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# 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*)

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ABSTRACT

The stress and immune-related effects of short-term (1, 6 and 24 h) air exposure stress (1 min), bath vaccination with *Vibrio anguillarum* bacterin, and both stressors combined were evaluated in liver and spleen of *Sparus aurata*, *Danio rerio* and *Onchorhynchus mykiss*. Expression profiles of immune (*interleukin 1 beta*: *il1β*; *tumor necrosis factor alpha*: *tnfα*; *interleukin 10*: *il10*; *tumor growth factor beta*: *tgfβ1*; *immunoglobulin M*: *igm*; lysozyme: *lys*; *complement protein c3*: *c3*) and stress-related genes (*glucocorticoid receptor*: *gr*; *heat shock protein 70*: hsp70; and *enolase*) were analysed by RT-qPCR. Cortisol level was assessed by radioimmunoassay. The gene expression patterns in liver and spleen were found to be differentially regulated in a time- and organ-dependent manner among species. In seabream, a higher *il1β*-driven inflammatory response was recorded. In zebrafish, air exposure stress but not bath vaccination alone modulated most of the changes in liver and spleen immune transcripts. Stressed and vaccinated trout showed an intermediate pattern of gene expression, with a lower upregulation of immune-related genes in liver and the absence of changes in the expression of hsp70 and enolase in spleen (as it was observed in seabream but not in zebrafish). Following air exposure, cortisol levels increased in plasma 1 h post-stress (hps) and then decreased at 6 hps in *O. mykiss* and *D. rerio*. By contrast, in *S.aurata* the cortisol level remained higher at 6 hps suggesting a greater degree of responsiveness to this stressor. When fish were exposed to combined air exposure plus bath vaccination cortisol levels were also augmented at 1 and 6 hps in *O. mykiss* and *S.aurata* and restored to basal level at 24 hps, whereas in *D. rerio* the response was higher in response to the combination of both stressors. In addition, *V. anguillarum* bacterin vaccination triggered cortisol secretion only in *D. rerio,* suggesting a greater responsiveness of *D. rerio* hypothalamic-pituitary-interrenal axis. Overall, comparing the tissue transcription responsiveness, liver was found to be more implicated in the response to handling stress compared to spleen. These results also indicate that a species-specific response accounts for the deviations of stress and immune onset in the liver and spleen in these fish species.

## 1. Introduction

The phenomenon by which an animal is subjected to an insult that may result in a real or symbolic danger affecting its integrity has been defined as stress [1,2]. In fish, once this stimulus is perceived, the hypothalamic-pituitary-interrenal axis (HPI) is activated, resulting in the release of cortisol which is considered the main glucocorticoid (GC) in fish [3] and participates in a wide range of secondary and tertiary responses such as immunomodulatory processes throughout receptors in several immune cell types and tissues [4]. Depending of the nature of the stimuli, an acute (short-term, high intensity) or a chronic (long-term, low intensity although persistent) neuro-immune-endocrine response takes place. Like mammals, the acute response has been shown to be stimulatory for the activation of some innate immune responses in fish, resulting in increased levels of lysozyme and complement proteins [5,6]. It is also assumed that a dual response is induced, first stimulatory and later suppressive, that would depend on the time course and the persistence of the stressor (Tort, 2011). It remains to be elucidated

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up to what extent this response pattern to an acute stressor is the same in different species of teleost fishes.

In daily aquaculture practice may be very common for fish to be subjected to stressors with distinct features, either acute (air exposure, catching, handling) or chronic (density, confinement, subordination) and also to the combination of different stressors, but few studies have evaluated the effect of combined stressors on immune-endocrine activation. Several studies in Atlantic salmon, rainbow trout and seabream subjected to vaccination and chronic/acute stress showed a clear allostatic overload, with elevated baseline levels of plasma/skin mucus cortisol, reduced systemic immune responses and/or enhanced mucosal immune activation [7,8]. Therefore, it is necessary to evaluate the impact of the combination of stressors on the activation of the HPI axis and the expression of cytokines in different organs implicated in the immune and stress response in different teleost species. We recently revealed that, although most fish show a generalized stress reaction *in vitro* and in mucosae, the pattern and magnitude of the response may be affected not only by environmental factors (such as temperature and salinity) but also by the nature of stressors and distinct evolutionary life stories [8,9]. Therefore, an experimental ground for the description of common and specific stress dynamics may require a comparative approach per species to short-term responsiveness to common aquaculture stressors.

The liver accounts for complement components production (a first weapon of the innate immune system against pathogens) and also plays a role in stress responses [10]. The spleen participates in antigen degradation and antibody production, and it serves as a secondary lymphatic and immune scavenging organ and potent weapon of the immune arm against parasite infection [11]. Both are considered systemic immune–related organs that usually respond to environmental pathogens in a medium-to-long-term activation dynamics. We selected specifically an opportunistic pathogen, *Vibrio anguillarum*, which uses the mucosal surfaces of fish as portals of entry and spreads to systemic organs usually in 24–48 h, to test the early immune reactiveness of these systemic organs.

Here we aim to provide evidences about how two stressors (bath vaccination with *Vibrio anguillarum* bacterin and acute air exposure), and the combination of both, can affect the short-term activation (after 1, 6 and 24 h) of immune-endocrine systems in liver and spleen from three phylogenetically separated fish species: *S. aurata* (a perciform marine cultured cypriniform fish and more phylogenetically recent species), *D. rerio* (a fish laboratory model, freshwater and older in evolution) and *O. mykiss* (a freshwater salmonid cultured fish model and bearing an extra genome duplication). It was intended to investigate the direction of the neuroendocrine and immune system responses after acute stress (enhancing *versus* suppressing), with the aim of identifying the responsiveness degree among the three species (seabream, zebrafish, rainbow trout) and confirming our hypothesis on the variability of those responses.

## 2. Materials and methods

### 2.1. Fish and rearing condition

Gilthead seabream (*Sparus aurata*) weighing 65 ± 5.0 g were obtained from Aquacultura els Alfacs,S.L. (Spain). Zebrafish (*Danio rerio*) weighing 0.45 ± 0.5 g were purchased from FLORAQUA (Barcelona, Spain). Rainbow trout (*Oncorhynchus mykiss*) weighing 130 ± 10 g were obtained from a local fish farm (TroutFactory, Peramola, Spain). Thus, all the fish included in the analysis were in the juvenile developmental stage [12–14]. Consequently, the immune system was mature and therefore able to mount a coordinated expression of genes involved in stress and immune response [15,16]. Fish were transferred to AQUAB fish facilities (Universitat Autonoma de Barcelona, UAB), and acclimatized for three weeks at species-specific thermoneutral temperatures (15 °C for trout, 20 °C for seabream and 22 °C, zebrafish), subjected to a 12L:12D photoperiod in a closed recirculation system and fed ad libitum with commercial diets (Skretting). Water parameters (dissolved oxygen, pH, ammonia, nitrites, nitrates, and salinity) were monitored and maintained at basal levels. The experiment complied with the Guiding Principles for Biomedical Research Involving Animals (EU2010/63), the guidelines of the Spanish laws (law 32/2007 and RD 53/2013) and authorized by the Ethical Committee of the UAB CEEAH (Spain) for the use of laboratory animals.

### 2.2. Stressors

Two types of stressors were applied: (1) air exposure stress by maintaining fish in a net subsequently 1 min out of water, and (2) bath vaccination by *V. anguillarum* formalin-killed bacterin (all pathogenic serotypes of the bacterium, O1, O2α, the most pathogenic serogroup, and O2β with RPS ≥ 60%, ICTHIOVAC $^{R}$ VR, Hipra). *V. anguillarum* is an opportunistic gram-negative pathogen that causes vibriosis in both marine and freshwater fish.

