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RESEARCH ARTICLE

Effects of acute and chronic stress on telencephalic neurochemistry and gene expression in rainbow trout (*Oncorhynchus mykiss*)

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ABSTRACT

By filtering relevant sensory inputs and initiating stress responses, the brain is an essential organ in stress coping and adaptation. However, exposure to chronic or repeated stress can lead to allostatic overload, where neuroendocrinal and behavioral reactions to stress become maladaptive. This work examines forebrain mechanisms involved in allostatic processes in teleost fishes. Plasma cortisol, forebrain serotonergic (5-HTergic) neurochemistry, and mRNA levels of corticotropin-releasing factor (CRF), CRF-binding protein (CRF-BP), CRF receptors (CRFR1 and CRFR2), mineralocorticoid receptor (MR), glucocorticoid receptors (GR1 and GR2) and serotonin type 1A (5-HT_{1A}) receptors (5-$HT_{1A\alpha}$ and 5-$HT_{1A\beta}$) were investigated at 1 h before and 0, 1 and 4 h after acute stress, in two groups of rainbow trout held in densities of 25 and 140 kg m^{-3} for 28 days. Generally, being held at 140 kg m^{-3} resulted in a less pronounced cortisol response. This effect was also reflected in lower forebrain 5-HTergic turnover, but not in mRNA levels in any of the investigated genes. This lends further support to reports that allostatic load causes fish to be incapable of mounting a proper cortisol response to an acute stressor, and suggests that changes in forebrain 5-HT metabolism are involved in allostatic processes in fish. Independent of rearing densities, mRNA levels of 5-$HT_{1A\alpha}$ and MR were downregulated 4 h post-stress compared with values 1 h post-stress, suggesting that these receptors are under feedback control and take part in the downregulation of the hypothalamic–pituitary–interrenal (HPI) axis after exposure to an acute stressor.

KEY WORDS: Allostatic load, HPI axis, 5-HT, Neurochemistry, Gene expression, Cortisol

INTRODUCTION

The brain is the central organ involved in stress coping and adaptation (McEwen, 2009). It constantly processes information, sorting out relevant sensory inputs and initiates bodily reactions to challenges. These reactions include activation of the neuroendocrine, autonomic and immune systems, which are vital for regaining homeostasis (Johnson et al., 1992). This process of achieving stability through change, or allostasis (McEwen, 1998, 2007; McEwen and Wingfield, 2003; Sterling and Eyer, 1988), is essential for stress resilience and survival (McEwen, 1998, 2007). Yet, when being exposed to chronic or repeated stress, these systems may become maladaptive, leading to allostatic overload, i.e. malfunctions in the way the brain and neuroendocrine systems respond to additional stress (McEwen, 1998, 2007).

In mammals, the hippocampus and amygdala in the telencephalon play central roles in the process of discriminating sensory inputs, which potentially will threaten the homeostasis of an individual (LeDoux, 2000, 2007). Moreover, they are part of the limbic system, which interacts with the hypothalamus–pituitary–adrenal axis (HPA axis) (De Kloet et al., 2005). This neuroendocrine stress axis includes corticotropin-releasing factor (CRF), which is the key neurotransmitter regulating the release of adrenocorticotropic hormone (ACTH) from the pituitary, which in turn induces the release of glucocorticoids from the adrenal medulla. Furthermore, the neurotransmitter serotonin (5-hydroxytryptamine; 5-HT) plays an important role in the neuroendocrine stress response by controlling CRF release in the hypothalamus (Dinan, 1996). Moreover, 5-HT and CRF transmission are under feedback control of glucocorticoids and interact with the stress response by affecting the processes in the limbic system. According to this, altered CRF and 5-HT transmission have been suggested to be involved in adaptive stress-coping processes, as well as in maladaptive processes underlying allostatic overload (McEwen, 1999).

The neuroendocrine regulation of the stress response is well conserved within the vertebrate linage. In the teleost homolog of the HPA axis, the hypothalamic–pituitary–interrenal axis (HPI axis), hypothalamic CRF controls the release of ACTH from the pituitary, which in turn stimulates synthesis and release of cortisol, the principal glucocorticoid in teleosts, from the interrenal cells in the head kidney (Wendelaar Bonga, 1997). In mammals and fish, the CRF signal is mediated by at least two receptors (CRFR1 and CRFR2). CRFR1 have been reported to mediate HPA/HPI axis activation, whereas CRFR2 takes part in the expression of several behavioral and physiological reactions in response to stress (for references, see reviews by Backström and Winberg, 2013; Flik et al., 2006). Furthermore, the CRF-binding protein (CRF-BP) may play a regulative role in the HPI axis by modulating the bioavailability of CRF and related peptides (Manuel et al., 2014; Seasholtz et al., 2002). Moreover, similarly to mammals, 5-HT in teleosts affects hypothalamic CRF release, where the 5-HT receptor type 1A (5-HT_{1A}) plays a central role in the regulation of the HPA/HPI axis (Dinan, 1996; Winberg et al., 1997; Höglund et al., 2001; Medeiros et al., 2010). In addition, the HPI axis is under feedback

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control by cortisol, through mineralocorticoid receptor (MR) and glucocorticoid receptor (GR) in the hypothalamus and the pituitary (Bury et al., 2003; Colombe et al., 2000; Sturm et al., 2005). Aside from the 5-HT and CRF systems having direct control of the HPI axis, studies suggests that other functions of these systems are evolutionarily conserved between species. For example, neurochemical and gene-expression studies indicate interactions with limbic functions in the teleost telencephalon (Alderman and Bernier, 2007; Silva et al., 2015). Moreover, associations between telencephalic 5-HT and HPI-axis activity (Höglund et al., 2000, 2001; Øverli et al., 2005, 1999; Silva et al., 2015; Winberg et al., 1997; Winberg and Lepage, 1998; Winberg and Nilsson, 1993) lends further support to similar involvement of this brain part in HPI-axis regulation as observed in mammals (De Kloet et al., 2005).

