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ORIGINAL ARTICLE

Differential effects of acute eustress and distress on gene regulation patterns in the carp (*Cyprinus carpio* L.) brain

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Abstract

The activities of certain genes as a consequence of exposure to stressors related to typical handling procedures in aquaculture have not been studied in sufficient detail in fish. A stress trial with koi carp was, therefore, conducted, aiming at identifying relevant gene expression patterns in different brain regions during stress responses. The following stressors were selected: tank manipulation, distress caused by exposure to air and eustress due to feed rewards. Responses to these stressors were evaluated 10, 30 and 60 min after their application. The exact determination of gene expression profiles in the carp brain required the comparison of several suitable reference genes, which is also highly recommended for other studies focusing on the fish brain. Moreover, and as expected, the mRNA expression of a number of early immediate genes indicated activity in different brain regions as a response to changes in rearing and experimental conditions. In addition, the mRNA expression of metabolic genes was investigated, since increased brain activity may also increase the metabolic demands of certain brain areas. Furthermore, genes related to the stress axis were included in the study. The mRNA expression patterns of genes belonging to the stress axis revealed that negative stress caused by exposure to air had broad-ranging effects on the gene regulation patterns in the fish brain, even if the fish were only treated for 1 min. This parallels the effects that have been observed on blood cortisol and glucose. In contrast, a limited number of genes allows discrimination of eustress and distress, which indicates that further research is needed in the future. Finally, the use of different control groups is highly recommended for fish experiments to correct for typical experimental procedures such as lid openings or social isolation.

KEYWORDS
aquaculture, brain gene regulation, stress responses, stressors

1 | INTRODUCTION

Rearing of fish in aquaculture often includes exposure of the fish to acute stress. Typical stressors that can lead to negative effects on fish – and are therefore called distressors – are, for example, unsuitable stocking density, water quality and handling processes such as grading, transportation and sampling. Distress interferes with the appetite and well-being of an animal (Höglund et al., 2007), while eustress also leads to increased locomotory activity and is therefore a stressor as well. A mildly stressful situation that can have beneficial or positive effects and is therefore categorized as eustress for fish is feed rewarding (Manuel et al., 2015). Interestingly, fish are clearly capable of distinguishing between different stressors to ensure that they react appropriately to their stressful environment.

Stress in fish is often assumed to be indicated by glucocorticoid responses in the blood stream. Glucocorticoids are important stress hormones that occur either in an active 11β-hydroxyl form (i.e., as cortisol or corticosterone) or in inactive forms, as 11-keto steroids (as cortisone or 11-dehydrocorticosterone). Cortisol or corticosterone increases allow individuals to cope with the prolonged energetic demands caused by exposure to stressors in a species-specific manner (Wendelaar Bonga, 1997). The balance between inactive and active forms of the glucocorticoids is maintained by 11β-hydroxysteroid dehydrogenase (11β-HSD) enzymes which thus determine the activation of glucocorticoid receptor (*gr*)- and mineralocorticoid receptor (*mr*)-mediated signalling pathways. Both receptor types thereby act as hormone response elements to regulate transcription of related genes. Measurement of stress hormones in plasma samples has commonly been used as an indicator for stress, but does not necessarily accurately reflect the stress status. This is because acute stress leads to only short-lived peaks of cortisol in the blood stream whereas de-sensitization of the stress axis can occur as a consequence of an allostatic overload during chronic stress exposure (Aerts et al., 2015; Bernier, 2006). In addition, specific glucocorticoids may play a different role in vertebrates. For example, in contrast to animals relying on corticosterone or 1α-hydroxycorticosterone as the main glucocorticoid, such as sharks, amphibians, birds, reptiles and several rodents (Palme et al., 2005; Ruiz-Jarabo et al., 2019), the major stress hormone in humans and fish is cortisol. In humans, cortisol induces transcriptional activities in both, the glucocorticoid (gr) and the mineralocorticoid receptor (mr), while the mineralocorticoid aldosterone activates mr, but not gr (Bentley, 1998; Rogerson et al., 2003). The two variants of gr1 in carp appear to differ in their transcriptional effects (Stolte et al., 2008). In addition, gr2 is more sensitive to corticoids than mr, gr1a and gr1b, although the sensitivity of gr and mr to cortisol may vary between fish species (Greenwood et al., 2003; Stolte et al., 2008). Moreover, the low circulating levels of aldosterone among teleosts (Baker, 2003; Jiang et al., 1998) has led to the assumption that its precursor, 11-deoxycorticosterone (DOC), is the dominant mineralocorticoid in teleosts (Sturm et al., 2005). What makes stress hormone analyses in fish also more complicated is the fact that the typical cortisol elevations shortly after exposure to acute stressors can differ by more than two orders of magnitude in different fish species (Barton, 2002). Furthermore, gender and reproductive tactics can influence *gr* and *mr* expression levels in different brain regions of teleosts (Arterbery et al., 2010).

A more detailed understanding of stress responses in fish is very important, since it has been shown that exposure to stressors can change the transcriptome after exposure to acute stressors also at early life stages (Robinson et al., 2019). The release of glucocorticoids into the blood stream requires the activation of different neurons and differential regulation of several neuropeptide networks in individual brain regions. This leads to the question: which region of the brain is most important for detecting stress responses? An initial study on carp indicated that there is involvement of different brain regions after exposure to acute distress (Burren & Pietsch, 2021). Responses to distress in fish are thought to involve the typical parts of the hypothalamus–sympathetic system–chromaffin tissue axis and the hypothalamus–pituitary–interrenal (HPI) axis (Conde-Sieira et al., 2018). In higher vertebrates, especially the amygdala plays an important role in being able to show fear and allows emotional learning, decision-making and social behaviour (Perathoner et al., 2016). Studies on primates and rodents clearly demonstrated that higher vertebrates are capable of distinguishing cues with either positive or negative values (Namburi et al., 2015; Paton et al., 2006; Schoenbaum et al., 1998, 1999). This was attributed to different neurons within the amygdala that exhibit selective activity in relation to either positive or negative cues. An amygdala-like area has been identified in ray-finned fish in the telencephalon (Maximino et al., 2013; von Trotha et al., 2014). However, whether an amygdala-like structure also regulates reward-seeking or fear-related behaviours in fish remains to be elucidated.

The telencephalon (tel) in vertebrates is subdivided into a dorsal and a ventral part, but different ontogenetic processes lead to profound differences in the organization of the amygdala in fish. Genetic markers in teleosts have indicated that the amygdala is located in the ventral tel (Ganz et al., 2012; Rohr et al., 2001), with the expression of some markers also extending to the dorsal zones of the ventral tel as well as to the subpallium (Alunni et al., 2004; Mueller et al., 2008). Similar to higher vertebrates, the amygdala in fish communicates with other parts of the brain, for example, the hypothalamus (hyp) or the brainstem, to achieve behavioural and physiological outputs (Folgueira et al., 2004; Kittelberger & Bass, 2013). The hyp plays an important role in energy homeostasis and appetite regulation. The activated or suppressed neurons then bring about behavioural and metabolic adjustments. In addition, genes that are often investigated in stress response studies are the proopiomelanocortins (pomc's), which belong to a complex gene family (Harris et al., 2014). The excitation of pomc cells in the hyp leads to activation of corticotropin releasing factor (*crf*) neurons (Cerdá-Reverter et al., 2003; Sanchez et al., 2009). Increased mRNA expression of *crf* in the tel and hyp, for example, caused by isolation, handling or hypoxia (Bernier, 2006),

is thought to be responsible for anorectic responses in fish under these stress conditions.

Crf and its receptors obviously play an important role in stress signalling via the HPI axis. In mammals, it is the crf-r2 subtype that is thought to exert the anorectic effects of crf (Richard et al., 2002). In fish, the crf receptor subtype functions that are responsible for anorectic effects have not been identified to date (Bernier & Peter, 2001; De Pedro et al., 1997). The intensity and duration of the stress application typically affect the magnitude of the changes in *crf* mRNA expression in the brain, as has been observed, for example, in carp and trout (Doyon et al., 2005; Huising et al., 2004). While a 30 min restriction had no effect on *crf* expression, confinement for 24 h caused increased *crf* expression in the hyp (Huising et al., 2004). Furthermore, a single chasing event did not affect *crf* mRNA levels in the preoptic area of trout, while chasing to exhaustion resulted in increased *crf* expression (Doyon et al., 2005). However, the biological activity of crf is also regulated by the crf-binding protein (*crf-bp*). In trout, it has been shown that *crf1* mRNA content was highest in the preoptic area and repeated stress application resulted in increased *crf-bp* expression in the pituitary (Doyon et al., 2003). In addition, 1 min air exposure resulted in a change in *crf 1* and *2* mRNA expression in carp relative to the expression of *crh-bp* (Burren & Pietsch, 2021). Furthermore, the expression of *crf* is not limited to the preoptic area and the pituitary in fish. While the cerebellum of fish is typically linked to spatial navigation (Durán et al., 2014) this brain region also shows expression of *crf 1* and *2*, *crf* receptors and *crh-bp* (Burren & Pietsch, 2021). Similarly, *crf-r1* expression has also been reported in the brainstem of catfish and goldfish (Arai et al., 2001; Bernier, 2006).

The activity of different brain regions can also be established by assessing the mRNA expression of other genes. Several immediate early genes (IEGs) are known to be used for this purpose. Immediate activation of *c-fos* is commonly used to indicate neuronal activity. Furthermore, *c-fos* as a component of transcriptional regulation networks acts on *erk1/2* mitogen-activated protein kinases increasing immune functions in murine macrophages (Hop et al., 2018). Light avoidance as an innate choice behaviour in adult zebrafish leads to increased expression of *c-fos* in tel within 30 min (Lau et al., 2011). In the same brain region, acute administration of D-amphetamine (a drug activating the reward system) and a behaviour assay based on the conditioned place preference also resulted in increased expression of *c-fos* in zebrafish (von Trotha et al., 2014).

