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The impact of social status on the erythrocyte β-adrenergic response in rainbow trout, *Oncorhynchus mykiss*

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Abstract

The objective of the present investigation was to determine whether chronic increases in circulating cortisol concentrations, resulting from the occupation of subordinate status in rainbow trout social hierarchies, resulted in an enhancement of the erythrocyte adrenergic response. Rainbow trout (*Oncorhynchus mykiss*) were confined in fork length matched pairs for 6 h, 18 h, 48 h or 5–7 days, and social status was assigned through observations of behaviour. Erythrocyte adrenergic responsiveness, determined in vitro as changes in water content following incubation with the β-adrenoreceptor agonist isoproterenol, was significantly greater in subordinate than dominant fish at 48 h of social interactions but not after 5–7 days, nor when assessed as changes in extracellular pH (pHe). However, the activity of the Na^+/H^+ exchanger (β-NHE), assessed in vitro as the pHe change following incubation with the permeable cyclic AMP analogue 8-bromo-cyclic AMP, was significantly lower in subordinate fish. The number of erythrocyte membrane-bound adrenergic receptors (B_{max}) was significantly higher in subordinate than dominant fish at 48 h, but had decreased by 5–7 days to a value that was not significantly different from that for dominant fish. The apparent dissociation constant (K_D) of these receptors was not significantly impacted by either social status or interaction time. Finally, the relative expressions of β-3_b adrenergic receptor (AR) and β-NHE mRNA were determined using real-time PCR and were found to be minimally affected by social rank. Relative to a control group, β-3_b AR mRNA was significantly up-regulated in both dominant and subordinate trout at all time periods, whereas the expression of β-NHE was in general significantly down-regulated. Unlike the situation in rainbow trout treated with exogenous cortisol, elevations in circulating cortisol resulting from low social status did not "pre-adapt" the erythrocyte adrenergic response, but rather may have served to offset the potentially adverse effects elicited by plasma catecholamines, which were elevated during social hierarchy formation.

Keywords: Social stress; Cortisol; Catecholamines; Rainbow trout; *Oncorhynchus mykiss*; Erythrocyte; β-3_b adrenoreceptor; β-NHE

1. Introduction

When confined to environments of limited resources, small groups of salmonid fish quickly establish linear dominance-based social hierarchies (Schreck, 1981; Sloman and Armstrong, 2002). Social status is determined through aggressive interactions between conspecifics, and all competitors initially experience acute stress as indicated by high circulating levels of the corticosteroid stress hormone, cortisol (Øverli et al., 1999a). As the social hierarchy is established, plasma cortisol concentrations in dominant fish drop, returning to levels similar to those of unstressed fish within a few hours (Øverli et al., 1999a), whereas prolonged elevation of circulating cortisol is characteristic of subordinate fish (Øverli et al., 1999a; Sloman et al., 2001). Subordinate social status is considered to be a chronic stressor (Sloman et al., 2002), and many of the adverse physiological consequences of low social status, including lowered growth rates, reduced condition factor and immunosuppression, may be attributed to responses that are secondary to the stress itself (Sloman and Armstrong, 2002; Gilmour et al., 2005).

Despite the many adverse physiological consequences associated with subordinate social status, chronic increases in circulating cortisol levels have the potential to enhance the acute stress response of subordinate salmonids, specifically the erythrocyte adrenergic response (Perry and Reid, 1993). In this response, catecholamines secreted from chromaffin cells

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during periods of severe acute stress bind to surface adenylate cyclase-coupled β-adrenergic receptors (β-3_b AR; Nickerson et al., 2003), resulting in the formation of the secondary messenger cyclic AMP. Subsequently, cyclic AMP stimulates a phosphorylation cascade within the erythrocyte that activates surface Na^+/H^+ exchangers (β-NHE; Nikinmaa and Huestis, 1984). The activation of β-NHE ultimately results in alkalisation of the erythrocyte interior (Nikinmaa, 1982; Nikinmaa and Huestis, 1984; Nikinmaa and Tufts, 1989), a reduction in the intracellular concentration of organic phosphates (Nikinmaa, 1983; Ferguson and Boutilier, 1988) and an increase in erythrocyte volume, all of which in turn enhance both haemoglobin-O_2 binding affinity and carrying capacity (reviewed by Thomas and Perry, 1992; Perry and Bernier, 1999).

Chronic increases in circulating cortisol concentrations have been proposed to "pre-adapt" the erythrocyte adrenergic stress response (Perry and Reid, 1993). In rainbow trout, cortisol treatment increased the internal pool of β-adrenergic receptors (β-AR) as well as the mobilization of such receptors to the cell surface following exposure to hypoxia (Reid and Perry, 1991; Perry and Reid, 1993). Because cortisol is chronically elevated in subordinate but not dominant trout, such processes could enhance the erythrocyte adrenergic response in low ranking individuals, thereby increasing the sensitivity of their erythrocytes to catecholamines released during exposure to acute stressors. Conversely, dominant individuals would be expected to lack a cortisol-mediated augmentation of erythrocyte adrenergic responsiveness. By contrast with the apparently beneficial effects of cortisol on the erythrocyte adrenergic response, repeated or prolonged elevation of circulating catecholamine levels desensitises the erythrocyte adrenergic response (Garcia-Romeu et al., 1988; Gilmour et al., 1994; Perry et al., 1996). Catecholamine secretion in vivo during social interactions has yet to be investigated in salmonid fish, but might be expected to accompany the aggressive encounters that characterise the early stages (initial 4–6 h; Øverli et al., 1999a) of hierarchy formation.

The objective of the present investigation was to test the hypothesis that social status in rainbow trout impacts the erythrocyte adrenergic response. Specifically, enhanced erythrocyte adrenergic responsiveness was predicted to occur in subordinate rainbow trout after several days (5–7 days) of confinement with a dominant conspecific owing to the prolonged elevation of plasma cortisol that typifies the chronic social stress of low rank. The acute stress attendant upon formation of the social hierarchy, on the other hand, was predicted to result in a desensitisation of the erythrocyte adrenergic response in subordinates during shorter periods (48 h) of social interaction. To investigate the extent to which dominant versus subordinate trout experience acute stress, plasma catecholamine levels were measured at different stages of hierarchy formation. The erythrocyte adrenergic response of dominant and subordinate rainbow trout was assessed at several levels. Cell swelling and the change in extracellular pH (pHe) following the incubation of erythrocytes with a β-adrenergic agonist were used as indices of overall adrenergic responsiveness. In addition, β-3_b AR surface numbers and mRNA expression were evaluated, as were β-NHE mRNA expression and, as an index of β-NHE activity specifically, the change in pHe following incubation of erythrocytes with a permeable cyclic AMP analogue.