### 2.3. Experimental design

For the experiment, fish were placed in 300L tanks (trout and seabream) or 15L boxes (zebrafish) with closed recirculating system provided with water pump, sand filter, and biofilter, 12L:12D photoperiod at species-specific thermoneutral temperatures, and divided in four groups and maintained in independent tanks. **(1) Vaccinated (VAC) group**: 48 fish were bath vaccinated (1 min) with formalin-killed *V. anguillarum* according to manufacturer's instructions (Hipra). Immediately after, fish were rinsed in clean water to discard vaccine excess. Fish were then equally distributed (n = 12) in four tanks, avoiding vaccine cross-contamination. **(2) Vaccinated and stressed (VAC + STR) group**: 24 h after vaccination, 24 fishes randomly selected from the vaccinated-group were stressed (air exposure stress, 1 min) and returned to two separated tanks. **(3) Stressed (STR) group**: 24 non-vaccinated fish were stressed (air exposure, 1 min) and returned to two separated tanks. **(4) Control fish** (n = 24) were mock-vaccinated (vaccine-free bathing) in the same conditions than vaccinated group, returned to two different separated tanks and sampled 24 h after. Concerning time-course utilized for the vaccine group, the preliminary data did not show any effect of vaccine immersion after 1h and 12 h post vaccine (hpv), (data not shown). Therefore, we decided to begin sampling 24 h after bath vaccination. Thus, “time 0” for vaccinated and vaccine + stress group represents 24 hpv, while in stress group represents the initial point of the air exposure stimuli.

### 2.4. Sampling

Fish (n = 8 fish per tissue, treatment, and time-point evaluated) were randomly sampled from the two separated tanks per treatment at 1, 6, and 24 h post-stress (air exposure) from each experimental group (control, VAC, V + S, STR) and sacrificed by over-anesthetization in MS222 (200 mg/L). Blood was collected with heparinized syringes from the caudal vein, transferred to Eppendorf tubes and centrifuged at 2500 rpm × 10 min x 4 °C. Plasma was carefully taken, transferred to a Eppendorf tube and immediately stored at −20 °C until analysis. In order to avoid cortisol rise induced by the manipulation, blood collection lasted less than 3 min. Liver and spleen were dissected out and immediately frozen in liquid nitrogen. Samples (organs and whole zebrafish) were stored at −80 °C until analysis.

### 2.5. Isolation of RNA and cDNA synthesis

Total RNA was isolated from individual fish sample tissues (n = 6 fish per tissue, treatment, and time-point evaluated) 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

**Table 1**
Primers used for gene expression analysis in *S. aurata*.

| Gene | GenBank Accession number | Sequence 5′–3′ | Product size | Primer efficiency |
|---|---|---|---|---|
| *18S* | AY587263.1 | **FW:** ACCAGACAAATCGCTCCACC<br>**RV:** AGGAATTGACGGAAGGGCAC | 172 | 2.02 |
| *Il1β* | AJ277166.2 | **FW:** TCAGCACCGCAGAAGAAAAC<br>**RV:** TAACACTCTCCACCCTCCAC | 115 | 1.97 |
| *tnfα* | AJ413189.2 | **FW:** TCGTTCAGAGTCTCCTGCAG<br>**RV:** AAGAATTCTTAAAGTGCAAACACACCAAA | 320 | 2.24 |
| *tgfβ1* | AF424703.1 | **FW:** AGACCCTTCAGAACTGGCTC<br>**RV:** ACTGCTTTGTCTCCCCTACC | 145 | 1.90 |
| *Il10* | JX976621.1 | **FW:** GAGCGTGGAGGAATCTTTCAA<br>**RV:** GATCTGCTGGATGGACTGC | 154 | 2.02 |
| *gr* | DQ486890.1 | **FW:** ACTGAGGAGGGAGGTCTATT<br>**RV:** GGACTCTGGGACTTCTAACA | 195 | 1.99 |
| *enolase* | AY263379.1 | **FW:** GATCTGACCGTGACCAACCC<br>**RV:** GACACCATCACACCCCATCC | 155 | 1.85 |
| *hsp70* | EU805481.1 | **FW:** AGGTTGGGTCTGAAAGGAAC<br>**RV:** TGAACTCTGCGATGAAGTGG | 174 | 1.96 |
| *c3* | HM543456.1 | **FW:** GTTCCACAACAACCCACAGC<br>**RV:** ACATACGCCATCCCATCCAC | 183 | 1.91 |
| *lys* | AM749959.1 | **FW:** TCATCGCTGCCATCATCTCC<br>**RV:** TGTTCCTCACTGTCCCATGC | 154 | 2.08 |
| *igm* | JQ811851.1 | **FW:** GATCGTGACATCGTCTGAGG<br>**RV:** TGTTGGGTTGTGGTTGTAGG | 187 | 1.91 |

use. The RNA concentration was quantified by NanoDrop ND-2000 spectrophotometer (Thermo Scientific). Total RNA (2 μg) was used as template to synthesize complementary DNA (cDNA) using iScript cDNA kit (Bio-Rad Laboratories) according to manufacturer's instructions.

### 2.6. Quantitative real time PCR (RT-qPCR)

Fish liver and spleen gene expression profiles of immune- (*interleukin 1 beta*: *il1β*; *tumor necrosis factor alpha*: *tnfα*; *interleukin 10*: *il10*; *tumor growth factor beta*: *tgfβ1*; *immunoglobulin M*: *igm*; lysozyme: *lys*; *complement protein c3*: *c3*) and stress-related genes (*glucocorticoid receptor*: *gr*; *heat shock protein 70*: hsp70; and *enolase*) were analysed using RT-qPCR. Several candidate reference genes were evaluated (*18s*, *ef1α* and *rpl27* in seabream; *ef1α* in zebrafish; *ef1α* and *β-actin* in trout) to elucidate which one had less variation among all samples included in the study and should be chosen for the normalization of gene expression. Thus, *18 S*, *ef1α* and *β-actin* were chosen for *S. aurata, D. rerio* and *O. mykiss* respectively. Specific primers used for *S. aurata* (Table 1), *D. rerio* (Table 2) and *O. mykiss* (Table 3), are indicated. All primers used in this study were designed with Primer-Blast. The primer secondary structure and primer specificity were checked with OligoAnalyzer (version 3.1) and Primer-Blast software, respectively. The primers used gave a pure PCR product which was verified by single peak in the real-time PCR melting curve. Only the expression of *il10* for zebrafish was not detected in the samples of this study. The amplification efficiency was determined individually for each organ by RT-qPCR and all of them 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 *O. mykiss*, *S. aurata* and *D. rerio* based on the efficiency curve and the detection limit for each primer set. 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 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

**Table 2**
Primers used for gene expression analysis in *D. rerio*.

| Gene | GenBank Accession number | Sequence 5′–3′ | Product size | Primer efficiency |
|---|---|---|---|---|
| *ef1α* | L23807.1 | **FW:** GGGCAAGGGCTCCTTCAA<br>**RV:** CGCTCGGCCTTCAGTTTG | 54 | 2.03 |
| *il1β* | NM_212844.2 | **FW:** TGGACTTCGCAGCACAAAATG<br>**RV:** GTTCACTTCACGCTCTTGGATG | 150 | 1.94 |
| *tnfα* | NM_212859.2 | **FW:** GGAGGGTGTTTGGGATCATTTTGG<br>**RV:** GTCTCAGCACACTTCCATCTTGTT | 108 | 1.98 |
| *tgfβ1* | NM_182873.1 | **FW:** TCTGGGAACTCGCTTTGTCTCCAA<br>**RV:** GCTGGTTTGCTTTACAGTCGCAGT | 146 | 1.95 |
| *il10* | NM_001020785.2 | **FW:** AGCACTCCACAACCCCAATC<br>**RV:** AGCAAATCAAGCTCCCCCATA | 181 | 1.98 |
| *gr* | NM_001020711.2 | **FW:** ACAGCTTCTTCCAGCCTCAG<br>**RV:** CCGGTGTTCTCCTGTTTGAT | 116 | 1.99 |
| *enolase* | XM_005162152.4 | **FW:** TAATGCCATCCTGGGCGTTT<br>**RV:** TAACGTTGAAGGCAGGCACT | 134 | 1.98 |
| *hsp70* | AB062116.1 | **FW:** TGAAAGGCAAGATCAGCGAG<br>**RV:** CATCCCTCCCTGGTAGAGTT | 173 | 1.82 |
| *c3* | NM_001037236.1 | **FW:** GTATTACTCACCCGATGCCCG<br>**RV:** AGATGGGGTTCACAGGCTTTAAT | 177 | 1.96 |
| *lys* | NM_139180.1 | **FW:** CGGGACATCTTACGACCGTT<br>**RV:** TCCGTCTCCAAATGCTGCAT | 178 | 2.01 |
| *igm* | AY643751.1 | **FW:** ACATGGAAGCAGGCAGAGAG<br>**RV:** ATGTCTTCAGGGGTGGGAGG | 149 | 2.08 |

