CHRONIC SOCIAL STRESS IMPAIRS THE THERMAL TOLERANCE OF RAINBOW TROUT (*Oncorhynchus mykiss*)

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Abstract

Juvenile rainbow trout (*Oncorhynchus mykiss*) held in pairs form dominance hierarchies, with subordinate individuals experiencing chronic social stress, as evidenced by prolonged elevation of the stress hormone cortisol. Prior work revealed that the thermal tolerance (measured as critical thermal maximum, CTmax) of subordinate fish was reduced, but the cause of this impairment was unknown. Here we tested the hypothesis that reduced thermal tolerance in subordinate trout is caused by prolonged elevation of circulating cortisol levels, affecting cardiac structure and function. In support of this hypothesis, subordinate trout that were allowed to recover from social stress for 48 h, a period sufficient to return cortisol to normal baseline levels, no longer showed a reduced CTmax. Furthermore, treatment of subordinates with cortisol to maintain elevated cortisol levels during the period of recovery from social stress prevented thermal tolerance from recovering. The possibility that prolonged elevation of cortisol levels induces cardiac remodelling in subordinate trout was explored by assessing heart histology and cardiac remodelling markers, and monitoring heart rate (f_H). Picrosirius red staining revealed lower collagen levels in the ventricles of subordinate relative to dominant trout, although this difference was not accompanied by changes in collagen type I transcript abundances or protein levels, or by changes in markers of collagen turnover. Transcript abundances of markers of cardiac remodelling and ventricle mass were not significantly altered by chronic social stress. Heart rate in subordinates during social interactions was comparable to that in dominant fish. However, differences in f_H responses of subordinate versus dominant fish were detected during acute warming. Specifically, peak heart rates tended to be observed at lower temperatures in subordinate fish relative to dominant. Thus, high baseline cortisol levels in subordinate trout

result in lowered thermal tolerance, and chronic social stress has only minor effects on cardiac structure and function.

Résumé

Les truites arc-en-ciel juvéniles (*Oncorhynchus mykiss*) forment des hiérarchies sociales agressive, quand tenue en paires. Les individus subordonnés subissant un stress social chronique, démontré par les élévations prolongées de l'hormone de stress, cortisol. Des travaux précédents ont établi que la tolérance thermique (mesurée comme maximum thermique critique, CTmax) des truites subordonnées était réduite. Ici, nous avons testé l'hypothèse que la tolérance thermique réduite chez les truites subordonnées est causée par l'élévation prolongée de cortisol plasmatique, affectant la structure et la fonction cardiaque. À l'appui de cette hypothèse, les truites subordonnées qui ont récupéré du stress social pendant 48 h, une période suffisante pour ramener le cortisol à des niveaux de base normaux, n'ont plus démontré une CTmax réduite. En outre, le traitement des subordonnés avec le cortisol durant la période de récupération, a empêché la tolérance thermique de récupérer. La possibilité qu'une élévation prolongée des niveaux de cortisol induise le remodelage cardiaque chez les truites subordonnées a été évalué par l'histologie cardiaque, les marqueurs du remodelage cardiaque, et en surveillant la fréquence cardiaque (f_H). Le colorant Picrosirius rouge a démontré des niveaux de collagène inférieurs dans les ventricules des subordonnés par rapport à les truites dominantes, mais cette différence n'ait pas accompagné avec des changements dans les niveaux de transcription ou de protéines du collagène de type I, ni de changements dans les marqueurs du renouvellement du collagène. L'abondance de la transcription des marqueurs du remodelage cardiaque et de la masse ventriculaire n'a pas été significativement modifiée par le stress social chronique. La fréquence cardiaque chez les subordonnés durant l'interaction sociale était comparable à celle des dominants. Cependant, des différences dans les réponses f_H des subordonnés par rapport aux truites dominants ont été détectées pendant le réchauffement. Plus précisément, les fréquences

cardiaques maximales avaient tendance à être observées à des températures plus basses chez les truites subordonnées par rapport aux poissons dominants. Pour conclure, des niveaux élevés de cortisol chez les truites subordonnées entraînent une baisse tolérance thermique, et le stress social chronique n'a que des effets mineurs sur la structure et la fonction cardiaque.

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List of abbreviations

[ ]	Concentration
ANCOVA	Analysis of covariance
ANOVA	Analysis of variance
AV	Atrioventricular
BCA	Bicinchoninic acid
Benzocaine	Ethyl-p-aminobenzoate
BSA	Bovine serum albumin
C	A control sample used for cross-blot normalization
cDNA	Complementary DNA
col1a1	Collagen type I alpha 1
col1a2	Collagen type I alpha 2
col1a3	Collagen type I alpha 3
CTmax	Critical thermal maximum
D	Day
D	Dominant
DO	Dissolved oxygen
Dom	Dominant
dpf	Days post-fertilization
ECG	Electrocardiogram
EDTA	Ethylenediaminetetraacetic acid
f_H	Heart rate
gr1	Glucocorticoid receptor 1

gr2	Glucocorticoid receptor 2
H	Hour
HPI	Hypothalamic-pituitary-interrenal
HR	High responding
HSP	Heat shock protein
ILT	Incipient lethal temperature
min	Minute
mlp	Muscle LIM protein
MMP	Matrix metalloproteinase
mmp2	Matrix metalloproteinase 2
mmp9	Matrix metalloproteinase 9
mmp13	Matrix metalloproteinase 13
mr	Mineralocorticoid receptor
mRNA	Messenger ribonucleic acid
N	Sample size
OCLTT	Oxygen and capacity limited thermal tolerance theory
P	P-value
PCR	Polymerase chain reaction
QI	Quality index
q-PCR	Quantitative polymerase chain reaction
rcan1	regulator of calcineurin I
RIA	Radioimmunoassay
RM	Repeated measures

S	Subordinate
SEM	Standard error of the mean
smlc2	Slow myosin light chain 2
SMR	Standard metabolic rate
Sub	Subordinate
T	Temperature
timp2	Tissue inhibitor of metalloproteinases 2
vmhc	Ventricular myosin heavy chain
TRP	Transient receptor potential
TRPV	Transient receptor potential vanilloid
VTmax	Voluntary thermal maximum
Δ	Delta

Chapter 1: General Introduction

Juvenile rainbow trout (*Oncorhynchus mykiss*) form dominance hierarchies when held in pairs, with subordinate individuals experiencing prolonged elevation of the stress hormone cortisol due to chronic social stress. The physiological consequences of social hierarchy formation and social status in fish have been reviewed several times (see reviews Backström and Winberg, 2013; Backström and Winberg, 2017; Gilmour et al., 2005; Johnsson et al., 2005; Sørensen et al., 2013; Winberg et al., 2016). Among the many physiological consequences of chronic social stress was the observation that thermal tolerance (based on the critical thermal maximum, CTmax) of subordinates is reduced, but what causes this impairment remains unknown (LeBlanc et al., 2011). The present thesis aimed to investigate the physiological factors contributing to lower thermal tolerance in subordinate rainbow trout. Specifically, we tested the hypothesis that reduced thermal tolerance in subordinate trout reflects prolonged elevation of circulating cortisol levels, which in turn alter cardiac structure and function. We predicted that the reestablishment of cortisol levels after a recovery period from social stress would lead to recovery of thermal tolerance in subordinates. To investigate potential mechanisms linking thermal tolerance to cortisol levels, cardiac morphology and function were investigated in socially stressed trout. The general introduction below provides context for the experimental chapters by briefly reviewing current research focused on social stress, thermal tolerance, and cardiac remodelling.

1.1 Social hierarchies and chronic social stress in rainbow trout

Social hierarchies typically arise owing to competition for limited resources in an environment, and have been observed among a wide variety of taxa, including vertebrates and

invertebrates (Backström and Winberg, 2013). Different types of social structures have been described (Oliveira and Gonçalves, 2008) with the present study being focused on linear or 'pecking order' social hierarchies, which have been particularly well studied in salmonid fish (Culbert and Gilmour, 2016; DiBattista et al., 2006; Gilmour et al., 2005; Gilmour et al., 2012; Jeffrey et al., 2014; LeBlanc et al., 2012; Lepage et al., 2005; Øverli et al., 1999a; Øverli et al., 2002; Pickering and Pottinger, 1989; Sloman et al., 2000; Sloman et al., 2001). Social stress arises in salmonids when a stress response is initiated as a result of intraspecific antagonistic behaviour (Øverli et al., 1999a; Sloman et al., 2008). Social stress frequently is studied by confining conspecific individuals in pairs or small groups (Øverli et al., 1999a), where the individuals that are confined together engage in chasing and nipping/biting until one fish retreats, thereby becoming the subordinate fish with the aggressor becoming the dominant. Similar aggressive behaviours by the dominant individual often continue throughout the interaction period (Øverli et al., 1999a). In the natural environment, these social interactions allow individuals to hold feeding territories, with dominant fish defending better territories and subordinate fish consigned to less desirable territories and/or forced to emigrate (Metcalfe et al., 1989). Similarly, juvenile salmonid fish will promptly form dominance hierarchies when placed in pairs or small groups in laboratory conditions (Adams et al., 1998; Noakes and Leatherland, 1977). The social hierarchies that are formed in a laboratory setting are thought to mimic the behaviour of a natural population, providing real-world applicability (Bachman, 1984). However, it has been argued that the social hierarchies formed in a laboratory setting are likely exaggerated in comparison to those that occur in the natural environment (Sloman et al., 2008), owing at least in part to the tendency to use pairs of conspecifics rather than groups (Barreto et al., 2015) and because the subordinate fish is unable to emigrate away from its dominant conspecific.

Although limiting the applicability of the results to natural environments, such effects facilitate the detection of physiological responses to social stress.

Among juvenile salmonids held in pairs, behaviours typical of dominant individuals include patrolling the water column, monopolizing food, and aggressive behaviours such as chasing and biting (Gilmour et al., 2005). By contrast, subordinate individuals can be identified by pronounced behavioural inhibition, including reduced activity, hiding in refuges, and a reduction in or cessation of feeding (Currie et al., 2010; Gilmour et al., 2005). Even when separated from the dominant fish for feeding, many subordinates will continue to not take food (DiBattista et al., 2006). Through these behavioral differences, social rank can be determined in an experimental setting (Culbert and Gilmour, 2016; Höglund et al., 2002; LeBlanc et al., 2011; Metcalfe et al., 1989; Øverli et al., 2004; Sloman et al., 2000; Sloman et al., 2001; Thomas and Gilmour, 2012). In addition to these behavioural differences, dominant and subordinate individuals also differ physiologically. Most importantly, subordinate fish experience chronic social stress as demonstrated by the prolonged elevation of circulating cortisol levels (Culbert and Gilmour, 2016; Sloman et al., 2001, see reviews by Gilmour et al., 2005; Johnsson et al., 2005; Sørensen et al., 2013; Winberg et al., 2016) . Cortisol is the main glucocorticoid stress hormone in teleost fish (Gorissen and Flik, 2016; Mommsen et al., 1999; Wendelarr Bonga, 1997). Acute increases in cortisol during a stressor contribute to regulation of metabolism and the release of energy reserves, ultimately benefitting the fish (Schreck and Tort, 2016; Schreibman et al., 1993). In contrast, chronic elevation of cortisol has deleterious effects, such as immunosuppression, reduced growth, and increased standard metabolic rate (Chan and Woo, 1978; Gregory and Wood, 1999; Pickering and Pottinger, 1989; Schreck and Tort, 2016).

Cortisol levels rise during hierarchy establishment in both fish that become dominant and in those that become subordinate, owing to the acute stress associated with this event (Øverli et al., 1999b). However, only subordinates experience chronic elevation of cortisol levels (Sloman et al., 2001), likely caused by on-going antagonistic interactions with the dominant fish (Sloman and Armstrong, 2002). The factors that contribute to this differential regulation of the stress axis have not yet been fully described (Jeffrey et al., 2012). In particular, it is not clear how prolonged elevation of circulating cortisol levels occurs given the availability of mechanisms that reduce cortisol levels, such as negative feedback regulation of the stress axis and cortisol metabolism and clearance (Mommsen et al 1999). A consequence of this chronic activation of the stress axis is attenuation of the acute cortisol response in subordinates when exposed to a subsequent stressor (Jeffrey et al., 2014; Øverli et al., 1999b). Several other physiological consequences of subordinate social status have been identified, and are generally thought to reflect, at least in part, the impact of prolonged elevation of cortisol. For example, subordinate fish exhibit reduced growth and changes in intermediary metabolism that favour reliance on energy reserves such as lipids and glycogen (DiBattista et al., 2006; Gilmour et al., 2012; Kostyniuk et al., 2018). Subordinate fish also exhibit increases in metabolic rate, which incurs a metabolic disadvantage (Sloman et al., 2000). Moreover, the ability to tolerate hypoxia is reduced, as subordinates fail to maintain arterial blood oxygen content under severely hypoxic conditions (Thomas and Gilmour, 2012). Subordinate trout appear to able to regulate blood pH when exposed to hypercapnia (Mussa and Gilmour, 2012), but exhibit higher uptake of heavy metals in the environment (Sloman et al., 2003). Importantly for the present study, subordinate rainbow trout have lower thermal tolerance than dominant fish (LeBlanc et al., 2011).

1.2 Thermal tolerance in fishes

Rising temperature can become a stressor for fishes owing to their ectothermic nature, in that temperature controls metabolism, affects locomotory activity and thus distribution of individuals, and ultimately can become a threat to survival (Beitinger et al., 2000; Tattersall et al., 2012). Populations are primarily constrained by warming by their ability to disperse, i.e. shift in distribution to find more suitable temperatures (Deutsch et al., 2008; Ficke et al., 2007). Alternatively, given rates of warming that are sufficiently slow, fish populations can also demonstrate the ability to acclimate to higher temperatures (Deutsch et al., 2008; Ficke et al., 2007; Komoroske et al., 2014; Lapointe et al., 2018; Tattersall et al., 2012; Yu et al., 2018). Acute warming induces an immediate stress response in an individual, in an attempt to immediately combat the disruption of physiological processes by elevated temperature (Schreck and Tort, 2016; Tattersall et al., 2012). For example, both catecholamine (Currie et al., 2013) and cortisol (Chadwick and McCormick, 2017; Chadwick et al., 2015; Mesa et al., 2002; Pérez-Casanova et al., 2008) levels rise when fish are exposed to acute warming, suggesting that the cortisol response plays a role in allowing fish to deal with thermal stress. Whether elevated cortisol levels (e.g. owing to social stress) prior to exposure to thermal stress influences the cortisol response to a thermal stressor has received little attention. Heat shock proteins (HSPs) can be induced in response to a multitude of stressors, including thermal stress (Barton, 2002; Chadwick and McCormick, 2017; Iwama et al., 1998; Iwama et al., 2004). HSPs constitute a family of proteins that are highly conserved over many species and can be found in two forms; constitutive and inducible (Iwama et al., 1998). Constitutive HSP expression maintains normal cellular functions such as protein folding and translocation (Iwama et al., 1998). Inducible HSPs are integral in protecting the individual from thermal stress at the cellular level because HSPs

function as chaperones, protecting the cell from abnormal protein aggregation by interacting with heat-damaged proteins (Iwama et al., 1998; Krebs and Feder, 1997).

The thermal tolerance of fishes tends to be tested in one of two ways, by measuring the incipient lethal temperature (ILT) or the critical thermal maximum (CTmax) (Becker and Genoway, 1979; Lutterschmidt and Hutchison, 1997). An ILT is determined by subjecting groups of fish to static test temperatures near the estimated upper thermal limit over a variety of acclimation temperatures (Beitinger et al., 2000). The temperature at which half of the group dies is considered the ILT. For the purposes of the present thesis, CTmax was the preferred measure of thermal tolerance because it is determined in a non-lethal fashion. In a CTmax test, individual animals are subjected to a constant linear increase in temperature until loss of equilibrium occurs (in fishes, this is typically indicated by the animal turning dorso-ventrally) (Becker and Genoway, 1979). Ultimately, the CTmax indicates the temperature at which the animal can no longer escape predation or find thermal refugia, and therefore is considered more ecologically relevant than the ILT (Vinagre et al., 2015). Prolonged exposure to CTmax or temperatures just 1-2°C higher will often cause death but survival is possible if the fish is rapidly returned to cooler water (Beitinger et al., 2000).

Although CTmax has been measured in many different fishes, the underlying physiology that results in the loss of equilibrium during acute warming remains unclear and indeed, the physiological factors that determine thermal tolerance remain heavily debated (Jutfelt et al., 2018; Motyka et al., 2017; Norin et al., 2014; Pörtner et al., 2017). It should also be noted that while CTmax is an index of thermal tolerance, the endpoint of CTmax (i.e. loss of equilibrium) is likely to be caused by disruption of neurological function and may not be reflective of the underlying cause of true thermal tolerance (Jutfelt et al., 2019). The oxygen and capacity limited

thermal tolerance theory (OCLTT) suggests that constraints on oxygen delivery to tissues at increasing temperatures limit thermal tolerance (Pörtner et al., 2017). This hypothesis posits that with increasing temperatures, the aerobic scope decreases to an unsustainable breadth, causing a mismatch between oxygen demand and oxygen delivery owing to compromised cardiorespiratory function. Support for the OCLTT has come from studies that reported a relationship between hematocrit and CTmax (Beers and Sidell, 2011; Muñoz et al., 2018), or demonstrated a lowering of CTmax under hypoxic conditions (Healy and Schulte, 2012; Zanuzzo et al., 2019). Additionally, hypoxia acclimation increases CTmax, which is thought to occur because acclimation to hypoxic conditions increases oxygen extraction efficiency (Burleson and Silva, 2011). Recent studies have also explored relationships between cardiovascular function and thermal tolerance, reporting that impaired cardiovascular function translates into the lowering of the upper thermal tolerance. It has long been established that heart rate increases in response to acute thermal stress (Ekström et al., 2014; Eliason and Anttila, 2017; Farrell, 2002; Farrell et al., 1996), which occurs due to increases in cardiac output and not stroke volume (Ekström et al., 2014; Eliason and Anttila, 2017). It is also common for cardiac arrhythmia to occur at higher temperatures (Clark et al., 2008; Gollock et al., 2006; Verhille et al., 2013). Although some species are able to maintain increases in heart rate until the loss of equilibrium (Clark et al., 2008), more commonly a plateau and/or decrease in heart rate is observed before CTmax is reached (Ekström et al., 2016; Ekström et al., 2019; Gollock et al., 2006; Mendonça and Gamperl, 2010; Penney et al., 2014). Recently, several studies have explored how impairing cardiac function results in changes in CTmax. For example, coronary-ligated trout demonstrated an elevated heart rate during acute warming and reduced thermal tolerance (Ekström et al., 2017). In a follow up study, these authors reported that the heart rate of

coronary artery-ligated trout peaked at a lower temperature than that in control animals, and these animals also exhibited lower thermal tolerance (Ekström et al., 2019).

At the same time, numerous studies oppose the OCLTT (Gräns et al., 2014; Lefevre et al., 2016; Mcdonnell et al., 2019; Motyka et al., 2017; Norin et al., 2014; Nyboer and Chapman, 2018; Wang et al., 2014), ultimately offering no consensus on the factors that determine thermal tolerance. For example, steelhead trout (*Oncorhynchus mykiss*) that were chronically acclimated to hypoxia showed no differences in CTmax despite reduced cardiac output, and were able to maintain oxygen delivery (Motyka et al., 2017). Additionally many studies have demonstrated that aerobic scope increases at higher temperatures, indicating that the capacity for oxygen delivery is not compromised, at least during acute thermal stress (Gräns et al., 2014; Norin et al., 2014; Nyboer and Chapman, 2018); these observations refute a key assumption of the OCLTT. An alternative hypothesis suggests that heat-induced impairment of neurological function determines thermal tolerance. It has long been thought that the endpoint of CTmax (i.e. loss of locomotory function) is a neurological endpoint, but until recently there has been little research to support this hypothesis. Jutfelt et al. (2019) reported that cooling the brain of Atlantic cod (*Gadhus morhua*) by 2-6°C below ambient water temperature increased CTmax by 0.5-0.6°C, supporting the role of neurological function in determining CTmax but at the same time suggesting that other neurological pathways, such as peripheral neurons and the spinal cord, also contribute to thermal tolerance. A second line of evidence supporting a neurological contribution to thermal tolerance has come from studies of neuronal membrane traits. In Antarctic notothenioid, the synaptic membranes of a more stenothermal species, *Chaenocephalus aceratus*, were found to be more fluid and their myelin contained higher cholesterol levels when compared to those of *Notothenia coriiceps*, a more thermotolerant species (Biederman et al., 2019). The

authors suggested that thermal tolerance in *C. aceratus* may be constrained by the extent to which synaptic membranes become fluid with warming.

As the debate over the factors that determine thermal tolerance continues, other aspects of CTmax have been investigated among fishes, including effects of photoperiod (Bulger, 1984; Healy and Schulte, 2012), ontogeny (Komoroske et al., 2014), and body size (Moyano et al., 2017; Zhang and Kieffer, 2014). Most notably, several studies have identified a positive relationship between thermal tolerance and acclimation temperature (Beitinger and Bennett, 2000; Beitinger et al., 2000; Healy and Schulte, 2012; Norin et al., 2019; Nyboer and Chapman, 2018; Zhang and Kieffer, 2014), that typically plateaus at higher acclimation temperatures (Lutterschmidt and Hutchison, 1997). Additionally, social stress appears to a modulator of CTmax as demonstrated by Leblanc et al. (2011).

1.3 Impacts of social stress on thermal tolerance

Recent work has identified the social environment as an important determinant of responses to temperature. For example, aggressive mangrove rivulus (*Kryptolebias marmoratus*) would remain in warming water longer when social cues were mimicked through the presence of a mirror, although CTmax remained unchanged (Currie and Tattersall, 2018). Additionally, individual three-spined stickleback (*Gasterosteus aculeatus*) would deviate from their preferred temperature to interact with a conspecific shoal (Cooper et al., 2018). The extent to which individual fish would stray from their preferred temperature depended on both sociability of the individual and whether the shoal was situated in an environment that was cooler or warmer than the preferred temperature of the individual (Cooper et al., 2018). A similar trade off was reported

in the Lake Tanganyikan cichlid *Neolamprologus pulcher*, a species that lives in permanent social groups consisting of a breeding pair and helpers of both sexes, where individual fish would tolerate lower oxygen levels to interact with a conspecific (Borowiec et al., 2018). Although the context of the social interaction reflects species differences (i.e. aggressive vs. social), all three of these studies demonstrated that fishes may chose less favourable environments to prioritize interactions with conspecifics. The social context may also influence stress responses in fish, and particularly the cortisol response to a stressor. It has been suggested that 'social buffering' can occur in fishes, which is the idea that the presence of conspecifics can attenuate the stress response within an individual, but it has been primarily examined in mammals (Hennessy et al., 2009; Hostinar et al., 2014; Kikusui et al., 2006). For example, the presence of conspecifics decreased the duration, without changing the magnitude, of the cortisol response to a stressor in lake sturgeon (*Acipenser fulvescens*) (Allen et al., 2009). Differences in secondary stress indictors, such as plasma glucose and ion concentrations were also detected (Allen et al., 2009). Similarly, in the group-living cichlid *Neolamprologus pulcher*, individuals exposed to an air exposure stressor exhibited lower peak cortisol levels when placed back into their group post-stressor compared to when held alone (Culbert et al., 2019). Social buffering was also demonstrated in zebrafish (*Danio rerio*), where the fear response (identified by freeze behaviours) was reduced when olfactory and visual cues of a conspecific shoal were present (Faustino et al., 2017). Of most direct relevance to the current study, lake sturgeon held in groups exhibited a reduced cortisol response to thermal stress, although CTmax was unaffected by the social context (Yusishen et al., 2020). Collectively, these studies highlight the potential for social context to be a factor modulating the tolerance of an individual fish to an environmental stressor like acute warming.

LeBlanc et al. (2011) demonstrated that the CTmax of subordinate rainbow trout was lower than that of dominant fish. However, the underlying cause of the observed difference in thermal tolerance between dominant and subordinate fish was not identified. Based on the importance of HSP responses in acute warming, and the role of cortisol in the responses to acute stress more broadly, it seems possible that social stress-induced changes in HSP and/or cortisol responses may contribute to the lowering of thermal tolerance in subordinate fish. Notably, HSP70 and HSP90 act as chaperones for and facilitate folding of the hormone-binding domain of the glucocorticoid receptor (GR), providing a mechanism for interactions between cortisol and HSPs (Basu et al., 2003). Indeed, in cortisol-treated rainbow trout and tilapia (*Oreochromis mossambicus*), levels of HSPs were suppressed during exposure to thermal stress (Basu et al., 2001; Basu et al., 2003). If the HSP response to thermal stress is impaired, then whole animal thermal tolerance (i.e. CTmax) also could be diminished during exposure to thermal stress. Cortisol-induced impairment of the HSP response therefore could contribute to the reduced CTmax of subordinate trout (LeBlanc et al. 2011). It is also important to note that Currie et al. (2010) reported differential induction of HSPs within certain tissues (liver, brain) in a time-specific manner in response to the stress of social interaction. Leblanc et al. (2011) observed similar HSP responses in dominant and subordinate fish in response to heat shock, suggesting that differential HSP responses did not contribute to the difference in CTmax, but these authors did not examine the HSP response of the heart, which may be important in thermal tolerance (see above). Thus, the possibility that differential cardiac HSP responses contribute to reduced thermal tolerance in subordinate fish was examined in the present study. More generally, investigation is warranted of the role of elevated cortisol in the reduced thermal tolerance of subordinate trout.

1.4 Impacts of social stress on the heart: a role in thermal tolerance?

Elevated cortisol levels have been implicated in eliciting cardiac remodelling in rainbow trout, resulting in a more fibrotic heart and decreased cardiac performance. Johansen et al. (2017) fed trout cortisol-coated food pellets for 45 d, which resulted in sustained circulating cortisol levels of ~20-30 ng ml^{-1}. In response to this prolonged elevation of cortisol, the ventricle of treated trout increased in mass and thickness of the compact myocardium, changes that were consistent with ventricular hypertrophy (Johansen et al., 2017). Accompanying these morphological changes was increased transcript abundance of several molecular markers of cardiac hypertrophy, as well as impaired cardiac function. In particular, resting heart rate was elevated and maximum cardiac output was reduced, in both cases lowering cardiac scope. In turn, these changes were associated with a reduction in aerobic swimming capacity as measured by U_{crit}, the critical swimming speed (Johansen et al., 2017). Subordinate rainbow trout typically experience cortisol levels of 120-400 ng ml^{-1}. These plasma cortisol values are higher than those used by Johansen et al. (2017), but are experienced for a shorter period, with pairs interacting for 4 d. However, indices of cardiac remodelling with dietary administration of cortisol were detected as early at 7 d (Nørstrud et al., 2018), suggesting that social stress-induced cardiac remodelling is a possibility. If elevated cortisol levels in subordinate rainbow trout cause remodelling of the heart, impairing cardiac function, an impact on thermal tolerance would be anticipated because blunted cardiac responses have been shown to result in decreases in CTmax (Ekström et al., 2017; Ekström et al., 2019; Gilbert et al., 2019).

