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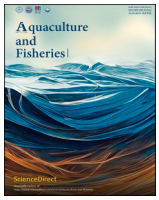

Original research article

Effects of an acute negative and positive stimulus on immune-related gene expression in the pituitary and head kidney of carp (*Cyprinus carpio* L.)

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ABSTRACT

The objective of this study was to investigate expression profiles of genes involved in the immune responses in the head kidney, as well as stress response-related genes in the pituitary in juvenile koi carp after exposure to an acute positive (feed reward) or negative cue (air exposure). Both, the head kidney and the pituitary transcriptome analysis, revealed distinct gene expression patterns in fish subjected to the different treatments. Notably, expression changes of genes with the greatest impact on the fish's stress status were identified, emphasizing their role in stress perception and immune regulation related to stress. This allows the identification of promising candidates for further investigations. Additionally, the application of ElasticNet regression enabled, with high accuracy, the classification of stimulus presence, absence and valence, based on the gene expression patterns, representing a novel and valuable tool for transcript analysis in stressed fish. To our knowledge, the ElasticNet regression was used for the first time in such an application. These findings further confirm the differential perception and response of fish to negative and positive stimuli, offering insights into the complex interplay between stress and immune responses.

1. Introduction

The stress responses in teleosts involve a variety of physiological processes in the organism including alterations of immune functions (Guo & Dixon, 2021; Wendelaar Bonga, 1997). Long-term chronic stress is commonly believed to have immunosuppressive effects in fish (Dai et al., 2023; Tort, 2011). On the other hand, being confronted with a short-lived, intense stimulus (acute stressor) is considered to either enhance the immune functions (Demers & Bayne, 1997; Klak et al., 2024) or to diminish them (Weyts et al., 1998). Especially for unpredictable stressors, it has been assumed that immune activity can increase, for example in parts of the body that require immune protection (Martin, 2009). In addition, glucocorticoid effects on the immune system can be modulated by increasing insensitivity of immune functions to stress hormones (Stark et al., 2001). Furthermore, contradictory evidence for stimulatory and downregulating effects of stress suggests a more complex response to acute stimuli, involving other, possibly more important immunomodulatory factors than the traditionally considered stress hormone cortisol. Therefore, Breuner, Delehanty, and Boonstra (2013) emphasized that cortisol measurements are not sufficient to assess physiological consequences of stress, and downstream metrics, such as immune status or body indices have been proposed as parameters allowing a better evaluation of stress effects. However, a more detailed knowledge about differential effects of acute stress on the immune system in fish is still scarce.

Activation of the hypothalamus-pituitary-interrenal (HPI) and the hypothalamus-sympathetic-chromaffin (HSC) axis following acute stress plays a substantial role in adaptation to challenges teleosts encounter. Initial studies assumed a non-specificity of stress responses regardless of the nature of the stressors (Cooper & Dewe, 2004; Selye, 1936). The effects of acute stress, however, have been reported to result in diverse physiological changes depending on the characteristics of stressors. If a fish can overcome the allostatic load caused by exposure to a moderate acute stressor and successfully return to homeostasis, so that a stressor may become adaptive and categorized as eustress. On the other hand, increased stress severity may be maladaptive (distress), meaning, that the regulatory mechanisms were unable to offset the impact of a stressor (Balasch & Tort, 2019; Schreck & Tort, 2016). Studies show, that both

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higher vertebrates and fish can distinguish positive and negative cues. This capability is linked to mammalian basolateral amygdala and its corresponding telencephalic dorsal pallium of teleosts (Broglio et al., 2005; Manuel et al., 2015; Namburi et al., 2015; Paton et al., 2006). This ability allows animals to tailor the responses to the nature of the encountered challenge. Stress is present in every aspect of life whether induced by social, reproductive, or intrinsic physiological factors (Birnie-Gauvin et al., 2023; Earley et al., 2006; Summers & Winberg, 2006; Takahashi et al., 2018). National Research Council Committee on Pain and Distress in Laboratory Animals, 1992 recommended to take the feeding ecology of a species into account, since the ignorance of the animals' needs may lead to conditions that contribute to stress and lower well-being. Even regular events such as feedings can be also stressful for animals as they have been linked to increased locomotion, especially when feeding is scheduled (Laguë & Reebs, 2000; Sánchez et al., 2009). Interestingly, meal timing has been shown to influence the cortisol and glucose levels plasma of sea bream, *Sparus aurata* (Sánchez et al., 2009), with fish exhibiting increased levels of both parameters when a single meal was applied randomly during the day compared with fish fed a single meal at a fixed time. Another reason for stress related to feeding of animal groups increased aggression levels (Huntingford & Adams, 2005; Oikonomidou et al., 2019; Sirot, 2000; Summers & Winberg, 2006). The occurrence of aggression in farmed fish is increasingly recognized since breeding often results in bolder fish that are willing to take greater risks when foraging and consequently, in some cases also show more aggressiveness (Huntingford & Adams, 2005). In higher vertebrates, there is increasing evidence that aggression traits and immune functions are interconnected (Takahashi et al., 2018). For feeding in groups, it was assumed that the level of aggressiveness increases with the animal density in the group and decreasing food availability (Sirot, 2000). Studies on European seabass (*Dicentrarchus labrax*) revealed that their aggressiveness is intensified not only during feeding but also before and after feeding (Oikonomidou et al., 2019). However, there are also species-specific differences in aggressiveness since juvenile cichlids (*Archocentrus nigrofasciatum*) showed lower aggression when food was scarce, higher aggression as food abundance increased, and again lower aggression when food was provided in excess (Grant et al., 2000). However, feeding has positive effects namely satiety. (Assan et al., 2021; Kulczykowska & Sánchez Vázquez, 2010). In addition, feed-deprivation has been shown to lead to anger-like states in vertebrates which also show changes in expression of the immediate early gene *c-fos* and the GABAergic system (Subhedar et al., 2023). In recent studies on fish, acute stress also involved changes of c-fos expression and GABA receptors at the brain level of common carp and zebrafish (Burren & Pietsch, 2021; Pawlak, Burren, Seitz, Glauser, & Pietsch, 2022; Pietsch et al., 2024).

