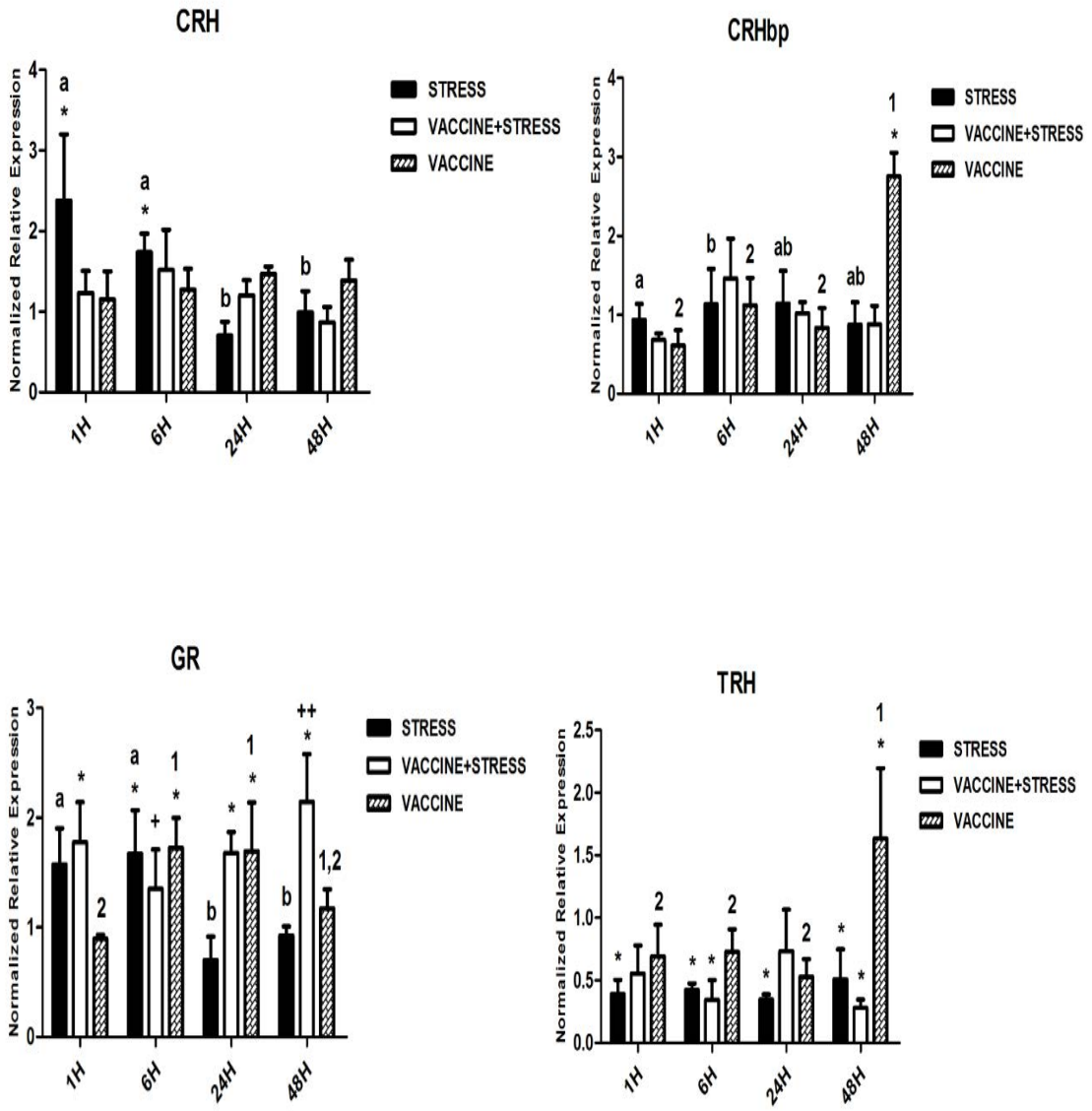

Fig.2. qPCR quantification of specific mRNA accumulation in gilthead seabream hypothalamus subjected at 1, 6, 24 and 48 h post challenge with acute handling stress and *Vibrio anguillarum* exposure. CRH, CRHbp, TRH and GR shown are mRNA relative abundance to 18S and RPL27 housekeeping genes. Date are presented as mean ±SE. significant differences are indicated by letters in stress group, vaccine+stress by ++ and vaccine group by number. * 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).

