

RESEARCH ARTICLE

Stress in Atlantic salmon: response to unpredictable chronic stress

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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 an unpredictable chronic stressor (UCS) paradigm for 3 weeks. The paradigm involves exposure of fish to seven 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 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 with the control group. In the preoptic area (POA) and pituitary gland, chronic stress resulted in decreased gene expression of *11βhsd2*, *gr1* and *gr2* in the POA and increased expression of those genes in the pituitary gland. POA *crf* expression and pituitary expression of *pomcs* 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*, *pomcs*, *11βhsd2*, *grs* and *mr* were down-regulated. In summary, our results provide a novel overview of the dynamic changes that occur at every level of the hypothalamic-pituitary gland–interrenal gland (HPI) axis as a result of chronic stress in Atlantic salmon.

KEY WORDS: *Salmo salar*, Parr, Cortisol, HPI axis, *crf*, Gene study, Brain, Hypothalamus, Pituitary gland, Head kidney

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 the definition of stress intensity. As opposed to wild fish, animals under farming conditions are 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 they have great resilience to a single stressor given repeatedly over long periods of time; however, knowledge on how ectotherms respond to multiple persistent stressors is very scant. One study on Atlantic salmon (*Salmo salar*, L.) has shown that chronic stress followed by an additional maze challenge resulted in a suppressed cortisol response, and 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 adrenaline and noradrenaline into the bloodstream 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 adrenocorticotrophic hormone (ACTH) and released into the bloodstream (Sumpter et al., 1986). In the interrenal gland, ACTH induces the synthesis and release of cortisol via the melanocortin 2 receptor (MC2R) (Wendelaar Bonga, 1997), which is expressed exclusively on cortisol-producing cells therein. MC2R activation results in the activation of steroidogenic 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 the 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; Bemier 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 (Mommensen et al., 1999). CRF binding protein (CRF-BP) provides yet another way of controlling 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.

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List of symbols and abbreviations

11 β HSD2	11- β -hydroxysteroid dehydrogenase 2
20S	20S ribosomal protein
ACTH	adrenocorticotrophic hormone
CRF	corticotropin-releasing factor
CRFBP	corticotropin-releasing factor binding protein
E	entopenduncular nucleus
EF1 α	elongation factor 1 α
GR	glucocorticoid receptor
HPI	hypothalamic-pituitary gland–interrenal gland
MC2R	melanocortin 2 receptor
MR	mineralocorticoid receptor
MSH	melanocyte-stimulating hormone
PM	preopticus magnocellularis
POA	preoptic area
POMC	pro-opiomelanocortin
PPa	preopticus parvocellularis pars anterior
SGR	specific growth rate
STAR	steroidogenic acute regulatory protein
UCS	unpredictable chronic stress

In the short term, the stress response mostly works to the benefit of the animal: faced with a danger or challenge, the concerted action of the catecholamines and cortisol increases heart rate and blood flow to the muscles and increases 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 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 with 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). In contrast, 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 has 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. Although effects of single, acute stressors have been studied in Atlantic salmon, knowledge of 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 food intake and growth throughout the experiment. Plasma cortisol levels were analysed at regular intervals throughout the experiment. After 3 weeks of UCS, all fish were subjected to yet another, novel stressor and sampled before and 1 h after this novel stressor. Total mRNA levels of *crf*, *crfbp*, *11 β hsd2*, *gr1*, *gr2* and *mr* in the POA, *crfr1*, *pomca1* and *pomcb1*, *11 β hsd2*, *gr1*, *gr2* and *mr* in the pituitary gland and *mc2r*, *star*, *11 β hsd2*, *gr1*, *gr2* and *mr* in interrenal tissue were analysed at both these time points.

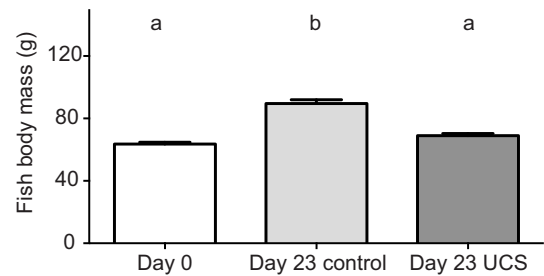


Fig. 1. Body mass of Atlantic salmon parr fed *ad libitum*, at the beginning and at the end of the experiment. Bars represent means \pm s.e.m.; different letters indicate a significant difference (one-way ANOVA followed by Sidak's *post hoc* test; $P < 0.025$). Number of fish: $N = 45, 60$ and 71 for control day 0, after 23 days in optimal breeding conditions and after 23 days under the effect of unpredictable chronic stress (UCS), respectively.

RESULTS**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 the study to 89.5 ± 2.4 g; the chronically stressed (hereafter, UCS) 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 UCS 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 food intake was observed in all groups.

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 with controls.

There was a significant interaction effect (UCS \times acute stressor: $F_{1,111} = 15.61$, $P = 0.0001$) on cortisol levels after 23 days. Cortisol increased in both groups after the stress test, but less so for UCS fish (91.26 ± 25.05 ng ml $^{-1}$) than for control fish (163.3 ± 21.98 ng ml $^{-1}$; Fig. 5).

Gene study**POA**

Fig. 6 shows the transcript abundance of selected genes in the POA of control and UCS fish before and 1 h following exposure to a novel stressor on day 23. A significant interaction (UCS \times acute stressor) was observed in *crf* expression ($F_{1,103} = 4.830$, $P = 0.03$).

