THE EFFECTS OF CHRONIC CORTISOL ELEVATION ON THERMAL TOLERANCE IN ZEBRAFISH (*Danio rerio*)

ALBRIGHTINE ORSAR, HBSc

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Department or Biology
Faculty of Science
University of Ottawa

Abstract

The present study tested whether chronic cortisol elevation influences thermal tolerance in zebrafish (*Danio rerio*), a species living near its upper thermal limit. Four days of cortisol treatment reduced CT_{max} by ~1°C, an effect that was alleviated once cortisol returned to baseline. Using glucocorticoid receptor (GR) and mineralocorticoid receptor (MR) knockout fish, as well as the GR antagonist RU486, revealed that the GR was the key mediator of cortisol's action on CT_{max}. To probe the mechanisms underlying this effect, temperature-sensing proteins (thermoTRPs) were examined. Blockade of TRPV1 using capsazepine, increased CT_{max}, and cortisol treatment elevated *trpv4* splice variant 3 transcripts in gill tissue, suggesting that cortisol's effects on CT_{max} may be mediated by changes in temperature sensing. Cortisol treatment during early development (0–5 days post-fertilization) produced adults with lower CT_{max} values than vehicle-treated control fish, suggesting that cortisol in early development has programming effects on thermal tolerance. Overall, our findings reveal that elevated cortisol, and by extension chronic stress, compromises thermal tolerance in zebrafish, with implications for fish health under climate warming.

Résumé

La présente étude a cherché à déterminer si une élévation chronique du taux de cortisol influence la tolérance thermique chez le poisson-zèbre (Danio rerio), une espèce vivant près de sa limite thermique supérieure. Quatre jours de traitement au cortisol ont réduit la CT_{max} d'environ 1 °C, un effet qui s'est atténué une fois que le cortisol est revenu à son niveau de base. L'utilisation de poissons knock-out pour le récepteur des glucocorticoïdes (GR) et le récepteur des minéralocorticoïdes (MR), ainsi que l'antagoniste GR, RU486, a révélé que le GR était le médiateur clé de l'action du cortisol sur la CT_{max}. Afin d'étudier les mécanismes sous-jacents à cet effet, les protéines sensibles à la température (thermoTRP) ont été examinées. Le blocage du TRPV1 à l'aide de la capsazépine a augmenté la CT_{max}, et le traitement au cortisol a élevé les transcrits de la variante d'épissage 3 du trpv4 dans le tissu branchial, suggérant que les effets du cortisol sur la CT_{max} pourraient être médiés par des changements dans la détection de la température. Le traitement au cortisol au cours du développement précoce (0 à 5 jours après la fécondation) a produit des adultes avec des valeurs de CT_{max} inférieures à celles des poissons témoins traités avec un véhicule, suggérant que le cortisol au cours du développement précoce a des effets programmants sur la tolérance thermique. Dans l'ensemble, nos résultats révèlent qu'une élévation du cortisol, et par extension un stress chronique, compromet la tolérance thermique chez le poisson-zèbre, ce qui a des implications pour la santé des poissons dans le contexte du réchauffement climatique.

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Table of Contents

List of Tables and Figures

List of abbreviations:

ACTH	Adrenocorticoptropic hormone
ANOVA	Analysis of variance
AP	Action potential
ATP	Adenosine triphosphate
BLAST	Basic Local Alignment Search Tool
Cas9	CRISPR associated protein 9
CCAC	Canadian Council of Animal Care
cDNA	Complementary DNA
Cort	Cortisol
CRF	Corticotropin releasing factor
CRISPR	Clustered regularly interspaced short palindromic repeats
CT_{max}	Critical thermal maximum
d	Day
dpf	Days post-fertilization
DNA	Deoxyribonucleic acid
EIA	Enzyme-linked immunosorbent assay
ELS	Early life stress
GH	Growth hormone
GR	Glucocorticoid receptor
GRE	Glucocorticoid response element
gRNA	Guide RNA
h	Hour

hpf	Hour post-fertilization
HPI	Hypothalamic-pituitary-interrenal
IGF	Insulin growth factor
KO	Knockout
LOE	Loss of equilibrium
m	Month
min	Minute
MR	Mineralocorticoid receptor
mRNA	Messenger ribonucleic acid
OCLTT	Oxygen and capacity limited thermal tolerance
PCR	Polymerase chain reaction
PID	Proportional-Integral- Derivative
POA	Pre-optic area
PT100	Platinum 100
qPCR	Semi-quantitative polymerase chain reaction
RM	Repeated measures
RNA	Ribonucleic acid
RU486	Roussel-Uclaf 486
SEM	Standard error of the mean
sgRNA	Single guide ribonucleic acid
TDEE	Temperature dependent deterioration of electrical excitability
TRP	Transient receptor potential

TRPA	Transient receptor potential ankyrin
TRPV	Transient receptor potential vanilloid

Chapter 1: Introduction

Overview

In this overview, I outline the ecological circumstances driving this study, provide context by briefly summarizing the empirical work that formed the foundations for my thesis, and present my overarching hypothesis.

Anthropogenic stressors including habitat fragmentation, overexploitation, pollution, and climate change severely impact the health and fitness of animals, often acting additively which intensifies their effects (Côté et al., 2016; Mallett et al., 2024). Freshwater habitats are particularly vulnerable, losing biodiversity at two to three times the rate of terrestrial and marine ecosystems (Birnie-Gauvin et al., 2023). Anthropogenic stressors disrupt physiological function by chronically activating the stress response, which can reduce growth and impair resilience to additional challenges (Segner et al., 2012). Research on salmonid fishes showed that prolonged cortisol elevation caused by the chronic stress of subordinate social status and that the elevation of cortisol alone in the absence of a stressor reduced thermal tolerance (LeBlanc et al., 2011; Bard et al., 2021; Bard, 2025). These findings suggest that any stressor that chronically elevates cortisol will impair thermal tolerance. However, the work to date has focused on salmonid fishes, raising the question of whether similar effects occur in other species. Thus, the goal of the present thesis was to investigate whether chronic elevation of cortisol lowers thermal tolerance in zebrafish (*Danio rerio*).

In the following sections, I further consider anthropogenic threats to freshwater fishes as well as how fish perceive and respond to these stressors. I present the evidence to date demonstrating the effects of stress on thermal tolerance and summarize current knowledge on how fish perceive their thermal environment and on how stress may affect thermal preference in

the context of a warming world. Finally, I detail the goals, hypotheses, and predictions that guided the research presented in this thesis.

1.1 Anthropogenic challenges and their effects

Freshwater ecosystems are experiencing persistent anthropogenic stressors that compromise fish performance from the individual to the population level. Habitat fragmentation restricts migration routes and reduces water flow, exacerbating the geographical constraints imposed by these habitats (Dudgeon, 2019; Barbarossa et al., 2021; Capon et al., 2021). Chemical pollutants, from industrial discharge to agricultural run-off, and microplastics, can also cause population declines, either directly through toxic effects or indirectly by altering ecosystem dynamics, suppressing fitness, and increasing mortality (Wen et al., 2017; Burns et al., 2018). Furthermore, sources of chemical pollution such as sewage effluent and agricultural run-off are major contributors to eutrophication in freshwater habitats, with sewage effluent representing over 70% of the total (Bunce et al., 2018; Akinnawo, 2023). Collectively, these stressors reduce water quality and/or increase the vulnerability of aquatic organisms to environmental change (Kazmi et al., 2022).

In addition to these pressures, freshwater ecosystems are simultaneously experiencing rapid climate change induced by anthropogenic activity (Meehl and Tebaldi, 2004; Woodward et al., 2010). Temperature, often termed the "master abiotic factor," regulates biochemical and physiological processes in ectothermic animals, including most fishes, across multiple levels of biological organization (Schulte, 2024). Because ambient water temperature is the main determinant of body temperature and hence metabolic processes in most fishes, chronic warming can have profound physiological consequences. Elevated temperatures downregulate genes

essential for brain development, reduce motor neuron differentiation, and consequently hinder muscle growth and overall development (Sheridan and Bickford, 2011; Pallotta et al., 2017; Beltrán et al., 2021). Furthermore, chronic warming also reduces water oxygen solubility, increasing the difficulty experienced by aquatic ectotherms in meeting their oxygen demands (Little et al., 2020). Moreover, many tropical freshwater ectotherms already inhabit environments nearing their upper thermal limits, and considerable warming events can exceed their physiological capabilities (Nash et al., 2021).

Stressors often act synergistically to amplify one another's impact, and chronic warming can exacerbate the impact of other anthropogenic factors. Rising temperatures can enhance the toxicity of pollutants by increasing metabolic rates, modifying toxicokinetics, and accelerating bioaccumulation (Buchwalter et al., 2003; Noyes et al., 2009; Wang et al., 2019). The effect of enhanced toxicity is further amplified in fragmented habitats as altered stream flow reduces pollutant dilution (Wen et al., 2017). Thermal stress also perturbs homeostasis, reducing the energetic capacity of organism to withstand pollutant exposure (Gandar et al., 2017). Moreover, increasing temperature can also exacerbate the effects of eutrophication (Yang et al., 2008; Zhang et al., 2025). As global warming and other stressors interact, fish are increasingly pushed beyond what (Romero et al. (2009) refer to as the "homeostatic range", the range of normal physiological function, resulting in chronic activation of stress responses and reduced resilience to ongoing challenges.

1.2 The stress response and the role of cortisol

Environmental (and other) challenges activate a stress response, a suite of coordinated behavioural, physiological, and cellular responses that help the animal cope with the challenge

(Schreck and Tort, 2016; Petitjean et al., 2019). Two neuroendocrine pathways are particularly important in responding to stressors. Increases in circulating catecholamines, stimulated by activation of the sympathetic pathway, enhance oxygen uptake and delivery and mobilize energy reserves in an acute fashion (Reid et al., 1998; Fabbri and Moon, 2016). Glucocorticoid hormones are the product of activation of the hypothalamic-pituitary-interrenal (HPI) axis and have both acute and longer-term actions (Mommsen et al., 1999; Faught and Schaaf, 2024). In fish, as in other vertebrates, activation of the HPI axis initiates a hormone cascade beginning with the secretion of corticotropin-releasing factor (CRF), which stimulates the corticotropes of the anterior pituitary to release adrenocorticotropic hormone (ACTH) (Mommsen et al., 1999; Faught et al., 2016). Circulation of ACTH to the head kidney allows it to bind to the steroidogenic interrenal cells, stimulating the synthesis of cortisol, which travels to target tissues via the bloodstream (Faught et al., 2016; Schreck and Tort, 2016).

Cortisol is the primary glucocorticoid in teleost fishes, and its actions are mediated primarily through two intracellular receptors that function as ligand-activated transcription factors, the glucocorticoid receptor (GR), and the mineralocorticoid receptor (MR) (Mommsen et al., 1999; Faught et al., 2016). Under basal conditions, the GR resides in the cytoplasm whereas MR is distributed across the cytoplasm and nucleus (Faught et al., 2016). These receptors are expressed in most tissues, including the liver, brain, gills, and red and white blood cells (Mommsen et al., 1999; Faught et al., 2016). Upon binding cortisol, the cortisol-receptor complex translocates to the nucleus, where it regulates gene transcription, altering the expression of target genes that contain glucocorticoid response elements (GRE) (Ramamoorthy and Cidlowski, 2016). Although both receptors mediate the actions of cortisol, their roles differ. The MR has higher binding affinity for cortisol and is fully occupied under baseline cortisol levels,

whereas GR is primarily activated during stress-induced elevation of cortisol (Faught et al., 2016).

The distinct contributions of the GR and MR have been examined using knockout (KO) models. Zebrafish (*Danio rerio*) lacking functional GR (GR-KO) display elevated baseline plasma cortisol, supporting the widely accepted role of GR in regulating cortisol levels via negative feedback mechanisms (Faught and Vijayan, 2018). The GR is also considered to be the principal receptor mediating the downstream effects of cortisol on metabolic pathways and ion balance (Vijayan et al., 2010; Cruz et al., 2013). In contrast, MR-KO zebrafish maintain normal baseline cortisol levels, although MR-KO larvae exhibited prolonged cortisol elevation after a stressor was removed (Faught and Vijayan, 2018). Similarly, MR has been implicated in suppressing genes associated with HPI axis regulation, indicating a role in negative feedback regulation of the HPI axis, albeit to a lesser extent than GR given the hypercortisolemic nature of GR-KO fish (Alderman and Vijayan, 2012; Alderman et al., 2012; Faught and Vijayan, 2018).