A factor required for neuronal differentiation and the survival of neuronal cells in vertebrates, including fish, is neurod (Olson et al., 2001; Thomas et al., 2012). Furthermore, neurod interacts with mr signalling in rodents (van Weert et al., 2019) which makes it an interesting marker also in stress studies. Similarly, after habituation to touristic zones, fish exhibited higher *neurod1* and *mr* expression compared with control sites (Geffroy et al., 2018). Another interesting stress marker, the eukaryotic translation initiation factor 4E (*eiF4E*), is known to play a central role in the control of post-transcriptional gene expression and has been reported to influence cell development in medaka, *Oryzias latipes* (Zhao et al., 2013). However, its main function in higher vertebrates is associated with the synapse-specific provision of proteins necessary for the strengthening of synaptic connections as a consequence of neuronal activation (Moon et al., 2009). Consequently, *eiF4E* was included in the present study as a potential marker for neuronal activity. In addition, metabolic genes can be included, since increased activity in a specific part of the brain often means that metabolic energy requirements are higher subsequently affecting regulation of metabolic genes.

This study investigated the early responses to different stressors in koi carp and aimed to show that different brain regions respond differentially to the application of stressors. The focus is, therefore, on effects on the mRNA expression of IEGs, metabolic genes, as well as genes belonging to the HPI axis.

2 | MATERIALS AND METHODS

2.1 | Rearing conditions

The rearing tank for the fish was a 290 L aquarium equipped with a biofilter in which 70 koi carp (*Cyprinus carpio*, purchased from a commercial supplier) were kept for 2 months and fed four times daily at a feeding rate of 2%–3% body weight per day. Approximately, 10%–20% of the system water were exchanged twice per week during the rearing phase. All experimental procedures were approved by the relevant cantonal authorities of Zurich (Switzerland) under permission number ZH-062-17. The fish were trained on a feed reward with frozen mosquito larvae for several weeks. The feed reward was given manually every day, between 8:00 and 8:30 CET. Of these fish, 60 carp (with an average weight of 78.71 ± 2.46 g, mean ± SEM) were used in a series of stress experiments. A graph showing experiment setup can be found in Figure S1. For this stress experiment, the fish were: (A) taken directly from the rearing tank and sampled (C0), or (B) kept in 50 L aquaria for three days with continuing feed rewards and curtains around the aquaria to prevent any effects caused by routine work. The 50 L aquaria for the experimental treatment were cleaned and filled with pre-conditioned fresh water before adding the fish. A continuous flow-through with pre-conditioned water ensured that the water quality in each tank remained stable with an average temperature of 22.83 ± 0.14°C, an average oxygen saturation of 85.74 ± 1.30%, and an average pH of 7.71 ± 0.03 (mean ± SD). After acclimatization, fish were treated as follows: by opening the curtains in front of the tanks and lifting the lid of the aquaria (C), receiving the feed reward (F) or by being exposed to air by netting for 1 min (A). Six individual fish were used for each treatment. The treatment selection had been randomized for each sampling day. Following the individual treatments, the animals were left undisturbed in the aquaria for further 10, 30 or 60 min leading to the treatment groups being named C10, F10, A10, C30, F30, A30 and C60, F60 and A60

depending on the treatment and the duration of time until being sampled. After the indicated time of being left in the aquaria, the fish were anesthetized and sampled immediately. Anaesthesia was performed with an overdose of tricaine methanesulfonate (MS-222; Sigma-Aldrich) for 70 ± 19 s. Anaesthesia and blood sampling from the caudal vein with heparinized syringes were performed on average within 158 ± 3.5 s (mean ± SEM) after starting to remove the fish from their aquaria. Following storage of the brains in RNAlater® (Sigma-Aldrich), the brains were cut into four regions (tel = telencephalon, hyp = hypothalamus, opt = optic tectum, rhomb = rhombencephalon which includes the cerebellum and the hindbrain). From each sample, total RNA was extracted using RNeasy Micro Kits (Qiagen AG) including an DNase treatment with the RNase-free DNase Set (Qiagen AG). The RNA content was confirmed using a spectrophotometer Q5000 (Quawell), and 20 µl of total RNA were reverse transcribed into cDNA using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, purchased from Thermo Fisher Scientific) according to the manufacturer's instructions. Thereafter, the cDNA content was adjusted to 50 ng/µl using nuclease-free water (Ambion®, purchased from Thermo Fisher Scientific).

2.2 | Blood analyses

Cortisol, cortisone, corticosterone, lactate and glucose were measured in plasma samples. For this, plasma was separated by centrifugation at 3000 *g* for 10 min and the samples were kept frozen until the subsequent analyses were carried out. The preparation of hormone samples from plasma followed a protocol developed by the Neuchatel Platform of Analytical Chemistry, University of Neuchatel (see details in the Appendix S1). Cortisol content was analysed by internal standardization using ultrahigh-performance liquid chromatography–tandem mass spectrometry (UHPLC–MS/MS), as described elsewhere (Reyes-Contreras et al., 2019). In brief, cortisol was separated on a reverse-phase C18 column and the mass spectrometer was operated in the multiple reaction monitoring mode using the transitions 363/121 and 367/121 for cortisol and cortisol-D4 respectively. The blood glucose and lactate measurements were conducted using commercial kits purchased from Sigma-Aldrich. Six animals were investigated in each group, except for the glucocorticoid analyses of the feed reward group, there were $n = 5$.

2.3 | PCR conditions

Gene expression studies were performed as described in Burren and Pietsch (2021). The primer pairs are given in Table S1. Four brain regions were investigated separately: telencephalon (tel), hypothalamus (hyp), optic tectum (opt) and rhombencephalon (rhomb). First, the mean quantitative cycle (mean ct) for the two technical replicates was calculated for each sample. Prior to this, all genes had been validated and 2–3 optimal reference genes were extracted from a set of 11 possible reference genes using the geNorm function in the QBASE$^+$ software, version 3.0 (Biogazelle – www.qbaseplus.com) established by Vandescompele et al. (2002), separately for each brain region.

The target genes included early immediate genes (*c-fos*, *eiF4E*, *egr-1*, *erk-1* and *erk-2*, *palld*, *neurod*) as well as metabolic genes (glyceraldehyde-3-phosphate dehydrogenase [*gapdh*], succinate-dehydrogenase [*succdh*] and pyruvate kinase [*pyrkin*]), to indicate active brain parts. The enzyme *succdh* is involved in the Krebs cycle, whereas *pyrkin* is an enzyme involved in aerobic glycolysis, similar to *gapdh*. The following genes related to the HPI axis were included: *crf1*, *crfr1*, *crfr2*, *crh-bp*, *pomc1*, *gr1*, *gr2* and *mr*. The PCR cycles on a LC480 Light Cycler II (Roche) were chosen as follows: 95°C, 10 min; [95°C, 15 s; 60°C, 30 s] × 40 cycles; and a melting curve [60–95°C, increasing by 0.5°C every 5 s]. All gene expression values were calculated relative to the expression of the selected reference gene (ΔCt method), as described in detail by Burren and Pietsch (2021), and were further calculated as fold-changes compared with the respective controls. The relative normalized expression for each target gene was used for data modelling as described in the next section.

2.4 | Calculations and statistics

Analyses of variance (ANOVA) with subsequent correction for multiple comparisons according to Bonferroni was used for the statistical calculation of differences between the means of the plasma parameters for each treatment group. The plasma parameters, as well as the genes investigated in the different parts of the brain, were also subjected to a principal component analysis (PCA) to show gene clusters that typically respond to the distinct treatments. This is the recommended method for developing new scales for subsequent experiments and field investigations. The PCA was run in R studio on individual sets of genes and separately on the plasma parameters, to prevent a sample to item ratio that was too low and would lead to incorrect factor structures and misclassification of items. Only the first two components of each PCA were used to prepare heatmaps of the $\cos^2$ values for each brain region using the *heatmap* function in R studio. The two-dimensional PCAs explained an average of 90.3% of the variance in the telencephalon, 72.2% in the hypothalamus, 71.9% in the optic tectum and an average of 78.4% of the variance in the rhombencephalon. More details on the contribution of each of the two components to the total variance are given for the individual treatment groups in the results section.

Assuming the tests are independent of each other, multiple testing leads to an inflated probability of false positive results. Consequently, mixed models with a fully Bayesian approach (as a part of the *brms* package in R STUDIO, Version 1.2.1335, (Bürkner, 2017)) were used. Pre-checks using 5000 iterations revealed

that a student's t-distribution shows better agreement with the data structure than a Gaussian distribution of the data. Hence, t-distributions were used for the models based on 10,000 iterations, which included gene-specific random effects for the constants (α), gene-specific random effects for the group differences (β) and animal-specific random effects for the constants (γ). The model fit was assessed through a comparison of graphical plots (QQ plots) showing the distribution of y and y_{rep}. The marginal R^2 considers only the variance of the fixed effects, while the *conditional* R^2 is based on both the fixed and random effects. To improve the handling of possible outliers, posterior predictive checks were carried out with the Markov Chain Monte Carlo approximation method, which yielded simulated replicated data under the fitted model that were then compared with the observed data. The point estimators, their SEMs, credibility intervals and posterior predictive p values are reported. Significance was determined using Wald χ^2-statistics for generalized linear models, and F-statistics for mixed models, and estimated marginal means were calculated where applicable. Effect estimates are presented as estimated marginal means, with corresponding 95% credible intervals (95% CI). A p value of <0.05 was considered statistically significant.

3 | RESULTS

3.1 | Blood parameters

3.1.1 | Effects of tank manipulation

The blood analyses revealed a significant difference in the corticosterone levels in plasma between the C0 group and the C60 group ($p = 0.036$, Figure 1a), but not for lactate, cortisol and cortisone. The plasma glucose levels exhibited a significant difference between the controls C10 and C30 ($p = 0.008$, Figure 1b).

3.1.2 | Effects of eustress and distress

The blood analyses revealed no differences in the levels of the steroids in the fish 10 min after the different treatments (Figure 2), while the glucose levels between the control fish and feed rewarded fish as well as air-exposed fish were significantly different ($p = 0.008$, Figure 3). Plasma cortisol levels were significantly different between control animals and those 30 min after air exposure ($p = 0.045$, Figure 2).