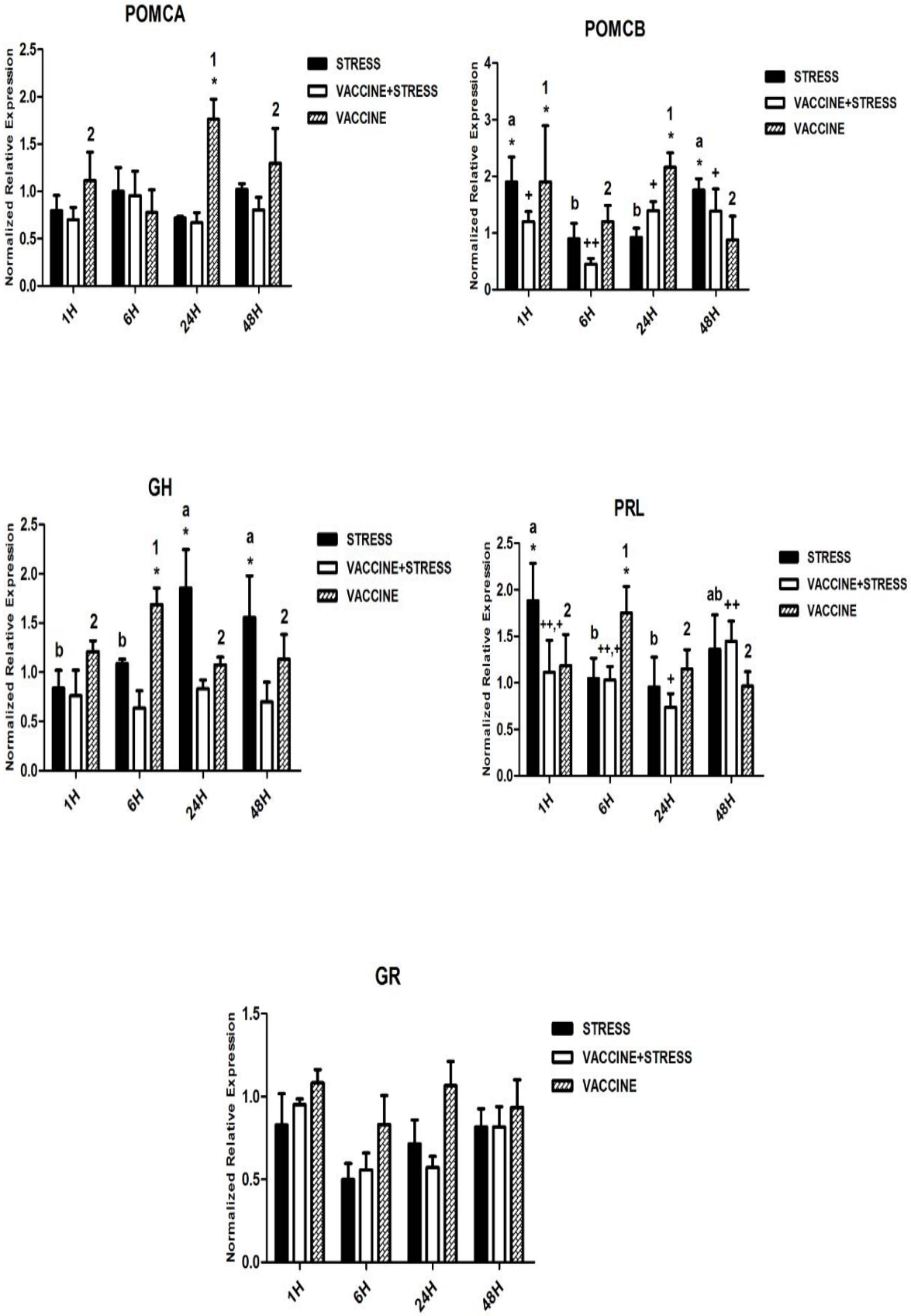

Fig.3. qPCR quantification of specific mRNA accumulation in gilthead seabream pituitary subjected at

1, 6, 24 and 48 h post challenge with acute handling stress and *Vibrio anguillarum* exposure. POMCa, POMCb, PRRL, GH and GR shown are mRNA relative abundance to 18S and RPL27 housekeeping genes. Date are presented as mean ±SE. significant differences are indicated by letters in stress group, vaccine+stress by ++ and vaccine group by number. * 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).

