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Stress in Atlantic salmon: response to unpredictable chronic stress

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Keywords

Salmo salar; parr; cortisol; HPI-axis; *crf*; gene study; brain; hypothalamus; pituitary gland; head kidney.

Abbreviations

11βhsd2: 11-β-hydroxysteroid dehydrogenase 2; **crf**: corticotropin-releasing factor; **crfbp**: corticotropin-releasing factor binding protein; **gr**: glucocorticoid receptor; **ISH**: in-situ hybridisation; **mr**: mineralocorticoid receptor; **PM** nucleus preopticus magnocellularis; **POA**: preoptic area; **pomc**: pro-opiomelanocortin; **mc2r**: melanocortin 2 receptor; **PPa** nucleus preopticus parvocellularis pars anterior; **SGR**: specific growth rate; **star**: steroidogenic acute regulatory protein; **UCS:** unpredictable chronic stress.

Abstract

Combinations of stressors occur regularly throughout an animal's life, especially in agriculture and aquaculture settings. If an animal fails to acclimate to these stressors, stress becomes chronic, and a condition of allostatic overload arises with negative results for animal welfare. In the current study we describe effects of exposing Atlantic salmon parr to the unpredictable chronic stressor (UCS) paradigm for three weeks. The paradigm involves exposure of fish to 7 unpredictable stressors three times a day. At the end of the trial, experimental and control fish were challenged with yet another novel stressor and sampled

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before and 1 h after that challenge. Plasma cortisol decreased steadily over time in stressed fish, indicative of exhaustion of the endocrine stress axis. This was confirmed by a lower cortisol response to the novel stressor at the end of the stress period in chronically stressed fish compared to the control group. In the preoptic area (POA) and pituitary gland, chronic stress resulted in decreased gene expression of *11βhsd2*, *gr1* and *gr2* in POA and increased expression of those genes in the pituitary gland. POA *crf* expression and pituitary expression of *pomc*s and *mr* increased, whereas interrenal gene expression was unaffected. Exposure to the novel stressor had no effect on POA and interrenal gene expression. In the pituitary, *crfr1*, *pomc*s, *11βhsd2*, *gr*s and *mr* were down-regulated. In summary, our results provide a novel overview of the dynamic changes that occur at every level of the HPI-axis as a result of chronic stress in Atlantic salmon.

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1. Introduction

The intensity, duration, predictability and controllability of a stressor are important aspects to assess the severity of a stressor. In addition, stressors seldom come alone, adding a complex extra dimension to definition of stress intensity. As opposed to wild fish, under farming conditions an animal is confined and cannot escape from stressors. In aquaculture, fish are exposed to several simultaneous stressors. Examples include suboptimal / poor water quality, repeated handling, transport and crowding. The effects of many of these challenges have been studied, albeit mostly as a single stressor (Barton and Peter, 1982; Gorissen et al., 2012; Di Marco et al., 2008; Pottinger, 2010; Remen et al., 2012). Studies on mammals (Aguilera and Rabadan-Diehl, 2000; Dhabhar and Mcewen, 1997; Grissom and Bhatnagar, 2009; Thorsell et al., 1999), as well as fish (Schreck, 2000), show great resilience to a single stressor given repeatedly over longer periods of time, however knowledge on how ectotherms respond to multiple persistent stressors is very scant. One study on Atlantic salmon has shown that chronic stress followed by an additional maze challenge resulted in a suppressed cortisol response, decreased neural plasticity and learning ability (Grassie et al., 2013). In this study, we aim to describe the effects of unpredictable chronic stress (UCS) on the stress axis of Atlantic salmon.

When a stressor is perceived, neuronal signals (visual, auditory and sensory) activate the hypothalamus and initiate a downstream activation of sympathetic fibres that in turn stimulate the chromaffin cells of the head kidney to release the catecholamines adrenalin and noradrenalin into the blood stream as the initial stress response. The catecholamines prepare the animal for fight or flight by increasing gluconeogenesis and glycogenolysis, lipid

degradation, etc. Secondly, the hypothalamic – pituitary gland - interrenal gland (HPI) axis (the equivalent of the hypothalamic – pituitary gland - adrenal axis in mammals) becomes activated (Arends et al., 1999; Wendelaar Bonga, 1997). In the hypothalamic preoptic area (POA), corticotropin-releasing factor (CRF) is released to activate the pituitary corticotropes (Huising et al., 2004). CRF, *via* its receptor CRF-R1, induces the synthesis of pro-opiomelanocortin (POMC) which is processed into adrenocorticotropic hormone (ACTH) and released into the blood stream (Sumpter et al., 1986). In the interrenal gland, ACTH induces synthesis and release of cortisol *via* the melanocortin 2 receptor (MC2R) (Wendelaar Bonga, 1997) expressed exclusively on cortisol producing cells therein. MC2R activation results in activation of steroidigenic acute regulatory protein (STAR), which is responsible for the transport of cholesterol into the mitochondrial membrane where it will be converted to corticosteroids including cortisol.

Cortisol acts as glucocorticoid and mineralocorticoid in teleosts as these animals do not produce aldosterone synthase (Wendelaar Bonga, 1997). Specific actions of cortisol are effected by receptor specificity, i.e. the mineralocorticoid (MR) and glucocorticoid receptor (GR) profile of the target cell. MR and GR are transcription factors, mediating activation or inhibition of target gene expression. They are also involved in the negative feedback regulation of the HPI axis at the level of hypothalamus and pituitary gland (Atkinson et al., 2008; Bury et al., 2003; Cole et al., 2000). Cortisol also exerts negative feedback on hypothalamic CRF synthesis (Bernier and Peter, 2001; Bernier et al., 1999) and ACTH secretion by the pituitary gland (Fryer et al., 1984), whereas 11β-hydroxysteroid dehydrogenase 2 (11β-HSD2) counteracts cortisol actions by converting cortisol into the inactive cortisone (Mommsen et al., 1999). CRF binding protein (CRF-BP) provides yet another way of control over the HPI axis. It modulates the effect of CRF and CRF-related peptides by binding these peptides and reducing their bioavailability (Geven et al., 2006; Huising et al., 2008; Manuel et al., 2014; Seasholtz et al., 2002) with a decreased release of ACTH as a result.

In the short term, the stress response mostly works to the benefit of the animal: faced with a danger or challenge, the consorted action of the catecholamines and cortisol increase heart rate and blood flow to the muscles and increase processes aimed at providing more energy. Less vital bodily functions including growth, the immune system, reproduction and digestion are put on hold. If this situation persists, and the balance is not restored, the animal's condition changes from eustress to distress. Eustress refers to the initial, beneficial function of

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the stress response, whereas distress reflects the situation in which the response is inadequate, and maladaptive (Kupriyanov and Zhdanov, 2014; Wendelaar Bonga, 1997).

Allostasis, or maintenance of stability through change (Sterling and Eyer, 1988), involves resetting of physiological and behavioural set points of regulatory mechanisms to optimize performance in accordance to predicted environmental demands at minimal costs (McEwen and Lasley, 2002). Low allostatic load, or eustress, can have a positive effect on the animal's performance (Kupriyanov and Zhdanov, 2014). On the other hand, long lasting and / or repeated stressors result in a chronic stress condition in which a lasting initial stress response proves to be inadequate and the alarm signals become deleterious (allostatic overload / distress) (McEwen and Seeman, 1999).

Much stress research addressed isolated hypothalamus tissue, pituitary gland or interrenal gland and, to the best of our knowledge, only Fuzzen and colleagues (2010) have conducted a study on zebrafish that covers all levels of the stress axis. And, although effects of single, acute stressors have been studied in Atlantic salmon, knowledge on the effects of unpredictable chronic stress in this commercially important species is scant.

The main objective of this study was to determine how freshwater Atlantic salmon parr respond to unpredictable chronic stress. Stressors were selected based on what salmon may experience in aquaculture. We measured feed intake and growth throughout the experiment. Plasma cortisol levels were analysed at regular intervals throughout the experiment. After three weeks of UCS, all fish were subjected to yet another and novel stressor and sampled before, and 1 h after this novel stressor. Total mRNA levels of *crf, crfbp, 11βhsd2, gr1, gr2* and *mr* in POA*, crfr1, pomca1* and *pomcb1, 11βhsd2, gr1, gr2* and *mr* in the pituitary gland and *mc2r, star, 11βhsd2, gr1, gr2* and *mr* in interrenal tissue were analysed at both these time points.

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2. Results

2.1. Effect of UCS on feeding and growth

After 23 days, control fish had grown by 41% from 63.6 ± 1.2 g at the start of study to 89.5 ± 2.4 g; the chronically stressed fish had grown by 8.5% to 69.0 ± 1.4 g ($F(2,173) = 54,2$ $P = 0.001$; **Fig 1**). This represents a daily growth rate of 1.42 and 0.53%, respectively ($t = 5,433$; df = 4; $P = 0.0056$; **Fig 2**). The reduced growth rate in the stressed group corresponds with a reduced appetite throughout the study (Fig 3). Fish were starved for 12 h before sampling

days (indicated by arrows in **Fig 3**). On the following day, a compensatory feed intake was observed in all groups.