2. Materials and methods

2.1. Experimental animals

Juvenile rainbow trout (*Oncorhynchus mykiss*; experimental $N=246$, mass $=204.4\pm4.2$ g, fork length $=266.1\pm1.6$ mm; mean ± SEM) were obtained from Linwood Acres Trout Farm (Campbellcroft, ON, Canada). Fish were maintained in large fibreglass aquaria supplied with flowing, dechloraminated, city of Ottawa tap water at 13 °C. A 12 h:12 h light:dark photoperiod was used. Trout were fed to satiation with commercial trout pellets on alternate days, and were acclimated to the holding conditions for at least 2 weeks before experiments commenced.

Social hierarchies were established within pairs of size-matched rainbow trout. Fish were lightly anaesthetised (i.e. to the point of losing equilibrium) in a solution of ethyl-*p*-aminobenzoate (0.065 g l^{-1}), fork length was measured, and fish were allocated to pairs according to comparable fork lengths (mean difference $=5.4\pm0.5$ mm, representing 2% of fork length; $N=114$ pairs in total). With the exception of fish used to determine circulating catecholamine levels during social interaction (see below), fish were confined in pairs in 60 l sections of a large fibreglass tank partitioned with perforated opaque Plexiglas, and social status was assigned through observations of behaviour (see below). After 6 h, 18 h, 48 h, or 5–7 days of confinement in pairs, fish were killed by immersion in a solution of ethyl-*p*-aminobenzoate (0.15 g l^{-1}) and blood (1–4 ml) was withdrawn by caudal puncture. These blood samples were then used to assess: erythrocyte adrenergic responsiveness ($N=16$–17 pairs at each sample time); β-NHE activity ($N=5$–6 pairs at each sample time); β-AR density and affinity ($N=5$–7 pairs at each sample time); or mRNA expression of β-3_b AR and β-NHE ($N=5$–10 pairs at each sample time); different pairs of fish were used in each experiment. The shorter times (6 and 18 h) were used only for evaluation of β-3_b AR and β-NHE mRNA expression, as changes in gene expression were expected to precede changes in protein levels or activity.

For evaluation of β-3_b AR and β-NHE mRNA expression by real-time PCR, blood samples were immediately centrifuged for 30 s. The plasma was then removed and stored at −80 °C for subsequent analysis of plasma cortisol concentrations (using a commercial radioimmunoassay kit; ICN; Gamperl et al., 1994), and erythrocytes were frozen in liquid nitrogen and stored at −80 °C until analysis. For all other experiments (erythrocyte adrenergic responsiveness, β-NHE activity, or β-AR density and affinity), blood was kept on ice in glass scintillation vials containing 100 IU ml $blood^{-1}$ ammonium heparin; a small aliquot of blood was removed and centrifuged to yield plasma that was subsequently assayed for cortisol levels. In all experiments, the sampling order of dominant and subordinate

fish was alternated between pairs to control for sequential sampling effects.

2.2. Behavioural observations

Social status was assessed by assigning points to each fish for position, food acquisition, fin damage and aggressive behaviour during the experimental period. The points system was a variation on similar approaches that have been used previously to assign social status to salmonid fish (Metcalfe et al., 1989; Johnsson et al., 1996; Sloman et al., 2000a,b).

Observations were carried out twice daily for 10 min; initial tank position was noted, and whether either fish was aggressive or fed during the observation period was recorded. Fish found to be in the water column received a score of 10, those on the bottom or in a black PVC refuge received a score of 5, and those at the surface of the tank were given a score of 0. Aggressive behaviour (attacks involving body contact or the displacement of the fish subjected to the aggressive behaviour, or counter-attacks in response to aggression) scored 1 point, while non-aggressive fish were given a score of 0. As the dorsal and caudal fins are generally the targets of aggressive behaviour (Moutou et al., 1998; Turnbull et al., 1998), fin damage at the end of the experimental period was also used as a measure of aggression (only fish exhibiting minimal or no fin erosion prior to pairing were used). Damage to both the caudal and dorsal fins was assessed as absent (scoring 3 points), minor (<30% of fin missing; 2 points), moderate (30–70% of fin missing; 1 point), or severe (>70% of fin missing; 0 points) according to Moutou et al. (1998). Observations of feeding were made following the addition of a few floating commercial fish pellets to each section of the tank. Fish that fed over the course of an observation period were given a score of 1, whereas non-feeding fish were given a score of 0.

Following the experimental period, a single behaviour score was calculated, by principal components analysis (using SPSS 10.0.5; SPSS Scientific Inc.), from the mean scores for each fish for tank position, aggression, feeding, and fin damage. Using this scoring system, dominant behaviours generated higher scores than subordinate behaviour. Thus, the fish with the higher score within a pair was assigned dominant social status. In one pair of fish, identical behaviour scores were obtained and the pair was removed from subsequent analyses.

2.3. Erythrocyte adrenergic responsiveness

The adrenergic responsiveness of rainbow trout erythrocytes was assessed by erythrocyte swelling and pHe changes following exposure to the synthetic adrenergic agonist isoproterenol, a non-selective β-AR ligand that was found to elicit maximal activation of the erythrocyte adrenergic response (Nickerson et al., 2003). Erythrocytes were incubated in the presence of either saline or one of up to 5 concentrations of isoproterenol (final nominal concentrations ranging from 10^{-8} to 10^{-5} mol l^{-1}). The number of isoproterenol concentrations that could be tested was constrained by the volume of blood that could be sampled by caudal puncture from a single fish. Blood samples were gassed with O_2 for 4 min and stored in the refrigerator for 24 h to allow endogenous catecholamines to decay (Perry et al., 1996). Aliquots of blood (0.5 ml) adjusted to 25% haematocrit (measured in duplicate using microcapillary tubes centrifuged at 6000×*g* for 5 min) were then pre-incubated for 30 min at 12 °C with a humidified, hypoxic gas mixture (PO_2=15.2 Torr, PCO_2=2.3 Torr) delivered at a rate of 2000 l min^{-1} by a gas mixing flow meter (GF-3/MP; Cameron Instruments). Pre-incubation of blood samples to hypoxic conditions was designed to maximise the magnitude of the erythrocyte adrenergic response (Reid and Perry, 1991; Motais et al., 1992; Reid et al., 1993; Perry et al., 1996; Nikinmaa et al., 2003).

Erythrocyte swelling was assessed by measuring cell water content. Following 30 min of incubation with saline or isoproterenol (chosen on the basis of previous work indicating that changes in cell water levels are detectable at this point; Salama and Nikinmaa, 1990; Salama, 1993), blood samples (0.5 ml) were centrifuged, and the supernatant and upper layer of erythrocytes were discarded. Erythrocyte pellets were weighed, dried at 60 °C to a constant mass, and re-weighed. Water content (erythrocyte [H_2O], %) was calculated as the percentage water of the total mass, and was not corrected for plasma water trapped in the erythrocyte pellet. A separate set of blood samples and a 5 min incubation period, selected on the basis of pilot experiments in which pHe changes were found to be maximal at this time, were used for the measurement of pHe, an index of H^+ extrusion. Measurements of pH were carried out on separated plasma (~0.2 ml) using E301 glass pH and E351 reference electrodes (Cameron Instruments) housed in a thermostatted (12 °C) cell and connected to a Radiometer PHM71b meter.

