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The effects of cortisol administration on social status and brain monoaminergic activity in rainbow trout *Oncorhynchus mykiss*

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Summary

The hypothesis that circulating cortisol levels influence the outcome of social interactions in rainbow trout was tested. Juvenile rainbow trout *Oncorhynchus mykiss* were given a single intraperitoneal (i.p.) implant containing either cortisol (110 mg kg^{-1} fish), or cortisol plus the glucocorticoid receptor antagonist RU486 (mifepristone; 1100 mg kg^{-1} fish), and sampled after 5 days of social interactions with either a similar sized (<1.5% difference in fork length) or smaller conspecific (>5% difference). Within size-matched pairs of fish, cortisol treatment significantly increased the probability that the treated fish within each pair became subordinate, an effect that was abolished by simultaneous administration of RU486. Cortisol treatment also reduced the usual success of the larger fish within a pair to preferentially become dominant from 86% to 40% of pairs. To investigate one potential mechanism underlying the apparent effect of cortisol in predisposing trout to low social status, fish were treated with cortisol or cortisol+RU486 for 5 days, after which brain monoamines [5-hydroxytryptamine (5-HT); dopamine (DA)] and their major metabolites [5-hydroxyindolacetic acid (5-HIAA); 3,4-dihydroxyphenylacetic acid (DOPAC)] were measured. Significant increases of serotonergic activity ([5-HIAA]/[5-HT] ratio) were detected in the telencephalon with cortisol treatment, an effect that was eliminated by simultaneous administration of RU486. Also, cortisol treatment significantly decreased dopaminergic activity in the telencephalon. Somewhat surprisingly, the effects of cortisol treatment on monoaminergic activity in the hypothalamus were opposite to those in the telencephalon. Moreover, in no case did administration of RU486 abolish these effects. These results suggest that the effects of cortisol on social status in rainbow trout may be mediated *via* the modulation of central signaling systems and subsequent changes in behaviour and/or competitive ability, although the exact site of action in the brain remains uncertain.

Key words: rainbow trout, *Oncorhynchus mykiss*, cortisol, social status, monoamines, serotonin, dopamine, RU486.

Introduction

Salmonid fish, such as rainbow trout *Oncorhynchus mykiss*, form linear, dominance-based, social hierarchies in both natural and artificial populations (Noakes and Leatherland, 1977; Bachman, 1984; Abbott and Dill, 1989). In the laboratory, confinement in pairs generally results in one fish becoming dominant over the other; subordinate fish, and as a result, subordinate individuals, experience chronic social stress (reviewed by Sloman and Armstrong, 2002). These subordinate fish are also generally excluded from preferential access to food (McCarthy et al., 1992) and experience increased standard metabolic rates (Sloman et al., 2000c), which result in decreased growth rates (Barton et al., 1987; Metcalfe et al., 1989, 1995; Winberg et al., 1992). Immunosuppression (Peters et al., 1988; Pottinger and Pickering, 1992) and increased mortality (Pickering and Duston, 1983; Pickering, 1993) are also observed in subordinate individuals. Finally, subordinate fish are characterized as having plasma concentrations of the corticosteroid stress hormone, cortisol, that are higher than those of dominant fish (reviewed by Sloman and Armstrong, 2002; Gilmour et al., in press). In fact, these marked elevations of plasma cortisol have been widely employed as an index of stress (Pottinger and Pickering, 1992) and are thought to contribute significantly to the poor overall physiological condition of subordinate fish (Gilmour et al., in press).

A number of social (e.g. prior residence, prior winning/losing experience) and inherent (e.g. size, aggressiveness) factors are known to influence the outcome of social interactions in salmonids (Huntingford and Turner, 1987; Fernandes and Volpato, 1993; Metcalfe, 1998; Rhodes and Quinn, 1998). Recent work has also raised the possibility that physiological factors, more specifically the physiological

condition of a fish, can impact on an individual's initial success during competitive interactions and affect its ultimate social status (e.g. Johnsson and Björnsson, 1994; Björnsson, 1997; Sloman et al., 2001). For example, Sloman et al. (2001) reported that plasma cortisol concentrations were significantly higher prior to pairing in size-matched rainbow trout that subsequently become subordinate, suggesting that individuals with high plasma cortisol levels are predisposed to become subordinate. In another study, experimental elevation of plasma cortisol concentrations reduced appetite, growth rate and condition in rainbow trout, and fin damage was greater in cortisol-treated trout held in mixed groups with untreated controls (Gregory and Wood, 1999). These data suggest that elevated cortisol concentrations might in fact be symptomatic of an individual fish's poor condition, placing it at a physiological and/or competitive disadvantage (Gregory and Wood, 1999). Thus, the main objective of the present study was to test the hypothesis that circulating cortisol concentrations affect the outcome of social interactions within pairs of rainbow trout. In particular, high plasma cortisol levels were predicted to predispose a fish to low social status. In accordance with the work of Gregory and Wood (1999), two possible mechanisms through which cortisol might influence social status can be envisaged. Elevated cortisol levels, by reducing physiological condition (Barton et al., 1987; Barton and Iwama, 1991; Gregory and Wood, 1999), could impact on competitive ability directly. Alternatively, interactions between cortisol and brain monoaminergic activity could affect competitive ability indirectly by modulating behaviour.

Many of the behavioural consequences of social status are thought to be the outcome of changes in brain monoaminergic activity that accompany victory or loss in competitive interactions (reviewed by Winberg and Nilsson, 1993). For example, subordinate fish generally exhibit significantly higher turnover of serotonin (5-HT), reflected by 5-hydroxyindolacetic acid (5-HIAA) accumulation and elevated 5-HIAA/5-HT ratios within the telencephalon, hypothalamus, and brain stem relative to dominant individuals (Winberg et al., 1991, 1992, 1993, 1997b). These social stress-induced increases of brain 5-HT activity are likely, at least in part, to be responsible for the marked behavioural inhibition commonly observed in subordinate fish; namely decreases in feeding, aggression, and spontaneous locomotor activity (Winberg and Nilsson, 1993; Winberg et al., 1997a; Øverli et al., 1998). In tetrapod vertebrates, experimentally elevated serotonergic activity causes a reversal of dominance relationships in a number of model systems (e.g. Sanchez and Hyttel, 1994; Villalba et al., 1997; Larson and Summers, 2001), suggesting that high brain 5-HT levels in these organisms have the capacity to act as antecedents for subordinance. Winberg et al. (1992) reported that the relationship between brain 5-HT turnover rate and social rank in fish developed through social interactions and was not caused by intrinsic differences in brain 5-HT activity. However, it is conceivable that high circulating cortisol levels could influence the outcome of social interactions by affecting central monoaminergic activity, specifically increasing serotonergic activity and/or decreasing dopaminergic activity. These changes, in turn, could alter behaviour (reducing aggression, locomotion, etc.) in such a way as to reduce competitive ability, resulting in low social status. Thus, in the present study the hypothesis that circulating cortisol levels influence brain monoaminergic activity was also tested as one potential mechanism underlying any observed relationship between cortisol treatment and the outcome of social interactions.