Through GR and MR signalling, cortisol influences a wide array of physiological processes that help to re-establish homeostasis following exposure to a stressor. Cortisol alters carbohydrate, protein, and lipid metabolism by enhancing the activity of glycolytic and gluconeogenic enzymes, actions that quickly mobilize energy reserves for the animal during acute stress (Vijayan et al., 2010; Faught et al., 2016). However, when stressors are prolonged and cortisol levels remain chronically elevated, the effects can be detrimental. Sustained cortisol exposure is immunosuppressive, reducing inflammatory responses and pathogen resistance (Maule and Schreck, 1990; Saeij et al., 2003; Vijayan et al., 2010). Interactions may also occur between the effects of cortisol and those of other metabolic hormones, including growth hormone (GH) and insulin-like growth factor (IGF), both of which are essential for growth,

reproduction, and osmoregulation (Sadoul and Vijayan, 2016). In fact, cortisol treatment has been shown to reduce growth in rainbow trout (*Oncorhynchus mykiss*), zebrafish larvae and Atlantic salmon (*Salmo salar*) (Philip and Vijayan, 2015; Faught and Vijayan, 2020; Vargas-Chacoff et al., 2021). Collectively, these effects of prolonged cortisol elevation can decrease fish fitness through various pathways.

Although there is abundant evidence that acute warming and/or exposure to elevated water temperatures serve as environmental challenges that elicit a cortisol response (King et al., 2006; Chadwick et al., 2015; Samaras et al., 2018; Alfonso et al., 2021), studies of the effects of cortisol on responses to acute warming are sparse.

1.3 Assessing thermal tolerance

A common measure of thermal tolerance owing to its high repeatability and low mortality rate is the critical thermal maximum, CT_{max} (Morgan et al., 2018; Desforges et al., 2023). First introduced in 1944 to assess the thermal limit of desert reptiles, measurement of CT_{max} involves subjecting the animal to acute thermal ramping at a rate considered ecologically relevant for the species being studied (Cowles and Bogert, 1944). The measurement is terminated when the individual being assayed is no longer able to maintain an upright position and experiences a loss of equilibrium (LOE) for at least two seconds (Desforges et al., 2023; Raby et al., 2025). Although fish generally regain equilibrium quickly after LOE (provided water temperature is lowered), few studies have explored recovery from CT_{max} or factors that might lead to variation in recovery. The temperature at which LOE occurs is defined as the CT_{max}, representing the temperature at which the animal cannot escape environmental conditions that will ultimately

result in death (Morgan et al., 2018). The underlying cause of LOE is not understood, but several hypotheses have been proposed (reviewed by Ern et al., 2023).

The temperature-dependent deterioration of electrical excitability (TDEE) hypothesis suggests that LOE occurs because of a mismatch between the source of the depolarizing ion current (Na^+) and the repolarizing current (K^+) in the receiving cell (sink) (Vornanen, 2020). For action potential (AP) propagation by excitable cells such as neurons, muscle cells and sensory cells, the source current must exceed the sink current (Spector, 2013). High temperatures accelerate inactivation of the Na^+ channels and shorten their open times while increasing sink current through faster K^+ activation (Volgushev et al., 2000; Vornanen, 2020). This source-sink mismatch can reduce AP frequency and block signal transmission, particularly at sites of structural discontinuity such as synapses and electrically coupled cells (Vornanen, 2020). These sites are common in cardiac tissue and nerves, and dysfunctions of these tissues are routinely studied to understand mechanisms of LOE (Andreassen et al., 2022; Chouinard-Boisvert et al., 2024; Morla et al., 2025). Another possible cause of LOE is summarized in the oxygen capacity limited thermal tolerance (OCLTT) hypothesis, which builds on earlier work linking the capacity for aerobic swimming to temperature in fishes (Brett, 1971). The OCLTT hypothesis suggests that at elevated temperatures, rising metabolic rates increase oxygen demand, and cardiovascular limitations restrict sufficient oxygen delivery to tissues creating an oxygen supply-demand mismatch (Pörtner, 2001; Gilbert et al., 2024). This leads to increased reliance on anaerobic metabolism as ATP depletes leading to the loss of motor function that is observed as the LOE (Pörtner, 2010).

Interestingly, empirical support for these hypotheses is mixed. For example, electrophysiological measurements in rainbow trout demonstrated that decay in visual perception

and locomotor control closely followed CT_{max}, supporting the TDEE hypothesis (Ekström et al., 2025). By contrast, *in vivo* recordings of neural activity in zebrafish larvae showed CT_{max} preceded brain wide depolarization, and hyperoxic conditions increased CT_{max}, rescuing neural activity in locomotor regions; these observations indicate that oxygen limitations impair neural function and cause LOE (Andreassen et al., 2022). However, increases in thermal tolerance under hyperoxia appear to be species and context specific (McArley et al., 2021; Ern et al., 2023). Recent work by Silva-Garay et al., (2025) used a broad range of oxygen levels to determine whether a particular concentration increased CT_{max} in zebrafish. Although hyperoxia increased the maximum metabolic rate, it did not increase thermal resilience, and hypoxia reduced CT_{max}, suggesting that oxygen limitation alone is insufficient to explain LOE. Ern et al. (2023) suggest that a single mechanism that is responsible for upper thermal limits across species is unlikely to exist.

1.4 Stress and thermal tolerance

Given the context of a warming world, it is critical to understand how chronic stress may influence fish performance and decrease thermal tolerance. Past research demonstrated that chronic stress impaired thermal tolerance in rainbow trout (LeBlanc et al., 2011; Bard et al., 2021). Juvenile salmonids readily form dominance hierarchies when housed in pairs (Gilmour et al., 2005). Subordinate trout within these hierarchies have a significantly lower CT_{max} relative to their dominant counterparts (LeBlanc et al., 2011). Bard et al. (2021) noted that subordinate fish allowed to recover from social interactions for two days, during which plasma cortisol returned to baseline levels, exhibited CT_{max} values comparable to those of dominant fish. However, subordinate trout that were not permitted to recover, but rather were treated with a cortisol

implant, had a significantly lower CT_{max} than dominant trout. These experiments indicated a key role for cortisol in lowering thermal tolerance. Subsequent experiments identified GR as the primary mediator of this effect, because co-treatment of trout with cortisol and the GR antagonist, RU486, prevented a reduction in CT_{max} (Bard, 2025). These studies support an impact of stress and specifically cortisol on thermal tolerance, but all were conducted on rainbow trout, raising the question of whether thermal tolerance in other fish species is also affected by stress and cortisol.

Research on mangrove rivulus (*Kryptolebias marmoratus*) suggested that social experience can influence thermal tolerance, although whether cortisol plays a causal role in this relationship has yet to be determined (Melanson et al., 2023). Fish reared in isolation were compared to those that had experienced prior social interactions, with both groups being subjected to acute thermal ramping. Fish with social experience tolerated higher temperatures before emerging from the water (a response that precedes CT_{max} in this species) when compared to their socially isolated conspecifics. Interestingly, a similar difference in threshold was observed when fish were exposed to an agonist of the heat-sensing ion channel, transient receptor potential vanilloid channel 1 (TRPV1). Fish with social experience emerged at higher capsaicin concentrations than socially naïve fish, suggesting that social experience alters the sensitivity of TRPV1 (Melanson et al., 2023).

Collectively, these findings suggest that chronic stress alters thermal tolerance, identify cortisol as the causative agent of the impact on thermal tolerance, and suggest that TRPV1 plays a role in mediating the effects of cortisol on thermal tolerance. In the present thesis, I asked whether similar relationships are present in the zebrafish, a species that is often found living near its thermal maximum (Dudgeon, 2019; Sundin et al., 2019). Specifically, I aimed to elucidate the

physiological mechanisms underlying effects of cortisol on thermal tolerance, focusing in particular on the role of TRPV1.

Thermal tolerance is influenced by multiple factors other than stress/cortisol. For example, acclimation temperature and thermal history can shape upper thermal limits. Exposure to thermal extremes and higher acclimation temperatures increased thermal tolerance in a variety of fish species, including stickleback (*Gasterosteus aculeatus*), Atlantic herring larvae (*Clupea harengus)*, Chinese sucker *Myxocyprinus asiaticus*, and juvenile rohu *(Labeo rohita)* (Das et al., 2005; Moyano et al., 2017; Zhou et al., 2019; McKenzie et al., 2021; De Bonville et al., 2025). Life stage and reproductive status can also influence thermal limits. A meta-analysis using observational, experimental and phylogenetic data to assess thermal tolerance at different life stages of over 694 marine and freshwater fish species found that embryos and spawning fish displayed more limited tolerance ranges when compared to adults and non-spawning individuals (Dahlke et al., 2020), highlighting the vulnerability of these stages. In particular, stress during early life stages is known to have impacts in later life (Best et al., 2017; Choi et al., 2024), but whether exposure to early life stress in early life impacts thermal tolerance later in life has not been investigated.

1.5 Early life stress and thermal tolerance

Early life stress (ELS) can have long lasting impacts on physiology and stress regulation (Lupien et al., 2009). Specifically, exposure to stress during early life has been linked to impaired ion regulation as fish age (Hare et al., 2021). Similarly, early exposure to heat stress and copper resulted in higher mortality rates and increased expression of several stress-related genes when stressors were combined (Dorts et al., 2016), indicating the sensitivity of developing

zebrafish to combined environmental stressors. More relevant to the work of the current study, where cortisol treatment was used as a proxy for chronic stress, cortisol-treatment of zebrafish embryos and larvae yields adults with a dysregulated stress response, compromising an individual's ability to cope with future stressors (Hartig et al., 2016; Nesan and Vijayan, 2016; Best et al., 2017; Choi et al., 2024; Nagpal et al., 2024). Given the evidence linking chronic stress and cortisol with impacts on thermal tolerance (LeBlanc et al., 2011; Bard et al., 2021; Bard, 2025), dysregulation of the HPI axis caused by ELS could, in turn make fish more susceptible to elevated temperatures.

There might also be direct impacts of ELS on thermal tolerance. The elevation of cortisol caused by ELS perturbs hypothalamic neurogenesis, feeding and growth, potentially diminishing fitness (Eachus et al., 2024). Current research suggests that the preoptic area (POA) serves as a key brain area in thermoregulation (Haesemeyer et al., 2018; Cutler and Haesemeyer, 2024; Palieri et al., 2024), and therefore effects of ELS on hypothalamic neurogenesis might also impact thermoregulatory pathways. Cortisol treatment has been shown to exacerbate the effects of high temperature in increasing metabolism (Pfalzgraff et al., 2022). As high temperatures reduce activity in locomotor brain centres (Andreassen et al., 2022), the combined activity of cortisol and high temperature may be a direct mechanism by which cortisol reduces thermal tolerance.

A survey of the literature did not yield studies that have examined the effects of elevated cortisol during sensitive early life stages on thermal tolerance in adulthood. A first step to address this knowledge gap is to evaluate whether early-life cortisol treatment as a proxy for ELS impacts thermal tolerance later in life.

1.6 Thermal sensing, thermoregulation and thermal preference

Although the evidence that chronic cortisol elevation, either experimentally or as a result of chronic stress, lowers thermal tolerance in rainbow trout is compelling (LeBlanc et al., 2011; Bard et al., 2021; Bard, 2025), the underlying mechanisms remain unclear. Potential effects of cortisol should be considered at all levels, from temperature detection to central processing, to behaviour. Temperature detection begins with thermoreceptors, which are afferent sensory neurons with their cell body located in the trigeminal or dorsal root ganglion and their axons extending to peripheral tissues (Butler and Hodos, 2005; Cutler and Haesemeyer, 2024). In larval zebrafish, signals from the peripheral tissue are integrated by local circuits in the lateral hindbrain, where differential processing of the trigeminal inputs allows neurons to detect the rate of temperature change (Haesemeyer et al., 2018). Hindbrain pre-motor cells use this information to initiate appropriate navigational behaviour, allowing the animal to seek out its preferred temperatures (Haesemeyer et al., 2018; Haesemeyer, 2020). Signal processing may also occur in the POA, which contains thermosensitive neurons (Prosser and Nelson, 1981) and receives temperature information from peripheral thermosensory neurons, suggesting that it acts as a central thermostat (Tan et al., 2016; Cutler and Haesemeyer, 2024; Palieri et al., 2024).