In a recent study, Laursen et al. (2013) demonstrated that being held at a high density (140 kg m^3) for a period of 28 days affected 5-HT neurochemistry in the telencephalon and brain stem of rainbow trout (*Oncorhynchus mykiss*). These changes in 5-HT signaling were suggested to be related to chronic stress and indications of compromised welfare. However, the molecular mechanisms and associated neurochemical changes underlying allostatic load in the telencephalon of teleost fishes still remains to be investigated. Furthermore, exposure to unavoidable stressors can be detrimental to animal welfare, depending on intensity and duration of the disturbance, and is therefore an important concern in aquaculture. Therefore, it has been suggested that the concept of allostasis should be included in animal welfare guidelines (Korte et al., 2007). Thus, potentially, changes in telencephalic neurochemistry and expression of genes associated with CRF and 5-HT may reveal the welfare status of farmed fish.

However, further studies on mechanisms involved in changes in stress resilience and HPI-axis reactivity is needed for verifying the effects of keeping fish in high densities on animal welfare. Thus, the aim of this study was to investigate whether the high-density holding conditions reported in the study performed by Laursen et al. (2013) would result in changes in HPI-axis reactivity and whether these changes are related to telencephalic 5-HT neurochemistry and expression of genes involved in 5-HT and CRF transmission. In order to do this, fish were held at two densities – 25 and 140 kg m^{-3} – whereupon they were exposed to a standardized crowding stress for 30 min. Fish were sampled 1 h before the stressor and at 0, 1 and 4 h after the standardized stressor. Blood samples were analyzed for cortisol concentrations, and one side of the telencephalon was analyzed for mRNA levels and the other for 5-HT neurochemistry.

MATERIALS AND METHODS

Experimental animals

The experimental fish were juvenile rainbow trout [*Oncorhynchus mykiss* (Walbaum 1792)] from Store Restrup fish farm, Denmark. The fish had an average mass of 171±6.9 g (means±s.d.) at the time they were used for the experiment. The experiment was performed at the Danish National Institute of Aquatic Resources (The Technical University of Denmark), in Hirtshals. All animal procedures used in this study followed the policy and ethics as described by FELASA (Federation of European Laboratory Animal Science Associations).

Experimental facilities

The experiment was carried out using the 12-tank experimental facility previously described in detail by Larsen et al. (2012); Laursen et al. (2013) and McKenzie et al. (2007, 2012), and is briefly outlined here.

The holding tanks were connected to a recirculation biofiltered system, which cleaned and aerated the water. Water inflow emerged at high pressure from a series of small apertures along an inlet pipe fixed vertically to the wall of each tank. This water inflow to each tank gave rise to a circulating movement of water around the tank, thereby generating a current. A slow water current of approximately 0.5 body lengths per second was provided to each tank. Each tank was 1 m in diameter, and held a volume of 600 liters.

Water quality parameters were measured daily to ensure that they were at optimal levels for the fish. The temperature in the system was controlled at 16°C. Light conditions were at 14.5 h light and 9.5 h dark, with the lights automatically switching on at 07:30 h and switching off at 22:00 h.

Experimental procedure

Fish were kept at two densities in six circular polythene-holding tanks, three tanks with low density (25 kg m^{-3}) and three tanks with high density (140 kg m^{-3}), for a period of 28 days. To adjust for growth, the biomass was weighed in each tank after 14 days and the number of fish was readjusted to the desired density. At the beginning, the low-density tanks were stocked with 89±2 (means±s.d.) fish and the high-density tanks with 475±24 fish. After 14 days, the number of fish in each tank was readjusted, resulting in 77±3 and 396±14 fish, respectively, with fish weighing 203±9 g (means±s.d.). Fish were fed a daily ration of 3 mm pellets (EFICO920, BioMar A/S), corresponding to 1.5% of the estimated biomass per day, with automated belt-feeders for a period of 6 h (for further details, see Laursen et al., 2013). After being kept at high or low densities for 28 days, fish were exposed to acute stress for 30 min. Acute stress was induced by water levels being dropped in the tanks and holding the fish for 30 min at an approximate density of 500 kg m^{-3}, after which tanks were refilled to previous water levels. Four fish from each tank were sampled by swiftly netting, and euthanized by an overdose of anesthetic (ethylene glycol monophenyl ether). Sampling was done at: 1 h before (baseline), and 0, 1 and 4 h after being exposed to the acute stressor. Blood samples were collected from the caudal vasculature, using a syringe pretreated with ethylenediaminetetraacetic acid (EDTA). The plasma was separated from the blood cells by centrifuging at 1500 ***g*** for 5 min and frozen at −80°C for later analysis. The forebrain was dissected out of each fish and the telencephalon was separated into two parts, wrapped in aluminum foil, frozen on dry ice and stored at −80°C. Serotonergic neurochemistry was analyzed using one of the telencephalic hemispheres and mRNA levels were analyzed using the other.

