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# Uncontrollable chronic stress reduces growth disparities in farmed Atlantic salmon

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## Abstract

Individual variation in behavior and physiological traits in a wide variety of animals has been the focus of numerous studies in recent years. In this context, early life experiences shape responses that individuals have to subsequent environments, *i.e.* developmental plasticity. In this experiment, we subjected 10-month old fish to an unpredictable chronic stress (UCS) regime or no stress (control) for 3 weeks. These individuals then underwent the parr-smolt transformation, when salmonids become adapted for the seawater environment, and were subsequently transferred into seawater before the final sampling. Biometric data was collected at the end of each period. Sampling on the final day was conducted in order to analyze basal monoaminergic activity in the brain stem and hypothalamus, as well as gene expression of target genes in the telencephalon. We found that post-hoc sorting of individuals by their serotonergic activity (high and low) resulted in the elucidation of growth and gene expression differences. UCS groups were found to have less growth disparities throughout the experiment, compared to control fish. Furthermore, we found brain serotonergic signaling and corticotropic releasing factor binding protein expression were positively associated with brain stem serotonergic activity, which is consistent with fish showing a stress reactivity neurophysiological profile. In conclusion, we here submit evidence that sorting individuals by their basal serotonergic activity levels may be a useful tool in the study of developmental plasticity. These results may thus apply directly to improving husbandry practices in aquaculture and elucidating neural mechanisms for coping behavior.

**Keywords:** stress, serotonin, coping style, SGR, developmental plasticity

## Introduction

Individual variation in behavior and physiological traits in a wide variety of animals has been the focus of numerous studies in recent years [1-4]. The term coping style characterizes a group of individuals that express consistent physiological and behavioral responses to stressful stimuli [3]. Much of the research leading to the characterization of the proactive and reactive coping styles in fish has been based upon the rainbow trout (*Oncorhynchus mykiss*) post-stress cortisol selected lines [5]. Notably, selection by post-stress cortisol levels is consistently proportional with the serotonergic system's reactivity. That is, while proactive (*i.e.* bold/aggressive) consistently exhibit low serotonergic activity, reactive (*i.e.* shy) have enhanced serotonergic responsiveness [6, 7]. The serotonergic system is phylogenetically ancient and anatomically well conserved across species [7]. The neurotransmitter serotonin (5-hydroxytryptamine; 5-HT) has been associated with energy regulation, neural plasticity, stress regulation and behavioral/emotional control [8-11]. Therefore, serotonergic reactivity has been crucial in the study of individual differences in stress responses. Interestingly, even though there is compelling evidence that early life experiences, particularly stress, shape how individuals cope with their present and future environments [12-14], this is not often taken into account when studying individual variation in animals. Even though there are consistent differences reported within individuals comprising a population, there is still a lack of consensus regarding the consistency of a given individual response throughout different contexts and across time [2]. For example, proactive individuals are often characterized as responding in an active and aggressive way, even when it appears to not be adaptive [15]. However, behavioral traits are not as fixed as once proposed, but highly dynamic and animals exhibit a series of plastic responses to stimuli that are based upon external and internal cues. For example, Ruiz-Gomez et al. [16] reported that reactive trout adopted a more proactive style for up to one year when they experienced a decrease in body fat after transport. This plasticity in behavioral outputs to exogenous stimuli may involve immediate responses (contextual plasticity) or it may involve responses shaped by past events (developmental plasticity) [2, 17]. Therefore, it is not always possible to find consistent responses across different situations. Importantly, since behavioral responses are regulated by physiological systems, it is fundamental to study differences in physiological traits. In this context, it has been particularly useful to apply strong artificial selection of extreme values of a given physiological trait in order to elucidate relationships between individual behavioral responses and physiological regulation [6, 18-22].

Here we explore the effect of an unpredictable chronic stress regime (UCS) on Atlantic salmon performance and physiology through different early life stages compared to control fish. Furthermore, via the post hoc sorting of individuals by their high (H) and low (L) serotonergic activity, we explore the regulation of gene expression of target serotonergic, neural plasticity and corticotropic genes. We hypothesize that long-term developmental plasticity of growth and neural responses to an early life stress regime will be elucidated in terms of the fish's basal brain serotonergic activity. We collected data at several time-points during development and analyzed the final monoamine neurochemistry in the hypothalamus and brain stem (which contain the main serotonergic nuclei innervating the brain [23]). In addition, we studied the telencephalic gene expression of the aforementioned genes, since this area has been associated with the top-down regulation of the serotonergic stress response [24, 25].

## Methods

### Ethics statement

This work was conducted in accordance with the laws and regulations controlling experiments and procedures on live animals in Norway and was approved by the Norwegian Animal Research Authority (NARA), following the Norwegian Regulation on Animal Experimentation of 1996.

### Experimental animals and facilities.

Atlantic salmon eggs (Aqua Gen strain, Aqua Gen AS, Trondheim, Norway) were hatched and reared at the Institute of Marine Research (IMR), Matre, Norway. Experimental fish were kept in 10000 L outdoor tanks under natural conditions (9°C). A month before the start of the experiment, 1110 fish (approx. 10 months old) were transferred into 9 indoor tanks (400 L; density: 7 kg fish /tank) supplied with flow-through freshwater. Fish were kept at 12 °C on a 12:12 photoperiod with a water flow of 15 L/min which provided an approximately a 92% oxygen saturation. Fish were fed with dry pellets (2 mm Skretting Nutra Olympic, Stavanger, Norway) distributed *ad libitum* three times a day with automatic feeders (Arvo-tec feeding units: Arvo-Tec T drum 2000. Huutokoski, Finland). Tank conditions were monitored and regulated by a fully automated system (SD Matre, Normatic AS, Nordfjordeid, Norway).

### Experimental procedure

At the beginning of the experiment, tank groups were randomly assigned to one of 2 treatments (3 replicates/treatment, 124 fish per tank), unpredictable chronic stress (UCS) or no stress (control). The UCS treatment consisted of stressing fish three times per day (at 8:30, 13:00, and 17:00) using 8 different stressors in a random and unpredictable order throughout the week for a total of 3 weeks, following the protocol previously described in Madaro et al. [26] and Vindas et al. [27]. Control fish were only subjected to routine practices of tank maintenance, but otherwise left undisturbed. The 3 feedings/day were maintained throughout the experiment starting approximately one hour after the stressors. Importantly, throughout this period fish were sequentially sampled terminally ( $n = 50$ ) in order to quantify their stress response through this period. These data were previously reported by Madaro et al. [26]. At the end of the stress regime, all fish were mildly sedated in metacaine (25 mg/L, Fiquel@vet, ScanAqua AS, Årnes, Norway, buffered with 25 mg/L sodium bicarbonate) and fork length and body weight recorded (Sampling 1). The remaining fish were individually tagged with a PIT-tag inserted into the abdominal cavity for individual recognition and distributed into two tanks per treatment (111 fish; 7kg/tank). The fish then underwent light controlled parr-smolt transformation (6 weeks L:D 24:0) At the end of this period all fish were mildly sedated, measured and weighed (sampling 2). To maintain a density of 7 kg/tank, the groups were reduced to 74 fish per tank. At this point, the water flow was switched into full strength seawater (35 ppt.) for a period of 4 weeks before the final sampling (sampling 3).