Materials and methods

Experimental animals

Juvenile female freshwater rainbow trout *Oncorhynchus mykiss* Walbaum (mean mass ± S.E.M., 86.79±2.2 g, *N*=132) were obtained from Linwood Acres Trout Farm (Campbellcroft, Ontario, Canada). All fish were held in large 1275 liter fibreglass stock tanks for several weeks at the University of Ottawa, supplied with flowing, aerated, dechloraminated city of Ottawa tapwater at a temperature of 13±1°C and under a 12 h:12 h L:D photoperiod. Fish were then transported to Carleton University and housed in 780 liter fibreglass holding tanks until use; tanks were supplied with flowing, aerated, wellwater (16.5±1.5°C) and a 12 h:12 h photoperiod was also used. Throughout this period, fish were hand-fed to satiation every second day with a commercial trout food diet (Purina Trout Chow).

Experiment 1: The effects of cortisol treatment on the outcome of social interactions

Fish were lightly (i.e. to the point of losing equilibrium) anaesthetized in a solution of benzocaine (0.05 g l^{-1} ethyl-*p*-aminobenzoate) and initial masses and fork lengths were measured (mass 76.8±2.4 g; fork length 186.1±2.1 mm, *N*=98). Abbott and Dill (1985) reported that an initial length difference of as little as 5% was sufficient to ensure dominant status to the larger individual within pairs of rainbow trout. Therefore, two separate series of experiments were carried out. In the first, fish were paired with a conspecific that was size-mismatched by 5–20% on the basis of fork length. Each pair of fish was then randomly assigned to either a control group (14 pairs) or a cortisol treatment group (11 pairs), with the larger fish within the pair in each case receiving the treatment. In the second experimental series, fish were size-matched by fork length (<1.5% difference), and each pair of fish was randomly assigned to either a cortisol treatment group (14 pairs), or a cortisol plus RU486 treatment group (10 pairs), with one fish within the pair receiving the treatment. Because the objective of these experiments was to investigate the effect of elevated cortisol concentrations on the outcome of social interactions, sham treatment groups (treatment with coconut oil or cocoa butter alone) were not included in the experimental design. Both scrutiny of the literature, and pilot trials using sham-treated fish, revealed that fish respond unpredictably to sham treatment, with cortisol concentrations being elevated in

some fish but not others. Such unpredictability would cloud comparisons between fish expected on the basis of their treatment group to have low cortisol levels, and those expected to have high cortisol levels, and therefore sham treatments were not employed.

Cortisol-treated fish received a coconut oil pellet (0.005 ml coconut oil g^{-1} fish) containing dissolved cortisol (110 mg hydrocortisone 21-hemisuccinate kg^{-1} fish) in the intraperitoneal cavity. The coconut oil was injected as a liquid but solidified rapidly within the fish and acted as a solid implant for the remainder of the experiment. Previous work has demonstrated that a coconut oil vehicle allows the delivery of a prolonged, slow-release dose of cortisol within each injected fish (reviewed by Gamperl et al., 1994); the cortisol dose used was selected on the basis of pilot trials that indicated that this dose provided a physiologically relevant elevation of plasma cortisol values over the desired experimental period. Cortisol plus RU486 treatment was achieved by implanting a cocoa butter pellet (0.01 ml cocoa butter g^{-1} fish) containing a combination of dissolved cortisol (110 mg hydrocortisone 21-hemisuccinate kg^{-1} fish) and the glucocorticoid receptor antagonist RU486 (1100 mg mifepristone kg^{-1} fish; Sigma, Oakville, ON, Canada). The concentration of RU486 was chosen on the basis of previous work indicating that treatment with this compound is most effective at a dose tenfold greater than that of cortisol (Vijayan et al., 1994). Control fish were untreated.

Following preparation, pairs of trout were placed in 40 l flow-through Plexiglas observation tanks. The fish were separated by an opaque perforated divider for a 48 h recovery and acclimation period, and the dividers were then removed to allow pairs of fish to interact; a small piece of PVC tubing was placed within each tank to provide shelter. Behavioural observations were carried out on all paired fish twice a day for 5 days, and the fish were then terminally sampled. During the experiment, fish were hand-fed to satiation with commercial trout food pellets once a day, after all observations had been carried out. Behaviour observations were first conducted 15 min after the opaque divider was removed, and then for 10 min each, once between 9:00 h and 11:30 h and once between 15:00 h and 17:30 h. The order of tank observation was randomized to account for any observational bias.

Social status was determined by assigning points to each fish based on its food acquisition, position, aggressive behaviour and fin damage; high scores in each case were indicative of dominant behaviour or characteristics. This method has been used previously for assigning social status among salmonids (Johnsson et al., 1996; Sloman et al., 2000a,b, 2001). In brief, to score fish on food acquisition, one pellet of food was dropped into the tank at the beginning of each observation period and the first fish to take the pellet was given a score of one, while the other fish scored zero points. Fish that maintained their position within the water column scored ten points, whereas fish that rested on the bottom of the tank or hid within the PVC tubing scored five points, and fish that attempted to swim at the surface (a behaviour indicative of subordinance; Sloman et al., 2000a) scored zero points. Fish directing five or more aggressive attacks towards the other individual within an observation period were given a score of two, fish performing between one and four aggressive attacks were given a score of one, and those individuals performing no aggressive attacks received a score of zero. Finally, fish were scored according to the extent of dorsal and caudal fin damage sustained during the 5 day interaction period. The mean dorsal and caudal fin damage scores were calculated and then combined into a total fin score. Previous work demonstrated that the severity of fin damage is likely to reflect the social rank of the individual (Abbott and Dill, 1985; Moutou et al., 1998). Therefore, fish having no fin damage were given a score of three, minor damage (<30% of the fin missing) a score of two, severe damage (30–70% of the fin missing) a score of one, and very severe damage (>70% of the fin missing) a score of zero. A single behaviour score was calculated from all observations by means of a principal components analysis (PCA; SPSS 10.1) (Sloman et al., 2000c). The fish with the higher overall behaviour score within each pair was assigned dominant social status, whereas that with the lower score was classified as subordinate.

Fish were rapidly killed by immersion in a lethal dose of anaesthetic solution (ethyl-*p*-aminobenzoate, 0.5 g l^{-1}). Pairs were removed simultaneously from their tanks and sampled within 1 min of each other; the sampling order within each pair was randomized to control for any sampling bias. Final masses and fork lengths were measured and a blood sample (~1 ml) was removed by caudal puncture. Following centrifugation (13 200 ***g*** for 3 min), plasma was removed, immediately frozen in liquid nitrogen, and subsequently stored at –80°C until analysis. Plasma cortisol concentrations were measured using a commercially available radioimmunoassay kit (ICN pharmaceuticals). The condition factor (CF) of each fish was calculated as $CF=100M_b/L^x$, where M_b=mass of fish in grams, L=length of fish in cm, and x=slope of regression line for all fish of $\log M_b$ *vs* $\log L$ (~3). The specific growth rate (SGR) of each fish was calculated as $SGR=[\ln(M_{bFinal})-\ln(M_{bInitial})]\times100/D$, where D=number of days elapsed.