Plasma cortisol assay

Corticosteroids were assessed by measurements of plasma cortisol levels determined by radioimmunoassay (RIA) as previously detailed by Pottinger and Carrick (2001) and is only briefly outlined here. An extraction of plasma samples was made and radioactively labeled cortisol was added to all samples, including all standards. The antibody used for this study was anti-cortisol, rabbit (from Cambio, CA-005). Cortisol concentrations were quantified by comparison with standard solutions of known cortisol concentrations.

Analysis of brain 5-HT neurochemistry

One of the telencephalon lobes was weighed and homogenized using an ultrasonic disintegrator in a homogenizing reagent [4% perchloric acid (PCA) containing 0.2% EDTA and 40 ng ml^{-1} dihydroxybenzylamine hydroxide (DHBA) solution]. The samples were then centrifuged at 10,000 ***g*** for 10 min at 4°C. The supernatants were analyzed by high-performance liquid

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chromatography (HPLC) with electrochemical detection to quantify the concentration of 5-HT and its metabolite 5-hydroxyindoleacetic acid (5HIAA). The HPLC system consisted of a solvent delivery system (Shimadzu, LC-10AD), an auto injector (Famod, Spark), a reverse phase column (4.6×100 mm, Hichrom, C18, 3.5 µm particle size) and an ESA Coulochem II detector (ESA, Bedford, MA, USA) with two electrodes at −40 mV and +320 mV. A conditioning electrode with potential of +40 mV was employed before the analytical electrodes to oxidize any contaminants. The ingredients in the mobile phase (HPLC buffer solution) were: 10.35 g l^{-1} monosodium dihydrogen phosphate (NaH_2PO_4), 0.3252 g l^{-1} sodium octyl sulphate (SOS), 0.0037 g l^{-1} EDTA, 70.0 ml l^{-1} acetonitril. pH was adjusted to 3.1 by adding concentrated phosphoric acid (H_3PO_4), approximately 4 ml, and MilliQ water was added until 5 liters in total. Telencephalic 5-HT and 5-HIAA content were quantified by comparing them with standard solutions of known concentrations and corrected for recovery of the internal standard using HPLC software (CSW, DataApex Ltd, Czech Republic). 5-HT turnover was calculated by dividing the concentration of monoamine metabolite 5-HIAA by the concentration of the main monoamine 5-HT.

Brain gene expression

mRNA levels were analyzed by real time quantitative reverse transcription PCR (RT-qPCR) in the other telencephalon lobe. Extractions of brain tissue mRNA levels were performed using the GenElute mammalian total RNA mini prep kit (Sigma, RTN70-1KT) followed by a DNAse digestion treatment using the Turbo DNA-free kit (Ambion) according to the manufacturers' instructions.

The total RNA concentration and purity was analyzed by spectrophotometry (Nanodrop, Thermo Scientific) for quality and quantity measures.

The RNA was transcribed to cDNA using the Maxima First Strand cDNA synthesis kit for RT-qPCR (Thermo Scientific) according to the manufacturer's instructions.

The cDNA solution was diluted 1:40 and used directly in the qPCR reaction.

The conditions for the qPCR was 50°C for 2 min; 95°C for 10 min; followed by 40 cycles at 95°C for 30 s; 60°C for 30 s and 72°C for 30 s and a dissociation stage. The reagent consisted of 5 µl 2× Maxima qPCR Master Mix including reference dye and 0.5 µl forward (F) and reverse (R) primers, and 4 µl cDNA. Primers designed for this study (Table 1) were 18–22 nucleotides in length with a melting point around 60°C and with a product size in the range of 69–240 bp. The relative mRNA levels were analyzed according to Vandesompele et al. (2002) using geNorm and three reference genes for normalization.

Statistics

Results are given as means±s.e.m. Two-way ANOVA with post-stress time and holding densities as independent factors was applied to investigate effects on plasma cortisol, 5-HIAA and 5-HT concentrations, 5-HIAA/5-HT, and mRNA levels of 5-$HT_{1A\alpha}$, 5-$HT_{1A\beta}$, CRF-BP, CRF, CRFR1, CRFR2, GR1, GR2 and MR. The Tukey's HSD (honest significant difference) *post hoc* test was used for detection of significant (P<0.05) differences between groups. Data were analyzed with STATISTICA v. 13 Dell.

RESULTS

Cortisol

Both post-stress time and holding densities had significant (P<0.001) independent effects on plasma cortisol; however, no significant interaction effect between them was observed (P=0.49) (Fig. 1). Generally, plasma cortisol values were significantly lower in fish held at 140 kg m^{-3} compared with fish held at 25 kg m^{-3} (P<0.001). On average, the acute stressor resulted in increased plasma cortisol, which was reflected in significantly higher post-stress levels at 0 h (P<0.001) and 1 h (P<0.001) compared with levels 1 h before the acute stressor. However, at 4 h post-stress, this effect was no longer visible and there were no significant differences compared with cortisol levels 1 h before the acute stressor (P>0.99).

Telencephalic 5-HT neurochemistry

Both post-stress time and holding densities affected 5-HIAA levels significantly (Table 2, Fig. 2A). However, there was no significant interaction effect between them ($F_{3,72}$=0.73, P=0.53). Generally, telencephalic 5-HIAA concentrations were lower in fish that were held in 140 kg m^{-3} compared with fish held in 25 kg m^{-3}. The acute stressor resulted in increased 5-HIAA concentrations. This was reflected in significantly higher levels at 0 h (P<0.001) and 2 h (P<0.001) post-stress as compared with levels 1 h before the acute stressor. However, 4 h post-stress levels did not differ significantly from telencephalic 5-HIAA levels 1 h before the acute stressor (P>0.99).