2.2. Plasma cortisol

Throughout the experiment, plasma cortisol levels were generally elevated in the stressed groups, but the amplitude of the response decreased towards the end of the study (interaction: $F(5,160) = 3.496$; $P = 0.005$; **Fig 4**). On day 5, cortisol production in the UCS group following chasing was not significantly different compared to controls.

There was a significant interaction effect (UCS × acute stressor: $F(1,111) = 15.61$; $P = 0.0001$) of cortisol levels after 23 days. Cortisol increased in both groups after the stress test, but less so for chronically stressed fish (91.26 ± 25.05 ng / ml) than for control fish (163.3 ± 21.98 ng / ml; **Fig 5**).

2.3. Gene study

2.3.1 Preoptic area

Figure 6 shows the transcript abundance of selected genes in the POA of controls and chronically stressed fish before and after 1 hr following exposure to a novel stressor on day 23. A significant interaction (UCS × acute stressor) was observed in *crf* expression ($F(1,103) = 4.830$; $P = 0.03$). Fish subjected to 23 days of chronic stress showed elevated basal levels of *crf* mRNA (Fig 6A); 1 h after the novel stressor expression levels of *crf* in control and chronically stressed fish had not changed. Also for *crfbp* a significant interaction effect was observed (UCS × acute stressor: $F(1,105) = 4.723$; $P = 0.032$). There were no differences in basal transcript abundance for *crfbp* between controls and chronically stressed fish. However 1 hr after stress *crfbp* expression was significantly higher in the control group compared with the chronically stressed group (Fig 6B). Messenger RNA for *11βhsd2* (Fig 6C) and the glucocorticoid receptors (*gr1* and *gr2*; Fig 6D and E) showed similar patterns: lower amount of transcripts in chronically stressed fish compared to controls but no effect of the novel stressor. An interaction was seen in *mr* mRNA levels (UCS × acute stressor: $F(1,105) = 17.9$; $P < 0.0001$): 23 days of chronic stress had no effect on basal *mr* transcript abundance, whereas the acute challenge increased *mr* mRNA in control fish, not in chronically stressed fish (Fig 6F).

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2.3.2 In-situ hybridisation

Transcript abundance of *crf* mRNA in the *nucleus preopticus parvocellularis pars anterior* (PPa) and the *nucleus preopticus magnocellularis* (PM) was visibly more pronounced in UCS fish (**Fig 7**C and D) compared to control (**Fig 7**A and B). The images shown are from a single fish per treatment.

2.3.3 Pituitary gland

An interaction effect (UCS × acute stressor: $F(1,106) = 25.78$; $P = 0.0001$) was observed for pituitary *crfr1* where the transcript abundance was similar in both groups before the novel stress but was down-regulated 1 h after in the UCS fish (**Fig 8**A). Differential effects were observed for *pomca1* and *pomcb1* in control and UCS fish (UCS × acute stressor: $F(1,108) = 26.70$; $P = 0.0001$ for *pomca1* and $F(1,106) = 16.49$; $P = 0.0002$ for *pomcb1*; Fig 8B and 8C). As an effect of UCS, transcript abundance of *pomc* paralogues was higher in stressed fish. Expression of both *pomc* paralogues increased as a result of the novel stressor in the control group, whereas expression decreased in the UCS fish. Basal pituitary transcript abundance of *11βhsd2*, *gr1*, *gr2* and *mr* was significantly higher in UCS fish compared to controls at t = 0. After the novel stressor, mRNA levels decreased significantly in the chronically stressed group only (Fig 8 D-F). Furthermore, *mr* expression was up-regulated in the control group 1 h after stress (Fig 8 G).

2.3.4 Interrenal gland

The interrenal tissue did not show any basal differences in the amounts of gene transcript (**Fig 9** A-F). However, interaction effects were observed for *mc2r*, and *11βhsd2* (Fig 9 A, and C): the new stressor resulted in a down-regulation in the control group whereas an up-regulation of these genes was observed in the UCS fish. No differences were seen post-stress for all other interrenal genes.

3. Discussion

We characterised the stress response of Atlantic salmon parr after unpredictable chronic stress (UCS) for three weeks. We made several major observations: (1) the UCS resulted in suppression of feed intake and growth; (2) the cortisol response abated over the three weeks of UCS, and the cortisol response to a novel stressor was more pronounced in control fish than in UCS fish; (3) in the POA, basal transcript abundance of genes involved in negative

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feedback of the stress-axis decreased in UCS fish, whereas the novel stressor resulted in limited effects in both control and UCS fish; (4) genes involved in negative feedback in the pituitary gland increased in UCS fish compared to controls. A novel stressor increased expression of *pomc* paralogues in the control group and decreased transcript abundance in the UCS fish; and (5) the novel stressor increased the transcript abundance of *mc2r* and *11βhsd2* transcripts in the UCS fish, while no differences were found for the other genes studied.

As a general remark, we would like to stress that we do not - as of yet - know how changes in the expression of genes, as measured by mRNA, are representative for the levels of proteins for which they encode. That means that our data should be interpreted with care (Maier et al. 2009; Schwanhäusser et al., 2011).

3.1 Growth and plasma cortisol

The UCS approach appeared to induce chronic stress as both growth and feed intake were reduced over the three weeks of the study. To the best of our knowledge, there are no similar studies on the effect of UCS in fish appetite and growth, although our results are in line with other studies where repeated stress reduced appetite and growth (McCormick et al., 1998)

We also observed a chronic down-regulation of the cortisol response during the trial. However, on day 5 of the experiment the UCS group displayed a remarkable low cortisol production post-stress. Perhaps the UCS together with the high frequency of samplings during the first days of the experiment resulted in an exhaustion of the HPI axis and thus a failure to mount a proper cortisol response following stress. Further, a lowered cortisol response was measured at the end of the trial when the fish were exposed to a novel stressor. These observations are in line with the general down-regulation of the HPI axis that is a common feature during chronic stress and stress adaptation in fish and mammals (Barton, 2002; Barton et al., 1987; Vijayan and Leatherland, 1990).

3.2 Preoptic area

Exposing Atlantic salmon to UCS resulted in a general down-regulation of the examined genes in the POA except for *crf*, which was up-regulated. The *crf* up-regulation measured by qPCR was also validated by ISH with more pronounced staining of hypothalamic *crf* mRNA in the *nucleus preopticus parvocellularis pars anterior* (PPa) and the *nucleus preopticus magnocellularis* (PM) in UCS fish compared to controls. This increase in *crf* seems at odds

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with the abated cortisol response, but the high levels of *crf*, together with the high cortisol levels compared to baseline cortisol, may explain the reduction of appetite that we observed in the UCS fish, as CRF and cortisol are known suppressors of appetite in fish (Bernier and Peter, 2001; Gregory and Wood, 1999).

POA *crfbp* transcript abundance was not affected by chronic stress. Studies on trout (Alderman et al., 2008; Doyon et al., 2005) reported that CRFBP may play a role in the inhibition of CRF signalling at its receptor in the acute stress response and this mechanism was confirmed by *in vitro* studies (Manuel et al., 2014). POA *crfbp* transcript amount was not affected by chronic stress in our study, which is in line with another experiment on rainbow trout (Jeffrey et al., 2012) where *crfbp* mRNA was unaffected by chronic stress caused by social interactions.

During chronic stress, HPI-axis down-regulation (Barton, 2002; Barton et al., 1987; Vijayan and Leatherland, 1990) in salmonids and in other teleostean fish results from negative feedback mediated by the two GRs, GR1 and GR2, of which GR2 has a similar to higher affinity for cortisol as MR (Bury et al., 2003; Greenwood et al., 2003; Prunet et al., 2006; Stolte et al., 2008). In the present study UCS induced a down-regulation of both *gr* mRNAs in the POA while *mr* transcript amount was unaffected. A reduced expression of the glucocorticoid receptors (*gr1* and *gr2*) and an increase in *crf* mRNA is in line with previous observations in zebrafish (Chakravarty et al., 2013;; Piato et al., 2011) and trout (Doyon et al., 2005). Low levels of *gr1* and *gr2* may protect neurones to high levels of cortisol during chronic stress. GR-containing cells are more prone to apoptosis by elevated levels of glucocorticoids (Aluru and Vijayan, 2006; Piato et al., 2011; Sapolsky et al., 2000). On the other hand, *11βhsd2* was down-regulated as a result of UCS. This enzyme catalyses the degradation of cortisol to cortisone, thus regulating cortisol availability to corticoid receptors (Funder et al., 1988). Lower number of *11βhsd2* transcripts may result in higher cortisol availability; we interpret these results combined as a search for a new balance in cortisol action under chronic stress conditions. Promotor analysis in zebrafish revealed putative sites for cortisol-mediated regulation of transcription of *11βhsd2* and (chemical) inhibition of *11βhsd2* activity resulted in increased POA *crf* expression and elevated cortisol levels (Alderman and Vijayan, 2012). Taken together, these findings indicate a pivotal role for *11βhsd2* in negative feedback control of the stress axis.

