Elevation of brain 5-HT activity, POMC expression, and plasma cortisol in socially subordinate rainbow trout

SVANTE WINBERG AND OLIVIER LEPAGE
Department of Animal Development and Genetics, Uppsala University, Norbyvägen 18A, S-752 36 Uppsala, Sweden

Winberg, Svante, and Olivier Lepage. Elevation of brain 5-HT activity, POMC expression, and plasma cortisol in socially subordinate rainbow trout. Am. J. Physiol. 274 (Regulatory Integrative Comp. Physiol. 43): R645–R654, 1998.—Agonistic behavior, brain concentrations of serotonin (5-hydroxytryptamine, 5-HT), and 5-hydroxyindoleacetic acid (5-HIAA, the main 5-HT metabolite), plasma cortisol levels, and the pituitary expression of pro-opiomelanocortin (POMC) A and B mRNA were determined in socially dominant and subordinate rainbow trout after 1 or 7 days of social interaction. Telencephalic and brain stem 5-HIAA/5-HT ratios, plasma cortisol levels, and pituitary POMC mRNA concentrations were elevated in fish being subordinate for 1 day. Furthermore, neither telencephalic 5-HIAA/5-HT ratios nor pituitary POMC A or POMC B mRNA expression showed any decline after 7 days of social interaction. By contrast, plasma cortisol concentrations of subordinate fish declined after 7 days but were still significantly higher than in dominant fish. Furthermore, in subordinate fish, hypothalamic 5-HIAA/5-HT ratios and plasma cortisol levels were highly correlated, suggesting an important role of hypothalamic 5-HT in the regulation of the teleost hypothalamic-pituitary-interrenal (HPI) axis. The number of aggressive acts received and plasma cortisol levels were highly correlated in 1-day subordinates, a relationship not seen in fish subjected to 1 wk of subordination. Thus the chronic stress experienced by subordinates in established dominance hierarchies appears to be more closely related to the threat imposed by the presence of the dominant fish than to actual aggressive encounters. The sustained elevation of pituitary POMC mRNA expression, an effect mainly related to an increase of melanotropic POMC expression, in subordinates could be a mechanism serving to maintain HPI axis excitability and promote acclimation in these individuals.

agonistic behavior; stress; social interaction

IN TELEOSTS, like in many other vertebrates, socially subordinate animals have scarce and unreliable access to food and other resources and experience a general lack of control and predictability as well as a constant threat of aggressive actions from dominants. These are all potential factors that make social subordination a stressful experience. Thus, not surprisingly, elevated levels of plasma cortisol and other indicators of sustained stress have repeatedly been observed in subordinate animals.

The hypothalamic-pituitary-interrenal (HPI) axis [the teleost homologue of the mammalian hypothalamic-pituitary-adrenal (HPA) axis] consists of a series of hormonal pathways, the major components being hypothalamic corticotropin-releasing factor (CRF), pituitary adrenocorticotropic (ACTH), and interrenal cortisol (20). Corticosteroid release is stimulated by ACTH secretion from the pituitary (rostral pars distalis), which in turn is regulated by CRF. As with most endocrine systems, the HPI axis is under feedback control, such that elevated cortisol concentrations inhibit subsequent HPI activity (20).

Subordinate fish display a sustained elevation of plasma cortisol levels (10) and increased interrenal cell sizes (17), suggesting a chronic HPI axis activation. In a fish exposed to chronic social stress, the interrenal stress response is probably subjected to two opposing drives: negative feedback imposed by a sustained elevation in plasma cortisol concentrations, and stimulation due to the continuous perception of stressful stimuli (3). However, in mammals there are indications of impaired sensitivity to glucocorticoid feedback in the brain of subordinate animals (23, 24). The stress-induced elevation in plasma cortisol is an adaptive response that serves to alert the organism to environmental or physiological changes and to defend homeostasis. Thus cortisol feedback resistance could perhaps be a mechanism serving to maintain the ability of subordinate individuals to activate the interrenal stress response should they be challenged by a superimposed stressor. Chronic stress might also result in a sensitization of the pituitary ACTH response to novel stressors as well as an upregulation of pituitary ACTH synthesis (1), effects that could further restore HPA-HPI axis excitability in chronically stressed animals. However, the mechanisms responsible for modulation of neuroendocrine responses to chronic stress and acclimation in teleosts are largely unknown.

ACTH and several other biologically active peptides, e.g., α-melanocyte-stimulating hormone (α-MSH), β-lipotropin, and the endogenous opioid β-endorphin, are all synthesized from a common precursor protein, pro-opiomelanocortin (POMC). In mammals, the effect of stress on pituitary POMC mRNA levels varies according to the nature of the stimulus, but chronic stress paradigms associated with hypersensitivity of the pituitary ACTH response to a novel stressor generally result in an elevation of POMC mRNA levels, whereas other stress paradigms not associated with sensitization of the stress response appear to have the opposite effect on POMC mRNA concentrations (1).

There are no previous studies on the effect of stress on POMC mRNA levels in fish. However, stress-induced effects on plasma levels of POMC-derived peptides seem to vary depending on the stimulus (20).

