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Modulation of hypothalamic–pituitary–interrenal axis function by social status in rainbow trout

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ABSTRACT

Juvenile rainbow trout (*Oncorhynchus mykiss*) form stable dominance hierarchies when confined in pairs. These hierarchies are driven by aggressive competition over limited resources and result in one fish becoming dominant over the other. An important indicator of low social status is sustained elevation of circulating cortisol levels as a result of chronic activation of the hypothalamic–pituitary–interrenal (HPI) axis. In the present study it was hypothesized that social status modulates the expression of key proteins involved in the functioning of the HPI axis. Cortisol treatment and fasting were used to assess whether these characteristics seen in subordinate fish also affected HPI axis function. Social status modulated plasma adrenocorticotropic hormone (ACTH) levels, cortisol synthesis, and liver glucocorticoid receptor (GR) expression. Plasma ACTH levels were lower by approximately 2-fold in subordinate and cortisol-treated fish, consistent with a negative feedback role for cortisol in modulating HPI axis function. Although cortisol-treated fish exhibited differences in corticotropin-releasing factor (CRF) and CRF-binding protein (CRF-BP) mRNA relative abundances in the preoptic area and telencephalon, respectively, no effect of social status on CRF or CRF-BP was detected. Head kidney melanocortin 2 receptor (MC2R) mRNA relative levels were unaffected by social status, while mRNA relative abundances of steroidogenic acute regulatory protein (StAR) and cytochrome P450 side chain cleavage (P450scc) enzyme were elevated in dominant fish. Liver GR2 mRNA and total GR protein levels in subordinate fish were lower than control values by approximately 2-fold. In conclusion, social status modulated the functioning of the HPI axis in rainbow trout. Our results suggest altered cortisol dynamics and reduced target tissue response to this steroid in subordinate fish, while the higher transcript levels for steroid biosynthesis in dominant fish leads us to propose an adaptive role for responding to subsequent stressors.

1. Introduction

Among juvenile salmonid fish, social hierarchies readily form in small groups of fish that compete over resources that are limited in the environment, such as food. Fish that outcompete their conspecifics for these resources achieve dominance [3,30,33,35]. The interactions between dominant and subordinate fish often are aggressive; dominant fish may chase and/or bite subordinate fish to attain and maintain high social rank [1,3,37]. Low social status can be disadvantageous for subordinate fish in that they reduce activity and feeding [1,33], and experience prolonged elevation of cortisol, the main stress hormone in fish, owing to continued activation of the hypothalamic–pituitary–interrenal (HPI) axis (reviewed by [24]).

Activation of the HPI axis results in the synthesis and release of cortisol (reviewed by [36,57]). Corticotropin-releasing factor (CRF) is released directly into the pituitary of teleost fish by neurons that have their cell bodies in the preoptic area (POA) of the brain [57]. Corticotropin-releasing factor-binding protein (CRF-BP) is highly localized to the central nervous system in fish and is thought to play a role in regulating CRF effects [4,46]. Adrenocorticotropic hormone (ACTH), a peptide derived from proopiomelanocortin (POMC) and the primary hormone that stimulates cortisol synthesis [57], is then released as a result of activation of corticotropes by CRF. Interrenal cells in the head kidney are stimulated by binding of ACTH to the melanocortin 2 receptor (MC2R), a G protein-

Abbreviations: ACTH, adrenocorticotropic hormone; BCA, bicinchoninic acid; BSA, bovine serum albumin; CR, corticosteroid receptor; CRF, corticotropin-releasing factor; CRF-BP, corticotropin-releasing factor-binding protein; P450scc, cytochrome P450 side chain cleavage enzyme; DEPC, diethylpyrocarbonate; GR, glucocorticoid receptor; HPI, hypothalamic–pituitary–interrenal; MC2R, melanocortin 2 receptor; MR, mineralocorticoid receptor; POA, preoptic area; POMC, proopiomelanocortin; S-QPCR, semi-quantitative real-time RT-PCR; StAR, steroidogenic acute regulatory protein; Tel, telencephalon.

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coupled receptor [9]. Binding of ACTH activates adenylate cyclase to initiate a cAMP-signaling cascade resulting in the movement of cholesterol to the inner mitochondrial membrane with the help of the transport protein, steroidogenic acute regulatory protein (StAR) [51]. In the mitochondrion, cytochrome P450 side chain cleavage enzyme (P450scc) then cleaves cholesterol to pregnenolone in the first and rate-limiting step of cortisol synthesis [40]. Secreted cortisol acts on corticosteroid receptors, which are ligand-activated transcription factors [43]. The corticosteroid receptors include glucocorticoid (GR) and mineralocorticoid receptors (MR). Most teleost species studied to date, with the exception of zebrafish, have multiple GR isoforms [5,6,14,25,43]. In addition, teleost fish do not produce aldosterone as a mineralocorticoid (reviewed by [43]), and, therefore, cortisol and potentially other corticosteroids (e.g., 11-deoxycorticosterone) are thought to act as MR ligands [16,31,32,34,43,52].

