EFFECTS OF CHRONIC CORTISOL ELEVATION ON THERMAL TOLERANCE, STRESS AXIS ACTIVITY AND HABITAT CHOICE IN RAINBOW TROUT (*Oncorhynchus mykiss*)

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Abstract

In a changing climate, salmonid fishes face increasing temperatures along with other stressors, and therefore it is important to understand how chronic stress influences their thermal biology. Rainbow trout (*Oncorhynchus mykiss*) treated with intraperitoneal implants to elevate cortisol showed reductions in thermal tolerance measured as the critical thermal maximum (CTmax). Co-treatment with the glucocorticoid receptor (GR) antagonist RU486 prevented the decrease in CTmax, suggesting that the effect is GR-dependent. Although acclimation to higher temperatures increased CTmax, the effects of chronic cortisol persisted (i.e. lower CTmax than control). To investigate the mechanisms through which cortisol alters thermal tolerance, I targeted TRPV1, a thermosensitive ion channel known to sense noxious warm temperatures. I confirmed that TRPV1 was involved in heat sensation in rainbow trout by altering TRPV1 activity and measuring CTmax. Trout exposed to the TRPV1 antagonist capsazepine showed an increase in CTmax, whereas CTmax decreased in trout exposed to the TRPV1 agonist capsaicin. In addition, cortisol-treated fish showed increased expression of *trpv4α*, which may be contributing to their heightened thermal sensitivity. Capsazepine treatment also blunted the cortisol response to acute elevation of temperature, and I therefore investigated the effects of cortisol treatment and thermal acclimation on the cortisol response to acute warming. Thermal acclimation increased the temperature at which the cortisol response to acute warming was detected, whereas cortisol-treated trout mounted a cortisol response at temperatures lower than those of their control counterparts, suggesting an increase in thermal sensitivity after chronic cortisol treatment. Measurement of the transcript abundances of key genes in the hypothalamic-pituitary-interrenal (HPI) axis supported

activation of the HPI axis by acute warming but there was little evidence of a role for cortisol in altering this response. Lastly, I asked whether these lab-based results would translate into effects in the natural environment, by investigating whether cortisol treatment altered the thermal habitat selection of rainbow trout in a lake. Cortisol-treated trout appeared to prefer warmer waters. Collectively, these data implicate TRPV1 in thermal sensing in rainbow trout, provide insight into the effects of chronic cortisol elevation on thermal tolerance, and emphasize the complexity of the relationships among chronic stress, stress axis activity and thermal tolerance.

Résume

Dans un climat changeant, les salmonidés sont confrontés à des températures croissantes ainsi qu'à d'autres facteurs de stress, et il est donc important de comprendre comment le stress chronique influence leur biologie thermique. Les truites arc-en-ciel (*Oncorhynchus mykiss*) traitées avec des implants intrapéritonéaux, pour augmenter le cortisol, ont montré des réductions de la tolérance thermique mesurée comme le maximum thermique critique (CTmax). Le co-traitement avec l'antagoniste du récepteur des glucocorticoïdes (GR), RU486, a empêché la diminution du CTmax, suggérant que l'effet est dépendant du GR. Bien que l'acclimatation à des températures plus élevées ait augmenté le CTmax, les effets du cortisol chronique ont persisté (c'est-à-dire un CTmax inférieur au contrôle). Pour étudier les mécanismes par lesquels le cortisol modifie la tolérance thermique, j'ai ciblé TRPV1, un canal ionique thermosensible connu pour détecter les températures chaudes nocives. J'ai confirmé que TRPV1 était impliqué dans la sensation de chaleur chez les truites arc-en-ciel en modifiant l'activité de TRPV1 et en mesurant le CTmax. Les truites exposées à la capsazépine, un antagoniste du récepteur TRPV1, ont montré une augmentation de CTmax, tandis que la valeur de CTmax a diminué chez les truites exposées à la capsaïcine, un agoniste du récepteur TRPV1. De plus, les poissons traités au cortisol ont montré une expression accrue de trpv4, ce qui peut contribuer à leur élevée sensibilité thermique. Le traitement à la capsazépine a également atténué la réponse du cortisol à une élévation de la température, et j'ai donc étudié les effets du traitement au cortisol et de l'acclimatation thermique sur la réponse du cortisol à un réchauffement aigu. L'acclimatation thermique a augmenté la température à laquelle la réponse du cortisol au réchauffement a été détectée, tandis que les truites

traitées au cortisol ont monté une réponse du cortisol à des températures inférieures à celles de contrôle, ce qui suggère une augmentation de la sensibilité thermique après un traitement chronique au cortisol. La mesure de l'abondance des transcriptions de gènes clés dans l'axe hypothalamo-pituitaire-interrénal (HPI) a soutenu l'activation de l'axe HPI par le réchauffement aigu, mais il y avait peu de preuves d'un rôle du cortisol dans la modification de cette réponse. Enfin, j'ai cherché à savoir si ces résultats obtenus en laboratoire pouvaient avoir des effets dans l'environnement naturel, en étudiant si le traitement au cortisol modifiait la sélection de l'habitat thermique des truites arc-en-ciel dans un lac. Les truites traitées au cortisol semblaient préférer les eaux plus chaudes. Collectivement, ces données impliquent TRPV1 dans la détection thermique chez la truite arc-en-ciel, donnent un aperçu des effets de l'élévation chronique du cortisol sur la tolérance thermique et soulignent la complexité des relations entre le stress chronique, l'activité de l'axe du stress et la tolérance thermique.

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List of Abbreviations

Abbreviation	Full Name
3N	triploid
ARR	acclimation response ratio
ACTH	adrenocorticotropic hormone
ANOVA	analysis of variance
BCA	bicinchoninic acid
bp	base pair
BSA	bovine serum albumin
cDNA	complementary DNA
Con	control
Cort	cortisol
CTmax	critical thermal maximum
CRF; *crf*	corticotropin releasing factor
CRFBP; *crfbp; crhbp*	corticotropin releasing factor binding-protein
d	day
dpf	days post-fertilization
EDTA	ethylenediaminetetraacetic acid
GR1; *gr1*	glucocorticoid receptor 1
GR2; *gr2*	glucocorticoid receptor 2
GRE	glucocorticoid response element
h	hour

HK	head kidney
HPI	hypothalamic-pituitary-interrenal
HRP	horseradish peroxidase
HSD11β	hydroxysteroid 11-beta dehydrogenase
HSP	heat shock protein
ID	identification
LOE	loss of equilibrium
min	minute
MR; *mr*	mineralocorticoid receptor
mRNA	messenger ribonucleic acid
N	sample size
OCLTT	oxygen and capacity limited thermal tolerance hypothesis
P	P-value
P450scc; CYP11A1; *p450scc*	cholesterol side-chain cleavage enzyme
PBS	phosphate buffered saline
PBST	PBS + 0.2% Triton X-100
PCR	polymerase chain reaction
PKA	protein kinase A
PCA	protein kinase C
ppm	parts per million
POA	pre-optic area
psi	pound per square inch

PTM	post-translational modification
PVDF	polyvinylidene fluoride
q-PCR	semi-quantitative real-time RT-PCR
RIA	radioimmunoassay
RU486	roussel-Uclaf 486
SF-1	steroidogenic factor 1
StAR; *star*	steroidogenic acute regulatory protein
SDS-PAGE	sodium dodecyl sulphate–polyacrylamide gel electrophoresis
SEM	standard error of the mean
T	temperature
TBST	tris-buffered saline with 0.05% Tween-20
TRP	transient receptor potential ion channel
TRPV; *trpv*	transient receptor potential vanilloid-type ion channel
VTmax	voluntary thermal maximum
WGD	whole genome duplication

Note for nomenclature: Gene and protein symbol nomenclature will be denoted by the following convention for the entirety of the thesis; uppercase (e.g. TRPV1) for general reference, lowercase italics for gene (e.g. *trpv1*), and title case for protein (e.g. Trpv1).

Chapter 1

General Introduction

1.1 Overview of thesis

Temperature is considered a master regulatory factor, particularly among ectotherms, owing to their body temperature being constrained by environmental conditions that vary not only daily but seasonally, and by depth in aquatic environments (Brett 1971). In fishes as in other ectotherms, environmental temperature controls many physiological processes and affects locomotion through its impact on body temperature, and thus can also alter and determine the distribution of individuals, communities and populations (Magnuson et al. 1979; Beitinger et al. 2000; Tattersall et al. 2012). Fishes, like other ectotherms, rely on behavioural thermoregulation to regulate body temperature (Ward et al. 2010; Goyer et al. 2014; Nay et al. 2020; Amat-Trigo et al. 2024). As such, fishes typically thermoregulate through changes in vertical water depth (Holland et al. 1992; Stehfest et al. 2017; Keefer et al. 2018a; Gallagher et al. 2019; Freitas et al. 2021; Amat-Trigo et al. 2023; Thomas et al. 2023; Winkler et al. 2023) or through horizontal migrations to find pockets of thermal refuge (Armstrong et al. 2013; Goyer et al. 2014; Raby et al. 2018; Amat-Trigo et al. 2023). This means that fish are generally limited to choosing available water temperatures, unlike terrestrial ectotherms that have a broad range of behavioural and physiological strategies to regulate body temperature (Stevenson 1985; Seebacher 2005; Rozen-Rechels et al. 2019; Carter et al. 2023).

Salmonid fishes are of ecological, cultural, and economic significance but continue to be under threat from a variety of environmental and anthropogenic factors

including climate change, over-exploitation, habitat loss, and water quality degradation (Martins et al. 2011; Almodóvar et al. 2019; Lehnert et al. 2019, 2020; Sinnatamby et al. 2020; Ulaski et al. 2023). In recent years, declines in salmonid populations have been reported across the Northern Hemisphere (Kendall et al. 2017; Almodóvar et al. 2019; Lehnert et al. 2019, 2020; Sinnatamby et al. 2020), highlighting the need for conservation efforts. Climate change will likely continue to expose fish to a variety of stressors occurring both simultaneously and in succession (Todgham et al. 2013). For example, fish may experience chronic stress owing to factors such as habitat loss or water quality degradation (King et al. 2016; Glassic and Gaeta 2019; Fakan et al. 2025), while also coping with increases in water temperature (Martins et al. 2011; Keefer et al. 2018b; Almodóvar et al. 2019; Ulaski et al. 2023). This scenario emphasizes the importance of studying how chronic stress alters the ability of salmonids to tolerate thermal stress. Previous research indicated that cortisol was a main contributor to the lowered thermal tolerance of subordinate trout experiencing chronic social stress (LeBlanc et al. 2011; Bard et al. 2021). However, it remains unknown how cortisol alters the physiological mechanism(s) that ultimately determine thermal tolerance. Additionally, it is not known whether chronic stress alters other aspects of thermal biology, such as thermal preference. The heightened sensitivity to high temperatures of trout experiencing stress-induced chronic cortisol elevation may also alter thermal preference. Ultimately, further research is needed to understand whether chronic elevation of cortisol is detrimental to the thermal biology of rainbow trout.

The present thesis used chronic cortisol manipulation (as per Sopinka et al. 2015) as a proxy for chronic stress to assess whether rainbow trout (*Oncorhynchus mykiss*) with

experimentally elevated cortisol exhibit heightened sensitivity to thermal stress, and to explore the underlying mechanisms. More specifically, Chapter 2 assessed whether chronic cortisol elevation on its own (i.e. in the absence of exposure to a stressor) alters thermal tolerance and investigated the role of thermosensitive transient receptor potential vanilloid (TRPV) ion channels in mediating the effects of cortisol on thermal sensing. Chapter 3 focused on the cortisol response to acute thermal stress, asking whether it is the result of HPI axis activation and whether the response is altered by thermal acclimation or chronic cortisol treatment. Lastly, Chapter 4 aimed to translate lab-based studies into a natural setting, by determining whether chronic cortisol elevation caused differences in thermal habitat use within a lake.

Below, I briefly review areas of the literature central to my thesis research to identify knowledge gaps and develop the hypotheses that were explored in Chapters 2-4.

1.2 Thermal tolerance

Thermal tolerance studies can be used to identify the ability of an individual organism (fish in the case of this thesis) to withstand variations in temperature within their environment. Such work has long been important for understanding animal-environment interactions and the thermal aspects of habitat, but in the last decade or so has become increasingly relevant for assessing vulnerability to climate change (Clark et al. 2017; Lapointe et al. 2018; Davis et al. 2019; Leclair et al. 2020). Upper thermal tolerance typically has been measured as the critical thermal maximum (CTmax). To determine CTmax, an individual fish is exposed to a linear and fairly rapid (e.g. 0.33°C min^{-1} - 1°C h^{-1}) increase in water temperature until loss of equilibrium (LOE) occurs

(Becker and Genoway 1979; Lutterschmidt and Hutchison 1997). The popularity of CTmax reflects the relative ease of measuring it and its widespread applicability across species. It provides a useful tool to make comparisons, not only between and within species (Fangue 2006; Chen et al. 2013; Bard et al. 2021; Nati et al. 2021; DeLiberto et al. 2022), but also within and among individuals (Morgan et al. 2018; Bard and Kieffer 2019; O'Donnell et al. 2020). Because CTmax trials are not lethal, they are useful when investigating endangered or at risk species (Bard and Kieffer 2019; Davis et al. 2019; Leclair et al. 2020; Potts et al. 2021; Reemeyer and Chapman 2024). However, CTmax is considered the temperature of 'ecological death', because a fish that has lost equilibrium can no longer find a thermal refuge and is at high risk of predation (Desforges et al. 2023).

Although CTmax has been used as an indicator of thermal tolerance for decades (Becker and Genoway 1979), the mechanism(s) underlying the LOE at elevated temperature has long been debated and remains uncertain (Ern et al. 2023). One school of thought focuses on oxygen delivery, which has been formalized as the oxygen- and capacity-limited thermal tolerance (OCLTT) hypothesis. The OCLTT hypothesis posits that at temperatures nearing CTmax, there is a mismatch between oxygen demand and oxygen delivery to the tissues, causing a decrease in performance and LOE (Pörtner 2010; Pörtner et al. 2017). Some empirical data support the OCLTT (Blasco et al. 2020; McArley et al. 2022), such as a strong positive relationship between CTmax and hematocrit (Beers and Sidell 2011; Muñoz et al. 2018; Anttila et al. 2023), decreased CTmax under hypoxic conditions (Healy and Schulte 2012; Zanuzzo et al. 2019), and correlations between hypoxia tolerance and CTmax (Akrokoh et al. 2024). Furthermore,

many studies have focused on the cardiorespiratory system, particularly cardiac function, to identify its role in determining thermal tolerance. In support of a cardiac contribution, studies have reported cardiac arrhythmias (Verhille et al. 2013), as well as a plateau and/or a decrease in heart rate (Mendonça and Gamperl 2010; Penney et al. 2014; Ekström et al. 2016, 2017, 2019; Anttila et al. 2023) at temperatures approaching the CTmax. Moreover, experimental impairment of cardiovascular function, for example through ligation of the coronary arteries in trout, also lowered CTmax (Ekström et al. 2017, 2019). Nevertheless, there are also empirical results that oppose the OCLTT hypothesis (Ern et al. 2016; Jutfelt et al. 2018; Nati et al. 2023; Ripley et al. 2023; Montgomery et al. 2024), such as exposure to hyperoxia during thermal ramping having no effect on CTmax (Devor et al. 2016), and aerobic scope not being reduced during thermal stress (Norin et al. 2014; Nati et al. 2023).

An alternative school of thought focuses on temperature effects on the nervous system, owing to the endpoint of a CTmax test being LOE. For example, cooling the brain of Atlantic cod (*Gadus morhua*) increased the CTmax, but not in a substantial fashion, which suggests that the peripheral nervous system may also be important in determining CTmax (Jutfelt et al. 2019). Similarly, Andreassen et al. (2022) suggested that gradual brain dysfunction causes CTmax. The authors demonstrated that CTmax preceded a brain-wide depolarization in larval zebrafish, and therefore depolarization could not be the cause of LOE; rather, they suggested it was likely that insufficient oxygenation of the brain causing slow neural dysfunction ultimately led to LOE (Andreassen et al. 2022). There is also long-standing evidence that both central and peripheral thermoreceptors may be involved in the control of ventilation, as direct heating

or cooling of the brainstem caused rapid increases or decreases, respectively, in ventilation (Crawshaw et al. 1973). This observation provides an important link between neural function and oxygen uptake, which could be critical to thermal tolerance. Furthermore, differences in thermal tolerance in two Antarctic notothenioid species were linked to differences in neuronal membrane composition (Biederman et al. 2019). More specifically, the less thermo-tolerant species, *Chaenocephalus aceratus*, demonstrated greater increases in membrane fluidity when warmed, suggesting that increases in membrane fluidity and their impact on neuronal function constrained thermal tolerance. It seems likely that multiple systems contribute to thermal tolerance (Ern et al. 2023).

While it remains unclear why acute warming results in LOE, researchers have identified several factors that alter CTmax, including environmental conditions such as photoperiod (Healy and Schulte 2012), seasonality (Hlohowskyj and Wissing 1985), hypoxia (Healy and Schulte 2012; Zanuzzo et al. 2019), and salinity (Sardella et al. 2008). A strong positive relationship between acclimation temperature and CTmax has also been reported (Beitinger and Bennett 2000; Rajaguru 2002; Moyano et al. 2017; da Silva et al. 2019; McDonnell et al. 2021; Mckenzie et al. 2021; Desforges et al. 2023; Ern et al. 2023), which is relevant to both interpreting field observations and designing lab experiments (Deforges et al. 2023). CTmax tend to increase in a linear fashion until a plateau is reached (Habary et al. 2017; McDonnell et al. 2019; Mckenzie et al. 2021). The strength of the relationship often reflects the thermal plasticity of an animal, with stenothermal fishes tending to have smaller increases in CTmax at higher acclimation temperature (i.e. less steep relationship) (Bilyk and DeVries 2011; Peck et al. 2014;

Vinagre et al. 2016; Spinks et al. 2019). Whether chronic stress alters an individual's ability to acclimate to higher temperatures remains to be determined.

Chronic social stress lowers thermal tolerance in rainbow trout (LeBlanc et al. 2011; Bard et al. 2021). Specifically, rainbow trout subjected to chronic social stress as a result of the formation of dominance hierarchies had lowered thermal tolerance, which was strongly linked to chronic elevation of cortisol in subordinate fish (LeBlanc et al. 2011; Bard et al. 2021). Subordinates given a recovery period sufficient for cortisol to fall to baseline values regained normal thermal tolerance, but when recovering subordinates were treated with cortisol to maintain elevated cortisol during the recovery period, thermal tolerance did not recover (Bard et al. 2021). The study of Bard et al. (2021) was the first to document a mechanistic link between elevated cortisol and thermal tolerance, although some prior work suggested the existence of such a relationship. For example, in threadfin shad (*Dorosoma petenense*), thermal tolerance was reduced after fish were subjected a netting stressor (Monirian et al. 2010), a protocol that would be expected to induce a cortisol response (Strange et al. 1977; Jeffrey et al. 2014). Additionally, exposure to acute changes in salinity (Sardella et al. 2008; Shaughnessy and McCormick 2018), which results in changes in cortisol for ionic and osmotic regulation, has been shown to lower thermal tolerance. These studies demonstrate that conditions that would elicit a stress response can modulate thermal tolerance, although linking these changes to cortisol specifically is more difficult. It remains to be determined whether the relationship between cortisol and thermal tolerance applies to stress conditions beyond chronic social stress, and whether the relationship can be attributed specifically to cortisol alone or

requires other aspects of the stress response to occur. These questions were assessed in Chapter 2 of the present thesis.

The pathway through which cortisol affects CTmax is not clear. Bard et al. (2021) investigated the role of the heart during acute warming in trout subjected to chronic social stress. However, evidence of a role for the heart was limited to cardiac arrhythmia occurring at lower temperatures in subordinate individuals (Bard et al. 2021). Alternatively, chronic cortisol elevation could cause differences in the central nervous system that lead to earlier LOE during acute warming. The brain is responsible for translating sensory information, including noxious thermal stress, into changes in behaviour (Haesemeyer et al. 2018). Additionally, the brain exhibits high expression of glucocorticoid (GR) and mineralocorticoid receptors (MR), and their expression differs based on an individual's stress responsiveness, which makes the brain highly susceptible to changes in cortisol (Johansen et al. 2011b). Indeed, chronic exposure to elevated cortisol reduced neurogenesis, leading to the potential of altering neural function (Sørensen et al. 2011, 2012). Moreover, long term exposure to thermal stress reduced protein expression in the brain of zebrafish (*Danio rerio*), which altered cognitive function related to exploratory behaviour (Toni et al. 2019). The brain expresses high levels of GR and MR in rainbow trout, which suggests a high sensitivity to cortisol and a possible pathway to enable changes in CTmax in response to chronic stress (Teitsma et al. 1998; Johansen et al. 2011b; Alderman et al. 2012; Teles et al. 2013).

1.3 The perception of temperature in fishes

Our understanding of the mechanisms underlying thermal perception in fishes remains incomplete (Haesemeyer 2020; Cutler and Haesemeyer 2024). The nervous system contains temperature-sensitive neurons in the lateral line (Rubin 1934), spinal cord (Iriki et al. 1976; Nagai et al. 1977), and the epithalamus and preoptic area (POA) of the brain (Greer and Gardner 1970, 1974). These afferent sensory neurons also have cell bodies in the trigeminal root ganglia and express thermo-sensitive transient receptor potential (TRP) ion channels, which serve in detecting ambient temperature (Palieri et al. 2024) (Fig. 1.1). These neurons ultimately transmit temperature information to the brain for further processing. Specific regions of the brain may be responsible for different responses – for example, the trigeminal ganglion appears to serve as the central temperature detector (Haesemeyer et al. 2018; Palieri et al. 2024). Temperature information is transmitted to the hypothalamic regions, such as the POA, which is thought to be the thermostat and appears to be responsible for signalling a change of swimming direction if the thermal conditions in the chosen path continue to worsen (Palieri et al. 2024). Lastly, the information is processed by the hindbrain, which may signal the initiation of an evasive behaviour (Haesemeyer et al. 2018).

The pineal organ also appears to be involved in nervous and neurohormonal responses that allow behavioural thermoregulation (Kavaliers and Ralph 1980; Kavaliers 1982a, 1982b; Yáñez et al. 2009; Herrera-Pérez et al. 2010). More specifically, it seems to control the diel rhythm of behavioural thermoregulation (Kavaliers and Ralph 1980; Kavaliers 1982b). As such, it was shown that white sucker (*Catostomus commersoni*) no longer chose cooler temperature during the day when the pineal organ was removed

(Kavaliers and Ralph 1980). However, there continues to be a knowledge gap pertaining to how these sites of thermal sensitivity interact to allow temperature perception and the generation of appropriate responses in teleost fishes (Nisembaum et al. 2015). Moreover, how these systems are adjusted in response to changing conditions (e.g. acclimation to different temperatures) remains unknown. Recently, a dynamic circuit model was generated to map and predict how heat-evoked brain stimuli translate into swimming behaviours in larval zebrafish (Haesemeyer et al. 2018). This paper was one of the first to begin to track the path of a thermal stimulus from peripheral detection all the way to a behavioural response in fishes. Coupled with the growing literature indicating that the nervous system may be involved in determining thermal tolerance (Jutfelt et al. 2019; Andreassen et al. 2022), investigating how elevated temperatures are perceived and signaled becomes important.

Thermosensation appears to rely primarily on thermo-sensitive TRP ion channels, which form a family of non-selective cation channels present in eukaryotes (Kashio 2021). Each thermoTRP channel has a specific range of activation temperatures, and animals typically express a range of thermoTRPs that spans noxious cool to noxious warm temperatures (Kashio and Tominaga 2022; Lezama-García et al. 2022; see Fig. 1.2). ThermoTRPs are located at the peripheral ends of somatosensory neurons and are responsible for thermal transduction (Kashio and Tominaga 2022). Across animals, thermoTRPs in the vanilloid (TRPV1-4) and melastatin (TRPM2-5) groups are involved in heat sensation, whereas the ankyrin TRPA1, the melastatin TRPM8, and the canonical TRPC5 are responsible for cold sensation (Lezama-García et al. 2022). Although not every channel is present in every animal, TRP channels appear to be well conserved

across animals and indeed, across eukaryotes (Saito and Shingai 2006; Saito et al. 2011; Venkatachalam et al. 2014; Gracheva and Bagriantsev 2015; Bagriantsev and Gracheva 2015; Morini et al. 2022). It should also be noted that TRP channels are responsible for roles beyond thermosensation, including mechanical and chemical signal transduction (Lezama-García et al. 2022). However, for the purpose of the present thesis, I will focus on the role of thermoTRPs in thermosensation.

Teleost fishes are thought to have about 28 TRP genes (York and Zakon 2022a; Huang et al. 2024; Li et al. 2024), of which 6 are thought to be thermosensitive (i.e. TRPV1, TRPV4, TRP1A, TRPM2, TRPM4, TRPM5) (Saito and Shingai 2006; Gau et al. 2013; Nisembaum et al. 2015, 2022; Boltana et al. 2018; Haesemeyer 2020; York and Zakon 2022a; Yoshimura et al. 2022; Melanson et al. 2023). TRPV1 is activated at noxious warm temperatures (Saito and Shingai 2006; Ashley et al. 2007; Gau et al. 2013; Yoshimura et al. 2022), whereas TRPV4 is thought to sense warm but not noxious temperatures (Saito and Shingai 2006; Boltana et al. 2018). These traits make TRPV1 and TRPV4 good targets for the research of the current thesis, specifically owing to our interest in noxious high temperatures (i.e. CTmax). TRPV1 and TRPV4 have been found in a range of tissues in fishes (see Table 1.1).

Salmonids express only two types of thermo-TRPV channels, TRPV1 and TRPV4 (Morini et al. 2022). There are two paralogues of TRPV1 in teleost fishes (i.e. TRPV1a and TRPV1b) owing to the teleost whole genome duplication (WGD) event, however salmonids have lost TRPV1b (Morini et al. 2022). Similarly, TRPV4 was duplicated during the teleost WGD event, however all teleosts seem to have lost TRPV4b (Morini et al. 2022). The remaining TRPVs in salmonids (i.e. TRPV1a and TRPV4a) both

underwent duplication in the salmonid-specific WGD (Morini et al. 2022). Thus, rainbow trout have a total of four thermosensitive TRPV channels (i.e. TRPV1aα, TRPV1aβ, TRPV4α and TRPV4β), which will be the focus for this thesis.

The TRPV channels are calcium-selective ion channels gated by changes in temperature (Nilius and Owsianik 2010; Saito et al. 2011; Saito and Tominaga 2017), and therefore allow detection of heat to be transduced into electrical signals, which are propagated as action potentials to the central nervous system to initiate responses (Patapoutian et al. 2003). Each TRPV channel has an activation threshold and once this temperature is reached, it causes the opening of the channel, with the resultant calcium influx promoting neuronal excitation (Bagriantsev and Gracheva 2015). It is likely that both TRPV1 and TRPV4 are of importance in responses to warm temperatures.

Several studies have implicated TRPV in thermal tolerance and/or preference in animals (Marics et al. 2014; Laursen et al. 2016; Saito et al. 2016; Fu et al. 2021; Ujisawa et al. 2022; Wang et al. 2023). For example, TRPV1 knockout mice do not avoid noxious warm temperatures compared to their control counterparts (Marics et al. 2014; Ujisawa et al. 2022, 2024), suggesting that TRPV1 aids in the avoidances of lethal temperatures. Similarly in embryonic turtles, it has been shown that TRPV1 plays a role in thermotaxis to avoid noxious heat within the egg (Ye et al. 2021). In heat-tolerant 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). 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 was restored in both squirrel and camel TRPV

channels through a single conserved amino acid substitution. Similarly, gerbils that have different heat tolerance differ in TRPV1 protein sequences but not expression (Wang et al. 2023). These observations imply that small changes in TRPV sequences can alter their activation threshold temperature and thus mediate thermal tolerance. In Pacific oysters, it was shown that TRP regulation to elevated temperatures is species-specific, with the more-thermally tolerant southern species having a far larger upregulation of TRP channels, most notably TRPV4 (Fu et al. 2021). This observation suggests that TRPV regulation may play a role in mediating thermal tolerance. Moreover, TRPV1 has also been implicated in other aspects of thermal biology such as thermal preference. For example, two closely-related species of clawed frog (*Xenopus* sp.) adapted to different thermal environments showed differences in TRPV responses to heat stimulation (Saito et al. 2016). Ultimately, these studies provide good evidence that TRPV channels are implicated in determining thermal tolerance and thermal preference in a range of animals.

Research on the role of thermoTRPVs in thermal sensing in teleost fishes has increased markedly in recent years. For example, TRPV1 was shown to be activated by heat in zebrafish, black rock cod (*Notothenia coriiceps*) and African lungfish (*Protopterus annectens*) (Gau et al. 2013; Hori and Saitoh 2023; York 2023). Moreover, larval zebrafish subjected to TRPV1 knockdown were less agitated at elevated temperatures, demonstrating that TRPV1 is essential for heat-induced locomotion (Gau et al. 2013). Together, these observations highlight the importance of TRPV1 in temperature sensing in teleost fishes. Interestingly, treatment of 5 and 7 days post-fertilization (dpf) zebrafish larvae with a TRPV4 antagonist (RN-1734) caused a significant decrease in seizure activity (Hunt et al. 2012), a response which has

historically been used as a endpoint for CTmax (Lutterschmidt and Hutchison 1997). In African lungfish (*Protopterus annectens*), which can survive long period out of water and are therefore heat-tolerant, TRPV1 remains inactivated even at 44°C (Hori and Saitoh 2023). Taken together, these findings provide evidence that both TRPV1 and TRPV4 channels may be involved in mediating thermal tolerance.

In mandarin fish, *Siniperca chuatsi*, transcript abundance of *trpv1* was upregulated after both acute warming and chronic exposure to elevated temperatures (Li et al. 2024). Similarly, *trpv1* and *trpv4* transcript abundances were upregulated following exposure to either acute or chronic high temperatures across various tissues in the black rockfish (*Sebastes schlegelii*) (Huang et al. 2024). These increases in expression appear to be time dependent in certain tissues, which raises the potential for primary and secondary TRPV responses, and differences in sensitivity to temperature across tissues within an individual (Huang et al. 2024; Li et al. 2024). These observations support the possibility that exposure to environmental conditions may modulate temperature sensing through changes in *trpv* expression.

In Atlantic salmon, *trpv4* transcript abundance was upregulated at high temperatures (Boltana et al. 2018). However, when the fish were infected with a virus, *trpv4* was downregulated and remained so even after acute warming (Boltana et al., 2018), indicating that prior stressors can modulate *tprv* expression during exposure to acute warming. In mangrove rivulus (*Kryptolebias marmoratus*), socially-experienced fish emerged from the water at higher temperatures than naïve fish, suggesting that social experience modulates thermal sensing (Melanson et al. 2023). Moreover, exposure to increasing concentrations of the TRPV1 agonist capsaicin elicited a comparable response,

where socially-experienced fish emerged from the water at higher capsaicin dosages, implicating TRPV1 in this response (Melanson et al. 2023). This finding highlights the potential that previous experience can modulate TRPV function and thresholds, altering the whole-animal response to temperature. Collectively, these studies implicate TRPV1 and/or TRPV4 in thermal sensing and suggest that changes in TRPV1 and/or TRPV4 transcript abundance or function may mediate environmental modulation of responses to temperature. In Chapter 2, I explore the possibility that chronic cortisol elevation modulates thermal tolerance in rainbow trout through effects on TRPV expression and/or function.

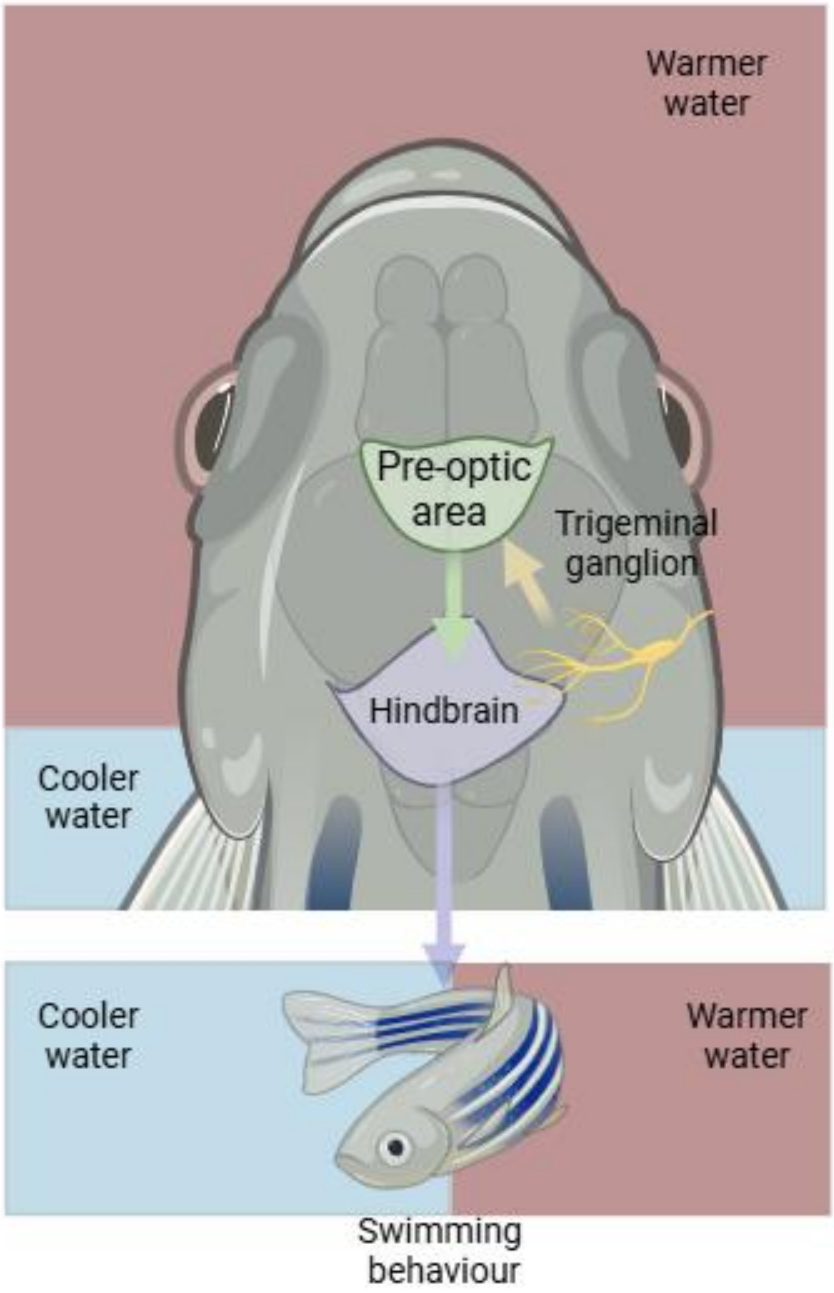


Figure 1.1. Neural basis of behavioural thermoregulation based on dynamic circuit model by Haesemeyer et al. (2018). Heat is firstly detected by the trigeminal ganglion. This information is then translated by the POA, which act as a thermostat. Lastly these signals are translated to the hindbrain, to evoke a swimming behaviour to evade the warmer water.

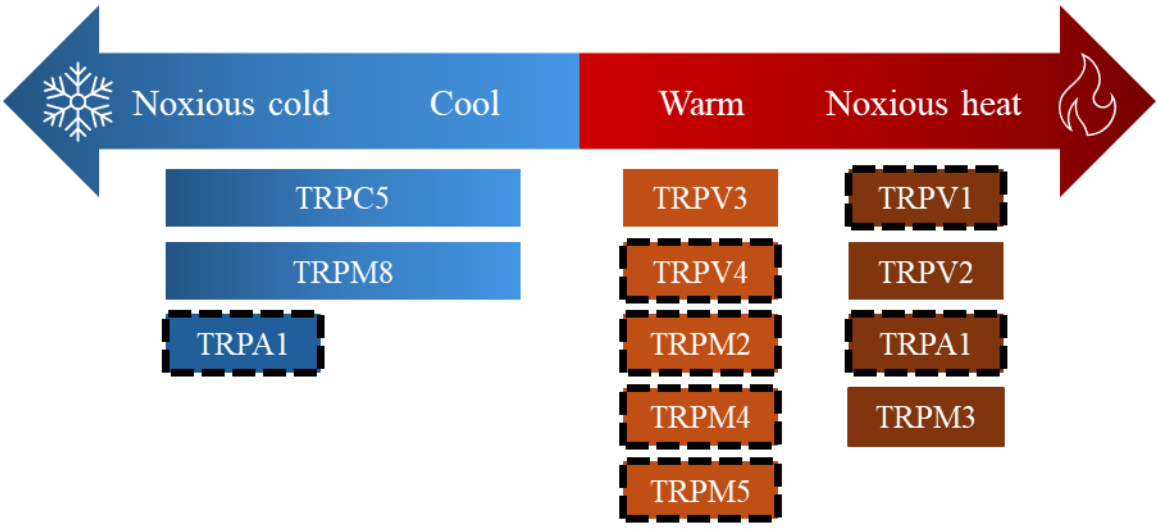


Figure 1.2. Temperature ranges known to activate thermoTRPs among vertebrates (Saito and Shingai 2006; Kashio and Tominaga 2022; Lezama-García et al. 2022). Boxes with dashed outlines represent thermoTRPs reported to be present in fishes.

Table 1.1. Tissue localization of thermoTRPVs in fishes.

TRPV channel	Common name (scientific name)	Tissues	Reference
TRPV1	Rainbow trout (*Oncorhynchus mykiss*)	Brain (telencephalon, cerebellum, diencephalon, optic tectum, pituitary); eye; gill; heart; spleen; stomach; intestine; kidney; liver; blood; skin; retina; intestine; spleen; pineal organ	(Nisembaum et al. 2015; Vercelli et al. 2023)
	Atlantic salmon (*Salmo salar*)	Brain (telencephalon, optic lobe, cerebellum, diencephalon, optic tectum, pituitary): fin; blood; brain; gill; heart; intestine; kidney; eye; skin; lateral line; pineal organ; spleen; saccus vasculosus	(Boltana et al. 2018; Nisembaum et al. 2022)
	Mandarin fish (*Siniperca chuatsi*)	Blood; gill; fin; skin; muscle; spleen; liver; brain; olfactory epithelium; intestine; heart; kidney, head kidney	(Li et al. 2024)
	Black rockfish (*Sebastes schlegelii*)	Gill; brain (hypothalamus); lateral line; skin	(Huang et al. 2024)
	Burton's Mouthbrooder (*Astatotilapia burtoni*)	Brain	(York and Zakon 2022b)
	Antarctic spiny plunderfish (*Harpagifer antarcticus*)	Brain	(York and Zakon 2022b)
	Masu salmon (*Oncorhynchus masou ishikawae*)	Gill; spinal cord	(Yoshimura et al. 2022)

	Zebrafish (*Danio rerio*)	Trigeminal ganglion; lateral line; sperm	(Gau et al. 2013; Chen et al. 2020)
	Rohu (*Labeo rohita*)	Sperm	(Majhi et al. 2013)
TRPV4	Rainbow trout (*Oncorhynchus mykiss*)	Brain (telencephalon, cerebellum, diencephalon, optic tectum, pituitary); heart; blood; skin; retina; liver; kidney; intestine; spleen; pineal organ	(Nisembaum et al. 2015)
	Atlantic salmon (*Salmo salar*)	Brain (telencephalon, optic lobe, cerebellum, diencephalon, optic tectum, pituitary); fin; blood; gill; heart; intestine; kidney; eye; skin; lateral line; pineal organ; spleen; saccus vasculosus; optic lobe	(Boltana et al. 2018; Nisembaum et al. 2022)
	Half-smooth tongue sole (*Cynoglossus semilaevis*)	Heart; spleen; testis; eye; kidney; liver; lateral line; free neuromasts	(Shang et al. 2020)
	European seabass (*Dicentrarchus labrax*)	Brain (pituitary); heart; gill; kidney; liver; spleen; intestine, muscle, skin	(Watanabe et al. 2012)
	Nile tilapia (*Oreochromis mossambicus*)	Brain (pituitary)	(Watanabe et al. 2012)
	Black rockfish (*Sebastes schlegelii*)	Gill; brain (hypothalamus); lateral line; skin	(Huang et al. 2024)

1.4 HPI axis responses to and regulation during acute warming

To gain insight into how chronic stress alters thermal tolerance, it is important to understand how hypothalamic-pituitary-interrenal (HPI) axis activity, which culminates in elevation of cortisol levels, is regulated during exposure to elevated temperature. Increases in circulating cortisol levels in response to elevated temperature have been well documented in fishes (Ryan 1995; Delaney et al. 2008; Pérez-Casanova et al. 2008; LeBlanc et al. 2011; Jaxion-Harm and Ladich 2014; Benítez-Dorta et al. 2017; Yang et al. 2018; Yusishen et al. 2020; Bard et al. 2021; reviewed by Alfonso et al. 2021). Cortisol increases during both acute warming (Basu et al. 2001; Pérez-Casanova et al. 2008; Steinhausen et al. 2008; LeBlanc et al. 2011; Benítez-Dorta et al. 2017; Cockrem et al. 2019; Bard et al. 2021; Alfonso et al. 2021) and chronic exposure to high temperatures (Benítez-Dorta et al. 2017; Hanke et al. 2019; Goikoetxea et al. 2021; Alfonso et al. 2021). Collectively, these studies suggest that elevated water temperature stimulates the production of cortisol in fish, but the mechanisms involved remain unclear.

In general, increases in circulating cortisol are achieved through activation of the HPI axis (Vijayan et al. 2010; Sørensen et al. 2012a; Faught et al. 2016; Gorissen and Flik 2016; See Fig. 1.3). Perception of a stressor causes the release of corticotropin releasing factor (CRF) from POA neurons onto the corticotropes of the anterior pituitary. The pituitary corticotropes then release adrenocorticotropic hormone (ACTH), which circulates to the interrenal cells of the head kidney to promote the production of cortisol. The rate limiting steps in cortisol steroidogenesis include the entry of cholesterol into the mitochondria, which is facilitated by steroidogenic acute regulatory protein (StAR), and its cleavage to pregnenolone, which is catalyzed by P450 side chain cleavage enzyme

(P450scc, CYP11A1) (Aluru and Vijayan 2008). Regulation of cortisol steroidogenesis may also be affected by steroidogenic factor 1 (SF-1), which is considered to be a key transcription factor for many steroidogenic genes (Chai et al. 2003; Hsu et al. 2003; Val et al. 2003; Quek and Chan 2009; Schimmer and White 2010; Ruggiero and Lalli 2016). In fishes, SF1 is encoded by the gene *ff1*, for which there are three paralogues in salmonids, *ff1b1, ff1b2* and *ff1d* (Best and Gilmour 2022). Cortisol exerts its effects on target tissues through GR and MR. Acute rises in cortisol are thought to aid the animal in regaining homeostasis, for example by mobilizing energy reserves to aid with extra energy expenditure (Schreck and Tort 2016). However, chronic elevation of baseline cortisol causes negative consequences such as reduced feeding and growth (Kammerer and Heppell 2013; Eldridge et al. 2015; Madeira et al. 2017), and immunosuppression (Ahmad et al. 2011; Schreck and Tort 2016; Teffer et al. 2019; da Costa et al. 2021, 2022).

Acute warming elicits responses such as increases in ventilation and heart rate to ensure proper oxygenation of tissues at high temperatures (Steinhausen et al. 2008; Ekström et al. 2017, 2019; Bard et al. 2021), and increases in lactate and glucose reflecting increased energy expenditure (Zhang and Kieffer 2014; Corey et al. 2017; Bard and Kieffer 2019; Gilbert et al. 2019, 2020; Morrison et al. 2020). These responses are an attempt to maintain physiological function until the stressor is removed or the individual acclimatizes to the new situation. If neither occurs, the individual becomes chronically stressed (Schreck and Tort 2016; Mackey et al. 2021; Goikoetxea et al. 2021). Individuals can also mount a cellular response to heat that includes induction of heat shock proteins (HSP). Inducible HSPs are integral in protecting the cell from thermal stress, as they act

as chaperones (Iwama et al. 1999; Currie 2011), protecting the cell from abnormal protein aggregations by identifying heat-damaged proteins (Krebs and Feder 1997; Iwama et al. 1998). Thus, fish employ a variety of mechanisms to withstand acute warming.