**Table 3**
Primers used for gene expression analysis in *O. mykiss*.

| Gene | GenBank Accession number | Sequence 5′–3′ | Product size | Primer efficiency |
|---|---|---|---|---|
| *βactin* | NM_001124235.1 | **FW:**GGACTTTGAGCAGGAGATGG<br>**RV:**ATGATGGAGTTGTAGGTGGTCT | 186 | 1.96 |
| *il1β* | NM_001124347.2 | **FW:**TGAGAACAAGTGCTGGGTCC<br>**RV:**GGCTACAGGTCTGGCTTCAG | 148 | 1.92 |
| *tnfα* | NM_001124357.1 | **FW:**CACACTGGGCTCTTCTTCGT<br>**RV:**CAAACTGACCTTACCCCGCT | 155 | 1.88 |
| *tgfβ1* | NM_001281366.1 | **FW:**GCCAAGGAGGTCCACAAGTT<br>**RV:**GTGGTTTTGATGAGCAGGCG | 146 | 1.94 |
| *il10* | NM_001245099.1 | **FW:**CCGCCATGAACAACAGAACA<br>**RV:**TCCTGCATTGGACGATCTCT | 105 | 1.91 |
| *gr* | NM_001124730.1 | **FW:**TTCCTTTCCTCCCTGTCAGT<br>**RV:**ATCCTCCTCCGTCTTGATGA | 171 | 1.95 |
| *enolase* | HM100654.1 | **FW:**CAAAGGTGTCTCAAAAGCCG<br>**RV:**GTTGACGTTCTGCCGTACAA | 73 | 1.91 |
| *hsp70* | AB176854 | **FW:**CGGGAGTTGTAGCGATGAGA<br>**RV:**CTTCCTAAATAGCACTGAGCCATAA | 140 | 2.01 |
| *c3* | L24433 | **FW:**GAGATGGCCTCCAAGAAGATAGAA<br>**RV:**ACCGCATGTACGCATCATCA | 91 | 1.96 |
| *lys* | X59491 | **FW:**TGCCTGTCAAAATGGGAGTC<br>**RV:**CAGCGGATACCACAGACGTT | 211 | 1.89 |
| *igm* | S63348.1 | **FW:**AAGAAAGCCTACAAGAGGGAGA<br>**RV:**CGTCAACAAGCCAAGCCACTA | 157 | 1.85 |

[17]. Values for each experimental condition were expressed as normalized relative expression.

### 2.7. Quantification of cortisol

Trout and seabream plasma cortisol levels were measured by radioimmunoassay (RIA) as has been previously described [18] (antibody from MO bio-medical LLC, USA, final dilution 1:4500, lower detection limit of the cortisol assay: 0.16 ng/mL, 100% antibody cross-reactivity with cortisol). Cortisol levels in whole-body zebrafish were measured according to [19].

### 2.8. Statistical analysis

A generalized linear model (GzLM) as implemented in SPSS v. 20 was used for statistical analysis. Stressors and time were considered as two factors. This statistical method is flexible since no homogeneity of variance is required. When a statistical significant interaction was found between stressors and time, the correlation between time and cortisol and gene expression modulation was evaluated (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. Gene expression modulation in seabream

A marked downregulation in *S. aurata* liver was observed in in several genes and times tested (Fig. 1A). Concerning pro-inflammatory cytokine transcripts, while the expression of *il1β* was upregulated in all stressed groups at different time-points (STR group: 1 hps, 6 hps; VAC + STR group: 6 hps, 24 hps; VAC group: 24 hps), the expression of *tnfα* was downregulated in all treatments and time-points evaluated. The expression of *tgfβ1* was also downregulated; however, *il10* was upregulated only in STR group at 24 hps. The upregulation of *c3* at 24 hps, a classical innate immunity marker, was also observed following bath vaccination, although its upregulation was also detected at 1 hps. The upregulation of *c3* was also noted in the air-exposed fishes. A marked downregulation in *S. aurata* liver was observed in all stress-related transcripts (*gr*, *hsp70*, and *enolase*) at all time-points evaluated, except at 24 hps when the expression of glucocorticoid receptor (*gr*) and *enolase* were upregulated in the vaccinated group. In sum, these antecedents indicate that the expression levels of *gr*, *hsp70*, *tnfα* and *tgfβ1* were most susceptible to be downregulated in *S. aurata* subjected to these stressors.

In *S. aurata* spleen (Fig. 1B) *il1β* showed a marked upregulation in response to air exposure stress and stress + vaccination. However, the upregulation of *il10* was only registered at 6 hps for both groups. No modulation was noted in the expression of *tnfα* and *tgfβ1*. *c3* transcripts showed an enhanced expression at 6 hps (VAC group) and 24 hps (VAC + STR group). The innate immunity-related *lysozyme* transcripts were found to be upregulated in VAC + STR and STR groups at 6 hps. On the other hand, the expression of *gr* was upregulated at 1 hps (VAC group) and 6 hps (VAC + STR and VAC groups). No modulation was observed on *hsp70* and *enolase*.

### 3.2. Gene expression modulation in zebrafish

In *D. rerio* liver (Fig. 2A), a punctual upregulation on *il1β* at 6 hps (VAC + STR group) was registered, meanwhile *tnfα* transcripts remained unaffected by stressors. Anti-inflammatory *tgfβ1* was downregulated for all treatments at different time-points (STR group: 1 hps; VAC + STR group: 1 hps, 24 hps; VAC group: 1hps, 6 hps, 24 hps). All the transcripts related to innate humoral-mediated immune response (*c3*, *lysozyme*, and *igm*) remained upregulated at 24 hps in the STR group. Concerning stress-related genes, the expression of *gr* was downregulated in all treatments at different time-points (STR group: 1 hps; VAC + STR group: 1 hps, 24 hps; VAC group: 1 hps, 6 hps, 24 hps). A very similar pattern was observed for *hsp70* (STR group: 1 hps; VAC + STR group: 24 hps; VAC group: 1hps, 6 hps, 24 hps). *Enolase* gene expression, on the contrary, was found to be upregulated at 6 hps in the STR group.

In seabream spleen (Fig. 2B), differences in the gene expression patterns were also observed between *il1β* and *tnfα*. A marked downregulation for *il1β* was observed in the VAC + STR group at all times analyzed, while a downregulation at early (1hps) and late (24 hps) times after the application of stress was observed in the vaccinated- and air exposure-stressed fish, respectively. This result contrasts with the enhanced expression of *tnfα* when both stressors were combined. No differences were observed in the other treatments and time-points for *tnfα* mRNA abundance, neither in the expression of *il10* and *tgfβ1*. Importantly, an opposite expression response was also observed between *c3* (upregulation) and *lysozyme* (downregulation) at 6hps in the

