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RESEARCH ARTICLE

Chronic social stress impairs thermal tolerance in the rainbow trout (*Oncorhynchus mykiss*)

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SUMMARY

When faced with limited resources, juvenile salmonid fish form dominance hierarchies that result in social stress for socially subordinate individuals. Social stress, in turn, can have consequences for the ability of the fish to respond to additional stressors such as pathogens or exposure to pollutants. In the present study, the possibility that social stress affects the ability of rainbow trout (*Oncorhynchus mykiss*) to tolerate acute increases in water temperature was investigated. To this end, we first evaluated physiological and cellular stress responses following a 1 h heat shock in juvenile fish in dominance hierarchies. We measured stress hormone (cortisol and catecholamines) concentrations and blood, brain and liver tissue levels of three heat shock proteins (HSPs), the stress inducible HSP70, the constitutive HSC70 and HSP90, in dominant and subordinate trout. No effects of social status on the hormonal response to the heat stress were detected, but the cellular heat shock response in the brain and liver of dominant and subordinate individuals was inhibited. We then assessed thermal tolerance in dominant and subordinate fish through critical thermal maximum temperature (CT_{max}) trials and measured HSPs following the heat shock. Subordinate fish were less thermally tolerant than their dominant counterparts. We conclude that social stress impacts the ability of fish to respond, on a cellular scale and in a tissue-specific manner, to increases in water temperature, with likely consequences for overall fitness.

Key words: dominance hierarchy, social stress, heat shock protein, cortisol, catecholamine, thermal tolerance, rainbow trout.

INTRODUCTION

When faced with limited access to resources, groups of juvenile salmonid fish readily establish social hierarchies where some fish become dominant and others become sub-dominant or subordinate (Kalleberg, 1958; Noakes and Leatherland, 1977; Metcalfe et al., 1989; Nakano, 1995). Rank in the 'pecking order' is established by the fish's ability to outcompete others through a series of agonistic encounters (Metcalfe et al., 1989; Adams et al., 1998). Dominance hierarchies established in a laboratory setting are comparable to those formed in natural or semi-natural environments (Kalleberg, 1958; Bachman, 1984) and are therefore suitable models for studying social stress in fish. Dominant fish typically monopolize essential resources such as food and shelter whereas subordinate fish may be excluded and consequently suffer penalties such as higher mortality rates (McCarthy et al., 1992; Elliott, 1994; Kadir et al., 1996). From a behavioural perspective, dominant fish are aggressive towards subordinate fish, have a choice of position in their environment and have preferential access to food (Fausch, 1984; Metcalfe et al., 1989; McCarthy et al., 1992; Sloman et al., 2000a). Consequently, subordinate fish exhibit reductions in activity, feeding and aggression (Abbott and Dill, 1985; McCarthy et al., 1992; Winberg et al., 1993; Moutou et al., 1998).

Differences in the physiological status of salmonids in dominance hierarchies have also been observed. For example, subordinate fish have higher levels of plasma cortisol and brain serotonergic activity and are immunosuppressed compared with their dominant counterparts (Winberg and Nilsson, 1993; Johnson et al., 1996; Sloman et al., 2001; Sloman and Armstrong, 2002). Although subordinate fish exhibit such indications of chronic stress, social interaction is an acute stress for both dominant and subordinate fish. Both experience a transient elevation of circulating catecholamines as the hierarchy is established (Thomas and Gilmour, 2006). Cortisol also increases initially in both dominant and subordinate fish but remains elevated only in subordinates (Øverli et al., 1999), again an indication of chronic stress in subordinate fish (Johnsson et al., 1996; Sloman et al., 2001; Gilmour et al., 2005). In addition to differences in the behaviour and physiology of fish in dominance hierarchies, the establishment of a dominance hierarchy has cellular-level effects on both dominant and subordinate fish. We recently demonstrated an induction of heat shock proteins (HSPs) early in hierarchy formation in both dominant and subordinate rainbow trout (Currie et al., 2010). This finding indicates that the formation of dominance hierarchies is stressful not only on a behavioural and physiological level, but also at a cellular level.

In addition to the social stress described above, fish may experience many forms of environmental stress on a daily and/or seasonal basis, such as exposure to contaminants, hypoxia and fluctuations in water temperatures. In salmonid-producing streams, dangerously high water temperatures in the summer months are now evident as a result of climate warming and human modification of fish habitat (Schindler, 2001). Across a wide geographical range, cool-water salmonid fishes experience water temperatures that approach or exceed lethal temperatures for several days of the year (e.g. Lund et al., 2002). In order to cope with the potential damaging and even lethal effects of high temperature, fish respond with behavioural (e.g. evasion) (Breau et al., 2007), physiological (e.g.

release of stress hormones) and cellular (e.g. induction of HSPs) strategies – strategies also used during social stress. Indeed, social stress has been shown to affect an animal's response to additional environmental stressors. For example, subordinate rainbow trout were more sensitive to waterborne toxicants and exhibited higher metal uptake rates than their dominant counterparts (Sloman et al., 2002a; Sloman et al., 2003) (reviewed by Sloman, 2007). A fish's ability to acclimate to a new environment can also be affected by social status. Subordinate rainbow trout exhibited little or no acclimation to novel surroundings whereas dominant fish acclimated as quickly as socially isolated fish (Pottinger and Pickering, 1992). Social status clearly impacts the stress response in fish; however, it is not known how socially stressed fish respond to an acute heat stress.

Given that dominant and subordinate fish differ in their cortisol levels (Gilmour et al., 2005) and in their response to other stressors (Peters et al., 1988; Sloman et al., 2003), and that social stress alone induces HSPs (Currie et al., 2010), we hypothesized that differences in the heat shock response would exist between rainbow trout of different social rank. We also predicted that subordinate fish would be less tolerant of a thermal stress than dominant fish as determined by their critical thermal maximum temperature (CT_{max}). CT_{max} is defined as the upper temperature at which a fish loses the ability to escape conditions that will ultimately lead to its death (Cox, 1974; Becker and Genoway, 1979; Beitinger et al., 2000). Thermal tolerance is affected by multiple factors including genetic differences (Elliott, 1991), acclimation temperature (Becker and Genoway, 1979; Elliott, 1991) and exposure to fluctuating temperatures (Feminella and Matthews, 1984). Furthermore, the expression of HSPs has been implicated in thermal tolerance (Berger and Woodward, 1983; Fangue et al., 2006). Thus, in an effort to relate physiological and cellular stress to CT_{max} in dominant and subordinate fish, we measured HSPs (HSP70, HSC70 and HSP90), cortisol, catecholamines and glucose before, during and after an acute heat shock in dominant, subordinate and unpaired fish. We also compared CT_{max} temperatures and HSP levels in these groups to gain insight into thermal tolerance, HSPs and social stress in fish.

MATERIALS AND METHODS

Experimental animals

Juvenile rainbow trout (*Oncorhynchus mykiss* Walbaum 1792; N=94, mass=218.1±4.1 g, mean ± s.e.m.) were obtained from Linwood Acres Trout Farm (Campbellcroft, ON, Canada). Fish were held in fibreglass aquaria supplied with flowing, aerated, dechlorinated city of Ottawa tap water. The water temperature was maintained at 13°C and a 12 h:12 h light:dark photoperiod was used. Fish were fed to satiation on alternate days with commercial trout pellets and were acclimated to the holding conditions for a minimum of 2 weeks before experiments commenced.

