

1 Communication

## 2 **Distress regulates different pathways in the brain of** 3 **common carp: an initial study**

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9 **Simple Summary:** The aquaculture sector provides for nearly half the world's seafood  
10 consumption, thanks to its large expansion over the last 30 years. Despite this intense growth, clear  
11 guidelines for responsible practices and animal wellbeing are lacking. Gene expression studies are  
12 a fundamental tool for understanding welfare, but stress-markers in aquaculture fish species are  
13 poorly studied. In addition, the biostatistical analyses of gene expression data is not trivial and the  
14 present study applies different statistical methods in order to evaluate potential differences in the  
15 gene expression levels between control fish and fish acutely stressed by air exposure.

16 **Abstract:** For the present study, a stress trial with common carp, which is one of the most important  
17 species in aquaculture world-wide, was conducted to identify relevant gene regulation pathways in  
18 different areas of the brain. Acute distress by air exposure significantly activated the expression of  
19 the immediate early gene *c-fos* in the telencephalon. In addition, evidence for regulation of the two  
20 *crf* genes in relation to their binding protein (*crh-bp*) have been highlighted in this initial study.  
21 Inference about the effects of distress by air exposure has been obtained by using point estimation  
22 which allows the prediction of a single value that is the best description of the up to know mostly  
23 unknown effects of stress in different brain regions of carp. Furthermore, principal component  
24 analyses have been performed to reveal possible regulation patterns in the different parts of the fish  
25 brain. In conclusion, these initial studies on gene regulation in the carp brain influenced by exposure  
26 to a stressor reveal that a number of genes may be successfully used as markers for exposure to  
27 unfavourable conditions.

28 **Keywords:** aquaculture; stressors; carp; early immediate genes; biostatistics

### 31 1. Introduction

32 Distress is defined as a condition that interferes with the well-being of an animal if adaptation  
33 processes of the organism fail to return the physiological and/or psychological homeostasis of the  
34 animal [1,2]. Fish in aquaculture are often subjected to distress for short periods (acute stress), but  
35 can also be exposed to stressors for longer time periods (chronic stress), meaning that the body's  
36 biological functions are sufficiently altered and its coping mechanisms overwhelmed [1].

37 Understanding the immediate effects of stressors, such as handling and crowding, on gene  
38 regulation in fish has been a focus of aquaculture research [3–5], in the interest of improving survival,  
39 growth, reproduction and fillet quality. The brain however, despite its role as first actor in the stress-  
40 cascade, has comparatively received little attention. Recently, a study in European seabass  
41 (*Dicentrarchus labrax* L.) and gilthead seabream (*Sparus aurata* L.) showed not only relevant differences  
42 between species, but the importance of studying the different fish brain areas separately [6]. In

43 addition, even certain brain areas may show significant differences in gene expression [7]. The main  
44 purpose of the present work is to identify stress-related biomarkers in different brain parts of  
45 common carp (*Cyprinus carpio*) to allow a precise evaluation of their rearing conditions.

46 The diversity of main functions between the regions of a fish brain have been already studied in  
47 the past. The telencephalon, for instance, has long been regarded solely with olfactory functions [8],  
48 but more recent research has confirmed its important role in the expression of emotional and  
49 motivational behaviour, as well as in fear conditioning in teleosts, including a pivotal role of these  
50 behaviours being attributed to the amygdala [9–11]. From mammals, it is known that the amygdala  
51 plays an essential role in mediating negative and positive emotions, which also involves the appraisal  
52 of incoming signals [12–15]. The optic tectum, directly connecting the efferent neurons with incoming  
53 retinal fibers in teleosts, has been recognized as essential for visually mediated behaviours already in  
54 early times [16,17]. However, the optic tectum is not solely required for perception of motions, but  
55 has more recently been proven to be essential for the correct pacing of saccades during optokinetic  
56 responses in zebrafish [18]. In addition, the hypothalamus plays an important role in energy  
57 homeostasis and appetite regulation. The activated or suppressed neurons then lead to adjustments  
58 in behaviour and metabolism. Proopiomelanocortin (*pomc*) neurons appear to drive satiety in the  
59 hypothalamic arcuate nucleus of mice [19]. The cerebellum of fish is responsible for the coordination  
60 of body movements [20], and has been linked to spatial navigation [21].

61 The effects of stressors on the brain can be assessed by analysing the activities of different gene  
62 sets. Firstly, brain activity can be assessed, for instance, by measuring the increased expression of  
63 stress-related immediate early genes (IEGs). For instance, *c-fos* is widely used as a functional marker  
64 of neuronal activity after a diversity of stimuli in vertebrates, due to its very rapid and robust  
65 expression [22]. In fish, different stimuli have been shown to induce varying levels of *c-fos* expression.  
66 Light avoidance as an innate choice behaviour involves rapid changes of the expression of *c-fos* in the  
67 medial zone of the dorsal telencephalon in adult zebrafish, *Danio rerio* [11]. In addition, the  
68 administration of D-amphetamine, known to activate the reward system, resulted in increased  
69 expression of *c-fos* in the same brain region 30 min after the injection of the substance [9]. Moreover,  
70 the sleep and wake behaviour of zebrafish also leads to typical differences in *c-fos* patterns in  
71 zebrafish [23]. Even caffeine has been proven to act as a stimulator of *c-fos* in the zebrafish brain [24].  
72 In addition, exposure to neurotoxins for 60 min resulted to rapid changes of the *c-fos* protein in  
73 different brain parts of killifish, *Fundulus heteroclitus* [25].

74 Another gene belonging to the group of the IEGs is *egr-1* (encoding for the early growth response  
75 protein 1) which has already been shown to be changing during the breeding cycle of sticklebacks  
76 [26]. Furthermore, the detection of phosphorylated extracellular signal-regulated kinase (*erk*) by  
77 immunohistochemistry has also been used as a readout of neural activity in fish at a whole-brain  
78 level [27]. The protein palladin (*pallid*) is essential for the organization of the actin cytoskeleton and a  
79 deficiency can lead to a failure of neurite outgrowth in rats [28]. Interestingly, in mirror carp (*Cyprinus*  
80 *carpio*) exposed to koi herpes virus, *pallid* has also been described as an immune-related gene [29]. The  
81 importance of this protein in cytoskeleton organization and kidney function has been confirmed in  
82 zebrafish [30,31]. Since it may also play an important role in the organization of the fish brain, this  
83 gene was included in the present study. In addition, one metabolic gene (*gadph*) was included in the  
84 present study, since its activity in tissues implicates higher energy demands and therefore increases  
85 of the metabolic gene expression.