### Final sampling protocol

During the final sampling (sample 3) a total of 60 fish were sampled directly from holding tanks and immediately killed with an overdose of MS-222 (1 g/L) which rendered them completely motionless (no opercular movement) within 10 s of immersion. Fish were rapidly

weighed, fork length measured and decapitated for brain dissection. The brain stem, hypothalamus and telencephalon were quickly excised within 2 min, snap-frozen in liquid nitrogen and stored at -80 °C for later analysis.

### The specific growth rate (SGR)

The percent of body weight gain per day (standardized growth into % body mass per time unit) may be studied by calculating the SGR which allows for comparison of growth rate and fish weight in a linear manner by correcting for fish size effects (although it needs to be considered that small fish grow faster in % of body mass). This is done by using the formula (1):

$$\text{SGR} = \left[ \frac{(\ln W_2 - \ln W_1)}{(t_2 - t_1)} \right] \times 100 \quad (1)$$

where  $W_1$  and  $W_2$  are the Mass (g) at the start ( $t_1$ ) and end ( $t_2$ ) of the specific growth period of interest [28].

### Serotonergic neurochemistry

Frozen brain stems and hypothalamus were analyzed by means of high-performance liquid chromatography (HPLC) as described by Vindas et al [27].

### Gene expression analysis

Total RNA was extracted from the telencephalon using TRIzol® reagent. All RNA concentrations were assessed using a NanoDrop® ND-1000 UV-Vis Spectrophotometer (NanoDrop Technologies, Rockland, DE, USA). The RNA quality was determined from RNA integrity numbers (RINs) calculated by a 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA). A RIN equal or above eight confirmed excellent RNA quality. First strand cDNA was synthesized from 1260 ng/μl DNase I (DNA-free™ Kit, Ambion Applied Biosystems)-treated total RNA using Superscript III reverse transcriptase (Invitrogen, Carlsbad, CA, USA) with oligo dT12–18 primers synthesized by Invitrogen.

Several of our target genes have previously been sequenced in Atlantic salmon and were retrieved using NCBI ([www.ncbi.nlm.nih.gov/](http://www.ncbi.nlm.nih.gov/); accession numbers are given in Table 1). Gene specific primers for Atlantic salmon for the remaining interest genes were designed using the web-based Primer3 program ([http://frodo.wi.mit.edu/cgi-bin/primer3/primer3\\_www.cgi](http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi)), and synthesized by Invitrogen. A minimum of four primer pairs were designed at exon junctions for each gene and the primers showing the lowest Cq values and a single peak melting curve were chosen and are listed in Supplementary Table S1. The qPCR products were also sequenced to verify that the primers amplified the right cDNA. The qPCRs were carried out using a Roche LC480 light cycler® (Roche Diagnostics, Penzberg, Germany). Reactions were 10 μL and included Light cycler® 480 SYBR Green I Master (Roche diagnostics GmbH, Mannheim, Germany), primers (5 μM) and cDNA. Cycling conditions were as follows: 10 min at 95 °C, then 42 cycles of 10 s at 95 °C, 10 s at 60 °C and 10 s at 72 °C followed by melting curve analysis. All reactions were run in duplicates and controls without DNA template were included to verify the absence of cDNA contamination. Individual crossing points (Cq) and priming efficiency were calculated for each qPCR reaction using LinRegPCR software (version 11.30.0) [29]. All Cq values ≥ 40 were eliminated since such high numbers imply low efficiency. Furthermore, all Cq values

above 35 were rejected based upon comparison between the Cq of the lowest concentration unknown and non-template controls, following procedures described by Bustin et al. [30]. All 3 reference genes were used to calculate a geometric average in order to accurately normalize the relative gene expression data following method by Vandesompele et al. [31].

### Statistical analyses

The R program v. 3.3.2 (R Development Core Team, <http://www.r-project.org>) and the statistical packages ‘nlme’ and ‘MuMIn’ were used for exploratory linear models (LM) and linear mixed effect models (LME). Body size was analyzed using an LME, with body weight as the dependent variable, treatment (control vs UCS) as a categorical independent variable, time (days) and brain stem serotonergic activity as continuous independent variables, and fish identification as the random effect. Weight data were missing from 6 control fish at sampling time 2 (4 low and 2 high responders); therefore, these individuals were not included in the growth and body size analysis. A similar LM was used to assess SGR, but following preliminary analysis, fish was not included as a random effect as in the SGR model (see supplementary Tables S2, S3 and S4). Here, it is noted that UCS fish weighed less than controls at the beginning of the individual growth trial. It is well established that smaller fish have a higher SGR than larger fish. To control for this, we included body mass at the start of the experiment as a main effect in the initial SGR model. Linear models were used for all monoaminergic neurochemistry and gene expression data, with treatment (control vs UCS) as a categorical independent variable and brain stem serotonergic activity as a continuous independent variable.

The initial LME/LM models allowed the independent variables *treatment* × *time* × *brain stem serotonergic activity* for body size/growth data or *treatment* × *brain stem serotonergic activity* for neurochemistry and gene expression to interact. However, the final model was selected based on a comparison of all possible model combinations, with the final model being the one with the lowest Akaike information criterion (AICc) score, *i.e.* the model with the best data fit (see supplementary table 2 for the final models). When significant interaction effects were observed, type III sum of squares were used to assess the main effects and the contrast values were used to identify effects within sampling time for growth/body size data, or treatment/brain stem serotonergic activity for brain neurochemistry and gene expression. An examination of the residual plots made sure that there were no systemic errors within the residuals of the final models. Here, we are aware that models with a delta value within 2 of the model with the lowest AICc score are considered to be of a similar fit as the model with the lowest AICc score and can be averaged. However, there is no defined practice for averaging models that contain interactions [32]. Therefore, we provide model average results for the benefit of the reader, but we report only the models with the lowest AICc score in the results. Body weight was natural log transformed prior to analysis, whereas the serotonin transporter (*5-HTT*) and *crf* data were natural log transformed +1 to improve data fit as judged by examination of the residual plots of the initial models. Initial exploratory statistics were used to assess all models with the addition of tank as a categorical independent variable, but tank was not found to influence any endpoint and was therefore excluded from the final analysis (see supplementary material). Significance was assigned at  $p < 0.05$ .

### Results

#### Selection of high and low serotonergic activity individuals (*i.e.* [5-HIAA]/[5-HT] ratios)

We used brain stem serotonergic activity at basal conditions as a proxy for high (H) and low (L) groups within treatment groups. From a total of 30 individuals we selected the fish exhibiting the highest and lowest ratios in each treatment ( $n = 10$  per coping style/ treatment, Fig 1A & 1B). We compared brain stem serotonergic activity between treatment groups and found no effect of UCS vs controls (LM, ss (type II) = 0.003,  $df = 1$ ,  $F = 0.77$ ,  $p = 0.384$ ). Interestingly, we found hypothalamic serotonergic activity was positively associated with brain stem activity, and UCS treatment led to a general increase in serotonergic activity (Fig 2).