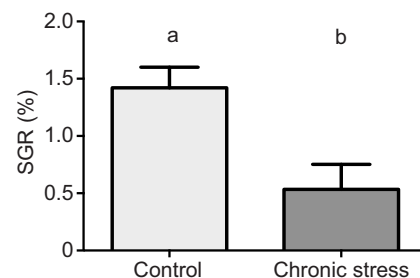


Fig. 2. Specific growth rate of salmon. Specific growth rate (SGR) is expressed as a percentage of body mass gain per day (g day $^{-1}$), of salmon parr fed *ad libitum* (control) and of fish exposed to UCS for 23 days. Values represent means \pm s.e.m. ($N = 3$; Mann–Whitney *U*-test, $P < 0.05$).

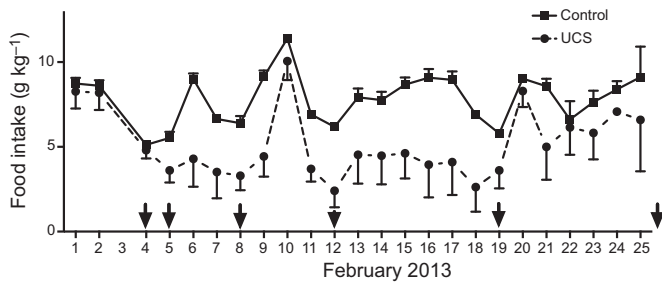


Fig. 3. Daily food consumption of salmon during the experiment. Food intake is expressed as food mass relative to the body mass of the fish for control and UCS groups. Each point represents the mean \pm s.e.m. of three replicates. The experiment started on the 4 February 2013 and samplings were finished on 26 February. Arrows indicate the sampling days.

Fish subjected to 23 days of chronic stress showed elevated basal levels of *crf* mRNA (Fig. 6A); 1 h after exposure to the novel stressor, expression levels of *crf* in control and UCS fish had not changed. A significant interaction effect was also observed for *crfbp* (UCS \times acute stressor: $F_{1,105}=4.723$, $P=0.032$). There were no differences in basal transcript abundance for *crfbp* between control and UCS fish. However, 1 h after exposure to the acute stress, *crfbp* expression was significantly higher in the control group compared with the chronically stressed group (Fig. 6B). mRNA for *11 β hsd2* (Fig. 6C) and the glucocorticoid receptors *gr1* and *gr2* (Fig. 6D, E) showed similar patterns: a lower amount of transcript in UCS fish compared with controls but no effect of the novel stressor. An interaction was seen in mineralocorticoid receptor (*mr*) mRNA levels (UCS \times acute stressor: $F_{1,103}=17.9$, $P<0.001$): 23 days of chronic stress had no effect on basal *mr* transcript abundance, whereas the acute challenge increased *mr* mRNA in control fish but not in UCS fish (Fig. 6F).

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. 7C, D) compared with control (Fig. 7A, B). The images shown are from a single fish per treatment.

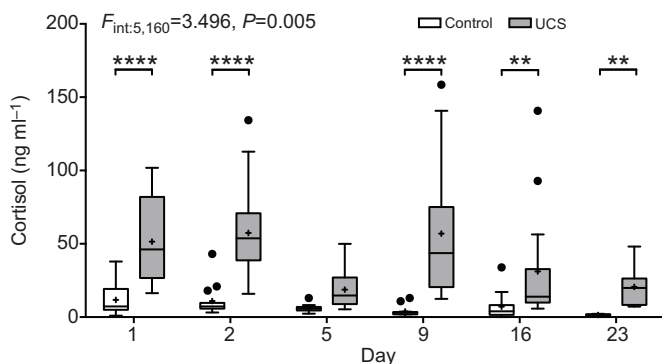


Fig. 4. Plasma cortisol levels of salmon parr under control and UCS conditions. Fish were sampled six times during the experiment: at days 1, 2, 5, 9, 16 and 23. Values are represented as Tukey box-and-whisker plots; the lines represent the medians, a '+' represents the mean and dots represent outliers ($N=15$). Two-way ANOVA analysis revealed a significant interaction effect (chronic stress \times day of the experiment): $F_{5,160}=3.496$, $P=0.005$. Asterisks indicate the degree of significance (**** $P<0.0001$, ** $P<0.01$) as assessed by Fisher's LSD *post hoc* test.

