



## OPEN ACCESS

## EDITED BY

Kang-le Lu,  
Jimei University, China

## REVIEWED BY

Carlo C. Lazado,  
Norwegian Institute of Food, Fisheries  
and Aquaculture Research (Nofima),  
Norway  
Dariel Tovar-Ramírez,  
Centro de Investigación Biológica del  
Noroeste (CIBNOR), Mexico

## \*CORRESPONDENCE

Lluís Tort  
Lluís.Tort@uab.es  
Felipe E. Reyes-López  
felipe.reyes.l@usach.cl

## SPECIALTY SECTION

This article was submitted to  
Aquatic Physiology,  
a section of the journal  
Frontiers in Marine Science

RECEIVED 24 June 2022

ACCEPTED 25 July 2022

PUBLISHED 17 August 2022

## CITATION

Vallejos-Vidal E, Sanz-Milián B,  
Teles M, Reyes-Cerpa S, Mancera JM,  
Tort L and Reyes-López FE (2022) The  
gene expression profile of the  
*glucocorticoid receptor 1 (gr1)* but not  
*gr2* is modulated in mucosal tissues of  
gilthead sea bream (*Sparus aurata*)  
exposed to acute air-exposure stress.  
*Front. Mar. Sci.* 9:977719.  
doi: 10.3389/fmars.2022.977719

## COPYRIGHT

© 2022 Vallejos-Vidal, Sanz-Milián,  
Teles, Reyes-Cerpa, Mancera, Tort and  
Reyes-López. This is an open-access  
article distributed under the terms of  
the [Creative Commons Attribution  
License \(CC BY\)](https://creativecommons.org/licenses/by/4.0/). The use, distribution  
or reproduction in other forums is  
permitted, provided the original  
author(s) and the copyright owner(s)  
are credited and that the original  
publication in this journal is cited, in  
accordance with accepted academic  
practice. No use, distribution or  
reproduction is permitted which does  
not comply with these terms.

# The gene expression profile of the *glucocorticoid receptor 1 (gr1)* but not *gr2* is modulated in mucosal tissues of gilthead sea bream (*Sparus aurata*) exposed to acute air-exposure stress

Eva Vallejos-Vidal<sup>1</sup>, Beatriz Sanz-Milián<sup>2</sup>, Mariana Teles<sup>2,3</sup>,  
Sebastián Reyes-Cerpa<sup>4,5</sup>, Juan Miguel Mancera<sup>6</sup>, Lluís Tort<sup>2\*</sup>  
and Felipe E. Reyes-López<sup>2,7\*</sup>

<sup>1</sup>Facultad de Medicina Veterinaria y Agronomía, Universidad de Las Américas, Santiago, Chile,

<sup>2</sup>Department of Cell Biology, Physiology, and Immunology, Universitat Autònoma de Barcelona, Bellaterra, Spain, <sup>3</sup>Institute of Biotechnology and Biomedicine, Universitat Autònoma de Barcelona, Barcelona, Spain, <sup>4</sup>Centro de Genómica y Bioinformática, Facultad de Ciencias, Universidad Mayor, Santiago, Chile, <sup>5</sup>Escuela de Biotecnología, Facultad de Ciencias, Universidad Mayor, Santiago, Chile, <sup>6</sup>Department of Biology, Faculty of Marine and Environmental Sciences, Instituto Universitario de Investigación Marina (INMAR), Campus de Excelencia Internacional del Mar (CEI-MAR), University of Cádiz, Cádiz, Spain, <sup>7</sup>Centro de Biotecnología Acuícola, Facultad de Química y Biología, Universidad de Santiago de Chile, Santiago, Chile

The perception of an acute stressor (short-duration; high-intensity) induces a physiological response that activates the hypothalamic-pituitary-interrenal (HPI) axis and the subsequent release of cortisol. Cortisol carries out its effect at the molecular level through its recognition by the mineralocorticoid receptor (MR) and the glucocorticoid receptor (GR). Recently, we unveiled the nucleotide sequence of the glucocorticoid receptor 1 (*gr1*) and *gr2* in gilthead sea bream (*Sparus aurata*). Importantly, GR1 and GR2 respond to different levels of cortisol concentration in fish and, consequently, play a differential role in the stress response. To date, and despite their relevance, no data describes the modulation of these receptors in response to an acute stressor in gilthead sea bream (*S. aurata*). In this study, we evaluated the kinetics of modulation of cortisol receptors expression (*gr1*, *gr2*, *mr*), and its similarity with the expression pattern of selected genes associated with stress (*hsp70*; *enolase*) and immune response (*lysozyme*; *c3*; *il-1β*; *tnf-α*; *il-10*; *tgf-β1*) in gilthead sea bream mucosal tissues (skin; gills; anterior gut). To do it, fish were acutely stressed by three-minute air exposure, and the expression profile was evaluated at zero, 1 h, 6 h, and 24 h post-stress (hps). The cortisol level in plasma and skin mucus peaked at 1 hps. All the mucosal tissues showed a time-dependent and tissue-specific upregulation of *gr1* and *mr*. The immune-related genes showed the upregulation of *il-1β* at 6 hps (gills; anterior gut), and *tnf-α* and *c3* at 24 hps (anterior gut). Taking together, our study concludes that fish subjected to three-minute air exposure modulated the expression of

*gr1* but not *gr2* in mucosal tissues (skin; gills; anterior gut). Furthermore, our data reinforce the idea of a stimulatory effect induced in genes associated with the innate immune response after acute stress but focused at the mucosal level and in a time- and tissue-dependent manner.

#### KEYWORDS

Mucosal-associated lymphoid tissues (MALT), cortisol, glucocorticoid receptors (GRs), fish skin mucosa, acute stress response, fish immune response

## Introduction

Stress is a situation in which an organism is subjected to an alteration (exposure to a stressor) that can result in real or symbolic challenge to its integrity (Tort, 2011). Depending on the time that the organism is exposed, stress can be classified as acute (short-term and high-intensity) or chronic (sustained over time). Acute stress situations provoke “fight or flight reactions” that imply the survival or death of the individual. In aquaculture, fish can be subjected to acute stressors due to intrinsic processes of production, and to the use of intensive systems associated to culture strategies (exposure to air, chase, catch, handling) (Iversen et al., 2014; Khansari et al., 2017c).

In teleosts, cortisol is the main corticosteroid and plays an important role in many physiological areas including growth, immunoregulation, and energy maintenance (Tsalafouta et al., 2018). A stress situation causes the activation of a coordinated system of two physiological axes: the HPI axis (Hypothalamus-Pituitary-Interrenal axis) where the head kidney interrenal cells produce corticosteroids (cortisol), and the HSC axis (Hypothalamus-Sympathetic-Chromaffin axis) where the chromaffin cells produce catecholamines (epinephrine and norepinephrine) (Khansari et al., 2019). Immediately after sensing the stressful stimulus, catecholamines activate cardiovascular, respiratory, and metabolic responses, aimed at mitigating the effects of the acute stressors. This mechanism of response is immediately activated once the stimulus is perceived, while the release of glucocorticoid hormones is slightly delayed by minutes (Schreck and Tort, 2016). Then, the HPI axis involves the immediate secretion of corticosteroid-releasing hormone (CRH) by the preoptic nucleus of the hypothalamus (Parra et al., 2015). Stimulated CRH receptors of corticotropic cells of the pituitary gland induce the release of adrenocorticotrophic hormone (ACTH) into the circulation. It subsequently stimulates the release of cortisol from the interrenal cells of the head kidney, as well as melanocyte-stimulating hormone ( $\alpha$ -MSH) (Tort, 2011).