4. Discussion

The purpose of this study was to ascertain how two potential stressors adiminstered through different pathways, activate the gene expression of the responsible peptides for the activation of the systemic response to stress and particularly whether vaccine administration may induce central responses in the stress HPI axis.

The results show that the vaccine does not activate the initial first hours of the response of hipothalamic peptides. Thus CRH does not show significant increase at 1 hour but it does increase at 48 hours after the delivery of the vaccine. At the same time the receptor for glucocorticoids in the hypothalamus does increase expression at any time after vaccine + anoxia. Therefore these data suggest that vaccine does not activate the central stress response unless a physical stressor is included. On the contray, in the pituitary, vaccine does induce the expression of some stress peptides like Prolactine, GH at 6 hours and POMCB and POMCA peptides after 24 hours. Therefore it seems that the pituitary is more sensitive to immune stimulation although at later time points (after 6 and 24 hours) which may indicate a slower stimulation dynamics or that the timulation comes from interaction between messsngers (cytokines and receptors at pituitary level). It is also worth of mention that the sensitivity at the level of the pituitary can be seen through the expression of POMC peptides, since POMCB increases the expression at 1 our and POMCA at 24 hours. At this point the research has not gone further as receptors for POMC have not been yet cloned in sea bream, although attempts have been made by several laboratories.

Compared to vaccine effects, anoxia stimulates CRH and Glucocorticoid receptor expression and down-regulation of TRH at the first hour, indicating a significant stimulation in th hypothalamus by this stressor which is correlated with the activation of POMCB and Prolactin in the pitutary.

Previous work on sea bream shows that salinity changes transferring fish from seawater to freshwater increases the expression of CRH and CRHBP at early stages and maintaining the difference through 24 hours whereas no relevant changes were shown after starvation and refeeding. The authors suggested that CRH could be regulated by CRHBP or that an equilibrium between these two factors would regulate the overall expression of stress hormones (Martos-Sitcha et al., 2014). In other studies the role of CRH in the overall stress

respoonse of fish has been assessed (Flik et al., 2006; Pepels, Meek, Wendelaar Bonga, & Balm, 2002) and it has been proposed more precisely that the interaction between CRH and CRHBP determines the CRH bioactivity and therefore the activation of the CRH receptor in the pituitary (Gorissen & Flik, 2016; Huising et al., 2004). Our present results would support this hypothesis as far as the CRH and CRHBP results are concerned.

The results for TRH show a significant decrease of expression following anoxia or anoxia plus vaccine, but not after vaccine treatment. This response may be related to an inhibition of the thyroid hormone axis regulating energetic responses, due to the anoxic challenge and thus saving energy resources while the anoxia allostatic load is present. This down-regulating response may be somehow related to the expression of POMCB at 6 hours, since a decrease of its expression is found at that time, although the increase of CRH can compensate for this decrease and therefore the dynamics along time would reflect the equilibrium between both peptides.

The glucocorticoid receptor seems to play a key role in the regulation of the stress response at the level of hypothalamus. Together with adrenaline, cortisol is the main stress hormone responsible for binding its receptor in many tissues thus exerting a plethora of effects which will help to cope with the stressors (Mommsen, Vijayan, & Moon, 1999; Rotllant et al., 2000). Among the tissues in which glucocorticoid receptors are found there is the hypothalamus and the pituitary, thus involving a relevant role of GR in terms of the modulation of the cortisol effects (Rotllant et al., 2000) to prevent, for instance, an excessive over stimulation of the HPI axis. Thus, changes of expression are detected either at short time (1 and 6 hours) particularly under anoxia or anoxia+vaccine, but also at longer time following the vaccine or vaccine+anoxia treatment. As mentioned before, this dynamics may reflect the sensitivity response of the GR initially to the anoxia treatment but also an eventual feed-back response to vaccine treatments at longer term (24 and 48 hours). On the other hand it is noteworthy that GR does not show significant differences in the pituitary. This seems to suggest that the main fedd-back action of cortisol, at very high levels after 1hour and 6 hours in plasma, is performed in the hypothalamus rather than in the pituitary, as far as the present results are concerned.