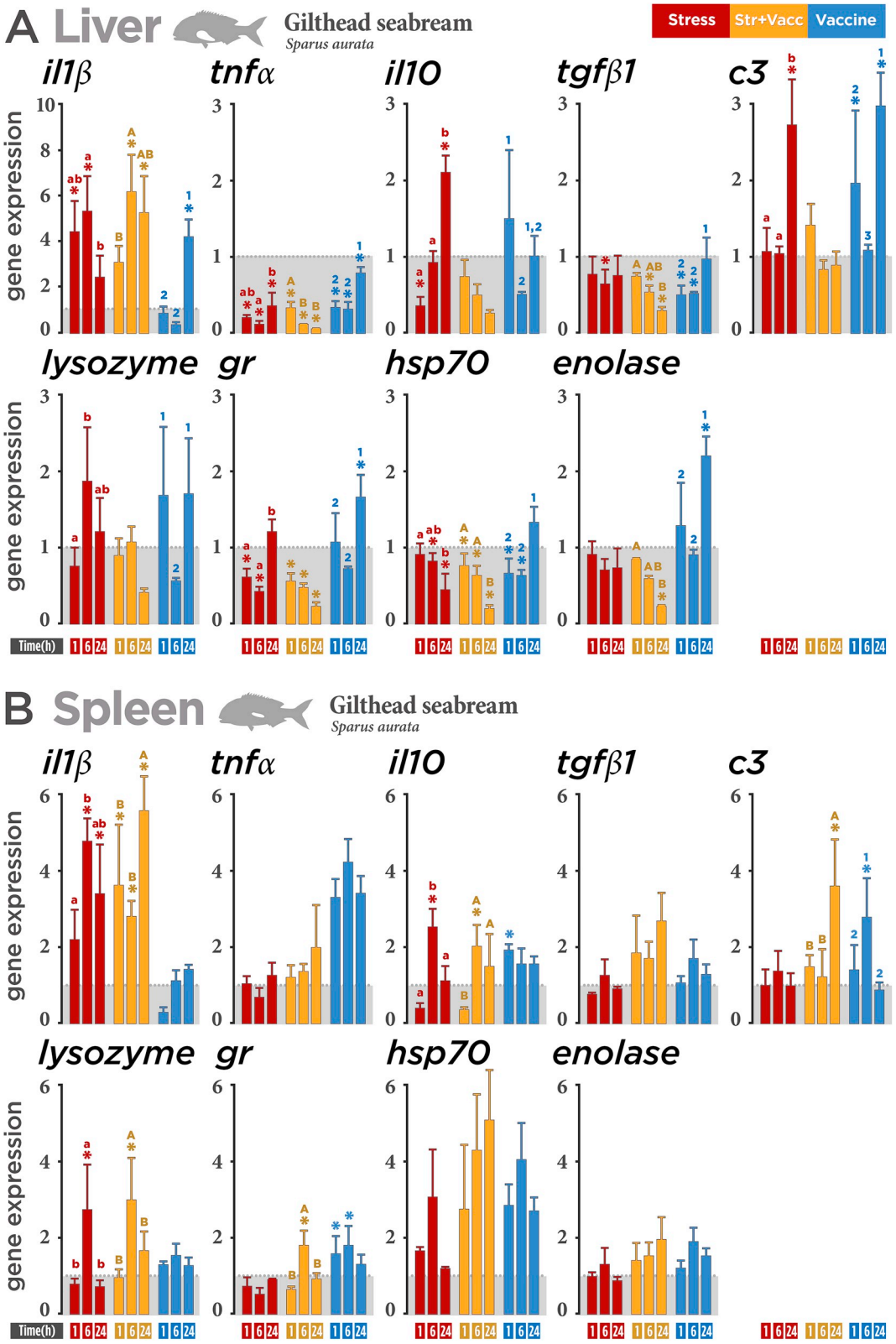


**Fig. 1.** Expression profiles for gilthead seabream in response to air exposure stress, bath vaccination by *Vibrio anguillarum* bacterin, and both stressors combined at 1, 6 and 24 h post stress. (A) Liver. (B) Spleen. The modulation of immune (*il1β*, *tnfα*, *il10*, *tgfβ1*, *igm*, *lys* and *c3*) and stress-related genes (*hsp70*, *gr* and *enolase*) was assessed. Date are presented as mean ± SE (n = 6 fish per tissue, treatment, and time-point evaluated). Different color bars indicate different treatments: red (air exposure; indicated as stress), orange (vaccine + air exposure; indicated as Str + Vacc), blue (vaccine). Dotted line represents the gene expression for control group. Time zero represents the end of the acute air exposure stress (for the air exposure and the vaccine + air exposure) or 24 h after bath vaccination (for vaccine group).Asterisk (*) indicates significant difference versus control. Significant differences into the air exposure group (lower case), vaccine + air exposure group (upper case), and vaccine group (numbers) are represented ($p < 0.05$; General-linear-Model test was performed for multiple comparisons). *interleukin 1 beta*: *il1β*; *tumor necrosis factor alpha*: *tnfα*; *interleukin 10*: *il10*; *tumor growth factor beta1*: *tgfβ1*; *immunoglobulin M*: *igm*; *lysozyme*: *lys*; *complement protein c3*: *c3*; *glucocorticoid receptor*: *gr*; *heat shock protein 70*: *hsp70*. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

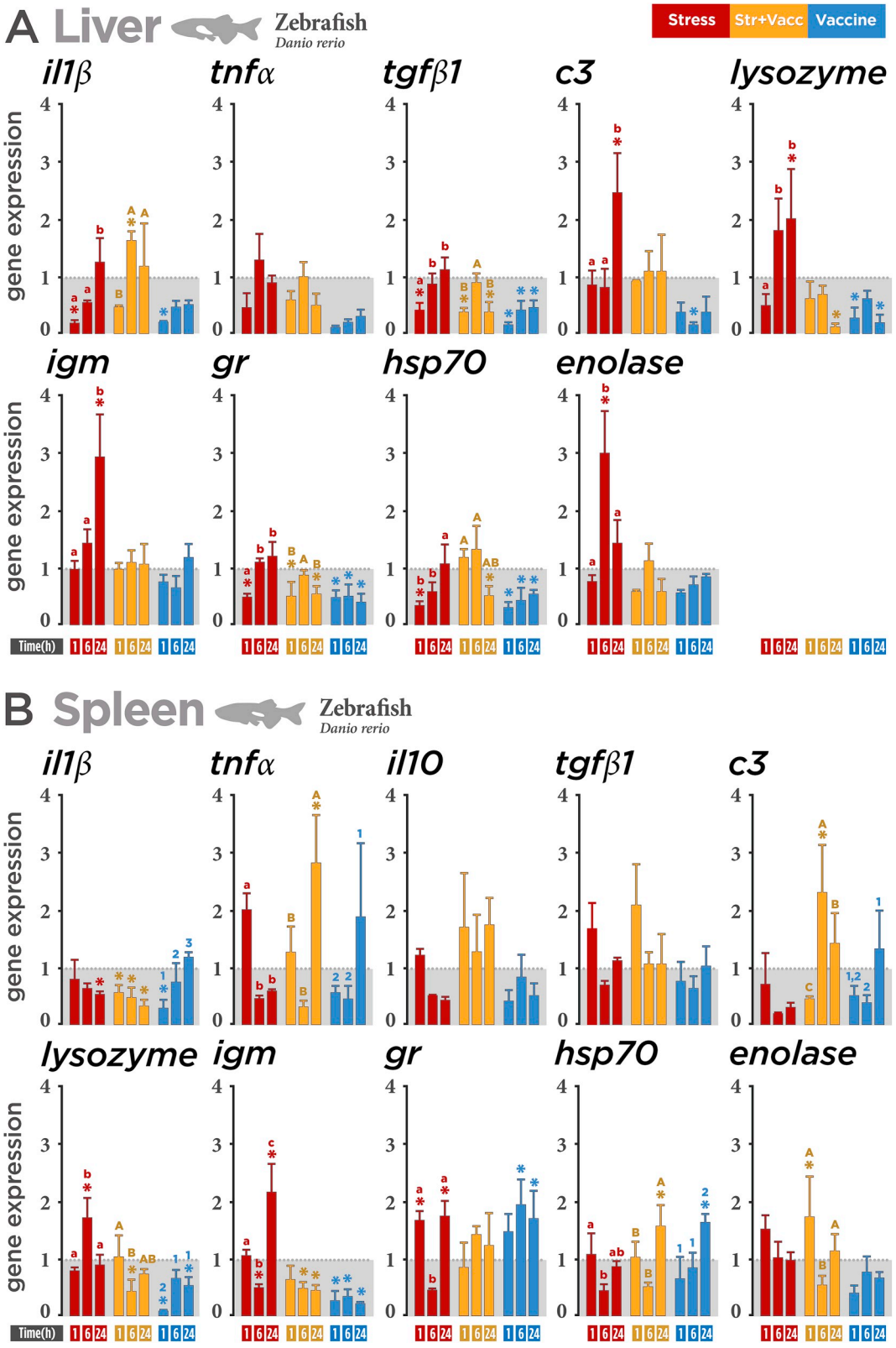


**Fig. 2.** Expression profiles for zebrafish in response to air exposure stress, bath vaccination by *Vibrio anguillarum* bacterin, and both stressors combined at 1, 6 and 24 h post stress. (A) Liver. (B) Spleen. The modulation of immune (*il1β*, *tnfα*, *il10*, *tgfβ1*, *igm*, *lys* and *c3*) and stress-related genes (*hsp70*, *gr* and *enolase*) was assessed. Date are presented as mean ± SE (n = 6 fish per tissue, treatment, and time-point evaluated). Different color bars indicate different treatments: red (air exposure; indicated as stress), orange (vaccine + air exposure; indicated as Str + Vacc), blue (vaccine). Dotted line represents the gene expression for control group. Time zero represents the end of the acute air exposure stress (for the air exposure and the vaccine + air exposure) or 24 h after bath vaccination (for vaccine group).Asterisk (*) indicates significant difference versus control. Significant differences into the air exposure group (lower case), vaccine + air exposure group (upper case), and vaccine group (numbers) are represented ($p < 0.05$; General-linear-Model test was performed for multiple comparisons). *interleukin 1 beta*: *il1β*; *tumor necrosis factor alpha*: *tnfα*; *interleukin 10*: *il10*; *tumor growth factor beta1*: *tgfβ1*; *immunoglobulin M*: *igm*; *lysozyme*: *lys*; *complement protein c3*: *c3*; *glucocorticoid receptor*: *gr*; *heat shock protein 70*: *hsp70*. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