Overall, the effects on telencephalic 5-HT concentrations followed the same pattern as the effects on 5-HIAA levels, with time following acute stress having significant effects ($F_{3,72}$=3.7, P=0.02) (Table 2, Fig. 2B). However, 5-HT concentrations 1 h before the acute stressor were not significantly different from levels at 0 h (P=0.15), 1 h (P>0.95) and 4 h (P=0.64) post-stress. Still, the

Table 1. Genes, accession numbers and primer sequences for qPCR analysis and three reference genes

Gene	Forward primer	Reverse primer	Size (bp)	Accession no.
CRF	CCGATGATCCGCCGATATC	TGTTCAGCTCTCTGGACATCTC	69	AY049980.1
CRF-BP	CACAGCAGACCGGCCTCAGC	CATCAACGGCAGGGCGTGGT	168	NM_001124631.1
CRFR1	CTGGCAGCCCTACTGGTGGC	ACCAGCCGGCACCACAACAC	182	AY363678.1
CRFR2	CCAAGTTGAGAGCTTCTACC	AACAGCATGTAGGTGATCCC	104	CCAF010002597
GR1	ACGACGATGGAGCCGAAC	ATGGCTTTGAGCAGGGATAG	107	NM_001124730.1
GR2	TGGTGGGCTGCTGGATTTCTGC	CTCCCTGTCTCCCTCTGTCA	240	NM_001124482.1
MR	AGACTCGACCCCCACCAAG	CGTTAGTGGGACTGGTGCTC	102	NM_001124483
5-$HT_{1A\alpha}$	TCCGCCCGTAGAGGACCAG	TCAGCCAGGACCCGGGCTAC	87	CCAF010069675
5-$HT_{1A\beta}$	GAGGACCAACGGGGACCCGA	AATCGCCGTGCTTGACCGCA	92	CCAF0100015582
EF1a	GCAGGAAAAGAACCCAACG	AGTTACCAGCAGCTTTCTTCC	133	NM_001124339.1
HH3	TCCGTCGTTACCAGAAGTCC	AGGTTGGTGTCCTCGAACAG	176	X01064.1
ActB	AGAGCTACGAGCTGCCTGAC	GTGTTGGCGTACAGGTCCTT	179	NM_001124235.1

CRF, corticotropin-releasing factor; CRF-BP, corticotropin-releasing factor binding protein; CRFR1, corticotropin-releasing factor receptor 1; CRFR2, corticotropin-releasing factor receptor 2; GR1, glucocorticoid receptor 1; GR2, glucocorticoid receptor 2; MR, mineralocorticoid receptor; 5-HT, 5-hydroxytryptamine; 5-$HT_{1A\alpha}$, 5-$HT_{1A\alpha}$ receptor; 5-$HT_{1A\beta}$, 5-$HT_{1A\beta}$ receptor. Reference genes: EF1a, elongation factor 1a; HH3, histone H3; ActB, actin beta.

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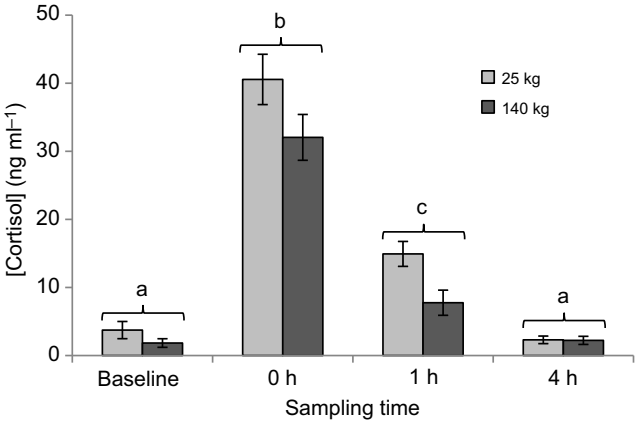

Fig. 1. Plasma cortisol content in rainbow trout kept at densities of 25 or 140 kg m^{-3} 1 h before (baseline) or 0, 1 and 4 h after exposure to an acute stressor. Acute stress was induced by dropping water levels in the tanks and holding the fish for 30 min at an approximate density of 500 kg m^{-3}. Different letters (a–c) indicate significant differences (P<0.05), which are independent of holding densities. See Results and Table 2 for complete statistics from the two-way ANOVA with densities and sampling time as independent variables. Values are means±s.e.m. and n=11 at each sampling time and density.

acute stressor resulted in elevated post-stress values at 0 h compared with 4 h (P=0.02). However, there was no significant difference between 5-HT levels 0 and 1 h after the acute stressor (P=0.35). There was no significant effect of holding densities on 5-HT concentrations, and no interaction between holding densities and time (Table 2).

The 5-HIAA/5-HT ratio followed the same pattern as 5-HIAA concentrations, with a significant effect of the post-stress time and holding densities separately (P<0.001), and with no significant interaction effect between these parameters (P=0.067) (Table 2, Fig. 2C). The 5-HIAA/5-HT ratio was significantly higher in fish held at the low density (P<0.001). Moreover, it was significantly higher at 0 and 1 h post-stress compared with values 1 h before stress (P<0.001) and 4 h post-stress (P<0.001). There were no significant differences in the 5-HIAA/5-HT ratio between 1 h before stress and 4 h post-stress (P>0.95), or 0 and 1 h post-stress (P=0.98).