Cortisol induces relevant effects on immune responses such as an increase in neutrophils, a reduction in the number of lymphocytes and antibody responses, or a decrease in the production of some cytokines, such as TNF- $\alpha$ , TGF- $\beta$ , and IL-6 (Parra et al., 2015). However, it seems difficult to conclusively state that acute stress has a definitive immunoenhancing effect in fish (Guo and Dixon, 2021). There is consensus that the greater energy demand required in response to the activation of the stress response produces a lack of energy resources destined to other processes, which compromises the immune response (Tort, 2011). In teleosts, the head kidney is a lymphohematopoietic organ with endocrine functions (Parra et al., 2015). The head kidney acts as an endocrine gland responsible for the secretion of stress hormones and also as an intermediary tissue for the regulation of immune processes in response to stressors (Khansari et al., 2017b; Khansari et al., 2017a). Thus, the activation of the stress response, and consequently the increase in cortisol, produces a series of secondary physiological responses in fish (changes in the metabolism and osmoregulation, and immunosuppression, among others) (Schreck and Tort, 2016).

Mucosal surfaces have a pivotal role in fish health. The mucosal surfaces play a key role in protecting the body from threats and infections. They act as the first body barrier when contacting with the external agents, with the benefits and threats that this implies, such as the interaction with pathogens. Thus, mucosal tissues register a wide repertoire of responses both at the innate and specific immunity level (Esteban and Cerezuela, 2015; Koppang et al., 2015; Merrifield and Rodiles, 2015; Salinas and Parra, 2015). For this reason, they have been described as mucosal-associated lymphoid tissues (MALT), including the nose-associated lymphoid tissue (NALT), skin-associated lymphoid tissue (SALT), gill-associated lymphoid tissue (GIALT), and gut-associated lymphoid tissue (GALT). The skin, gills, and gut are the mucosal tissues in which special emphasis has been placed on the evaluation of the stress response at the hormone and gene expression levels. The presence of stress hormones in

mucosa-associated tissues has attracted special interest because of its relevance as a potential non-invasive biomarker but also due to the modulation of genes associated with stress and immune response at the local level (Khansari et al., 2018). In this way, the measurement of cortisol in blood has been the traditional method for assessing the fish stress response performance. In the last years, new sample collection strategies have been used to measure cortisol including skin mucus (Khansari et al., 2018; Franco-Martinez et al., 2022). Importantly, it has been shown in rainbow trout that the measurement of cortisol levels in skin mucus accurately reflects plasma responses to stress at early times of exposure (Carbajal et al., 2019). In other words, the cortisol detected in skin mucus would be the reflection of the high circulating concentrations at systemic (bloodstream) level (Carbajal et al., 2019). Importantly, it has been recently proposed that the cortisol secreted after an intense activation of the HPI axis but not the exogenous cortisol sensed from the environment would be responsible of the changes in the expression profile in fish mucosa (Vallejos-Vidal et al., 2022). This acquires special relevance when considering the immunoprotective and defense role of mucosa-associated tissues (Parra et al., 2015), and the repercussions of the presence of cortisol in the modulation over time of the expression of genes involved in the immune response to a given stressor stimulus.

Cortisol is recognized by the mineralocorticoid receptor (MR) and the glucocorticoid receptors 1 (GR1) and 2 (GR2), thus carrying out its mechanism of action at the physiological level. The identification of glucocorticoid receptors in the different species of teleost has been a challenge and a matter of interest given their relevance in the activation of the stress response by different levels of cortisol (Bury et al., 2003; Stolte et al., 2006). Thus, GR1 would be responsible for the recognition of cortisol at higher concentrations, meanwhile the GR2 would act at a very low concentration of cortisol (Vazzana et al., 2010). In the case of gilthead sea bream, our research group reported in 2007 (Acerete et al., 2007) a single GR. Years later, in 2018, another study indicated that such *gr* nucleotide sequence corresponded to *gr1* (Tsalafouta et al., 2018). A recent study made by our research team determined that the difference at nucleotide sequence level between *gr1* and *gr2* resides in an insertion/deletion in the DNA binding region of gilthead sea bream (*S. aurata*), thus unveiling the uncertainty that existed around the glucocorticoid receptors in gilthead sea bream. At the same time, this finding evidences the lack of knowledge regarding the pattern of gene expression of *gr1* and *gr2* in gilthead sea bream in the presence of a stressor. Thus, in this study, we aimed to evaluate the gene expression profile for glucocorticoid receptors (*gr1*, *gr2*) in gilthead sea bream. We subjected fish to air exposure as a model of stress response

induction by the activation of the HSC and HPI axes (Arends et al., 1999; Khansari et al., 2018; Skrzynska et al., 2018; Khansari et al., 2019; Liu et al., 2019). We also evaluated the expression pattern of mineralocorticoid receptor (*mr*) and genes associated with stress (*hsp70*; *enolase*) and immune response (*lysozyme*; *c3*; *il-1 $\beta$* ; *tnf- $\alpha$* ; *il-10*; *tgf- $\beta$ 1*) in gilthead sea bream mucosal tissues (skin; gills; anterior gut). The acute stress response was analyzed at zero, 1 h, 6 h, and 24 h post-stress (hps). Taking together, our study concludes that fish subjected to three-minute air exposure modulated the expression of *gr1* but not *gr2* in mucosal tissues (skin; gills; anterior gut).

## Materials and methods

### Fish and acclimatization

Gilthead sea bream (*S. aurata*, Linnaeus, 1758) were obtained from a local fish farm (Culmarex, Puerto de Sagunto, Valencia, Spain), with a length of  $23.59 \pm 0.83$  cm (mean  $\pm$  standard deviation) and a body weight of  $150 \pm 5.2$  g (n total= 32). The fish were acclimatized to the laboratory conditions in the same tank for 30 days. Fish were kept in a closed recirculating seawater system at 21–22°C, on a 12-h light/12-h dark cycle, and fed a commercial diet at 1.5% of total body weight per day (Skretting). They were kept in 300-liter tanks at a density of 2 kg/m<sup>3</sup>. The fish were fasted 24 h before the experimental procedures. Dissolved oxygen ( $7.1 \pm 0.1$  mg/l), pH ( $7.1 \pm 0.1$ ), nitrite (<0.5 mg/l), nitrate (<10 mg/l), and total ammonia ( $\leq 0.5$ ) were analyzed periodically.

### Ethics Statement

All procedures were carried out in accordance with the ethical guidelines for animal experimentation (EEC regulation 86/609), and authorized and supervised by the ethics committee of the “Universitat Autònoma de Barcelona” (Ref. OH4218\_4219).

### Experimental design

Fish were fasted 24 h before the experimental procedure. Gilthead sea bream (total n = 24) were stressed by exposure to the air for 3 minutes using a net because it has been shown to be effective in inducing the stress response (Arends et al., 1999; Khansari et al., 2018; Skrzynska et al., 2018; Khansari et al., 2019; Liu et al., 2019). Then, fish were placed in three independent tanks (n=8 fish in each tank; one tank per sampling time-point) at a culture density of 2 kg/m<sup>3</sup>. Each tank has a closed recirculation system consisting of a water pump, solid filter,

and a mature biofilter. Non-stressed fish (n=8) were used as a control group and maintained at a density of 2 kg/m<sup>3</sup>.