Conclusion:

In conclusion, our results indicate that first, the stress response of the hypothalamus and the pituitary is clearly dependent on the stressor, that the GR has an important feed-back role, specifically at the hypothalamus and that the vaccine is not able to initiate a central stress response at short time, but it shows significant changes at longer times.

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Table 1: Primers used in real-time PCR for gene expression analysis for gilthead seabream

Gene	Gene bank accession number	Sequence 5′–3′	Product size
CRH	KC195964.1	**FW:** ATGGAGAGGGGAAGGAGGT	176
		RV: ATCTTTGGCGGACTGGAAA	
CRHbp	KC195965.1	**FW:** GCAGCTTCTCCATCATCTACC	147
		RV: ACGTGTCGATACCGCTTCC	
GH	U01301.1	**FW:** CGTCTCTTCTCAGCCGAT	131
		RV: GCTGGTCCTCCGTCTGC	
GR	DQ486890.1	**FW:** TGCTGGCGGAGATCATCACCA	182
		RV: GCAGGCCAAGCGAAGGCTTA	
pomca	HM584909.1	**FW:** AGCCAGAAGAGAGAGCAGTGAT	120
		RV: ATCGGGTCAGAAAACACTCA	
pomcb	HM584910.1	**FW:** AGCTCGCCAGTGAGCTGT	81
		RV: CCTCCTGCATCACTTCCTG	
PRL	AF060541.1	**FW:** TGACATCGGCGAGGACAACATT	111
		RV: CGGCAGCGGAGGACTTTCAG	
Sl1	Y11144.1	**FW:** GCCAGCGAGGAGGAATACAC	126
		RV: GGCAACAGAGGGAAAACTCC	
Sl2	L49205.1	**FW:** CAACAGAGGGCAAAGTGGA	120
		RV: AGAGCCAGCGAGGAATACAA	
TRH	KC196277.1	**FW:** GAAACGCTTTTGGGATAACTCC	131
		RV: CGGCGTGACTCTTGTTTATGTT	
18S	AY587263.1	**FW:** ACCAGACAAATCGCTCCACC	172
		RV: AGGAATTGACGGAAGGGCAC	
RPL27	AY188520	**FW:** AAGAGGAACACAACTCACTGCCCCAC	160
		RV: GCTTGCCTTTGCCCAGAACTTTGTAG	

9. General discussion

The accumulation of the investigations concerning immune system has revealed the presence in teleosts of the main immune mechanisms described for mammals. Teleosts are armed by both innate and adaptive immunity in which the innate immune arm provides the immediate first line of defence (Medzhitov, 2007). The immune system is considered to respond to danger signals both internal and external that may cause damage rather than to those signals that are simple foreign (Matzinger, 1994). In fact substantial progress has recently been made in the characterization of the fish immune system and its relationship with other regulatory networks such as the endocrine system; nevertheless there are still important gaps in the knowledge of the modulation of the immune system through hormones.

In the current thesis we tried to investigate the effect of stress hormones on head kidney of rainbow trout and gilthead seabream. Modulation of the immune system by those hormones secreted by hypothalamic-pituitary-interrenal axis (HPI) and sympathetic-adreno-medullar (SAM) including cortisol, ACTH and catecholamine has been demonstrated. Meanwhile other authors showed that other hormones such as growth hormone (GH), prolactin (PRL) and proopiomelanocortin (POMC)are also effective regarding interaction with the immune system (Harris and Bird, 2000). Thus, the existence of bidirectional interaction between neuro-endocrine and immune system appears to be indispensable. Indeed fish are an intriguing model in this respect, as head kidney is the main producer of hematopoietic cells and also is the producer of cortisol by interrenal cells and catecholamines by chromaffin cells (Chadzinska et al., 2012; Carl B. Schreck and Tort, 2016).

