Comparative Biochemistry and Physiology, Part A xxx (2015) xxx–xxx

Contents lists available at ScienceDirect

Comparative Biochemistry and Physiology, Part A

journal homepage: www.elsevier.com/locate/cbpa

# A comparative study of the response to repeated chasing stress in Atlantic salmon (*Salmo salar* L.) parr and post-smolts

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ARTICLE INFO

*Article history:*
Received 10 June 2015
Received in revised form 22 October 2015
Accepted 3 November 2015
Available online xxxx

*Keywords:*
*Salmo salar*
Pituitary gland
Cortisol
HPI-axis
Gene expression
Brain
Allostasis

ABSTRACT

When Atlantic salmon parr migrate from fresh water towards the sea, they undergo extensive morphological, neural, physiological and behavioural changes. Such changes have the potential to affect their responsiveness to various environmental factors that impose stress. In this study we compared the stress responses in parr and post-smolt salmon following exposure to repeated chasing stress (RCS) for three weeks. At the end of this period, all fish were challenged with a novel stressor and sampled before ($T_0$) and after 1 h ($T_1$). Parr had a higher growth rate than post-smolts. Plasma cortisol declined in the RCS groups within the first week suggesting a rapid habituation/desensitisation of the endocrine stress axis. As a result of the desensitised HPI axis, RCS groups showed a reduced cortisol response when exposed to the novel stressor. In preoptic area (POA) *crf* mRNA levels were higher in all post-smolt groups compared to parr. *11βhsd2* decreased by RCS and by the novel stressor in post-smolt controls ($T_1$), whereas no effect of either stress was seen in parr. The *grs* were low in all groups except for parr controls. In pituitary, parr controls had higher levels of *crf1r* mRNA than the other parr and post-smolt groups, whilst *pomcb* was higher in post-smolt control groups. Overall, *11βhsd2* transcript abundance in parr was lower than post-smolt groups; after the novel stressor *pomcs*, *grs* and *mr* were up-regulated in parr control ($T_1$). In summary, we highlight differences in the central stress response between parr and post-smolt salmon following RCS.



## 1. Introduction

The life cycle of Atlantic salmon (*Salmo salar* L.) is characterised by two major migrations, from fresh water where the fish are born, to the rich feeding grounds in sea, and back again into fresh water to breed. From hatch to the parr stage, Atlantic salmon can spend up to four years in fresh water. Then, in spring, due to external cues like natural photoperiod (Duston and Saunders, 1990) and water temperature (Stefansson et al., 1998), the fish undergo parr-smolt transformation. This involves a plethora of behavioural, morphological and physiological changes to prepare the smolt to live in seawater (Carey and McCormick, 1998; Ebbesson et al., 2003, 2007; Handeland et al., 2003a). These changes include silvering of the skin, darkening of the fin margins, increased seawater tolerance by changes in gill $Na^+/K^+$-ATPase activity and gene expression (Nilsen et al., 2007) and structural and chemical reorganisation of the neuronal circuits (Ebbesson et al., 2003, 2007, 2011; Lorgen et al., 2015). Several endocrine changes drive this parr-smolt transformation. For example, prior to seawater migration plasma cortisol levels rise, as do the levels of growth hormone, insulin-like growth factor I (IGF-I) and thyroid hormone (Ebbesson et al., 2011; Lorgen et al., 2015).

Before smoltification, parr are aggressive, territorial 'sit and wait' predators (Yamamoto and Keenleyside, 1962; Wańkowski, 1981) and defending available food resources against competitors. After smoltification, the fish become non-aggressive and form schools in which they start migration downstream. A structural and biochemical reorganisation of the brain during smoltification is likely to explain the behavioural differences between parr and post-smolt fish (Ebbesson et al., 2003).