All experimental procedures and holding conditions complied with requirements outlined by the Canadian Council on Animal Care and were approved by the University of Ottawa Animal Care Committee.

Establishment of dominance hierarchies

Dominant and subordinate fish were generated by confining size-matched rainbow trout in pairs as described previously (Currie et al., 2010). Briefly, fish were lightly anaesthetized in an aerated solution of ethyl-*p*-aminobenzoate (0.065 g l^{-1}, Sigma-Aldrich, St Louis, MO, USA), fork length was measured (25.3±0.1 cm, mean ± s.e.m.) and fish were assigned to pairs according to comparable fork length (fork length difference averaged 0.47±0.05 cm or <2% of body length). Each pair of fish was placed in a ~60 l section of a large fibreglass tank partitioned with perforated opaque Plexiglas® and supplied with flowing, aerated water. Social status was assigned through observations of behaviour that took place 3 times per day for 5 min each time. Behaviours were scored using a modification of a point system used previously to assign social status (Metcalfe et al., 1989; Johnsson et al., 1996; Sloman et al., 2000b), in which behaviours associated with dominance, such as patrolling the water column, initiating acts of aggression or taking a food item, were given higher scores than behaviours associated with subordinance. Within each pair, the fish with the higher overall score at the end of the interaction period (determined by a principal components analysis) was assigned dominant social status whereas the fish with the lower behaviour score was assigned subordinate social status. Single (sham) fish were handled as described above except that they were placed in the tank section alone rather than in a pair. After ~54 h, dominant, subordinate and single (sham) fish were used in heat shock (Series 1) or CT_{max} (Series 2) experiments as described below (see also Fig. 1).

Validation experiment: the effect of surgery on social status

Because the stress associated with surgery and handling could have an impact on social hierarchies, the influence of surgery itself on social status was assessed. Following 48 h of social interaction, fish (four pairs) were anaesthetized in an oxygenated solution of ethyl-*p*-aminobenzoate (0.1 g l^{-1}) and were placed on a surgery table that allowed continuous irrigation of the gills with the oxygenated anaesthetic solution. An indwelling cannula of flexible polyethylene tubing (Clay-Adams PE50 polyethylene tubing; VWR, Mississauga, ON, Canada) was placed in the dorsal aorta according to the method of Soivio et al. (Soivio et al., 1975) and was then cut short to prevent tangling when the trout was subsequently re-paired with its conspecific. Fish were revived on the operating table, transferred to individual black Plexiglas® boxes supplied with continuously flowing, aerated 13°C water, and allowed to recover for 24 h. Pairs were then placed into their original experimental tank sections for an additional 48 h observation period. Behavioural observations were initiated 10 min following the confinement of pairs in tank sections. Observations were carried out over 5 min periods for a minimum of 20 min per day.

Experimental protocols

Series 1: heat shock

Fish (N=50) were anaesthetized and fitted with a dorsal aortic cannula as described above, with the exception that the cannula was not cut short. Cannulae were rinsed with heparinised (100 IU ml^{-1} ammonium heparin; Sigma-Aldrich), modified (4.5 mmol l^{-1} $NaHCO_3$, Sigma-Aldrich) Cortland saline (Wolf, 1963). After surgery, fish were placed in individual experimental chambers served with flowing, aerated water for an overnight recovery period.

The heat shock protocol began (approximately 15 h after the end of confinement in pairs; Fig. 1) with the withdrawal of an initial control blood sample (0.4 ml). The water temperature was then raised over 1 h to 25°C where it was maintained for 1 h before being returned to 13°C over the course of 15–20 min. Blood samples (0.4 ml) were withdrawn at 1, 8 and 24 h after the initiation of the heat shock. Sampling at the end of the heat shock was selected to measure changes in rapidly induced physiological stress responses (i.e. glucose, cortisol and catecholamines) whereas the 8 and 24 h sampling points were selected to measure HSP induction. HSPs are detectable 8–24 h following heat shock in rainbow trout (Rendell et al., 2006; Fowler et al., 2009). The heat shock temperature of

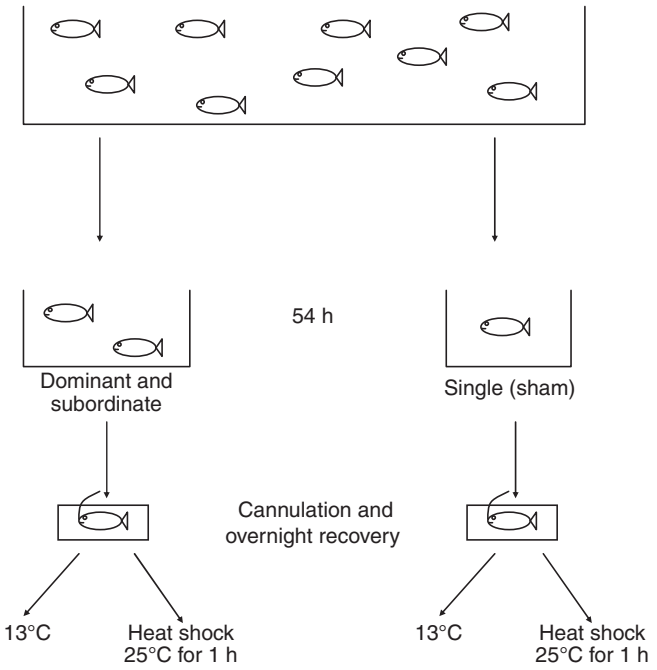

Fig. 1. Summary of experimental design for Series 1. Fish were taken from the main holding tank and either paired with a size-matched conspecific in an experimental chamber (dominant and subordinate) or placed alone into an experimental chamber [single (sham)]. After 54 h in the experimental chambers, fish were cannulated and placed into individual boxes for an overnight recovery period. Fish were then left at the acclimation temperature of 13°C for the remainder of the experiment or were subjected to a heat shock (25°C for 1 h). See Materials and methods for more details.

25°C was selected because it is known to induce HSPs in red blood cells (RBCs) and other tissues of rainbow trout acclimated to 13°C (e.g. Currie et al., 2008; Fowler et al., 2009). To control for the effect of blood sampling and reduction in haematocrit (Soivio et al., 1975), the same sampling regime was performed on fish maintained at 13°C throughout the experiment. Haematocrit and glucose measurements were carried out on whole blood (see below); the remaining blood was centrifuged at 10,000 ***g*** to separate plasma and RBCs. Plasma was separated into two aliquots (for measurement of cortisol and catecholamine concentrations) and these, as well as the RBCs (for HSP analysis), were immediately frozen in liquid N_2 and stored at –80°C for later processing and analysis. After the final blood sample was withdrawn, fish were euthanized by immersion in a solution of ethyl-*p*-aminobenzoate (0.15 g l^{-1}, Sigma-Aldrich), and liver and brain tissues were quickly dissected and immediately freeze-clamped in liquid N_2 for storage at –80°C until further processing and HSP analysis.