As a result of the tetraploid status of their common ancestor, salmonid fish possess two nonallelic copies of the POMC gene. These two forms, in the rainbow trout referred to as POMC A and POMC B, show relatively low sequence homology despite the high conservation of some peptide sequences (α-MSH, β-MSH, and β-endorphin).
Both forms are expressed by melanotrophs and corticotrophs of the pituitary, whereas only POMC A is expressed by hypothalamic neurons of juvenile rainbow trout (22). By contrast, both POMC A and POMC B are expressed in the hypothalamus of sexually mature trout. On the basis of these observations, Salbert et al. (22) hypothesized that regulatory mechanisms differ between the two POMC genes.

Recent studies have shown that several hormones and/or neurotransmitters are involved in the regulation of ACTH secretion from the teleost pituitary. Fish occupying low positions in the dominance hierarchy are characterized by a stress-induced increase in brain serotonin (5-hydroxytryptamine, 5-HT) turnover, as indicated by elevated brain levels of 5-hydroxyindoleacetic acid (5-HIAA, the major 5-HT metabolite) and 5-HIAA/5-HT ratios (31). The 5-HT system of the mammalian brain has been suggested to play a role in integrating autonomic, behavioral, and neuroendocrine stress responses (5), even though its effects on HPA axis regulation have been debated (12). However, 5-HT has repeatedly been found to stimulate the release of CRF and ACTH from the mammalian hypothalamus and pituitary, respectively (9).

In addition, the 5-HT system of the mammalian brain also appears to be inhibitory to behavioral responsiveness (6, 27). Socially subordinate fish frequently display a pronounced behavioral inhibition, an effect that could be mediated by brain 5-HT (7, 27, 31) and represent a passive coping strategy in subordinate animals, lowering the frequency of agonistic interactions with dominant individuals and thus possibly reducing the stress in subordinates. Thus brain 5-HT could play a role in the activation of the interrenal stress response as well as in coping and habituation to chronic stress, presumably by acting through separate neuronal pathways. However, even though there are several studies describing effects of social interaction on 5-HT activity in various regions of the teleost brain, these effects have never been related to other neuroendocrine stress responses.

In the present study, we report the effects of short (1 day) and long-term (7 days) social interaction on brain 5-HT activity, pituitary POMC mRNA expression, and plasma cortisol concentrations in juvenile rainbow trout. Furthermore, plasma cortisol levels in this study have been directly correlated to the frequency of agonistic behavior and to hypothalamic 5-HT activity. In addition, by using two oligonucleotide probes complementary to POMC A and POMC B mRNA, respectively, we were able to quantify each mRNA separately.

**MATERIALS AND METHODS**

**Subjects.** The fish (weighing 90.9 ± 21.1 g, mean ± SD, n = 48) were 2-yr-old juvenile rainbow trout (Oncorhynchus mykiss). They were kept indoors in a holding tank continuously supplied with aerated Uppsala tap water (8-11°C) for >3 mo before the experiment. The light-dark regimen was continuously and automatically adjusted to conditions at 51° north latitude. The fish were fed daily with commercial trout pellets (EWOS ST40, Astra-EWOS) at 1-2% of their body weight.

**Behavioral observations.** Behavioral observations were made in seven glass aquariums (1,000 × 500 × 500 mm) continuously supplied with aerated Uppsala tap water (0.80 l/min, 8-10°C). Each aquarium was divided into four 50-liter chambers by inserting black polyvinyl chloride (PVC) walls, and one fish (tagged by small clips at the dorsal or ventral side of the caudal fin) was put into each of these chambers. In this way, each fish was kept visually isolated from other fish for at least 7 days in an attempt to diminish the effect of previous social experience. After this isolation period, fish were put together in groups of three, at 0930, by removing the PVC wall that kept them separated. One fish in each aquarium was kept isolated and served as a control. Experimental fish were kept together in groups for 1 or 7 days.

Aggressive acts in the groups were counted during three daily observation sessions, at 0930, 1300, and 1700, each 5 min long. The first observation was made 5–10 min after the fish were put together in pairs, and the last was made immediately before the fish was killed. Behavioral acts considered aggressive were attack (in which the fish made a rapid approach often culminating in a bite), charge (a direct but slow approach toward the other individual), and nip (a bite without a prior approach).

A black plastic screen with small holes (50 × 50 mm) for observation, was placed in front of the aquariums to minimize disturbance of the fish. Light was provided by fluorescent tubes (2 × 20 W, warm white) placed 100 mm above the water surface. The photoperiod was 12:12-h light-dark, with light on between 0800 and 2000.

Fish were fed (~2% of body fresh weight) once a day at 1000 by hand during both the initial isolation period and group rearing.