86 Secondly, the response to stressors also commonly involves activation of genes of the  
87 hypothalamus-pituitary-interrenal (HPI) axis [32] and a number of these genes has therefore also

88 been included in the present study. *Crf* and its receptors play an important role in the stress signalling  
89 via the HPI axis [33]. The abundance of the *crh*-binding protein (*crh-bp*) determines the availability of  
90 *crh* to its receptors, although also other biological functions of *crh-bp* have been proposed [34]. Finally,  
91 the release of stress hormones such as cortisol and 11-deoxycorticosterone lead to the activation of  
92 glucocorticoid receptor (*gr*)- and mineralocorticoid receptor (*mr*)-mediated signaling pathways in  
93 teleosts [35]. In carp, *gr2* is the most sensitive corticoid receptor, followed by the *mr* and *gr1a* and *gr1b*  
94 [36].

95 Thirdly, other brain networks are also known to be involved and/or affected by stress responses  
96 in other fish species. As one example, the serotonergic pathways are affected by stress in trout [37].  
97 Serotonin also plays a role in the habituation to startling acoustic stimuli in zebrafish [38]. While  
98 serotonin agonists have anxiolytic effects in humans [39], chemicals including ethanol have shown  
99 that the acute anxiolytic effects by these substances are likely mediated by  $\gamma$ -aminobutyric acid  
100 receptors A (*gaba<sub>A</sub>*, [40]). The same psychoactive compounds have also been able to influence the  
101 normal behaviour of zebrafish [41]. In addition, isotocin is, together with vasotocin, a  
102 neurotransmitter and neuromodulator that is produced in distinct neurosecretory neurons in the  
103 hypothalamic nuclei [42]. Both influence the result of different behaviours, the establishment of the  
104 social status, but are also involved in osmoregulation and stress responses in fish [42].  
105 Osmoregulation is also regulated by prolactin in fish [43]. In addition, early development, behaviour,  
106 growth, and immunoregulation depend on prolactin and prolactin receptor expression [30,44]. In  
107 neuronal tissue, prolactin is also involved in the activation of neurons that evoke action potentials  
108 and/or calcium influx in neurons [45]. These reactions culminate in the release of neurotransmitters,  
109 e.g. dopamine in the hypothalamus of rats, being able to exert a negative feedback on the prolactin  
110 release [46–48]. Interestingly, an inhibition of the prolactin release from the pituitary of trout by  
111 GABA mediated probably by both, GABA receptors A and B has been described by Prunet et al. [49].  
112 However, the influence of stressors on these brain regulation pathways in other fish species is mostly  
113 unknown, which is the reason why a wider range of genes was included for the present study.

114 The present study was conducted to yield initial data on the differential gene expression patterns  
115 in the carp brain of stressed fish compared with non-stressed animals, as controls, and to apply  
116 different biostatistical methods that allow the identification of a set of potential genes suitable as  
117 biomarkers of distress in fish.

118

## 119 2. Materials and Methods

### 120 2.1. Rearing conditions and sampling

121 The fish were reared at 23 – 24°C in a 290 L aquarium equipped with a settler and a moving-bed  
122 biofilter. The carp (*Cyprinus carpio*) were kept for two months and fed 4 times daily at a feeding rate  
123 of 2 to 3 % body weight per day. During the experiment, the mean weight of fish was 28.3 g and the  
124 mean standard length was 8.9 cm. For stress treatment, fish were exposed to the air for 1 min in a  
125 net, returned to the tank and anaesthetised 30 min after that. After the acclimatization period, the  
126 control fish were taken directly from the rearing tank. Anaesthesia was performed with an overdose  
127 of tricaine methanesulfonate (MS-222, Sigma, Switzerland). The brains were sampled and stored in  
128 RNAlater® (Sigma-Aldrich, Buchs, Switzerland) for at least 24 h and afterwards divided into the 4  
129 brain areas (tel = telencephalon, hyp = hypothalamus, opt = optic tectum, rho = rhombencephalon  
130 comprising the corpus cerebelli and the medulla oblongata). All experimental procedures have been  
131 approved under permission number ZH-062-17 by the according Cantonal veterinarian authorities  
132 of Zurich (Switzerland).

133

## 134 2.1. PCR conditions

135 Gene expression studies have been performed by means of qPCR on a LC480 Light Cycler II  
136 from Roche (Basel, Switzerland). The total RNA from each of the four parts of each brain tel, hyp,  
137 opt, and rho has been extracted using RNeasy Micro Kits (Qiagen AG, Hombrechtikon, Switzerland).  
138 The RNA content was confirmed using the spectrophotometer Q5000 (Quawell, San Jose, USA).  
139 Subsequently, 20 µl of total RNA were reverse transcribed into cDNA using a High-Capacity cDNA  
140 Reverse Transcription Kit (Applied Biosystems, distributed by Thermo Fisher Scientific, Basel,  
141 Switzerland) according to the manufacturer's instructions. Thereafter, the cDNA content was  
142 adjusted to 50 ng per µl using nuclease-free water (Ambion®, distributed by Thermo Fisher Scientific,  
143 Switzerland) and used for real-time PCR using the LightCycler® SYBR® Green I Master mix (Roche,  
144 Switzerland). All primer pairs that were used are shown in the Table S1 in the Supplement. Prior to  
145 the PCR runs all primer reads have been validated, the respective PCR products confirmed by Sanger  
146 sequencing and the optimal reference genes have been extracted from a set of 8 possible reference  
147 genes by using the three genes with the best value for the expression stability M (i.e. *eIF4E*, *bactin*,  
148 and *ef*, for more details see Table S2 in the Supplement) extracted by geNorm function in the qbase+  
149 software, version 3.0 (Biogazelle, Zwijnaarde, Belgium - [www.qbaseplus.com](http://www.qbaseplus.com)) established by  
150 Vandesompele et al. [51]. The target genes included early immediate genes (*c-fos*, *egr-1*, *erk-1*, *erk-2*,  
151 and *palld*) as well as the metabolic gene glyceraldehyde-3-phosphate dehydrogenase (*gapdh*) in order  
152 to indicate active brain parts. The following genes related to the HPI axis have been included: *crfl*,  
153 *crf2*, *crfr1*, *crfr2*, *crhbp*, *pomc1*, *pomc2*, *gr1*, *gr2*, and *mr*. In addition, genes of the serotonergic pathway  
154 (*5-ht-r*, *serotr*) as well as *gaba<sub>a</sub>*, *iso-pre* and *prolr* have been investigated.