2.4. Assessment of β-NHE activity

To assess the activity of the β-NHE specifically, pHe was measured following 30 min incubation of blood samples (0.5 ml; prepared and pre-incubated as described above) with saline or the permeable cyclic AMP analogue 8-bromoadenosine-3′,5′-cyclic monophosphate (8-bromo-cyclic AMP; 10^{-4}, 10^{-3}, and 10^{-2} mol l^{-1} final nominal concentrations).

2.5. Characterisation of erythrocyte β-AR

The characterisation of erythrocyte β-ARs from dominant and subordinate fish was carried out on duplicate samples of intact erythrocytes incubated with the hydrophilic β-AR radioligand [(−)-4-93-butylamino-2-hydroxypropoxy-[5,7-3H] benzimidazol-2-1][^{3}H]CGP-12177 ([^{3}H]CGP, Amersham, specific activity 52 Ci $mmol^{-1}$), essentially as described by Reid et al. (1991), with the exception that alprenolol rather than isoproterenol was used to assess non-specific binding (e.g. Dugan et al., 2003). In brief, [^{3}H]CGP (6 final nominal concentrations ranging from 0.25–10 nmol l^{-1}) was added to 50 µl of blood in 90 µl of Cortland saline alone, or saline containing the non-specific β-adrenergic receptor antagonist alprenolol (10^{-5} mol l^{-1}). The number of erythrocytes added to

the assay was determined by cell counting using a haemocytometer. Erythrocyte samples were incubated in the dark for 45 min at room temperature, following which ligand binding was terminated by harvesting cell membranes (Brandel 24R cell membrane harvester) onto glass fibre filters (#32, Schleicher and Schuell) and washing four times with ice-cold 0.9% (w/v) NaCl. Glass fibre filters were placed in scintillation vials containing 4 ml of scintillation cocktail (Ready Safe; Beckman Coulter), and after 24 h of incubation in the dark, radioactivity was determined using a Packard 2500TR liquid scintillation counter. The maximum number of alprenolol-displaceable binding sites (β-receptor density; B_{max}) and apparent dissociation constant (K_D) were determined by Scatchard plot analysis. Blood samples were prepared as described above but not pre-incubated under hypoxic conditions prior to β-AR characterisation. Although acute exposure to hypoxia in vitro has been reported to increase erythrocyte β-AR numbers in several studies (e.g. Martilla and Nikinmaa, 1988; Reid and Perry, 1991; Reid et al., 1993), other studies have failed to detect a statistically significant effect of acute hypoxic exposure (Perry and Reid, 1992; Perry et al., 1996; Koldkjaer et al., 2004). Because the magnitude of the hypoxic increase in β-AR number, when present, is relatively small and its physiological relevance has been questioned (Perry et al., 1996), hypoxic pre-incubation was not utilised in assessing β-AR characteristics in the present study.

2.6. Quantification of β-3b AR mRNA and β-NHE mRNA expression

Frozen erythrocyte samples were ground to a fine powder under liquid N_2, and total RNA was extracted from 30 mg aliquots of tissue using the RNeasy Mini Kit (Qiagen) or Absolutely RNA® RT-PCR Miniprep Kit (Stratagene). To remove any remaining genomic DNA, RNA was treated on-column using RNase-free DNase (30 μl, Qiagen) for 20 min at room temperature (Qiagen kit) or 30 min at 37 °C (Stratagene kit). The RNA was eluted in 40 μl of nuclease-free H_2O and its quality was assessed using gel electrophoresis and spectrophotometry (Eppendorf Biophotometer). cDNA was synthesised from 10 μg of RNA using random hexamer primers (Boehringer Mannheim) and Stratascript reverse transcriptase (Stratagene).

Nickerson et al. (2003) demonstrated that the trout erythrocyte β-AR most closely resembles the β_3-AR of mammals, and termed the trout form β-3_b. β-3_b AR and β-NHE mRNA levels were assessed by real-time PCR on 0.5 μl samples of cDNA using a Hot StarTaq Master Mix kit (Qiagen) and a Stratagene MX-4000 multiplex quantitative PCR system. SYBR Green (Molecular Probes Inc.) and ROX (Stratagene) were used as DNA and reference dyes, respectively. The PCR conditions for a final reaction volume of 25 μl were: cDNA template = 0.5 μl; forward and reverse [primer] = 300 nmol l^{-1}; [Mg^{2+}] = 2.0 mmol l^{-1}; CYBR green = 1 : 50,000 final dilution; ROX = 1 : 30,000 final dilution; dNTPs = 200 μmol l^{-1}. The annealing and extension temperatures over 40 cycles were 58 °C (30 s) and 72 °C (30 s), respectively. The gene-specific primer pairs used in the present study were those designed and tested by Koldkjaer et al. (2004). They included: β-actin forward 5′-CCAACAGATGTGGATCAGCAA-3′; β-actin reverse 5′GGTGGCAGAGCTGAAGTGGTA-3′; β-3b forward 5′-CCAGCGCCGACGACAAC-3′; β-3b reverse 5′-CCTTGTGCTCCTTGACAACG-3′; β-NHE forward 5′-GGGTAATGCGTCAGACAACC-3′; and β-NHE reverse 5-AACAGGACATGAAGGGATCG-3′. To ensure that CYBR green was not being incorporated into primer dimers or non-specific amplicons during real-time PCR reactions, CYBR green dissociation curves were constructed after the completion of 40 PCR cycles and always revealed the presence of a single amplicon for each primer pair. Experiments in which reverse transcriptase was omitted during cDNA synthesis served as a control against the amplification of any residual genomic DNA. Using β-actin as an endogenous standard, relative expressions of β-3_b AR and β-NHE mRNA were determined through modification of the delta-delta Ct method (Perry et al., 2003). Relative changes in mRNA expression for dominant and subordinate fish were compared to those for unstressed control fish (N = 6) sampled directly from holding tanks.

2.7. Determination of catecholamine release during social interaction

Size-matched rainbow trout were placed into separate halves of a 40 l flow-through tank partitioned by a removable perforated black PVC divider. Smaller tanks were utilised in this set of experiments to minimise capture times and reduce the extent of catecholamine release during netting (Reid et al., 1994; Perry et al., 1996). Following a 24 h recovery period, dividers were removed and fish were allowed to interact. A pair of fish was observed until a hierarchy was clearly established (i.e. one fish repeatedly fleeing from the other). At this point, or at 8 h or 24 h after removal of the tank divider, fish were rapidly netted and killed by anaesthetic overdose (ethyl-*p*-aminobenzoate; 0.5 g l^{-1}), and a blood sample (~1 ml) was withdrawn via caudal puncture. Blood samples were immediately centrifuged, and the plasma was then frozen in liquid nitrogen and stored at −80 °C until analysis for catecholamine concentrations. For 8 h and 24 h pairings, pairs were observed for an additional 5 min period prior to sampling to ensure that the initial hierarchy was maintained. Samples from single (unpaired) fish exposed to the same experimental conditions (i.e. removal of the divider after a 24 h acclimation period, and netting) were used to take into account catecholamine mobilization elicited by handling.