Our data in the head kidney of rainbow trout and gilthead seabream revealed the distinct effect of glucocorticoids on cytokine expression including IL-β1, IL-6, TNF-α, IL-10 and TGF-β1 in both species. No modulation occurred on cytokines by cortisol treatment (200 ng/ml, mimicking the acute stress response in fish), while the transcriptional level of all seabream cytokines except TGF-β1 was found to be suppressed by cortisol (Khansari et al., 2017), the findings is supported by previous results obtained by our research group (Castillo et al., 2009). This suppressive effect of cortisol was abolished by incubation with RU-486, a well-known glucocorticoid receptor (GR) blocker. On the other hand, cortisol was not able to induce any alteration in rainbow trout head kidney. Cortisol up-regulated rainbow trout hepatocyte IL-β1 under repeated handling stress and consequently cortisol increment enhanced IL-β1 expression, whereas suppression of cytokines was observed in seabream (Castillo et al., 2009; Fast et al., 2008; Philip et al., 2012). Adrenaline as the main product of the SAM axis in the head kidney chromaffin cells has not been studied as much as other products of the endocrine system. Interestingly, data showed an inverse effect of adrenaline suppressing IL-β and IL-6 in rainbow trout, while enhancing them in gilthead seabream and the effect of adrenaline was block via propranolol administration. Overall, we demonstrate

enhancing versus suppressing effect of adrenaline on cytokines in two species and also indicating the main effect of catecholamine is mediated through β-Adrenoceptor. Furthermore, previous studies in vertebrates have shown that stress hormones enhance immune system function under some circumstances, while suppressing under others (Dhabhar, 2009). *Vibrio anguillarum* is described as one of the ubiquitous and prevalent pathogens in aquaculture that cause vibriosis in both fresh and sea water fish (Zhang et al., 2012), thus the bidirectional interaction between local endocrine and immune system was investigated utilizing the bacterin. When looking at the effect of stress hormones following immune activation by using *Vibrio anguillarum* exposure and antagonist receptor, the mRNA level of rainbow trout IL-β1 and IL-6, as they are implicated in pro-inflammatory reaction for clearance of pathogen (Wang and Secombes, 2013), were markedly increased by Vibrio, while in much less magnitude in seabream. On the other hand, it was not a surprise the up-regulation of anti-inflammatory agents such as IL-10 and TGFβ1 in rainbow head kidney and IL-10 in seabream, as the induced inflammation should be regulated and terminated in order to avoid tissue injury (Delves et al., 2006). Thereby our data suggest higher sensitivity of rainbow trout head kidney than gilthead seabream. Our findings also showed no effect of cortisol treatment (200 ng/ml) on induced-cytokines response in trout. In contrary cortisol as the major GCs was able to suppress cytokine expression in seabream. This is supported by previous results obtained in seabream and other species illustrating the suppressive effect of GCs (Castillo et al., 2009; Flory and Bayne, 1991; Saeij et al., 2003). The difference in the cytokine response appears to be species-specific as it was already explained above. Regarding GR signalling which has been shown to mediate the effect of cortisol on cells (Aluru and Vijayan, 2007), we found that only for seabream IL-β1 and TNF-α cortisol effect could be fully blocked through incubation with RU-486, and spironolactone was not able to antagonise GR, although both antagonists have already been shown to bind GR and MR receptor respectively (McCormick et al., 2008). Adrenocorticotropic hormone (ACTH) plays a key role in cortisol secretion pathway and also the expression of ACTH receptor MC2R has been found in immune cells (Mola et al., 2005), thus modulating the immune function both in mammals and fish (Castillo et al., 2009; Csaba and Pallinger, 2007). In gilthead seabream, not in rainbow trout ACTH was seen to be effective on cytokines suppression, thereby indicating species-specific response mediated by this neuro-peptide. In contrary to the first study, rainbow trout stimulated-cytokines was not affected by adrenaline, whereas adrenaline enhanced expression of IL-6 as well as TGF-β1. It suggests also species-dependent response following bacterin stimulation, though different GCs regulatory pathways on immune system, as mentioned already.

We have also studied the mucosal immunity in rainbow trout and gilthead seabream. As the physical barrier mucosae have been described to be the first line of defence to environmental challenges (Salinas, 2015). According to mammalian nomenclatures mucosal-associated-lymphoid tissue are classified into three different organs including gut associated-lymphoid-tissue (GALT), skin-associated-lymphoid-tissue (SALT) and gills –