155

## 156 2.1. Calculations and statistics

157 As reference genes *bactin*, *eIF4E*, and *ef* have been used. The calculation of the normalized fold  
158 expression of each target gene was calculated according to Taylor et al. [52]. First, the mean  
159 quantitative cycle (mean ct) of the three technical replicates for each sample have been calculated.  
160 The average ct of all control samples for each target gene were calculated and the relative difference  
161 ( $\Delta$ ct) between the average ct for the control group and the mean ct for each sample within each target  
162 was assessed. Subsequently, relative quantities are calculated from the  $\Delta$ ct values. For each biological  
163 group (i.e. control group versus air-exposed group), a normalization factor was derived from the  
164 geometric mean of the relative quantities of each reference gene. Then the relative quantity of each  
165 target gene is divided by the normalization factor followed by log transformation. The obtained  
166 values have then been used to calculate the geometric means for each treatment group. The standard  
167 deviation (SD) and the standard error of the mean (SEM) have then been calculated from the log  
168 transformed normalized expression data. The figures show the average relative normalized  
169 expression for each target gene  $\pm$  SEM. For the statistical calculation of the differences between the  
170 means in gene expression per treatment group, non-parametric tests (Man-Whitney U tests) have  
171 been run in IBM SPSS Statistics (version 26, IBM Schweiz), since it has previously been shown that  
172 non-parametric calculation methods may have better control of false discovery of significant  
173 differences between expression levels for example after RNA sequencing [53]. Differences between  
174 treatment groups were considered statistically significant when  $p < 0.05$ . Assuming independence of  
175 tests, multiple testing leads to an inflated probability of a false positive results. To address this  
176 problem the following mixed models with a fully Bayesian approach (as a part of the *brms* package

177 [54] in R studio, Version 1.2.1335, RStudio Team 2018) and assuming a Gaussian distribution of the  
 178 data have been used to invest potential differences between the two treatment groups:

179

$$180 \quad y_{ij} \sim N(\mu_{ij}, \sigma^2) \quad (1)$$

$$181 \quad \mu_{ij} \sim \alpha_j + \beta_j x_i + \gamma_i \quad (2)$$

$$182 \quad \alpha_j \sim N(0, \sigma_\alpha^2), 1, \dots, n_{gen} \quad (3)$$

$$183 \quad \beta_j \sim N(0, \sigma_\beta^2) \quad (4)$$

$$184 \quad \gamma_i \sim N(0, \sigma_\gamma^2), i = 1, \dots, n_{animal} \quad (5)$$

185

186 The models include gene specific random effects for the constants ( $\alpha$ ) and gene specific random  
 187 effects for the group differences ( $\beta$ ) and animal specific random effects for the constants ( $\gamma$ ). The  
 188 model fit has been assessed by comparison of graphical plots (QQ plots) showing the distribution of  
 189  $y$  and  $y_{rep}$ . To be better able to handle possible outliers, posteriori predictive checks based on the  
 190 Markov Chain Monte Carlo (MCMC) approximation method have been applied which yielded  
 191 simulated replicated data under the fitted model that have subsequently been compared to the  
 192 observed data. The point estimators, their standard errors of the means, credibility intervals and  
 193 posteriori p values are reported.

194 For an initial description of the genes mostly contributing to the common variance within the  
 195 gene expression patterns in the different brain parts a principle component analysis (PCA) as a data  
 196 reduction method was performed on the log transformed normalized expression data in R studio  
 197 (Version 1.2.1335, RStudio Team 2018). The representation of the variables for the principle  
 198 components is calculated as a cos2 value. For a given variable, the sum of the cos2 on all the principal  
 199 components is equal to one.

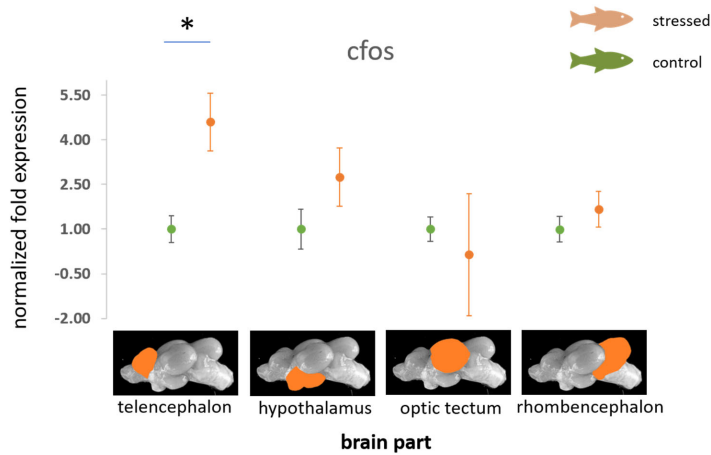
### 200 3. Results

#### 201 3.1. Immediate early genes, (*c-fos*, *egr-1*, *erk-1* and *erk-2*, *palld*) and *gapdh*

202 A significant difference between control fish and distressed fish was observed for the IEG *c-fos* in  
 203 the telencephalon ( $p < 0.05$ ), but not in the other brain sections that have been investigated (Figure 1).  
 204 In addition, an increased probability for a reduction of the expression of this gene was observed in  
 205 the optic tectum in relation to other genes that have been included in the present study (Table 1 and  
 206 Figure S4). However, the other IEGs have not significantly been influenced by the stress treatment

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208



209

210 **Figure 1.** Gene expression profile of the immediate early gene c-fos in each of the 4 brain parts in the  
 211 control fish and fish 30 min after the air exposure, mean ± SEM; n = 4 per treatment, significance  
 212 according to the Mann-Whitney U tests, p < 0.05.