Experiment 2: The effect of cortisol administration on brain monoamine levels

Fish were lightly anaesthetized in a solution of benzocaine (0.05 g l^{-1} ethyl-*p*-aminobenzoate), initial masses were measured (mass 92.5±2.3 g, N=34) and fish were randomly placed within groups of twelve in 780 liter holding tanks. Following a 5 day acclimation period, fish were again lightly anaesthetized and injected intraperitoneally with a cocoa butter pellet (0.005 ml coconut oil g^{-1} fish) containing dissolved cortisol (50 mg hydrocortisone 21-hemisuccinate kg^{-1} fish; N=10) or a combination of dissolved cortisol (50 mg hydrocortisone 21-hemisuccinate kg^{-1} fish) and RU486 (500 mg mifepristone kg^{-1} fish; N=12); an additional group of untreated fish served as controls (N=12). The group and tank sizes were chosen to avoid the formation of

dominance hierarchies, as hierarchy formation in this experiment would have a confounding effect on brain monoaminergic activities. In addition, fish were fed by scattering food on the water surface. Examination of the control group suggested that hierarchies did not form, in that the group exhibited positive growth and plasma cortisol concentrations in all cases were typical of unstressed fish (<10 ng ml^{-1}).

Fish were sampled 5 days after receiving treatment in groups of four, to minimize disturbance of the fish remaining within each tank. Fish were killed by immersion in a lethal dose of anaesthetic (ethyl-*p*-aminobenzoate, 0.5 g l^{-1}), mass was measured and the brain was rapidly removed. Two discrete brain regions were dissected out (on ice) for analysis, the telencephalon (excluding the olfactory bulbs), and the hypothalamus (excluding the pituitary gland). These brain areas were selected on the basis of earlier studies showing that monoamine activity within these regions was particularly influenced by social stress (Winberg et al., 1991, 1992, 1997b). Brain samples were frozen in liquid nitrogen and stored at –80°C. Blood samples (~1 ml) were removed *via* caudal puncture, and separated plasma was frozen in liquid nitrogen and stored at –80°C until analysis for plasma cortisol concentration using a commercial RIA kit (ICN pharmaceuticals). All blood and tissue samples were collected between 11:30 h and 14:30 h to control for any diurnal variations in either plasma cortisol and/or brain monoamine concentrations.

Frozen brain samples were sonicated in a homogenizing solution comprising 0.1 mmol l^{-1} Na_2EDTA, 0.3 mol l^{-1} ClCHCOOH, 10% methanol, and 12.5 pg µl^{-1} DHBA (the internal standard). Brain monoamines were then quantified by high performance liquid chromatography (HPLC) using electrochemical detection. The HPLC consisted of a solvent-delivery system (Waters590/WaterPump, Mississuaga, ON, Canada), an autoinjector (Waters712WISP), a reverse-phase column (8 mm×100 mm, Waters, NovaPak, 4 µm) kept at 30°C and a 5100A Coulochem detector (ESA, Bedford, MA, USA) with two electrodes at oxidizing potentials of –330 mV and +350 mV. The mobile phase consisted of 1.3 g l^{-1} heptanesulphonic acid sodium salt, 0.1 g l^{-1} disodium ethylene tetracycline, and 7.3 ml triethyloamine adjusted to pH 2.45 with orthophosphoric acid. Sample monoamine levels were indexed to standard solutions of known concentration, corrected for recovery of the internal standard, and expressed relative to total tissue protein content.

The monoamines measured were 5-hydroxytryptamine (5-HT) and dopamine (DA), as well as their major metabolites, 5-hydroxyindolacetic acid (5-HIAA) and 3,4-dihydroxyphenylacetic acid (DOPAC), respectively. The ratio of [metabolite]/[parent monoamine] was used as an index of brain monoaminergic activity. This index reduces variance related to tissue sampling and provides a more direct measure of brain monoaminergic activity than do levels of monoamine metabolites on their own (Shannon et al., 1986).

Statistical analysis

All data are presented as means ± 1 standard error of the mean (S.E.M.). χ^2 analysis was used to evaluate the effects of treatment group on social status and behaviour scores for all pairs of fish. Behaviour scores in Experiment 1 were compared between treatment groups for fish in the same category (dominant treated, subordinate treated, dominant untreated or subordinate untreated). Plasma cortisol concentrations were analysed by two-way analysis of variance (ANOVA) followed by Bonferroni-corrected *t*-tests, or by Student's *t*-tests, as appropriate. A two-way ANOVA followed by Bonferroni-corrected *t*-tests, as appropriate, was carried out on specific growth rate and final condition factor in Experiment 1, using social status and treatment group as factors. The statistical significance of differences in mean brain monoamine concentrations in Experiment 2 were assessed using one-way ANOVA on ranks followed by Dunn's *post hoc* pairwise multiple comparisons test, as appropriate. Non-normally distributed plasma cortisol concentrations were log transformed as needed. The level for significance for all tests was set at P=0.05 and all statistical analyses were performed using SigmaStat v3.0 (SPSS, Inc) or SPSS v10.1 (SPSS, Inc) software.

Results

Experiment 1: The effect of cortisol treatment on the outcome of social interactions

Plasma cortisol concentrations and behaviour scores

Socially subordinate rainbow trout exhibited marked behavioural changes, including decreases in feeding and aggression as well as selection of tank positions not occupied by the other fish, during the 5-day interaction period. Through the scoring scheme and PCA analysis, these differences in behaviour were translated into behaviour scores that were typically high positive values for dominant fish and low negative scores for subordinate individuals, regardless of treatment group or experiment (scores ranged from 1.7 to –1.9; Fig. 1A,B). Rarely, the behaviour scores for fish within a pair were not sufficiently different (i.e. identical behaviour scores) to enable social status to be assigned; the one pair for which this was the case was eliminated from further analysis. Treatment group had no impact on behaviour scores within a category (i.e. dominant treated, subordinate treated, dominant control or subordinate control; Student's *t*-test, P>0.05).

In general, plasma cortisol concentrations at the end of the interaction period were also indicative of social status in most treatment groups, with subordinate fish tending to exhibit higher circulating cortisol levels than dominant fish (Fig. 2A,B). Within the size-matched group, mean cortisol concentrations in subordinate fish examined as a whole (121.1±30.8, N=24) were significantly higher (rank sum test, P=0.002) than those in dominant fish (70.3±37.0, N=24); note that the dominant fish included cortisol-treated individuals. Similarly, subordinate fish within the control treatment group of the size-mismatched pairs (17.5±7.8, N=11) exhibited