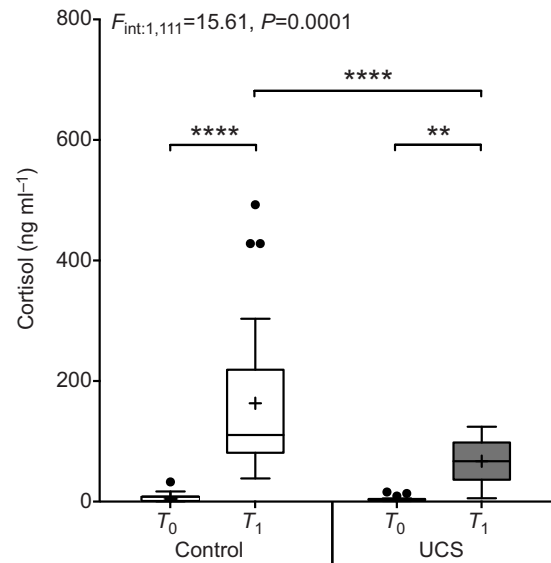


Fig. 5. Plasma cortisol levels of control and UCS salmon parr before and after a novel stressor on the final day of the experiment. Plasma cortisol was measured for fish from both the UCS and control groups before the stress (T_0) and 1 h after a novel stressor (T_1) that consisted of handling and confinement ($N=5$ fish in 10 l). Values are represented as Tukey box-and-whisker plots; the lines represent the medians, a '+' represents the mean and dots represent outliers ($N=30$). The α -level was adjusted to correct for increased Type-I error. Two-way ANOVA analysis revealed a significant interaction effect (chronic stress \times novel stressor): $F_{1,111}=15.61$, $P=0.0001$. Asterisks indicate the degree of significance (**** $P<0.0001$, ** $P<0.01$) as assessed by Tukey's *post hoc* test.

Pituitary gland

An interaction effect (UCS \times acute stressor: $F_{1,106}=25.78$, $P=0.0001$) was observed for pituitary *crfr1*, where the transcript abundance was similar in the two groups before the novel stress but was down-regulated 1 h afterwards in the UCS fish (Fig. 8A). Differential effects were observed for *pomca1* and *pomcb1* in control and UCS fish (UCS \times acute stressor: $F_{1,109}=26.70$, $P=0.0001$ for *pomca1* and $F_{1,106}=16.49$, $P=0.0001$ for *pomcb1*; Fig. 8B, C). 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 *11 β hsd2*, *gr1*, *gr2* and *mr* was significantly higher in UCS fish compared with controls at (T_0). After the novel stressor, mRNA levels decreased significantly in the chronically stressed group only (Fig. 8D–F). Furthermore, *mr* expression was up-regulated in the control group 1 h after stress (Fig. 8G).

Interrenal gland

The interrenal tissue did not show any basal differences in the amount of transcript (Fig. 9). However, interaction effects were observed for *mc2r* and *11 β hsd2* (Fig. 9A,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.

DISCUSSION

We characterised the stress response of Atlantic salmon parr after exposing them to UCS for 3 weeks. We made several major observations: (1) the UCS resulted in suppression of food intake and growth; (2) the cortisol response abated over the 3 weeks of UCS

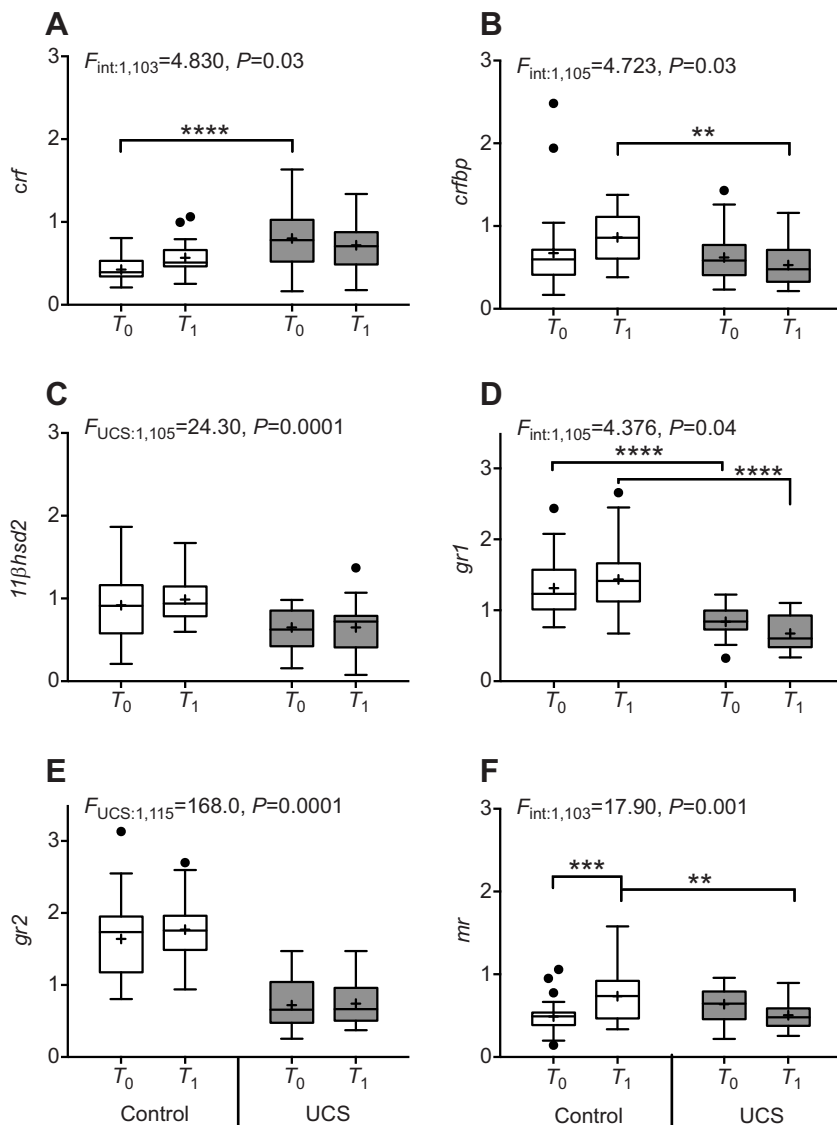


Fig. 6. Gene study in the preoptic area (POA). Control and UCS parr, before (T_0) and 1 h after (T_1) exposure to a novel stressor. The selected genes were: *crf* (A), *crfbp* (B), *11βhsd2* (C), *gr1* (D), *gr2* (E) and *mr* (F) (see Introduction for description). Values were normalised to the reference genes *20S ribosomal protein (20S)* and *elongation factor 1α (ef1α)* and are represented as Tukey box-and-whisker plots; the lines represent the medians, a '+' represents the mean and dots represent outliers ($N=30$). The α -level was 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's *post hoc* test.