213 For the IEGs, the first two components extracted in the PCA for each brain part explained 77.1 %  
 214 of the variance in the IEG data in the telencephalon, 83.8 % in the hypothalamus, 82.8 % in the optic  
 215 tectum, and 81.1 % of the variance in the IEG data from the rhombencephalon (Figure S1 in the  
 216 supplement). However, the genes that indicated a good representation on the principle component,  
 217 i.e. displayed by a high cos2 value in Figure S1 (in the supplement), differed for each brain part.

218 **Table 1.** Probabilities for potential group differences between the control animals and the distressed  
 219 fish (n = 4 each) in the different brain parts, the table shows for each of the genes the point estimator,  
 220 the SEM in brackets, and the credibility interval and the posteriori p value in the second row.

Gene	Tel	Hyp	Opt	Rho
<i>18S RNA</i>	0.16 (0.59)	0.29 (0.46)	0.75 (0.83)	0.35 (0.46)
	-1 - 1.39, p = 0.602	-0.5 - 1.31, p = 0.740	-0.76 - 2.47, p = 0.824	-0.52 - 1.28, p = 0.780
<i>5-ht-r</i>	0.22 (0.60)	0 (0.41)	0.09 (0.82)	0.03 (0.45)
	-0.9 - 1.51, p = 0.646	-0.87 - 0.86, p = 0.492	1.57 - 1.77, p = 0.540	-0.87 - 0.91, p = 0.526
<i>eIF4E</i>	-0.01 (0.60)	-0.48 (0.51)	-0.66 (0.85)	0.03 (0.46)
	-1.22 - 1.22, p = 0.496	-1.66 - 0.31, p = 0.162	-2.41 - 0.98, p = 0.210	-0.90 - 0.96, p = 0.520
<i>b2m</i>	0.01 (0.58)	0.09 (0.42)	-0.85 (0.82)	0.15 (0.44)
	-1.15 - 1.18, p = 0.515	-0.75 - 1.01, p = 0.580	-2.58 - 0.64, p = 0.144	-0.72 - 1.07, p = 0.630
<i>bactin</i>	0.13 (0.60)	0.14 (0.43)	0.33 (0.82)	-0.08 (0.45)
	-1.06 - 1.38, p = 0.584	-0.67 - 1.12, p = 0.621	-1.23 - 2.03, p = 0.650	-1.00 - 0.80, p = 0.432
<i>c-fos</i>	0.91 (0.70)	0.37 (0.48)	-1.5 (0.93)	0.33 (0.46)
	-0.26 - 2.40, p = 0.912	-0.40 - 1.49, p = 0.783	-3.41 - 0.21, p = 0.045	-0.53 - 1.28, p = 0.758
<i>crf-1</i>	-0.05 (0.57)	0.12 (0.43)	-0.31 (0.82)	0.25 (0.47)
	-1.26 - 1.07, p = 0.467	-0.70 - 1.08, p = 0.611	-1.97 - 1.28, p = 0.350	-0.64 - 1.22, p = 0.698
<i>crf-2</i>	-0.87 (0.69)	0.05 (0.44)	1.16 (0.87)	0.30 (0.47)