Plasma noradrenaline and adrenaline levels were determined on alumina-extracted samples (200 μl) by high performance liquid chromatography (HPLC) with electrochemical detection (Woodward, 1982). 3,4-Dihydroxybenzylamine hydrobromide (DHBA) was used as an internal standard, and detection limits for noradrenaline and adrenaline were 0.1 nmol l^{-1}.

2.8. Statistical analysis

All data are presented as mean values ± 1 standard error of the mean (SEM). Erythrocyte swelling and pHe data were expressed relative to the control value to reduce inter-individual

variation. Statistical significance was assessed by one-way repeated measures analysis of variance (RM ANOVA) or one-way ANOVA followed by either the Bonferroni test or Dunn's test for post hoc multiple comparisons, by two-way ANOVA followed by the Bonferroni test, or using two-tailed unpaired Student's *t*-tests, as appropriate. All analyses used a significance level of 5% and were carried out with commercial software (SigmaStat v3.0; SPSS Scientific Inc.).

3. Results

Social hierarchies, in which dominant and subordinate fish were readily distinguished on the basis of their behaviour, were formed for all interaction periods (Table 1). As in previous studies, circulating plasma cortisol levels were significantly higher in subordinate fish than in dominant individuals (Table 1). Moreover, data for a separate group of fish revealed that social interaction also resulted in significant elevation of circulating catecholamine levels (Fig. 1). As noradrenaline and adrenaline exhibited similar patterns of elevation, total catecholamine values are presented. In both dominant and subordinate fish, catecholamine levels were significantly elevated immediately after hierarchy establishment in comparison to those measured for control (unpaired) fish (one-way ANOVA, $P<0.001$ for both dominants and subordinates). Catecholamine levels then fell in fish that achieved dominant status, and were not significantly different from the control value by 8 h after the initiation of social interactions. However, circulating catecholamine levels in subordinate fish remained significantly elevated at 8 h, returning to the control value only at 24 h. At 24 h after the onset of social interactions, but not immediately after hierarchy formation or at 8 h after the onset of social interactions, plasma catecholamine values were significantly higher in subordinate fish than in dominants (unpaired Student's *t*-test, $P=0.006$).

The erythrocyte β-adrenergic response was impacted subtly, but significantly, by social status. Cell swelling and pHe following incubation with isoproterenol were used as indices of erythrocyte adrenergic responsiveness (Fig. 2). Significant

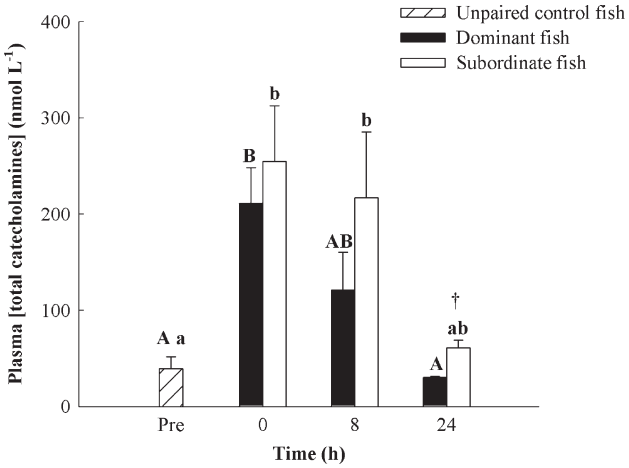

Fig. 1. The effect of social interactions on total plasma catecholamine levels (noradrenaline+adrenaline) in dominant and subordinate rainbow trout (*Oncorhynchus mykiss*). Values for unpaired control fish represent total catecholamine values for rainbow trout prior to social interaction. For paired trout, blood samples for catecholamine analysis were collected immediately after hierarchy establishment (0 h), and at 8 h and 24 h after removal of tank dividers to allow social interaction to occur. Data are presented as mean values ±1 SEM, with $N=12$ for the control group, $N=9$–11 for dominants and subordinates at 0 h and 8 h, and $N=5$–6 for 24 h pairings. Within a rank (dominant or subordinate), bars that do not share a letter are significantly different from one another (one-way ANOVA on each group, $P<0.001$ in both cases). A dagger (†) denotes a significant impact of social rank on plasma catecholamine levels at a given sampling time (unpaired Student's *t*-test, $P<0.05$).

Table 1
Behaviour scores and circulating plasma cortisol concentrations for dominant and subordinate rainbow trout following 6 h, 18 h, 48 h, or 5–7 days of social interaction

	Rank	Behaviour scores	[Cortisol] (ng ml^{-1})
6 h	Dominant	0.73±0.76 (11)	52.7±13.0 (6)
	Subordinate	−0.73±0.60 (11)*	143.3±35.7 (7)*
18 h	Dominant	0.76±0.42 (6)	34.2±12.1 (6)
	Subordinate	−0.76±0.80 (6)*	234.4±53.8 (6)*
48 h	Dominant	0.80±.12 (34)	27.2±6.8 (24)
	Subordinate	−0.76±0.9 (34)*	81.1±15.7 (25)*
5–7 days	Dominant	0.79±0.10 (34)	7.4±2.6 (21)
	Subordinate	−0.79±0.53 (34)*	96.3±23.8 (21)*

Behaviour scores were calculated using a principal components analysis on observations of tank position, aggressive behaviour, feeding, and accumulated damage to the dorsal and caudal fins.
Data are mean values±1 SEM (*N*).
A significant effect of social status is indicated by an asterisk (unpaired Student's *t*-test, $P<0.05$).

effects of isoproterenol dose were apparent (one-way RM ANOVA, $P<0.001$ for dominants and subordinates for $\Delta[H_2O]$ and ΔpHe at 48 h, and $\Delta[H_2O]$ at 5–7 days, $P=0.003$ and $P>0.05$ for ΔpHe for dominants and subordinates, respectively, at 5–7 days) with the exception of ΔpHe in subordinate fish at 5–7 days. However, the only significant effect of social status on erythrocyte adrenergic responsiveness occurred for cell swelling at 48 h, where subordinates exhibited significantly greater cell swelling than dominant fish at the highest dose of isoproterenol (Fig. 2A; unpaired Student's *t*-test, $P=0.01$). The maximal changes in erythrocyte water content and pHe were also compared between dominant and subordinate fish, as indicators of the magnitude of the adrenergic response. Analysis of these data indicated that neither social status nor interaction time significantly impacted upon the magnitude of adrenergic responsiveness (2-way ANOVA, $P>0.05$ for both interaction time and social status in both cases, with non-significant interactions; Table 2).