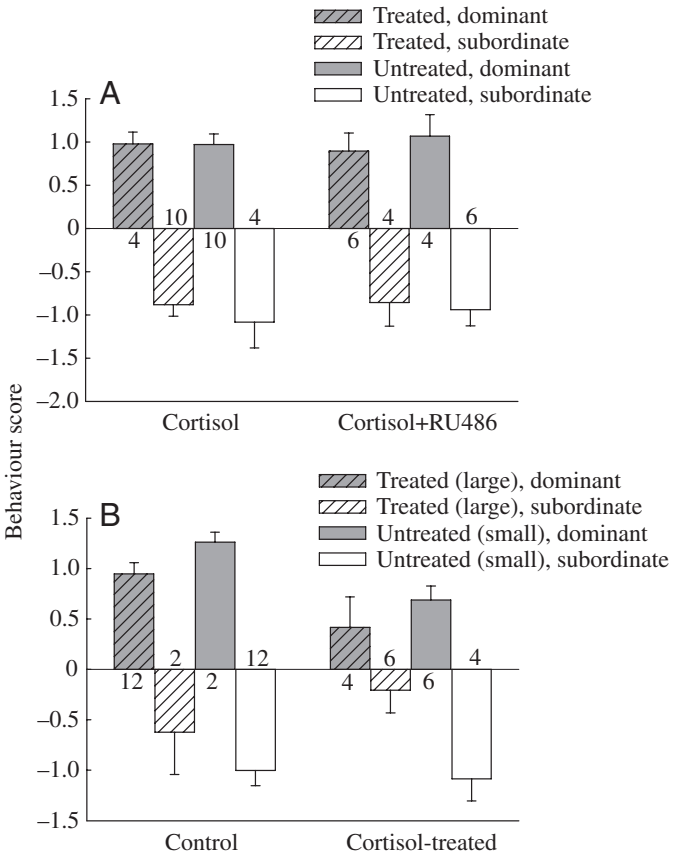

Fig. 1. The effects of cortisol and cortisol+RU486 treatments (single dose injections) on the behaviour scores of (A) size-matched (respectively, 14 and 10 pairs) and (B) size-mismatched (respectively, 14 and 10 pairs) pairs of rainbow trout *Oncorhynchus mykiss* confined together for 5 days. Values are means ± 1 S.E.M.; *N* values are given in the figure. Means were calculated for each combination of social status and treatment within a treatment group to give dominant treated, subordinate treated, dominant untreated, and subordinate untreated categories. There was no significant effect of treatment group on behaviour score within a category (Student's *t*-test, $P>0.05$).

significantly higher (rank sum test, $P=0.030$) cortisol levels than did dominant fish (4.63±2.9, $N=11$). This difference was eliminated, however, in the cortisol treatment group of the size-mismatched trial (dominant mean=29.5±15.7, $N=10$, subordinate mean=145.8±73.5, $N=10$; rank sum test, $P=0.121$) owing to high variability in circulating cortisol concentrations within cortisol-treated fish. In addition, cortisol administration was generally effective in raising plasma cortisol levels, with cortisol-treated fish in either size-matched pairs (overall mean=154.7±43.2, $N=24$) or the cortisol treatment group of size-mismatched pairs (153.0±72.4, $N=10$) exhibiting significantly higher (rank sum test, $P=0.004$ and 0.021, respectively) circulating cortisol concentrations than the corresponding untreated groups (size-matched, overall mean=36.7±14.4, $N=24$; size-mismatched cortisol treatment group mean=22.2±14.4, $N=10$).

An experimental protocol in which cortisol-treated or cortisol+RU486 treated individuals were paired with a conspecific that was <1.5% different in fork length revealed significant differences in behaviour as a result of treatment. χ^2 analysis indicated that cortisol-treated fish became subordinate more often than expected by chance alone (Table 1). This effect of cortisol was eliminated by simultaneous treatment with RU486; untreated and cortisol+RU486-treated fish were equally likely to be relegated to subordinate social status (Table 1). In an attempt to elucidate the importance of circulating cortisol concentrations relative to a factor that is known to affect the outcome of social interactions in rainbow trout (i.e. body size; Abbott and Dill, 1985), an experiment was carried out in which untreated or cortisol-treated individuals were paired with a conspecific that was at least 5% (range 4.6–17.4%) smaller in fork length. The larger fish became dominant in 86% of size-mismatched pairs of trout in which both fish were untreated ($N=14$ pairs), a result that was confirmed to be significantly different than that expected by chance alone *via* χ^2 analysis (Table 2). This size effect was eliminated by cortisol treatment ($N=10$ pairs), in which only 40% of larger (treated) fish became dominant. χ^2 analysis indicated that cortisol treatment ($\chi^2=0.8$, d.f.=1, $P>0.25$) decreased the probability, to the point where it was not significantly different than that expected by chance alone, of larger fish within each pair becoming dominant (Table 2).

Table 1. *A summary of the χ^2 analysis of the effects of cortisol treatment on social status in size-matched pairs of rainbow trout* Oncorhynchus mykiss *confined together for 5 days*

Treatment	χ^2	$\chi^2_{0.05,1}$	Conclusion
Cortisol	5.14 (14)	3.84	Reject H_o ($P<0.025$)
Cortisol+RU486	0.8 (10)	3.84	Fail to reject H_o ($P>0.25$)

Numbers in parentheses are *N* values. The null hypothesis (H_o) was that there was a 50% probability that the treated fish within a pair would become subordinate. The alternative (H_A) was that the treatment tested had a significant effect on this probability.

Condition factor and specific growth rate

Prior to the onset of social interactions, there were no significant differences in initial condition factor (CF_i) for either

Table 2. *A summary of the χ^2 analysis of the effects of cortisol treatment on social status in size-mismatched pairs of rainbow trout* Oncorhynchus mykiss *confined together for 5 days*

Treatment	χ^2	$\chi^2_{0.05,1}$	Conclusion
Control	14.28 (14)	3.84	Reject H_o ($P<0.001$)
Cortisol	0.8 (10)	3.84	Fail to reject H_o ($P>0.25$)

Numbers in parentheses are *N* values. The null hypothesis (H_o) was that there was a 50% probability that the larger fish within a pair would become dominant. The alternative (H_A) was that the treatment tested had a significant effect on this probability.

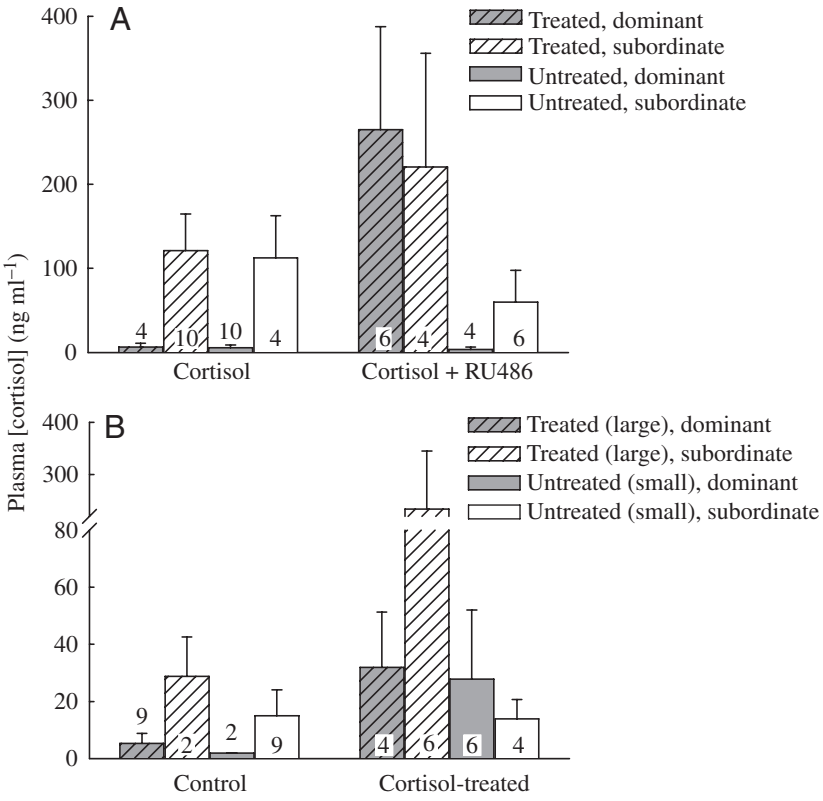

Fig. 2. The effects of cortisol and cortisol+RU486 treatments on plasma cortisol concentrations in (A) size-matched (respectively, 14 and 10 pairs) and (B) size-mismatched (respectively, 11 and 10 pairs) rainbow trout *Oncorhynchus mykiss* confined in pairs for 5 days. Values are means ± 1 S.E.M.; *N* values are given in the figure. Means were calculated for each combination of social status and treatment within a treatment group to give dominant treated, subordinate treated, dominant untreated, and subordinate untreated categories. See text for statistical analysis of these data.

size-matched or size-mismatched pairs of rainbow trout (size-matched pairs CF_i=1.08±0.014, *N*=48; size-mismatched pairs CF_i=1.11±0.021, *N*=48). Similarly, neither social status (two-way ANOVA, *P*=0.141) nor treatment group (*P*=0.937) had a significant effect on final condition factor (CF_f) within size-matched pairs of fish at the end of the 5-day interaction period (Table 3). By contrast, significant effects of social status (two-way ANOVA, *P*=0.006) were present within pairs of size-mismatched fish for CF_f (Table 4), with dominant fish exhibiting significantly higher CF_f values than subordinate fish (*P*=0.006).