The stress concept of Smyth et al. (2023) distinguished between three stress-response components: the baseline state, the reactivity state and the pile-up phase if several stressors act together over-time. Although the downstream responses to either positive or negative cues differ, initially, exposure to a stressor eventually leads to activation of the HPI axis in the reactivity state if a certain threshold value is exceeded. This typically causes release of a corticotropin-releasing hormone (Crh) from the hypothalamic preoptic area (Huising et al., 2004), that stimulates corticotrophs in the pituitary gland to synthetize pro-opiomelanocortin (Pomc1 and Pomc2) – precursors of the adrenocorticotropic hormone (ACTH) (Metz et al., 2004). ACTH stimulates the interrenal tissue in the head kidney through melanocortin 2 receptor (Mc2rec) and activation of steroidogenic acute regulating protein (Star) to synthetize and release cortisol that in turn mediates numerous biological processes, including immune response (Aluru & Vijayan, 2008; Faught et al., 2016; Wendelaar Bonga, 1997). The corticotropin releasing hormone-binding protein (Crh-bp) in mammals is known to reduce further ACTH release as a consequence of binding Crh (McClennen et al., 1998). Moreover, Doyon et al. (2005) suggested that Crh-bp found in the pituitary of rainbow trout, *Oncorhynchus mykiss*, plays a role in regulating HPI axis activity by reducing accessible Crh which in turn accelerates the homeostasis recovery after stress. Furthermore, corticotropin releasing factor receptor 1 (Crf-r1) and corticotropin releasing factor receptor 2 (Crf-r2) mediate the effects of crh in teleosts (Arai et al., 2001; Manuel et al., 2014; Pohl et al., 2001). Furthermore, mammalian urocortin, homologous with teleostean urotensin1 (Uro1), a neuropeptide belonging to the Crh family, binds to Crf-r1 and Crf-r2 (Suda et al., 2004) contributing to anti-inflammatory immune responses in mammals (Tsatsanis et al., 2005). However, detailed knowledge on Uro1 in teleosts is still lacking. Furthermore, teleost fish stress responses involve nonapeptides arginine vasotocin and isotocin, regulating numerous processes, including osmoregulation, metabolism, and social behaviour through their receptors – the arginine vasotocin receptor 1 (Vasor1), arginine vasotocin receptor 2 (Vasor2) and isotocin receptor 1 (Itr1) in target tissues and organs, including the head kidney (Mahlmann et al., 1994; Skrzynska et al., 2018). A study using goldfish (*Carassius auratus*) showed that arginine vasotocin and isotocin can stimulate cortisol release (Fryer & Leung, 1982). On the other hand, Cadiz et al. (2015) found that both pathways are regulated by cortisol in gilthead sea bream (*Sparus aurata*), which can be an indication of a negative feedback on cortisol synthesis. Nevertheless, besides a clear role in stress responses, the head kidney has crucial functions in fish immunity, and due to its organization enables direct signaling between the endocrine and the immune system (Weyts et al., 1999). In contrast to its mammalian analogue, the adrenal gland, the head kidney lacks its typical structures, as cortex and medulla cannot be differentiated. The interrenal cells responsible for cortisol production and chromaffin cells producing catecholamines are integrated in the hematopoietic head kidney tissue of teleosts (Gallo & Civinini, 2003). Likewise, cytokines are an important link between the immune system and the central nervous system in both fish and mammals. They are synthesized mainly by granulocytes, macrophages and lymphocytes. Pro-inflammatory cytokines initiate and amplify the immune response, while anti-inflammatory cytokines resolve inflammation to prevent tissue damage (Grayfer et al., 2011; Piazzon, et al., 2015). Nevertheless, the classification of cytokines can be challenging due to their numerous functions, which can overlap and even contradict each other. Interleukin 1beta (Il-1 β) as a proinflammatory cytokine plays an influential role in the innate immune response (Dinarello, 1997; Secombes et al., 1999) and similarly to mammals, it modulates the activity of the HPI axis in fish (Holland et al., 2002). Accordingly, in the head kidney of common carp (*Cyprinus carpio*), its increased expression, along with the modulation of other proinflammatory cytokines such as tumor necrosis factor α (TNF- α) and neutrophil-activating chemokine 8 (Il-8) (Zhonghua et al., 2008) was observed after 4 h of transport in high and low stocking density groups (Hoseini et al., 2019). Similarly, upregulation of *Il-1β* and its receptor was observed in the head kidney 24 h after acute confinement (Metz et al., 2006). In addition, interferon gamma (*IFNg*) is thought to be involved in modulating several immune factors in fish, including interleukin 17 (Il-17), increasing the release of other proinflammatory cytokines, e.g., Il-1 β and TNF- α. However, it also affects the activation of macrophages, along with an increase in phagocytosis and the release of nitric oxide (López-Muñoz, Roca, Meseguer, & Mulero, 2009; Grayfer et al., 2010; Du et al., 2015; Gan et al., 2020). The latter is produced by the inducible nitric oxide synthase (iNOS) as a response to the exposure to cytokines, such as Il-1β, bacterial components or parasites (Kunz et al., 1994; Nathan, 1992; Stuehr & Marletta, 1985). Accordingly, bacterial lipopolysaccharides can induce *iNOS* expression thus leading to increased production of nitric oxide in isolated leucocytes of *C. carpio* (Pietsch et al., 2009, 2011). The endocrine system can influence immune functions of common carp, as shown by lower *iNOS* expression and consequently reduced production of nitric oxide in cultured head and trunk kidney leucocytes after exposure to medroxyprogesterone acetate, dihydroxy progesterone and cortisol (Pietsch et al., 2009). To maintain immune homeostasis and prevent excessive inflammation that can lead to tissue damage, suppression of the

pro-inflammatory response is necessary. This regulation can be achieved through the action of anti-inflammatory cytokines, such as interleukin 10 (Il-10) (Grayfer et al., 2011; Piazzon et al., 2015), and interleukin 4 (Il-4) (Bottiglione et al., 2020; Wang et al., 2016). Furthermore, arginase, the last enzyme in the urea cycle plays a substantial role in oxidative stress-related reactions by converting arginine to ornithine and urea. Arginine serves as a substrate for proliferation of pathogens, as well as for iNOS for catalyzing NO production, and by reducing its availability, arginase exhibits anti-inflammatory effects (Vincendeau et al., 2003). There are two arginase isoforms known, the cytosolic arginase 1 (Arg1) and the mitochondrial arginase 2 (Arg2), differing in their expression in various organs. Joerink et al. (2006) suggested that fish Arg2 may be involved in alternative macrophage activation which is important in type 2 immune responses.

The initial activation of the HPI axis, as well, as differential downstream regulation of the cortisol release were observed in our previous study on carp (Pawlak, Burren, Seitz, Glauser, & Pietsch, 2022). We investigated the impacts of a positive stimulus (an expected feed reward), a negative stimulus (netting the fish above water surface for 1 min), as well as tank manipulation (opening and closing curtains around the tanks and tank lids), 10, 30 and 60 min following the exposures on the expression patterns of stress-related genes in the brain of the juvenile koi carp used also in the current study. Results showed a significant increase in plasma cortisol levels 30 min after air exposure, as well as plasma glucose levels, however, 10 min after both, the air exposure and feed reward. Furthermore, tank manipulation resulted in elevated plasma corticosterone and glucose levels at different time points. Even though cortisol plasma levels were not significantly elevated after receiving a feed reward, the initial activation of the HPI axis was observed, where several Crf-pathway-related genes were similarly expressed after both experimental treatments. Similarly treated zebrafish (*Danio rerio*) confirmed these results (Pietsch et al., 2024).

Advancing these findings, this study aims to examine the gene expression patterns associated with immune responses in the head kidney and stress response-related genes in the pituitary of the juvenile koi carp, 10, 30 and 60 min following the abovementioned treatments.

2. Materials and methods

2.1. Rearing conditions

Seventy juvenile koi carp (*Cyprinus carpio*), reared for two months in a 290 L aquarium fitted with a biofilter, as specified previously in (Pawlak, Burren, Seitz, Glauser, & Pietsch, 2022, 2023), were fed four times a day with a feed amount corresponding to 2–3 % body weight per day. Furthermore, the koi carp were trained on a daily feed reward with mosquito larvae provided manually between 8:00 and 8:30 CET, as one of the daily feed portions. To conduct the stress experiment, a total of 60 koi carp weighting on average 78.7 g were selected from the rearing tank and either sampled immediately (C0) or transferred to 50 L aquaria with a lid where they continued to receive the daily feed reward. Curtains were placed around the aquaria to minimize the impact of routine maintenance. After a three-day acclimatization period, six fish were subjected to one of three treatments: opening the curtains and lifting and closing the lid (C), receiving the feed reward (F), or netting the fish above the water level for 1 min and put back in the aquaria (A). Following the treatments, the koi carp were left for 10, 30 or 60 min without disturbance. Subsequently, they were euthanized using an overdose of tricaine methanesulfonate (MS-222, Sigma-Aldrich, Buchs, Switzerland) and promptly sampled. A schematic representation of the experimental procedures can be found in S1. The process of anesthetization, blood sampling from the caudal vein, and sampling of the pituitary gland and head kidney took on average 13.24 min (±2.24 min). Prior to further processing, the collected tissue samples were stored in RNAlater® (Sigma-Aldrich, Buchs, Switzerland) for at least 24 h. The measured blood plasma parameters (cortisol, cortisone, corticosterone, lactate and glucose) have been shown previously (Pawlak, Burren, Seitz, Glauser, & Pietsch, 2022). The experimental procedures were authorized by the cantonal veterinary council of Zurich (Switzerland) under permission number ZH-062-17. All experimental animal protocols were carried out by following the guidelines of the Swiss Council of Animal Care on the husbandry and use of animals in scientific research.