The final stress test had no major influences on the gene expression in the POA; only in control fish a rapid up-regulation of *mr* was seen. Possibly, MR is the main corticoid receptor responsible for the mediation of cortisol actions in the POA in Atlantic salmon after acute

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stress. In mammals, corticosteroid effects mediated by MRs are involved in a suppression of the basal and stress-induced ACTH secretion (De Kloet et al., 2005). Studies in rats support the idea that even when MRs are occupied in resting conditions by basal levels of corticosteroids, they still have important roles for the sensitivity and feedback responses when high levels of corticosteroids are present (Ratka et al., 1989; Dallman 1993, Oitxl et al., 1994). In contrast, the UCS group displayed no effects on *mr* transcript abundance despite a persistent *gr* downregulation. De Kloet (1991) hypothesised that an increased amount of MRs relative to GRs may be predictive for a reduced responsiveness of HPI axis to stress. If this holds for fish POA should be tested by measurement of the number of receptor proteins. Furthermore, additional regulatory mechanisms may be present in other tissues. For example, Stolte and colleagues (2008), showed that carp, when repeatedly given a temperature shock, displayed a down-regulation of the glucocorticoid receptors in the brain but not in the POA and pituitary gland, suggesting a central initiation by telencephalic pallial areas homologous to the mammalian hippocampus, amygdala and prefrontal cortex (Mueller, 2012; Mueller et al., 2011) of stress axis control, rather than direct feedback via the POA or pituitary gland.

3.3 Pituitary gland

We analysed the transcript abundance of *pomca1* and *pomcb1*, paralogues that originate from the salmonid genome duplication (Arends et al., 1998; Leder and Silverstein, 2006; Macqueen and Johnston, 2014; Salbert et al., 1992). No changes in *pomca1* and *pomcb1* mRNA transcripts were observed when fish were subjected to either UCS or the novel stressor. In the pituitary gland, *pomc* is expressed in both the corticotrope cells (*pars distalis*) and in the melanotrope cells (*pars intermedia*). In the *pars distalis*, POMC is processed by prohormone convertase 1 to ACTH and β-endorphin, whereas in the *pars intermedia* POMC, through an additional action of prohormone convertase 2, POMC is processed into α-MSH and β-endorphin (Takei and Loretz, 2006). We isolated the mRNA of the entire pituitary gland. Therefore we are unable to discern where in the pituitary gland the measured *pomc* was produced. Both *pomc* paralogues possess the required dibasic cleavage sites to produce ACTH and α-MSH. Plasma concentrations of α-MSH (data not shown) were not affected by UCS treatment or by exposure to the novel stressor. Unfortunately, ACTH was not analysed as a reliable assay for plasma ACTH in salmon is lacking. In our study we observed an increase of *pomca1* and *pomcb1* mRNA in UCS fish compared to controls: in line with these results, in a trout study Winberg and Lepage (1998) found increased *pomc* expression levels as a result of

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chronic stress induced by social interactions. In our study, the additional novel stressor resulted in increased *pomca1* and *pomcb1* expression in control fish but not in the UCS fish, which is corroborated by the elevated plasma cortisol levels.

In contrast to what we observed in POA tissue, all examined genes involved in negative feedback (*gr1, gr2*, *mr*, and *11βhsd2)*, were up-regulated in the pituitary gland following chronic stress. However, the capacity to metabolise cortisol into the inactive cortisone may have increased as indicated by the enhanced expression of *11βhsd2* and taken together this could indicate an enhanced turnover of cortisol under stress conditions. Studies on transgenic mice homozygous for a target disruption of the *gr* gene (Cole et al. 1995) and for a mutation of the dimerisation loop domain in GR (Reichardt et al. 1998) suggest that the down-regulation of *Crf* expression by negative feedback triggered by glucocorticoids are GR-DNA binding independent, whereas *Pomc* transcription is under negative control by the GR-DNA binding (Newton, 2000). In our study, we observed a decrease in POA and increase in pituitary gland *gr* transcripts of chronically stressed fish, pointing to a stronger feedback control over the pituitary gland.

The effects of the acute novel stressor on the pituitary gland were significantly different in control and chronically stressed fish. In control fish, the novel stressor did not affect *crfr1*, while *pomc* mRNA increased, in agreement with studies in zebrafish (Fuzzen et al., 2010) and trout (Gilchriest et al., 2000). We underline that in naive fish the novel stressor resulted in up-regulation of *mr*, but not *gr1* and *gr2*. The regulation of *mr* expression in POA and pituitary gland indicates a role of *mr* in corticosteroid-regulated processes, particularly after an acute stressor (De Kloet et al., 1998; Stolte et al., 2008)

In UCS salmon, *pomc* expression is up-regulated but did / could not further respond to the novel stressor. This might indicate a failure to respond to the novel stressor following the chronic stress and may explain why the chronically stressed fish had an impaired ability to mount a proper cortisol response after the last stress test. Furthermore, after the novel stressor, the drop of the cortisol receptors mRNA suggested a temporary reduction of the glucocorticoid-mediated feedback mechanism(s). Taken together, the down-regulation of *crfr1*, *11βhsd2, gr1, gr2* and *mr* after the novel stressor results in a low responsiveness (and protection) of the pituitary gland to the circulating levels of cortisol. Such situation, if prolonged, may expose fish to the maladaptive and deleterious effects caused by elevated levels of glucocorticoids following chronic stress.

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3.4 Interrenal gland

Surprisingly, we did not observe any differences in transcript abundance of genes involved either in cortisol production and release (*mc2r* and *star)*, inactivation (*11βhsd2)* and cortisol receptors (*gr1*, g*r2*, and *mr*) between the groups. An ultra-short loop, auto-feedback for cortisol has been suggested in the teleostean head kidney (Bradford, 1992; Rotllant et al., 2001); however our results are in line with former studies (McEwen, 2006; Rotllant et al., 2000) and indicate that the main site of modulation of the stress response is in the POA and pituitary gland.

After exposure to a novel acute stressor, the two groups displayed significant differences in transcript abundance of *mc2r* and *11βhsd2*, which are higher in UCS fish compared to the controls. The transcript abundance of *star* follows the same pattern. Fuzzen et al. (2010) observe a transient increase in *mc2r*, *star* and *11βhsd2* in the zebrafish head kidney after the onset of a stressor and a return to basal levels around 60 min post-stress, which could explain why no up-regulation of *mc2r, star* and *11βhsd2* was observed following the UCS treatment of parr. Noteworthy, in trout, an up-regulation of *mc2r* and *star* 4 h and 1 h after acute stress respectively has been observed (Aluru and Vijayan, 2008). The lack of effect following the acute and chronic stress on both the control and the chronically stressed groups in the present study can lead to two different hypotheses: first, sampling 1 h post-stress may have been too late to observe up-regulation of *mc2r* and *star*. A second hypothesis, also suggested by Geslin and Auperin (2004) for trout, could be that the low transcription in the interrenal gland is explained by activation and of the residing (over-) capacity of protein in cortisol production. Alternatively, transcript abundance of *mc2r* and *11βhsd2* in control fish could have been depleted post-stress due to the induction of protein production, whereas in the UCS group transcription of these genes was already activated in order to support steroidogenesis in response to the UCS. Additional studies should clear the doubts about the time course and dynamics of stress axis related gene expression in the head kidney.

3.5 Conclusions & perspectives

Whereas the acute stress response of fish has been extensively reviewed (Barton, 2002; Wendelaar Bonga, 1997), the effects of long-term stress on fish remain relatively unexplored. The purpose of the present study was to show the effects of unpredictable chronic stress on Atlantic salmon parr and explore the mechanisms that drive and control the HPI axis under these conditions. We established a new UCS protocol, based on multiple stressors, applied

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randomly, to avoid habituation. In these conditions the fish could experience allostatic overload or "wear and tear" of the body if they cannot attenuate the mediators involved in the stress response (McEwen, 1998; McEwen and Stellar, 1993). We also examined genes relevant to all levels of the HPI axis before and after a novel acute stressor between chronically stressed fish and controls. As predicted, gene expression data on POA and pituitary gland demonstrate that control fish respond readily to an acute stressor (Wendelaar Bonga, 1997). On the other hand, UCS fish were characterised by a dampened cortisol response that correlates with changes in stress-axis related gene expression at the levels of POA and pituitary gland. We conclude that, despite an increased *crf* expression in the POA, UCS treatment reduced the pituitary capacity to mount a proper stress response. Before the novel stressor the expression of genes involved in the feedback systems as well as the initiation of the interrenal cortisol production increased. After the novel stressor, mRNA levels of the stress axis regulators in the central part of the HPI's axis were depleted. The interval time between stress events is critical for the physiological response (Schreck, 2000). Therefore a higher frequency of UCS-episodes may reduce the time available for the fish to cope with the stressors: in such situation the effect of each stress episode may become cumulative and push the fish tolerance capabilities until disease and death occur.