Series 2: CT_{max}

Fish (*N*=36) were lightly anaesthetized (to the point of losing equilibrium), weighed and then placed in individual experimental chambers supplied with aerated, flowing water for an overnight recovery period. The next morning at approximately 09:30 h, CT_{max} trials were performed. The CT_{max} protocol involved raising water temperature at a rate of 0.33°C min^{-1} [as suggested by Becker and Genoway (Becker and Genoway, 1979)] until the fish lost equilibrium. The water temperature was then quickly (over 20 min) returned to the recovery temperature of 13°C. The mortality rate for this experiment was <10%; two single (sham) fish and one dominant fish did not survive the CT_{max} protocol. A group of single (sham) fish [hereafter termed the 27°C single (sham) group] was subjected to the same time and temperature profile as the subordinate fish regardless of whether the 27°C single (sham) fish lost equilibrium (only two did) to determine whether differences between dominant and subordinate fish were a result of social status or the heat-shock protocol. After 24 h, fish were euthanized by immersion in a solution of ethyl-*p*-aminobenzoate (0.15 g l^{-1}, Sigma-Aldrich), a blood sample (~1 ml) was withdrawn *via* caudal puncture, and liver and brain tissues were collected and stored as described above. Blood samples were centrifuged at 10,000 ***g*** to separate plasma and RBCs, and RBCs were immediately frozen in liquid N_2 and stored at –80°C for later processing and HSP analysis.

Analytical techniques

Blood variables

Whole blood glucose concentrations were measured as an index of physiological stress (Moon and Foster, 1995) using a OneTouch Ultra 2 blood glucose monitoring system (LifeScan, Milpitas CA, USA) (Clarkson et al., 2005) with ~5 µl of blood. Haematocrit was measured in duplicate using microcapillary tubes centrifuged at 6000 ***g*** for 5 min in an Autocrit Ultra 3 centrifuge (BD, Mississauga, ON, Canada). Plasma cortisol concentrations were measured using a commercial RIA kit (MP Biomedical, Solon, OH, USA) as per the manufacturer's instructions. Plasma noradrenaline and adrenaline levels were determined on alumina-extracted samples (200 µl) by high-performance liquid chromatography (HPLC) with electrochemical detection (Woodward, 1982). We used 3,4-dihydroxybenzylamine hydrobromide (DHBA; Sigma-Aldrich) as an internal standard, and detection limits for noradrenaline and adrenaline were 0.1 nmol l^{-1}.

HSP analysis

Using the procedure described in Currie et al. (Currie et al., 2010), soluble protein was extracted and quantified from frozen liver and brain samples. Protein was extracted from RBCs by rapid thawing to lyse the cells followed by the addition of 2 µl of 0.1 mmol l^{-1} protease inhibitor cocktail (Sigma-Aldrich), and a second freeze–thaw cycle. All samples were then pushed through a 27½ G needle to shear the DNA, and spun at 14,000 ***g*** for 10 min in a Sorvall Legend RT microcentrifuge (Mandel, Guelph, ON, Canada) at 4°C. The supernatant containing the soluble protein fraction was removed and stored at –80°C. The soluble protein content of all samples was measured using the BioRad (Hercules, CA, USA) DC protein assay for microtitre plates based on the Lowry method (Lowry et al., 1951), as in Currie et al. (Currie et al., 2010).

HSP levels were determined by immunodetection using western blotting and the Novex Midi Gel System (Invitrogen, Carlsbad CA, USA), as described previously (Currie et al., 2010). Fifteen micrograms of soluble protein was electrophoresed and then transferred to a polyvinylidene difluoride (PVDF) membrane using the iBlot system (Invitrogen) as per the manufacturer's instructions. For HSP70 analysis, a RBC sample from a heat-shocked fish was loaded on every gel to allow direct comparison across gels. Purified human protein (75 ng; Stressmarq, Victoria, BC, Canada) was used for HSC70 (SPP-751) and HSP90 (SPP-770) as standards to permit comparison of protein levels among gels. Rabbit anti-salmonid inducible HSP70 (AS05061), constitutive HSC70 (AS05062) or HSP90 (AS05063) polyclonal antibodies (Agrisera, Vännäs, Sweden) were used for immunodetection at final concentrations of 1:50,000. The HSP70 antibody is specific for the inducible isoform of HSP70 and does

not detect constitutive isoforms, whereas the HSC70 antibody detects the constitutive isoform and does not cross-react with the inducible HSP70 (Rendell et al., 2006). Although HSP90 has both constitutive (HSP90βa, HSP90βb) (Ojima et al., 2005) and inducible (HSP90α) family members in fish, the antibody does not distinguish among these isoforms. The secondary antibody, goat anti-rabbit IgG (SAB-300, Stressmarq), was used at a final concentration of 1:50,000. Secondary antibody was detected using the ECL Advance Chemiluminescent Western Blotting Detection Kit (GE Healthcare, Baie d'Urfe, QC, Canada) and protein bands were visualized using a FluorSMax MultiImager High Sensitivity Digital Light Capture Camera (BioRad). Protein levels were quantified using Quantity One software (BioRad) and divided by the standard sample (RBCs from heat-shocked fish or commercial standard) band density to give relative band density.

Statistical analysis

All data were analyzed using PASW software (version 17.0; IBM, Chicago, IL, USA). Prior to analysis, data were checked for violation of assumptions of parametric tests using the Kolmogorov–Smirnov test for normality and Levene's test for equal variance. Where these assumptions were violated, data were transformed or non-parametric tests were used. In all cases, α was set to 0.05 and data are presented as means ± s.e.m.

Behaviour scores in the preliminary cannulation experiment were analyzed by paired *t*-tests between the before-surgery and after-surgery behaviour scores within each social status. In the heat shock experiment (Series 1), blood HSP levels, blood glucose concentrations, haematocrit, plasma cortisol concentrations and plasma catecholamine levels were analyzed by three-way repeated measures (RM)-ANOVA using social status (dominant, subordinate or sham) and treatment (heat shock or 13°C) as between-subject factors and time as a within-subject factor. Where a significant interaction between the factors was detected, the data were split by treatment or social status according to where the interaction occurred and separate two-way RM-ANOVAs were performed. Bonferroni's *post hoc* test with correction of α for multiple comparisons was used to determine the source of significant differences as needed. HSPs in brain and liver from Series 1 were analyzed using a two-way ANOVA with social status (dominant, subordinate or sham) and treatment (heat shock or 13°C) as fixed factors. The data were split by treatment (heat shock *versus* no heat shock) if the two-way ANOVA showed a significant interaction between treatment and social status and separate one-way ANOVAs were performed. Bonferroni's test was again used for *post hoc* comparisons. For CT_{max} experiments (Series 2), loss of equilibrium temperatures and HSP levels were analyzed by one-way ANOVA and Bonferroni's test was used where *post hoc* analysis was warranted.

RESULTS

Dominance hierarchies were successfully established as indicated by the strongly polarized behaviour scores determined for dominant (0.98±0.09; *N*=29) and subordinate (–0.9±0.05; *N*=25) fish.

Validation experiment

To assess the impact on social hierarchies of the stress associated with surgery and handling, behaviour scores were compared prior to and following implantation of a dorsal aortic cannula. In no case did surgery result in the reversal of the social hierarchy and behaviour scores for dominant and subordinate fish did not change significantly as a result of surgery (Fig. 2).

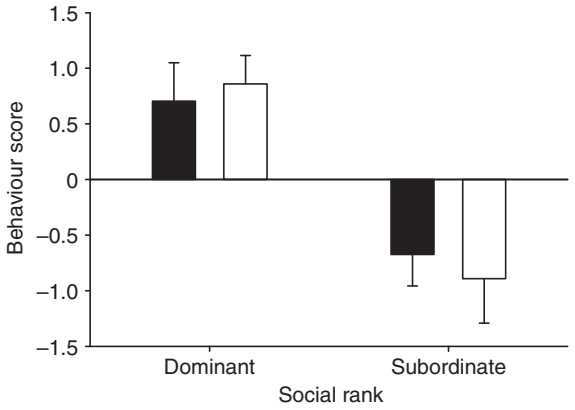

Fig. 2. Behaviour scores of dominant and subordinate rainbow trout (*Oncorhynchus mykiss*) after 48 h of social interaction (filled bars) and during a second 24 h social interaction period that followed cannulation of the dorsal aorta and 24 h of recovery from surgery (unfilled bars). Data are means ± s.e.m. for *N*=4 pairs and were analyzed by paired Student's *t*-tests for effects of surgery on behaviour score within dominants (*P*=0.573) and subordinates (*P*=0.185).