**Blood and tissue sampling.** At the end of each experimental period, fish in individual groups were netted simultaneously and anesthetized (500 mg/l ethyl m-anisobenzoate methanesulfonate) between 1000 and 1100. Blood was collected from the caudal vasculature using a heparinized syringe (within 2 min after netting). The blood was spun at 1,500 g to separate plasma, which was subsequently aliquoted, frozen on dry ice, and stored at −80°C. The fish was weighed and thereafter killed by decapitation. The telencephalon (excluding olfactory bulbs) and the brain stem (including medulla, cerebellum, and part of the spinal cord) was rapidly removed. These brain samples were wrapped in aluminum foil, frozen in liquid nitrogen (within 2 min after decapitation), and kept at −80°C. Subsequently, in controls, dominant, and one of the subordinates from each tank, the rest of the brain, including the hypothalamus with the pituitary attached, was carefully dissected, embedded in Tissue Tek (Miles), frozen on dry ice (within 5 min after decapitation), and stored at −80°C. From one of the subordinates in each group, the hypothalamus (excluding the pituitary) was removed as described above for telencephalon and brain stem and used for analysis of 5-HT and 5-HIAA concentrations.

**Brain 5-HT and 5-HIAA analysis.** The frozen brain samples were homogenized in 4% (wt/vol) ice-cold perchloric acid containing 0.2% EDTA, 0.05% sodium bisulfite, and 40 ng/ml epinephrine (deoxyepinephrine, the internal standard), using a Potter-Elvehjem homogenizer (brain stem) or an MSE 100-W ultrasonic disintegrator (telencephalon and hypothalamus).

5-HT and 5-HIAA were quantified using high-performance liquid chromatography with electrochemical detection as described by Nilsson (16). As a measure of serotonergic activity, the 5-HIAA/5-HT ratio was calculated for each individual (35).

**Plasma cortisol assay.** Cortisol analysis was performed directly on rainbow trout plasma without extraction, using a radioimmunoassay described by Olsen et al. (18). In short,
plasma samples were diluted twofold with 0.1 M phosphate buffer, pH 7.5, containing 1% bovine serum albumin. Standards were prepared from hydrocortisone (Sigma, St. Louis, MO) serially diluted in the same phosphate buffer. To prevent protein binding, a solution of trichloroacetic acid (7.5 g/l) and sodium hydroxide (2.25 g/l) was added. The antibody (anticortisol rabbit antiserum, lot 345–10–22–80, Endocrine Sciences) was added to give ~33% binding of total radioactivity. The tracer was [1,2,6,7-3H]cortisol (NEN Research Products, Dreieich, Germany) diluted with ethanol to an activity of 10 µCi/ml. Tubes were incubated overnight at 4°C. Free and bound tracer were separated by a 30-min incubation with charcoal on ice, followed by centrifugation. Samples were counted in AquaSafe scintillation fluid (Zinser Analytic, Frankfurt, Germany).

The sensitivity of the standard curve (r² = 0.964 ± 0.022) was 0.92 ± 0.25 ng/ml, the intra-assay coefficient of variation (CV) was 2.1%, and the inter-assay CV was 7.1%. Cortisol-free blanks gave a count of 1–2% of the total count.

In situ hybridization. The tissue was sectioned at 14 µm in a cryostat (Leica, Wetzlar, Germany) at –20°C and thaw mounted onto Superfrost Poly-lysine slides (Menzel Glaser). Sections were allowed to air dry for 20 min at room temperature before being transferred to the −80°C freezer. The sensitivity of the standard curve (r² = 0.964 ± 0.022) was 0.92 ± 0.25 ng/ml, the intra-assay coefficient of variation (CV) was 2.1%, and the inter-assay CV was 7.1%. Cortisol-free blanks gave a count of 1–2% of the total count.

For in situ hybridization, tissue series were removed from the −80°C freezer, allowed to attain room temperature, and immersed in 4% phosphate-buffered paraformaldehyde for 5 min. Slides were then rinsed in phosphate-buffered saline for 5 min, dehydrated in 70 and 95% ethanol, and allowed to air dry for 5 min at room temperature.

35S-Labeled oligonucleotide probes were diluted in hybridization buffer [50% formamide, 4 × saline sodium citrate (SSC), and 10% dextran sulfate] to yield a concentration of ~6 fmol/100 µl (corresponding to 75.800 and 108.000 disintegrations min⁻¹.100 µl⁻¹ for the POMC A and POMC B probes, respectively). Aliquots of 100 µl were applied to each slide, and Parafilm coverslips were applied to each section. Adjacent sections were hybridized with probes complementary to rainbow trout POMC A and POMC B, respectively. All slides were incubated overnight at 37°C in sealed plastic boxes containing filter paper moistened with 50% formamide:4× SSC.

Hybridized slides were washed in 1× SSC at room temperature for 15 min, 1× SSC at 60°C for 30 min, and finally in 1× SSC at room temperature for 5 min. After washing, slides were taken through ice-cold solutions of 0.1× SSC, 70% ethanol, and 95% ethanol (2 min each) before being allowed to air dry for at least 30 min.

For analysis of mRNA levels, sections hybridized with the POMC A and POMC B probe were placed in cassettes, together with 14C-labeled standards of known radioactivity (Amersham International, Buckinghamshire, UK), and exposed to a phosphoimaging (IP) plate (BAS-111S, Fuji) for 2 or 3 h, respectively. After exposure, the IP plate was immediately read in an IP reader (BAS 1500, Fuji), and the resulting digitized image was analyzed by image processing (MacBas ver. 2.2, Fuji). The amount of probe hybridized to individual pituitaries was measured as photostimulated luminescence (PSL) per square millimeter within the labeled area of the pituitary on 15–20 consecutive sections per fish. An average for each fish was calculated from individual PSL values and corrected for background (<5%). The average PSL value for each fish was subsequently converted to percent of control (divided by group mean for controls) and used to calculate group means.