	-2.31 – 0.27, p = 0.088	-0.88 – 1.00, p = 0.546	-0.45 – 2.93, p = 0.918	-0.57 – 1.29, p = 0.734
<i>crf-r1</i>	<b>0.04 (0.57)</b>	<b>0.21 (0.44)</b>	<b>0.39 (0.80)</b>	<b>-0.37 (0.45)</b>
	-1.12 – 1.21, p = 0.520	-0.58 – 1.20, p = 0.670	-1.17 – 2.06, p = 0.686	-1.32 – 0.47, p = 0.204
<i>crf-r2</i>	<b>-0.11 (0.58)</b>	<b>0.28 (0.46)</b>	<b>0.10 (0.81)</b>	<b>-0.31 (0.47)</b>
	-1.31 – 1.02, p = 0.420	-0.53 – 1.34, p = 0.727	-1.50 – 1.68, p = 0.546	-1.29 – 0.57, p = 0.260
<i>crh-bp</i>	<b>0.39 (0.61)</b>	<b>0 (0.41)</b>	<b>0.08 (0.80)</b>	<b>-0.15 (0.46)</b>
	-0.72 – 1.72, p = 0.737	-0.89 – 0.84, p = 0.494	-1.49 – 1.67, p = 0.545	-1.10 – 0.72, p = 0.372
<i>ef</i>	<b>-0.13 (0.59)</b>	<b>0.12 (0.43)</b>	<b>0.38 (0.81)</b>	<b>-0.1 (0.46)</b>
	-1.33 – 1.06, p = 0.414	-0.68 – 1.09, p = 0.606	-1.24 – 2.07, p = 0.688	-1.03 – 0.81, p = 0.411
<i>egr-1</i>	<b>-0.08 (0.59)</b>	<b>-0.04 (0.43)</b>	<b>-0.81 (0.85)</b>	<b>0.16 (0.46)</b>
	-1.31 – 1.07, p = 0.444	-0.95 – 0.84, p = 0.468	-2.55 – 0.76, p = 0.160	-0.77 – 1.12, p = 0.632
<i>erk-1</i>	<b>-0.15 (0.57)</b>	<b>-0.23 (0.44)</b>	<b>0.14 (0.79)</b>	<b>-0.22 (0.45)</b>
	-1.34 – 0.97, p = 0.395	-1.25 – 0.52, p = 0.312	-1.38 – 1.75, p = 0.567	-1.15 – 0.63, p = 0.318
<i>erk-2</i>	<b>0.10 (0.57)</b>	<b>0.27 (0.47)</b>	<b>0.65 (0.82)</b>	<b>0.02 (0.45)</b>
	-1.02 – 1.24, p = 0.570	-0.55 – 1.36, p = 0.717	-0.88 – 2.34, p = 0.788	-0.90 – 0.92, p = 0.513
<i>gabaa</i>	<b>-0.07 (0.58)</b>	<b>-0.08 (0.42)</b>	<b>-1.62 (0.93)</b>	<b>-0.36 (0.46)</b>
	-1.22 – 1.09, p = 0.454	-1.01 – 0.75, p = 0.426	-3.55 – 0.07, p = 0.035	-1.30 – 0.49, p = 0.214
<i>gapdh</i>	<b>0.09 (0.58)</b>	<b>0.41 (0.50)</b>	<b>0.53 (0.82)</b>	<b>0.04 (0.47)</b>
	-1.05 – 1.30, p = 0.564	-0.38 – 1.56, p = 0.807	-1.00 – 2.16, p = 0.745	-0.93 – 0.99, p = 0.539
<i>gr1</i>	<b>-0.07 (0.59)</b>	<b>-0.33 (0.46)</b>	<b>-0.90 (0.85)</b>	<b>-0.15 (0.45)</b>
	-1.33 – 1.04, p = 0.456	-1.37 – 0.44, p = 0.230	-2.59 – 0.68, p = 0.140	-1.06 – 0.75, p = 373
<i>gr2</i>	<b>0 (0.59)</b>	<b>0.16 (0.44)</b>	<b>0.30 (0.81)</b>	<b>-0.24 (0.45)</b>
	-1.21 – 1.24, p = 0.490	-0.66 – 1.12, p = 0.631	-1.29 – 1.96, p = 0.649	-1.17 – 0.65, p = 0.299
<i>isopre</i>	<b>1.21 (0.79)</b>	<b>0.12 (0.44)</b>	<b>-0.79 (0.85)</b>	<b>-0.02 (0.46)</b>
	-0.07 – 2.87, p = 0.953	-0.74 – 1.08, p = 0.599	2.47 – 0.80, p = 0.170	-0.07 – 2.87, p = 0.953
<i>mr</i>	<b>-0.31 (0.61)</b>	<b>-0.31 (0.47)</b>	<b>0.20 (0.82)</b>	<b>-0.25 (0.47)</b>
	-1.64 – 0.89, p = 0.306	-1.39 – 0.45, p = 0.256	-1.42 – 1.83, p = 0.603	-1.22 – 0.65, p = 0.298
<i>palld</i>	<b>0.11 (0.55)</b>	<b>0.20 (0.45)</b>	<b>-0.40 (0.83)</b>	<b>-0.16 (0.46)</b>
	-0.96 – 1.26, p = 0.577	-0.61 – 1.22, p = 0.656	-2.08 – 1.18, p = 0.313	-1.10 – 0.76, p = 0.363
<i>pomc1</i>	<b>0.09 (0.60)</b>	<b>-0.39 (0.50)</b>	<b>1.47 (0.93)</b>	<b>1.68 (0.73)</b>
	-1.08 – 1.38, p = 0.546	-1.58 – 0.39, p = 0.221	-0.22 – 3.40, p = 0.952	0.28 – 3.13, p = 0.997
<i>pomc2</i>	<b>0.75 (0.71)</b>	<b>-0.22 (0.44)</b>	<b>2.05 (1.12)</b>	<b>1.09 (0.63)</b>
	-0.41 – 2.31, p = 0.867	-1.23 – 0.56, p = 0.316	0.03 – 4.45, p = 0.977	-0.01 – 2.40, p = 0.974
<i>prolr</i>	<b>-0.45 (0.61)</b>	<b>0.14 (0.43)</b>	<b>-0.99 (0.89)</b>	<b>0.11 (0.46)</b>
	-1.78 – 0.66, p = 0.233	-0.67 – 1.07, p = 0.618	-2.84 – 0.63, p = 0.118	-0.77 – 1.07, p = 0.586
<i>serotr</i>	<b>-1.47 (0.87)</b>	<b>-0.61 (0.58)</b>	<b>-0.74 (0.85)</b>	<b>-0.32 (0.47)</b>
	-3.27 – 0.02, p = 0.033	-1.96 – 0.21, p = 0.117	-2.44 – 0.87, p = 0.194	-1.30 – 0.54, p = 0.244

221

222

### 3.2. HPI axis-related genes

223

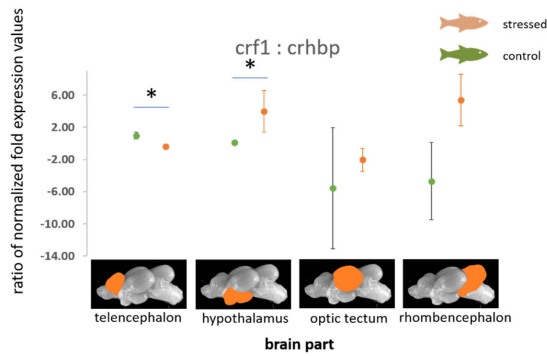
224

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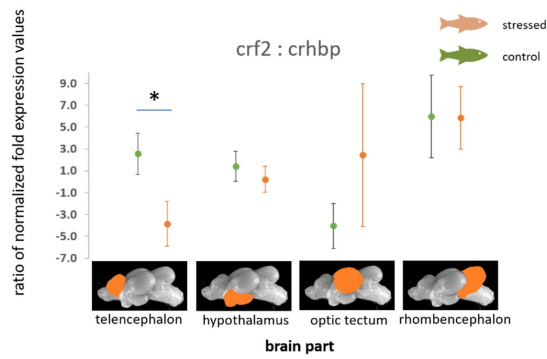
For stress responses, it is essential to consider how the mRNA expression values of hormones, their binding proteins and receptors change relative to each other. A significant decrease of the ratio of *crf1:crh-bp* in the telencephalon and an increase in the hypothalamus was observed in stressed fish