1.5 Hypothesis and predictions

The present thesis aims to the test the hypothesis that chronically high cortisol is responsible for the lower thermal tolerance in subordinates, by inducing cardiac remodelling, ultimately hindering cardiac function. It is predicted that subordinates that have undergone a recovery period will demonstrate a normal thermal tolerance, which will reflect the reestablishment of cortisol. Additionally, we predict that subordinates will demonstrate differences in cardiac function, which will be tested by monitoring heart rate, during both social interaction and acute warming. Lastly, we expect to see differences in morphology and mass of the heart after 4 days of social interaction.

Chapter 2: Does cortisol play a role in determining the thermal tolerance of subordinate rainbow trout?

2.1 Introduction

Juvenile rainbow trout held in pairs or small groups form social hierarchies, in which socially subordinate fish experience chronic social stress as evidenced by sustained elevation of baseline cortisol levels (Culbert and Gilmour, 2016; Gilmour et al., 2005; Jeffrey et al., 2014). Among other consequences of chronic social stress (see Gilmour et al., 2005; Johnsson et al., 2005; Sørensen et al., 2013 for review), subordinate rainbow trout exhibit reduced tolerance to environmental stressors, such as hypoxia (Thomas and Gilmour, 2012), hypercapnia (Mussa and Gilmour, 2012), and thermal stress (LeBlanc et al., 2011). Leblanc et al. (2011) assessed thermal tolerance using the critical thermal maximum, CTmax, which is the upper temperature at which a fish loses equilibrium, i.e. loses the ability to escape conditions that will ultimately lead to its death (Becker and Genoway, 1979; Beitinger et al., 2000; Rajaguru, 2002). Subordinate rainbow trout had a lower CTmax than their dominant counterparts (LeBlanc et al. 2011). Because HSPs are elevated during social interactions (Currie et al. 2010) as well as thermal stress (Blair and Glover, 2019; Fowler et al., 2009; Li et al., 2019; Liu et al.), the potential role of HSPs in mediating the effects of chronic social stress on thermal tolerance was investigated. However, differences in HSPs between dominant and subordinate trout were limited and did not offer a clear mechanism to explain the lowered thermal tolerance of subordinate fish (LeBlanc et al. 2011). An alternative possibility is that chronic elevation of cortisol levels lowers thermal tolerance in subordinate rainbow trout.

Cortisol is the primary glucocorticoid stress hormone in teleost fish (Mommsen et al., 1999; Schreck and Tort, 2016). Whereas the cortisol response to an acute stressor may be advantageous to the organism's immune capacity and energy mobilization (Mommsen et al., 1999; Schreck and Tort, 2016), chronic cortisol elevation is detrimental, resulting in negative impacts such as

reduced growth and increased susceptibility to pathogens (Pickering and Duston, 1983; Pickering and Pottinger, 1989; Schreck and Tort, 2016). However, how persistently elevated cortisol levels could be affecting the ability to handle acute heat stress is not known. It is well documented that cortisol increases in response to thermal stress in fishes (Delaney et al., 2008; Jaxion-Harm and Ladich, 2014; Pérez-Casanova et al., 2008; Ryan, 1995; Yang et al., 2018; Yusishen et al., 2020). In Senegalese sole (*Solea senegalensis*), chronic thermal stress induced increases in cortisol in both short (1 h) and long term (7 d) trials, and transcript abundances of glucocorticoid receptors and HSPs also increased (Benítez-Dorta et al., 2017). Similarly, increases in cortisol in response to chronic thermal stress were demonstrated in juvenile milkfish (*Chanos chanos*) (Hanke et al., 2019). Changes in the expression of stress-axis related genes such as *crfbp*, *pomc a* and *pomc b*, also were detected in sole, suggesting that negative feedback processes were activated at the level of the stress axis (Benítez-Dorta et al., 2017). Both adrenocorticotropic hormone (ACTH), the hormone that stimulates cortisol production, and cortisol itself increased for up to two days after thermal stress in Wuchang bream (*Megalobrama amblycephala*) (Liu et al., 2016). These observations indicate that elevated temperature stimulates the production of cortisol to cope with thermal stress (Benítez-Dorta et al., 2017; Hanke et al., 2019). Previously, the cortisol response of subordinate rainbow trout to a subsequent netting stressor was reported to be attenuated compared to that of dominant fish (Jeffrey et al., 2014). A similar attenuation of the cortisol response to a handling stressor was recorded in subordinate Arctic charr (*Salvelinus alpinus L.*) (Øverli et al., 1999b). If subordinate trout experience a similar attenuation of the cortisol response when subjected to thermal stress, this factor could potentially contribute to the lowered thermal tolerance of subordinates. Leblanc et al. (2011) found that cortisol values did not differ between subordinate and dominant fish during a heat shock treatment, which suggests that the

cortisol response in subordinates was suppressed because their baseline cortisol values are significantly higher than those of dominant fish.

Given this framework, the objective of the present study was to test the hypothesis that chronic elevation of cortisol in subordinate rainbow trout lowers thermal tolerance. This hypothesis generates the prediction that thermal tolerance will return to normal if cortisol levels in subordinate trout are reduced. Recovery from social stress was used to lower cortisol levels in subordinate fish (Culbert and Gilmour 2016). When subordinate trout are separated from their dominant tank-mate by an opaque perforated divider that prevents physical interaction, their circulating cortisol levels fall to normal baseline levels within 48 h, although aspects of their behaviour (e.g. feeding) remain typical of subordinate status (Culbert and Gilmour 2016). If elevated cortisol lowers thermal tolerance, then CTmax in 'recovered' subordinates should be restored to values typical of dominant fish. Further, CTmax in 'recovered' subordinates treated with exogenous cortisol to maintain elevated cortisol levels during the recovery period should remain lower than that of dominant fish.

2.2 Materials and methods

2.2.1 Experimental animals

Juvenile rainbow trout, *Oncorhynchus mykiss* (mass = 97.2 ± 2.2 g, fork length = 20.5 ± 0.2 cm, mean ± SEM, N = 158), were purchased from Linwood Acres Trout Farm (Campbellcroft, Ontario). Fish were held at the University of Ottawa in 1275 L fibreglass tanks supplied with flowing, aerated, dechloraminated city of Ottawa tap water at a temperature of 13°C. A 12L:12D 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, homogenous 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) and were in compliance with the guidelines of the Canadian Council on Animal Care (CCAC) for the use of animals in research and teaching.

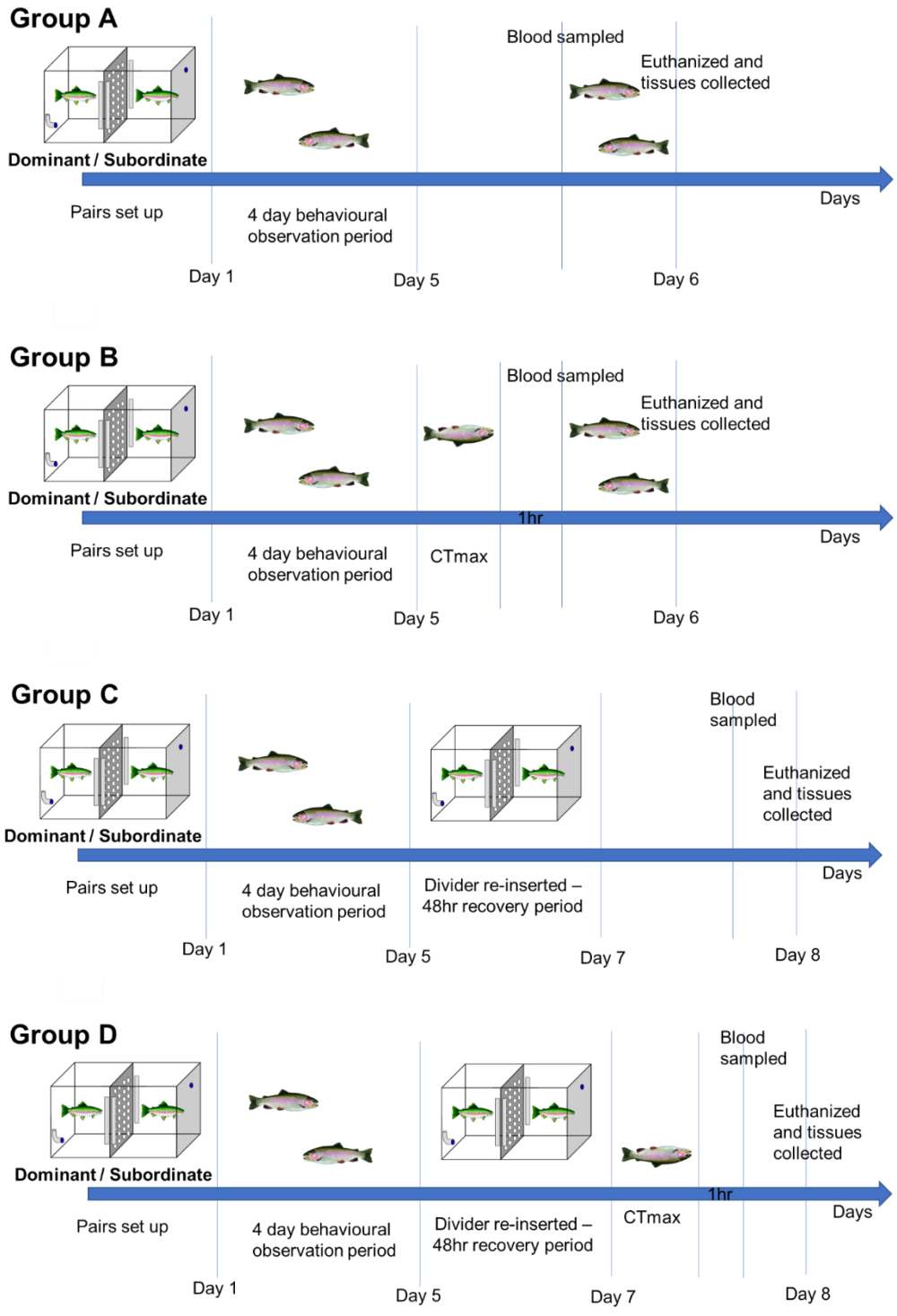
2.2.2 Experimental protocol

Rainbow trout were allocated in pairs based on similar length (Δ_{Length} = 0.26 ± 0.03 cm, N = 59 pairs) and mass (Δ_{Mass} = 5.19 ± 0.3 g, N = 59 pairs) according to established protocols (Culbert and Gilmour, 2016; Jeffrey et al., 2014; LeBlanc et al., 2011; Mussa and Gilmour, 2012; Thomas and Gilmour, 2012). 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, CA) for the measurement of fork length, mass, and fin damage (as per Moutou et al., 1998). The members of a pair were placed into 40 L flow-through plexiglass tanks separated by an opaque perforated divider for an overnight recovery period. Tanks were supplied with flowing, aerated, dechorlaminated 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 d. Behaviour observations were carried out twice daily for 5 min each time, noting position in the tank, acts of aggression such as charges, chases and nips, and willingness to take a single pellet of food. Points were awarded for each behaviour, with higher scores for more dominant behaviours (Culbert and Gilmour, 2016; LeBlanc et al., 2011; Øverli et al., 1999b; Sloman et al., 2000). At the end of the 4-d 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 experiments (a total of 10 pairs). 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 were fed beginning on the second day of observation with a ration of 0.5% body mass.

Pairs were then allocated to one of eight treatment groups (Fig. 2.1). In group A (N = 8 pairs), fish were sampled for blood on day 5, and euthanized on day 6 for collection of tissue samples (see below). Fish of group B (N = 6 pairs) were subjected to a CTmax trial at ~9 am on day 5 (see below). Following the CTmax trial, a blood sample was collected 1 h after return of water temperature to the acclimation temperature, and fish were euthanized on day 6 for collection of tissue samples. Blood was sampled 1 h after the thermal stress to obtain peak plasma cortisol concentrations, and tissues were collected 24 h after thermal stress to obtain peak HSP70 concentrations (LeBlanc et al., 2012). In group C (N = 7 pairs), the members of a pair were separated after the 4-d interaction period by re-insertion of the opaque divider, and were allowed to recover from social interactions for 2 d. A blood sample was collected at the end of the recovery period and fish were euthanized the following day for the collection of tissue samples. The protocol for group D (N = 6 pairs) matched that of group C, except that at the end of the 2 d recovery period, pairs were subjected to a CTmax trial on day 7. A blood sample was collected 1 h after return of water temperature to the acclimation temperature, and fish were euthanized on day 8 for collection of tissue samples. In group E (N= 6 pairs), the protocol used for group C was followed, except that subordinates were given cocoa butter implants impregnated with cortisol (see below) immediately prior to the 2 d recovery period. Similarly,

group F (*N*= 6 pairs) followed the protocol for group D with the additional of cortisol-containing cocoa butter implants. The protocol for group G (*N* = 6 pairs), followed that of group E, but subordinates were given an implant of cocoa butter alone. Group H (*N* = 6 pairs) followed the protocol for group F but used an implant of cocoa butter alone.


Group A
Blood sampled
Euthanized and tissues collected
Dominant / Subordinate
Days
Pairs set up
4 day behavioural observation period
Day 1
Day 5
Day 6
Group B
Blood sampled
Euthanized and tissues collected
Dominant / Subordinate
1hr
Days
Pairs set up
4 day behavioural observation period
CTmax
Day 1
Day 5
Day 6
Group C
Blood sampled
Euthanized and tissues collected
Dominant / Subordinate
Days
Pairs set up
4 day behavioural observation period
Divider re-inserted – 48hr recovery period
Day 1
Day 5
Day 7
Day 8
Group D
Blood sampled
Euthanized and tissues collected
Dominant / Subordinate
1hr
Days
Pairs set up
4 day behavioural observation period
Divider re-inserted – 48hr recovery period
CTmax
Day 1
Day 5
Day 7
Day 8

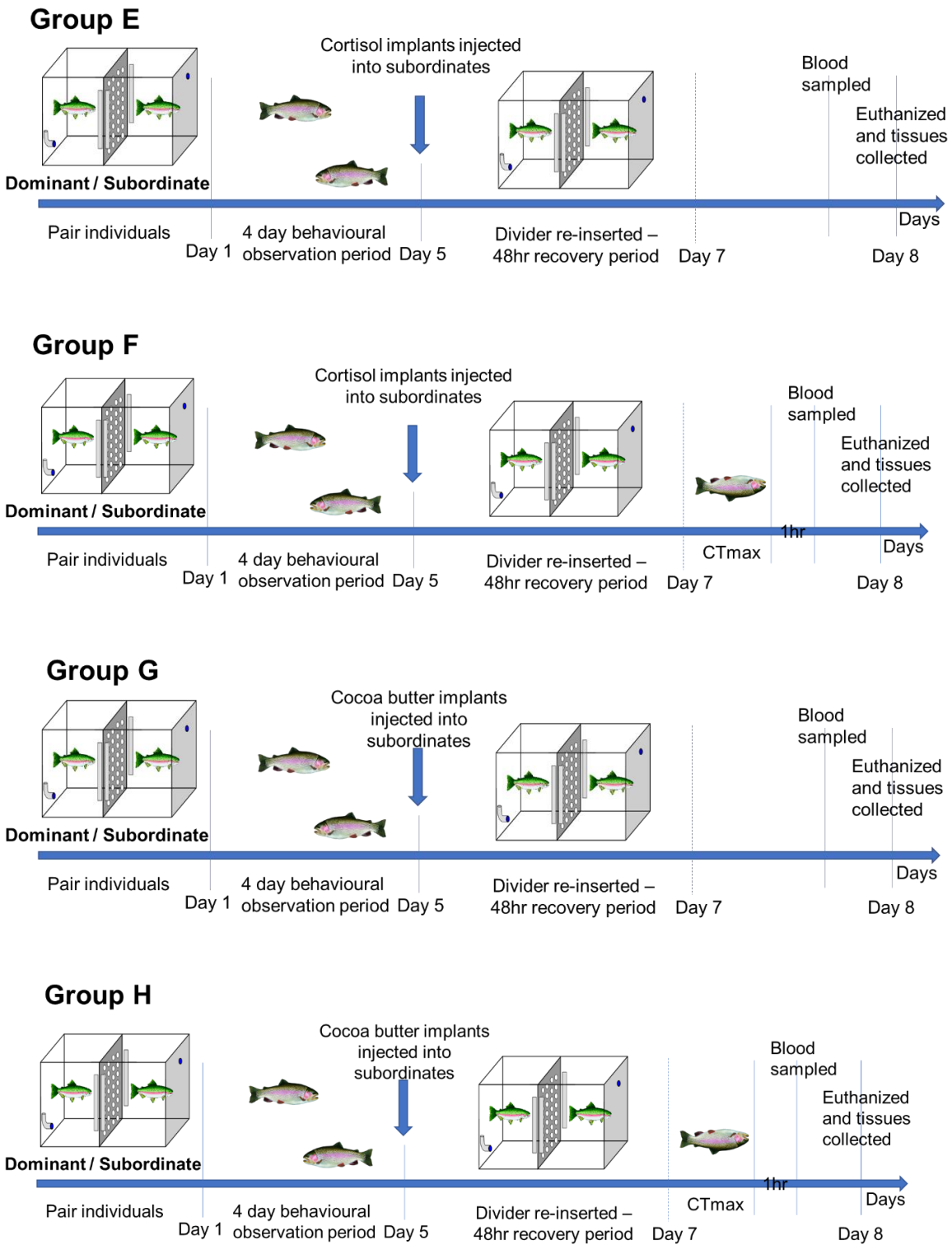


Figure 2.1. A schematic of the experimental design (see text for details).

2.2.3 Measurement of CTmax and voluntary thermal tolerance (VTmax)

Measurement of CTmax was carried out between 9 am and 11am 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 loss of equilibrium. The temperature at which the fish lost equilibrium, indicated by the fish turning dorso-ventrally and being unable to right itself within 3 s, was noted as CTmax. Once CTmax was reached, water temperature was returned to the acclimation temperature (13°C). The fish that first reached its CTmax was temporarily removed from the behaviour tank to immediately begin the return to acclimation temperature. A VTmax was also recorded for each fish, as a behavioural measure of thermal tolerance (Mcdonnell et al., 2019; McDonnell and Chapman, 2015). The VTmax was identified as the temperature of onset of irregular behaviours, including erratic swimming (quickly swimming up and down water column, swimming into corners, quick changes in direction) and surfacing, which ultimately represents an escape response. Water temperature was altered using a series 440 Fotopanel thermostatic mixing valve (POWERS™, Burlington, ON), and water flowing to the experimental tanks was vigorously aerated using an equilibration column to ensure maintenance of air saturation with increasing temperature (the efficacy of this approach was tested by monitoring dissolved oxygen levels during a CTmax trial). All trials were recorded using a video camera (Canon VIXIA HF R500). Video recordings were scored independently by two observers who were blind to the treatment group of the pair for CTmax and VTmax as well as surfacing, aggressive and unpredictable thrashing behaviours using the event-logging software BORIS (https://www.boris.unito.it/).

2.2.4 Tissue sample collection and analysis

For collection of blood samples, the members of a pair were lightly anesthetized together as described above and 0.3 ml of blood was withdrawn by caudal venipuncture using a 23 G needle and syringe rinsed with 0.5 M ethylenediaminetetraacetic acid (EDTA). 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 except for pairs allocated to recovery treatment groups (i.e. groups C, D, E, F, G and H). Blood samples were centrifuged at 10,000 *g* for 2 min. An aliquot (10 µl) of plasma was used immediately for the analysis of plasma lactate concentration, and the remaining plasma was flash frozen in liquid N_2 and stored at -80°C for later analysis of cortisol concentrations.

Fish were euthanized 24 h later using terminal anaesthesia (0.5 g L^{-1} ethyl-p-aminobenzoate), and the heart was dissected out. Hearts were blotted dry and the bulbus arteriosus and atrium were removed, after which the mass of the ventricle was measured (see Ch. 3). 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 (see Ch. 3) or protein abundance by real-time RT-PCR. Fixed ventricle tissue was dehydrated in 70% ethanol and stored in 70% ethanol at 4°C until histological analysis (see Ch. 3).

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) according to the manufacturer's instructions. Intra-assay variation was 4.1% and inter-assay variation was 3.5% (% CV).

Plasma lactate concentrations were analyzed using a hand-held lactate meter (Lactate Plus Lactate Analyzer, Nova Biomedical, USA) and corresponding lactate test strips (Lactate Plus test strips, https://lactate-testing.myshopify.com/collections/all/products/lactate-plus-analyzer) as described previously (Brown et al., 2008; Serra-Llinares et al., 2012).

Western analysis was used to measure HSP70 protein levels in heart tissue. 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 2x 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,CA) 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, CA) in 2% skim milk in TBST. The following day, membranes were washed with TBST (3x 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 (3x for 15 min each time) to remove excess antibody, incubated with 1 ml of Luminata Classico HRP substrate (MilliporeSigma™; Oakville, ON, CA), and imaged using chemiluminescence

(ChemiDoc XRS+, Bio-Rad). Relative HSP70 protein abundance was normalized to total protein using ImageLab 6.0 according to Taylor and Posch (2014).

2.2.5 Cortisol implant injections

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 weight; BW) or cocoa butter alone (5 ml kg^{-1} BW) as a sham control (Lawrence et al., 2019; Pickering and Duston, 1983). 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 then added to melted cocoa butter and the ethanol was evaporated off by heating the solution (Lawrence et al., 2019). To administer the implants, fish were lightly anesthetized as described above and liquid cocoa butter 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 tanks with the divider inserted to separate the dominant and subordinate fish for the 2 d recovery period.

2.2.6 Statistical analysis

All statistical analyses were performed using R-studio. 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. In all cases, α was set to 0.05 and data are

presented as means ± SEM in tables. In figures, data are presented in boxplots where the upper and lower limits of the box represent the 75th and 25th percentiles, respectively, the red line across the box represents the mean value, the whiskers represent the maximum and minimum values, and each red circle presents the value for an individual fish.

Effects within a treatment group of social status on CTmax, VTmax, plasma lactate and HSP70 protein abundance were analyzed by Student's *t*-tests. Aggression before and after VTmax was analyzed by a paired Wilcoxon signed-rank test. Plasma cortisol concentrations were analyzed according to treatment group by two-way ANOVA using status (dominant or subordinate) and acute warming (underwent CTmax trial or not) as factors.

2.3 Results

Rainbow trout held in pairs in an experimental chamber formed social hierarchies in which dominant fish could be distinguished from subordinate fish on the basis of behaviour (Table 2.1). Following 4 d of interaction, subordinate fish exhibited a significantly lower CTmax than dominant fish (Fig. 2.2A; Student's *t*-test, $P = 0.0013$). When the divider was inserted between the fish within a pair to physically separate them and minimize visual contact for a 48 h recovery period, the thermal tolerance of (recovering) subordinates was similar to that of dominant fish (Fig. 2.2B; Student's *t*-test, $P = 0.35$). The treatment of recovering subordinates with cortisol by means of a cortisol-containing cocoa butter implant prevented the recovery of thermal tolerance; CTmax values in these fish (recovering subordinates + cortisol) were significantly lower than those of dominant fish (Fig. 2.2C; Student's *t*-test, $P = 0.0003$). The CTmax of sham-treated recovering subordinates, which received a cocoa butter implant lacking

cortisol, did not differ from that of dominant fish (Fig. 2.2D; Student's *t*-test, $P = 0.2$). There were no differences in VTmax, the temperature at which a set of behaviours associated with agitation (i.e. rapid swimming, surfacing and thrashing), were first observed between subordinate and dominant fish regardless of experimental group (Fig. 2.3; Student's *t*-tests, $P = 0.49$, 0.11, 0.97 and 0.17). Notably, aggressive interactions continued to occur during acute warming until VTmax was reached (Fig 2.4; Wilcoxon signed-rank test, $P = 0.031$).

Table 2.1. Morphometric data and behaviour scores for dominant and subordinate rainbow trout (*Oncorhynchus mykiss*).

Experimental group	Status	Mass (g)	Fork length (cm)	Behaviour score
A: 4 d interaction	Dominant (*N* = 8)	114.4 ± 7.7	21.4 ± 0.5	1.4 ± 0.5
	Subordinate (*N* = 8)	111.8 ± 9.0	21.0 ± 0.5	-1.5 ± 0.3
B: 4 d interaction + CTmax	Dominant (*N* = 6)	111.8 ± 12.2	21.1 ± 0.7	0.9 ± 0.5
	Subordinate (*N* = 6)	107.3 ± 9.6	21.1 ± 0.6	-1.8 ± 0.1
C: Recovery	Dominant (*N* = 7)	103.7 ± 4.0	21.1 ± 0.4	1.1 ± 0.2
	Subordinate (*N* = 7)	103.9 ± 4.2	21.1 ± 0.4	-1.4 ± 0.2
D: Recovery + CTmax	Dominant (*N* = 6)	117.3 ± 7.6	22.0 ± 0.6	2.1 ± 0.5
	Subordinate (*N* = 6)	114.7 ± 11.4	22.1 ± 0.7	-1.0 ± 0.2
E: Recovery with cortisol treatment	Dominant (*N* = 6)	76.7 ± 5.3	18.8 ± 0.5	1.7 ± 0.5
	Subordinate (*N* = 6)	76.2 ± 7.3	18.9 ± 0.7	-1.6 ± 0.2
F: Recovery with cortisol treatment + CTmax	Dominant (*N* = 6)	81.5 ± 2.8	19.4 ± 0.3	1.7 ± 0.2
	Subordinate (*N* = 6)	78.7 ± 3.2	19.6 ± 0.4	-1.4 ± 0.2

G: Recovery with sham treatment	Dominant (*N* = 6)	94.2 ± 9.8	20.4 ± 0.8	1.0 ± 0.3
	Subordinate (*N* = 6)	87.3 ± 9.7	20.3 ± 0.8	-1.7 ± 0.2
H: Recovery with sham treatment + CTmax	Dominant (*N* = 6)	86.0 ± 9.2	19.6 ± 0.8	1.8 ± 0.6
	Subordinate (*N* = 6)	87.8 ± 9.7	19.8 ± 0.9	-1.5 ± 0.2

Values are means ± SEM, with *N* numbers indicated in parentheses next to social status.