2.2. PCR conditions

Total RNA was extracted separately from each pituitary and head kidney sample using RNeasy Micro and Mini Kits (Qiagen Basel, Switzerland), respectively, with an on-column DNase 1 treatment (RNase-free DNase Set, Qiagen, Basel Switzerland) during the RNA extraction. The concentration and quality of the extracted RNA was assessed on a Tecan Infinite M Plex (Tecan Instruments, Switzerland). All mRNA samples were reverse transcribed to cDNA using ProtoScript® II First Strand cDNA Synthesis Kit (BioConcept AG, Allschwil, Switzerland). Both head kidney and pituitary samples were diluted to a concentration of 50 ng cDNA/ul. To examine gene expression patterns in pituitaries and head kidneys of each fish, real-time quantitative PCR (qPCR) on a CFX Connect Real-Time System using the SsO fast® SYBR®Green Master mix (BioRad, Switzerland) were performed with cycling conditions as described previously (Burren & Pietsch, 2021) using two replicates for each run. The primer pairs used for the qPCR are listed in Table S1 in the supplement. Optimal reference genes sets were identified using RefFinder (http://blooge.cn/RefFinder/) separately for pituitary and head kidney. The results from the reference gene evaluation can be found in the supplement (Tables S2 and S3). The reference gene of choice for both, the pituitary and the head kidney, was beta-actin (*bact*) as other potential reference genes showed significant differences in expression among treatments. All gene expression values of the two technical replicates for each sample have been calculated relative to the expression of the selected reference genes (ΔCt method) and were further calculated as fold-changes compared with the respective controls as described previously in detail (Burren & Pietsch, 2021). The target genes for the pituitary gland included *ef, gapdh, tub, crf-r2, crh-bp, gr1, gr2, pomc1, pomc2* and *ef, gapdh, tub*, *iNOS,* arg*1,* arg*2, Il-1β, Il-8, Il-10, TNF-a, itr-1, IFNg, mc2rec, star, uro1, vasor2, Il-4, Il-17*, and *vasor1* for the head kidney.

2.3. Calculations and statistics

The gene expressions investigated in pituitaries and head kidneys have been used to build mixed models with a fully Bayesian approach using the *brms* package (Bürkner, 2017) in R studio (Version 1.2.1335, RStudio Team, 2018). Pre-checks performed with 5000 iterations revealed that the student's t-distribution shows the highest agreement with the data structure. Hence, t-distributions were used for the models based on 10,000 iterations that included gene-specific random effects for the constants (α), gene-specific random effects for differences between groups (β), as well as animal-specific random effects for the constants (γ) as explained earlier by Burren and Pietsch (Burren & Pietsch, 2021). Improved handling of possible outliers was achieved by using posterior predictive checks based on Markov Chain Monte Carlo (MCMC) approximation. This resulted in simulated replicated data under the fitted model which were subsequently compared to the observed data. Finally, the point estimators, their SEMs, credibility intervals and posterior predictive p values were extracted. Significance was determined using Wald χ2-statistics for generalized linear models, F-statistics for mixed models, and estimated marginal means were calculated if applicable. The effect estimates presented in the results section are, thus, estimated marginal means with their corresponding 95% credible intervals (95% CI). A $p < 0.05$ was considered statistically significant. Additionally, the gene expression data were subjected to a principal component analysis (PCA) to show gene clusters that typically respond to the distinct treatments. The PCA was run in R studio on

individual sets of genes as described earlier (Pawlak, Burren, Seitz, Glauser, & Pietsch, 2022). More details on the contribution of each of the two main components to the total variance are given for the individual treatment groups in the results section.

In order to confirm the hypothesis that the selected stressors and genes for stress evaluation allow a classification, gene expression values as log 2 of the normalized expression have been used to generate bootstrapped datasets with n = 2000 data points separately for each treatment group and sampling time point for each gene using the R package *boot* (Canty & Ripley, 2021) and *resample* (Hesterberg T., 2015) in R studio. These data have been used for further analyses whereby 75 % of the data were randomly chosen to train individual ElasticNet regression models followed by classification using logistic regression and odd's ratio calculation with the R package *caret* (Kuhn, 2008) and *glmnet* (Friedman et al., 2010) in R studio. The remaining 25 % of the data have subsequently been used to test the model fit and calculate misclassification rates. The alpha and lambda parameter values for model performance optimization were selected by a five-fold cross validation method individually for each comparison (Tables S4 and S5). Finally, the odd's ratios calculated from the logistic regression models have been visualized using the *heatmap2* function from *ggplots* package in R (Warnes et al., 2009).

3. Results

3.1. Effects of tank manipulation

The expression of the investigated genes in pituitary and head kidney was not influenced by tank manipulation, i.e., curtain and lid opening, between control animals after 0, 10, 30 and 60 min (C0, C10, C30, C60) (Figs. 1 and 2). The two-component PCA explained 90.15 % of the total variance in the control fish in the pituitary and 81.03 % in the head kidney considering the potential housekeeping genes, 82.56 % of the total variance for the HPI axis-related genes in the pituitary gland (Fig. 4), 80.17 % of the total variance of immune response-related genes in the head kidney, and 83.1 % of the remaining genes examined in the head kidney (Fig. 3).

3.2. Effects of feed reward and air exposure in the pituitary gland

Significant differences in gene expression were observed only 30 min after the treatments (Fig. 2). The mRNA expression of *18S RNA* and *gapdh* decreased after air exposure compared with the C30 group ($p = 0.004$ and $p = 0.046$, respectively), as well as with the feed reward group ($p = 0.042$ and $p = 0.012$). The mRNA expression of the genes *crf-r2* and *crh-bp* was elevated after both treatments compared with the respective control group, and simultaneously the expression of both genes was considerably higher after air exposure than after feed rewarding ($p < 0.001$).

3.3. Effects of feed reward and air exposure in the head kidney

Genes investigated in the head kidney were influenced by the experimental treatments at different time points (Fig. 1). Differences in gene expression were seen already 10 min after the experimental treatments. The mRNA expression of *Il-1 β* and *vasor1* was elevated after feed rewarding than in the C10 group ($p = 0.036$ and $p = 0.006$, respectively). The expression of *vasor1* was also higher after the air exposure treatment than in the control group ($p = 0.042$).

In the group 30 min after feed rewarding, the mRNA expression of *iNOS* and *vasor1* was lower than in the C30 group ($p = 0.046$ and $p = 0.048$, respectively). The expression of *star* was the highest after feed rewarding, compared with both, C30 ($p = 0.002$) and air exposure group ($p = 0.012$).

At 60 min after the experimental treatments more differences in gene expression in the head kidney were observed. The gene *18S RNA* was downregulated after feed rewarding, compared to animals exposed to air ($p = 0.02$). In contrast, *mc-r2* was upregulated in the head kidneys of animals receiving the feed reward compared with the air exposure group ($p = 0.016$). The expression of *ef* was lower in the feed reward group than in the C60 ($p = 0.004$), as well as in the air exposure group ($p < 0.001$). Higher expression of *iNOS*, *uro1* and *vasor1* was observed 60 min after the feed reward than in the respective control group. *Uro1* and *vasor1* were also upregulated in the head kidneys of fish receiving the feed reward compared with fish after the acute stressor. In contrast, *Il*-4 mRNA expression levels showed a significant decrease in the feed reward group compared with the respective control group ($p = 0.01$) and the air exposure group ($p = 0.042$).

3.4. Principal component analysis

The two-component PCA explained 88.3 % of the total variance of the potential reference genes and 86.9 % of the total variance for the stress-related genes 10 min after both treatments in the pituitary (Fig. 4). At the same timepoint, the two-component PCA explained 25.2 % of the total variance for the potential reference genes, 16.0 % for the immune response genes, and 85.1 % for the other examined genes in the head kidney after each treatment (Fig. 3).

For the groups 30 min after the treatments, the two-component PCA explained 73.6 % of the total variance of potential reference genes and 78.1 % of the stress-related genes in the pituitary gland, whereas in the head kidney, 86.7 % of the total variance was explained for the potential housekeeping genes, 82.3 % for the immune response genes and 84.6 % for the other examined genes. In the pituitary gland, the two-component PCA explained 78.3 % of the total variance of potential reference genes and 83.0 % of the stress-related genes for the 60 min data set. In the same data set for the head kidney, 85.2 % of the total variance was explained for the potential reference genes, 86.6 % for immune-related genes and 89.3 % of the total variance in the other analyzed genes.

The highest cos2 values for the pituitary were observed for *gr2, pomc2* and *crh-bp* in the 10 min data set, for *crh-bp*, *crf-r2* and *pomc1* in the 30 min data set and for *pomc1*, *gr1 gr2 pomc2* and for *crf-r2* in the 60 min data set. Moreover, the feature loading plots (Fig. 4) suggest similar loading patterns for *pomc2* and *gr2* in the pituitary. On the other hand, the loading plot for the pituitary data set 10 min after treatments suggests a opposite loading pattern for *pomc1* and *gr1*. Similar results, including also *pomc2* and *gr2* against *pomc1* can be seen in the 30 min data set (Fig. 4).

