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RESEARCH ARTICLE

Elevated cortisol lowers thermal tolerance but results in limited cardiac remodelling in rainbow trout (*Oncorhynchus mykiss*) experiencing chronic social stress

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ABSTRACT

Juvenile rainbow trout (*Oncorhynchus mykiss*) held in pairs form dominance hierarchies in which subordinate individuals experience chronic social stress accompanied by lowered thermal tolerance (assessed as the critical thermal maximum, CT_{max}). Here, we tested the hypothesis that chronic elevation of circulating cortisol levels reduces thermal tolerance in subordinate trout. In support of this hypothesis, subordinate trout that recovered from social stress for 48 h, a period sufficient to return cortisol to normal baseline levels, no longer showed reduced CT_{max}. Further, thermal tolerance was not restored in subordinates treated with cortisol during recovery from social stress. To explore possible mechanisms underlying the effect of chronic stress on CT_{max}, we also tested the hypothesis that chronic cortisol elevation induces cardiac remodelling in subordinate trout, as previously reported for cortisol-treated rainbow trout. Ventricle mass and cardiac hypertrophy markers were unaffected by social stress. Picrosirius Red staining revealed a trend for lower collagen levels in the ventricles of subordinate relative to dominant trout. However, collagen type I transcript and protein levels, and markers of collagen turnover were unaffected. Indicators of cardiac function, including ventricle passive stiffness and intrinsic heart rate (f_H), similarly were unaffected. *In vivo* f_H was also similar between subordinate and dominant fish. Nevertheless, in keeping with their lower CT_{max}, subordinate fish exhibited cardiac arrhythmia at significantly lower temperatures than dominant fish during CT_{max} trials. Thus, high baseline cortisol levels in subordinate trout result in lowered thermal tolerance, but 5 days of social stress did not greatly affect cardiac structure or function.

KEY WORDS: CT_{max}, Cortisol, Cardiac remodelling, Heart rate

INTRODUCTION

Juvenile rainbow trout (*Oncorhynchus mykiss*) held in pairs or small groups form social hierarchies through agonistic interactions. The resulting dominant and subordinate individuals differ not only in their behaviour, with dominant fish patrolling the water column, monopolizing food and exhibiting aggression, but also physiologically. Most importantly, subordinate fish experience chronic social stress as demonstrated by the prolonged elevation of circulating levels of the glucocorticoid stress hormone cortisol (Ejike and Schreck, 1980; Øverli et al., 1999a; Pottinger and Pickering, 1992; Sloman et al., 2001; Culbert and Gilmour, 2016; see reviews by Gilmour et al., 2005; Johnsson et al., 2005; Sørensen et al., 2013; Winberg et al., 2016). The use of these social hierarchies as a model to study the consequences of chronic stress has enabled the mechanisms underlying effects such as reduced growth and changes in intermediary metabolism to be probed, with elevated cortisol in combination with limited food intake emerging as causative factors (DiBattista et al., 2006; Gilmour et al., 2012; Kostyniuk et al., 2018). Subordinate trout also exhibit reduced tolerance of environmental challenges such as hypoxia (Thomas and Gilmour, 2012) and elevated temperature (LeBlanc et al., 2011), yet the mechanisms underlying these effects remain to be determined. The goal of the present study was to investigate whether and how chronic cortisol elevation contributes to the reduced thermal tolerance of subordinate rainbow trout.

A widely used measure of acute thermal tolerance is the critical thermal maximum (CT_{max}), the temperature at which an animal loses the ability to maintain physical equilibrium (Becker and Genoway, 1979; Lutterschmidt and Hutchison, 1997; Beitinger et al., 2000). Although CT_{max} has been measured in many different fishes, the underlying physiology that results in the loss of equilibrium (LOE) during acute warming remains unclear, and indeed, the physiological factors that determine thermal tolerance remain heavily debated (Fangue et al., 2011; Norin et al., 2014; Lefevre et al., 2016; Motyka et al., 2017; Pörtner et al., 2017; Jeffries et al., 2018; Jutfelt et al., 2018). The oxygen- and capacity-limited thermal tolerance (OCLTT) hypothesis suggests that thermal tolerance is limited by the capacity of the cardiorespiratory system to meet tissue oxygen demands (Pörtner, 2010). Empirical support for the OCLTT hypothesis has been mixed, with evidence both for (e.g. Beers and Sidell, 2011; Devor et al., 2016; Muñoz et al., 2018; Blasco et al., 2020) and against (e.g. Wang et al., 2014; Ern et al., 2016, 2017; Jutfelt et al., 2019; Joyce and Perry, 2020) limitations in tissue oxygen supply as the cause of LOE at CT_{max}. In accordance with the OCLTT hypothesis, impaired cardiac function translates into lowering of CT_{max} (Ekström et al., 2017, 2019; Gilbert et al., 2019). Acute warming increases cardiac output in fishes, a response that is driven largely by increases in heart rate (f_H) because stroke volume is maintained or increases relatively little during acute warming (e.g. Gollock et al., 2006; Steinhausen et al., 2008; Mendonça and Gamperl, 2010; Ekström et al., 2017, 2019; Joyce and Wang, 2020). As water temperature approaches CT_{max}, a plateau and/or decrease in f_H typically is observed (Gollock et al., 2006; Mendonça and Gamperl, 2010; Ekström et al., 2016, 2019; Joyce et al., 2018). In rainbow trout in which the coronary arteries of the

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heart were ligated, resting f_H was elevated, f_H peaked at a lower temperature than in control animals during acute warming, and CT_{max} was reduced (Ekström et al., 2019). Thus, factors that elevate resting f_H and/or limit maximum cardiac output may constrain cardiac performance, and hence CT_{max}, during acute warming.

Thomas and Gilmour (2012) reported significantly higher resting f_H in subordinate relative to dominant trout. In addition, Johansen et al. (2017) reported that chronic cortisol treatment increased resting f_H and lowered maximum cardiac output in rainbow trout, effects that were attributed to pathological ventricular hypertrophy. Specifically, dietary cortisol administration for 45 days elevated circulating cortisol concentrations to ∼20 ng ml^{-1}, increasing ventricular mass as well as transcript abundances of specific molecular markers associated with cardiac remodelling in mammals (Johansen et al., 2017). Ventricular hypertrophy also was detected in a line of rainbow trout selected for a high cortisol response to a standardized stressor [‘high responsiveness’ (HR) trout; Pottinger and Carrick, 1999], in which it was accompanied by increased collagen deposition consistent with fibrosis (Johansen et al., 2011). Ventricular hypertrophy and fibrosis in this case were thought to be associated with the high cortisol levels routinely experienced by these fish during a stress response. Subordinate trout exhibit substantially higher cortisol levels than those used by Johansen et al. (2017), although for a relatively brief duration (few days). However, even 7 days of cortisol treatment were sufficient to induce molecular markers of ventricular hypertrophy (Nørstrud et al., 2018), suggesting a potential for cardiac remodelling in response to chronic social stress.

The present study aimed to identify the physiological mechanisms contributing to lower CT_{max} in subordinate rainbow trout. To test the hypothesis that elevated cortisol in subordinate fish lowers CT_{max}, we measured CT_{max} in subordinates recovering from social stress, where cortisol returns to normal baseline values, and in recovering subordinates treated with cortisol to prevent this fall in circulating cortisol levels. We predicted that the reestablishment of normal baseline cortisol levels during recovery from social stress would lead to recovery of CT_{max}, and that this recovery would be prevented if high cortisol levels were maintained. To investigate potential mechanisms linking CT_{max} to cortisol levels, f_H responses to social interactions and acute warming were measured together with markers of ventricular hypertrophy and fibrosis.

MATERIALS AND METHODS

Experimental animals

Juvenile rainbow trout, *Oncorhynchus mykiss* (Walbaum 1792) (mass=109.4±2.2 g, fork length=21.3±0.2 cm, mean±s.e.m., *N*=165; see Table 1), were purchased from Linwood Acres Trout Farm (Campbellcroft, ON, Canada). Fish were held at the University of Ottawa in 1275 litre fibreglass tanks supplied with flowing, aerated, dechloraminated city of Ottawa tap water at a temperature of 13°C. A 12 h:12 h light:dark photoperiod was maintained, and fish were fed a ration of 0.5% body mass daily by scattering commercial trout pellets on the water’s surface. Trout were acclimated to these holding conditions, which served to minimize hierarchy formation (e.g. use of scatter feeding, homogeneous tanks with a mild current; Jeffrey et al., 2014; Kostyniuk et al., 2018), for at least 2 weeks prior to experimentation. All experimental protocols were approved by an institutional animal care committee (protocol BL-2118, University of Ottawa) and were in compliance with the guidelines of the Canadian Council on Animal Care (CCAC) for the use of animals in research and teaching.

Rainbow trout were allocated to pairs based on similar fork length (mean length difference=0.3±0.03 cm, *N*=80 pairs) and mass (mean mass difference=5.0±0.4 g, *N*=80 pairs) according to established protocols (LeBlanc et al., 2011; Jeffrey et al., 2014; Culbert and Gilmour, 2016). Fish were lightly anaesthetized (to the point of losing equilibrium) in a solution of benzocaine (0.05 g l^{-1} ethyl-p-aminobenzoate; Sigma-Aldrich, Oakville, ON, Canada) for the measurement of fork length and mass, and fin damage was assessed as per Moutou et al. (1998). The members of a pair were placed in 40 litre flow-through Plexiglas ‘behaviour’ tanks separated by an opaque, perforated divider for an overnight recovery period. Tanks were supplied with flowing, aerated, dechloraminated city of Ottawa tap water at 13°C. The following morning, the divider was removed and the fish in a pair were allowed to interact for 4 days. Behaviour observations were carried out twice daily (morning and afternoon). Tank covers were adjusted to allow the fish to be observed. Following 10 min recovery from this minor disturbance, position in the tank, acts of aggression such as charges, chases and nips, and willingness to take a single pellet of food were recorded for 5 min. Points were awarded for each behaviour, with higher scores for more dominant behaviours (Øverli et al., 1999a; Sloman et al., 2000; LeBlanc et al., 2011; Culbert and Gilmour, 2016). At the end of the 4-day interaction period, mean scores for individual behaviours over the interaction period were combined using a principal components analysis, and the fish within a pair with the higher behaviour score was assigned dominant social status. Pairs in which behaviour scores did not diverge by at least 0.5 were excluded from further analysis (a total of 10 pairs) (Jeffrey et al., 2014; Culbert and Gilmour, 2016). Shelters were added to the tank after the first observation period to provide a refuge and tanks were covered between observation periods to avoid disturbance of the fish. Fish

Table 1. Morphometric data and behaviour scores for dominant and subordinate rainbow trout (*Oncorhynchus mykiss*)

Experimental group	Status	Mass (g)	Fork length (cm)	Behaviour score
4 day interaction (groups A+B)	Dominant (*N*=12)	113.5±6.2	21.3±0.4	1.2±0.4
	Subordinate (*N*=12)	110.3±6.6	21.0±0.4	−1.6±0.2
Recovery (groups C+D)	Dominant (*N*=13)	110.0±4.4	21.5±0.3	1.6±0.3
	Subordinate (*N*=13)	108.8±5.7	21.6±0.4	−1.2±0.2
Recovery with cortisol treatment (groups E+F)	Dominant (*N*=12)	79.1±3.8	19.1±0.3	1.7±0.3
	Subordinate (*N*=12)	77.4±3.8	19.3±0.4	−1.5±0.1
Recovery with sham treatment (groups G+H)	Dominant (*N*=12)	90.1±6.5	20.0±0.6	1.4±0.4
	Subordinate (*N*=12)	87.6±6.6	20.0±0.6	−1.6±0.2
Heart rate biologger trial	Sham (*N*=6)	154±4.9	23.7±0.3	NA
	Dominant (*N*=10)	138.2±4.9	23.3±0.3	1.5±0.3
	Subordinate (*N*=10)	138.2±5.0	23.3±0.3	−1.5±0.7
Isolated heart preparations	Dominant (*N*=14)	121.1±4.6	22.4±0.3	1.5±0.2
	Subordinate (*N*=14)	120.2±4.1	21.8±0.3	−1.5±0.1

Values are means±s.e.m. NA, not applicable.

were fed daily (after the afternoon observation period) from the second day of observations with a ration of 0.5% body mass.