associated lymphoid-tissue (GIALT) all of them being immunological active surfaces (Salinas et al., 2011). In this current work the effect of acute handling stress (anoxia) and *Vibrio anguillarum* exposure has been investigated at 1, 6 and 24 h post stress in rainbow trout and gilthead seabream. It has been widely shown that stress result in activation of HPI axis and thus cortisol secretion (Castillo et al., 2008; Cortés et al., 2013; Fierro-castro et al., 2015; Ramsay et al., 2009). Although there is no evidence on whether secreted cortisol in mucosal surfaces, particularly in skin mucus, is delivered from systemic compartments our data show an increase of cortisol level by anoxia in the skin mucus of both species. The elevation was shown to be time-dependent, reaching the maximum in seabream with delay. Interestingly *Vibrio anguillarum* exposure did stimulate cortisol secretion in skin mucus of rainbow trout. It should be noted that the inverse communication between immune and neuro-endocrine has been documented elevating cortisol level with the presence of pathogens (Bilodeau et al., 2003; Bowers et al., 2000). Hence, Vibrio was not able to provoke cortisol secretion in seabream skin mucus under the present conditions. On the other hand combination of both stressors was able to induce HPI axis activation in both species. IgM at protein level, one of the major immunoglobulins coating microbiota and providing protection against pathogens (Parra et al., 2015), was also examined by using ELISA method. IgM shows no regulation by *Vibrio anguillarum* which is reasonable since activation of adaptive immunity and secretion of suitable antibodies versus pathogens needs time, at least couple of weeks (Raida and Buchmann, 2008, 2007). Interestingly our data showed transcription of IgM at protein level in seabream induced by acute stress. In fact IgM is the only adaptive immune component which has been evident to be regulated by environmental changes even 3 h post stress in several organs (Cui et al., 2010), thus indicating the specific response of IgM at protein level in seabream skin mucus. Cytokines and other genes related to stress and innate immune responses were also examined by qPCR to determine how acute stress and *Vibrio anguillarum* could drive mRNA transcription after exposure. Altogether, stress and immune genes were found to be clearly expressed in gilthead seabream skin in greater magnitude by anoxia or combination of both stressors than inrainbow trout skin, and also seabream gut appears to be affected mainly by acute stress. Contrarily rainbow trout gills have been shown to be chiefly affected by vaccine. By taking into consideration the result of cortisol and IgM at protein level, it is clearly observed that seabream is more sensitive than rainbow trout to stressors. In addition, we highlight the result of our study that TGF-β1 seems to be in charge of the anti-inflammatory response in trout, while IL-10 acts as an anti-inflammatory agent in seabream.

Regarding the effect of anoxia stress and vaccination at systemic level, the investigation focused in spleen and liver. Both challenges anoxia and anoxia+vaccine (vaccine+stress) induced HPI axis activation in all the species utilized in the study, so that cortisol level reached the maximum level at 1 h and depending on fish species levels slightly decreased and returned to the basal level at 24 h except for zebrafish, since the combination of both stressors induced longer activation of neuroendocrine system. This activation is in

agreement with previous result showing cortisol release into circulation within few minutes until 1 h after stimulation (Grutter, 2000). The presented results suggest that the marine fish seabream produced higher amounts of cortisol than rainbow trout, that could indicate a higher susceptibility to stressors. In other words, as a response to the same intensity and duration of an acute stressor we conclude that trout, a freshwater fish, is less stressed than seabream, a seawater fish. Vaccine immersion was capable of producing cortisol only in zebrafish in which cortisol was elevated at 6 h. Therefore the zebrafish endocrine system appears to be the only specie that was affected via bacterin. Furthermore, the expression pattern of pro and anti-inflammatory cytokines (IL-β1, TNF-α, IL-10 and TGF-β1), stress genes (enolase, HSP70 and GR) and innate immune genes (C3, Lys and IgM) were found to be suppressed by stressors at systemic level, though the gene expression pattern appear to be up-regulation at mucosal surfaces.

When looking at the response of the central regulatory organs, hypothalamus and pituitary, our work show that anoxia stimulates primarily the hypothalamic peptides and that the vaccine treatment affects hypothalamus and pituitary at later stages, signaling a relevant contribution of cortisol and glucocorticoid receptors that would be involved in the central regulation of the response via feed-back action.