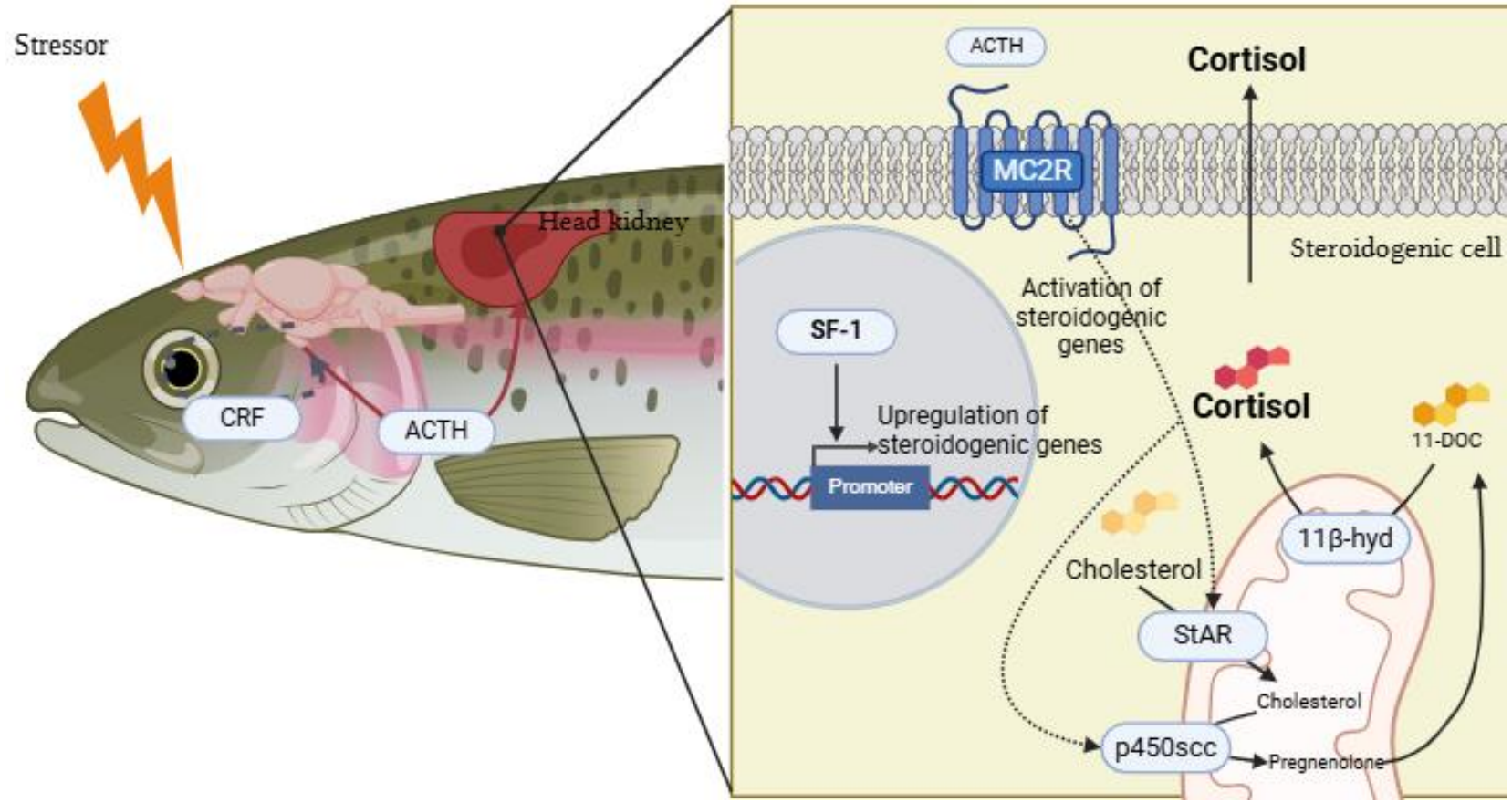
Regulation of the HPI axis in response to elevated temperatures remains relatively understudied in fishes. To date, studies that have measured HPI axis targets after thermal stress are limited (Benítez-Dorta et al. 2017; Goikoetxea et al. 2021; Tang et al. 2022a). In post-smolt Atlantic salmon, thermal acclimation significantly downregulated *crf, mr* and *gr2*, key genes along the HPI axis, but expression remained unaltered after an acute warming (Tang et al. 2022a). In Senegalese sole (*Solea senegalensis*), expression of *crfbp* was elevated by exposure to acute warming, but only a week after the acute thermal stressor (Benítez-Dorta et al. 2017). Corticotropin releasing factor-binding protein (CRFBP) plays an inhibitory role on CRF by binding to its corresponding receptors, and therefore can halt CRF-signalling (Flik et al. 2006). Although changes in the transcript abundance of HPI axis targets with exposure to elevated temperature suggests that cortisol elevation during acute warming reflects activation of the HPI axis, the mechanism through which acute warming triggers HPI axis activity has not been described. Initial experiments to probe this question were carried out (Chapters 2 and 3). Moreover, the cortisol response has been shown to dynamically change based on the magnitude of the prolonged thermal stressor (Mahmoud et al. 2020). These findings suggest that thermal acclimation to elevated temperatures may alter regulation of HPI axis activity and/or the threshold temperature to activate a cortisol response, a possibility that was investigated in Chapter 3.

Chronic elevation of temperature can lead to damaging effects (Alfonso et al. 2021). For example, chronic exposure to high temperature reduces feeding and growth (Kammerer and Heppell 2013; Eldridge et al. 2015; Madeira et al. 2017), increases parasitism and causes immunosuppression (Ahmad et al. 2011; Macnab and Barber 2011; Teffer et al. 2019; da Costa et al. 2021, 2022). These effects can lead to increased mortality (Teffer et al. 2019). Chronic exposure to elevated temperatures has also been shown to increase circulating cortisol (Benítez-Dorta et al. 2017; Madaro et al. 2018; Kim et al. 2019; Hanke et al. 2019; Goikoetxea et al. 2021; Alfonso et al. 2021). This would suggest chronic exposure to high cortisol levels. It has been previously shown that induction of HSP70 was suppressed in fish with chronically elevated cortisol levels (Basu et al. 2001), an effect that implies that chronically stressed fish are likely to be more susceptible to increases in temperature. Furthermore, individuals experiencing chronic stress often exhibit a blunted cortisol response to an acute stressor (Barton et al. 1987; Barcellos et al. 1999; Wunderink et al. 2011; Jeffrey et al. 2014; Madaro et al. 2015; Culbert and Gilmour 2016; Samaras et al. 2018b, 2021; Best and Gilmour 2022). With chronic social stress, this blunting of the cortisol response to acute stress is thought to be largely because the interrenal cells become less responsive to ACTH (Jeffrey et al. 2014; Best and Gilmour 2022). However, subordinate trout subjected to an acute thermal stressor were able to mount a cortisol response comparable to that of their dominant counterparts (Bard et al. 2021). This finding raises questions about regulation of the HPI axis in response to high temperature, with one possibility being that increases in the rate of cortisol production elicited by high temperature offset the impact of chronic stress in reducing responsiveness to ACTH (Burel et al. 1996; Pang et al. 2011; Alfonso et al.

2021). In Chapter 3, I assessed HPI axis regulation in cortisol-treated fish after exposure to high temperature to address this question.

Figure 1.3. Hypothalamic-pituitary-interrenal (HPI) axis of rainbow trout (*Oncorhynchus mykiss*). Once the stressor (external or internal) is perceived, this causes the release of CRF onto the corticotropes of the anterior pituitary. The corticotropes then release ACTH which circulates through the bloodstream to the interrenal cells of the head kidney. This will promote the production of cortisol within steroidogenic cells. MCR2 and SF-1 can cause the activation of StAR and p450scc, which facilitates the rate limiting steps of cortisol synthesis. ACTH, adrenocorticotropic hormone; SF-1, steroidogenic factor 1; StAR, steroidogenic acute regulatory protein; p450scc, cholesterol side-chain cleavage enzyme; 11 β-hyd, 11β-hydroxysteroid dehydrogenase; 11-DOC, 11-deoxycortisol. Adapted from Best & Gilmour (2022).

Figure 1.3.


Stressor
Head kidney
CRF
ACTH
ACTH
Cortisol
MC2R
Steroidogenic cell
Activation of steroidogenic genes
SF-1
Upregulation of steroidogenic genes
Promoter
Cortisol
11-DOC
11β-hyd
Cholesterol
StAR
Cholesterol
p450scc
Pregnenolone

1.5 Thermal preference and behavioural thermoregulation

Although CTmax is a non-lethal endpoint, it represents the highest temperature an individual can withstand before losing locomotory abilities, and survival would be threatened if the individual was not returned to cooler water (Lutterschmidt and Hutchison 1997). As such, the ecological relevance of CTmax often comes under scrutiny, owing both to the rapid heating rates that are used (e.g. 0.33°C/min) (Beitinger et al. 2000; Terblanche et al. 2011; Vinagre et al. 2015; Gallant et al. 2017; Åsheim et al. 2020; Desforges et al. 2023), and because the LOE endpoint is considered to be "ecological death" (Beitinger et al. 2000; Currie and Tattersall 2018; Blasco et al. 2020). In recent years, measures of thermal tolerance that may be more ecologically relevant have been proposed, such as emersion temperature in mangrove rivulus (Currie and Tattersall 2018) and mudskippers (*Periophthalmus argentilneatuis*) (Nay et al. 2018). Emersion temperature works well for amphibious fishes, because the ability to leave the water can be used as an ecologically-relevant endpoint. For aquatic species, a similar approach is the onset-of-agitation temperature, which is the temperature at which swimming becomes erratic (McDonnell and Chapman 2015; McDonnell et al. 2019; Kochhann et al. 2021). Thus, these measures represent the maximum temperature an individual will tolerate before attempting escape to find a thermal refuge. As with CTmax, these measures of thermal tolerance can be altered by environmental conditions. For example, African cichlids (*Pseudocrenilabrus multicolor victoriae*) that were reared in warm and hypoxic (29°C, 15% air saturation) water had lower agitation temperatures than fish reared under warm, normoxic conditions (McDonnell et al. 2019).

Another ecologically-relevant measure of thermal biology is temperature preference. Indeed, when the thermal preferences of two fish species were compared to their realized thermal niches in the wild, no significant differences were found, supporting the ecological relevance of this measure (Ángeles-González et al. 2020). Thermal preference is usually identified by placing an individual in a dynamic thermal gradient and determining the temperature at which it spends the most time (Khan and Herbert 2012; Macnaughton et al. 2018; Christensen et al. 2021). Theoretical grounds suggest that thermal preference represents the temperature within a thermal performance curve at which an animal's performance peaks (Martin and Huey 2008; Dillon et al. 2012) (Fig. 1.4). However, empirical data suggest this may not be the case, because thermal preference often falls below the thermal performance optimum (Martin and Huey 2008; Schram et al. 2013), at least in ectotherms other than fishes, where most of the research attention has been focused (Huey and Bennett 1987; Martin and Huey 2008; Dillon et al. 2012; Tomlinson 2019). Nevertheless, the gap between preferred temperature and optimum temperature indicates that factors beyond performance alone must determine thermal preference, although these factors are poorly understood in fishes (Beitinger and Fitzpatrick 1979; Killen 2014). If thermal tolerance is lowered, we might predict that thermal preference also would be lowered. In this context, it is noteworthy that Cull et al. (2015) reported that cortisol-treated wild pufferfish (*Sphoeroides testudineus*) in their natural habitat preferred cooler temperatures, suggesting that prolonged exposure to elevated cortisol may render fish more susceptible to high temperatures such that they actively seek out cooler water. To our knowledge, the work of Cull et al. (2015) is the only study to have focused on the thermal preference of

cortisol-treated or chronically stressed fish. There is a clear need to determine how elevated cortisol affects thermal preference in other fish species, particularly within the natural environment - this was explored in Chapter 4.

The mechanism through which stress or elevated cortisol alters thermal preference has yet to be identified. A range of possibilities exists, including both direct effects of cortisol on heat sensing mechanisms and indirect effects of cortisol in modulating a variable that in turn impacts thermal preference. For example, well-fed cod seek higher temperatures to optimize growth, whereas cod that receive less food search out colder temperature to reduce metabolic demand (Björnsson 2019). A similar effect was demonstrated in bluegill sunfish (*Lepomis macrochirus*) (Wildhaber and Crowder 1990). Chronic stress and/or cortisol treatment can cause growth depression and reduced food intake (Barton et al. 1987; Pickering et al. 1991; DiBattista et al. 2006; Sadoul and Vijayan 2016), suggesting that stressed fish may seek cooler temperatures to lower metabolic demand. Similarly, the effects of chronic stress in causing immunosuppression are well known (Pickering and Pottinger 1989), and can increase susceptibility to pathogens. In turn, some pathogens may alter thermal preference. For example, parasitic infection in three-spined stickleback (*Gasterosteus aculeatus*) altered thermal preference in favour of warmer temperatures, thereby increasing parasite proliferation (Macnab and Barber 2011). A similar effect on thermal preference was observed in Trinidadian guppies (*Poecilia reticulata*), but in this case, parasitic load was reduced (Mohammed et al. 2016). These examples support the possibility of indirect effects of cortisol elevation on thermal preference.

Thermal preference also appears to vary with animal personality; bolder or more aggressive zebrafish tended to be found at higher temperatures than shy, reactive fish (Rey et al. 2015a). Similar results were obtained in Nile tilapia (*Oreochromis niloticus*) (Cerqueira et al. 2016). Social context may also modulate thermal preference, with fish choosing temperatures outside of their preferred range under some conditions. For example, aggressive mangrove rivulus remained in warmer water (as opposed to emerging from the water) than they otherwise would have when a mirror was used to mimic the presence of a conspecific (Currie and Tattersall 2018). Similarly, group-living three-spined stickleback deviated from their preferred temperature to interact with conspecifics (Cooper et al. 2018).

Fishes use behavioural thermoregulation to regulate body temperature (Freitas et al. 2021). For aquatic ectotherms like fishes, behavioural thermoregulation typically involves actively seeking an environmental temperature that allows the animal to remain at or near its thermal preference. For example, fish often use water column migrations (Biro 1998; Armstrong et al. 2016), but other possibilities include burrowing in the mud (Tytler and Vaughan 1983), sun-basking (Nordahl et al. 2018), and emerging from the water (Gibson et al. 2015). In fact, thermal preference was a main driver of behaviour and habitat choice for fishes within a southern Norwegian fjord (Freitas et al. 2021). However, in a natural setting the preferred temperature of an individual may not always be available, or factors such as avoidance of other stressors may outweigh being at their preferred temperature (Stehfest et al. 2017; Björnsson 2019). These factors may result in individuals shuttling between different thermal regimes, creating daily migrations. For salmonid fishes, it is becoming increasingly difficult to find optimal thermal

environments as temperature ranges narrow and summer water surface temperatures often approach or surpass their thermal tolerance (Keefer et al. 2018a; Mejia et al. 2020). Alterations of depth are an important behavioural thermoregulation mechanism in these freshwater fishes (Biro 1998; Armstrong et al. 2016), because individuals can experience marked changes in temperature (~5°C) below the thermocline, ultimately providing an essential thermal refuge (Biro 1998; Mejia et al. 2020).

In rainbow trout, diel migrations are more common during the summer and vary among individuals (Morbey et al. 2006; Watson et al. 2019). The higher frequency of migrations in the summer likely reflects active avoidance of warm summer surface water temperatures, which makes the summer a good time of year to investigate possible mechanisms underlying individual variation in the use of thermal gradients. Interestingly, 41% of behavioural differences in habitat use in Atlantic cod could be explained by differences in personality (Villegas-Ríos et al. 2017). As previously mentioned, personality types also seem to be related to stress-coping style (Øverli et al. 2004), providing a potential link between cortisol and behavioural thermoregulation.

High temperatures can also alter behaviour. For example, increases in environmental temperature increased exploratory behaviour in zebrafish (Angiulli et al. 2020). When zebrafish were exposed to chronic temperature elevation (i.e. 34°C for 21 days), boldness and instances of individuals being at the top of the tank increased (Angiulli et al. 2020) - notably, these types of behaviour can increase predation risk in the wild. Furthermore, some types of fishing target bolder individuals (Biro and Post 2008). Salmonids are important commercially, ecologically, and culturally, and are in some cases stocked in lakes and rivers (Aas et al. 2018; Lehnert et al. 2020). Given the

importance of salmonid fishes, it is of interest to understand whether chronic stress and/or cortisol elevation alter their habitat use. In Chapter 4, I investigated this possibility in a field study.

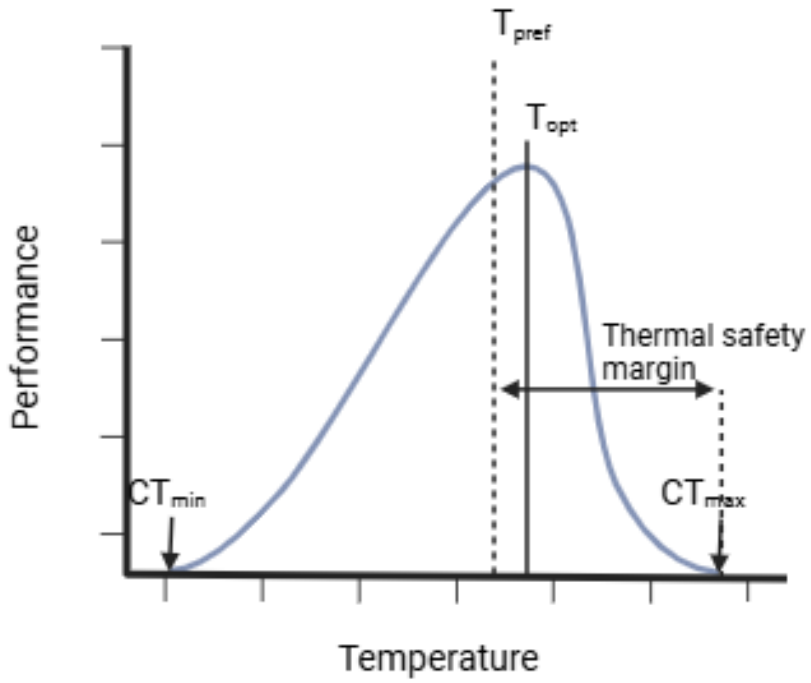


Figure 1.4. Hypothetical schematic of a thermal performance curve (TPC). The temperature at which peak performance occurs is T_{opt}. The temperatures at which performance is zero indicate the lower and upper thermal tolerance of an individual (CT_{min} and CT_{max}, respectively). The temperature that is actively chosen by an individual is T_{pref}. The difference between T_{pref} and CTmax is the upper thermal safety margin. Adapted from Schulte et al. (2011).

1.6 Overarching hypotheses and goals of the thesis

The objective of the present thesis was to elucidate mechanisms underlying thermal tolerance and perception in chronically stressed fish. A review of the literature (see above) indicates that many knowledge gaps remain in our understanding of the relationships between chronic stress and thermal biology in fishes. An overarching hypothesis for my thesis work is that the prolonged elevation of cortisol elicited by chronic stress lowers thermal tolerance, thereby altering the thermal biology of the fish. Chapter 2 assessed whether chronic cortisol elevation on its own is sufficient to alter thermal tolerance (measured as CTmax). I also asked whether chronic cortisol treatment would alter the capacity of an individual to acclimate to elevated temperature, predicting that cortisol-treated fish would experience a lower increase in CTmax upon acclimation to high temperature. In addition, I investigated whether TRPV1 plays a role in sensing noxious high temperature in rainbow trout, through a series of experiments in which TRPV1 activation was manipulated. To test the hypothesis that chronic elevation of cortisol alters thermal perception, causing heightened sensitivity to high temperatures, I evaluated *trpv1* and *trpv4* paralogues in cortisol-treated fish.

In Chapter 3, I investigated the effects of high temperature on HPI axis regulation and the dynamics of the cortisol response by measuring cortisol levels during acute warming together with the expression of key genes in the HPI axis. I also explored the effects of thermal acclimation on the dynamics of the cortisol response. I predicted that cortisol-treated fish would activate a cortisol response to acute warming at lower temperatures whereas trout acclimated to higher temperatures would activate a cortisol response to acute warming at higher temperatures.

Lastly, Chapter 4 investigated potential effects of cortisol treatment on thermal habitat choice in rainbow trout in a field experiment. I predicted that cortisol-treated fish would seek out cooler waters owing to their increased thermal sensitivity. Collectively, these experiments aimed to determine how cortisol may serve as a driver in regulating the thermal biology of rainbow trout. This study also attempted to implicate TRPVs in thermosensation in rainbow trout for the first time. Thus, this thesis aimed to contribute novel information on the mechanism(s) underlying thermal tolerance and thermal perception in fishes.

Chapter 2

The effects of chronic cortisol elevation on thermal tolerance in rainbow trout (*Oncorhynchus mykiss*)

2.1 Introduction

Salmonid fishes are exposed to a multitude of stressors owing to climate change and other anthropogenic effects, such as habitat loss, invasive species, over-exploitation and pollution (Kendall et al. 2017; Almodóvar et al. 2019; Lehnert et al. 2019, 2020; Sinnatamby et al. 2020). Previous research reported that chronic social stress lowered thermal tolerance (measured as the critical thermal maximum, CTmax) in rainbow trout (*Oncorhynchus mykiss*) (Bard et al. 2021), and established a causal relationship between chronic cortisol elevation in socially subordinate fish and decreased CTmax (LeBlanc et al. 2011; Bard et al. 2021). Given rising water temperatures caused by climate change and the economic, ecological and cultural significance of salmonid fishes, it is increasingly important to establish how chronic stress alters their ability to tolerate thermal stress.

Although Bard et al. (2021) demonstrated a mechanistic link between elevated cortisol and lowered thermal tolerance in subordinate trout, social stress has a range of impacts, including appetite loss, metabolic remodelling, and growth suppression (Johnsson et al. 2005; Kostyniuk et al. 2018). Thus, it is important to determine whether elevated cortisol alone lowers thermal tolerance, or whether this effect is specific to chronic social stress. Moreover, increases in CTmax with acclimation temperature are well-established (Beitinger et al. 2000; Zhang and Kieffer 2014; Morrison et al. 2020; McDonnell et al. 2021; Adams et al. 2022; Zhu et al. 2022; Stewart et al. 2023), but

whether chronic stress or cortisol elevation alters an individual's capacity to acclimate to increasing temperature remains unknown. If acclimation capacity is impaired by chronic stress or cortisol elevation, then chronically stressed fish would experience heightened risk as temperature increases.

The mechanism through which cortisol acts to reduce CTmax is not clear. One possibility is that prolonged elevation of cortisol causes detrimental physiological changes that impair the ability of the fish to respond appropriately to high temperature. Based on evidence that chronic cortisol elevation can impair cardiac function (Johansen et al. 2011a, 2017) and research linking heart function and CTmax (Mendonça and Gamperl 2010; Verhille et al. 2013; Ekström et al. 2014, 2016, 2017, 2019; Penney et al. 2014; Eliason and Anttila 2017; Anttila et al. 2023), Bard et al. (2021) tested the hypothesis that lowered CTmax in socially subordinate trout reflected impaired cardiac function. However, support for this hypothesis was limited; most notably, cardiac arrhythmia occurred at lower temperatures in subordinate relative to dominant fish during acute warming (Bard et al. 2021). An alternative hypothesis is that chronic stress/cortisol elevation modulates temperature sensing and/or perception. Under this hypothesis, cardiac arrhythmia and CTmax occur at lower temperatures in subordinate trout because they sense or perceive these temperatures as being warmer than do dominant trout.

Current models suggest that ambient temperature is detected by afferent sensory neurons that express thermosensitive transient receptor potential ion channels (thermoTRPs) (Castillo et al. 2018; Haesemeyer 2020; Kashio 2021; Lezama-García et al. 2022; Cutler and Haesemeyer 2024). These neurons, which have their cell bodies in the trigeminal or dorsal root ganglia, relay signals to several brain regions which integrate

temperature information and produce appropriate behaviours, such as swimming away from temperatures that are too warm (Haesemeyer et al. 2018; Haesemeyer 2020; Cutler and Haesemeyer 2024). Among the thermoTRPs that have been identified, members of the vanilloid (TRPV) and melastatin (TRPM) groups, particularly TRPV1-4 and TRPM2-5, are activated by heat (Saito and Shingai 2006; Castillo et al. 2018; Kashio 2021; Lezama-García et al. 2022). Ray-finned fishes express only TRPV1 and TRPV4 (Morini et al. 2022), with rainbow trout expressing two paralogues each of TRPV1 (TRPV1aα and TRPV1aβ) and TRPV4 (TRPV4α and TRPV4β), likely arising from the salmonid-specific genome duplication event (Saito and Shingai 2006; Saito and Tominaga 2017; Morini et al. 2022). In mammals, TRPV1 in particular is well-documented as being responsible for the detection of noxious high temperatures (Caterina et al. 1997; Saito and Shingai 2006; Saito et al. 2011; Laursen et al. 2016; Saito and Tominaga 2017; Castillo et al. 2018). However, in fishes, limited research has investigated the involvement of TRPV1 in thermoreception (Hunt et al. 2012; Gau et al. 2013; Boltana et al. 2018; Yoshimura et al. 2022). Of importance to the present study, exposure of masu salmon (*Oncorhynchus masou*) to the TRPV1 agonist capsaicin potentiated heat-induced locomotory responses, including loss of equilibrium, such that these responses occurred at lower temperatures than in salmon that were not exposed to capsaicin (Yoshimura et al., 2022). This observation not only implicates TRPV1 in responses to noxious heat in a salmonid but also suggests that the temperature at which the responses are activated reflects the temperature at which TRPV1 is activated.

In the present study, I investigated the role of cortisol itself (rather than chronic stress) in altering thermal tolerance. Based on the hypothesis that chronic cortisol

elevation is sufficient to lower thermal tolerance, I predicted that cortisol treatment would lower CTmax in rainbow trout, and this effect would be blocked by co-treating trout with the glucocorticoid receptor (GR) antagonist RU486. I carried out similar experiments in trout acclimated to 16, 19 or 22°C to explore whether chronic elevation of cortisol decreased the acclimation capacity of trout. Using two-temperature choice tests, I assessed thermal preference in cortisol-treated rainbow trout, to understand whether cortisol treatment altered behavioural thermoregulation. Through TRPV1 antagonist and agonist experiments, I evaluated the role of TRPV1 in activating two key responses to acute warming, increases in circulating cortisol and loss of equilibrium. I also quantified transcript abundances of TRPV1 and TRPV4 paralogues to investigate the effects of cortisol treatment on thermosensation. Collectively, these experiments examined the hypothesis that chronic cortisol elevation in rainbow trout alters thermal tolerance through effects on thermal sensing and/or perception.

2.2 Material and Methods

2.2.1 Experimental animals

Juvenile rainbow trout, *Oncorhynchus mykiss* (Walbaum 1792) (N = 234; mass = 127.2 ± 3.8 g, fork length = 22.4 ± 0.2 cm, mean ± SEM; see Table 2.1), were purchased from Izumi Aquaculture, formerly Linwood Acres Trout Farm (Campbellcroft, ON, Canada), Cedar Crest Trout Farms (West Grey, ON) and Lyndon Fish Hatcheries (New Dundee, ON). Fish were held at the University of Ottawa in 1,275 litre fibreglass tanks supplied with flowing, aerated, dechloraminated city of Ottawa tap water at a temperature of 13°C (“system” water). A 12 h:12 h light:dark photoperiod was maintained, and fish

were fed a ration of 0.5% body mass daily by scattering commercial trout pellets (Skretting, BC) on the water's surface. 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-3668, University of Ottawa) and were in compliance with the guidelines of the Canadian Council on Animal Care (CCAC) for the use of animals in research and teaching.

2.2.2 Experiment 2.1: Effects of cortisol treatment on CTmax

The goal of this experiment was to determine whether prolonged elevation of cortisol in the absence of a stressor was sufficient to decrease CTmax. Trout were allocated haphazardly to one of three treatment groups, control (N = 8), cortisol-treated (N = 13), or co-treatment with cortisol and the GR blocker RU486 (cortisol+RU486; N = 8). Following treatment, fish were held individually in 40 L experimental tanks supplied with flowing system water for 4 d and then subjected to a CTmax trial. In a second, independent trial, fish were allocated to control (N = 6) or cortisol treatment (N = 7) groups and held for 2 d before being subjected to a CTmax trial. Fish were not fed during the treatment period because cortisol treatment typically supresses feeding behaviour (Van Weerd and Komen 1998).

2.2.2.1 Administration of cocoa butter cortisol implants

Fish were lightly anaesthetized (to the point of losing equilibrium) in a solution of benzocaine (0.05 g L^{-1} ethyl-p-aminobenzoate; E1501, Sigma-Aldrich, Oakville, ON, Canada), and mass and fork length were measured. Fish in the control group were then placed in their experimental tank to recover, whereas fish in the cortisol or cortisol+RU486 treatment groups were first given an intraperitoneal implant of cocoa

butter (NOW Health Group Inc., Bloomingdale, IL, USA; 0.5mL per 100 g body mass) containing cortisol (hydrocortisone 21-hemisuccinate; H2270, Sigma- Aldrich; 80 mg kg^{-1} body mass) or cortisol and RU486 (M8046, Sigma-Aldrich; 500 mg kg^{-1} body mass). The doses of cortisol and RU486 were chosen on the basis of previous studies (DiBattista et al. 2005, 2006; Hoogenboom et al. 2011; Lawrence et al. 2017; Bard et al. 2021). Cortisol and cortisol+RU486-impregnated cocoa butter implants were prepared by first dissolving the cortisol or cortisol+RU486 in 99% ethanol. This solution was added to melted cocoa butter and the ethanol was evaporated off by heating the solution (Hoogenboom et al., 2011; Lawrence et al., 2019). The liquid cocoa butter (~32°C) was injected into the peritoneal cavity using a 22G sterile needle and 1 mL syringe, where it quickly solidified. After administration of the implant, fish were placed in their experiment tanks to recover.

2.2.2.2 Measurement of CTmax

Measurement of CTmax was carried out between 09:00 and 11:00 h on individual trout in 40 L experimental tanks. Fish were subjected to a linear increase in water temperature of 0.33 ± 0.01°C min^{-1} (Becker and Genoway, 1979) until LOE. Loss of equilibrium was indicated by the fish turning dorso-ventrally and being unable to right itself within 3 s. The temperature of LOE was then noted as CTmax. Following CTmax, the water temperature was returned to the acclimation temperature (13°C) for 1 h. At the end of the hour, fish were euthanized using terminal anesthesia (0.5 g L^{-1} ethyl-p-aminobenzoate, Sigma Aldrich) followed by spinal severance. Water temperature was monitored using a digital thermometer (Digi-sense, Cole Palmer, Quebec, QC, Canada) and regulated using a series 440 Fotopanel thermostatic mixing valve (POWERS™,

Burlington, ON, Canada). Incoming water flow to the experimental tanks was vigorously aerated using an equilibration column, which ensured the maintenance of air saturation as temperature increased; this was confirmed by monitoring dissolved oxygen levels during a CTmax trial.

2.2.3 Experiment 2.2. Effects of thermal acclimation and cortisol treatment on CTmax

Owing to the well-established relationship between acclimation temperature and CTmax (Beitinger et al. 2000; Zhang and Kieffer 2014; Morrison et al. 2020; McDonnell et al. 2021; Adams et al. 2022; Zhu et al. 2022; Stewart et al. 2023), I investigated how acclimation to higher temperatures altered CTmax in trout experiencing chronic cortisol elevation. The hypothesis was that chronic cortisol treatment reduces plasticity for acclimation to elevated temperatures. For this experiment, three acclimation temperatures were used (16°C, N = 24; 19°C, N = 24; 22°C, N = 23). Individual fish were fitted with mini-osmotic pumps containing a cortisol solution, to keep cortisol levels high for the entirety of the three-week acclimation period. Fish were fed daily during the acclimation period, and following the acclimation period, individuals were subjected to a CTmax trial.

2.2.3.1 Thermal acclimation

Fish were transferred into 250 L circular holding tanks supplied with system water at 15°C and left to recover overnight. Water temperature was increased by 1°C per day until the desired acclimation temperature was achieved (16°C, 19°C, or 22°C). After 24 h at the acclimation temperature, fish (N = 24) were selected haphazardly from the tank and fitted with mini-osmotic pumps (described below). Each tank held 30 fish, with

12 of the fish fitted with mini-osmotic pumps and the remaining being used for measurement of *trpv* transcript abundance (described below) and/or an experiment for another study (see Chapter 3). This approach was chosen to ensure appropriate densities in holding tanks, because low densities can give rise to social hierarchies that elicit stress in some fish (Ellis et al. 2002; Brockmark and Johnsson 2010; Roy et al. 2021). The fish were then held at the acclimation temperature for three weeks, with the choice of three weeks reflecting both thermal acclimation times in previous studies (Healy and Schulte 2012; Anttila et al. 2015; Adams et al. 2022; Stewart et al. 2023) and the limits of the mini-osmotic pumps (Madison et al. 2015). Fish were fed once daily at 0.5% of their body mass by scattering commercial food pellets on the water's surface. Following the acclimation period, fish were transferred individually to 40 L experimental tanks for an overnight recovery period (at the acclimation temperature) and subjected to a CTmax trial as described above (Section 2.2.2.2).

2.2.3.2 Implantation of mini-osmotic pumps

Fish were lightly anesthetized in a solution of benzocaine (0.05 g L^{-1} ethyl-p-aminobenzoate; Sigma-Aldrich) until LOE and then transferred to a surgical table that allowed constant irrigation of the gills with the benzocaine solution. An ~2 cm incision was made midline on the ventral surface posterior to the pectoral girdle and a mini-osmotic pump (Alzet, model 1007D, Durect Corporation, Cupertino, CA, USA) was inserted into the peritoneal cavity. The incision was sutured closed (using 5-0 suture silk) and the fish were transferred to 40 L tanks containing the corresponding acclimation water (i.e. 19 or 22°C) to recover for 1 h. The fish were then returned to their acclimation tank (250 L). The mini-osmotic pumps of cortisol-treated fish were filled with 100 µL of

a solution of cortisol dissolved in 65% 2-hydroxypropyl-β-cyclodextrin (H107, Sigma-Aldrich) to achieve a concentration of 80 μg g^{-1} body weight as per Madison et al. (2015). Sham-treated fish received mini-osmotic pumps filled with 100 μL of the cyclodextrin solution alone.

2.2.4 Experiment 2.3. Effects of cortisol treatment on thermal choice

As a test of whether cortisol treatment alters thermal perception, behavioural thermoregulation was investigated by monitoring the time spent within each chamber of a two-chamber thermal choice arena. Cortisol treated individuals (N = 13) were given a cocoa butter cortisol implant as in Experiment 2.1. Control individuals (N = 14) were untreated. Thermal preference was determined by allowing fish to actively choose between two static chambers held at different temperatures.

2.2.4.1 Static two-chamber thermal choice test

A two-chamber shuttle-box based on the plans provided by Tattersall et al. (2020) but sized to accommodate rainbow trout of 50-300g (the volume of each chamber was ~30L; total length of tank was ~0.9m) was constructed from opaque, black Plexiglas. Each chamber of the shuttle-box was supplied with system water via a series 440 Fotopanel thermostatic mixing valve, and water temperature at each end of the shuttle-box was recorded every 10 s using a data logging thermometer (Digi-sense, Cole Palmer, Quebec, QC, Canada). A temperature difference of 4-6°C could be reliably maintained between the two chambers with this system. The shuttle-box was used in a static mode owing to limitations on the control of water temperature that would be needed for dynamic temperature changes (Chistensen et al. 2021). Three temperature choices were used in separate trials: (a) cool chamber 10°C vs warm chamber 16°C, (b) 16°C vs 20°C,

and (c) 20°C vs 25°C, with the designated cool chamber being chosen randomly for each trial. For each trial, the overnight acclimation temperature in both chambers was midway between the test temperatures (i.e. 13°C, 18°C, and 23°C, respectively, for the three choice trials). A video camera (Vixia HF R500, Canon) was placed directly over the shuttle-box to record each trial.

Fish were allocated randomly to one of the three choice trials and were placed into the shuttle-box at 13°C on day 4 of control or cortisol treatment. Water temperatures were slowly ramped (0.33°C/min) to the appropriate temperature for an overnight habituation and exploratory period. The static thermal choice test began at 09:00 h the following morning with adjustment of water temperatures at each end of the shuttle-box to the set temperatures (~10 min). Once the set temperatures had stabilized, recording was initiated and fish were monitored for 2 or 4 h, after which water temperature was returned to the acclimation value. Fish were euthanized using terminal anesthesia and spinal severance.

Video recordings were analyzed by an observer who was blind to the treatment group of the fish being analyzed. The event-logging application BORIS™ (https://www.boris.unito.it/) was used to determine time spent in the warm chamber as a percentage of the total trial time (Nati et al. 2018; Chistensen et al. 2021).

2.2.5 Experiment 2.4: Effects of manipulating TRPV1 activation

To implicate TRPV1 in thermoreception in rainbow trout, effects of TRPV1 activation on thermal tolerance and cortisol release were investigated. Activation of TRPV1 was manipulated using capsazepine (TRPV1 antagonist; $N = 8$) or capsaicin (TRPV1 agonist; $N = 8$), with corresponding shams ($N = 8$) for each. Following

treatment, fish were subjected to a CTmax trial. An additional experiment focused on whether TRPV1 activation is linked to the cortisol response to acute warming. In this experiment, fish were treated with capsazepine (N = 5) or capsaicin (N = 8), along with respective and shams (N = 6-8), and then exposed to acute warming to monitor their cortisol response.

2.2.5.1 Effect of TRPV1 inhibition by capsazepine on CTmax and the cortisol response to acute warming

Fish were individually placed in 3 L experimental chambers supplied with flowing system water at 13°C for an overnight recovery period. The following morning, fish were lightly anesthetized using a benzocaine solution (0.05g L^{-1} ethyl-p-aminobenzoate, Sigma Aldrich) and given an intraperitoneal injection of 50 µL of capsazepine (C191, Sigma Aldrich; 5mg kg^{-1} body mass) dissolved in DSMO (5 mg capsazepine per 1 mL^{-1} DMSO; final circulating concentration of DMSO was ~ 99%; the concentration of capsazepine was based on that used by Rocha Barreto et al. (2022)). Sham fish were injected with 50 µL of DSMO only. The anesthetic solution was quickly flushed out of the tanks with a high flow of 13°C system water, and 10 min after the fish had regained equilibrium, it was subjected to a CTmax trial under flowthrough conditions, as described above (Section 2.2.2.2).

A separate group of fish was treated with capsazepine or vehicle alone as described above. After the 10 min recovery period, water temperature was raised at a rate of 0.3°C min^{-1} to 27°C, at which temperature it was held for 10 min. The choice of 27°C was based on the observation (CTmax data of Fig. 2.1, as well as the data of Fig. 3.1 in Chapter 3) that fish could be reliably held for 10 min at this temperature without losing

equilibrium. After the 10 min exposure, water temperature was returned to 13°C and fish were left for 1 h of recovery. Fish were then euthanized using terminal anesthesia and spinal severance as described above and a blood sample (~1 mL) was withdrawn from the caudal vasculature using a 23G needle and 1 mL syringe rinsed with 0.5 mol L^{-1} ethylenediaminetetraacetic acid (EDTA; E6758, Sigma Aldrich) as an anticoagulant. Blood samples were centrifuged at 10,000 *g* for 2 min, and the resulting plasma was flash frozen in liquid N_2 and stored at -80°C for later analysis of plasma cortisol concentrations. Plasma cortisol concentrations were analyzed using a commercial radioimmunoassay (RIA; MP Biomedical, LLC, USA) previously used for the analysis of trout plasma samples (Gamperl et al. 1994). All samples were assessed in a single assay with an intra-assay coefficient of variation of 13.0%. Preliminary trials comparing cortisol concentrations between control (untreated) and sham-treated fish suggested that DMSO alone had no significant effect on the cortisol response. Moreover, cortisol production by head kidney preparations *in vitro* was measured in the presence and absence of capsazepine (5 mg/1 mL of DMSO; 5 µL per well) to confirm that DMSO did not inhibit cortisol production (Section 2.5.1; Fig. S.2.2).

2.2.5.2 Effect of TRPV1 activation by capsaicin on CTmax and the cortisol response to acute warming

Fish were individually placed in 3 L experimental chambers supplied with flowing system water at 13°C for an overnight recovery period. The following morning, fish were exposed to waterborne capsaicin (PHR1450, Sigma Aldrich) dissolved in 1% ethanol to a final concentration of 100 µM (Yoshimura et al. 2022). Sham fish were

exposed to the 1% ethanol solution alone. Fish were exposed to treatment for 10 min and then subjected to a CTmax trial as described above (Section 2.2.2.2).

A separate group of fish was treated with capsaicin or vehicle alone as described above. After the 10 min exposure period, water temperature was raised at a rate of 0.3°C min^{-1} to 13°C, 25°C or 27°C and held for 10 min before being returned to 13°C for 1 h. The 13°C exposure temperature allowed baseline cortisol levels to be determined. The temperature of 25°C was chosen because it typically did not elicit a cortisol response under control conditions (Chapter 3), whereas 27°C was the lowest temperature that reliably elicited a cortisol response under control conditions. At the end of the 1 h recovery period, fish were euthanized using terminal anesthesia and spinal severance and a blood sample (~1 mL) was withdrawn from the caudal vasculature using a 23G needle and 1 mL syringe rinsed with 0.5 mol L^{-1} EDTA as an anticoagulant. Blood samples were centrifuged at 10,000 *g* for 2 min, and the plasma was flash frozen in liquid N_2 and stored at -80°C for later analysis of plasma cortisol concentrations together with the samples from the capsazepine trails (as described above, Section 2.2.5.1). Preliminary trials comparing cortisol concentrations between control (untreated) and sham-treated fish suggested that ethanol alone had no significant effect on the cortisol response.

2.2.6 Experiment 2.5: Effects of cortisol treatment and acute warming on *trpv1* and *trpv4* paralogue transcript abundances

To examine the potential for cortisol elevation to modulate thermoreception by altering the abundance of thermoTRPs, the promoter regions of *trpv1* and *trpv4* paralogues were evaluated for the presence of glucocorticoid response elements (GREs), and transcript abundances of the paralogues were measured in gill and brain tissue of

control and cortisol-treated fish prior to acute warming (13°) or following acute warming to 27°C. Gill was chosen as a tissue that plays a key role in sensing other environmental variables (e.g. water O_2 and CO_2 tensions; Zachar and Jonz 2012; Perry and Tzaneva 2016), and the POA and hypothalamus were assessed because of their roles in regulation of the HPI axis and internal environment, respectively (Vijayan et al. 2010; Faught et al. 2016; Haesemeyer 2020). Transcript abundances of *trpv* paralogues were also measured in gill tissue of trout that were acclimated to higher temperatures to determine whether transcriptional regulation of thermoTRPs accompanies long-term exposure to elevated temperatures.

2.2.6.1 Identification of GREs in TRPV promoter sequences

To identify GREs in the promoter regions of the *trpv1* paralogues, *trpv1aα* and *trpv1aβ*, and the *trpv4* paralogues, *trpv4α* and *trpv4β*, JASPAR 2022 (https://jaspar.elixir.no/) was used (Fornes et al. 2020; Jiang et al. 2021; Best et al. 2024). The promoter region was considered to be the 2000 base pairs (bp) upstream of the start of the mRNA strand [i.e. 2000 bp within the 5' untranslated region (UTR)]. A GRE was identified as the imperfect palindrome GGTACAnnnTGTTCT. The promoter region was then scanned by JASPAR 2022 for the GRE (*nr3c1*, matrix id. MA0113.1) with a filter of a relative profile score threshold of 80%, chosen based on previous literature to reliably identify GRE sequences (Best et al. 2024).

2.2.6.2 Measurement of trpv paralogue transcript abundances using real-time RT-PCR

Fish were individually placed in 40 L experimental tanks for overnight recovery. The following morning, individuals (N = 4) were sampled at the acclimation temperature (13°C) or following acute warming in which fish were subjected to linear increases in

water temperature of 0.3 ± 0.01°C min^{-1} until water temperature reached 27°C. Fish were held at this temperature for 10 min, and water temperature was then returned to 13°C for 1 h of recovery. Fish were then euthanized with terminal anesthesia followed by spinal severance, and POA, hypothalamus and the first two gill arches on the left side were dissected out, flash frozen with liquid nitrogen, and stored at -80°C for further analysis.

A separate group of fish (N = 36) underwent a thermal acclimation treatment (as described in Section 2.2.3.1). Fish were then individually transferred to 40 L tanks for an overnight recovery period. The following morning, fish were exposed to the thermal ramping protocol described above with the final temperatures being the acclimation temperature (19°C or 22°C, i.e. no change in temperature), 27°C or 29°C. Fish were euthanized with terminal anesthesia and spinal severance and the first two gill arches from the left side were dissected out and flash frozen in liquid nitrogen. Gill tissue was stored at -80°C until further analysis.

Frozen tissues were ground to a powder on dry ice using a mortar and pestle. To extract total RNA, powdered brain (approximately 10-40 mg) and gill (100-200 mg) tissues were sonicated (Sonic Dismembrator Model 100, Fisher Scientific) on ice in TRIzol® reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. The RNA was re-suspended in nuclease-free water, and the quantity and quality of RNA were assessed using a NanoDrop® (NanoDrop™ 2000, ThermoFisher Scientific). Genomic DNA was removed, and cDNA was synthesized from total RNA using the QuantiTect®reverse transcription kit (Qiagen, Montreal, QC, Canada) according to the manufacturer's directions.