Figure 2.2. Critical thermal maxima (CTmax) of dominant and subordinate rainbow trout (*Oncorhynchus mykiss*) following (A) social interactions for 4 d (N = 22 pairs), (B) 48 h of recovery from a 4 d social interaction period (N = 6 pairs), (C) 48 h of recovery from 4 d 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 d 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 = 0.0013, 0.35, 0.0003 and 0.2 for panels A to D, respectively). Data are presented as a box plot where the upper and lower limits of the box represent the 75^{th} and 25^{th} percentiles, respectively, the red line across the box represents the mean value, the whiskers represent the maximum and minimum values, and each red circle presents the value for an individual fish.

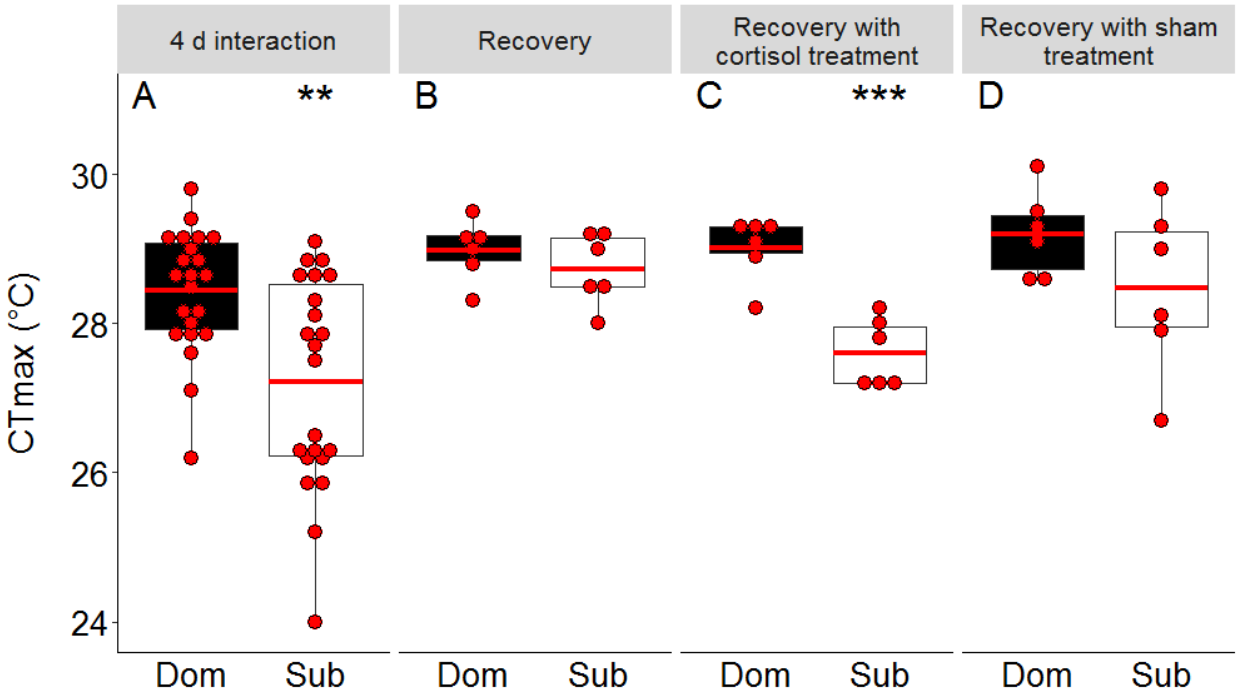

4 d interaction
Recovery
Recovery with cortisol treatment
Recovery with sham treatment
A
**
B
C
***
D
CTmax (°C)
30
28
26
24
Dom
Sub
Dom
Sub
Dom
Sub
Dom
Sub

Figure 2.3. Voluntary thermal maxima (VTmax) of dominant and subordinate rainbow trout (*Oncorhynchus mykiss*) following (A) social interactions for 4 d (N = 6 pairs), (B) 48 h of recovery from a 4 d social interaction period (N = 6 pairs), (C) 48 h of recovery from 4 d of social interaction but with cortisol treatment of subordinate fish during the recovery period (N = 7 pairs), and (D) 48 h of recovery from 4 d of social interactions but with sham treatment (cocoa butter implant) of subordinate fish during the recovery period (N = 6 pairs). No significant differences were detected between dominant and subordinate fish (Student's t-tests, P = 0.49, 0.11, 0.97 and 0.17 for panels A to D, respectively). Data are presented as a box plot; please see the legend of Fig. 2.2 for details.

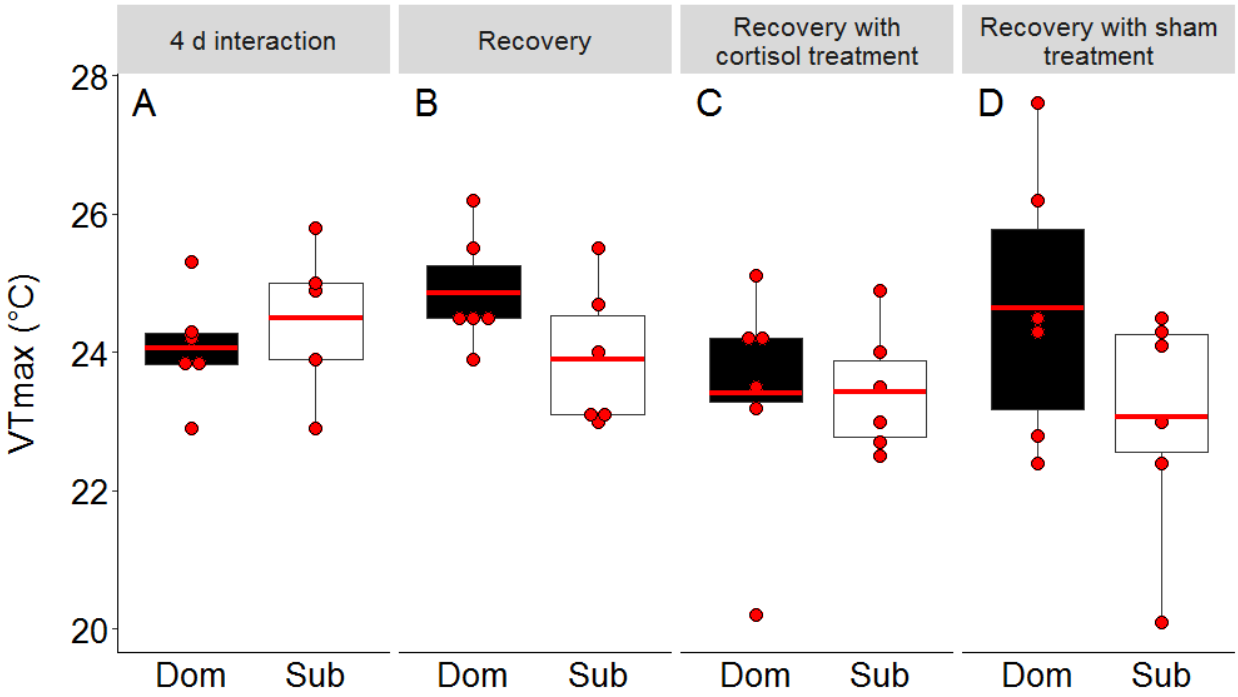
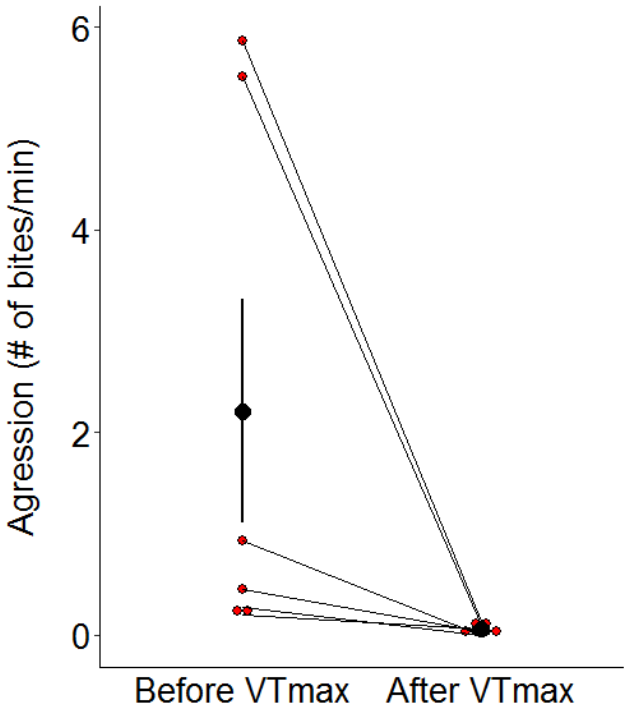
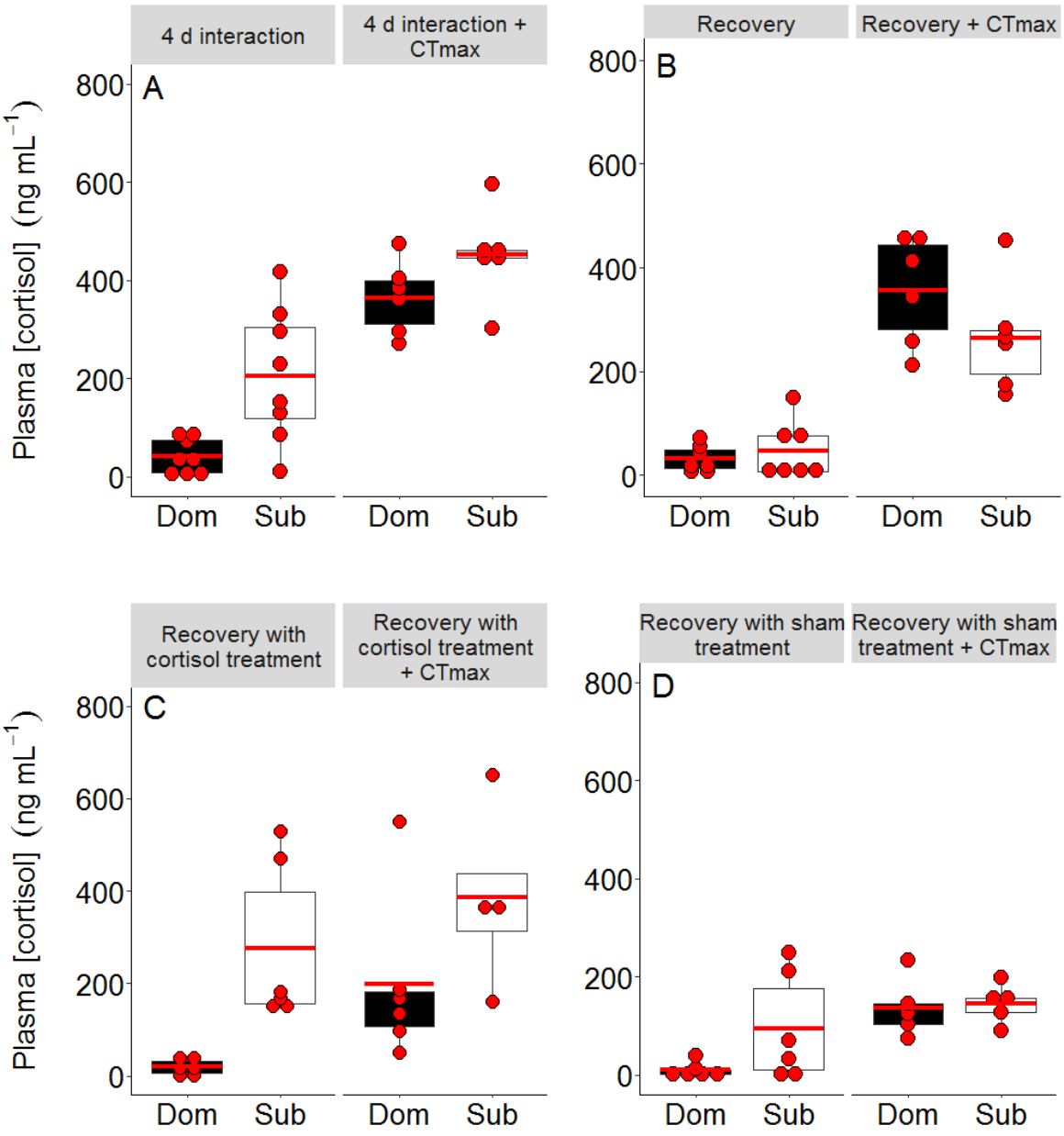

4 d interaction
Recovery
Recovery with cortisol treatment
Recovery with sham treatment
A
B
C
D
VTmax (°C)
28
26
24
22
20
Dom
Sub
Dom
Sub
Dom
Sub
Dom
Sub

Figure 2.4. Aggressive interactions before and after VTmax in dominant (N = 6) rainbow trout (*Oncorhynchus mykiss*) during acute warming after 4 days of interaction (Paired Wilcoxon test, P = 0.031). Number of bites is stadardized by the time that the subordinate fish could be bitten.


Agression (# of bites/min)
6
4
2
0
Before VTmax
After VTmax

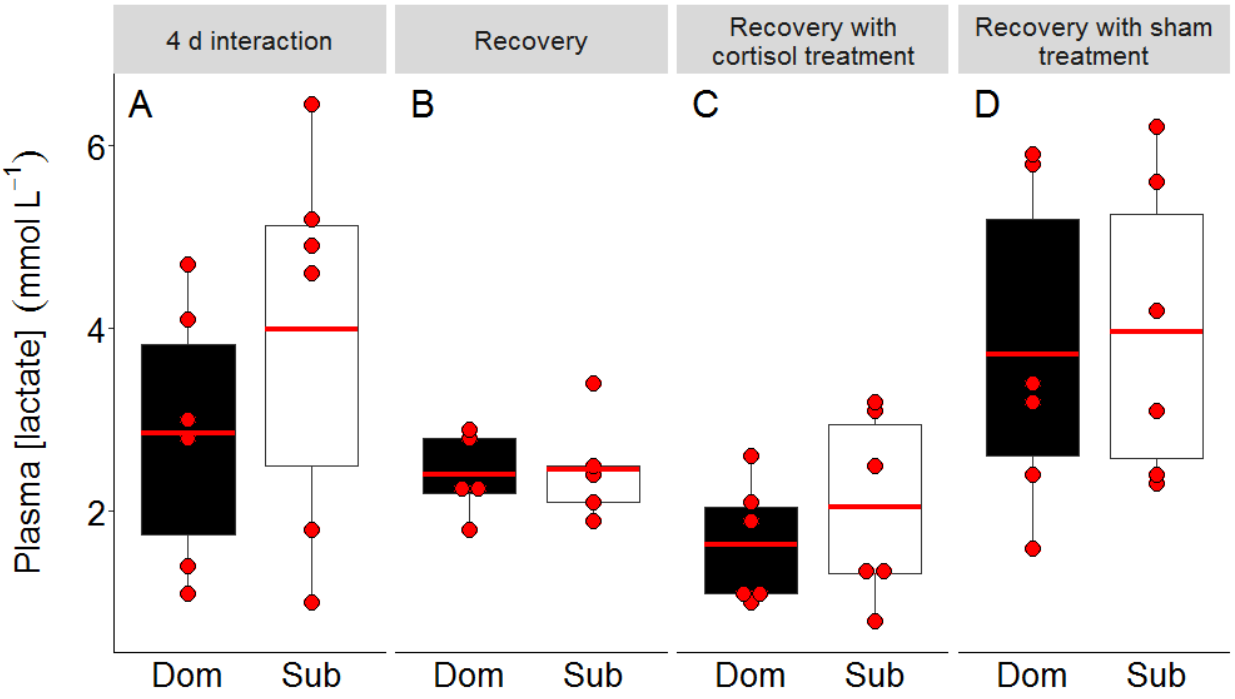
Plasma cortisol levels were higher in subordinate fish compared to dominants after a 4 d interaction period (Fig. 2.5A; 2-way ANOVA; status $P = 0.001$, acute warming $P < 0.0001$, status x acute warming $P = 0.289$), but returned to baseline after a 48 h recovery period (Fig. 2.5B; 2-way ANOVA; status $P = 0.271$, acute warming $P < 0.0001$, status x acute warming $P = 0.089$). Cortisol levels of recovering subordinates injected with cortisol implants were higher than those of dominant fish (Fig. 2.5C; 2-way ANOVA; status $P = 0.0009$, acute warming $P = 0.01$, status x 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. 2.5D; 2-way ANOVA; status $P = 0.09$, acute warming $P = 0.006$, status x acute warming $P = 0.20$). Plasma cortisol levels increased after thermal stress (CTmax trial) similarly in subordinate and dominant fish in all trials (Fig. 2.5).

Figure 2.5. Plasma cortisol concentrations of subordinate and dominant rainbow trout (*Oncorhynchus mykiss)* following (A) social interactions for 4 d (*N* = 8 pairs, 6 pairs; 2-way ANOVA; status P = 0.001, acute warming P < 0.0001, status x acute warming P = 0.289), (B) 48 h of recovery from a 4 d social interaction period (*N* = 7 pairs, 6 pairs; 2-way ANOVA; status *P* = 0.271, acute warming *P* < 0.0001, status x acute warming *P* = 0.089), (C) 48 h of recovery from a 4 d social interaction period with cortisol treatment of subordinate fish during the recovery period (*N* = 6 pairs, 6 pairs; 2-way ANOVA; status *P* = 0.0009, acute warming *P* = 0.01, status x acute warming *P* = 0.62) , and (D) 48 h of recovery from a 4 d social interaction period with sham treatment of subordinate fish during the recovery period (*N* = 6 pairs, 5 pairs; 2-way ANOVA; status *P* = 0.09, acute warming *P* = 0.006, status x acute warming *P* = 0.20). Data are presented as a box plot; please see the legend of Fig. 2.2 for details.


4 d interaction
4 d interaction + CTmax
Recovery
Recovery + CTmax
Recovery with cortisol treatment
Recovery with cortisol treatment + CTmax
Recovery with sham treatment
Recovery with sham treatment + CTmax
A
B
C
D
Plasma [cortisol] (ng mL^{-1})
0
200
400
600
800
Dom
Sub

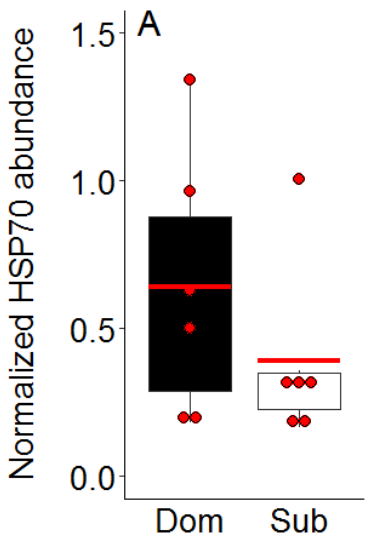
No differences in plasma lactate concentrations were detected between thermally stressed (i.e. after the CTmax trial) subordinate and dominant fish, regardless of experimental group (Fig. 2.6; Student's *t*-tests, $P = 0.30$, 0.96, 0.42 and 0.81).

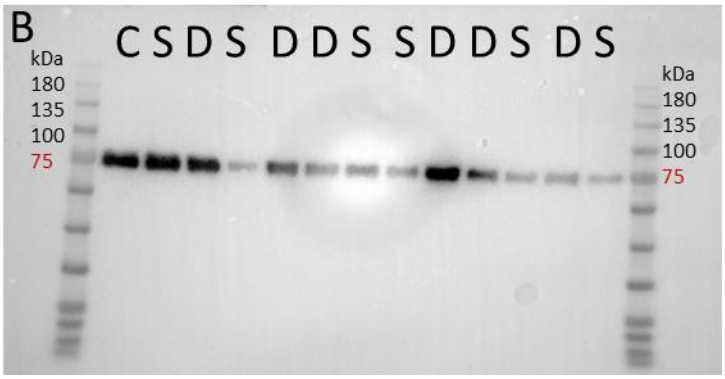
Figure 2.6. Plasma lactate concentrations following the acute warming of a CTmax trial in rainbow trout (*Oncorhynchus mykiss*) subjected to (A) social interactions for 4 d (N = 6 pairs), (B) 48 h of recovery from a 4 d social interaction period (N = 6 pairs), (C) 48 h of recovery from 4 d of social interaction but with cortisol treatment of subordinate fish during the recovery period (N = 6 pairs), and (D) 48 h of recovery from 4 d of social interactions but with sham treatment (cocoa butter implant) of subordinate fish during the recovery period (N = 6 pairs). No significant differences were detected between dominant and subordinate fish (Student's t-tests, P = 0.30, 0.96, 0.42 and 0.81 for panels A to D, respectively). Data are presented as a box plot; please see the legend of Fig. 2.2 for details.


4 d interaction
Recovery
Recovery with cortisol treatment
Recovery with sham treatment
A
B
C
D
Plasma [lactate] (mmol L^{-1})
6
4
2
Dom
Sub
Dom
Sub
Dom
Sub
Dom
Sub

Finally, no differences in HSP70 protein levels were detected between subordinate and dominant trout following exposure to acute warming in a CTmax trial (Fig. 2.7; Student's *t*-test, $P = 0.29$).

Figure 2.7. The effect of social stress on HSP70 protein abundance in the ventricle of rainbow trout (*Oncorhynchus mykiss*) following a CTmax trial. (A) Mean ventricular HSP70 levels expressed relative to total protein abundance. No significant difference was detected between subordinate ($N = 6$) and dominant ($N = 6$) fish (Student's t-test, $P = 0.29$). The data are presented as a box plot; see the legend of Fig. 2.2 for details. (B) An image of the western blot from which HSP70 band densities were measured. The HSP70 band appears at 75 kDa. *S*, subordinate; *D*, dominant; *C*, a pooled sample used for cross-blot normalization.


A
Normalized HSP70 abundance
1.5
1.0
0.5
0.0
Dom
Sub


B
C S D S D D S S D D S D S
kDa
180
135
100
75
kDa
180
135
100
75

2.4 Discussion

The objective of the present study was to determine whether the prolonged elevation of circulating cortisol levels experienced by subordinate rainbow trout contributes to their lower thermal tolerance. As reported by LeBlanc et al. (2011), the CTmax of subordinate trout was significantly lower than that of dominant fish. When subordinates were allowed to recover from social stress for 48 h by placing an opaque perforated divider in the tank to physically separate the members of a pair, cortisol levels in subordinate fish decreased to levels that were on par with those of dominant fish, confirming the findings of Culbert and Gilmour (2016). This lowering of cortisol levels was accompanied by recovery of thermal tolerance, i.e. no significant difference in CTmax between dominant and subordinate fish. The use of cortisol-impregnated cocoa butter implants to elevate cortisol levels in subordinates during recovery from social stress confirmed the critical role of cortisol in modifying thermal tolerance, because CTmax was significantly lower in cortisol-treated recovering subordinates than in dominant trout. In recovering subordinates treated with sham implants (i.e. cocoa butter alone), neither cortisol levels nor thermal tolerance differed from those of dominant fish. Collectively, these data provide strong support for the hypothesis that elevated cortisol levels lower thermal tolerance as indicated by CTmax. Although few, if any, other studies have focused specifically on cortisol as a modulator of CTmax in fish, there is support in the literature for stress-induced changes in CTmax. For example, threadfin shad (*Dorosoma petenense*) showed reduced thermal tolerance (lower CTmax) shortly after being subjected to a netting stressor (Monirian et al., 2010), a protocol that would be expected to elicit a cortisol response (Jeffrey et al., 2014; Øverli et al., 1999b; Strange et al., 1977). Exposure to hypoxia (Rutledge & Beitinger 1989; Healy & Schulte 2012) as well as acute changes in salinity (Shaughnessy & McCormick 2018) also have been

associated with reductions in CTmax, although in these cases it is more difficult to link changes in CTmax to cortisol specifically. In mammals, it appears that elevation in cortisol during acute warming contributes to their ability to tolerate heat. For example in humans, cortisol levels mimic rectal temperatures, with cortisol peaking at temperatures that cause serious discomfort (Follenius et al., 1982), allowing cortisol to be utilized as an indicator of heat intolerance. Furthermore, the taurine breed of cattle were less heat tolerant and had significantly higher cortisol concentrations than other breeds (Pires et al., 2019), with similar trends also seen among pigs (Vashi et al., 2018). Additionally, in small-tail Han sheep, female sheep coped with heat stress better than male sheep owing to differential cortisol responses (Li et al., 2018).

The voluntary thermal maximum (VTmax) was also investigated, because it is a behavioural measure of the temperature an individual will tolerate before initiating an escape response (Currie and Tattersall, 2018; McDonnell and Chapman, 2015). In mangrove rivulus, individuals emersed from the water at higher temperatures when socially stressed indicating that individuals would tolerate temperatures closer to their thermal limits when challenged by an apparent conspecific (Currie and Tattersall, 2018). However, in the present study differences in VTmax were not detected between subordinate and dominant trout even where CTmax differed. Similarly, the African cichlid *Pseudocrenilabrus multicolor victoriae* exhibited differences in CTmax when acclimated to a variety of temperatures, but VTmax remained unchanged (McDonnell and Chapman, 2015). In contrast to the study of Currie and Tattersall (2018), where mirror reflections were used to simulate a social challenge, VTmax in the present study was measured for two fish simultaneously, and it is possible that the presence of a tank mate modified behaviour. More specifically, the onset of erratic behaviour for one fish may have elicited escape behaviour by its tank mate, yielding similar VTmax for both fish in the pair.

Chronic elevation of cortisol during social stress could be associated with a reduction in CTmax through several possible mechanisms. Acute warming elevates circulating cortisol levels in rainbow trout and other fishes (LeBlanc et al. 2011, LeBlanc et al. 2012, Yusishen et al. 2020). Factors that attenuate this heat-induced cortisol response could potentially impair thermal tolerance, and chronic social stress is associated with attenuation of the cortisol response to handling or netting stressors (Overli et al. 1999, Jeffrey et al. 2014). 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). Although the reasons why chronic social stress attenuates the cortisol response to some stressors but not others remain to be determined, the lower CTmax of subordinate relative to dominant fish despite comparable cortisol responses to acute warming suggests that the acute cortisol response to warming does not determine thermal tolerance. A similar conclusion can be drawn from a recent study of social buffering in lake sturgeon (*Acipenser fulvescens*), where sturgeon held in groups had a reduced cortisol response when subjected to an acute thermal stress, but thermal tolerance (CTmax) was unchanged (Yusishen et al., 2020).