Prior to the establishment of social hierarchies, a subset of trout (N=10 pairs plus N=6 shams; see Table 1) was fitted with commercially available heart rate biologgers (DST micro-HRT, 8.3×25.4 mm, 3.3 g or 2.35±0.05% body mass, Star-Oddi, Iceland; http://www.star-oddi.com/) that were programmed to record temperature, f_H and electrocardiogram (ECG) traces. Fish were anaesthetized by immersion in a solution of benzocaine as above, and after measuring mass, fork length and fin damage, fish were transferred to a surgical table that allowed continuous irrigation of the gills with the aerated anaesthetic solution. A 2 cm incision was made midline on the ventral surface just posterior to the pectoral girdle. Following Prystay et al. (2017), heart rate loggers were inserted into the peritoneal cavity through the incision to rest against the pericardial membrane, and sutured to the body wall with two stitches (2-0 surgical silk; Fisher Scientific, Nepean, ON, Canada). The incision was closed using two single interrupted sutures. Fish were then transferred to the behaviour tanks as described above for a 20-h recovery period. Fish in the sham treatment group were prepared in the same way as fish that were paired, but were placed individually in the behaviour tank (with the divider in place) to serve as a control for effects of handling associated with the establishment of social hierarchies.

Experimental protocols

Three experiments were carried out. Experiment 1 investigated the effects of chronic social stress and elevated cortisol levels on CT_{max}. Experiment 2 aimed to provide more information on differential responses of dominant versus subordinate fish to acute warming by investigating f_H during a CT_{max} trial. Experiment 3 investigated whether chronic social stress causes cardiac remodelling in subordinate trout.

Experiment 1

Pairs of trout established as described above were allocated to one of eight treatment groups (Fig. S1). In group A (N=6 pairs), fish were subjected to a CT_{max} trial (see below) at ∼09:00 h on day 5. A non-lethal blood sample was collected 1 h after the return of water temperature to the acclimation temperature (13°C), and fish were euthanized on day 6 for the collection of heart tissue (see below). Blood was sampled 1 h after the thermal stress to assess the cortisol response to acute warming, and heart tissue was collected 24 h after thermal stress to assess the heat shock protein 70 (HSP70) response to acute warming; these sampling times were selected on the basis of a previous study that reported elevated cortisol concentrations and HSP70 protein abundances to a heat shock protocol of comparable length to the present CT_{max} trial (LeBlanc et al., 2011). The fish of group B (N=8 pairs) followed the same protocol as group A but were not subjected to a CT_{max} trial. Thus, samples collected from these fish reflected the effects of social interactions alone, whereas those collected from the fish of group A reflected the effects of thermal stress on top of social interactions. In group C (N=6 pairs), the members of a pair were separated after the 4-day interaction period by re-insertion of the opaque divider, and were allowed to recover from social interactions for 2 days, following which they were subjected to a CT_{max} trial on day 7, with subsequent blood sample collection as for group A. The 2-day recovery period was chosen based on the findings of Culbert and Gilmour (2016) that circulating cortisol levels in subordinate fish had returned to normal baseline values within 2 days of physical separation from their dominant tank-mate. The protocol for group D (N=7 pairs) matched that of group C but lacked the CT_{max} trial. In groups E (N=6 pairs) and F (N=6 pairs), the protocols used for groups C and D were followed, except that subordinates were given cortisol suspended in cocoa butter implants (see below) immediately prior to the 2 day recovery period. The implant served to maintain elevated cortisol concentrations in subordinate fish during the recovery period. Finally, the protocols for groups G (N=6 pairs) and H (N=6 pairs) followed those of groups E and F, but subordinates were given an implant of cocoa butter alone as a control for the handling and stress associated with implant administration.

Measurement of CT_{max}

Measurement of CT_{max} was carried out between 09:00 and 11:00 h on pairs of trout in their behaviour tank to achieve consistency and to avoid further handling. Fish were subjected to a linear increase in water temperature of 0.33±0.01°C min^{-1} (Becker and Genoway, 1979) until LOE. The temperature of LOE, indicated by the fish turning dorso-ventrally and being unable to right itself within 3 s, was noted as CT_{max}. Once CT_{max} was reached, water temperature was returned to the acclimation temperature (13°C). The fish that first reached its CT_{max} was temporarily removed from the behaviour tank to immediately begin the return to acclimation temperature. Water temperature (measured using a digital thermometer) was altered using a series 440 Fotopanel thermostatic mixing valve (POWERS™, Burlington, ON, Canada), and water flowing to the experimental tanks was vigorously aerated using an equilibration column to ensure maintenance of air saturation with increasing temperature (tested by monitoring dissolved oxygen levels during a CT_{max} trial). The CT_{max} trials on fish instrumented with heart rate biologgers (see Experiment 2 below) revealed that body temperature (as measured by the biologger) lagged water temperature by ∼0.3±0.1°C. Therefore, CT_{max} values based on water temperature likely over-estimated actual CT_{max} values. Importantly, CT_{max} values for dominant and subordinate fish were always determined simultaneously for size-matched pairs of fish held in the same tank such that any differences between dominant and subordinate fish were robust.

Administration of cortisol implants

Subordinate rainbow trout were given an intraperitoneal implant of cocoa butter (NOW Health Group Inc., Bloomingdale, IL, USA) containing cortisol (hydrocortisone 21-hemisuccinate, Sigma-Aldrich; 65 mg kg^{-1} body mass; groups E and F) or cocoa butter alone (5 ml kg^{-1} body mass; groups G and H) as a sham control (Pickering and Duston, 1983; Lawrence et al., 2019). The cortisol dose was chosen on the basis of a pilot trial to achieve circulating cortisol concentrations typical of subordinate fish. Cortisol-impregnated cocoa butter implants were prepared by dissolving the hydrocortisone 21-hemisuccinate in 99% ethanol; the cortisol–ethanol solution was added to melted cocoa butter and the ethanol was evaporated off by heating the solution (Hoogenboom et al., 2011; Lawrence et al., 2019). To administer the implants, fish were lightly anaesthetized as described above and liquid cocoa butter (∼32°C) was injected into the peritoneal cavity using a 22 G sterile needle and 1 ml syringe, where it quickly solidified. After administration of the implant, fish were returned to their behaviour tanks with the divider inserted to separate the dominant and subordinate fish for the 2-day recovery period.

Blood sample collection and analysis

For collection of blood samples, the members of a pair were together lightly anaesthetized as described above and 0.3 ml of blood was withdrawn from the caudal vasculature using a 23 G needle and

syringe rinsed with 0.5 mol l^{-1} ethylenediaminetetraacetic acid (EDTA). Blood samples were collected within 2–3 min of netting the fish to avoid handling-induced elevation of cortisol (Lawrence et al., 2018). The fish were then returned to their tank, separated by the opaque divider, until both individuals had regained equilibrium, upon which the divider was removed (groups A and B) or left in place (groups C–H) until the experiment was terminated. Blood samples were centrifuged at 10,000 ***g*** for 2 min, and plasma was flash-frozen in liquid N_2 and stored at −80°C for later analysis of cortisol concentrations.

Plasma cortisol concentrations were analyzed using a commercial radioimmunoassay (RIA; MP Biomedical, LLC, USA) previously validated for analysis of trout plasma samples (Gamperl et al., 1994). The intra- and inter-assay coefficients of variation were 4.1% and 3.5%, respectively.

Heart tissue collection and analysis

Fish of groups A and B were euthanized by terminal anaesthesia (0.5 g l^{-1} ethyl-p-aminobenzoate), and the heart was dissected out. The hearts of fish from group A were blotted dry and the bulbus arteriosus and atrium were removed, after which the mass of the ventricle was measured. Ventricles were sectioned longitudinally using a fresh razor blade and a 20 mm section was fixed in 4% paraformaldehyde overnight at 4°C. The remainder of the ventricle was flash-frozen in liquid N_2 and stored at −80°C for later analysis of transcript abundance by real-time RT-PCR or protein abundance by western blot analysis. Fixed ventricle tissue was dehydrated in 70% ethanol and stored in 70% ethanol at 4°C until histological analysis (see Experiment 3). Ventricle tissue from group B fish was flash-frozen in liquid N_2 and stored at −80°C for later analysis of HSP70 abundance by western blotting.

Soluble protein was extracted from frozen ventricle tissue that was ground to a powder on dry ice using a mortar and pestle. Approximately 10–30 mg of powdered ventricle tissue was sonicated (Sonic Dismembrator Model 100, Fisher Scientific) in a RIPA buffer containing protease inhibitors. Sample protein concentrations were determined using the bicinchoninic acid method (BCA; Sigma Aldrich) with bovine serum albumin (BSA; Sigma-Aldrich) as a standard. Samples of extracted soluble protein were diluted 2× with Laemmli buffer (Sigma-Aldrich) and boiled at 95°C for 5 min. Protein samples (15 µg) were separated by size on 10% polyacrylamide gels and then transferred onto Immuno-Blot LF PVDF membranes (Bio-Rad, St-Laurent, QC, Canada) using a semi-dry transfer apparatus (Trans-blot® SD; Bio-Rad). Membranes were blocked with 5% skim milk in Tris-buffered saline containing 0.1% Tween 20 (TBST) for 1 h at room temperature, and then incubated overnight at 4°C with primary antibody (1:50,000 rabbit anti-salmon inducible HSP70; Cedarlane AS05061, Burlington, ON, Canada) in 2% skim milk in TBST. The following day, membranes were washed with TBST (3× for 5 min each time) and then incubated with secondary antibody [1:5000 goat anti-rabbit IgG conjugated to horseradish peroxidase (HRP) in 2% skim milk in TBST] for 1 h at room temperature. Membranes were washed with TBST (3× for 15 min each time) to remove excess antibody, incubated with 1 ml of Luminata Classico HRP substrate (MilliporeSigma™, Oakville, ON, Canada), and imaged using chemiluminescence (ChemiDoc XRS+, Bio-Rad). Relative HSP70 abundance was normalized to total protein using ImageLab 6.0 according to Taylor and Posch (2014).