Series 1: Heat shock

Control plasma cortisol levels (Table 1) in all social status groups were higher than those typically observed in unstressed fish (i.e. 10 ng ml^{-1}) (Gamperl et al., 1994) and remained elevated throughout the experiment. Heat shock resulted in an increase in plasma cortisol that was evident after the 1 h heat stress (*P*=0.021). However, in both heat shock and 13°C groups, cortisol levels were elevated within 8 h post-heat shock. Social status, however, had no effect on cortisol levels regardless of whether fish were heat shocked.

Overall, total catecholamine concentrations (Table 1) significantly increased during the heat shock (*P*=0.016) and, as was the case with cortisol levels, there were no significant effects of social stress. Adrenaline was significantly higher (*P*<0.001) at 1 h in fish in the heat-shocked groups (11.8±5.3 nmol l^{-1}, *N*=23) *versus* the control time point (0.68±0.32 nmol l^{-1}, *N*=23). Similarly, noradrenaline levels were higher during heat shock (1 h time point, 17.68±5.31 nmol l^{-1}, *N*=23) compared with the control time point (7.86±2.8 nmol l^{-1}, *P*=0.047, *N*=23) in subordinate fish but no other differences were detected. No significant differences in plasma total catecholamine concentrations were detected in fish held at 13°C (*P*=0.705).

In all heat-shocked fish, whole blood glucose concentration (Table 2) increased significantly during the heat shock (*P*<0.001) but had decreased to levels significantly lower than the control value by 24 h (*P*=0.013). Blood glucose concentrations did not change significantly over the course of the experiment for the groups of fish maintained at 13°C except at 24 h (2.11±0.36 mmol l^{-1}, *N*=24), when glucose levels were lower than those at control (3.65±0.86 mmol l^{-1}, *N*=24) and 8 h (3.63±0.78 mmol l^{-1}, *N*=24). Our resting blood glucose values are on the low end of 'normal' for plasma glucose in salmonid fish (Iwama, 1998), possibly as a result of the measurement of whole blood instead of plasma. No statistically significant effects of social status on blood glucose were detected in either the heat-shocked groups or the groups of fish maintained at 13°C. Haematocrit (Table 2) followed a trend very similar to that of the blood glucose concentrations in that haematocrit increased during heat shock (*P*=0.044) and then decreased over the recovery period. In the fish held at 13°C, haematocrit also significantly decreased over time (Table 2). There were no significant

Table 1. Plasma cortisol and total catecholamine (noradrenaline and adrenaline) concentrations in single (sham), dominant and subordinate rainbow trout (*Oncorhynchus mykiss*) from Series 1 maintained at the acclimation temperature (13°C) or exposed to a heat shock (HS)

					Time		
	Treatment	Social status	*N*	Control	1 h	8 h	24 h
				a	a	b	a
Cortisol (ng ml^{-1})	13°C	Single (sham)	7	48.5±16.5	75.4±33.2	186.2±39.7	48.9±14.4
		Dominant	8	57.6±13.2	34.5±13.1	115.3±33.2	9.5±2.4
		Subordinate	8	43.5±14.1	40.2±16.9	144.4±45.2	32.7±12.2
				a	b	a,b	a,b
	HS	Single (sham)	7	33.2±9.5	130.6±37.9	225.5±94.8	79.7±41.3
		Dominant	9	47.4±14.8	119.9±32.6	171.0±49.2	81.1±42.1
		Subordinate	7	99.2±32.3	131.5±19.2	189.5±52.8	54.8±11.0
				a	a	a	a
Catecholamines (nmol l^{-1})	13°C	Single (sham)	7	12.7±5.5	6.2±2.8	12.3±6.3	5.2±2.6
		Dominant	8	10.1±4.1	14.3±8.0	17.0±12.8	15.2±4.7
		Subordinate	8	3.6±1.1	6.5±1.8	7.6±4.5	20.4±6.3
				a	b	a,b	a,b
	HS	Single (sham)	7	8.7±4.9	9.6±3.6	27.6±11.2	4.2±1.1
		Dominant	9	8.8±2.2	25.2±6.2	20.1±5.7	22.3±7.3
		Subordinate	7	9.2±2.5	38.7±15.9	22.7±11.6	25.1±5.0

Values are means ± s.e.m.

We detected no significant effect of social status, so dominant, subordinate and single (sham) fish were grouped at each time point.

Groups [i.e. single (sham), dominant and subordinate fish] that do not share a letter are significantly different from one another (three-way RM-ANOVA followed by two-way RM-ANOVA); see text for further details.

effects of social status on haematocrit in heat-shocked fish or those kept at 13°C.

Relative levels of HSP70 (inducible) in the blood were undetectable for dominant and subordinate fish kept at 13°C (data not shown). In heat-shocked fish, RBC HSP70 was not significantly induced until 8 h and increased further by 24 h after the heat shock in all groups (P<0.001). We did not observe any significant differences in HSP70 among dominant, subordinate and single (sham) fish (Fig. 3). Neither heat shock nor social status significantly impacted RBC HSC70 or HSP90 levels (data not shown).

HSP70 levels in the brain were barely detectable in fish kept at 13°C and there was no effect of social status (Fig. 4A). Heat shock resulted in a significant increase in brain HSP70 in single (sham) fish (P<0.001) but, surprisingly, this heat shock response was completely inhibited in both dominant and subordinate fish. Neither social status nor heat shock had significant effects on relative levels of HSC70 or HSP90 in the brain (data not shown). Similar to brain, liver HSP70 was barely detectable in fish kept at 13°C and there was no effect of social status on these levels (Fig. 4B). Heat shock also resulted in a significant increase in liver HSP70 in single (sham) fish (P=0.003) but, as observed in the brain, there was no induction in dominant or subordinate fish.

HSC70 levels (Fig. 5) were significantly higher in the livers of heat-shocked dominant (P=0.018) and subordinate fish (P=0.028)

Table 2. Blood glucose concentrations and haematocrit in single (sham), dominant and subordinate rainbow trout (*Oncorhynchus mykiss*) from Series 1 maintained at the acclimation temperature (13°C) or exposed to an acute heat shock (HS)

					Time		
	Treatment	Social status	*N*	Control	1 h	8 h	24 h
				a,b	a	a	b
Glucose (mmol l^{-1})	13°C	Single (sham)	7	3.09±0.74	3.51±1.24	3.39 ±1.09	2.13±0.61
		Dominant	8	2.90±0.85	2.83±0.71	1.91±0.51	1.51 ±0.21
		Subordinate	8	3.10±0.84	3.26±1.04	3.91±1.15	1.86±0.38
				a	b	b	c
	HS	Single (sham)	7	2.37±0.25	4.31±0.52	3.28±0.59	1.67±0.27
		Dominant	9	3.26±1.09	5.63±1.88	5.10±1.70	3.04±1.01
		Subordinate	7	6.80±1.27	7.57±1.32	8.14±1.97	4.07±1.23
				a	a	b	c
Haematocrit (%)	13°C	Single (sham)	7	21.9±1.9	20.1±3.0	13.4±2.1	12.4±2.1
		Dominant	8	21.1±1.8	18.9±1.5	15.8±0.9	13.9±1.3
		Subordinate	8	20.9±1.7	20.4±1.5	14.6±1.3	13.3±1.1
				a	b	c	d
	HS	Single (sham)	7	20.1±1.6	22.4±2.6	14.4±1.5	11.1±1.3
		Dominant	9	20.8±1.4	25.9±2.2	16.0±1.4	12.1±1.1
		Subordinate	7	21.4±1.2	23.5±1.6	13.6±1.0	10.7±1.2

Values are means ± s.e.m.