Gene-specific primer sequences were identified from the literature for target genes, *trpv1aα* and *trvp4α*, as well as *β-actin* as a housekeeping gene that was used for normalization (Table 2.2). Custom primers were created with Primer-BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi?GROUP_TARGET=on) for *trpv1aβ* and *trpv4β* using the gene sequences identified by Morini et al. (2022) (Table 2.2). Primer pairs were chosen based on length (100-200bp) and annealing temperature (~60°C). Real-time RT-PCR was carried out using the Rotor-Gene SYBR Green PCR kit (Qiagen) and a Rotor- Gene Q real-time PCR machine (Qiagen). Reactions were carried out according to the manufacturer's instructions, but volumes were scaled to 10 µl. Samples were run in duplicate, together with negative controls (a no-template sample in which cDNA was replaced with water, and a no-RT sample in which the cDNA synthesis reaction was carried out without reverse transcriptase). Cycling conditions were as follows: 5 min at 95°C, 42 cycles of 10 s at 95°C, 10 s at 60°C and 10 s at 72°C, followed by melt curve analysis to confirm amplification of a single product, as per the manufacturer's instructions. Standard curves were generated for gill and brain tissues separately using serially diluted pooled samples and relative transcript abundances were calculated according to Pfaffl (2001), using ß-actin as the housekeeping gene, and expressed as fold-change relative to the control fish sampled at 13°C.

2.2.7 Statistical analysis

All statistical analyses were performed using R-studio. Prior to analysis, data were checked for violation of assumptions of parametric tests; normality with the Kolmogorov–Smirnov test, and equal variance with Levene's test. For all statistical analyses, α was set to 0.05 and data are presented as means ± SEM in tables. In figures,

data are presented as box plots. The upper and lower limits of the box indicate the 75th and 25th percentiles, respectively, the black line across the box designates the median. The whiskers highlight the maximum and minimum values, the red plus sign indicates the mean value, and each circle represents an individual data point.

CTmax data was analyzed using a Student's *t*-test for 2 d and a one-way analysis of variance (ANOVA) for 4 d (Experiment 2.1). Multiple comparisons were carried out using Tukey's test. The effects of thermal acclimation and cortisol treatment on CTmax and thermal safety margin were assessed by two-way ANOVA (Experiment 2.2). Thermal safety margin was calculated as the difference between CTmax and acclimation temperature for every individual. Acclimation response ratios (ARR) were also calculated as ΔCTmax/Δacclimation temperature. Similarly, thermal choice data were analyzed by two-way ANOVA on arcsine-transformed data, with treatment group and temperature choice as the two factors (Experiment 2.3). The effects of capsazepine and capsaicin treatment on CTmax were analyzed by Student's *t*-tests (Experiment 2.4). The effect of capsazepine treatment on plasma cortisol was analyzed using a Welch Student's *t*-test owing to unequal variances, whereas plasma cortisol values after capsaicin treatment were analysed using a two-way ANOVA with treatment and temperature as factors (Experiment 2.4). Relative mRNA abundances of *trpv* paralogues were analyzed by two-way ANOVA using treatment or acclimation temperature as one factor, and exposure temperature as the second factor; data for each tissue and paralogue were analyzed independently (Experiment 2.5). If the data were non-normal and/or heteroscedastic, they were initially log-transformed but if the transformed data failed to meet the assumptions,

a non-parametric two-way ANOVA, Scheirer–ray–hare test was used, with treatment and temperature as factors (Experiment 2.5).

Table 2.1. Morphometric data of rainbow trout (*Oncorhynchus mykiss*) by experiment.

Experiment	Treatment	Mass (g)	Fork length (cm)
2.1 Effects of cortisol treatment on CTmax			
	Control (*N* = 15)	127.9 ± 9.7	23.2 ± 0.7
	Cortisol treated (*N* = 20)	149.3 ± 13.2	23.8 ± 0.7
	Cortisol & RU486 treated (*N* = 8)	115.0 ± 8.9	21.8 ± 0.5
2.2 Effects of thermal acclimation and cortisol treatment on CTmax			
	Sham (*N* = 35)	105.7 ± 4.7	21.4 ± 0.4
	Cortisol treated (*N* = 36)	103.1 ± 3.6	21.0 ± 0.3
2.3: Effects of cortisol treatment on thermal choice			
	Control (*N* = 13)	192.6 ± 25.6	25.7 ± 0.9
	Cortisol treated (*N* = 14)	191.4 ± 16.0	26.1 ± 0.7
2.4: Effects of manipulating TRPV1 activation			
	Sham DSMO (*N* = 13)	92.6 ± 14.1	20.1 ± 1.0
	Capsazepine-treated (*N* = 16)	63.5 ± 2.9	18.1 ± 0.3
	Sham ethanol (*N* = 24)	122.4 ± 11.6	22.1 ± 0.6
	Capsaicin-treated (*N* = 24)	126.5 ± 10.5	22.4 ± 0.6
2.5: Effects of cortisol treatment and acute warming on *trpv1* and *trpv4* paralogue transcript abundances			
	Control (*N* = 8)	190.0 ± 6.2	26.3 ± 0.4

	Cortisol treated (N = 8)	193.0 ± 13.3	26.3 ± 0.4
	Control acclimation (N = 36)	76.1 ± 4.6	18.6 ± 0.3
Total (N = 270)		120.3 ± 3.5	21.9 ± 0.2

Table 2.2. Gene-specific primers used for real-time RT-PCR (qPCR).

Gene	Primer sequence (5' to 3')	Amplicon size (bp)	Efficiency* (%)	Accession number	Reference
trpv1aα	F: CGTCCTGCTGAAGGCTCTA	121	97,97,86	XM_021618024.2	(Nisembaum et al. 2015)
	R: TGTCTGTGTATGCAGCATTTACTA				
trpv1aβ	F: ATCGGAGGACCAATATGCGG	111	109, 112, 96	ENSOMYG00000026242	
	R: TGGGTTTCGGTCATTCCACG				
trpv4α	F: GAGAATCGCCATGAGATGC	155	96, 105, 108	ENSOMYG00000019921	(Nisembaum et al. 2015)
	R: TGGGATGGGCGGTAGTA				
trpv4β	F: GGGGTTTGCACATTTGGGAC	153	96, 105	ENSOMYG00000006364	
	R: CCATGTTTGGCTGGTTGGTG				
β-actin	F: AGAGCTACGAGCTGCCTGAC	197	103, 103, 104	NM_001124235.1	(Moltesen et al., 2016)
	R: GTGTTGGCGTACAGGTCCTT				

*Efficiency values are presented for gill, hypothalamus and POA, in that order.

Where no reference is provided, the primers were designed for the present study.

2.3 Results

2.3.1 Experiment 2.1: Effects of cortisol treatment on CTmax

Although no significant difference in CTmax was detected between control and cortisol-treated fish after 2 d of cortisol treatment (Fig. 2.1A), CTmax was significantly lower (~1°C) than the control value after 4 d of cortisol treatment (Fig. 2.1B). Co-treatment with cortisol and RU486 eliminated the effect of cortisol treatment on CTmax (Fig. 2.1B).

2.3.2 Experiment 2.2: Effects of thermal acclimation and cortisol treatment on CTmax

Both acclimation temperature and cortisol treatment had significant effects on CTmax, but there was no significant interaction between these factors (Fig. 2.2A). Sham fish, which received a mini-osmotic pump filled with vehicle, had higher CTmax values than cortisol-treated fish (Fig. 2.2A). Regardless of treatment group, CTmax increased with acclimation temperature (Fig. 2.2A). Similarly, both acclimation temperature and cortisol treatment had significant effects on thermal safety margin, but there was no significant interaction between these factors (Fig. 2.2B). Thermal safety margins significantly decreased with increasing acclimation temperatures, and cortisol-treated fish had lower thermal safety margins than control fish (Fig. 2.2B). Acclimation response ratios were 0.14 and 0.11 for sham and cortisol-treated fish, respectively.

2.3.3 Experiment 2.3: Effects of cortisol treatment on thermal choice

Cortisol treatment had a significant effect on thermal choice, in that cortisol-treated fish spent significantly more time in the warmer chamber regardless of the

thermal choice they were offered (Fig. 2.3). At the same time, as water temperature increased across choice temperatures, individuals spent significantly less time in the warmer chamber regardless of treatment (Fig. 2.3).

2.3.4 Experiment 2.4: Effects of manipulating TRPV1 activation in rainbow trout

Fish treated with the TRPV1 antagonist capsazepine had a significantly higher CTmax than sham-treated fish exposed to vehicle alone (Fig. 2.4A). Correspondingly, fish exposed to the TRPV1 agonist capsaicin had a significantly lower CTmax than sham fish exposed to vehicle alone (Fig. 2.4B). Acute warming to 27°C appeared to elevate plasma cortisol compared to values for fish that were held at 13°C (Fig. 2.5). However, fish treated with capsazepine exhibited significantly lower plasma cortisol at 27°C than sham-treated fish (Fig. 2.5). It should also be noted that ACTH-stimulated cortisol production by *in vitro* head kidney preparations was not affected by exposure to capsazepine (Fig. S.2.1). Plasma cortisol was unaffected by exposure to capsaicin in fish held at 13°C or following acute warming to 25°C or 27°C (Fig. 2.6). However, fish acutely warmed to 25°C or 27ºC exhibited significantly higher cortisol levels than fish held at 13°C (Fig. 2.6).

2.3.5 Experiment 2.5: Effects of cortisol treatment and acute warming on trpv1 and trpv4 paralogue transcript abundances

A GRE was found within the promoter sequence of *trpv1aβ* starting at -567 bp and ending at -584 bp (relative to the transcription start site on the negative strand; Fig. 2.7A; Fig. S.2.4B). A GRE was also found within the promoter of *trpv4α* at -469 bp to -486 bp (Fig. 2.7B; Fig. S.2.4C).

Examination of *trpv* paralogue transcript abundances in gill tissue (Fig. 2.8) revealed a significant effect of cortisol treatment on *trpv4α* expression regardless of exposure temperature (Fig. 2.8C). No significant differences were detected in *trpv1* and *trpv4* in the brain (POA and hypothalamus), at either temperature or between treatment groups (Fig. 2.9; Fig. 2.10). A trend for *trpv1aα* expression to be lower at high temperatures in hypothalamus, regardless of treatment, was detected (Fig. 2.9A). There was also a significant interaction between treatment and temperature for POA *trpv4α* and a trend for POA *trvp1aβ*; however, multiple comparisons failed to reveal the origin of this significant difference (Fig. 2.10).

Acclimation to higher temperatures resulted in significantly higher *trpv1aα* and *trpv1aβ* transcript abundances in the gill, with no observed effect for *trpv4* paralogues (Fig. 2.11). In addition, fish subjected to acute warming exhibited significantly higher abundance of *trpv1aβ* only (Fig. 2.11B). No significant interactions between acclimation temperature and exposure temperature were detected for any paralogue.

2.4 Figures

Figure 2.1. Critical thermal maxima (CTmax) of rainbow trout (*Oncorhynchus mykiss*) after (A) 2 days or (B) 4 days of cortisol treatment. For (A), no significant difference was detected (Student's *t*-test, $P = 0.127$; $N = 6\text{-}7$ for each group). For (B), the CTmax of cortisol-treated fish ($N = 13$) was significantly lower than that of control fish ($N = 8$) or fish treated with a combination of cortisol and the GR antagonist RU486 ($N = 8$) (one-way ANOVA, $P = 0.001$). Treatment groups that share a letter are not significantly different from one another (Tukey's Multiple Comparisons). Data are presented as boxplots (see Section 2.2.6 for details).

Figure 2.1.

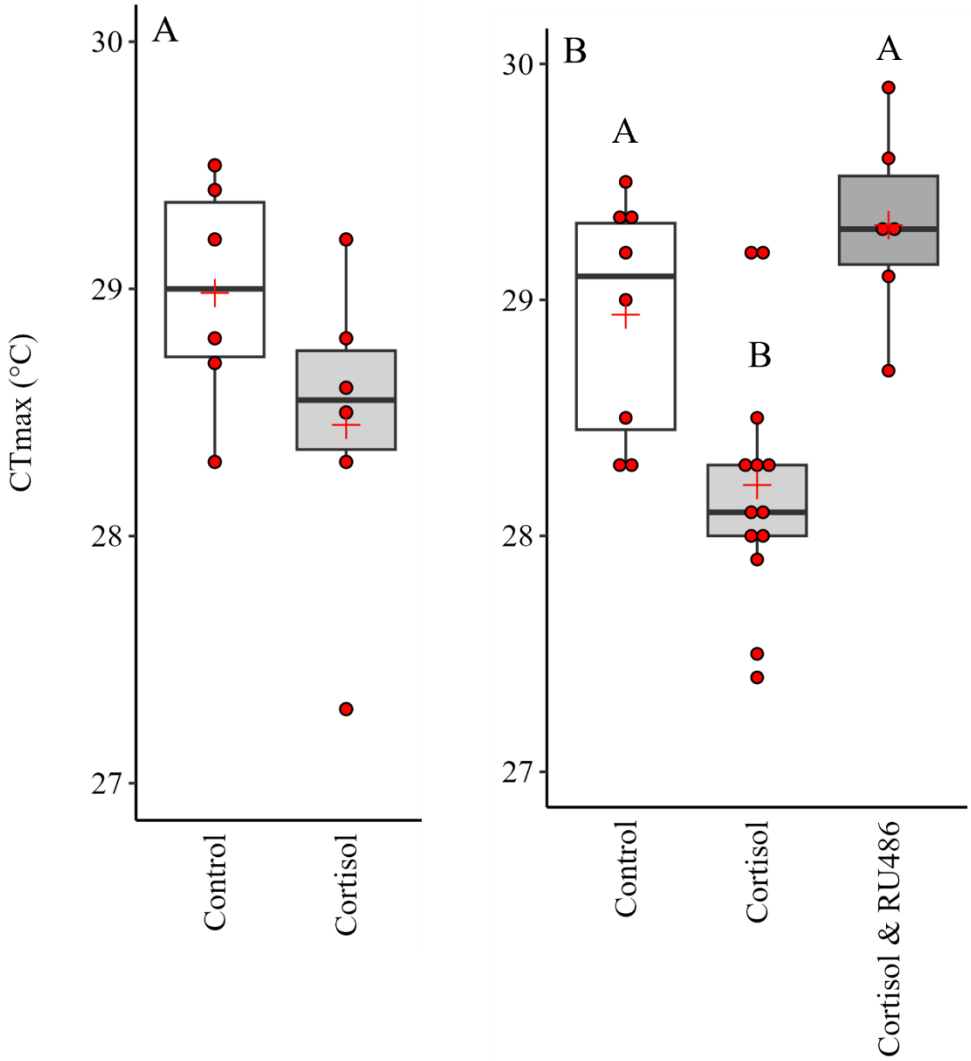

CTmax (°C)
30
29
28
27
A
Control
Cortisol
B
A
B
A
Control
Cortisol
Cortisol & RU486

Figure 2.2. (A) Critical thermal maxima (CTmax) and (B) thermal safety margins of sham and cortisol-treated rainbow trout (*Oncorhynchus mykiss*) after acclimation to 16°C (*N* = 12 per group), 19°C (*N* = 12 per group), or 22°C (*N* = 11-12 per group). Thermal acclimation and cortisol treatment but not the interaction of these two factors had significant effects on CTmax and thermal safety margins (two-way ANOVA, for panel A, $P_{\text{treatment}} < 0.0001$, $P_{\text{acclimation temperature}} < 0.0001$, $P_{\text{treatment x acclimation temperature}} = 0.812$; for panel B, $P_{\text{treatment}} < 0.0001$, $P_{\text{acclimation temperature}} < 0.0001$, $P_{\text{treatment x acclimation temperature}} = 0.812$). Acclimation temperatures that share a letter are not significantly different from one another, and the effect of cortisol treatment is represented on the figure using the bar fills. Thermal safety margin was calculated as the difference between CTmax and acclimation temperature. Data are presented as boxplots (see Section 2.2.6 for details).

Figure 2.2.

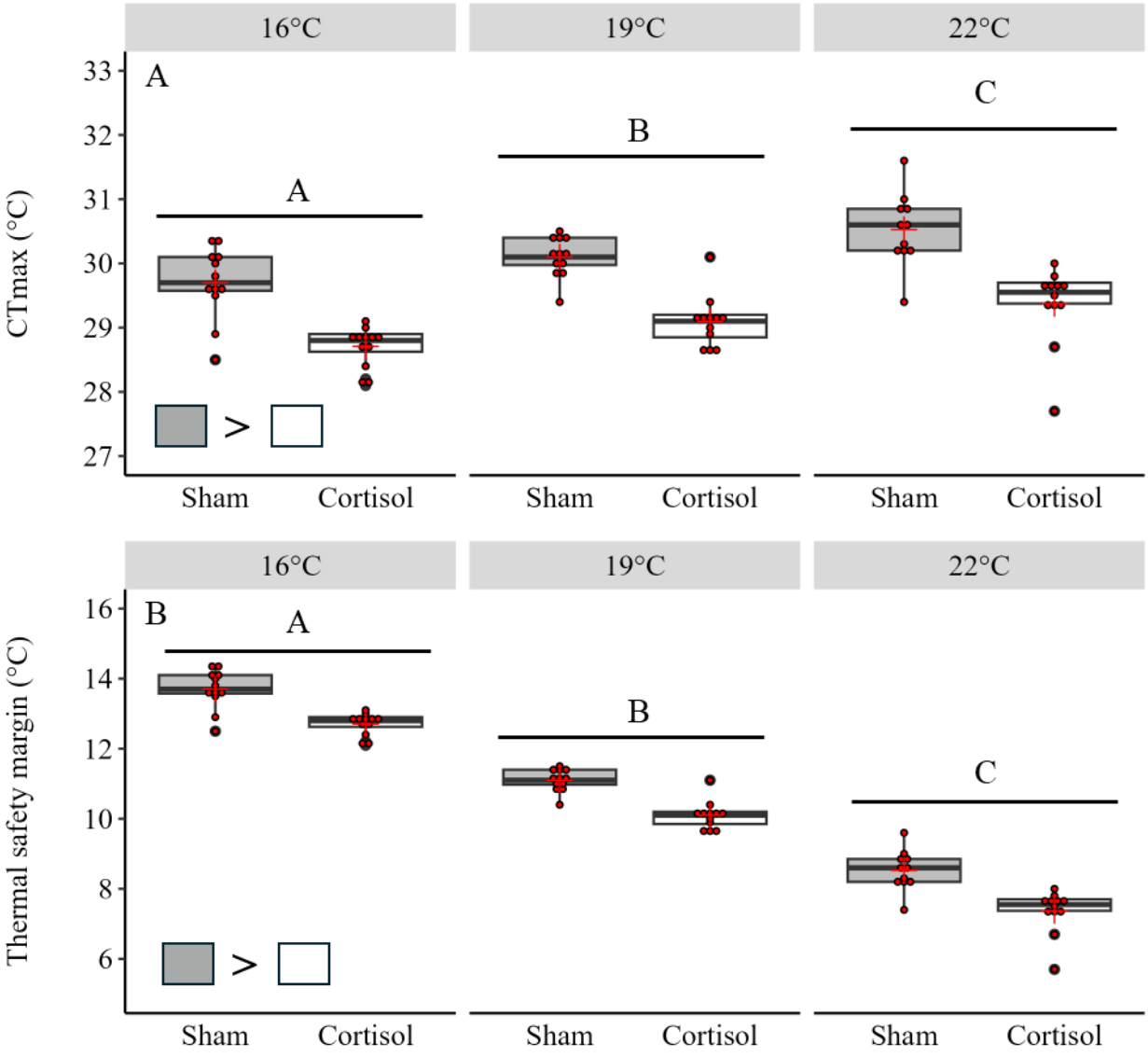
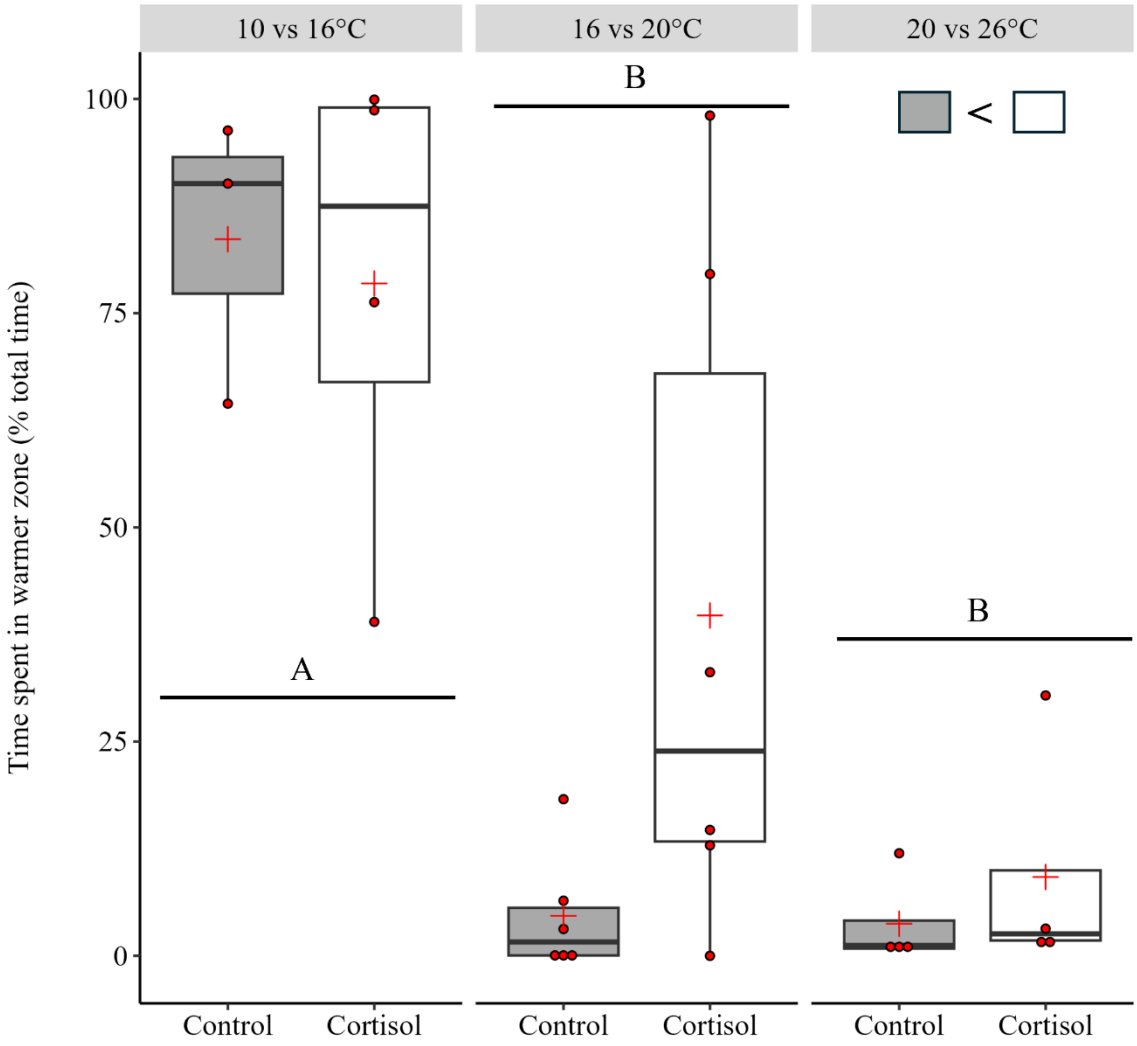

16°C
19°C
22°C
CTmax (°C)
33
32
31
30
29
28
27
A
A
B
C
>
Sham
Cortisol
Thermal safety margin (°C)
16
14
12
10
8
6
B
A
B
C

Figure 2.3. Time spent at the warmer temperature for control and cortisol-treated rainbow trout (*Oncorhynchus mykiss*) given a choice between two temperatures, 10 vs 16°C (N = 3-4 per group), 16 vs 20°C (N = 6 per group), or 20 vs 26°C (N = 4 per group). Both the temperature choice and cortisol treatment significantly affected time spent in the warmer chamber, but the interaction of these factors was not significant (two-way ANOVA on arcsine-transformed data, $P_{\text{treatment}} = 0.031$, $P_{\text{temperature choice}} < 0.0001$, $P_{\text{treatment x temperature choice}} = 0.234$). Temperature choices that do not share a letter are significantly different from one another, and the effect of cortisol treatment is represented on the figure using the bar fills. Data are presented as boxplots (see Section 2.2.6 for details).

Figure 2.3.


10 vs 16°C
16 vs 20°C
20 vs 26°C
Time spent in warmer zone (% total time)
100
75
50
25
0
A
B
B
<
Control
Cortisol
Control
Cortisol
Control
Cortisol

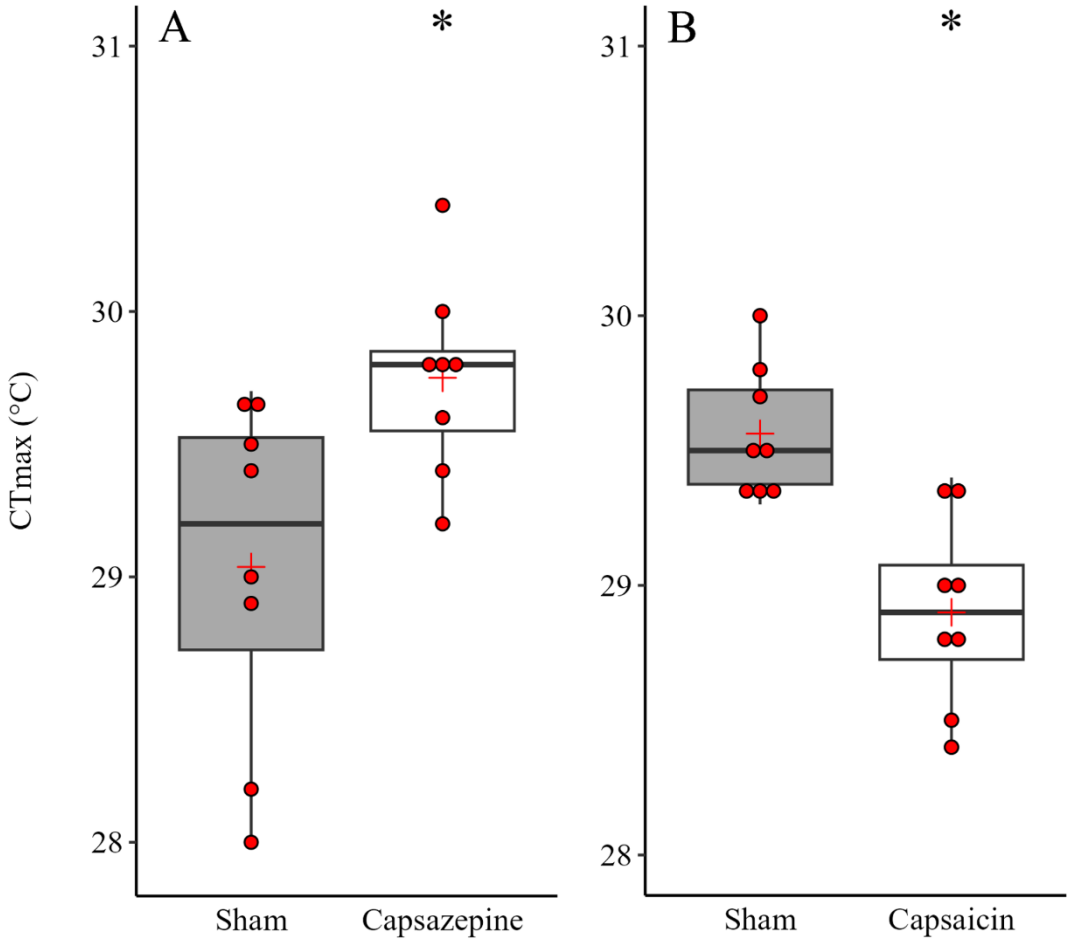


Figure 2.4. Critical thermal maxima (CTmax) of rainbow trout (*Oncorhynchus mykiss*) treated with (A) the TRPV1 antagonist capsazepine ($N = 8$ per group; Student's *t*-test, $P = 0.0197$), or (B) the TRPV1 agonist capsaicin ($N = 8$ per group; Student's *t*-test $P = 0.0008$). An asterisk indicates a significant difference between the sham and experimental groups. Data are presented as boxplots (see Section 2.2.6 for details).

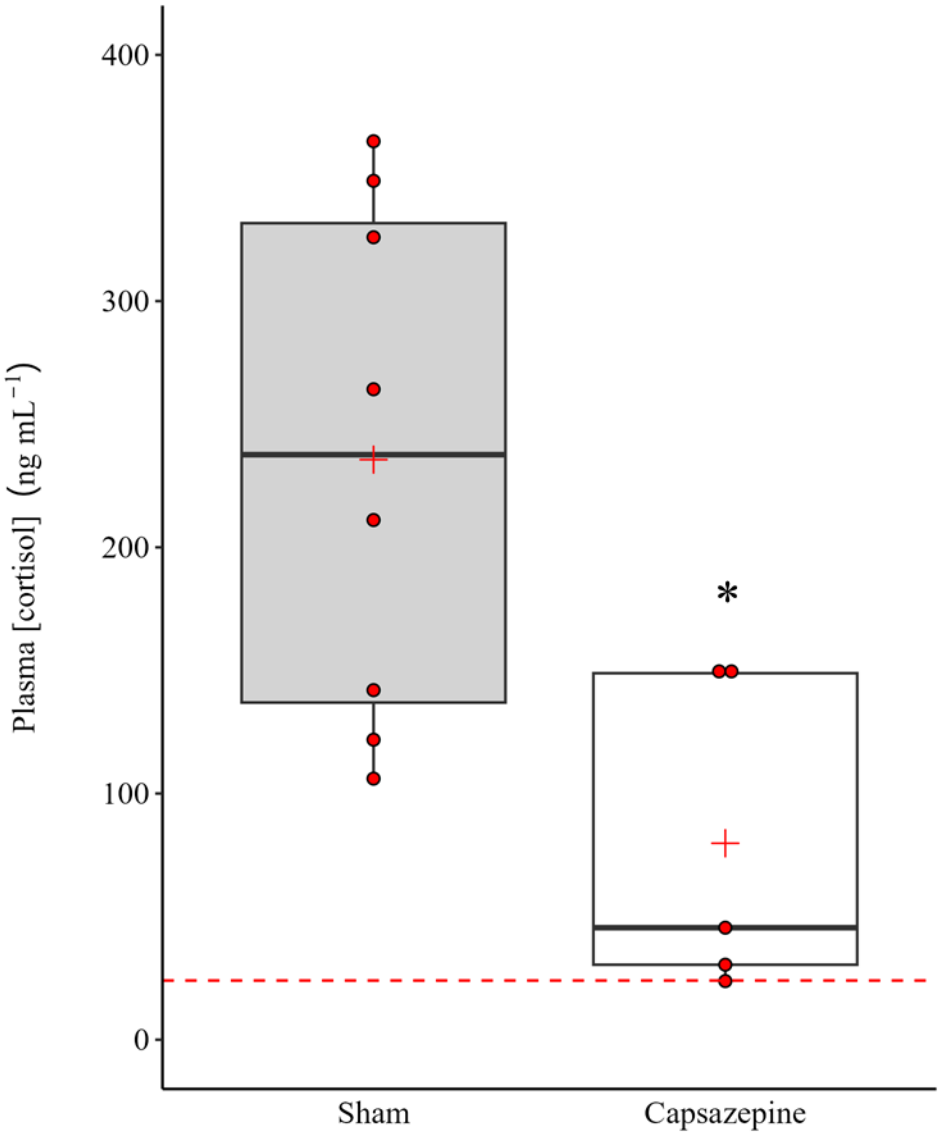


Figure 2.5. Plasma cortisol concentrations of rainbow trout (*Oncorhynchus mykiss*) following acute warming to 27°C. Trout were treated with vehicle alone (sham) or the TRPV1 antagonist capsazepine (N = 5-8 per group; Welch Student's t-test, P = 0.006). An asterisk indicates a significant difference from the sham group. The dashed red line represents baseline (unstressed) plasma cortisol for rainbow trout held at 13°C (from pilot trial data). Data are presented as boxplots (see Section 2.2.6 for details).

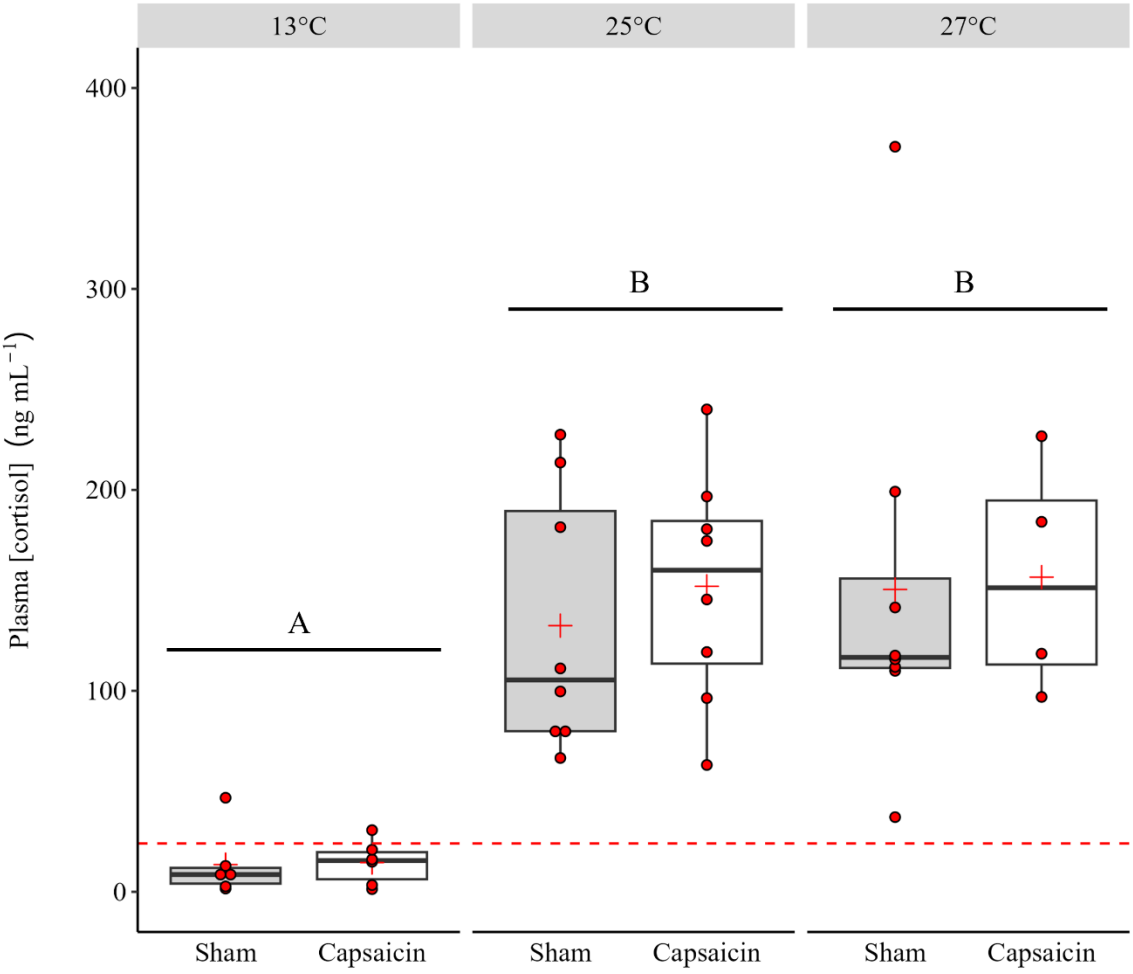


Figure 2.6. Plasma cortisol concentrations of rainbow trout (*Oncorhynchus mykiss*) at 13°C or following acute warming to 25 or 27°C. Trout were exposed to vehicle alone (sham) or treated with the TRPV1 agonist capsaicin (N = 6-8 per group). No significant effects of treatment were detected, but cortisol levels in fish exposed to 25 or 27°C were significantly higher than those held at 13°C; values for temperatures that share a letter were not significantly different from one another (two-way ANOVA, $P_{\text{treatment}} = 0.975$, $P_{\text{temperature}} < 0.0001$, $P_{\text{treatment x temperature}} = 0.922$). Data are presented as boxplots (see Section 2.2.6 for details).

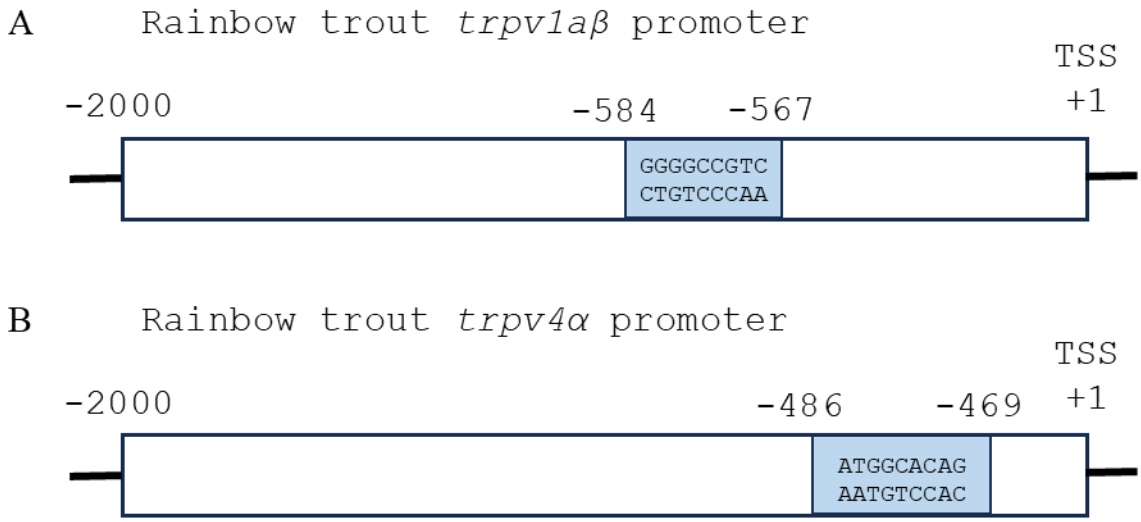


Figure 2.7. Schematic representation of the promoter regions of (A) trpv1aβ and (B) trpv4α, 2000 bp upstream of the transcription start site (TSS). The regions highlighted (in blue) are full glucocorticoid response element (GRE) sites. Sequences of each GRE are shown within the blue box.

Figure 2.8. Thermo*TRPV* transcript abundances in the gill tissue of rainbow trout (*Oncorhynchus mykiss*; *N* = 3-4 per temperature; Con, Control; Cort, Cortisol treated) exposed to thermal ramping to 13° (i.e. no change in temperature) or 27°C. Temperature had no significant effect on transcript abundances, but an effect of cortisol treatment is represented on the figure panel using the bar fills (two-way ANOVA; for panel A, $P_{\text{treatment}} = 0.375$, $P_{\text{temperature}} = 0.324$, $P_{\text{treatment x temperature}} = 0.661$; for panel B, $P_{\text{treatment}} = 0.162$, $P_{\text{temperature}} = 0.069$, $P_{\text{treatment x temperature}} = 0.424$; for panel C, $P_{\text{treatment}} = 0.018$, $P_{\text{temperature}} = 0.\ 649$, $P_{\text{treatment x temperature}} = 0.627$; Scheirer–ray–hare test, for panel D, $P_{\text{treatment}} = 0.385$, $P_{\text{temperature}} = 0.385$, $P_{\text{treatment x temperature}} = 0.999$). For panels B and C, data were log-transformed for statistical analysis. All values were normalized to β-actin as a reference gene and expressed as fold-change relative to the control group at 13°C. Data are presented as box plots (see Section 2.2.6 for details).

Figure 2.8.

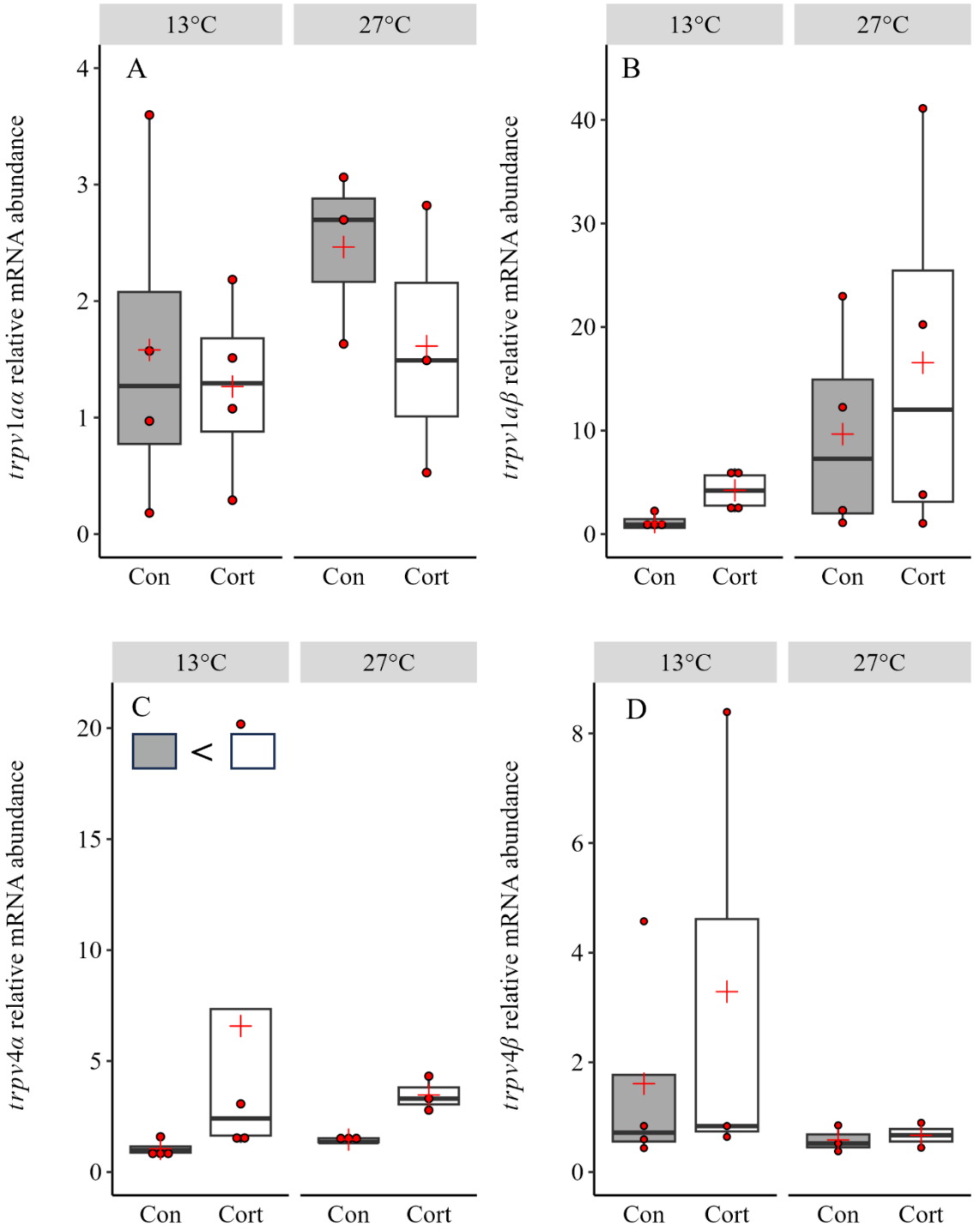

13°C
27°C
A
B
C
D
trpv1aα relative mRNA abundance
trpv1aβ relative mRNA abundance
trpv4α relative mRNA abundance
trpv4β relative mRNA abundance
Con
Cort
<

Figure 2.9. Thermo*TRPV* transcript abundances in the hypothalamus tissue of rainbow trout (*Oncorhynchus mykiss*; *N* = 2-4 per temperature; Con, Control; Cort, Cortisol treated) exposed to thermal ramping to 13° (i.e. no change in temperature) or 27°C. Neither cortisol treatment nor temperature had a significant effect on transcript abundance (two-way ANOVA, for panel A, $P_{treatment} = 0.815$, $P_{temperature} = 0.067$, $P_{treatment\ x\ temperature} = 0.946$; for panel B, $P_{treatment} = 0.339$, $P_{temperature} = 0.331$, $P_{treatment\ x\ temperature} = 0.704$; for panel C, $P_{treatment} = 0.326$, $P_{temperature} = 0.628$, $P_{treatment\ x\ temperature} = 0.680$; for panel D, $P_{treatment} = 0.380$, $P_{temperature} = 0.897$, $P_{treatment\ x\ temperature} = 0.163$). For panels B, C, D, data were log-transformed for data analysis. All values were normalized to β-actin as a reference gene and expressed as fold-change relative to the control group at 13°C. Data are presented as box plots (see Section 2.2.6 for details).

Figure 2.9.