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 the genes involved in negative 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 with 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 yet – know how changes in the expression of genes, as measured by mRNA, are representative of 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).

Growth and plasma cortisol

The UCS approach appeared to induce chronic stress as both growth and food intake were reduced over the 3 weeks of the study. To the best of our knowledge, there are no similar studies on the effect of UCS on 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).

POA

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 *in situ* hybridisation with more pronounced staining of hypothalamic *crf* mRNA in the PPa and the PM in UCS fish compared with controls. This increase in *crf* seems at odds with

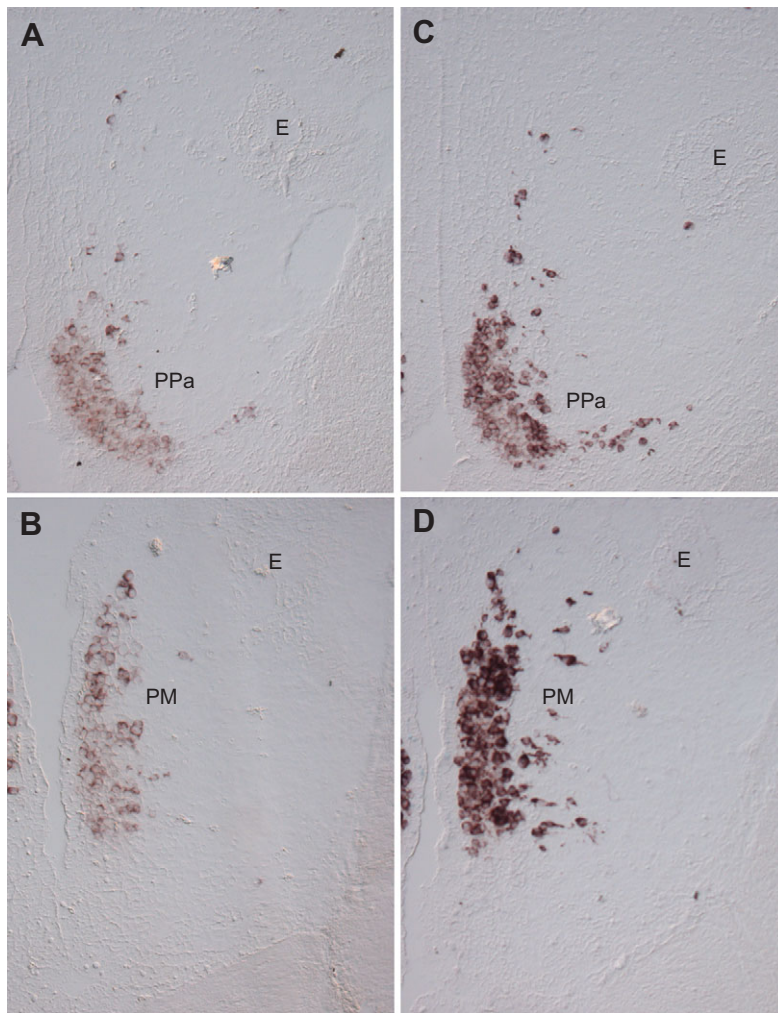


Fig. 7. *crf* mRNA localisation in the brain of control and UCS salmon parr in resting condition (T_0). *In situ* hybridisation shows *crf* mRNA in the nucleus preopticus parvocellularis pars anterior (PPa; A,C) and the nucleus preopticus magnocellularis (PM; B,D) of the POA. Staining in chronically stressed fish (C,D) is dramatically increased compared with that in control fish (A,B). Images shown are from a single fish. E (Entopeduncular nucleus) is indicated in every panel as a reference landmark.

the abated cortisol response, but the high levels of *crf*, together with the high cortisol levels compared with 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). The effect of chronic stress in our study is in line with the findings of 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 glucocorticoid receptors, GR1 and GR2, of which GR2 has an affinity for cortisol that is similar to/higher than that of 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 *gr1* and *gr2* and an increase in *crf* mRNA is in line with previous observations in zebrafish (Chakravarty et al., 2013; Piatto et al., 2011) and trout (Doyon et al., 2005). Low levels of *gr1* and *gr2* may protect

neurons from high levels of cortisol during chronic stress. GR-containing cells are more prone to apoptosis by elevated levels of glucocorticoids (Aluru and Vijayan, 2006; Piatto et al., 2011; Sapolsky et al., 2000). In contrast, *11 β hsd2* 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). The lower *11 β hsd2* transcript level 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 *11 β hsd2* and (chemical) inhibition of *11 β hsd2* activity resulted in increased POA *crf* expression and elevated cortisol levels (Alderman and Vijayan, 2012). Taken together, these findings indicate a pivotal role for *11 β hsd2* in negative feedback control of the stress axis.