We detected no significant effect of social status, so dominant, subordinate and single (sham) fish were grouped at each time point.

Groups [i.e. single (sham), dominant and subordinate fish] that do not share a letter are significantly different from one another (three-way RM-ANOVA followed by two-way RM-ANOVA); see text for further details.

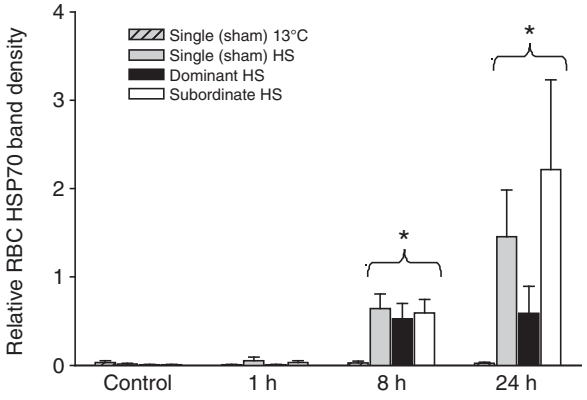

Fig. 3. Relative levels of HSP70 (inducible) in red blood cells of single (sham) [grey; *N*=7 for both 13°C and heat-shocked (HS) fish], dominant (black; *N*=8) and subordinate (white; *N*=8) rainbow trout in Series 1. Solid bars represent fish that were heat-shocked for 1 h at 25°C whereas hatched bars represent fish maintained at 13°C. Blood samples were collected prior to the heat shock (control) as well as 1, 8 and 24 h after the beginning of the heat shock. Values are means ± s.e.m. An asterisk indicates a significant difference from control and 1 h (P<0.001; three-way RM-ANOVA followed by separate two-way ANOVA for each treatment because of a significant interaction between time and treatment, P<0.05). There were no significant effects of social status.

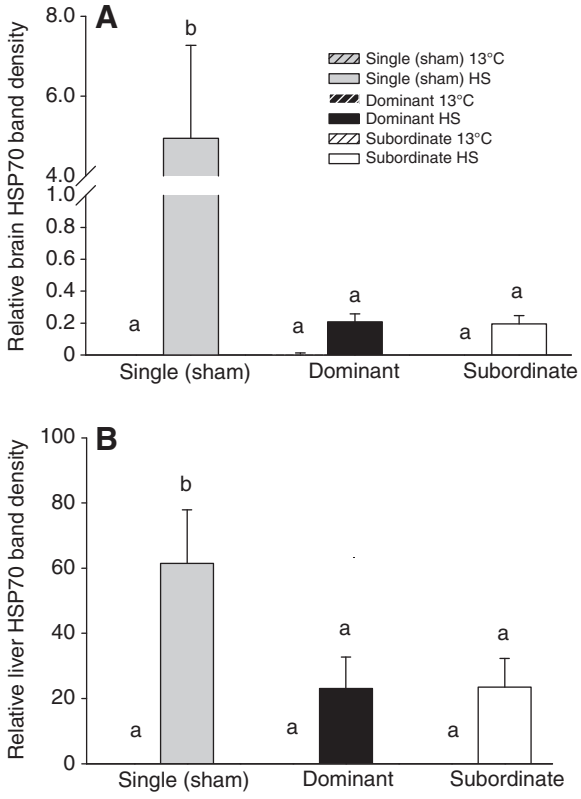

Fig. 4. Relative levels of HSP70 (inducible) in (A) brain and (B) liver of single sham [grey; *N*=7 for both 13°C and heat-shocked (HS) groups], dominant (black; *N*=11 for 13°C and *N*=9 for HS) and subordinate (white; *N*=8 for both 13°C and HS groups) rainbow trout in Series 1. The solid bars represent fish that were heat-shocked for 1 h at 25°C and the hatched bars represent fish maintained at 13°C. Samples were collected 24 h after the beginning of the heat stress. Values are means ± s.e.m. Groups that do not share a letter are significantly different from one another (brain: two-way ANOVA followed by one-way ANOVAs on the recoded data with Bonferroni *post hoc* testing; see Materials and methods for details).

when compared with single (sham) fish kept at 13°C. Again, HSP90 levels were not significantly affected by either heat shock or social status (data not shown).

Series 2: CT_{max}

Social status had a significant effect on the thermal tolerance of rainbow trout (Fig. 6). When exposed to an acute temperature increase of 0.33°C min^{-1}, dominant fish were able to tolerate a significantly higher temperature (28.6±0.2°C) than subordinate fish (27.2±0.2°C) before losing equilibrium (P=0.009).

Twenty-four hours following the thermal stress, RBC HSP70 was lower in the subordinate fish than in dominant or in single (sham) fish (Fig. 7A). To determine whether these differences between dominant and subordinate fish were a result of social status or the heat-shock protocol (i.e. lower maximum exposure temperature for subordinate fish), an additional single (sham) group (27°C sham) was added to the experiment. This group was exposed to a temperature increase of 0.33°C min^{-1} until water temperature reached the loss of equilibrium value for subordinate fish (i.e. 27.2°C), even though these (sham) fish did not lose equilibrium. HSP70 levels in the RBCs of the 27°C sham fish were not significantly different from those in the subordinate fish (Fig. 7A). The low HSP70 levels at 24 h post-heat shock in both the 27°C sham fish and the subordinate fish indicated that the absence of RBC HSP70 induction in subordinate fish was most likely a result of the shorter heat stress and lower heat shock temperature to which they were exposed, as opposed to an effect of social status *per se*. We observed no significant differences in RBC HSC70 and HSP90 levels among dominant, subordinate and single (sham) fish (data not shown).

In brain tissue of fish exposed to their CT_{max}, single (sham) fish exhibited significantly higher levels of HSP70 than 27°C sham and subordinate fish (P=0.003 and 0.033, respectively; Fig. 7B) but not dominant fish 24 h following the heat stress. This HSP70 profile was very similar in liver where single (sham) fish had significantly higher levels of HSP70 than 27°C sham and subordinate fish (P<0.001 and P=0.03, respectively). However, liver HSP70 levels in 27°C sham fish were significantly lower than those of subordinate fish.

Brain HSC70 levels were not different among single (sham), dominant or subordinate fish 24 h following a critical thermal stress, but HSC70 levels in the brain of 27°C sham fish were significantly lower than those in single (sham) fish (P=0.01; Fig. 8), indicating again that HSP induction is dependent on the magnitude and length of the heat stress. There were no significant differences in liver HSC70 among the social or single (sham) groups (data not shown).

HSP90 levels were significantly lower in the brain of 27°C sham fish than in all other groups (Fig. 9A). Although brain HSP90 levels were lower in subordinate compared with single (sham) fish (P=0.044), no significant differences were observed between dominant and subordinate fish. It is notable that in both brain and liver (P<0.001 and P=0.01, respectively; Fig. 9), HSP90 levels were significantly lower in 27°C sham fish than in subordinate fish but levels between dominant and subordinate fish did not differ.