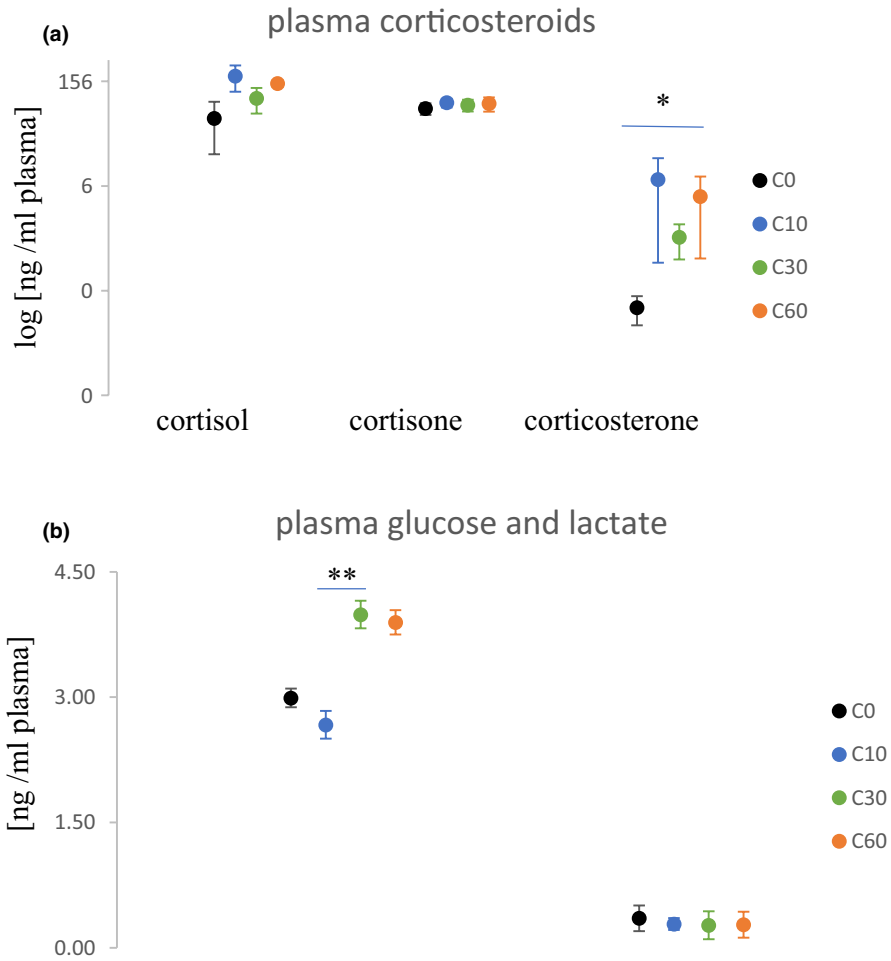

FIGURE 1 Plasma cortisol, cortisone and corticosterone (a) and plasma glucose and lactate levels (b) in the different control treatments, mean ± SEM; $n = 6$ per treatment, $^*p < 0.05$, $^{**}p < 0.01$, for better visualization of the differences between treatment groups the y axis was log-scaled for figure a.

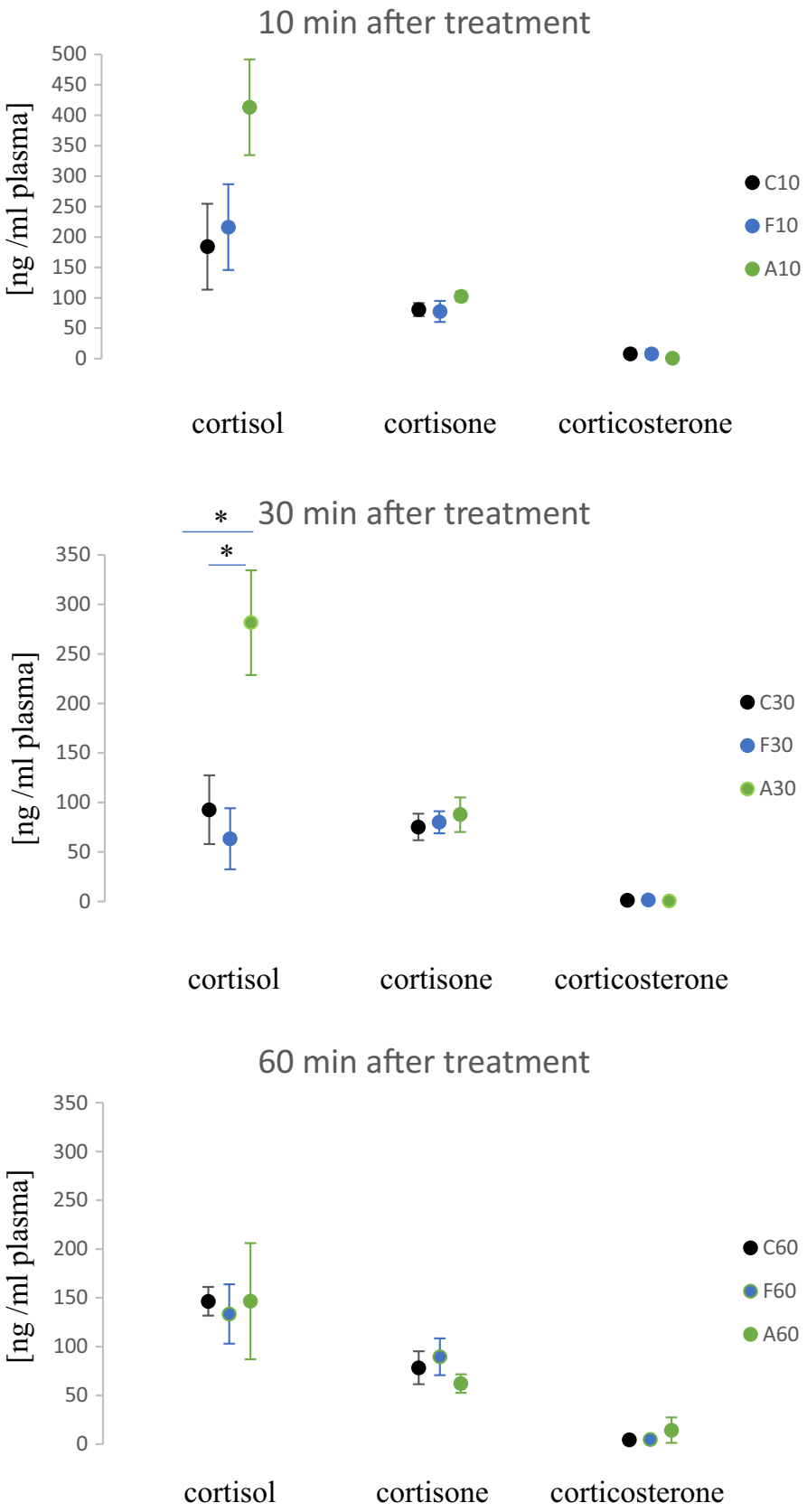

FIGURE 2 Plasma cortisol, cortisone and corticosterone levels in fish 10, 30 and 60 min after the different treatments, mean ± SEM; $n = 6$ per treatment, except for the feed reward group with $n = 5$, $^{*}p < 0.05$.

Similarly, cortisol levels between the animals receiving feed rewards and air-exposed fish were different at the same sampling time point ($p = 0.018$, Figure 2). These differences were not accompanied by differences in glucose or lactate levels (Figure 3). Moreover, 60 min after the different treatments, none of the plasma parameters exhibited any differences between the treatment groups (Figures 2 and 3).

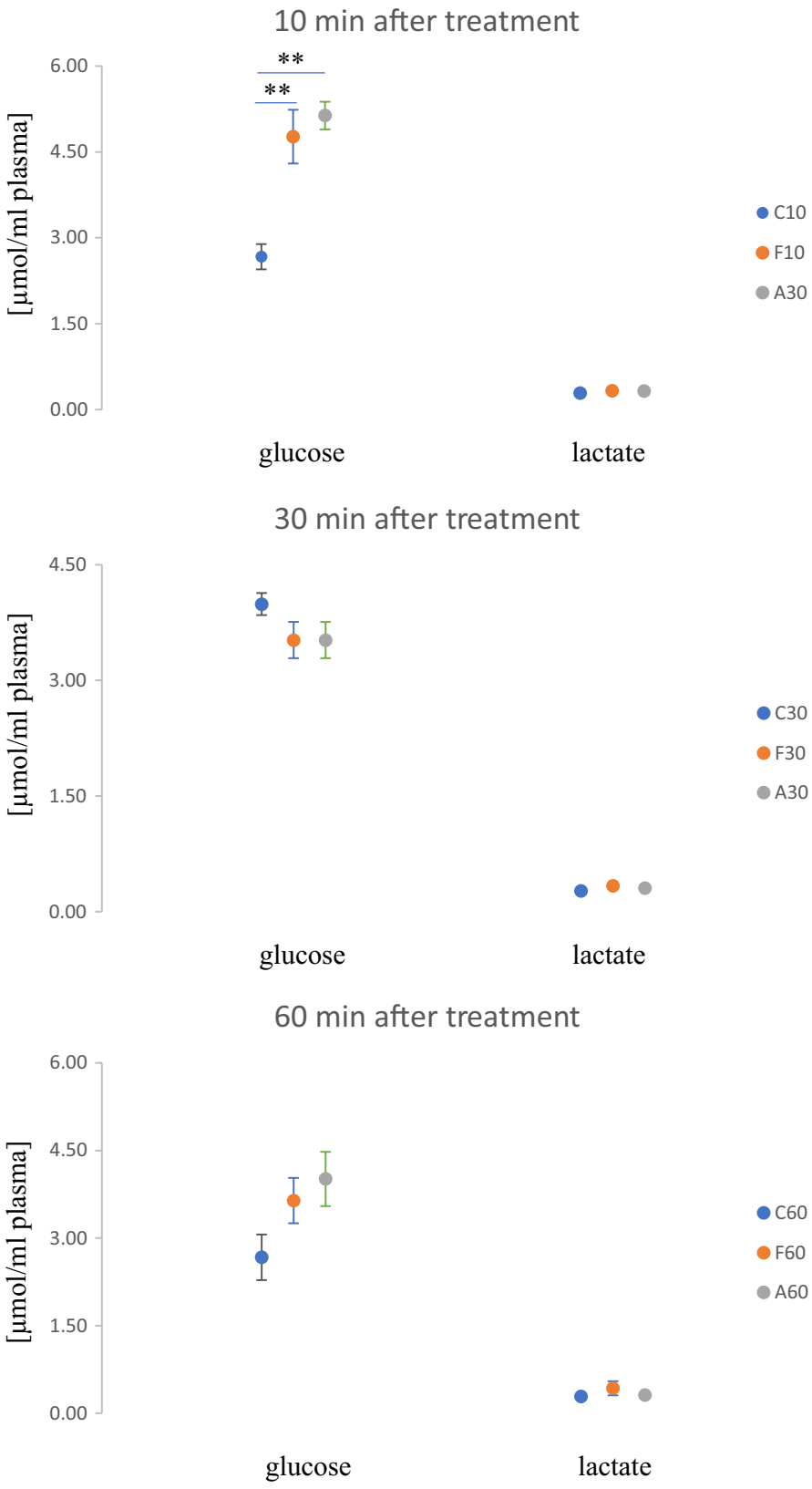

FIGURE 3 Plasma glucose and lactate levels in the fish 10, 30 and 60 min after the different treatments, mean ± SEM; $n = 6$ per treatment, $^{**}p < 0.01$.