Fish were sampled at zero (control group), and 1 h, 6 h, and 24 hours post-stress (hps) (n=8 per time-point, coming all sampled fish from the same tank). All animals were sacrificed by anesthetic overdose (MS222 (Sigma), 200 mg/l). For the collection of plasma samples, the tubes and syringes were previously heparinized. Once collected, the blood sample was centrifuged at 3000 x g and 4°C for 10 minutes. Each sample of plasma obtained (supernatant) was stored at -20°C. On the other hand, the skin mucus samples were collected as described elsewhere (Khansari et al., 2018), and immediately placed on ice. The samples were homogenized in a 1:1 volume of TBS buffer and centrifuged at 3500 x g at 4°C for 10 min. The collected supernatant was stored at -80°C. Skin tissue samples were taken from the upper lateral line area behind the dorsal fin, the left side, and avoiding muscle contamination (Vallejos-Vidal et al., 2022). Then, the body cavity was opened laterally and the anterior gut was sampled removing the feces and mucus carefully with forceps, and then rinsed once with PBS. For gills, the first lamella from the left side was sampled (Khansari et al., 2018). Tissue samples were immediately frozen in liquid nitrogen and stored at -80°C for further processing.

## Quantification of cortisol in plasma and skin mucus

Cortisol level was measured by radioimmunoassay (RIA) and radioactivity was quantified using a liquid scintillation counter (Scintillation Counter Wallac 1409; PerkinElmer), as described elsewhere (Vargas et al., 2018). Anti-cortisol antibody (ref. 07-121016, MP Biomedicals, Solon, OH, USA) was used for the assay at the final dilution of 1:4,500. The cross-reactivity of the antibody with cortisol was 100%, and the lowest detection limit of the assay was 0.16 ng/ml. Cross-reactivity with other steroid hormones ranged from 1.6% for corticosterone to less than 0.7% for other steroids tested. The analysis was conducted in duplicate for each sample analyzed (n=8).

## RNA extraction and complementary DNA synthesis

The total RNA was extracted from each individual tissue sample using the TriReagent (Sigma) according to the manufacturer's instructions. In brief, 50-100 mg of tissue was homogenized in 1 ml TriReagent<sup>®</sup> (Sigma) with a Polytron and then incubated at room temperature for 5 min. Subsequently, 0.1 mL of BCP (Sigma) was added to each sample, vortexed, and incubated at room temperature

for 15 min. The samples were centrifuged at 12,000 x g for 15 minutes at 4°C to separate the phases. After transferring the aqueous phase to a new tube, the RNA was precipitated by adding 0.5 ml of 2-propanol (Sigma). The samples were incubated at -20°C overnight and subsequently centrifuged at 12,000 x g for 10 min at 4°C. Finally, the RNA pellet was washed twice with 1 ml of 75% ethanol (prepared with DEPC-water), air-dried for 10 min, resuspended in DEPC-water, and then incubated in a thermoblock at 55°C for 10 min. The RNA sample was quantified with a Nanodrop ND-1000 (Thermo Scientific) and the quality of the RNA was verified by Bioanalyzer (Agilent Technologies). All the RNA integrity number (RIN) values obtained were > 7.0, which indicates the integrity and quality of the RNA suitable for transcriptomics analysis. Subsequently, cDNA was prepared from 1000 ng of total RNA, using the iScript cDNA kit (Bio-Rad Laboratories) according to the manufacturer's instructions.

## Real-time quantitative PCR

The immunological gene expression profiling in gilthead sea bream subjected to air-exposure acute stress was assessed in the skin, gills, and anterior gut. Thus, the transcript level of *interleukin 1-beta (il-1β)*, *tumor necrosis factor-alpha (tnf-α)*, *interleukin 10 (il-10)*, *tumor growth factor-beta 1 (tgf-β1)*, *lysozyme (lys)*, *complement protein c3 (c3)*, the *glucocorticoid receptor (gr) 1 (gr1)* and *gr2*, the *mineralocorticoid receptor (mr)*; and stress-related genes including the *heat shock protein 70 (hsp70)*, and *enolase* were analyzed by real-time PCR (RT-qPCR). The reference genes *elongation factor 1-alpha (ef1-α)* and the *ribosomal protein L27 (rpl27)* were evaluated in gilthead sea bream based on previous evidence (Khansari et al., 2018; Khansari et al., 2019) to determine the gene with the lower variation among all the samples included in the analysis. Thus, in our study, the *rpl27* was used as a reference gene. All primers used in this study were designed and checked for their specificity with the Primer-Blast Primer software. Table 1 details the list of primers. The potential development of secondary structure and self/heterodimer were verified with OligoAnalyzer (version 3.1). The primer efficiency was checked for all the primer sets included in the study. The RT-qPCR reactions were performed with iTaq universal sybr green supermix (Bio-Rad Laboratories) using 2 μl of 1:40 dilution of cDNA (for the reference genes) and 2 μl of 1:10 dilution of cDNA (for the rest of the genes) as optimum cDNA dilution for each tissue based on the set of serial dilutions contained in the primer efficiency curve. Primers for all genes were used at a final concentration of 500 nM. The thermal conditions used were 3 min at 95°C for preincubation followed by 40 cycles at 95°C for 30 s, and 60°C for 30 s. All reactions were performed in duplicate using the CFX384

TABLE 1 List of primers for RT-qPCR analysis.

Gene name	Gene acronym	GenBank accession number	Primer Sequence (5'-3')	Amplicon size (bp)	Primer efficiency
<i>Interleukin 1-beta</i>	<i>il-1<math>\beta</math></i>	AJ277166.2	FW: TCAGCACCGCAGAAGAAAAC RV: TAACACTCTCCACCCCTCCAC	150	1.94
<i>Tumor necrosis factor-alpha</i>	<i>tnf-<math>\alpha</math></i>	AJ413189.2	FW: TCGTTCAGAGTCTCCTGCAG RV: AAGAATTCTTAAAGTGCAAACACACCAAA	108	1.98
<i>Interleukin 10</i>	<i>il-10</i>	JX976621.1	FW: GAGCGTGGAGGAATCTTTCAA RV: GATCTGCTGGATGGACTGC	181	1.98
<i>Transforming growth factor-beta 1</i>	<i>tgf-<math>\beta</math>1</i>	AF424703.1	FW: AGACCCTCAGAACTGGCTC RV: ACTGCTTTGTCTCCCCTACC	146	1.95
<i>Lysozyme</i>	<i>lys</i>	AM749959.1	FW: TCATCGCTGCCATCATCTCC RV: TGTTCTCACTGTCCCATGC	178	2.01
<i>Complement component c3</i>	<i>c3</i>	HM543456.1	FW: GTTCCACAACAACCCACAGC RV: ACATACGCCATCCCATCCAC	177	1.96
<i>Heat shock protein 70</i>	<i>hsp70</i>	EU805481.1	FW: AGGTTGGGTCTGAAAGGAAC RV: TGAACCTCTGCGATGAAGTGG	173	1.82
<i>Enolase</i>	<i>enolase</i>	AY263379.1	FW: GATCTGACCGTGACCAACCC RV: GACACCATCACACCCCATCC	134	1.98
<i>Glucocorticoid receptor 1</i>	<i>gr1</i>	XM_030437675.1	FW: GAAGGATGGAGAGCAGACAAAA RV: GCTTCCAAGTTCATTCCGGC	149	2.06
<i>Glucocorticoid receptor 2</i>	<i>gr2</i>	DQ486890.1	FW: AAGAGAGCAGTGAAGGGCAGCATAATTA RV: CCGCAAGTGTCTGATGGCT	147	2.00
<i>Mineralocorticoid receptor</i>	<i>mr</i>	XM030418022.1 (from (Tsalafouta et al., 2018))	FW: CGCCTGGCTGGAAAGCAGATG RV: GAGGTCAGGGGCAAAGTAGAGCAT	189	1.97
<i>Elongation factor 1 alpha</i>	<i>ef1-<math>\alpha</math></i>	AF184170.1	FW: TCAACATCGTGGTTCATTGGC RV: CTTCAAGTTTGCCAGCACCC	169	2.00
<i>Ribosomal protein L27</i>	<i>rpl27</i>	AY188520.1	FW: AAGAGGAACACAACCTCACTGCCCCAC RV: GCTTGCCTTTGCCAGAACTTTGTAG	160	2.00

Touch real-time PCR detection system (Bio-Rad Laboratories). Quantification was performed according to the Pfaffl method and corrected for the efficiency of each set of primers (Pfaffl, 2001). The value of the normalized relative expression for each experimental condition was calculated in relation to values obtained for the control group and the rpl27 (reference gene).