It is also possible that the prolonged elevation of cortisol during chronic social stress causes cardiac hypertrophy and fibrosis, as demonstrated by Johansen et al. (2017) for cortisol-treated rainbow trout. In the study of Johansen et al. (2017), trout treated with cortisol for 45 d experienced an increase in ventricle mass and compact myocardium associated with lower maximum cardiac output, reduced scope of cardiac output, and a reduction in aerobic swimming capacity. Although the physiological factors determining thermal tolerance remain controversial, limitations on oxygen delivery to tissues as temperature increases have been suggested as a determinant of thermal tolerance (the oxygen and capacity limited thermal tolerance theory,

OCLTT; Pörtner et al. 2017), and blunting of cardiac responses to acute warming reduce CTmax (Ekström et al. 2019, Gilbert et al. 2019). In the present study, heart rate increased in response to acute warming to a similar extent in both dominant and subordinate fish but tended to peak at lower temperatures in subordinate fish (Ch. 3). It was previously thought that the HSP70 response in subordinates would be attenuated by chronic elevation of cortisol, because cortisol treatment in trout and tilapia reduced the HSP70 response to heat in the liver and gill (Basu et al., 2001). In the present study, however, the cardiac HSP70 response to acute warming was similar in dominant and subordinate fish, indicating comparable stress responses at the cellular level.

It has long been thought that the endpoint of a CTmax trial (i.e. loss of locomotory function) is neurological (Bilyk et al., 2012; Jutfelt et al., 2019). Recent support for this mechanism was provided by Jutfelt et al. (2019), who cooled the brain of Atlantic cod (*Gadus morhua*) by 2-6°C, and saw increases in CTmax of 0.2-0.6°C. These data suggest that the brain is involved in determining thermal tolerance but given that the increase in CTmax was smaller than the decrease in brain temperature, it is most likely in concert with other systems (i.e. peripheral neurons, spinal cord, heart, etc.). The fish brain is rich in both the mineralocorticoid and glucocorticoid receptors that mediate the physiological effects of cortisol on target tissues, making it very responsive to changes in cortisol (Alderman and Vijayan, 2012; Alderman et al., 2012; Kiilerich et al., 2018; Teitsma et al., 1998). Indeed, impairment of reflex responses is used as an indicator of stress in fish (Davis, 2010), although the pathways through which cortisol alters reflex responses remain uncertain. Significant interactions also exist between the serotonergic system and the stress axis (Winberg et al., 1997). Trout fed diets supplemented with L-tryptophan (the precursor of serotonin) exhibited elevated baseline cortisol levels but a decreased cortisol response to acute stress (Lepage et al., 2002). Elevated brain serotonergic

activity also is observed in subordinate trout (Backström and Winberg, 2017; Lepage et al., 2005), Winberg and Lepage 1998). Interestingly, when the diet of fingerling Mgrial carp (*Cirrhinus mrigala*) was supplemented with L-tryptophan, CTmax was increased (Tejpal et al., 2014). These findings suggest a link between brain serotonergic activity and CTmax, although in the opposite direction to that which would have been predicted by the results of the present study, where subordinate trout, which are presumed to have elevated brain serotonergic activity, had lower CTmax than dominant trout. Thus, while it seems likely that chronic elevation of cortisol has effects on the central nervous system that contribute to loss of equilibrium (i.e. the endpoint of CTmax), the specific mechanisms remain to be identified.

Chapter 3: Does chronic social stress induce cardiac remodelling and affect heart function?

3.1 Introduction

Chronic elevation of cortisol has a range of deleterious effects such as reduced growth and condition factor (Barton et al., 1987; Gregory and Wood, 1999), suppression of gonadal steroidogenesis (Poursaeid et al., 2012), and increased metabolic rate (Chan and Woo, 1978; Morgan and Iwama, 1996). Recently, Johansen et al. (2017) reported that chronic cortisol treatment promoted cardiac remodelling in rainbow trout. Specifically, elevation of circulating cortisol concentrations to ~20 ng ml^{-1} for 45 d induced ventricular hypertrophy, and impaired cardiac function (Johansen et al., 2017). Cortisol treated fish exhibited a reduction in maximum cardiac output and a smaller scope for changes in heart rate (f_H) (Johansen et al., 2017). Moreover, this reduction in cardiac function lowered the critical swimming speed (Ucrit), demonstrating that changes in cardiac function can impact whole-animal performance (Johansen et al., 2017). The ventricles of cortisol-treated fish exhibited hypertrophy based on increased expression of molecular markers of hypertrophy coupled with the unchanged expression of cell-proliferation markers (Johansen et al., 2017). In a follow-up study, 7 d of cortisol treatment still induced some aspects of cardiac remodelling, which largely included the upregulation of pro-hypertrophic genes (Nørstrud et al., 2018). However, differences in relative ventricular mass were not yet present, demonstrating that cardiac remodelling is time dependent (Nørstrud et al., 2018). Using trout that were selected for either a high (HR) or low (LR) cortisol response to a standardized stressor, changes in cardiac collagen deposition were detected. The HR trout exhibited increased collagen deposition, which causes fibrosis (i.e. increases stiffness of the heart) (Johansen et al., 2011a). In mammalian hearts, fibrosis is a common response to injury and can cause cardiac impairment (Schnitt et al., 1993), but in fishes most research has been focused

on the plasticity of collagen deposition in relation to thermal acclimation (Johnson et al., 2014; Keen et al., 2015; Keen et al., 2018).

Although subordinate trout exhibit substantially higher circulating plasma cortisol values, ranging from 120 to 400 ng ml^{-1}, than were used by Johansen et al. (2017), the duration of exposure to these elevated levels is brief (4 d) relative to even the shorter exposure used by Norstrud et al. (2018). Thus, one goal of the present study was to determine whether chronic social stress induces cardiac remodelling in subordinate rainbow trout. Subordinate rainbow trout exhibited reduced thermal tolerance (Ch. 2; LeBlanc et al. 2011), and several lines of evidence suggest that cardiac function is an important determinant of thermal tolerance in fish. Acute warming increases heart rate and cardiac output (Ekström et al., 2016; Motyka et al., 2017; Penney et al., 2014), presumably to increase oxygen delivery (Ekström et al., 2014; Sandblom et al., 2016), and the peak heart rate response coincides with the upper thermal limit (Ekström et al., 2014; Ekström et al., 2017; Ekström et al., 2019; Gilbert et al., 2019). In Nile perch, *Lates niloticus*, larger relative ventricular mass was correlated with increased CTmax (Nyboer and Chapman, 2018). Nyboer and Chapman (2018) also demonstrated that the percent compact myocardium was positively related to aerobic scope and standard metabolic rate (Nyboer and Chapman, 2018). A similar relationship between CTmax and relative ventricular mass was detected in Atlantic salmon, *Salmo salar* (Anttila et al., 2013). Additionally, several recent studies reported that impaired cardiac function translates into lowered thermal tolerance in salmonid fish. For example, coronary artery-ligated rainbow trout exhibited increased heart rate during warming and reduced CTmax (Ekström et al., 2017). In a follow up study, the same authors reported that the heart rate of coronary artery-ligated trout reached a maximum at a lower temperature than that of control animals, and this difference was accompanied by lowered

thermal tolerance (Ekström et al., 2019). Similarly, inhibition of cardiac autonomic reflexes in rainbow trout resulted in the occurrence of the peak heart rate at a lower temperature and reduced thermal tolerance (Gilbert et al., 2019). Collectively, this evidence suggests that cardiac function plays a role in determining thermal tolerance.

If sustained elevation of baseline cortisol levels during chronic social stress induces cardiac remodelling in subordinate trout, then impaired cardiac function may contribute to the negative effects of subordinate status, including hindering the ability of subordinate fish to withstand higher water temperatures. Thus, the present study tested the hypothesis that subordinate trout experience cardiac remodelling by investigating heart morphology, together with molecular markers of hypertrophy and collagen turnover. In addition, heart rate responses to social interaction and acute warming were assessed to address the hypothesis that impaired cardiac function contributes to the lower thermal tolerance of subordinate trout.

3.2 Materials and methods

3.2.1 Experimental animals

Juvenile rainbow trout, *Oncorhynchus mykiss* (mass = 141.9 ± 3.1 g, fork length = 23.3 ± 0.2 cm, mean ± SEM, N = 26), were obtained from Linwood Acres Trout Farm (Campbellcroft, Ontario). At the University of Ottawa, trout were held in 1275 L fibreglass tanks supplied with flowing, aerated, dechloraminated city of Ottawa tap water. Water temperature was maintained at 13°C, a 12L:12D photoperiod was used, and fish were fed commercial trout pellets at 0.5% body mass daily. Trout were acclimated to these holding conditions for at least 2 weeks prior to experimentation. All experimental protocols were approved by an institutional animal care

committee (protocol BL-2118) and were in compliance with the guidelines of the Canadian Council on Animal Care (CCAC) for the use of animals in research and teaching. These trout were fitted with bio-heart loggers prior to the establishment of social hierarchies as described previously (see section 2.2.1). In addition to 10 dominant and 10 subordinate fish, heart rate data were collected for 6 sham fish. Sham-treated fish are handled in the same fashion as fish that were paired but are housed individually and serve as a control for effects of handling associated with the establishment of social hierarchies.

Ventricles used for assessment of heart morphology and molecular markers were obtained from experimental fish of groups A (N = 8 pairs) and B (N = 6 pairs) of Chapter 2 (see section 2.2.4).

3.2.2 Measurement of heart rate during social interactions and acute warming

To measure f_H during social interactions and acute warming, trout were implanted with commercially available heart rate loggers (DST milli HRT, 13mm × 39.5 mm, Star-Oddi, Iceland; http://www.star-oddi.com/) that were programmed to record f_H and electrocardiogram (ECG) traces. Prior to heart logger implantation, fish were anaesthetized by immersion in a solution of benzocaine (0.05 g L^{-1} ethyl-p-aminobenzoate; Sigma-Aldrich, Oakville, ON, CA). Once loss of equilibrium had occurred, fish were weighed, fork-length was measured, fin damage was assessed, and 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. Heart loggers were inserted into the peritoneal cavity through the incision, placed against the pericardial membrane, and sutured to

the body wall with two stitches (2-0 suture silk; Fisher Scientific, Nepean, ON, CA). The incision was closed using two single interrupted sutures. Fish were transferred to one side of a behaviour tank (40 L) supplied with flowing, aerated dechloraminated city of Ottawa tap water at 13°C and given an overnight recovery period. A length- and weight-matched conspecific fitted with a heart biologger was placed in the other side of the behaviour tank; the fish were separated from each other by a removable opaque, perforated divider. Fish in the sham treatment group were prepared in the same way but placed individually in the behaviour tank (with the divider in place).

The following morning, f_H measurements were collected every 5 min for 2 h from 9 am (Fig. 3.1). The divider was removed at 11 am, 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 following 3 d, heart rate measurements (but not ECG traces) were recorded at 5 min intervals every morning for 2 h, from 9 to 11 am. Over this 4-d period, behaviour observations were collected twice daily for 5 min each time to assess social status based on position in the tank, aggressive behaviour and willingness to take a single pellet of food (see section 2.2.2). Fish were fed 0.5% body mass after the second observation period each day. On the morning of day 5 (see Fig. 3.1), paired and sham fish were subjected to a CTmax trial (see section 2.2.3) during which heart rate measurements and ECG recordings were collected every 3 min through both acute warming and the return to the acclimation temperature of 13°C. Fish were then euthanized using terminal anaesthetic (0.5 g L^{-1} ethyl-p-aminobenzoate, Sigma Aldrich).

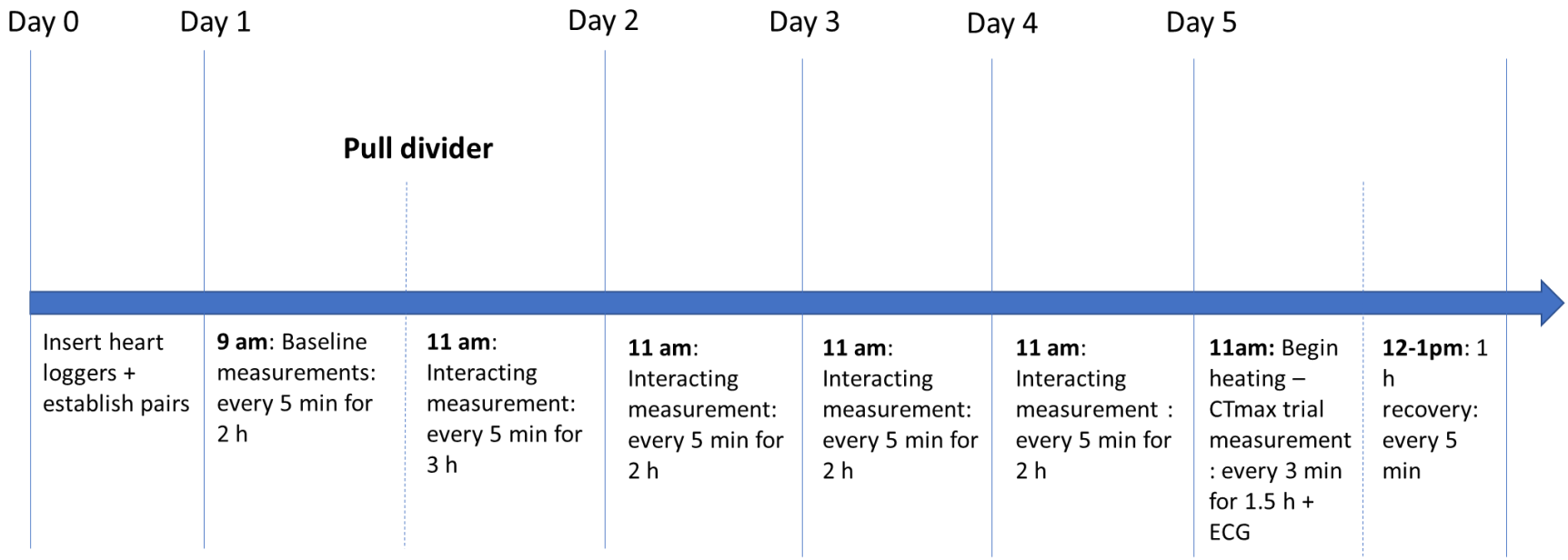


Figure 3.1. A schematic depicting the frequency of heart rate and electrocardiogram measurements in trout fitted with heart biologgers.

3.2.3 Histological assessment of cardiac collagen abundance

Fixed heart tissue was embedded in paraffin wax and sectioned transversely at 5 µm thickness to yield approximately 6-10 sections per heart; sections were placed onto positively charged microscope slides (Fisherbrand™ Superfrost™ Plus; Fisher Scientific). The slides were heated at 65°C for 30 min. The paraffin was removed through a series of xylene washes (3x for 5 min), and the tissue was rehydrated using a series of ethanol washes (100%, 95%, 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, location). Red staining was an indicator for collagen. Three random areas of one heart section were photographed, and average relative collagen content was quantified as the area of red staining within the total area of tissue (see Fig. 3.2 for protocol details). In addition, compact myocardium thickness was estimated by as the mean of five measurements per image (Fig. 3.3). All image analysis was carried out using Image J (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.

Figure 3.2. Determination of collagen content using Image J of ventricles stained with picrosirius red (*N* = 3). (A) Original photo taken with Zeiss microscope; all microscope parameters were maintained within pairs. (B) Photo with background subtracted (50 pixels; Process → Subtract background → ☒ Light background). (C) Photo split by colour hues (Green, Blue, Red; Image → Colour → Split channels); Green was chosen for analysis because green contrasts the most against red collagen staining. (D) Red indicates pixels counted as collagen (Image → Adjust → Threshold, Analyze → Set measurements → ☒ Area and Limit to threshold → Measure, Area). Black represents pixels removed from collagen counts because pixels are determined to be artificial staining. Threshold range counted as collagen was maintained within pairs. (E) Gray indicates pixels counted as tissue to calculate percentage of tissue in image displaying red staining.

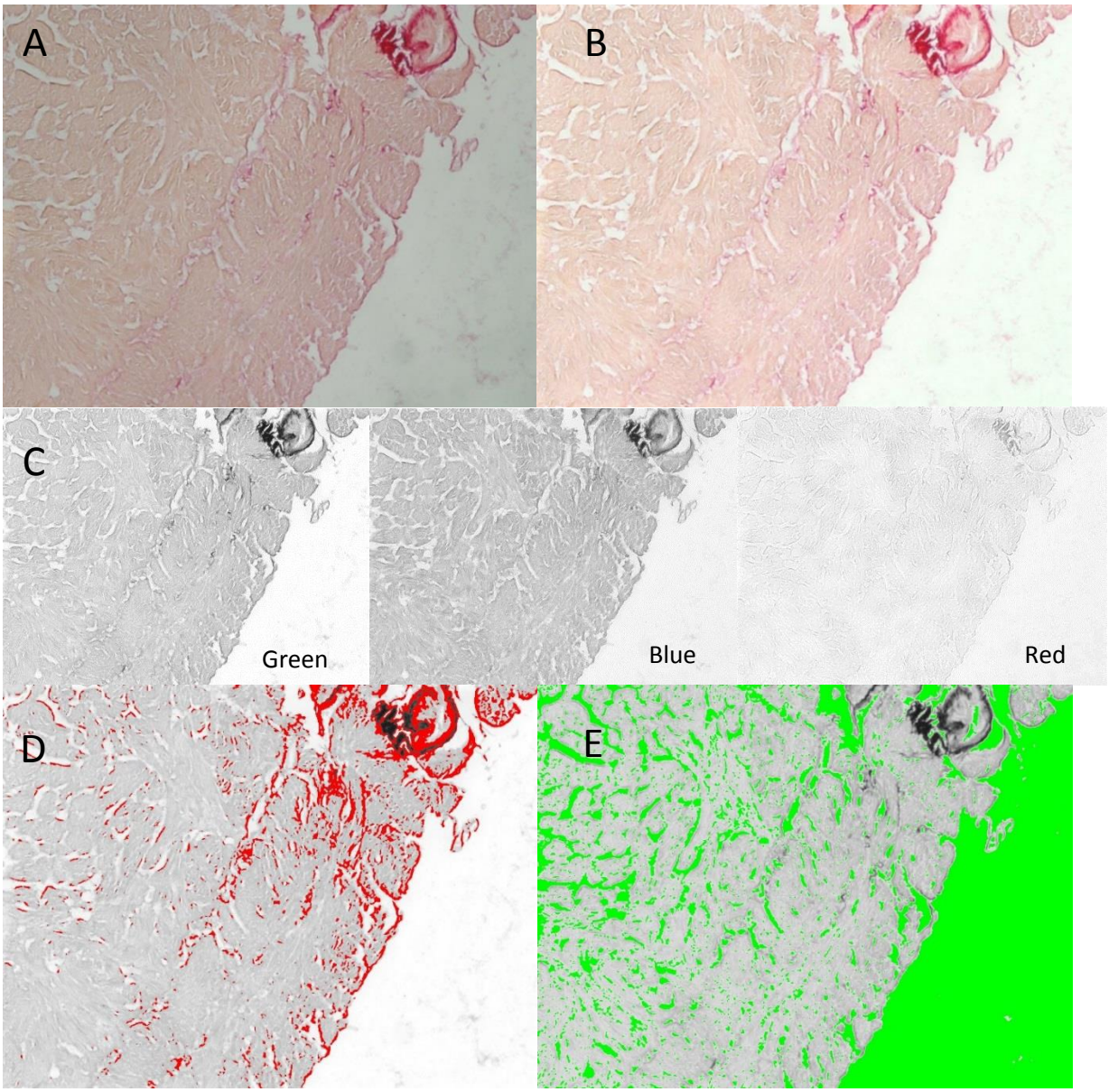
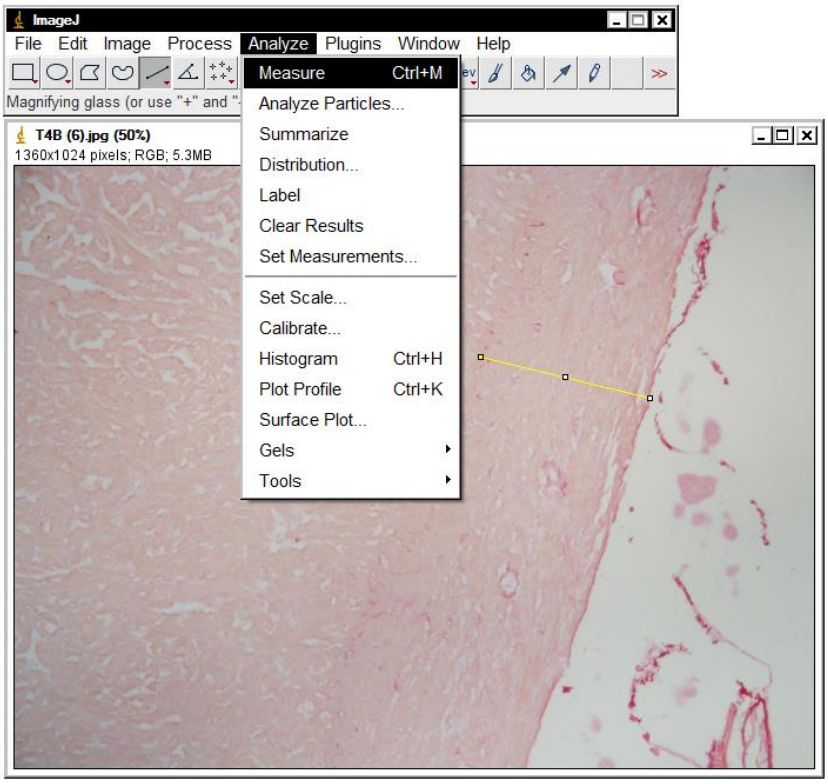

A
B
C
Green
Blue
Red
D
E

Figure 3.3. Quantification of relative compact myocardium using Image J software. Yellow line drawn across compact myocardium with straight line tool, and then measured (Analyze → Measure, Length). Five such measurements were made per image, and the mean was calculated.


ImageJ
File Edit Image Process Analyze Plugins Window Help
Measure Ctrl+M
Analyze Particles...
Summarize
Distribution...
Label
Clear Results
Set Measurements...
Set Scale...
Calibrate...
Histogram Ctrl+H
Plot Profile Ctrl+K
Surface Plot...
Gels
Tools
Magnifying glass (or use "+" and "
T4B (6).jpg (50%)
1360x1024 pixels; RGB; 5.3MB

3.2.4 Western blotting for measurement of collagen type I protein abundances

Collagen type I protein abundance was measured by western analysis according to the protocol described previously (see section 2.2.5) using 40 µg of protein sample and 1:1000 rabbit anti-salmon collagen type 1(Cedarlane CL50171AP, Burlington, ON, CA) as the primary antibody.

3.2.5 Measurement of transcript abundances by real-time RT-PCR

Ventricles stored at -80°C were ground to a powder on dry ice using a mortar and pestle. Powdered ventricle tissue (approximately 10-40mg) was sonicated (Sonic Dismembrator Model 100, Fisher Scientific) on ice in TRIzol® reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol for the extraction of RNA. 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, CA) according to the manufacturer's directions. Gene-specific primer sequences (Table 3.1) were identified from the literature for rainbow trout *β-actin* (as a housekeeping gene), ventricular myosin heavy chain (*vmhc*), slow myosin light chain 2 (*smlc2*), muscle LIM protein (*mlp*), regulator of calcineurin I (*rcan1*), mineralocorticoid receptor (*mr*), glucocorticoid receptors 1 and 2 (*gr1* and *gr2*), collagen type I isoforms, collagen type I alpha 1 (*col1α1*), alpha 2 (*col1α2*), and alpha 3 (*col1α3*), and connective tissue regulators, matrix metalloproteinase 2 (*mmp2*), matrix metalloproteinase 9 (*mmp9*), and tissue inhibitor of metalloproteinases 2 (*timp2*). 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 (5 µl

SYBR Green real-time PCR master mix, 1 µl primer pair mix, 3 µl nuclease-free water, and 1 µl template). 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 sample and relative transcript abundances were calculated based on previously establish protocols (see Pfaffl, 2001).

Table 3.1. Genes of interest and primers used for quantitative real-time PCR

Gene	Primer pair	GenBank accession number	Function/marker	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	BC076439.1	Cardiomyocyte	1.92; 136	Johansen et al. (2011)

	R: GGGGACTGCCTATGAGGAC	(*Danio rerio*)	hypertrophy		
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 R: GCAGATAACTTCGTCGCACA	NM001124177.1	Fibrosis	1.97; 154	Keen et al. (2015)
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 (*Danio rerio*)	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 (*Danio rerio*)	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*, and collagen type 1 alpha 3: *mmp2*, matrix metalloproteinase 2: *mmp9*, matrix metalloproteinases 9: *timp2*, tissue inhibitor of metalloproteinases 2

Annealing temperature 60°C for all primers

3.2.6 Data processing and statistical analysis

Electrocardiograms (ECG) and f_H data were obtained from the biologgers using Mercury software (Star Oddi, Iceland). The ECG traces were used to verify whether the f_H data were reliable, and to identify the temperature at which arrhythmia first occurred (see Appendix 2). Only individuals with clear ECG traces were used for analysis (i.e. a clear QRS complex was present, with a P wave to the left of the QRS complex). The Mercury software automatically transforms ECGs into f_H measurements along with a quality index (QI), which indicates the reliability of the signal. A QI of 0 indicates a reliable value whereas a QI of 3 indicates a poor data value, so all f_H with QI of 3 were removed from analysis. Based on previous literature, a range of 20-150 bpm was adopted for f_H (Ekström et al., 2014; Ekström et al., 2018; Farrell, 2002; Farrell et al., 1996; Kurt Gamperl et al., 2011; Sandblom and Axelsson, 2007; Verhille et al., 2013; Wood et al., 1979); values outside of this range were removed from the analysis. Heart rate during the interaction period was calculated by averaging f_H over the 2 or 3 h measurement period for each day. For the CTmax trial, heart rate at 13° was calculated as the mean of f_H values for water temperatures of 13-13.5°C. Peak Δf_H was the largest difference between f_H during acute warming and f_H at 13°C.

All statistical analyses were carried out using R-studio. 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. In all cases, α was set to 0.05 and data are presented as boxplots where the upper and lower limits of the box represent the 75^{th} and 25^{th} percentiles, respectively, the red line across the box represents the mean value, the whiskers represent the maximum and minimum values, and each red circle presents the value for an individual fish.