226 compared with the control fish ( $p = 0.021$ , Figure 2). Furthermore, the ratio of *crf2:crh-bp* was  
 227 decreased in the telencephalon by stress application ( $p = 0.029$ , Figure 2).  
 228

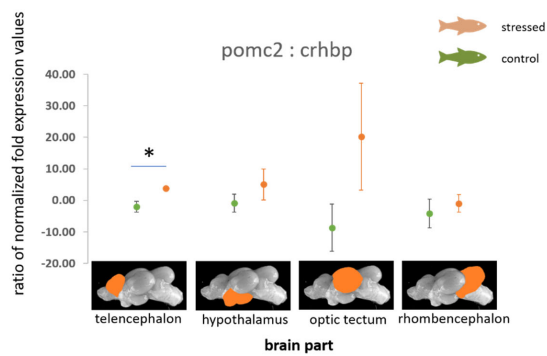
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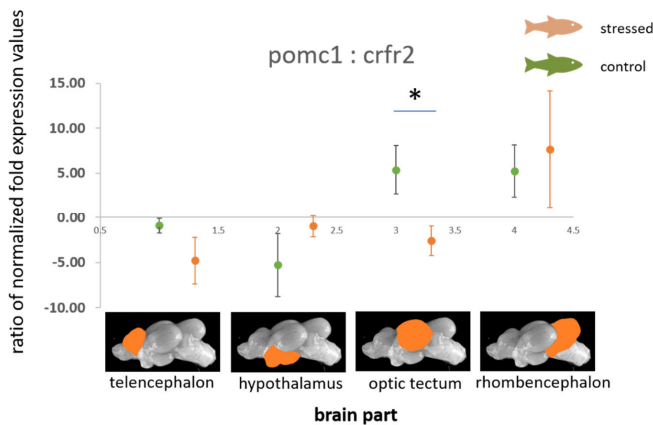
231



232 **Figure 2.** Ratios of the normalized fold expression of *crf1*, *crf2*, and *pomc2* relative to *crh-bp* in each of the 4  
 233 brain parts in the control fish and fish 30 min after the air exposure, mean  $\pm$  SEM;  $n = 4$  per treatment, significance  
 234 according to the Mann-Whitney U tests,  $p < 0.05$ .  
 235



236 In addition, the ratios of the normalized fold expression of *pomc2* relative to *crh-bp* were found to  
 237 be increased in the telencephalon of stressed fish compared with the control fish ( $p = 0.034$ , Figure 2).  
 238 The ratio of the normalized fold expression of *pomc1* to the *crf* receptor 2 was found to be significantly  
 239 decreased ( $p = 0.043$ , Figure 3).  
 240



241 **Figure 3.** Ratios of the normalized fold expression of *pomc1* compared with the expression of *crfr2* in each of  
 242 the 4 brain parts in the control fish and fish 30 min after the air exposure, mean  $\pm$  SEM;  $n = 4$  per treatment.  
 243  
 244

Kommentiert [PC1]: Achtung change figure

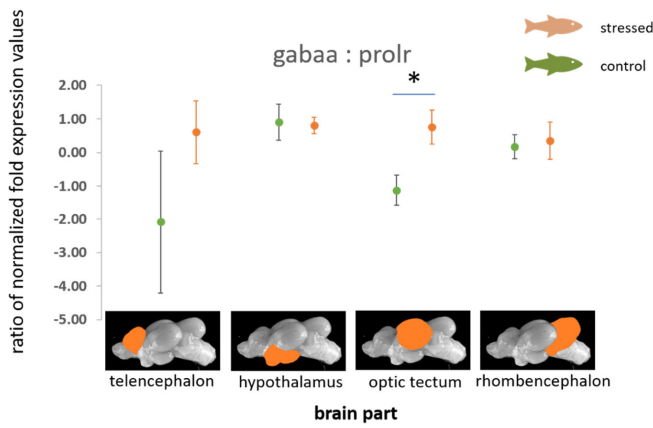
245 The PCA showed that the first two components of the PCA represented 74.5 % of the total variance  
 246 in the HPI genes in the telencephalon, 74.2 % of the variance in the same genes in the hypothalamus,  
 247 and 78.8 % and 60.6 % of the variance in these genes in the optic tectum and the rhombencephalon  
 248 (Figure S2 in the supplement). A high  $\cos^2$  value for *crf-r2* was observed in all four brain parts which  
 249 indicates a good representation of this variable on the principal component. In these cases, the  
 250 variables are positioned close to the circumference of the correlation circle in Figure S2. In contrast, a  
 251 low  $\cos^2$  indicates that the variable is not perfectly represented by the principle components. In this  
 252 case the variable is close to the center of the circle which, for example, can be seen for *pomc2* in the  
 253 telencephalon and rhombencephalon (Figure S2).  
 254

### 255 3.3. The gene expression patterns of the serotonergic genes, *gaba*, isotocin precursor and the prolactin receptor

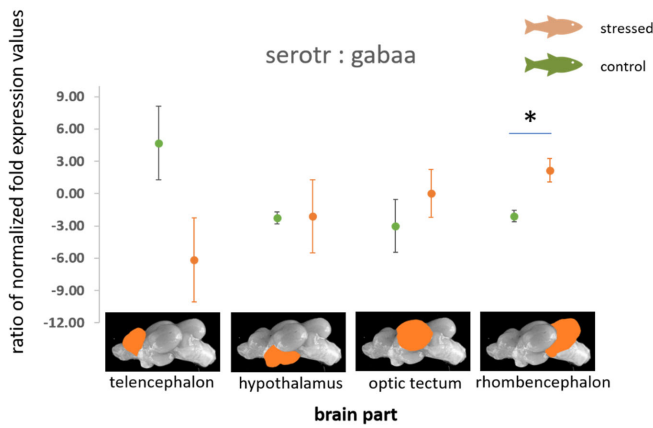
256 The ratios of the normalized fold expression of *gaba* relative to *prolr* were found to be increased  
 257 in the optic tectum of stressed fish compared with the control fish ( $p = 0.021$ , Figure 4). Furthermore,  
 258 an increased probability of a reduction of *gaba* expression in the optic tectum in relation to other  
 259 genes that have been calculated in the present study (Table 1 and Figure S4). In addition, the ratio of  
 260 the normalized fold expression of *serotr* relative to *gaba* were found to be increased in the  
 261 rhombencephalon of stressed fish compared with the control fish ( $p = 0.021$ , Figure 4), and increased  
 262 probability of a reduction of *serotr* in the telencephalon in relation to other genes that have been  
 263 included in the present study (Table 1 and Figure S4).