The PCA conducted on the plasma parameters (Figure 4) revealed that the highest $\cos^2$ values for cortisol and cortisone occurred in the control fish comparison, as well as the 10 min data set. This changed profoundly in the 30 and 60 min data sets, which revealed the highest influence of the corticosterone values on the outcome of the PCA.

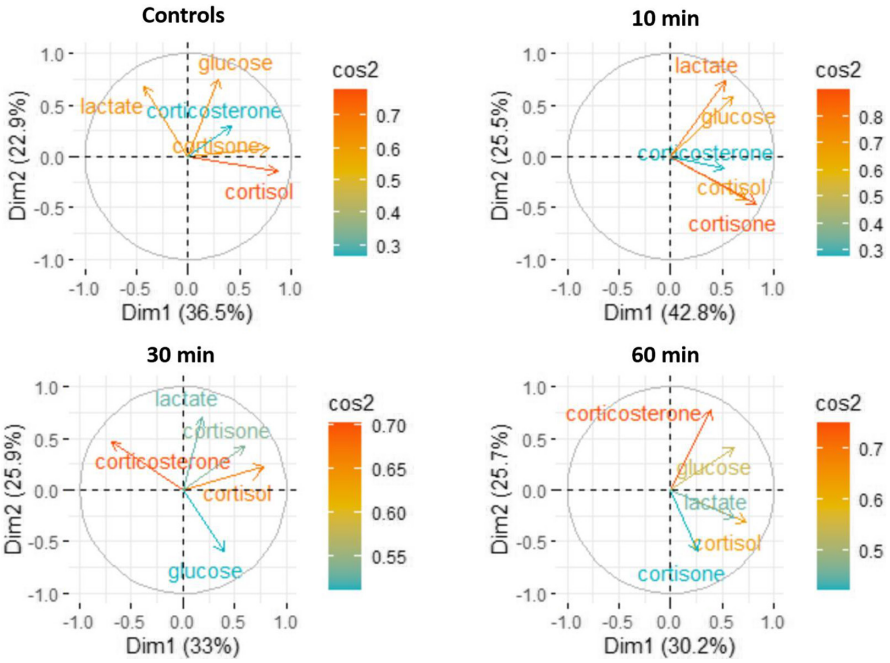

FIGURE 4 Quality of representation of the plasma parameters of controls and fish 10, 30, and 60 min after treatment on the factor map $\cos^2$ (the numbers next to Dim1 and Dim2 indicate the percentage of the variance in the data sets that is explained by the first two components of the PCA), $n = 6$ per treatment, except for the glucocorticoid analyses from the feed reward group with $n = 5$.

3.2 | Gene expression studies

3.2.1 | Effects of tank manipulation

The estimated marginal means for the genes often referred to as functionally distinct 'housekeeping' genes in PCR analyses, including *18S RNA*, *bactin*, *ef* and *tub* (Faheem et al., 2018; Mahanty et al., 2017; Rojas-Hernandez et al., 2019; Wang et al., 2018), demonstrated that the mRNA expression in different brain regions is influenced by tank manipulation (Figure 5). A reduction in expression of *18S RNA*, *bactin* and *tub* also occurred in the rhomb in the C30 group compared with the other control groups. The expression of *ef* was reduced in the same region in the C30 group compared only to C10 and C60.

Further investigations concentrated on gene expression patterns for three metabolic enzymes in the different brain parts. In the tel, opt and rhomb, a reduction of the *succdh* expression occurred in C30 compared with the other control groups, whereas the expression of *succdh* was significantly higher in the hyp in the C30 than in the C0 group ($p < 0.05$). The *gapdh* expression was found to be lower in the tel and hyp of animals from each of the control groups compared with C0 ($p < 0.001$). Conversely, *pyrkin* expression in tel, hyp and rhomb was found to be higher in each control group after tank manipulations ($p < 0.001$, Figure 5). A down-regulation of *gapdh* was observed in the rhomb, showing significant differences in C10 and C60 compared with C0 ($p < 0.01$). A decrease in the expression of *pyrkin* occurred in the opt in each control group compared with C0 ($p < 0.05$), and only in the C10 ($p < 0.01$) and C60 groups ($p < 0.05$) compared with C0 for *gapdh*.

Moreover, the IEG c-*fos* showed a significant increase in the mRNA expression in the tel in C10 compared with C0 ($p < 0.05$), whereas decreased expression of *c-fos* was observed in the rhomb in C30 compared with the other controls ($p < 0.05$). A decreased expression of *erk-1* and *erk-2* occurred in the same brain region in the C30 group compared with the other control groups ($p \leq 0.002$). A significant increased mRNA expression of *neurod* occurred in the tel, hyp, and rhomb in all controls compared with C0 ($p < 0.001$). However, a significant decrease in *neurod* expression was observed in the rhomb in group C30 compared with C10 and C60, but was still higher than in the C0 group. A similar pattern was found for *palld*, including in the opt, however, with a significantly increased mRNA expression in the C30 group in the rhomb ($p = 0.002$). The mRNA expression of *egr1* in the opt was found to be higher in the C60 group than in C30 ($p < 0.05$).

Furthermore, HPI axis–related genes were investigated (Figure 5). The expression of *crf-1* in the tel was higher in the C30 than in the C10 group ($p = 0.006$). In contrast, the gene was up-regulated in the opt in the C10 and C30 groups compared with C0 ($p < 0.05$). In the rhomb, *crf-1* was down-regulated in the C10 group compared with C0 ($p = 0.006$), up-regulated 30 min after tank manipulation ($p < 0.05$), and down-regulated again in the C60 group ($p = 0.004$).

The expression of *gr2* was higher in all control groups in the opt compared with C0 ($p < 0.05$). In the rhomb, the *gr2* expression was higher in C60 compared with C0 ($p < 0.05$), and decreased 30 min after tank manipulation compared with C10 and C60 ($p \leq 0.002$). The expression of *gr1* in the rhomb was significantly lower in each control group compared with C0 ($p \leq 0.004$), reaching its lowest point in the C30 group. Furthermore, 60 min after tank manipulation, expression of the same gene was lower in the tel compared with C0 ($p < 0.05$). In addition, there was a significant increase in the *mr* expression and a decrease in *pomc1* expression in each of the controls in the tel and hyp compared with C0. The expression of *pomc1* in the opt was higher in C10 and C60 compared with C0 ($p < 0.001$). The same pattern was observed for the *mr* in the rhomb. In the same brain region, the expression of *pomc1* was lower in each control compared ith C0 ($p < 0.001$). Up-regulation of *crf-1* was observed in the opt in the C10