The final stress test had no major influences on gene expression in the POA; only in control fish was a rapid up-regulation of *mr* seen. Possibly, MR is the main corticoid receptor responsible for the mediation of cortisol action in the POA in Atlantic salmon after acute stress. In mammals, corticosteroid effects mediated by MRs are involved in suppression of 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 in the sensitivity and feedback responses when high levels of corticosteroids are present (Ratka et al., 1989; Dallman, 1993; Oitzl et al., 1994). In contrast,

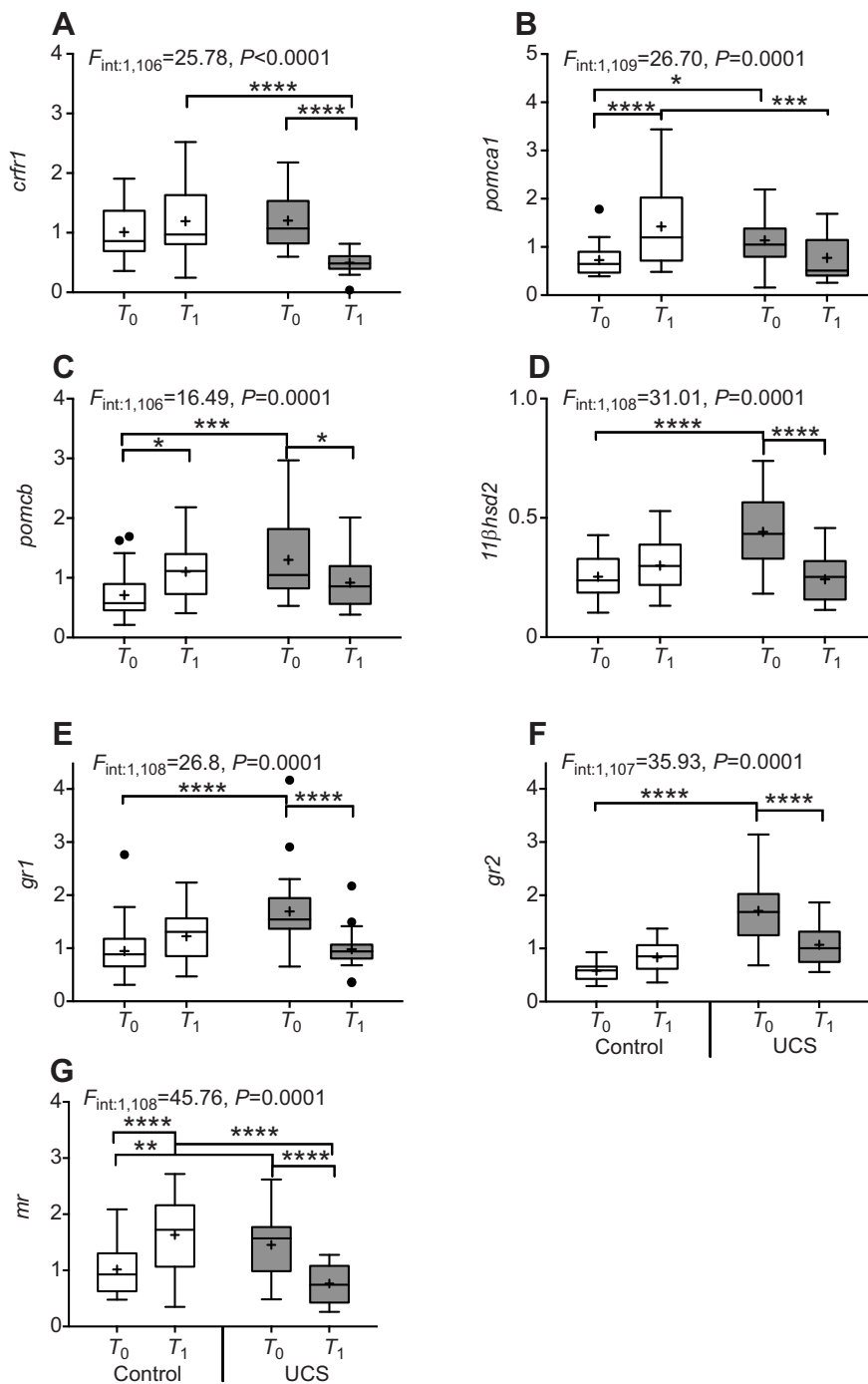


Fig. 8. Gene study in the pituitary gland. Control and UCS parr, before (T_0) and 1 h after (T_1) exposure to a novel stressor. The elected genes were: *crfr1* (A), *pomca1* (B), *pomcb1* (C), *11βhsd2* (D), *gr1* (E), *gr2* (F) and *mr* (G) (see Introduction for description). Values were normalised to the reference genes *20S* and *ef1α* and are represented as Tukey box-and-whisker plots; the lines represent the medians, a '+' represents the mean and dots represent outliers ($N=30$). The α -level was adjusted to correct for increased Type-I error. Asterisks indicate the degree of significance (* $P<0.05$, ** $P<0.01$, *** $P<0.001$; **** $P<0.0001$) as assessed by Tukey's *post hoc* test.

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 the 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 GRs 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.