The ability to detect temperature, peripherally or centrally, reflects the activity of thermoTRP proteins, a conserved family of temperature-gated ion channels that, collectively, are responsive to a broad range of temperatures, from cold to noxious heat. For instance, mammalian TRPV1 is activated at 40°C *in vitro* and TRPA1-mediated cold avoidance occurs at 15°C in *Caenorhabditis elegans* (Jordt et al., 2004; Chatzigeorgiou et al., 2010; Haesemeyer, 2020). In zebrafish, TRPV1 activity increases with rising temperatures and is required for heat-induced locomotion (Gau et al., 2013). Similarly, TRPV4 is activated by heat and is necessary for

hyperthermia-induced seizures in zebrafish (Patapoutian et al., 2003; Hunt et al., 2012). Interestingly, zebrafish TRPV1 is also activated by low pH (Gau et al., 2013), hinting that environmental stress may be able to alter thermal perception. For the purposes of this thesis work, I focused on TRPV1 and TRPV4, which at the gene level can exist as multiple paralogues and/or splice variants. In rainbow trout, manipulation of TRPV1 activity resulted in changes in CT_{max} – treatment of trout with the TRPV1 inhibitor capsazepine increased CT_{max} whereas treatment with the TRPV1 agonist capsaicin lowered CT_{max}, results that are consistent with a role for TRPV1 in sensing temperatures that lead to LOE (Bard, 2025). In addition, cortisol-treated trout exhibited increases in *trpv4* transcript abundances in gill tissue, suggesting a mechanism through which cortisol may alter thermal sensitivity (Bard, 2025). This altered thermal sensitivity may also have influenced thermal preferences, because cortisol-treated trout appeared to seek out warmer waters (Bard, 2025).

Thermoregulatory behaviours are influenced by thermal preference and shaped by environmental and physiological cues (Beitinger and Lutterschmidt, 2011; Reynolds, 2011; Killen, 2014; Skandalis et al., 2020). For instance, the spatial distribution of Atlantic cod (*Gadus morhua*) shifted towards colder regions in response to climate change (Engelhard et al., 2014). Infection with the ectoparasite (*Gyrodactylus turnbulli*), led to a preference for warmer temperatures in the Trinidadian guppy (*Poecilia reticulata*), likely because immune responses increase with temperature (Bowden et al., 2007). In another study, hypoxia-exposed wild caught carmine shiner (*Notropis percobromus*) sought out colder temperatures (Enders et al., 2019), likely as an attempt to reduce metabolic rates. These studies demonstrate how fish behaviourally thermoregulate to help align their internal physiological states with water temperatures that optimize performance. Thus, it might be expected that fish always prefer temperatures that

maximize their physiological performance. However, zebrafish do not appear to show a preference for temperatures that maximize their aerobic scope (Ripley et al., 2022), raising questions as to how environmental factors affect thermoregulation in the zebrafish. Whether stress alters thermal preference has been the subject of some debate in zebrafish. Evidence of stress-induced hyperthermia, a preference for warmer temperatures in response to a stressor, was reported by Rey et al. (2015), but a subsequent study using more robust experimental approaches failed to detect stress-induced hyperthermia (Jones et al., 2019). However, the recent finding that chronic exposure to exogenous cortisol increases transcript abundances of *trpv4* paralogues (Bard, 2025) suggests the possibility of an interaction between chronic stress and thermal preference. If alterations in the expression of TRPV channels change thermal sensitivity, then these changes may alter behavioural responses causing changes in thermal preference. My thesis work aimed to explore this possibility by assessing the impacts of chronic cortisol elevation on thermal preference at the whole animal level and on *trpv1* and *trpv4* transcript abundances at the molecular level.

1.7 Hypotheses and predictions

I hypothesized that chronic stress lowers thermal tolerance in zebrafish. Based on this hypothesis, I predicted that prolonged elevation of plasma cortisol would reduce CT_{max} and that returning cortisol to baseline levels would allow CT_{max} to recover. To test this hypothesis, I subjected zebrafish to 4 d of cortisol treatment. This duration was chosen because it was effective in eliciting changes in CT_{max} in rainbow trout (Bard, 2025).

Given the role of GR in mediating key effects of cortisol in fish, particularly in relation to stress (Faught and Vijayan, 2018), I hypothesized that cortisol exerts its effects on CT_{max} via the

GR. This hypothesis yielded the prediction that co-treatment with cortisol and the GR antagonist, RU486, would block cortisol from lowering CT_{max} (see also Bard, 2025). Moreover, I predicted that MR-KO fish, but not GR-KO fish, would experience lowering of CT_{max} in response to elevated cortisol. Although MR-KO fish were treated with exogenous cortisol, GR-KO fish are hypercortisolemic owing to the loss of negative feedback and even without cortisol treatment, they exhibit circulating cortisol levels comparable to those of WT zebrafish subjected to cortisol treatment (Faught and Vijayan, 2018).

I hypothesized that cortisol treatment would exert its effect on CT_{max} by altering thermal sensing. To test this hypothesis, I first asked whether TRPV1 is involved in thermal sensing by treating zebrafish with capsazepine to block TRPV1 and measuring CT_{max}. I then predicted that cortisol treatment would increase transcript abundances of *trpv* paralogues, thereby increasing the sensitivity of cortisol-treated fish to high temperatures. I also predicted that cortisol-treated fish would prefer lower temperatures than sham-treated fish and measured thermal preference using a shuttle box set-up.

Finally, I hypothesized that ELS lowers thermal tolerance in later life. Based on this hypothesis, I predicted that cortisol-treated embryos and larvae would have lower CT_{max} values as adults. To test this hypothesis, fertilized eggs were treated with cortisol to 5 dpf, after which they were raised to adulthood in untreated waters; CT_{max} was measured in adult fish at 3 and 8 months of age. Collectively, the work presented in this thesis attempts to address key gaps in our understanding of how stress affects thermal tolerance in fish.

Chapter 2: Materials and Methods

2.1 Overview of experimental series

Several experimental series were carried out. Experimental series 1 used a paired design to assess the effects of sham or cortisol treatment on CT_{max} and evaluated how rapidly CT_{max} recovered following cortisol treatment. Experimental series 2 investigated the role of cortisol receptors in mediating the effect of cortisol treatment on CT_{max}. Experimental series 3 probed the role of TRPV in mediating the effect of cortisol on CT_{max}. Experimental series 4 asked whether early life exposure to cortisol impacted CT_{max} later in life. Experimental series 5 evaluated thermal preference in sham versus cortisol-treated fish.

2.2 Experimental animals

Adult zebrafish, *Danio rerio*, were purchased initially from the pet trade (Big Al's, Ottawa, ON, Canada) and then maintained at the University of Ottawa aquatics facility. Wild-type (WT) adult zebrafish provided by the in-house breeding program were housed in tanks supplied with flowing, dechloraminated city of Ottawa tap water ("system water") kept at 28 ± 1°C. Fish were held at a density of 4 fish L^{-1} in 3 L or 10 L tanks and were fed a commercial fish diet (GEMMA Micro 300, Skretting, Vancouver, British Columbia, Canada) twice a day. A 14h:10h light:dark photoperiod was maintained. Unless otherwise stated, male and female zebrafish aged 6-12 months were used for experiments (Table 2.1).

Zebrafish lacking functional expression of either the MR (MR-KO; *nr3c2*$^{-/-}$) or the GR (GR-KO; *nr3c1*$^{-/-}$) together with sibling WT fish (sWT; *nr3c1*$^{+/+}$*nr3c2*$^{+/+}$) were used for Experimental series 2. These fish were generated through a full gene deletion of NP_001018547 and NC_007112.7 for GR and MR, respectively, using CRISPR/Cas9 gene-editing technology (Hong, 2024). Four CHOPCHOP-designed guide RNAs (gRNA) alongside short-guide oligos

(sgRNA) flanking both ends of the target sequences were synthesized via a cloning-free method (Talbot and Amacher, 2014) and microinjected into *roy*$^{-/-}$; *nacre*$^{-/-}$ (*casper*) embryos at the one-cell stage. The use of *casper* embryos facilitated the detection of founder fish carrying the *nr3c1*$^{-/-}$ or *nr3c2*$^{-/-}$ gene deletion. Founder fish were crossed with WT fish to obtain heterozygous F1 fish which were then crossed to generate an F2 population of KO and sWT fish. Genotypes were confirmed using Sanger sequencing (Genome Quebec, McGill University, Montreal, QC, Canada). These fish were held and maintained as described above, and male fish of 6-12 months old were used in experiments (Table 2.1). Female fish were not used because they were retained for breeding.

For Experimental series 4, embryos were obtained by breeding two male and three female WT fish in an acrylic breeding trap with a perforated bottom insert that allowed for embryo collection. Embryos were randomly allocated to either sham or cortisol treatment groups (see below) and reared in an incubator at 28.5°C in Petri dishes with 30-40 embryos per dish. At ~20-24 hours post-fertilization (hpf), all embryos were bleached using standard protocols and transferred to new Petri dishes in their respective conditions. Water was changed daily (50%), and dead embryos or larvae were removed. After 5 d of sham or cortisol treatment, larvae were transferred to a "nursery" holding system supplied with system water and were raised under the standard conditions of the in-house breeding program until they reached sexual maturity. The fish were then moved to standard holding conditions until they were used for measurement of CT_{max} at 3 or 8 months post-fertilization.

All holding and experimental protocols adhered to the guidelines of the Canadian Council on Animal Care (CCAC) for the use of animals in teaching and research and were approved by

the institutional animal care committee of the University of Ottawa (protocols BL-2118, BL-3675).

2.3 Validation of cortisol treatment

Adult zebrafish were treated with cortisol or vehicle alone (sham) using the waterborne cortisol exposure of Faught and Vijayan (2022). Based on the findings of Bard (2025) that 4 d but not 2 d of cortisol treatment was sufficient to lower CT_{max} in rainbow trout, a 4 d exposure period was chosen. A preliminary trial was carried out to confirm that circulating cortisol levels in treated fish were at physiological levels, i.e. those typical of zebrafish exposed to a chronic stressor for 4 d (Tea et al. 2019). Fish were haphazardly allocated to treatment groups and held at 28 ± 1°C in breeding baskets inserted into 3 L tanks containing 2 L of system water. Control fish were held in untreated water. Sham fish were held in tanks containing 0.05% ethanol. Cortisol-treated fish were held in water containing 5 μg mL^{-1} cortisol (hydrocortisone 21-hemisuccinate sodium salt, Sigma-Aldrich, Oakville, ON, Canada) dissolved in 0.05% ethanol, as described by Faught and Vijayan (2022). Water was renewed daily (100% water change).

During the exposure, fish were fed once a day (GEMMA Micro 300), about 10-15 min before a water change to reduce fecal build-up between changes. At the end of the 4 d treatment period, fish were terminally anaesthetized by immersion in ice-cold, buffered tricaine methane sulfonate (MS-222; 4.2 g L^{-1}; Syndel, Nanaimo, BC, Canada). Blood was collected via caudal severance using the approach of Babei et al. (2013) and ethylenediaminetetraacetic acid (EDTA; 0.5 M) as an anti-coagulant. Blood samples were centrifuged at 14,000 *g* for 3 min. The separated plasma was diluted 30-40-fold, flash frozen in lN_2, and stored at -80°C for later analysis of cortisol concentrations. Cortisol concentrations were analysed using a commercial

enzyme-linked immunosorbent assay (EIA) according to the manufacturer's instructions (Neogen, Lansig, MI, USA). The intra-assay coefficient of variation was 0.84% and all samples were analyzed in a single assay.

2.4 Measurement of CT_{max}

The measurement of CT_{max} occurred in a custom-made Plexiglas tank (Figs. 2.1, 2.2; overall dimensions 38 cm L x 30 cm W x 26 cm H) containing 9 L of system water, which provided a water depth of 8 cm. The tank was made of white Plexiglas and had inner partitions of white Plexiglas with mesh inserts that divided the area into two compartments. The larger compartment (28 cm L x 22 cm W) housed the (single) fish for the duration of the trial and contained a PT100 resistance temperature probe for the measurement of water temperature. The smaller compartment contained an air stone for vigorous aeration of the water, to ensure maintenance of air saturation as water temperature increased (tested by monitoring dissolved oxygen levels in preliminary trials). It also contained an aquarium heater (HiTauing, Amazon, Seattle, WA, USA; 500 W, 32.9 cm long), and two submersible pumps (Mimouse, Sicce, Coconut Creek, FL, USA, 300 L h^{-1}) to ensure uniform water temperature throughout the tank. The aquarium heater was connected to a PID temperature controller (Solo 4848, Automation Direct, Cumming, GA, USA), which regulated power to the immersed heater based on measured temperature and the desired heating rate. The desired rate was set using the Solo Configuration software (Automation Direct) which was installed on a laptop computer and connected to the controller. The software was configured to produce a linear temperature increase of 0.33 ± 0.01 °C min^{-1} (Fig. 2.3; Becker and Genoway, 1979) and to automatically record water temperature during each run.