DISCUSSION

Our data indicate that the cellular response to heat shock is different in socially stressed fish compared with single (sham) fish. We found

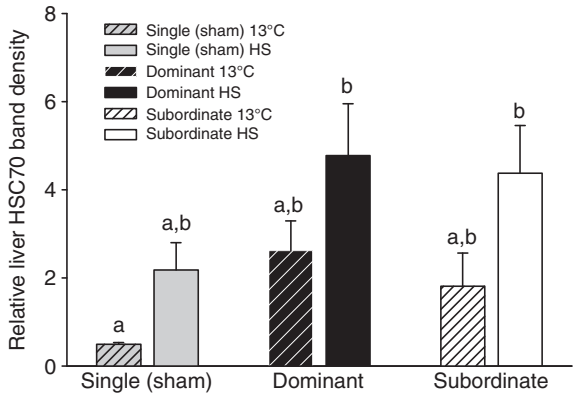

Fig. 5. Relative levels of HSC70 (constitutive) in liver of single (sham) (grey), dominant (black) and subordinate (white) rainbow trout in Series 1. Other details are the same as in Fig. 4. Groups that do not share a letter are significantly different from one another (two-way ANOVA followed by one-way ANOVAs with Bonferroni *post hoc* testing; see Materials and methods for details).

that social stress inhibited the classic heat shock response in brain and liver. In contrast, in blood, a classic induction of HSPs was observed in response to heat shock in both single (sham) and socially stressed fish together with increases in haematocrit, cortisol, catecholamine and blood glucose concentrations. Furthermore, the formation of dominance hierarchies resulted in changes in thermal tolerance as well as tissue-specific HSP induction in fish exposed to a critical acute heat stress. Our work is the first to describe how socially stressed fish respond to a subsequent acute heat shock.

Heat shock

The heat shock protocol used in Series 1 permitted us to examine how fish of different social status responded to the same stressor, and also allowed comparisons with previous work that used the same heat shock protocol (e.g. Currie et al., 2008). Using cannulated fish also enabled us to describe the physiological and cellular responses to heat stress in an individual fish over time. Control levels of plasma cortisol were elevated in all groups compared with values reported

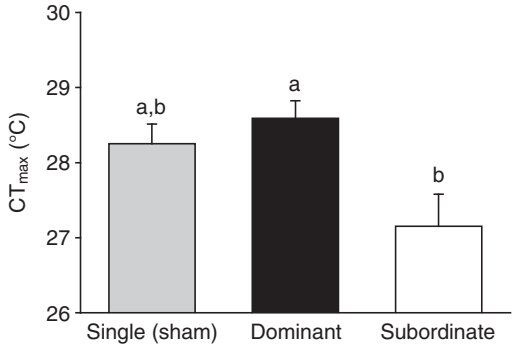

Fig. 6. Temperatures at which single (sham), dominant and subordinate rainbow trout lost equilibrium [i.e. critical temperature maximum (CT_{max})] during a rapid increase in water temperature in Series 2. Fish were exposed to a water temperature increase of 0.33°C min^{-1} until loss of equilibrium. Data are means ± s.e.m. (N=11 for dominant and N=10 for both subordinate and sham). Groups that do not share a letter are significantly different from one another (one-way ANOVA followed by Bonferroni *post hoc* testing; P=0.009 between dominant and subordinate fish).

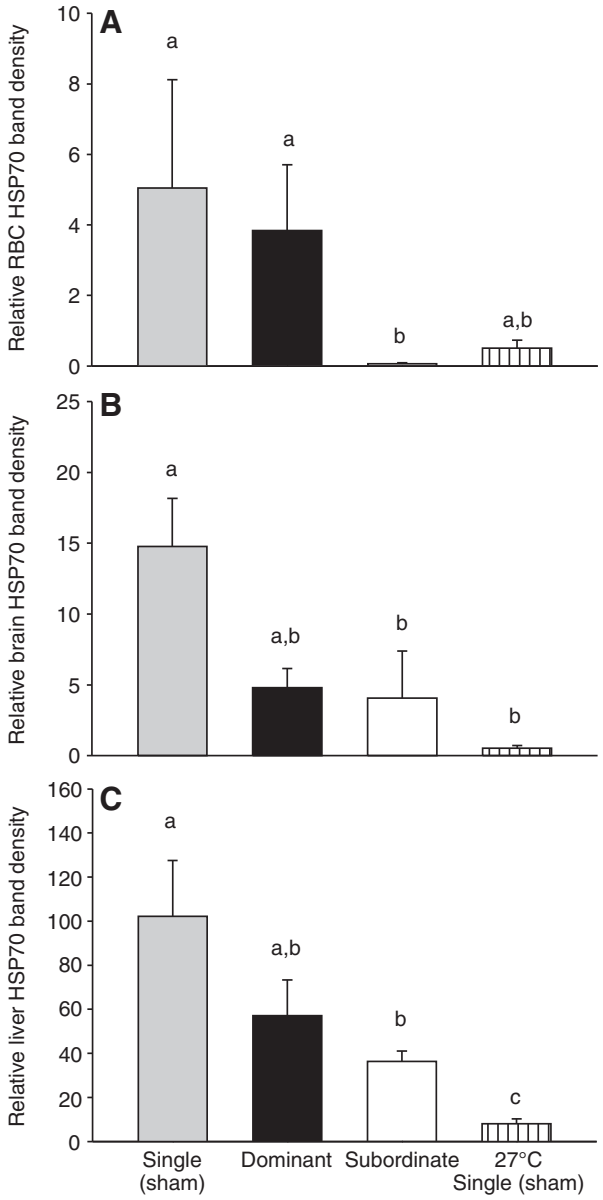

Fig. 7. Relative levels of HSP70 (inducible) in (A) red blood cells (RBCs), (B) brain and (C) liver of single (sham) (grey; N=6), 27°C sham (striped; N=8), dominant (black; N=9) and subordinate (white, N=9) rainbow trout in Series 2. Fish were exposed to a water temperature increase of 0.33°C min^{-1} until loss of equilibrium (LOE), with the exception of the 27°C single (sham) fish, where the temperature increase was halted at 27°C, the CT_{max} of the subordinate fish. Samples were collected 24 h after the beginning of the heat shock. Data are means ± s.e.m. Groups that do not share a letter are significantly different from one another (RBCs: Kruskal–Wallis test followed by multiple Mann–Whitney tests to compare multiple groups with α-level corrections; brain and liver: one-way ANOVA followed by Bonferroni *post hoc* tests; see Materials and methods for details).

in the literature for unstressed fish (Gamperl et al., 1994), suggesting that our fish may not have fully recovered from the cannulation surgery and/or that confinement in a black box imposes a certain degree of stress (Brown et al., 1986). In many studies, subordinate fish exhibit higher levels of plasma cortisol than their dominant counterparts (e.g. Sloman et al., 2001), but this was not the case in

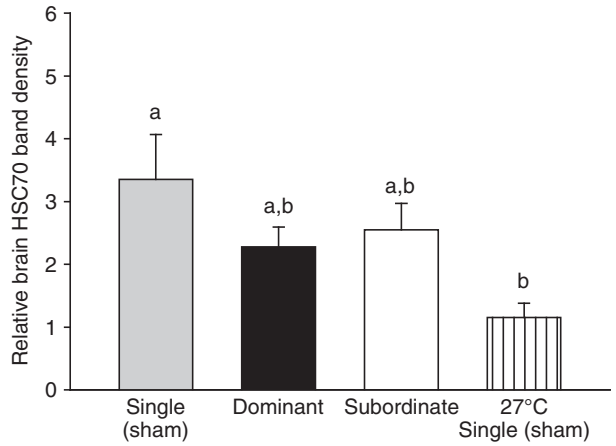

Fig. 8. Relative levels of HSC70 (constitutive) in brain tissue of single (sham) (grey), 27°C sham (striped), dominant (black) and subordinate (white) rainbow trout in Series 2. Other details are the same as in Fig. 7. Data are means ± s.e.m. Groups that do not share a letter are significantly different from one another (one-way ANOVA followed by Bonferroni *post hoc* testing; see Materials and methods for details).