Determination of specific growth rates (SGR) revealed significant effects of both social status (two-way ANOVA, *P*<0.001) and treatment group (*P*=0.036), as well as significant interactions (*P*=0.003), within size-matched pairs of fish (Table 3). Dominant individuals demonstrated significantly higher growth rates than subordinate fish in the cortisol (*P*<0.001) but not the cortisol+RU486 treatment group (*P*=0.08). Furthermore, growth rates for dominant fish from the cortisol+RU486 treated group were significantly lower than those of dominants from the cortisol treatment group (*P*=0.01). Finally, SGR for cortisol-treated fish was significantly lower (*P*=0.005) than that of non-injected fish with which they were paired, while a similar analysis in the cortisol+RU486 group revealed no difference (*P*=0.72, data not shown), a finding that suggests that the observed differences in growth might be a cortisol-mediated effect. Within size-mismatched pairs of rainbow trout, both social status (two-way ANOVA, *P*<0.001) and treatment group (*P*=0.012) had a significant effect on SGR, although there were no significant interactions between these factors (*P*=0.154; Table 4). Growth rates were significantly higher for dominant over subordinate fish (*P*<0.001), and for control over cortisol-treated fish (*P*=0.012).

Experiment 2: The effect of cortisol administration on brain monoamine levels

To assess the impact of circulating cortisol concentrations on brain monoaminergic activity, groups of trout were injected

Table 3. *Specific growth rates and final condition factors of dominant and subordinate rainbow trout* Oncorhynchus mykiss *from cortisol and cortisol+RU486 treatment groups confined in size-matched pairs for 5 days*

Treatment	Social status	SGR (% growth/day)	CF_f ($100\times g\ cm^{-3}$)
Cortisol	Dominant	0.25 ± 0.12^a (14)	1.09±0.03 (14)
	Subordinate	-1.16 ± 0.19^b (14)	0.99±0.03 (14)
Cortisol+RU486	Dominant	-0.58 ± 0.13^b (10)	1.04±0.03 (10)
	Subordinate	-1.01 ± 0.13^b (10)	1.05±0.04 (10)

SGR, specific growth rate; CF_f, final condition factor.

Values are means ± 1 S.E.M. (*N*). For SGR, groups that do not share a letter are significantly different from one another (two-way ANOVA with treatment group and social status as factors, *P*=0.036 for treatment group, *P*<0.001 for social status, and *P*=0.003 for interactions between these two factors); there were no significant effects of treatment group or social status on CF_f (two-way ANOVA, *P*=0.937 for treatment group, *P*=0.141 for social status, and *P*=0.088 for interactions).

Table 4. *Specific growth rates and final condition factors of dominant and subordinate rainbow trout* Oncorhynchus mykiss *from control and cortisol treatment groups confined in size-mismatched pairs for 5 days*

Treatment	Social status	SGR (% growth/day)	CF_f ($100\times g\ cm^{-3}$)
Control	Dominant	0.70±0.28 (14)	1.08±0.03 (14)
	Subordinate	–0.76±0.16* (14)	0.99±0.03* (14)
Cortisol	Dominant	–0.30±0.22† (10)	1.04±0.03 (10)
	Subordinate	–1.05±0.29*,† (10)	1.05±0.04* (10)

SGR, specific growth rate; CF_f, final condition factor.

Values are means ± 1 S.E.M. (*N*). The data were analysed by two-way ANOVA with treatment group and social status as factors. In neither case was there a significant interaction term between these two factors. Thus, *significant effect of social status (dominant *vs* subordinate); †significant effect of treatment group (control *vs* cortisol-treated) (two-way ANOVA; for SGR, P=0.012 for treatment group, P<0.001 for social status and P=0.154 for interactions between these two factors; for CF_f, P=0.129 for treatment group, P=0.006 for social status and P=0.113 for interactions).

intraperitoneally with a slow-release pellet of cortisol. Circulating plasma cortisol concentrations increased significantly (one-way ANOVA on ranks, P<0.001) with cortisol administration (Fig. 3). The levels attained in the plasma in both cortisol and cortisol+RU486 injected fish were similar to those associated with moderately stressed salmonids (Gamperl et al., 1994; Wendelaar Bonga, 1997), and marginally lower than those observed in previous studies that adopted similar methods (e.g. Vijayan et al., 2003; McDonald and Wood, 2004).

Brain monoaminergic activity was evaluated as a function of cortisol administration. Outlier tests were used to exclude any values that were greater than 10 standard deviations from the mean, thus identifying and accounting for HPLC measurement errors (in total, 5 data points were removed from subsequent analyses: 3 cortisol+RU486 injected 5-HT values in the telencephalon, one control DA value in the telencephalon, and one cortisol injected 5-HT value in the hypothalamus; three 'zero' DA values were also excluded from the data set). Within the telencephalon, mean serotonergic activity was significantly increased by the cortisol implant treatment (one-way ANOVA on ranks, P=0.008), an effect that was eliminated by simultaneous administration of the glucocorticoid receptor antagonist RU486 (Fig. 4C). This enhanced serotonergic activity observed in trout given cortisol implants reflected the significant lowering of telencephalon 5-HT concentration (one-way ANOVA on ranks, P<0.001; Fig. 4A) in the absence of significant change in 5-HIAA level (Fig. 4B). Interestingly, significantly lower levels of both 5-HT and 5-HIAA (one-way ANOVA on ranks, P<0.001) were measured in the telencephalon of trout treated with implants containing both cortisol and RU486. Significant differences in telencephalon dopaminergic activity also occurred (one-way ANOVA on ranks, P=0.021; Fig. 4F), although neither DA nor DOPAC concentrations in the telencephalon differed significantly among treatment groups (one-way ANOVA on ranks, P=0.086 and 0.057, respectively; Fig. 4D,E). While the effect of cortisol treatment in decreasing telencephalon dopaminergic activity was not quite statistically significant, a significant reduction from the control value was observed when cortisol was administered with RU486 (Fig. 4F). The absence of difference between the cortisol and cortisol+RU486 treatment groups (Fig. 4F) suggested that cortisol did in fact have a significant impact on telencephalon dopaminergic activity.