To measure CT_{max}, a single fish was transferred to the tank and given 45 min to acclimate. Water temperature was then increased at the rate of 0.33 ± 0.01°C min^{-1} until LOE. Loss of equilibrium was deemed to occur when the fish turned dorso-ventrally and was unable to right itself within 2 s. Immediately following LOE, the fish was transferred to a tank containing aerated system water at 28-30°C to recover. The tank was emptied, washed and refilled before the next CT_{max} trial.

2.5 Experimental series 1: Effect of cortisol treatment on CT_{max}

To assess the effect of cortisol treatment on CT_{max}, a paired experimental design was used. Fish were haphazardly allocated to sham or cortisol treatment groups (n = 11 fish per group; Table 2.1) and CT_{max} was measured as described above. Following a 20 min recovery period, fish were lightly anaesthetized in 0.12 g L^{-1} buffered MS-222 and photographed to allow differences in caudal and/or anal fin markings to be used in identifying individual zebrafish. Fish were then returned to the holding facility for 9 d. On day 10, the fish were transferred to treatment tanks for 4 d of cortisol or sham treatment, as described above, and on day 14, CT_{max} was measured for the second time, after which fish were terminally anaesthetized, fish mass and length were measured, and fish sex was confirmed. Morgan et al. (2018) reported that heat hardening, an increase in CT_{max} following exposure to elevated temperatures, persisted for a week following measurement of CT_{max}. Therefore, a 2-week gap between trials was used to overcome this thermal acclimation response. Initial work with control fish that underwent the protocol described above showed no significant difference in CT_{max} between trials (Fig. 2.4). For the duration of the experimental period, fish were fed their normal diet daily.

A second experiment evaluated how rapidly CT_{max} recovered following cortisol treatment. Fish were haphazardly allocated to sham or cortisol treatment groups, and within each treatment group, to recovery times of 0, 48 or 96 h (n = 9 fish per group; Table 2.1). Fish in the 0 h recovery group were exposed to sham or cortisol treatment for 4 d, after which CT_{max} was measured. Fish in the 48 and 96 h recovery groups were exposed to sham or cortisol treatment for 4 d and then returned to system water for 48 or 96 h, after which CT_{max} was measured. The experiment was then repeated (n = 6-9 fish per group; Table 2.1), but instead of measuring CT_{max}, fish were terminally anaesthetized at the end of the exposure/recovery period and blood samples were collected for the measurement of plasma cortisol concentrations. Exposure to sham or cortisol treatment, measurement of CT_{max}, collection, and processing of blood samples and measurement of plasma cortisol were as described above.

2.6 Experimental series 2: The role of cortisol receptors

To investigate the receptor through which cortisol exerts its action on thermal tolerance, MR-KO, GR-KO and sWT fish were used together with sWT fish treated with the GR antagonist RU-486. The sWT fish were haphazardly allocated to sham, cortisol, or cortisol + RU-486 treatment groups (n = 8-9 fish per group; Table 2.1), and MR-KO fish were haphazardly allocated to sham or cortisol treatment groups (n = 9 fish per group; Table 2.1). The GR-KO fish were untreated (i.e. held in system water) because these fish experience elevated baseline plasma cortisol levels as a result of the loss of GR-mediated negative feedback of HPI axis activity (Faught and Vijayan, 2018). Measurement of plasma cortisol levels in the GR-KO and sWT lines developed at uOttawa revealed that the GR-KO fish were also hypercortisolemic (Fig. 2.5). Because the GR-KO line had a strong male bias, experiments with KO lines and their sWT controls were

conducted only in males. Sham and cortisol treatments were as described above. For cortisol + RU-486, fish were exposed to 5 μg mL^{-1} cortisol and 2.57 μg mL^{-1} RU-486 (Ziv et al., 2013) in 0.05% ethanol. Following the 4 d exposure period, CT_{max} was measured as described above.

To measure the effects of cortisol treatment on recovery, fish were video recorded for the initial 8 min immediately after LOE and transfer to recovery tanks. Frames were extracted from these recordings using Deep Lab Cut, an open-source software that employs user-defined labels for tracking (Mathis et al., 2018). The labelled frames were then processed in Google Colab with open-source code from the Deep Lab Cut website to extract positional data allowing for the calculation of swimming speed and distance travelled during recovery.

2.7 Experimental series 3: A role for TRPV?

Two experiments were carried out in this series. First, the effect on CT_{max} of inhibiting transient receptor potential cation channel subfamily V1 (TRPV1) activity using capsazepine was assessed. Fish were haphazardly allocated to sham or capsazepine treatment groups (n = 6 fish per group; Table 2.1). Following the 45 min acclimation period to the CT_{max} set-up, capsazepine or vehicle (sham) was administered by intraperitoneal (i.p.) injection according to the methods of Rocha Barreto et al. (2022). Fish were anaesthetized (in 0.12 g L^{-1} of buffered MS-222) and 5 μL of 0.5 mg mL^{-1} capsazepine dissolved in DMSO and added to 0.9% saline was delivered i.p. using a 10 μL Hamilton syringe fitted with a 27 G needle; sham-treated fish received DMSO in 0.9% saline. After being injected, fish were given 10 min to recover in standard 2 L holding tanks before transfer to the CT_{max} tank for measurement of CT_{max} as described above.

A second experiment assessed the effect of cortisol treatment on transcript abundances of *trpv* paralogues. For *trpv1*, no splice variant was identified. In contrast, three splice variants have

been established for *trpv4.* Sequence alignment using protein BLAST revealed that *trpv4* splice variants 1 and 2 were nearly identical, differing only by one amino acid. Given this minimal difference, a single primer set was designed to target both variants (Table 2.2).

For these trials, fish were once again haphazardly allocated to either sham or cortisol treatment for 4 d, at the end of which they were terminally anaesthetized (as described above), and the brain and gills were collected and frozen in lN_2. Tissues were stored at -80°C until processed for the measurement of transcript abundances. Total RNA was extracted from gill tissue following the addition of 1 mL of cold TRIzol® reagent (Invitrogen, Burlington, ON, Canada) per 30-50 mg of tissue and homogenization with a sonicator (Sonic Dismembranator, Model 100; Fisher Scientific, Waltham, MA, USA). The resulting RNA pellet was dissolved in nuclease-free water and RNA quality, and concentration were assessed using a Nanodrop® spectrophotometer (Fisher Scientific). To eliminate genomic DNA contamination, RNA was treated with DNAse I using the DNAse Amplification Grade kit (Invitrogen, Catalog No.18068015) according to the manufacturer's instructions. Subsequently, DNAse-treated RNA was reverse transcribed into cDNA using a high-capacity cDNA synthesis kit (Applied Biosystems, Waltham, MA, USA).

The relative expression of target genes was assessed by semi-quantitative real-time RT-PCR (qPCR) using a CFX Opus 96 real-time PCR machine (Bio-Rad Laboratories, Mississauga, ON, CA). The PCR cycling conditions used the following steps: an initial denaturation of three min at 95°C, followed by 40 cycles of 15 s at 95°C, followed by annealing for 30 s at 58°C, and an extension of 2 s at 72°C. The reactions consisted of 0.5 µL of each forward and reverse primer (Table 2.2) and 5 µL of Blast Taq 2x qPCR Master Mix, containing the DNA polymerase (Applied Biological Materials Inc, Richmond, BC, CA) added to 2 µL of cDNA template. A

standard curve was generated for each primer set from pooled cDNA samples to assess primer efficiency (Table 2.2). Quality controls included no template controls (reactions in which cDNA was not added), and no reaction mix wells, where only cDNA was added, to monitor for contamination. Melt curve analyses following each run confirmed the specificity of amplification. The relative mRNA abundance of target genes was normalized to the arithmetic mean of two housekeeping genes, elongation factor (*ef1α*) and *β*-actin (*actb,* Table 2.2) and expressed as fold-change relative to the sham treatment group using the method of Pfaffl (2001).

2.8 Experimental series 4: Effects of early-life cortisol exposure

To determine whether early life exposure to cortisol influences thermal tolerance later in life, fish were treated with cortisol from 0 to 5 days post-fertilization (dpf). Fertilized eggs were collected from two separate breeding events using WT adult zebrafish from the University of Ottawa breeding program. Immediately after collection, the fertilized eggs were divided into two treatment groups, with half being placed in embryo medium containing 10 μg mL^{-1} cortisol in 0.065% ethanol and the other half being placed in embryo medium containing ethanol alone, as described by Best and Vijayan (2018). When the fish reached 3 or 8 months of age, CT_{max} was measured as described above (n = 9 fish per group; Table 2.1).

2.9 Experimental series 5: Measurement of thermal preference

Fish were haphazardly allocated to sham or cortisol treatment groups (n = 9 fish per group; Table 2.1) and treated for 4 d as described above. At the end of the 4 d treatment, fish were transferred individually to a shuttle box system (Loligo Systems, Viborg, Denmark) to assess the effects of cortisol treatment on thermal preference.

The shuttle box system was located in a quiet room with a 14L:10D photoperiod to avoid disturbance of the fish during the experiment. The shuttle box arena contained ~21 L of system water and consisted of two circular compartments of equal sizes (each 30 cm diameter) that were initially set to and maintained at 28 and 30°C, based on the protocol of Ripley et al. (2022). To prevent mixing between the chambers, water from each chamber was continuously circulated through a buffer tank using recirculating pumps, creating mild currents of opposite directions in the two chambers. Temperature regulation was achieved by passing water through titanium coils submerged in temperature-controlled baths (12°C for cooling and 37°C for heating), then pumping it into the appropriate buffer tanks and chambers. The compartments were connected by a short passageway, allowing the fish to move freely between the chambers and sample both temperatures during the first hour of the trial.

After this acclimation period, the system was switched to the 'dynamic' mode, in which the water temperature was adjusted according to the fish's location, for 24 h of recording. When the fish entered the warmer compartment, the temperature in both compartments increased at a rate of 4°C h^{-1}. Conversely, when the fish moved to the cooler compartment, the water temperatures in both chambers decreased at the same rate of 4°C h^{-1}. Throughout the experiment, the 2°C temperature difference between the compartments was maintained. By shuttling between the compartments, the fish could alter water temperature until its preferred temperature was achieved. The chamber designated as the warmer compartment was alternated between fish, and the water in the shuttle tank was replaced after each trial.

The location of the zebrafish was tracked by means of an overhead camera connected to ShuttleSoft software (Loligo Systems). The water temperatures in each chamber were recorded every second. The thermal preference of each fish was determined from the initial 12 h of

tracking during the dynamic phase. This duration was selected to maximize the number of fish that could be included in the analysis; unexpected system shutdowns truncated data collection for several fish. Fish that failed to exhibit shuttling during the acclimation phase were eliminated from further consideration (2 fish).

2.10 Statistical analysis

Data analysis was carried out using SigmaPlot v13.0 (Grafiti LLC, Palo Alto, CA, USA) with a fiducial limit of significance (a) of 0.05. Data are presented as means ± standard error of the mean (SEM). Prior to statistical analysis, data sets were assessed for normality and equal variance using Shapiro-Wilk and Brown-Forsythe tests, respectively. Where data did not meet these assumptions, they were transformed to achieve normality and equal variance. Data that could not be transformed to meet the assumptions were analyzed with an equivalent non-parametric test.

A repeated measures (RM) two-way analysis of variance (ANOVA) was used to assess the effects of treatment and trial on CT_{max}. Two-way ANOVAs were used to assess effects of treatment and sex on CT_{max}, effects of treatment and recovery duration on CT_{max} and plasma cortisol, and to analyze interactions between age and early life cortisol exposure on adult CT_{max}. A one-way ANOVA was used to assess the effects of corticosteroid receptor knockout or inhibition on CT_{max} and the effect or treatment of recovery after LOE. Student's *t*-tests were used to determine whether significant differences existed between sham and cortisol-treated fish in plasma cortisol, transcript abundances of *trpv4* splice variants and thermal preference. The Mann-Whitney rank sum test was used to determine whether differences existed between *trpv* abundances of sham and cortisol-treated fish. A Student's *t*-test was also used to assess the

impact of capsazepine treatment on CT_{max}. Finally, the repeatability of CT_{max} ($R = 0.62 \pm 0.21$) was estimated from the data for sham-treated fish from Experimental series 1 using a Linear Mixed Model.