The Atlantic salmon parr used in the present study (AquaGen strain) proved to be a fish with a high tolerance to multiple, unpredictable stressors. The procedure did not harm the fish physically and no fish died during the experiment. Accordingly, Solberg et al. (2013) showed that farmed salmon are characterised by a reduced responsiveness to stress compared with wild strains. Solberg and colleagues suggest that, during the last four decades of intensive salmon breeding programs, the selection of individuals with increased growth rate in captivity environments is likely to have involuntarily led to the selection of fish with reduced stress responsiveness and / or higher resilience, as indeed individuals affected by stress would display impaired growth rates and therefore would not be selected as brood stock for the next generation. A comparative study on the effects of the UCS protocol on the AquaGen strain and wild salmon seems indicated.

4. Materials and Methods

4.1. Fish and experimental facilities

Atlantic salmon (Salmo salar L., AquaGen strain) eggs obtained from a commercial farm (Aqua Gen AS, Trondheim, Norway) were hatched (March 2012) and reared at the Institute of Marine Research (IMR), Matre, Norway. Experimental fish were kept in freshwater with light and temperature according to natural winter conditions (12L:12D, 9°C). On January 11th

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2013, 740 fish (average weight 57 g) were transferred from a 10,000-L circular outdoor tank to six 400-L square indoor tanks (± 7 kg fish / tank) supplied with flow-through freshwater. The tanks were furnished with lids that contained fluorescent light tubes and automatic feeders (Arvo-tec feeding units: Arvo-Tec T drum 2000. Huutokoski, Finland). Feeding (ad libitum three times per day), temperature (12°C), water flow (15 L / min) and oxygenation (92%) of the water were automatically regulated by customised computer software (SD Matre, Normatic AS, Nordfjordeid, Norway). One week before the start of the experiment, the fish bulk weight was recorded for each tank following mild sedation in oxygenated water (25 mg / L, Finquel®vet, ScanAqua AS, Årnes, Norway buffered with sodium bicarbonate 25 mg / L) to reduce handling stress.

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4.2. Experimental design

At the beginning of the experiment (February 4, 2013) six tanks were divided over two groups of three tanks (replicates) each, receiving unpredictable chronic stress or left undisturbed (control groups). The UCS group was stressed three times per day (at 8:30, 13:00, and 17:00) using a total of 8 types of stressors given in random and unpredictable order throughout one week, and this protocol was then repeated over the next 2 weeks (Table 1). All stressors were chosen such that there would be no physical damage to the fish (such as scale loss or fin damage). Disturbance for the control group was reduced to a minimum and limited to routine practices of tank maintenance and sampling. Fish were fed with dry feed for the duration of one hour (2mm Skretting Nutra Olimpic, Stavanger, Norway) 30 - 60 min after each stress event (i.e. at 9:00 - 10:00, 13:30-14:30 and 17:30-18:30). To study the fish stress response along the experiment, on day 1, 2, 5, 9, 16 and 23, the first stressor of each day was 5 min chasing stress, which was followed by sampling of 5 fish per tank (n = 15) 1 h later. Undisturbed fish from the control tanks served as controls (5 fish per tank, n = 15). We chose to use the same stressor before these samplings in order to avoid confounding effects of different stressor intensities.

On the last day of the experiment (day 23), 10 fish per tank (n = 30) were collected from both control and stressed groups before stress (T_0) to assess basal cortisol levels and gene expression, while two extra fish per tank (n = 6) were collected for in-situ hybridisation analysis. Immediately thereafter, another 10 fish per tank (n = 30) were collected and exposed to a novel stressor which consisted of netting, air exposure for 15 s and confinement in a 10-L bucket for 5 min before being transferred to a new 400-L tank for recovery for one hour, after

which the fish were sampled for analysis (T_1). All experiments were approved by the Norwegian Experiment Animal Committee (Forsøksdyrutvalget, 11.12.2012).

Table 1: Description of the stressors randomly given to Atlantic salmon parr throughout the experiment, three times per day, for 23 days.

Stressor	*Time*	*Description*	*References*
Chasing	5	Stirring in the tank with a net	Pavlidis et al., 2015 Tsalafouta et al., 2014
Netting	3	Net and release fish with a dip net including brief air exposure (± 1 sec)	Barton et al., 1980
Temperature shock 12°C to 4°C	120	Reduction of the water temperature from 12°C to 4°C and up to 12°C	Foss et al., 2012
Temperature shock 12°C to 19°C	120	Rise of the water temperature from 12°C to 19°C and down to 12°C	Templeman et al., 2014
Noise	5	Knockingon the tank with a metal object	Slabbekoorn, 2012
Darkness + flash light	5	Turn off the tank lighting and use of a white intermittent led light	
Brief hypoxia	5	Closure of water inflow until the oxygen saturation of the water reaches 40%	Remen et al., 2012
Emptying the tank	5	Removal of the tank plug while leaving the water flow open with a constant 3 cm deep layer of water as a result	Einarsdóttir and Nilssen 1996.

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4.3. Sampling

Fish were starved for 12 hrs before sampling. Fish received an overdose anaesthesia (100 mg / L, Finquel®vet., ScanAqua AS, Årnes, Norway) buffered with 100 mg / L sodium bicarbonate (Finquel®vet.). Fork length and body weight were recorded for each individual fish. Blood was collected using 1 ml heparinised syringes fitted with a 23G needle and plasma was separated immediately by centrifugation at 13,000 rpm for 3 min and stored at -80°C until cortisol analysis. Pituitary glands, brains and head kidneys were collected and stored in RNAlater (RNA*later*® RNA Stabilization Solution, Life Technology, Oslo, Norway) at 4°C for one night and subsequently stored at –80°C until RNA isolation. The sex of the fish was

recorded. POAs were isolated from brains immediately before the RNA purification (Bernier et al., 2008).

4.4. Feed consumption and growth rate

Feed uneaten after 15 min was collected per tank after each feeding and left to dry in a colander 10 min before recording the weight. Feed consumption was calculated following the method described by Helland and colleagues (1996). The specific growth rate (SGR) was calculated using the equation 1:

$$\text{Eqn 1: SGR (\% body weight gain } \times \text{day}^{-1}) = \left[\frac{(\text{Log } W_2 - \text{Log } W_1)}{(t_2 - t_1)}\right] \times 100$$

in which W_1 is the bulk weight at the start of the growth period (t_1) and W_2 the bulk weight at the end (t_2) (Houde and Schekter, 1981). Both feed consumption and final SGR were calculated per tank (n = 3).

4.5. Blood analyses

Plasma cortisol concentrations were quantified by radioimmunoassay (Gorissen et al., 2012) using a highly specific and sensitive commercially available antibody (cortisol antibody [xm210]; Abcam, Cambridge, United Kingdom) and ^{3}H-cortisol (Perkin Elmer, Groningen, The Netherlands). The primary antibody shows a 100% cross reactivity with cortisol, 0.9% with 11-deoxycortisol, 0.6% with corticosterone, and < 0.01% with 11-deoxycorticosterone, progesterone, 17-hydroxyprogesterone, testosterone and oestradiol. Inter- and intra-assay variations were 12.5 and 3.5%, respectively.

4.6. Gene expression analysis

Preoptic area, pituitary and head kidney total RNA was isolated with Trizol reagent (Invitrogen, Carlsbad, USA) according to manufacturers' instructions and then dissolved in DEPC-treated H_2O. RNA concentration and purity was determined by spectrophotometry (Nanodrop Wilmington DE, US). 500 ng of total mRNA was incubated for 15 minutes at room temperature with 1 µl (1U) DNase I (Invitrogen, Carlsbad, USA) in a volume of 10 µl to remove possible genomic DNA contamination. DNase was inactivated by the addition of 1 µl 15 mM EDTA and incubation at 65°C for 10 min. To each sample, 250 ng random hexamer primers, 4 µl 5 × First Strand buffer, 1 µl (10 mM) dNTP mix, 1 µl 0.1 M DTT, 1 µl RNase OUT (10 U / µl) and 100 U of SuperscriptII Reverse Transcriptase (200 U / µl; all from

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Invitrogen) were added and incubated for 10 min at 25°C, 50 min at 42°C, 15 min at 70°C for the synthesis of first strand cDNA. The cDNA was diluted 5 times and stored at -20°C until measurement of the relative mRNA levels by real-time quantitative PCR (RT-qPCR). Each RT-qPCR reaction consisted of 4 µl template and 10 µl SYBR Green Mastermix (BioRad, Hercules, CA, USA), 0.7 µl (10 µM) forward primers, and 0.7 µl (10 µM) reverse primers and 4.6 µl DEPC-treated H_2O. RT-qPCR (3 min 95°C, 40 cycles of 15 sec at 95°C and 1 min 60°C) was carried out on a CFX96 Touch™ real-time PCR detection system (BioRad, United). The relative gene expression was analysed according to Vandesompele et al. (2002) and normalised to an index of two reference genes, *viz. 20S ribosomal protein* (*20S*) and *elongation factor 1 α* (*elf1α*). Sequences of primers used for the qPCR analysis are shown in Table 2.