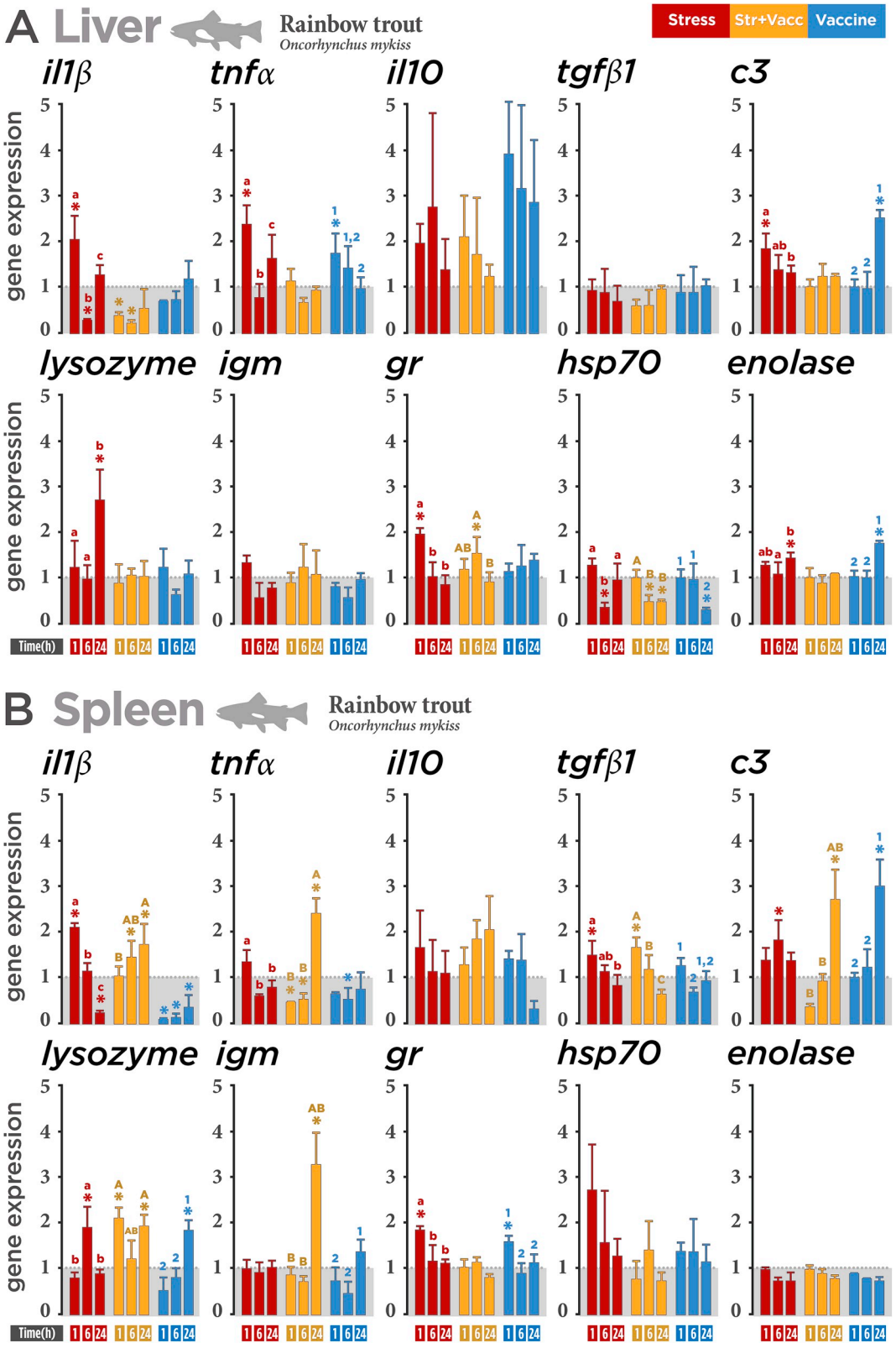


**Fig. 3.** Expression profiles for rainbow trout in response to air exposure stress, bath vaccination by *Vibrio anguillarum* bacterin, and both stressors combined at 1, 6 and 24 h post stress. (A) Liver. (B) Spleen. The modulation of immune (*il1β*, *tnfα*, *il10*, *tgfβ1*, *igm*, *lys* and *c3*) and stress-related genes (*hsp70*, *gr* and *enolase*) was assessed. Date are presented as mean ± SE (n = 6 fish per tissue, treatment, and time-point evaluated). Different color bars indicate different treatments: red (air exposure; indicated as stress), orange (vaccine + air exposure; indicated as Str+Vacc), blue (vaccine). Dotted line represents the gene expression for control group. Time zero represents the end of the acute air exposure stress (for the air exposure and the vaccine + air exposure) or 24 h after bath vaccination (for vaccine group).Asterisk (*) indicates significant difference versus control. Significant differences into the air exposure group (lower case), vaccine + air exposure group (upper case), and vaccine group (numbers) are represented ($p < 0.05$; General-linear-Model test was performed for multiple comparisons). *interleukin 1 beta*: *il1β*; *tumor necrosis factor alpha*: *tnfα*; *interleukin 10*: *il10*; *tumor growth factor beta1*: *tgfβ1*; *immunoglobulin M*: *igm*; *lysozyme*: *lys*; *complement protein c3*: *c3*; *glucocorticoid receptor*: *gr*; *heat shock protein 70*: *hsp70*. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

VAC + STR group. *Lysozyme* also remained downregulated at 1hps and 24 hps in the VAC group, but not in the STR group on which its expression was upregulated at 6hps. At stress response level, the expression of *gr* was upregulated when fish suffered air exposure (1 hps, 24 hps) and bath vaccination (6 hps, 24 hps). VAC + STR induced an upregulation of *hsp70* transcripts at 24 hps. Importantly, this gene expression modulation observed in *gr* and *hsp70* was only observed in seabream spleen but not in seabream nor in rainbow trout. The expression of *enolase* was only upregulated at 1hps when fish were subjected to the combination of both stimuli.

### 3.3. Gene expression modulation in trout

In *O. mykiss* (Fig. 3A), the upregulation of the pro-inflammatory-related genes *il1β* (STR group) and *tnfα* (STR group; VAC group) was only observed at 1 hps. No modulation of the anti-inflammatory-related transcripts *il10* and *tgfβ1* was observed in any group at all time-points tested. *c3* (1 hps in STR group; 24hps in VAC group) and *lysozyme* (24 hps in STR group) transcripts were also upregulated. Regarding stress-related modulation, the expression of *gr* was upregulated at 1 hps (STR group) and 6 hps (VAC + STR group). By contrast, the expression of *hsp70* was downregulated at 6 hps (STR group; vaccine + air exposure group) and also at 24 hps (VAC + STR group; VAC group). Enolase transcripts were upregulated only 24 hps (STR group; VAC group).

In *O. mykiss* spleen (Fig. 3B), bath vaccination + air exposure stress combined and air exposure stress enhanced the expression pattern of *il1β* but a marked downregulation was observed in non-stressed vaccinated trout at all times evaluated. The expression of *tnfα* diminished at 1 hps and 6 hps in VAC + STR group but increased at 24 hps. In VAC group, *tnfα* was downregulated only at 6 hps. Anti-inflammatory *tgfβ1* was found to be upregulated only at 1hps (STR and VAC + STR groups). No variation was registered for *il10* in all treatments and time-points analyzed. The upregulation of *c3* and *lysozyme* transcripts at 6hps in the STR group contrasts with the upregulation of *c3* and *lysozyme* at 24 hps in the VAC and VAC + STR groups. Similarly, the augmented gene expression of *igm* at 24 hps only in the VAC + STR group was also determined. In terms of stress-related genes, only *gr* showed an upregulation at 1 hps (STR and VAC groups). No significant differences were noted in the expression pattern of *enolase* and *hsp70*.