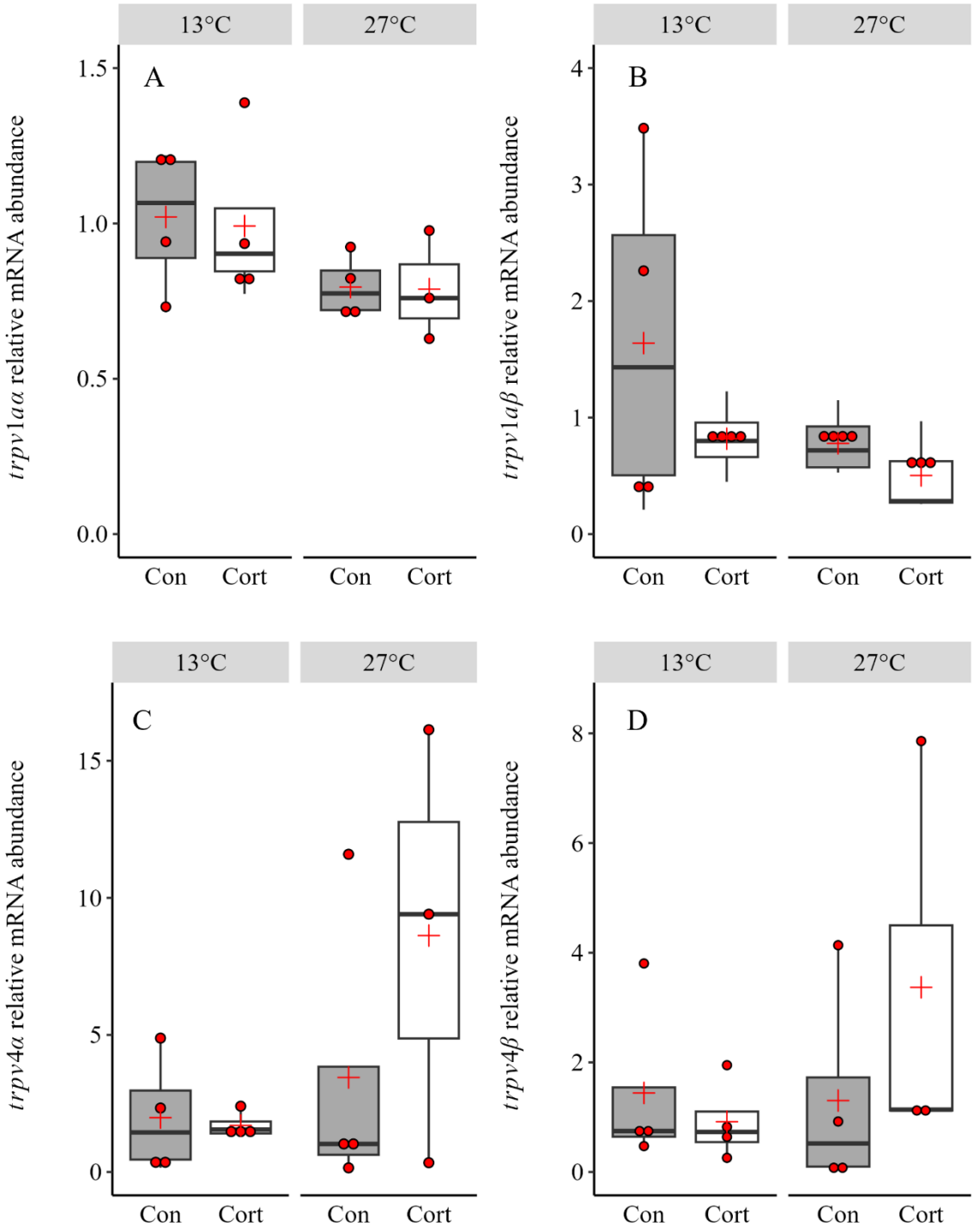

13°C
27°C
A
B
C
D
trpv1aα relative mRNA abundance
trpv1aβ relative mRNA abundance
trpv4α relative mRNA abundance
trpv4β relative mRNA abundance
Con
Cort

Figure 2.10. Thermo*TRPV* transcript abundances in the pre-optic area tissue of rainbow trout (*Oncorhynchus mykiss*; *N* = 3-4 per temperature; Con, Control; Cort, Cortisol treated) exposed to thermal ramping to 13°C (i.e. no change in temperature) or 27°C. Neither cortisol treatment nor temperature had a significant effect on transcript abundance (two-way ANOVA, for panel A, $P_{treatment} = 0.374$, $P_{temperature} = 0.124$, $P_{treatment\ x\ temperature} = 0.862$; Scheirer–ray–hare test, for panel B, $P_{treatment} = 0.753$, $P_{temperature} = 0.462$, $P_{treatment\ x\ temperature} = 0.059$; two-way ANOVA, for panel C, $P_{treatment} = 0.576$, $P_{temperature} = 0.657$, $P_{treatment\ x\ temperature} = 0.043$). For panel C, data was log-transformed for data analysis. All values were normalized to β-actin as a reference gene and expressed as fold-change relative to the control group at 13°C. Data are presented as box plots (see Section 2.2.6 for details).

Figure 2.10.

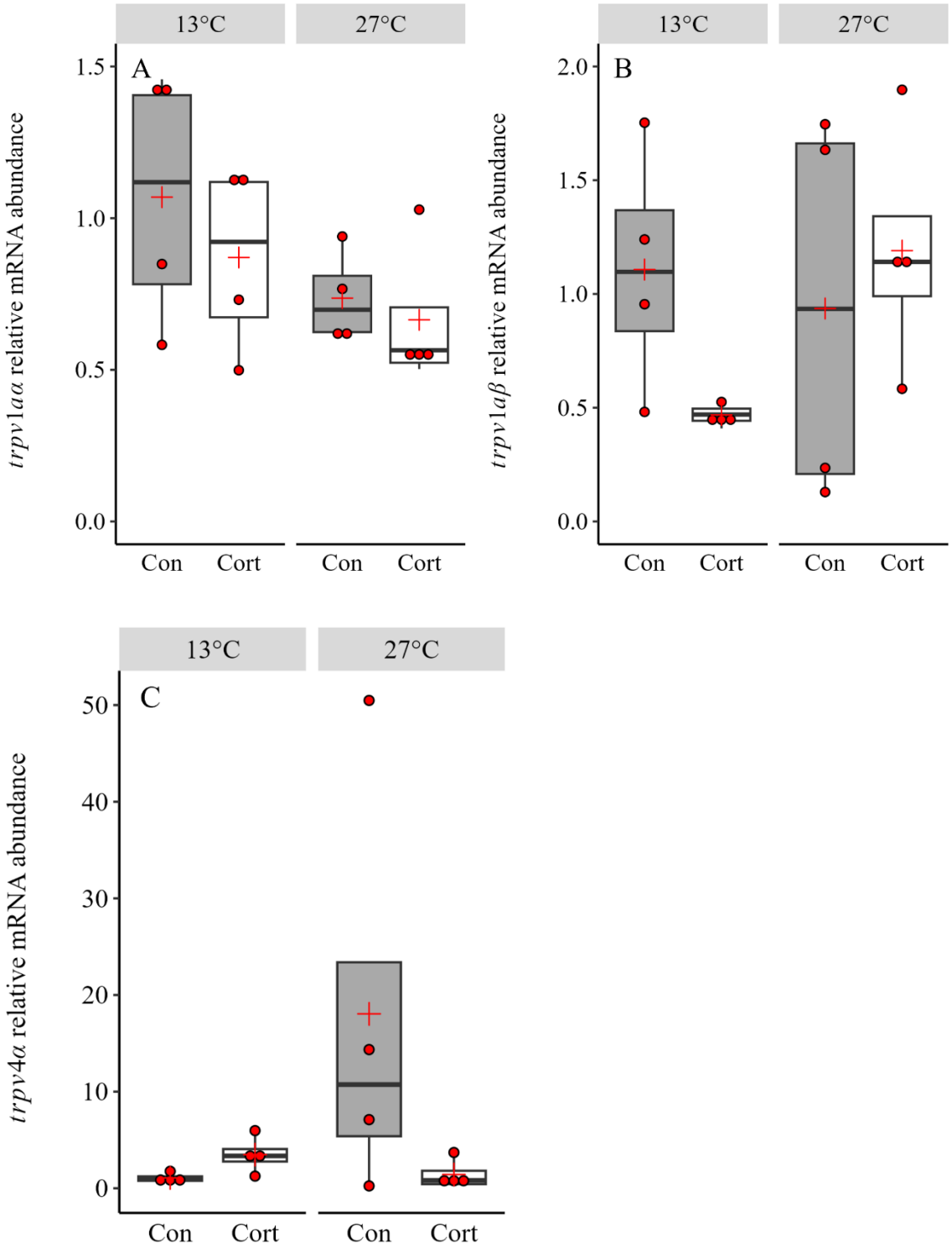
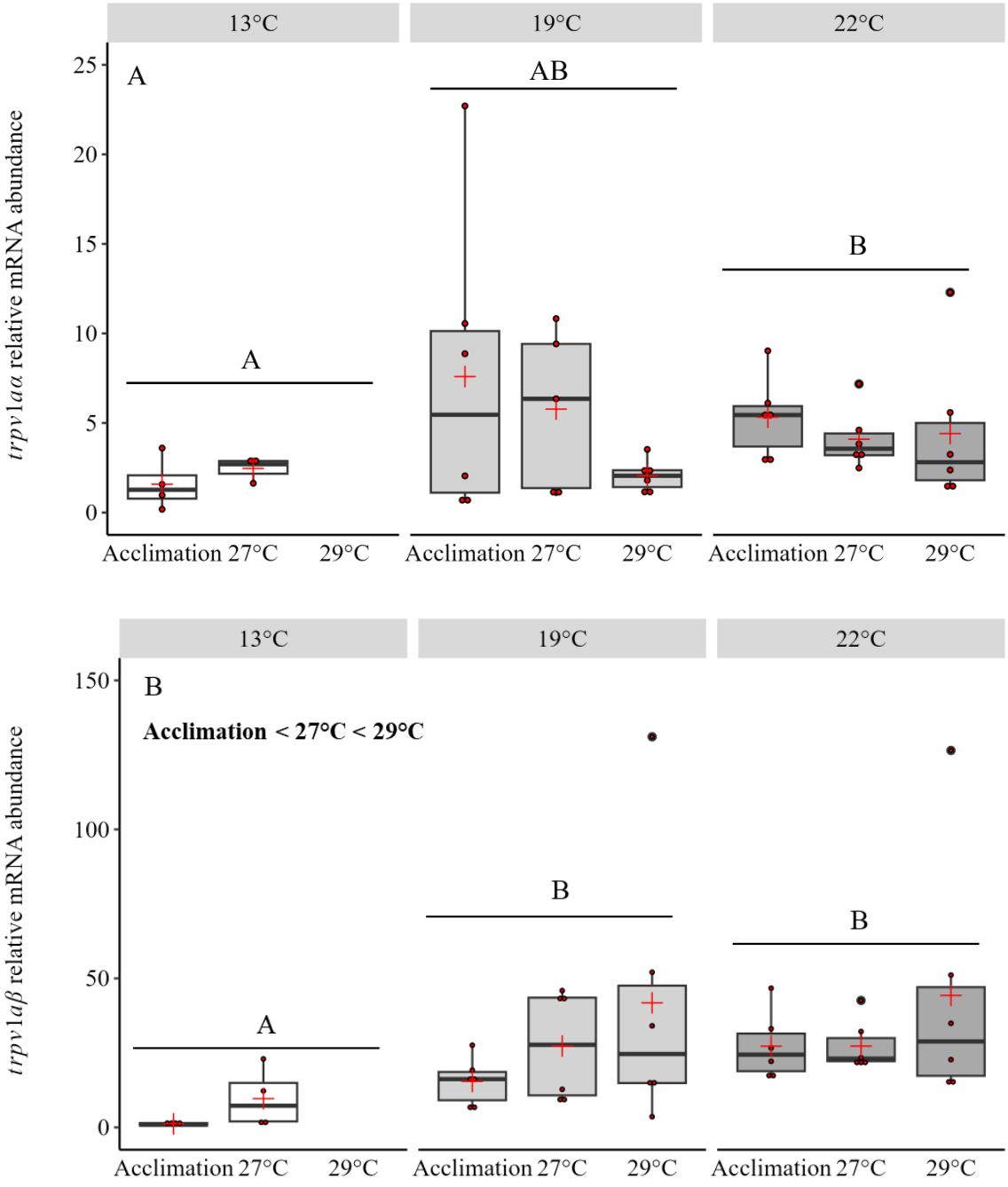
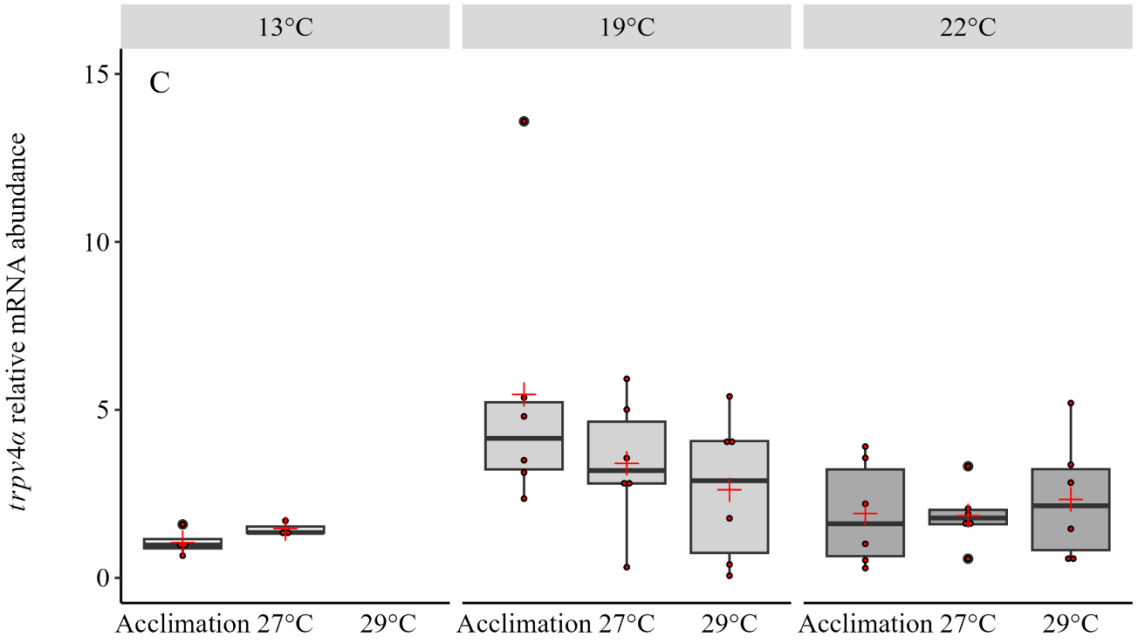
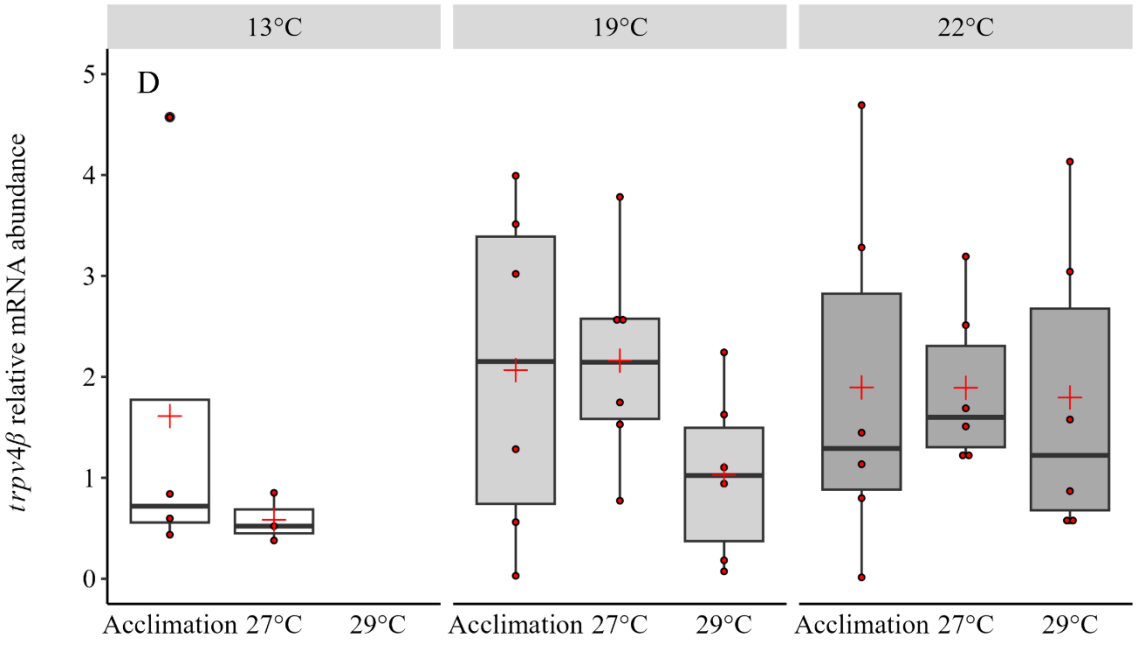

13°C
27°C
A
trpv1aα relative mRNA abundance
1.5
1.0
0.5
0.0
Con
Cort
Con
Cort
B
trpv1aβ relative mRNA abundance
2.0
1.5
1.0
0.5
0.0
C
trpv4α relative mRNA abundance
50
40
30
20
10
0

Figure 2.11. Thermo*TRPV* transcript abundances in gill of rainbow trout (*Oncorhynchus mykiss*; N = 3-6 per temperature) after thermal acclimation alone or thermal acclimation followed by acute warming to 27°C or 29°C. Acclimation temperature and/or acute warming, but not the interaction of these two factors, had significant effects on transcript abundances (two-way ANOVA, for panel A, $P_{\text{acclimation}} = 0.031$, $P_{\textit{acute}\ \text{warming}} = 0.638$, $P_{\text{acclimation x acute warming}} = 0.576$; for panel B, $P_{\text{acclimation}} < 0.0001$, $P_{\text{acute warming}} = 0.003$, $P_{\text{acclimation x acute warming}} = 0.206$; for panel C, $P_{\text{acclimation}} = 0.137$, $P_{\text{acute warming}} = 0.717$, $P_{\text{acclimation x acute warming}} = 0.273$; for panel D, $P_{\text{acclimation}} = 0.649$, $P_{\text{acute warming}} = 0.535$, $P_{\text{acclimation x acute warming}} = 0.495$). Acclimation temperatures that share a letter are not significantly different from one another, and the effect of acute warming is represented on the figure using bold text. Data were log-transformed for statistical analysis. "Acclimation" refers to the acclimation temperature indicated at the top of each panel. All values were normalized to β-actin as a reference gene and expressed as fold-change relative to the control group at 13°C. Data are presented as box plots (see Section 2.2.6 for details). It should be noted that 13°C acclimated fish were not exposed to 29°C.

Figure 2.11.


13°C
19°C
22°C
trpv1aα relative mRNA abundance
25
20
15
10
5
0
A
AB
B
A
Acclimation 27°C
29°C
trpv1aβ relative mRNA abundance
150
100
50
0
B
Acclimation < 27°C < 29°C
B
B
A


13°C
19°C
22°C
C
trpv4α relative mRNA abundance
15
10
5
0
Acclimation 27°C
29°C


13°C
19°C
22°C
D
trpv4β relative mRNA abundance
5
4
3
2
1
0
Acclimation 27°C
29°C

2.5 Discussion

The current study demonstrated that chronic elevation of cortisol alone is sufficient to lower thermal tolerance, and the effects of cortisol seem to be mediated by GR. Moreover, elevated cortisol did not prevent thermal acclimation from raising CTmax, although the effect of chronic cortisol elevation in lowering thermal tolerance remained. These findings suggest that chronic elevation of cortisol impairs thermal tolerance. Prolonged increases in circulating cortisol also appear to alter other aspects of thermal biology, with cortisol-treated fish seeming to prefer warmer temperatures in a choice test. This observation may indicate that cortisol treatment alters thermal perception. The present study confirmed that TRPV1 plays a role in determining CTmax as well as the onset of the cortisol response to acute warming. Additionally, *trpv*4α transcript abundance was elevated in gill tissue, suggesting that transcriptional regulation of TRPV could be contributing to the mechanism through which cortisol treatment increases sensitivity to high temperatures.

The present study confirmed that chronic elevation of cortisol lowers thermal tolerance, with CTmax in cortisol-treated fish decreasing by approximately 1°C. Moreover, the effect of cortisol was mediated by GR, because co-treatment of fish with cortisol and the GR antagonist RU486 prevented a fall in CTmax. Previous work had demonstrated that chronic elevation of cortisol was responsible for the lowered thermal tolerance of subordinate trout, with CTmax decreasing by ~1°C following 4 d of subordinate social status where cortisol was elevated to ~100-400 ng mL^{-1} (Bard et al. 2021). Cortisol treatment in the present study was expected to elevate circulating cortisol to levels comparable to those observed by Bard et al. (2021) in subordinate fish, likely

accounting for the similar reduction of CTmax between the two studies. The use of cortisol treatment in the present study confirms that the lowering of CTmax is not limited to social stress but rather is a consequence of prolonged elevation of cortisol. Thus, any stressor that chronically elevates circulating cortisol would be predicted to lower thermal tolerance. The persistent elevation of cortisol is clearly necessary, because 2 d of cortisol treatment did not lower thermal tolerance significantly. However, it does not seem that thermal tolerance is further reduced after more prolonged cortisol treatment (i.e. 3 weeks) because CTmax decreased by ~1°C in cortisol-treated trout across all three acclimation temperatures. Moreover, ARR were similar for control and cortisol-treated trout, suggesting that chronic elevation of cortisol did not hinder thermal acclimation. However, increases in CTmax with acclimation temperature appeared to be plateauing in both control and cortisol-treated fish, as demonstrated by the decreasing thermal safety margins as acclimation temperature increased. Cortisol treatments in the current study were chosen to stay within physiologically-relevant cortisol levels (Bard et al. 2021), but higher plasma cortisol levels may cause greater reduction in CTmax; further work is needed to address this question.

The use of mini-osmotic pumps allowed cortisol levels to be elevated for longer periods (i.e. 3 weeks). In previous work, mini-osmotic pumps elevated cortisol levels for 28 d, with cortisol falling back to baseline by 42 d (at 12°C; Madison et al. 2015). In a pilot study by the same authors, mini-osmotic pumps were found to deliver cortisol for as long as 34 d, although the cortisol levels achieved at this time were not measured (Madison et al. 2015). Because the rate of cortisol release from the mini-osmotic pump is dependent on temperature, fish acclimated to higher temperatures may have experienced

higher cortisol levels for shorter periods of time. Despite this possibility, the effects of cortisol treatment on CTmax were consistent across acclimation treatments.

The effect of cortisol in lowering CTmax was maintained regardless of acclimation temperature (between 16 and 22°C). As expected from the well-documented effect of higher acclimation temperatures raising CTmax (Beitinger et al. 2000; Zhang and Kieffer 2014; Morrison et al. 2020; McDonnell et al. 2021; Adams et al. 2022; Zhu et al. 2022; Stewart et al. 2023), three weeks of acclimation increased CTmax from ~29°C in trout held at 13°C to ~29.7°C in (sham) trout acclimated to 16°C and ~30.5°C in (sham) trout acclimated to 22°C. Although thermal acclimation increased CTmax in cortisol-treated fish, it did not overcome the effect of chronic cortisol elevation in lowering CTmax. Thus, chronically stressed fish are likely to remain more vulnerable to climate change-induced warming than unstressed fish, even when given sufficient time to acclimate to higher temperatures. Both *trpv1aα* and *trpv1aβ* were significantly upregulated in the gill of fish acclimated to higher temperatures, suggesting a potential role for TRPV1 in thermal acclimation. However, work is needed to determine how higher transcript abundances of *trpv1* paralogues contributes to higher thermal tolerance.

Although the data of the current study and Bard et al. (2021) make a compelling case for the role of elevated cortisol in lowering thermal tolerance, the mechanism through which cortisol acts to reduce CTmax has not yet been elucidated. The current study put forward the hypothesis that cortisol modulates thermosensation and/or perception. As a first step in supporting this hypothesis, CTmax was measured under conditions of TRPV1 inhibition (via capsazepine) or activation (via capsaicin). Blockade of TRPV1 with capsazepine increased CTmax, whereas activation of TRPV1 with

capsaicin decreased CTmax. A similar effect was reported previously in masu salmon, where exposure to capsaicin during acute warming initiated locomotory responses and loss of equilibrium at lower temperatures than in untreated fish (Yoshimura et al. 2022). Building on earlier work that used knockdown approaches to demonstrate that TRPV1 is necessary for heat-induced locomotory responses in zebrafish (Gau et al. 2013), the findings of the present study and Yoshimura et al. (2022) show that the onset of heat-induced responses can be altered by manipulating TRPV1 activity. The effect of capsaicin observed in the present study appeared to be smaller than that reported by Yoshimura et al. (2022) for the same concentration of capsaicin. Trout in the present study were treated with capsaicin for only 10 min for logistical reasons – use of the mixing valve to increase water temperature required flow-through conditions. By contrast, in the experiments of Yoshimura et al. (2022), salmon were exposed to capsaicin throughout the trial, which likely achieved a more sustained activation of TRPV1 than in the present study.

In addition to raising CTmax, treatment of trout with capsazepine appeared to blunt the plasma cortisol response to acute warming, an observation that is consistent with altered thermal sensation or perception of heat. However, exposure to capsaicin did not alter the cortisol response to acute warming, nor did exposure to capsaicin elicit a cortisol response in trout held at 13°C. Similarly, capsaicin potentiated locomotory responses to heat in masu salmon, but did not evoke these responses in the absence of a temperature stimulus (Yoshiumura et al. 2022). The effects of capsaicin in the present study were tested at 27°C, a temperature that reliably elicits a cortisol response in rainbow trout (Chapter 3), as well as 25°C, a temperature that typically does not elicit a

cortisol response. However, a cortisol response was observed at 25°C in this particular group of trout. Thus, it remains to be determined whether capsaicin treatment can lower the threshold temperature to evoke a cortisol response in trout. Collectively, however, these data suggest that activation of TRPV1 alone is not sufficient to stimulate heat-evoked responses.

There is abundant evidence of regulation of thermoTRPs by steroid hormones (Kumar et al. 2015), suggesting that transcriptional regulation of TRPV paralogues is a potential mechanism through which chronic cortisol elevation could modulate thermosensing. In support of this possibility, a GRE was identified in the promoter regions of *trpv1aβ* and *trpv4α*, although not in those of *trpv1aα* or *trpv4β.* In agreement with the presence of a GRE in its promotor, transcript abundance of *trpv4α* was significantly higher in the gills of cortisol-treated compared to control trout; however, no evidence of changes in *trpv1* or *trpv4* transcript abundances was detected in the brain. Whether *trpv* paralogues are differentially regulated in the POA remains ambiguous, with more work needed to identify effects of cortisol treatment vs temperature. Higher transcript abundances are anticipated to translate into increased abundance of TRPV, a prediction that needs to be confirmed (see Section 2.5.2). Increased abundance of TRPV4 in gill tissue might heighten sensitivity to temperature, although empirical data are needed to support this possibility. Moreover, cortisol elevation might alter TRPV function. For example, cholesterol is an important regulator of TRPV1 function in mammals (Morales-lázaro and Rosenbaum 2019). A recent study on TRPV1 from Antarctic notothenioids expressed in *Xenopus* oocytes reported a significantly lower temperature threshold for TRPV1 activation under conditions of reduced cholesterol

(York 2023), suggesting that cholesterol also regulates TRPV1 function in fishes. Subordinate rainbow trout experiencing chronic social stress exhibited lowered plasma cholesterol, and both subordinate status and cortisol treatment resulted in changes in a microRNA that is thought to be involved in regulating hepatic cholesterol abundance (Kostyniuk et al. 2018). These observations suggest that cortisol elevation could modulate TRPV1 activation thresholds via changes in cholesterol, but additional studies are needed to investigate this mechanism.

At the organismal level, cortisol treatment altered behavioural thermal avoidance responses, with cortisol-treated rainbow trout of the current study spending more time in warmer water when given a choice between two water temperatures. This response appears to be inconsistent with the effect of cortisol in lowering thermal tolerance – fish that have lower thermal tolerance might also be expected to prefer cooler water to be further from their upper thermal limit. Indeed, cortisol-treated pufferfish preferred cooler waters (Cull et al. 2015). However, pathogen infection was found to induce temporary increases in ambient temperature choice in fishes, an effect termed “behavioural fever” (Mohammed et al. 2016; Boltana et al. 2018; Huntingford et al. 2020). It is possible that cortisol elevation mimics a physiological state that promotes behavioural fever. Consistent with this possibility, a study reported stress-induced hyperthermia in zebrafish, but methodological issues and inconsistent results have raised questions about this finding (Rey et al. 2015b, 2017a, 2017b; Key et al. 2017; Jones et al. 2019).

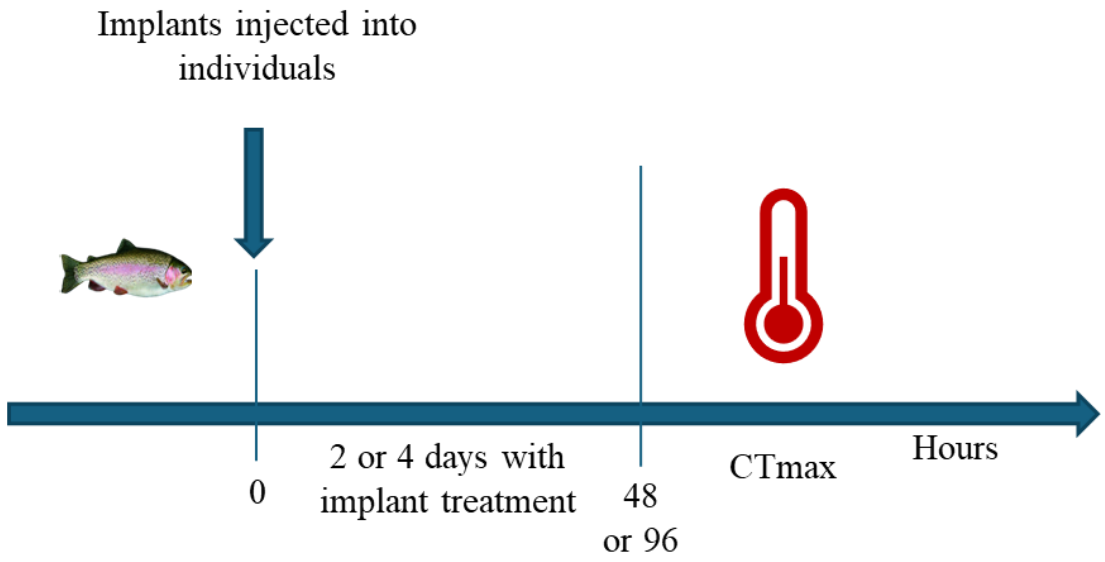
The current study had limitations in measuring the true thermal preference of cortisol-treated trout. It is probable that the static thermal choice test used here does not properly reflect individual thermal preference as measured in a dynamic shuttle-box test

(Chistensen et al. 2021). However, it is intriguing that when given the choice of cooler temperatures, cortisol-treated fish chose to be in warmer temperatures. Given differences between the current experiment and the findings of Cull et al (2015), there is a clear need for further research in this area.

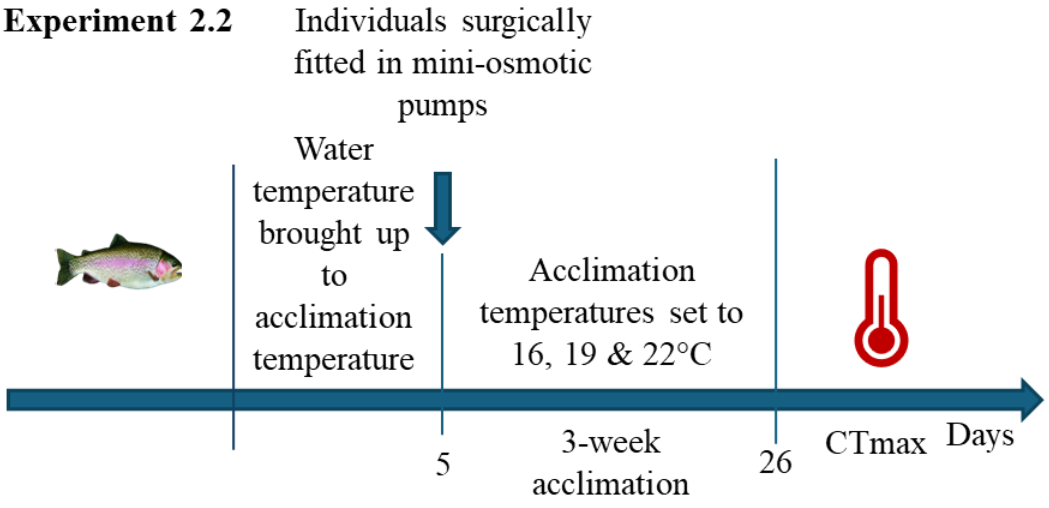
In summary, the present study demonstrated that prolonged cortisol elevation, even in the absence of a stressor, is sufficient to lower thermal tolerance and that the effect of cortisol in lowering CTmax persists even after thermal acclimation. Modulation of TRPV1 activity altered the temperatures at which key responses to acute warming (LOE and a cortisol response) occurred, suggesting changes in TRPV1 abundance or function as a mechanism through which cortisol could alter thermal tolerance. Correspondingly, cortisol treatment increased transcript abundances of *trpv4α* in the gills, a response that could potentially contribute to increasing thermal sensitivity in cortisol-treated fish. Future research should focus on whether chronic cortisol elevation alters TRPV activation temperatures, ultimately changing the physiological response to heat. The links between thermal perception, the HPI axis and thermal tolerance require further elucidation.

2.6 Supplemental materials

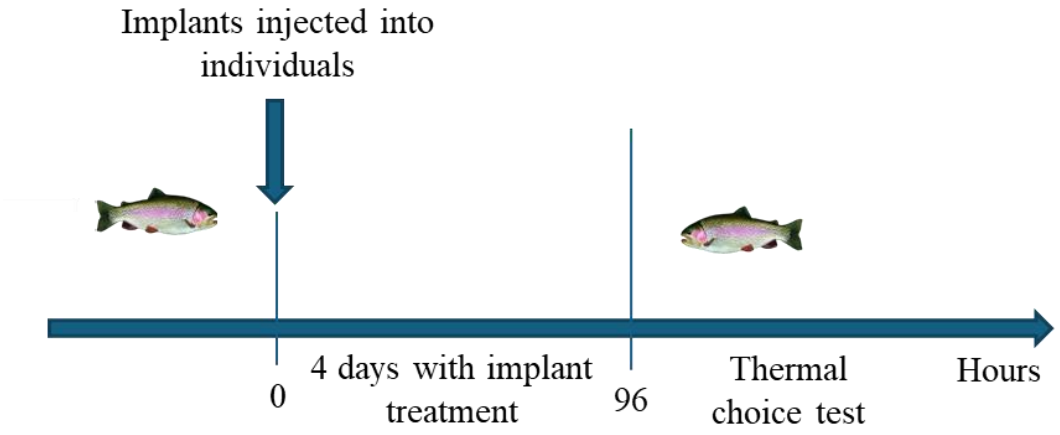
Experiment 2.1

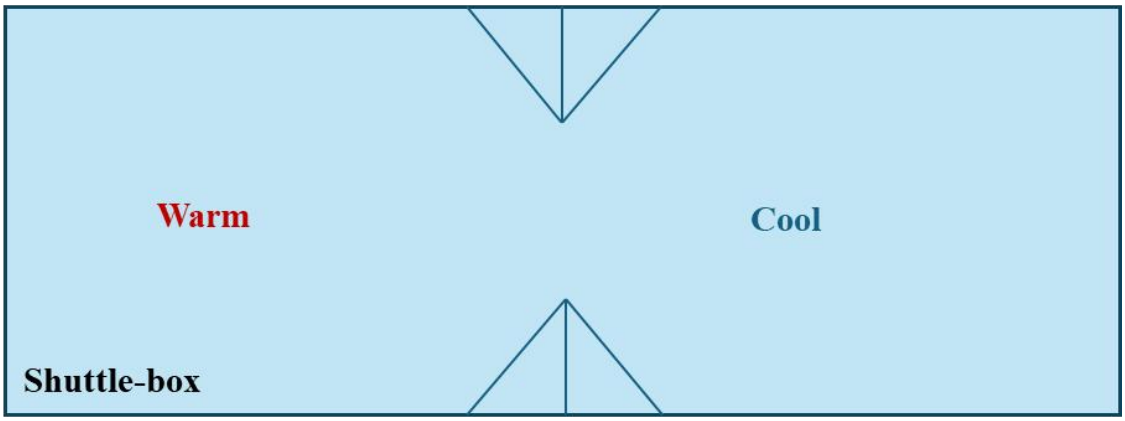


Experiment 2.2



Experiment 2.3





Experiment 2.4

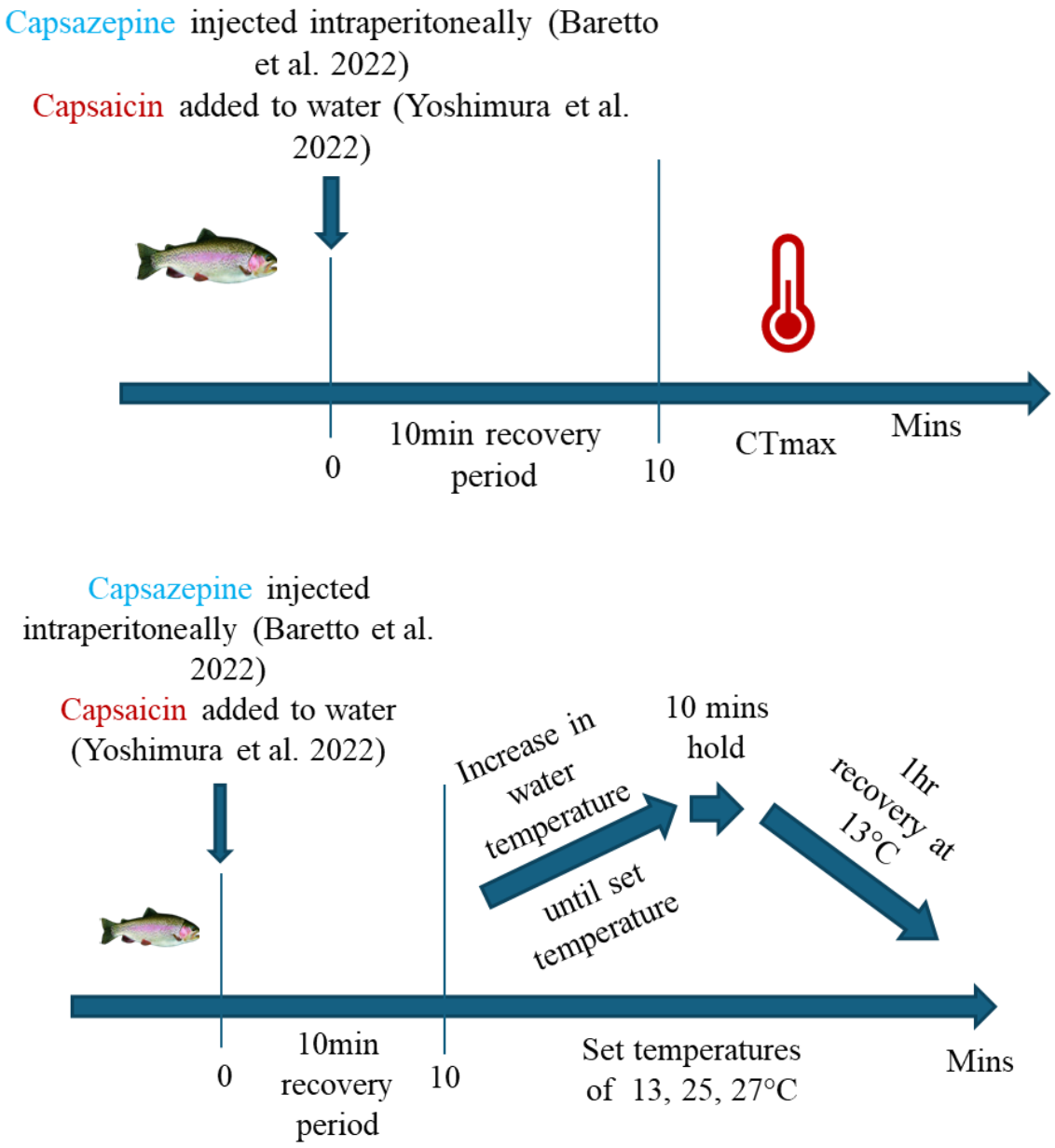


Figure S2.1. Schematics of experimental timelines.

2.6.1 In vitro head kidney preparations treated with capsazepine

To verify that cortisol production was not impaired by capsazepine exposure, cortisol production by *in vitro* head kidney preparations was measured in the presence and absence of capsazepine. Head kidney tissue was prepared according to the protocols of Best and Gilmour (2022), Aluru and Vijayan (2006), and Jeffrey et al (2014). In brief, head kidney tissue was from large (~500 g) trout and rinsed in chilled Leibovitz's L-15 medium (Gibco, Thermo Fisher Scientific). Total tissue mass was measured. Tissue was transferred to chilled glass Petri dishes containing 100 mL of L-15 medium per gram of tissue and minced to 1 mm^3 pieces. The minced tissue was incubated at 13°C on a shaker for 3 h and then evenly distributed across wells of a 48-well microplate (Corning Costar, Corning, NY, USA). Approximately 25 mg of tissue was added to L-15 medium in each well, but final weights were measured after the trial for normalization. The treatments (assayed in duplicate) included 5 µL of capsazepine in DSMO or 5 µL of DSMO alone, and 1 UL ml^{-1} ACTH was added to each well. The tissue was incubated at 13°C on a shaker for 3 h after which the content of each well was centrifuged for 2 min at 13,000 *g*. The resulting supernatant was flash frozen and stored in -80°C for cortisol analysis. After removing the supernatant, any remaining tissue was weighed, and tissue weight was used to standardize cortisol production.

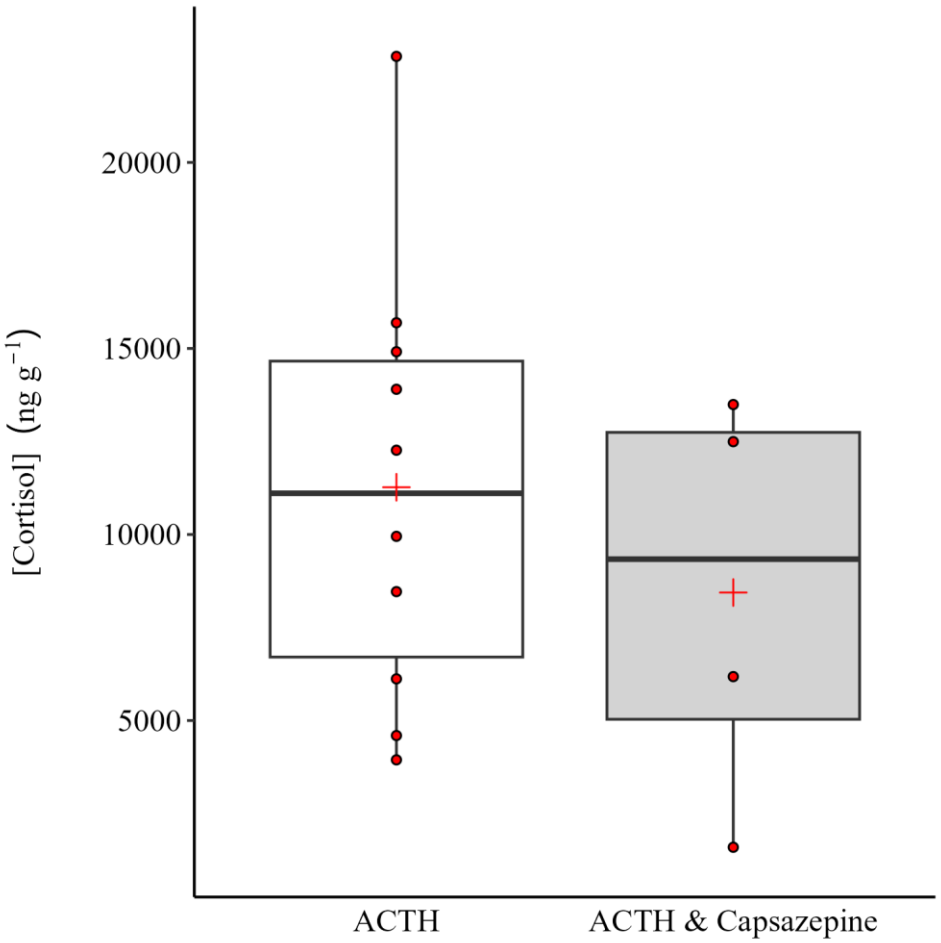


Figure S.2.2. Stimulated cortisol production when treated with capsazepine at 13°C in rainbow trout (*Oncorhynchus mykiss*) (Student's *t*-test, $P = 0.43$). No significant differences detected. Data are presented as box plots, see Section 2.2.6 for details.