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 the melanotrope cells (pars intermedia). In the pars distalis, POMC is processed by prohormone convertase 1 to ACTH and β -endorphin,

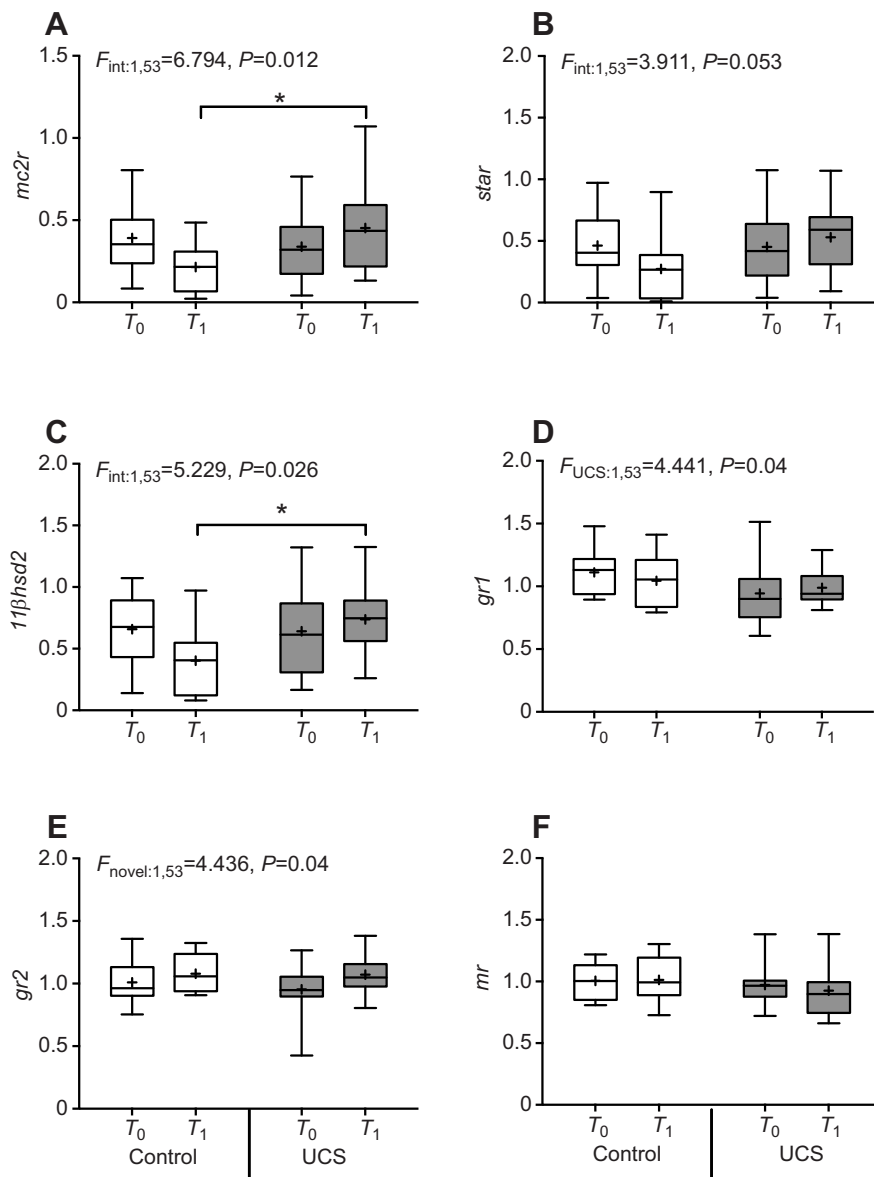


Fig. 9. Gene study in the head kidney. Control and UCS parr, before (T_0) and 1 h after (T_1) exposure to a novel stressor. Selected genes are: *mc2r* (A), *star* (B), *11βhsd2* (C), *gr1* (D), *gr2* (E) and *mr* (F). Values were normalised to the reference genes *20S* and *ef1α* and are represented as Tukey box-and-whisker plots; the lines represent the medians, a '+' represents the mean and dots represent outliers ($N=15$). The α -level was adjusted to correct for increased Type-I error. Asterisks indicate the degree of significance ($*P<0.05$) as assessed by Tukey's *post hoc* test.

whereas in the pars intermedia POMC, through an additional action of prohormone convertase 2, POMC is processed into α -MSH (melanocyte-stimulating hormone) 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 *pomc1* and *pomc1* mRNA in UCS fish compared with controls: in line with these results, in a trout study, Winberg and Lepage (1998) found increased *pomc* expression levels as a result of chronic stress induced by social interactions. In our study, the additional novel stressor resulted in increased *pomc1* and *pomc1* 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 targeted 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 is GR–DNA binding independent, whereas *Pomc* transcription is under negative control by GR–DNA binding (Newton, 2000). In our study, we observed a decrease in POA and an 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 *crf1*, while *pomc* mRNA increased, in agreement with studies in zebrafish (Fuzzen et al., 2010) and trout (Gilchrist 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 was up-regulated but did not/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 receptor 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 a situation, if prolonged, may expose fish to the maladaptive and deleterious effects caused by elevated levels of glucocorticoids following chronic stress.

Interrenal gland

Surprisingly, we did not observe any differences in transcript abundance of genes involved in cortisol production and release (*mc2r* and *star*), inactivation (*11βhsd2*) or cortisol receptors (*gr1*, *gr2* and *mr*) between the groups. An ultra-short, auto-feedback loop for cortisol has been suggested in the teleostean head kidney (Samuel Bradford et al., 1992; Rotllant et al., 2001); however, our results are in line with previous 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*, with higher levels in UCS fish compared with controls. The transcript abundance of *star* followed the same pattern. Fuzzen et al. (2010) observed 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 salmon 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 in both the control and UCS groups in the present study leads to two different hypotheses: first, sampling 1 h post-stress may have been too late to

observe up-regulation of *mc2r* and *star*; second, as also suggested by Geslin and Auperin (2004) for trout, the low transcription in the interrenal gland could be explained by activation 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 as a result of 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.