4.7. crf mRNA in-situ hybridisation:

In-situ hybridisation (ISH) of *crf* mRNA was performed according to (Ebbesson et al., 2011) In brief, three juvenile Atlantic salmon from control and stressed groups each were deeply anaesthetised with buffered Finquel®vet. and fixed by vascular perfusion with 4% paraformaldehyde (PF) in 0.1 M Sørensens phosphate buffer (PB; 28 mM NaH_2PO_4, 71 mM Na_2HPO_4, pH 7.2). The brains were dissected out and post fixed in the same fixative for 16 hours at 4°C. The tissue was washed 3 × 20 minutes in PB, cryopreserved overnight in 25% sucrose in PB at 4°C, embedded in Tissue-Tek O.C.T -Compound (Sakura Fintek, Zoeterwoude, Netherlands) and stored at -80°C until sectioning. Adjacent transversal 12 µm sections were cut using a Leica CM 1850 cryostat, collected on SuperFrost Ultra Plus glasses (Menzel Glaser, Braunschweig, Germany) and dried at 60 ºC for 10 min. Digoxigenin labelled riboprobes were prepared using a digoxigenin (DIG)-RNA labelling mix according to the manufacturer's instructions (Roche Diagnostics, Mannheim, Germany). The ISH probe was 469 nucleotides long with a high homology to Atlantic salmon *crf I* (99%) and *crf II* (93%) transcripts, and a low homology to *urotensin I* (51%) transcripts (Ebbesson et al unpublished results). The following forward 5'-TTTCCTCGTCACCACCGTG-3' and reverse 5'-ATCATTTTTCTGTTGCTATGGGC-3' primers were used to clone the probe. The quality and quantity of the synthesised riboprobes were assessed by agarose gel electrophoresis. Prior to in-situ hybridization, the tissue was air dried at room temperature for 1 h and at 65°C for 10 min, rehydrated in a graded ethanol series (95-50%), washed 1 min with 2 × SSC, then permeabilised with proteinase K (10 µg/ml in 0.1M Tris-HCl, pH 8.0) for 3.5 min, post-fixed in 4% PF in KPBS (137 mM NaCl, 1.4 mM KH_2PO_4, 2.7 mM KCl, 4.3 mM Na_2HPO_4, pH

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7.3) for 5 min, followed by rinsing 2 times 2 min in KPBS. Tissue was then treated with 0.1 M triethanolamine (TEA, pH 8.0, Sigma) for 3 min and then with 0.25% acetic anhydride (Sigma) in 0.1 M TEA for 10 min. Finally, tissue was dehydrated in a graded ethanol series (50-100%) and air dried for one h. For hybridisation, 200 ng digoxigenin labelled probe in 100 µl hybridisation solution was applied to each slide. The composition of the hybridisation solution was: 10 mM Tris-HCl, 300 mM NaCl, 20 mM EDTA, 0.2% tween-20, 1% blocking solution (Roche Diagnostics), 0.1% Dextransulphate (Simga-Aldrich) 50% deionised formamide (Sigma-Aldrich). Incubation was carried out at 65 ºC for 16 h, using humidity chambers and hybri-slips (Sigma-Aldrich; Carlsbad, CA, USA) to prevent evaporation. The sense probe was applied as a control for non-specific staining. After hybridisation, tissue was washed 2 times 30 min in 2 × SSC, 30 min in 50% deionized formamide in 2 × SSC at 65ºC, and 2 times 10 min in 2 × SSC at 37°C. The tissue was treated for 20 min with RNase A (0.02 mg ml^{-1}, Sigma) at 37°C, and washed 20 min at 65 °C. The sections were incubated one hour with 2% blocking solution in 2 × SSC with 0.05%Triton X-100 and then overnight with alkaline phosphatase-conjugated sheep anti-DIG goat antibody (1:2000, Roche Diagnostic). The tissue was washed 2 times 10 min in 1 × maleate buffer and then 10 min in visualisation buffer (100 mM Tris-HCl, 100 mM NaCl, pH 9.5). The staining reaction with chromogen substrate (3.4 µl nitroblue tetrazolium, 3.5 µl 5-bromo-4-chloro-3-indoylphosphate (Roche Diagnostics) and 0.24 mg / ml levamisole in visualisation buffer) was carried out for 3 hours in darkness at room temperature. The reaction was terminated with stop solution 10 mM Tris-HCl, 1 mM EDTA, 150mM NaCl, pH 8.0) and tissue was mounted in ProLong®Gold (Invitrogen). Photographs were taken using a digital camera (Leica DFC 320, Leica 350 FX) attached to a Leica DM 6000B microscope using the Leica Application Suite V 3.0.0 image acquisition and processing software.

4.8. Statistics

Values are represented as mean + s.e.m. or as a Tukey box and whiskers plot. Differences in SGR were assessed with Mann Whitney *U*-test, while body weight data were analysed with one-way analysis of variance (ANOVA) followed by Sidak post-hoc test. Prior to analysis, data were checked for outliers, and the replicate tanks compared by ANOVA. If there were no differences between the tanks, they were pooled for further comparison of groups. Plasma cortisol levels and gene expression were evaluated using two-way ANOVA followed by a Tukey post-hoc test with Bonferroni correction for multiple comparisons applied where necessary, or Fisher's LSD test when no correction for multiple comparisons was needed (i.e.

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in case of comparison of cortisol between control and UCS between one day). Differences were considered to be statistically different from one another when $P < 0.05$, unless otherwise stated (i.e. in case of correction for multiple comparisons).

All statistical analyses were carried out using GraphPad Prism (version 6 for Windows, GraphPad Prism Software; La Jolla, CA, USA).

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Table 2: Primer sequences used in the real-time qPCR assays.

Gene		Primer sequences (5' → 3')	Amplicon (bp)	Accession number	References
elf1a	fw	CCCCTCCAGGACGTTTACAAA	57	AF321836	Olsvik et al., 2005
	rev	CACACGGCCCACAGGTACA			
20S	fw	GCAGACCTTATCCGTGGAGCTA	85	BG936672	Olsvik et al., 2005
	rev	TGGTGATGCGCAGAGTCTTG			
crf	fw	AACCAGCTCGACGACTCGATGG	135	BT057824	Lars O. E. Ebbesson, unpublished sequence
	rev	GCTATGGGCTTGTTGCTGTAACTG			
crfbp	fw	TGAGCCCAACCAGGTCATCAATGT	85	BT059529	Leong et al., 2010
	rev	TCCCTTCATCACCCAGCCATCAAA			
crfr1	fw	TGACCATCTGGGCTGTTGTGATCT	81	GBRB01035702	Lars O. E. Ebbesson, unpublished sequence
	rev	TAAGATTGGTGGACAGCAGGAGCA			
pomca1	fw	CCCATCCAGCACTGTAGGAC	112	NM_001198575.1	Leong et al., 2014 direct submission
	rev	CATTGCTGGGTCAGAGGACA			
pomcb1	fw	CAATCCCACAAGCCCCTGAT	75	NM_001128604.1	Designed by authors
	rev	TTCTGCTGCCCTCCTCTACT			
gr1	fw	ACGACGATGGAGCCGAAC	106	AF209873.1	Kiilerich et al., 2007
	rev	ATGGCTTTGAGCAGGGATAG			
gr2	fw	TGGTGGGCTGCTGGATTTCTGC	240	AY495372.1	Kiilerich et al., 2011
	rev	CTCCCTGTCTCCCTCTGTCA			
Mr	fw	TCGTCCACAGCCAAAGTGTG	148	AF209873	Designed by authors
	rev	TTCTTCCGGCACACAGGTAG			
11βhsd2	fw	GCTGCCTATACTCTGCCA	74	G934620	Kiilerich et al., 2007
	rev	GCCTGTGATGAAGACAGC			
Star	fw	AGGATGGATGGACCACTGAG	163	DQ415678.1	Arukwe 2005
	rev	TTCTCCCATCTGCTCCATGT			
mc2r	fw	AACCTCCACTCCCCGATGTA	95	NM_001124680.1	Designed by authors
	rev	ACCATCATCAGGGTCTCCCA			

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Author contribution statement

A.M., R.E.O., T.S.K., L.O.E.E., G.F. and M.G. conceived and designed the experiments; A.M., R.E.O., T.S.K., L.O.E.E., T.O.N. and M.G. carried out the experiments and data acquisition; A.M., R.E.O., T.S.K., L.O.E.E., G.F. and M.G. analysed and interpreted the data; A.M., R.E.O. and M.G. drafted the manuscript; all authors critically revised the manuscript.