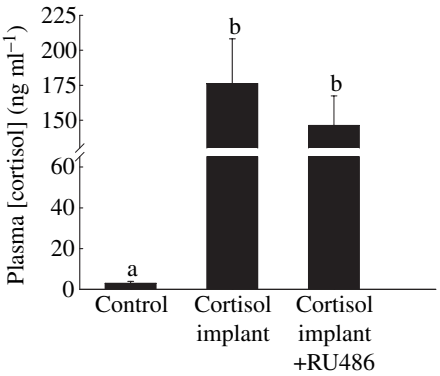

Fig. 3. Mean plasma cortisol concentrations in untreated rainbow trout *Oncorhynchus mykiss* (control; N=12) as well as those given an intraperitoneal implant containing either cortisol (50 mg hydrocortisone 21-hemisuccinate kg^{-1} fish; N=10) or cortisol+RU486 (500 mg mifepristone kg^{-1} fish; N=12). Values are means ± 1 S.E.M. Groups that do not share a letter were significantly different from one another (one-way ANOVA on ranks followed by Dunn's *post hoc* multiple comparisons test, P<0.001).

Surprisingly, the observed effects of cortisol treatment on serotonergic and dopaminergic activities in the hypothalamus were opposite to those in the telencephalon; hypothalamic serotonergic activity was significantly reduced by cortisol implants (one-way ANOVA on ranks, P<0.001; Fig. 5C), while dopaminergic activity was significantly increased (one-way ANOVA on ranks, P<0.001; Fig. 5F). The lower serotonergic activity occurred because of a significant reduction in 5-HIAA concentrations (one-way ANOVA on ranks, P<0.001; Fig. 5B) even though hypothalamic 5-HT was unchanged (one-way ANOVA on ranks, P=0.078; Fig. 5A). The higher dopaminergic activity reflected significant increases of hypothalamic DOPAC levels (one-way ANOVA on ranks, P<0.001; Fig. 5E) in combination with significantly

lower dopamine concentrations (one-way ANOVA on ranks, $P<0.001$; Fig. 5D). However, in no case did simultaneous administration of RU486 abolish these effects.

Discussion

The findings of the present study implicate circulating cortisol concentrations as one factor that determines the outcome of competitive social interactions between pairs of rainbow trout. Specifically, high plasma cortisol levels predisposed individual trout towards low social status. Although the physiological mechanisms underlying the effect of cortisol on the outcome of agonistic encounters remain to be fully elucidated, the data support a role for interactions between circulating cortisol concentrations and brain monoaminergic activity as an indirect modulator of competitive ability.

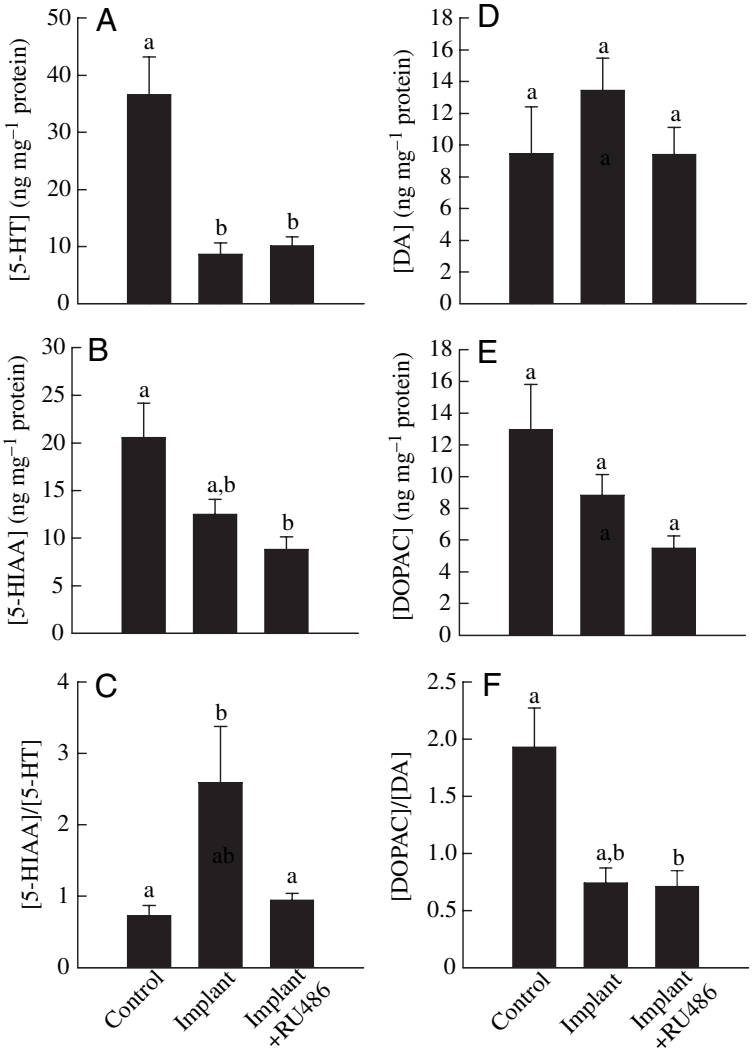

Fig. 4. Monoamine concentrations (A,D), main monoamine metabolite concentrations (B,E) and the ratio of concentrations of main metabolite to parent monoamine (monoaminergic activity; C,F) for serotonin (A–C) and dopamine (D–F) in the telencephalon of rainbow trout *Oncorhynchus mykiss* treated with cortisol. Cortisol was administered by intraperitoneal implant on its own ('implant', 50 mg hydrocortisone 21-hemisuccinate kg^{-1} fish) or simultaneously with the glucocorticoid receptor blocker RU486 (500 mg mifepristone kg^{-1} fish); an additional group of untreated fish served as a control group. Values are means ± 1 S.E.M.; N=10–12 for the control group, N=10 for the group given cortisol implants, and N=9–10 for the cortisol implant + RU486 treatment group. Treatment groups that do not share a letter were significantly different from one another (one-way ANOVA on ranks followed by Dunn's *post hoc* multiple comparisons test; P values, A<0.001, B=0.014, C=0.008, D=0.086, E=0.057, F=0.021).

Competitive ability is probably the key determinant of the winner of agonistic contests within juvenile salmonid fish, although factors such as prior residence can also play a role (Bachman, 1984; Rhodes and Quinn, 1998; Cutts et al., 1999). Competitive ability, in turn, reflects numerous factors including innate aggressiveness (Adams et al., 1998; Cutts et al., 1999), prior experience of winning or losing social contests (Abbott and Dill, 1985; Rhodes and Quinn, 1998), body size in some cases (Abbott and Dill, 1985; Rhodes and Quinn, 1998) and, presumably, physiological condition (e.g. Guderley and Couture, 2005). Physiological parameters such as abundant energy reserves, good condition and perhaps high metabolic capacity might be expected to correlate with competitive strength. For example, among several salmonid species, fish with higher metabolic rates prior to social interactions tended to achieve higher social status (Metcalfe et al., 1995; Yamamoto et al., 1998; Cutts et al., 1999; McCarthy, 2001). High metabolic rate was associated with greater levels of aggression in juvenile Atlantic salmon (Cutts et al., 1998), suggesting a mechanism through which high metabolic rate could translate into competitive success, and emphasizing the complexity of factors that determine competitive ability.