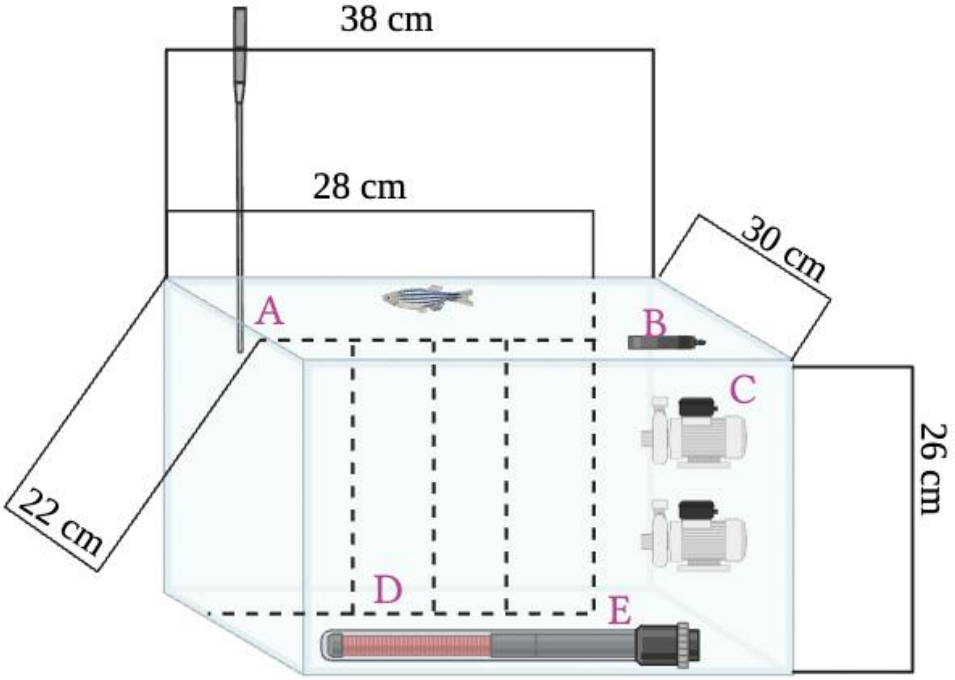
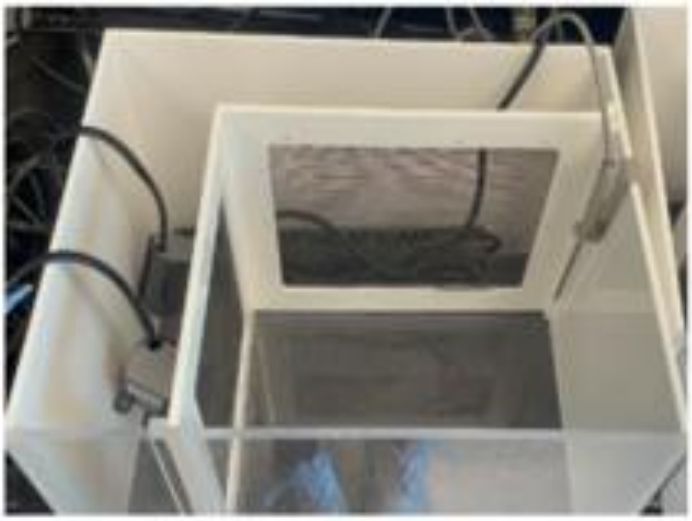


Figure 2.1. Schematic of CT_{max} experimental set-up. (A) Temperature probe to measure water temperature; (B) Air stone to ensure sufficient aeration; (C) submersible pumps ensuring homogenous temperature across the tank; (D) mesh insert to separate equipment from fish on trial; (E) aquarium heater (500W).

A



B

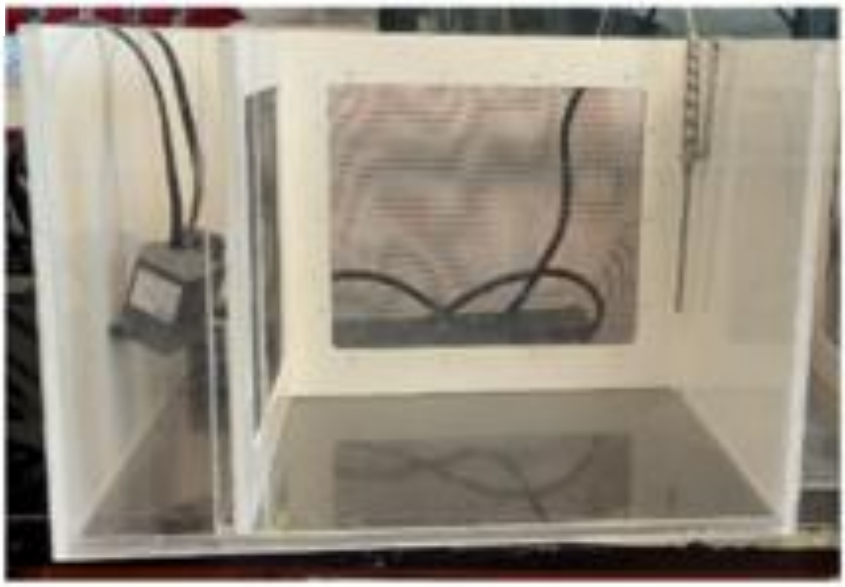


Figure 2.2. Photographs of the CT_{max} experimental set-up. (A) Overhead view of the CT_{max}, tank; (B) Side view of the CT_{max} tank.

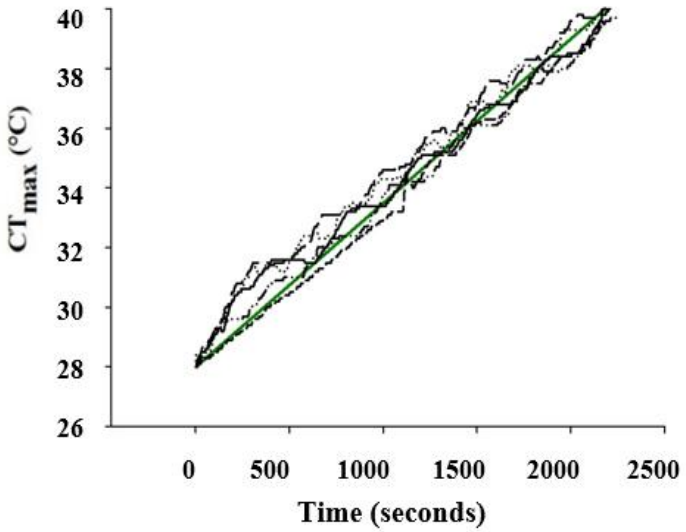


Figure 2.3. Water temperature increases over time for a selection of CT_{max} trials. The black lines present water temperatures during five representative CT_{max} measurements and the green line illustrates a linear heating rate of 0.33 ± 0.01°C min^{-1}.

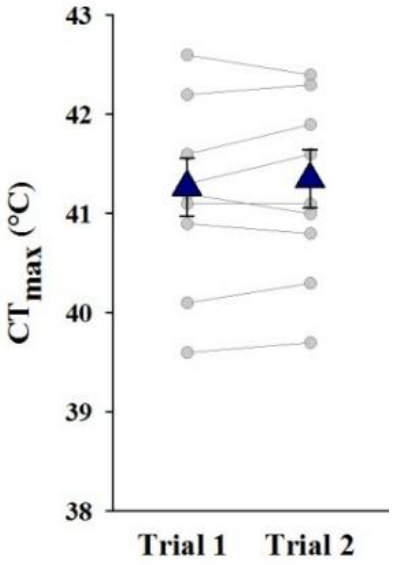


Figure 2.4. Pilot CTmax trials carried out in the same zebrafish (Danio rerio) with a two-week gap between measurements. The triangles present mean values ± SEM and grey circles represent values for individual fish. No significant differences were detected (paired Student's t-test, P = 0.235, n = 10).

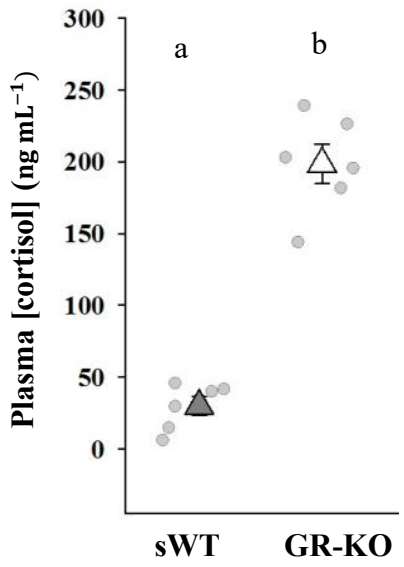


Figure 2.5. Measurement of baseline plasma cortisol concentrations in GR-KO and sWT zebrafish (Danio rerio). The triangles indicate mean values ± SEM and grey circles represent values for individual fish. Groups sharing a letter are not significantly different from one another (Student's t-test, $P < 0.001$, $n = 6$). These data were collected by Michael Tea.

Table 2.1. Fish mass and length for each experimental series

Experimental series	Treatment groups	Mass (g)	*P* value	Fork length (cm)	*P* value
Pilot series	Control ($n = 6$)	0.371 ± 0.03	0.398	3.4 ± 0.133	0.302
	Sham ($n = 6$)	0.333 ± 0.024		3.2 ± 0.07	
Series 1: Effect of cortisol treatment on CT_{max}	Sham ($n = 6$ recorded)	0.367 ± 0.038	0.600	3.3 ± 0.115	0.419
	Cortisol ($n = 11$)	0.397 ± 0.04		3.5 ± 0.11	
Series 1: Effect of recovery on CT_{max}	Sham ($n = 27$)	0.267 ± 0.019	0.001	3.1 ± 0.079	0.065
	Cortisol ($n = 27$)	0.370 ± 0.025		3.3 ± 0.087	
Series 1: Effect of recovery on plasma cortisol	Sham ($n = 21$)	0.435 ± 0.028	0.924	3.6 ± 0.067	0.394
	Cortisol ($n = 21$)	0.432 ± 0.019		3.4 ± 0.133	
Series 2: The role of cortisol receptors	sWT Sham ($n = 9$)	0.325 ± 0.02	0.07	3.3 ± 0.025	0.001
	sWT Cortisol ($n = 9$)	0.291 ± 0.04		3.2 ± 0.143	
	sWT Cortisol + RU486 ($n = 8$)	0.329 ± 0.02		3.5 ± 0.025	
	GR-KO ($n = 9$)	0.421 ± 0.05		3.7 ± 0.08	
	MR-KO Sham ($n = 9$)	0.259 ± 0.03	0.480	3.0 ± 0.089	0.257
	MR-KO Cortisol ($n = 9$)	0.297 ± 0.04		3.1 ± 0.084	
Series 3: A role for TRPV? The effects of capsazepine on CT_{max}	Sham ($n = 6$)	0.227 ± 0.029	0.545	3.0 ± 0.13	0.137
	Capsazepine ($n = 6$)	0.249 ±0.016		3.2 ± 0.08	
Series 3: A role for TRPV? Effects of cortisol on TRPV paralogues	Sham ($n = 9$)	0.309 ± 0.018	0.002	N/A	N/A
	Cortisol ($n = 9$)	0.551 ± 0.067		3.8 ± 0.105	
Series 4: Effect of early-life cortisol exposure	8-month Sham ($n = 9$)	0.379 ± 0.032	0.151	3.4 ± 0.107	1
	8-month Cortisol ($n = 9$)	0.444 ± 0.027		3.4 ± 0.102	

Series 5: Measurement of thermal preference	Sham (n = 9)	0.416 ± 0.038	0.400	N/A	N/A
	Cortisol (n = 9)	0.371 ± 0.035		3.65 ± 0.117	

Values are means ± SEM. Comparisons of mass or fork length between treatment groups were carried out using Student's *t*-tests or ANOVA, as appropriate. Morphological data were not recorded for 3 month old fish in Series 4.

Table 2.2. Primers used for semi-quantitative real-time RT-PCR

Primer sequence (5' to 3')	GenBank Accession number	Amplicon Size	Efficiency	R^2 value	Source
F: tgtccctgtatgcctctggt R: aagtccagacggaggatgg	NM_181601.5	121	104.8	0.961	Alsop & Vijayan, 2008
F: ctggaggccagctcaaacat R: atcaagaagagtagtaccgctagcattac	NM_131263.1	87	94.0	0.973	Tang et al., 2007
F: acctcaagccaagttactcaca R: gtgacgccttcttagtctcaca	XM_005165327.5	115	97.6	0.965	
F: tgactgacggtttttctgcttc R: gtggtgaggagacagacaagg	XM_005165152.5	81	107.0	0.991	
F: gagcttctggtggagaaggg R: gagaggggcagttcaccaaa	XM_068221210.2	107	91.5	0.985	

Chapter 3: Results

3.1 Pilot series and validation of cortisol treatment

Preliminary experiments revealed that CT_{max} in control (untreated) fish did not differ significantly from that in sham-treated fish (Fig. 3.1A). Based on these findings, experiments to test the effect of cortisol on CT_{max} used a two-week gap between measurements of CT_{max} and did not include control (untreated) fish. In a final preliminary experiment, 4 d of cortisol treatment resulted in plasma cortisol levels that were significantly higher than those in sham-treated fish (Fig. 3.1B).