## Statistical analysis

All statistical analyzes were performed with GraphPad Prism 9 software. Statistical differences between all experimental groups were determined by one-way ANOVA, and specific differences for multiple comparisons were performed with Turkey's test ( $p < 0.05$ ). These differences

were considered statistically significant when a p-value  $< 0.05$  between the groups.

## Results

### Cortisol level in plasma and skin mucus of gilthead sea bream subjected to the acute air exposure stress

In our study, exposure of gilthead sea bream to air caused a rapid increase in plasma cortisol levels at 1 hps. The cortisol value remained high at 6 hps to subsequently decrease at 24 hps. Like in the plasma, in the skin mucus the cortisol level increased at 1 hps. However, a rapid decrease in concentration was observed at 6 hps and then returned to basal level at 24 hps (Figure 1).

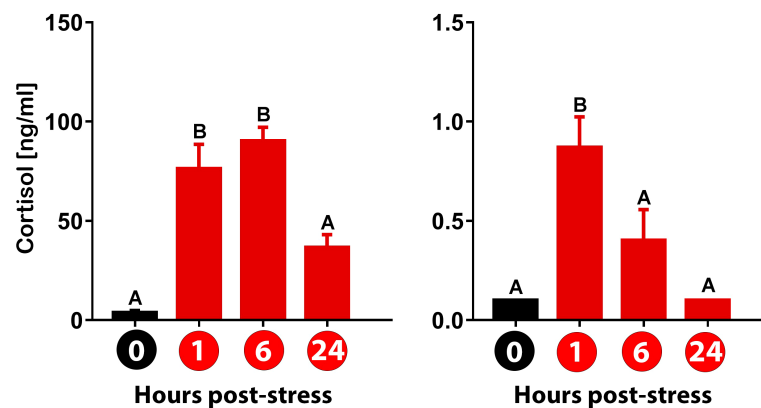


FIGURE 1

Detection of cortisol levels in plasma and skin mucus for gilthead sea bream (*Sparus aurata*) subjected to three-minute air exposure stress. Values for (A) plasma and (B) skin mucus at 0, 1, 6, and 24 hours post-stress (hps) are plotted. Values (ng/ml) are presented as mean  $\pm$  standard error ( $n = 8$  fish per sample type and time evaluated). Significant differences between times are represented in capital letters (One-way Anova;  $p < 0.05$ ). Different colored bars indicate different treatments: black (control group) and red (air exposure-stressed group).

## Modulation of gene expression in the skin

The expression of *gr1* registered a slight but non-significant increase at 1 hps in gilthead sea bream subjected to air exposure acute stress. Then, the value returns to the basal level of expression. The same expression pattern was observed for the *mr*, noting an upregulation at 1 hps to then decrease to the control level at 6 hps. A time-dependent increase in *gr1* was again detected at 24 hps. This modulation was also registered on *mr* at 24 hps compared with 6 hps. Importantly, the *gr2* showed no sign of modulation at any time-point evaluated (Figure 2).

The immune-related genes showed no differences between the time points analyzed. However, the same modulatory behavior observed for *gr1* and *mr* was identified for the pro-inflammatory genes *il-1 $\beta$* , lysozyme, *c3*, and the anti-inflammatory genes *il-10* and *tgf- $\beta$ 1*. No modulation was either observed for *hsp70* and *enolase* (Figure 2).

## Modulation of gene expression in gills

The glucocorticoid receptors in gills showed the same trend of expression over time as those observed in the skin. In detail, the *gr1* was upregulated at 1 hps compared to time-zero (control group). The *mr* also registered an upregulation at 1 hps, although not significant. Then, the kinetics of expression showed the downregulation of *gr1* at 6 hps, to then increase (but not significantly) its value at 24 hps. This time-dependent pattern is also observed for *mr*, noting a diminishing in the expression at 6 hps, to then increase its value at 24 hps. Like in the skin, no modulation of *gr2* was observed (Figure 3).

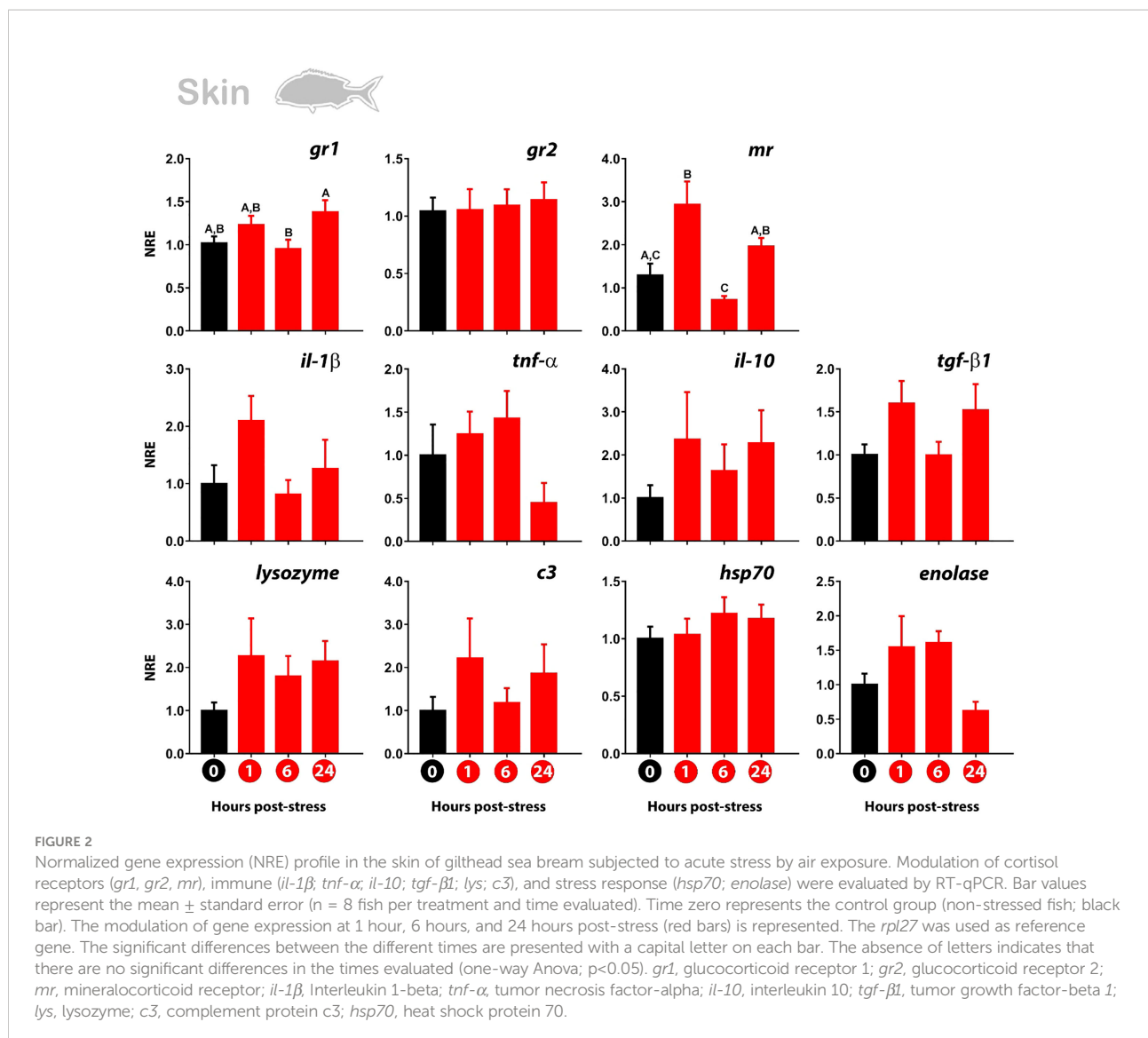
At the immunity level, a marked upregulation of *il-1 $\beta$*  was identified at 6 hps to then return to the basal expression value of the control. No modulation was registered for the other immune genes analyzed. From them, only *tgf- $\beta$ 1* represented a similar trend of expression than the observed for *gr1* and *mr*. The stress-related genes *hsp70* and *enolase* registered no modulation of their expression profile (Figure 3).