Variables that contributed the most to the two dimensions of the immune-related genes in the head kidney were *IlL-8*, arg*2* and *TNF- α* for each data set, however, *Il-17* and *Il-10* also contributed within the 10 min data set, *iNOS* and *Il-10* in the 30 min data set, and *iNOS* in the 60 min data set. For the other examined genes in the head kidney, *itr1* and *uro1* shared high contribution among all datasets. Moreover, *mc2rec* in the 10 min data set, *star* and *mc2rec* in the 30 min data set, and *star* in the 60 min data set had the highest cos2 values. The feature loading plots (Fig. 3) suggest a similar expression pattern of *mc2rec, uro1, itr1* and *vasor1* in the other examined genes' group, as well as *Il-8,* arg*2, TNF- α* and *Il-10* in the immune-related genes. Furthermore, different expression patterns of *pomc1* and *pomc2* were observed in the loading plots of each data set of the pituitary (Fig. 4).

3.5. Regression and classification with bootstrapped gene expression data

To evaluate whether the selected stressors and genes for stress evaluation allow a classification if a sufficient number of data points has been compiled, bootstrapped datasets have been generated and subjected to an ElasticNet regression analysis, since this is optimal for handling multicollinearity, reducing overfitting, and selecting relevant features. ElasticNet regression is able to evaluate both, the usage of a Lasso regression model and a Rigde regression model at the same time. In the head kidney, the ElasticNet regression favored Ridge regression,

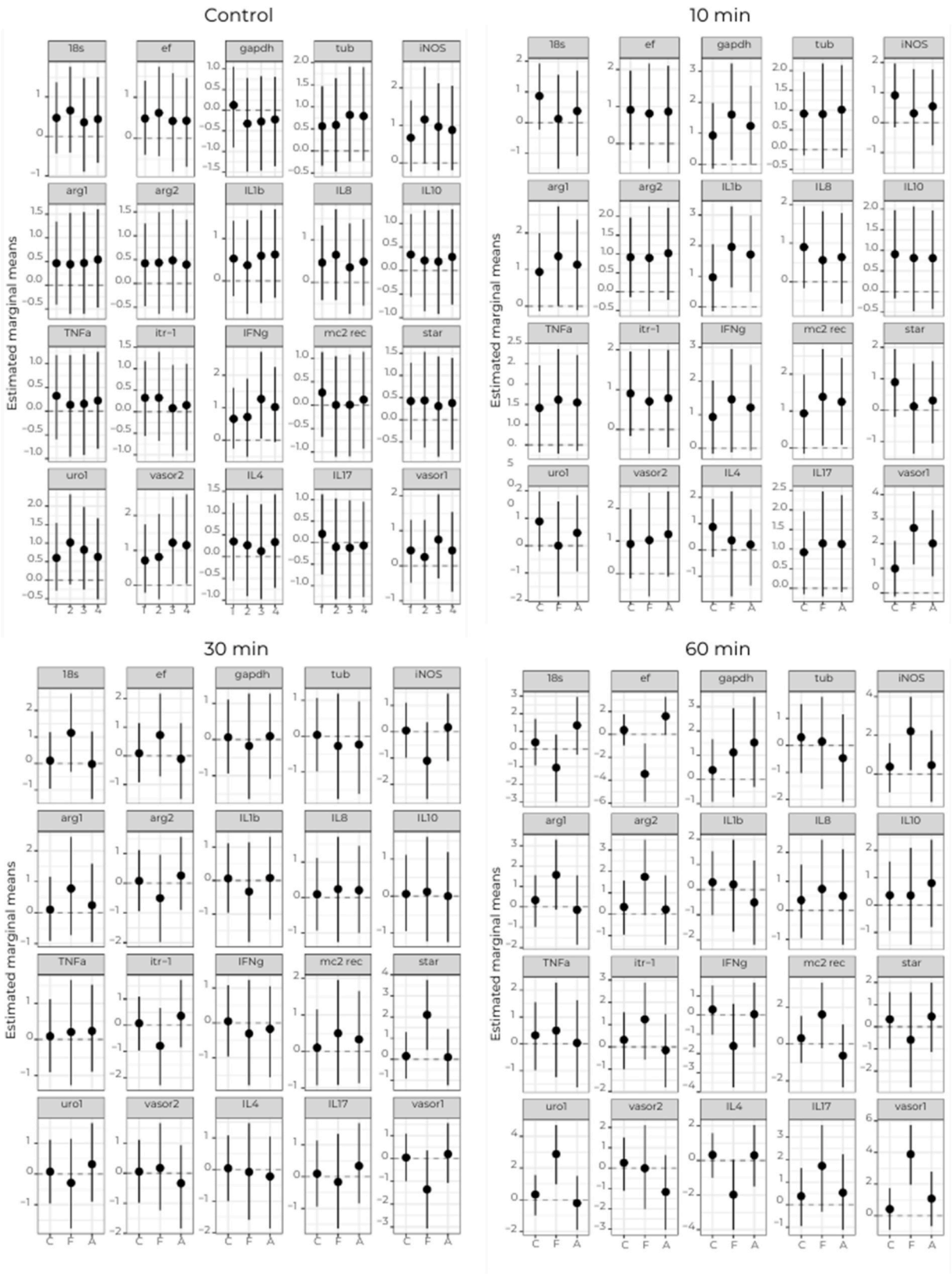

Fig. 1. Estimated marginal means of the genes tested in the head kidney of control fish (for which group 1 = C0, group 2 = C10, group 3 = C30, and group 4 = C60), as well as of the treated fish, 10 min, 30 min and 60 min after the experimental treatments (for which group C = respective control, group F = feed reward, and group A = air exposure), n = 6 per group.

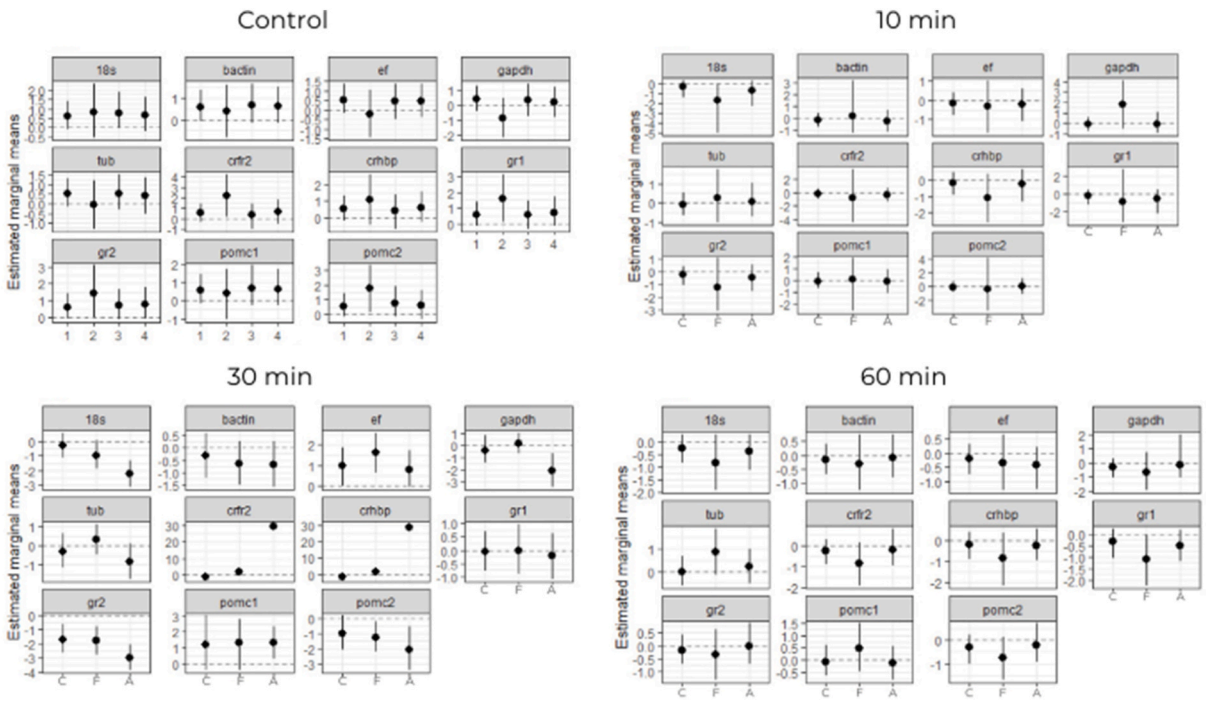

Fig. 2. Estimated marginal means of the genes tested in the pituitary of control fish (for which group 1 = C0, group 2 = C10, group 3 = C30, and group 4 = C60), as well as of the treated fish, 10 min, 30 min and 60 min after the experimental treatments (for which group C = respective control, group F = feed reward, and group A = air exposure), n = 6 per group.