Isolation of β-NHE activity by the measurement of pHe following incubation with 8-bromo-cyclic AMP revealed a different pattern of responses (Fig. 3). Significant dose-dependent increases in β-NHE activity (measured as the decrease in pHe) with 8-bromo-cyclic AMP concentration were detected for both ranks at 48 h (Fig. 3A; one-way RM ANOVA, $P<0.001$ for dominants, $P=0.002$ for subordinates) and 5–7 days (Fig. 3B; one-way RM ANOVA, $P=0.003$ for dominants, $P=0.007$ for subordinates), but no significant differences between dominant and subordinate fish were apparent (unpaired Student's *t*-test, $P>0.05$). However, the

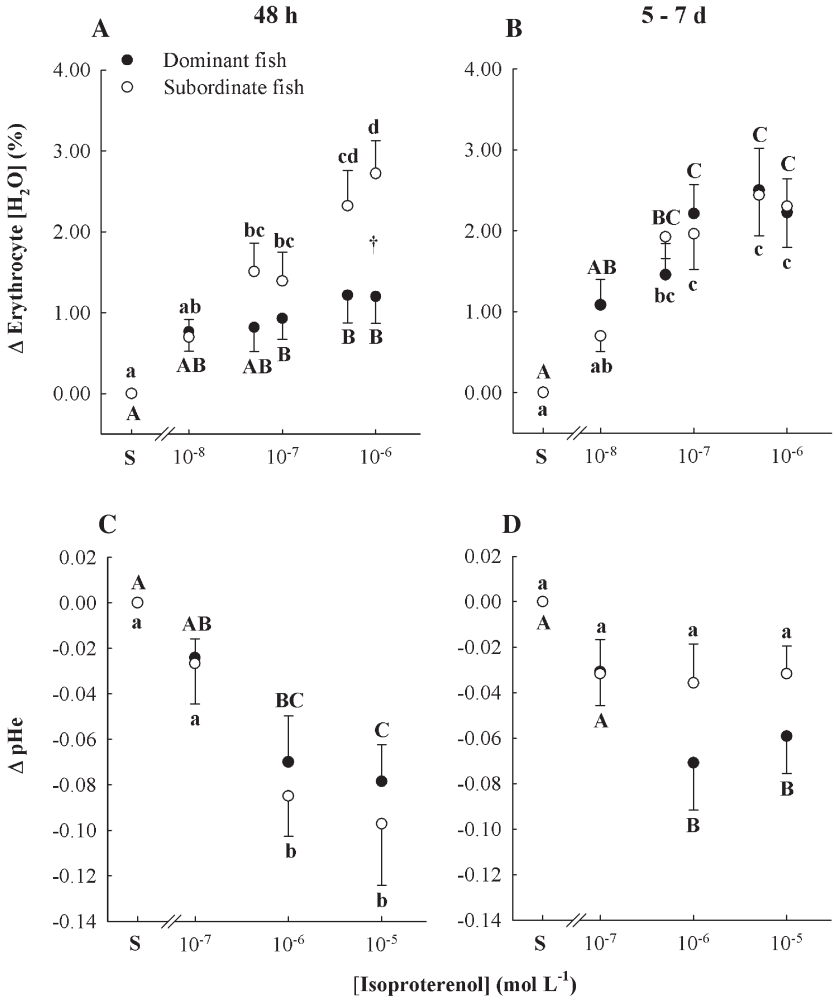

Fig. 2. The effect of isoproterenol concentration on the change in erythrocyte water content (Δerythrocyte [H_2O], %; A and B) and decrease in plasma pH (ΔpHe; C and D) of dominant and subordinate rainbow trout (*Oncorhynchus mykiss*) exposed to 48 h (A and C) or 5–7 days (B and D) of social interaction. Blood samples were incubated in vitro, under hypoxic conditions (PO_2=15.2 Torr), in the presence of the β-AR agonist isoproterenol. Data are expressed relative to the control value (incubation with saline, S, in which [isoproterenol] was assumed to be $<10^{-9}$–10^{-8} mol l^{-1}), and are presented as means±1 SEM, with N=10 in panels A and B, 7 in panel C, and 6 in panel D for each of dominant and subordinate fish. Each data set was analysed by one-way RM ANOVA for effects of isoproterenol dose within rank, and by unpaired Student's *t*-tests for effects of rank within isoproterenol dose. Within a rank, points that do not share a letter are significantly different from one another (for dominants, $P<0.001$ for A–C, $P=0.003$ for D; for subordinates, $P<0.001$ for A–C, $P>0.05$ for D). A dagger (†) denotes a significant difference between dominant and subordinate fish at a particular isoproterenol dose ($P=0.01$).

magnitude of the overall response to 8-bromo-cyclic AMP was significantly greater in dominant (mean maximum ΔpHe= −0.18±0.02 pH units, N=11) than subordinate fish (mean maximum ΔpHe=−0.11±0.02 pH units, N=11; two-way ANOVA with social status and interaction time as factors, P=0.02 for social status, $P>0.05$ for interaction time and the interactions between rank and interaction time).

Trout erythrocytes characteristically possess two fractions of β-AR, an internalised low-affinity fraction, as well as a high-affinity cell surface fraction that is coupled to adenylate cyclase and capable of activating β-NHE. Previous work demonstrated that β-ARs detected using [^{3}H]CGP are of the high-affinity fraction (Reid et al., 1991). β-AR densities (B_{max}; Fig. 4A) were similar in all groups with the exception of subordinate fish at 48 h, which exhibited a B_{max} value that was significantly higher than that of the dominant fish at 48 h, as well as that of subordinate fish at 5–7 days (two-way ANOVA with social status and interaction time as factors, $P>0.05$ for effects of social status and interaction time, P=0.041 for the interaction term; N=5 for both ranks in 48 h pairs, N=7 for both ranks in 5–7 days pairs). The apparent dissociation constant of β-AR, an index of binding affinity (K_D; Fig. 4B), was not affected by either social status or interaction time (two-way ANOVA, $P>0.05$ for both factors and interactions).

Real-time PCR was used to quantify β-3_b AR and β-NHE mRNA expression for dominant and subordinate fish after 6 h, 18 h, 48 h or 5–7 days of social interaction, relative to values for unstressed control fish sampled directly from the holding tank (Fig. 5). Social status was without significant effect on the relative expression of either β-3_b AR mRNA (Fig. 5A) or β-NHE mRNA (Fig. 5B). Interestingly, however, interaction time significantly influenced the relative expression of β-3_b AR

Table 2
Initial values (for saline-incubated samples) and maximal changes in erythrocyte water content and pHe for blood samples from dominant and subordinate rainbow trout after 48 h or 5–7 days of social interaction

	Rank	Initial [H_2O] (%)	Maximal Δ[H_2O] (%)	Initial pHe	Maximal ΔpHe
48 h	Dominant	74.4±0.7	1.7±0.3 (10)	7.64±0.07	−0.09±0.016 (7)
	Subordinate	74.9±0.9	2.9±0.3 (10)	7.58±0.03	−0.11±0.025 (7)
5–7 days	Dominant	72.8±0.9	2.8±0.5 (10)	7.58±0.04	−0.08±0.018 (6)
	Subordinate	73.8±0.9	2.7±0.5 (10)	7.53±0.04	−0.05±0.015 (6)