2.6.2 Measurement of TRPV1 protein abundances using western blotting

I aimed to use western analysis to measure TRPV1 protein levels in gill tissue with a custom anti-trout TRPV1 antibody.

Soluble protein was extracted from frozen gill tissue (first two arches on the left side) that was powdered on dry ice using a mortar and pestle. Approximately 200-300 mg of powdered gill tissue was sonicated (Sonic Dismembrator Model 100, Fisher Scientific) in a RIPA buffer containing protease inhibitors (A32955, Thermo Fisher Scientific, Mississauga, ON, Canada). Samples were then processed for transmembrane proteins only, by firstly centrifuging them at 700 *g* for 10 min (Eppendorf, Mississauga, ON, Canada). The supernatant was transferred then centrifuged at 10, 000 *g* for 30 min. The resulting pellet contained only transmembrane proteins and was reconstituted in 50 µl of RIPA buffer containing protease inhibitors (Thermo Fisher Scientific). Sample protein concentrations were determined using the bicinchoninic acid method (BCA) with bovine serum albumin (BSA; 10711454001, Sigma Aldrich) as a standard.

Samples of extracted transmembrane soluble protein were diluted 2x with Laemmli buffer (Sigma Aldrich). Protein samples (50 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 wet transfer apparatus (Bio-Rad) overnight. Membranes were blocked with fish gelatin (Biotum, CA#22010, USA) in Tris-buffered saline containing 0.1% Tween 20 (TBST) for 1 h at room temperature and then incubated for 24 h at 4°C with primary antibody (1:500 chicken anti-salmonid TRPV1; Genetel, USA) 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 (1:1000 goat

anti-chicken 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 Forte HRP substrate (MilliporeSigma™; Oakville, ON, CA), and imaged using chemiluminescence 27 (ChemiDoc XRS+, Bio-Rad).

Although bands of the appropriate size were detected sporadically, but ultimately, they could not be detected reliably and therefore were not quantifiable (Fig. S.2.3).

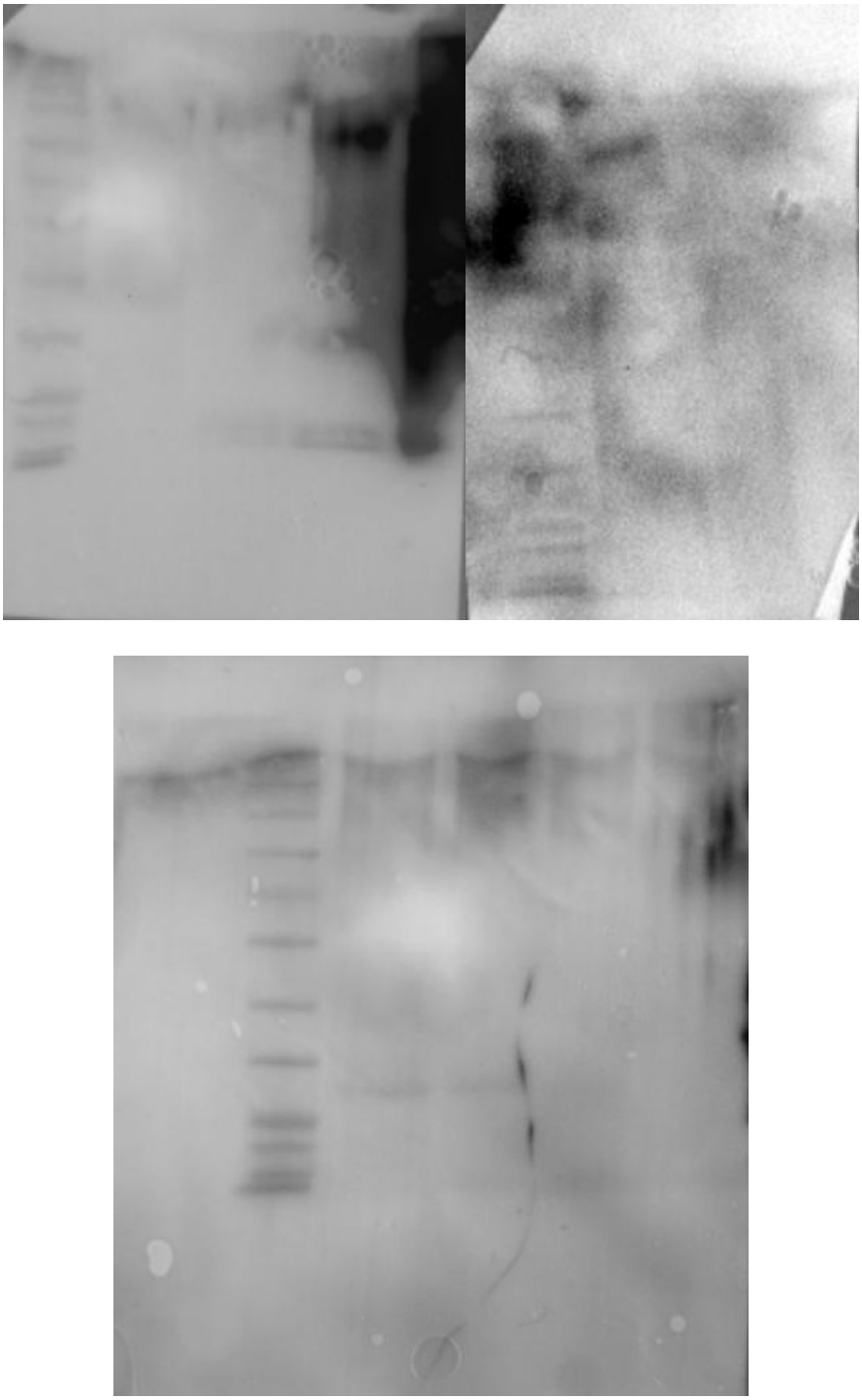


Figure S.2.3. Representative westerns of TRPV1 abundance in the gills using custom gernerated antibody.

(A) Rainbow trout *trpv1aα* promoter region:

TAAGCAAAAAACAAAAACATTTGTATTTATTAACAGCATTAATACAGACTGGTCGACTGG
CAGGAGAGCTTGATAAACTGATGAAAGAGAAGGGTGTCAGAGTAAGTAGAGGTTAGTCCA
CAGTCACTCCATGGGAAGCTCCATTTGTCACTATGGGCAAAATGTGTGTTCTGGTTGATC
TTTTGGTTACTAAACATACACTACATGACCAAAGGTATGTGGACACCTGCTCGTCGAACA
TCTCATTCCAAAATCCTGGGCATTAATTTGGAGTTGGTCCGCCCTTTGCTGGTATTACAG
CTTCCATTATTCTGGGAAGGCTTTCCATTTGGTGATGCTGCGAGGACCTGCTTCCATTCA
GCCACAAGAGCATTAGTGAGGTTGGGCACTGATGTTGGAGCGATTAGGCCTGGCTCGCAG
TCGGCGTTCCAATTCATCCCAAAGGTGTTCGATGGGGTTGAGGTCAGGGCTTTGTGCAGG
CCAGTCAAGTTCTTCCACACCGATCTCGACAAACCATTTCTGTATGGACCTCGCTTTGTG
CACGAGGGCATTGTGATGTTGAAACAGGAAAGGGCCTTCCCTAAACTGTTGTCACAATGT
TGGAAGCACAGAATCGCCTAGAATGCCGTTGTATGCTGTAGTGTTAAGATTTCCCTTCAC
AGGAACTAATGGTCCTAGCCCGAACCATGAAAAACAGCTCCAGACCATTAATCCTCCTCC
ACCAAACTTTACAGTTAGCACTATGTATTCGGGCAGGTAAAGTTATCCTGGCATCCACCA
AACACAGATTTGTCCGTCAGACTGTCAGATGTGAAGCGTGATTTATCACTCCAGAGAACG
CATTTCCACTGCTCCAGAGTCCAATGGAAACCCATTTCATGAAGCTCCAGAAGACCAGTT
CTTGTGCTGACATTGCTTCCAGAGGCAGTTTGGAACTCGGTAGTGAGTGTTGCAACCGAG
GACAAACTATTTTTATGCTACATGCTTCAGCACTCGGCGGTCCTGTTCTGTGAGCTTGTG
TGGCCTACCACTTTGCGGCTGAGCTGTTGTTGCTCCTAGACGTTTCCACTTCACAATAAC
AGCACTCACAGTTGACCGGGGAAGCTGTAGCAGGACAAACATTTTACGAACTGACTTGTT
GGAAAGGTGGTATCCTCTCGGTATGGGCATTCTATTGCCAGTGTTTGTCTATGGAGATTG
CATGGCGGTGTGCTTGATTTTATACACCTGTCAGCAATGGGTGTGGCTGATATAGCCGAA
TCCACTAATTTGAAGGGGTGTCCACATACTTTTGTATATATAGTGTCGCTTTGCACAAAT
CAAGCTGTATCCTGTTGGGGAATCATTGGAAACCCAAGTTAAGGTCTCCTGTCAGAATCA
ACCATACAGTTGCGGGCTGTGTCTGGCCTTTACTATGACAAATCCAGCAACATATTTTTG
TTTCAGAGATACACATGCATGCACTTGCACACACACACAGACAAGCACGCACGCGCACAC
ACAGACACACACACACAGAGAGAGACAGCAATGCTGTGTCATCATTCTGGATTCCCCAGG
ATGTAGTCACCTTGTCTAGGCATTCCTTTAACAGTGAGTTATACAGTACATCCTTGGTAT
CAACTACAAACATCATAAACTGTAGCATGTCCTTGGCTGTAGAATGTTCTCACTCTCTCT
GACAGCACATGGGGTTGACATGTAAACTTCCTCCAAGAGGAGCAACACAAATAGAGAGCT
GCAATCCAGTCAGGGAAAAGAGAGTGCCTCTAGTACACTCCAACAAATAGGATTATCAGA
CTTTACTGTGGAAGGTGAAAAGATGAAAGGATGAAGAGCGGCTTTCTTTGATAAACTCTC
CAAACAGAGGGGTGACAAATGAACGAGTCATCCACCAGGCGTGTTTGAGACTACTCTGTT
TGAGTTCAAACAAAGAATGAGTCCAGAACCAGGAGCTAATACTATTGTGTGTTCACACCA
GTGGAAGCTGGCTTCAATTT

(B) Rainbow trout *trpv1aβ* promoter region:

GTGGGTTGTCAGGACTGGTGGTTAGTAGACTGACGATGTTGAGCGCCAGAGTGGGAGTGA
CAGCCTTGGGGAGGTGGAGGAGTGTTGCTAGGGATGGCAACAGCCAGGGGACCAGGAACC
ATTGGCCTGAGGAGGGAAACCTTGTTGACCCTCCGGAGGACCTTGAAGAGGCTGACTAGA
AGTGAGGCTGACTGTGACCATTTGTCCTCCGCGAAACACTTCTTCTTCACATTGCTCACT
TAACCCAGAAGCCAGCCACTCCAATGTGTCGGACGAAAGACCGTACAACTGGTGACCATG
TCAGCGTGCATGCGCCCGGCCCACCACAGGAGTCGCTAGAGCACTATGGCACTAGGACAT
CCCGGCCGGCCAAACCCTCCCCTAACATAGACAATGCTGTGTCAATTGTTTGTCACCTCA
TGGGTCTCCCAGTTGCTGCAACACAGCCGGGATCGTAGTGATGCATCAAGCACTGCAGTG
CCTTAGACCCCTGTGCCACTTGGGAGGCCCACGGAAGTTTTTGAGTAGTATGCACATGGA
TACAATTTCTGTGGGTTACCATGACGTTGGGACAGGACGGCCCCAACGCCCACTTCTGAA
GCATCCACCTACACCATGAAGGGTGACGTAGGATCTGGGTGTTTCGGCAACGGGGTGGAG
GTGAAACGTCCATTCAGTATACGGAAGGCCTTATCGGCTGCTGGACTCCACACCAACTTA
TGGGGACCACCCTTGAGGAGAGAGGTGAGAGGAGAAGCGATAGAGCTGAAGTTCCTAATG
AAGCGGCAGTAGAAGTTGGCAATCCTCAAAACATTTTGTAGCCCCTTTATGGTGGTTGGG
ACTGGCCATGACCTGATTGTATCGACCTTCCTCTCCTTCATCGTCACTCCCTGTGGACTG
ATCTGGTATCCTAAGAAGTAGAAAGCGGCCTGATAGAACTGCCACTTCTCCGCTTTCACA
CACAGATGATTCTCCAGGAGGTGTACCAGGACTACTCTGACGTGGGTGACGTGATCCTCC

AAGGTGGCCCTCTTGACCACAAAGAAGAAGCCTGCTAATGCAGGGGAGGTGGACATAAAA
CCCTTTTGTTGAACCTCCTGGATGTGCTCCTCCATAGCCTTGATTTCAGCTTCCGACAAA
GGGTAGATGTGGCTGCACGGAGGCGCAGAGCCTGCAAGCAGGTCGATGGCACAGTCCCAA
GGGCGATGAGGAGGGTGAATGTGTCAAGGACTTGGGGCTGGATCTAATCCATATAGCGGA
AGATCCGTGTTTAAATTGAAGGTTGTTTATGATTGAGCTGACAAATGCAGCATTTACAGT
GAATCAGTGAGGAAGAGGCGAAGTCTGTTCACGTGGTATTACAGCGATAAGCAGACTTTT
TTTTACAAGCATGCCTTTGGGATGACGTCTCCCACTGTTGGGGCGGAGACAAGACCATCT
CATCATTATATACAGTATCTCTGCGTGAATGTGGTCTCCACGAGCTCGGGAACAACTCTC
CCTTAGAACCAACTTCTCTGGTTTTAAACGGCCTACGGTATGTGGCATCGATAAGTCAGA
AACATCAATTCTAGTGTCAAAATTGACTACAAAGTGTAAATAGGATATTATTGGTCATAA
AGTAAGTCTTGTCCAAAACAGTTTTGTGAGCAAGTTCATCACGACTTACTGGAGGGTTGG
GTATGGATTACAATCATGCCCACTTGCCCAGTACCCACTGACACGAGAGAAGCAGCTGTG
CTGATTGTGCTCTACACCATAGTCCTCTCCAAGCTCATCACCAAGCTGGGGACCTTTTAA
AAAATATAGCTGATATGGCTGACTTGCTTAAGCAAATGTGATTTCTACTGACAATCGAGA
TGTACAAATTATGGCATAAGGGGACGACAAGCGGATAGGAGGCAATCCGTAATTTCGCTT
AAGACATTAATGAGCGGGCTAGGACGGACGTAGTCAATATAACTATTTGTTCAGCACTTT
TGAAATGTATAGTGACAGAA

(C) Rainbow trout *trpv4α* promoter region:

GGTCATAGGTCAAAGGCACGGGAGAGAAACTTAGCAGTCCAATCATAAACCTTGTATTTG
GTCTCACTCAACTGTGAATGACCTGGGTCGTGTTCATTTGGGCACACAAGCAGAAAATAG
TTTTGCCACAGAAAACGAATAAAAAGTTTCCTGTTTCAGTCTGTTTTCTGTGCTTAATGA
ACAGGACCCTGAACAAGTATCACTGATTGCACGACAAACAGTTACTTGAATAGAATGAGC
CTTTGGGTGATTGGACTGTGTATTTCAAGAAAGAAAATGTATGTCTAAAAGTGATTCTCC
TGTATGTAGCCTTGATCCTGAAGTAGCCAGGGCTAAAATGGTACCCTATTTCCTTTCTAG
TGCACTACGGGGGTAATACCCTATGGGCCCTGGTCAAATGTAGTGCACTGCAACCCAGAT
GTGTGTGGGCAGAGCTCTGGCATCCTGCTCATCTTGTGGGCCACGTTGATGGCACAGAAT
GTCCACCAGTCAGCCATTTGCAGCCGCTGGCAGCCTGGGAACATCATCTGTAAATATTGA
GCAATGCATTGCCTGACCATTACTGGCCCAAGATCAGTGCATGGTAGTGGTGCCCAGATT
TTTCCATTTGGGGTCGGAGAACAGAGTGGGATGCCTCTGAATTTCCAACCAGTTTACTGA
CCAGACGGAGAACACAACAGAGGATGACTTCAAATACACTTTTCCCATGACTGAGTATTT
TCTCCAAAGCATTTTCTCTTCTTTCCAACAGACACTGGGAGACAAATTCACCTTTCAGCA
GGACAATAACCTAAAAGGCCAAATCTACACTGGATTTGCTTACCGAGACCACAATATTTC
TGAGTGGCCTAGTTTTTACTTAAATCATCTTGAAAAAGTATGGCAAGACATGAAAATGTT
TTTAAAAATGTTAGCAATGATCAACAACCAACTTGACAGAGCTTGAAGAATTTAAATAAG
CATAATGGGCAAATATTGTATAATCCAGGTGTGCAAATATCTCAAGAGACGTACCCAAAA
AGACTCCCAGCTGTAATCGCTGCCAAAGGTGATTCTACCATATATTGTCTCCGGGGGTTG
AATACTTATCTAATCAAGATATATTAGTGTTGGTCGTTGCCGATCTACACAAGATGTTAA
ACAACAACATCTGGAAAAAGTAAAGCGGTGTGAATACTTCCTGAAGTCACTGTACACAAG
TAACCCATTACTTACCCATTACTTACATTATAGTAACGCTATGTAATTTGTGTCTCCCTT
TCTTTTGCCGATGCTAACCTGACTAAAACAGAGAGGGAACTACCTGGTTCAAGTAAGGAA
AAAAAAACAATTTTGTGCTGCAAAACATGTATCTAATGGCCACGGCCCAGAAGCCTTTTC
AAGTGGGGAAACATGCAACCGCCACCTGACAAATTCTGAATGGCTACACAGCTAAATGGT
GCATTTTCTACTCGGGGATGACAGAAACAGAAAGCTGGTAAAGACCTATGACCCCTGCGA
GTGGAAAGGGCAAATCCCACGTGTGTTCCCCTGGGGTCCTGGGTTCTATTCAGATGGTCC
CTTAATCCCTCCGTACCTCATTCATCCCTTATGTCACGCTGAGGTGCTGAGTTCTTCTTC
AGGGGAAACTGGAAGTGACGGGGGGGGGGGGGGGGGGGGGGTCCTGTTTAAGCAAAAA
ACAAAAACATTTGTATTTATTAACAGCATTAATACAGACTGGTCGACTGGCAGGAGAGCT
TGATAAACTGATGAAAGAGAAGGGTGTCAGAGTAAGTAGAGGTTAGTCCACAGTCACTCC
ATGGGAAGCTCCATTTGTCACTATGGGCAAAATGTGTGTTCTGGTTGATCTTTTGGTTAC
TAAACATACACTACATGACCAAAGGTATGTGGACACCTGCTCGTCGAACATCTCATTCCA
AAATCCTGGGCATTAATTTGGAGTTGGTCCGCCCTTTGCTGGTATTACAGCTTCCATTAT
TCTGGGAAGGCTTTCCATTT

(D) Rainbow trout *trpv4β* promoter region:

ACCTTCTCAGTAGAGTGGCAGCGGAGCAGCCCTAAAACACATCACAGTAAAACATGCAGG
TTAAAACTCCACCCAGGTCATAGGTCAAAGACATGGGAGAGAAACAGTGATCCAAACATA
TCCCTTGCATTTGGTCTCACTCAACTGTGAATGACCTGGGTTGTGAACATTCGGGCACGT
TAGCTGAATTTGTTTGCCGCAGAAAACAAGAAGGTCCAGTTAGTCCCTCCCTGTTTCAGT
CTGTTTTTTTCCATCTGGTTCTTCATGAACACAACCCTGAACAACTATCACTAATTACTC
TAAAACAGTAATTTGAATAAAATAAGTCTTTGACCAGAGCTGAGATAAGAGACTGAGAAA
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GTCCGAAATGACACACTATTCCCTTTATAGTGCACTACAGGGATTATACCCCAAGGGCCC
TGGTCAAGCGCTATGCACTGCAGCCCAGGTGTGTGTTGGTAGAGCTCTTGCATCCTACTC
ATCTTGTGGGCAACATTGATGGCACAGGAAGTACACCAGTCAGCCATTTGCAGCCACTGG
CAGCCTGGGAACATAATCTGTAAATACTGAGCACTGTATTGCCTGACACTTCCTGGCCCA
AAGCTCATGTGAAGGAAGTGGTACCGGAATTTTCAATTTGGGGTTGAAGAACAGAGTGGG
ATGATTCTGAATTTCCAACCAGTTTACTGACCAGACAGAGGACAGCTTACTGACCAGACA
GAGAACAGCTTACTGACCAGACAGAGAATAGCTTACTGACCAGACAGAGAACAGCTTACT
GACCAGACAGAGAATAGCTTACTGACCAGACAGAGAACAGCTTACTGACCAGACAAAGAA
CAGCTTACTGACCAGACAAAGAACAGCTTACTGACCAGGCAAAGAACAGCTTACTGACCA
GACAGAGAACAGCCTACTGACCAGACAGAGAACAGCTTACTGACCAGACAGAGAACAGCT
TACTGACCAGACAGAGAACAGCTTACTGACCAGACAGAGAACAGCTTACTGACCAGACAG
AGAACAGCTTACTGACCAGACAGAGAACAGCTTACTGACCAGACAGAGAACAGCTTATTG
ACCAGACAAAGAACAGCTTACTGGCCAAACAGAGAACAGCTTATTGACCAGACAGAACAG
CTTACTGACCAGACAGAACAGCTTATTGACCAGACAGAACAGCTTACTGACCAAACAGAG
AACAGCTTACTGACCAGACAGAGAACAATAAGCTATTTGTTAGTCAGAAGAGCTGTAAGT
CAATAACCTCTCACATAGTCTTAAACAGCTTACTGACCAGACAAAGAACAGCTTACTGGC
CAAACAGAGAACAGCTTATTGACCAGACAGAACAGCTTACTGACCAGACAGAACAGCTTA
TTGACCAGACAGAATAGCTTATTGACCAGACAAAGAACAGCTTACTGGCCAAACAGAGAA
CAGCTTATTGACCAGACAGAACAGCTTACTGACCAGACAGAACAGCTTACTGACCAGACA
AAGAACAGCTTACTGACCAGACAAAGAGCAGCTTACTGGCCAAACAGAGAACAGCTTATT
GACCAGACAAAGAACAGCTTACTGGCCAAACAGAGAACAGTTTATTGACCAGACAAAGAA
CAGCTTACTGGCCAAACAGAGAACAGCTTACTGGCCAAACAGAGAACATCTTATTGACCA
GACAAAGAACAGCTTACTGGCCAAACAGAGAACAGCTTACTGACCAGACAAAGAACAGCT
TACTGGCCAAACAGAGAACAGCTTATTGACCAGACAGAGAACAGCTTACTGACCAGACAA
AGAACAGCCTACTGACCAGACAAAGAATAGCTTACTGACCAGACAAAGAGCAGCTTACTG
GCCAAACAGAGAACAGCTTATTGACCAGACAAAGAACAGCTTACTGGCCAAACAGAGAAC
AGCTTACTGACCAGACAGAG

GRE full sites

Figure S.2.4. GRE identification within promoter sequences of (A) *trpv1aα,* (B) *trpv1aβ,* (C) *trpv4α,* and (D) *trpv4β.*

Chapter 3

The cortisol response to acute warming in rainbow trout (*Oncorhynchus mykiss*)

3.1 Introduction

Acute increases in water temperature activate a suite of physiological and cellular responses in fishes that are aimed at maintaining homeostatic function as body temperature, and hence metabolic rate, increases. These responses include increases in heart rate and cardiac output to increase oxygen delivery to tissues (Clark et al. 2008; Steinhausen et al. 2008; Ekström et al. 2017, 2019; Bard et al. 2021), increases in antioxidant enzymes to protect the cell from damage induced by reactive oxygen species (ROS) (Sopinka et al. 2016; Rossi et al. 2017; Li et al. 2019; Anttila et al. 2023), and increases in plasma glucose to support the higher rate of energy expenditure (Zhang and Kieffer 2014; Corey et al. 2017; Bard and Kieffer 2019; Gilbert et al. 2019, 2020; Morrison et al. 2020). Induction of heat shock proteins (HSPs) is the most common cellular response to heat; this response is activated when temperatures surpass certain thresholds that are species-specific (Iwama et al. 1999; Pirkkala et al. 2001; Currie 2011). Catecholamine mobilization (Whiteley et al. 2006; LeBlanc et al. 2011, 2012) and increases in circulating cortisol (Basu et al. 2001; Pérez-Casanova et al. 2008; Steinhausen et al. 2008; LeBlanc et al. 2011; reviewed by Alfonso et al. 2021) may also occur. However, although increases in cortisol with acute warming have been well documented (Alfonso et al. 2021, 2023), little work to date has examined the proximate pathways involved or the activity of the HPI axis in response to temperature. In

particular, it is not clear whether there is a threshold temperature at which the cortisol response is induced, nor whether the threshold can be altered by environmental conditions and/or the physiological state of the fish.

Increases in circulating cortisol in fishes are the product of activation of the HPI axis (Wendelarr Bonga 1997; Faught et al. 2016; Schreck and Tort 2016; Winberg et al. 2016). This cascade begins with perception of a stressor, which causes release of corticotropin-releasing factor (CRF) from neurons of the POA directly to the pituitary, in turn causing the release of adrenocorticotropic hormone (ACTH). The production of cortisol by the interrenal cells located in the head kidney is stimulated by ACTH. Steroidogenic acute regulatory protein (StAR) and P450 side chain cleavage enzyme (P450scc, CYP11A1) mediate rate liming components of cortisol steroidogenesis in the head kidney (Aluru and Vijayan 2008). Moreover, steroidogenic factor 1 (SF-1) is thought to be a key transcription factor regulating the expression of several steroidogenic genes (Hu et al. 2001; Quek and Chan 2009). In fishes, SF1 is transcribed by the gene *ff1*, with salmonids having several paralogues, including *ff1b1, ff1b2* and *ff1d* (Best and Gilmour 2022). Cortisol is delivered by the circulatory system to target tissues and its effects are mediated by glucocorticoid (GR) and mineralocorticoid (MR) receptors, which serve as ligand-activated transcription factors (Wendelarr Bonga 1997; Faught et al. 2016; Schreck and Tort 2016; Winberg et al. 2016).

In rainbow trout (*Oncorhynchus mykiss*), the prolonged elevation of cortisol caused by chronic social stress lowers thermal tolerance (LeBlanc et al. 2011; Bard et al. 2021). Recent work reported that chronic cortisol treatment alone, in the absence of a stressor, decreased the critical thermal maximum (CTmax) of rainbow trout by ~1°C, an

effect that was blocked by co-treating the fish with the GR antagonist RU486 (Chapter 2). Whether chronic cortisol elevation also alters the threshold for other heat-induced responses, such as the cortisol response, remains to be determined. A potential complication is that chronic social stress attenuates the acute cortisol response to a netting stressor (Jeffrey et al. 2014), likely through the inhibition of ACTH-stimulated cortisol production at the head kidney (Best and Gilmour 2022). However, subordinate rainbow trout experiencing chronic social stress mounted an acute cortisol response to warming that was comparable to that of their dominant counterparts (Bard et al. 2021).

Thermal tolerance is also influenced by acclimation temperature, with well-documented increases in CTmax following acclimation to higher temperature (Beitinger and Bennett 2000; Rajaguru 2002; Moyano et al. 2017; da Silva et al. 2019; McDonnell et al. 2021; Mckenzie et al. 2021; see also Chapter 2). Again, however, few studies have evaluated whether thermal acclimation alters the threshold for other heat-induced responses (Dalvi et al. 2012; Madaro et al. 2018; Samaras et al. 2018a; Zhu et al. 2022), such as the cortisol response. Research on the cortisol response to thermal acclimation has tended to focus on cortisol levels and expression of HPI axis genes (Alzaid et al. 2015; Mahmoud et al. 2020; Goikoetxea et al. 2021; Tang et al. 2022a, 2022b). As such, there is good evidence of elevated cortisol during acclimation to higher temperatures (Benítez-Dorta et al. 2017; Madaro et al. 2018; Kim et al. 2019; Hanke et al. 2019; Goikoetxea et al. 2021; Alfonso et al. 2021). In terms of HPI axis regulation, acclimation to higher temperature in Atlantic salmon caused a downregulation of *hsd11b2α* and *hsd11b2β*, as well as *gr2α* and *mr* (Tang et al. 2022a). Hydroxysteroid 11-beta dehydrogenase 2 (HSD11B2) catalyzes the conversion of cortisol into cortisone,

transforming bioactive cortisol into an inactive metabolite, which can protect glucocorticoid-sensitive tissues during stress (Alderman and Vijayan 2012). Moreover, the expression of *star* was dynamically regulated when Nile tilapia were acclimated to elevated temperatures, which mimicked the patterns of circulating cortisol (Mahmoud et al. 2020). This finding suggests that the temperature threshold for activation of the cortisol response can be altered by acclimation, and this effect may be mediated by the regulation of *star*. In sea bass reared at high temperatures to adulthood, scale cortisol was nearly 10x higher relative to scales taken from fish held at control temperatures (Goikoetxea et al. 2021). Corresponding to these increases in scale cortisol, *crhbp* was upregulated and *mc2r* was downregulated (Goikoetxea et al. 2021). Corticotropin releasing factor-binding protein (CRFBP, CRHBP) can bind to corresponding CRF receptors and therefore can play an inhibitory role on CRF by diminishing CRF-signalling (Flik et al. 2006). Collectively, these data suggest that acclimation to warmer temperatures alters HPI axis regulation, which may also alter the temperature at which a cortisol response is induced.

Recent work has implicated the thermosensitive transient receptor potential (thermoTRP) ion channel TRPV1 in activating responses to noxious heat in fishes, as in other vertebrates (Saito et al. 2011; Laursen et al. 2016; Haesemeyer 2020; Cutler and Haesemeyer 2024). For example, knockdown of TRPV1 in zebrafish (*Danio rerio*) larvae prevented heat-induced locomotion (Gau et al. 2013), while treatment of masu salmon (*Oncorhynchus masou*) with the TRPV1 agonist capsaicin potentiated locomotory responses to acute warming (Yoshimura et al. 2022). Heat-induced emergence of mangrove rivulus (*Kryptolebias marmoratus*) from water could be reproduced by

exposure to capsaicin in the absence of heat (Melanson et al. 2023). In rainbow trout, treatment with the TRPV1 antagonist capsazepine increased the temperature at which loss-of-equilibrium (LOE) occurred, and the cortisol response was initiated, whereas capsaicin lowered the temperature of LOE without impacting the cortisol response (Chapter 2). These data support the hypothesis that TRPV1 is a key component of the heat-sensing mechanism that induces a cortisol response.

The primary objective of the present study was to elucidate the mechanism which initiates the cortisol response during acute warming. As such, I evaluated the cortisol response to acute warming over a gradient of water temperatures in hatchery-raised juvenile rainbow trout. It was also possible that increases in temperature could increase cellular rates of cortisol production through the effects of temperature on reaction rates (Schulte 2015; Mckenzie et al. 2021). To explore the impact of this effect on circulating cortisol levels, cortisol production was measured *in vitro* in response to changes in incubation temperature using isolated head kidney tissue preparations. I also explored the interacting effects of cortisol treatment and acute warming on HPI axis regulation, focusing on key HPI axis targets (i.e. *crf*, *star*, *p450scc* and *ff1*). As such, transcript abundances of these HPI axis targets were evaluated in trout at their acclimation temperature and following acute warming sufficient to evoke a cortisol response. Under the hypothesis that acute warming activates the HPI axis, leading to increases in cortisol, I predicted that transcript abundances of key genes of the HPI axis would be elevated at high temperature. A secondary objective was to further examine the potential role of TRPV1 in inducing the activation of the cortisol response through heat-sensation; to this end, key HPI axis targets were assessed following acute warming in fish treated with

capsazepine. Lastly, to investigate the plasticity of the cortisol response, the cortisol response to acute warming was assessed over a range of water temperatures in trout that had been acclimated to higher temperatures.

3.2 Methods

3.2.1 Experimental animals

Juvenile rainbow trout, *Oncorhynchus mykiss* (Walbaum 1792) (N = 172, mass = 159.8 ± 9.8 g, fork length = 23.2 ± 0.4 cm, mean ± SEM; see Table 3.1), were purchased from Izumi Aquaculture formerly know as Linwood Acres Trout Farm (Campbellcroft, ON, Canada), Cedar Crest Trout Farms (West Grey, ON) and Lyndon Fish Hatcheries (New Dundee, ON). Fish were held at the University of Ottawa in 1,275 L fibreglass tanks supplied with flowing 13°C city of Ottawa tap water. Incoming water was aerated and dechloraminated before reaching the holding tanks. Trout were fed by scattering commercial trout pellets on the water's surface, at a ration of 0.5% body mass daily. A 12 h:12 h light:dark photoperiod was utilized. Fish were held under these conditions for at least 2 weeks prior to experimentation. Experimental protocols followed the guidelines of the Canadian Council on Animal Care (CCAC) for the use of animals in research and teaching. All experimental protocols were authorized by an institutional animal care committee (protocol BL-3668, University of Ottawa).

3.2.2 Experimental protocols

Four experiments were carried out. Experiment 3.1 assessed whether there was a threshold temperature for the cortisol response to acute warming and explored the effect of chronic cortisol elevation on this threshold. Experiment 3.2 investigated the effects of

temperature of cortisol production *in* vitro. Experiment 3.3 evaluated transcript abundances of key genes in the HPI axis under baseline conditions (13°C) and at threshold temperatures for the cortisol response in control as well as cortisol-treated trout; this experiment used a subset of the fish from Experiment 3.1. Experiment 3.4 explored the consequences of thermal acclimation on the cortisol response to acute warming.

3.2.3 Experiment 3.1 The cortisol response to thermal ramping

To investigate the cortisol response to thermal ramping, individuals (N = 50) were exposed to acute warming to a designated temperature, which included 13, 23, 25, 27°C and loss of equilibrium. The effect of chronic elevation of cortisol on this acute response was studied by using the same protocol in cortisol-treated individuals (N = 42). For the purpose of this study, thermal ramping refers to acute warming where water temperature is raised in a linear fashion at 0.33 ± 0.01°C min^{-1}.

3.2.3.1 Administration of cocoa butter cortisol implants

Rainbow trout were injected with an intraperitoneal implant of cocoa butter (NOW Health Group Inc., Bloomingdale, IL, USA; 0.5mL per 100g body mass) containing cortisol (hydrocortisone 21-hemisuccinate, Sigma- Aldrich, Oakville, ON, Canada; 80 mg kg^{-1} body mass). Cocoa butter implants containing cortisol were made by dissolving the hydrocortisone 21-hemisuccinate in 99% ethanol; the solution was then added to liquid cocoa butter and the ethanol was evaporated off using heat (Hoogenboom et al., 2011; Lawrence et al., 2019). Trout were lightly anaesthetized in a solution of benzocaine (0.05 g L^{-1} ethyl-p- aminobenzoate; Sigma-Aldrich). Liquid cocoa butter (~32°C) was injected into the peritoneal cavity using a 22G sterile needle and 1 mL syringe. The cocoa butter solution solidified almost immediately after administration.

Following placement of the cocoa butter implant, fish were placed individually in 40 L flow-through Plexiglas tank for 4 d. Tanks were supplied with 13°C system water and fish were not fed during the 4d treatment period. It should be noted that control fish were untreated, as the administration of a sham implant can elevate cortisol levels in an unpredictable fashion, which would of conflated plasma cortisol results (DiBattista et al. 2005).

3.2.3.2 Thermal ramping

Individual trout in their 40 L experimental tanks were exposed to acute warming between 09:00 and 11:00 h to avoid potential impacts of daily cortisol rhythms (Rance et al. 1982) on the values that were measured. Fish were subjected to a linear increase in water temperature of 0.33 ± 0.01°C min^{-1} to a set temperature of 13, 23, 25, 27 or 29°C (Fig S3.1). Fish were held at the designated temperature for 10 min and water temperature was then returned to 13°C for a 1 h recovery period, following which fish were euthanized for the collection of tissues. If an individual lost equilibrium prior to reaching the designated temperature or during the 10 min exposure period, LOE temperature was noted, and water was immediately cooled to 13°C to begin the 1 h recovery period. Water temperature was adjusted using a series 440 Fotopanel thermostatic mixing valve (POWERS^{TM}, Burlington, ON, Canada) and monitored using a digital thermometer (Digi-sense, Cole Palmer, Quebec, QC, Canada). Water flowing to the experimental tanks was vigorously aerated using an equilibration column to ensure the maintenance of air saturation as temperature increased.

3.2.3.3 Tissue collection and analysis

Individuals were euthanized by terminal anesthesia (0.5 g L^{-1} ethyl-p-aminobenzoate) and spinal severance. Following euthanasia, 1 mL of blood was withdrawn from the caudal vasculature using a 23G needle and a 1 mL syringe rinsed with 0.5 mol L^{-1} ethylenediaminetetraacetic acid (EDTA). Blood samples were collected within 2–3 min of netting the fish to avoid handling-induced elevation of cortisol (Lawrence et al., 2018). Blood samples were centrifuged at 10,000 *g* for 2 min. The resulting plasma was flash-frozen in liquid N_2 and stored at -80°C for later measurement of cortisol concentrations. Plasma cortisol levels were measured using a commercial radioimmunoassay (RIA; MP Biomedical, LLC, USA), which has been validated in trout (Gamperl et al., 1994). All samples were measured in a single assay, and the intra-assay coefficient of variation was 8.0%. Following collection of blood samples, the brain was dissected out and the POA and hypothalamus were collected. The head kidney and the first two gill arches from the left side were also collected. All tissues were flash frozen in liquid N_2 and stored at -80°C for later analysis. Head kidney and POA were used in the present study, and POA, hypothalamus and gill tissue were used in a separate study (see Chapter 2).

3.2.4 Experiment 3.2 The effects of high temperature on cortisol steroidogenesis

3.2.4.1 In vitro head kidney preparations

To examine the effect of temperature on cortisol production, *in vitro* cortisol production by isolated head kidney tissue was measured at 13, 23 and 27°C. The preparation of head kidney tissue was based on the protocols of Best and Gilmour (2022), Aluru and Vijayan (2006), and Jeffrey et al. (2014). A set of larger trout (N = 10; Table 3.1) that had been held under the standard holding conditions described above was used

for this experiment. Head kidney tissue was dissected out and rinsed in chilled Leibovitz's L-15 medium (Gibco, Thermo Fisher Scientific). The tissue was weighed before being transferred to glass Petri dishes that were held on ice and contained 100 mL of L-15 medium per gram of tissue. The head kidney tissue was minced to 1 mm^3 pieces and incubated at 13°C on a shaker for 3 h. Tissue pieces were then allocated evenly among wells across three 48-well microplates (Corning Costar, Corning, NY, USA), one for each temperature. Each well received ~25 mg of tissue but final tissue weights were determined at the end of the experiment. Treatments (1 IU ml^{-1} ACTH; A6303, Sigma-Aldrich; or saline, in L-15 medium) were added to the appropriate wells in duplicate. The tissue was incubated at 13°C, 23°C or 27°C on a shaker for 3 h. Both ACTH concentration and incubation time were based on previous studies (Aluru and Vijayan 2006; Jeffrey et al. 2014; Best and Gilmour 2022). Following incubation, the contents of each well were centrifuged for 2 min at 13,000 *g*. The resulting supernatant was flash frozen in N_2 and stored at -80°C until measurement of cortisol concentrations, and the remaining tissue was weighed and used to normalize cortisol production to tissue weight. Cortisol concentrations were measured using a commercial RIA (MP Biomedical) and intra- and inter-assay coefficients of variation were 7.3% and 6.1%, respectively.

3.2.5 Experiment 3.3 Transcript abundances of key HPI genes in response to thermal ramping

To evaluate how the HPI axis is regulated at high temperatures, key genes of the HPI axis were investigated in fish exposed to 13°C (N = 4) and 27°C (N = 4). The 13°C temperature was chosen for baseline values (i.e. unstressed, control temperature) and 27°C (i.e. stressed, acute warming) was chosen because it is a temperature that is

expected to produce an acute cortisol response. Therefore, I predicted that the HPI axis would be regulated differently between these two temperatures. An additional group of fish was treated with cortisol to assess how chronic cortisol elevation alters HPI axis regulation at 13°C (N = 4) and 27°C (i.e. elevated temperatures; N = 4). The fish used for Experiment 3.3 were a subset of the fish from Experiment 3.1.

A separate group of fish were used to measure *crf* in fish treated with the TRPV1 antagonist, capsazepine. Individual fish were left overnight in 3 L experimental chambers supplied with flowing system water at 13°C. Following overnight recovery, fish were lightly anesthetized using a benzocaine solution (0.05g L^{-1} ethyl-p- aminobenzoate, Sigma Aldrich). Individuals were given an intraperitoneal injection of 50 µL of capsazepine (C191, Sigma Aldrich) dissolved in DSMO (5 mg capsazepine per 1 mL^{-1} DMSO; Rocha Barreto et al. 2022). Sham fish were given an injection with 50 µL of DSMO only. The tanks were then quickly flushed of the anesthetic solution using a high flow of 13°C system water. After the fish had regained equilibrium for 10 min, water temperature was raised at a rate of 0.3°C min^{-1} to 27°C, and held at this temperature for 10 min. Water temperature was then returned to 13°C and fish were left for 1 h of recovery. Fish were then euthanized using terminal anesthesia and spinal severance. The POA was removed, flash frozen and stored at -80°C until analysis. The fish used were a subset of fish from Experiment 2.4.

To evaluate differences in HPI axis regulation, transcript abundances of key HPI axis genes were measured (Table 3.2). The POA and HK tissues were powdered by mortar and pestle on dry ice. Total RNA was extracted from powdered aliquots (~10-40 mg)) of POA and HK to which chilled TRIzol® reagent was added (Invitrogen, Carlsbad,

CA, USA) based on the manufacturer's protocol. The tissue-TRIzol® solution was then sonicated on ice (Sonic Dismembrator Model 100, Fisher Scientific). The quantity and quality of the resulting RNA were measured using a NanoDrop® (NanoDrop™ 2000, ThermoFisher Scientific). Genomic DNA removal and subsequent cDNA production was carried out with the QuantiTect®reverse transcription kit (Qiagen, Montreal, QC, Canada) according to the manufacturer's instructions.

All gene-specific primer sequences for the target genes, *crf*, *star*, *p450scc*, *ff1b1*, *ff1b2*, *ff1d*, were obtained from published studies, as was that for β-actin, which served as a housekeeping gene (Table 3.2). Real-time RT-PCR was completed using the Rotor-Gene SYBR Green PCR kit (Qiagen) and a Rotor- Gene Q real-time PCR machine (Qiagen) according to the manufacturer's instructions, with the exception that reactions were adjusted to 10 µl. Samples were assayed in duplicate together with negative controls (a no-template sample where cDNA was replaced with water, and a no-RT sample, which is where cDNA synthesis reaction was completed without reverse transcriptase). The cycling conditions were 5 min at 95°C, 42 cycles of 10 s at 95°C, 10 s at 60°C and 10 s at 72°C. Following cycling, melt curve analysis was performed to ensure the amplification of a sole product, based on the manufacturer's protocols. Standard curves were produced for each tissue (i.e. head kidney and POA, separately) through the serial dilution of pooled samples and relative transcript abundances were calculated according to Pfaffl (2001). All relative abundances were presented as fold-changes compared to the 13°C control fish.

3.2.6 Experiment 3.4 Effects of thermal acclimation on the cortisol response to thermal ramping

To determine whether thermal acclimation alters the cortisol response to elevated temperatures, fish were subjected to a 3-week acclimation to 19°C (N = 34) or 22°C (N = 36). Following thermal acclimation, fish were exposed individually to a thermal ramping protocol using the approach of Experiment 3.1 but with the temperatures being the acclimation temperature (i.e. 19°C or 22°C) as well as 23, 25, 27, or 29°C.

3.2.6.1 Thermal acclimation

Fish were allocated haphazardly to two 250 L flow-through tanks supplied with 15°C water and left to recover overnight. Each tank contained a total of 30 fish, with approximately half of the fish being used for a different study (see Chapter 2). This approach was used to maintain appropriate densities so as to avoid the formation of dominance hierarchies that subject some fish to social stress (Ellis et al. 2002; Brockmark and Johnsson 2010; Roy et al. 2021). Water temperature was elevated by 1°C per day until the desired acclimation temperature was reached (19°C or 22°C), and fish were then held under these conditions for a minimum of 3 weeks. Fish were fed commercial food pellets daily throughout the 3-week period at 0.5% of their body mass. The 3-week acclimation period reflected values used in previous studies (Healy and Schulte 2012; Anttila et al. 2015; Adams et al. 2022; Stewart et al. 2023). At the end of the acclimation period, fish (N = 36) were transferred into individual 40 L tanks for overnight recovery and subjected to thermal ramping (as described in Section 3.2.3.2) the following morning. The experimental tanks were supplied with flowing water of the appropriate acclimation temperature, the lowest temperature tested was the acclimation temperature for that group instead of 13°C, and fish were returned to their acclimation temperature for the 1 h recovery after thermal ramping. Fish were then euthanized by terminal anesthesia and

spinal severance, and a blood sample was collected (as described above in Experiment 3.1). Plasma cortisol was measured using an RIA (as described in Experiment 3.1), with the intra-assay coefficient of variation being 10.3% (all samples were measured in a single assay). It should be noted that I twice attempted acclimation to 16°C; however, in both cases the fish were subjected to an unanticipated warm thermal stressor over several days owing to equipment malfunction. Unfortunately, in both cases individual fish showed evidence of stress under acclimation conditions (i.e. high cortisol values at acclimation temperature; Section 3.5, Fig S3.2), prior to thermal ramping, and these trials therefore were excluded from this thesis.