Experiment 2

To complement previous work on the responses of dominant and subordinate trout to acute warming (LeBlanc et al., 2011) and further explore potential mechanisms underlying differences in CT_{max}, f_H data and ECG recordings were collected from dominant, subordinate and sham-treated trout during the 4-day social interaction period and the subsequent CT_{max} trial. The protocol was similar to that for group A of Experiment 1 (Fig. S1), but used fish fitted with heart rate biologgers and tissue samples were not collected. Following the 20-h recovery period, f_H measurements were collected every 5 min for 2 h from 09:00 h (recording frequency=150 Hz). The divider was removed at 11:00 h, allowing the members of a pair to interact, and f_H measurements and ECG recordings were collected every 5 min for 3 h during the initial stages of hierarchy formation. For the next 3 days, f_H (but not ECG traces) was recorded at 5 min intervals every morning for 2 h, from 09:00 to 11:00 h, a period that coincided with the morning observation period. On the morning of day 5, paired and sham fish were subjected to a CT_{max} trial (as described above) during which temperature, f_H and ECG recordings were collected every 3 min. Fish were then euthanized using terminal anaesthesia.

Measurements of f_H were also carried out on hearts isolated from a separate group of dominant and subordinate trout (N=6 pairs; see Table 1). This trial served to assess intrinsic f_H of the heart itself and the capacity for f_H to increase in response to stimulation, to investigate whether differences in f_H responses to temperature between dominant and subordinate fish (see Results) reflected effects at the level of the heart itself versus nervous control of f_H. Fish were euthanized by anaesthetic overdose on day 5. The intact heart was removed from the fish and placed in a thermostatted (to 13°C) bath of Ringer's solution (in mmol l^{-1}: 150 NaCl, 2.5 KCl, 1.5 $MgSO_4$, 0.4 NaH_2PO_4, 10 glucose, 10 HEPES, 1 $CaCl_2$, pH 7.7 at room temperature). The bath contained two tin-copper electrodes that were ∼2 cm apart and connected to a custom-built amplifier that allowed the electrical activity of the heart to be recorded (without direct contact) via a data acquisition system (BIOPAC Systems; Harvard Apparatus Canada, Montreal, QC, Canada) and software (AcqKnowledge, BIOPAC Systems; sampling rate set to 200 Hz). A cannula (PE 90 polyethylene tubing; Clay-Adams) was inserted into the ventricle lumen through the bulbus arteriosus and secured in place with 2-0 surgical silk. The cannula was connected to a syringe pump (model KDS220, kdScientific, Holliston, MA, USA) that retrograde perfused the heart with aerated, 13°C Ringer's solution at a rate of 0.05 ml min^{-1}, which preliminary experiments established was necessary to maintain heart viability. Following a 10 min recovery period, recording commenced for a 10 min control period. Noradrenaline (a general adrenergic receptor agonist) was then added to the bath to achieve 1 µm final (nominal) concentration and recording continued for an additional 10 min. Heart rate (in beats min^{-1}) was calculated from 10 s periods of the ECG trace at 5 min (control period) or 1 min (post-noradrenaline addition) intervals.

Experiment 3

The goal of this experiment was to determine whether the elevation of circulating cortisol levels caused by chronic social stress elicits cardiac hypertrophy and fibrosis in subordinate rainbow trout. To assess cardiac hypertrophy, ventricle mass and thickness of the compact myocardium were measured together with transcript abundances of commonly used markers of muscle growth (Johansen et al., 2011, 2017; Keen et al., 2016, 2017; Nørstrud et al., 2018), including ventricular myosin heavy chain (*vmhc*), slow myosin light chain 2 (*smlc2*) and muscle LIM protein (*mlp*), and the pro-hypertrophic factor regulator of calcineurin I (*rcan1*).

To evaluate fibrotic remodelling, histological assessment of collagen deposition was carried out, and collagen type I protein abundance was measured together with transcript abundance of the collagen type I isoforms, collagen type I alpha 1 (*col1α1*), alpha 2 (*col1α2*) and alpha 3 (*col1α3*). Transcript abundances of the connective tissue regulators matrix metalloproteinase 2 (*mmp2*), matrix metalloproteinase 9 (*mmp9*) and tissue inhibitor of metalloproteinases 2 (*timp2*) were also measured (Johnson et al., 2014; Johnston et al., 2019). Transcript abundances of the corticosteroid receptors mineralocorticoid receptor (*mr*) and glucocorticoid receptors 1 and 2 (*gr1* and *gr2*) were measured to assess the potential for the ventricle to respond to cortisol.

Histological assessment of cardiac collagen abundance

Fixed ventricle tissue from the fish of group A was embedded in paraffin wax and sectioned transversely at 5 µm thickness to yield approximately six to 10 sections per heart. Sections were placed on positively charged microscope slides (Fisherbrand™ Superfrost™ Plus; Fisher Scientific) and heated at 65°C for 30 min. The paraffin was removed through a series of xylene washes (3× for 5 min), and the tissue was rehydrated using a series of ethanol washes (100%, 95% and 80% for 1 min each). Sections were stained for collagen using Picrosirius Red (Fisher 5030077) as described by Johnson et al. (2014), and then rinsed with acetic acid.

The stained heart sections were examined using brightfield light microscopy (Axiophot; Zeiss, North York, ON, Canada). Three randomly selected areas of one section per heart were photographed, and average relative collagen content was quantified as the area of red staining (indicating collagen presence) within the total area of tissue. In addition, compact myocardium thickness was estimated as the mean of five measurements per image. All image analysis was carried out using ImageJ (https://imagej.nih.gov/ij/) by an observer who was blind to the social status of the fish from which the heart tissue was collected.

Measurement of transcript abundances by real-time RT-PCR

Ventricles from the fish of group A stored at −80°C were ground to a powder on dry ice using a mortar and pestle. To extract total RNA, powdered ventricle tissue (approximately 10-40 mg) was sonicated (Sonic Dismembrator Model 100, Fisher Scientific) on ice in TRIzol® reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. The quantity and quality of RNA were assessed using a NanoDrop® (NanoDrop™ 2000, ThermoFisher Scientific). Following genomic DNA removal, cDNA was synthesized from total RNA using the QuantiTect® reverse transcription kit (Qiagen, Montreal, QC, Canada) according to the manufacturer's directions. Gene-specific primer sequences were identified from the literature for target genes as well as *β-actin* as a housekeeping gene (Table S1). Real-time RT-PCR was carried out using the Rotor-Gene SYBR Green PCR kit (Qiagen) and a Rotor-Gene Q real-time PCR machine (Qiagen). Reactions were carried out according to the manufacturer's instructions, but volumes were scaled to 10 µl. Samples were run in duplicate, together with negative controls (a no-template sample in which cDNA was replaced with water, and a no-RT sample in which the cDNA synthesis reaction was carried out without reverse transcriptase). Cycling conditions were as follows: 5 min at 95°C, 42 cycles of 10 s at 95°C, 10 s at 60°C and 10 s at 72°C, followed by melt curve analysis, as per the manufacturer's instructions. Standard curves were generated using serially diluted pooled samples and relative transcript abundances were calculated according to Pfaffl (2001).

Measurement of protein abundance by western blotting

Collagen type I protein abundance was measured in ventricle tissue from the fish of group A by western analysis according to the protocol described above using 40 µg of protein sample and 1:1000 rabbit anti-salmon collagen type 1 (Cedarlane CL50171AP, Burlington, ON, Canada) as the primary antibody.

Ex vivo passive pressure–volume curves

In addition to the histological analysis and measurements of transcript or protein abundance, a functional measure of fibrotic remodelling was obtained by generating *ex vivo* pressure–volume curves. Data were generated for a separate group of pairs (N=8; see Table 1) following the 4-day interaction period. The basic approach of Keen et al. (2016) was used. Intact hearts isolated from the dominant and subordinate fish making up a pair were placed in 19°C Ringer's solution containing 20 mmol l^{-1} 2,3-butanedione monoxime (BDM; Sigma-Aldrich) to prevent cardiac contraction. A cannula (PE 90 tubing; Clay-Adams) was inserted through the bulbus arteriosus into the lumen of the ventricle and tied in place with 2-0 surgical silk around the bulbus–ventricle junction. The atrial–ventricular junction was closed with 2-0 silk to seal the ventricle. The cannula was attached to a syringe pump (model KDS220, kdScientific), while a sidearm constructed from PE 50 tubing (Instech Laboratories, Plymouth Meeting, PA, USA) was connected to a pressure transducer (TSD104A, BIOPAC Systems). The pressure transducer was connected to a data acquisition system (BIOPAC Systems, AcqKnowledge software, sampling rate=200 Hz), and was calibrated daily against a static column of water. The ventricle was filled at a rate of 0.02 ml min^{-1} with Ringer's solution containing BDM until its maximum capacity was reached (as determined by a drop in pressure as the ventricle developed a leak).

Data processing and statistical analysis

ECG traces, f_H and temperature data were obtained from the biologgers using Mercury software (Star Oddi). The ECG traces were used to verify whether f_H data were reliable, and to identify the temperature at which arrhythmia first occurred. Only individuals with acceptable ECG traces were included in analyses. To be deemed acceptable, a clear P wave and QRS complex were present, and the quality index (QI0 to QI3; where QI0=great and QI3=poor) generated automatically by the Mercury software was <3. Although inclusion of QI1 and QI2 values can increase variability in the f_H data, inclusion of only QI0 values substantially reduces the data available for analysis (Brijs et al., 2019). Finally, f_H values below 20 beats min^{-1} or above 150 beats min^{-1} at 13°C were eliminated based on the range of f_H reported in the literature (Wood et al., 1979; Farrell et al., 1996; Farrell, 2002; Sandblom and Axelsson, 2007; Gamperl et al., 2011; Verhille et al., 2013; Ekström et al., 2014, 2018); these cut-off values were adjusted according to temperature using a Q_{10} value of 2.1 (Gamperl et al., 2011). For the 4-day interaction period, f_H was reported as the mean value for an individual over the 2 or 3 h measurement period for each day. For the CT_{max} trial, f_H at 13°C was calculated as the mean of f_H values for water temperatures of 13–13.5°C, immediately prior to thermal ramping. Peak Δf_H was the largest difference between f_H observed during acute warming and f_H at 13°C.

All statistical analyses were carried out using R-studio (https://rstudio.com/). Data were checked for normality using the Kolmogorov–Smirnov test and for equal variances using Levene's test. In all cases, α was set to 0.05. In most cases (CT_{max}, ventricle mass, compact myocardium thickness, collagen deposition, protein

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and transcript abundances), Student's *t*-tests were used to determine the statistical significance of differences between dominant and subordinate fish. Where sham-treated fish were included in the experiment, ANOVA was used. Plasma cortisol concentrations were analyzed by two-way ANOVA using status (dominant or subordinate) and whether the fish underwent a CT_{max} trial as factors. Heart rate during the interaction period was analyzed by two-way repeated-measures (RM) ANOVA with social status (dominant, subordinate or sham) as a between-subject factor and time (day) as a within-subject factor. Heart rate during the CT_{max} trial was analyzed by linear regression for each group separately (dominant, subordinate, sham), with comparisons of slopes between dominant and subordinate fish by analysis of covariance (ANCOVA). Heart rates for isolated hearts were analyzed by two-way RM ANOVA with social status (dominant or subordinate) and time (baseline or post-noradrenaline) as factors. Pressure–volume curves were analyzed by two-way RM ANOVA on rank-transformed data with social status (dominant or subordinate) and volume as factors.