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Figures

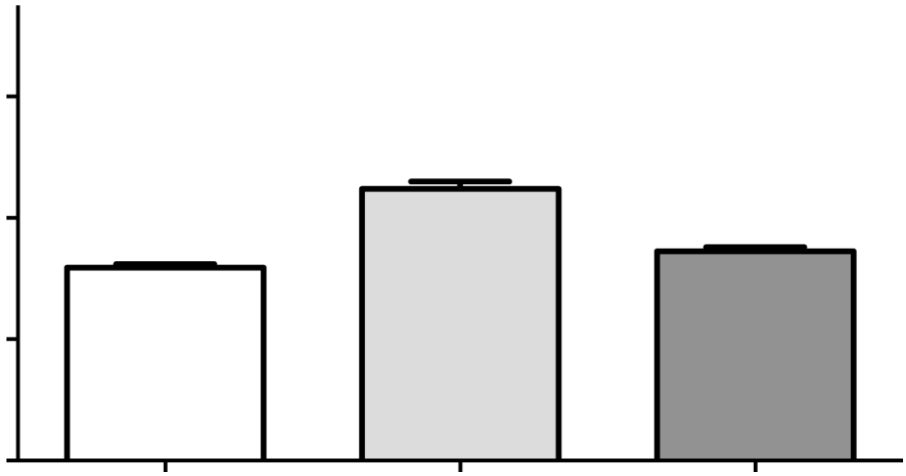

Fig 1: Body weight of Atlantic salmon parr fed *ad libitum*, at the beginning and at the end of the experiment. Bars represent mean + s.e.m, columns with different letters are significantly different from one another (one-way ANOVA followed by Sidak's post-hoc test; $P < 0.025$). Number of fish are $n = 45$, 60 and 71 for control day 0, after 23 days in optimal breeding conditions and after 23 days under the effect of chronic stress respectively.

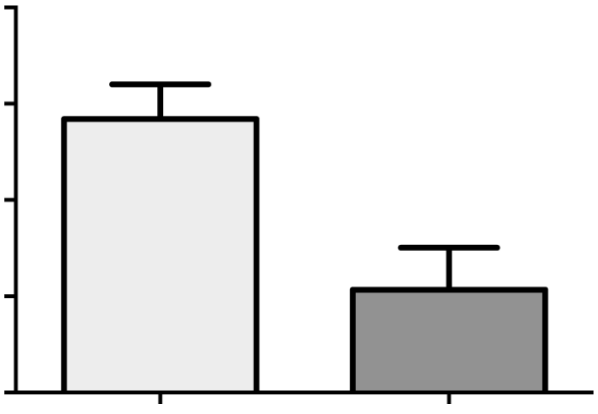

Fig 2: Specific growth rate (SGR) of Atlantic salmon. SGR is expressed as % body weight gain per day (g / day), of salmon parr fed *ad libitum* (control) and of fish exposed to unpredictable chronic stress for 23 days. Values represent means ± s.e.m. ($n = 3$; Mann Whitney *U*-test; $P < 0.05$).

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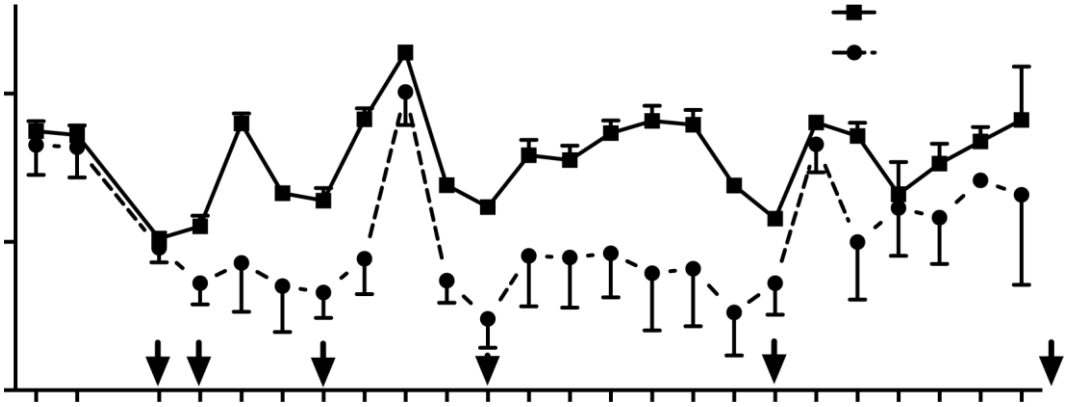

Fig 3: Daily food consumption of Atlantic salmon during the experiment. Food intake is expressed as gram dry food per kg of fish. Control groups are indicated with a solid line and chronically stressed salmon with a dashed line. Each point represents the mean value ± s.e.m. of three replicates. The experiment started on the 4th of February 2013 and samplings were finished on February 26th. Arrows indicate the sampling days.

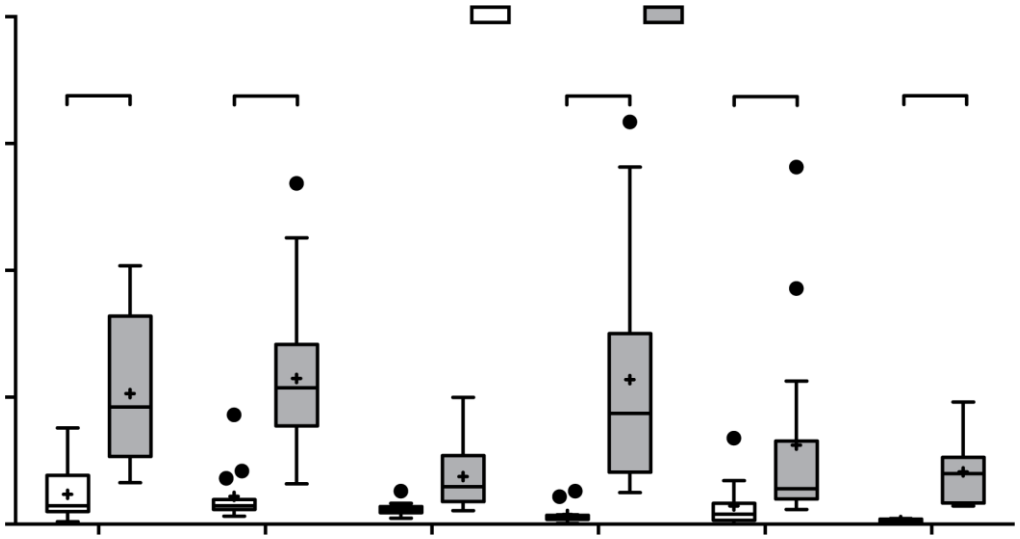

Fig 4: Plasma cortisol levels of Atlantic salmon parr control (white boxes) and chronically stressed for 23 days (grey boxes). Fish were sampled six times along the experiment, *viz*. at day 1, 2, 5, 9, 16 and 23. Values are represented as Tukey box-and-whisker plots; the lines represent the medians, a '+' represents the mean, dots outside the box-and-

whiskers represent outliers (n = 15). Two-way ANOVA analysis revealed a significant interaction effect (chronic stress × day of the experiment): F = (5, 152) = 4.860; P = 0.0004. Asterisks indicate the degree of significance (****: $P < 0.0001$; **: $P < 0.01$) as assessed by Fisher's LSD post-hoc test.

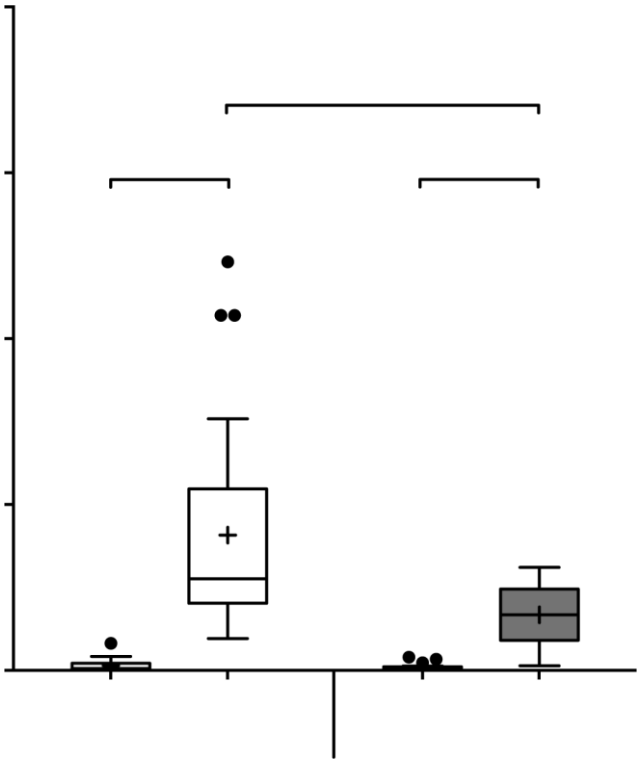

Fig 5: Plasma cortisol levels of control and chronically stressed Atlantic salmon parr before and after a novel stressor on the final day of the experiment. Plasma cortisol was measured for both unpredictable chronic stressed (grey boxes) and control (white boxes) groups before the stress (T0) and 1 h after a novel stressor (T1) that consisted of handling and confinement (5 fish in 10 litre). Values are represented as Tukey box-and-whisker plots; the lines represent the medians, a '+' represents the mean, dots outside the box-and-whiskers represent outliers (n = 30). The α-level is adjusted to correct for increased Type-I error. Two-way ANOVA analysis revealed a significant interaction effect (chronic stress × novel stressor): F = (1, 112) = 4.819; P = 0.0302. Asterisks indicate the degree of significance (****: $P < 0.0001$; **: $P < 0.01$) as assessed by Tukey post-hoc test.