1P-autoradiography has several advantages, such as high sensitivity, a linear relationship between amount of radioactivity and image density over a wide range, and the omission of wet processes. However, the spatial resolution of the equipment used in the present study was limited and did not allow separate quantification of POMC hybridization in the pars intermedia (melanotrophs) and pars distalis (corticotrophs). Thus the values for pituitary POMC mRNA expression reported are based on the hybridization signals from the total labeled area of the pituitary. Similarly, hypothalamic POMC A mRNA expression could not be quantified.

Addition of a 100-fold quantity of the unlabeled probe to the hybridization medium markedly reduced hybridization signals. However, when a 100-fold quantity of unlabeled mismatching probe was added to the hybridization medium, i.e., the unlabeled POMC A probe was added to labeled POMC B probe and vice versa, hybridization signals were not altered, confirming the specificity of the hybridization signal. After IP-autoradiography, sections were dipped in NTB-2 nuclear emulsion (Eastman Kodak, Rochester, NY) for cellular resolution. To visualize pituitary POMC mRNA, emulsion-dipped slides were exposed for 18 days. The emulsion was developed (D 19 Kodak) for 3 min at 20°C, fixed in 30% sodium thiosulfate for 5 min at 20°C, and counterstained with thionin.

Statistics. All data are presented as means ± SE. Statistical significance between the control and experimental groups was determined by analysis of variance followed by Fisher’s test. Correlations were tested using Spearman rank correlation coefficients (r).

RESULTS

Agonistic behavior. When PVC walls separating fish in each group were removed, each fish began performing agonistic behavior within a few minutes. An initial phase of mutual displays was often followed by a series of very violent attacks and circling, lasting for 5–45 min. Fish losing this first period of encounters either swam to the surface or took a position close to the walls of the aquarium (seemingly hiding). Thereafter, subordinate fish spent most of the time in a corner of the aquarium, usually with their heads at the water surface. However, even after attaining this submissive position, subordinates were frequently attacked and nipped by their dominant opponent. The latter individual was active and moved freely in the tank. In no case did the dominance-subordination relationship evident after the first period of encounters change during the experiment. Furthermore, in none of the groups did the fish losing the first encounter and becoming subordinate perform any agonistic acts during the rest of the experiment. Thus, even though the number of agonistic acts received differed between the two subordinates within a group, the individual rank of these individuals could not be determined.

The number of aggressive acts performed by dominant fish declined with time until reaching only a few aggressive acts per 5 min at the end of the 7-day period (Fig. 1).
Brain 5-HIAA/5-HT ratios. Social rank had significant effects on 5-HIAA/5-HT ratios (Fig. 2, A and B) in telencephalon ($F_{4,39} = 6.118, P = 0.0006$) and brain stem ($F_{4,42} = 13.296, P < 0.0001$). The effects were reflected in very similar effects on telencephalic and brain stem 5-HIAA concentrations ($F_{4,39} = 3.675, P = 0.0124$ and $F_{4,42} = 6.442, P = 0.0004$, respectively), whereas 5-HT concentrations were not affected in either telencephalon ($F_{4,39} = 1.133, P = 0.3553$) or brain stem ($F_{4,42} = 1.008, P = 0.4146$) (Table 1). Thus, because social interaction did not affect 5-HT concentrations, the differences observed in 5-HIAA/5-HT ratios were mainly caused by effects on telencephalic and brain stem 5-HIAA concentrations. However, brain 5-HIAA/5-HT ratios provide a more direct index of central 5-HT activity than brain 5-HIAA concentrations per se, because variance related to tissue sampling and to differences in total levels of 5-HT and 5-HIAA are reduced. Specifically, subordinate fish showed a significant elevation of telencephalic and brain stem 5-HIAA/5-HT ratios after 1 as well as 7 days of social interaction compared with controls and dominant individuals (Fig. 2, A and B).

Brain stem 5-HIAA/5-HT ratios in subordinate fish declined with time and were significantly lower in subordinates sampled after 7 days of social interaction than in those allowed to interact for only 1 day (Fig. 2B). Telencephalic 5-HIAA/5-HT ratios, on the other hand, did not decline with time and did not differ between 1- and 7-day subordinates (Fig. 2A).

Telencephalic and brain stem 5-HIAA/5-HT ratios in dominant fish did not differ from those of controls after either 1 or 7 days of social interaction (Fig. 2, A and B).

Plasma cortisol concentrations. Social rank also had a significant effect on plasma cortisol concentrations ($F_{4,43} = 11.490, P < 0.0001$). Subordinate fish displayed significantly higher plasma cortisol than dominants and controls after 1 as well as 7 days of social interaction (Fig. 3). However, plasma cortisol levels in subordinates were significantly higher after 1 day than after 7 days of group rearing (Fig. 3).