RESULTS

Experiment 1: Impact of elevated cortisol on the CT_{max} of subordinate rainbow trout

Rainbow trout held in pairs formed social hierarchies in which dominant fish were distinguished from subordinate fish by their behaviour (Table 1). The CT_{max} of subordinate fish was significantly lower than that of dominant fish (Fig. 1A; Student's *t*-test, P=0.015). Following the physical separation of the fish in a pair for a 48 h recovery period, the CT_{max} of (recovering) subordinates was similar to that of dominant fish (Fig. 1B; Student's *t*-test, P=0.35). Cortisol treatment of recovering subordinates using a cortisol-containing cocoa butter implant prevented the recovery of thermal tolerance; CT_{max} in cortisol-treated recovering subordinates was significantly lower than that of dominant fish (Fig. 1C; Student's *t*-test, P=0.0003). The CT_{max} of sham-treated recovering subordinates, which received a cocoa butter implant lacking cortisol, did not differ from that of dominant fish (Fig. 1D; Student's *t*-test, P=0.2).

Plasma cortisol levels were significantly higher in subordinate than dominant fish after a 4-day interaction period (Fig. 2A; two-way ANOVA; status P=0.001, acute warming P<0.0001, status×acute warming P=0.289), but returned to baseline levels after a 48 h recovery period (Fig. 2B; two-way ANOVA; status P=0.271, acute warming P<0.0001, status×acute warming P=0.089). Cortisol levels of cortisol-treated recovering subordinates were significantly higher than those of dominant fish (Fig. 2C; two-way ANOVA; status P=0.0009, acute warming P=0.01, status×acute warming P=0.62). However, when recovering subordinates were treated with cocoa butter alone, cortisol levels returned to baseline values within the 48 h recovery period (Fig. 2D; two-way ANOVA; status P=0.0009, acute warming P=0.01, status×acute warming P=0.62). In all treatment groups, plasma cortisol levels after a CT_{max} trial were significantly higher than those in fish that had not undergone a CT_{max} trial, regardless of social status. No difference in HSP70 protein abundance in the heart was detected between subordinate and dominant trout following exposure to acute warming in a CT_{max} trial (normalized HSP70 abundance=1.00±0.70 for dominants, 0.61±0.48 for subordinates, means±s.e.m., N=6; Student's *t*-test, P=0.29).

Experiment 2: Heart rate during social interactions and acute warming

Fish instrumented with f_H biologgers and held in pairs formed social hierarchies in which dominant fish were distinguished from

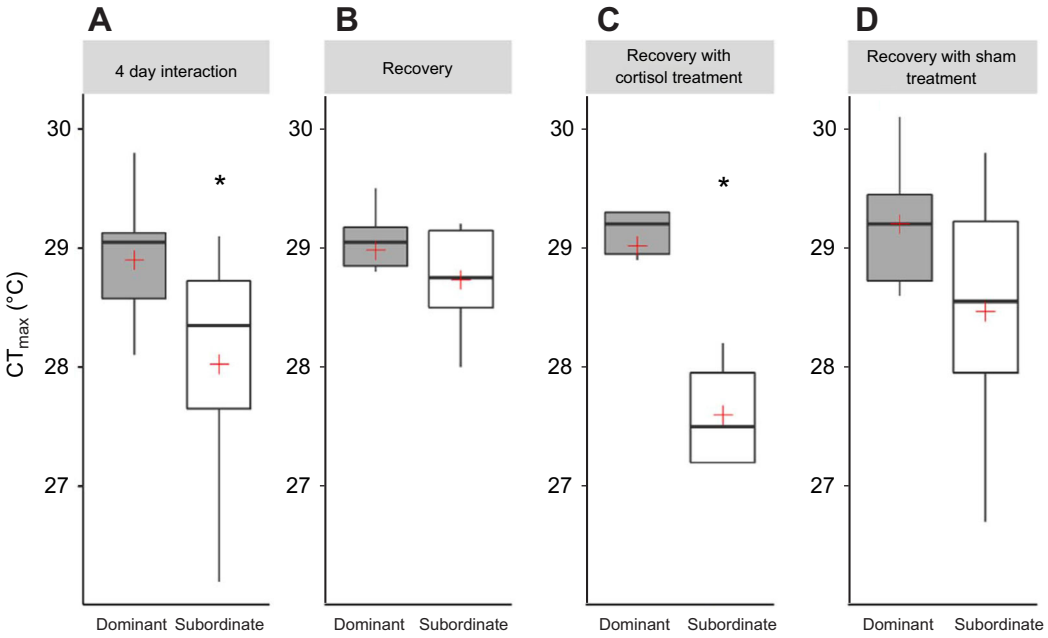

Fig. 1. Critical thermal maxima (CT_{max}) of dominant and subordinate rainbow trout (*Oncorhynchus mykiss*). Data are following (A) social interactions for 4 days (N=12 pairs), (B) 48 h of recovery from a 4-day social interaction period (N=6 pairs), (C) 48 h of recovery from 4 days of social interaction but with cortisol treatment of subordinate fish during the recovery period (N=6 pairs) and (D) 48 h of recovery from 4 days of social interactions but with sham treatment (cocoa butter implant) of subordinate fish during the recovery period (N=6 pairs). An asterisk indicates a significant difference between dominant and subordinate fish within a treatment group (Student's *t*-tests, P=0.015, 0.35, 0.0003 and 0.2 for A to D, respectively). Data are presented as box plots where the upper and lower limits of the box indicate the 75th and 25th percentiles, respectively, the black line across the box indicates the median, the whiskers indicate the maximum and minimum values, and the red plus sign indicates the mean value.

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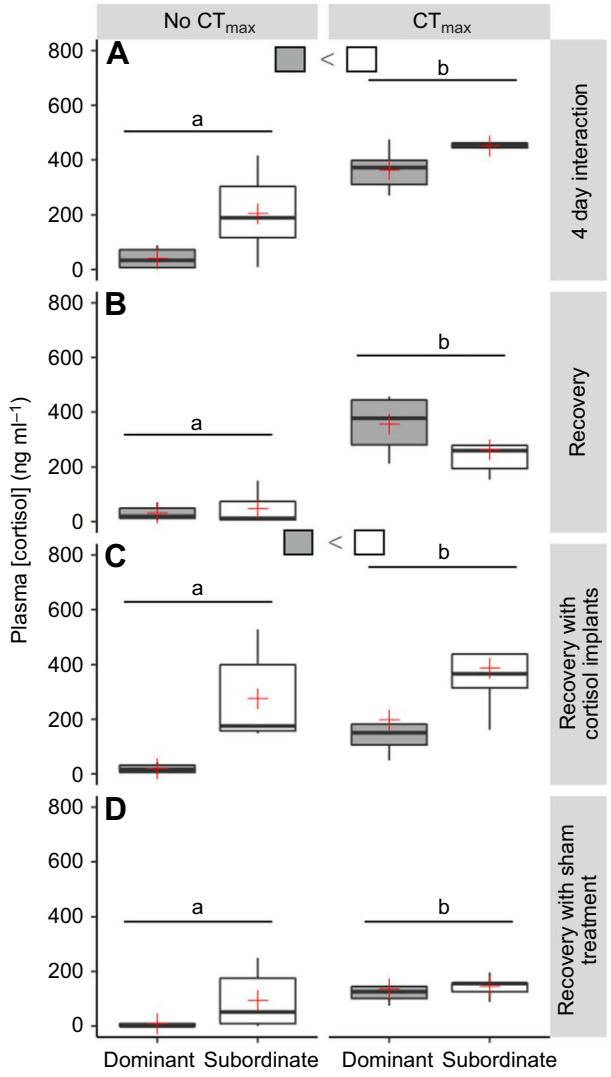

Fig. 2. Plasma cortisol concentrations of subordinate and dominant rainbow trout (*O. mykiss*). Data are following (A) social interactions for 4 days (N=8 and 6 pairs; two-way ANOVA; status P=0.001, acute warming P<0.0001, status×acute warming P=0.289), (B) 48 h of recovery from a 4-day social interaction period (N=7 and 6 pairs; two-way ANOVA; status P=0.271, acute warming P<0.0001, status×acute warming P=0.089), (C) 48 h of recovery from a 4 day social interaction period with cortisol treatment of subordinate fish during the recovery period (N=6 and 6 pairs; two-way ANOVA; status P=0.0009, acute warming P=0.01, status×acute warming P=0.62) and (D) 48 h of recovery from a 4 day social interaction period with sham treatment of subordinate fish during the recovery period (N=6 and 5 pairs; two-way ANOVA; status P=0.09, acute warming P=0.006, status×acute warming P=0.20). A significant effect of acute warming is indicated by pairs of bars (linked by a horizontal line) that do not share a lowercase letter. A significant effect of social status is indicated on the panel using the fill symbols (grey for dominant fish and white for subordinate fish). Data are presented as box plots; please see the legend of Fig. 1 for details.

subordinate fish by their behaviour (Table 1). Measurement of f_H during social interactions revealed a significant interaction between the effects of time and social status (Fig. 3; two-way RM ANOVA, P=0.184 for status, P<0.001 for time, P=0.020 for status×time). Heart rate differed among sham, dominant and subordinate fish only on day 2 of the interaction period, where f_H in sham-treated fish was significantly lower than that in subordinate fish. Among all groups, f_H decreased over the interaction period, being significantly higher on day 1 following removal of the divider than on days 3 or 4.

On day 5, f_H values prior to warming were similar to values for day 4 and did not differ among dominant, subordinate and sham fish (Fig. 4A; one-way ANOVA, P=0.475). During acute warming, f_H increased with temperature in all groups (Fig. 5). Regressions of f_H on temperature were significant, with the slope at which heart rate increased with warming being significantly lower in subordinate relative to dominant trout (ANCOVA, P=0.040). Although there were no significant differences in the magnitude of the f_H response (Fig. 4B; one-way ANOVA, P=0.850), there was a trend for the temperature of peak f_H response to be lower in subordinate than dominant or sham-treated trout (Fig. 4C; one-way ANOVA, P=0.077). Similarly, arrhythmia was first detected in subordinate trout at significantly lower temperatures than in dominant or sham-treated trout (Fig. 4D; one-way ANOVA, P=0.002). Correspondingly, CT_{max} of subordinate trout (26.5±0.9, N=9) was significantly lower than that of sham-treated fish (28.2±0.1, N=6), although the difference between subordinate and dominant fish (27.8±0.2, N=9) did not quite reach statistical significance (one-way ANOVA on ranks, P=0.012; Student's t-test on subordinate versus dominant fish, P=0.093).

For isolated hearts, neither baseline f_H (dominant 63.0±3.7, subordinate 60.3±2.0, mean±s.e.m., N=6) nor peak f_H post noradrenaline addition (dominant 70.0±3.7, subordinate 71.0±2.4, N=6) differed significantly between dominant and subordinate fish (two-way RM ANOVA on log-transformed data, P=0.878 for status, P<0.001 for sample time, P=0.183 for status×time). The fish from which hearts were isolated formed pairs with divergent behaviour (Table 1) and cortisol concentrations (dominant [cortisol]= 4.7±2.1 ng ml^{-1}, subordinate [cortisol]=137.6±43.9 ng ml^{-1}, N=14 pairs; Student's t-test, P=0.006).

Experiment 3: Does cardiac remodelling occur in subordinate rainbow trout?