### Telencephalic mRNA gene expression

We found a significant positive association between brain stem serotonergic activity and 5-*HT*<sub>1A $\beta$</sub>  (Fig 3A) and *crf**bp* (Fig 4A) mRNA expression. For *bdnf*, there was a significant interaction effect, whereby there was a positive association with brain stem serotonergic activity in controls, but a negative association in UCS fish (Fig 4B). We found an increase in 5-HTT expression following UCS treatment (Fig 3B), but no other treatment effects were found in any of the other studied genes (Supplementary Figs 1S and 2S).

### Mass and SGR

Fish groups did not differ in body mass ( $t_{(28)} = -1.39$ ,  $p = 0.18$ ; mean:  $63 \pm 1$  and  $63 \pm 2$  for UCS and control, respectively) at the start of the experiment (sample 0). For further details please refer to Vindas et al. [27]. There was a significant interaction between treatment and time on body mass, as UCS were smaller than controls, but this difference decreased over time (LME,  $\text{Chisq} = 15.7$ ,  $df = 1$ ,  $p < 0.001$ ). Brain stem serotonergic activity was also negatively associated with body mass (LME,  $\text{Chisq} = 4.86$ ,  $df = 1$ ,  $p = 0.027$ ). There was a significant interaction between brain stem serotonergic activity, treatment, and time on SGR. Here, SGR for control fish was positively associated with brain stem serotonergic activity at time 2, but the association was negative at time 3. In contrast, brain stem serotonergic activity was not associated with SGR in UCS fish at either time point (Fig 5).

### Discussion

We studied the effects of early life stress on growth, neurochemistry, and telencephalic gene expression in farmed Atlantic salmon. Of most interest, we found significant associations in growth rates with brain stem serotonergic activity throughout the experiment in control fish, but not in UCS groups, which suggest a more dynamic regulation of growth rates in control groups. Furthermore, there was a positive association between hypothalamic and brain stem 5-HT ratios, which shows that hypothalamic serotonergic activity follows the same pattern of activation as that of the brain stem. In addition, we found positive associations between telencephalic gene expression of the 5-*HT*<sub>1A $\beta$</sub>  receptor and corticotropin releasing factor binding protein (*crf**bp*) abundance and brain stem serotonergic activity, which is indicative of a reactive neuroendocrine profile in fish showing high serotonergic activity.

The main serotonergic nuclei, the raphe, are located in the brain stem [23], therefore serotonergic activity in this area has been commonly used as a proxy of total brain 5-HT activity [11, 33, 34]. In this context, proactive fish have been found to have lower serotonergic levels than reactive individuals [7]. We used basal levels of brain stem serotonergic activity (measured as the catabolite to neurotransmitter ratio; 5-HIAA/5-HT



[35]) to sort the highest (H) and lowest (L) groups within each treatment in order to establish an indicator of individual differences, similar to what has been done in other studies [2, 36]. Interestingly, we found in all fish a positive association between brain stem and hypothalamic serotonergic activity, which shows that these two brain areas have the same activation pattern regarding serotonergic neurochemistry. This, in turn, corroborates the robustness of our sorting method since often brain regions are highly dynamic and often show different activation patterns, as we have previously reported in salmon [37]. Furthermore, by sorting fish in this manner, we were able to indirectly infer previous growth differences related to long-term effects of stress conditions. That is, there were marked treatment-related differences relating to serotonergic activity in the controls, but not in UCS fish. We found that in the control group, serotonergic activity was positively associated with growth rate after smoltification, but negatively associated during seawater transfer. No association between growth rates and serotonergic activity were found in UCS fish. These associations suggest a higher growth discrepancy within the population in control groups. Here, it is noted that UCS treated fish were smaller at the end of the stress regime period and may therefore have expressed compensatory growth. Although we corrected for the initial differences in body mass in the statistical model, we cannot exclude any possible confounding effect of growth history on brain stem serotonergic activity. Nevertheless, classically, low serotonergic responding fish have been associated with a proactive coping style [7], and these individuals are characterized as bolder, more aggressive, and dominant. Therefore, proactive fish have been proposed to perform better in environments with high competition for resources [3, 36, 38, 39], such as those usually encountered in aquaculture environments, particularly during the seawater period where food distribution promotes competition over areas closest to the middle and the water surface of the seacages [40]. Our results are certainly in agreement with this literature, particularly in the control group at the end of the seawater period, where the growth rate was the highest in the fish with the lowest serotonergic activity. However, we found that UCS treated fish do not show the same pattern as control groups, in fact, all UCS fish show less growth disparities and this exemplifies how important it is to consider previous environmental conditions experienced by animals when studying group differences to environmental stimuli. Notably, since it is not ideal to have high growth disparity within the population, the growth in the UCS group highlights some of the possible benefits that early life stress may confer in artificial environments. That is, although speculative, it is possible that the stress treatment may decrease the growth rate of proactive fish throughout their lives, while the more reactive fish may cope better with this unpredictability early in life and therefore grow at a similar rate than proactive fish. Importantly, we found that UCS fish were smaller than control fish at sampling 1, after the stress regime. This size difference is a direct result of the stress regime, as discussed in Vindas et al [27]. Unfortunately, due to logistical reasons fish were not individually tagged before the initiation of the stress regime and we were, therefore, unable to include this time point within our statistical analysis. However, we found no general significant differences in mass between fish tanks assigned to control and UCS treatments at the start of the experiment (Sampling 0).

The  $5\text{-HT}_{1A}$  receptor is both a somatic autoreceptor and a postsynaptic heteroreceptor, which has been highly associated with alterations in mood and emotions [9]. However, since there are no serotonergic nuclei in the fish's telencephalon [23], this implies that the  $5\text{-HT}_{1A}$  mRNA expression obtained in our experiment is indicative of the heteroreceptor abundance in this brain area. Importantly, we only found significant effects in the  $5\text{-HT}_{1A\beta}$  abundance

and not of its paralog  $5-HT_{1A\alpha}$ . Since duplicate genes may exhibit different functions [41], it is possible that in the case of salmonid fish the  $5-HT_{1A\beta}$  is more involved in stress regulation and individual differences, as evidenced from several recent experiments in our lab ([37] and Riise et al. unpublished). We found that low expression of both the  $5-HT_{1A\beta}$  receptor the 5-HT transporter genes are indicative of an overall lower serotonergic activity, as evidenced by the serotonergic activity ratios in the brain stem and hypothalamus. Interestingly, in mammals, low postsynaptic 5-HT<sub>1A</sub> levels in telencephalic areas (particularly in the telencephalon and amygdala) have been associated with non-human-specific anxious behavior (hereafter referred to as anxiety/anxious behavior) [9, 42]. However, in our experiment we did not observe a downregulation of  $5-HT_{1A\beta}$  to the stress regime, in fact there are no significant differences between control and UCS groups. Instead we found that lower  $5-HT_{1A\beta}$  abundance characterized fish with an overall lower serotonergic activity at least at basal conditions. It would be interesting to analyze this relationship post-stress, to see what is the general response in serotonergic signaling under these conditions. In addition, region-specific studies of telencephalic 5-HT<sub>1A</sub> regulation need to be conducted in salmonid fishes in order to corroborate the role of serotonergic signaling in stress and anxiety in more specific telencephalic neuronal populations, particularly since neural gene expression in individuals exhibiting opposite coping styles, has been shown to be highly dynamic exhibiting rapid region-specific changes to stress (e.g. [37, 43]).