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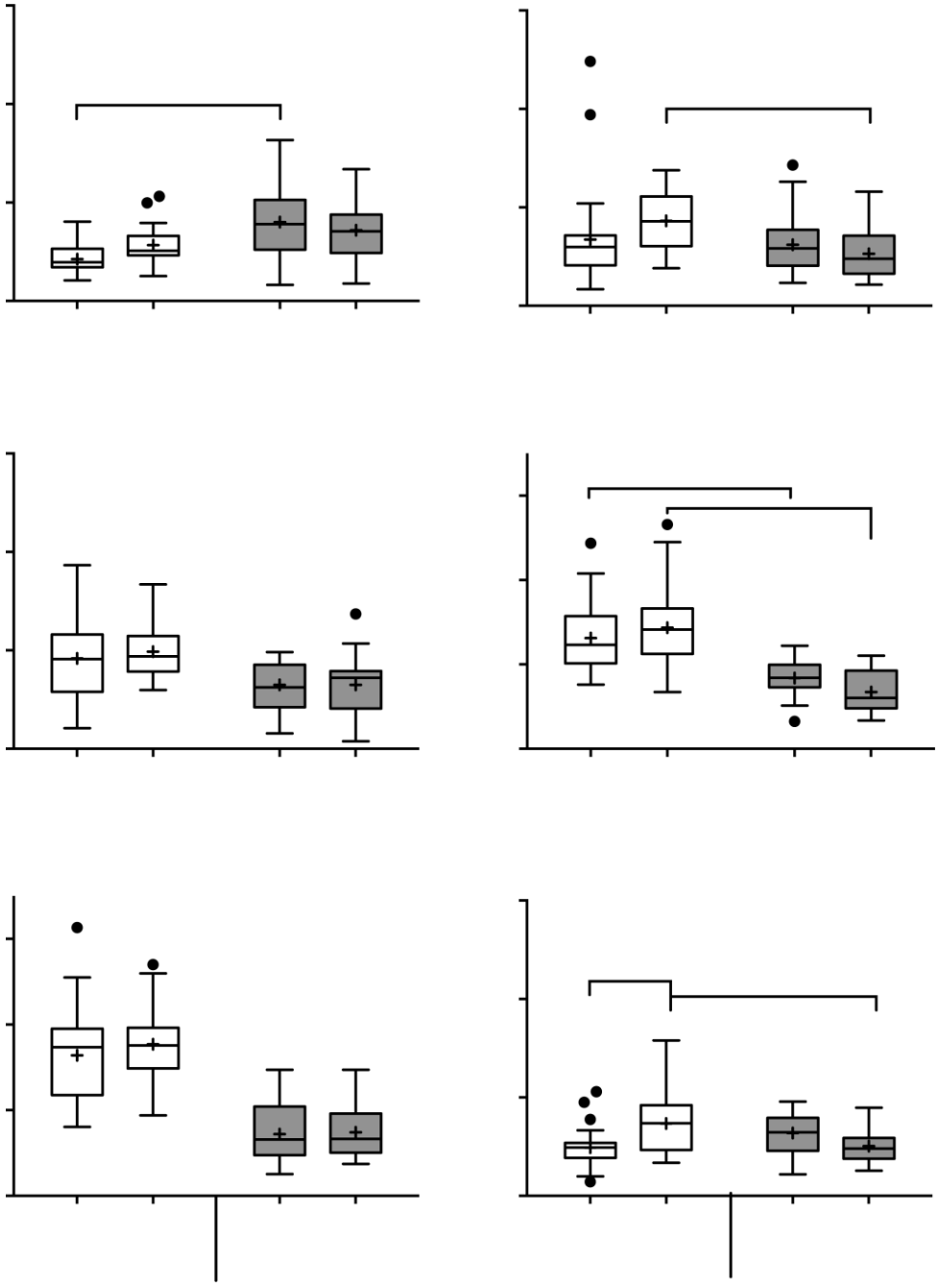

Fig 6: Gene study in the preoptic area (POA). Control (white boxes) and chronically stressed parr (grey boxes), before (T0) and one hour after (T1) being exposed to a novel stressor. Selected genes were: *crf* (A), *crfbp* (B), *11βhsd2* (C), *gr1* (D), *gr2* (E) and *mr* (F). Values are represented as Tukey box-and-whisker plots; the lines represent the medians, a '+' represents the mean, dots outside the box-and-whiskers represent outliers (n = 30). The α-level is adjusted to correct for increased Type-I error. Asterisks indicate the degree of significance (****: $P < 0.0001$; ***: $P < 0.001$; **: $P < 0.01$) as assessed by Tukey post-hoc test.

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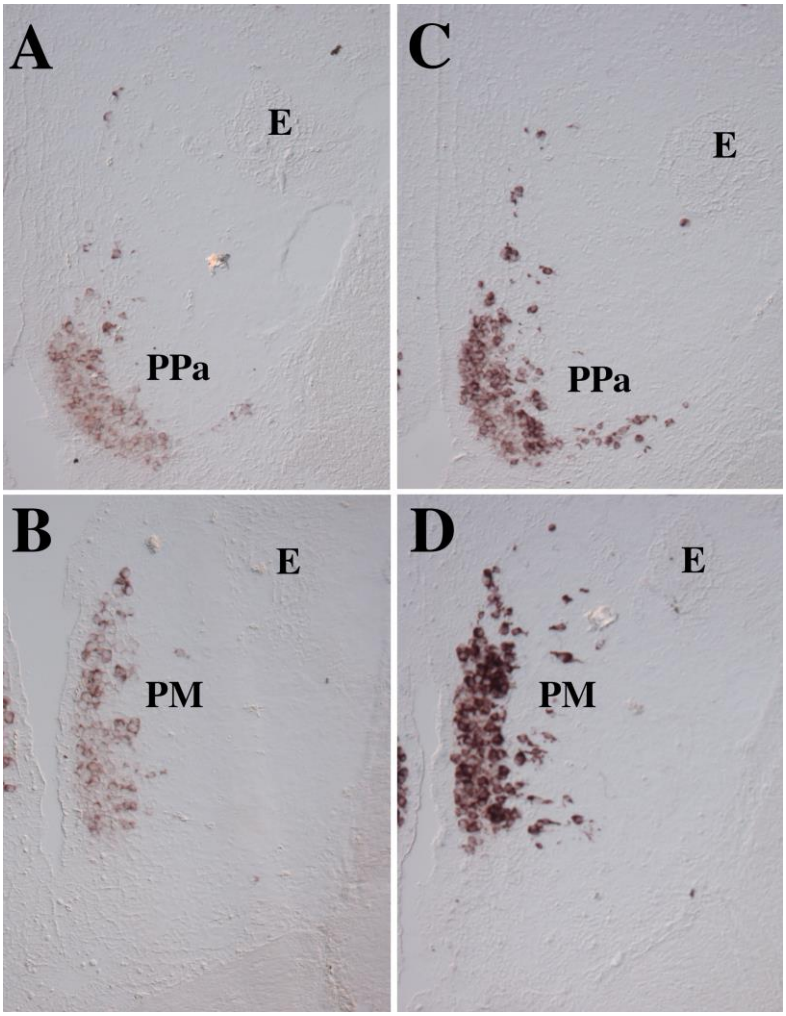

Fig 7: *crf* mRNA localisation in salmon parr brain of control and UCS fish in resting condition (T0). In-situ hybridisation shows *crf* mRNA in the *nucleus preopticus parvocellularis pars anterior* (PPa; A and C) and the *nucleus preopticus magnocellularis* (PM; B and D) of the pre-optic area (POA). Staining in chronically stressed fish (C and D) is dramatically increased compared to control fish (A and B). Images shown are from a single fish.