Plasma cortisol concentrations of dominant individuals did not differ from those of controls after either 1 or 7 days of social interaction (Fig. 3).

In 1-day subordinates, there was a significant correlation between the mean number of aggressive acts received per 5-min observation and plasma cortisol levels (Fig. 4A). In contrast, plasma cortisol levels in fish being subordinate for 7 days showed no such relationship with the mean number of aggressive acts received per observation (Fig. 4B). In fact, neither the mean number of aggressive acts received per observation during the 7-day period (Fig. 4B) nor the frequency of aggressive acts received during observations on the first ($r_s = 0.483, P = 0.110$) or the last day ($r_s = 0.294, P = 0.272$) showed any significant relationship with plasma cortisol concentrations.

Pituitary POMC mRNA levels. In agreement with the results by Salbert et al. (22), POMC A and POMC B probe hybridization was detected in the pars intermedia and pars distalis of the pituitary (Fig. 5).

**Table 1.**

<table>
<thead>
<tr>
<th>Condition</th>
<th>$F$ Value</th>
<th>$P$ Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Telencephalon</td>
<td>3.675</td>
<td>0.0124</td>
</tr>
<tr>
<td>Brain stem</td>
<td>6.442</td>
<td>0.0004</td>
</tr>
<tr>
<td>Controls</td>
<td>1.133</td>
<td>0.3553</td>
</tr>
<tr>
<td>Dominants</td>
<td>1.008</td>
<td>0.4146</td>
</tr>
</tbody>
</table>

**Fig. 1.** Mean number ± SE of aggressive acts performed by socially dominant rainbow trout during individual 5-min observations. Fish were allowed to interact in groups, each group consisting of 3 juvenile rainbow trout, during 1 (n = 8) or 7 days (n = 8).

**Fig. 2.** 5-Hydroxyindoleacetic acid/5-hydroxytryptamine (5-HIAA/5-HT) ratios in telencephalon (A) and brain stem (B) of juvenile rainbow trout having 1- or 7-days experience of a dominant or subordinate position in a group consisting of 3 individuals. Controls are isolated fish. Values are means ± SE from 8 animals. *P < 0.05, **P < 0.01, ***P < 0.001 analysis of variance (ANOVA) followed by Fisher's post hoc test.

**Fig. 3.** Plasma cortisol concentrations of juvenile rainbow trout after 1- or 7-days of social interaction with either dominant or subordinate fish. Values are means ± SE from 8 animals. *P < 0.05, **P < 0.01, ***P < 0.001 analysis of variance (ANOVA) followed by Fisher's post hoc test.

**Fig. 4.** Mean number ± SE of aggressive acts performed by socially dominant rainbow trout during individual 5-min observations. Fish were allowed to interact in groups, each group consisting of 3 juvenile rainbow trout, during 1 (n = 8) or 7 days (n = 8).
Social rank had significant effects on pituitary POMC A (F\textsubscript{4,29} = 5.174, P = 0.0029) as well as POMC B (F\textsubscript{4,29} = 5.992, P = 0.0012) probe hybridization. Subordinate fish displayed a significant elevation of POMC A and POMC B probe hybridization compared with dominants and controls after 1 as well as 7 days of social interaction, suggesting increased pituitary POMC A and B mRNA levels in subordinates (Fig. 6, A and B). There was no difference in either POMC A or POMC B probe hybridization between subordinate fish sampled after 1 day and subordinates allowed to interact for 7 days (Fig. 6, A and B).

In dominant individuals after 1 as well as 7 days of social interaction, neither POMC A nor POMC B probe hybridization in the pituitary differed from controls. There was a significant correlation between pituitary POMC A and POMC B probe hybridization in pooled data from dominant and subordinate fish sampled after 1 and 7 days of social interaction and controls (Fig. 7). Relationships between 5-HIAA/5-HT ratios, pituitary POMC A and B probe hybridization, and plasma cortisol concentrations. In the 10 subordinate individuals after 1 as well as 7 days of social interaction, neither POMC A nor POMC B probe hybridization in the pituitary differed from controls.

Table 1. Concentrations of 5-HT and 5-HIAA in telencephalon and brain stem of control rainbow trout kept in isolation or trout occupying dominant or subordinate positions in dominance hierarchy for 1 or 7 days

<table>
<thead>
<tr>
<th></th>
<th>Telencephalic</th>
<th>Hypothalamic</th>
<th>Brain Stem</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5-HT</td>
<td>5-HIAA</td>
<td>5-HT</td>
</tr>
<tr>
<td>Isolated controls</td>
<td>127 ± 24</td>
<td>37 ± 7</td>
<td>96 ± 16</td>
</tr>
<tr>
<td>(12) (12)</td>
<td>(12)</td>
<td>(12)</td>
<td>(12)</td>
</tr>
<tr>
<td>1-Day dominants</td>
<td>82 ± 13</td>
<td>22 ± 3</td>
<td>108 ± 14</td>
</tr>
<tr>
<td>(6)</td>
<td>(6)</td>
<td>(6)</td>
<td>(6)</td>
</tr>
<tr>
<td>1-Day subordinates</td>
<td>146 ± 16</td>
<td>73 ± 15</td>
<td>107 ± 11</td>
</tr>
<tr>
<td>(12)</td>
<td>(12)</td>
<td>(6)</td>
<td>(6)</td>
</tr>
<tr>
<td>7-Day dominants</td>
<td>117 ± 19</td>
<td>28 ± 4</td>
<td>92 ± 12</td>
</tr>
<tr>
<td>(12)</td>
<td>(12)</td>
<td>(6)</td>
<td>(6)</td>
</tr>
<tr>
<td>7-Day subordinates</td>
<td>137 ± 19</td>
<td>57 ± 6</td>
<td>124 ± 11</td>
</tr>
<tr>
<td>(12)</td>
<td>(12)</td>
<td>(6)</td>
<td>(6)</td>
</tr>
</tbody>
</table>

