



# Rapid recovery of the cortisol response following social subordination in rainbow trout



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## HIGHLIGHTS

- Subordinate trout regained a normal cortisol response when separated from a dominant conspecific.
- 'Recovered' subordinates retained aspects of subordinate behaviour, such as low food intake.
- 'Recovered' subordinates failed to achieve metabolic recovery.
- Thus, stress axis recovery precedes behavioural recovery from social subordination.

## ARTICLE INFO

### Article history:

Received 12 March 2016

Received in revised form 29 May 2016

Accepted 13 June 2016

Available online 14 June 2016

### Keywords:

Social stress

HPI axis

Food intake

Specific growth rate

Subordinate behaviour

*Oncorhynchus mykiss*

## ABSTRACT

Rainbow trout (*Oncorhynchus mykiss*) confined in pairs form social hierarchies in which distinctive behavioural and physiological phenotypes distinguish dominant from subordinate fish. In particular, subordinate fish are characterized by inhibition of behaviours such as feeding and activity, by low growth rates, and by chronic elevation of circulating glucocorticoid stress hormone (cortisol) concentrations. To evaluate the ability of trout to recover from chronic social stress, pairs of fish were allowed to interact for 4 d, and subordinate fish were then separated from dominant fish. Recovery was assessed using behavioural (position in the tank, latency to feed, and food consumed) and physiological (plasma cortisol and glucose concentrations, liver glycogen content, hepatosomatic index, specific growth rate, and gall bladder mass) indices. During 48 or 96 h of recovery from the 4 d interaction period, only plasma cortisol and glucose levels of subordinates returned to baseline values consistent with those of dominant and sham trout (fish that were handled like paired fish but housed singly). All other physiological variables failed to recover, likely owing to the absence of behavioural recovery, including continued inhibition of food intake even following separation from the dominant fish. Whereas subordinate fish exhibited an attenuated cortisol response to an acute netting stressor, 'recovered' subordinates mounted a cortisol response that was equivalent to those of dominant and sham fish. However, 'recovered' subordinates that were paired with a socially naïve conspecific were unable to achieve non-subordinate status. Collectively, these results indicate that recovery of the cortisol response precedes behavioural recovery from social subordination.

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## 1. Introduction

Juvenile salmonid fish held in pairs interact to form social hierarchies with one fish becoming dominant over the other, subordinate, fish [2,19,45; reviewed in 24,33,52,58]. These social interactions reflect agonistic competition for access to limited resources, such as food [3,4,40,41] and territory [27,34]. Dominant and subordinate fish can be distinguished by characteristic behavioural differences in activity, feeding and aggression, with subordinates being less active and aggressive,

and consuming less food [e.g. 1,16,39,48]. Differences in brain serotonergic activity (the ratio of 5-hydroxyindoleacetic acid, 5-HIAA, to 5-hydroxytryptamine, 5-HT, where 5-HIAA is the main metabolite of 5-HT, which is also known as serotonin) can be used to further distinguish dominant and subordinate fish. Following initial interaction, brain serotonergic activity rises in both dominant and subordinate fish [50]. While serotonergic activity quickly returns to baseline in dominant fish, elevated levels persist in subordinate fish [50,65,66].

Other physiological differences also distinguish dominant from subordinate fish, particularly with respect to activity of the HPI axis. During the initial stages of interaction, the HPI axis is activated in both fish, resulting in elevated levels of the glucocorticoid stress hormone cortisol [50]. Circulating cortisol levels return to baseline within a few hours in

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dominant fish, but remain elevated in subordinate fish, indicative of chronic stress [19,36,50,53,55,65]. The mechanisms responsible for this chronic activation of the HPI axis in subordinate fish remain unclear. Circulating levels of adrenocorticotropic hormone (ACTH), which stimulates cortisol biosynthesis by interrenal cells, were reduced in subordinate fish, suggesting that negative feedback is operational [31]. At the same time, elevated expression of other components of the HPI axis, including the main ACTH secretagogue, corticotropin releasing factor (CRF) [9,17], argue in favour of enhanced HPI axis activity, perhaps stimulated by the on-going presence of the dominant fish [6,65]. Chronically elevated cortisol levels, in turn, likely contribute to many of the widespread negative consequences of subordinate social status [reviewed in 24], including reduced hepatic glycogen content [19], a reduction in the size of the liver [56], a decreased immune response [51], and attenuation of the cortisol response to an acute stressor [32,49,57]. Together with reduced food intake, which leads to accumulation of bile in the gall bladder [16,18,59], chronic elevation of cortisol remodels metabolic pathways [25], reduces energy stores [7,25] and lowers growth rate [1,7,16,66].

Although the physiological and behavioural changes experienced by subordinate fish during chronic social stress have been well documented [reviewed in 24,33], few studies have assessed the timeline or extent of recovery from chronic social stress [21,48]. Food intake by subordinate fish gradually increased after separation from a dominant conspecific, and brain serotonergic activity fell [48]. However, the capacity for HPI axis recovery from chronic social stress remains to be determined and could provide insight into the mechanisms responsible for chronic HPI axis activation in subordinate fish. Based on the hypothesis that the physiological consequences of social stress are dependent upon continuing contact with a dominant fish, separation of subordinate from dominant trout was predicted to allow subordinate fish to recover, i.e. to return to the physiological condition prior to hierarchy formation, including low baseline cortisol levels and a robust cortisol response to an acute stressor. To test this hypothesis, rainbow trout (*Oncorhynchus mykiss*) were confined with a size-matched conspecific for 4 d to allow social hierarchies to become established, after which the members of a pair were separated and their behaviour and physiology were evaluated for 48 or 96 h to determine the extent of recovery of subordinate fish.