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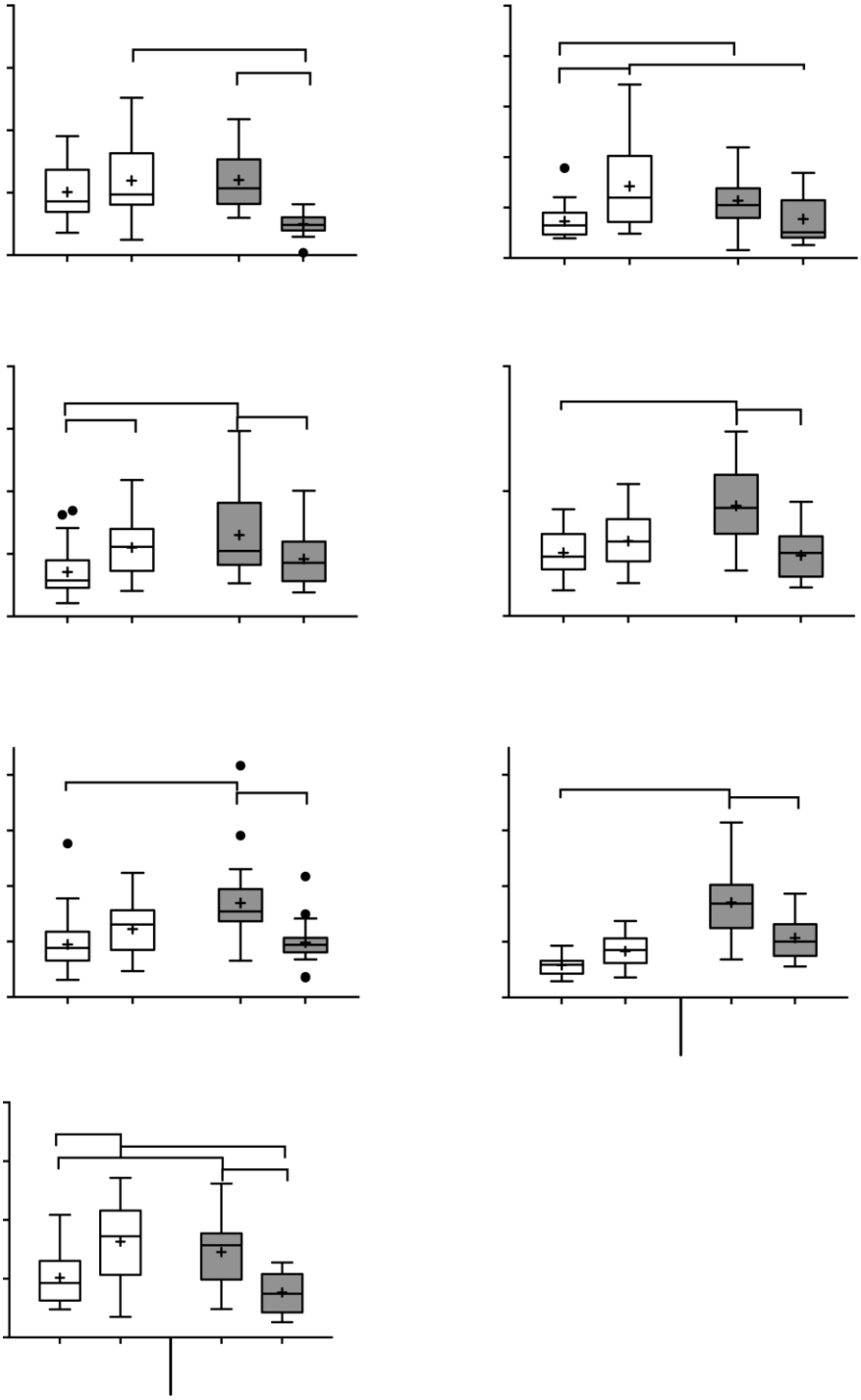

Fig 8: Gene study in the pituitary gland. Control (white boxes) and chronically stressed parr (grey boxes), before (T0) and one hour after (T1) being exposed to a novel stressor. Selected genes are: *crfr1* (A), *pomca1* (B), *pomcb1* (C), *11βhsd2* (D), *gr1* (E), *gr2* (F) and *mr* (G). Values are represented as Tukey box-and-whisker plots; the lines represent the medians, a '+' represents the mean, dots outside the box-and-whiskers represent outliers (n = 30). The α-level is adjusted to correct for increased Type-I error. Asterisks indicate the degree of significance (**: $P < 0.01$; ****: $P < 0.0001$) as assessed by Tukey post-hoc test.

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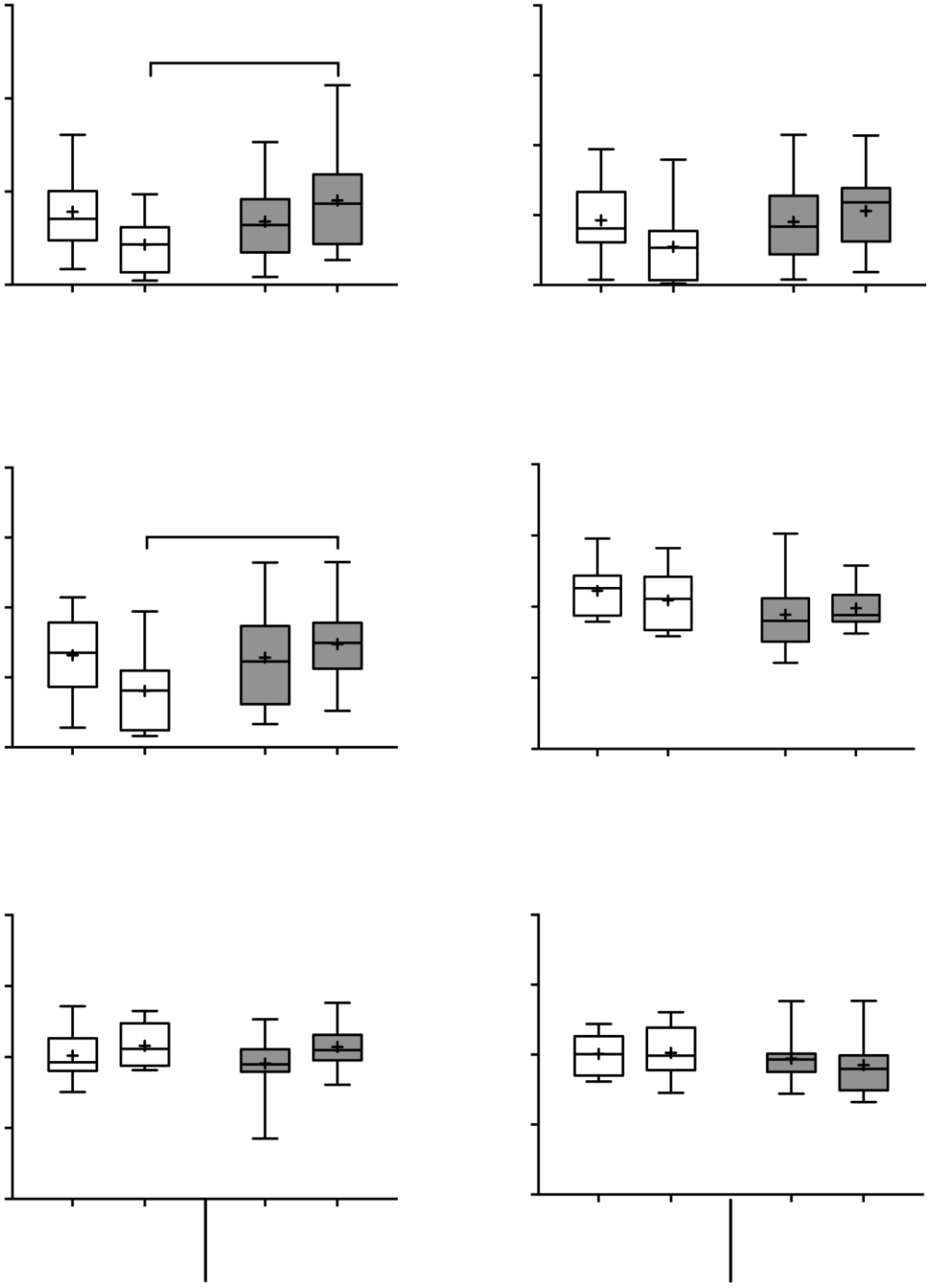

Fig 9: Gene study in the in the head kidney. Control (white boxes) and chronically stressed parr (grey boxes), before (T0) and one hour after (T1) being exposed to a novel stressor. Selected genes are: *mc2r* (A), *star* (B), *11βhsd2* (C), *gr1* (D), *gr2* (E) and *mr* (F). Values are represented as Tukey box-and-whisker plots; the lines represent the medians, a '+' represents the mean, dots outside the box-and-whiskers represent outliers (n = 15). The α-level is adjusted to correct for increased Type-I error. Asterisks indicate the degree of significance (****: $P < 0.0001$) as assessed by Tukey post-hoc test.