based on the optimal alpha and lambda values extracted for each model for the 10 min data set (Table S4). In the 30 min data set, the ElasticNet regression showed preference of Lasso as well as Ridge regression, especially favoring the Ridge regression in the control vs feed reward comparison. For the 60 min data set, the ElasticNet regression, chose alpha parameter of 1.0 in the control vs distress comparison, giving all weight to the L1 Lasso penalty. For the remaining comparisons, there was a strong preference towards Ridge regression (Table S4). For pituitary data, the ElasticNet regression, the comparison of control and distress gene expression values, the chosen alpha value of 0.9 indicates a preference of Lasso regression for the 10 min data set. Control and feed reward comparison showed small preference towards Lasso regression, however, for the remaining comparison of the feed reward and air exposure data sets, Ridge regression was favored. In the 30 min dataset, the ElasticNet regression in the control versus distress comparison chose an alpha of 0.5, providing equal contribution of both, Lasso and Ridge regression penalties. Similarly, the feed reward versus air exposure comparison also revealed an alpha of 0.6. However, in the remaining comparison, the Ridge regression was strongly favored. In the 60 min data set, the ElasticNet regression favored both, Lasso and Ridge regression, choosing alphas of 0.2 for control versus air exposure comparison, 0.4 for feed reward and air exposure comparison, and 0.9 for control vs feed reward data set (Table S5).

The classification accuracies showed high values for the head kidney data sets with levels ranging from 95.3 % to 100 % (Table 1) and for the pituitary data sets with levels from 97.2 % to 100 % (Table 2). The highest misclassification occurred in the 10 min datasets for both head kidney (false distress classification 1.9 % and false feed reward classification 2.8 %) and pituitary (false distress classification 1.8 %, false feed reward classification 1%), when comparing feed reward and air exposure. Moreover, feed reward was falsely classified in the 30 min datasets in both head kidney (0.5 %) and pituitary (1.7 %) when compared with the control groups, whereas distress was misclassified 0.3% in the head kidney and 0.1 % in the pituitary when compared with control groups. These results, nevertheless, support the hypothesis that the selected genes allow a classification across treatment groups.

To evaluate the contribution of certain features, odd's ratios have been calculated for each regression analysis. For the head kidney data, the heatmap (Fig. 5) displaying the odd's ratios of the different genes belonging to the 10 min data set shows that when comparing the feed reward and air exposure treatments, the genes *vasor2* and *star* are associated with the distress treatment. Contrary to that, *gapdh* and *vasor1* appear to be more related to the feed reward treatment. The heatmap depicting the odd's ratios of the data collected 30 min after each treatment does not demonstrate strong differences in gene association to the two treatments. Moreover, similar expression patterns can be seen when both treatments are compared to the control group in this data set. Furthermore, in the heatmap showing the odd's ratios for the 60 min data set, more profound differences could be seen in the control group versus distress, where the three potential reference genes appear to be more associated with the air exposure group than with the control group.

For the pituitary data, the heatmap (Fig. 6) for the odd's ratios for the 10 min data set suggests that the potential reference gene *tub* is strongly associated with the distress treatment compared with the control group. The metabolic gene *gapdh* appears to relate more to the feed reward than to the air exposure treatment. On the other hand, *pomc2* and *pomc1* may be linked to the air exposure treatment. The 30 min odd's ratio data set shows an association of *crfr2* and *gr1* with the feed reward treatment. Similarly, to the 10 min data set, *pomc1*, but not *pomc2,* indicates a relation to the air exposure treatment. In the odd's ratios' heatmap for the 60 min data set, the potential reference gene *tub* shows a strong association with both treatments compared with the control group. Moreover, two other potential reference genes, *bactin* and *gapdh,* appear to be linked to the distress treatment. Similarly, *crfr2*, *gr1*, *gr2,* and *pomc2* also show an association with air exposure. On the other hand, *pomc1* indicates a connection to the feed reward treatment.

4. Discussion

The objective of this study was to investigate gene expression profiles involved in the immune responses in the head kidney, as well as stress

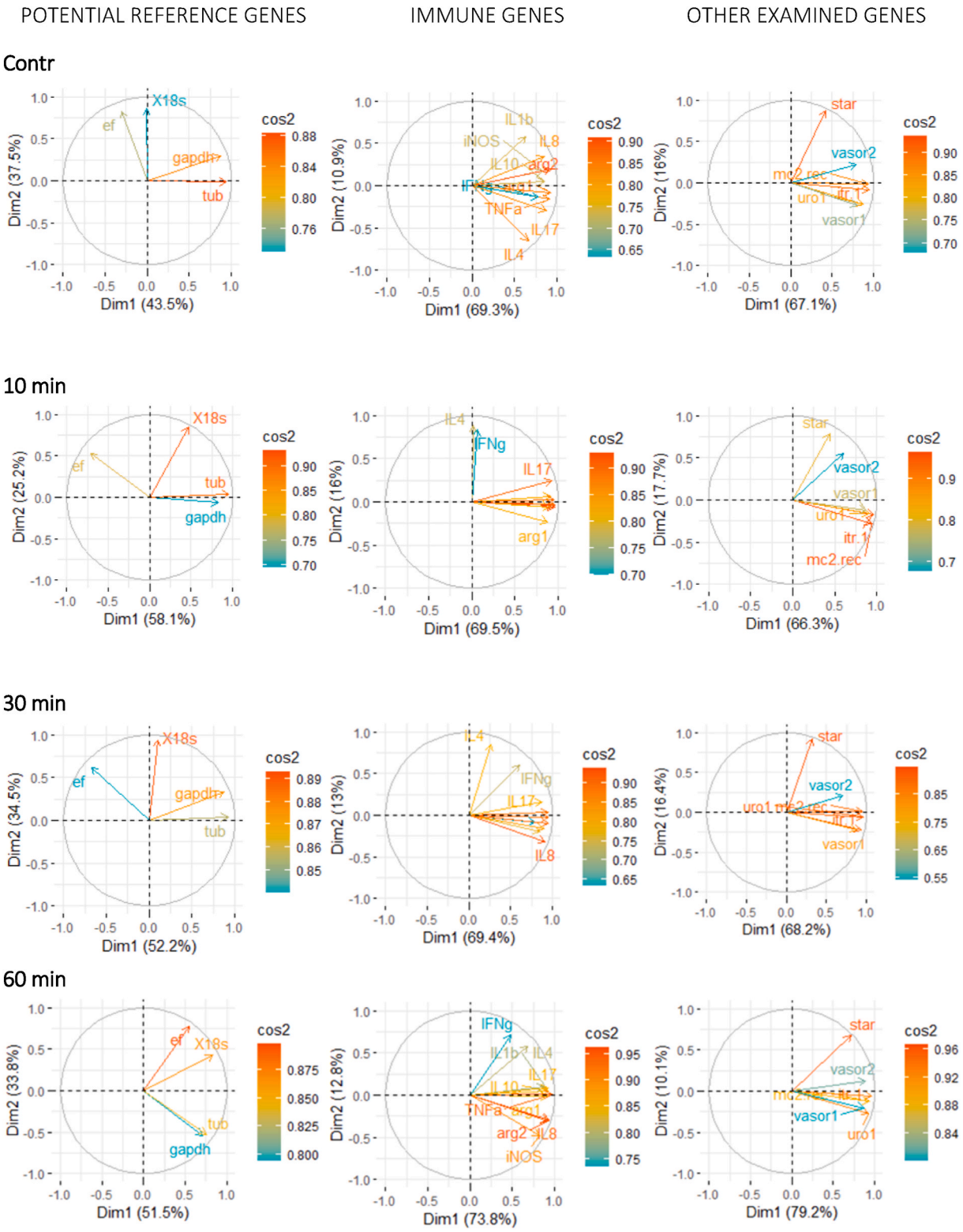

Fig. 3. Quality of representation of the expression levels of potential reference genes (*18S, ef, gapdh, tub*), immune-related genes (*iNOS,* arg*1,* arg*2, Il-1β, Il-8, Il-10, TNF-α, IFNγ, Il-4, Il-17*) and other examined genes (*itr1, mc2rec, star, uro1, vasor1, vasor2*) in head kidneys of control fish and fish 10, 30, and 60 min after both treatments on the factor map cos2 (the numbers next to Dim1 and Dim2 indicate the percentage of the variance in the data sets that is explained by the first two components of the PCA), n = 6 per treatment.

response-related genes in the pituitary in juvenile koi carp after exposure to an acute positive and negative stimulus.