264 The PCA for the mRNA levels of the genes *5-ht-r*, *serotr*, *gaba*, *isopre* and *prolr* in the telencephalon  
 265 allowed the explanation of 72.2 % of the variance in the data set, whereas the same calculations in the  
 266 hypothalamus revealed that 85.9 % of the variance are related to the selected genes (Figure S3 in the  
 267 Supplement). Similarly, 83.6 % and 73.9 % of the variance was attributed to the selected genes in the

268 optic tectum and in the rhombencephalon, respectively (Figure S3 in the supplement). Consequently,  
 269 an optimal set of genes was desired for a final PCA, for which *gaba<sub>a</sub>*, *crfr1*, *crfr2*, *mr*, *egr-1*, *5-ht-r* and  
 270 *c-fos* have been selected for each of the brain parts separately (Figure 5). The PCA for the  
 271 telencephalon showed that 74.0 % of the variance in the data set could be explained when these genes  
 272 have been selected as variables. Compared to this, the PCA for the hypothalamus showed that 89.5  
 273 % of the variance was covered by the selected genes. Similarly, the respective variance levels were as  
 274 high as 93.5 % in the optic tectum and 86.1 % in the rhombencephalon.  
 275



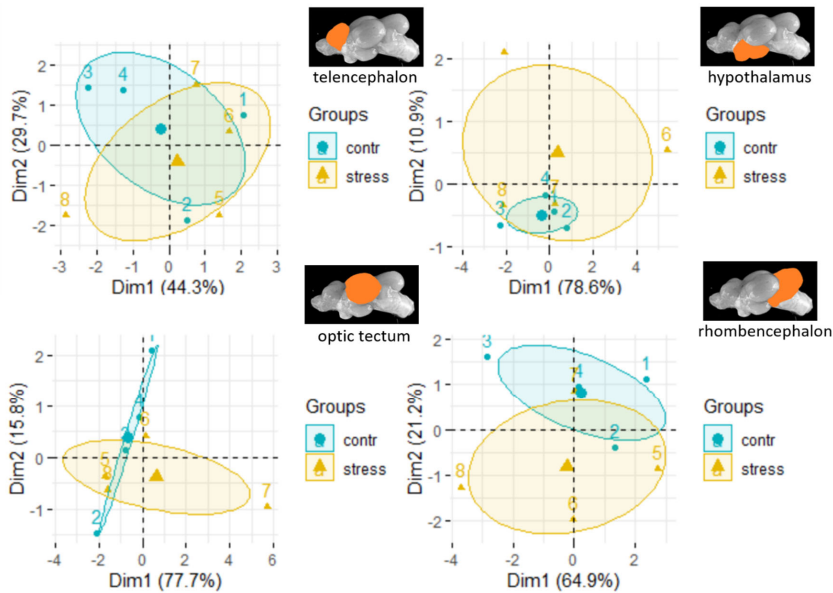
276



277

278 **Figure 4.** Ratios of the normalized fold expression of *gaba* to *prolr* and the normalized fold expression  
 279 of *serotr* to the expression of the *gaba* gene in each of the 4 brain parts in the control fish and fish 30  
 280 min after the air exposure, mean ± SEM; n = 4 per treatment, significance according to the Mann-  
 281 Whitney U tests, p < 0.05.

282



**Figure 5.** Results for the first two components (Dim1 and Dim2) of the PCA including confidence ellipses for the selected genes *gaba*, *crfr1*, *crfr2*, *erg-1*, *mr*, *erg-1*, *5-ht-r* and *c-fos* in each in the 4 brain parts of control fish and fish 30 min after the air exposure (the numbers in the brackets indicate the percentage of the variance in the data sets that is explained by each components, mean  $\pm$  SEM; n = 4 per treatment).

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#### 289 4. Discussion

290 Fish reared in aquaculture systems are continuously exposed to different stimuli, some of them  
291 being able to reach a distress situation once they overcome the physiological limits of the animals.  
292 With a continuous growth of the fish farming industry, ways to define and quantify welfare become  
293 vital to generate recommendations for best practice and legislation adaptations. In this study, we aim  
294 to identify the diversity of gene expression profiles between the four brain parts (telencephalon, optic  
295 tectum, hypothalamus and rhombencephalon) of distressed common carp.

296 Reference genes are fundamental for the exact determination of changes in gene expression  
297 [52,55]. Usually these are chosen from previous studies but only a limited number has selected  
298 reference genes according to their stable expression pattern in distinct tissues [56,57]. Typical  
299 reference genes are related to maintenance of cell structures and metabolism. For fish brain no such  
300 detailed investigation of suitable reference genes has been performed before. The reason is that that  
301 even acute stress, for example in trout, can alter the gene expression of a number of genes involved  
302 in intracellular signalling and cytoskeleton changes [58]. Thus, the selection of housekeeping genes  
303 that typically have these functions in cells might be impaired. Other genes, such as *gapdh*, may show  
304 a high variability which makes them unsuitable as reference genes [59,60]. Here, only three genes  
305 have been shown to be suitable as reference genes. Given the limited number of animals per treatment  
306 group and in order to confirm this hypothesis that for the fish brain region-specific reference genes

307 are needed, it is recommend to perform an additional study using a higher sample size to confirm  
308 the suitability of the analysed reference genes in brain regulation studies of fish.