Telencephalic gene expression

The two-way ANOVAs indicated significant effects of the acute stressor in expression of 5-HT$_{1A\alpha}$ (Table 2, Fig. 3A) and MR (Table 2, Fig. 3B). The expression of 5-HT$_{1A\alpha}$ was significantly lower at 4 h compared with values at 1 h post-stress (P=0.02). Moreover, mRNA levels of this receptor at 4 h post-stress tended to be lower compared with values 1 h post-stress (P=0.08), but it did not differ significantly compared with values at 0 h post-stress (P=0.27). Furthermore, there were no significant differences in mRNA levels of 5-HT$_{1A\alpha}$ between 0 and 1 h post-stress (P=0.64), or between 1 h before stress and 0 (P=0.91) and 1 h (P>0.95) post-stress. The mRNA levels of MR followed the same pattern, with significantly lower levels at 4 h compared with 1 h post-stress (P=0.02). Moreover, mRNA levels of MR at 4 h did not differ from values at 1 h before stress (P=0.40) and 0 h post-stress (P=0.17). Furthermore, the mRNA level of this receptor did not differ significantly between 1 h before stress and 0 h post-stress (P=0.97), 1 h before stress and 1 h post-stress (P=0.54) or between 0 h and 1 h post-stress (P=0.79). There were no significant effects of density or the interaction between the acute stressor and holding densities in expression of any of the investigated genes (Tables 2 and 3).

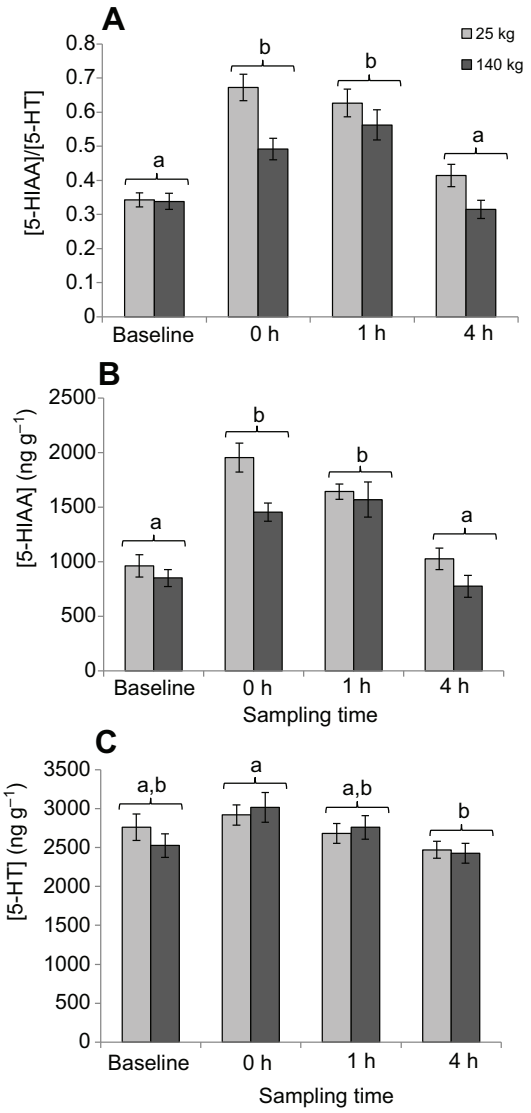

Fig. 2. Effects of chronic and acute stress on telencephalic 5-HT neurochemistry. (A) Serotonergic activity [5-HIAA/5-HT (5-hydroxyindoleacetic acid/5-hydroxytryptamine) ratio]; (B) 5-HIAA concentration; and (C) 5-HT concentration in rainbow trout held in 25 kg m^{-3} or 140 kg m^{-3} 1 h before (baseline) or 0, 1 and 4 h after being exposed to an acute stressor. Acute stress was induced by dropping water levels in the tanks and holding the fish for 30 min at an approximate density of 500 kg m^{-3}. Different letters (a,b) indicate significant differences (P<0.05), which are independent of holding densities. See Results and Table 2 for complete statistics from the two-way ANOVA with densities and sampling time as independent variables. Values are means±s.e.m. and n=11 at each sampling time and density.

DISCUSSION

Effects of acute stress

The acute crowding stressor resulted both in increased plasma cortisol levels and 5-HT turnover in the telencephalon. This effect was present 0 and 1 h post-stress, with both plasma cortisol and 5-HT turnover returning to basal levels at 4 h post-stress. Similar time spans (3–8 h) for downregulation of the neuroendocrine stress response after intense acute stressors has been reported in rainbow trout (Øverli et al., 1999; McKenzie et al., 2012) and in gilt-head sea bream (*Sparus aurata*) (Barton et al., 2005).

The acute stressor also affected mRNA levels of the receptors MR and 5-HT$_{1A\alpha}$ in the telencephalon. Previous studies in fish demonstrate that treatment with the 5-HT$_{1A}$ receptor agonist

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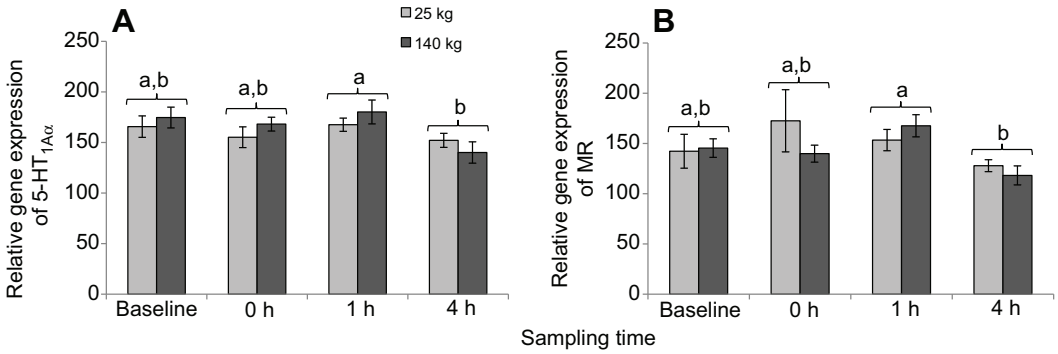