The signaling molecules CRF and CRFBP are important in the regulation of stress, appetite and modulation of the immune response [44, 45]. Specifically, in telencephalic areas CRF mediates anxious behavior, increased arousal and altered locomotor activity [44, 46]. We found a positive association between serotonergic activity and *crfbp* mRNA expression. The biological effect of CRF is mediated through its receptors (CRF<sub>1</sub> and CRF<sub>2</sub>) and its binding protein [47]. Specifically, CRFBP has been proposed to be involved in the negative feedback of CRF signaling, since it inhibits the CRF activation of both its receptor isoforms. Notably, the magnitude of this receptor inhibition is receptor and isoform-specific. Therefore, cell populations exhibiting only one type of receptor isoform may be selectively inhibited by CRFBP, which provides a very localized regulation of CRF signaling [47]. On the other hand, other functions of CRFBP have been proposed in addition to CRF sequestering, such as CRF<sub>2</sub> potentiating by an accessory protein /escort protein action [48]. The exact interpretation for increased CRFBP will thus depend on affinity and relative concentration of both CRFBP and specific receptor subtypes. Conceivably, the increased expression of *crfbp* in fish showing high serotonergic activity could be part of a mechanism aimed at attenuating the release of cortisol, although in order to conclude further on this matter it would be necessary to obtain information on CRF receptor expression as well as protein levels in the hypothalamus and pituitary, since they play a key role on CRF activity and regulation [44, 45].

Curiously, we found that there were opposite association patterns between serotonergic activity and the brain derived neurotrophic factor (*bdnf*) mRNA abundance in control (positive) and UCS (negative) groups. The negative regulation of *bdnf* in UCS fish suggests that the stress regime may have long-term consequences on learning and neural plasticity, since *bdnf* is highly associated with promoting neurogenesis, cell survival, and the strengthening of learning and memory [49]. However, further research is needed in order to

understand the possible effects of an early life stress regime on learning, memory and neural plasticity.

In conclusion, we here submit evidence that high and low 5-HT activity-based post-hoc sorting of individuals may be a useful tool in the study of long-term effects of early life stress. Grouping fish in this manner allowed us to discern significant differences in neuroendocrine and growth metabolism. Notably, we found that fish exposed to an early unpredictable stressful environment appear to have less variability in their growth throughout several life stages. Furthermore, we found that individuals showing high brain stem serotonergic activity, were also characterized by both enhanced serotonergic signaling in the hypothalamus and the telencephalon (*i.e.* increased  $5-HT_{1A\beta}$  receptor) as well as *crfbp* expression, which is in agreement with a stress reactive neuroendocrine profile. Importantly, this profile should not be interpreted as a possible constraint for these individuals, since this could be a tradeoff to a life history strategy. In other words, the profile exhibited by these fish may yield the highest survival in an unpredictable environment and should therefore convey an advantage, so long as it does not mismatch their current environment [50]. The methodology described in this paper may help promote novel research into the study of phenotypic developmental plasticity not only in fish, but also in other vertebrates. Results from these endeavors may help elucidate vulnerable phenotypes in specific environments and help reduce disease, mortality and increase welfare.

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### **Competing financial interests**

The authors declare no competing financial interests

## References

- [1] B.J. Ellis, D.F. Bjorklund, Beyond mental health: An evolutionary analysis of development under risky and supportive environmental conditions: An introduction to the special section, *Develop Psychol* 48(3) (2012) 591-597.
- [2] J.A. Stamps, Individual differences in behavioural plasticities, *Biol Rev* (2015) n/a-n/a.
- [3] J.M. Koolhaas, S.M. Korte, S.F. De Boer, B.J. Van Der Vegt, C.G. Van Reenen, H. Hopster, I.C. De Jong, M.A.W. Ruis, H.J. Blokhuis, Coping styles in animals: current status in behavior and stress-physiology, *Neurosci Biobehav Rev* 23(7) (1999) 925-935.
- [4] Ø. Øverli, C. Sørensen, K.G.T. Pulman, T.G. Pottinger, W. Korzan, C.H. Summers, G.E. Nilsson, Evolutionary background for stress-coping styles: Relationships between physiological, behavioral, and cognitive traits in non-mammalian vertebrates, *Neurosci Biobehav Rev* 31(3) (2007) 396-412.
- [5] T.G. Pottinger, A.D. Pickering, M.A. Hurley, Consistency in the stress response of individuals of two strains of rainbow trout, *Oncorhynchus mykiss*, *Aquaculture* 103(3-4) (1992) 275-289.
- [6] Ø. Øverli, T.G. Pottinger, T.R. Carrick, E. Øverli, S. Winberg, Brain monoaminergic activity in rainbow trout selected for high and low stress responsiveness, *Brain Behav Evol* 57(4) (2001) 214-224.
- [7] S. Winberg, P.-O. Thörnqvist, Role of brain serotonin in modulating fish behavior, *Curr Zool* (2016).
- [8] L. Lanfumey, R. Mongeau, C. Cohen-Salmon, M. Hamon, Corticosteroid-serotonin interactions in the neurobiological mechanisms of stress-related disorders, *Neurosci Biobehav Rev* 32(6) (2008) 1174-1184.
- [9] P.W. Andrews, A. Bharwani, K.R. Lee, M. Fox, J.A. Thomson Jr, Is serotonin an upper or a downer? The evolution of the serotonergic system and its role in depression and the antidepressant response, *Neurosci Biobehav Rev* 51 (2015) 164-188.
- [10] C.H. Summers, S. Winberg, Interactions between the neural regulation of stress and aggression, *J Exp Biol* 209(23) (2006) 4581-4589.
- [11] S. Winberg, G.E. Nilsson, Roles of brain monoamine neurotransmitters in agonistic behaviour and stress reactions, with particular reference to fish, *Comp Biochem Physiol* 106(3C) (1993) 597-614.
- [12] W.E. Frankenhuis, M. Del Giudice, When do adaptive developmental mechanisms yield maladaptive outcomes?, *Develop Psychol* 48(3) (2012) 628-642.
- [13] C.K. Ghalambor, J.K. McKay, S.P. Carroll, D.N. Reznick, Adaptive versus non-adaptive phenotypic plasticity and the potential for contemporary adaptation in new environments, *Func Ecol* 21(3) (2007) 394-407.
- [14] C.A. Oomen, H. Soeters, N. Audureau, L. Vermunt, F.N. van Hasselt, E.M.M. Manders, M. Joëls, P.J. Lucassen, H. Krugers, Severe early life stress hampers spatial learning and neurogenesis, but improves hippocampal synaptic plasticity and emotional learning under high-stress conditions in adulthood, *J Neurosci* 30(19) (2010) 6635-6645.
- [15] A. Sih, A. Bell, J.C. Johnson, Behavioral syndromes: an ecological and evolutionary overview, *Trend Ecol Evol* 19(7) (2004) 372-378.
- [16] M.L. Ruiz-Gomez, S. Kittilsen, E. Höglund, F.A. Huntingford, C. Sørensen, T.G. Pottinger, M. Bakken, S. Winberg, W.J. Korzan, Ø. Øverli, Behavioral plasticity in rainbow trout (*Oncorhynchus mykiss*) with divergent coping styles: When doves become hawks, *Horm Behav* 54(4) (2008) 534-538.
- [17] J. Stamps, T.G.G. Groothuis, The development of animal personality: Relevance, concepts and perspectives, *Biol Rev* 85(2) (2010) 301-325.
- [18] Ø. Øverli, T.G. Pottinger, T.R. Carrick, E. Øverli, S. Winberg, Differences in behaviour between rainbow trout selected for high- and low-stress responsiveness, *J Exp Biol* 205(3) (2002) 391-395.
- [19] Ø. Øverli, C. Sørensen, G.E. Nilsson, Behavioral indicators of stress-coping style in rainbow trout: Do males and females react differently to novelty?, *Physiol Behav* 87(3) (2006) 506-512.