Erythrocyte water content ([H_2O], %) and pHe were measured in vitro in blood samples incubated under hypoxic incubation (PO_2=15.2 Torr) in the presence of saline or various concentrations of the β-AR agonist isoproterenol.
Data are mean values±1 SEM (*N*). Initial values are provided for reference. Neither social status nor interaction time significantly influenced maximal changes in erythrocyte water content or pHe (2-way ANOVA with social status and interaction time as factors, $P>0.05$ for both factors and the interaction term).

mRNA in both dominant (one-way ANOVA, $P=0.008$, $N=26$) and subordinate fish (one-way ANOVA, $P=0.006$, $N=24$), with β-3_b AR mRNA levels at 6 h of social interaction being significantly higher than levels at 48 h (Fig. 5A). Moreover, compared to the relative expression of β-3_b AR mRNA in control fish (which was set to 1), both dominant and subordinate fish demonstrated a significant 12- to 215-fold up-regulation of β-3_b AR gene expression at all times (one-sample Student's *t*-tests, $P<0.05$ in all cases). A significant effect of interaction time was also apparent in subordinate (one-way ANOVA, $P=0.016$, $N=25$) but not dominant fish (one-way ANOVA, $P>0.05$, $N=27$) for β-NHE mRNA relative expression, which was significantly higher in subordinate fish at 5–7 days than at any other time point (Fig. 5B). In comparison to the relative expression of β-NHE mRNA in a control group of fish (which

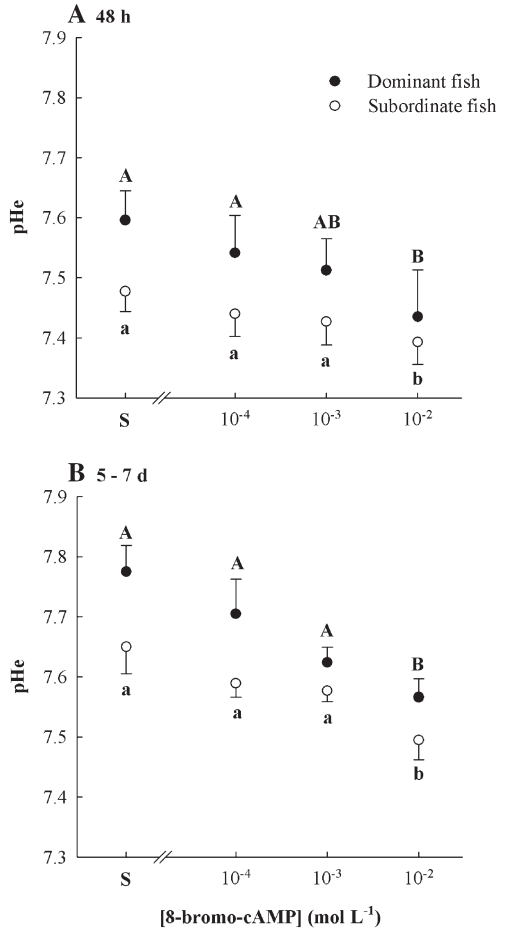

Fig. 3. The effect of 8-bromo-cyclic AMP concentration on extracellular pH (pHe) for erythrocytes of dominant and subordinate rainbow trout (*Oncorhynchus mykiss*) exposed to 48 h (A) or 5–7 days (B) of social interaction. Blood samples were incubated in vitro, under hypoxic conditions (PO_2=15.2 Torr), in the presence of saline (S) or different concentrations of 8-bromo-cyclic AMP. Data are presented as mean values±1 SEM with N=5–6 for both dominants and subordinates at each interaction time. Within a rank, points that do not share a letter are significantly different from one another (one-way RM ANOVA on each group; for dominants, $P<0.001$ for A, $P=0.003$ for B; for subordinates, $P=0.002$ for A, $P=0.007$ for B). Unpaired Student's *t*-tests were used to determine whether an effect of social status occurred at a given dose (in all cases, $P>0.05$).

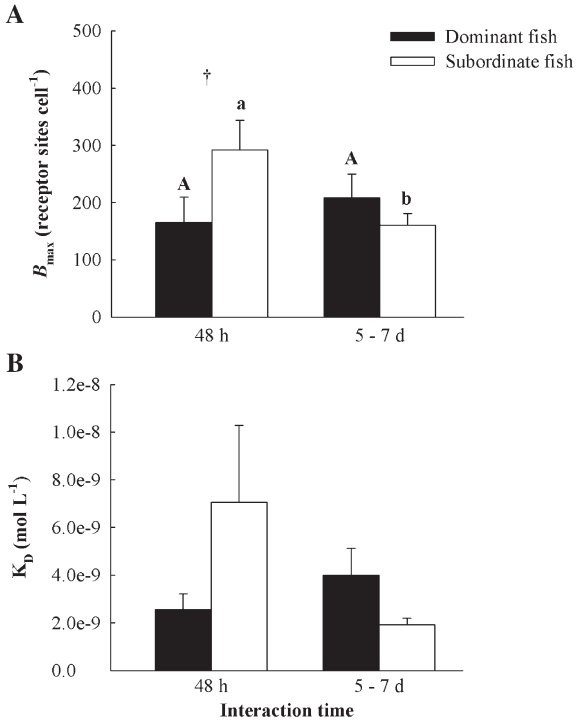

Fig. 4. The impact of 48 h or 5–7 days of social interaction on the number of erythrocyte surface β-adrenoreceptors (B_{max}; A) and their apparent dissociation constant (K_D; B) determined in vitro under normoxic conditions in whole blood samples from dominant and subordinate rainbow trout (*Oncorhynchus mykiss*). Data are mean values±1 SEM with N=5–7 for each group. Two-way ANOVA with social status and interaction time as factors revealed a significant interaction term for B_{max} ($P>0.05$ for social status and interaction time, $P=0.041$ for the interaction term) but not K_D ($P>0.05$ for both factors and the interaction term). Within a social rank, bars that do not share a letter differ significantly from one another, while a dagger (†) denotes a significant effect of social status at a given interaction time.