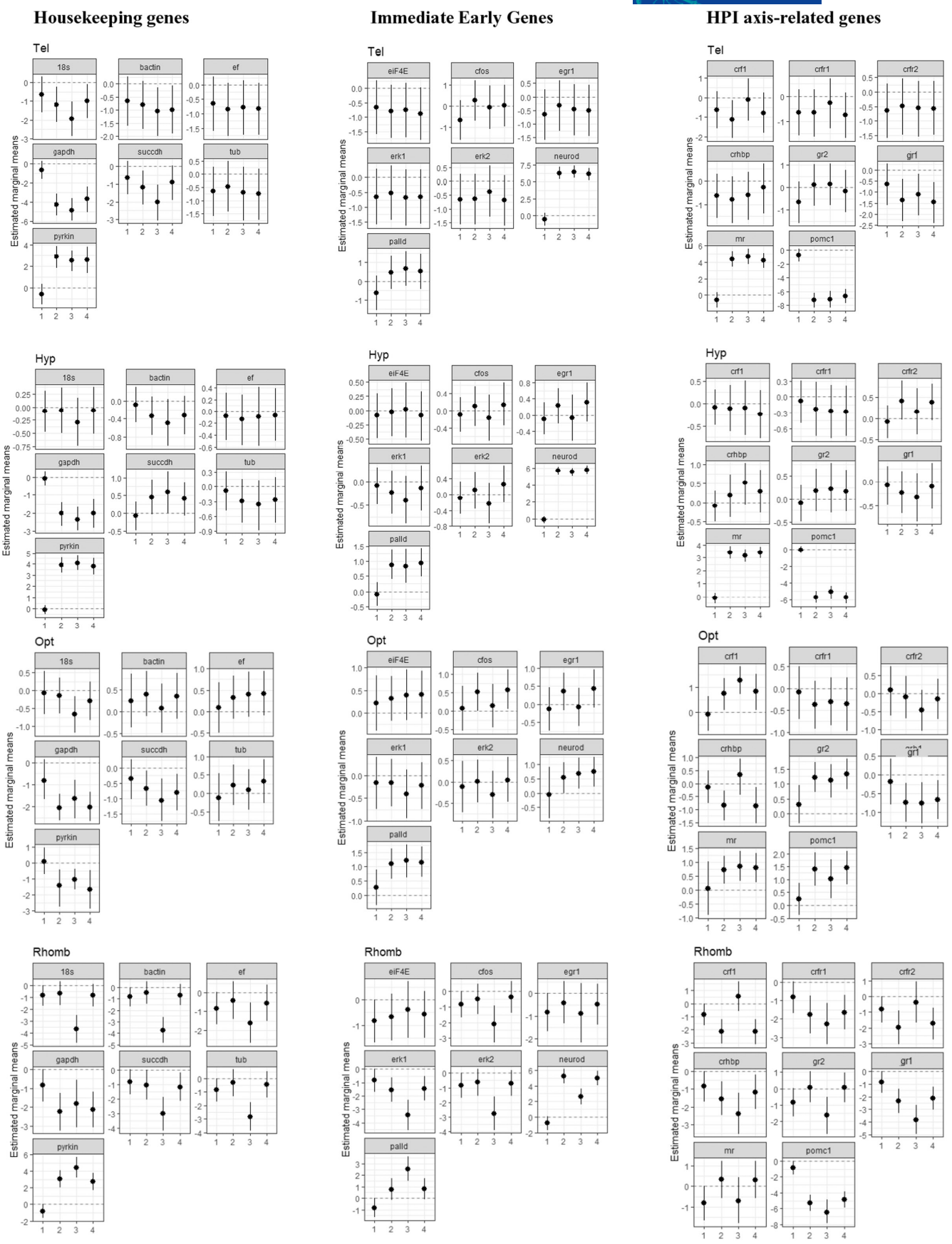

FIGURE 5 Marginal means of the potential housekeeping genes, immediate early genes, and hypothalamus–pituitary–interrenal axis–related genes in each of the four brain regions in the control fish 0 (= group 1), 10 (= group 2), 30 (= group 3) and 60 min (= group 4) after lid opening, mean ± SEM; $n = 6$ per treatment.

and C30 groups compared with C0 ($p < 0.05$). The *crh-bp* expression in the opt was found to be lower in the fish in group C10 than in C0 ($p < 0.05$), higher in C30 compared with C10 ($p = 0.002$), and once again lower in C60 compared with C30 ($p = 0.002$). A similar pattern was observed for the *crf-1* gene in the rhomb. However, in this case, all the control groups were significantly different from C0 ($p < 0.05$). The expression of *crf-r1* decreased in the rhomb after 30 min compared with C0 ($p < 0.05$). The expression of *crf-r2* in the rhomb was higher in the animals from the C30 group than in C10 ($p < 0.05$), and lower in C10 compared with the C0 group ($p < 0.05$). The *crh-bp* expression in the rhomb was lower in fish from C30 than in the C0 ($p = 0.006$) and C60 groups ($p < 0.05$).

Changes in the mRNA expression of genes that have not been mentioned in different brain regions were not significantly different between the treatment groups.

3.2.2 | Effects of eustress and distress

Genes often assumed to be suitable as housekeeping genes were influenced by the experimental treatments (Figures 6–8). The mRNA expression of *18S RNA* increased in the rhomb after 30 min in air-exposed fish compared with C30 ($p < 0.05$). Up-regulation of *tub* was observed in the rhomb, in the air-exposed group 30 min after the treatment compared with C30 ($p < 0.05$).

As a gene involved in metabolic pathways *pyrkin* showed a reduced expression after 10, 30 and 60 min after feed rewarding or air exposure in each brain region ($p \leq 0.004$), except in the opt at 10 and 60 min. Up-regulation was observed for *gapdh* in the opt at 10 min after both treatments ($p < 0.006$), whereas expression of this gene decreased in the same brain region and in the hyp at 30 min compared with C30 ($p \leq 0.004$). *Succdh* was down-regulated in the opt 10 min after receiving the feed reward compared with the C10 ($p < 0.05$). However, the expression of this gene was higher in the rhomb in both experimental groups 30 min after treatments ($p \leq 0.02$). The *succdh* expression was also increased in the rhomb in air-exposed fish compared with the feed reward group at 60 min after the treatment ($p = 0.02$). In contrast, a decrease in *succdh* expression was noted in the tel in air-exposed fish at the same time point ($p < 0.05$).

Immediate early genes were also influenced by the experimental treatments. The mRNA expression of the IEGs *eiF4E* and *c-fos* was higher in the rhomb in both experimental groups than C10 ($p < 0.05$ and $p \leq 0.01$ respectively). The IEG *c-fos* was also increased in each brain region 30 min after both treatments ($p \leq 0.004$). Up-regulation was also observed for *egr-1* in the hyp in the fish from F30 and A30 ($p < 0.05$). The IEG *erk-1* exhibited significantly higher expression in the rhomb 10 min after both experimental treatments, similar to *erk-2*, being up-regulated in the hyp after 30 min ($p < 0.05$ for each). A decreased *palld* expression was observed in the tel and opt in both experimental groups 10 min after the treatment ($p < 0.05$), in the hyp, opt and rhomb in both experimental groups 30 min after the treatment ($p \leq 0.002$), and in each brain region 60 min after both treatments ($p < 0.05$). Furthermore, the expression of *neurod* in the rhomb was lower in fish exposed to air than in the feed reward group 30 min after the treatment ($p < 0.05$). The expression of *neurod* increased in the opt 60 min after the feed reward compared with the air-exposed fish ($p < 0.05$). *Neurod* was, however, down-regulated in the tel, hyp and rhomb 10 min after both treatments ($p < 0.001$), in tel, hyp and opt after 30 min ($p < 0.05$), as well as in the tel, hyp and rhomb 60 min after both treatments ($p < 0.001$).

As for the HPI axis–related genes, *crf1* was up-regulated in the tel 10 min after both treatments ($p < 0.05$), as well as after 30 min in the hyp and opt ($p < 0.05$ and $p \leq 0.002$ respectively). However, the gene was only up-regulated in the opt in the feed reward group 60 min after the treatment compared with the control group ($p = 0.002$). The mRNA expression of *crf-r1* increased in the rhomb in the feed reward group after 10 min compared with C10 ($p < 0.05$). In contrast, the expression of *crf-r2* in the same brain region at the same time point increased after both treatments compared with the control group ($p < 0.05$). Expression of *crf-r1* was increased in the hyp in both treatment groups compared with C30 ($p < 0.05$). Up-regulation of *crf-r1* and *crf-r2* was observed in the rhomb 60 min after air exposure compared with the feed reward group ($p < 0.05$). In parallel, *pomc1* was up-regulated in each brain region and at each time point in both treatment groups ($p \leq 0.002$), except in the opt where its expression decreased.

Estimated marginal means revealed a decrease in *gr2* expression in the tel and opt 10 min after both treatments ($p < 0.05$), and only in the opt 30 and 60 min after both treatments compared with the control group ($p < 0.05$). Conversely, the gene was only down-regulated in the rhomb in the feed reward group 60 min after the treatment compared with the controls ($p < 0.05$). The expression of *gr1* was increased in the tel, opt and rhomb 10 min after both treatments ($p < 0.05$), as well as in the opt and rhomb after 30 min ($p \leq 0.004$), and only in the rhomb after 60 min in both experimental groups compared with the controls ($p \leq 0.002$). Up-regulation of *gr1* was also observed in the hyp after 10 min but only in the air-exposed group compared with the control ($p < 0.05$).

The gene *mr* was down-regulated in the opt after 10 min ($p < 0.05$), in the hyp and opt after 30 min ($p \leq 0.01$), and in the hyp after 60 min of both treatments compared with the controls ($p < 0.05$). The expression of this gene was also decreased in the opt 60 min after the treatment ($p < 0.05$), but only in the air-exposed group relative to the feed reward group. Similarly, *mr* expression was also decreased in the tel after 30 min ($p < 0.05$), but only in the fish exposed to air compared with the controls. However, its expression increased in the rhomb in this group after 60 min compared with the controls ($p < 0.05$).

3.3 | Principal component analyses

A PCA was conducted to reveal the presence of regulation patterns in the genes that were investigated. The PCA exhibited that the first two components that were selected for the PCA calculations showed a number of strong loadings on both components.