## Modulation of gene expression in the anterior gut

The glucocorticoid receptors showed a different expression pattern of modulation in the anterior gut than in the skin and gills. The *gr1* was not modulated at 1 and 6 hps, noting the upregulated only at 24 hps. The *mr* also showed an upregulation that took place at 6 hps to then drop to the value of the non-stressed fish (Figure 4).

When we look at genes associated with immune response, three types of modulation are observed. First, there is an upregulation of pro-inflammatory genes only at 6 hps (*il-1 $\beta$* ), like the *mr* expression profile. Other genes (*il-10*; *hsp70*) showed the same trend although not significant. Secondly, in the same way as *gr1*, we observed an upregulation exclusively at 24 hps for pro-inflammatory genes (*tnf- $\alpha$* ; *c3*). Third, the other genes were not significant (*tgf- $\beta$ 1*; *enolase*), although lysozyme registered a similar not significant profile to *gr2* (Figure 4).

Taking together, the data suggest that the stress response in gilthead sea bream could modulate the expression of immune- and stress-related genes preferentially by the action of one of these glucocorticoid receptors at the mucosal level.



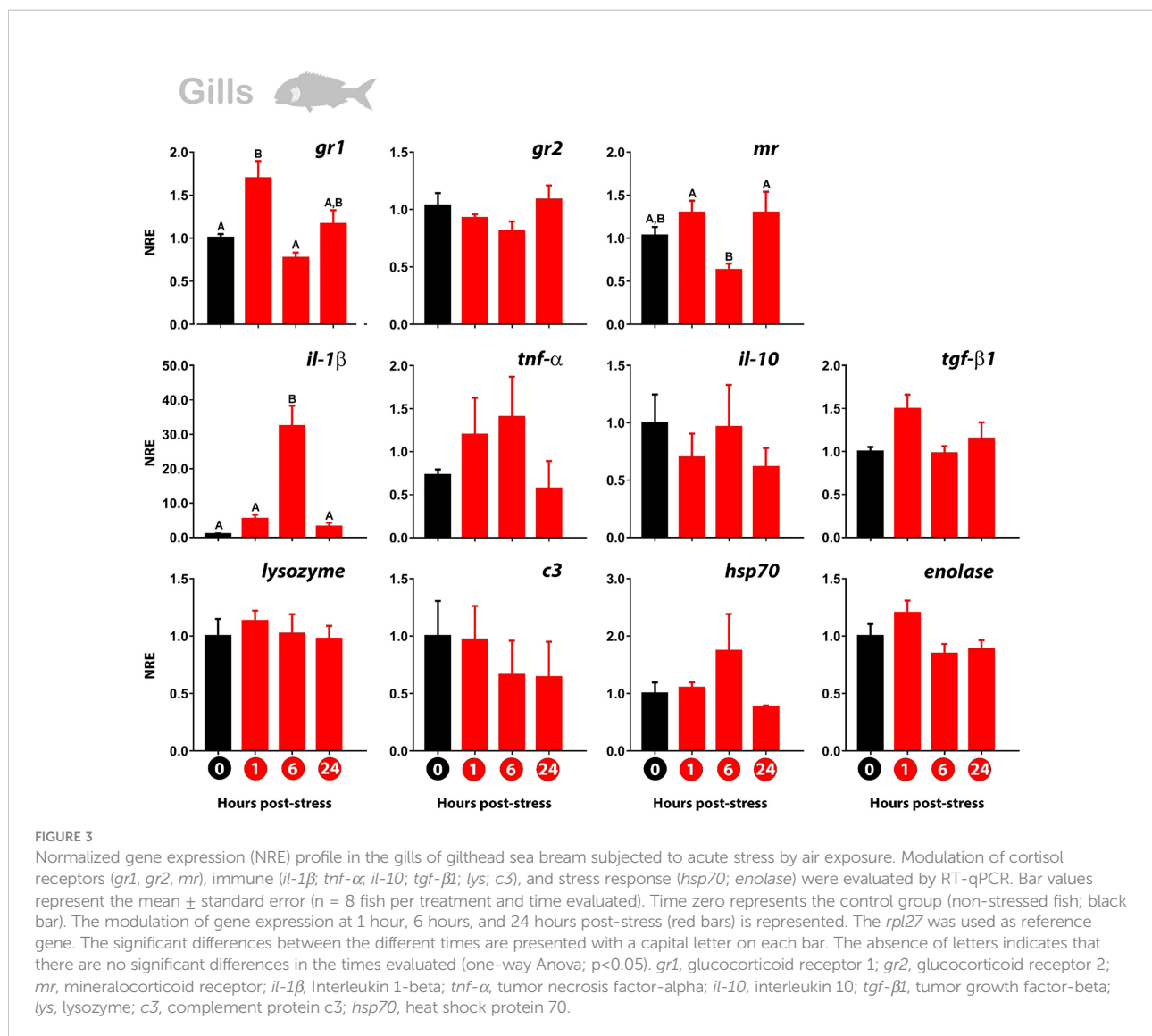
## Discussion

Gilthead sea bream (*S. aurata*) is one of the most relevant Mediterranean fish species in terms of volume and economic value (Salomón et al., 2021). Thus, the analysis of the stress response and the modulatory mechanisms of the glucocorticoid receptors deserve attention. In this study, we evaluated for the first time the modulation of the expression of cortisol receptors (*gr1*; *gr2*; *mr*) in gilthead sea bream subjected to an acute stressor (three minutes of air exposure), and its eventual similarity with the expression profile of a set of stress- and immune-related genes in mucosal tissues (skin; gills; anterior gut).

Our data indicate that *gr1* but not *gr2* is modulated in a time-dependent manner in gilthead sea bream subjected to air exposure. This modulation is also tissue-specific, confirming

previous evidence of differential responses between mucosal tissues subjected to the same stressor (Khansari et al., 2018).

In this study, we reported the differential expression profile of the *gr1* and *gr2* in response to three-minute air exposure stress for the first time in gilthead sea bream. We registered the upregulation of *gr1* in all the mucosal tissues evaluated (skin; gills; anterior gut) in a time- and tissue-specific dependent manner. By contrast, no modulation of *gr2* was detected in any of the tissues and the time-points evaluated. The glucocorticoid receptors are responsible of the cortisol recognition and its subsequent effect. The cortisol-GR complex is transported to the nucleus, where it binds to DNA by glucocorticoid response elements (GREs) present in the promoter region of several genes, interacting with co-activator molecules. In this way, the cortisol concentration is of great relevance. In fact, GR1 and GR2 can respond to different levels of