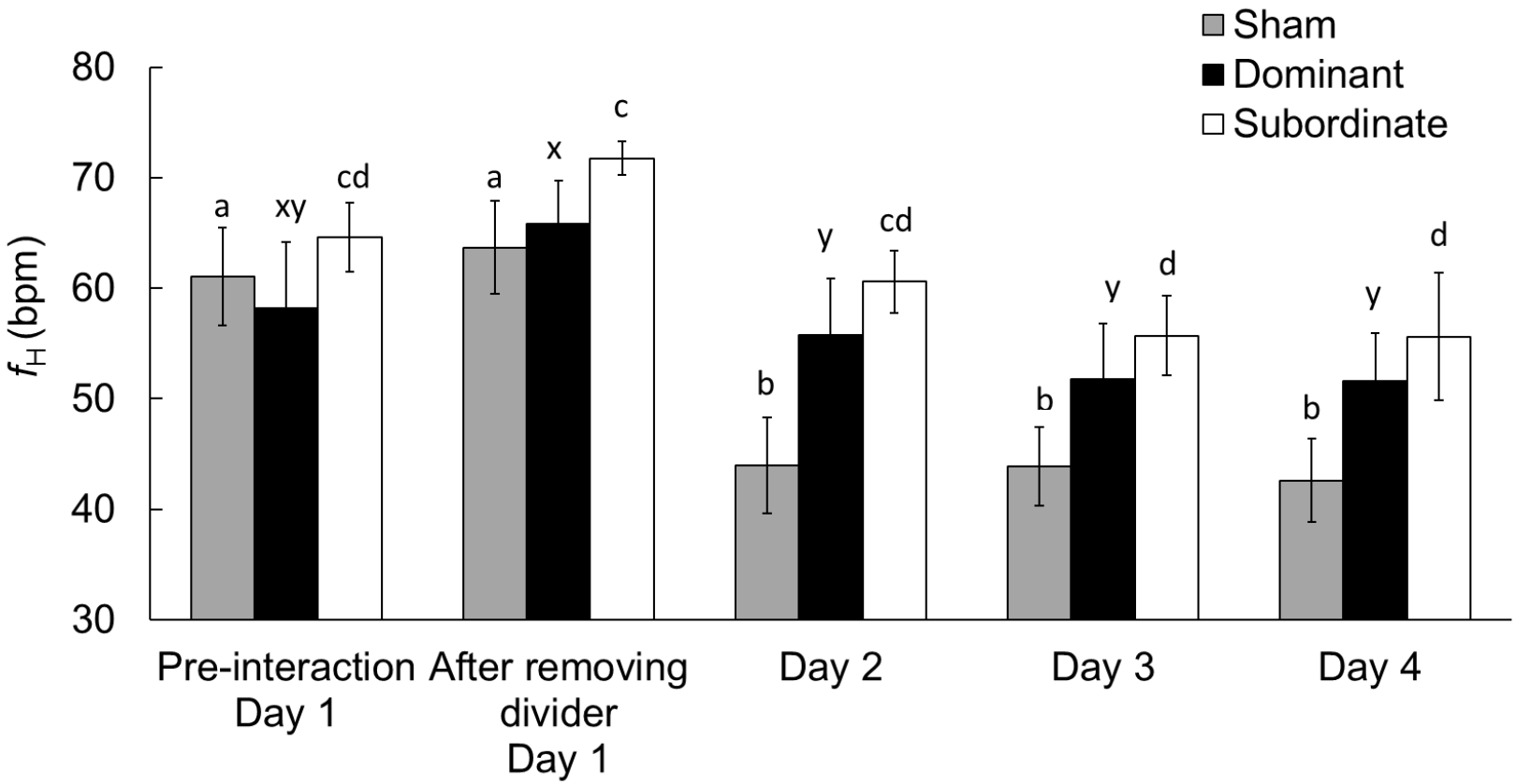
Heart rate during the interaction period was analyzed by a two-way repeated measures (RM) analysis of variance (ANOVA) using social status (dominant, subordinate or sham) as a between-subject factor and time (day) as a within-subject factor. For f_H at 13°C, peak Δf_H, and temperature at peak Δf_H, one-way ANOVA was used to assess effects of social status. Heart rate during the CTmax trial were analyzed by linear regressions. The slopes of regressions of f_H during a CTmax trial on temperature between dominant and subordinate were compared using analysis of covariance (ANCOVA). The statistical significance of differences between dominant and subordinate fish in transcript abundances, ventricular mass and relative ventricular mass, relative compact myocardium thickness and collagen protein abundance were determined using Student's *t*-tests. Collagen content was analyzed by one-way ANOVA.

3.3 Results

3.3.1 Heart rate during social interactions and acute warming

Measurements of f_H using biologgers during social interactions revealed significant effects of time but not social status, with a significant interaction between these factors (2-way RM ANOVA, $P = 0.184$ for status, $P < 0.001$ for time, $P = 0.020$ for status x time). Heart rate did not differ among sham, dominant and subordinate fish at any time point (Fig. 3.4; Holm-Sidak *post hoc*, $P > 0.1$). Among all groups, f_H was significantly higher on day 1, following the removal of the divider, than on the final day (Fig. 3.4; Holm-Sidak *post hoc*, $P \leq 0.002$).

Figure 3. 4. Heart rate (f_H) of dominant, subordinate and sham rainbow trout (*Oncorhynchus 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 ± SEM (*N*= 6 sham, 8 dominant and 8 subordinate). Bars that share a letter within a social status are not significantly different from one another (2-way RM ANOVA; Status $P = 0.184$, time $P < 0.001$, Status x time $P = 0.020$).


Sham
Dominant
Subordinate
80
70
60
50
40
30
f_H (bpm)
a
xy
cd
a
x
c
b
y
cd
b
y
d
b
y
d
Pre-interaction Day 1
After removing divider Day 1
Day 2
Day 3
Day 4

Heart rate was also measured during a CTmax trial after the 4 d interaction period. Resting f_H values prior to warming were similar to those on day 4 of the social interaction period and did not differ among dominant, subordinate and sham fish (Fig. 3.5A; one-way ANOVA, $P = 0.475$). During acute warming, there were no significant differences in the magnitude of the f_H response (Fig 3.5B; one-way ANOVA, $P = 0.813$), but there was a trend for the temperature at which the peak difference in f_H occurs, which tended to be lower in subordinate trout (Fig 3.5C; one-way ANOVA, $P = 0.059$). The temperature that arrhythmias in f_H first occurs is also significantly lower in subordinates (Fig 3.6; one way ANOVA, $P = 0.002$). Regressions of f_H on temperature were significant, with the slope at which heart rate increases with warming being significantly lower in subordinate relative to dominant animals (Fig 3.7; ANCOVA, $P = 0.049$).

Figure 3. 5. Measurements of heart rate (f_H) of sham (N = 6), dominant (N = 6) and subordinate (N = 6) rainbow trout during a CTmax trial. (A) f_H at 13°C, (B) peak Δf_H, and (C) temperature at which the peak Δf_H occurred (one-way ANOVA; P = 0.475, 0.813, 0.059 for panels A to C, respectively). Data are presented as a box plot where the upper and lower limits of the box represent the 75^{th} and 25^{th} percentiles, respectively, the red line across the box represents the mean value, the whiskers represent the maximum and minimum values, and each red circle presents the value for an individual fish.

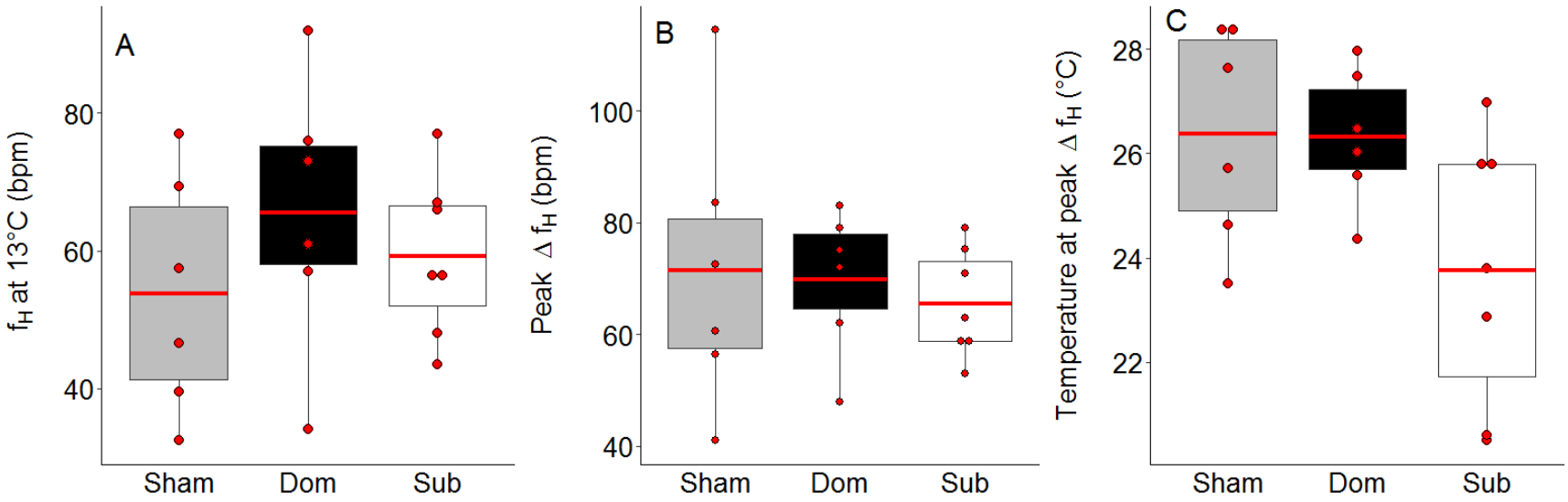

A
f_H at 13°C (bpm)
80
60
40
Sham
Dom
Sub
B
Peak Δ f_H (bpm)
100
80
60
40
Sham
Dom
Sub
C
Temperature at peak Δ f_H (°C)
28
26
24
22
Sham
Dom
Sub

Figure 3. 6. Temperature at which cardiac arrhythmia was first detected in sham (N = 5), dominant (N = 5), and subordinate (N = 6) rainbow trout (*Oncorhynchus mykiss*) exposed to acute warming during a CTmax trial. The appearance of arrhythmias was detected from scrutiny of ECG traces collected every 3 min; a representative series of ECG traces is presented in panel B (see section 3.2.2 for details). Bars that share a letter are not significantly different from one another (one-way ANOVA, P = 0.002). Data are presented as a box plot; see the legend of Fig. 3.5 for details. Note that one sham and one dominant fish did not exhibit arrhythmia.

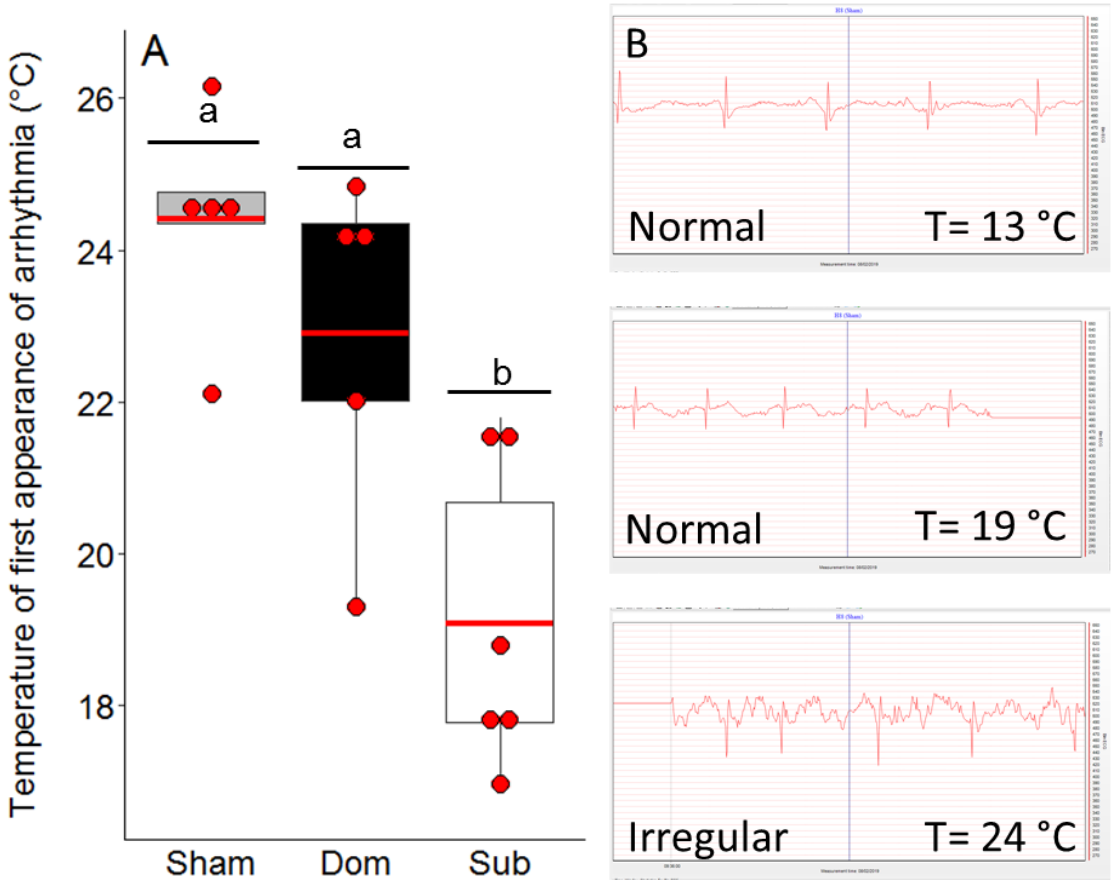
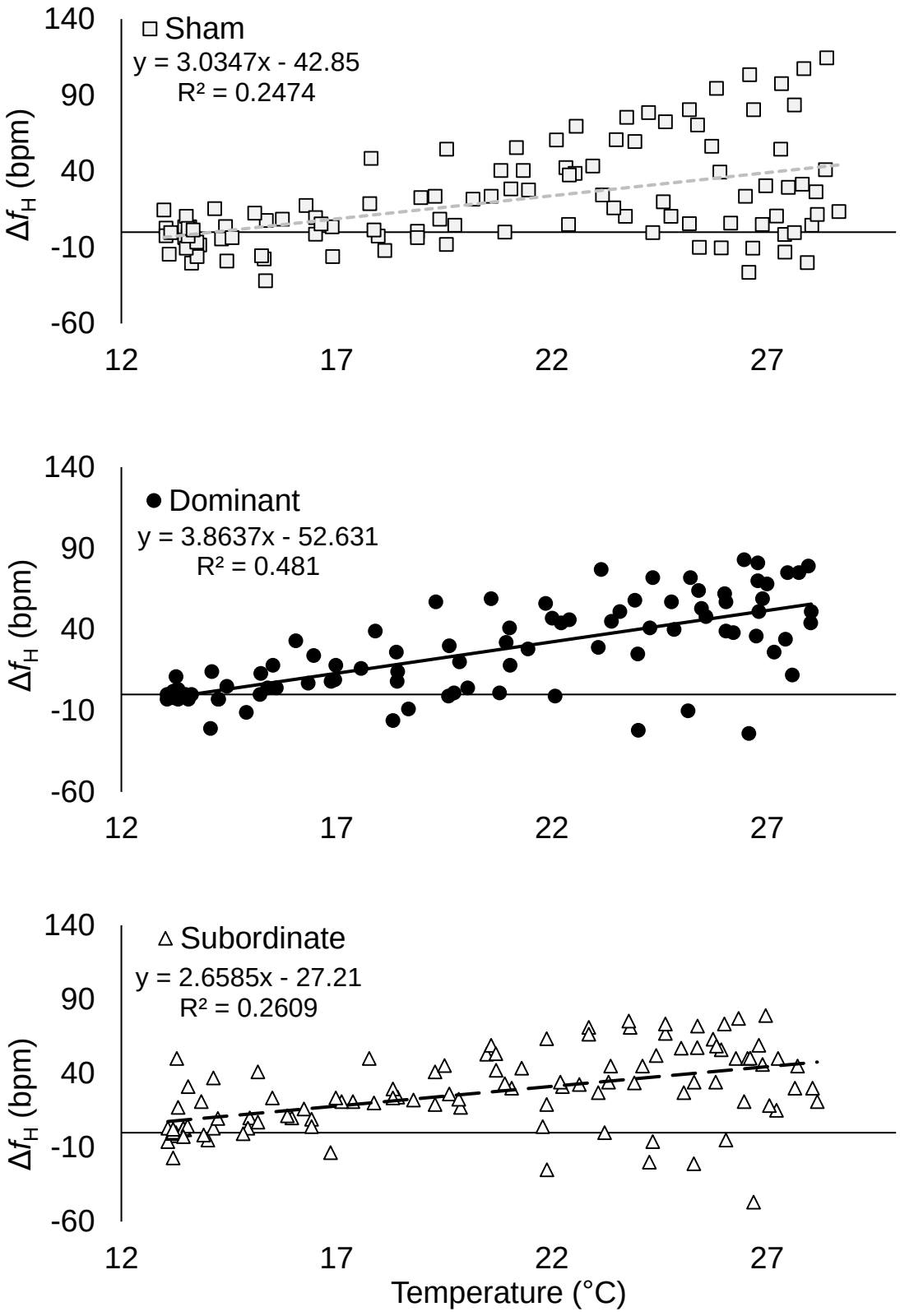

A
Temperature of first appearance of arrhythmia (°C)
26
24
22
20
18
a
a
b
Sham
Dom
Sub
B
Normal
T= 13 °C
Normal
T= 19 °C
Irregular
T= 24 °C

Figure 3.7. Change in heart rate (f_H) of rainbow trout (*Oncorhynchus mykiss*) during acute warming for sham (108 points for $N = 6$ fish; Linear regression, $P < 0.0001$), dominant (90 points for $N = 8$ fish; Linear regression, $P < 0.0001$) and subordinate (109 points for $N = 8$ fish; Linear regression, $P < 0.0001$).


Sham
y = 3.0347x - 42.85
R² = 0.2474
Dominant
y = 3.8637x - 52.631
R² = 0.481
Subordinate
y = 2.6585x - 27.21
R² = 0.2609
Δf_H (bpm)
140
90
40
-10
-60
12
17
22
27
Temperature (°C)

3.3.2 Cardiac remodelling in rainbow trout

To examine the potential for cardiac remodelling as a consequence of the cortisol response to chronic social stress, transcript abundances of the three cortisol receptors in rainbow trout, *gr1*, *gr2* and *mr*, were assessed in ventricle after 4 d of social interaction. Although no significant differences were detected between dominant and subordinate trout, *gr2* exhibited a trend towards higher transcript abundance in subordinate fish (Fig. 3.8; 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 either absolute ventricle mass or ventricular mass expressed relative to body mass (Fig. 3.9; Student's *t*-tests, P = 0.31 and 0.21, respectively), or in the relative thickness of the compact myocardium (Fig. 3.10 and Appendix 3; Student's *t*-test, P = 0.78). Because the 4-d interaction period 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 (Fig. 3.11). 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 between dominant and subordinate trout in transcript abundances of these molecular markers (Student's *t*-tests, P = 0.39 for *mlp*, 0.37 for *smlc2* and 0.55 for *vmhc*).

Figure 3.8. Relative mRNA abundance of glucocorticoid receptors (A) *gr1*, and (B) *gr2*, and (C) the mineralocorticoid receptor (*mr*) in the ventricle of dominant (N = 5) and subordinate (N = 6) rainbow trout (*Oncorhynchus mykiss*). Transcript abundances were normalized to the housekeeping gene *β-actin* and expressed relative to the value for the dominant fish. No significant differences were detected (Student's t-tests, P = 0.53, 0.062, 0.71, for panels A to C, respectively). Data are presented as box plots; see the legend of Fig. 3.5 for details.

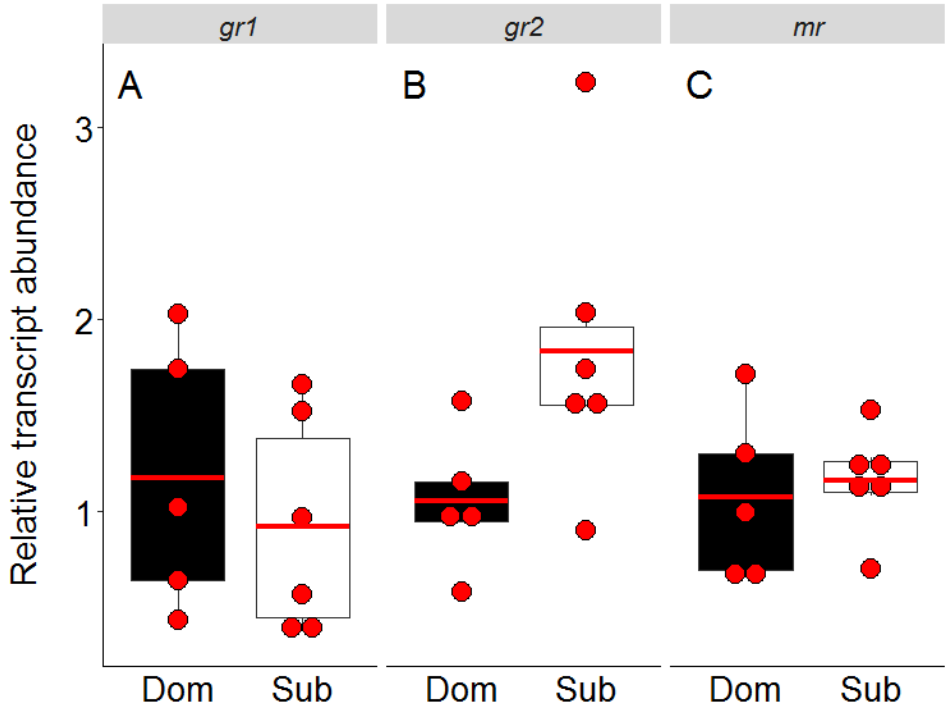

gr1
gr2
mr
A
B
C
Relative transcript abundance
3
2
1
Dom
Sub
Dom
Sub
Dom
Sub

Figure 3.9. (A) Absolute and (B) relative ventricular wet mass of dominant (N = 8) and subordinate (N = 8) rainbow trout (*Oncorhynchus mykiss*) after 4 d of social interaction. Relative mass was standardized using the mass of the individual fish after 4 d of interaction. No significant differences were detected (Student's t-tests, P = 0.31 and 0.21 for panels A and B, respectively). Data are presented as box plots; see the legend of Fig. 3.5 for details.

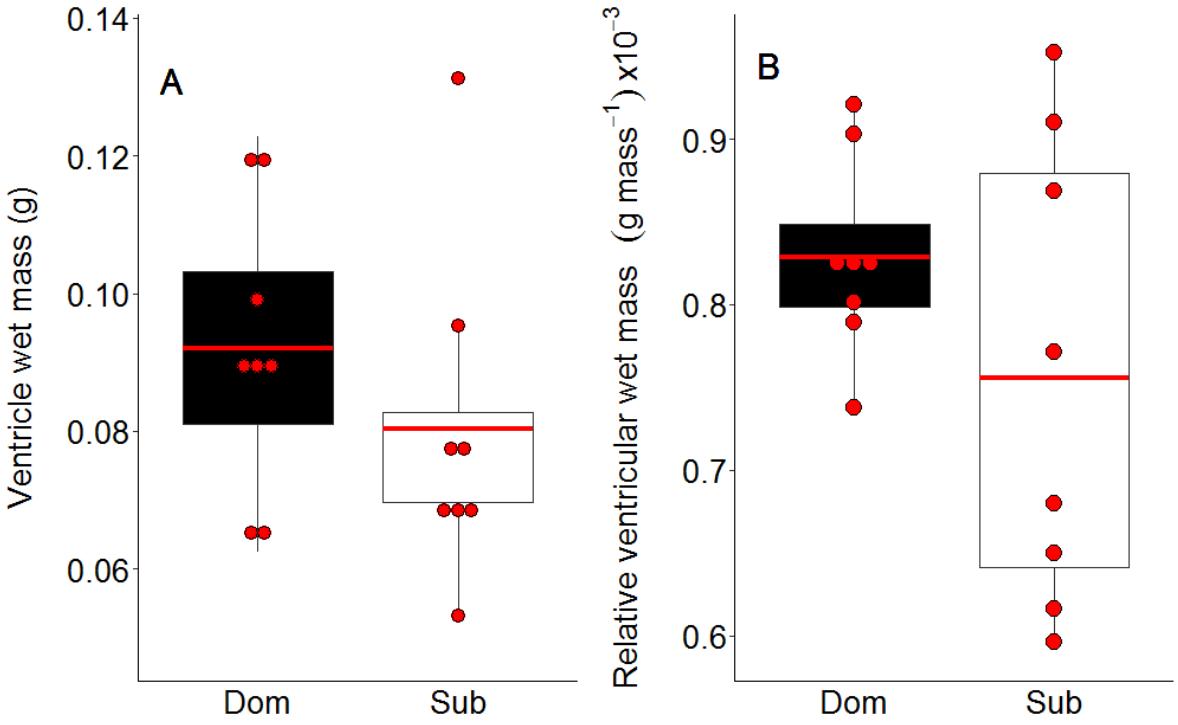

A
Ventricle wet mass (g)
0.14
0.12
0.10
0.08
0.06
Dom
Sub
B
Relative ventricular wet mass (g mass^{-1}) x10^{-3}
0.9
0.8
0.7
0.6
Dom
Sub

Figure 3.10. Relative compact myocardial thickness assessed histologically from sections of ventricle tissue stained with Picrosirius red for dominant (N = 5) and subordinate (N = 5) rainbow trout (*Oncorhynchus mykiss*) after 4 d of social interaction. No significant difference was detected between dominant and subordinate fish (Student's t-test, P = 0.78). Data are presented as a box plot; see the legend of Fig. 3.5 for details. Representative images of tissue sections from (B) dominant and (C) subordinate trout; arrows indicate the compact myocardial thickness.