- [20] J. Schjolden, T. Backström, K.G.T. Pulman, T.G. Pottinger, S. Winberg, Divergence in behavioural responses to stress in two strains of rainbow trout (*Oncorhynchus mykiss*) with contrasting stress responsiveness, *Horm Behav* 48(5) (2005) 537-544.
- [21] J. Schjolden, S. Winberg, Genetically Determined Variation in Stress Responsiveness in Rainbow Trout: Behavior and Neurobiology, *Brain Behav Evol* 70 (2007) 227-238.
- [22] I.B. Johansen, C. Sørensen, G.K. Sandvik, G.E. Nilsson, E. Höglund, M. Bakken, Ø. Øverli, Neural plasticity is affected by stress and heritable variation in stress coping style, *Comp Biochem Physiol* 7(2D) (2012) 161-171.
- [23] C. Lilleaar, The serotonergic system in fish, *Journal of Chem Neuroanat* 41(4) (2011) 294-308.
- [24] S. Winberg, A. Nilsson, P. Hylland, V. Söderström, G.E. Nilsson, Serotonin as a regulator of hypothalamic-pituitary-interrenal activity in teleost fish, *Neurosci Lett* 230(2) (1997) 113-116.
- [25] L.R. Medeiros, E.M. Mager, M. Grosell, M.D. McDonald, The serotonin subtype 1A receptor regulates cortisol secretion in the Gulf toadfish, *Opsanus beta*, *Gen Comp Endocrinol* 168(3) (2010) 377-387.
- [26] A. Madaro, R.E. Olsen, T.S. Kristiansen, L.O.E. Ebbesson, T.O. Nilsen, G. Flik, M. Gorissen, Stress in Atlantic salmon: response to unpredictable chronic stress, *J Exp Biol* 218(16) (2015) 2538-2550.
- [27] M.A. Vindas, A. Madaro, T.W.K. Fraser, E. Höglund, R.E. Olsen, Ø. Øverli, T.S. Kristiansen, Coping with a changing environment: the effects of early life stress, *R Soc Open Sci* 3(10) (2016).
- [28] M. Jobling, Growth studies with fish—overcoming the problems of size variation, *J Fish Biol* 22(2) (1983) 153-157.
- [29] J.M. Ruijter, C. Ramakers, W.M.H. Hoogaars, Y. Karlen, O. Bakker, M.J.B. van den Hoff, A.F.M. Moorman, Amplification efficiency: linking baseline and bias in the analysis of quantitative PCR data, *Nucl Acid Res* 37(6) (2009) e45.
- [30] S.A. Bustin, V. Benes, J.A. Garson, J. Hellems, J. Huggett, M. Kubista, R. Mueller, T. Nolan, M.W. Pfaffl, G.L. Shipley, J. Vandesompele, C.T. Wittwer, The MIQE guidelines: Minimum information for Publication of Quantitative Real-Time PCR Experiments, *Clin Chem* 55(4) (2009) 611-622.
- [31] J. Vandesompele, K. De Preter, F. Pattyn, B. Poppe, N. Van Roy, A. De Paepe, F. Speleman, Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes, *Genom Biol* 3(7) (2002) research0034.1 - research0034.11.
- [32] C.E. Grueber, S. Nakagawa, R.J. Laws, I.G. Jamieson, Multimodel inference in ecology and evolution: challenges and solutions, *J Evol Biol* 24(4) (2011) 699-711.
- [33] S. Winberg, G.E. Nilsson, K.H. Olsén, Changes in brain serotonergic activity during hierarchic behavior in Arctic charr (*Salvelinus alpinus* L.) are socially induced, *J Comp Physiol* 170(1A) (1992) 93-99.
- [34] Ø. Øverli, C.A. Harris, S. Winberg, Short-term effects of fights for social dominance and the establishment of dominant-subordinate relationships on brain monoamines and cortisol in rainbow trout, *Brain Behav Evol* 54(5) (1999) 263-275.
- [35] N.J. Shannon, J.W. Gunnet, K.E. Moore, A comparison of biochemical indices of 5-hydroxytryptaminergic neuronal activity following electrical stimulation of the dorsal raphe nucleus, *J Neurochem* 47(3) (1986) 958-965.
- [36] J.M. Koolhaas, S.F. de Boer, C.M. Coppens, B. Buwalda, Neuroendocrinology of coping styles: Towards understanding the biology of individual variation, *Front Neuroendocrinol* 31(3) (2010) 307-321.
- [37] M.A. Vindas, M. Gorissen, E. Höglund, G. Flik, V. Tronci, B. Damsgård, P.-O. Thörnqvist, T.O. Nilsen, S. Winberg, Ø. Øverli, L.O.E. Ebbesson, How do individuals cope with stress? Behavioural, physiological and neuronal differences between proactive and reactive coping styles in fish, *J Exp Biol* (2017).
- [38] J.M. Koolhaas, S.F. de Boer, B. Buwalda, K. van Reenen, Individual variation in coping with stress: A multidimensional approach of ultimate and proximate mechanisms, *Brain Behav Evol* 70(4) (2007) 218-226.