3.2 Experimental series 1: Effect of cortisol treatment on CT_{max}

To test the effect of cortisol on CT_{max}, CT_{max} was measured before and after 4 d of sham or cortisol treatment, with the treatment period occurring for the final 4 d of the two-week gap between measurements. In cortisol-treated but not sham fish, the second CT_{max} was significantly lower than the first (Fig. 3.2A). The effect of cortisol treatment on CT_{max} was not sex-dependent (Fig. 3.2B), and therefore most subsequent experiments included both male and female fish.

To test whether recovery of plasma cortisol levels would restore normal CT_{max} values, fish were given 0, 48 or 96 h of recovery from 4 d of cortisol treatment, and CT_{max} or plasma cortisol levels were measured. Although analysis of CT_{max} values did not detect significant differences between groups, it did reveal a trend for a significant effect of treatment (Fig. 3.3A). Use of Student's *t*-tests to compare CT_{max} values between cortisol-treated and sham fish independently at each recovery time revealed a significant effect of cortisol treatment only in the 0 h recovery group ($P = 0.0250$) and not in the 48 h and 96 h recovery groups ($P = 0.521$ and $P = 0.523$, respectively). Similar results were obtained for plasma cortisol. Again, no significant differences were detected with analysis by two-way ANOVA (Fig. 3.3B). However, using Student's *t*-tests to compare cortisol levels between cortisol-treated and sham

fish at each recovery time revealed a significant difference only in the 0 h recovery group (P = 0.0191). Plasma cortisol levels did not differ at 48 h or 96 h (P = 0.936 and P = 0.310, respectively).

3.3 Experimental series 2: The role of cortisol receptors

To assess the role of cortisol receptors in mediating the effect of cortisol on CT_{max}, GR-KO, MR-KO and sWT fish were employed together with the GR antagonist, RU486. Cortisol-treated sWT fish had significantly lower CT_{max} than sham-exposed sWT fish, or sWT fish co-treated with cortisol and RU486 (Fig. 3.4A). The CT_{max} of GR-KO fish did not differ from that of sWT fish (Fig. 3.4A). Independently, CT_{max} was measured in sham and cortisol-treated MR-KO fish (Fig. 3.4B), revealing significantly lower values in cortisol-treated fish.

To investigate whether cortisol treatment affects recovery from the LOE caused by acute warming to CT_{max}, swimming speed and distance travelled were determined from video recordings of the initial 10 min in 28°C water after LOE. Neither average speed nor distance travelled differed between sham and cortisol-treated sWT fish and GR-KO fish (Fig. 3.5A, B).

3.4 Experimental series 3: A role for TRPV?

To investigate whether TRPV1 plays a role in determining CT_{max}, TRPV1 activity was manipulated using the antagonist capsazepine. Capsazepine-treated fish had a significantly higher CT_{max} than sham-treated fish exposed to the vehicle DMSO alone (Fig. 3.6A). Transcript abundances of *trpv* paralogues in gill tissue were compared between sham and cortisol-treated fish. No significant differences in the relative mRNA abundances of *trpv1* and *trpv4 (*splice variants 1,and 2) were found between treatment groups (Figs. 3.6B, C).

However, a significant difference in relative mRNA abundance of *trpv4* splice variant 3 was detected between treatment groups (Fig. 3.6D).

3.5 Experimental series 4: Effects of early-life cortisol exposure

To investigate the effect of early-life stress on thermal tolerance later in life, zebrafish were treated with cortisol or vehicle only (sham) from 0 to 5 dpf and then raised under normal holding conditions to 3 or 8 months of age, when CT_{max} was measured. Zebrafish treated with cortisol during early development had significantly lower CT_{max} values than their sham counterparts regardless of the adult age at which CT_{max} was measured (Fig. 3.7).

3.6 Experimental series 5: Measurement of thermal preference

A shuttlebox system was used to assess thermal preference in cortisol-treated zebrafish. No significant difference in thermal preference was detected between cortisol-treated and sham fish (Fig. 3.8).

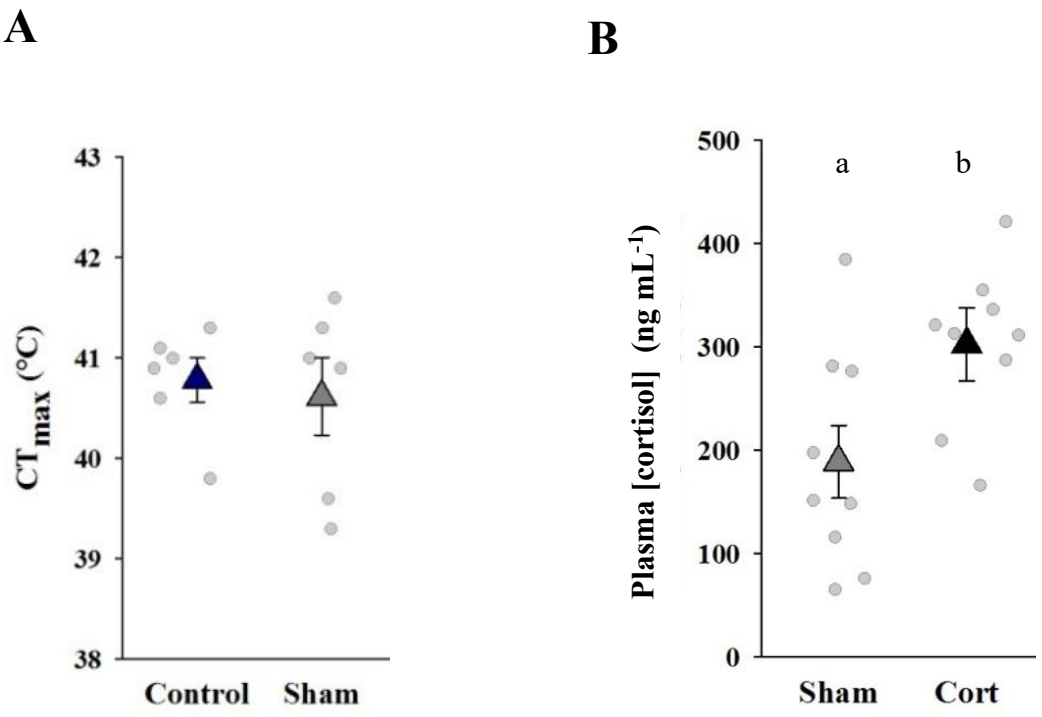


Figure 3.1. Measurements of CT_{max} in zebrafish (*Danio rerio*) carried out in untreated (control) versus sham-treated fish (A). Panel (B) presents plasma cortisol following 4 d of cortisol or vehicle alone (sham) treatment. The triangles present mean values ± SEM and grey circles represent values for individual fish. No significant differences were detected in panel A (Student's *t*-test, $P = 0.714$, $n = 6$ fish per group). In panel B, groups that share a letter are not significantly different from one another (Student's *t*-test, $P = 0.0191$, $n = 9$ fish per group**).**

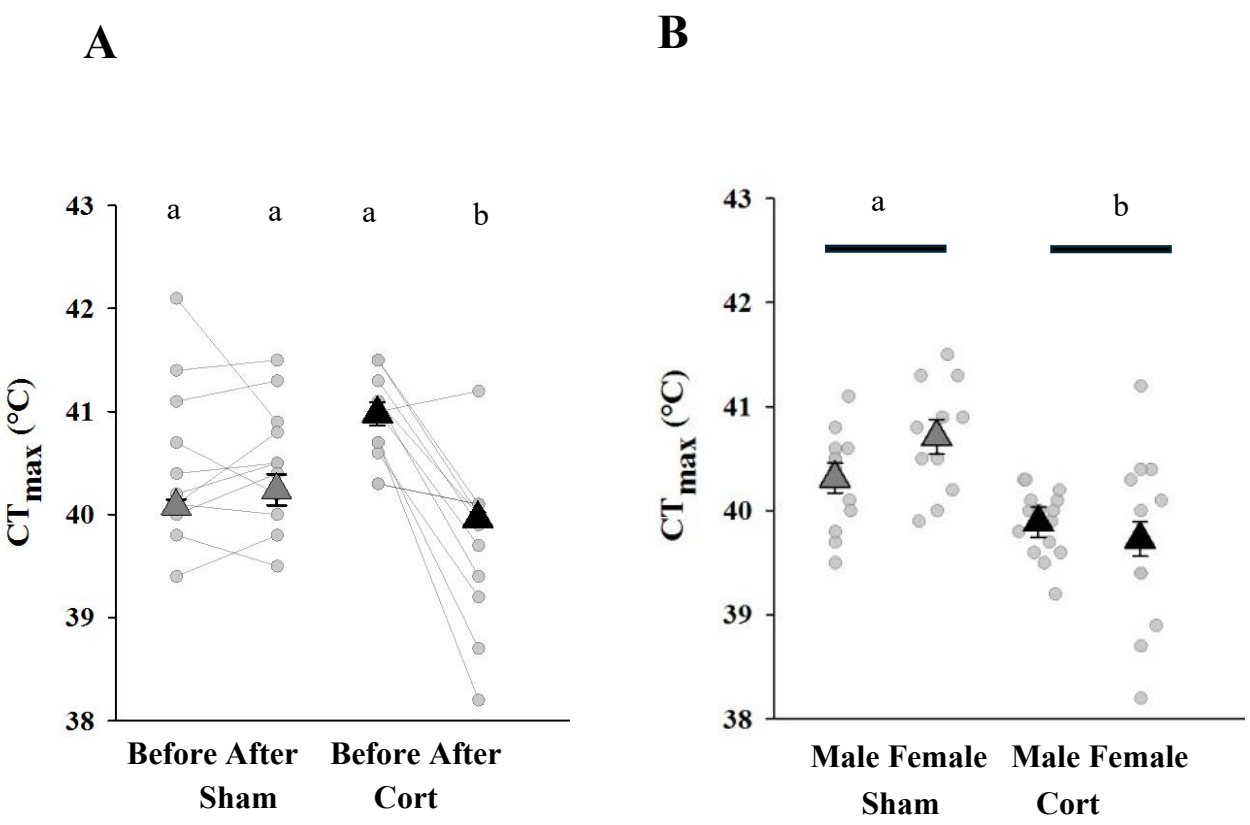
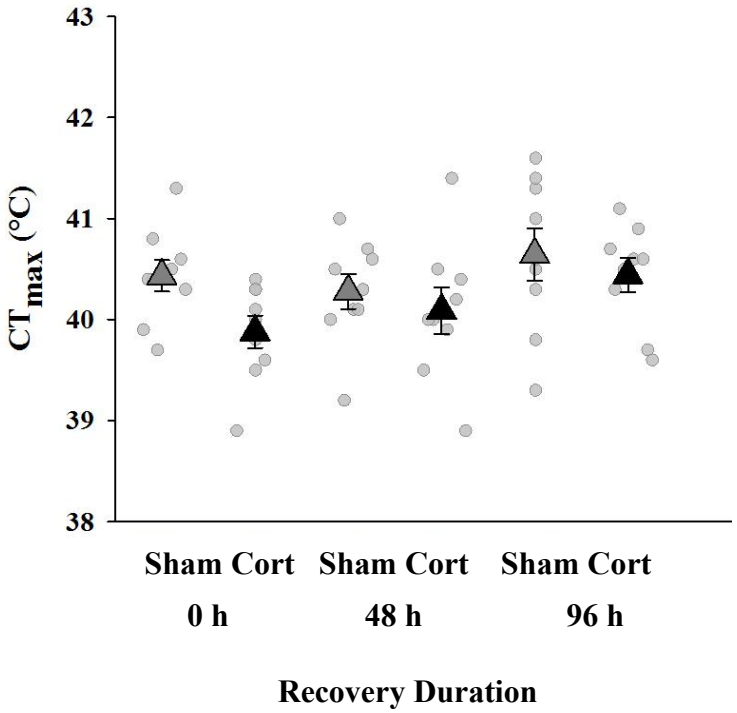


Figure 3.2. Measurements of CT_{max} in zebrafish (*Danio rerio*) before and after cortisol or sham treatment (A). Measurements of CT_{max} were also compiled across trials in male versus female fish (B). The triangles indicate mean values ± SEM and grey circles represent values for individual fish. Values that share a letter are not significantly different from one another (panel A; 2-way RM ANOVA, $P_{trial} < 0.001$, $P_{treatment} = 0.539$, $P_{trial \times treatment} < 0.001$, $n = 11$ fish per group; panel B; 2-way ANOVA, $P_{treatment} < 0.001$, $P_{sex} = 0.451$, $P_{treatment \times sex} = 0.081$, $n = 22$-30 fish per group).