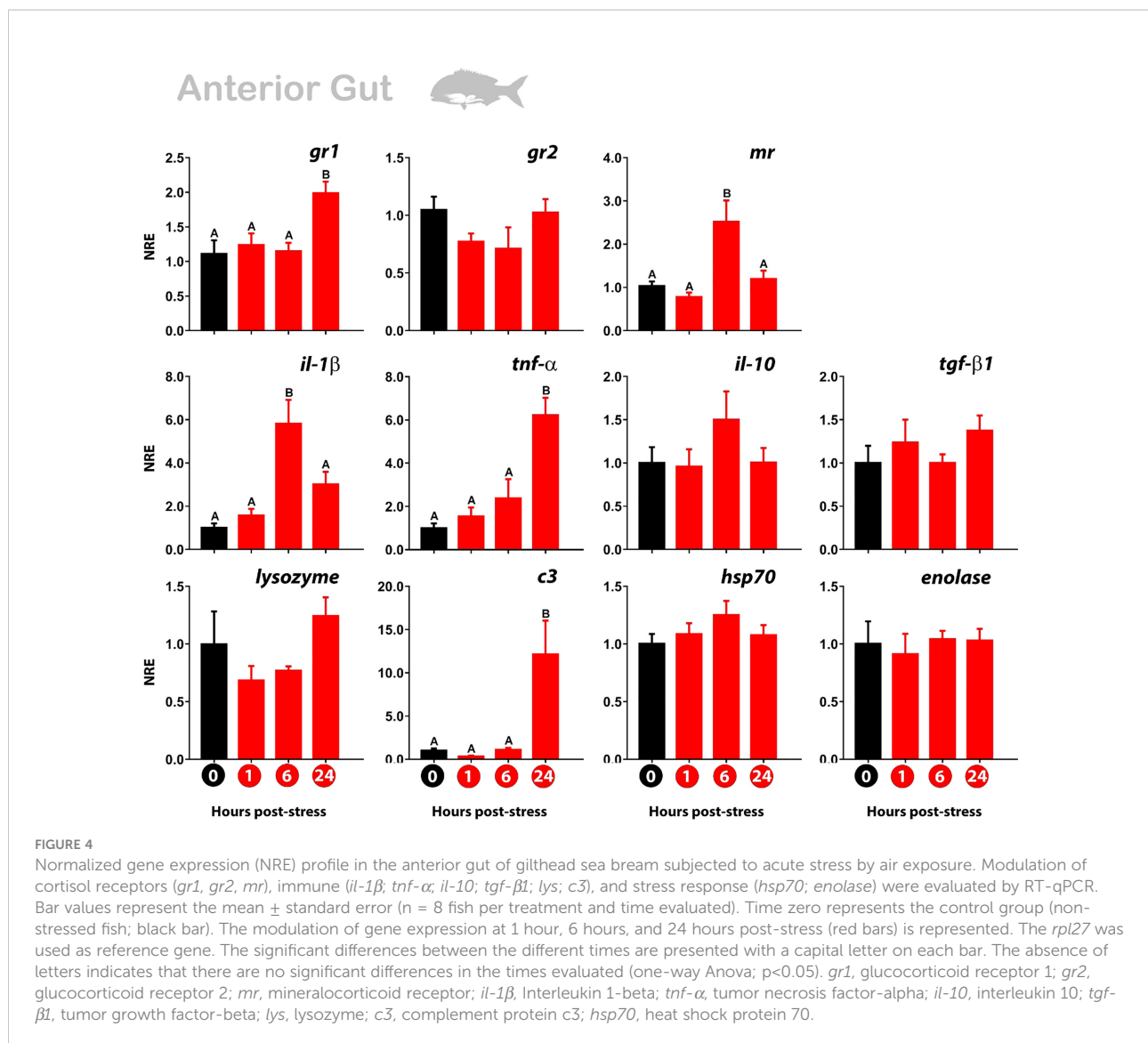


cortisol concentration in fish (Bury et al., 2003; Stolte et al., 2006). It has been postulated that GR2 and MR have a higher affinity for cortisol, making possible the ligand-receptor interaction at a very low concentration of cortisol; by contrast, GR1 would be responsible for the recognition of cortisol at higher concentrations (Vazzana et al., 2010). Thus, the modulation of *gr1* but not *gr2* in our study might reside in the marked increase of cortisol after 1 hps, which even persists high at 6 hps in plasma. Thus, we speculate that the high level of cortisol could stimulate its recognition of cortisol by GR1 but not by GR2 in our stress model. In this way, the differential expression pattern between *gr1* and *gr2* observed in our study is in line with the hypothesis that neo-functionalization likely facilitated the maintenance of duplicate GRs in teleosts (Arterbery et al., 2011). The effect of the early stress response

within the first hour (0-1hps) on the modulation of *gr1* and *gr2* remains to be elucidated.

On the other hand, the expression of *mr* was also modulated in a time- and tissue-dependent manner. This result should be expected taking into consideration the role of MR in the ion and water transport (McCormick and Bradshaw, 2006; Takahashi and Sakamoto, 2013). This evidence rises particular consideration taking into account the relevance of such functional processes and the permeability characteristic of the mucosal tissues (Parra et al., 2015). On the other hand, special attention requires the similarity in the expression pattern observed for *gr1* and *mr*, and some of the genes associated with the immune and stress response. The association in the expression pattern of such genes should be analyzed in future functional works.





The measurement of cortisol in plasma and skin mucus was assessed as an indicator of the presence of the hormone at systemic and local level, respectively. The quantification of plasmatic cortisol has been the traditional method used to evaluate the response to stress in fish (Schreck and Tort, 2016). Previous evidence demonstrated that air exposure stimulates both the HPI and HSC axes in gilthead sea bream, activating the transcriptomic response and the transitory enhancement of cortisol (Skrzynska et al., 2018). The analysis of cortisol in skin mucus appears as a very useful tool to less-invasively evaluate the activity of the HPI axis of fish (Carbajal et al., 2019; Franco-Martinez et al., 2022). The identification of cortisol in skin mucus is possible because of the lipophilic property that makes it feasible to diffuse it easily through cell membranes, thus reaching different biological matrices (i.e. skin mucus) in proportion to the amount of

circulating hormone when high amounts of cortisol are released in the bloodstream (Carbajal et al., 2019). In a previous study, it was observed that exposing fish to air for one minute induces the release of cortisol in plasma and skin mucus, with a peak of maximum release after 1h and 6hps, respectively (Khansari et al., 2018). In this way, the comparative stress response of gilthead sea bream with other fish species indicates that gilthead seabream has a lower activation threshold to acute stress by one-minute air exposure (Khansari et al., 2018). Thus, it is not surprising that in our results the highest duration of the acute stressor stimulus (3-minutes air exposure) could keep augmented the level of cortisol at 1 and 6 hps. In skin mucus, the cortisol peaked at 1hps and then began to decrease to reach the control level at 24 hps. The augmented but not significant cortisol level at 6 hps could be also the result of a delay in the peak of

cortisol in epidermal mucus, as it has been suggested in previous studies for gilthead sea bream (Guardiola et al., 2016) and other fish species (Kroska et al., 2021). In line with this idea, the very low concentrations of cortisol detected in skin mucus makes it very sensitive to the determination of significant differences. In any case, the cortisol release profile in skin mucus registered in our study matches the level previously found in this fish species under acute stress (Khansari et al., 2019).