## 2. Materials and methods

### 2.1. Experimental animals

Juvenile female rainbow trout, *Oncorhynchus mykiss* (mass =  $94.0 \pm 1.9$  g, fork length =  $20.2 \pm 0.1$  cm, mean  $\pm$  SEM,  $N = 124$ ), were purchased from Linwood Acres Trout Farm (Campbellcroft, Ontario). All fish were held at the University of Ottawa in 1275 L fibreglass tanks supplied with flowing, aerated, dechloraminated City of Ottawa tap water at a temperature of 13 °C. A 12L:12D photoperiod was maintained, and fish were fed a ration of 0.5% body mass every day by scattering commercial trout pellets on the water's surface. Trout were acclimated to these holding conditions, which served to minimize hierarchy formation (e.g. use of scatter feeding, homogenous tanks with a mild current), for at least 2 weeks prior to experimentation. All experimental protocols were approved by an institutional animal care committee (protocol BL-2118), and were in compliance with the guidelines of the Canadian Council on Animal Care (CCAC) for the use of animals in research and teaching.

### 2.2. Experimental protocols

Three experimental series were carried out. The first compared the behaviour and physiological condition of trout that had been confined in pairs for 4 d ( $N = 6$  pairs, 6 shams), with those that were confined in pairs for 4 d and then separated for 48 h ( $N = 8$  pairs, 6 shams) or

96 h ( $N = 5$  pairs, 6 shams) recovery periods. In the second experimental series, the cortisol response to an acute stressor was examined in fish following 4 d of interaction ( $N = 6$  pairs, 6 shams) or 4 d of interaction plus 48 h of recovery ( $N = 6$  pairs, 6 shams). The goal of the final experimental series was to assess the behaviour of 'recovered' subordinate fish (4 d of social interactions plus 48 h of recovery) during social interactions with a naïve conspecific ( $N = 6$ ).

#### 2.2.1. Social interactions and recovery from social interactions

Fish were lightly anaesthetized to the point of losing equilibrium in a solution of benzocaine ( $0.05 \text{ g L}^{-1}$  ethyl-*p*-aminobenzoate; Sigma-Aldrich), initial mass and fork length were measured, and fin damage was scored (see below). When existing physical differences did not allow for identification of individual fish, clipping of a pectoral fin was used for identification. The fish to form a pair were matched on fork length, with differences not exceeding 5% (fork length difference averaged  $3.0 \pm 0.2$  mm or 1.5% of fork length;  $N = 43$  pairs). After initial assessment, the members of a pair were placed into a 40 L flow-through Plexiglass observation tank, separated from each other by an opaque, perforated divider. Tanks were supplied with flowing, aerated 13 °C water.

Following an overnight recovery period, the divider was removed and the fish were allowed to interact for 4 d. A PVC tube (*t*-shaped,  $11 \times 13$  cm long, 6 cm diameter) was added at the end of the first day of interaction to provide a shelter. Behavioural observations were carried out twice per day at 0900–1200 h and 1500–1800 h, for 5 min each time. Sham-treated animals were included in the experimental design. Trout in the sham group underwent the same handling and treatment as paired fish except that they were held individually. Fish were fed to satiation daily following the final observation period (except on the initial day of interaction).

Social status was assessed by assigning points to each fish for position within the tank, food acquisition, aggressive acts, and fin damage acquired during the interaction period [as previously described in 15, 16,55]. The scoring system awards more points for more dominant behaviours. A principle components analysis (Minitab, v16) was used to calculate a behaviour score for each fish based on the mean scores of each parameter. Within a given pair, the fish with the higher score was assigned dominant status, while the fish with the lower score was deemed to be subordinate (scores averaged  $1.43 \pm 0.10$  for dominant fish and  $-1.54 \pm 0.10$  for subordinate fish; the difference within a pair averaged  $2.97 \pm 0.16$ ,  $N = 43$  pairs). Pairs with similar scores ( $\approx 2\%$  of all pairs) were excluded from further analysis.

Following the interaction period, fish were either euthanized immediately or the opaque, perforated divider was replaced, separating the members of the pair for a recovery period of 48 or 96 h. Behavioural observations were carried out daily, as described above, on each fish during the recovery period, and consisted of noting the position of the fish within the water column, the latency to take a single pellet of food, and the mass of food consumed.

Fish were rapidly euthanized via terminal anaesthesia ( $0.5 \text{ g L}^{-1}$  ethyl-*p*-aminobenzoate), mass and length were measured, and fin damage was scored. Blood samples were collected via caudal venipuncture into heparin-coated syringes ( $2500 \text{ IU mL}^{-1}$  heparin sodium salt, Sigma-Aldrich) and centrifuged ( $10,000g$  for 2 min). Plasma was extracted and flash frozen in liquid nitrogen before being stored at  $-80$  °C for later analysis of cortisol and glucose concentrations. Cortisol levels were measured using a commercially-available radioimmunoassay (RIA; MP Biomedical) that had previously been validated for trout plasma [22]. The kit has a detection limit of  $0.17 \mu\text{g dL}^{-1}$ . Intra-assay variation was 10.5% and inter-assay variation was 14.5% (% CV). Plasma glucose levels were determined in triplicate according to the method described by Keppler and Decker [35]; intra-assay variation was 4.2% and inter-assay variation was 4.7%. Liver and gall bladder masses were measured and a sample ( $\approx 150$  mg) of liver tissue was collected, flash frozen in liquid nitrogen and stored at  $-80$  °C for later analysis of

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