309

### 310 3.1. Immediate Early Genes (IEGs)

311 Different IEGs have been investigated in the present study to identify brain activity in the  
312 different brain parts. The increased expression of *c-fos* in the telencephalon of distressed carp and the  
313 increased probability of a reduction of this gene in the optic tectum in relation to other genes (see  
314 Figure S4 for this) confirms that *c-fos* is not only a suitable indicator of brain activity in higher  
315 vertebrates and fish species such as zebrafish and goldfish [11,48,61], but also in carp. *In situ*  
316 hybridization has already shown that light avoidance leads to changes of the expression of *c-fos* in  
317 the medial zone of the dorsal telencephalon in adult zebrafish 30 min after the induction of neuronal  
318 activity [11]. Together with the present results from carp, it becomes clear that *c-fos* is capable of  
319 indicating changes in brain activity after exposure to acute stressors, in this case air exposure.

320

### 321 3.2. HPI axis-related Genes

322 That acute stress involves the *crf* system, e.g. preoptic area in the forebrain in fish, has already  
323 been reviewed in the past [61,62]. The fact that *crf* and *crh-bp* are widely distributed in the fish brain  
324 supports the assumption that the *crf* system has important functions even outside the cerebral system  
325 [63]. For example, changes in the *crf* system caused by stressors, including hypoxia, have also been  
326 observed in the caudal neurosecretory system and the heart of zebrafish [64,65]. *Crh-bp* is known to  
327 inhibit the *crf*-mediated activation of the *crf* receptors in a receptor subtype-specific fashion [66]. For  
328 this reason, the ratios of the normalized fold expression of the *crf* genes in the present study have  
329 been compared to the level of *crh-bp*, and the application of an acute stressor appears to influence this  
330 ratio, proving the assumption that the *crh-bp* actions are receptor-specific. Previous investigations  
331 have indicated that *crh-bp* is a more potent inhibitor of the *crfr2* activation of than for *crfr1* in fish [66].  
332 Similarly, the *crf2* receptors in humans have been described as being coupled to the cAMP-PKA  
333 signaling pathways similar to *crfr2*, but they mediate effects opposite to those of *crfr1* receptors during  
334 arthritis [67]. This reflects that *crf1* and *crf2* and also the two *crf* receptors have different functions  
335 which is probably also the case in fish. The differential effects of distress on the ratio of the normalized  
336 fold expression of the *crf* receptors compared with the fold expression of *crh-bp* in the present study  
337 support the assumption that both receptors have different functions in carp as well.

338 In the present study, also the ratio of the normalized fold expression of *pomc1* to *crfr2* is reduced  
339 in the optic tectum. The hypothalamic circuit includes two populations of neurons: one co-expressing  
340 orexigenic neuropeptides, such as neuropeptide Y, and the second one expressing pro-  
341 opiomelanocortin (*pomc*) and anorexigenic neuropeptides thus regulating feed intake in fish [68]. The  
342 functions of *pomc* neurons in the optic tectum are less well described. Nevertheless, the results of the  
343 present study support the hypothesis that the optic tectum is more than a dominantly retinorecipient  
344 structure.

345

### 346 3.3. Genes of the serotonergic and the gaba-ergic pathway

347 A connection of prolactin release and GABA receptor signalling has previously been shown in  
348 the hypothalamus of rodents as well as in the pituitary in rodent and rainbow trout [47,49,50].  
349 Surprisingly, the change of the ratio of the normalized fold expression of the GABA A receptor (*gaba<sub>a</sub>*)  
350 relative to the expression of the *prolr* was observed in the optic tectum, but the role of the *prolr* in this  
351 brain part remains unclear so far. The levels of GABA receptor mRNA expression together with the

352 expression of other important receptors, for example membrane receptors for serotonin and  
353 dopamine, affect memory loss in rats [69].

354 Serotonin transporter expression and activity which is required for returning serotonin to the  
355 presynaptic neuron where it can be degraded or retained for later re-use. In higher vertebrates,  
356 selective serotonin reuptake inhibitors can lead to increased GABA concentrations [70] which  
357 confirms an interaction of serotonin pathways and GABA levels. The present study on carp also  
358 indicated that the ratio of *serotr* to *gaba* is influenced by acute distress. In trout, acute stress resulted  
359 in downregulation of a serotonin receptor subtype and *mr* in the telencephalon 4 h post stress  
360 compared with the levels 1 h post-stress which indicated that a negative feedback exists for these  
361 receptors that aims at downregulating the HPI axis after activation by stress [71]. More sampling time  
362 points would have been required in the present study to show the dynamics of the activation of  
363 similar feedback mechanisms in carp.

364 In rodents, maternal care increases the 5-HT turnover at the serotonin receptor increasing the  
365 activity of this receptor which leads to the activation of the expression of factors such as *egr-1* further  
366 downstream [72,73]. The PCA in the present study indicated that *egr-1* is a suitable gene to indicate  
367 changes in the brain regulation in carp as a result of exposure to distress.

## 368 5. Conclusions

369 This section is mandatory.

370 **Supplementary Materials:** The following are available online at [www.mdpi.com/xxx/s1](http://www.mdpi.com/xxx/s1), Figure S1: "Variable  
371 correlation plot of the PCA for the IEG-related genes in each in the 4 brain parts", Figure S2: "Variable correlation  
372 plot of the PCA for the HPI axis-related genes in each in the 4 brain parts", Figure S3: "Variable correlation plot  
373 of the PCA for the serotonin- and *gaba*-related genes in each in the 4 brain parts", Figure S4: "Posteriori  
374 probability plots of all genes in each in the 4 brain parts of control fish and fish 30 min after the air exposure",  
375 Table S1: "Primer pairs selected for the gene expression studies", Table S2: "Average expression stability (M  
376 value) of the potential reference genes",

377 **Author Contributions:** Conceptualization, methodology, investigation, funding acquisition, project  
378 administration, writing—original draft preparation, software, formal analysis and data curation, visualization,  
379 writing—review and editing: A.B. and C.P. All authors have read and agreed to the published version of the  
380 manuscript.

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383 **Conflicts of Interest:** The authors declare that there are no conflicts of interest regarding the publication of this  
384 paper.

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