There is a consensus that when the stressor is acute and short-term, the response pattern is somehow stimulatory and the fish immune response shows an activating phase that especially enhances innate responses (Tort, 2011), including the upregulation of pro-inflammatory genes like *complement component c3* (Demers and Bayne, 2020; Qian et al., 2020), *il-1 $\beta$*  and *tnf- $\alpha$*  (Metz et al., 2006; Hoseini et al., 2019; Su et al., 2020). Accordingly, in our study we observed the upregulation of the complement protein *c3* (anterior gut), and the pro-inflammatory genes *il-1 $\beta$*  (gills; anterior gut) and *tnf- $\alpha$*  (anterior gut) in mucosal tissues. Importantly, the same *il-1 $\beta$*  expression pattern, as observed in our study at 6 hps for the anterior gut, was also registered in another study using one-minute air exposure as acute stressor (Khansari et al., 2018). However, the magnitude of the upregulation detected in our study was much higher, probably due to the application of a stressor of greater intensity (three-minute of air exposure). In the same way, our study determined a higher expression value for *c3* in the anterior gut at 24 hps than the study subjecting gilthead sea bream to one-minute air exposure stress (upregulated at 6 hps) (Khansari et al., 2018). This displacement of the upregulated expression profile observed in our study might explain the marked upregulation trend but not the significant differences registered for skin at the time-points evaluated. Moreover, the higher expression magnitude of pattern for *il-1 $\beta$*  was even more marked at 6 hps for gills applying three-minute of air exposure stress. We hypothesize that this much higher magnitude of upregulation observed in our study for gills could be due to the vascular anatomy and blood-flow because of its multifunctional characteristics (i.e., respiratory, osmoregulatory, and immune processes). Thus, the high cortisol content detected in plasma at 1 hps and slightly higher at 6 hps in gilthead sea bream subjected to three-minute of air exposure stress, might promote a stronger stimulatory effect upon *il-1 $\beta$*  in gills. Anyway, it is important to take into consideration that this augment on the expression of increase of *il-1 $\beta$*  does not necessarily increase the protection against pathogenic challenges (Fast et al., 2008).

Collectively, our results suggest that gilthead sea bream subjected to an acute stressor of short duration (three minutes of air exposure) and high intensity (high cortisol level at 1hps and peaked at 6 hps) modulated the expression of *gr1* but not *gr2*. This result agrees with previous evidence that assigns GR1

the responsibility for the recognition of cortisol at higher concentrations (Vazzana et al., 2010). In addition, our data reinforce the stimulatory effect that acute stress induces mainly in genes associated with the innate immune response but at the mucosal level, and in a time- and tissue-dependent manner.

## Data availability statement

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding authors.

## Ethics statement

The animal study was reviewed and approved by Universitat Autònoma de Barcelona.

## Author contributions

EV-V, LT, and FER-L performed the conceptualization of the study. EV-V, BS-M, MT, LT, and FER-L carried out the experiments. EV-V, BS-M, and FER-L conducted the sampling processing for RT-qPCR. EV-V, BS-M, SR-C, JM, LT, and FER-L performed the data analysis. EV-V and FER-L conceptualized the figures. EV-V and FER-L wrote the original draft. All authors contributed to the article and approved the submitted version.

## Funding

The authors thank the funding by the AGL2016-76069-C2-2-R, PID2020-117557RB-C21, and PID2020-117557RB-C22, AEI-MINECO (Spain). EV-V thanks the support of Fondecyt iniciación grant (project number 11221308; Agencia Nacional de Investigación y Desarrollo de Chile, Government of Chile). MT thanks the support of the post-doctoral fellowship “Ramón y Cajal” (ref. RYC2019-026841-I) (Ministerio de Ciencia e Innovación, Spanish Government). FER-L thanks the support of Fondecyt regular grant (project number: 1211841; Agencia Nacional de Investigación y Desarrollo de Chile, Government of Chile).