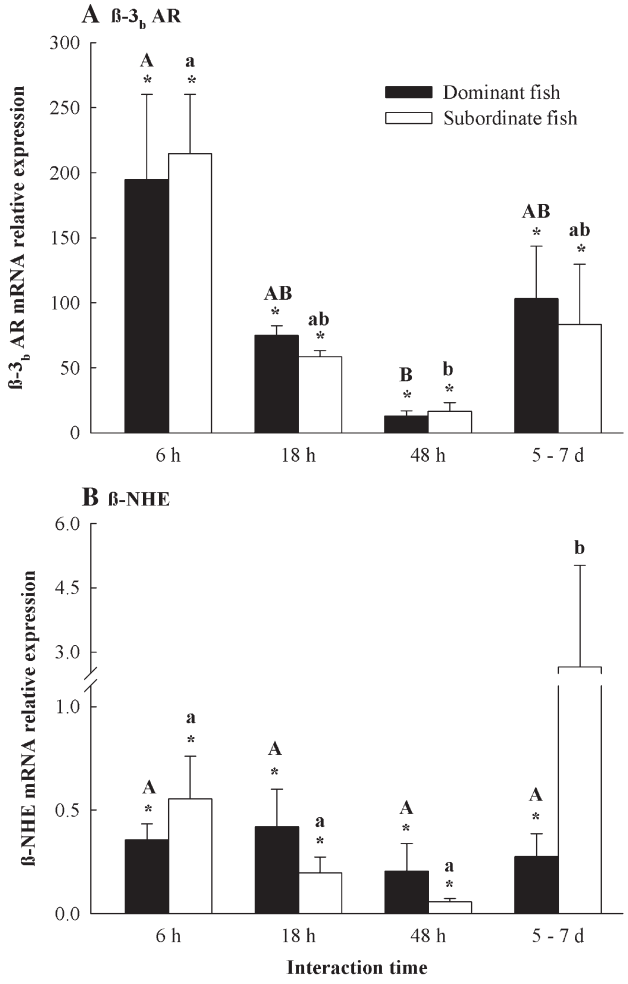

Fig. 5. The expression of erythrocyte β-3_b AR mRNA (A) and β-NHE mRNA (B) relative to erythrocyte β-actin in dominant and subordinate rainbow trout exposed to 6 h, 18 h, 48 h, or 5–7 days of social interaction. For β-3_b AR mRNA expression, N=6–8 for dominants and 5–6 for subordinates, while for β-NHE mRNA expression, N=6–10 for dominants and 5–7 for subordinates. For statistical analysis, values were compared to corresponding mRNA values for fish sampled directly from the holding tank (N=6); mRNA expression in this control group was given a relative value of 1. Within a social rank, bars that do not share a letter are significantly different from one another (one-way ANOVA; for dominants, P=0.008 for A and P>0.05 for B; for subordinate fish, P=0.006 for A and 0.016 for B). No significant effect of social status was identified at any given interaction time (unpaired Student's t-test, P>0.05 in all cases). One-sample Student's t-tests were used to determine whether mRNA expression in a given group was significantly different from the corresponding value (1) for the control group. An asterisk denotes β-3_b AR mRNA relative expression that was significantly greater than 1 (A), or β-NHE mRNA relative expression that was significantly lower than 1 (B) (P<0.05 in all cases).

was set to 1), β-NHE mRNA in paired fish was significantly down-regulated in all groups (one-sample Student's t-test, P<0.05) apart from the 5–7 days subordinate group.

4. Discussion

Pairs of size-matched rainbow trout confined in a resource-limited environment in the present study rapidly formed strong social hierarchies, as evidenced by the marked polarisation of behaviour scores for dominant versus subordinate individuals (Table 1). As in previous work (Sloman et al., 2001, 2002), subordinates were characterised by elevated circulating cortisol levels at all time points (Table 1). Plasma cortisol concentrations in dominant fish tended to be higher in the initial stages after pairing (i.e. short-term pairings of 6 h or 18 h) than typical resting levels (~10 ng ml^{-1}; Gamperl et al., 1994). Aggressiveness tends to be higher in shorter-term pairings (Winberg et al., 1991), as would-be dominant fish try to assert their position in the hierarchy, and subordinates attempt to challenge for dominant rank. With time and hierarchy establishment, however, subordinates behave submissively in an attempt to avoid contact with dominant fish (Winberg et al., 1991). The stress hormone profiles of dominant and subordinate fish reflect these changes. Cortisol levels are high initially in all fish, and although they fall in both dominant and subordinate individuals over time, a return to resting values occurs only in dominant fish; cortisol remains elevated in subordinate individuals (Øverli et al., 1999a; Sloman et al., 2001).

In the present study, a similar pattern was documented for circulating catecholamine levels in socially-interacting trout (Fig. 1). Relative to values for unpaired control fish, circulating catecholamine levels were significantly higher immediately after hierarchy establishment in both dominant and subordinate fish. Catecholamine levels remained elevated for a longer period in subordinate fish, and even though levels at 24 h did not differ significantly from the control value in either dominant or subordinate trout, catecholamines were significantly higher in subordinate than in dominant fish at this time. The impact of social interactions on circulating catecholamine concentrations in salmonid fish has not previously been evaluated because of the conflicting demands of obtaining blood for such measurements (which typically involves cannulation of the fish) and social behaviour (which requires that fish be able to interact unencumbered by trailing cannulae). Fish in the present study were not cannulated, and the netting and handling associated with blood withdrawal did result in catecholamine mobilization as indicated by the total catecholamine concentrations of ~40 nmol l^{-1} in the unpaired control fish (cf. resting values of <10 nmol l^{-1}; Randall and Perry, 1992; Thomas and Perry, 1992; Gamperl et al., 1994). This background level did not, however, obscure the elevation of circulating catecholamines that accompanied acute social stress.

The time-dependent differences in stress hormone profiles formed the basis upon which 48 h and 5–7 days were selected as social interaction periods. In the shorter-term pairings (48 h), high aggressiveness clearly elicited mobilization of both catecholamines and cortisol in subordinate fish. Subordinate fish in longer-term pairings, on the other hand, were likely exposed predominantly to the effects of elevated cortisol levels. High cortisol levels alone are expected to enhance, or "pre-adapt," the erythrocyte adrenergic response (Perry and Reid, 1993). Reid and Perry (1991) demonstrated that both chronic in vivo and acute in vitro cortisol treatment significantly increased the in vitro responsiveness of trout erythrocytes to catecholamines under hypoxic conditions. The enhanced response, detected as a larger decrease in pHe, was attributed to a cortisol-mediated increase in internalised β-ARs which were then mobilized to the erythrocyte surface by hypoxia (Reid and

Perry, 1991). The results of the present study do not, however, support the hypothesis that social stress-induced prolonged elevations of plasma cortisol pre-adapt the erythrocyte adrenergic response. Five to seven days of social interaction was without effect on β-AR kinetics (Fig. 4). In addition, the lack of an isoproterenol dose effect within subordinate fish on the erythrocyte pHe adrenergic response (Fig. 2D), coupled with the significantly lower β-NHE activity in subordinate compared to dominant fish, were effects opposite to that expected with pre-adaptation. Indeed, these data suggest that salmonids experiencing prolonged social stress may suffer from a compromised erythrocyte adrenergic response. The apparent absence of pre-adaptation at 5–7 days of social stress may reflect differences between exogenous cortisol administration into otherwise unstressed fish (Reid and Perry, 1991), and elevation of endogenous cortisol concentrations by chronic behavioural stress; such differences have been reported previously (e.g. Balm and Pottinger, 1995; Sloman et al., 2002). Alternatively, intermittent bouts of aggression by dominant fish may have continued, beyond the initial establishment of the hierarchy, to invoke catecholamine mobilization in subordinate fish at a frequency sufficient to impact on the erythrocyte adrenergic response.