Values are means ± SE; n (no. of fish) in parentheses. Hypothalamic 5-hydroxytryptamine (5-HT) and 5-hydroxyindoleacetic acid (5-HIAA) concentrations were analyzed and 5-HIAA/5-HT ratios were calculated in 1 subordinate fish from each group only. All values are in ng/g except 5-HIAA/5-HT ratio.

Fig. 3. Cortisol concentrations in plasma of juvenile rainbow trout having 1- or 7-days experience of a dominant or subordinate position in a group consisting of 3 individuals. Controls are isolated fish. Values are means ± SE from 8 animals. “P < 0.01, “P < 0.001 ANOVA followed by Fisher’s post hoc test.

Fig. 4. Relationships between plasma cortisol concentrations and mean number of aggressive acts received per 5-min observation in juvenile rainbow trout having 1 (A) or 7-days (B) experience of a subordinate position in a group consisting of 3 individuals. Mean number of aggressive acts received by 1- and 7-day subordinates is based on 3 and 21 observations, respectively. Relationships were tested using Spearman rank correlation test (rₜ).
als (1 from each group) in which hypothalamus was analyzed for 5-HT and 5-HIAA concentrations, hypothalamic 5-HIAA/5-HT ratios were highly correlated with plasma cortisol concentrations (Fig. 8B). Furthermore, in these fish hypothalamic and brain stem 5-HIAA/5-HT ratios were correlated ($r_s = 0.648$, $P = 0.05$), whereas no such relationship was indicated between telencephalic and brain stem 5-HIAA/5-HT ratios or hypothalamic and telencephalic 5-HIAA/5-HT ratios.

In fish in which hypothalamus was used for in situ hybridization (1 subordinate from each group, dominants and controls), correlations between brain stem 5-HIAA/5-HT ratios and pituitary POMC A and B probe hybridization and plasma cortisol were tested. In subordinate fish (pooled data from 1- and 7-day subordinates), a significant correlation was found between brain stem 5-HIAA/5-HT ratios and plasma cortisol concentrations (Fig. 8C), but neither POMC A ($r_s = -0.315$, $P = 0.30$) nor POMC B probe hybridization ($r_s = 0.187$, $P = 0.52$) showed any significant correlation with brain stem 5-HIAA/5-HT ratios. In dominant fish and controls, brain stem 5-HIAA/5-HT ratios did not show any significant relationship with either plasma cortisol or POMC A or POMC B probe hybridization.

Plasma cortisol concentrations of subordinate fish (pooled data from 1- and 7-day subordinates) showed a nonsignificant negative correlation with POMC A ($r_s = -0.525$, $P = 0.0582$) and a weak, but again nonsignificant, positive correlation ($r_s = 0.396$, $P = 0.170$) with POMC B probe hybridization.

**DISCUSSION**

Social stress is more complex than stress in general, and most likely the stress experienced by subordinate individuals initially results from losing fights, whereas later on it probably also relates to being constantly threatened and having to inhibit one’s own aggression (38). In the present study, the frequency of agonistic interactions was high during the development of social hierarchies, whereas it decreased over time and reached a low and constant level after 3–4 days. The vigorous aggressive interactions occurring during the formation of a dominance hierarchy are an intense stressor. This is reflected by the strong positive correlation found between the number of aggressive acts received and plasma cortisol levels in 1-day subordinates, a relationship that was not found in fish subjected to 1 wk of subordination. Even though the amount of aggression received by subordinates was low and constant at the end of the 7-day period, plasma cortisol concentrations in subordinate fish varied considerably. Thus possibly the chronic stress experienced by subordinates in an established dominance hierarchy is more related to the threat imposed by the sheer presence of the dominant fish than it is to actual aggressive encounters. Most likely, the intense and acute stress experienced during the initial formation of a dominance hierarchy results in an immediate activation of the sympatho-chromaffin system as well as of the HPI axis, even in future dominants. However, as soon as the contests are settled, stress responses in dominants seem to be rapidly turned off (unpublished observations). By contrast, in fish becoming subordinate, plasma cortisol concentrations remain elevated even though circulating plasma cortisol had declined by $\sim36\%$ in 1-wk subordinates compared with subordinates sampled after 1 day of social interaction.