Fig. 3. Effects of acute stress on gene expression. mRNA levels of (A) 5-HT$_{1Aα}$ (5-HT 1Aα receptor) and (B) MR (mineralocorticoid receptor) in relation to three reference genes in rainbow trout held at 25 kg m^{-3} or 140 kg m^{-3} 1 h before (baseline) or 0, 1 and 4 h after being exposed to an acute stressor. Acute stress was induced by dropping water levels in the tanks and holding the fish for 30 min at an approximate density of 500 kg m^{-3}. Different letters (a,b) indicate significant differences (P<0.05), which are independent of holding densities. See Results and Table 2 for complete statistics from the two-way ANOVA with densities and sampling time as independent variables. Values are means±s.e.m. and n=11 at each sampling time and density.

8-hydroxy-2-(di-*N*-propylamino)-tetralin (8-OHDPAT) results in elevated plasma cortisol levels, suggesting a stimulatory role of this receptor on HPI-axis activity (Winberg et al., 1997; Höglund et al., 2001; Medeiros et al., 2010). Moreover, Medeiros and McDonald (2013) demonstrated decreased expression of the 5-HT$_{1A}$ receptor gene in the brain in cortisol-treated Gulf toadfish (*Opsanus beta*), indicating that this receptor is under negative feedback of cortisol. Our results showed a reduction in 5-HT$_{1Aα}$ receptor mRNA level at 1–4 h post-stress. Taken together with the aforementioned studies, this suggests that 5-HT$_{1Aα}$ is under feedback control of cortisol and takes part in the downregulation of the HPI axis in teleosts. This is consistent with what has been reported in mammals, where transcription of the 5-HT$_{1A}$ receptor gene is negatively regulated by corticosteroids (review by Lanfumey et al., 2008). Furthermore, in mammals, this effect of glucocorticoids seems to target the limbic system, which plays a pivotal role in the regulation of the HPA axis (see review by Jankord and Herman, 2008). Moreover, the downregulation of the MR mRNA levels, observed in this study, is in accordance with a study performed by Johansen and coworkers (2011), in which 2 h of confinement induced a downregulation of MR expression in the telencephalon in rainbow trout, and it was suggested that this autoregulation of MR reflected limbic functions of the telencephalon. Potentially, studies of regional 5-HT neurochemistry and mRNA levels of 5-HT$_{1Aα}$ and MR will shed light on the functional integrity of the teleost telencephalon and its involvement in regulation of the HPI axis.

Table 2. Effects of acute stress and being held at different densities for 28 days

	Density	Time	Density×Time
Cortisol	**$F_{1,79}$=7.60, P=0.01**	**$F_{3,79}$=83.0, P=0.00**	$F_{3,79}$=0.81, P=0.49
5-HIAA/5-HT	**$F_{1,79}$=12.6, P=0.00**	**$F_{3,79}$=32.2, P=0.00**	$F_{3,79}$=2.49, P=0.07
5-HIAA	**$F_{1,79}$=10.1, P=0.00**	**$F_{3,79}$=33.4, P=0.00**	$F_{3,79}$=0.81, P=0.49
5-HT	$F_{1,79}$=0.10, P=0.71	**$F_{3,79}$=3.80, P=0.01**	$F_{3,79}$=0.50, P=0.66
5-HT$_{1Aα}$	$F_{1,80}$=0.72, P=0.40	**$F_{3,80}$=3.43, P=0.02**	$F_{3,80}$=0.80, P=0.50
5-HT$_{1Aβ}$	$F_{1,80}$=0.05, P=0.82	$F_{3,80}$=2.23, P=0.09	$F_{3,80}$=0.50, P=0.68
CRF	$F_{1,80}$=0.79, P=0.38	$F_{3,80}$=0.53, P=0.66	$F_{3,80}$=0.75, P=0.53
CRF-BP	$F_{1,80}$=0.00, P=0.99	$F_{3,80}$=0.53, P=0.66	$F_{1,80}$=0.70, P=0.56
CRFR1	$F_{1,80}$=0.01, P=0.94	$F_{3,80}$=1.08, P=0.36	$F_{3,80}$=0.06, P=0.98
CRFR2	$F_{1,79}$=0.49, P=0.49	$F_{3,79}$=1.21, P=0.31	$F_{3,79}$=2.12, P=0.11
GR1	$F_{1,80}$=1.10, P=0.30	$F_{3,80}$=0.68, P=0.57	$F_{3,80}$=0.52, P=0.67
GR2	$F_{(1,79)}$=0.00, P=0.98	$F_{3,79}$=0.52, P=0.67	$F_{3,79}$=2.13, P=0.10
MR	$F_{(1,80)}$=0.04, P=0.84	**$F_{3,80}$=3.14, P=0.03**	$F_{3,80}$=0.69, P=0.56

The acute stress was induced by the water levels being dropped in the tanks and the fish held for 30 min at an approximate density of 500 kg m^{-3}. Significant effects (P<0.05) in the two-way ANOVA are marked with bold. 5-HIAA, 5-hydroxyindoleacetic acid; 5-HT, 5-hydroxytryptamine; 5-HT$_{1Aα}$, 5-HT$_{1Aα}$ receptor; 5-HT$_{1Aβ}$, 5-HT$_{1Aβ}$ receptor; CRF, corticotropin-releasing factor; CRF-BP, corticotropin-releasing factor binding protein; CRFR1, corticotropin-releasing factor receptor 1; CRFR2, corticotropin-releasing factor receptor 2; GR1, glucocorticoid receptor 1; GR2, glucocorticoid receptor 2; MR, mineralcorticoid receptor.