## Conflict of interest

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

## Publisher's note

All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated

organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

## References

- Acerete, L., Balasch, J. C., Castellana, B., Redruello, B., Roher, N., Canario, A. V., et al. (2007). Cloning of the glucocorticoid receptor (GR) in gilthead seabream (*Sparus aurata*): differential expression of GR and immune genes in gilthead seabream after an immune challenge. *Comp. Biochem. Physiol. - B Biochem. Mol. Biol.* 148, 32–43. doi: 10.1016/j.cbpb.2007.04.015
- Arends, R. J., Mancera, J. M., Muñoz, J. L., Wendelaar Bonga, S. E., and Flik, G. (1999). The stress response of the gilthead sea bream (*Sparus aurata* L.) to air exposure and confinement. *J. Endocrinol.* 163, 149–157. doi: 10.1677/joe.0.1630149
- Arterbery, A. S., Fergus, D. J., Fogarty, E. A., Mayberry, J., Deitcher, D. L., Kraus, W. L., et al. (2011). Evolution of ligand specificity in vertebrate corticosteroid receptors. *BMC Evol. Biol.* 11, 14. doi: 10.1186/1471-2148-11-14
- Bury, N. R., Sturm, A., Le Rouzic, P., Lethimonier, C., Ducouret, B., Guiguen, Y., et al. (2003). Evidence for two distinct functional glucocorticoid receptors in teleost fish. *J. Mol. Endocrinol.* 31, 141–156. doi: 10.1677/jme.0.0310141
- Carbajal, A., Reyes-López, F. E., Tallo-Parra, O., Lopez-Bejar, M., and Tort, L. (2019). Comparative assessment of cortisol in plasma, skin mucus and scales as a measure of the hypothalamic-pituitary-interrenal axis activity in fish. *Aquaculture* 506, 410–416. doi: 10.1016/j.aquaculture.2019.04.005
- Demers, N., and Bayne, C. (2020). Immediate increase of plasma protein complement C3 in response to an acute stressor. *Fish. Shellfish Immunol.* 107, 411–413. doi: 10.1016/j.fsi.2020.10.022
- Esteban, M.Á., and Cerezuela, R. (2015). "Fish mucosal immunity: Skin," in *Mucosal health in aquaculture*. Eds. B. H. Beck and E. Peatman (Elsevier Inc), 67–92. doi: 10.1016/B978-0-12-417186-2.00005-4
- Fast, M. D., Hosoya, S., Johnson, S. C., and Afonso, L. O. B. (2008). Cortisol response and immune-related effects of Atlantic salmon (*salmo salar* Linnaeus) subjected to short- and long-term stress. *Fish. Shellfish Immunol.* 24, 194–204. doi: 10.1016/j.fsi.2007.10.009
- Franco-Martínez, L., Brandts, I., Reyes-López, F., Tort, L., Tvarijonavičute, A., and Teles, M. (2022). Skin mucus as a relevant low-invasive biological matrix for the measurement of an acute stress response in rainbow trout (*Oncorhynchus mykiss*). *Water* 14, 1754. doi: 10.3390/w14111754
- Guardiola, F. A., Cuesta, A., and Esteban, M. A. (2016). Using skin mucus to evaluate stress in gilthead seabream (*Sparus aurata* L.). *Fish. Shellfish Immunol.* 59, 323–330. doi: 10.1016/j.fsi.2016.11.005
- Guo, H., and Dixon, B. (2021). Understanding acute stress-mediated immunity in teleost fish. *Fish. Shellfish Immunol. Rep.* 2, 100010. doi: 10.1016/j.fsirep.2021.100010
- Hoseini, S. M., Yousefi, M., Hoseinifard, S. H., and Van Doan, H. (2019). Cytokines' gene expression, humoral immune and biochemical responses of common carp (*Cyprinus carpio*, Linnaeus, 1758) to transportation density and recovery in brackish water. *Aquaculture* 504, 13–21. doi: 10.1016/j.aquaculture.2019.01.049
- Iversen, M.H., and Eliassen, R.A. (2014). The effect of allostatic load on hypothalamic-pituitary-interrenal (HPI) axis before and after secondary vaccination in Atlantic salmon postsmolts (*Salmo salar* L.). *Fish Physiol. Biochem.* 40, 527–538. doi: 10.1007/s10695-013-9863-x
- Khansari, A. R., Balasch, J. C., Reyes-López, F. E., and Tort, L. (2017a). Stressing the inflammatory Network: Immuno-endocrine responses to allostatic load in fish. *J. Mar. Sci. Res. Technol.* 1–14.
- Khansari, A. R., Balasch, J. C., Vallejos-Vidal, E., Parra, D., Reyes-López, F. E., and Tort, L. (2018). Comparative immune- and stress-related transcript response induced by air exposure and vibrio anguillarum bacterin in rainbow trout (*Oncorhynchus mykiss*) and gilthead seabream (*Sparus aurata*) mucosal surfaces. *Front. Immunol.* 9. doi: 10.3389/fimmu.2018.00856
- Khansari, A. R., Balasch, J. C., Vallejos-Vidal, E., Teles, M., Fierro-Castro, C., Tort, L., et al. (2019). Comparative study of stress and immune-related transcript outcomes triggered by vibrio anguillarum bacterin and air exposure stress in liver and spleen of gilthead seabream (*Sparus aurata*), zebrafish (*Danio rerio*) and rainbow trout (*Oncorhynchus mykiss*). *Fish. Shellfish Immunol.* 86, 436–448. doi: 10.1016/j.fsi.2018.11.063
- Khansari, A. R., Parra, D., Reyes-López, F. E., and Tort, L. (2017b). 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. *Gen. Comp. Endocrinol.* 250, 122–135. doi: 10.1016/j.ygcen.2017.06.005
- Khansari, A. R., Parra, D., Reyes-López, F. E., and Tort, L. (2017c). Modulatory *in vitro* effect of stress hormones on the cytokine response of rainbow trout and gilthead sea bream head kidney stimulated with vibrio anguillarum bacterin. *Fish. Shellfish Immunol.* 70, 736–749. doi: 10.1016/j.fsi.2017.09.009
- Koppang, E. O., Kvellestad, A., and Fischer, U. (2015). "Fish mucosal immunity: Gill," in *Mucosal health in aquaculture*. Eds. B. H. Beck and E. Peatman (Elsevier Inc), 93–133. doi: 10.1016/B978-0-12-417186-2.00005-4
- Kroska, A. C., Wolf, N., Planas, J. V., Baker, M. R., Smeltz, T. S., and Harris, B. P. (2021). Controlled experiments to explore the use of a multi-tissue approach to characterizing stress in wild-caught pacific halibut (*Hippoglossus stenolepis*). *Conserv. Physiol.* 9, 1–9. doi: 10.1093/conphys/coab001
- Liu, X. H., Khansari, A. R., Teles, M., Martínez-Rodríguez, G., Zhang, Y. G., Mancera, J. M., et al. (2019). Brain and pituitary response to vaccination in gilthead seabream (*Sparus aurata* L.). *Front. Physiol.* 10. doi: 10.3389/fphys.2019.00717
- McCormick, S. D., and Bradshaw, D. (2006). Hormonal control of salt and water balance in vertebrates. *Gen. Comp. Endocrinol.* 147, 3–8. doi: 10.1016/j.ygcen.2005.12.009
- Merrifield, D. L., and Rodiles, A. (2015). "The fish microbiome and its interactions with mucosal tissues," in *Mucosal health in aquaculture* (Elsevier Inc), 273–295. doi: 10.1016/B978-0-12-417186-2/00010-8
- Metz, J. R., Huising, M. O., Leon, K., Verburg-van Kemenade, B. M. L., and Flik, G. (2006). Central and peripheral interleukin-1 $\beta$  and interleukin-1 receptor I expression and their role in the acute stress response of common carp, *Cyprinus carpio* L. *J. Endocrinol.* 191, 25–35. doi: 10.1677/joe.1.06640
- Parra, D., Reyes-López, F. E., and Tort, L. (2015). Mucosal immunity and b cells in teleosts: Effect of vaccination and stress. *Front. Immunol.* 6. doi: 10.3389/fimmu.2015.00354
- Pfaffl, M. W. (2001). A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res.* 29, e45. doi: 10.1093/nar/29.9.e45
- Qian, B., Xue, L., Qi, X., Bai, Y., and Wu, Y. (2020). Gene networks and toxicity/detoxification pathways in juvenile largemouth bass (*Micropterus salmoides*) liver induced by acute lead stress. *Genomics* 112, 20–31. doi: 10.1016/j.ygeno.2019.06.023
- Salinas, I., and Parra, D. (2015). *Fish mucosal immunity: intestine* (Elsevier Inc). doi: 10.1016/B978-0-12-417186-2/00006-6
- Salomón, R., Reyes-López, F. E., Tort, L., Firmino, J. P., Sarasquete, C., Ortiz-Delgado, J. B., et al. (2021). Medicinal plant leaf extract from sage and lemon verbena promotes intestinal immunity and barrier function in gilthead seabream (*Sparus aurata*). *Front. Immunol.* 12. doi: 10.3389/fimmu.2021.670279
- Schreck, C. B., and Tort, L. (2016). *Biology of stress in fish*. Eds. A. P. Farrell and C. J. Brauner (London: Academic Press).
- Skrzynska, A. K., Maiorano, E., Bastaroli, M., Naderi, F., Míguez, J. M., Martínez-Rodríguez, G., et al. (2018). Impact of air exposure on vasotocinergic and isotocinergic systems in gilthead sea bream (*Sparus aurata*): New insights on fish stress response. *Front. Physiol.* 9. doi: 10.3389/fphys.2018.00096
- Stolte, E. H., Van Kemenade, B. M. L. V., Savelkoul, H. F. J., and Flik, G. (2006). Evolution of glucocorticoid receptors with different glucocorticoid sensitivity. *J. Endocrinol.* 190, 17–28. doi: 10.1677/joe.1.06703
- Su, M., Zhang, R., Liu, N., and Zhang, J. (2020). Modulation of inflammatory response by cortisol in the kidney of spotted scat (*Scatophagus argus*) *in vitro* under different osmotic stresses. *Fish. Shellfish Immunol.* 104, 46–54. doi: 10.1016/j.fsi.2020.05.060
- Takahashi, H., and Sakamoto, T. (2013). The role of 'mineralocorticoids' in teleost fish: Relative importance of glucocorticoid signaling in the osmoregulation and 'central' actions of mineralocorticoid receptor. *Gen. Comp. Endocrinol.* 181, 223–228. doi: 10.1016/j.ygcen.2012.11.016
- Tort, L. (2011). Stress and immune modulation in fish. *Dev. Comp. Immunol.* 35, 1366–1375. doi: 10.1016/j.dci.2011.07.002

Tsalafouta, A., Sarropoulou, E., Papandroulakis, N., and Pavlidis, M. (2018). Characterization and expression dynamics of key genes involved in the gilthead Sea bream (*Sparus aurata*) cortisol stress response during early ontogeny. *Mar. Biotechnol.* 20, 611–622. doi: 10.1007/s10126-018-9833-5

Vallejos-Vidal, E., Khansari, A. R., Soliva-Dueso, L., Balasch, J. C., Tort, L., and Reyes-López, F. E. (2022). The direct exposure of cortisol does not modulate the expression of immune-related genes on tissue explants of mucosal surfaces in rainbow trout (*Oncorhynchus mykiss*) nor in gilthead Sea bream (*Sparus aurata*). *Front. Mar. Sci.* 9. doi: 10.3389/fmars.2022.828050

Vargas, R., Balasch, J. C., Brandts, I., Reyes-López, F., Tort, L., and Teles, M. (2018). Variations in the immune and metabolic response of proactive and reactive *Sparus aurata* under stimulation with *Vibrio anguillarum* vaccine. *Sci. Rep.* 8, 17352. doi: 10.1038/s41598-018-35863-w

Vazzana, M., Vizzini, A., Sanfratello, M. A., Celi, M., Salerno, G., and Parrinello, N. (2010). Differential expression of two glucocorticoid receptors in seabass (teleost fish) head kidney after exogenous cortisol inoculation. *Comp. Biochem. Physiol. - A Mol. Integr. Physiol.* 157, 49–54. doi: 10.1016/j.cbpa.2010.05.003