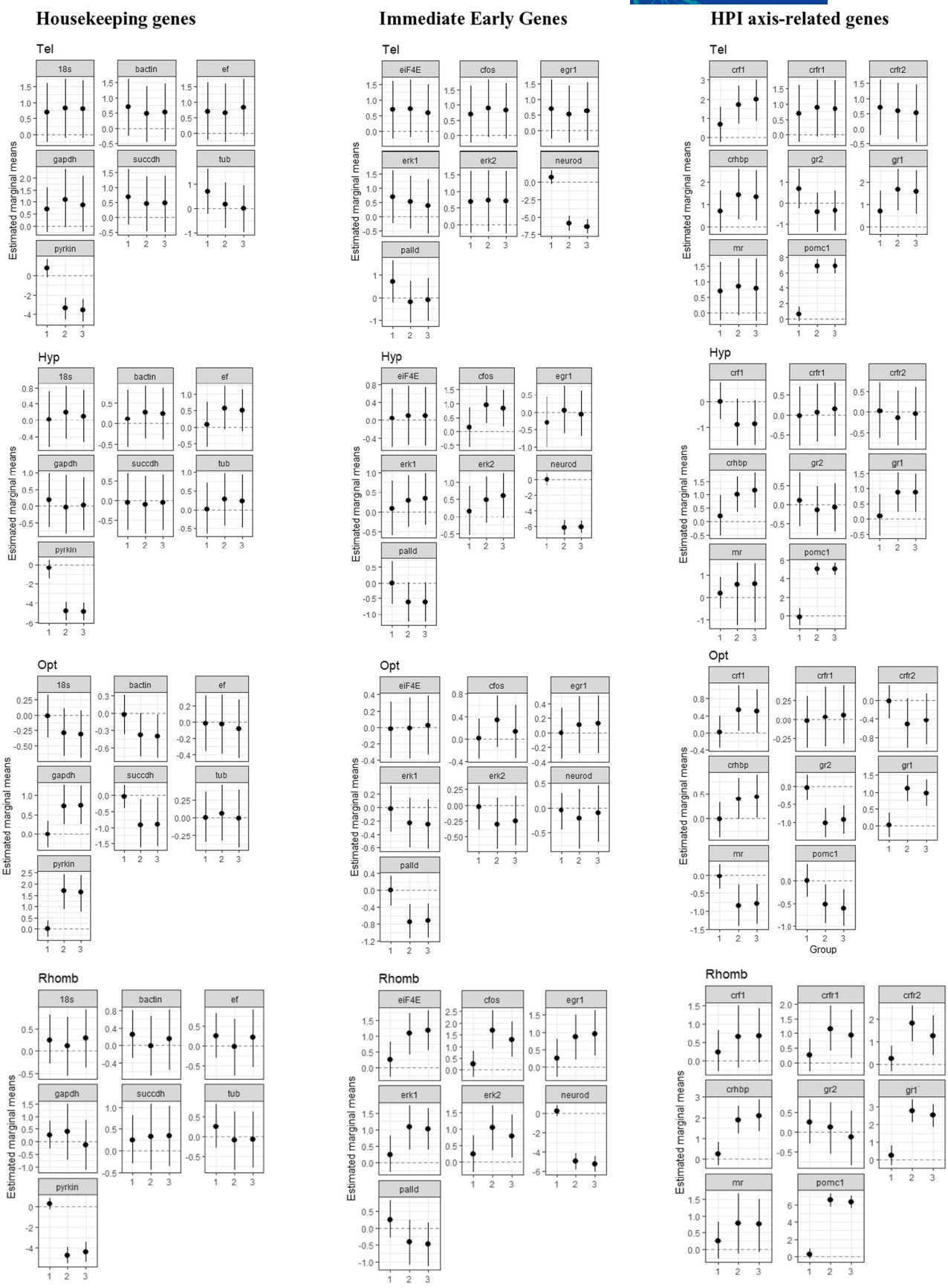

FIGURE 6 Estimated marginal means of the potential housekeeping genes, immediate early genes, and hypothalamus–pituitary–interrenal axis–related genes in each of the four brain regions in fish 10 min after the different treatments (group 1 = C, group = F, group 3 = A), mean of 5000 iterations ± credible intervals; models based on $n = 6$ per treatment.

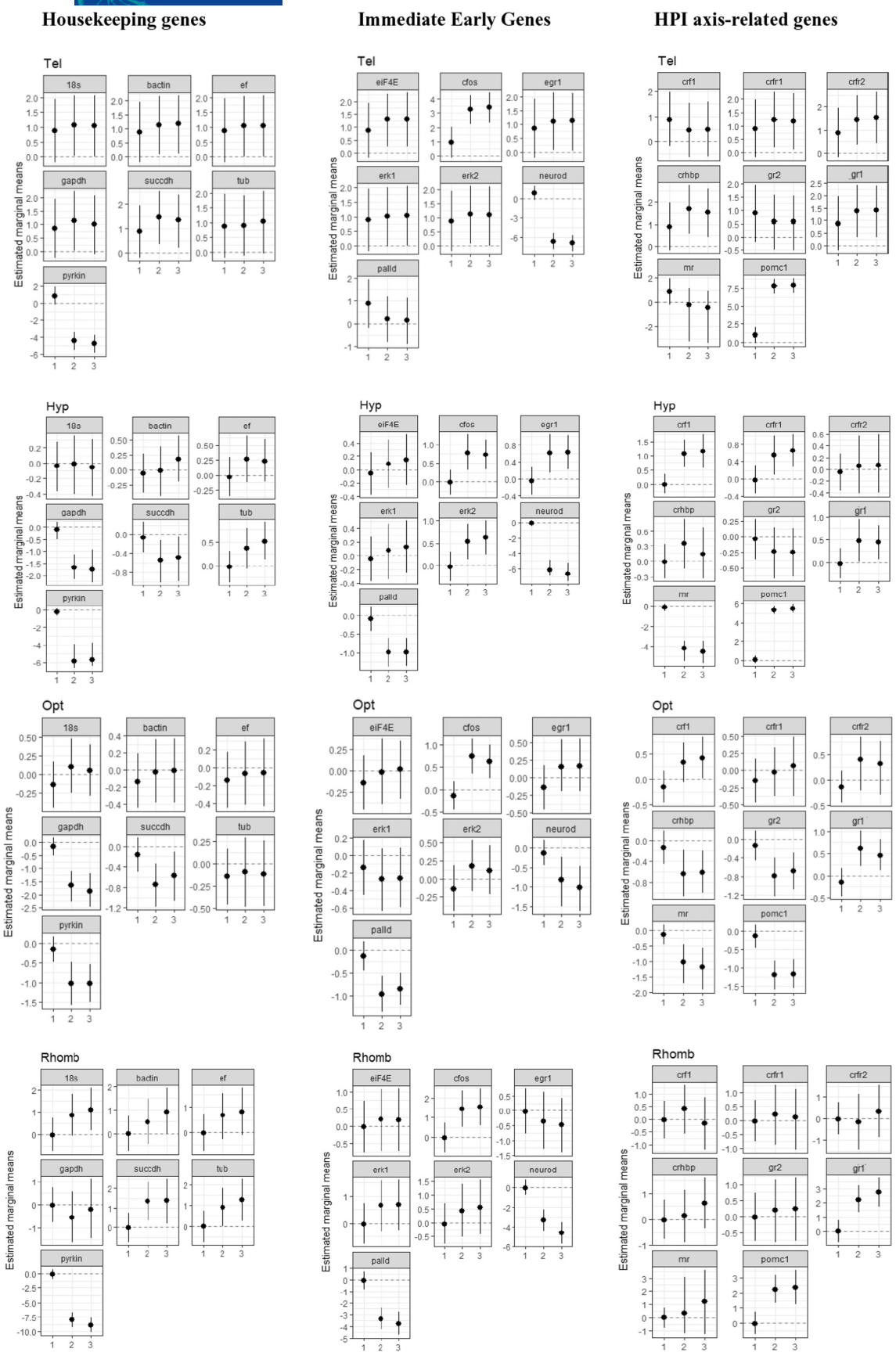

FIGURE 7 Estimated marginal means of the potential housekeeping genes, immediate early genes, and hypothalamus–pituitary–interrenal axis–related genes in each of the four brain regions in fish 30 min after the different treatments (group 1 = C, group = F, group 3 = A), mean of 5000 iterations ± credible intervals; models based on $n = 6$ per treatment.

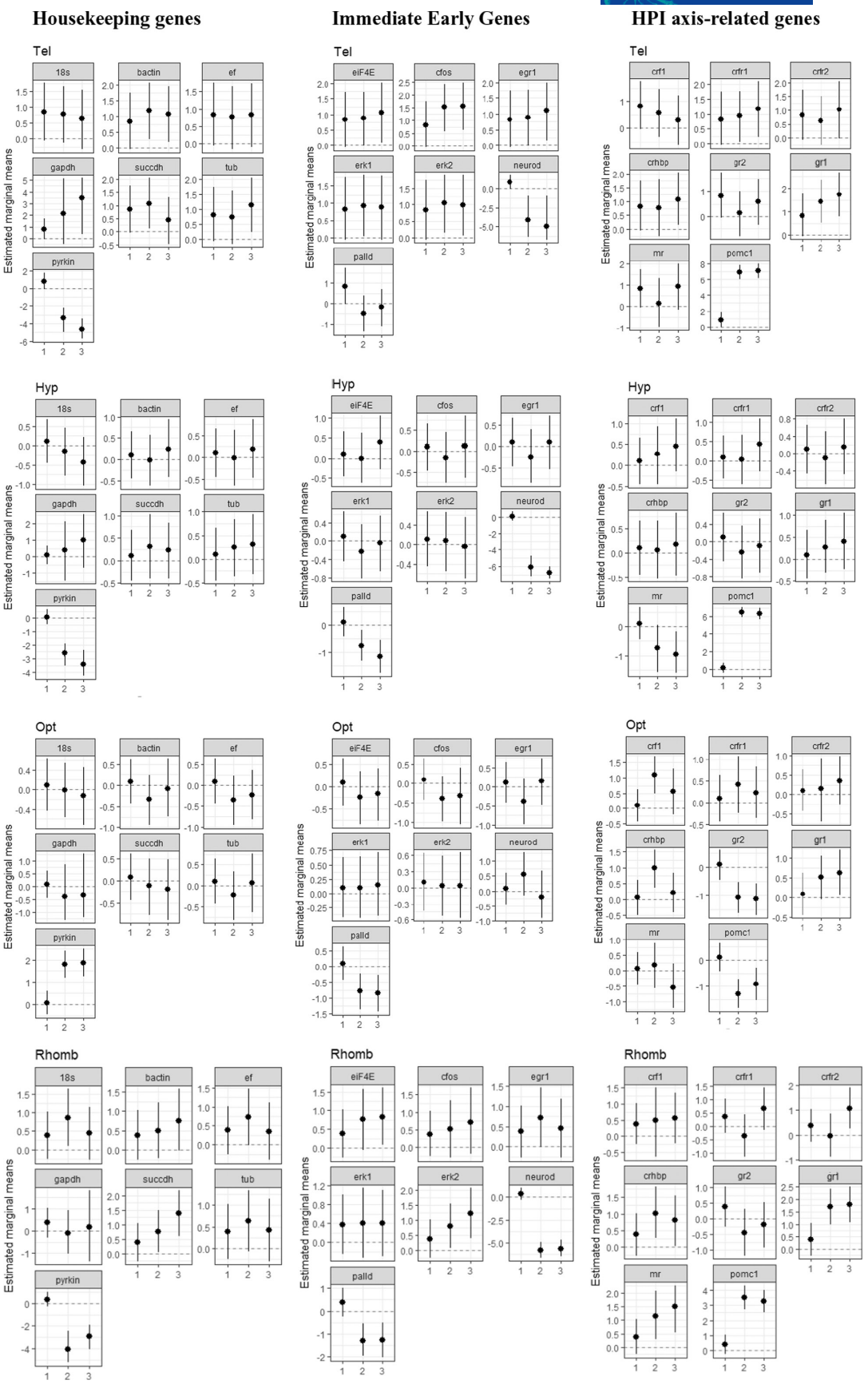

FIGURE 8 Estimated marginal means of the potential housekeeping genes, immediate early genes and hypothalamus–pituitary–interrenal axis–related genes in each of the four brain regions in fish 60 min after the different treatments (group 1 = C, group = F, group 3 = A), mean of 5000 iterations ± credible intervals; models based on $n = 6$ per treatment.