### 3.4. Cortisol levels

In the three fish species included in our study, air exposure elicited a rapid increase at 1 h post-stress (1 hps) compared to control (Fig. 4). Notably in *S. aurata*, the plasma cortisol level peaked at higher values than compared to *O. mykiss* and *D. rerio*. Then, cortisol level dropped at 6 hps in all species except in *S.aurata*, whose values remained high compared to the control group. Cortisol values at 24 hps reached the basal level in all the teleost species evaluated. Although both basal and peak cortisol are different in *S. aurata* and *O. mykiss* after air exposure, the differential increase is similar in both species (between 6 and 9 fold for sea bream and 5–6 fold for trout). Zebrafish is not directly comparable as it is the whole body cortisol, but the levels increased in a similar range of 3–7 fold.

The exposure to *V. anguillarum* bacterin bath did not induce significant changes in plasma cortisol levels in *O. mykiss* and *S. aurata* at the three time-points evaluated, though a slight tendency to increase at 6 h was observed in seabream and rainbow trout. By contrast, in bath-vaccinated *D. rerio* the cortisol levels peaked at 6 hps and then diminished to control level at 24 hps.

When fish were exposure to vaccine + air stimuli, a similar cortisol level dynamics was observed in the three species evaluated. However, only for *O. mykiss* and *S. aurata* the cortisol level was significantly upregulated showing the upmost peak at 1 hps with a slight decrease at 6 hps to then dropping to basal level at 24 hps. In the case of zebrafish, its secretion kinetics was found to be different from those of *O. mykiss* and *S. aurata*, showing at upmostcortisol value peak at 6 hps and remaining greater than control at 24 hps.

## 4. Discussion

In this study, stress and immune-related transcript responses to air-exposure and bath vaccination stress in liver and spleen of three phylogenetically distant fish species were evaluated at 1, 6 and 24 h post-stress. In fish, MALT (mucosa-associated lymphoid tissues) and head-kidney (HK) chromaffin and hematopoietic cell populations modulate the short-term responses (minutes to hours) to physiological imbalances. The correlated activation of MALT and HK tissues upon common aquaculture-related stressors, such as hypoxia and vaccination, has been described extensively [8]. Instead, here we aimed to describe the short-term changes in pro-/anti-inflammatory (*il1β*, *tnfα*, *igm*, *lys*, *c3, il10* and *tgfβ1*,) and stress-related (*hsp70*, *gr* and *enolase*) transcripts to those stressors, focusing in a true secondary lymphatic organ, the spleen, and also in a multifunctional metabolic toolbox, the liver. In fish, both organs participate in the transition from local to systemic immune outcomes, and can be properly described as a medium-to-long-term responsive organs in terms of full immune activation and distress onset. However, as suggested by our cross-species study, we cannot describe a set of minimum common short-term stress and immune transcript responses related to acute (1 min) air-exposure and/or bath vaccination with *Vibrio anguillarum* bacterin (Fig. 5). A classical currency for stress states, plasmatic level of cortisol, was also evaluated as a mean to refine the description of the effects of combined stressors.

### 4.1. Inflammatory responses

As expected by the dynamics of short-term responses in these medium-to-long responsive organs, no severe changes at 1, 6 and 24 h were observed in the gene expression levels in trout and zebrafish liver (most genes were downregulated or overexpressed 2–3 fold relative to control). Fish inflammatory responses are characterized by a first wave of expression of innate components and pro-inflammatory cytokines such as *il1β* and *tnfα* [20,21]. Anti-inflammatory components such as *il10* and *tgfβ1* are also required to regulate the inflammation, otherwise this may lead to tissue damage and immune dysfunction [22,23]. Interestingly, the expression of pro-inflammatory *il1β* in liver and spleen of stressed or vaccinated seabream increased up to 8-fold but only up to 2-fold in zebrafish and trout (Fig. 5). This illustrates a species-specific pattern of short-term gene activation for *il1β* that has been described previously [9,24].

Seabream responded to bath vaccination, but also to air exposure stress and both stressors combined, with an all-stressors global pro-inflammatory and regulatory reactivity, the liver being the most responsive organ in terms of magnitude and number of differentially modulated genes. In this species, the inflammatory response seems to be enhanced by *il1β*, complement component *c3* and *lysozyme* transcripts, and regulated by the expression of *il10*. In seabream spleen the effects of bath vaccination enhanced only the expression of c3 and *tgfβ1*, and our results indicate a more pronounced influence of air exposure stress and also the combination of both stressors in a *il1β*, *c3, lysozyme* and *il10*-mediated inflammatory response. Surprisingly, immune-related transcript responsiveness to *Vibrio anguillarum* bath vaccination is widespread in livers of challenged seabream but scarce in spleen, less than 24 h post-vaccination. This indicates that an *il1β*-driven inflammatory response, together with the expression of *c3*, defined the short-term immune response to *V. anguillarum* bacterin in the liver of *S. aurata*. In this species, for the time and treatments tested, the spleen sensed mainly the effects of air exposure stress. Moreover, the activation of *il10*, a key regulatory cytokine, suggest that an ongoing and effective immune response unfolded in both organs during the first 24 h post-stress.