Similarly to our previous study (Pawlak, Burren, Seitz, Glauser, & Pietsch, 2022), an assessment of five potential reference genes (Table S2. & S3.) was conducted, and once more, several of them, including the commonly used *18S RNA*, were found to be influenced by the experimental treatments. This emphasizes the need for careful selection and validation of reference genes to ensure accurate normalization in gene

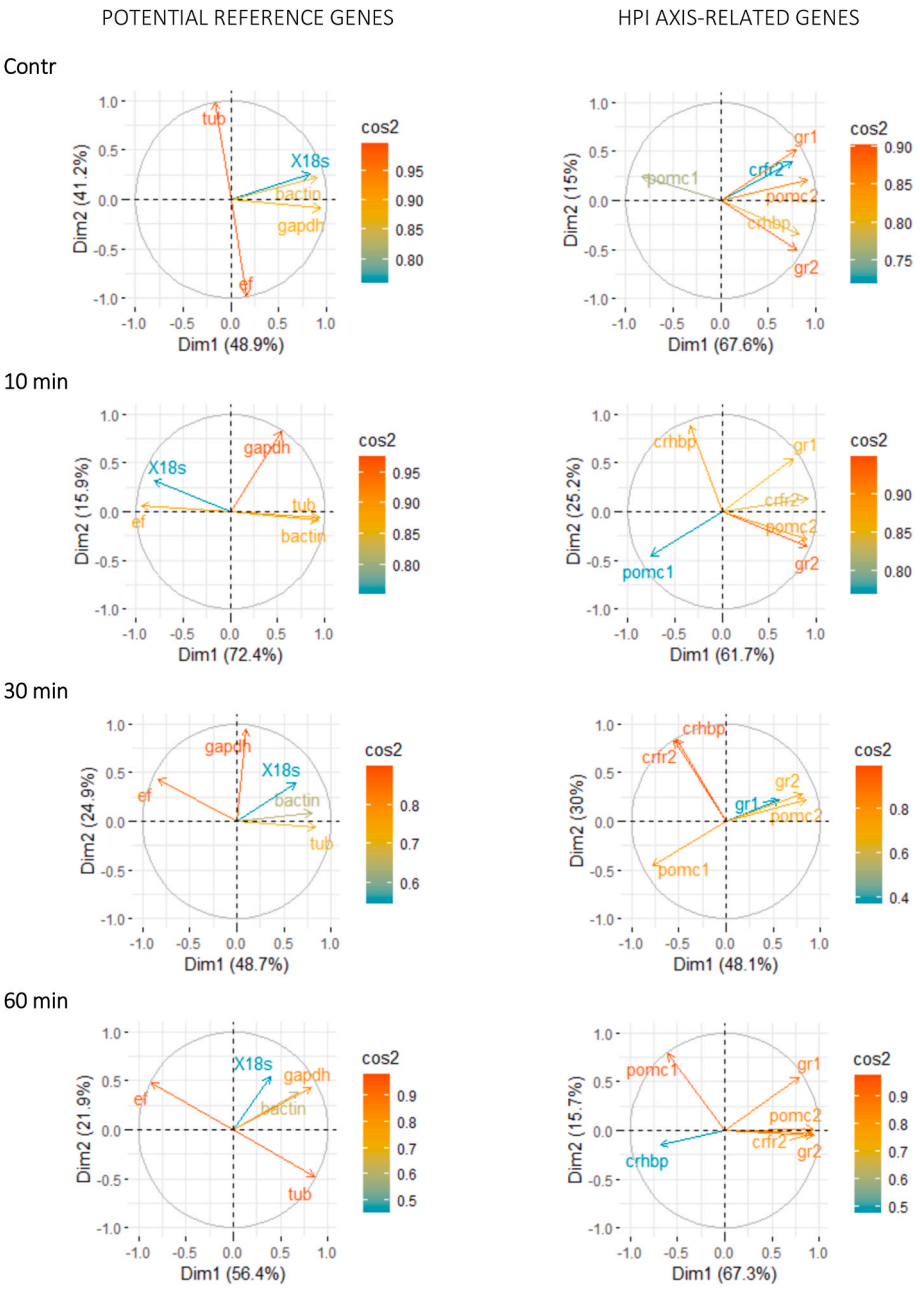

Fig. 4. Quality of representation of the expression level of potential reference genes (*18S, bactin, gapdh, ef, tub*) and HPI axis-related genes (*crfr2, crhbp, gr1, gr2, pomc1, pomc2*) in pituitaries of the control fish and fish 10, 30, and 60 min after both treatments on the factor map cos2 (the numbers next to Dim1 and Dim2 indicate the percentage of the variance in the data sets that is explained by the first two components of the PCA), n = 6 per treatment.

expression studies.

Stress can be defined as the body's physiological reaction to perceived threats or challenges (Schreck & Tort, 2016; Wendelaar Bonga, 1997)Click or tap here to enter text. Initially stress was described as a nonspecific response (Selye, 1936), however, studies show that reactions differ based on the specific stressor (Bernier et al., 2008; Bowers et al., 2008; Pacak et al., 1998). The brain processes stress by evaluating stimuli and determining an appropriate physiological response (Lazarus, 1993; McEwen, 2007). Acute negative stressors are known to impact cortisol levels in plasma of teleosts (Pankhurst, 2011). An increase in cortisol concentration but only in fish exposed to air 30 min after the treatment was shown in our previous study (Pawlak, Burren, Seitz, Glauser, & Pietsch, 2022). Furthermore, an increased mRNA expression of *crf-r2* and *crh-bp* was observed in the pituitary gland 30 min after both experimental treatments compared with the control group in the present study. However, expression of these two genes was significantly higher in fish exposed to air than in the feed reward group. Similarly, the expression of the *crf-r1* was increased in in the hypothalamus at the same time point following both treatments, as reported previously (Pawlak, Burren, Seitz, Glauser, & Pietsch, 2022). Crf being released from neurons of the hypothalamus, may act on its receptors Crf-r1 and -r2, eventually leading to the release of cortisol (Faught et al., 2016). Crh-bp found in pituitary may play a role in regulating HPI axis activity by reducing accessible Crf which in turn accelerates the homeostasis recovery after stress (Doyon et al., 2005; Ji et al., 2024). The regulating actions of the pituitary Crh-bp on Crf1 can be seen in the current study and our previously reported results (Pawlak, Burren, Seitz, Glauser, & Pietsch, 2022), where higher *crf1* expression was observed in the hypothalamus but also in the optic tectum 30 min after both treatments, however increased *crf1* expression levels

Table 1
Accuracy of the ElasticNet regression models for the gene expression data in kidney based on 5-fold cross validation and additional tuning in R Studio.

data set		Classification results		
		accuracy	false positives	false negatives
10 min	comparison control vs distress	1.000	0 %	0 %
	comparison control vs feed reward	1.000	0 %	0 %
	comparison feed reward vs air exposure	0.953	1.9 %	2.8 %
30 min	comparison control vs air exposure	0.991	0.3 %	0.6 %
	comparison control vs feed reward	0.992	0.5 %	0.3 %
	comparison feed reward vs air exposure	1.000	0. %	0 %
60 min	comparison control vs air exposure	0.997	0 %	0.3 %
	comparison control vs feed reward	1.000	0 %	0 %
	comparison feed reward vs air exposure	1.000	0 %	0 %

Table 2
Accuracy of the ElasticNet regression models for the gene expression data in pituitary gland based on 5-fold cross validation and additional tuning in R Studio.

data set		Classification results		
		accuracy	false positives	false negatives
10 min	comparison control vs distress	0.998	0 %	0.2 %
	comparison control vs feed reward	1.000	0 %	0 %
	comparison feed reward vs air exposure	0.972	1.8 %	1 %
30 min	comparison control vs air exposure	0.999	0.1 %	0 %
	comparison control vs feed reward	0.975	1.7 %	0.8 %
	comparison feed reward vs air exposure	1.000	0 %	0 %
60 min	comparison control vs air exposure	0.998	0 %	0.2 %
	comparison control vs feed reward	0.999	0 %	0.1 %
	comparison feed reward vs air exposure	1.000	0. %	0 %

remained only in the optic tectum 60 min after feeding. Furthermore, Star and Mc2rec are important mediators of cortisol synthesis. ACTH binds to the Mc2rec in the interrenal tissue, activating intermembrane transport of cholesterol in mitochondria by Star and conversion of cholesterol to cortisol by relevant enzymes (Aluru & Vijayan, 2008; Faught et al., 2016; Wendelaar Bonga, 1997). In the current study, an increased expression of *star* in the head kidney, 30 min after feed reward, as well as the *mc2rec* after 60 min may suggest that ACTH signaling has reached the head kidney activating Mc2rec and Star in fish from the feed reward group. However, the absence of increased plasma cortisol levels (Pawlak, Burren, Seitz, Glauser, & Pietsch, 2022) suggests a differential downstream regulation of cortisol synthesis and release, as well as a lack of negative feedback of cortisol on the stress axis. Moreover, existence of other factors regulating the expression levels of these genes should not be excluded.