TABLE 1 The first two components of the principal component analysis (PCA) with the highest eigenvalue in the tel, hyp, opt and rhomb for the control groups (C0, C10, C30 and C60). The % variance in relation to the total variance in the data sets that is explained by the individual components (variance exp.) is shown. The gene sets that have been used for the PCA include genes that had been considered as potential reference genes, immediate early genes (IEGs) and hypothalamus–pituitary–interrenal (HPI) axis–related genes, $n = 6$ animals per group.

	tel		hyp		opt		rhomb	
Component	**1**	**2**	**1**	**2**	**1**	**2**	**1**	**2**
Housekeepings								
Eigenvalue	5.409	0.782	3.247	1.400	3.825	1.577	4.294	1.608
Variance exp.	77.3	11.2	46.4	20.0	54.6	22.5	61.3	23.0
IEGs								
Eigenvalue	4.684	0.841	3.128	1.303	4.026	0.908	4.163	1.011
Variance exp.	78.1	14.0	52.1	21.7	67.1	15.1	69.4	16.9
HPI axis								
Eigenvalue	6.240	1.300	3.592	1.697	4.592	1.324	5.696	0.797
Variance exp.	78.0	16.3	44.9	21.2	57.4	16.5	71.2	10.0

The two-component PCA explained 88.5% of the total variance in the control fish in the tel (C0, C10, C30, C60) when looking at the potential reference genes, 92.1% of the total variance for the IEGs, and 94.3% of the total variance in the HPI axis–related genes (Table 1).

In the data set for the 10 min treatment, the two-component PCA explained 91.9% of the total variance in potential reference genes, 92.9% of the total variance in the IEGs and 90.7% of the total variance in the HPI axis–related genes in the tel (Table 2). For the 30 min data set, the total variance explained by the two-component PCA was 87.9% for the potential reference genes, 91.5% for the IEGs and 90.7% for the HPI-related genes. For the 60 min data set, the potential reference genes explained 86.4% of the total variance in the data, whereas the IEG and HPI axis–related genes explained 93.8% and 84.3% of the total variance within the data. Similar to the control data sets alone, lower levels of variance could be explained by the gene set in the hyp, opt and rhomb.

The $\cos^2$ values for all five dimensions of each PCA from the control data sets (C0, C10, C30 and C60) have been summarized separately as heatmap profiles for each brain region in Figure S1. This figure shows that for each brain region there is a different profile of genes showing higher $\cos^2$ values than the other genes. Similarly, the five dimensions of each PCA have also been summarized separately as heatmap profiles for each brain region in Figure S2 for the control, feed reward and air exposure (C, F, A) data sets after 10, 30 and 60 min following exposure.

4 | DISCUSSION

4.1 | Blood parameters and metabolic genes as stress indicators

The glucose levels in the control fish were comparable with values for unstressed carp reported previously, with exception of the C30 treatment (Hoseini & Ghelichpour, 2013; Pietsch et al., 2014; Svobodova et al., 2006). In addition, carp that are completely at rest and under normoxic conditions exhibit <0.5 μmol/ml lactate in their plasma (Vianen et al., 2001). This may indicate that exposure to air for 1 min did not result in hypoxia of the carp in the current experiment, as no increase in lactate was detected in the plasma samples of air-exposed fish.

Several studies have shown that asphyxia due to brief exposure to air is accompanied by a pronounced increase in cortisol levels in the body, but these return to baseline levels within 1–2 h after application of acute stressors (Fuzzen et al., 2010; Pavlidis et al., 2015; Ramsay et al., 2009). Hypoxia also increases *crf* gene expression in the forebrain and chronically activated *crf* exerts a negative feedback on the cortisol release into the bloodstream in rainbow trout (Bernier & Craig, 2005). Our results are in agreement with previous reports on increased cortisol levels in fish exposed to acute negative stressors (Bernier, 2006; Pankhurst, 2011). Cortisol is known to act on *gr* to prevent further *crf* release (e.g., Stolte et al., 2008; Ziv et al., 2012). Contrary to this, the blocking of *gr* down-regulates *crf* and *gr* expression in different fish species, for example, in the preoptic area in rainbow trout (Alderman et al., 2012) and the telencephalon-preoptic brain region in goldfish, *Carassius auratus* (Bernier et al., 2009). In this study, significant changes in gene expression in all tested brain regions occurred after 60 min. Several studies have shown, however, that cortisol is not the best indicator of stress in fish concerning a response to chronic stress (Martínez-Porchas et al., 2009; Vijayan & Leatherland, 1990). This problem is partly due to the fact that a number of environmental factors are known to modify the clearance of cortisol from the body, including stress, ambient salinity, maturity and fitness as well as the nutritional state of the fish (summarized by Mommsen et al., 1999). Cortisol levels in teleosts are controlled by 11β-HSD2 (Jiang et al., 2003; Kusakabe et al., 2003; Meyer et al., 2012; Miura et al., 1991), which forms cortisone which is assumed to be an inactive glucocorticoid in cyprinids (Tsachaki et al., 2017). Nevertheless, cortisone may also

TABLE 2 The first two components of the PCA with the highest eigenvalue in the tel, hyp, opt and rhomb for the three treatment groups (C, F, and A) 10, 30 and 60 min after treatment. The % variance in relation to the total variance in the data sets that is explained by the individual components (variance exp.) is shown. The gene sets that have been used for the PCA include genes that had been considered as potential reference, immediate early genes (IEGs) and hypothalamus–pituitary–interrenal (HPI) axis–related genes, $n = 6$ animals per group.

10 min treatment								
	tel		**hyp**		**opt**		**rhomb**	
Component	**1**	**2**	**1**	**2**	**1**	**2**	**1**	**2**
Housekeepings								
Eigenvalue	4.450	1.063	3.504	1.126	2.763	1.564	1.952	1.521
Variance exp.	74.2	17.7	58.4	18.8	46.1	26.1	32.5	25.3
IEGs								
Eigenvalue	5.568	0.936	4.568	1.354	4.308	1.002	4.674	1.497
Variance exp.	79.5	13.4	65.3	19.3	61.5	14.3	66.8	21.4
HPI axis								
Eigenvalue	6.298	0.963	4.370	1.206	3.399	1.368	4.935	1.338
Variance exp.	78.7	12.0	54.6	15.1	42.5	17.1	61.7	16.7
30 min treatment								
	tel		**hyp**		**opt**		**rhomb**	
Component	**1**	**2**	**1**	**2**	**1**	**2**	**1**	**2**
Housekeepings								
Eigenvalue	4.298	0.981	2.045	1.334	2.410	1.229	3.187	1.799
Variance exp.	71.6	16.3	34.1	22.2	40.2	20.5	53.1	30.0
IEGs								
Eigenvalue	5.296	1.103	3.211	1.729	3.640	1.284	3.930	1.727
Variance exp.	75.7	15.8	45.9	24.7	52.0	18.3	56.1	24.7
HPI axis								
Eigenvalue	6.376	0.878	2.480	1.943	2.943	1.777	3.713	1.169
Variance exp.	79.7	11.0	31.0	24.3	36.8	22.2	46.4	14.6
60 min treatment								
	tel		**hyp**		**opt**		**rhomb**	
Component	**1**	**2**	**1**	**2**	**1**	**2**	**1**	**2**
Housekeepings								
Eigenvalue	4.043	1.140	2.714	1.832	3.485	1.025	2.963	1.680
Variance exp.	67.4	19.0	45.2	30.5	58.1	17.1	49.4	28.0
IEGs								
Eigenvalue	5.275	1.290	5.006	1.113	4.930	0.852	4.236	1.596
Variance exp	75.4	18.4	71.5	15.9	70.4	12.2	60.5	22.8
HPI axis								
Eigenvalue	5.682	1.061	5.460	1.246	4.816	1.155	5.379	0.943
Variance exp.	71.0	13.3	68.2	15.6	60.2	14.4	67.2	11.8

increase in the plasma of teleosts as a response to stress (Patiño et al., 1987; Pottinger et al., 1992; Weisbart & McGowan, 1984) and can be re-activated through the reductive activity of 11β-HSD1. However, this enzyme appears to be absent in ray-finned fish and the catalytic activity of 11β-HSD3 in fish remains to be elucidated (Baker, 2010; Diederich et al., 2002). In the current study, were insufficiently intense or prolonged to yield cortisone responses in the blood. Or as an alternative, cortisone metabolization to 20β-hydroxycortisone has taken place, which is thought to be excreted (Tokarz et al., 2012, 2013).

Corticosterone levels in control animals 60 min after tank manipulation were elevated compared with the control fish in the group tank. To date, the ability of corticosterone to activate *gr* or *mr* signaling in fish remains unclear. Further metabolization of corticosterone leads to the formation of aldosterone, but the role of the latter in fish is still being debated (Baker, 2003; Jiang et al., 1998). Nevertheless, the stronger binding of aldosterone than DOC to mr in several fish species (Arterbery et al., 2010) allows even low levels of aldosterone to activate mr. In addition, in cats, which also have cortisol as the dominant glucocorticoid (Möstl, 2014), effects on glycogen phosphorylation in skeletal muscle have been influenced by DOC treatment, whereas its acetate form DOCA, corticosterone and cortisol (used as the acetate in that study) were less effective (Montigel & Verzár, 1943). Nevertheless, this shows that we are probably currently underestimating glucocorticoid functions in fish and that the relationship between steroid hormone structure and physiological functions should be investigated in greater detail in the future.

The role of DOC as a mineralcorticoid has been evaluated in several studies in fish and the sensitivity of mr to DOC is thought to be an ancestral function of this receptor (Arterbery et al., 2010; Sturm et al., 2005). In addition, *gr2* is sensitive to aldosterone as well as DOC of midshipman fish, *Porichthys notatus* (Arterbery et al., 2010). The fact that an increasing number of steroids binds to *gr* is assumed to be a broader functionalization of the ancestral receptors and allows species-specific adaptations of receptor-dependent responses. However, it was not possible to include, for example, the metabolite 11-DOC, in the current study, to further elucidate the importance of DOC metabolization in teleosts.