In teleostean fishes, the stress response is mainly conveyed by the hypothalamus–pituitary–interrenal axis (HPI axis). When a fish is subjected to a stressor, neuronal signals from the hypothalamus initiate a downstream activation of sympathetic fibres releasing catecholamines from the chromaffin cells of the head kidney into the blood stream. The catecholamines have an immediate effect on glycogen stores to increase glucose availability, and also increase heart rate, gill blood flow and oxygen uptake (to facilitate a fight/flight response). Stressors

*Abbreviations:* POA, preoptic area; RCS, repeated chasing stress; SGR, specific growth rate.

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http://dx.doi.org/10.1016/j.cbpa.2015.11.005
1095-6433/

activate the HPI axis and sequentially the release of Corticotropin Releasing Factor (CRF) from the hypothalamic preoptic area (Wendelaar Bonga, 1997). In the pituitary, CRF via its receptor CRF-R1 induces the synthesis of pro-opiomelanocortin (POMC), which is then processed into adrenocorticotropic hormone (ACTH). ACTH induces the synthesis and release of cortisol from the interrenal gland.

In fish, cortisol exerts both glucocorticoid and mineralocorticoid actions (Wendelaar Bonga, 1997) which are mediated through either glucocorticoid receptors (GR) or mineralocorticoid receptors (MR). These receptors operate as transcription factors and, after cortisol binding, activate or inhibit transcription of target genes. GR and MR are also involved in the modulation of the HPI axis as these receptors convey the cortisol negative feedback at multiple levels including hypothalamus and pituitary gland (Bernier et al., 1999; Cole et al., 2000; Bury et al., 2003; Doyon et al., 2006; Atkinson et al., 2008). For instance, cortisol affects CRF synthesis (Bernier et al., 1999; Bernier and Peter, 2001) and ACTH secretion from the pituitary gland. Cortisol can be deactivated by 11β-hydroxysteroid dehydrogenase 2 (11β-HSD2), an enzyme that converts cortisol into the inactive cortisone (Mommsen et al., 1999). Another mechanism of control over the HPI axis is represented by CRF binding protein (CRF-BP), which modulates the effect of CRF and CRF-related peptides by binding and reducing their bioavailability (Seasholtz et al., 2002; Geven et al., 2006; Huising et al., 2008; Manuel et al., 2014).

The complex nature of the neural, behavioural and physiological changes that take place in the "smoltification window", affects the fish' sensitivity to stress (Barton et al., 1985). Carey and McCormick (1998) suggested that smolts in fresh water (defined as fish older than one year) are more susceptible to stress than parr due to developmental differences reflected in ion balance, osmotic regulation and higher cortisol concentrations post-stress. Damsgård and Arnesen (1998) showed that Atlantic salmon smolts transferred from fresh water into seawater (post-smolts) had reduced food intake after acclimation to seawater. It has accordingly been suggested that smolts or early post-smolts may be particularly sensitive to stress and should be handled with care when exposed to transportation (Iversen et al., 1998), handling or crowding (Carey and McCormick, 1998), particularly for the risk of scale loss and subsequent infections (Bruno et al., 2013).

The aim of this study was to compare the stress response of fresh water parr with that of seawater post-smolts. Therefore, fish were subjected to 23 days of repeated chasing stress (RCS; 5 min twice daily). We analysed plasma cortisol levels at regular intervals and calculated the growth and the specific growth rate (SGR) for 23 days. At the end of the study, all groups of fish were subjected to a novel stressor (netting, air exposure for 15 s and confinement for 5 min in a 10-L container) and sampled before (0 h) and after (1 h) stress. Further, transcript abundance of *crf*, *crfbp*, *11βhsd2*, *gr1*, *gr2* and *mr* in preoptic area (POA), and *crfr1*, *pomc-a1* and *pomc-b1*, *11βhsd2*, *gr1*, *gr2* and *mr* in the pituitary gland was analysed at both these time points.