3.2.7 Statistical analysis

All statistical analyses were conducted using R-studio. Data were checked for violation of the assumptions of parametric tests before analysis. Normality was verified using the Kolmogorov–Smirnov test and equal variance was verified using Levene's test. For all analyses, α was set to 0.05. Data are presented as means ± SEM in tables. In figures, data are presented as box plots where the upper and lower limits of the box indicate the 75th and 25th percentiles, respectively, the black line across the box indicates the median, the whiskers indicate the maximum and minimum values, and the red plus sign indicates the mean value. Symbols represent individual data points.

Plasma cortisol concentrations during thermal ramping (Experiments 3.1 and 3.4) and transcript abundances (Experiment 3.3) were analyzed by two-way analysis of variance (ANOVA), with temperature and treatment (either cortisol treatment in Experiment 3.1 and Experiment 3.3 or acclimation temperature in Experiment 3.4) as factors. Following a significant result for the two-way ANOVA, further analysis of the

data from Experiment 3.1 was carried out on data split by treatment, because cortisol treatment elevated baseline cortisol values, which skewed the cortisol values higher for one treatment. Plasma cortisol data were then analyzed with Kruskal Wallis one-way ANOVA and multiple comparisons were carried out with Student *t*-tests corrected for multiple comparisons. Multiple comparisons were carried out using Tukey's tests for Experiments 3.3 and 3.4. Transcript abundance of *crf* in capsazepine-treated vs sham fish was analyzed with a Student's *t*-test. Cortisol production by head kidney preparations (Experiment 3.2) was analyzed using two-way ANOVA, with ACTH treatment and temperature as factors.

Table 3.1. Morphometric data of rainbow trout (*Oncorhynchus mykiss*) by experiment.

Experiment	Treatment	Mass (g)	Fork length (cm)
3.1 The acute cortisol response to thermal ramping			
	Control (N = 50)	176.5 ± 4.6	25.4 ± 0.3
	Cortisol-treated (N = 42)	165.9 ± 5.3	25.1 ± 0.3
3.2 The effects of high temperature on cortisol steroidogenesis			
	Control (N = 10)	619.2 ± 25.8	35.0 ± 0.7
3.4: Effects of thermal acclimation on the cortisol response to thermal ramping			
	19°C thermal acclimation (N = 34)	82.9 ± 5.3	19.2 ± 0.4
	22°C thermal acclimation (N = 36)	74.4 ± 4.7	18.6 ± 0.4
Total (N = 172)		159.8 ± 9.8	23.2 ± 0.4

Table 3.2. Gene-specific primers used for real-time RT-PCR.

Gene	Primer sequence	Amplicon size (bp)	Efficiency (%)	Accession number	Reference
crf	F: ACAACGACTCAACTGAAGATCTCG	54	98	AF296672.1	(Bernier et al. 2008)
	R: AGGAAATTGAGCTTCATGTCAGG				
star	F: TGGGGAAGGTGTTTAAGCTG	101	104	AB047032.2	(Aluru and Vijayan, 2008)
	R: AGGGTTCCAGTCTCCCATCT				
p450scc	F: GCTTCATCCAGTTGCAGTCA	140	108	S57305.1	(Aluru and Vijayan, 2008)
	R: CAGGTCTGGGGAACACATCT				
ff1d	F: CTACAGACCCTACAGGCGGA	81	102	XM_014143565.1	(Best & Gilmour, 2022)
	R: CAGTAGCTTCACATCGGGGT				
ff1b1	F: ACGTCTGGCCTACCTCATAG	64	103	NM_001124537.1	(Best & Gilmour, 2022)
	R: TTTCAAAGGGACGCCTTGT				
ff1b2	F: TGGTAGGCGTAATGGACTACA	131	106	XM_021605623.1	(Best & Gilmour, 2022)
	R: TGTCCTCTTGAAGAAGCCCTTA				
β-actin	F: AGAGCTACGAGCTGCCTGAC	197	104, 82	NM_001124235.1	(Moltesen et al., 2016)
	R: GTGTTGGCGTACAGGTCCTT				

3.3 Results

3.3.1 Experiment 3.1 The acute cortisol response to thermal ramping

Trout exposed to thermal ramping exhibited increases in plasma cortisol that reflected the interaction of exposure temperature and cortisol treatment (Fig. 3.1A). There were significant differences in plasma cortisol with increasing temperature in both control and cortisol-treated fish. In control fish, there was a significant elevation in cortisol at 27°C and LOE when compared to baseline cortisol levels at 13°C (Fig. 3.1B). In cortisol-treated fish, plasma cortisol levels were significantly higher at 25°C, 27°C and LOE, compared to 13°C (Fig. 3.1C).

3.3.2 Experiment 3.2 The effects of high temperature on cortisol steroidogenesis

Although the temperature to which fish were exposed during thermal ramping significantly influenced plasma cortisol, *in vitro* cortisol production by head kidney tissue was not significantly influenced by incubation temperature (Fig. 3.2).

3.3.3 Experiment 3.3 Transcript abundances of key HPI genes in response to thermal ramping

Transcript abundance of *crf* in the POA was significantly affected by the interaction of exposure temperature and cortisol treatment (Fig. 3.3A). Whereas cortisol treatment resulted in significantly higher transcript abundance of *crf* at 13°C, no effect of cortisol treatment was detected after thermal ramping to 25 or 27°C (Fig. 3.3A). Transcript abundances were significantly higher in fish exposed to 27°C than in those exposed to 25°C (Fig. 3.3A). Capsazepine-treated fish had significantly lower *crf* abundance than sham fish when exposed to 27°C (Fig. 3.3B).

In head kidney, transcript abundances of *star* and *p450scc* were significantly lower in cortisol-treated fish regardless of exposure temperature but were unaffected by exposure temperature (Fig. 3.4). No significant effects of cortisol treatment or exposure temperature were detected for transcript abundances of *ff1b1* and *ff1b2* (Fig. 3.5A, B). However, *ff1d* abundance was significantly lower in cortisol-treated trout (Fig. 3.5C). Additionally, *ff1d* abundance was significantly higher at elevated temperature irrespective of treatment group (Fig. 3.5C).

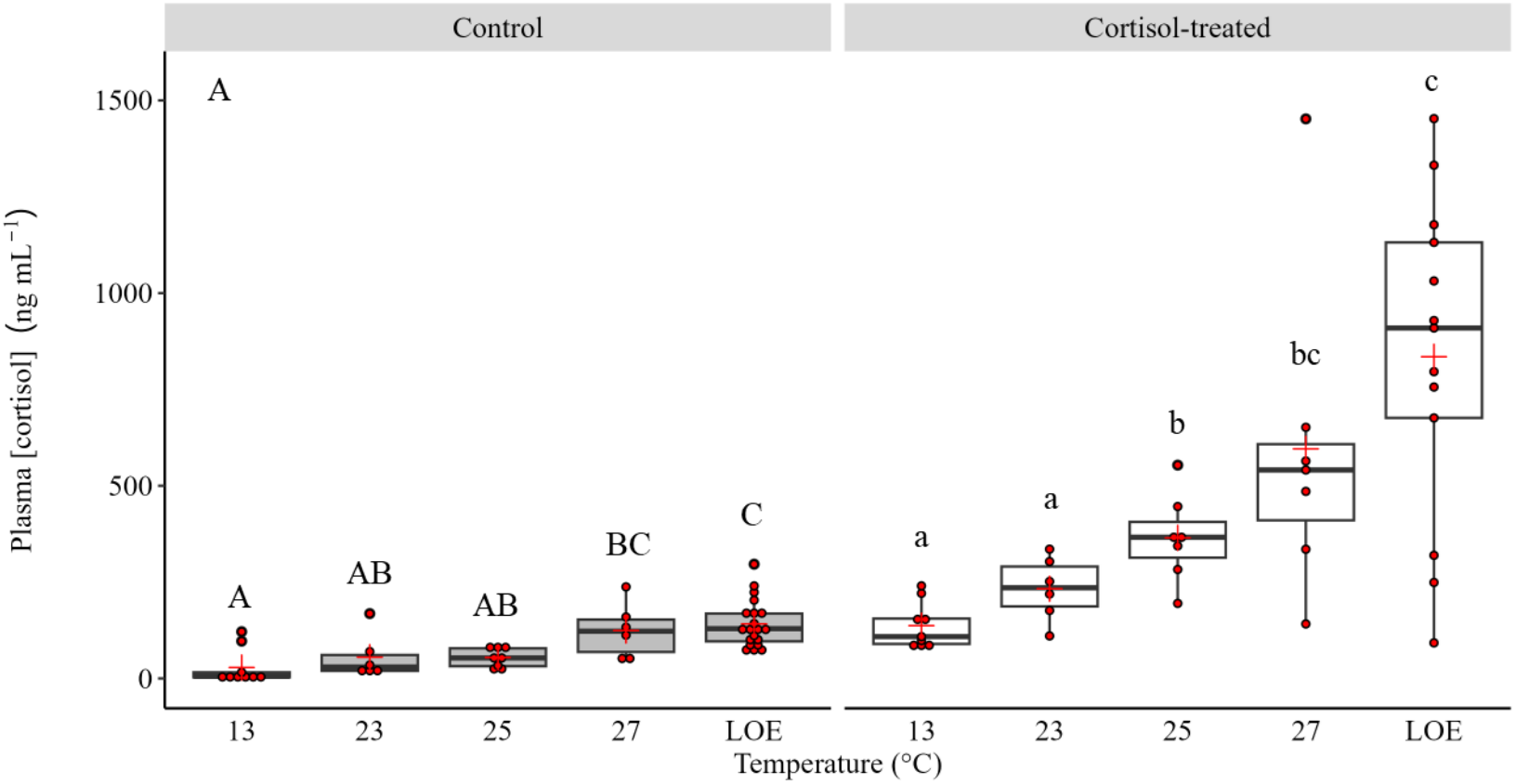
3.3.4 Experiment 3.4 Effects of thermal acclimation on the cortisol response to thermal ramping

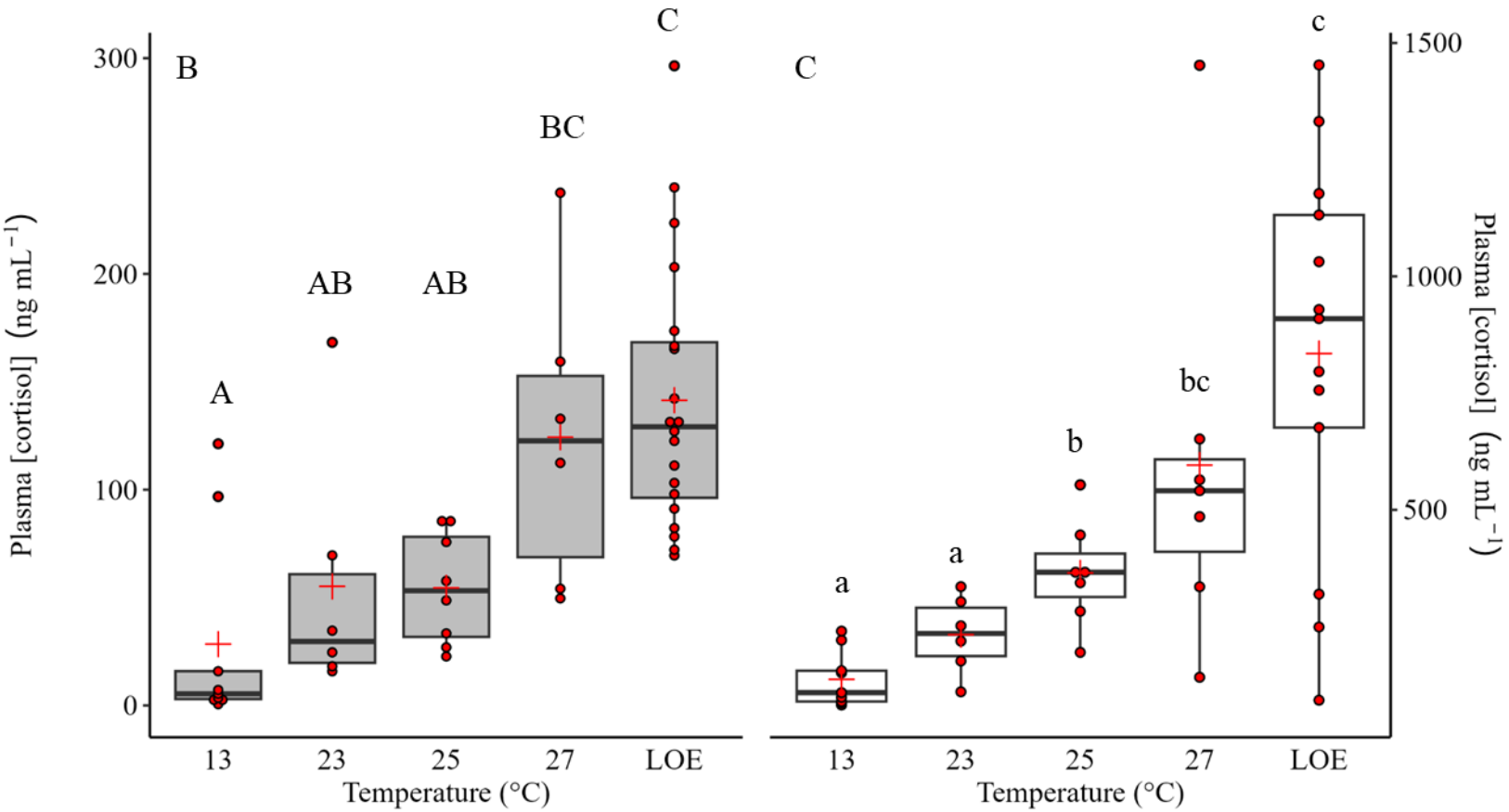
Plasma cortisol reflected a significant interaction between exposure temperature and acclimation temperature (Fig. 3.6). Fish acclimated to 19°C showed significant elevation of cortisol at 27°C and 29°C, whereas fish acclimated to 22°C showed significant elevation of cortisol only at 29°C (Fig 3.6).

3.4 Figures

Figure 3.1. The cortisol response to thermal ramping in control (N = 7-16 per temperature) versus cortisol-treated (N = 7-13 per temperature) rainbow trout (*Oncorhynchus mykiss*), using the same y-axis range (A) or y-axis ranges scaled to (B) control vs (C) cortisol-treated fish. Fish that lost equilibrium during thermal ramping or the 10-min exposure to the set temperature were placed in the loss-of-equilibrium (LOE) group. A significant interaction between the effects of temperature and cortisol treatment was observed (two-way ANOVA, $P_{\text{temperature}} < 0.0001$, $P_{\text{treatment}} < 0.0001$, $P_{\text{temperature x treatment}} < 0.0001$) and the data set was therefore split by treatment group for further analysis. Data was subset by treatment for further analysis due to cortisol treatment elevating circulating cortisol artificially, skewing cortisol levels unnaturally. Boxplots that share a letter within a treatment status category are not significantly different from one another (Kruskal-Wallis one-way ANOVA, for panel b, $P = 0.0001$; for panel c, $P < 0.0001$, for control and cortisol-treated respectively). Data are presented as box plots (see Section 3.2.7 for details).

Figure 3.1.


Control
Cortisol-treated
Plasma [cortisol] (ng mL^{-1})
1500
1000
500
0
A
A
AB
AB
BC
C
a
a
b
bc
c
13
23
25
27
LOE
13
23
25
27
LOE
Temperature (°C)


Plasma [cortisol] (ng mL⁻¹)
300
200
100
0
B
A
AB
AB
BC
C
13
23
25
27
LOE
Temperature (°C)
C
a
a
b
bc
c
1500
1000
500
Plasma [cortisol] (ng mL⁻¹)
13
23
25
27
LOE
Temperature (°C)

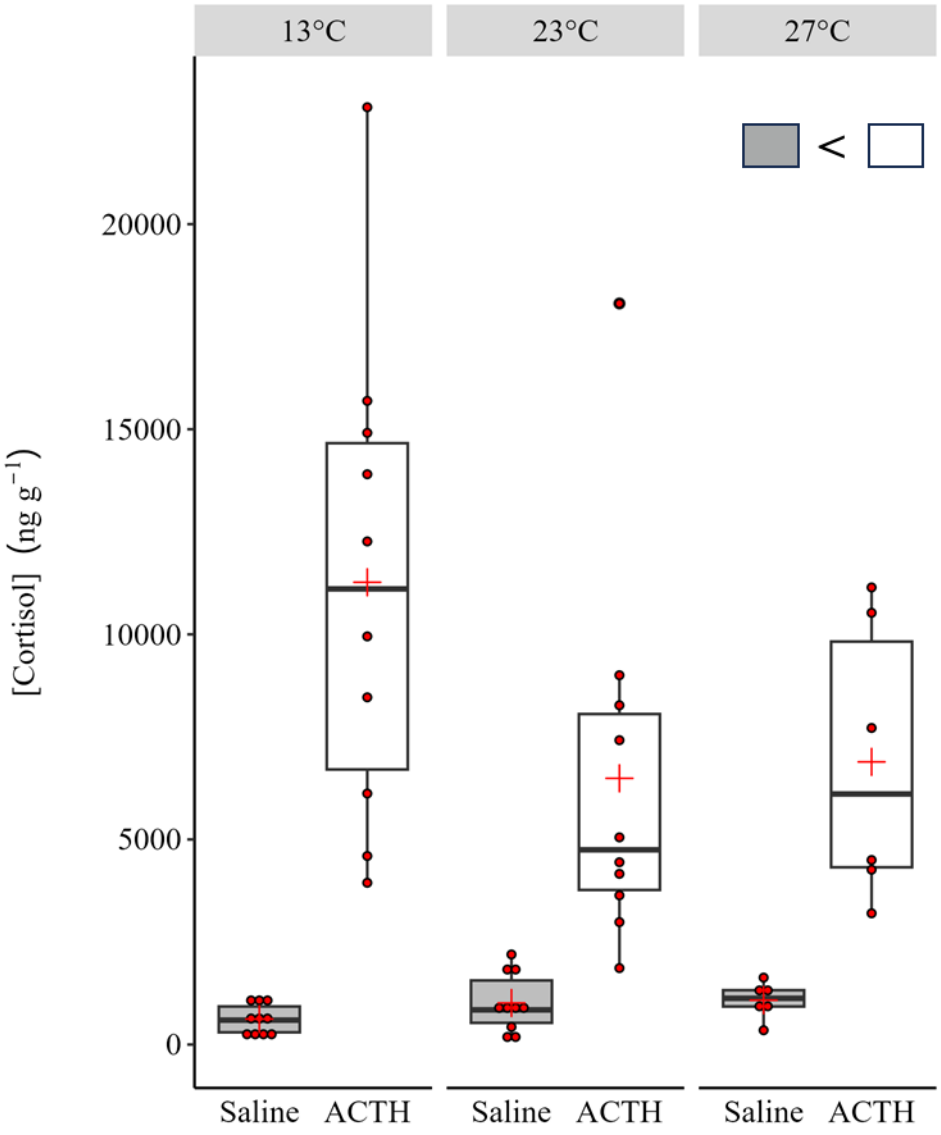


Figure 3.2. Basal and ACTH-stimulated cortisol production as a function of incubation temperature for in vitro head kidney preparations from rainbow trout (*Oncorhynchus mykiss*). A significant effect of ACTH but not temperature on cortisol production was detected (two-way ANOVA, $P_{treatment} < 0.0001$, $P_{temperature} = 0.1211$, $P_{treatment\ x\ temperature} = 0.0518$; $N = 10$). The significant effect of treatment (incubation with ACTH) is indicated on the panel using the fill symbols. Data are presented as box plots (see Section 3.2.7 for details).

Figure 3.3. Transcript abundance of *crf* in the pre-optic area of (A) control (N = 4 per temperature) and cortisol-treated (N = 4 per temperature) rainbow trout (*Oncorhynchus mykiss*) exposed to thermal ramping to 13°C (i.e. no change in temperature), 25°C or 27°C, or (B) sham (N = 4) and capsazepine-treated (N = 4) rainbow trout exposed to 27°C. For panel (A), a significant interaction of cortisol treatment and thermal ramping temperature on transcript abundance of *crf* was detected (two-way ANOVA, $P_{\text{treatment}}$ = 0.125, $P_{\text{temperature}}$ = 0.0004, $P_{\text{treatment x temperature}}$ = 0.0004). Boxes that share a letter are not significantly different from one another. For (B), fish subjected to capsazepine treatment had significantly lower *crf* abundance (Student's *t*-test, P = 0.033). An asterisk denotes a significant difference. All values were normalized to β-actin as a reference gene and expressed as fold-change relative to the control group at 13°C in panel (A), or the control group in panel (B). Data are presented as box plots (see Section 3.2.7 for details).

Figure 3.3.

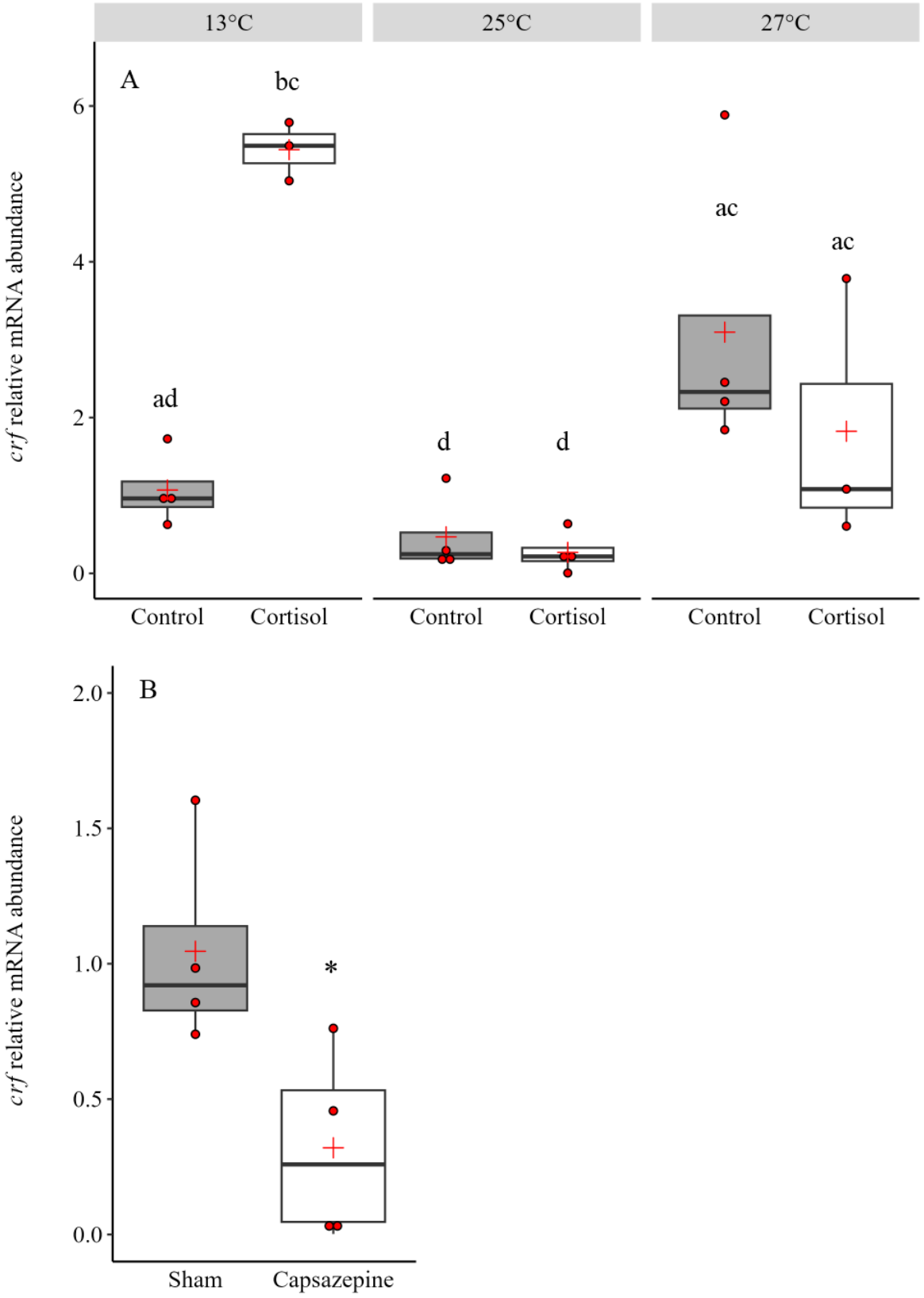

13°C
25°C
27°C
A
bc
ad
d
d
ac
ac
crf relative mRNA abundance
0
2
4
6
Control
Cortisol
Control
Cortisol
Control
Cortisol
B
*
crf relative mRNA abundance
0.0
0.5
1.0
1.5
2.0
Sham
Capsazepine

Figure 3.4. Transcript abundances of (A) *star* and (B) *p450scc* in the head kidney of control (N = 4 per temperature) and cortisol-treated (N = 4 per temperature) rainbow trout (*Oncorhynchus mykiss*) exposed to thermal ramping to 13° (i.e. no change in temperature) or 27°C. Cortisol treatment but not temperature had significant impacts on transcript abundances (two-way ANOVA; for panel A, $P_{\text{treatment}} = 0.02$, $P_{\text{temperature}} = 0.65$, $P_{\text{treatment x temperature}} = 0.75$; for panel B, $P_{\text{treatment}} = 0.04$; $P_{\text{temperature}} = 0.87$, $P_{\text{treatment x temperature}} = 0.83$), which are indicated on the figure using the fill symbols. Values were normalized to β-actin as a reference gene and expressed as fold-change relative to the control group at 13°C. Data are presented as box plots (see Section 3.2.7 for details).

Figure 3.4.

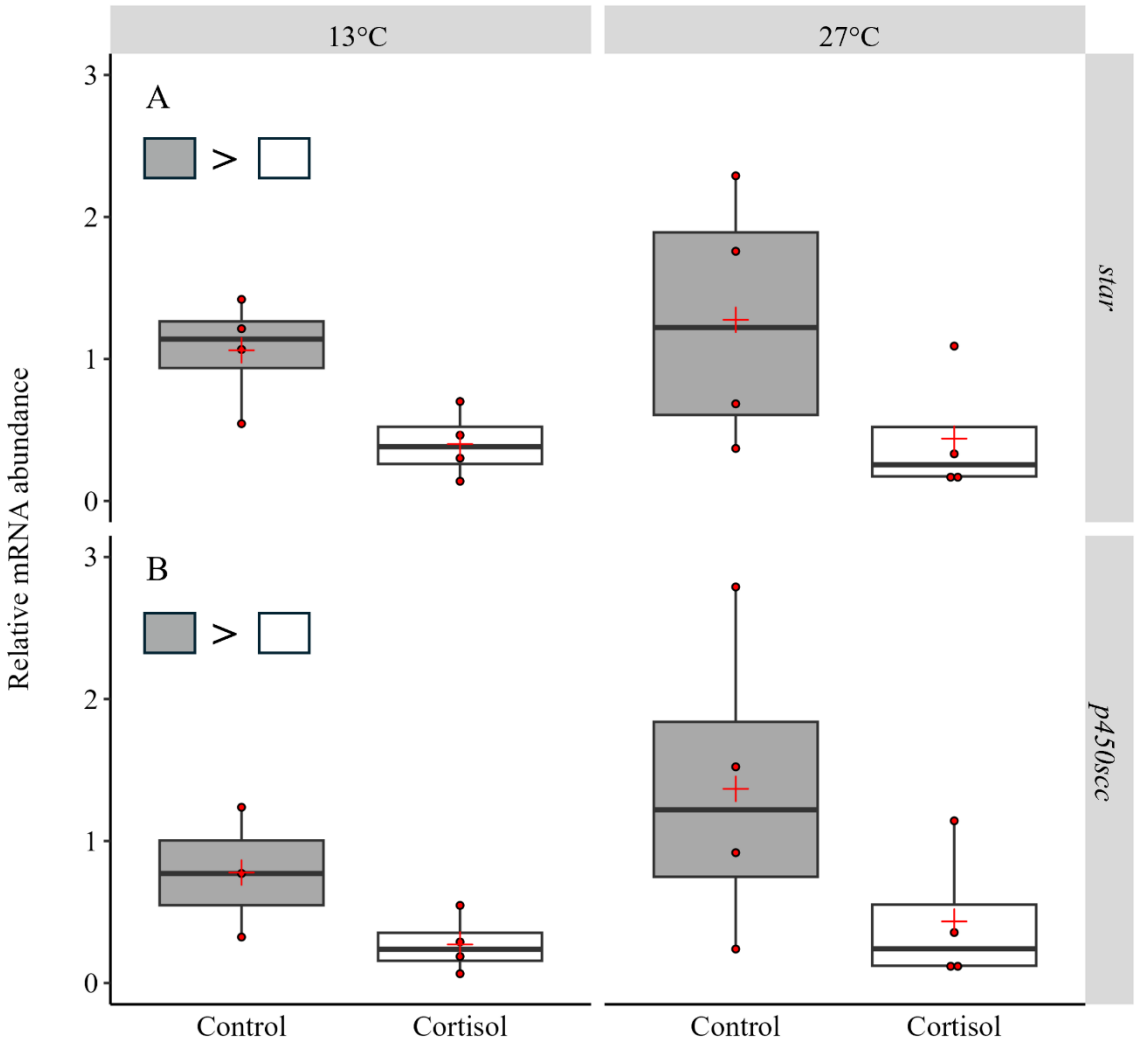

13°C
27°C
star
p450scc
A
B
>
>
Relative mRNA abundance
0
1
2
3
Control
Cortisol
Control
Cortisol

Figure 3.5. Transcript abundance of SF-1 paralogues (A) *ff1b1*, (B) *ff1b2* and (C) *ff1d* in the head kidney of control (N = 4 per temperature), and cortisol-treated (N = 4 per temperature) rainbow trout (*Oncorhynchus mykiss*) exposed to thermal ramping to 13° (i.e. no change in temperature) or 27°C. Cortisol treatment and temperature had significant impacts on expression of *ff1d* only (two-way ANOVA; for panel A, $P_{\text{treatment}}$ = 0.12, $P_{\text{temperature}}$ = 0.57, $P_{\text{treatment x temperature}}$ = 0.63; for panel B, $P_{\text{treatment}}$ = 0.11; $P_{\text{temperature}}$ = 0.054, $P_{\text{treatment x temperature}}$ = 0.66; for panel C, $P_{\text{treatment}}$ = 0.019; $P_{\text{temperature}}$ = 0.023, $P_{\text{treatment x temperature}}$ = 0.10). Temperatures that do not share a letter are significantly different from one another, and the effect of cortisol treatment is represented on the figure using the bar fills. Values were normalized to β-actin as a reference gene and expressed as fold-change relative to the control group at 13°C. Data are presented as box plots (see Section 3.2.7 for details).

Figure 3.5.

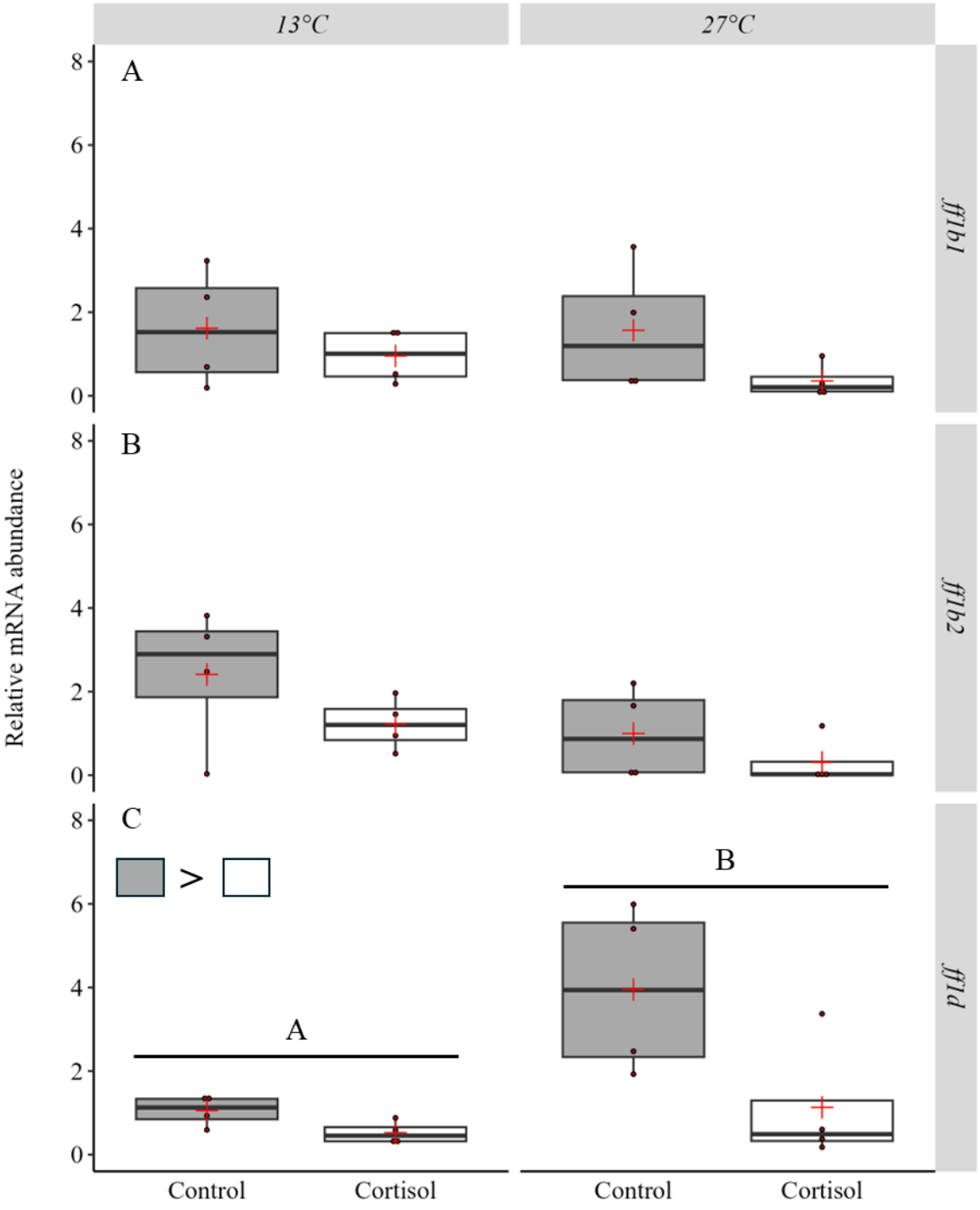
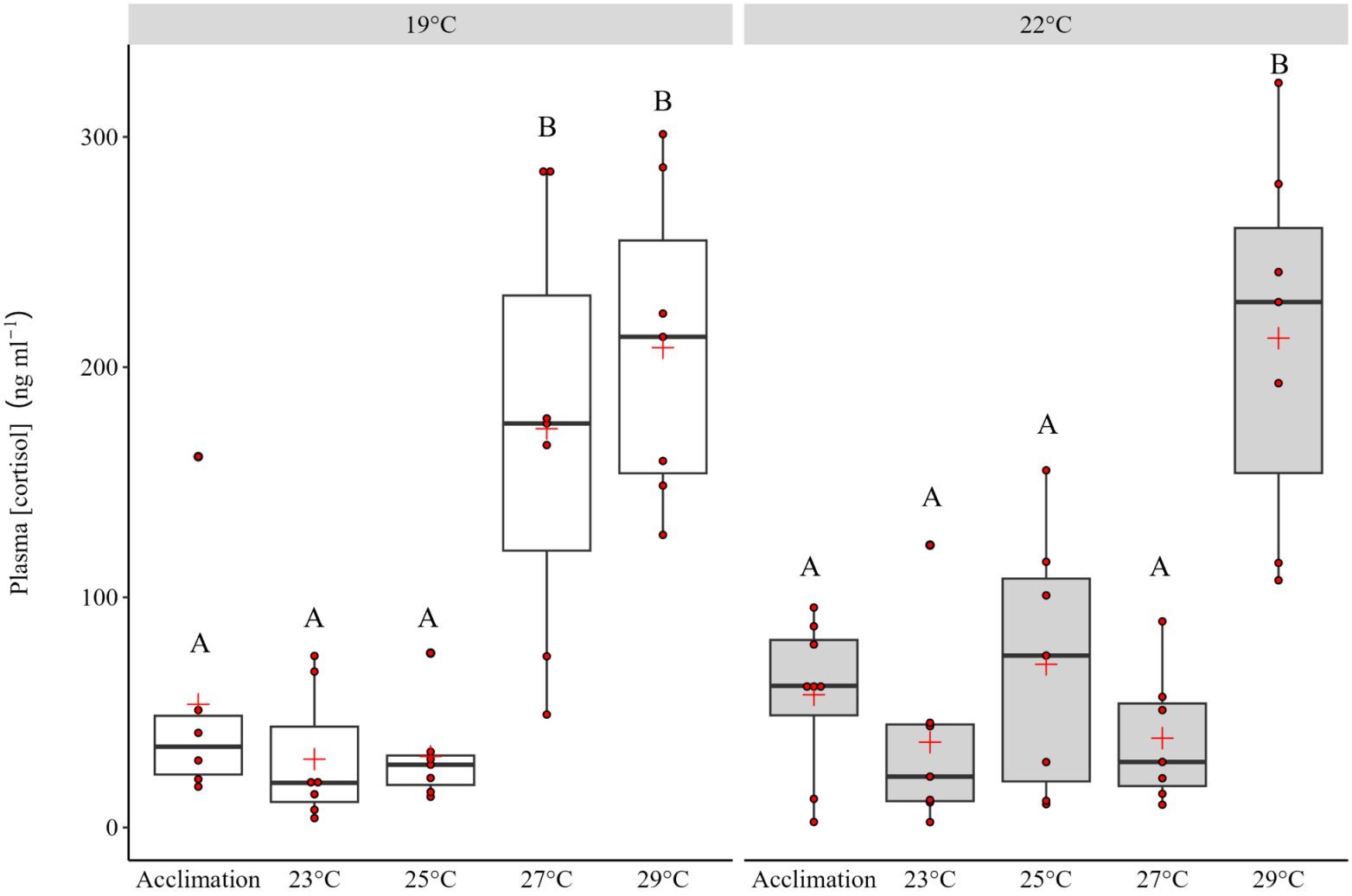

13°C
27°C
A
B
C
Relative mRNA abundance
0
2
4
6
8
>
A
B
Control
Cortisol
Control
Cortisol
fff1b1
fff1b2
fff1d

Figure 3.6. The cortisol response to acute warming in rainbow trout (*Oncorhynchus mykiss*) acclimated to 19°C (*N* = 6-7 per temperature) or 22°C (*N* = 6-7 per temperature) and then acutely warmed to temperatures from 23 to 29°C (two-way ANOVA, $P_{\text{acute temperature}} < 0.0001$, $P_{\text{acclimation temperature}} = 0.18$, $P_{\text{acute temperature x acclimation temperature}} = 0.0009$). Boxes that share a letter are not significantly different from one another. "Acclimation" refers to the acclimation temperature indicated in the grey band at the top of the figure. Data are presented as box plots (see Section 3.2.7 for details).

Figure 3.6.


19°C
22°C
Plasma [cortisol] (ng ml^{-1})
0
100
200
300
A
A
A
B
B
A
A
A
A
B
Acclimation
23°C
25°C
27°C
29°C
Acclimation
23°C
25°C
27°C
29°C

3.5 Discussion

The present study demonstrated that acute warming evokes a cortisol response that reflects the activation of the HPI axis, as the increases in circulating cortisol cannot be attributed to the biochemical effect of heat on cortisol production. Moreover, the threshold at which the cortisol response is induced was altered by cortisol treatment as well as thermal acclimation. These findings are consistent with the effects of cortisol treatment and thermal acclimation on CTmax (see Chapter 2). Interestingly, the observation of lowered *crf* transcript abundance in capsazepine-treated trout supports the involvement of TRPV1 in the heat-sensing mechanism that initiates the cortisol response.

Exposure to high temperature appears to increase circulating cortisol by activating the HPI axis. Measurement of cortisol following acute warming to a range of temperatures where fish were held for 10 min revealed a transition from no significant change in cortisol at temperatures at or below 25°C to a significant elevation of cortisol at temperatures at or above 27°C, a pattern suggestive of a threshold for HPI axis activation around 27°C, which is ~2°C below the CTmax for these fish, which was 28.9 ± 0.2°C (see Chapter 2). I am aware of only one other study that investigated cortisol during thermal ramping; it was conducted on goldfish, and researchers documented extensive variation in cortisol within each temperature point (Cockrem et al. 2019) and were not able to identify key temperatures for the activation of the cortisol response. Cortisol production measured *in vitro* in isolated head kidney tissue was not altered by increasing incubation temperature, providing support for the involvement of HPI axis activation in the cortisol response to temperature *in vivo*. In addition, transcript abundance of *crf* was significantly higher in control fish at 27°C than at 25°C. Interestingly, in Senegalese sole

(*Solea senegalensis*), no differences in *crf* expression were detected after 1 h of heat shock (Benítez-Dorta et al. 2017). It should be noted that CRF plays a key role in activating the HPI axis, because it initiates the cascade that stimulates cortisol production (Wendelarr Bonga 1997; Vijayan et al. 2010; Gorissen and Flik 2016). Due to the importance of CRF to HPI axis activation, *crf* transcript abundance was also measured in fish treated with the TRPV1 antagonist, capsazepine. Trout treated with capsazepine had lower *crf* transcript abundance during acute warming to 27°C than sham fish. This finding aligns well with the previously demonstrated blunting of the acute cortisol response at 27°C in capsazepine-treated trout (Chapter 2). These data provide further support for a role for TRPV1 in activating the HPI axis in response to acute warming.

Perhaps surprisingly, transcript abundances of *star* and *p450scc*, which facilitate the rate-limiting steps in cortisol biosynthesis in the interrenal cells, did not differ significantly between fish exposed to 13 and 27°C. However, transcript abundances in control fish exposed to 27°C were more variable than in fish exposed to 13°C and may warrant further examination. A role for SF-1 in regulating HPI axis activity in response to temperature is likely. A marked elevation of *ff1d* transcript abundance was apparent in fish at 27°C relative to 13°C. Interestingly, SF-1 is thought to regulate the expression of *star* and *p450scc* (Hu et al. 2001; Chai et al. 2003; Quek and Chan 2009), but unexpectedly no differences in these genes were detected at elevated temperatures. This apparent discrepancy may reflect a time-lag in translating increases in *ff1d* into corresponding increases in *star* and *p450scc*. How SF-1 is activated during stress remains unknown – it is considered to be an "orphan" nuclear receptor (Schimmer and White 2010; Meinsohn et al. 2019) – but trout subjected to chronic social stress for 4 d also

showed a marked elevation in *ff1d* (Best and Gilmour 2022), supporting regulation of *ff1d* expression by cortisol.

Cortisol-treated rainbow trout showed significant elevation of cortisol at a lower temperature (25°C) than their control counterparts (27°C). This finding suggests that cortisol-treated trout are more sensitive to high temperature and is consistent with the lowered thermal tolerance reported in trout subjected to chronic stress or cortisol treatment (LeBlanc et al. 2011; Bard et al. 2021; Chapter 2). Cortisol-treated trout exhibited elevated *crf* transcript abundance at 13°C, but not at 25°C or 27°C. Similar upregulation of *crf* in cortisol-treated trout was documented previously (Jeffrey et al. 2012; Madison et al. 2015). However, the absence of a cortisol effect on *crf* transcript abundance at higher temperatures was unexpected. In addition, *crf* transcript abundance was not elevated at 25°C, the temperature at which a cortisol response was detected, although it was elevated at 27°C. Cortisol treatment also downregulated *star* and *p450scc* regardless of temperature, which contrasted with the lack of effect of cortisol treatment on mRNA abundance of these genes reported by Jeffrey et al. (2012). Finally, cortisol-treated trout had lower *ff1d* transcript abundance than control fish regardless of temperature. Given that trout subjected to chronic social stress displayed marked elevation of *ff1d* transcript abundance (Best and Gilmour 2022), the absence of a cortisol effect was unexpected. Although these data provide a snapshot of HPI axis responses to high temperature in cortisol-treated trout, additional work is needed to understand the time course of the responses to both cortisol and high temperature.