Previous work suggested that circulating cortisol levels might also be a physiological factor that affects competitive ability (Gregory and Wood, 1999; Sloman et al., 2001). Specifically, Sloman et al. (2001) documented significantly higher plasma cortisol levels prior to social interaction in rainbow trout that were identified as subordinate following pairing with a conspecific. Similarly, Gregory and Wood (1999) attributed the greater fin damage sustained by cortisol-treated trout held together with untreated trout to a cortisol-related competitive disadvantage. The results of the present study confirmed and extended these observations by revealing a causal relationship between high plasma cortisol concentrations prior to social interactions, and subsequent subordinate social status within pairs of rainbow trout. Experimental elevation of plasma cortisol levels was associated with a statistically significant increase in the probability of relegation to subordinate rank, an effect that was eliminated by blocking cortisol receptors using the glucocorticoid receptor antagonist RU486 (Table 1). Notably, the few (4 of 14) cortisol-treated fish that did achieve dominant status failed to exhibit elevated

plasma cortisol concentrations (Fig. 2). It is possible that these fish simply did not receive an effective dose of cortisol *via* the implant. As cortisol levels in the plasma reflect the balance between cellular biosynthesis and secretion into the blood (i.e. production), as well as clearance of the hormone from circulation (Mommsen et al., 1999), these fish may alternatively have been able to combat cortisol treatment by increasing cortisol clearance from the plasma, although this possibility remains to be tested.

Cortisol treatment also countered the effect of large size in determining dominance (Table 2). Larger fish became dominant in 86% of pairs in which both fish were untreated, a result that was in agreement with previous studies that reported a positive correlation between body size and dominant social status in rainbow trout (Bachman, 1984; Abbott and Dill, 1985). This significant effect of size in assuring dominant status was lost when the larger fish were treated with cortisol; only 40% of cortisol-treated large fish became dominant. The effect of cortisol treatment on the outcome of social interactions observed in the present study fits well with previous reports in which social status was closely linked with the magnitude of the cortisol response to an acute stressor (Pottinger and Carrick, 2001). Pottinger and Carrick (2001) found that within genetically maintained lines of rainbow trout selected for divergent cortisol responses to an acute confinement stress, high responding (HR) trout (i.e. greater elevation of cortisol levels) preferentially became subordinate when paired with size-matched, low-responsive (LR) trout in staged social encounters. Although Pottinger and Carrick (2001) were unable to determine whether the link between social status and cortisol responses to an acute stress was causal or circumstantial, the results of the present study point to a causal relationship.

A causal relationship between high plasma cortisol levels and low social status in rainbow trout could be the result of one or more underlying physiological mechanisms. One possibility is that high circulating cortisol levels affect competitive ability directly by depressing physiological condition so that fish are not able to compete effectively. Prolonged experimental administration of cortisol lowers growth rate and condition factor, and increases mortality (Barton et al., 1987; Pickering and Pottinger, 1989; Gregory and Wood, 1999), effects that have been attributed to appetite suppression, the mobilisation of energy reserves, changes in digestive tract morphology, reduced food conversion efficiency, increased metabolic rate, and immune function suppression (Barton et al., 1987; Pickering and Pottinger, 1989; Morgan and Iwama, 1996; Gregory and Wood, 1999; De Boeck et al., 2001). For example, the mean specific growth rate of cortisol-treated fish in the present study was significantly lower over the 5 day interaction period than that of the untreated fish with which they were paired, an effect that was cortisol-specific since it was eliminated by co-administration of RU486. Similarly, the chronic elevation of plasma cortisol attendant upon low social status probably accounted for, at least in part (see Gilmour et al., 2005), the generally significantly lower growth rates of subordinate fish relative to dominant fish (Tables 3, 4). These findings are in agreement with previous reports in which lower growth rates

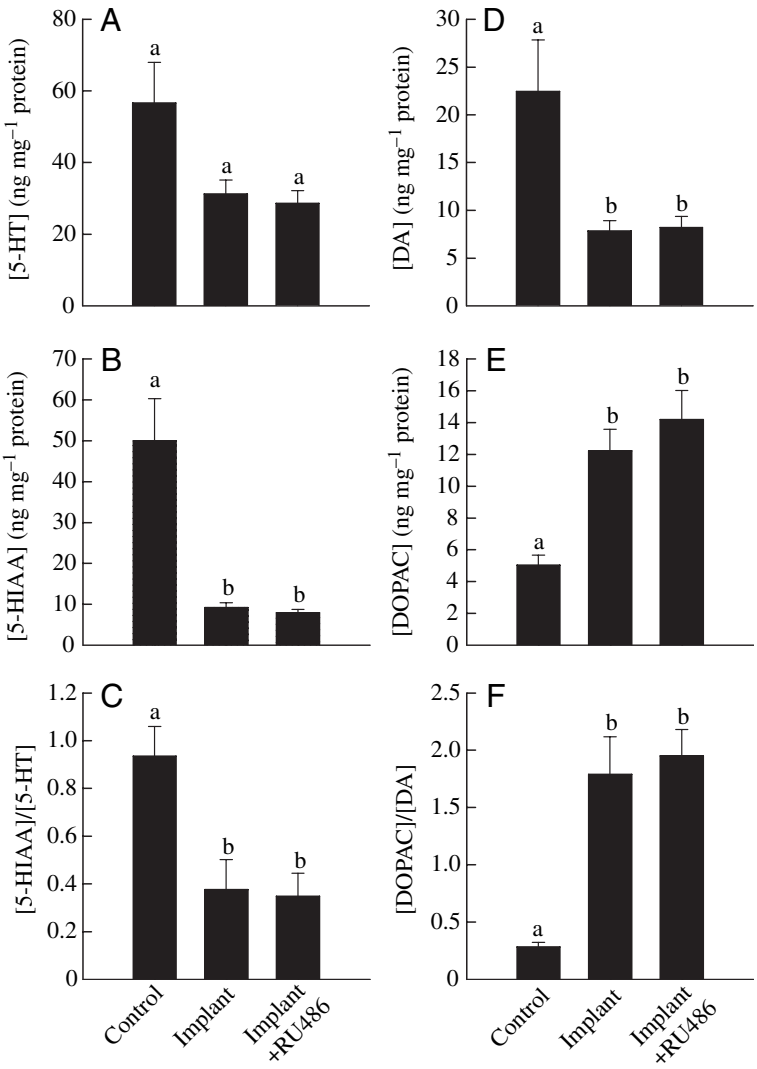

Fig. 5. Monoamine concentrations (A,D), main monoamine metabolite concentrations (B, E) and the ratio of concentrations of main metabolite to parent monoamine (monoaminergic activity; C,F) for serotonin (A,B,C) and dopamine (D,E,F) in the hypothalamus of rainbow trout *Oncorhynchus mykiss* treated with cortisol. Cortisol was administered by intraperitoneal implant either on its own ('implant', 50 mg hydrocortisone 21-hemisuccinate kg^{-1} fish) or simultaneously with the glucocorticoid receptor blocker RU486 (500 mg mifepristone kg^{-1} fish); an additional group of untreated fish served as a control group. Values are means ± 1 S.E.M.; *N*=12 for the control group, *N*=9–10 for the group given cortisol implants, and *N*=12 for the cortisol implant+RU486 treatment group. Treatment groups that do not share a letter were significantly different from one another (one-way ANOVA on ranks followed by Dunn's *post hoc* multiple comparisons test; *P* values, A=0.078, B<0.001, C<0.001, D<0.001, E<0.001, F<0.001).

were exhibited by fish of low social status (Abbott and Dill, 1989; Sloman et al., 2000a,b). However, the deleterious impact of cortisol on physiological condition reflects prolonged elevation of the hormone, whereas cortisol levels were raised in the present study only 48 h prior to the initiation of social interactions. Thus, while cortisol-induced physiological depression may diminish competitive ability, and may have contributed to the association reported by Sloman et al. (2001) between higher plasma cortisol prior to pairing and subsequent subordinate status, it is unlikely to be the sole explanation for the results of the present study.