The effect of acute stress was not reflected in alterations of 5-HT$_{1Aβ}$ mRNA levels in our study. Our results are coherent with a study performed by Thörnqvist et al. (2015) showing that 1 h of confinement resulted in elevated mRNA levels of 5-HT$_{1Aα}$ but had no effect on mRNA levels of 5-HT$_{1Aβ}$ in the forebrain (the hypothalamus and telencephalon together) of Atlantic salmon (*Salmo salar*). These two 5-HT$_{1A}$ receptor subtypes are paralogous and a result of genome duplication early in teleost evolution. They show different expression patterns in the brain (Lillesaar, 2011) but whether they have different functions is currently not known. The results from this study and the study performed by Thörnqvist et al. (2015) suggest a specific role of 5-HT$_{1Aα}$ in regulation of the HPI axis.

Effects of acute stress on GR mRNA levels in the telencephalon were not detected in this study. This is in accordance with previous studies showing that exposure to acute stressors did not alter GR transcript abundance in the teleost brain (Alderman et al., 2012; Alderman and Vijayan, 2012). Because cortisol is the main glucocorticoid and binds to both MRs and GRs in teleosts (Chester Jones et al., 1980), one might expect that acute stress would affect mRNA levels of both these receptors. However, in rainbow trout, it has been shown that MRs have a higher affinity to cortisol than GRs (Bury and Sturm, 2007; Greenwood et al., 2003; Sturm et al., 2005). According to this, GR mRNA expression seems

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Table 3. Relative gene expression values in the telencephalon and density and sampling time 1 h before (baseline) and 0, 1 and 4 h after an acute stressor in rainbow trout kept at low and high density

	Baseline		0 h post-stress		1 h post-stress		4 h post-stress	
	25 kg m^{-3}	140 kg m^{-3}	25 kg m^{-3}	140 kg m^{-3}	25 kg m^{-3}	140 kg m^{-3}	25 kg m^{-3}	140 kg m^{-3}
5-$HT_{1A\alpha}$	166±11	175±10	155±10	168±6.8	168±6.5	180±12	152±6.9	140±11
5-$HT_{1A\beta}$	171±10	184±14	204±20	205±13	207±19	221±14	197±17	178±17
CRF	134±15	177±23	159±19	163±12	152±16	190±21	160±9.3	163±37
CRFR1	149±27	142±18	136±21	139±20	129±21	128±22	170±19	147±15
CRFR2	197±19	183±14	188±12	224±14	162±13	205±18	200±28	169±18
CRF-BP	169±25	191±29	211±41	192±29	185±28	222±25	193±33	151±32
GR1	165±16	166±6.5	179±15	180±12	166±10	188±5.5	177±12	171±6.7
GR2	177±25	153±14	137±18	164±14	131±13	160±17	167±14	137±27
MR	142±17	145±9.2	173±31	140±8.5	153±11	168±11	128±5.9	118±9.5

The acute stress was induced by the water levels being dropped in the tanks and the fish held for 30 min at an approximate density of 500 kg m^{-3} before returning to previous levels. n=11. 5-$HT_{1A\alpha}$, 5-$HT_{1A\alpha}$ receptor; 5-$HT_{1A\beta}$, 5-$HT_{1A\beta}$ receptor; CRF, corticotropin-releasing factor; CRF-BP, corticotropin-releasing factor binding protein; CRFR1, corticotropin-releasing factor receptor 1; CRFR2, corticotropin-releasing factor receptor 2; GR1, glucocorticoid receptor 1; GR2, glucocorticoid receptor 2; MR, mineralcorticoid receptor.

to be affected by more intense prolonged stress than does MR (Stolte et al., 2008), and it is possible that a longer period of intense crowding than applied in this study would have affected GR levels. Furthermore, we could not detect any effects of acute crowding stress on the expression of genes encoding for proteins involved in CRF transmission (CRF, CRFR1, CRFR2 and CRF-BP). Previous studies in rainbow trout demonstrate an increase in preoptic area (POA) CRF mRNA levels in response to stress (Bernier and Craig, 2005; Doyon et al., 2003, 2005). In mammals, limbic CRF and related peptides play an important role in stress coping; for example, glucocorticoids increase CRF mRNA expression in the central nucleus of the amygdala (Brunson et al., 2001). *In situ* hybridization studies of telencephalic mRNA expression patterns in zebrafish indicate similar limbic functions of the CRF system in teleost fishes (Alderman and Bernier, 2007). Still, relatively few studies have examined the effect of acute stress on telencephalic CRF signaling in teleosts. The results from our study show that acute stress does not affect the gross production of telencephalic CRF mRNA, accentuating the need for investigating effects of stress on CRF signaling in regions with limbic functions in the teleost telencephalon. In addition, there are investigations suggesting effects of acute stress on genes encoding for proteins involved in CRF, cortisol and 5-HT signaling outside the time span of our study. For example, a tendency for increased POA CRF mRNA appeared 8 h post-stress (Sopinka et al., 2016) and Alderman and Vijayan (2012) reported elevated MR mRNA levels in the POA 24 h after an acute stressor. Taken together, this suggests that further studies, including a wider time span and regional differences, are needed to clarify the telencephalic mechanisms involved in the acute stress response.