Several studies have investigated the physiological causes and consequences of social status in salmonid fish (reviewed by [24]). However, how chronic social stress modifies HPI axis functioning is not well understood. Filling this information gap is important in understanding the potential impact of chronic stress on a fish's ability to respond to additional environmental stressors (i.e., acute stress response). Thus, the present study investigated the effects of social status on the functioning of the HPI axis using pairs of juvenile rainbow trout (*Oncorhynchus mykiss*). It was hypothesized that prolonged elevation of plasma cortisol levels in subordinate fish involves modulation of key proteins of the HPI axis and affects corticosteroid receptors in target tissues. During periods of chronic stress such as social stress, fish experience two conflicting demands: continual activation of the HPI axis resulting in elevated plasma cortisol levels and the associated activation of the negative feedback mechanism to regulate this steroid level [10]. As such, we predicted that socially-subordinate fish would attenuate HPI axis functioning to regulate plasma cortisol levels. Furthermore, social subordinance also was predicted to down-regulate corticosteroid receptors to protect against overstimulation of target tissues by cortisol. To test this hypothesis, we used semi-quantitative real-time RT-PCR (S-QPCR) and immunodetection to examine gene and protein expressions, respectively, at multiple levels of the HPI axis in control, fasted, cortisol-treated, dominant and subordinate fish. Fasted and cortisol-treated groups were included in the experimental design to tease out the roles of specific characteristics of subordinate fish; subordinates often do not feed [33,39] and have elevated circulating plasma cortisol levels (reviewed by [24]).

2. Materials and methods

2.1. Experimental animals

Juvenile female freshwater rainbow trout (85.0 ± 1.9 g, mean ± SEM; $N = 123$) were obtained from Linwood Acres Trout Farm (Campbellcroft, Ontario) and were kept at the University of Ottawa Aquatic Facility. Fish were housed in 1275 L fiberglass stock tanks under a 12L:12D photoperiod; the tanks were supplied with 13 °C dechloraminated, aerated city of Ottawa tap water. Fish were fed every second day to satiation by scattering commercial trout pellets on the water's surface. Trout were allowed to acclimate to these holding conditions for at least 2 weeks prior to experimentation. Fish were chosen from the stock tanks at random and placed into appropriate experimental tanks for a 5 day-period (see below) with the exception of control fish that were sampled directly from stock tanks. Fish masses did not differ significantly among treatment groups ($P = 0.342$, one-way ANOVA; data not shown). The holding conditions (e.g., use of scatter feeding, homogenous tanks with a mild current) tended to minimize hierarchy formation.

Social pairing experiments were performed as described by DiBattista et al. [17,18]. Briefly, rainbow trout were lightly anesthetized (to the point of losing equilibrium) in a solution of benzocaine (0.05 g L^{-1} ethyl *p*-aminobenzoate) to assess fish weight, fork length and fin damage. Each fish was paired with a conspecific that was no more than 0.1 ± 0.3% (mean ± SEM) different in fork length [2,17]. The fish in a pair were placed individually on either side of an opaque divider in a 40 L flow-through observation tank for an overnight recovery period. The divider was removed the following morning (day 1), and fish were allowed to interact for a period of 5 days (representing a chronic stress scenario). A PVC tube (*t*-shape, 13 × 11 cm long, 6 cm diameter) was added on the afternoon of day 1 to provide a shelter.

Fish were observed twice daily for 5 min per observation period and were scored on the occurrence of acts of aggression, position within the tank (e.g., patrolling the water column versus at the surface, bottom or in the shelter), and feeding; the scoring system awarded more points for more dominant behaviors such as initiating aggressive attacks, patrolling the water column and taking food. Fin damage was assessed on the final day and compared to initial fin damage observed. This method of scoring behavior is similar to methods used previously to assign social status in salmonid fish for examples see [17,18,35,47–49]. Principal component analysis (SPSS, v 16.0) was used to generate an overall behavior score for each fish. Within a pair, the fish with the higher score was assigned dominant status. Only pairs for which the behavior scores differed by at least 0.5 were used for subsequent analysis.

To generate fasted and cortisol-treated groups, fish were placed in 115–230 L holding tanks in groups of 10; this group size (together with the use of scatter-feeding, where appropriate, and homogenous tanks with a mild current) was sufficient to minimize unwanted hierarchy formation. Food was withheld from fasted fish for a period of 5 days. Cortisol-treated fish were lightly anaesthetized as above, and given an intraperitoneal injection of 0.005 ml g^{-1} body mass cocoa butter (Now Personal Care) containing 110 mg kg^{-1} body mass hydrocortisone 21-hemisuccinate (Sigma–Aldrich). This dose was chosen on the basis of previous work in which the circulating cortisol levels achieved were similar to those in subordinate rainbow trout [17]; also as in previous work, sham-injected fish (i.e., fish injected with vehicle alone) were not used because the injection itself raises circulating cortisol levels in an unpredictable fashion, confounding interpretation of the effects of cortisol elevation [17].

2.2. Collection of tissue samples

Fish in all experimental groups were terminally anaesthetized with an overdose of benzocaine (0.5 g l^{-1} ethyl *p*-aminobenzoate) on day 5 and tissue samples were collected. Gill, liver and white muscle were collected for analysis of corticosteroid receptor mRNA expression and protein levels. Liver was chosen because of the well documented role of cortisol in regulating metabolism (reviewed by [36,57]), white muscle because of the impact of social status on growth [18], and gill because it is a key site of ionic regulation, which is also regulated by cortisol in fish (reviewed by [36,57]). Head kidney tissue was collected for MC2R, P450scc and StAR mRNA analysis. Brain tissue, specifically POA and telencephalon, was collected for analysis of CRF and/or CRF-BP mRNA expression. The POA is the major site of CRF production for regulation of pituitary ACTH secretion [4]. Telencephalon was isolated in addition to POA for CRF-BP analysis owing to the possible regulation of CRF-BP by social stress in these tissues [4]. In addition, blood samples were collected by caudal puncture. Blood samples were centrifuged (10,000g for 2 min) and plasma was extracted for later analysis of ACTH and/or cortisol concentrations using radioimmunoassay kits (MP Biomedical) previously validated for analysis of trout