A


43
42
41
40
39
38
CT_{max} (°C)
Sham Cort
Sham Cort
Sham Cort
0 h
48 h
96 h
Recovery Duration

B

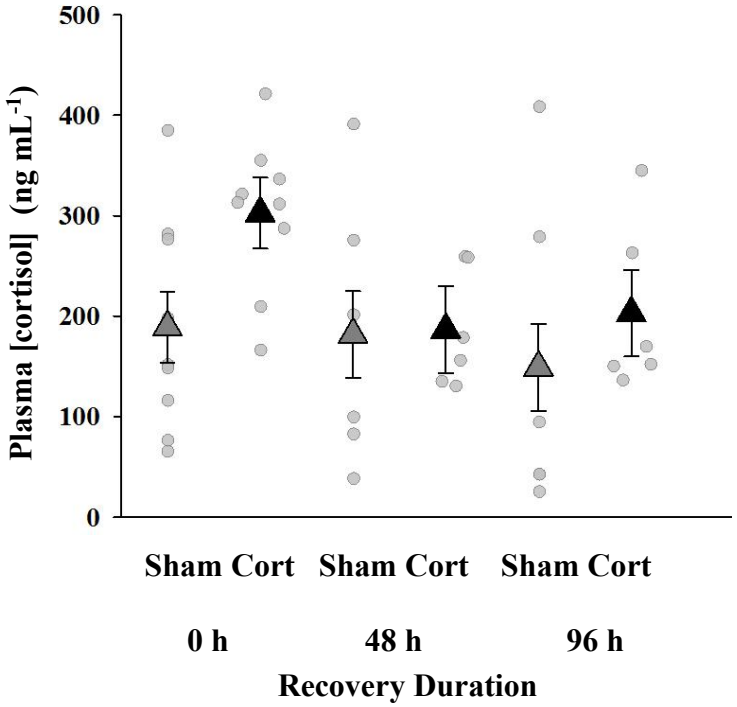
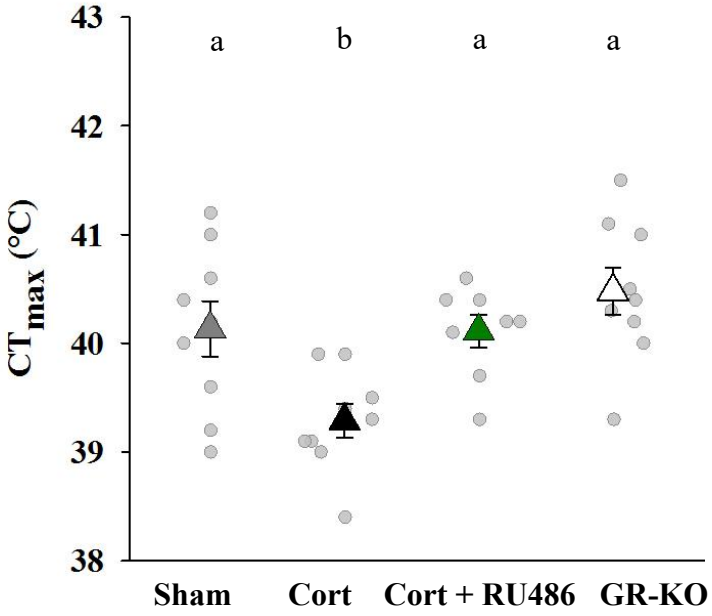

500
400
300
200
100
0
Plasma [cortisol] (ng mL^{-1})
Sham Cort
Sham Cort
Sham Cort
0 h
48 h
96 h
Recovery Duration

Figure 3.3. Measurements of CT_{max} (A) and plasma cortisol (B) for sham or cortisol-treated zebrafish (*Danio rerio*) given 0, 48 or 96 h of recovery from treatment. The triangles indicate mean values ± SEM and grey circles represent values for individual fish. No significant effects of cortisol treatment or recovery time were detected (2-way ANOVA, panel A, $P_{treatment} = 0.053$, $P_{recovery} = 0.094$, $P_{treatment\ x\ recovery} = 0.567$, $n = 9$ fish per group; panel B, $P_{treatment} = 0.093$, $P_{recovery} = 0.150$, $P_{treatment\ x\ recovery} = 0.388$, $n = 6$-9 fish per group).

A


43
42
41
40
39
38
CT_{max} (°C)
a
b
a
a
Sham
Cort
Cort + RU486
GR-KO

B

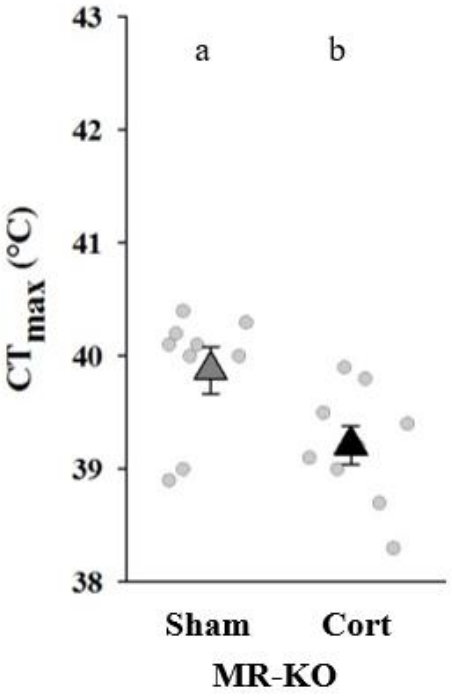

43
42
41
40
39
38
CT_{max} (°C)
a
b
Sham
Cort
MR-KO

Figure 3.4. Measurements of CT_{max} in sibling wild-type (sWT) zebrafish (*Danio rerio*) treated with cortisol or cortisol and RU486, and in zebrafish lacking functional expression of the glucocorticoid receptor (GR-KO; A). Panel (B) presents CT_{max} in zebrafish lacking functional expression of the mineralocorticoid receptor (MR-KO) following sham or cortisol treatment. The triangles indicate mean values ± SEM and grey circles represent values for individual fish. Groups that share a letter are not significantly different from one another (A, ANOVA, $P = 0.002$, $n = 8$-9 fish per group; B, Student's *t*-test, $P = 0.006$, and $n = 9$ fish per group).

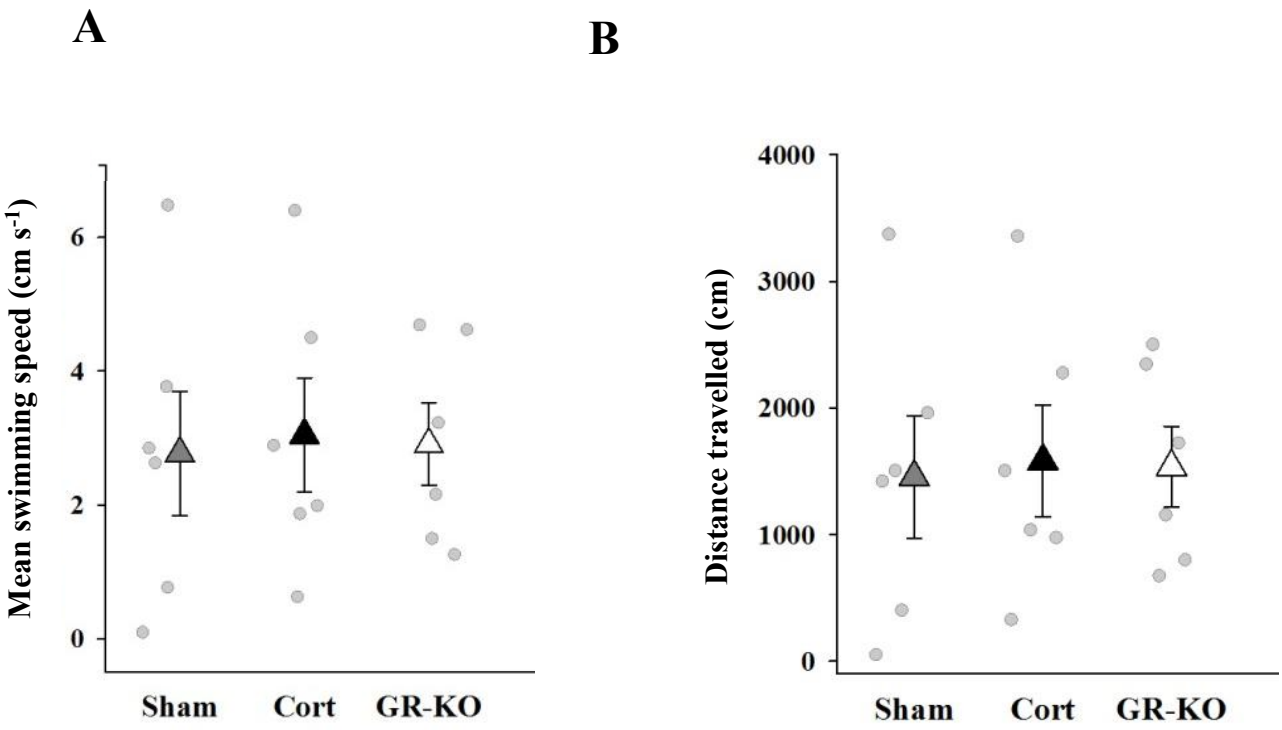


Figure 3.5. Mean swimming speed (A) and distance travelled (B) during the initial 15 min of recovery in 28°C water following loss of equilibrium (LOE) during the measurement of CT_{max} for sibling wild-type (sWT) zebrafish (*Danio rerio*) exposed to 4 d of cortisol or sham treatment and zebrafish lacking functional expression of the glucocorticoid receptor (GR-KO). Triangles indicate mean values ± SEM and grey circles represent values for individual fish. No significant differences were detected (ANOVA, $P = 0.971$ and 0.977 for panels A and B, respectively, $n = 6$ fish per group).

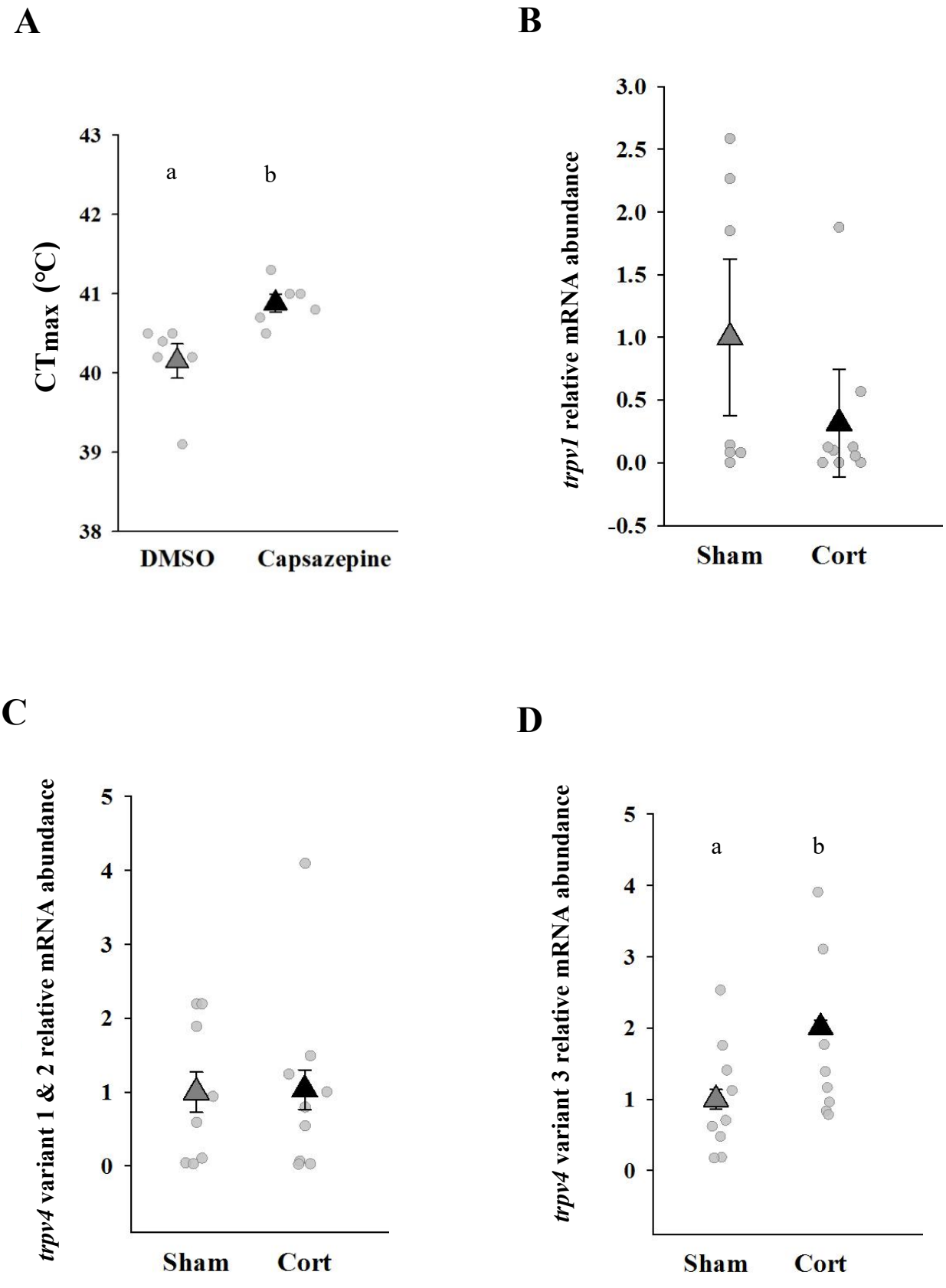

A
B
C
D
CT_{max} (°C)
43
42
41
40
39
38
a
b
DMSO
Capsazepine
trpv1 relative mRNA abundance
3.0
2.5
2.0
1.5
1.0
0.5
0.0
-0.5
Sham
Cort
trpv4 variant 1 & 2 relative mRNA abundance
5
4
3
2
1
0
Sham
Cort
trpv4 variant 3 relative mRNA abundance
5
4
3
2
1
0
a
b
Sham
Cort