To examine the potential for cardiac remodelling in response to the elevated cortisol of chronic social stress, transcript abundances of the three cortisol receptors in rainbow trout, *gr1*, *gr2* and *mr*, were assessed in the ventricle of dominant versus subordinate trout after 5 d of social interaction (Table 2). No significant differences were detected, although *gr2* exhibited a trend towards higher transcript abundance in subordinate fish (Student's t-tests, P=0.53, 0.062, 0.71 for *gr1*, *gr2* and *mr*, respectively). Subordinate fish did not differ from dominant fish in ventricle mass (Fig. 6A; Student's t-test, P=0.21) or thickness of the compact myocardium (Fig. 6B; Student's t-test, P=0.78). Because the 5-day interaction period (Fig. S1) might not have been long enough for ventricular hypertrophy to become apparent at the organ level, transcript abundances of known molecular markers for hypertrophy were assessed in ventricular tissue (Table 2). The markers *mlp*, *smlc2* and *vmhc* are associated with muscle growth and are direct indicators of hypertrophy. The marker *rcan1* activates the calcineurin-NFAT signalling cascade, which promotes hypertrophic growth, and is considered a pathological marker of hypertrophy (Keen et al., 2018). Although *rcan1* transcript abundance tended to be higher in the ventricle of subordinate relative to dominant trout (Student's t-test, P=0.075), no significant differences were detected (Student's t-tests, P=0.39 for *mlp*, 0.37 for *smlc2* and 0.55 for *vmhc*).

Histological assessment of ventricular collagen content using Picrosirius Red revealed lower collagen content in subordinate than dominant trout, a difference that did not quite reach statistical significance (Fig. 7A; Student's t-test, P=0.062). To further explore this trend, collagen type I protein levels and transcript abundances were evaluated together with transcript abundances of two matrix

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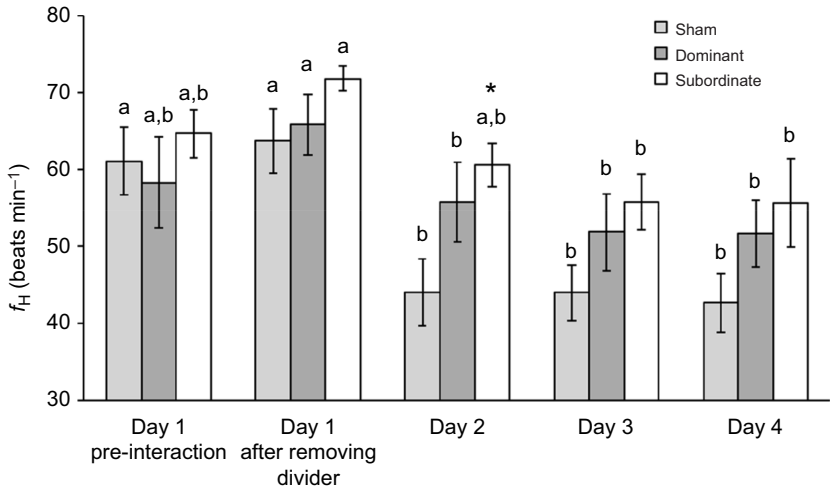

Fig. 3. Heart rate (f_H) of dominant, subordinate and sham rainbow trout (*O. mykiss*) prior to (pre-interaction) and during social interactions. Heart rate was measured using biologgers for a 2 h period each morning except for the period immediately after removal of the divider, where f_H was collected for 3 h. Values are means±s.e.m. (*N*=6 sham, 8 dominant and 8 subordinate fish). Bars that share a lowercase letter within a social status category are not significantly different from one another, and within a day, an asterisk indicates a significant difference from the sham fish (two-way RM ANOVA; status *P*=0.184, day *P*<0.001, status×day *P*=0.020).

metalloproteinases (*mmp2* and *mmp9*) and their inhibitor (*timp2*) involved in collagen degradation (Table 3). Type I collagen is the major contributor to total collagen content in mammalian hearts (Medugorac, 1982) and in fish is composed of three alpha-helical chains, α1, α2 and α3 (Keen et al., 2016). However, neither type I collagen protein abundance (Student's *t*-test, *P*=0.80) nor transcript abundances of the three alpha-helical chains, *col1a1*, *col1a2* and *col1a3* (Student's *t*-tests, *P*=0.41, 0.25 and 0.27, respectively), differed between the ventricles of dominant and subordinate trout. Similarly, no significant differences in the transcript abundances of *mmp2*, *mmp9* and *timp2* were detected (Student's *t*-tests, *P*=0.55, 0.78 and 0.15, respectively). Additionally, no differences were detected in the passive stiffness of the ventricle, which was assessed as a functional measure of potential differences in collagen deposition (Fig. 7D; two-way RM ANOVA; status *P*=0.812, volume *P*<0.001, status×volume *P*=0.472).

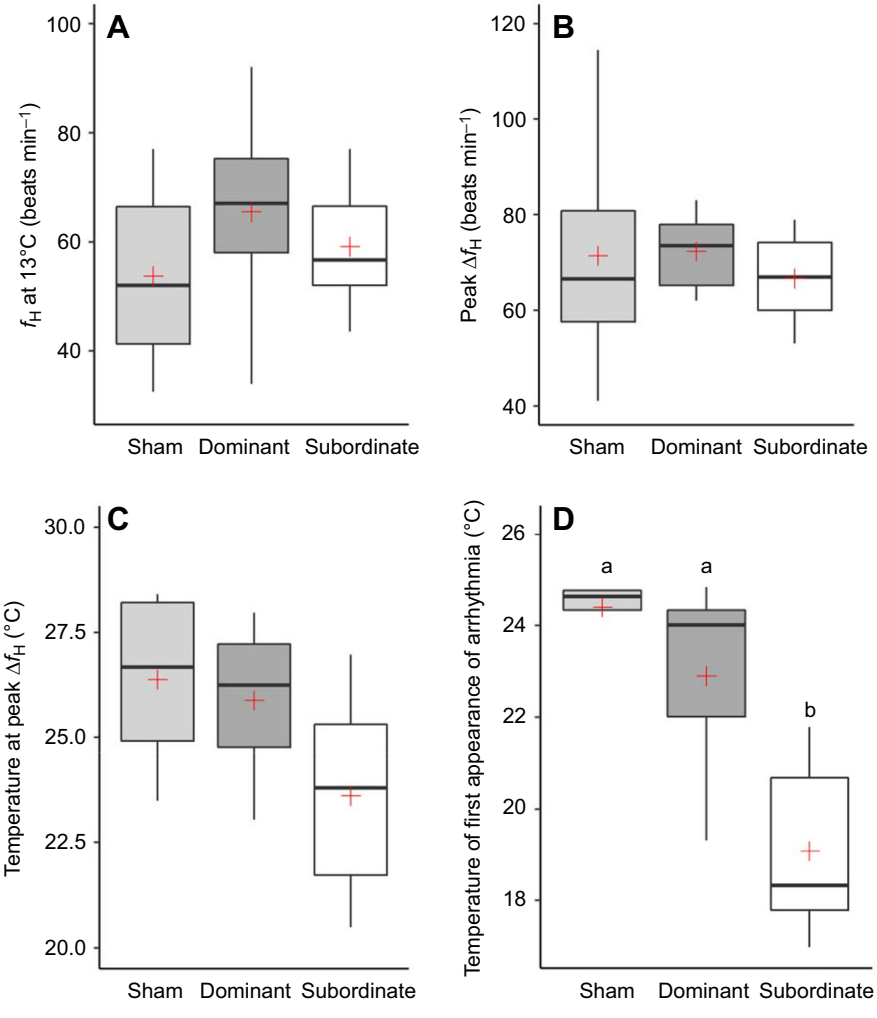

Fig. 4. Measurements of heart rate (f_H) of sham (*N*=6), dominant (*N*=6) and subordinate (*N*=6) rainbow trout (*O. mykiss*) during a CT_{max} trial. (A) f_H at 13°C, immediately prior to the CT_{max} trial, (B) the peak increase in heart rate (Δf_H), (C) the temperature at which the peak Δf_H occurred and (D) the temperature at which arrhythmia was first detected in ECG traces. Groups that share a letter are not significantly different from one another (one-way ANOVA; *P*=0.475, 0.850, 0.077 and 0.002 for A to D, respectively). Data are presented as box plots; please see the legend of Fig. 1 for details.

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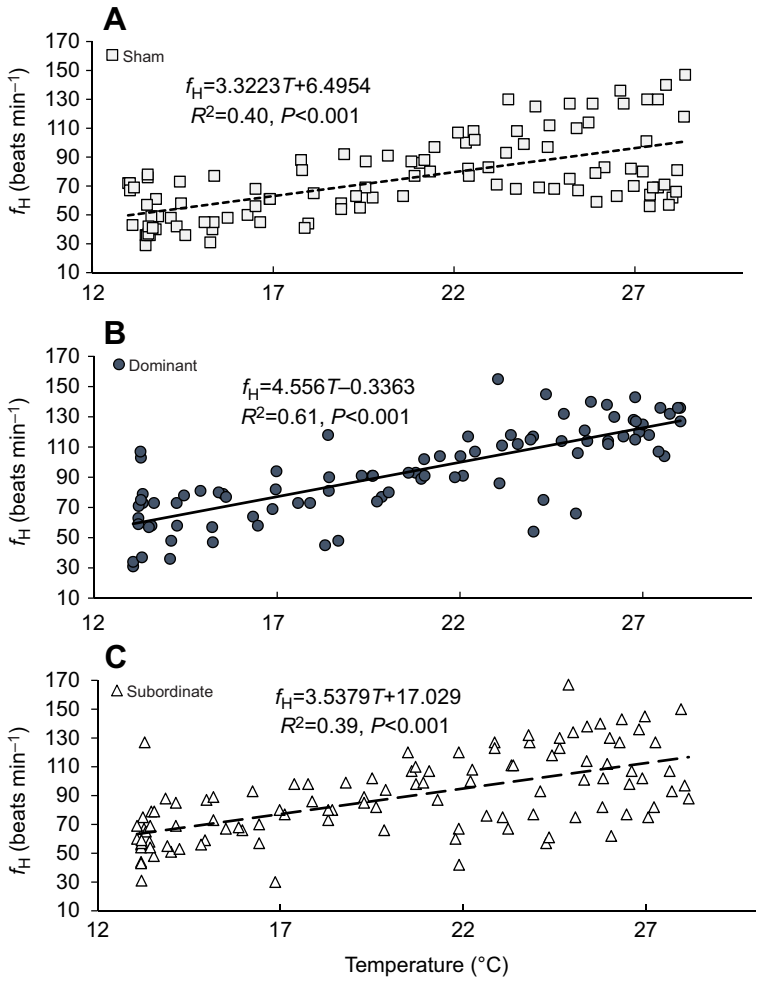

Fig. 5. Heart rate (f_H) of rainbow trout (*O. mykiss*) during acute warming for sham, dominant and subordinate fish. (A) Sham (105 points for *N*=6 fish), (B) dominant (89 points for *N*=6 fish) and (C) subordinate fish (109 points for *N*=7 fish). The equation of the line determined by linear regression is indicated on the plot, together with the R^2 and *P*-values for the regression. *T*, temperature.