Somewhat surprisingly, a greater adrenergic cell swelling response (Fig. 2A) was detected in subordinate fish at 48 h of social interactions, a point at which high plasma catecholamines were expected to offset the benefits of elevated cortisol levels in subordinates (see below). The enhanced adrenergic responsiveness occurred despite significantly lower β-NHE activity in subordinate fish relative to dominants, and may have reflected the significantly higher β-AR density in subordinate compared to dominant fish at this time (Fig. 4A). The results of previous studies suggest that the consequences of the concomitant elevation of catecholamines and cortisol on adrenergic responsiveness are complex. Elevated catecholamine concentrations reduce erythrocyte β-AR numbers (Gilmour et al., 1994), an effect opposite to that of cortisol. This down-regulation probably occurs via a β-AR kinase/β-arrestin regulatory pathway, rather than by lowering β-AR transcription (Nickerson et al., 2002). Under at least some conditions where both catecholamines and cortisol are elevated, catecholamine-mediated receptor down-regulation appears to dominate over any effect of cortisol in increasing erythrocyte β-AR numbers. For example, Perry et al. (1996) used a 7 days exposure to a daily 5 min bout of exhaustive exercise to repeatedly mobilize catecholamines while also raising plasma cortisol in rainbow trout and found that β-AR numbers decreased with this treatment. In the present investigation, however, β-AR numbers were significantly higher in subordinate fish than in dominants at 48 h (Fig. 4A), a difference that may reflect a protective effect of high cortisol levels against catecholamine-mediated down-regulation of erythrocyte β-AR numbers in subordinate fish. Differences in stress hormone profiles may account for the different impacts on β-AR density between the two studies, with high cortisol and catecholamine levels occurring simultaneously during repeated exhaustive exercise (Perry et al., 1996), but high catecholamines occurring transiently on a background of high cortisol levels during the initial 48 h of social subordinance. By 5–7 days of social interactions, the β-AR numbers of subordinate fish were no longer significantly different from those of dominant fish (Fig. 4A), suggesting that the protective effect of high cortisol levels is of limited duration.

High-affinity receptor numbers measured in the present study for both 48 h and 5–7 days experiments (160 to 336 receptor sites cell^{-1}; Fig. 4A) were somewhat lower than previously reported (500 to 2600 receptor sites cell^{-1}; Koldkjaer et al., 2004; Perry et al., 1996; Reid et al., 1993; Reid and Perry, 1991). The generally low β-AR numbers may reflect the stress of social interaction and particularly the mobilization of plasma catecholamines during hierarchy formation. The elevation of β-3$_b$ AR mRNA expression in both dominant and subordinate trout over that of unstressed control fish (Fig. 5A), in turn, suggests the activation of a mechanism to counteract a general down-regulation of β-3$_b$ AR during the early stages of hierarchy formation. The increased β-AR transcription was probably not cortisol mediated, as cortisol treatment on its own does not appear to promote β-AR transcription (Nickerson et al., 2002), and the increases were consistent between dominant and subordinate trout even though plasma cortisol levels were significantly higher in subordinate individuals (Table 1). Somewhat surprisingly, erythrocyte β-3$_b$ AR mRNA expression remained elevated even after 5–7 days of social interaction. The conditions that promote strong social hierarchies in pairs of rainbow trout may also affect the regulation of β-3$_b$ AR. For example, both dominant and subordinate trout fed only sporadically. Whether nutritional status affects erythrocyte β-ARs is unclear, but numerous factors, including season and different types of stressors (Cossins and Kilbey, 1989; Perry et al., 1996; Koldkjaer et al., 2004), are known to influence β-AR characteristics.

Although Perry et al. (1996) reported a decrease in erythrocyte β-AR numbers in trout subjected to repeated physical stress, the magnitude of the erythrocyte adrenergic response was unaffected. Subtle changes were detected in the sensitivity of erythrocytes from chased fish to catecholamines and to 8-bromo-cyclic AMP, implying modulation of β-NHE activity (Perry et al., 1996). Similarly, seasonal decreases in erythrocyte β-AR numbers did not translate into a reduced adrenergic response. Rather, seasonal attenuation of the adrenergic response was attributed to changes at the level of the β-NHE itself, probably including lowered β-NHE transcription (Koldkjaer et al., 2004). In the present study, reduced β-NHE transcription relative to (unpaired) control fish occurred in most fish subjected to social interactions, but trout of high and low social status did not differ in β-NHE mRNA expression (Fig. 5B). Modulation of β-NHE activity by social status did occur, a finding reminiscent of those of previous studies (Perry et al., 1996; Koldkjaer et al., 2004), with erythrocytes from subordinate individuals exhibiting a smaller response to 8-bromo-cyclic AMP than those of dominants. However, the overall erythrocyte adrenergic responsiveness of subordinate fish was generally equivalent to or even exceeded that of dominant fish (Fig. 2), implying that compensation for the depression of β-NHE activity occurred at one or more levels of

the adrenergic response in subordinate individuals, with the elevation of β-AR density at 48 h of social interactions in subordinate fish being one example. A similar conclusion was reached by Perry et al. (1996) for the effect of repeated physical stress on the erythrocyte adrenergic response.

The impact of chronic stress, and particularly chronic behavioural stress, on the ability of fish to respond to acute stress remains poorly understood. Chronic behavioural stress attenuates the cortisol response to an acute stressor (Øverli et al., 1999b), at least in part because of reduced sensitivity of the cortisol-secreting interrenal tissue to stimulation by the secretagogue adrenocorticotropic hormone (Sloman et al., 2002). On the other hand, catecholamine secretion, measured using acetylcholine as a secretagogue in an in situ perfused posterior cardinal vein preparation, did not differ between dominant and subordinate rainbow trout (Sloman et al., 2002), although previous work suggested that, like the erythrocyte adrenergic response, catecholamine secretion by chromaffin cells would be enhanced by high circulating cortisol levels (Reid et al., 1996) but desensitised by repeated catecholamine release (Perry et al., 1996). Investigation of catecholamine mobilization during social interactions in the present study revealed that high catecholamine levels occur in fish of both ranks during the initial stages of hierarchy formation, but levels fall more rapidly in dominant fish. Whether catecholamine release in vivo in response to additional stressors is affected by social status remains to be determined, as does the impact of social status on other physiological responses to elevated catecholamines. However, the picture that is emerging from the available data is one in which the function of the acute adrenergic stress response is largely maintained in subordinate rainbow trout, despite the significant physiological disadvantages, including reduced growth, elevated plasma cortisol levels, increased plasma glucose, and decreased disease resistance, that accompany low social status particularly in settings where the subordinate is unable to emigrate away from the dominant (reviewed by Sloman and Armstrong, 2002; Gilmour et al., 2005).

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