- [39] N.J. Dingemans, A.J.N. Kazem, D. Réale, J. Wright, Behavioural reaction norms: animal personality meets individual plasticity, *Trend Ecol Evol* 25(2) (2010) 81-89.
- [40] A. Fernö, G. Huse, P.J. Jakobsen, T.S. Kristiansen, J. Nilsson, Fish behaviour, learning aquaculture and fisheries, in: C. Brown, K. Laland, J. Krause (Eds.), *Fish Cognition and Behavior*, Wiley-Blackwell, Oxford, 2011, pp. 359-404.
- [41] J. Zhang, Evolution by gene duplication: an update, *Trends in Ecol Evol* 18(6) (2003) 292-298.
- [42] H. Matsuzaki, T. Izumi, T. Horinouchi, S. Boku, T. Inoue, T. Yamaguchi, T. Yoshida, M. Matsumoto, H. Togashi, S. Miwa, T. Koyama, M. Yoshioka, Juvenile stress attenuates the dorsal hippocampal postsynaptic 5-HT<sub>1A</sub> receptor function in adult rats, *Psychopharmacology* 214(1) (2011) 329-337.
- [43] I.B. Johansen, G.K. Sandvik, G.E. Nilsson, M. Bakken, Ø. Øverli, Cortisol receptor expression differs in the brains of rainbow trout selected for divergent cortisol responses, *Comp Biochem Physiol* 6(2D) (2011) 126-132.
- [44] A.F. Seasholtz, H.L. Burrows, I.J. Karolyi, S.A. Camper, Mouse models of altered CRH-binding protein expression, *Peptides* 22(5) (2001) 743-751.
- [45] R.J. Denver, Evolution of the corticotropin-releasing hormone signaling system and its role in stress-induced phenotypic plasticity, *Ann NY Acad Sci* 897(1) (1999) 46-53.
- [46] M.J. Owens, C.B. Nemeroff, Physiology and pharmacology of corticotropin-releasing factor, *Pharmacol Rev* 43 (1991) 425-473.
- [47] R. Manuel, J.R. Metz, G. Flik, W.W. Vale, M.O. Huising, Corticotropin-releasing factor-binding protein (CRF-BP) inhibits CRF- and urotensin-I-mediated activation of CRF receptor-1 and -2 in common carp, *Gen Comp Endocrinol* 202 (2014) 69-75.
- [48] P.G. Slater, C.A. Cerda, L.A. Pereira, M.E. Andrés, K. Gysling, CRF binding protein facilitates the presence of CRF type 2 $\alpha$  receptor on the cell surface, *Proc Nat Acad Sci* 113(15) (2016) 4075-4080.
- [49] M.P. Mattson, S. Maudsley, B. Martin, BDNF and 5-HT: a dynamic duo in age-related neuronal plasticity and neurodegenerative disorders, *Trend Neurosci* 27(10) (2004) 589-594.
- [50] E. Nederhof, M.V. Schmidt, Mismatch or cumulative stress: Toward an integrated hypothesis of programming effects, *Physiol Behav* 106(5) (2012) 691-700.

**Figure 1.** Fish selection by means of basal brain stem serotonergic activity ([5-HIAA]/[5-HT]) in control (**A**) and UCS (**B**) groups.

**Figure 2.** Relationship between brain stem and hypothalamic serotonergic activity ([5-HIAA]/[5-HT]) for high and low groups previously subjected to an unpredictable chronic stress (UCS) regime or at control conditions. Significant linear model results: ss (type II) = 0.003,  $df = 1$ ,  $F = 20.8$ ,  $p < 0.001$ ; UCS: ss (type II) = 0.007,  $df = 1$ ,  $F = 52.7$ ,  $p < 0.001$  and

**Figure 3.** Relationship between brain stem serotonergic activity ([5-HIAA]/[5-HT]) and the relative telencephalic mRNA expression of (**A**) the serotonin receptor  $5-HT_{1A\beta}$  and (**B**) the serotonin transporter  $5-HTT$ , for high and low groups previously subjected to an unpredictable chronic stress (UCS) regime or at control conditions. Significant linear model results for (**A**)  $5-HT_{1A\beta}$ : ss (type II) = 9.5,  $df = 1$ ,  $F = 10.5$ ,  $p = 0.003$  and (**B**)  $5-HTT$ : ss (type II) = 0.31,  $df = 1$ ,  $F = 7.7$ ,  $p = 0.010$ ; brain stem 5-HIAA/5-HT, ss (type II) = 0.11,  $df = 1$ ,  $F = 2.8$ ,  $p = 0.104$ .

**Figure 4.** Relationship between brain stem serotonergic activity ([5-HIAA]/[5-HT]) and the relative telencephalic mRNA expression of the corticotropin releasing factor binding protein (*crfbp*, **A**), and the brain derived neurotrophic factor (*bdnf*, **B**) for high and low groups previously subjected to an unpredictable chronic stress (UCS) regime or at control conditions. Significant linear model results for (**A**) brain stem 5-HIAA/5-HT: ss (type II) = 10.6,  $df = 1$ ,  $F = 9.87$ ,  $p = 0.004$  and (**B**) brain stem 5-HIAA/5-HT x UCS: ss (type III) = 10.4,  $df = 1$ ,  $F = 5.41$ ,  $p = 0.027$ .

**Figure 5.** Relationship between brain stem serotonergic activity ([5-HIAA]/[5-HT]) and the specific growth rate (SGR) for high and low groups previously subjected to an unpredictable chronic stress (UCS, **A**) regime or at control (**B**) conditions at sampling point 2 (**S2**), after smoltification triggered by continuous light and at sampling point 3 (**S3**), after seawater transfer. Significant linear model results, brain stem 5-HIAA/5-HT x Time x UCS: ss (type III) = 0.76,  $df = 1$ ,  $F = 9.3$ ,  $p < 0.003$ .