DISCUSSION

The results of the present study identified cortisol as a major contributor to the lowered CT_{max} in rainbow trout experiencing chronic social stress. Although CT_{max} is known to be modulated by factors such as acclimation temperature (e.g. Beitinger and Bennett, 2000; Fangue, 2006; Zhang and Kieffer, 2014; Nyboer and Chapman, 2018), body size (Zhang and Kieffer, 2014), life history stage (Komoroske et al., 2014) and chronic social stress (LeBlanc et al., 2011), the physiological mechanisms underlying these effects often remain unclear. Thus, the results of the present study constitute an important step forward in understanding how CT_{max} is modulated by chronic stress. However, additional work is needed to identify the mechanisms linking changes in CT_{max} to circulating cortisol levels. Because chronic cortisol elevation can induce pathological ventricular hypertrophy with associated negative impacts on cardiac function in rainbow trout (Johansen et al., 2011, 2017), the present study assessed markers of ventricular hypertrophy and fibrosis, and f_H responses to acute warming in subordinate trout. The results of these experiments did not support chronic social stress-induced cardiac remodelling as a mechanism underlying the effects of cortisol on CT_{max}.

Effects of cortisol on CT_{max}

The findings of the present study support the hypothesis that prolonged elevation of cortisol in subordinate rainbow trout lowers thermal tolerance. As reported by LeBlanc et al. (2011), the CT_{max} of subordinate trout was significantly lower than that of dominant fish after 4 days of interaction. In recovering subordinates that were allowed to recover from social stress for 48 h by placing a partition in the tank to separate the members of a pair, cortisol levels were on par with those of dominant fish, in agreement with the findings of Culbert and Gilmour (2016). The lowering of cortisol levels in recovering subordinates was accompanied by a return of CT_{max} to values similar to those in dominant fish. A similar recovery of both

Table 2. Relative mRNA abundances of corticosteroid receptors and genes used as indicators of cardiac hypertrophy in the ventricle of subordinate and dominant rainbow trout (*O. mykiss*)

Gene	Dominant (*N*=5)	Subordinate (*N*=6)	*P*-value
	Corticosteroid receptors		
gr1	1.17±0.31	0.92±0.23	0.53
gr2	1.05±0.16	1.84±0.32	0.062
mr	1.07±0.20	1.16±0.11	0.71
	Indicators of cardiac hypertrophy		
vmhc	1.04±0.16	0.89±0.17	0.55
smlc2	1.93±1.09	0.78±0.42	0.37
mlp	1.29±0.38	0.87±0.25	0.39
rcan1	1.08±0.20	3.17±0.93	0.075

gr1, glucocorticoid receptor 1; *gr2*, glucocorticoid receptor 2; *mr*, mineralocorticoid receptor; *vmhc*, ventricular myosin heavy chain; *smlc2*, slow myosin light chain 2; *mlp*, muscle LIM protein; *rcan1*, regulator of calcineurin I. Values are means±s.e.m. Transcript abundance of the gene of interest was normalized using β-actin and expressed relative to the value for dominant fish. Relative transcript abundances were compared using Student's *t*-tests.

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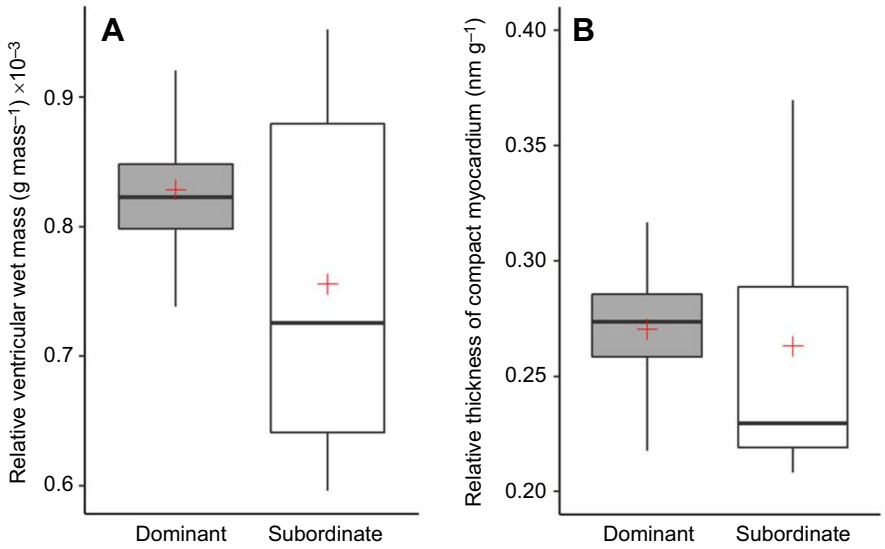

Fig. 6. Relative ventricular wet mass and relative compact myocardial thickness of dominant and subordinate rainbow trout (*O. mykiss*). (A) Relative ventricular wet mass of dominant (*N*=8) and subordinate (*N*=8) rainbow trout after 5 days of social interaction. (B) Relative compact myocardial thickness assessed histologically from sections of ventricle tissue stained with Picrosirius Red for dominant (*N*=5) and subordinate (*N*=5) trout after 5 days of social interaction. Relative mass and relative thickness were standardized using the mass of the individual fish. No significant differences were detected between dominant and subordinate fish (Student's *t*-tests, *P*=0.21 and 0.78 for A and B, respectively). Data are presented as box plots; see the legend of Fig. 1 for details.

CT_{max} and cortisol levels was observed in recovering subordinates treated with sham implants (i.e. cocoa butter alone). Compellingly, however, when the fall in cortisol levels was prevented by treating recovering subordinates with cortisol, CT_{max} also failed to recover. Collectively, these data indicate that elevated cortisol is a key driver contributing to lower CT_{max} in trout exposed to chronic social stress. To our knowledge, no other study has focused specifically on cortisol as a modulator of CT_{max} in fish, but there is support in the literature for stress-induced changes in CT_{max}. For example, threadfin shad (*Dorosoma petenense*) showed reduced thermal tolerance (lower CT_{max}) shortly after being subjected to a netting stressor (Monirian et al., 2010), a protocol that would be expected to elicit a cortisol response (Strange et al., 1977; Øverli et al., 1999a; Jeffrey et al., 2014). Exposure to hypoxia (Rutledge and Beitinger, 1989; Healy and Schulte, 2012), as well as acute changes in salinity (Shaughnessy and McCormick, 2018), also have been associated with reductions in CT_{max}, although in these cases it is more difficult to link changes in CT_{max} to cortisol specifically. A related observation that is also consistent with cortisol-mediated changes in thermal tolerance is that in a field study carried out in their natural habitat, cortisol-treated checkered pufferfish (*Sphoeroides testudineus*) selected cooler temperatures than control animals (Cull et al., 2015).

The mechanisms through which chronic elevation of cortisol during social stress reduces CT_{max} remain to be determined. Acute warming elevates circulating cortisol levels in rainbow trout and other fishes (Pérez-Casanova et al., 2008; LeBlanc et al., 2011, 2012; Yang et al., 2018; Yusishen et al., 2020), and factors that

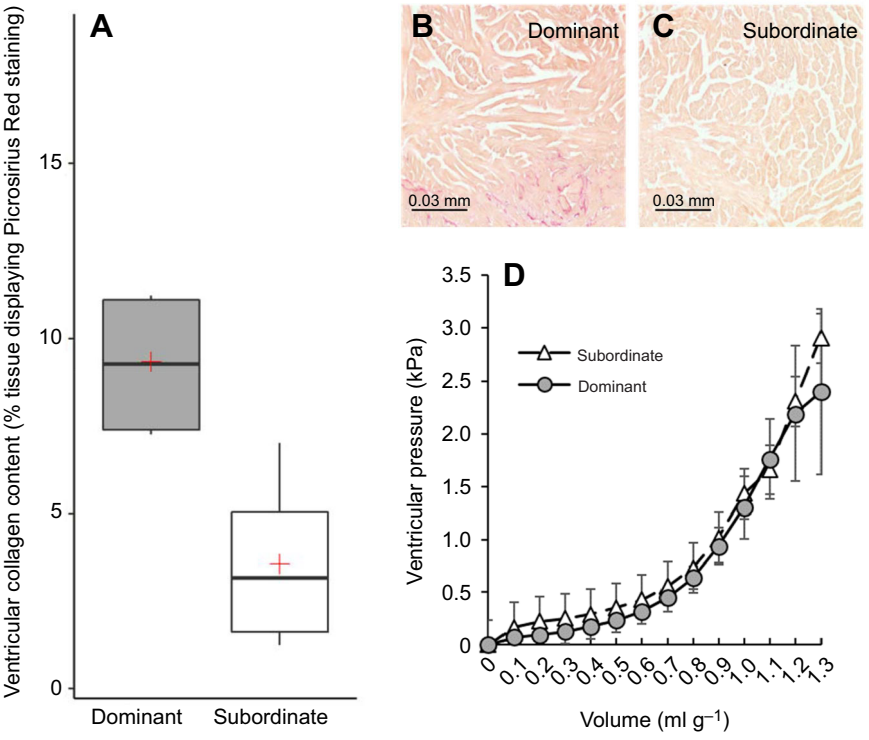

Fig. 7. Relative ventricular collagen content assessed histologically from tissue sections stained with Picrosirius Red for dominant (*N*=6) and subordinate (*N*=6) rainbow trout (*O. mykiss*) after 5 days of social interaction. (A) Collagen content was quantified as the percentage of the tissue in the micrograph showing red staining. The difference between dominant and subordinate fish did not reach statistical significance (Student's *t*-test, *P*=0.062). Data are presented as a box plot; please see the legend of Fig. 1 for details. Representative images of ventricle tissue sections from (B) dominant and (C) subordinate trout stained with Picrosirius Red also are presented. (D) Passive pressure–volume relationships for ventricles isolated from dominant and subordinate fish. Values are means±s.e.m., and each data point represents a mean of *N*≥4 individual fish (maximum 8). Pressure was normalized to start at 0 kPa for graphical representation (as per Keen et al., 2016). Ventricular pressure was significantly affected by volume but not social status (two-way RM ANOVA; status *P*=0.812, volume *P*<0.001, status×volume *P*=0.472).

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Table 3. Relative protein and mRNA abundances of collagen type I protein and genes involved in collagenous cardiac remodelling in the ventricle of subordinate and dominant rainbow trout (*O. mykiss*)

Protein or gene	Dominant	Subordinate	*P*-value
Collagen type I	1.00±0.15 (6)	1.06±0.17 (6)	0.80
col1α1	1.32±0.55 (5)	0.77±0.29 (6)	0.41
col1α2	1.51±0.76 (5)	0.49±0.06 (6)	0.25
col1α3	1.58±0.85 (5)	0.48±0.07 (6)	0.27
mmp2	1.44±0.68 (5)	0.92±0.43 (6)	0.55
mmp9	1.85±0.74 (5)	2.17±0.80 (6)	0.78
timp2	1.10±0.26 (4)	0.48±0.27 (4)	0.15

col1α1, collagen type I alpha 1; *col1α2*, collagen type I alpha 2; *col1α3*, collagen type 1 alpha 3; *mmp2*, matrix metalloproteinase 2; *mmp9*, matrix metalloproteinases 9; *timp2*, tissue inhibitor of metalloproteinases 2. Values are means±s.e.m. (*N*). Protein abundance was expressed relative to the value for the dominant fish. Transcript abundance of the gene of interest was normalized using β-actin and expressed relative to the value for dominant fish. Relative abundances were compared using Student's *t*-tests.

attenuate this heat-induced cortisol response potentially could impair thermal tolerance. Chronic social stress was associated with attenuation of the cortisol response to handling or netting stressors (Øverli et al., 1999b; Jeffrey et al., 2014; Culbert and Gilmour, 2016). However, subordinate trout in the present study mounted cortisol responses to acute warming that were comparable to those of dominant fish, a finding also reported by LeBlanc et al. (2011). The lower CT_{max} of subordinate relative to dominant fish despite comparable cortisol responses to acute warming suggests that the cortisol response to acute warming does not influence thermal tolerance. This conclusion is supported by a recent study of lake sturgeon (*Acipenser fulvescens*), where the cortisol response to acute warming was reduced in sturgeon held in groups versus singly, but CT_{max} was unchanged (Yusishen et al., 2020). Similarly, CT_{max} values for HR and LR (low cortisol responsiveness to a standardized stressor) trout did not differ despite strongly divergent cortisol responses to acute warming (LeBlanc et al., 2012).