Alternatively, cortisol could affect competitive ability by modulating behaviour in either a direct or indirect fashion. For example, time- and context-dependent effects of cortisol administration were observed on aggressive behaviour and locomotory activity in rainbow trout (Øverli et al., 2002a). The locomotory response to an intruder was enhanced after 1 h of cortisol treatment, whereas both aggressive behaviour and activity in an intruder test were inhibited following 48 h of cortisol treatment. Locomotory activity in the absence of an intruder was unchanged by cortisol treatment, suggesting an indirect role for cortisol in modifying behaviour, through interactions with other signaling systems activated under particular circumstances (Øverli et al., 2002a). Similarly, studies involving HR and LR rainbow trout found that HR trout reacted to stress-induced increased plasma cortisol concentrations by marked changes in locomotor activity, whereas LR trout did not (Overli et al., 2001, 2002b). With respect to social interactions in salmonid fish, brain monoamines, specifically serotonin and dopamine, represent a signaling system of particular interest because the behaviours characteristic of high or low social status are thought to result in large part from the changes in brain monoaminergic activity that accompany victory or loss in competitive encounters (reviewed by Winberg and Nilsson, 1993). The results of the present study support, albeit not conclusively, a role for cortisol in modifying brain monoaminergic activity, and hence suggest that the causal link between high cortisol and low social status may reflect an indirect modulatory action of cortisol on behaviour mediated through brain monoaminergic systems.

Relative to control fish, serotonergic activity was markedly higher and dopaminergic activity was lower in the telencephalon of cortisol-treated trout (Fig. 4). This result is consistent with work on other vertebrate groups in which corticosteroids have been found to affect brain serotonergic activity (reviewed by Chaouloff, 1993, 2000). For example, intraperitoneal injection of corticosterone in male *Anolis carolinesis* lizards significantly enhanced serotonergic activity within 20 min in two separate brain regions (Summers et al., 2000), and intracorticol infusion evoked transient, dose-dependent increases in serotonin overflow from neurons in the hippocampus (Summers et al., 2003). The effects of cortisol administration on telencephalon serotonergic and dopaminergic activity observed in the trout of the present study seemed to mimic those produced by defeat in an agonistic contest (Winberg et al., 1991, 1992). Experimental treatments designed to increase brain 5-HT levels and/or serotonergic activity generally have been reported to elicit behavioural inhibition in fish (but see Stoddard et al., 2003), whereas high brain dopaminergic activity, on the other hand, seems to facilitate aggressive behaviour (Winberg and Nilsson, 1993). For example, aggressive behaviour in rainbow trout was suppressed by dietary administration of the 5-HT precursor L-tryptophan, a treatment that also increased brain serotonergic activity (Winberg et al., 1991). Similarly, territorial aggression in a coral reef fish was depressed by intraperitoneal injection of the 5-HT selective reuptake inhibitor fluoxetine (Perrault et al., 2003), while intracranial injection of either 5-HT or fluoxetine inhibited aggressive 'chirping' behaviour in a weakly electric fish (Maler and Ellis, 1987). Aggressive behaviour in several salmonid species was increased following treatment with the DA receptor agonist apomorphine (Tiersch and Griffith, 1988) or DA itself (Nechayev and Musatov, 1992), and oral administration of the DA precursor, L-dopa, increased the probability of winning dominant social status in Arctic charr (Winberg and Nilsson, 1992). Thus, high circulating cortisol levels may be linked to low social status through a pathway in which cortisol-induced increases in brain serotonergic activity and/or decreases in dopaminergic activity result in the inhibition of the aggressive behaviour critical for success in agonistic encounters.

Within the hypothalamus, cortisol treatment resulted in a significant decrease of serotonergic activity and an increase of dopaminergic activity (Fig. 5), effects opposite to those observed in the telencephalon of cortisol-treated fish (Fig. 4), and opposite also to the impact of social defeat on hypothalamic serotonergic activity (Winberg and Nilsson, 1993). The hypothalamus is a key component of the hypothalamic–pituitary–interrenal (HPI) stress axis in fish (Wendelaar Bonga, 1997; Mommsen et al., 1999). Hypothalamic corticotropin releasing factor acts on the pituitary to stimulate the secretion of adrenocorticotropic hormone, which in turn elicits cortisol synthesis and mobilisation from interrenal cells. Cortisol secretion *via* this pathway may be modulated by the negative feedback actions of cortisol at the levels of the hypothalamus and pituitary (Mommsen et al., 1999), and several lines of evidence suggest that hypothalamic 5-HT also may be involved in the regulation of the HPI axis (e.g. Winberg et al., 1997a; Lepage et al., 2002, 2003; Hoglund et al., 2002). Experimental elevation of plasma cortisol would be expected to downregulate endogenous cortisol secretion *via* negative feedback. It is conceivable that the lowering of hypothalamic serotonergic activity observed in cortisol-treated fish reflected such a downregulation of cortisol secretion pathways.

The changes of monoaminergic activity in the telencephalon and hypothalamus of cortisol-treated rainbow trout were in general not abolished by co-administration of the glucocorticoid receptor antagonist RU486 (Figs 4, 5), except in the case of telencephalon serotonergic activity (Fig. 4C). While these findings raise concerns about whether the responses were cortisol-specific, at least two plausible explanations exist. First, the responses may be mediated *via* a

mineralocorticoid receptor (MR) rather than a glucocorticoid receptor (GR). In mammals, MRs and GRs exhibit different expression patterns in the brain and play different roles in mediating the effects of corticosteroids (Chaouloff, 2000; Korte, 2001). GRs are widely distributed in the forebrain of rainbow trout (Teitsma et al., 1997, 1998) and the recently identified fish MR (Colombe et al., 2000; Greenwood et al., 2003; Sturm et al., 2005) also appears to be present in the brain (Greenwood et al., 2003; Sturm et al., 2005). However, the relative distributions and roles of the two corticosteroid receptors in fish brains remain to be explored. Alternatively, cortisol may exert effects in the brain *via* non-genomic mechanisms, a route of action that has been well documented in mammals (reviewed by Makara and Haller, 2001). For example, Mikics (2004) suggested that glucocorticoids rapidly increased aggressive behaviour in rats *via* non-genomic mechanisms. The non-genomic effects of corticosteroids are much more rapid than the genomic responses, and resistant to both GR and MR blockade (Makara and Haller, 2001).

In conclusion, the results of the present study revealed a causal association between high plasma cortisol concentrations and low social status, and in general supported cortisol-induced changes in brain monoaminergic activity as a potential regulatory pathway for this effect. Clearly, however, additional work is required to validate the hypothesis that high circulating cortisol levels modify brain serotonergic activity and/or dopaminergic activity in trout, resulting in the suppression of aggressive behaviour and a consequent lowering of competitive ability that increases the likelihood of relegation to subordinate social status during agonistic contests.

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