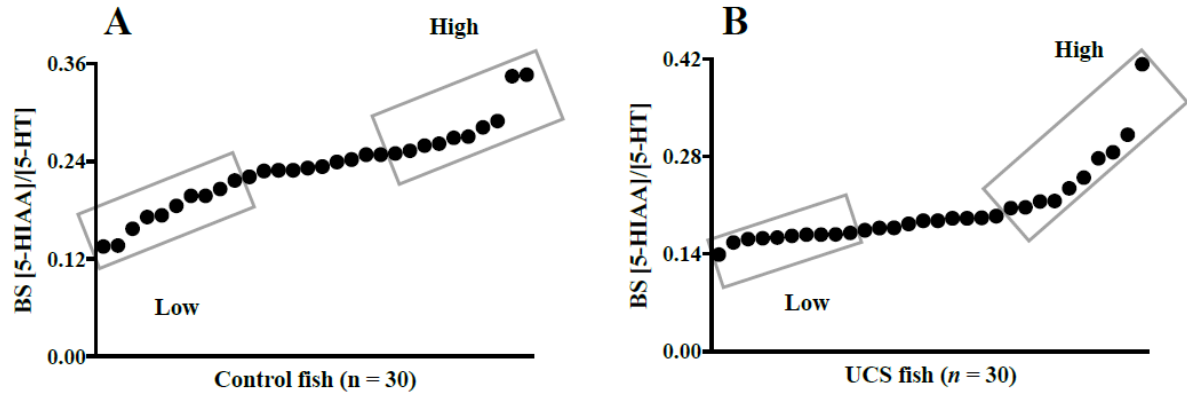


Fig. 1



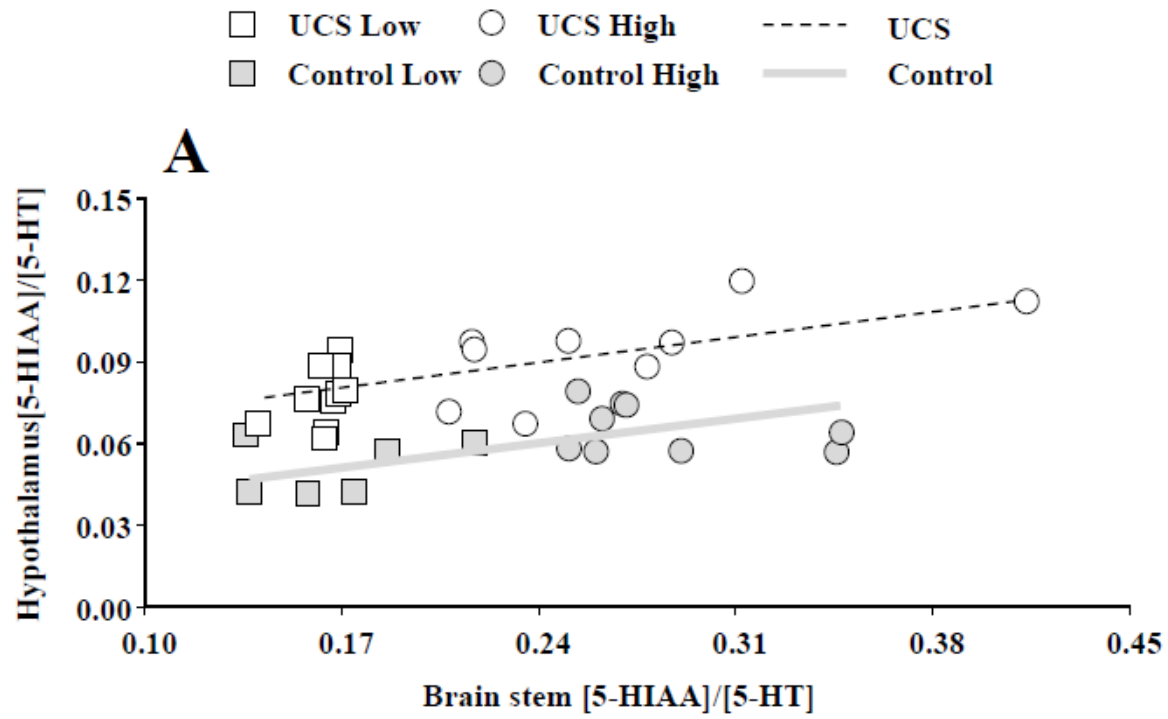


Fig. 2

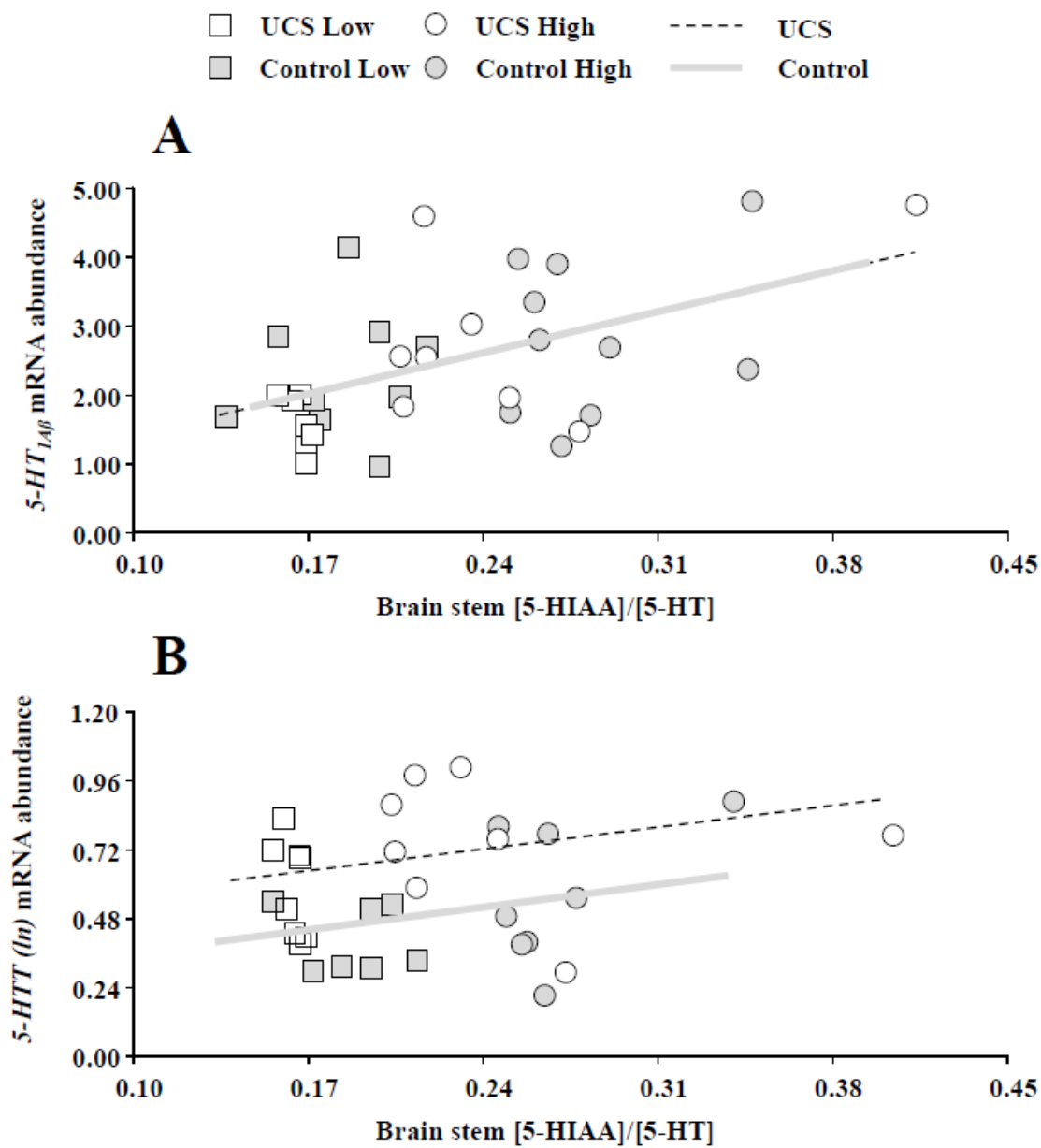


Fig. 3

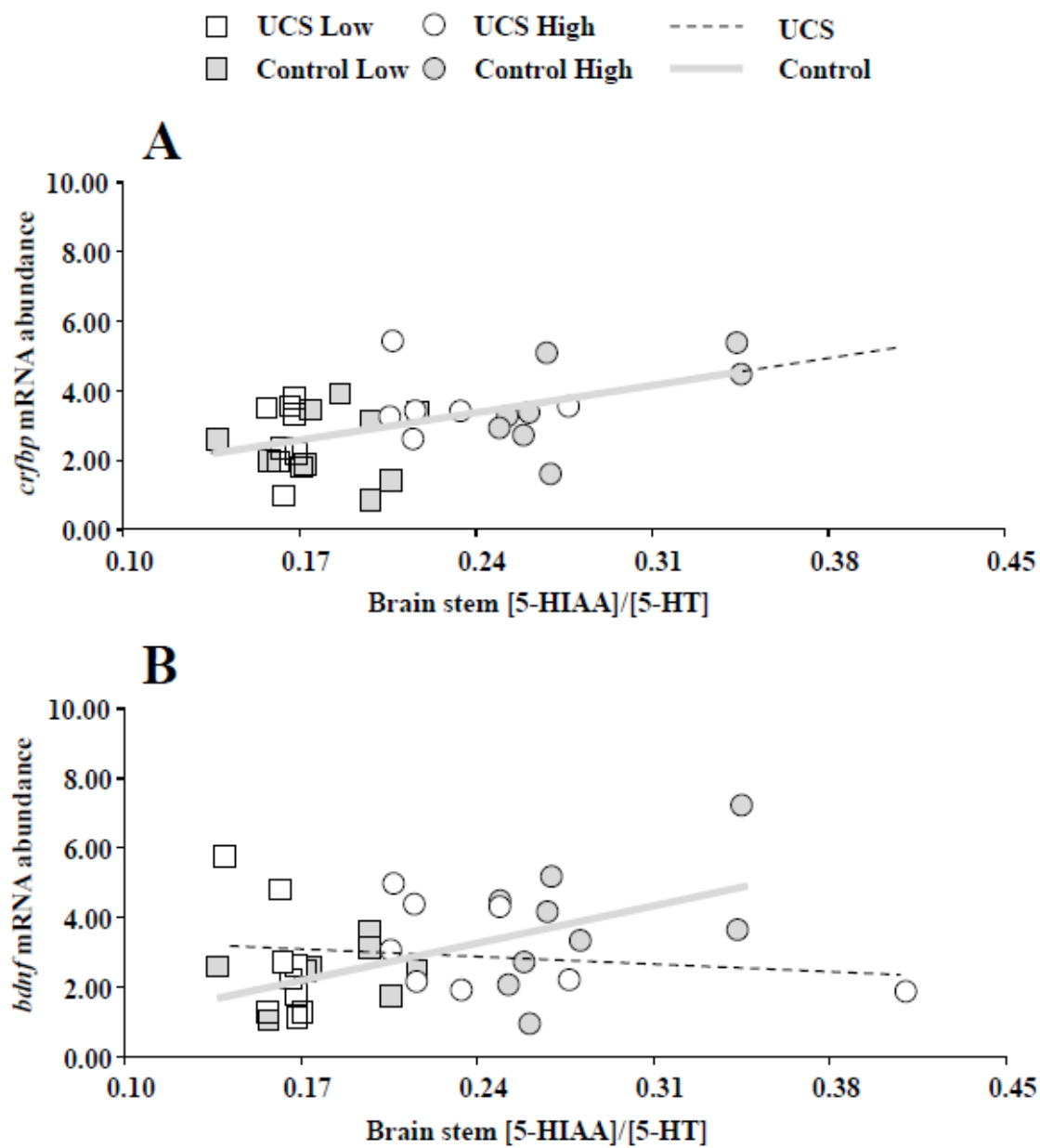


Fig. 4

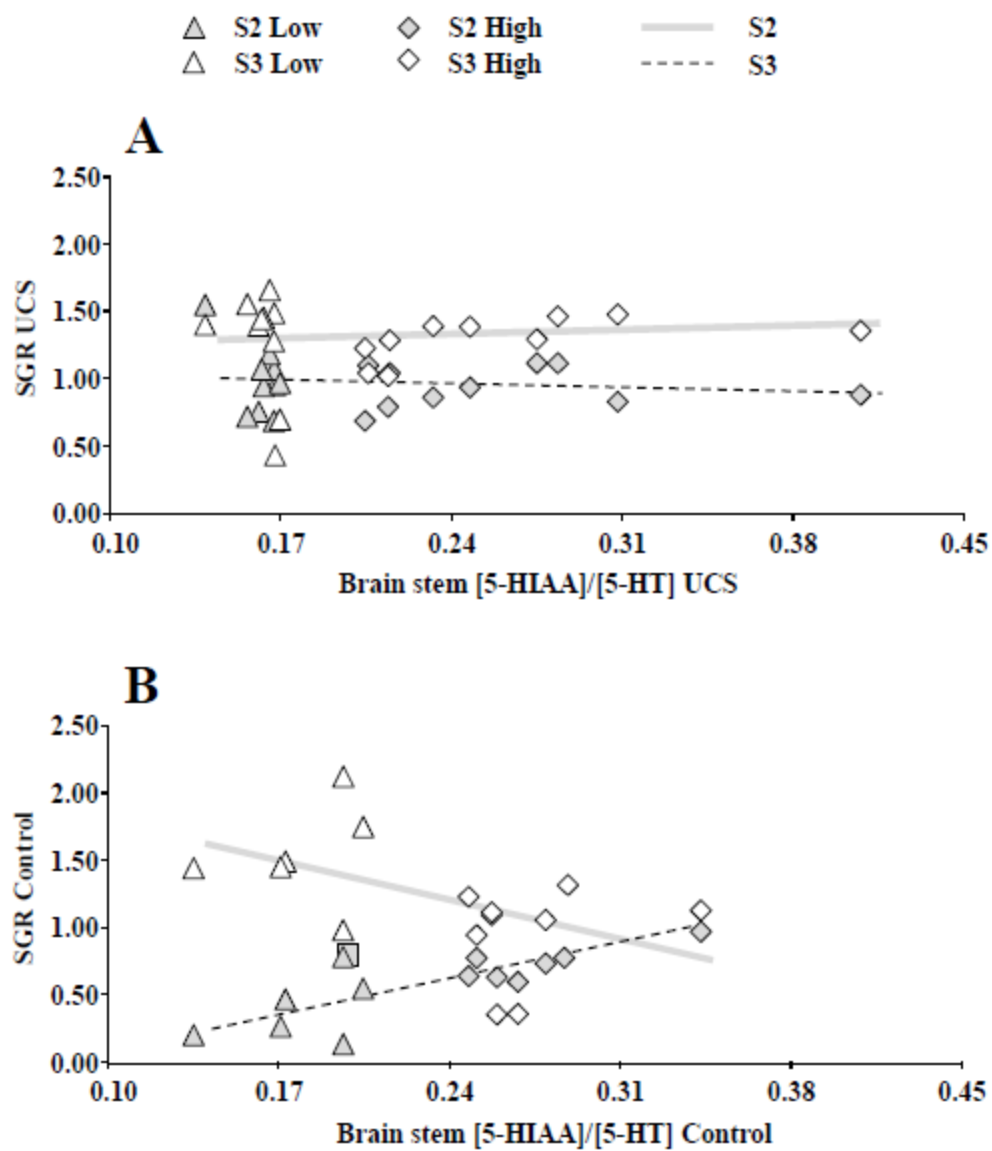


Fig. 5

## Highlights

- Early life stress decreases growth disparities in farmed salmon
- Post-hoc serotonergic activity sorting was used to study developmental plasticity
- An early life stress regime may increase welfare in farmed fish

ACCEPTED MANUSCRIPT