Measurement of f_H provided additional insight into the physiological responses to acute warming of subordinate versus dominant fish. The expected increase in f_H with increasing temperature (Farrell et al., 1996; Clark et al., 2008; Steinhausen et al., 2008; Gamperl et al., 2011) was apparent in all groups, with the magnitude of the f_H increase being comparable across dominant, subordinate and sham-treated trout. However, peak increases in f_H tended to occur at higher temperatures in dominant and sham-treated trout than in subordinate trout, resulting in a significant difference between dominant and subordinate trout in the slope of the relationship between f_H and temperature. That is, dominant fish appeared to be able to maintain increases in f_H to higher temperatures than subordinate fish. Thus, increases in f_H tended to plateau or peak near the CT_{max} regardless of social status, with peak f_H and CT_{max} values in subordinate trout being lower than those in dominant or sham-treated trout. This pattern is in agreement with observations more broadly of peak f_H responses coinciding with the upper thermal limit (Ekström et al., 2014, 2017, 2019; Gilbert et al., 2019). Correspondingly, irregularities in the electrical activity of the heart occurred at significantly lower temperatures in subordinate than dominant or sham-treated trout. Recent work suggests that rising temperature alters the electrical excitability of the trout heart, ultimately disrupting the frequency of heart beats (Vornanen, 2016, 2020; Haverinen and Vornanen, 2020). In particular, it appears that at high temperature, the ventricle fails to rhythmically contract in response to the atrium owing to an atrioventricular block. The atrioventricular block is caused by reduced excitability in the ventricle, which, in turn, reflects differential changes in Na^+ and K^+ conductance with temperature (Vornanen et al., 2014; Vornanen, 2016; Badr et al., 2018; Haverinen and Vornanen, 2020; reviewed by Vornanen, 2020). Whether the prolonged elevation of cortisol experienced by subordinate fish directly contributes to changes in cardiac ion conductances that alter the temperature sensitivity of the ventricle, or whether changes in cardiac ion conductances are an indirect effect of the actions of cortisol elsewhere remains to be determined. In this regard, it is worth considering that the cardiac HSP70 response to acute warming was similar in dominant and subordinate fish, even though subordinate fish were exposed to a lower temperature (by virtue of their lower CT_{max}) than dominant fish.

Additional work clearly is needed to identify the mechanisms through which chronic elevation of cortisol during social stress reduces CT_{max}. Given that the endpoint of a CT_{max} trial is loss of motor function/equilibrium (Beitinger et al., 2000), effects of temperature on brain neural function may determine CT_{max}. Recent support for this possibility was provided by Jutfelt et al. (2019), who reported that CT_{max} in Atlantic cod (*Gadus morhus*) could be increased, albeit to a limited extent, by local cooling of the brain. The fish brain is rich in both the mineralocorticoid (MR) and glucocorticoid receptors (GR1 and GR2) that mediate the physiological effects of cortisol on target tissues, making it very responsive to changes in cortisol (Teitsma et al., 1998; Alderman and Vijayan, 2012; Alderman et al., 2012; Teles et al., 2013; Kiilerich et al., 2018). Further, experimental elevation of cortisol levels as well as the chronic elevation of cortisol associated with social stress reduce neurogenesis in the brain of rainbow trout, providing an additional mechanism through which cortisol may influence nervous function (Sørensen et al., 2011, 2012; Johansen et al., 2012). Thus, effects of cortisol on brain neural function warrant consideration as a mechanism through which chronic elevation of cortisol may impair thermal tolerance in fishes. Further study is needed to directly manipulate cortisol levels as well as explore the impacts of stressors beyond social stress that chronically elevate cortisol.

Chronic social stress and cardiac remodelling

Support for the hypothesis that subordinate trout experience cardiac remodelling was limited. Neither ventricle mass nor thickness of the compact myocardium was elevated in subordinate fish, unlike the situation in HR rainbow trout (Johansen et al., 2011) or trout treated with cortisol for 21–45 days (Johansen et al., 2017; Nørstrud et al., 2018). Because cortisol treatment for shorter periods altered the transcript abundance of molecular markers of hypertrophic signalling and remodelling without significantly affecting ventricle mass (Nørstrud et al., 2018), several of these markers were compared between dominant and subordinate trout. Transcript abundances of slow myosin light chain 2 (*smlc2*), ventricular myosin heavy chain (*vmhc*), muscle LIM protein (*mlp*) and regulator of calcineurin 1 (*rcan1*) did not differ significantly between dominant and subordinate trout, although ventricles from subordinate trout tended to show higher transcript abundance of *rcan1*. Transcript abundances of the cortisol receptors *gr1*, *gr2* and *mr* did not differ between the ventricles of dominant and subordinate trout, suggesting that the capacity for cortisol signalling was maintained in the heart of subordinate trout. Thus, it seems likely that a longer exposure to elevated cortisol than the 5-day interaction period of the present study is needed to elicit ventricular hypertrophy.

In the HR rainbow trout studied by Johansen et al. (2011), ventricular hypertrophy was accompanied by an increase in collagen content that resulted in a more fibrotic heart, a situation associated with increased risk of dysfunction. The hearts of cold-acclimated trout are both larger and have greater collagen content than the hearts of warm-acclimated trout, although in this case the cardiac remodelling is not viewed as pathological (Graham and Farrell, 1989; Klaiman et al., 2011; Keen et al., 2016). In contrast to our prediction, histological analysis of ventricle tissue sections revealed a tendency for lower collagen content in the hearts of subordinate relative to dominant trout. However, this difference was not accompanied by differences in collagen type I protein or transcript abundances, nor were differences detected in the transcript abundances of collagen regulators. The apparent fall in collagen abundance in subordinate fish therefore may represent a change in collagen organization from densely packed (which appears red when stained with Picrosirius Red and viewed under polarized light) to thin and/or disorganized (Rich and Whittaker, 2005).

Indices of cardiac function also were assessed in subordinate rainbow trout. In cold-acclimated trout, the higher collagen content of the heart increases stiffness (Keen et al., 2016). Based on ventricular pressure–volume relationships for isolated hearts, subordinate and dominant fish did not differ in passive stiffness of the ventricle, a finding that is consistent with the lack of difference in collagen type I protein abundance. Similarly, neither baseline f_H nor the f_H response to adrenergic stimulation differed between the isolated hearts of dominant and subordinate fish. Somewhat unexpectedly, given previous reports of elevated f_H in subordinate trout (Thomas and Gilmour, 2012), *in vivo* f_H also did not differ between dominant and subordinate trout in the present study. The use of f_H loggers in the present study enabled effects of hierarchy formation on f_H to be evaluated. Previous studies of f_H responses to social interaction were limited to a single fish observing another of known social status, because non-invasive approaches that could not differentiate between individuals were used to measure f_H (Höjesjö et al., 2007, 2015). In the present study, f_H decreased significantly by day 2 in both sham and dominant fish from a high reached on day 1 after removal of the divider in the tank, but f_H was slightly slower to decrease in subordinate fish, perhaps suggesting greater metabolic expenditure in these fish during the early stages of hierarchy establishment.

Although 5 days of social stress had little effect on cardiac structure or function in the present study, it is possible that different results would be obtained if sexually mature fish were examined rather than the juvenile trout used in the present study. For example, Klaiman et al. (2011) reported stronger effects of cold acclimation on heart morphology in male versus female rainbow trout, and attributed the differences to the pro-hypertrophic effects of testosterone versus the anti-hypertrophic effects of estrogen. Thus, the resilience of cardiac structure and function to the effects of cortisol in subordinate trout of the present study may reflect, at least in part, the use of juvenile fish. Longer exposure to cortisol than the 5-day interaction period of the present study appears to be necessary to induce ventricular hypertrophy in juvenile salmonids (Johansen et al., 2011, 2017; Nørstrud et al., 2018).

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Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: K.M.G.; Methodology: B.B., A.D., W.J., M.J.L., S.J.C., K.M.G.; Validation: B.B., A.D., W.J., M.J.L., K.M.G.; Formal analysis: B.B., A.D., W.J., K.M.G.; Investigation: B.B., A.D., W.J.; Resources: S.J.C., K.M.G.; Data curation: B.B., K.M.G.; Writing - original draft: B.B.; Writing - review & editing: B.B., A.D., W.J., M.J.L., S.J.C., K.M.G.; Visualization: B.B., K.M.G.; Supervision: K.M.G.; Project administration: K.M.G.; Funding acquisition: S.J.C., K.M.G.

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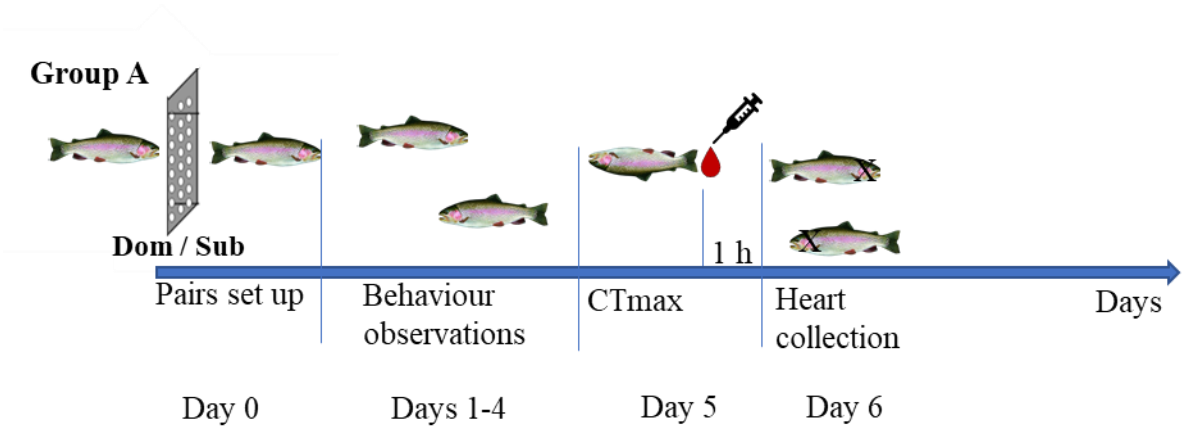