Pituitary POMC mRNA expression. The results of the present study show that pituitary POMC expression is upregulated in subordinate rainbow trout after 1 day of social interaction and shows no decline even after 1 wk of subordination. Cortisol is believed to inhibit POMC synthesis and the release of POMC-derived peptides through negative feedback (1, 3). However, the pituitary POMC mRNA expression remained elevated in subordinate fish after 1 wk of social interaction despite a chronic increase in plasma cortisol. This could be explained by a dampened sensitivity to cortisol feedback and/or a strong continuous central drive of the HPI axis in subordinates.

The effect on pituitary POMC A and POMC B mRNA expression was very similar. Furthermore, pituitary POMC A and POMC B probe hybridization showed a
significant correlation, suggesting that social stress had similar effects on the pituitary expression of the two POMC genes. However, it is worth mentioning that plasma cortisol levels appeared to show a positive relationship with POMC B expression but a negative relationship with that of POMC A in subordinate fish. However, in neither case did these correlations reach the level of statistical significance.

Elevated pituitary POMC mRNA levels in subordinate fish are likely to reflect enhanced synthesis and release of POMC-derived peptides in these individuals. POMC is the precursor of several biologically active peptides, i.e., ACTH, α-MSH, and β-endorphin.

Our results show that pituitary POMC A and POMC B mRNA levels remain high in subordinate individuals even after 1 wk of social interaction, whereas plasma cortisol concentrations decline, possibly suggesting a concomitant decline in plasma ACTH concentrations. As in mammals (6, 25), the habituation of the stress response in fish seems to be regulated at the hypothalamic-pituitary level by a decrease in plasma levels of ACTH (3).

In the present study, POMC A as well as POMC B were highly expressed in the intermediate lobe. Most likely, the overall increase in pituitary POMC probe hybridization observed in subordinate fish mainly reflects an elevation of melanotropic POMC mRNA concentrations, because the area represented by pars distalis corticotrophs is rather small compared with that of the neurointermediate lobe (Fig. 5).

Sumpter et al. (30) showed that handling and confinement in combination with thermal shock elevates plasma levels of α-MSH and β-endorphin in brown trout (Salmo trutta). In contrast, in the study by Pickering et al. (21), confinement stress alone had no effect on plasma levels of α-MSH in rainbow trout. In fact, Balm and Pottinger (3) reported that confinement alone reduced levels of circulating α-MSH and β-endorphin in rainbow trout. However, restraint out of water elevated plasma concentrations of α-MSH in this species (30), as did exposure to acidified water (14) and exposure to low pH in the presence of aluminum in tilapia (Oreochromis mossambicus) (2). Thus the effect of stress on plasma levels of POMC-derived peptides of melanotropic origin seems to vary according to the nature and/or the intensity of the stressor. Interestingly, the results of the present study suggest that social subordination stimulates the release of melanotrop POMC-derived peptides. However, the effects of social stress on the synthesis and release of individual POMC-derived peptides will have to be confirmed by
measuring peptide concentrations in the pituitary and plasma.

Social position and brain 5-HT activity. In accordance with the results from previous studies, subordinate rainbow trout displayed elevated 5-HIAA/5-HT ratios in telencephalon and brain stem. In subordinate trout, the telencephalic 5-HIAA/5-HT ratio was elevated after 1 day and did not show any decline after 1 wk of social interaction. Brain stem 5-HIAA/5-HT ratios were also drastically increased in fish being subordinate for 1 day but declined after 1 wk of social interaction, although still being significantly higher than in dominants and controls. Hypothalamic 5-HIAA/5-HT ratios, which were analyzed in only one of the subordinates from each group, were also higher in 1-day subordinates than in fish subjected to subordination for 1 wk. In contrast, Winberg and Nilsson (32) showed that 5-HIAA/5-HT ratios in telencephalon, hypothalamus, and brain stem of subordinate Arctic charr (Salvelinus alpinus) did not decline during 21 days of social interaction. This discrepancy could be related to the fact that the juvenile rainbow trout used in the present experiment were extremely aggressive. Thus possibly subordinate rainbow trout were exposed to a much more severe stress during the initial hierarchy formation than the subordinate Arctic charr in the experiment by Winberg and Nilsson (32). In fact, in the present experiment, brain stem 5-HIAA/5-HT ratios of subordinate fish were elevated by 97% after 1 day and by 53% after 7 days of social interaction compared with controls. Brain stem 5-HIAA/5-HT ratios of subordinate Arctic charr were elevated by ~40% compared with controls (32). In the present experiment, the effect of subordinate experience on telencephalic 5-HIAA/5-HT ratios was less pronounced (60 and 50% elevations after 1 and 7 days, respectively) but did not decline over time.

Early activation (by 1 h) of the brain 5-HT system has been reported in subordinate male Anolis carolinensis. However, after longer periods of social interaction, subordinate males of this species no longer displayed elevated brain 5-HT activity (28). By contrast, similar to teleosts, chronic activation of the brain 5-HT system in subordinate animals has been reported in mammals (4), including primates (37).