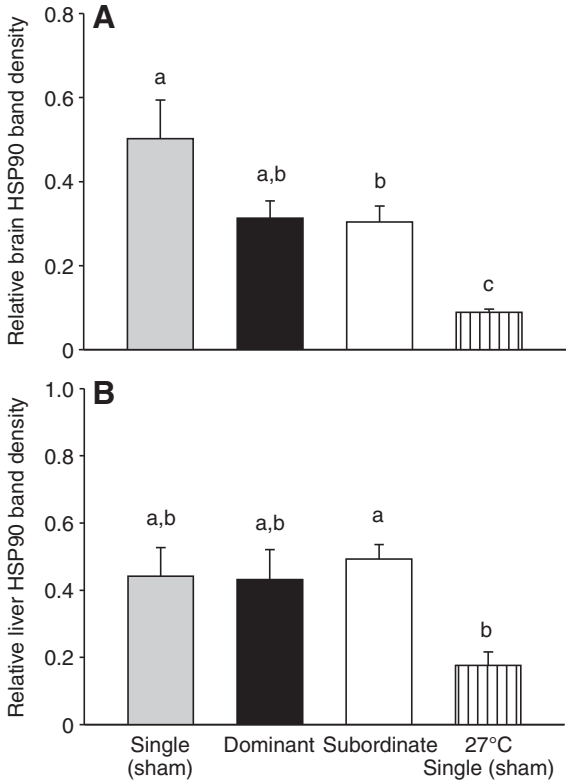

Fig. 9. Relative levels of HSP90 in (A) brain and (B) liver of single (sham) (grey), 27°C sham (striped), dominant (black) and subordinate (white) rainbow trout in Series 2. Other details are the same as in Fig. 7. Data are means ± s.e.m. Groups that do not share a letter are significantly different from one another (one-way ANOVA followed by Bonferroni *post hoc* testing; see Materials and methods for details).

our experiment where no differences in cortisol levels were detected between fish of different social status. Notably, cortisol responses in small groups of salmonids are variable where factors such as group size, environment or degree of aggressiveness may eliminate the difference typically observed in plasma cortisol between dominant and subordinate fish (Gilmour et al., 2005). It is worth pointing out, however, that plasma levels of both cortisol ($71.3\pm23.2\,ng\,ml^{-1}$) and glucose ($4.95\pm1.1\,mmol\,l^{-1}$) in subordinate fish tended to be higher than those in dominant ($52.5\pm14.0\,ng\,ml^{-1}$ and $3.08\pm1.94\,mmol\,l^{-1}$) or single (sham) ($40.9\pm13.0\,ng\,ml^{-1}$ and $2.73\pm0.50\,mmol\,l^{-1}$) fish when the 13°C and heat-shocked fish are combined at the control time point. It is possible that the stress of cannulation raised cortisol levels in both single (sham) and dominant fish whereas the chronically stressed subordinates were unable to further increase plasma cortisol levels with cannulation. In support of this possibility, long-term exposure of fish to pollutants inhibits the cortisol response to handling stress (Hontela et al., 1992; Hontela et al., 1995; Hontela et al., 1997), suggesting that chronic stress may inhibit the response to other stressors. Furthermore, Øverli et al. (Øverli et al., 1999) observed a higher cortisol response to handling stress in dominant compared with subordinate fish. Similarly, cortisol secretion in response to application of the secretagogue adrenocorticotropic hormone (ACTH) in a perfused head kidney preparation was attenuated in subordinate relative to dominant rainbow trout (Sloman et al., 2002b). Nevertheless, the absence of a marked cortisol difference between subordinate and dominant or single (sham) fish in our study suggests that the physiological consequences of subordinate status were reduced in the subordinate fish.

To our knowledge, this study is the first to document the cortisol response to an acute heat shock in individual fish over time. Cortisol levels increased with heat shock and there were no differences between fish of different social status. We had predicted that subordinate fish would exhibit an attenuated cortisol response to heat stress compared with dominant fish. Although circulating cortisol concentrations during and after heat stress were similar among social groups, cortisol increased from control to 1 h of heat shock by $97.4\,ng\,ml^{-1}$ in single (sham) fish and $72.5\,ng\,ml^{-1}$ in dominant fish, but only by $32.3\,ng\,ml^{-1}$ in subordinate fish, suggesting that subordinate fish may indeed have a decreased capacity to raise cortisol levels after an acute stress. Heat shock resulted in a typical mobilization of catecholamines (Currie et al., 2008) and this adrenergic response was not affected by social status. Similarly, Sloman et al. (Sloman et al., 2002b), using a perfused head kidney preparation, found no difference in the rate of catecholamine secretion elicited by acetylcholine between dominant and subordinate rainbow trout. Thus, our findings are in agreement with those of previous studies (Sloman et al., 2002b; Thomas and Gilmour, 2006) that have suggested that the function of the acute adrenergic stress response is largely maintained in subordinate rainbow trout despite social stress-induced modulation of the hypothalamic–pituitary–interrenal (cortisol) stress axis.

For all groups [i.e. single (sham), dominant and subordinate], including fish kept at 13°C, plasma cortisol levels were elevated within the 8 h following heat shock (Table 1). This finding was surprising given that even the single (sham) fish maintained at 13°C exhibited elevated cortisol levels at the 8 h time point. Plasma cortisol in fish varies according to circadian cycles and is highest during the dark period (Rance et al., 1981). Our 8 h samples coincided with the onset of the dark period and this factor may explain the high levels of cortisol observed in all groups. Total plasma catecholamine concentrations also tended to be elevated (although not significantly) at 8 h, particularly in fish kept at 13°C. It is possible that circulating levels of catecholamines follow a circadian cycle similar to that of cortisol. Although Le Bras (Le Bras, 1984) reported that plasma

catecholamine levels in the eel (*Anguilla anguilla*) peaked during the light cycle, Sauerbier and Meyer (Sauerbier and Meyer, 1976) found that catecholamines in the goldfish (*Carassius auratus*) heart peaked twice during the day, once shortly after the light period started and again shortly after the dark period commenced, exactly when our 8 h samples were taken.

As was the case with stress hormones, blood glucose concentrations and haematocrit also increased with heat shock in single (sham), dominant and subordinate fish and there were no differences observed among these groups (Table 2). These data are consistent with the heat-shock-induced elevation of stress hormones in that, in situations of stress, cortisol and catecholamines are responsible for the mobilization of energy reserves, thereby increasing blood glucose levels as well as oxygen delivery to the tissues (Gamperl et al., 1994; Reid et al., 1998). The latter is achieved at least in part through increases in haematocrit by sympathetic release of RBCs from the spleen (Gallaugher et al., 1992; Vermette and Perry, 1998) and/or an increase in RBC cell division (Lewis et al., 2010). The decreases in blood glucose and haematocrit observed 24 h after the heat shock in all groups likely reflected repeated blood sampling (Soivio et al., 1975).