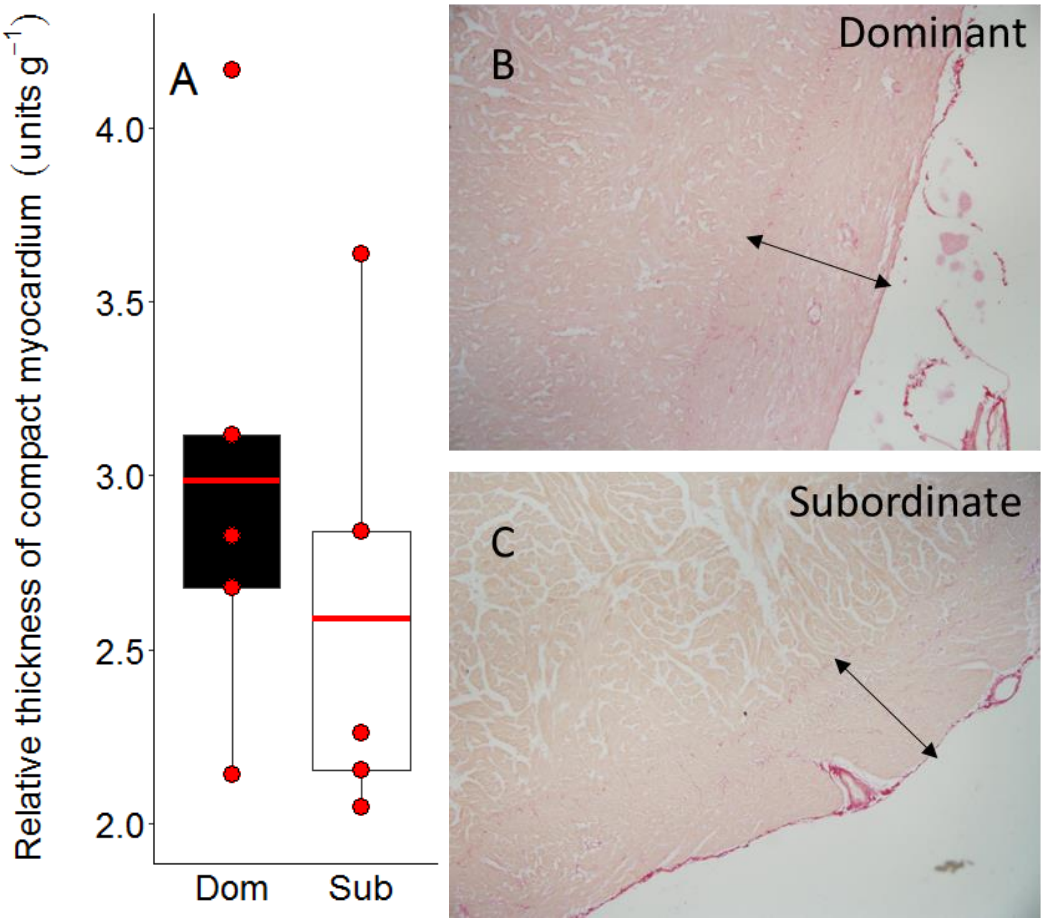
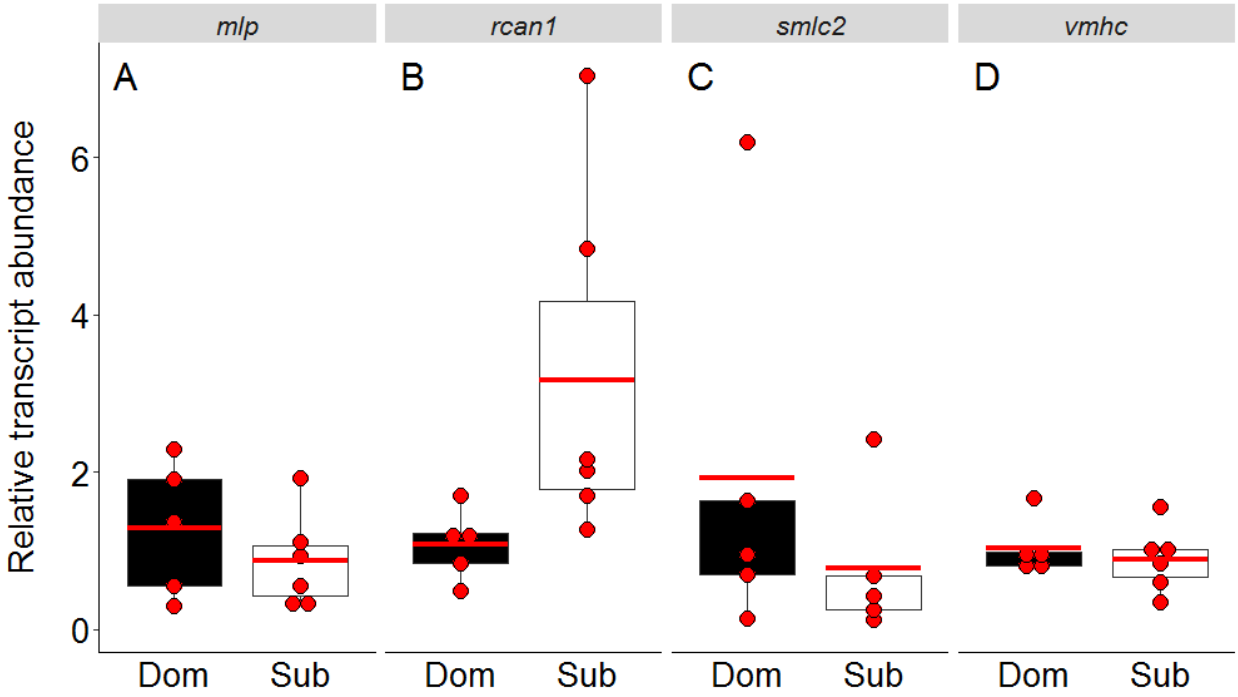

A
B
Dominant
C
Subordinate
Relative thickness of compact myocardium (units g^{-1})
4.0
3.5
3.0
2.5
2.0
Dom
Sub

Figure 3.11. Relative mRNA abundances of molecular markers associated with cardiomyocyte hypertrophy, (A) *mlp*, (B) *rcan1*, (C) *smlc2*, and (D) *vmhc* in the ventricle of dominant (N = 5) and subordinate (N = 6) rainbow trout (*Oncorhynchus mykiss*). Transcript abundances were normalized to the housekeeping gene *β-actin* and expressed relative to the value for the dominant fish. No significant differences were detected (Student's t-tests, P = 0.39, 0.075, 0.37, 0.55, for panels A to D, respectively). Data are presented as box plots; see the legend of Fig. 3.5 for details. *mlp*, muscle LIM protein; *rcan1*, regulator of calcineurin I; *smlc2*, slow myosin light chain 2; *vmhc*, ventricular myosin heavy chain.


mlp
rcan1
smlc2
vmhc
A
B
C
D
Relative transcript abundance
0
2
4
6
Dom
Sub
Dom
Sub
Dom
Sub
Dom
Sub

Histological assessment of ventricular collagen content using Picrosirius red revealed a significant effect of social status, with subordinate fish exhibiting lower collagen content than dominant or sham-treated trout (Fig. 3.12 and Appendix 4; one-way ANOVA, $P = 0.044$). Sham-treated trout were included in this analysis as a benchmark of what would be considered to be typical collagen content. To explore the physiological basis of this difference in collagen content, collagen type I protein levels (Fig. 3.13) and transcript abundances (Fig. 3.14) were evaluated together with transcript abundances of two matrix metalloproteinases (*mmp*) and their inhibitor (*timp2*) involved in collagen degradation (Fig. 3.15). 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, α 2 and α 3 (Keen et al. 2016). However, neither type I collagen protein abundance (Student's *t*-test, $P = 0.98$) nor transcript abundances of the three alpha-helical chains, *col1a1*, *col1a2* and *col1a3* (Student's *t*-tests, $P = 0.41$, 0.25, 0.27, respectively), differed between the ventricles of dominant and subordinate trout after 4 d of social interaction. Similarly, no significant differences in the transcript abundances of *mmp2*, *mmp9* and *timp2* were detected (Student's *t*-tests, $P = 0.55$, 0.78, 0.15, respectively).

Figure 3.12. Relative ventricular collagen content assessed histologically from tissue sections stained with Picrosirius red for dominant (N = 6) and subordinate (N = 6) rainbow trout (*Oncorhynchus mykiss*) after 4 d of social interaction (A). Collagen content was quantified as the percentage of the tissue in the micrograph showing red staining (see section 3.2.3 for details). An asterisk indicates a significant effect of social status (one-way ANOVA, P = 0.044). Data are presented as a box plot; see the legend of Fig. 3.5 for details. Representative images of ventricle tissue sections from (B) dominant and (C) subordinate trout stained with Picrosirius red are also presented.

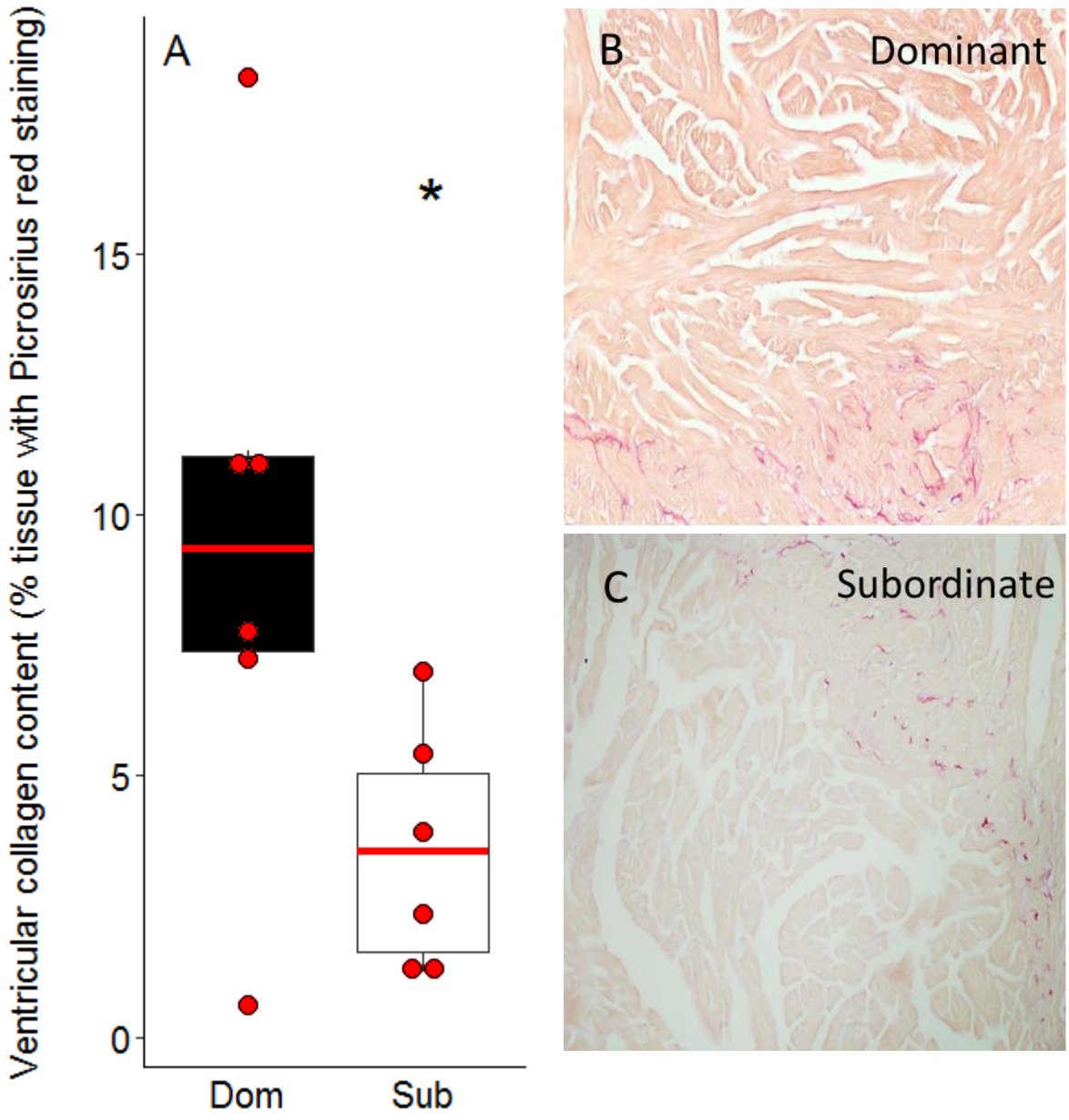

A
Ventricular collagen content (% tissue with Picrosirius red staining)
15
10
5
0
Dom
Sub
*
B
Dominant
C
Subordinate

Figure 3.13. The effect of social interaction on collagen type I protein levels in the ventricle of dominant (N = 6) versus subordinate (N = 6) rainbow trout (*Oncorhynchus mykiss*). (A) Normalized collagen type I abundance in ventricular tissue. The band at 130 kDa was quantified (Johnston et al. 2019) and expressed relative to total protein. No significant effect of social status was detected (Student's t-test, P = 0.98). Data are presented as a box plot; see the legend of Fig. 3.5 for details. (B) An image of the western blot from which collagen type I protein abundance was measured by densitometry. *S*, subordinate; *D*, dominant; *C*, a sample used for cross-blot normalization.

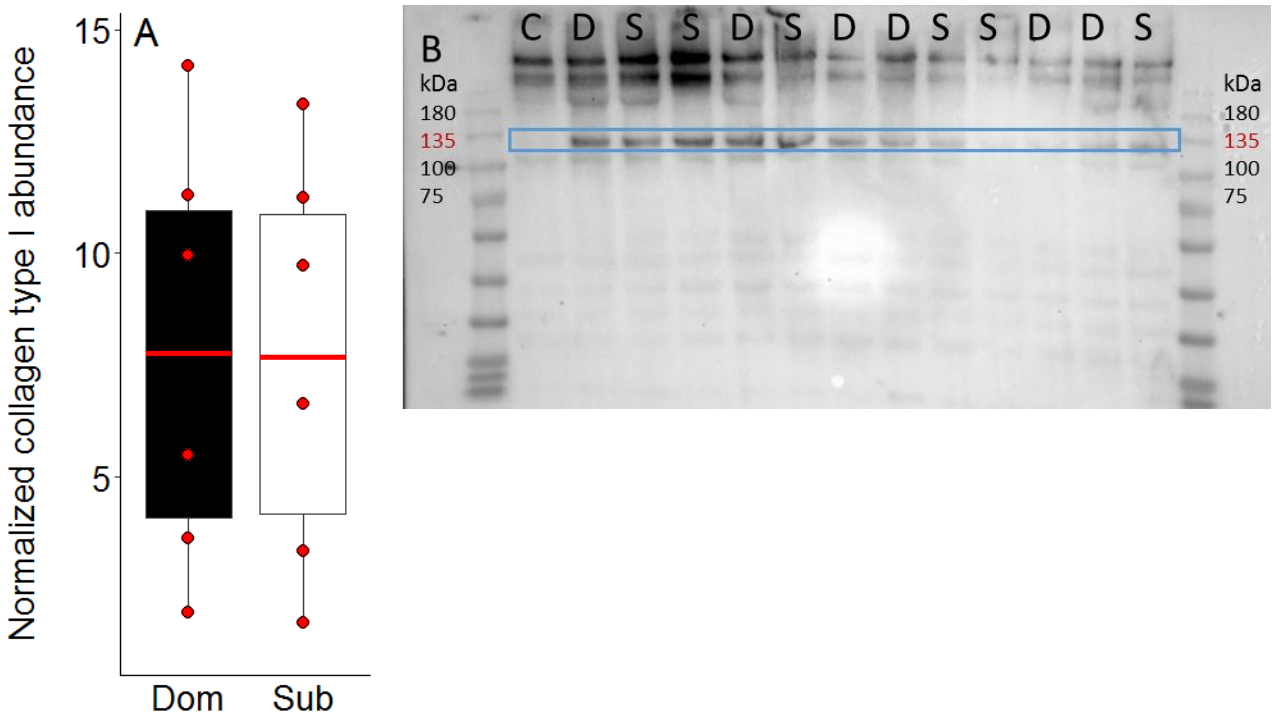

A
Normalized collagen type I abundance
15
10
5
Dom
Sub
B
kDa
180
135
100
75
C D S S D S D D S S D D S
kDa
180
135
100
75

Figure 3. 14. Relative mRNA abundances of collagen alpha-chain isoforms (A) *col1a1*, (B) *col1a2*, and (C) *col1a3* in ventricular tissue of dominant (N = 5) and subordinate (N = 6) rainbow trout (Oncorhynchus mykiss) after 4 d of social interaction. Transcript abundances were normalized to the housekeeping gene *β-actin* and expressed relative to the value for the dominant fish. No significant differences were detected (Student's t-tests, P = 0.41, 0.25, 0.27, for panels A to C, respectively). Data are presented as box plots; see the legend of Fig. 3.5 for details.

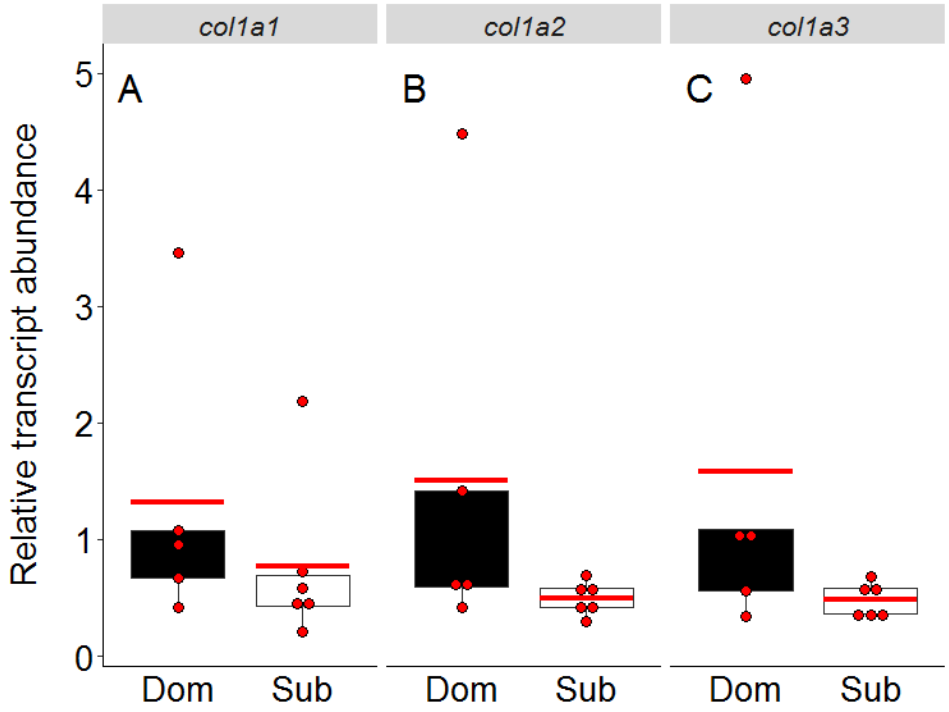
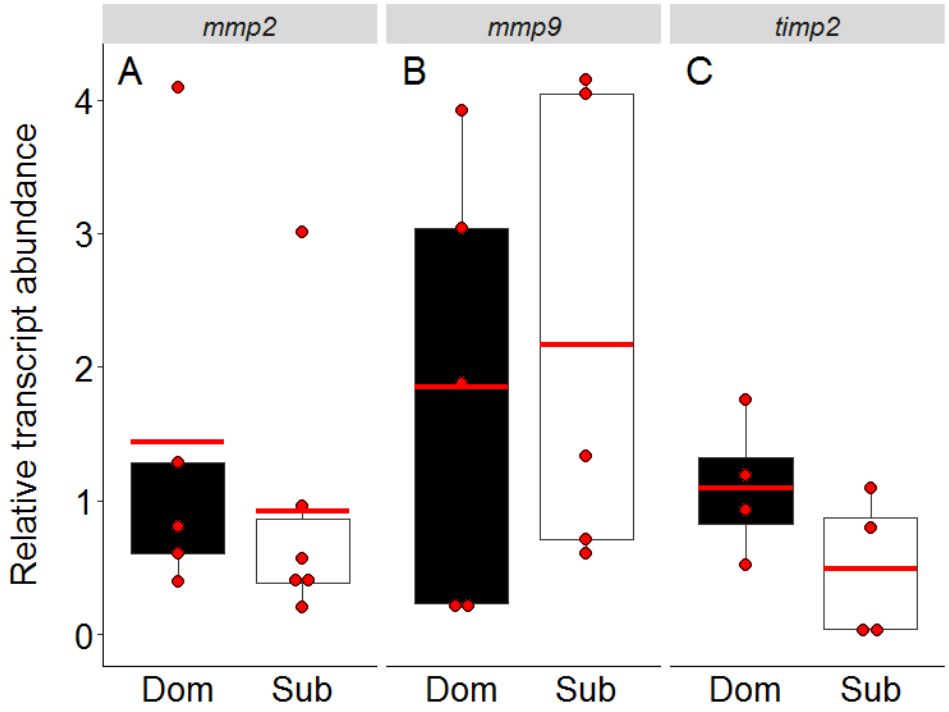

col1a1
col1a2
col1a3
A
B
C
Relative transcript abundance
0
1
2
3
4
5
Dom
Sub
Dom
Sub
Dom
Sub

Figure 3.15. Relative mRNA abundances of matrix metalloproteinases (A) *mmp2*, and (B) *mmp9*, and (C) the tissue inhibitor of metalloproteinases 2 (*timp2*) in ventricular tissue of dominant (N = 5) and subordinate (N = 6) rainbow trout (*Oncorhynchus mykiss)* after 4 d of social interaction. Transcript abundances were normalized to the housekeeping gene *β-actin* and expressed relative to the value for the dominant fish. No significant differences were detected (Student's t-tests, P = 0.55, 0.78, 0.15, for panels A to C, respectively). Data are presented as box plots; see the legend of Fig. 3.5 for details. *mmp2*, matrix metalloproteinase 2; *mmp9*, matrix metalloproteinases 9; *timp2*, tissue inhibitor of metalloproteinases 2


mmp2
mmp9
timp2
A
B
C
Relative transcript abundance
0
1
2
3
4
Dom
Sub
Dom
Sub
Dom
Sub

3.4 Discussion

The use of f_H loggers allowed f_H to be monitored in pairs of rainbow trout engaged in social interactions. 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; Höjesjö et al., 2015). Heart rates of dominant and subordinate trout were also determined from blood pressure traces, but in this case the fish were separated after the establishment of a hierarchy for instrumentation and measurements (Thomas and Gilmour, 2012). Thus, in neither case was it possible to monitor f_H during hierarchy formation. The use of f_H loggers in the present study revealed differential effects of social status on f_H during hierarchy formation. Whereas 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, f_H was slower to decrease in subordinate fish, suggesting greater metabolic expenditure in these fish during the early stages of hierarchy establishment. Similarly, Thomas and Gilmour (2012) reported elevated f_H in subordinate fish.

Chronic social stress also impacted the f_H response to acute warming. During acute warming to measure CTmax, the expected increase in f_H with increasing temperature was apparent in all groups (Clark et al., 2008; Farrell et al., 1996; Kurt Gamperl et al., 2011; Steinhausen et al., 2008), 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 significant differences between dominant and subordinate trout in the slope of the relationship between f_H and temperature. That is, dominants appeared to be able to maintain increases in f_H to higher temperatures than subordinate fish. Correspondingly, irregularities in the electrical

activity of the heart were detected at significantly lower temperatures in subordinate than dominant or sham-treated trout.

These differences in the f_H response to thermal stress suggest that differences in cardiac function may exist between subordinate and dominant trout that potentially could contribute to differences in thermal tolerance (Ch. 2). To date, experimental evidence that explains the plateau in f_H during warming remains sparse. Alterations in cellular electrical excitability occur at higher temperatures and may set thermal limits for cardiac action potentials, ultimately disrupting the frequency of heart beats (Vornanen, 2016). In particular, it appears that at high temperature, the ventricle fails to rhythmically contract in response to the atrium owing to an atrioventricular (AV) block. The AV block is caused by reduced excitability in the ventricle, which, in turn, reflects differential changes in Na^+ and K^+ conductance with temperature (Badr et al., 2018; Vornanen, 2016; Vornanen et al., 2014). However, how the prolonged elevation of cortisol experienced by subordinate fish could contribute to changes in cardiac ion conductance that alter the temperature sensitivity of the ventricle remains to be determined. More detailed ECG data during acute warming of dominant versus subordinate trout (Badr et al., 2016; Vornanen et al., 2014), as well as analysis of the ventricular myocytes of dominant versus subordinate fish would be useful in this regard. One possibility is that social stress-induced fibrotic remodelling of the ventricle leads to alterations in conduction properties. Hypertrophy accompanied by elevated collagen expression and deposition was detected in the ventricle of a strain of rainbow trout selected for a high cortisol response to stress (HR trout; Johansen et al. 2011). This type of fibrotic hypertrophy occurs in mammalian hearts in response to ageing and pathological stressors such as hypertension, altering the conduction properties of the heart and leading to cardiac arrhythmia (Bernardo et al., 2010; Horn and Trafford, 2016). In rainbow trout, cold acclimation

initiates fibrotic cardiac hypertrophy accompanied by a reduction in f_H, although in this case the cardiac remodelling is not viewed as pathological (Klaiman et al. 2011; Keen et al. 2016; Vornanen et al. 2002). Given these associations among chronic stress, cardiac remodelling and arrhythmogenesis, the possibility that social stress induces cardiac remodelling in subordinate rainbow trout was investigated.

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 d (Johansen et al. 2017; Norstrud et al. 2018). Because cortisol treatment for shorter periods altered transcript abundance of molecular markers of hypertrophic signalling and remodelling without significantly affecting ventricle mass (Norstrud 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*), and muscle LIM protein (*mlp*) did not differ significantly between dominant and subordinate trout, but ventricles from subordinate trout tended to show higher transcript abundance of regulator of calcineurin 1 (*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 4 d interaction period of the present study is needed to elicit cardiac 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. Similarly, the hearts of cold-acclimated trout are both larger and have greater collagen content than the

hearts of warm-acclimated trout (Keen et al., 2015). In cold-acclimated trout, the higher collagen content of the heart increases stiffness, increasing contractile force and hence cardiac output (Keen et al., 2015). By contrast, histological analysis of ventricle tissue sections revealed 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. Western analysis of collagen type I protein levels yielded four bands, including the ~135 kDa band analyzed by Johnston et al. (2019) and in the present study (see Appendix 5). Investigations of collagen type I in zebrafish (*Danio rerio*) (Gistelinck et al., 2016), Japanese flounder (*Paralichthys olivaceus*) (Kimiya et al., 2005), and red sea bream (*Pagrus major*) (Touhata et al., 2002) suggest that the variety of bands produced in western analysis represents a mixture of different α and β amino acids chain of collagen type I caused by collagen degradation. Analysis of protein abundance using these bands also did not yield differences between dominant and subordinate trout (Appendix 5). The absence of differences in the transcript abundances of collagen type 1 genes or genes involved in the regulation of collagen degradation supports the analysis of protein abundance. The apparent fall in collagen abundance in subordinate fish revealed by histological analysis 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). Alternatively, the abundance of collagen isoforms other than type I may have decreased. Despite the significant reduction in collagen abundance detected by histological analysis, a functional analysis of ventricular pressure-volume relationships in isolated hearts did not reveal a difference in the passive stiffness of the ventricle between dominant and subordinate fish (A. Dodge, W. Joyce, B. Bard, K. Gilmour, unpublished

data). Thus, the physiological significance of the decrease in collagen abundance remains unclear.