Conclusions and 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 UCS 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 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 in both chronically stressed fish and controls. As predicted, gene expression data from the POA and pituitary gland demonstrate that control fish respond readily to an acute stressor (Wendelaar Bonga, 1997). In contrast, UCS fish were characterised by a dampened cortisol response that correlates with changes in stress axis-related gene expression at the level of the 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 interrenal cortisol production increased. After the novel stressor, mRNA levels of the stress axis regulators in the central part of the HPI axis were depleted. The interval 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 a situation, the effect of each stress episode may become cumulative and push the fish tolerance capabilities until disease and death occur.

Table 1. Description of the stressors randomly given to Atlantic salmon parr throughout the experiment

Stressor	Time (min)	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 s)	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	Knocking on the tank with a metal object	Slabbekoom, 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

Stressors were given three times per day, for 23 days.

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

Gene	Primer sequences (5'→3')	Amplicon (bp)	Accession no.	References
<i>ef1a</i>	F CCCCTCCAGGACGTTTACAAA	57	AF321836	Olsvik et al., 2005
	R CACACGGCCACAGGTACA			
20S	F GCAGACCTTATCCGTGGAGCTA	85	BG936672	Olsvik et al., 2005
	R TGGTGATGCGCAGAGTCTTG			
<i>crf</i>	F AACCAAGCTCGACACTCGATGG	135	BT057824	L.O.E.E., unpublished sequence
	R GCTATGGGCTTGTGCTGTAAGT			
<i>crfbp</i>	F TGAGCCCAACCAGGTCATCAATGT	85	BT059529	Leong et al., 2010
	R TCCCTTCATCACCCAGCCATCAAA			
<i>crfr1</i>	F TGACCATCTGGGCTGTTGTGATCT	81	GBRB01035702	L.O.E.E., unpublished sequence
	R TAAGATTGGTGGACAGCAGGAGCA			
<i>pomca1</i>	F CCCATCCAGCACTGTAGGAC	112	NM_001198575.1	Leong et al., 2014 direct submission
	R CATTGCTGGGTCAGAGGACA			
<i>pomcb1</i>	F CAATCCACAAGCCCTGTAT	75	NM_001128604.1	Designed by authors
	R TTCTGCTGCCCTCCTCTACT			
<i>gr1</i>	F ACGACGATGGAGCCGAAC	106	AF209873.1	Kiilerich et al., 2007
	R ATGGCTTTGAGCAGGGATAG			
<i>gr2</i>	F TGGTGGGCTGCTGGATTCTGC	240	AY495372.1	Kiilerich et al., 2011
	R CTCCTGTCTCCCTCTGTCA			
<i>Mr</i>	F TCGTCCACAGCCAAAGTGTG	148	AF209873	Designed by authors
	R TTCTTCCGGCACACAGGTAG			
<i>11βhsd2</i>	F GCTGCCTATACTCTGCCA	74	G934620	Kiilerich et al., 2007
	R GCCTGTGATGAAGACAGC			
<i>Star</i>	F AGGATGGATGGACCACTGAG	163	DQ415678.1	Arukwe, 2005
	R TTCTCCCATCTGCTCCATGT			
<i>mc2r</i>	F AACCTCCACTCCCCGATGTA	95	NM_001124680.1	Designed by authors
	R ACCATCATCAGGGTCTCCCA			

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 programmes, 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.

MATERIALS AND METHODS

Fish and experimental facilities

Atlantic salmon (*S. salar*, 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 (12 h light:12 h dark, 9°C). On 11 January 2013, 740 fish (average mass 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⁻¹) 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 mass was recorded for each tank following mild sedation in oxygenated water (25 mg l⁻¹, Finquel[®]vet., ScanAqua AS, Årnes, Norway, buffered with 25 mg l⁻¹ sodium bicarbonate) to reduce handling stress.

Experimental design

At the beginning of the experiment (4 February 2013), the six tanks were divided into two groups of three tanks (replicates) each, receiving UCS or left undisturbed (control group). The UCS group was stressed three times per day (at 08:30 h, 13:00 h and 17:00 h) using a total of eight types of stressors given in random and unpredictable order throughout 1 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 1 h (2 mm Skretting Nutra Olimpic, Stavanger, Norway) 30–60 min after each stress event (i.e. at 09:00 h–10:00 h, 13:30 h–14:30 h and 17:30 h–18:30 h). To study the fish stress response during the experiment, on days 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 1 h, after which the fish were sampled for analysis (T_1). All experiments were approved by the Norwegian Animal Experiment Committee (Forsøksdyrutvalget, 11.12.2012).

Sampling

Fish were starved for 12 h before sampling. Fish received an overdose of anaesthesia (100 mg l⁻¹ Finquel[®]vet.) buffered with 100 mg l⁻¹ sodium bicarbonate (Finquel[®]vet.). Fork length and body mass were recorded for each individual fish. Blood was collected using 1 ml heparinised syringes fitted with a 23 G 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 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 RNA purification (Bernier et al., 2008).