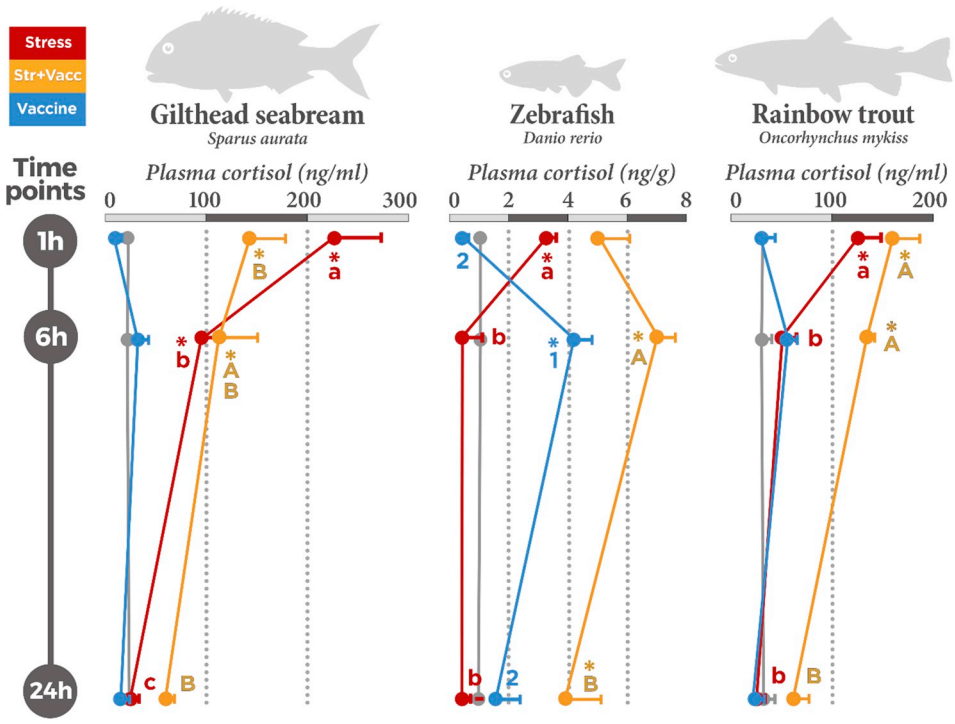


**Fig. 4.** Cortisol dynamics for gilthead seabream, zebrafish and rainbow trout in response to air exposure stress, bath vaccination by *Vibrio anguillarum* bacterin and both stressors combined at 1, 6 and 24 h post stress. Date are presented as mean ± SE (n = 8 fish per tissue, treatment, and time-point evaluated). Different color lines indicate different treatments: red (air exposure; indicated as stress), orange (vaccine + air exposure; indicated as Str + Vacc), blue (vaccine), and grey (control group). Significant differences are indicated by lower case in the air exposure group, by upper case in the vaccine + air exposure group, and by numbers in the vaccine group. Asterisk (*) indicates significant difference of each treatment versus control ($p < 0.05$; General-linear-Model test was performed for multiple comparisons). Figure originally modified from Khansari et al. (2018) [8]. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Inflammatory responses in zebrafish liver, on the contrary, relied in the enhanced expression of *c3*, *lysozyme* and *igm*, in an air-exposure-only reactivity pattern that indicate a preferential recruitment of components of the classical innate immune activation. In fact, Zhang, et al. [25] showed that vaccination of *D. rerio* with attenuated *V. anguillarum* induced expression of *il1β* in liver 7 days post vaccination, therefore suggesting different effect of vaccine at short-term. In this species, the spleen showed a minor enhancement of *c3* and *tnfα* transcript levels in response to bath vaccination and of *lysozyme* and *igm* for air-exposed fish. The focus on innate immune transcripts and the unchanged expression levels of regulatory/inhibitory cytokines (*il10* and *tgfβ1*) suggests a lack of complex immune responses to bath vaccination for this species less than 24 h post-stress. The differences in *il10* expression levels have been previously shown in other works [26], but the rate of organ colonization of waterborne *V. anguillarum* in zebrafish may play a role. It has been described that following the initial pathogen attachment to mucosal surfaces, the spreading of *V. anguillarum* bacteria reaches the liver and spleen at the later stages (> 24 h) of infection [27]. In *O. mykiss*, an intermediate pattern directed the inflammatory reaction to stressors: the expression of *il1β*, *tnfα*, c3 and lysozyme liver responded preferentially to air exposure and bath vaccination while the regulators (*il10* and *tgfβ1*) remained unchanged. In the spleen, bath vaccination enhanced only the expression of *c3*, *lysozyme* and *tgfβ1,* whereas air exposure and both stressors combined influenced the overall pro-inflammatory and innate immune transcript responses. *Igm* transcripts were modulated by air exposure stress and the combination of air exposure and bath vaccination. Short-term non-pathogenic stressors (notably heat stress) have been described to change the main antibody in head kidney, spleen, and intestine of orange-spotted grouper and Nile tilapia, *Oreochromis niloticus* [28,29]. However, pathogen-induced expression of *igm* seems to be highly species-specific, as suggested by our data and other studies describing the effects of *Ichthyophthirius multifiliis* and *Yersinia ruckeri* in trout [30,31]. Our results indicate that the mechanisms of attachment and spreading of *V. anguillarum*, albeit common to affected fish species, may be strongly species-specific once the pathogen reaches immune-related organs, and it would be advisable a functional description of short-term immune and stress responses on a per species base.

### 4.2. The stress response and its effects upon the liver and spleen gene expression

Bath-vaccination and air exposure induced changes in the expression levels of stress-related genes (*hsp70*, *gr* and *enolase*) in the liver of the three species tested and also in the spleen of zebrafish. The expression of *enolase* in liver of *O. mykiss*, *S. aurata* and *D. rerio* was also differentially regulated. Aside from its function for glucose catabolism and energetics, it has been documented that enolase acts as a cell-associated stress protein and also protecting cells after hypoxia [32,33]. This result is supported with our previous finding showing up-regulation of *enolase* in *S. aurata* brain after *in vivo* LPS challenge [34]. The type of vaccination and the rate of spreading of *V. anguillarum* bacterin once attached to mucosal surfaces [27] may account for the lower levels of *hsp* transcripts described in the liver and spleen of our fish. In *Larimichthys crocea* injected but not bath vaccinated intraperitoneally with *Vibrio alginolyticus*, the expression of *hsp70* peaked at 48 h post-injection (hpi) in liver, and at 24 hpi in spleen, declining at 72 hpi [35]. A similar study in *Vibrio alginolyticus*-infected seabream determined a peak of *hsp70* transcripts at 36 h post-infection in the liver [36].

Unexpectedly, only the glucocorticoid (GC) receptor (*gr*) showed expression changes in both seabream and trout spleen. The effect of GCs (mainly cortisol) on target tissues has been reported to be predominantly mediated by the glucocorticoid receptor (*gr*) [37], inducing reduction or upregulation at the promotors of their target genes. The expression patterns of *gr* have been shown to be highly diverse and tissue- and species-specific [38–40]. From our data, an elevated *gr* mRNA after air exposure is in agreement with the increased *gr* expression after cortisol treatment found in *O. mykiss* hepatocytes [41]. Nevertheless, temporal elevation of *gr* in the three species by vaccine, correlates with a rapid transient induction for *gr1*(a,b)/*gr* by LPS and also Zymosan in *Cyprinus carpio* and *S.aurata* [42,43].

A direct witness of stress states, plasma cortisol has been frequently used as an indicator of stress response in fish and other vertebrates [44–46], and the effects of pathogen colonization on cortisol secretion have been extensively studied. *Lepeophtheirus salmonis* increased the level of cortisol in Atlantic salmon [47,48], and *E. ictaluri* and *V. anguillarum* infection raised cortisol levels in channel catfish and *O.*