To conclude, it appears that 4 d of elevated cortisol is not sufficient to induce pathological cardiac hypertrophy or fibrosis in subordinate rainbow trout. However, some evidence of differences in cardiac function in response to acute warming between subordinate and dominant fish was obtained, in particular a tendency for heart rate increases to peak at lower temperatures in subordinate fish. These data contribute to the evidence that thermal tolerance is reduced by the prolonged elevation of cortisol levels associated with chronic social stress.

Chapter 4: General Discussion

The current study tested whether high baseline cortisol levels impair the thermal tolerance of subordinate rainbow trout by inducing cardiac remodelling, ultimately hindering cardiac function. Data collected in the present thesis strongly supported the hypothesis that cortisol plays a role in determining thermal tolerance in subordinate trout, as demonstrated by subordinates that experienced a fall in cortisol levels as they recovered from social stress also show recovering CTmax values that were comparable to those of dominant fish. Perhaps most compelling was the observation that when recovering subordinates were treated with cortisol during the recovery period so as to maintain elevated cortisol levels, their CTmax was not restored. Because recent studies demonstrated that prolonged exposure to elevated cortisol induced cardiac remodelling in rainbow trout that impaired cardiac function, by lowering the capacity for aerobic exercise (Johansen et al., 2017; Nørstrud et al., 2018), and because several studies have linked CTmax and cardiac function (Ekström et al., 2019; Gilbert et al., 2019), the cardiac responses to social stress and acute warming were explored in subordinate trout.

During acute warming, f_H of subordinate trout demonstrated differences to dominant or sham-treated trout, with peak increases in f_H tending to occur at lower temperatures in subordinate fish. Thus, increases in f_H tended to plateau or peak near the CTmax regardless of social status, with peak f_H and CTmax 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; Ekström et al., 2017; Ekström et al., 2019; Gilbert et al., 2019). However, the change in f_H response to acute warming in subordinate trout did not appear to be a consequence of chronic stress-induced cardiac remodelling of the ventricle, because neither ventricle mass nor molecular markers used as indicators of cardiac hypertrophy exhibited differences between dominant and subordinate

trout. Only one difference was detected, a decrease in collagen abundance in subordinates quantified through histological analysis, although it was not associated with differences in transcript or protein abundances of collagen type I, or with differences in the transcript abundance of proteins involved in collagen dynamics. Given that the capacity for cortisol responsiveness was maintained in the heart (at least at the transcript level), the simplest explanation for the apparent absence of cortisol-induced cardiac hypertrophy in subordinate trout is that the exposure to elevated cortisol is not of sufficient length. Indeed, in a time-course study of cortisol-induced cardiac hypertrophy, Norstrud et al. (2018) reported effects of 7 d but not 2 d of cortisol treatment.

Collectively, the data reported in this thesis provide strong support for the impact of elevated cortisol on CTmax, but little support for social stress-induced cardiac remodelling within a 4 d period or for cardiac remodelling as a determinant of CTmax. On the latter point it is noteworthy that although Johansen et al. (2011) found cardiac hypertrophy and fibrosis in the high-responding (HR) trout line selected for a robust cortisol response to a standardized stressor (Pottinger and Carrick 1999), a separate study of thermal tolerance in these fish found no difference in CTmax between HR and low-responding (LR) trout (LeBlanc et al. 2012). This observation, like others, calls into question the importance of oxygen delivery limitations in determining thermal tolerance (Gräns et al., 2014; Lefevre et al., 2016; Mcdonnell et al., 2019; Motyka et al., 2017; Norin et al., 2014; Nyboer and Chapman, 2018; Wang et al., 2014). A key question arising from the current study therefore concerns the underlying physiological mechanisms through which cortisol acts to lower CTmax, and presumably thermal tolerance.

It is important to note that the endpoint of CTmax likely reflects neurological effects (Bilyk et al., 2012; Jutfelt et al., 2019), which suggests that identifying differences in the nervous

system of dominant versus subordinate trout would be a logical continuation of the present research. Both the glucocorticoid (GR1 and GR2) and mineralocorticoid (MR) receptors that mediate the effects of cortisol in fish are expressed in the brain of rainbow trout, providing a mechanism through which cortisol can influence nervous function (Alderman et al., 2012; Johansen et al., 2011b; Teitsma et al., 1998; Teles et al., 2013). In addition, 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 (Johansen et al., 2012; Sørensen et al., 2011; Sørensen et al., 2012).

An attractive target for additional research is to explore the role of transient receptor potential vanilloid (TRPV) channels, which are responsible for thermo-sensing among other sensory functions (Nilius and Owsianik, 2010). TRPV channels are among a family of TRP ion channels, which are involved in numerous sensory functions and ion homeostasis (Nilius and Owsianik, 2010). The thermally-activated TRPV channels allow the reception of heat to be converted into electrical signals that can be propagated to the peripheral and central nervous system to induce responses to maintain homeostasis (Patapoutian et al., 2003). Although research on TRPV channels in teleost fish to date has been limited, it is thought that teleost fish contain only TRPV1 and TPV4 channels (Boltana et al., 2018; Gau et al., 2013; Hunt et al., 2012; Kastenhuber et al., 2013; Nisembaum et al., 2015; Perálvarez-Marín et al., 2013; Saito and Shingai, 2006; Saito et al., 2011). TRPV1 channels have been identified along the lateral line in zebrafish larvae (Gau et al., 2013) and in the sperm of rohu (*Labeo rohita*) (Majhi et al., 2013), whereas both TRPV1 and TRPV4 have been demonstrated in the pineal organ of rainbow trout (Nisembaum et al., 2015) and the telencephalon and optic lobe of Atlantic salmon (*Salmo salar*)

(Boltana et al., 2018). It has also been confirmed that TRPV1 channels in zebrafish are activated by heat, suggesting TRPVs are involve in temperature sensing in teleost fish (Gau et al., 2013). Moreover, it has been demonstrated that *TRPV1* and *TRPV4* expression increases in developing zebrafish (2 to 7dpf), however this did not result in differences in the onset temperature of hyperthermia induced seizures (Hunt et al., 2012). Although, when 5 and 7dpf larvae were treated with a TRPV4 antagonist (RN-1734), there was a significant decrease in seizure activity, whereas a TRPV1 antagonist (capsazapine) had no effect on seizure activity. In virus-infected Atlantic salmon, TRPV4 remains downregulated even when exposed to increasing temperatures (Boltana et al., 2018), demonstrating prior stressors can induce changes in TPRV expression to heat. Among other vertebrates, TRPV channels are better characterized and studied (see reviews Saito and Tominaga, 2017; Tominaga and Tominaga, 2005), and there is evidence that differences in TRPV may be associated with differences in thermal preference and/or tolerance. For example, two closely-related species of clawed frog (*Xenopus* sp.) that are adapted to different thermal environments, showed differences in TRPV responses to heat stimulation (Saito et al., 2016). In mammals that show a wide degree of thermal tolerance, such as thirteen-lined ground squirrels (*Ictidomys tridecemilineatus*) and Bactrian camels (*Camelus ferus*), molecular differences in TRPV channels have been associated with decreased channel responses to heat (Laursen et al., 2016). More specifically, cloned TRPV1 channels of squirrels and camels failed to produce an electrical response to ramping temperatures from 22-46°C, in vitro. However, heat sensitivity could be restored in both squirrel and camel TRPV channels through a single conserved amino acid substitution. While in invertebrates, more specifically silverleaf whiteflies (*Bemisia tabaci*) it was demonstrated that decreased expression in *TRP* resulted in a higher mortality rate (lower heat tolerance) when exposed to high temperatures (Lü et al., 2014).

Collectively, these studies provide support for the idea that thermal tolerance can be linked to TRPV channel structure and function. Thus, study of the potential role of TRPV in cortisol-induced changes in thermal tolerance seems warranted, both to establish whether differences in thermal tolerance can be linked to changes in TRPV structure or function in rainbow trout, and to determine how cortisol affects this relationship.

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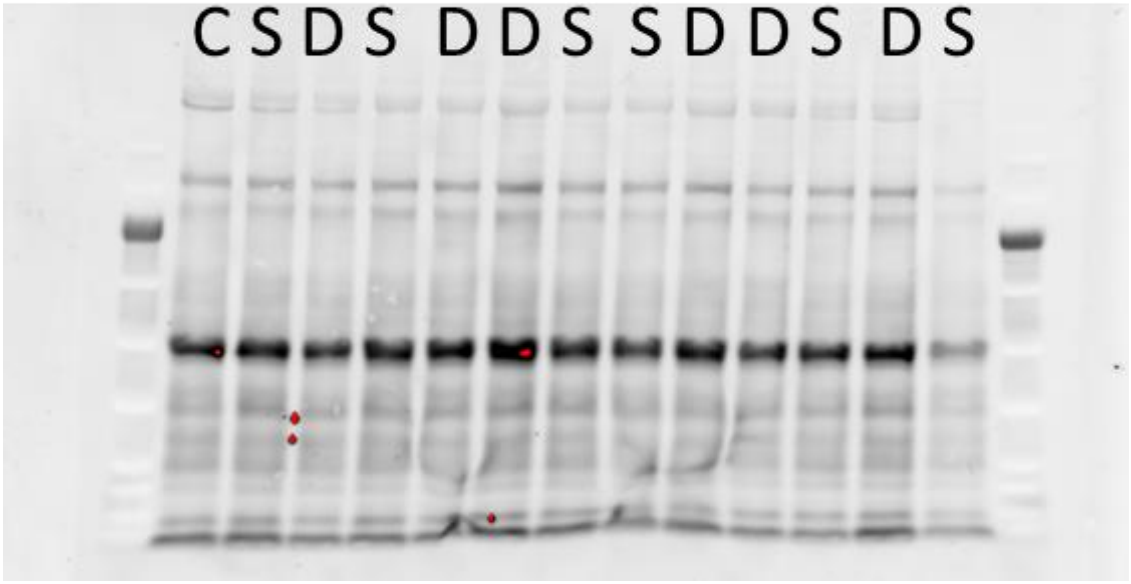
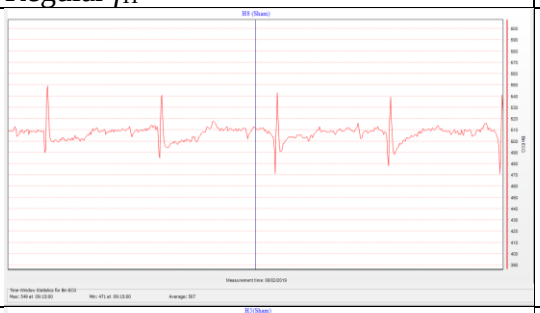
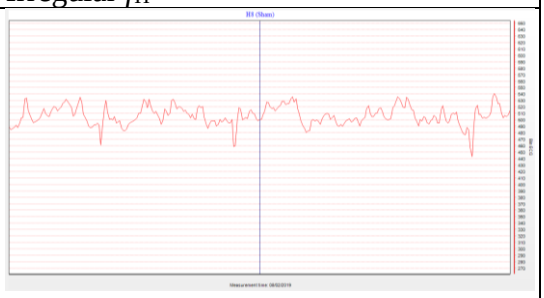
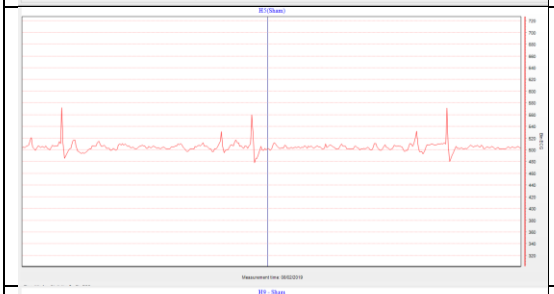
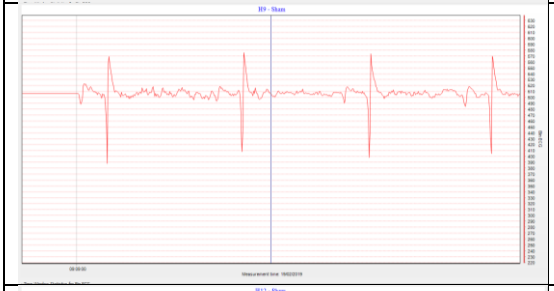
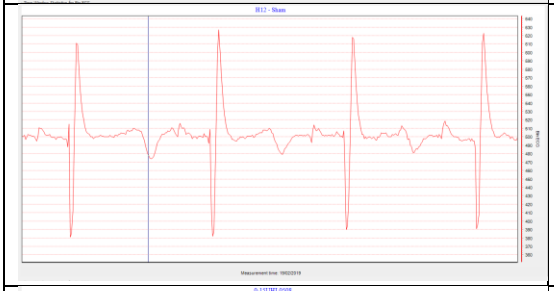
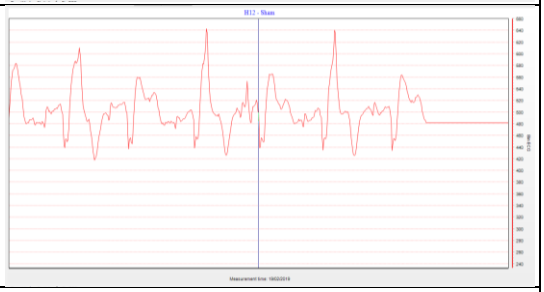
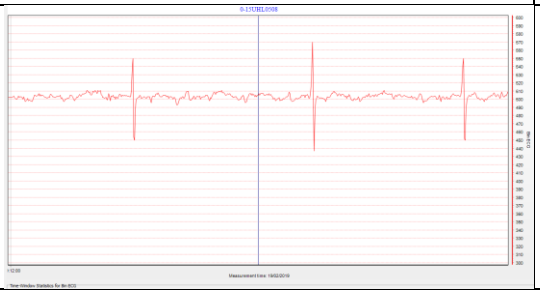
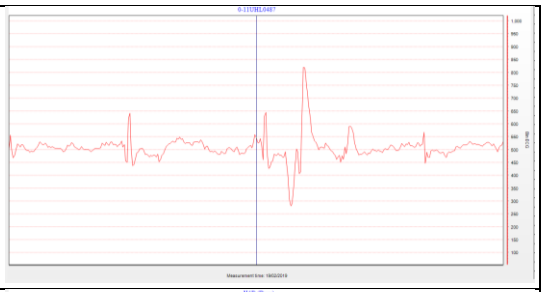
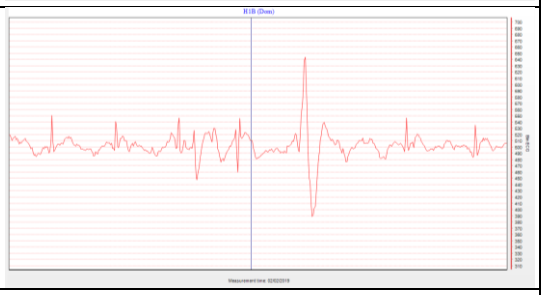
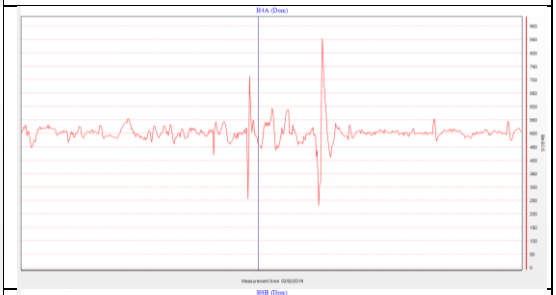
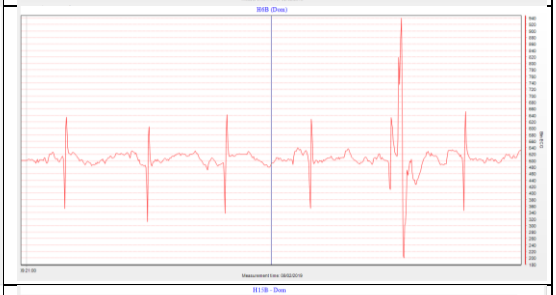
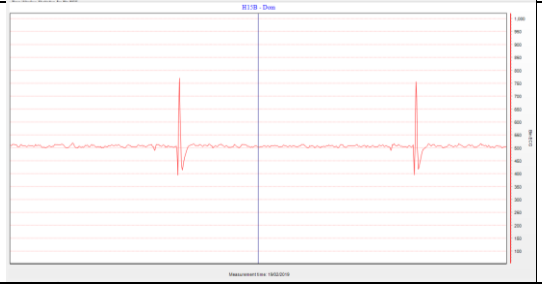
Appendices

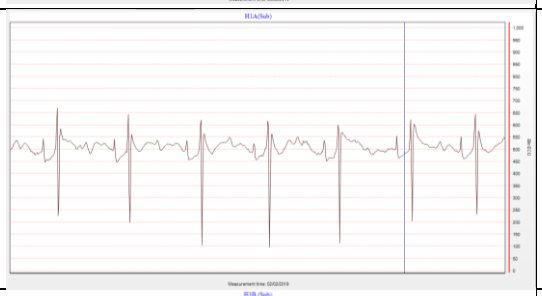
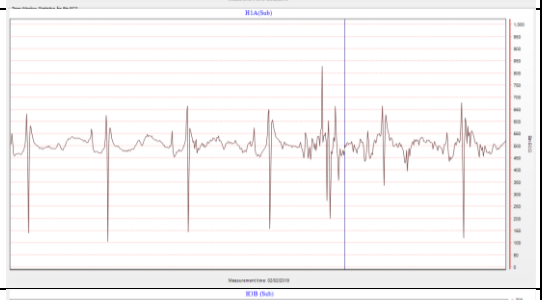
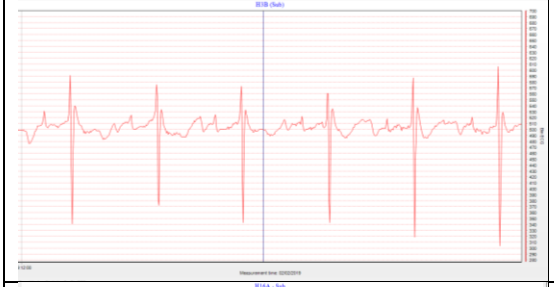
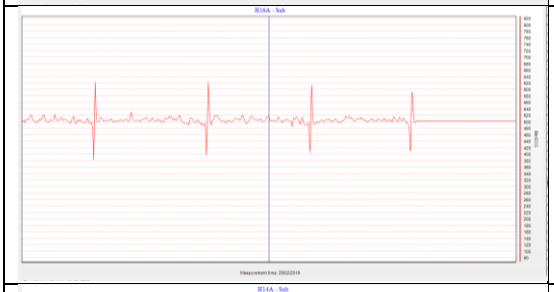
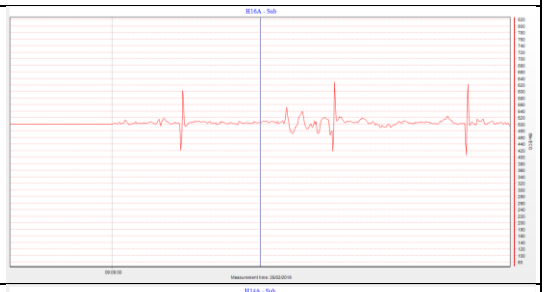
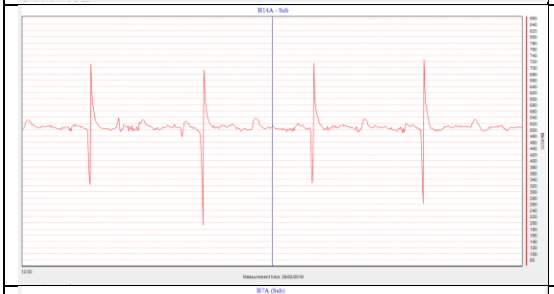
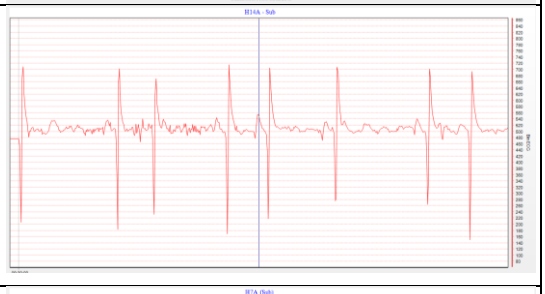
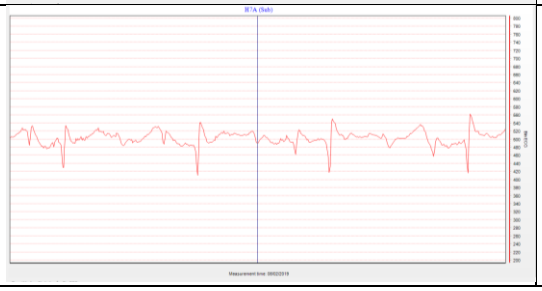
Appendix 1. An image of the western blot demonstrating total protein abundance utilized to normalize HSP70 protein abundance. *S*, subordinate; *D*, dominant; *C*, a sample used for cross-blot normalization.

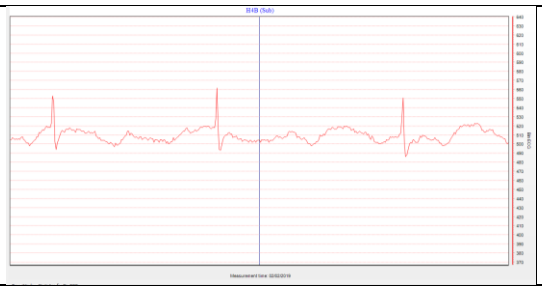
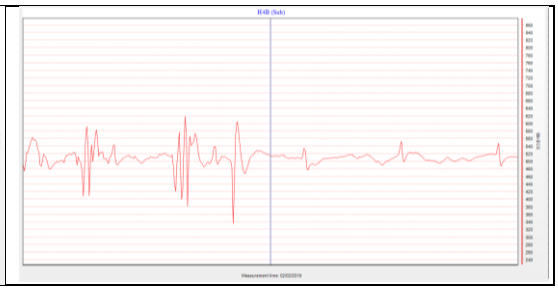


Appendix 2. Electrocardiogram (ECG) utilized to determine temperature at which irregular f_H occurred.

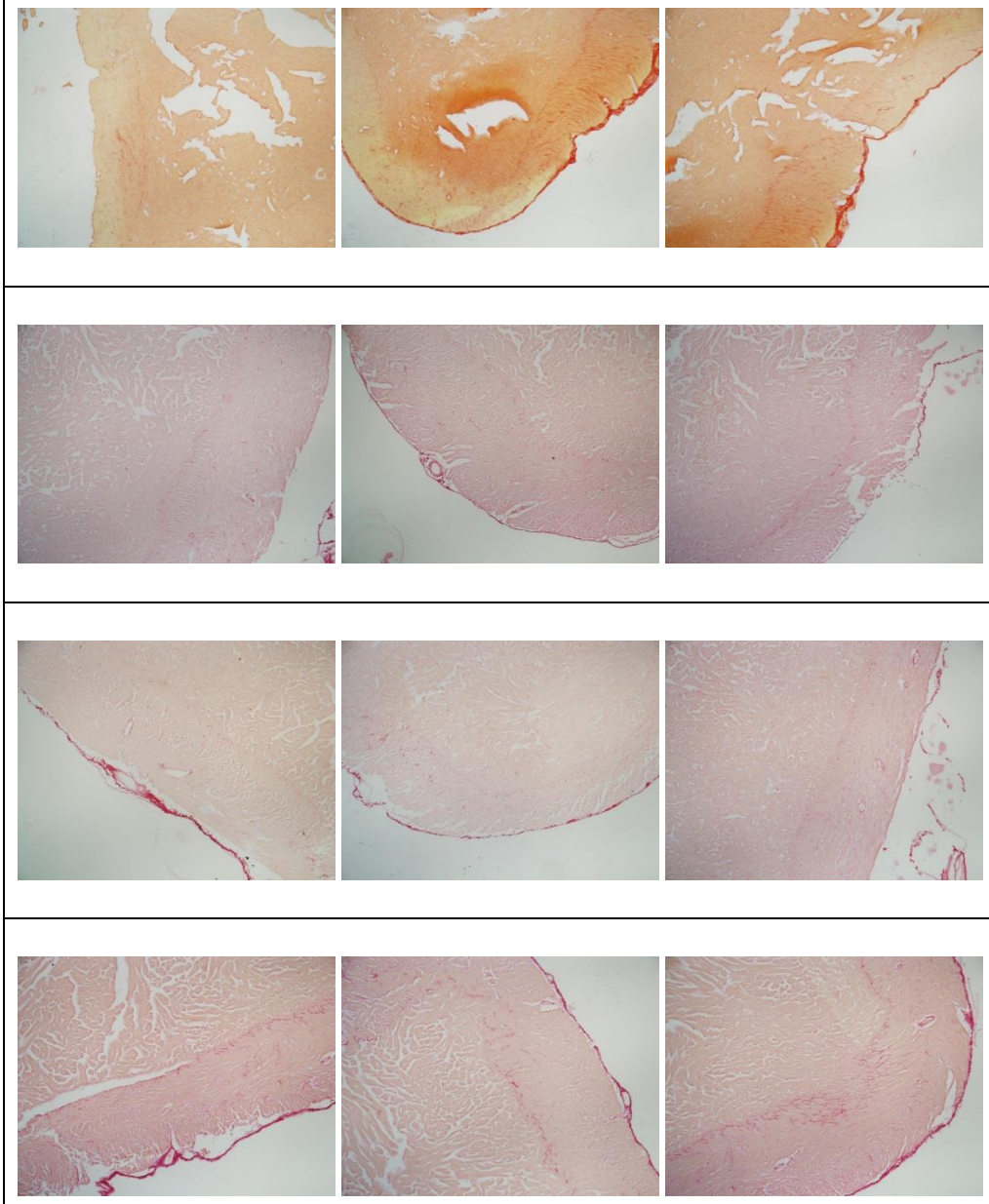
Group	Regular f_H	Irregular f_H
Sham		
		
		
		