Urocortin, is involved in anti-inflammatory processes in mammals (Agnello et al., 1998; Tsatsanis et al., 2005). Tsatsanis et al. (2005) have shown that in human, urocortins act pro-apoptotic on macrophages and this effect is regulated by the Crf-r2 receptors. Also in mice, it was observed, that urocortin reduced the serum levels of TNF, Il-1 β and Interleukin 6 (Agnello et al., 1998). In the current study, *uro1* showed increased expression in the head kidney 30 min after feed reward, together with an increased expression of *crf-r2* in the pituitary at the same time point. Furthermore, acute stress can have both activating and suppressing effects on the immune system. The activation of immune responses is commonly related with the release of characteristic cytokines, and thus synthesis of acute phase proteins. On the other hand, suppressive effects are mediated by HPI axis-related corticosteroids, especially cortisol (Tort, 2011). The initial high transcript levels of *Il-1β* in the head kidney 10 min after feed reward were not observed at the later time points. The early increase of *Il-1β* expression is, however, consistent with other studies (Hoseini et al., 2019; Metz et al., 2006). Furthermore, and as mentioned previously, in mammals the *iNOS* expression can be detected after exposure to bacterial products or cytokines (Kunz et al., 1994; Stuehr & Marletta, 1985), e.g., Il-1 β but also through stimulation by prolactin, as it is observed in mammals (Kunz et al., 1994; Nathan, 1992; Stuehr & Marletta, 1985). It has been shown, that besides its role in osmoregulation in both mammals and fish, prolactin is known to function as a hormone and cytokine in humans (Borba et al., 2019). Furthermore, it exhibits immuno-enhancing effects that include stimulating macrophage phagocytic activity (Narnaware et al., 1998), inhibiting cortisol-related apoptosis and reduced mitotic activity in leukocytes (Yada et al., 2004), as well as increasing immunoglobulin M production (Yada et al., 1999). As shown in our previous study (Pawlak et al., 2023), expression of prolactin receptor was increased in each brain region and in every time point of both treatments, implying higher supply of prolactin. After an initial decline 30 min following feed reward, a raise in *iNOS* expression was seen 60 min after the treatment, suggesting a delayed response to an increased leucocyte and macrophage activity, due to their potential activation by prolactin and Il-1 β. This finding may suggest stimulation of the immune system in the treated fish, that was however, suppressed in the air exposed fish due to cortisol release and could not be counteracted or neutralized by actions of prolactin. Consequently, the relevance of prolactin's impact on immune response appears to be still unclear and needs to be further elucidated.

On the other hand, the changes of expression of *iNOS* in the fed fish may indicate a complex regulation of NO production, possibly involving arginine metabolism. Chironomid larvae fed to the fish contain the amino acid arginine (Czeczuga & Gierasimow, 1973) and thereby influence the body's arginine level after ingestion. Arginine, amongst other functions, serves as a precursor for the synthesis of NO.

Il-4 plays a key role in the later stages of type 2 immune response by limiting activity of neutrophiles and therefore preventing potential tissue damage. In this study a decreased expression of *Il-4* was observed in the head kidney, 60 min after feed reward, suggesting a shift towards a pro-inflammatory state.

The rapid increase of *vasor1* expression in the head kidney in both treatment groups and its fluctuations after 30 and 60 min only in the feed reward group, as well as a decreased expression of *vasor2* 60 min after air exposure may support a hypothesis of Skrzynska and Cadiz (Cadiz et al., 2015; Skrzynska et al., 2018) that these receptors play a role in regulation of the endocrine responses after experiencing stress. In our previous study (Pawlak et al., 2023), the Isotocin precursor was downregulated in several brain regions after tank manipulation in fish kept in pairs (C10, C30 and C60), compared to animals reared in a group tank, suggesting that isolation but also fasting may have affected its expression. Furthermore, *itr1*, among the genes *mc2rec*, *star* and *uro1* impacted the head kidney PCA the most in the group of other examined genes in controls, as well as both treatments, suggesting an involvement of isotocin system in our study. However, further isotocin signalling was not reflected in significant changes in expression of *itr1* in the head

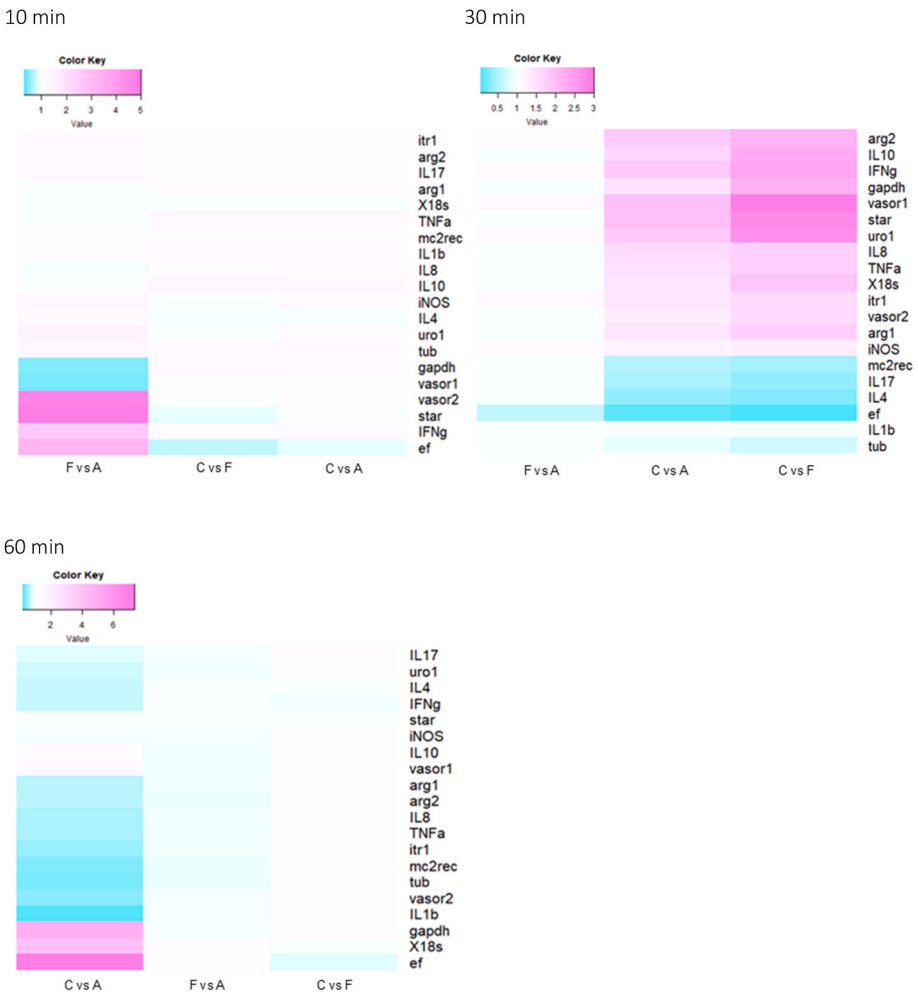

Fig. 5. Heatmaps for the odd's ratios calculated from bootstrapped head kidney gene expression data 10 min, 30 min and 60 min after exposure to the treatments. Classified genes are listed on the Y axis, whereas different treatments (C – control group, A – group stressed by air exposure, F – feed rewarded group) are on the X axis. A ratio below 1 indicates that a different regulation of a gene increases the probability that a sample belonged to a fish among the first listed treatment in the comparison, whereas a ratio above 1 for a gene indicates that a different regulation of a gene increases the probability that a sample belonged to a fish among the treatment listed as second in the comparison.

kidney in the current study.