## 2. Materials and methods

### 2.1. Fish and experimental facilities

Atlantic salmon (*S. salar* L., AquaGen strain) eggs were obtained from AquaGen Ltd. (Sunndalsøra, Norway), hatched (March 2012) and reared at the Institute of Marine Research (IMR; Matre, Norway). Parr were kept in freshwater with light and temperature according to simulated winter conditions (12L:12D, 9 °C). On January 8th 2013, 740 parr (average body mass 57 g) were transferred from a 10,000-L circular outdoor tank into six 400-L square indoor tanks (~7 kg fish/tank) supplied with flow-through freshwater. Post-smolt production started 12 weeks earlier by light-controlled smoltification (6 weeks 12L:12D followed by 6 weeks 24L:0D, 9 °C) and, on the same day as the parr salmon, 400 post-smolt (average body mass 105 g) were divided into six 400-L square indoor tanks (~7 kg fish/tank; tanks and density were identical as for parr) and supplied with full strength seawater (35 ppt). The tanks were furnished with lids that contained fluorescent light tubes and automatic feeders (Arvo-Tec, Huutokoski, Finland). Feeding (1.5% of the body mass per day, divided over three meals 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). Fish were allowed to acclimatise to the experimental setup for three weeks before the start of the experiment.

### 2.2. Experimental design

The experiment started on February 4th, 2013. The experimental groups (triplicate tanks) were parr control, post-smolt control, parr stressed, and post-smolt stressed. Two groups of parr and two groups of post-smolt were subjected to a RCS protocol that consisted of 5 min chasing with a dip net (Pavlidis et al., 2015) twice a day (at 8:30 h and 17:00 h) for 23 days. Control fish were undisturbed except for routine cleaning of tanks and sampling.

Fish were fed dry pellets for 1 h (Skretting Nutra Olympic 2 mm (parr) and 3 mm (post-smolt)) 30–90 min after each stress episode (9:00–10:00 h and 17:30–18:30 h). To study possible habituation to RCS, on days 1, 2, 5, 9, 16 and 23, 5 fish per tank were collected (3 replicate tanks; $n = 15$) 1 h after the first stress episode of that day (Barton, 2002; Pankhurst et al., 2008). Fish from the undisturbed tanks served as controls (5 fish per replicate tank; $n = 15$).

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 mRNA abundances. Simultaneously, another 10 fish per tank were collected and exposed to a novel stressor that 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 recovery tank (1 h), after which the fish were sampled for analysis ($T_1$). All experiments were approved by the Norwegian Experimental Animal Committee (Forsøksdyrutvalget, 11.12.2012).

### 2.3. Sampling

Fish were fasted for 12 h before each sampling. Fish were sacrificed with an overdose of anaesthesia (100 mg $L^{-1}$, Finquel®vet., ScanAqua AS, Årnes, Norway). For freshwater parr, the anaesthesia solution was 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 23 G needles. Plasma and blood cells were separated immediately by centrifugation (Eppendorf Centrifuge 5415 R, Hamburg, Germany) at 13,000 rpm (i.e. 15,682 ×*g*) for 3 min and stored at −80 °C until cortisol analysis. Pituitary glands and brains were collected and stored in RNAlater (RNA*later*® RNA Stabilization Solution, Life Technology, Oslo, Norway) at 4 °C overnight and subsequently stored at −80 °C until isolation of total RNA. POAs were isolated from brains immediately before the RNA purification according to Bernier et al. (2008).

### 2.4. Specific growth rate

SGR was calculated using Eq. (1):

$$\mathrm{SGR}\left(\%\text{body weight gain} \times \mathrm{day}^{-1}\right) = \left[\frac{\mathrm{Log}\,M_2 . - \mathrm{Log}\,M_1 .}{(t_2 - t_1)}\right] \times 100 \qquad (1)$$

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). The final SGR was calculated per tank ($n = 3$).



Please cite this article as: Madaro, A., et al., A comparative study of the response to repeated chasing stress in Atlantic salmon (*Salmo salar* L.) parr and post-smolts, Comp. Biochem. Physiol., A (2015), http://dx.doi.org/10.1016/j.cbpa.2015.11.005