The magnitude of the cortisol response was similar in control (~ 120 ng ml^{-1}) and cortisol-treated (~ 230 ng ml^{-1}) trout during acute warming (i.e. at the temperature where

the cortisol response was first detected to be significantly different, 27 and 25°C respectively), which is in agreement with the previously reported similarity of cortisol responses in subordinate and dominant trout exposed to elevated temperatures (Bard et al. 2021). By contrast, the cortisol response to a netting stressor was blunted in subordinate relative to dominant trout (Jeffrey et al. 2014; Culbert and Gilmour 2016). The reason for the different responses to these different stressors remains uncertain, but the finding of the present study that *crf* transcript abundance at 27°C was similar between control and cortisol-treated trout may contribute to maintenance of the cortisol response in cortisol-treated trout. However, cortisol-treated fish mounted a cortisol response at temperatures 2°C lower than control fish. This finding suggests that trout experiencing chronic stress will be more susceptible to thermal stress even at temperatures below their thermal tolerance (i.e. CTmax). In turn, this finding serves as a caution in relying on CTmax as an index of susceptibility to warming (Strowbridge et al. 2021; Desforges et al. 2023), because underlying negative physiological changes may occur even at lower temperatures.

Acclimation to higher temperatures appears to shift the threshold for a cortisol response. In the present study, acclimation of trout raised the temperature at which a cortisol response was detected from 27°C in trout acclimated to 19°C to 29°C in trout acclimated to 22°C. However, the shift was limited to higher temperatures, with no difference in the threshold temperature for a cortisol response between trout acclimated to 13° or 19°C. The 2°C intervals between temperatures limited the resolution at which changes could be detected. Nevertheless, these findings suggest that activation of the HPI axis is plastic and responds not only to changes in the physiological condition of the fish

(e.g. with cortisol treatment; see above), but also to acclimation to different environmental conditions. Although the threshold for activating a cortisol response shifted by 2°C, thermal acclimation was less effective in increasing CTmax, with increases limited to ~0.5-1°C (see Chapter 2). This difference may reflect the fact that CTmax is the limit of the fish's thermal tolerance, whereas the temperature at which the cortisol response is activated is lower and therefore has a larger margin for plasticity. Although the present study appears to be the first to have examined the effects of thermal acclimation on the threshold temperature for a cortisol response, other aspects of the cortisol response have been examined in relation to thermal acclimation. For example, acclimation temperature altered both the magnitude and duration of the cortisol response to a confinement stressor in Atlantic salmon (*Salmo salar*), with higher acclimation temperatures associated with a faster time to reach peak cortisol and more rapid return to baseline (Madaro et al. 2018). A similar effect was reported in European sea bass (*Dicentrarchus labrax*), where increases in cortisol after chasing occurred more quickly and the magnitude of the cortisol response was larger with increasing acclimation temperature (Samaras et al. 2018a). On the other hand, acclimation of Atlantic salmon to warm temperatures did not alter their cortisol response to a confinement stressor (Tang et al., 2022a). In the present study, the magnitude of the cortisol response was unchanged with acclimation temperature, suggesting that acclimation effects may be stressor- and/or species-specific. The impacts of acute warming and how they are modulated by acclimation to higher temperature warrant additional study.

In summary, the present study provides insight into the mechanisms responsible for an acute cortisol response to heat. The cortisol response to acute warming reflects

activation of the HPI axis, perhaps mediated by the effect of noxious heat in stimulating TRPV1. Moreover, HPI axis responsiveness to acute warming was altered by the physiological condition of the fish as well as environmental conditions. That is, cortisol treatment increased the sensitivity of trout to acute warming, even at temperatures below CTmax, and thermal acclimation can increase tolerance of high temperature, shifting the acute cortisol response to higher temperatures. Importantly, the evidence suggests that trout experiencing chronic stress will be more vulnerable to warming caused by climate change, owing to heightened sensitivity to high temperatures.

3.6 Supplementary materials

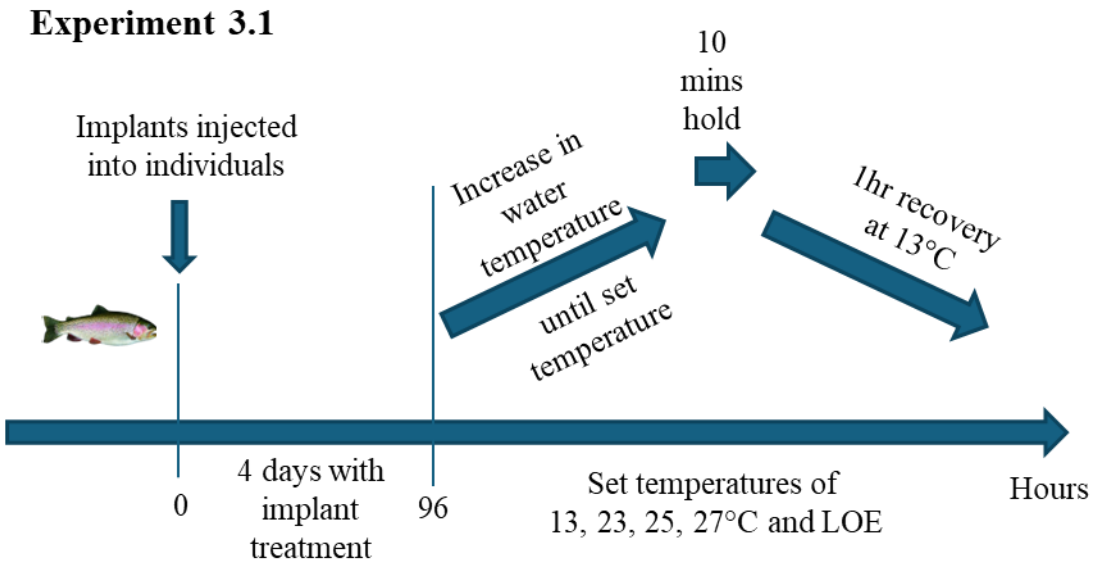
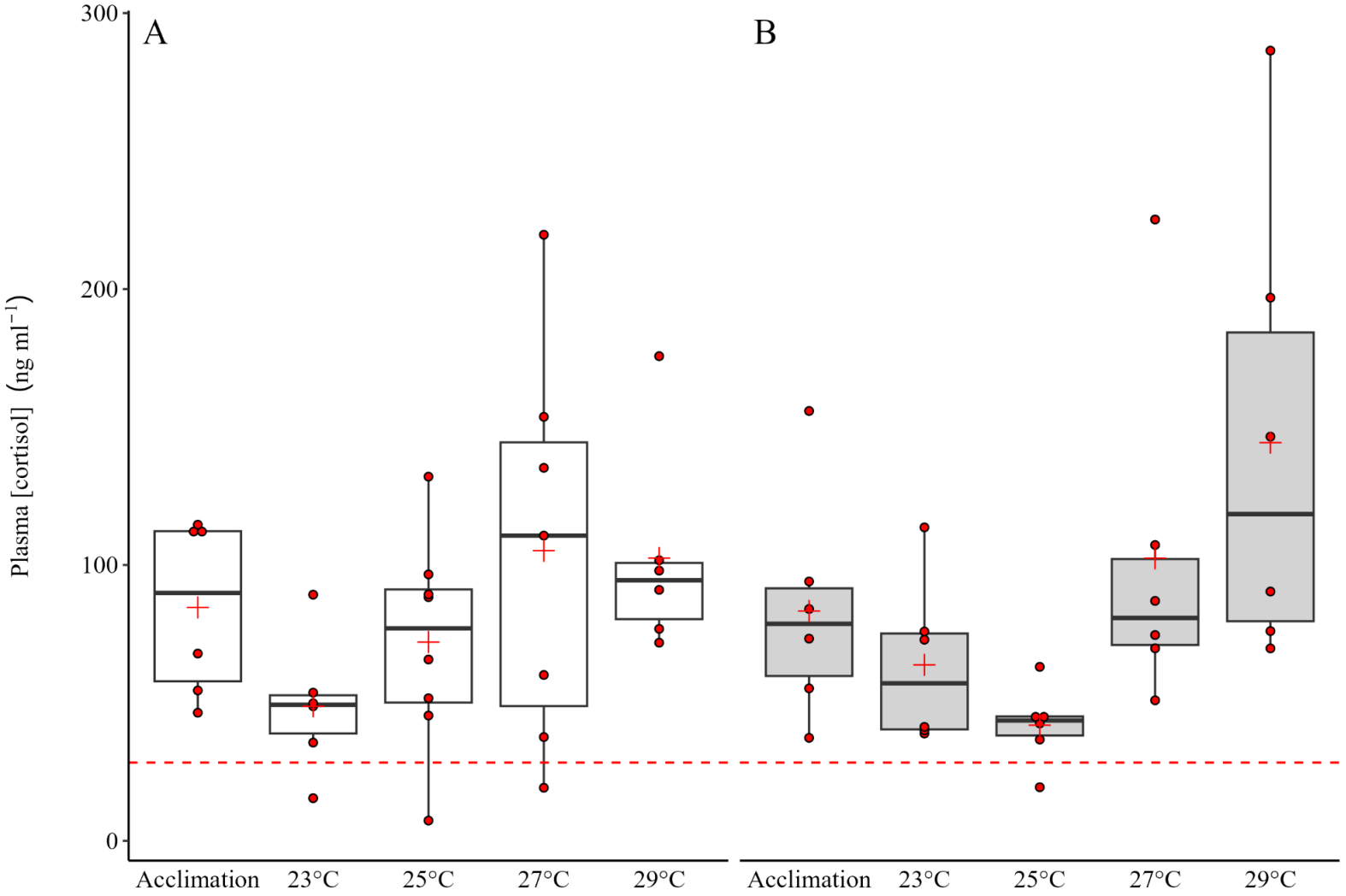


Figure S3.1. Schematic of the experimental timeline for Experiment 3.1

Figure S3.2. Plasma cortisol in rainbow trout (*Oncorhynchus mykiss*) acclimated to 16°C for three weeks and then exposed to acute thermal ramping with (A) and (B) presenting data from two different trials. No significant effect of thermal ramping was detected in the first trial (panel A, one-way ANOVA, $P = 0.172$). Although a significant effect of thermal ramping was detected in the second trial (panel B, Kruskal -Wallis one-way ANOVA, $P = 0.013$), pairwise Student's *t*-tests did not identify the source of this effect. Control fish (no change in temperature during "thermal ramping") in both trials exhibited elevated plasma cortisol relative to unstressed values for fish held at 13°C (dashed red line), suggesting that these fish experienced chronic stress, likely owing to equipment failure that resulted in both groups of fish experiencing elevated temperatures for several days. Data are presented as box plots, see Section 3.2.7 for details.

Figure S3.2.


A
B
300
200
100
0
Plasma [cortisol] (ng ml^{-1})
Acclimation
23°C
25°C
27°C
29°C
Acclimation
23°C
25°C
27°C
29°C

Chapter 4

Effects of chronic cortisol treatment on the summer habitat choice of rainbow trout (*Oncorhynchus mykiss*) in a natural lake

4.1 Introduction

Ectothermic animals, such as fishes, rely on behavioural thermoregulation to adjust body temperature (Reynolds 1977; Reynolds and Casterlin 1979; Crawshaw and Podrabsky 2011). Behavioural thermoregulation refers to the dynamic movements made by an individual to control body temperature (Crawshaw and Podrabsky 2011; Raby et al. 2018; Amat-Trigo et al. 2023). If a range of temperatures is accessible, then behavioural thermoregulation allows fishes to choose a temperature that may be optimal for a specific physiological function (Amat-Trigo et al. 2023). Similarly, if temperatures near or exceed the thermal tolerance of an individual fish, it will use behavioural thermoregulation to seek a thermal refuge, so long as such refuges exist in the habitat occupied by the fish (Magnuson et al. 1979). This example highlights the limits faced by fish in thermoregulating – they are restricted to finding water that reflects their preferred temperature, which may not always be available.

Fishes use a variety of strategies for behavioural thermoregulation, the most common being changes in depth, i.e. vertical migration (Holland et al. 1992; Stehfest et al. 2017; Keefer et al. 2018a; Gallagher et al. 2019; Freitas et al. 2021; Amat-Trigo et al. 2023; Thomas et al. 2023; Winkler et al. 2023), and horizontal migration (Armstrong et al. 2013; Goyer et al. 2014; Raby et al. 2018; Amat-Trigo et al. 2023). However, in some instances fish have been shown to bury themselves in the substrate (Ziegler and Frisk

2019) or to emerge from the water (Sayer and Davenport 1991; Gibson et al. 2015; Nay et al. 2018). Rainbow trout (*Oncorhynchus mykiss*) typically use vertical changes in depth, with cooler water being found deeper in the water column (Amat-Trigo et al. 2023). This approach provides them with temperature refuges during the hot summer months, where differences in water temperatures of up to 15-20°C can be measured from the top to the bottom of the water column (Thomas et al. 2023). As such, during summer months, trout were more likely to be found within the thermocline or slightly below. Vertical migrations also occur daily (Thomas et al. 2023). For example, lake trout were found closer to the surface at night and in deeper waters during the day (Thomas et al. 2023). However, these migrations are not thought to be caused by thermoregulation alone, but rather coincide with feeding (Mazur and Beauchamp 2006; Hansen and Beauchamp 2014).

Thermal preference can be described as the body temperature an individual actively choses when there are no thermal ecological constraints (Crawshaw and Podrabsky 2011). Surprisingly, fish populations that differ in environmental temperatures do not necessarily show differences in thermal preference (Siikavuopio et al. 2014; Pilakouta et al. 2023). However, it appears that thermal preference can be altered in a matter of hours, with many species demonstrating daily rhythms, preferring warmer temperatures during the day and cooler temperatures at night (Beitinger 1975; Reynolds and Casterlin 1978; Casterlin and Reynolds 1982; Vera et al. 2023; de Alba et al. 2024). Interestingly, acclimation to higher temperature appears to increase thermal preference only in some cases (Ihnat and Bulkley 1984; Hernández et al. 2002; Fangue et al. 2009; Schram et al. 2013; Barker et al. 2018). Moreover, there appears to be a threshold at

which acclimation temperature becomes maladaptive to thermal preference. As such, acclimation temperatures of 20 and 23°C caused a 30% increase in thermal preference in common molly (*Poecilia sphenops*), whereas acclimation temperatures of 32 and 35°C causes 20 and 30% reductions, respectively, in thermal preference (Hernández et al. 2002). There may also be a relationship between animal personality and thermal preference (Rey et al. 2015a; Cerqueira et al. 2016), where bolder individuals prefer warmer temperatures. Nonetheless, how thermal preference is regulated and altered remains poorly understood. A further complication is that thermal preference may not always be the key factor regulating habitat choice. For example, five bearded rockling (*Ciliata mustela*) strayed from their preferred temperature to have access to shelter (Dye et al. 2024), and individuals drifted from their environmental preferences to remain with conspecifics (Cooper et al. 2018; Currie and Tattersall 2018). Collectively, these findings suggest that thermal preference is plastic, but that it can also be constrained by other ecological factors.

Few studies have asked how chronic stress alters these behaviours. Chronic stress and chronic cortisol treatment lowered thermal tolerance (Bard et al., 2021; Chapter 2), and therefore might also alter thermal preference. Moreover, cortisol-treated puffer fish (*Sphoeroides testudineus*) chose to occupy cooler temperatures (Cull et al. 2015), supporting the possibility that thermal preference shifts in response to chronic stress. Indeed, cortisol may serve as an endocrine regulator of behavioural thermoregulation during chronic stress. The apparent capacity of cortisol to alter TRPV1 expression (Chapter 2) supports this possibility, although the mechanistic pathway remains unknown.

Field-based studies are needed to confirm how chronic stress may be altering thermoregulatory patterns of wild fish. Research to date has largely been focused on the tagging of wild-caught fish and monitoring their thermal habitat use (Mazur and Beauchamp 2006; Armstrong et al. 2016; Watson et al. 2019; Littlefair et al. 2020; Wilbur et al. 2020; Amat-Trigo et al. 2023; Morgan and O'Sullivan 2023) without experimental manipulation. However, migration is not always a thermoregulatory option for salmonids, particularly for those stocked in small lakes. Little is also known about how chronic stress may alter thermal habitat choice of salmonids in a natural environment. I hypothesized that chronic stress would alter behavioural thermoregulation in rainbow trout. Specifically, I predicted that individuals experiencing chronic elevation of cortisol would occupy thermal habitats of lower temperature, both daily and hourly, owing to the effect of cortisol in lowering thermal tolerance.

4.2 Material and Methods

4.2.1 Experimental animals

Juvenile rainbow trout, *Oncorhynchus mykiss* (Walbaum 1792) (total length = 27.1 ± 0.3 cm, mean ± s.e.m., N = 30), were provided by and held on-site at the Kenauk Hatchery (Montebello, QC, Canada) until used for experimentation. Fish were fed daily by scattering commercial feed pellets over the water surface. Tank water temperature is drawn from a nearby lake and therefore varies with season, with summer water temperatures typically ~14°C. Fish were held in covered outdoor enclosures, which followed the natural environmental photoperiod. All experimental protocols were approved by the Québec Ministère des Forêts, de la Faune et des Parcs under provincial

scientific permits (PM_23-07-SP-001-GR-0) and were in compliance with the guidelines of the Canadian Council on Animal Care (CCAC) for the use of animals in research and teaching.

Fish were fitted with a telemetry tag (4.2 x 0.9 cm) and a mini-osmotic pump (1.5 x 0.6 cm). Fish were lightly anesthetized by immersion in a water containing 60 ppm clove oil (18601771, Fisher Scientific, Ottawa, ON) until loss of equilibrium. After measuring fork length, fish were transferred to a surgical table that allowed constant irrigation of the gills with water containing 30 ppm clove oil. A 2.5 cm incision was made midline on the ventral surface posterior to the pectoral girdle. A telemetry tag was inserted into the peritoneal cavity (Lotek, model MMR8SOTP, temperature and pressure, or MMR8SOT, temperature alone, Newmarket, ON, CA) together with a mini-osmotic pump (Alzet, model 1007D, Durect Corporation, Cupertino, CA, USA), and the incision was closed using two single interrupted sutures with 3-0 Vicryl sutures (VCP423, Ethicon, NJ, USA). Fish were transferred to individual 40 L tanks containing fresh hatchery water to recover. After regaining equilibrium, fish were placed in 40 L coolers (~ 1 h) with fresh hatchery water containing up to 4 individuals for transport to Otter Lake.

Rainbow trout (N = 30) were allocated haphazardly to control (N = 15) and cortisol-treated (N = 15) groups. Cortisol-treated fish were fitted with mini-osmotic pumps containing a solution of 100 µL cortisol (hydrocortisone 21-hemisuccinate, H2270, Sigma- Aldrich, Oakville, ON) dissolved in 65% 2-hydroxypropyl-β-cyclodextrin (H107, Sigma-Aldrich) to achieve a nominal concentration *in vivo* of 80 µg g^{-1} body weight. Sham fish were implanted with mini-osmotic pumps containing 100 µL of the

cyclodextrin solution alone, following the approaches of Madison et al. (2015). The cortisol concentration was chosen based on pilot trials that assessed dose and the duration of elevated cortisol (Fig S4.1). Within each group, six fish were fitted with telemetry tags that measured depth and temperature, and nine fish were fitted with telemetry tags that measured temperature alone. The tags provided readings every 5 min.

Fish were transported to Otter Lake from the hatchery (~10-15 mins) in groups of four in 40 L coolers filled with fresh hatchery water at ~18°C. Once at Otter Lake, coolers were transported by boat to the middle of the lake (~5 min) and the fish were released; this release location was chosen to provide the released fish with access to deeper, cooler water.

4.2.2 Field telemetry

The experiment was conducted in Otter Lake (~7.5 hectares, max depth 15.2 m, average depth 7.6 m) of the Kenauk Nature Reserve, Montebello, QC. Otter Lake was chosen because it is bowl-shaped, providing ample depths and a range of temperature choices for the fish. Additionally, Otter Lake is privately owned within the Kenauk Nature Reserve and therefore is closed to public fishing, so there was no risk of fish being caught and removed from the system. The lake is stocked annually with rainbow trout but also contains largemouth bass (*Micropterus nigricans*) and a variety of small-bodied forage fishes. Receivers (Lotek, model WHS 6000) were deployed at a depth of ~9 m across the middle (from east to west) of the lake (east receiver, N 45°48.216', W 074°47.695'; middle receiver, N 45°48.260', W 074°47.683'; west receiver, N 45°48.302', W 074°47.635'). Thermo-loggers (AquaMeasure DO, Innovasea, MA, USA) were deployed at 3 m intervals along the line of the middle receiver. The loggers

provided temperature readings every 5 min. Data collection began on July 10th, 2023, and data were downloaded from the receivers on September 14th, 2023.

4.2.3 Data analysis

4.2.3.1 Raw detection filtering

Telemetry data were filtered and analyzed using R-studio. A series of filters was used to identify and remove false positives (based on Bergman et al. 2022). First, data for tag IDs that did not exist were removed, as were data for valid tag IDs detected before a fish was released into the lake. Detection of non-valid tags occurs when the receiver picks up a "natural" frequency in the environment that happens to correspond to a tag within the Lotek system. Next, an "interval method" filter was applied, in which data collected within the 5 min programmed tag transmission interval following a data read were removed. Lastly, data detected with a power of less than five were removed, where power quantifies the strength of the signal for each detection. This criterion was based on the tag manufacturer's recommendation as well as consideration of outliers from a histogram of power data generated for the present study. Using these criteria, ~19% of detected data were considered to be false positives and were removed from further consideration.

Following visualization of the initial data set, additional criteria were applied to eliminate fish that were likely dead even though their telemetry tag was still being detected. Fish were considered dead if depth or temperature remained stable (±1 psi or ± 1°C) for 24 h. Data for each fish were then removed starting from the beginning of the 24 h period of stability. Additionally, if temperatures remained below 10°C or above 27°C for 24h, fish were removed from the analysis from that point onwards. These

temperatures were chosen based on expected temperatures at the surface and at depth for Otter Lake from recorded air temperatures and the thermo-loggers deployed in the lake, as well as observations from other field studies that monitored rainbow trout thermal habitat choice (Baird and Krueger 2003; Huff et al. 2005). Following these criteria, ~94% of detected data were considered to be from dead fish and were removed from further consideration. Because of fish loss caused by death or lack of detection, the number of fish included in the data set varied per day. In addition, the data filtering parameters likely removed some valid data as well as false positives. For the analyses over multiple days, the data for a 24 h period (i.e. an entire day) were averaged into a single value for each fish, whereas for analyses of a 24 h period, the data for each 1 h period were averaged into a single value for each fish. Depth data were converted from psi to meters for analysis (1 psi = ~ 0.703 m).

4.2.4 Statistical analysis

Statistical analyses were carried out using R-Studio. Survival was analyzed with Cox proportional regression. Daily temperature and depth use were analyzed with general linear mixed models with day and temperature or depth as factors and individual as a random factor. Hourly temperature and depth use were analyzed with general linear mixed models with hour and temperature or depth as factors and individual as a random factor. General linear mixed models were chosen because the data were not normally distributed and to take into account the unbalanced design (not every individual was detected every day). Multiple pairwise comparisons were made with Least squares means between control and cortisol-treated fish. In figures, data are presented as box plots where the upper and lower limits of the box indicate the 75th and 25th percentiles, respectively,

the black line across the box indicates the median, the whiskers indicate the maximum and minimum values, and the red plus sign indicates the mean value. Symbols represent individual data points.

4.3 Results

After filtering, the data set consisted of 29,258 true detections on 29 of 30 tagged rainbow trout (14 control and 15 cortisol-treated) from July 10th to July 31st, 2023. The data set included 23,232 temperature recordings and 6,026 depth recordings. It should be noted that there are fewer depth recordings since only six trout in each group were fitted with depth tags. For statistical analysis of the data, only recordings to July 19th, 2023, were considered owing to limited post-release survival in each group (i.e. two or lower) after this time. Cortisol treatment did not significantly affect survival (Fig. 4.1).

Control and cortisol-treated fish were found at similar temperatures for the first 5 days post release, largely choosing temperatures of 14-17°C (Fig. 4.2). From day 6 onwards, cortisol-treated fish were found at significantly warmer temperatures (~20-25°C) (Fig. 4.2). However, it should be noted that the number of fish dropped substantially on day 6 and beyond (Fig. 4.1). Control and cortisol-treated fish remained at similar depths for the first 3 days and were found ~3-4 m below the surface. On day 4, cortisol-treated fish were found to be significantly closer to the surface (~1-2 m) (Fig. 4.3). Again, however, the number of fish decreased markedly over time (Fig. 4.1). Individual traces of temperature and depth for each fish can be found in supplementary materials (Figs. S4.2-S4.5).

Because previous work revealed influences of photoperiod on thermal preference, I evaluated thermal habitat choice of individuals hourly and asked whether chronic elevation of cortisol altered these patterns. Two days were selected for analysis; July 12th, because at this time cortisol had only been elevated for two days, which was likely not sufficient time to exert effects, and July 14th, where effects of chronic cortisol elevation were expected to have occurred. On July 12th, 2023, cortisol-treated fish occupied significantly warmer temperatures than control fish during the early hours of the morning (0:00 – 02:00) and again late at night (23:00) (Fig. 4.4). A different pattern was apparent on July 14th, 2023, where cortisol-treated fish occupied warmer temperatures than control fish for much of the day (07:00 – 22:00) (Fig. 4.5).

4.4 Figures

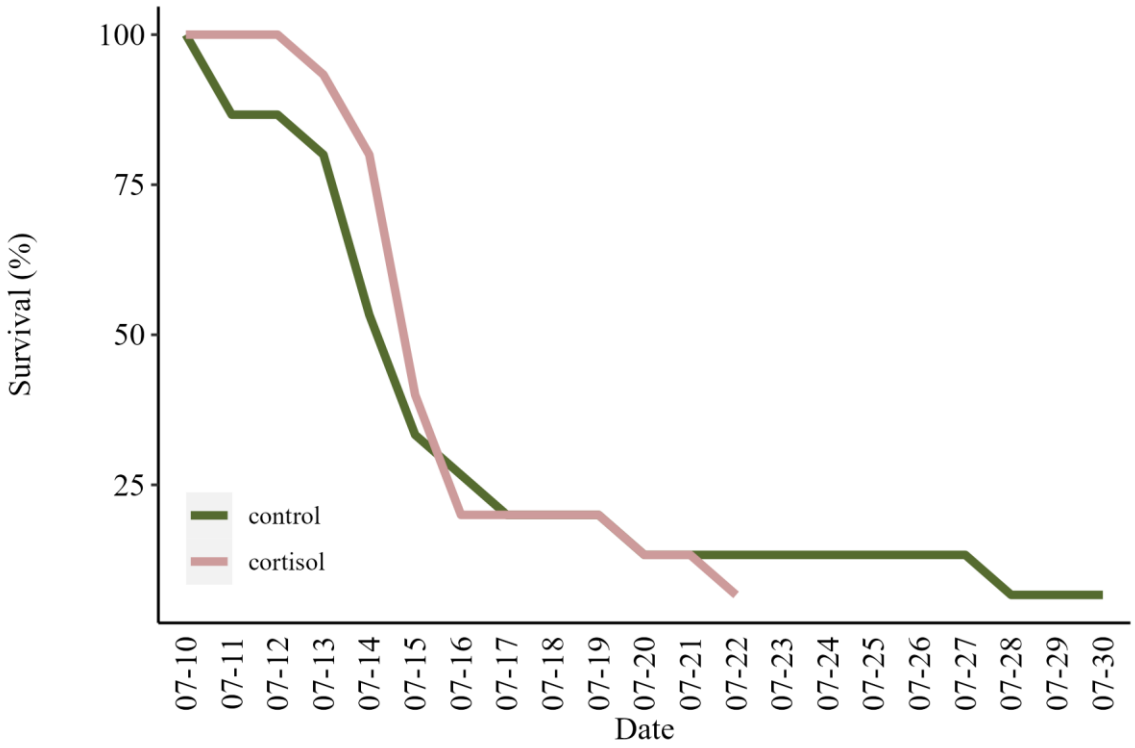


Figure 4.1. Survival of control (green, N = 15) and cortisol-treated (pink, N = 14) rainbow trout (*Oncorhynchus mykiss*) implanted with telemetry tags to record depth and/or temperature released in Otter Lake, located within the Kenauk Nature Reserve, Montebello, QC. Survival did not differ between control and cortisol-treated fish (Cox proportional regression, $P_{treatment}$ = 0.837). Recording began immediately after releasing the fish into the lake on July 10th, 2023.

Figure 4.2. Mean daily temperature of control (N = 3-13) and cortisol-treated (N = 3-15) rainbow trout (*Oncorhynchus mykiss*) released into Otter Lake on July 10th, 2023. Asterisks identify significant differences between control and cortisol-treated fish (generalized linear mixed model, $P_{treatment} < 0.0001$, $P_{day} = 0.005$, $P_{treatment\ x\ day} < 0.0001$ followed by least square mean multiple pairwise comparisons). Recording began immediately after releasing the fish into the lake on July 10th, 2023. It should be noted that the number of individuals decreases markedly starting July 15th (see Fig. S4.3; Fig. S4.4 for individual traces). Data analysis ends on July 19th due to only two fish surviving past this date. Data are presented as box plots (see Section 4.2.4 for details).

Figure 4.2.

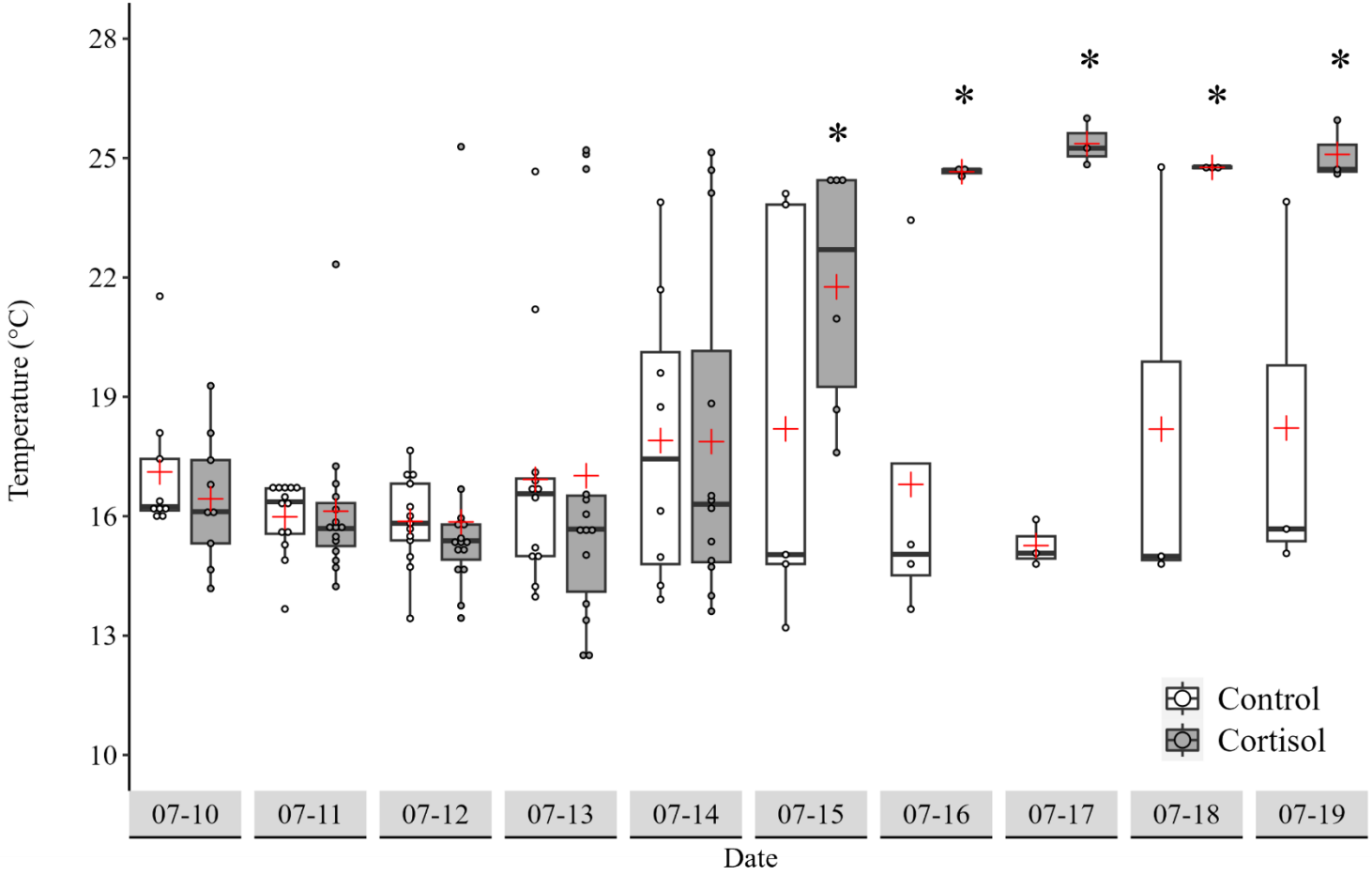

Temperature (°C)
28
25
22
19
16
13
10
07-10
07-11
07-12
07-13
07-14
07-15
07-16
07-17
07-18
07-19
Date
Control
Cortisol
*
*
*
*
*
*

Figure 4.3. Mean daily depth of control (N = 3-6) and cortisol-treated (N = 3-6) rainbow trout (*Oncorhynchus mykiss*) released into Otter Lake on July 10th, 2023. Asterisks identify significant differences between control and cortisol-treated fish (generalized linear mixed model, $P_{treatment}$ = 0.020, P_{day} = 0.049, $P_{treatment\ x\ day}$ = 0.0001 followed by least square mean multiple pairwise comparisons), with negative values being relative to the surface of the water. Recording began immediately after releasing the fish into the lake on July 10th, 2023. It should be noted that the number of control fish decreases markedly on July 13th, and only one control fish survived past this date, ending the analysis (see Fig. S4.5; Fig. S4.6 for individual traces). Data are presented as box plots (see Section 4.2.4 for details).

Figure 4.3.

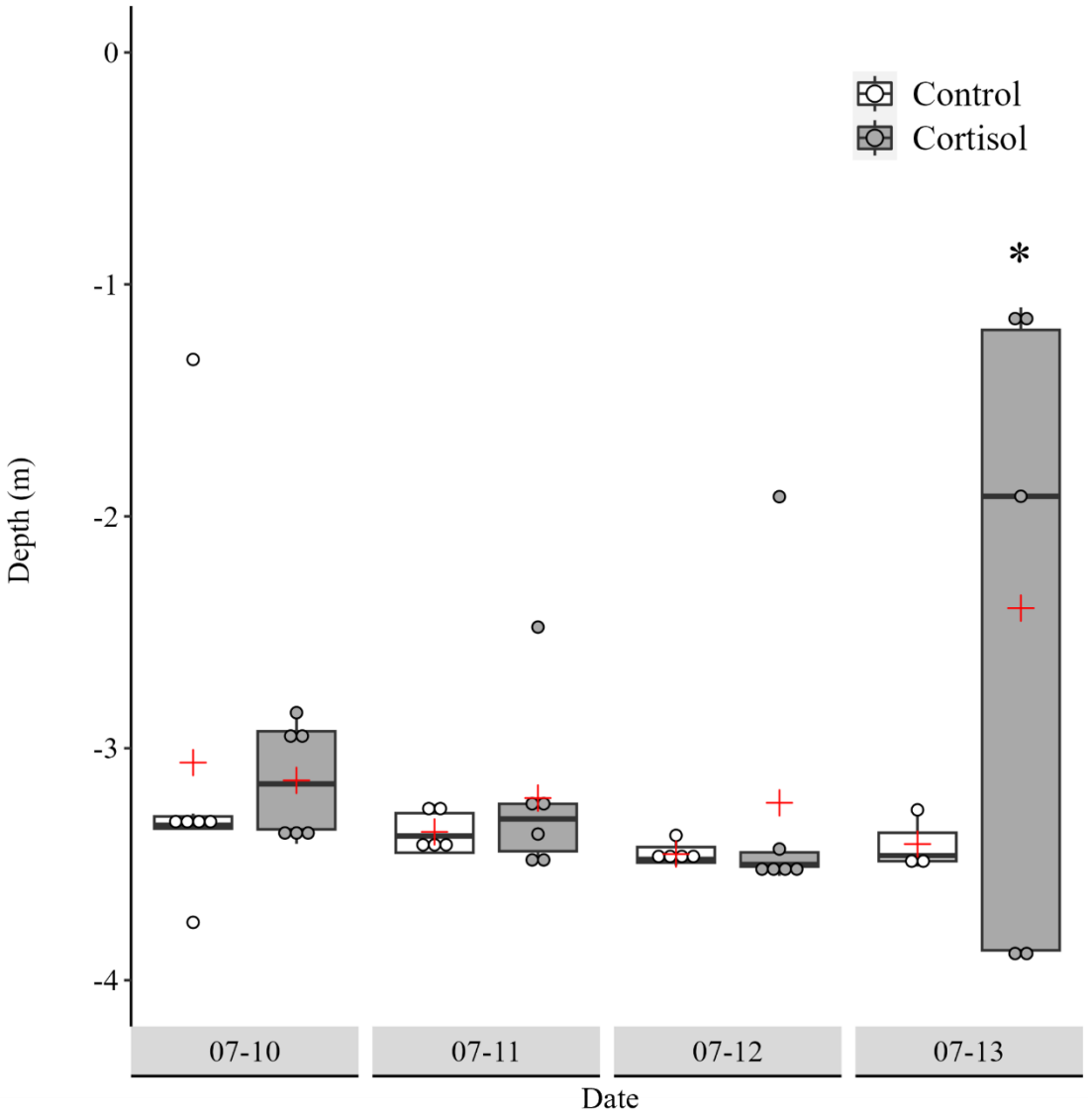


Figure 4.4. Mean hourly temperature in control (N = 9-13) and cortisol-treated (N = 12-15) rainbow trout (*Oncorhynchus mykiss*) on July 12th, 2023, with 0 being midnight on July 11th. Asterisks identify significant differences between control and cortisol-treated fish (generalized linear mixed model, $P_{treatment}$ = 0.006, P_{hour} = 0.93, $P_{treatment\ x\ day}$ = 0.014, followed by least square mean multiple pairwise comparisons). Data are presented as box plots (see Section 4.2.4 for details).

Figure 4.4.

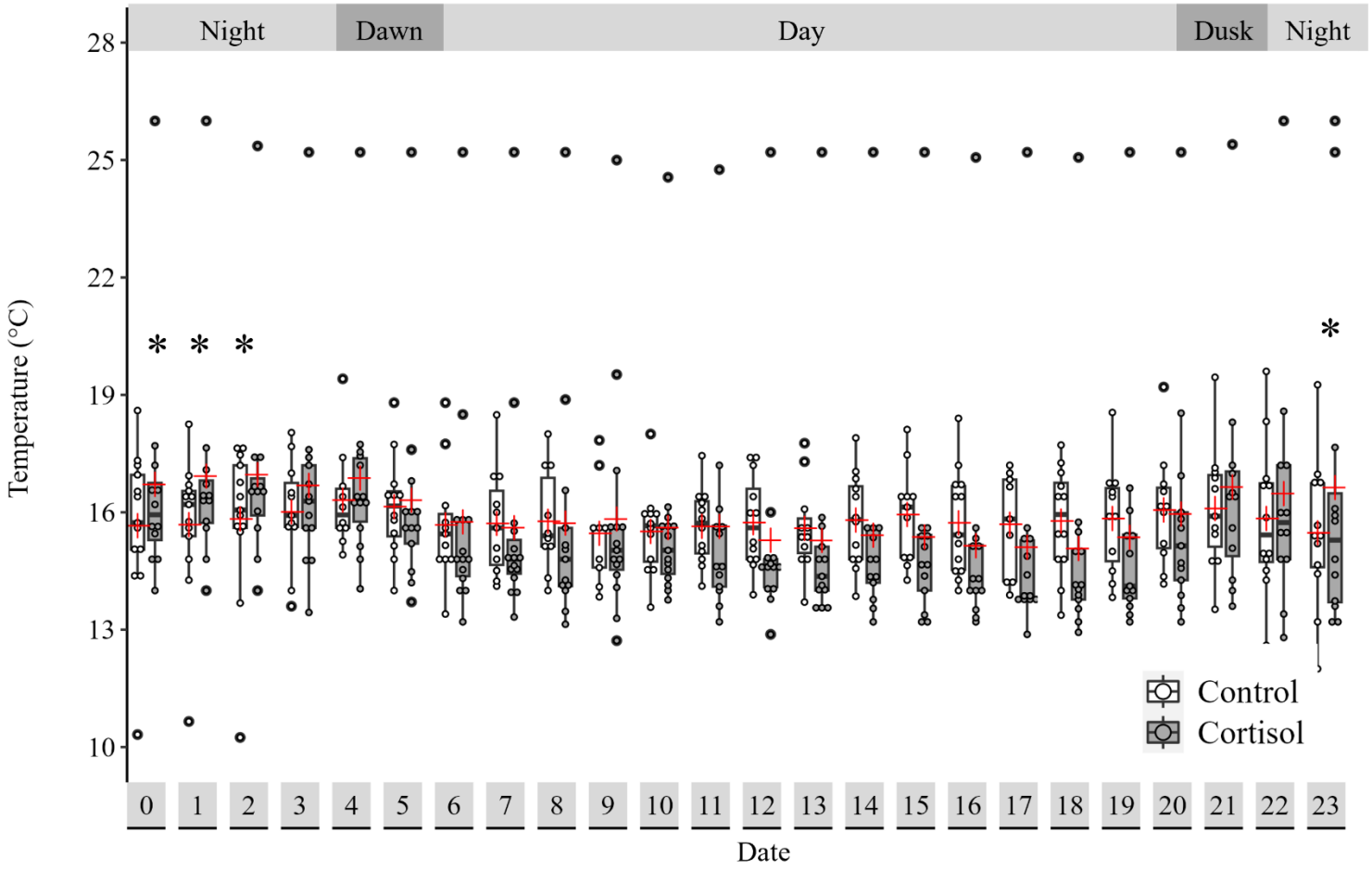
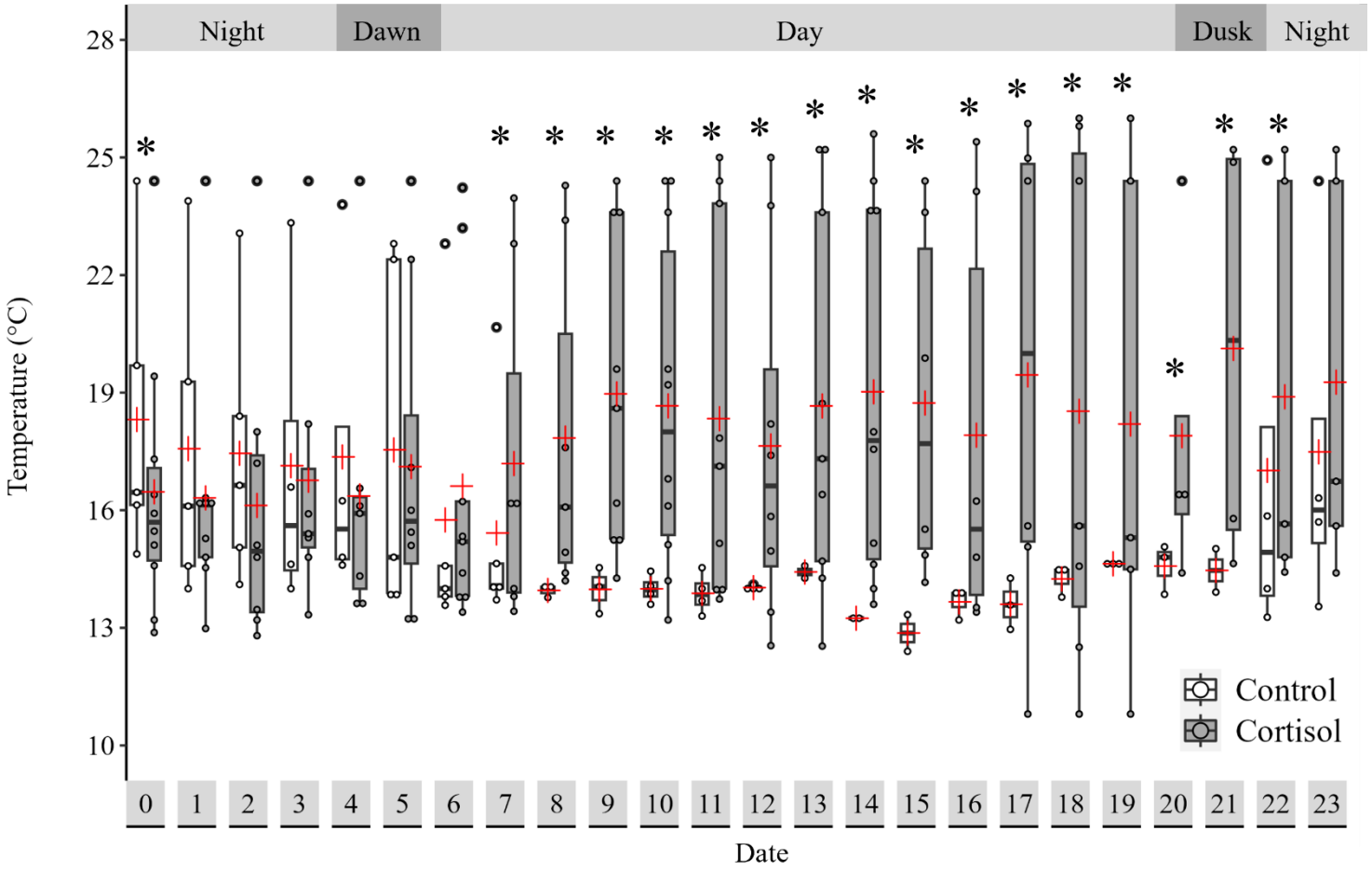

Night
Dawn
Day
Dusk
Night
28
25
22
19
16
13
10
Temperature (°C)
Control
Cortisol
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
Date

Figure 4.5. Mean hourly temperature of control (N = 2-5) and cortisol-treated (N = 4-10) rainbow trout (*Oncorhynchus mykiss*) on July 14th, 2023, with 0 being midnight on July 13th. Asterisks identify significant differences between control and cortisol-treated fish (generalized linear mixed model, $P_{treatment} < 0.0001$, $P_{hour} < 0.0001$, $P_{treatment\ x\ day} < 0.0001$, followed by least square mean multiple pairwise comparisons). Data are presented as box plots (see Section 4.2.4 for details).