The immune-related genes, including mediators of the inflammatory response *Il-8*, *TNF- α, Il-17* and *iNOS*, as well as of the anti-inflammatory response *Il-10* and arg*2,* contributed to the PCA outcome the most (Fig. 3). Previous studies have demonstrated the involvement of these genes in immune regulation in relation to acute distress, however, data about acute eustress is lacking. In a study conducted on carp subjected to an acute transportation stress, *Il-8* and *TNF- α* were upregulated after the stressor (Hoseini et al., 2019). Additionally, Du et al. (2015) found that Il-17 can induce other proinflammatory cytokines, including TNF- *α* in the head kidney leucocytes and Il-8 in head kidney cells. Moreover, as mentioned earlier, iNOS, an enzyme involved in the production of NO in response to a pro-inflammatory stimulus, is also indirectly involved in the immune response. During stress, however, the balance between Arg2 and iNOS activity can influence the availability of arginine for NO, thereby modulating immune functions and inflammatory responses. In addition, Banerjee (Banerjee et al., 2021) suggests a potential role of Arg2 in hypoxic stress. Interestingly, among the pro-inflammatory cytokines, also an increased expression of a gene coding for the Il-10, a potent anti-inflammatory cytokine was observed after air exposure in the sea bream gut (Khansari et al., 2018). The functions of these genes and their responsiveness to acute stress support their significant impact on the PCA outcome.

Even though the gene expression analysis did not indicate significant differences in the transcript levels of the abovementioned genes (except for *iNOS* in the feed reward group 30 and 60 min after the treatment), they are likely involved in the regulation and modulation of the immune response under different stress conditions.

Similarly, the PCA of the pituitary gene expression values suggest that several genes closely involved in the stress axes, namely *gr2, crh-bp, crf-r2 and pomc1* may help distinguish the stress status (whether a cue was perceived positively or negatively) of the fish (Fig. 4). Indeed, when looking at the gene expression in the pituitaries (Fig. 2), especially after 30 and 60 min, differential expression of those genes can be observed between the treatments, even though the differences were statistically significant only for *crf-r2* and *crh-bp* after 30 min.

The ElasticNet regression is a machine learning technique used for predictive modeling and variable selection. It is particularly useful for high-dimensional datasets with a large number of predictors. To our knowledge, the ElasticNet regression analysis has not been performed yet for classifying genes in stressed fish. In this study, the ElasticNet regression analysis was able to successfully classify treatments based on the gene expression levels and emphasized genes that influenced the classification. Several of those genes (e.g., *star*, *Il-8* and *iNOS* in the head kidney data set, Figs. 3 and 5) also strongly contributed to the PCA results, prioritizing them for further investigation. This makes the ElasticNet regression a promising tool for identifying potential biomarkers of fish stress status.

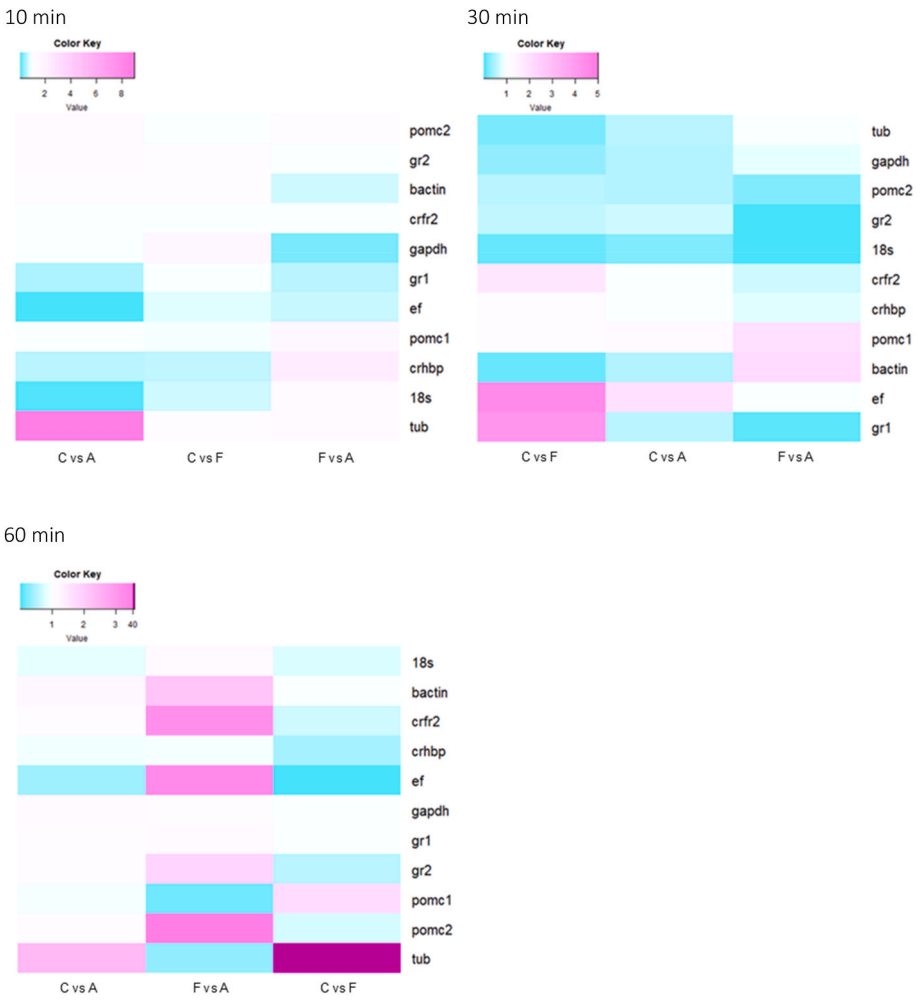

Fig. 6. Heatmaps for the odd's ratios calculated from bootstrapped pituitary gene expression data 10 min, 30 min and 60 min after exposure to the treatments. Classified genes are listed on the Y axis, whereas different treatments (C – control group, A – group stressed by air exposure, F – feed rewarded group) are on the X axis. A ratio below 1 indicates that a different regulation of a gene increases the probability that a sample belonged to a fish among the first listed treatment in the comparison, whereas a ratio above 1 for a gene indicates that a different regulation of a gene increases the probability that a sample belonged to a fish among the treatment listed as second in the comparison.

Because various factors are involved and interact with each other in the stress reaction mechanisms, the interpretation of their influence on the immune system is challenging and should not be limited to the immunosuppressive effect of cortisol and influences of the usually investigated stress hormones. Our results highlight the complexity of the immune responses and the interplay between inflammatory and anti-inflammatory factors. To fully grasp impacts of those factors on the stress-immune interactions, further research is essential.

5. Conclusion

This study aimed to investigate the molecular mechanisms occurring in the head kidney and pituitary of juvenile koi carp after exposure to a negative or positive stimulus. The pituitary transcriptome profile showed differences in the gene expression levels between fish receiving the two treatments. Moreover, the head kidney expression analysis showed that the acute positive and negative cue induced differential immune-related responses, depending on the quality of the stimulus. Furthermore, the ElasticNet regression was able to classify with high accuracy the tested gene sets to the respective treatments. We thus have confirmed the results of our previous studies showing that fish perceive negative and positive cues differently, however, both induced the activation of the Crf system. Furthermore we were able to identify genes that had the biggest impact in the stress status of the fish. However, based on this set of genes, we were not able to clearly determine the character of the immune response and their pro- or anti-inflammatory potential needs further investigation. A better understanding of immune responses induced by mechanisms of stress in teleosts is a prerequisite for this all authors gave final approval for publication.

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CRediT authorship contribution statement

Paulina Pawlak: Writing – review & editing, Writing – original draft, Visualization, Formal analysis, Data curation, Conceptualization. **Jonathan Konrad:** Writing – review & editing. **Andreas Seitz:** Writing – review & editing. **Constanze Pietsch:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Ethics statement

All experimental animal protocols were carried out by following the guidelines of the Swiss Council of Animal Care on the husbandry and use of animals in scientific research.

Declaration of competing interest

The authors declare that they have no known competing interests.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aaf.2024.09.004.

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