The overall results suggest that first, under stress circumstances, mucosal immunity looks more sensitivity than systemic organs in response to the studied stimuli, and concomitantly the reaction at systemic level shows lower intensity. Second, that our results show a very clear differential response among the species studied. Therefore we can speculate that other fish species, particularly thos with very different environmental conditions, different coping strategies or different feeding habits or diets, should show significant differences with the species that we have studied. This would be supported by the fact that fish species evolved early in the evolution thus occupying a wide range of natural niches with the result of being around 40% of the vertebrate species. Third, that the relationship between the neural, endocrine and immune systems is very intense and that each one of these systems is stimulated under stress circumstances, not only to cope individually with the stressors, but to signal the other systems for a better and more effective coping with the allostatic load involved and to regain homeostasis(C.B. Schreck and Tort, 2016) . In summary, the stress response, as studied in this work, shows a wider and more complex picture that the one found in the classical literature on this subject, with contributions from central (hypothalamus, pituitary, head kidney), peripheral (mucosal surfaces), and systemic compartments (blood, tissues) working at similar or different time points. This picture includes also an intrincate network of relationship and mediators that connect the different compartments and regulatory centers to better cope with the stressor challenges.

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Research paper

Cytokine modulation by stress hormones and antagonist specific hormonal inhibition in rainbow trout (*Oncorhynchus mykiss*) and gilthead sea bream (*Sparus aurata*) head kidney primary cell culture

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ABSTRACT

A tight interaction between endocrine and immune systems takes place mainly due to the key role of head kidney in both hormone and cytokine secretion, particularly under stress situations in which the physiological response promotes the synthesis and release of stress hormones which may lead into immunomodulation as side effect. Although such interaction has been previously investigated, this study evaluated for the first time the effect of stress-associated hormones together with their receptor antagonists on the expression of cytokine genes in head kidney primary cell culture (HKPCC) of the freshwater rainbow trout (*Oncorhynchus mykiss*) and the seawater gilthead sea bream (*Sparus aurata*). The results showed a striking difference when comparing the response obtained in trout and seabream. Cortisol and adrenocorticotropic hormone (ACTH) decreased the expression of immune-related genes in sea bream but not in rainbow trout and this cortisol effect was reverted by the antagonist mifepristone but not spironolactone. On the other hand, while adrenaline reduced the expression of pro-inflammatory cytokines (IL-1β, IL-6) in rainbow trout, the opposite effect was observed in sea bream showing an increased expression (IL-1β, IL-6). Interestingly, this effect was reverted by antagonist propranolol but not phentolamine. Overall, our results confirm the regional interaction between endocrine and cytokine messengers and a clear difference in the sensitivity to the hormonal stimuli between the two species.

1. Introduction

Stressors may compromise the overall health status in fish including increased susceptibility to pathogens and reduced disease resistance. The stress response, as a general, non-specific and widespread reaction, involves all physiological systems, and particularly the neuroendocrine and immune systems which are tightly connected (Engelsma et al., 2002). In fish, the head kidney plays a principal role in this network since, not only is crucial for the organization of the systemic stress response in fish, secreting corticosteroids and catecholamines, but also for its main role in the immune response as lymphopoietic tissue, and in energetics as the producer and supplier of oxygen carrying red blood cells.

It has been demonstrated that the Hypothalamus-Pituitary-Interrenal (HPI) and Sympathetic Adreno-Medullar (SAM) axes, as the two major pathways by which the endocrine response is organized, modify the immune function in mammals and fish (MacKenzie et al., 2006; Padgett and Glaser, 2003). Cortisol is secreted by head kidney interrenal cells in a concatenated response involving the hypothalamic corticotrophin-releasing hormone (CRH) and the adrenocorticotropic hormone (ACTH) secretion. Thus, secreted ACTH is recognized by melanocrotin receptor 2 (MC2R) on the surface of the interrenal cells and activates a signalling cascade which mediates the secretion of cortisol. Cortisol is the major glucocorticoid (GC) in teleost fish and the final product of HPI axis activation. It plays essential roles in energy homeostasis, including balance maintenance, modulation of the immune response, and regulates behaviour through genomic (slow) and non-genomic (fast) mechanisms in the central nervous system (Castro et al., 2011; Cortés et al., 2013; Mommsen et al., 1999).

Abbreviations: HPI, Hypothalamus-Pituitary-Interrenal; SAM, Sympathetic-Adreno-Medullar; CRH, corticotrophin-releasing hormone; ACTH, adrenocorticotropic hormone; MC2R, melanocrotin receptor 2; GCs, glucocorticoids; GREs, glucocorticoid response elements; GR, glucocorticoid receptor; MR, mineralocorticoid receptor; AR, adrenergic receptor; HKPCC, head kidney primary cell culture.

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