Plasma glucose concentrations commonly rise following stress application, with a maximum level a few hours after the cortisol increases (Costas et al., 2011; Skrzynska et al., 2018). The regulation in blood glucose levels was at least partly attributed to the actions of cortisol via membrane-associated binding sites in sea bream, *Sparus aurata* (Aedo et al., 2019). Moreover, the increase of blood glucose is thought to be a leptin-dependent pathway in tilapia treated with salt water and cortisol (Baltzegar et al., 2014). *Pyrkin* is the enzyme in the glycolysis pathway that forms lactate anaerobically, which can lead to accumulation of lactate in the white musculature in fish (Wood, 1991), and increased plasma lactate concentrations in elasmobranchs (Frick, Reina, et al., 2010; Frick, Walker, et al., 2010) and neopterygians after exhaustive exercise (Thomas et al., 1999). In the study carried out by Barragán-Méndez et al. (2019), changes in plasma lactate levels paralleled those in muscle after air exposure, but returned to basal levels 5 h after stress application. Coinciding with the increased lactate levels, gluconeogenesis can occur together with increased activity of lactatedehydrogenase and fructosebisphosphatase in the liver (Moon & Mommsen, 1987), which convert lactate and other non-carbohydrate molecules into glucose (Suarez & Mommsen, 1987). Consequently, it was important to measure both lactate and glucose in plasma samples in the current study, but only increased glucose levels were observed in the blood stream. In parallel, decreased expression of *pyrkin* was observed after 10 and 60 min in every brain region and each experimental treatment, except in the optic tectum after 10 and 60 min (Figures 6–8).

In fish, the opt participates in the processing of numerous external stimuli, for example, visual and auditory stimuli, as well as signals from the lateral line (Farrell, 2011), and is known to be influenced by the hyp in larval zebrafish (Heap et al., 2018). The metabolic genes that were investigated clearly show the influence of different stressors (opening the curtains in front of the tanks and lifting the lid of the aquaria [C], receiving a feed reward [F] and air exposure for 1 min [A]) compared with fish taken directly from their rearing tank without the influence of a stressor (C0). Rapid non-genomic cortisol signalling has been associated with decreased activity of *gapdh* in sea bream liver (Aedo et al., 2019). Decreased expression of *gapdh* was observed in this study in all brain regions that were tested in fish from the controls kept in pairs compared with fish from the group tank (Figure 5). Air exposure also increased *gapdh* activity in catshark liver (*Scyliorhinus canicula*) 5 h after the stressor (Ruiz-Jarabo et al., 2019). This was also observed in this study in two different brain regions (tel and hyp), 60 min after air exposure compared with the controls. However, up-regulation of this gene was also observed in the tel and rhomb in the feed reward group 10 min after the treatment.

4.2 | Immediate early genes

In the current study, the IEG *c-fos* was confirmed to play an important role in stress responses (Burren & Pietsch, 2021; Kovacs, 2008). In contrast to the study by Burren and Pietsch (2021), we not only used air exposure but also the feed reward, and *c-fos* expression was up-regulated in both groups compared with the controls, especially after 30 min, while there were no big differences between F and A. In this study, an increase in *c-fos* activity was observed in each brain region 30 min after both treatments compared with the controls. In the feed reward group, the expression of *c-fos* was only higher in the opt and the rhomb 10 min after treatment. This may suggest that expression of *c-fos* might not be a good marker for distinguishing between eustress and distress in fish.

As shown by Kim et al. (2017), knock-out of the Down syndrome gene in zebrafish, in combination with acute social isolation, results in reduced expression levels of *c-fos* and *crf* in the hyp compared with the wild type (Herget et al., 2014). *Crf* is known to be secreted by neurons in the paraventricular nucleus, which links stress-related emotional responses and social interaction behaviours in mammals (Elliott et al., 2010; Ziv et al., 2012). *Erk-1* and *-2* are known to act on target genes, such as *crf* or *pomc* (Vasconcelos et al., 2020). However, in the current study, the expression patterns of these two genes were not comparable in all brain regions, which still makes *c-fos* a preferable indicator of stress in the carp brain. Similarly, further differences between the brain regions have also been identified for other IEGs. For example, *palld*, a regulator of *mTOR* (Umegaki et al., 2018), exhibited lower mRNA expression in the rhomb 30 min after air exposure, whereas expression of *neurod* was found to be higher in the opt 60 min after feed rewarding, and lower in both

groups in other brain regions. The expression of *eiF4E* was found to be higher after 60 min in the tel and hyp in the air exposure group. In addition, *egr-1* was higher after 60 min in the tel and hyp in the air exposure group. In contrast, it was lower in the feed reward group than in the air exposure and control groups in the hyp and opt after 60 min and higher in rhomb.

4.3 | Hypothalamus–pituitary–interrenal axis–related genes

Cortisol is known to increase the mRNA expression of *crf* (Bernier et al., 1999). However, the stress intensity and duration also play a clear role, since a single chasing event did not affect *crf* mRNA levels in the preoptic area of trout while chasing to exhaustion increased *crf* expression (Doyon et al., 2005). Juvenile cichlids, *Neolamprologus pulcher*, treated with cortisol, however, showed down-regulation of *crf* mRNA expression and *mr* up-regulation in the tel but not in the hyp, whereas the *gr1* expression was unaffected (Reyes-Contreras et al., 2019). In the current study, *mr* expression was significantly increased compared with C0, but this was not paralleled by a decrease in *crf-1* expression. Reyes-Contreras et al. (2019) concluded that the down-regulation of *crf* and the up-regulation of *mr* in the tel by early-life exposure to cortisol may be caused by increased *mr* expression, resulting in higher sensitivity of limbic stress responses and faster initial stress responses mediated by mr (Kasper et al., 2018). Alternatively, it is possible that lower *crf* expression decreases the activity of the HPI axis after exposure to a stressor (Backström & Winberg, 2013). By contrast, the application of a stressor appears to be strong enough to increase mRNA expression in both *crf* and *mr* in koi carp in the present study suggesting that exposure to acute stress did not allow the individuals to use physiological mechanism that enables them to cope better with these stressors. However, it is to be expected that more chronic exposure to the stressors that were used would have resulted in coping mechanisms and eventually led to less pronounced stress responses in the fish. Adaptation to stressors is assumed to prevent physiological damage caused by increased allostatic loads in the fish (Goymann & Wingfield, 2004). Unfortunately, elevations of cortisol due to social deprivation can also lead to re-programming of the stress axis genes (Sandi & Haller, 2015), which is also indicated in the current study by the comparison of the fish reared in groups and the control treatments, C10, C30 and C60. According to Doyon et al. (2005), increased *crf-bp* expression promotes a more rapid return to homeostasis after stress. A similar response in *crf-bp* expression was not observed in the current study, perhaps because the stress application and the respective time frame until sampling of the fish was shorter than in the study by Doyon et al. (2005). The fact that cortisol elevations, for example due to stressful treatment at early life stages, had long-term behavioural consequences, including more aggressive behaviour, increased time needed for contest completion and increased energy expenditure of the fish, has been demonstrated in cichlids (Grantner & Taborsky, 1998; Reyes-Contreras et al., 2019; Sloman, 2010). If the same is true for other fish species, this could also have important implications for fish breeding in aquaculture. The long-term effects of exposure to stress will therefore have to be investigated in further aquaculture-relevant fish species in the future.

According to Sakamoto et al. (2016) adult medaka show high *mr* mRNA levels in telencephalic regions. However, the authors proposed a different role for mr in medaka, according to which *mr* expression in the brain is less important for osmoregulation, but is necessary for typical responses to visual motion stimuli. Furthermore, evidence for membrane-located actions of cortisol leading to down-stream effects on liver *gr1* expression, but not *gr2* or *mr* expression, has been observed in seabream (Aedo et al., 2019). It is thus not only glucocorticoid effects via nuclear receptors that should be considered.

Crf and *pomc* also reacted to distress caused by air exposure in seabream (Skrzynska et al., 2018), and our previous work on carp also confirmed that the ratios of these HPI axis–related genes can be important stress markers (Burren & Pietsch, 2021). However, in the current study, *pomc1* mRNA expression was found to be even lower in the opt in the feed reward group than in the air exposure group 60 min after the treatments, while being up-regulated in the other brain regions compared with the controls. This fact emphasizes the importance of investigating the stress response pattern in the different brain regions separately, whereas another marker of changes in brain gene expression due to exposure to distress, for example, *c-fos*, is capable of indicating changes in brain activity in each brain region 30 min after exposure to the acute stressor.

5 | CONCLUSIONS

More differences in the stress responses in carp were revealed compared with the initial study by Burren and Pietsch (2021). In contrast to this earlier research, the current study allowed us to separately investigate suitable reference genes for the different brain parts. The assessment of suitable reference genes is highly recommended, since even commonly used reference genes, such as 18S RNA, exhibited an influence depending on the experimental treatment and a lack of stability within the current study. With respect to the subsequently calculated gene expression data, feed rewards and distress are stressors for koi carp, and these stressors are perceived differently in carp. The rearing of the koi carp in pairs also had effects on brain gene expression levels. In addition, it was evident that there is more differential expression of most of the genes in each of the investigated parts of the brain and probably also more crosstalk between the different brain regions. The distinct functions of each brain region have so far not been sufficiently understood, as was already stated by Heap et al. (2018) when investigating the interaction of the hyp and the tectum in zebrafish. Our results indicate that genes with a high impact on the gene expression pattern in the four brain regions, but especially in

the tel, can be used to determine the effects of different stressors on the fish. This is an important step towards the selection of marker genes for fish welfare.

AUTHOR CONTRIBUTIONS

Conceptualization and methodology, CP; fish husbandry, AS; blood and brain sampling, AS, CP; analysis and writing – original draft preparation, AB, CP, PP; writing – review and editing, GG, AB, AS, PP, CP; visualization, CP; project administration and funding acquisition, CP.

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CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest regarding the publication of this paper.

DATA AVAILABILITY STATEMENT

The data can be made available on request.

ETHICS STATEMENT

All experimental procedures were approved by the relevant cantonal authorities of Zurich (Switzerland) under permission number ZH-062-17.

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