Effects of stocking densities

Previous studies in rainbow trout and gilt-head sea bream demonstrate that being held at high densities results in decreased HPI-axis reactivity following exposure to an acute stressor (McKenzie et al., 2012; Barton et al., 2005). This is in accordance with our study, showing generally lower plasma cortisol levels in fish held in the highest density for 28 days when exposed to an acute stressor. Madaro et al. (2015) reported a similar suppression of HPI-axis reactivity in salmon exposed to repeated unpredictable chronic stress, and they suggested that this type of change was related to increased allostatic load. However, allostatic load may affect HPA/HPI activity in different ways, including decreased resilience and hyperreactivity (Korte et al., 2007; McEwen, 1998). In this scenario, the higher cortisol levels induced by acute crowding stress in fish held at low density might be an effect related to chronic stress. The fact that indications of a higher level of aggressive behavior, such as elevated oxygen consumption and scale loss, have been observed in densities of 25 kg m^{-3} (Laursen et al., 2015) might put some support to increased allostatic load in fish kept at low densities. Still, our study demonstrates that, generally, lower plasma cortisol levels in trout held at the highest density are associated with reduced 5-HT turnover in the telencephalon. This alludes to mammalian studies that show that chronic stress results in decreased brain serotonergic (5-HTergic) responsiveness and metabolism, which have been suggested to indicate allostatic load (reviewed by Beauchaine et al., 2011). Taken together, this suggests that reduced HPI-axis reactivity and changes in telencephalic 5-HT production and/or metabolisms are associated with allostatic load in fish.

Generally, chronic-stress-induced lowering of HPI-axis reactivity seems to be associated with decreased GR1 and GR2 mRNA levels in the POA (Madaro et al., 2015). Furthermore, studies in mammals show that these receptors are involved in allostatic processes. For example, the neurodegenerative effects of glucocorticoids in limbic structures are mediated by GRs (for references, see Zhang et al., 2006). In line with this, Madaro et al. (2015) suggested that the suppressive effect of chronic stress on GR receptors in teleosts was related to feedback mechanisms protecting against the apoptotic effects of cortisol. Moreover, in mammals, the transcription of the 5-HT_{1A} receptor gene is negatively regulated by glucocorticoids in the limbic system, an effect that is mediated both by MRs and GRs (Lanfumey et al., 2008). Interestingly, stimulation of 5-HT_{1A} has been associated with increased neurogenesis in limbic structures (Dranovsky and Hen, 2006). Moreover, the positive relationship between stress coping ability, learning and hippocampal neurogenesis puts further support to a central role of 5-HT in allostatic processes (Lyons et al., 2010). In addition, effects on 5-$HT1_A$ and GR mRNA levels of allostatic load and chronic stress have been associated with increased levels of CRF mRNA in limbic structures of mammals (Fuchs and Flügge, 2003). In alignment with this, chronic stress has been shown to increase CRF mRNA levels in the POA (Chakravarty et al., 2013; Piato et al., 2011; Doyon et al., 2005).

In view of the above studies, our result showing no effect of crowding on mRNA levels in the telencephalon of MR, GR or 5-HT_{1A}, or on expression of genes encoding for proteins involved in CRF transmission, may seem surprising. However, it is important to bear in mind that this study did not include regional differences in

gene expression and neurochemistry. One reason why the lowered cortisol levels observed in fish kept at high densities was only reflected in 5-HT turnover and metabolism could be that this neurotransmitter also acts as a neuromodulator, diffusing through large areas of the nervous system. Further studies, of neurochemistry and gene expression in regions with limbic functions, may reveal important functions of proteins involved in cortisol, 5-HT and CRF transmission in allostatic processes in fish. Moreover, it has been shown that chronic social stress resulted in decreased ACTH-stimulated cortisol production in rainbow trout (Jeffrey et al., 2014), accentuating that effects downstream of the pituitary also have to be included when investigating allostatic processes in fish.

Conclusions

In this study, we report increased plasma cortisol levels and 5-HT turnover in the telencephalon 0–1 h after an acute stressor. At 4 h post-stress, these measurements returned back to basal levels. At this time point, 5-$HT_{1A\alpha}$ and MR mRNA levels in the telencephalon showed lower levels compared with at 1 h post-stress. This downregulation of mRNA indicates that 5-$HT_{1A\alpha}$ and MR are under feedback control and take part in post-stress HPI-axis downregulation in fish.

In addition, we report a desensitization of the HPI axis in rainbow trout held at 140 kg m^{-3}. This desensitization was reflected by lowered 5-HT turnover in the telencephalon and an attenuation of the cortisol response to a crowding stressor. Together with results of previous studies on fish and mammals, this suggests that high holding densities induce chronic stress and allostatic load in rainbow trout. A desensitization of the stress response induced by allostatic load may lead to poor animal welfare, preventing fish from mounting an adequate cortisol response when confronted with unavoidable stressors, such as size grading and transport in aquaculture. Furthermore, this emphasizes the importance of controlled challenges, to activate the stress axis, when using physiological stress parameters for investigating the welfare status of animals.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

E.H. designed the experiment; D.C.L., E.H., M.M., M.Å.A. and P.-O.T. performed the experiments and laboratory work; D.C.L., E.H., M.Å.A., P.-O.T. and S.W. participated in writing and revising the manuscript; M.M. participated in planning the experiment, data analysis and drafted the manuscript.

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