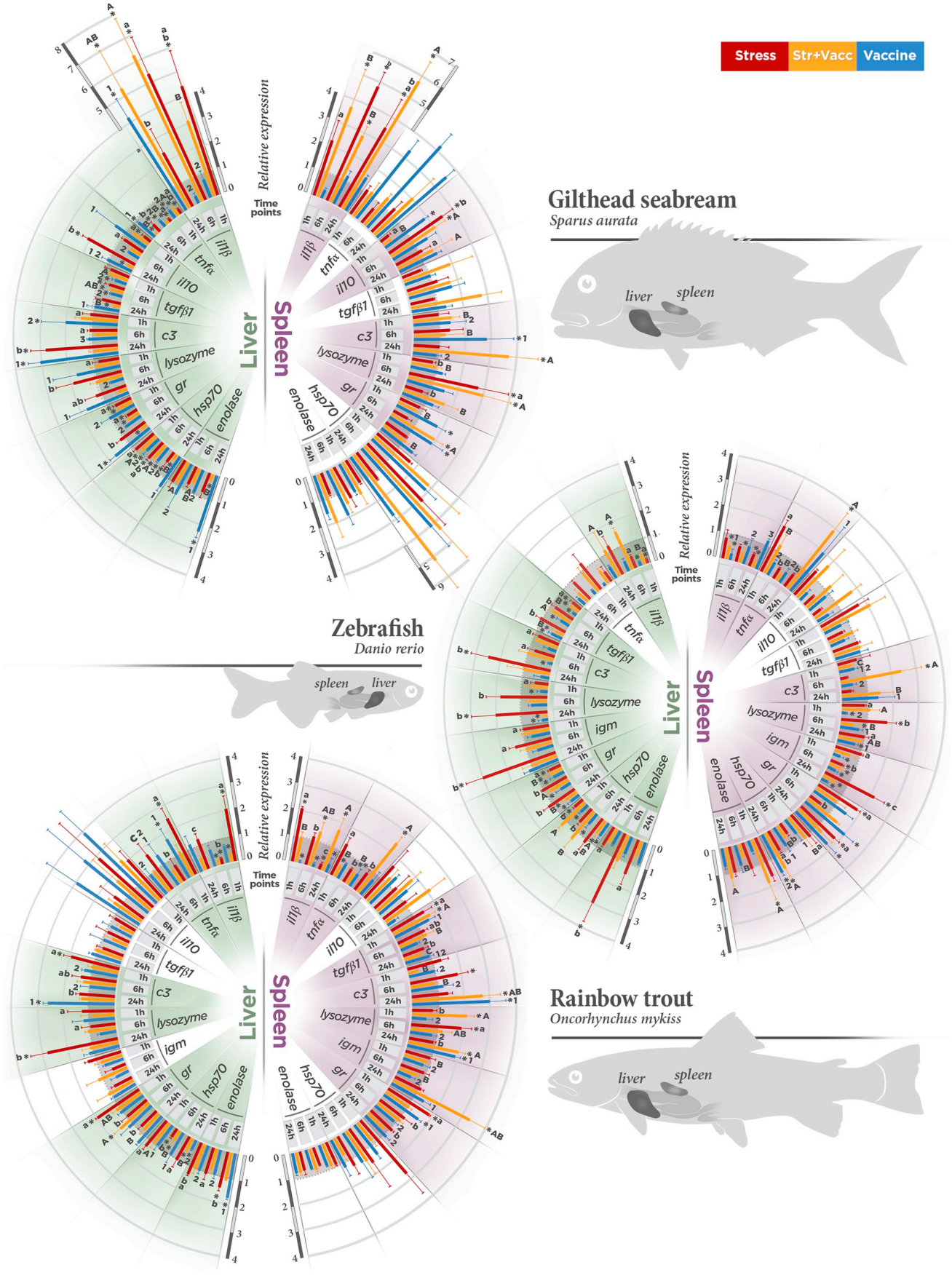


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**Fig. 5.** Integrated comparative immune and stress-related responses to air exposure stress, bath vaccination by *Vibrio anguillarum* bacterin, and both stressors combined at 1, 6 and 24 h post stress for seabream, zebrafish and trout. Coloured shaded areas (both on gene name and bars) indicate significant transcript expression differences. White areas show transcripts with no significant differences in expression levels. Date are presented as mean ± SE (n = 6 fish per tissue, treatment, and time-point evaluated). Different color bars indicate different treatments: red (air exposure; indicated as stress), orange (vaccine + air exposure; indicated as Str +Vacc), blue (vaccine). Dotted line represents the gene expression for control group. Time zero represents the end of the acute air exposure stress (for the air exposure and the vaccine + air exposure) or 24 h after bath vaccination (for vaccine group). Asterisk (*) indicates significant difference versus control. Significant differences into the air exposure group (lower case), vaccine + air exposure group (upper case), and vaccine group (numbers) are represented ($p < 0.05$; General-linear-Model test was performed for multiple comparisons). *interleukin 1 beta*: *il1β*; *tumor necrosis factor alpha*: *tnfα*; *interleukin 10*: *il10*; *tumor growth factor beta1*: *tgfβ1*; *immunoglobulin M*: *igm*; *lysozyme*: *lys*; *complement protein c3*: *c3*; *glucocorticoid receptor*: *gr*; *heat shock protein 70*: *hsp70*. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

*mykiss*, respectively [49,50]. Our results indicate that in the three fish species subjected to acute air exposure stress, cortisol levels raised at 1 h post-stress (hps), diminishing its plasmatic content at 6 hps and returning to the basal levels 24 h later in fish stressed by air exposure. The acute augment of cortisol at 1 hps was much higher in seabream than zebrafish and rainbow trout. In a recent previous report, we showed that this increase on the cortisol level could be associated to a lower activation threshold to air exposure in seabream [8] compared to the other two fish species evaluated in this current study. Accordingly, the cortisol level at 6 hps provided more evidence about a higher responsiveness of seabream to this abiotic stressor, suggesting a longer activation of the hypothalamic-pituitary-interrenal (HPI) axis, as it has been previously described for several species [19,51–53]. On the other hand, a high cortisol level was also registered for seabream, zebrafish and rainbow trout when both stimuli were applied (VAC + STR group). Contrary to the higher cortisol level observed at 1 hps in seabream subjected to air exposure, the magnitude of response in the VAC + STR group at 1 hps was in its upmost peak in rainbow trout but not seabream. This suggests that *V. anguillarum* bacterin positively influences the higher responsiveness to the combination of air exposure and *V. anguillarum* bacterin stressors. Recently, we have shown the augment of cortisol after 1h exposure to *V. anguillarum* bacterin bath in rainbow trout skin mucus (but not in seabream) [8]. This higher responsiveness of rainbow trout provide evidence of a greater activation of the HPI axis in response to exposure to *V. anguillarum* bacterin. Importantly, in zebrafish subjected to the VAC + STR stimuli the high cortisol level observed at 1 hps was even greater at 6 hps. Accordingly, the highest peak for the vaccinated group was also registered at 6 hps, thus providing evidence of a higher responsiveness to *V. anguillarum* bacterin bath exposure in zebrafish. One reason for such higher responsiveness to the vaccine may be related to the surface/volume ratio of zebrafish compared to the other two species also evaluated. Thus, zebrafish shows a much higher ratio due to its small size (0.45) compared to trout and seabream (65 g and 130 g mean value, respectively). This means that the degree of vaccine intake should be also much higher in zebrafish and therefore the response to such stressor may be also greater, with the subsequent higher cortisol release. In accordance with this hypothesis, the level of cortisol for zebrafish was elevated even at 24 hps VAC + STR group compared to control. Thus, *D. rerio* appears to be more responsiveness to the combination of both stressors probably because of the *V. anguillarum* bacterin contribution.

Taking a look to the transcript abundance analyzed in our study, zebrafish was the only fish species whose most of their downregulated liver genes (*il1β*, *tgfβ1*, *c3, lysozyme*, *gr*, *hsp70*) were differentially modulated because of the *V. anguillarum* bacterin. In the same direction, no upregulated genes in response to the vaccine bath were registered in zebrafish liver. This suggests that the high cortisol zebrafish release in response to *V. anguillarum* bacterin could have a majoritarian downregulatory effect on immune- and stress-related genes in liver. This downregulatory effect could be related to the primary metabolic role of liver (but not the immune one) and its key function to provide energy resources demanded by the host to respond successfully against the stressor stimuli. Contrary to the expression in zebrafish liver, only a slight upregulation on stress- (*gr* and *hsp70*) and the downregulation on immune-related genes (*il1β*, *lysozyme*, *igm*) was observed in spleen. In accordance with the higher cortisol level observed for the VAC + STR group, in zebrafish spleen subjected to this combination of stimuli (VAC + STR) most of the genes were differentially upregulated (*tnfα*, *c3*, *hsp70*, *enolase*). Importantly, the same trend (upregulation of *il1β*, *tnfα*, *tgfβ1*, *c3, igm, lysozyme*) was also observed in rainbow trout for the VAC + STR group. This indicates that the combination of both stressors (VAC + STR) promote a higher cortisol release compared to the separated effect of each stressor. This high plasmatic cortisol content may have a stimulatory effect on some of the genes evaluated in zebrafish and rainbow trout spleen. On the other hand, it is also important to take in consideration that the gene modulation observed in zebrafish and rainbow trout spleen could be also be a natural mechanism of response to an exogenous pathogenic antigen such as *V. anguillaum* bacterin. In line with this hypothesis, it is not surprising the upregulation also observed in seabream spleen both on immune-(*il1β*, *il10*, *c3*, *lysozyme*) and stress-related genes (*gr*). However, there is no a clear time-dependent correlation between the expression profile of VAC + STR and VACC group. By contrast, most of the differentially expressed genes in the STR group (*il1β*, *il10*, *lysozyme*) show a correlated upregulation with the expression profile of the VAC + STR group. This antecedent provides more evidence that in seabream air exposure (STR group) has a preponderant effect upon the combination of both stressors (VAC + STR group) with the concomitant higher cortisol release and, in consequence, their relationship over the gene expression profile observed in spleen. More studies will be needed to address the mechanisms of the modulation of punctual genes and the consequences of their expression on the stress response.

## 5. Conclusions

From our data, *S. aurata* relies on *il1β*-driven pro-inflammatory reactions when subjected to bath vaccination with *Vibrio anguillarum* or stressed by 1 min of air exposure, in both liver and spleen, whereas *D. rerio* and *O. mykiss* engage preferentially components of the innate immune response, such as *c3* and *lysozyme*. The immune response seems to be stronger in seabream or its sensitivity is higher after air exposure; the combination of both stressors shows higher effects in zebrafish and the influence of air exposure and also bath vaccination affects trout, albeit less organized and regulated at the times tested. The spleen shows a more robust stress response in terms of gene expression of glucocorticoid receptor, *enolase* and *hsp70* in *D. rerio* than in seabream or trout, defined by elevated levels of cortisol at 6 h post stress in bath vaccinated zebrafish, rendering a more pronounced sensibility to stress in this species. When comparing our results with previous description of vaccination stress upon mucosal surfaces [8], it seems that mucosal epithelia react more intensively, in terms of gene expression levels, than systemic organs such as liver and spleen. Seabream and rainbow trout have been increasingly used for field analyses [54], being the zebrafish the archetypical laboratory fish model and therefore, the gold standard by which physiological responses are compared. However, as our and other studies recognize, the evolutionary life story of a species limits and permits the temporal and spatial dynamics of homeostatic reactiveness. In this sense, our analysis of short-term stress and immune-related gene expression in metabolic or immune reactive organs advocates for a comparative species-specific approach to study the onset

of immune responses following typical aquaculture procedures and disease outbreaks. Altogether, these results indicate that the stress and immune response in liver and spleen is clearly very species-specific.

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