		Did not show any f_H irregularities

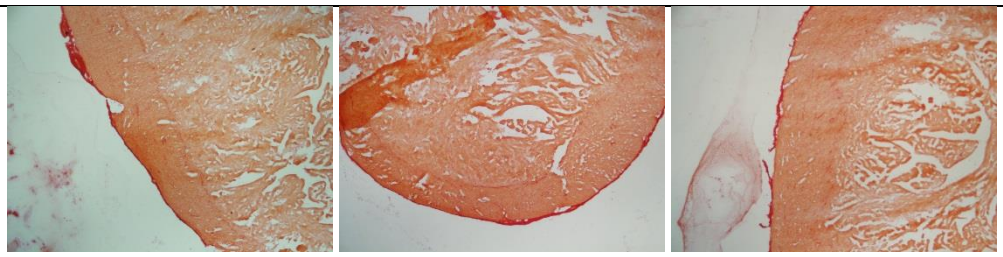
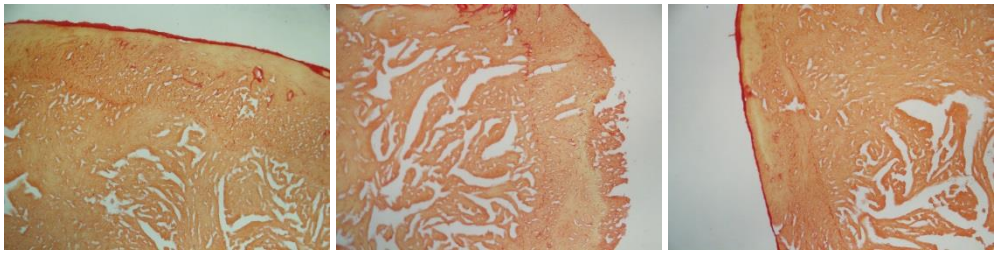
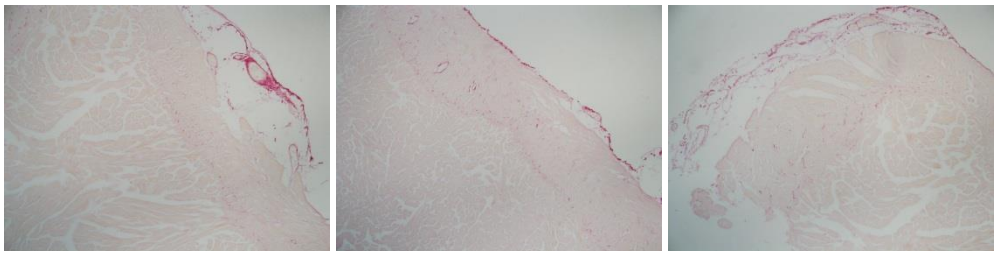
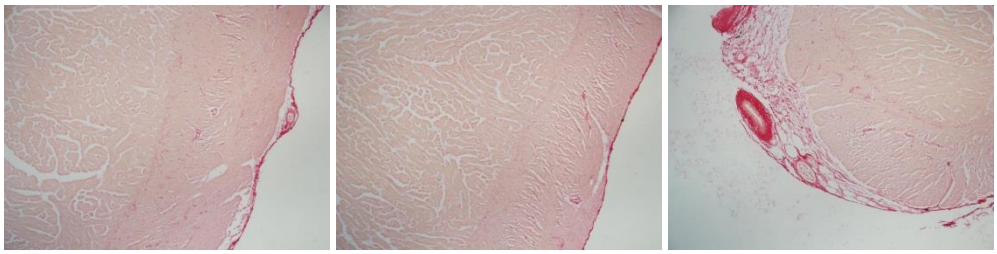
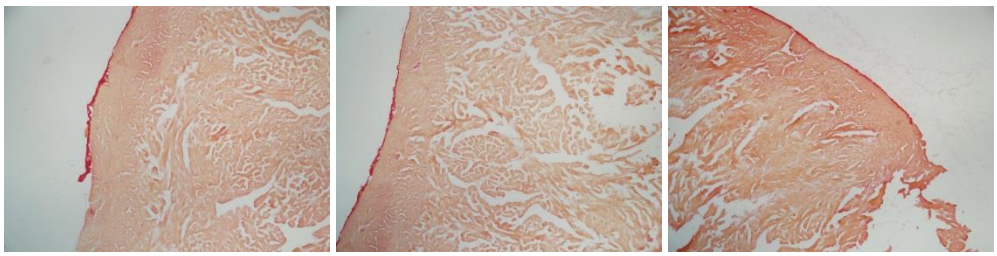
		
Dominant		
		Did not show any f_H irregularities
		
		
		

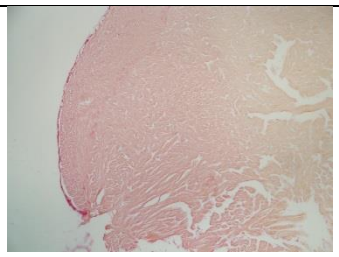
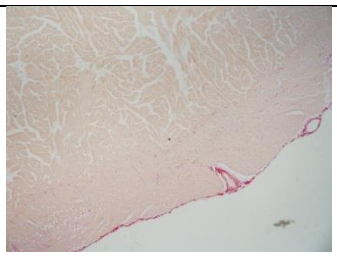
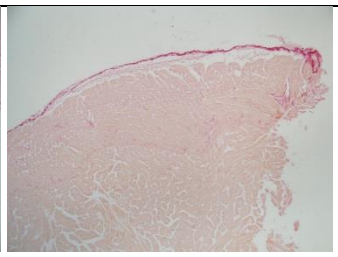
		
Subordinate		
		
		
		
		

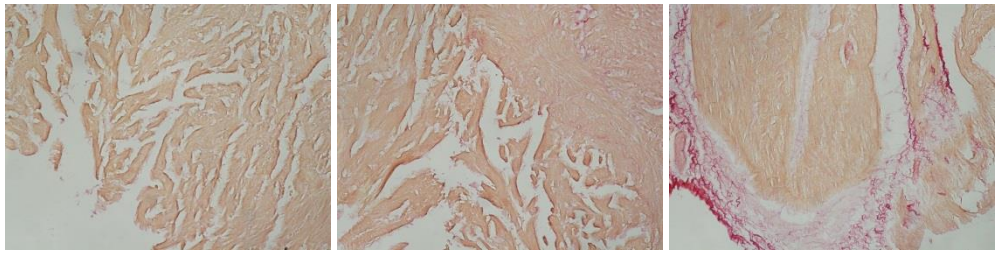
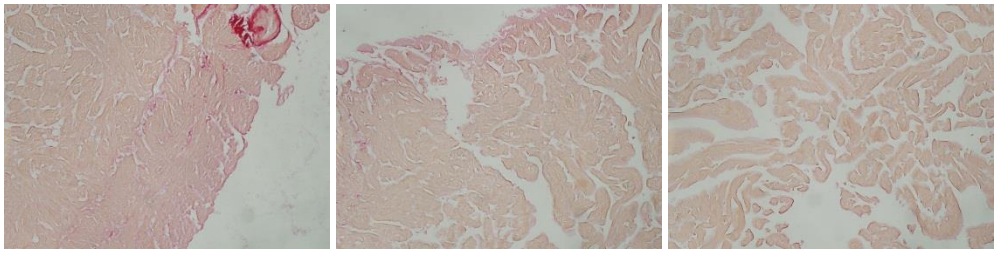
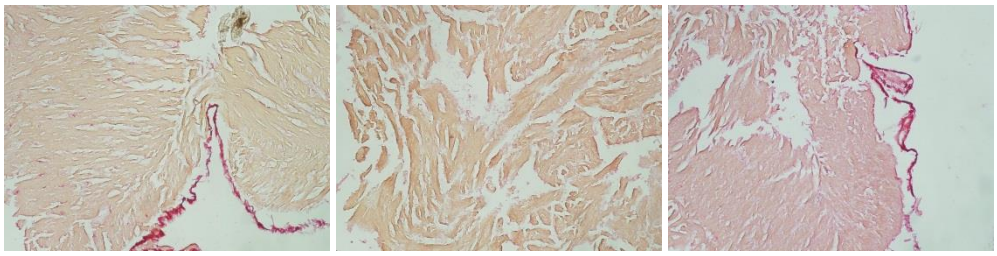
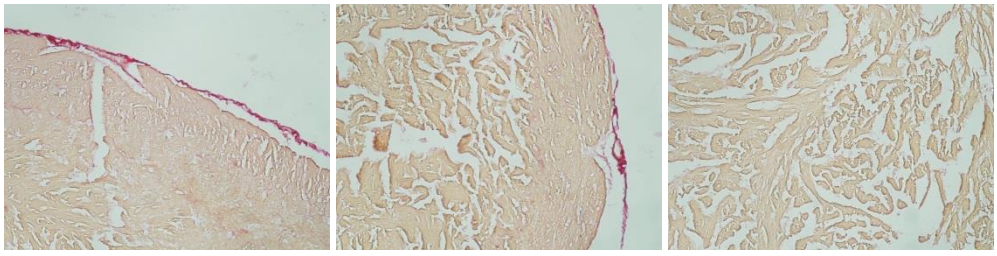
Appendix 3. Images of ventricle tissue stained with Picrosirius red staining utilized for relative myocardium thickness in dominant (*N* = 5) and subordinate rainbow trout (*N* = 5) (*Oncorhynchus mykiss*).

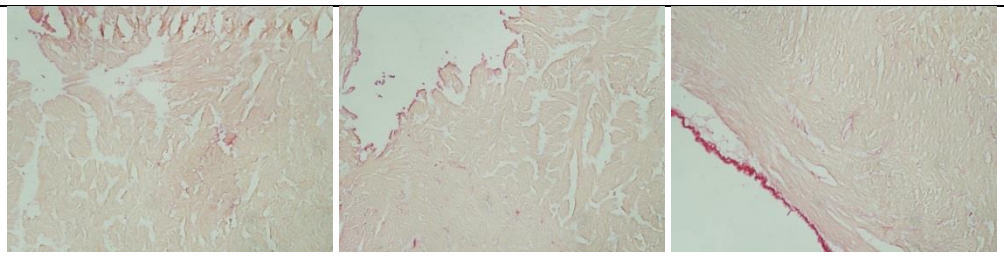
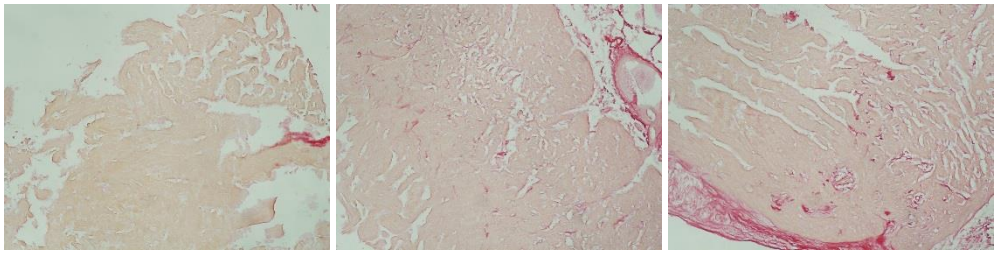
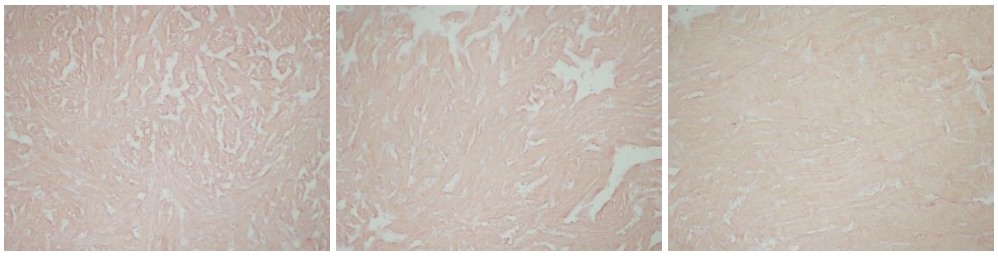
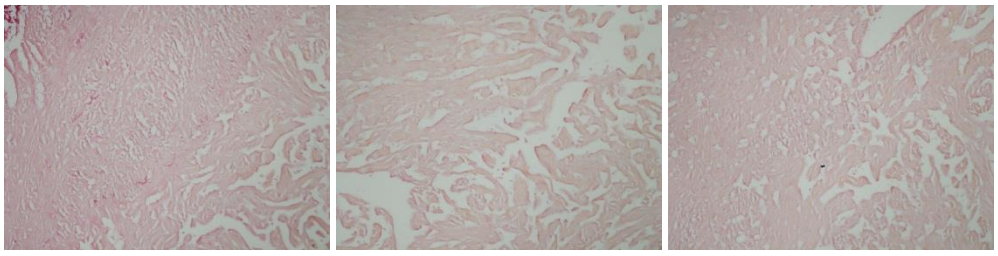
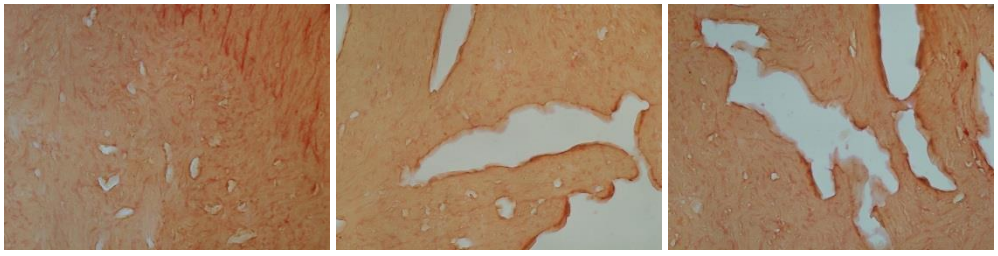
Dominant	

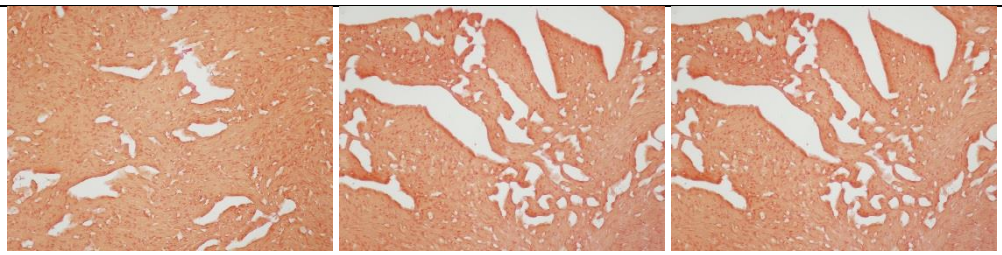
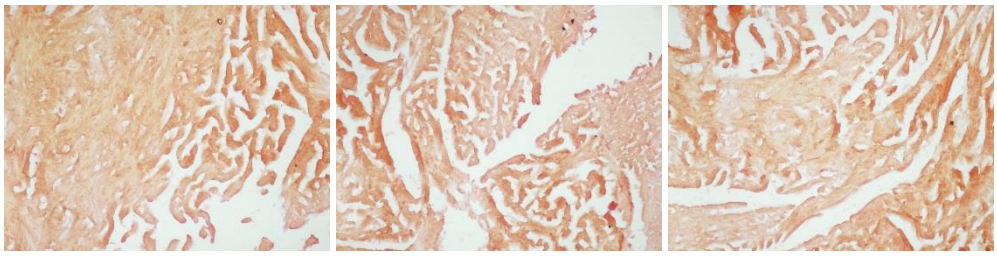
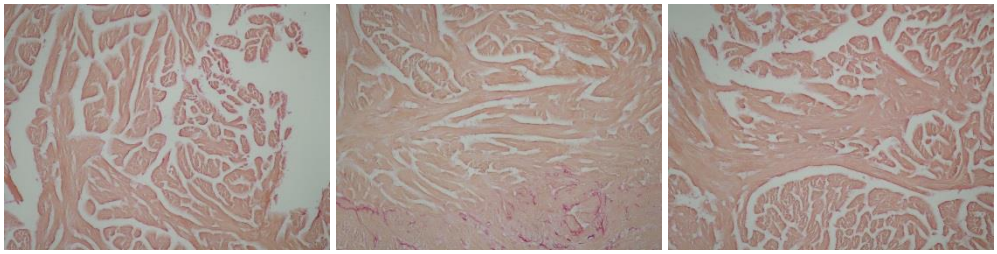
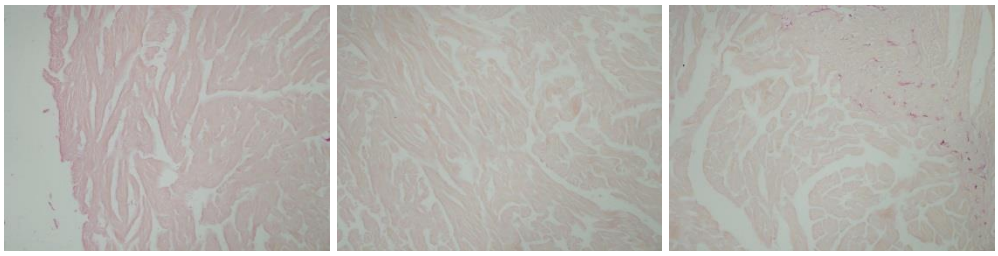
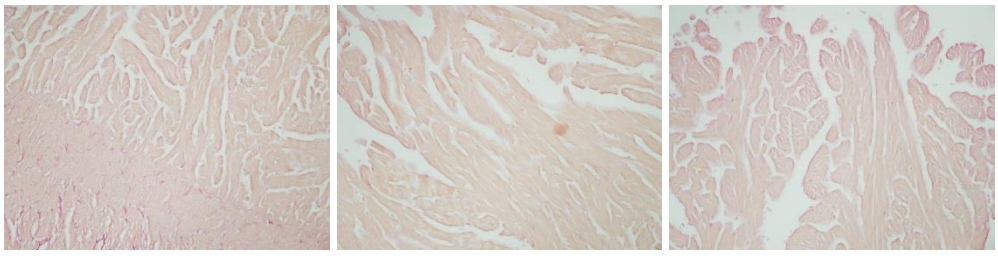
	
Subordinate	
	
	
	

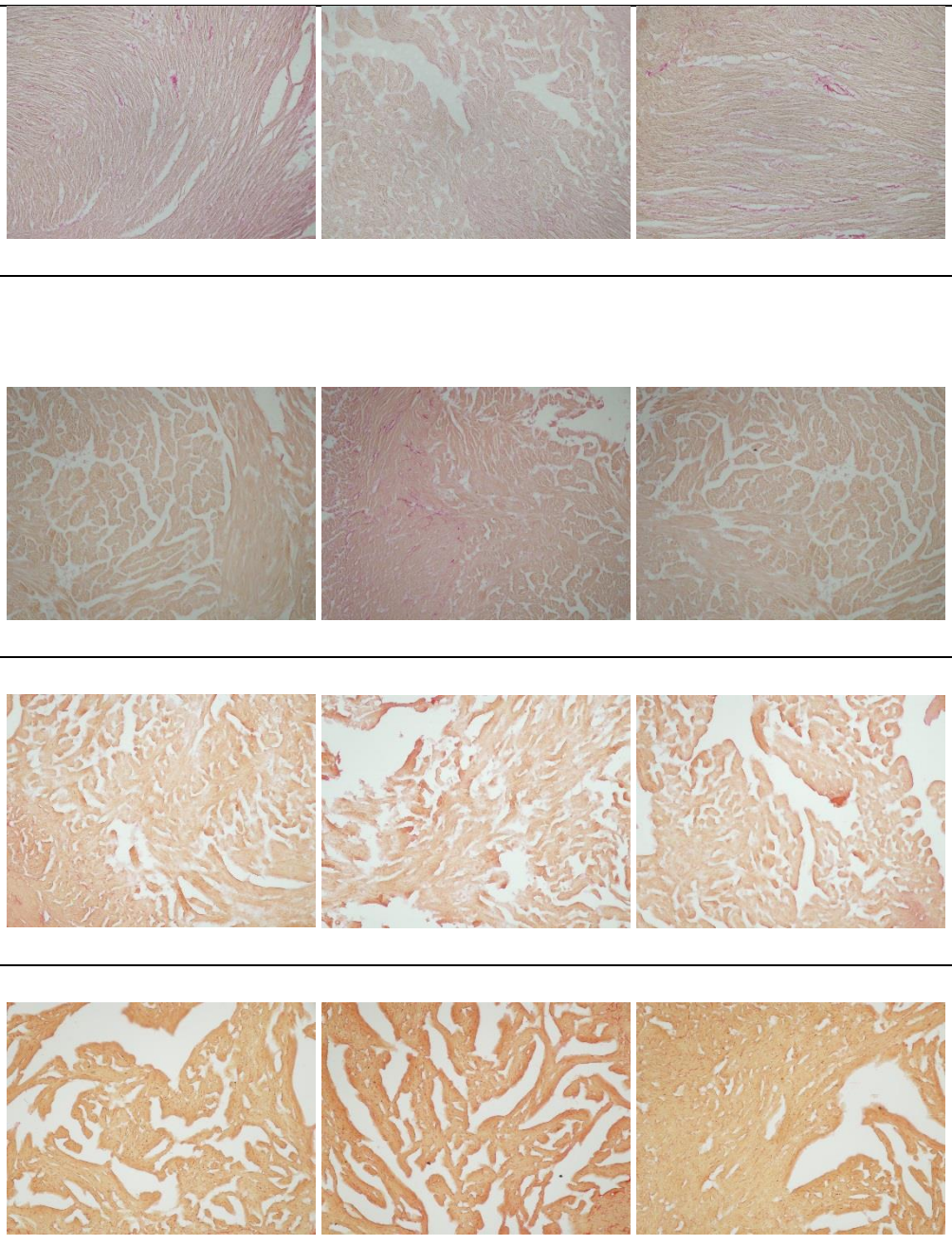
	  

Appendix 4. Images of ventricle tissue dyed with Picrosirius red staining that were utilized for quantifying collagen content in sham (*N* = 6), dominant (*N* = 6), and subordinate (*N* = 6) rainbow trout (*Oncorhynchus mykiss*).

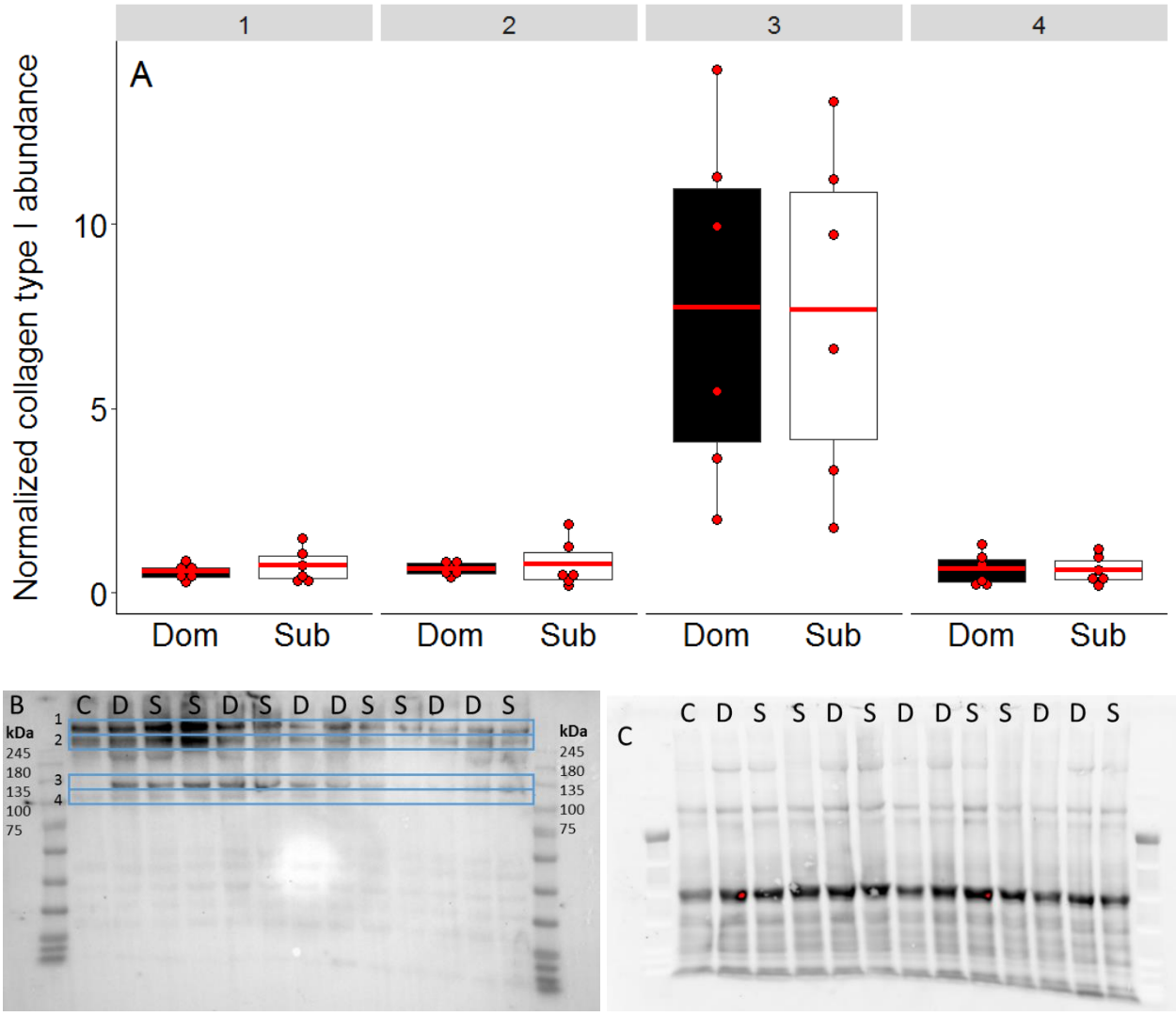
Sham	
	
	
	

	
	
Dominant	
	
	

	
	
	
Subordinate	
	



Appendix 5. The effect of social interaction on collagen type I protein levels in the ventricle of dominant (N = 6) versus subordinate (N = 6) rainbow trout (*Oncorhynchus mykiss*). (A) Normalized collagen type I abundance in ventricular tissue and expressed relative to total protein. No significant effect of social status was detected (Student's *t*-test, P = 0.49, 0.71, 0.98, 0.95 for bands 1 to 4 respectively). Data are presented as a box plot; see the legend of Fig. 3.5 for details. (B) An image of the western blot from which collagen type I protein abundance was measured by densitometry. *S*, subordinate; *D*, dominant; *C*, a sample used for cross-blot normalization. Each band encased in blue. (C) An image of the western blot showing total protein. *S*, subordinate; *D*, dominant; *C*, a sample used for cross-blot normalization.


A
1
2
3
4
Normalized collagen type I abundance
10
5
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C D S S D S D D S S D D S
kDa
245
180
135
100
75
1
2
3
4
C
C D S S D S D D S S D D S