Possible endocrine and behavioral effects of elevated brain 5-HT activity. An elevation of central 5-HT activity seems to be a phylogenetically old response to stress, the function of which is still not well understood. The mammalian telencephalon and diencephalon are largely innervated by 5-HT cell bodies in the midbrain raphe area (13). However, 5-HT innervation of the hypothalamic paraventricular nucleus, a brain region that appears to be the crucial focus for central regulation of the mammalian HPA axis, is limited (26), although Liposits et al. (15) showed that 5-HT terminals make direct synaptic contact with CRF-containing neurons in this area. The 5-HT system of the mammalian brain has been suggested to stimulate the release of CRF, which in turn activates POMC synthesis (1) and ACTH release from the pituitary. Furthermore, 5-HT has also been suggested to act directly at the corticotrophs of the mammalian pituitary to stimulate ACTH release (6). The organization of the brain 5-HT system seems to be remarkably constant throughout the vertebrate subphylum (19), and in the present study, hypothalamic 5-HIAA/5-HT ratios and plasma cortisol concentrations of subordinate fish were highly correlated, suggesting a role of hypothalamic 5-HT in the regulation of the teleost HPI axis. In mammals, the
stimulatory role of 5-HT on the HPA axis has been attributed to 5-HT_{1A} and 5-HT_{2} receptors (9). Treatment with 8-hydroxy-2(di-n-propylamino)tetralin, a specific 5-HT_{1A} receptor agonist, elevates plasma cortisol concentrations in catheterized rainbow trout in a dose-dependent manner (34). Recently, Winberg and Nilsson (33) characterized three 5-HT receptor subtypes in the Arctic char brain. One of these receptors shows a pharmacological profile strikingly similar to the mammalian 5-HT_{1A} receptor, suggesting the presence of this receptor subtype in the salmonid brain.

The brain 5-HT system is also believed to exert an inhibitory effect on sensory-motor reactivity to environmental stimuli (7, 27), which might be a mechanism allowing the animal to cope with stress (11). Subordinate animals are frequently characterized by a behavioral inhibition (4, 31) and it is tempting to speculate that the socially induced increase in telencephalic 5-HT activity reflects such a modulatory role of 5-HT, the telencephalon being involved in the regulation and integration of agonistic behavior in fish (8). In the present experiment, telencephalic 5-HIAA/5-HT ratios of subordinate fish were drastically elevated but did not show any relationship with plasma cortisol levels. In contrast to brain stem and hypothalamic 5-HIAA/5-HT ratios, telencephalic 5-HIAA/5-HT ratios did not decline in fish being subordinate for 1 wk. Furthermore, telencephalic 5-HIAA/5-HT ratios showed no correlation with brain stem or hypothalamic 5-HIAA/5-HT ratios.

In conclusion, the results of the present study show that subordinate stress causes a rapid and sustained elevation of telencephalic 5-HT activity and pituitary POMC mRNA expression in juvenile rainbow trout, as indicated by 5-HIAA/5-HT ratios and in situ hybridization signals, respectively. However, plasma cortisol concentrations, as well as brain stem 5-HIAA/5-HT ratios, which were drastically elevated in subordinate fish after 1 day, declined after 1 wk of social interaction, although remaining significantly higher than in dominants and controls. Hypothalamic 5-HIAA/5-HT ratios of subordinate fish, after 1 and 7 days of social interaction, were highly correlated with plasma cortisol concentrations, suggesting that hypothalamic 5-HT plays an important role in HPI axis regulation. On the other hand, the chronic elevation of telencephalic 5-HT activity in subordinate fish could mediate the behavioral inhibition displayed by these individuals. Most likely the sustained elevation of brain 5-HT activity and pituitary POMC mRNA expression in subordinate fish represents mechanisms to restore HPI axis excitability and cope with chronic social stress.

**Perspectives**

In general, animals losing an aggressive encounter and becoming socially subordinate display a striking behavioral inhibition, preventing these animals from making useless attempts to regain social status. Furthermore, subordination frequently results in a chronic activation of the interrenal stress response, often in parallel with impaired cortisol feedback regulation and an elevation of brain 5-HT activity.

Physiological and behavioral stress responses are to a large extent integrated by control mechanisms in the brain, and the brain 5-HT system appears to play a key role in this integration. For instance, in the present study, we obtained a strong correlation between hypothalamic 5-HT activity and plasma cortisol levels in subordinate rainbow trout, providing a strong argument for 5-HT as a regulator of the HPI axis.

We also observed an elevation of POMC mRNA expression in the pituitary intermediate lobe of subordinate rainbow trout, possibly reflecting a mechanism to modulate and restore HPI axis excitability in fish exposed to chronic stress. However, the melanotropic stress response appears to be variable and stressor specific, and the role of pars intermedia POMC products during stress is poorly understood. α-MSH is best known for its effect on the body pigmentation in teleost fish, stimulating chromatophore dispersal. Interestingly, subordinate salmonids display a dark body coloration which might provide a social signal serving to reduce attacks from dominants. In addition, POMC-derived peptides of melanotropic origin could be involved in stress-induced effects on the reproductive function and immune system.

This study was financially supported by the Swedish Council for Forestry and Agricultural Research.

Address reprint requests to S. Winberg.

Received 30 May 1997; accepted in final form 7 November 1997.

**REFERENCES**