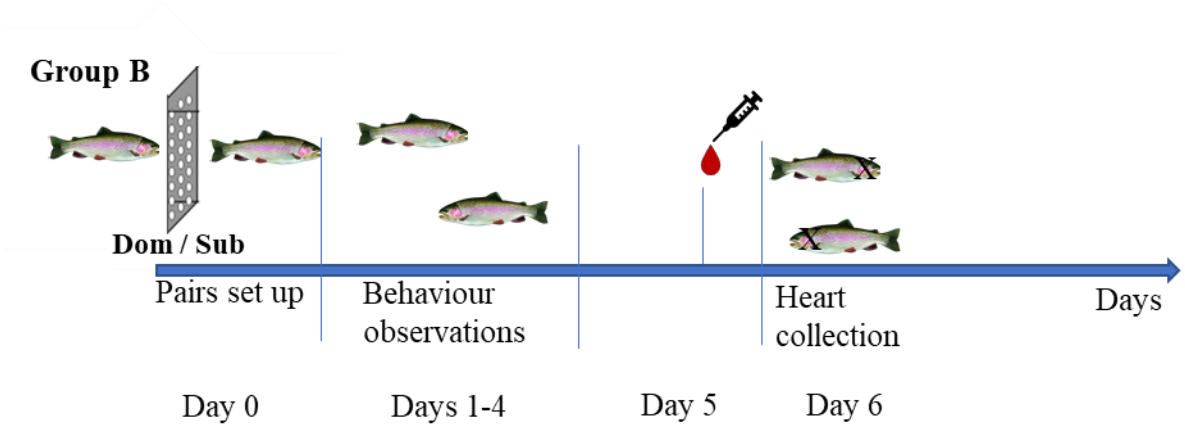

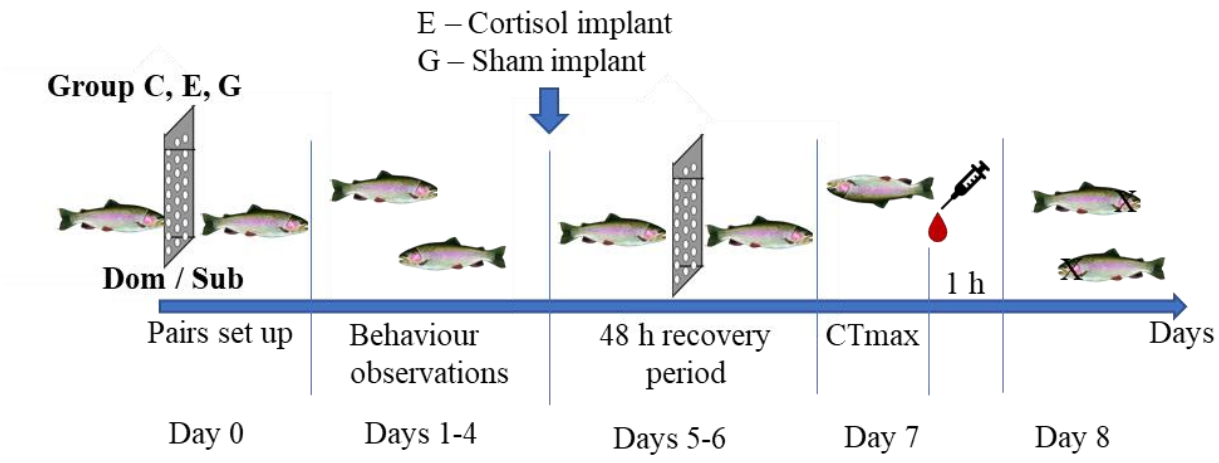

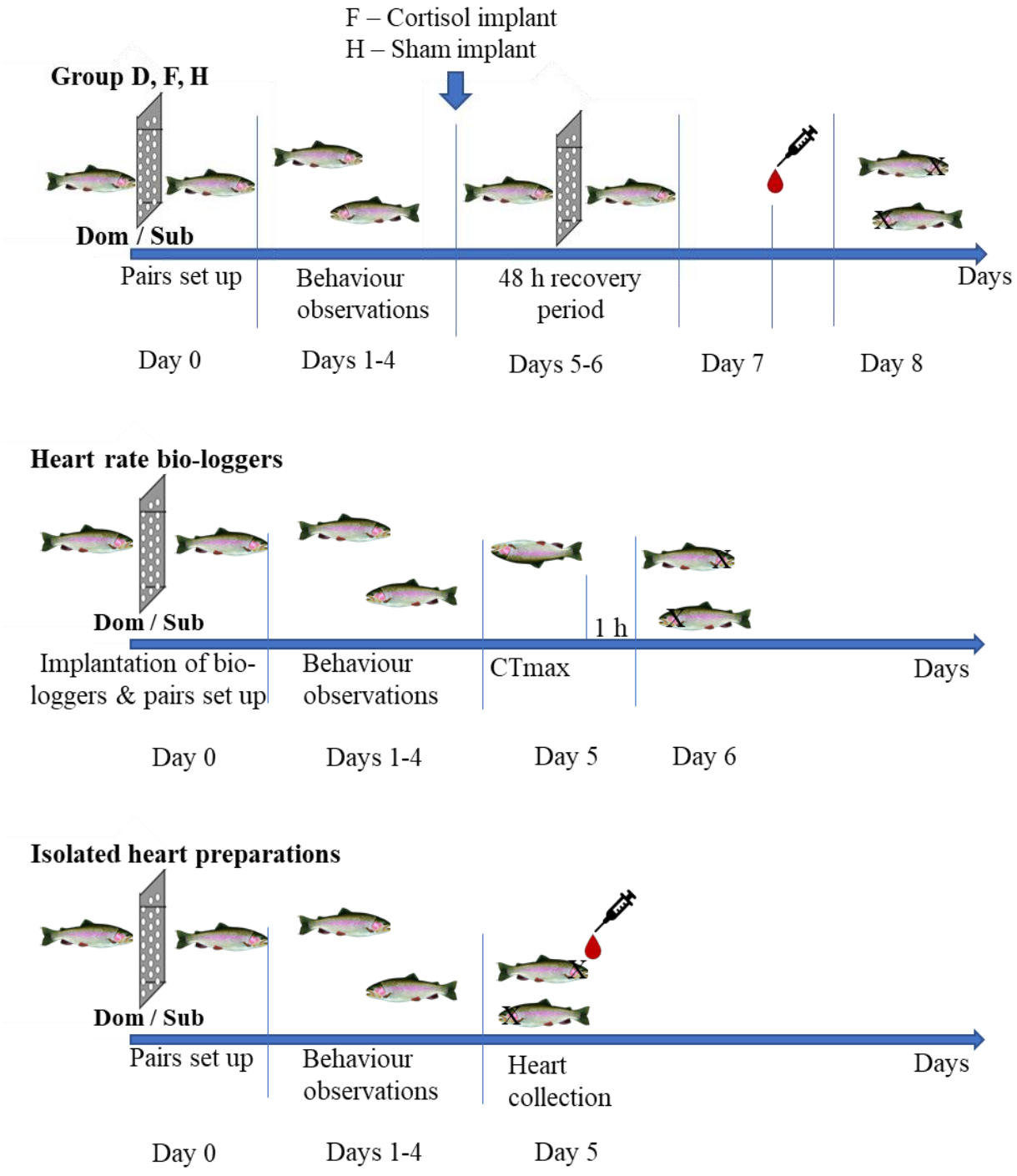

Figure S1. A schematic of experimental protocols (see text for details). Arrowheads indicate when implants were administered for Groups E through H. The syringe and red droplet represent withdrawal of a blood sample. *Dom*, dominant; *Sub*, subordinate

Table S1. Primers used for semi-quantitative real-time RT-PCR.

Gene	Primer pair	GenBank accession number	Function or indicator	Efficiency; Amplicon size	Source
β-actin	F: AGAGCTACGAGCTGCCTGAC R: GTGTTGGCGTACAGGTCCTT	NM_001124235.1	Housekeeping gene	2; 179	Johansen et al. (2011)
vmhc	F: TGCTGATGCAATCAAAGGAA R: GGAACTTGCCCAGATGGTT	AY009126.1	Cardiomyocyte hypertrophy	1.99; 191	Johansen et al. (2011)
smlc2	F: TCTCAGGCGGACAAGTTCA R: TAGCACAGGTTCTTGTAGTCC	NM001124678.1	Cardiomyocyte hypertrophy	2.01; 100	Johansen et al. (2011)
mlp	F: AGTTCGGGGACTCGGATAAG R: CGCCATCTTTCTCTGTCTGG	NM001124725.1 BC076439.1	Cardiomyocyte hypertrophy	1.95; 156	Johansen et al. (2011)
rcan1	F: AGTTTCCGGCGTGTGAGA R: GGGGACTGCCTATGAGGAC	BC076439.1*	Cardiomyocyte hypertrophy	1.92; 136	Johansen et al. (2011)
mr	F: GGCAGCGTTTGAGGAGATGA R: CATGGCGTCCAGTAGCTTGG	AF209873.1	Cortisol sensitivity	2; 127	Johansen et al. (2011)
gr1	F: TTCCAAGTCCACCACATCAA R: GGAGAGCTCCATCTGAGTCG	NM_001124730.1	Cortisol sensitivity	1.88; 115	Johansen et al. (2011)
gr2	F: GGGGTGATCAAACAGGAGAA R: CTCACCCCACAGATGGAGAT	NM_001124482.1	Cortisol sensitivity	1.95; 140	Johansen et al. (2011)
col1a1	F: GCTTTTGGCAAGAGGACAAG	NM001124177.1	Fibrosis	1.97; 154	Keen et al. (2015)

	R: GCAGATAACTTCGTCGCACA				
col1a2	F: GGCTGATCGGCTCTGTACTC R: TGGCTCTGCTGGTATCACTG	NM001124207.1	Fibrosis	1.96; 290	Keen et al. (2015)
col1a3	F: CCCTGCTTTTTATGGTTGGA R: GCAGGGTTCTGGTTTCCATA	NM001124206.1	Fibrosis	2.03; 235	Keen et al. (2015)
mmp2	F: TGTATTGGGCAACATCAGGA R: CCCAGGAGACGATAGTCCAA	NM_198067.1*	Inhibits fibrosis	1.94; 219	Keen et al. (2015)
mmp9	F: GGTCCAGTTTTCGTCATCGT R: AGACATGGGAGCCTCTCTGA	NM001124370.1	Inhibits fibrosis	1.99; 116	Keen et al. (2015)
timp2	F: CAGGCCATCCACCTACTGTT R: TGTTGCTCTCTTGCATACGG	NM_182874.1*	Inhibits MMPs	1.81; 113	Keen et al. (2015)

vmhc, ventricular myosin heavy chain; *smlc2*, slow myosin light chain 2; *mlp*, muscle LIM protein; *rcan1*, regulator of calcineurin I; *mr*, mineralocorticoid receptor; *gr1*, glucocorticoid receptor 1; *gr2*, glucocorticoid receptor 2; *col1a1*, collagen type I alpha 1; *col1a2*, collagen type I alpha 2; *col1a3*, collagen type I alpha 3; *mmp2*, matrix metalloproteinase 2; *mmp9*, matrix metalloproteinases 9; *timp2*, tissue inhibitor of metalloproteinases 2; * indicates where GenBank sequences for zebrafish (*Danio rerio*) were used for primer design. The annealing temperature was 60°C for all primers.