Heat-shocked fish exhibited a typical HSP response with peak induction of HSP70 in blood occurring 8–24 h after the heat shock (Fowler et al., 2009) and again, there were no significant differences amongst single (sham), dominant or subordinate fish. The lack of an effect of social status on the RBC heat shock response suggests that even though the cortisol response of subordinate fish may be impaired, the cellular stress response remained intact, at least in blood. Surprisingly, and unlike the situation in blood, the HSP70 heat shock responses in brain and liver were completely inhibited in socially stressed fish (Fig. 4). Brain and liver HSP70 levels in heat-shocked dominant and subordinate fish were not different from levels in their 13°C, non-heat-shocked counterparts. This inhibition is all the more interesting given the clear induction of HSP70 in the blood of heat-shocked dominant and subordinate fish as well as in the brain and liver of single (sham) fish, which collectively indicated that the heat stress was sufficiently severe to elicit a cellular heat shock response. It is not clear why these tissue differences in the heat shock response of dominant and subordinate fish exist, but one possibility is that the heat shock response was maintained in the blood of socially stressed fish by the presence of catecholamines. Whereas cortisol has been reported to decrease the HSP response to a stressor (Basu et al., 2001), catecholamines enhance the response (Currie et al., 2008). Adrenaline is found in tissues other than blood only at low levels (Sebert et al., 1986). It is conceivable that the mobilization of adrenaline and noradrenaline into the blood during heat shock potentiated HSPs sufficiently to counter the suppression of HSP induction by social stress that was observed in the brain and liver. Alternatively, blood exhibits one of the more robust heat shock responses relative to other tissues in rainbow trout (Currie et al., 2000), and this strong response may have masked the HSP-inhibitory effects of social stress. In contrast to HSP70, neither HSC70 nor HSP90 was induced by heat shock in blood, liver or brain tissue in dominant, subordinate or single (sham) fish. The lack of HSP90 or HSC70 induction after heat shock is not surprising given that these proteins are only weakly inducible after heat stress in rainbow trout (Rendell et al., 2006).

Thermal tolerance

CT_{max} trials, useful for understanding an animal's thermal physiology, revealed that subordinate fish were significantly less heat tolerant than their dominant counterparts (Fig. 6). These findings are consistent with a pattern in which chronically stressed subordinate fish are more susceptible to an additional acute stressor [e.g. opportunistic bacterium (Peters et al., 1988) and waterborne toxicants (Sloman et al., 2002a; Sloman et al., 2003)]. There are many biotic and abiotic factors that influence CT_{max} in fish in general and salmonids in particular; acclimation temperature (Beitinger et al., 2000) and genetics are probably the most important (Carline and Machung, 2001). However, photoperiod and diel seasonal cycles are also crucial in the determination of CT_{max} (Lutterschmidt and Hutchison, 1997), as are salinity and diet. For example, Sardella et al. (Sardella et al., 2008) recently demonstrated that salinity affects the acute temperature tolerance of green sturgeon (*Acipenser medirostris*), and the presence of microbial levan in the diet of rohu fish (*Labeo rohita*) increased CT_{max} temperatures (Gupta et al., 2010). Acute temperature tolerance may also be affected by prior exposure to aquatic contaminants. For example, CT_{max} temperatures decreased when rainbow trout were previously exposed to sublethal concentrations of the agricultural pesticides endosulfan and chlorpyrifos (Patra et al., 2007). Social stress can now be added to the numerous factors that influence thermal tolerance in fish.

As HSPs are not induced until 8–24 h following acute heat shock in rainbow trout (Fowler et al., 2009), we did not expect these proteins to influence the CT_{max}. However, given the observed differences in thermal tolerance between dominant and subordinate fish, we wished to determine whether the ensuing heat shock response would be different in fish in dominance hierarchies following this acute thermal trial. Dominant and single (sham) fish in the CT_{max} experiment exhibited a classic HSP70 response in blood 24 h post-heat stress. However, low levels of HSP70 were observed in subordinate fish compared with the other groups (Fig. 7A). This apparent lack of HSP70 induction can be explained, at least in part, by the fact that subordinate fish were subjected to a shorter, less severe heat stress than dominant or single (sham) fish because of their earlier loss of equilibrium, at a lower absolute temperature. In support of this explanation, single (sham) fish subjected to the same thermal stress as subordinate fish (i.e. 27°C sham fish) also exhibited low HSP70 levels (Fig. 7). It appears that HSP70 is induced only at temperatures ≥24°C in trout acclimated to 13°C (S.C. and N. Templeman, unpublished data); subordinate and 27°C sham fish were exposed to temperatures above 24°C for a shorter time (~9 min) than dominant and single (sham) fish (>12 min). The shorter heat stress endured by subordinate fish (and 27°C sham fish) may not have been enough to cause protein damage, the putative trigger for HSP induction (Anathan et al., 1986). Thus, our results indicate that the induction of at least HSP70 depends not only on temperature but also on the duration of the heat stress.

It is noteworthy that in liver, HSP70 levels were significantly higher in subordinate fish than in 27°C sham fish (Fig. 7C), despite the equivalent heat stress experienced by these groups. This observation suggests that a prior social stress likely has some impact on the later induction of HSPs, at least in the liver. Moreover, appreciable levels of liver HSP70 were detected in dominant and subordinate fish in the CT_{max} trial (Series 2), whereas HSP70 induction was completely inhibited in both dominant and subordinate fish in the first heat shock experiment (Series 1; Fig. 4B). These results suggest that the HSP70 inhibition resulting from social stress (as observed in Series 1) can be overcome, at least in part, in dominant and subordinate fish by exposure to higher (CT_{max}) temperatures (Series 2).

As in the heat shock experiment (Series 1), brain HSC70 and brain and liver HSP90 levels in the CT_{max} trial did not differ among single (sham), dominant and subordinate fish. Interestingly,

however, protein levels in 27°C sham fish were significantly lower than those in single (sham) fish, and for HSP90, lower also than those in subordinates. Because the 27°C sham and subordinate fish were exposed to the same heat shock profile, the implication of these findings is that the subordinate response to the short, acute heat shock was larger than expected based on temperature alone. These results suggest that subordinate fish may be relying to a greater extent on constitutive HSPs (HSC70 and HSP90) to deal with increases in temperature, as opposed to the single (sham) or dominant fish strategy of relying on the stress-inducible HSP70. Indeed, Currie et al. (Currie et al., 2010) found that social stress alone induced HSC70 in subordinate fish.

In conclusion, social stress affected the ability of fish to mount a cellular stress response to an acute heat stress. The lack of a heat-shock response in specific tissues of socially stressed fish may have profound consequences for overall fitness. Future work focused on the mechanism(s) underlying inhibition of heat shock responses will be important to improve our understanding of the ability of fish to cope with stressors in nature. We have also demonstrated that subordinate trout are less thermally tolerant than their dominant counterparts, but this tolerance cannot be simply explained by induction of HSPs, given that the response to an acute heat shock is not different between dominant and subordinate fish (Series 1). The differences we observed in thermal tolerance add to the body of literature supporting the greater susceptibility of subordinate fish to a variety of stressors and indicating that not all fish will be able to tolerate rises in water temperature to the same degree.

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