Food consumption and growth rate

Food uneaten after 15 min was collected from each tank after each feeding and left to dry in a colander for 10 min before the mass was recorded. Food consumption was calculated following the method described by Helland and colleagues (1996). The specific growth rate (SGR, % body mass gain day⁻¹) was calculated using Eqn 1:

$$\text{SGR} = \left[\frac{(\log M_2 - \log M_1)}{(t_2 - t_1)} \right] \times 100, \quad (1)$$

in which M_1 is the bulk mass at the start of the growth period (t_1) and M_2 the bulk mass at the end (t_2) (Houde and Schekter, 1981). Both food consumption and final SGR were calculated per tank ($N=3$).

Blood analyses

Plasma cortisol concentrations were quantified by radioimmunoassay (Gorissen et al., 2012) using a highly specific and sensitive commercially available antibody (xm210 cortisol antibody, Abcam, Cambridge, UK) and ³H-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 variation was 12.5% and 3.5%, respectively.

Gene expression analysis

POA, pituitary and head kidney total RNA was isolated with Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions and then dissolved in DEPC-treated H₂O. RNA concentration and purity were determined by spectrophotometry (Nanodrop, Wilmington, DE, USA). A 500 ng sample of total mRNA was incubated for 15 min at room temperature with 1 µl (1 U) DNase I (Invitrogen) in a volume of 10 µl to remove possible genomic DNA contamination. DNase was inactivated by the addition of 1 µl 15 mmol l⁻¹ 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 mmol l⁻¹) dNTP mix, 1 µl 0.1 mol l⁻¹ DTT, 1 µl RNase OUT (10 U µl⁻¹) and 100 U of SuperscriptII Reverse Transcriptase (200 U µl⁻¹; all from Invitrogen) were added and incubated for 10 min at 25°C, 50 min at 42°C and 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 µmol l⁻¹) forward primers, 0.7 µl (10 µmol l⁻¹) reverse primers and 4.6 µl DEPC-treated H₂O. RT-qPCR (3 min at 95°C, 40 cycles of 15 s at 95°C and 1 min at 60°C) was carried out on a CFX96 Touch™ real-time PCR detection system (BioRad). The relative gene expression was analysed according to Vandesompele et al. (2002) and normalised to an index of two reference genes: *20S ribosomal protein (20S)* and *elongation factor 1a (ef1a)*. Sequences of primers used for the qPCR analysis are shown in Table 2.

crf mRNA in situ hybridisation

In situ hybridisation 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 in 0.1 mol l⁻¹ Sørensen phosphate buffer (PB; 28 mmol l⁻¹ NaH₂PO₄, 71 mmol l⁻¹ Na₂HPO₄, pH 7.2). The brains were dissected out and post-fixed in the same fixative for 16 h at 4°C. The tissue was washed 3×20 min 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°C 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 *in situ* hybridisation 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 *wrotensin I* (51%) transcripts (L.O.E.E., T.O.N., M.G. and V. Tronci, unpublished results). The probe was cloned using the following primers: forward 5'-TTTCCTCGTCACCACCGTG-3' and reverse 5'-ATCATTCTTCTGTTGCTATGGGC-3'. The quality and quantity of the synthesised riboprobes were assessed by agarose gel electrophoresis. Prior to *in situ* hybridisation, 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⁻¹ in 0.1 mol l⁻¹ Tris-HCl, pH 8.0) for 3.5 min, post-fixed in 4% paraformaldehyde in KPBS (137 mmol l⁻¹ NaCl, 1.4 mmol l⁻¹ KH₂PO₄, 2.7 mmol l⁻¹ KCl, 4.3 mmol l⁻¹ Na₂HPO₄, pH 7.3) for 5 min, followed by rinsing 2 times 2 min in KPBS. Tissue was then treated with 0.1 mol l⁻¹ triethanolamine (TEA, pH 8.0, Sigma) for 3 min and then with 0.25% acetic anhydride (Sigma-Aldrich) in 0.1 mol l⁻¹ TEA for 10 min. Finally, tissue was dehydrated in a graded ethanol series (50–100%) and air dried for 1 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 mmol l⁻¹ Tris-HCl, 300 mmol l⁻¹ NaCl, 20 mmol l⁻¹ EDTA, 0.2% Tween-20, 1% blocking solution (Roche Diagnostics), 0.1% dextran sulphate (Sigma-Aldrich), 50% deionised formamide (Sigma-Aldrich). Incubation was carried out at 65°C for 16 h, using humidity chambers and hybri-slips (Sigma-Aldrich) 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 65°C, 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-Aldrich) at 37°C, and washed 20 min at 65°C. The sections were incubated for 1 h 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 Diagnostics). The tissue was washed 2 times 10 min in 1× maleate buffer and then 10 min in visualisation buffer (100 mmol l⁻¹ Tris-HCl, 100 mmol l⁻¹ 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⁻¹ levamisole in visualisation buffer] was carried out for 3 h in darkness at room temperature. The reaction was terminated with stop solution (10 mmol l⁻¹ Tris-HCl, 1 mmol l⁻¹ EDTA, 150 mmol l⁻¹ 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.

Statistics

Values are represented as means±s.e.m. or as a Tukey box-and-whiskers plot. Differences in SGR were assessed with Mann–Whitney *U*-test, while body mass data were analysed with one-way ANOVA followed by Sidak's *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. in the case of comparison of cortisol levels between control and UCS between days). 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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Competing interests

The authors declare no competing or financial interests.

Author contributions

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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