Figure 4.5.


Night
Dawn
Day
Dusk
Night
Temperature (°C)
28
25
22
19
16
13
10
Control
Cortisol
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
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21
22
23
Date

4.5 Discussion

Unfortunately, fish did not survive prolonged periods following mid-summer stocking into the lake, and this factor constrains interpretation of the data. However, survival did not differ between control and cortisol-treated fish, which suggests that mortality reflected environmental conditions rather than cortisol treatment. Overall, it appears that cortisol treatment had little effect on temperature use by rainbow trout. Nevertheless, where effects were observed, cortisol-treated fish seemed to select higher temperatures than control fish.

One limitation of this study is that I wanted to explore the thermal habitat chosen by individual fish. However, I measured body temperature and therefore had to assume that body temperature reflected the preferred temperature of the fish within the range of environmental temperatures available. There is likely some thermal lag between body temperature and water temperature (Lutterschmidt and Hutchison 1997; Baird and Krueger 2003), particularly if the fish only briefly occupies water of a particular temperature. Nevertheless, this approach is commonly used (Snucins and Gunn 1995; Baird and Krueger 2003; Stehfest et al. 2017; Raby et al. 2018), and likely the best available at present. Other approaches include the use of cameras and thermo-loggers along the experimental site, or remote sensing with drones, but these approaches are poorly suited for detecting individual preferences and usually used to identify locations where species are congregating (O'Sullivan et al. 2022, 2023; Linnansaari et al. 2023).

The high mortality observed within a short period of time post-stocking was a substantial limitation of the present study. The rapid decline in individuals with time limited my ability to detect differences between treatments, particularly because cortisol

was only expected to exert effects after several days. For example, CTmax was significantly lowered after 4 d but not 2 d of cortisol treatment (Chapter 2). Moreover, the high mortality raises questions about the condition of the fish during the period of data collection. Most stocking occurs early or late in the season when surface water temperatures are typically cooler (Wagner 1962; Kennedy et al. 1984; Baer et al. 2007; Gatch et al. 2022). Although surface water temperatures in the present study were likely above the preferred range for rainbow trout, the fish had access to cooler temperatures at depth. The high mortality despite the availability of cooler water in the environment may suggest that factors beyond temperature alone proved challenging.

Nevertheless, the data set obtained is only the second, to my knowledge, to assess how elevated cortisol impacts temperature selection in the natural environment (the other is Cull et al. 2015). Regardless of treatment, trout typically chose temperatures within 14-17°C, a range that aligns well with thermal preference measured in rainbow trout in laboratory studies (Schurmann et al. 1991; Crawshaw and Podrabsky 2011; Golovanov 2013; Macnaughton et al. 2021) as well as other field monitoring studies (Ebersole et al. 2001; Baird and Krueger 2003; Huff et al. 2005). Over the first five days, cortisol-treated fish occupied temperatures similar to those of their control counterparts. This behaviour was counter to my prediction that cortisol-treated fish would prefer cooler temperatures. It is possible that cortisol did not have time to exert its effects – as noted above, 4 d of cortisol treatment were required to have a significant impact on CTmax (Chapter 2). By day 5, however, cortisol-treated fish diverged from the control fish and, exhibited significantly warmer temperatures. Correspondingly, treatment-related differences in depth were not detected until 4 d, at which time cortisol-treated fish were closer to the

surface, which would be expected to result in warmer temperatures (Jacobson et al. 2010; Missaghi et al. 2017). From day 6 onwards, data were available for only three cortisol-treated individuals, and therefore caution is needed in interpreting trends. It is intriguing that the fish that survived for longer periods were those with warmer temperatures. The apparent preference for warmer temperatures in cortisol-treated trout was consistent with the results of a thermal choice test (Chapter 2), in which cortisol-treated fish chose to stay in warmer waters for longer periods than control fish. A similar finding was reported for polyploid rainbow trout, where 3N trout occupied warmer waters than diploid rainbow trout (Verhille and Farrell 2023). Like cortisol-treated trout, 3N trout have been shown to have lower thermal tolerance and their performance at warm temperatures is reduced (Myers and Hershberger 1991; Simon et al. 1993; Ojolick et al. 1995; Altimiras et al. 2002; Hyndman et al. 2003; Verhille et al. 2013). Contrasting results were reported for puffer fish, where cortisol-treated individuals selected cooler temperatures than control fish (Cull et al. 2015). The effects of cortisol treatment on thermal biology may be species-specific, but differences in life history may also come into play – puffer fish are warm water stenotherms whereas rainbow trout are cold water stenotherms (Baird and Krueger 2003; Huff et al. 2005; Cull et al. 2015; Uiuiu et al. 2019; Nati et al. 2021; Linnansaari et al. 2023).

Examination of temperature over a finer scale (i.e. hourly over 24 h) also suggested that cortisol-treated fish preferred warmer temperatures. On day 2, cortisol-treated fish exhibited significantly higher temperatures than control fish for 4 h, whereas they exhibited higher temperatures for 16 h on day 4. This trend suggests that the longer duration of elevated cortisol caused cortisol-treated individuals to seek out warmer

temperatures more frequently, implying increasing dysregulation of behavioural thermoregulation. Previous work reported modulation of thermal preference with photoperiod (Macnaughton et al. 2018; Vera et al. 2023; de Alba et al. 2024), and fish have also been observed to be at warmer temperatures at night, often because of night-time migration to the surface for feeding (Mazur and Beauchamp 2006; Hansen and Beauchamp 2014; Gallagher et al. 2019; Thomas et al. 2023; Winkler et al. 2023). The trends observed in the present study did not follow an obvious circadian rhythm, perhaps owing to challenges the fish experienced upon introduction to the lake environment (see below).

It is also possible that cortisol treatment elicits behavioural fever, in which fish choose higher temperatures, often because of inflammation or pathogen exposure (Huntingford et al. 2020). For example, rainbow trout injected with bacterial lipopolysaccharide selected higher temperatures in an annular thermal choice experiment, with higher temperatures enhancing the inflammatory response (Gräns et al. 2012). Chronic elevation of cortisol increases susceptibility to pathogens (Pickering and Pottinger 1989), so it is possible that cortisol-treated fish are experiencing higher pathogen exposure, which is causing them to seek out warmer waters. Regardless, it is cause for concern that cortisol-treated trout have lowered thermal tolerance but appear to be choosing to be at warmer temperatures. Indeed, the temperatures chosen by fish in the present study appeared at least in some cases to be suboptimal for their survival. This observation suggests that chronic cortisol elevation may impair behavioural thermoregulation.

Interestingly, wide variation in thermal habitat choice (~10°C) occurred in the present study, regardless of treatment. The trout of the present study were raised together under hatchery conditions and therefore share the same thermal history. Thermal preference can vary with size (Morita et al. 2010; McDermid et al. 2013; Schakmann et al. 2023), but the trout of the present study were of similar size. Differences in personality may play a role, as proactive fish tend to be bolder and at least in some cases prefer higher temperatures than reactive individuals (Rey et al. 2015a; Cerqueira et al. 2016; Enders et al. 2019). Some species, such as cod, seek out warmer temperatures when feeding regularly to optimize growth (Björnsson 2019; Volkoff and Rønnestad 2020), so individual differences could reflect differences in feeding behaviour or growth rate. Additionally, warmer temperatures after exhaustive exercise have been found to favour metabolic recovery (Wilkie et al. 1997; Kieffer 2000; Galloway and Kieffer 2003), whereas cooler temperatures result in faster oxygen debt repayment (Kieffer 2000; Zeng et al. 2010; Zhang and Kieffer 2017). Taken together, there appear to be various conditions that might contribute to the individual differences in thermal habitat choice that were observed. Nevertheless, the specific factors that influenced differences in habitat choices both within and across treatments in the present study remain to be determined.

Understanding the preferred temperatures and movements of stocked fish is useful for management because it can inform stocking decisions. If water temperatures deviate from the thermal profiles previously recorded, stocking may be unsuccessful. In the current study, for example, stocking at the temperatures observed in July would not be effective given the high mortality that was observed. Previous research reported that

summer stocking is less successful than autumn stocking in brown trout (*Salmo trutta*), with ~10% lower survival and high mortality overall (>90%; Kennedy et al., 1984). Similarly, lake trout (*Salvelinus namaycush*) exhibited high survivability (73.7%) when stocked in May, before temperature increased to summer values (Gatch et al. 2022). Stocking procedures typically involve handling, high density holding and transport, sometimes over long distances, which can lead to stress (Harmon 2009; Luz and Favero 2024; Yang et al. 2024) and likely contribute to mortality. In the present study, it is likely that the combination of surgery, transport and warm environmental temperatures provided significant challenges. Exposure to a novel environment that was different and more variable than the consistent hatchery conditions the fish had experienced prior to being introduced into the lake may have reduced their capacity to cope with a suboptimal, warm environment (Morgan et al. 2022). Despite the limitations of the current study, the apparent trend for cortisol-treated fish to exhibit higher temperature warrants further investigation because it suggests that fish experiencing chronic stress may choose suboptimal thermal environments that could hinder their survival given their lower thermal tolerance.

4.6 Supplementary materials

Juvenile rainbow trout, *Oncorhynchus mykiss* (Walbaum 1792) (N = 10, mass = 265.1 ± 13.5 g, fork length = 23.9 ± 0.5 cm, mean ± SEM), were purchased from Izumi Aquaculture (Campbellcroft, ON, Canada). Fish were held in 1,275 L fibreglass tanks located at the University of Ottawa, with a 12 h:12 h light:dark photoperiod. Tanks were filled with continuously flowing, aerated and dechloraminated, city of Ottawa tap water at 13°C. Fish were fed 0.5% body mass daily, by scattering commercial trout pellets on the water's surface. Fish were held under these conditions for at least 2 weeks to acclimate before experimentation. Experimental protocols followed the guidelines of the Canadian Council on Animal Care (CCAC) for the use of animals in research and teaching and were authorized by an institutional animal care committee (protocol BL-3668, University of Ottawa).

Fish were lightly anesthetized using a solution of benzocaine (0.05 g L^{-1} ethyl-p-aminobenzoate; E1501, Sigma-Aldrich, Oakville, ON, Canada) and were fitted with a mini-osmotic pump containing cortisol as described in Section 4.2.2. The cortisol concentrations were chosen to achieve 40 or 80 µg g^{-1} body mass. Fish were placed individually in 40 L tanks supplied with flowing water at 13°C (for 40 µg g^{-1} body mass) or 21°C (for 80 µg g^{-1} body mass), and blood samples were collected at 10 and 20 d of cortisol treatment. Prior to blood collection, fish were anaesthetized using benzocaine (0.05 g L^{-1}) and blood (0.3 mL) was withdrawn using a 1 mL syringe with a 27G needle from the caudal vasculature. Blood samples were centrifuged at 10,000 *g* for 2 min. The resulting plasma was collected, flash froze and stored at -80°C until cortisol analysis. Plasma cortisol concentrations were analyzed using a commercial radioimmunoassay

(RIA; MP Biomedical, LLC, USA) previously validated for analysis of trout plasma samples (Gamperl et al., 1994). The intra-assay and inter-assay coefficients of variation were 11.0 and 4.9%, respectively.

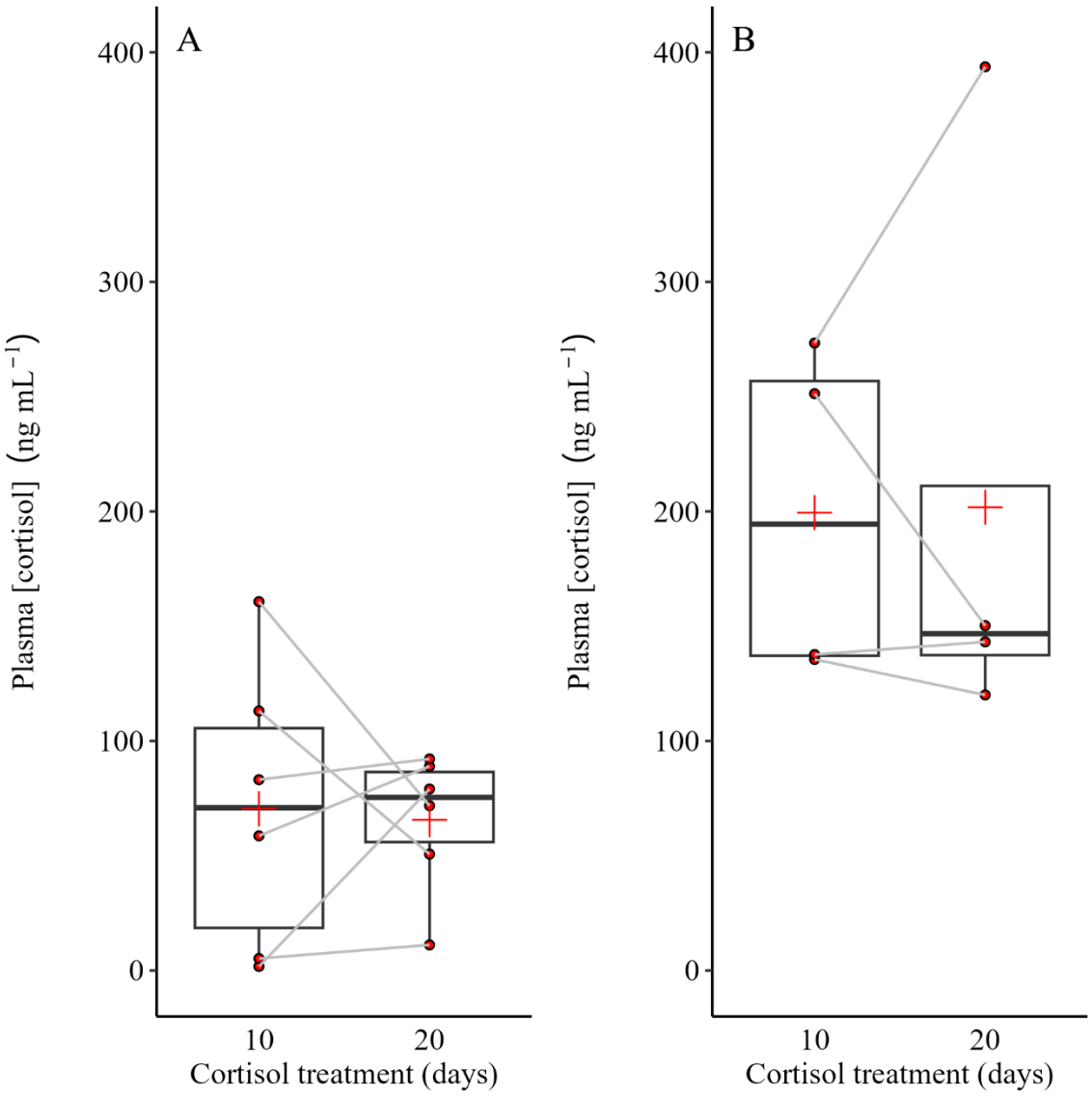

Figure S4.1. Plasma cortisol concentrations at 10 and 20 days of cortisol treatment in rainbow trout (*Oncorhynchus mykiss*) for (A) fish held at 13°C and treated with 40 µg g^{-1} body mass cortisol (paired Student's *t*-test, $P = 0.856$) and (B) fish held at 21°C and treated with 80 µg g^{-1} body mass cortisol (paired Student's *t*-test, $P = 0.962$). Data are presented as box plots (see Section 4.2.4 for details). Solid grey lines indicate values for the same individual.

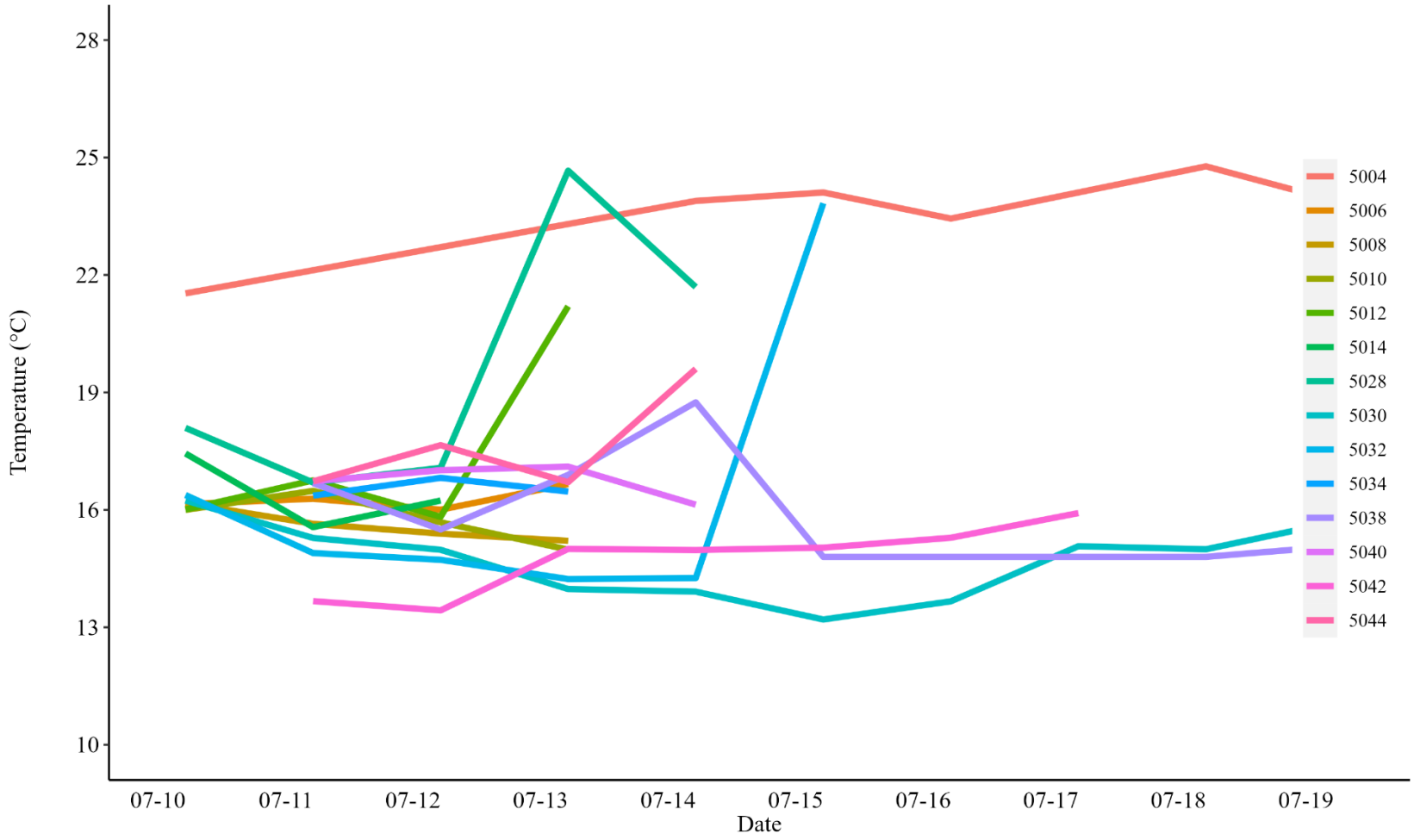

Figure S4.2. Daily average temperature of individual control rainbow trout (*Oncorhynchus mykiss*; *N* = 14) released into Otter Lake on July 10th, 2023.

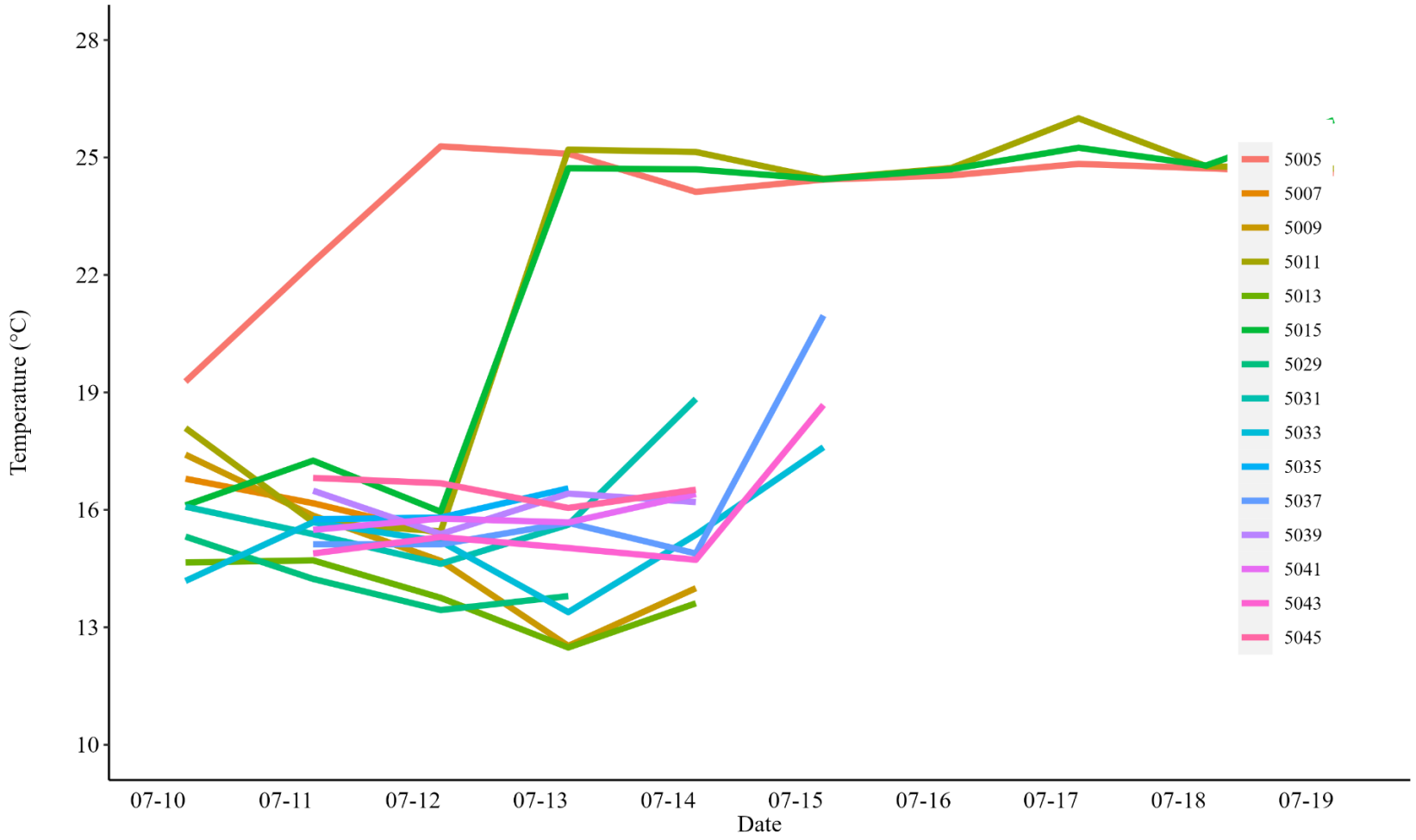

Figure S4.3. Daily average temperature of individual cortisol-treated rainbow trout (*Oncorhynchus mykiss*; *N*=15) released into Otter Lake on July 10th, 2023.

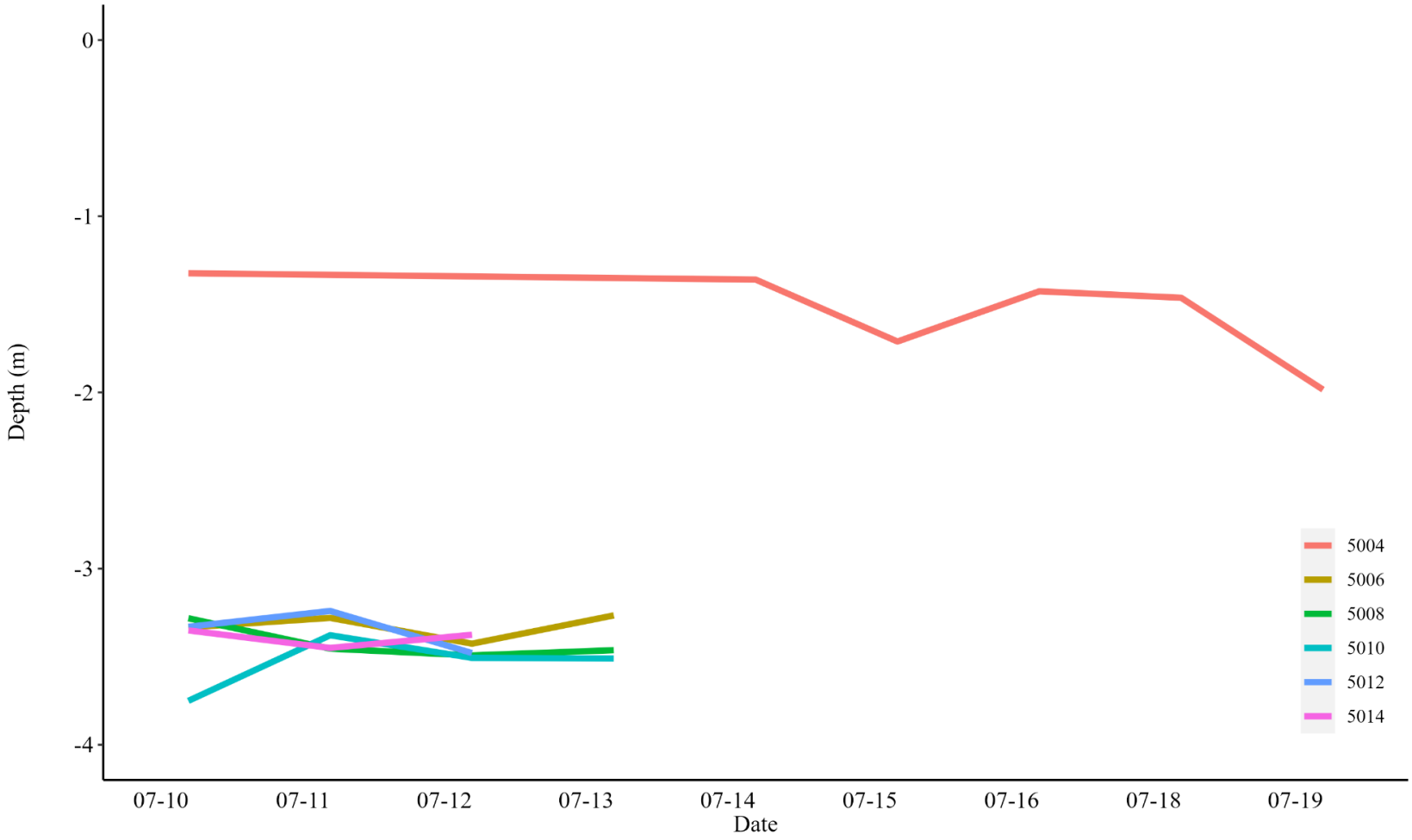

Figure S4.4. Daily average depth of individual control rainbow trout (*Oncorhynchus mykiss*; *N*=6) released into Otter Lake on July 10th, 2023.

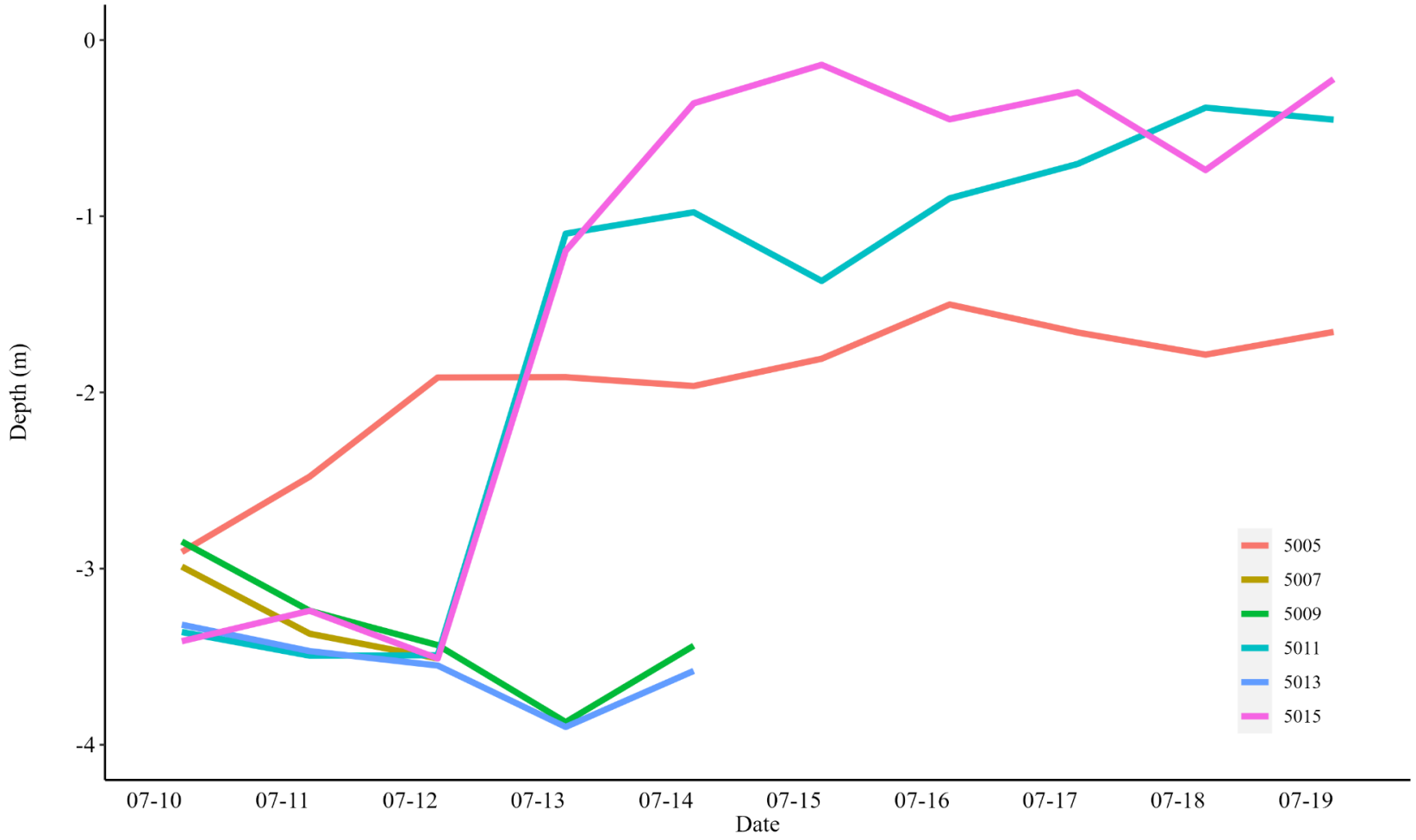

Figure S4.5. Daily average depth of individual cortisol-treated rainbow trout (*Oncorhynchus mykiss*; *N*=6) released into Otter Lake on July 10th, 2023.

Chapter 5

General discussion

5.1 Overview

The present thesis investigated the impact of chronic cortisol treatment on the thermal biology of rainbow trout (*Oncorhynchus mykiss*). My research revealed that chronic cortisol elevation lowered CTmax, regardless of acclimation temperature. This confirmed that elevated cortisol, and by extension chronic stress, can play a role in determining thermal tolerance in rainbow trout and presumably other teleost fishes. To examine the hypothesis that thermal perception is hindered by chronic stress, I targeted TRPV1 and TRPV4. My research provided strong evidence that TRPV1 is involved in thermosensing in rainbow trout, because CTmax was altered by treating fish with a TRPV1 antagonist or agonist. I demonstrated that *trpv4α* was elevated in gill tissue of cortisol-treated trout, which may contribute to their increased thermal sensitivity (i.e. lower CTmax). However, the mechanism(s) through which cortisol altered thermal tolerance remain to be fully described.

Because cortisol-treated rainbow trout have lower thermal tolerance, I asked whether other aspects of their thermal biology are altered. As reported by Bard et al. (2021), the magnitude of the cortisol response to high temperature in cortisol-treated trout was comparable to that in control animals, unlike the blunted cortisol response to a netting stressor observed in chronically-stressed trout (Jeffrey et al. 2014; Culbert and Gilmour 2016). However, cortisol-treated trout mounted a cortisol response to acute warming at lower temperatures compared to their control counterparts. This observation

is in keeping with the finding that chronically stressed trout are more thermally sensitive. To further examine the cortisol response to acute warming, the expression of key genes of the HPI axis was explored as a function of cortisol treatment and temperature. In control trout, I determined that acute thermal stress (warming to 27°C) caused increases in *crf* expression. By contrast, cortisol-treated trout exhibited elevated *crf* transcript abundance at their acclimation temperature (13°C) as well as at 27°C, but not at 25°C, the temperature at which a cortisol response was initiated in cortisol-treated trout. These observations suggest that *crf* may be playing an important role in modulating the cortisol response to acute warming in cortisol-treated trout. Finally, my research revealed that cortisol-treated fish preferred warmer temperatures, both in the lab and in the natural environment. This finding implies that cortisol treatment hinders behavioural thermoregulation and/or the ability to sense temperature. Taken all together, the present thesis suggests that chronic elevations of cortisol are detrimental to the thermal biology of rainbow trout. In this chapter, I will consider first the limitations of my research and then highlight its contributions to the field.

5.2 Limitations

It is important to acknowledge the limitations of a study to avoid over-reaching the significance of the results, and to open a discussion of important future directions for research. I used changes in transcript abundance to investigate the responses of thermoTRPVs to cortisol treatment and temperature, and to assess key genes of the HPI axis to understand how cortisol release is controlled during exposure to elevated temperatures. However, changes in transcript abundance do not always translate into

changes in protein abundance or activity that impact animal function (Vijayan et al. 2003; Lackner and Bähler 2008; Vogel and Marcotte 2012). Measurements of protein abundance can provide more insight into impacts on animal function, but unfortunately, homologous antibodies for rainbow trout are often not commercially available. To address this problem, I used a custom antibody raised against trout TRPV1 with the goal of investigating whether increases in transcript abundances of *trpv1* paralogues translated into increases in protein abundance. However, despite considerable troubleshooting, reliable protein detection was never achieved with the antibody. Although reliance on transcript abundances is a constraint, it should be noted that trout often have multiple paralogues for a gene (Alexandrou et al. 2013; Macqueen and Johnston 2014; Warren et al. 2014; Morini et al. 2022) and real-time RT-PCR allows the response of each paralogue to be assessed separately in a straightforward manner.

Equipment and water supply issues limited my lab-based study of thermal preference to a static choice chamber. A dynamic shuttlebox would have been a more powerful approach because it can be used to deduce the temperature an individual prefers. In this approach the subject is actively tracked as it moves between the chambers, altering water temperature according to its movements (Chistensen et al. 2021). Thus, small differences in thermal preference between individuals can be identified (Macnaughton et al. 2018, 2021; Nay et al. 2020; Pilakouta et al. 2023). Similarly, a static tank with a gradient of temperature choices would have provided more information on thermal preference (Vera et al. 2023). However, gradient tanks can be difficult to establish, particularly given the relatively large size of the trout used in the present study. Although I detected an effect of cortisol treatment on thermal preference, most fish

regardless of group spent ~75% or more of their time in the zone that was closer to their 13°C acclimation temperature. A broader range of temperature choices as well as a smaller difference in water temperature between the chambers in a given choice test might have yielded a clearer picture of how cortisol treatment alters thermal preference. However, the temperature ranges and the temperature difference between chambers were chosen on the basis of previous thermal preference literature (Cooper et al. 2018; Macnaughton et al. 2018; Ripley et al. 2023), as well as the capacity to maintain temperatures reliably with little or no mixing. Despite the limitations of the lab-based choice experiment, it yielded a finding similar to that of the field-based experiment, in that cortisol-treated trout chose warmer temperatures.

Lastly, the field study showed unexpected high mortality, hindering statistical analysis because of low numbers of individuals in each group as the experiment progressed. Although my initial plan had been to track the tagged fish for multiple months, the high mortality limited useful observations essentially to the first four to six days, necessitating short term assessments. As such, there is still a need for research on longer-term consequences of chronic elevation of cortisol on habitat use. It is probable that both the control and cortisol-treated fish experienced elevation of cortisol, making it difficult to discern differences caused by cortisol treatment. Moreover, while cortisol delivered by the mini-osmotic pumps would rapidly increase plasma cortisol, loss of fish meant that fish experiencing chronic cortisol elevation (typically at least 4 d in a lab setting) were limited to a few individuals. Strategies such as holding fish post implantation of the tags and mini-osmotic pumps for several days to ensure full recovery from surgery before transporting them to the lake system likely would be helpful for

future work. Additionally, I would recommend conducting future studies earlier in the summer before lake temperatures reach their summer highs. For example, lake trout stocked in May showed a ~74% survival rate (Gatch et al. 2022).

A final limitation of the present study was the likely impact of temperature on the rate of cortisol release from the cocoa butter implants or mini-osmotic pumps. Although both approaches have been used previously to chronically elevate cortisol (DiBattista et al. 2005; Madison et al. 2015; Lawrence et al. 2017; Bard et al. 2021), changes in temperature provide an additional challenge to achieving the desired circulating concentration of cortisol. As such, the rate of cortisol release was likely accelerated at elevated temperatures because cortisol is released from both cocoa butter implants and mini-osmotic pumps by diffusion. Thus, fish acclimated to 22°C likely were exposed to higher rates of cortisol release, emptying the pumps earlier than in fish acclimated to 16 or 19°C. In the field study, individual fish may have experienced variation in cortisol owing to their behavioural choice of temperature. Nevertheless, at least in the laboratory experiments, the effects of cortisol treatment on CTmax remained consistent across acclimation temperatures.

5.3 Significance

Animals, including fishes, are increasingly experiencing chronic stress in their natural habitats owing to the effects of climate change and other anthropogenic factors (Ficke et al. 2007; Lefevre et al. 2021). Because salmonid fishes are of great importance ecologically, culturally and economically, owing to aquaculture and fisheries (Stefansson et al. 2003; Healey 2009; Solar 2009; Martins et al. 2011; Noble et al. 2016; Johnsson

and Näslund 2018; Almodóvar et al. 2019; Lehnert et al. 2019, 2020; Sinnatamby et al. 2020; Ulaski et al. 2023), it is important to understand how chronic stress or chronic cortisol elevation may be affecting the thermal biology of these species. It is worth noting that many salmonid species also face the challenges of moving from oceans to freshwater rivers and streams to spawn (Armstrong et al. 2016; Keefer et al. 2018a; Amat-Trigo et al. 2023). During these migrations, salmonids are likely to be exposed to temperatures in river and stream systems that are elevated compared to the relatively stable temperatures of the open ocean (Keefer et al. 2018a; Mottola et al. 2020). This thesis provides multiple lines of evidence suggesting that chronically stressed fish are more sensitive to high temperatures yet surprisingly seem to select higher temperatures.

First, cortisol-treated fish exhibited lower thermal tolerance (measured as CTmax) than control fish. The effect of cortisol persisted even after acclimation of trout to higher temperatures, which increased CTmax. Interestingly, cortisol-treated trout had similar ARR to controls, suggesting that chronic elevations in circulating cortisol did not hinder thermal acclimation. In addition, cortisol-treated trout showed increases in cortisol in response to acute warming at lower temperatures than their control counterparts. These findings build on previous work showing that chronic stress lowers thermal tolerance, and that the effect is linked to the chronic elevation of cortisol (LeBlanc et al. 2011; Bard et al. 2021). Moreover, cortisol-treated trout tended to spend more time in warmer waters both when tested in a choice chamber in a laboratory setting and in a natural lake system, suggesting that prolonged elevation of cortisol alters behavioural thermoregulation. An important implication of these findings is that fish experiencing chronic stress are likely to select warmer temperatures despite their lower thermal tolerance, reducing thermal

safety margins. Thermal safety margins represent the capacity to be exposed to further elevations in temperature before reaching the upper thermal limit (Sinclair et al. 2016; Madeira et al. 2017; McArley et al. 2017). Although the mechanistic basis of this difference remains unclear, with two possibilities being a behavioural fever response or differences in thermal sensing prompted by changes in thermoTRPV expression, the findings of this thesis imply that chronically stressed fish will be at greater risk from climate change-caused warming owing to their increased thermal sensitivity, lowered thermal tolerance and their choice to occupy warmer temperatures. Notably, climate change is also likely causing these fishes to experience chronic stress (Whitney et al. 2016; Islam et al. 2022; Franke et al. 2024).

Although it has been well established that circulating cortisol increases in response to high temperature in fishes (Alfonso et al. 2021), the present study is one of the few that has investigated HPI axis activity during thermal stress (Benítez-Dorta et al. 2017; Goikoetxea et al. 2021; Tang et al. 2022a), and the first to my knowledge to explore this in cortisol-treated individuals. Understanding HPI axis function during warming and the impact of chronic cortisol on this relationship is a necessary first step in trying to identify and apply mitigation strategies. The research of this thesis suggests that salmonid fishes that are more resilient to chronic stress would experience fewer impacts of high temperature. There have already been calls for hatcheries that produce fish for stock enhancement to prioritize the breeding of resilient fish over maximizing yield (Healey 2009; Sae-Lim et al. 2017). Given the link between chronic stress and thermal tolerance, this is a selective breeding strategy that warrants further research and development, particularly within salmonid fisheries and aquaculture, because salmonids

are very important economically (Solar 2009; Buschmann and Muñoz 2019; Gallagher et al. 2022).

A novel contribution of this thesis was to implicate TRPV1 in thermal perception leading to activation of the cortisol response or loss of equilibrium in rainbow trout. I reported that blocking TRPV1 with capsazepine increased CTmax as well as the temperature at which the cortisol response was activated. Using capsaicin to stimulate TRPV1 lowered CTmax but did not change the threshold temperature of the cortisol response. These observations demonstrate that TRPV1 is involved in thermal perception, extending previous work carried out on masu salmon (Yoshimura et al. 2022) and zebrafish (Hunt et al. 2012; Gau et al. 2013). However, the tissue location of the TRPV1 receptors central to these effects remain to be determined. The thermoTRPVs are widely distributed in the tissues of salmonid fishes, with *trpv1* showing highest expression in the kidney and intestines, whereas *trpv4* expression is highest in the heart, pineal organ, kidney and intestines (Nisembaum et al. 2015, 2022). However, the key tissues involved in thermoreception remain uncertain. In the present study, the highest *trpv* transcript abundances were detected in the gill, a location that is well suited to detecting changes in water temperature given the high rates of water flow over the gill (Saunders 1961; Randall et al. 1991; Strother 2013). I focused largely on the potential role of TRPV1 in temperature sensing because it is known to be activated by noxious high temperatures (Gau et al. 2013), but the findings of the present study suggest that TRPV4 could also play a role. It would be useful to measure thermal tolerance in trout treated with a TRPV4 antagonist (e.g. ruthenium red) or agonist (e.g. 4-α-phorbol- 12,13-didecanoate; 4αPDD) (Nisembaum et al. 2015, 2022), as in the experiments of the current study using TRPV1

agonists and antagonists, to verify whether activation of TRPV4 also plays a key role in determining thermal tolerance. My work also suggests that cortisol treatment may alter thermal tolerance through effects on TRPV4, in that cortisol-treated fish exhibited higher *trpv4α* transcript abundance in the gill, which may increase thermal sensitivity. Although the picture is far from complete, greater sensitivity of thermal perception could lower the threshold at which temperature-induced responses are activated, providing a mechanism through which prolonged elevation of cortisol alters thermal tolerance. Further work is needed to determine whether cortisol treatment impacts TRPV activity as well as abundance. This will increase our knowledge of thermal perception in fishes and how it translates into responses at the organismal level (Haesemeyer et al. 2015, 2018; Haesemeyer 2020). Ultimately, this thesis adds insights to efforts to understand the physiological mechanisms through which fish tolerate thermal stress (Currie 2011; Tattersall et al. 2012; Lefevre et al. 2021; Alfonso et al. 2021, 2023; Ern et al. 2023).

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