Figure 3.6. Measurement of CT_{max} in capsazepine-treated zebrafish (*Danio rerio*) and their sham counterparts (A). Panels B to D present transcript abundances of *trpv* paralogues in gill tissue of sham and cortisol-treated fish. Groups that share a letter are not significantly different from one another (Student's *t*-tests, $P = 0.00530$, $n = 6$ fish per group for panel A; $P = 0.452$, $n = 6$ fish per group for panel B; Mann-Whitney Rank Sum test, $P = 0.736$, $n = 8$-9 fish per group for panel C; Student's *t*-test, $P = 0.0432$, $n = 9$ fish per group for panel D).

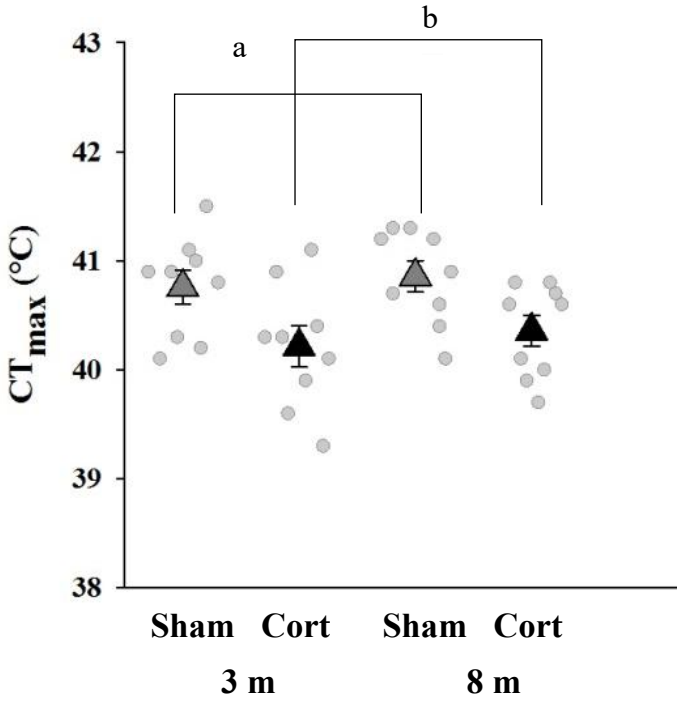


Figure 3.7. Measurement of CT_{max} in 3- and 8-month old adult zebrafish (*Danio rerio*) that were exposed to cortisol or sham treatment from 0 to 5 dpf. Triangles indicate mean values ± SEM and grey circles represent values for individual fish. Groups that share a letter are not significantly different from one another (2-way ANOVA, $P_{treatment} = 0.003$, $P_{age} = 0.449$, $P_{treatment\ x\ age} = 0.890$, $n = 9$ fish per group).

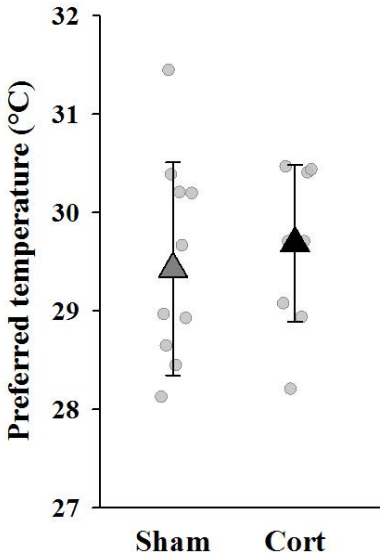


Figure 3.8. The effect of cortisol treatment on thermal preference in cortisol-treated and sham zebrafish (*Danio rerio*). Triangles indicate mean values ± SEM and grey circles represent values for individual fish. No significant difference was detected (Student's *t*-test, $P = 0.573$, $n = 9$ fish per group).

Chapter 4: Discussion

The present research revealed that zebrafish respond to waterborne cortisol treatment with a pronounced reduction in thermal tolerance, a finding that was similar to earlier work in rainbow trout (LeBlanc et al., 2011; Bard et al., 2021; Bard, 2025). Additionally, I demonstrated that this effect was mediated by the GR but not the MR using GR-KO and MR-KO zebrafish lines and the GR antagonist, RU486. The effect of cortisol was reversible, because allowing cortisol to return to baseline levels restored CT_{max}. Cortisol treatment increased the transcript abundance of a single *trpv4* paralogue in gill tissue, suggesting that cortisol may exert its effects by altering thermal sensing. Cortisol treatment did not affect recovery from LOE, nor did it change thermal preference. Finally, treatment of developing fish with cortisol from 0 to 5 dpf resulted in lower CT_{max} in 3- and 8-month old adult fish, suggesting that early exposure to cortisol 'programs' the fish to be less tolerant of high temperatures.

4.1 Methodological considerations

The initial studies of stress and thermal tolerance in rainbow trout used social interactions to achieve chronic elevation of endogenous cortisol levels (LeBlanc et al. 2011, Bard et al. 2021). Bard et al. (2021) also treated subordinate fish with a cortisol-impregnated cocoa butter implant in the peritoneal cavity to maintain elevated cortisol levels in fish recovering from social stress. Bard (2025) continued to use cocoa butter implants, but in addition used mini-osmotic pumps placed in the peritoneal cavity, which allowed for a prolonged release of cortisol, maintaining elevated levels for up to 28 d. In contrast, my study employed waterborne delivery of cortisol and a sham group treated with the vehicle (ethanol) alone, as in other work on zebrafish (Faught and Vijayan, 2022), ensuring that the only difference between groups was the presence of cortisol. This approach yielded plasma cortisol levels in cortisol-treated fish that were comparable to or higher than those observed in subordinate

zebrafish (Filby et al., 2010; Tea et al. 2019), but plasma cortisol levels in sham-treated fish were also somewhat elevated, albeit they were significantly lower than those in cortisol-treated fish. The waterborne cortisol treatment required that water be renewed daily because the ethanol vehicle caused the water to be clouded. It is possible that this cloudiness, along with the repeated water changes, contributed to the elevation of baseline cortisol levels observed in the sham fish. However, the absence of a difference in CT_{max} between sham and (untreated) control fish diminished concerns that stress in sham fish may have impacted thermal tolerance.

Measurements of CT_{max} were carried out using a custom-built automated temperature control system developed in partnership with the Electronics shop of the Faculty of Science, University of Ottawa. Repeated trials of the system confirmed that water temperature increased linearly at a rate of 0.3°C per minute, the commonly used rate for small-bodied fishes (Raby et al., 2025). This warming rate is used for fish species in which body temperature tracks changes in water temperature with little or no lag, an assumption that was tested empirically and found to hold for zebrafish (Morgan et al., 2018). In trout, however, Bard et al. (2021) found that a lag can occur when larger fish (e.g. >150 g) are used. The absence of such a lag in zebrafish provided confidence that the recorded CT_{max} values accurately reflected the actual body temperatures experienced by the fish at LOE.

4.2 The effect of cortisol on thermal tolerance and recovery

A key finding of the present study was that prolonged elevation of plasma cortisol lowered CT_{max}, a widely used measure of thermal tolerance. This observation extends the previous work in rainbow trout (LeBlanc et al., 2011; Bard et al., 2021; Bard, 2025) to a second species in a different order (Cypriniformes vs Salmoniformes), suggesting that it may be a widespread consequence of chronic cortisol elevation. In zebrafish, CT_{max} was reduced in

some individuals by ~2°C, although the mean decrease tended to be ~1°C; in trout the reduction was ~1°C, following increases in plasma cortisol that were comparable in the two species (~100 ng mL^{-1} in both species; Fig. 3.1; Bard et al 2021; Bard, 2025). The use of a paired design for assessing CT_{max} before and after cortisol treatment in the same individual provided greater insight into the impact of cortisol in zebrafish than the comparison of different treatment groups used in rainbow trout (Bard, 2025). However, it is also possible that zebrafish are more vulnerable to cortisol-induced decreases in thermal tolerance than rainbow trout, possibly because they naturally inhabit warmer environments that elevate metabolic demands (Schulte, 2015; Morgan et al., 2019; Sundin et al., 2019). The metabolic demands of living at warm temperature in combination with the effects of cortisol, which also increases metabolic load (Morgan and Iwama, 1996; Haese et al., 2016; Pfalzgraff et al., 2022), may contribute to a stronger effect of cortisol on CT_{max} in zebrafish.

The use of zebrafish rather than rainbow trout also allowed me to ask whether male and female zebrafish differ in CT_{max} and whether sex influenced the effect of cortisol on thermal tolerance. Studies that have assessed effects of sex on CT_{max} in fishes are sparse and have yielded mixed results, with Johnson (1976) reporting higher thermal tolerance in female over male mosquitofish (*Gambusia affinis*), but Wheeler et al. (2022) finding no effect of sex on CT_{max} in an elasmobranch, the epaulette shark (*Hemiscyllium ocellatum*). Interestingly, work by Ishihara et al (2015) demonstrated that steroid hormones, particularly estrogen, can protect neuronal activity against oxidative stress suggesting a potential mechanism by which female fish might maintain higher thermal tolerance under stressful conditions relative to male fish. It is also worth noting that reproductively active fish tend to have lower thermal tolerance than fish that are not reproductively active, perhaps because of the metabolic demands of reproduction (Dahlke et al., 2020; Moffett et al., 2022). In the present study, the reproductive status of female fish was not tracked. Although CT_{max} did not differ between

male and female zebrafish, a trend for the interaction of sex and cortisol treatment to influence CT_{max} was observed although it did not reach statistical significance. Thus, male and female fish were generally included in the experiments of the present study and no further sex-based analyses were pursued.

Cortisol treatment did not, however, affect recovery from thermal stress after the fish experienced LOE, because no significant differences were observed in swimming speed or distance travelled between treatment groups. This finding is consistent with previous work demonstrating that whether rainbow darter (*Etheostoma caeruleum*) had undergone thermal or hypoxic stress to the point of LOE, they recovered similarly as measured by their aerobic scopes, recovery time and post-warming oxygen consumption (Borowiec et al., 2024).

4.3 Mechanisms of action underlying the effect of cortisol on CTmax

Although the effect of prolonged cortisol treatment on CT_{max} appears to be robust, the mechanisms through which cortisol lowers thermal tolerance have yet to be fully elucidated. Similarly to rainbow trout (Bard, 2025), cortisol reduces thermal tolerance in zebrafish by acting through the GR, because effects of cortisol on CT_{max} were eliminated by cotreatment with the GR antagonist RU486. In agreement with this observation, CT_{max} in GR-KO zebrafish did not differ from that in sham-treated sWT fish, despite chronic hypercortisolemia of GR-KO fish stemming from the lack of negative feedback regulation of the HPI axis (Faught and Vijayan, 2018). Indeed, plasma cortisol levels in the GR-KO fish were on par with those in cortisol-treated WT fish, even in the absence of exogenous cortisol treatment. Cortisol-treated MR-KO fish experienced a significant reduction in CT_{max} compared to MR-KO sham-treated fish. Importantly, CT_{max} in sWT sham-treated fish did not differ from that in MR-KO sham-treated fish, confirming that loss of the MR did not alter thermal tolerance. The dominant role of the GR in mediating the effects of cortisol on CT_{max} is consistent with

the higher affinity of the MR for cortisol relative to the GR, which results in the MR being fully occupied by cortisol at baseline cortisol levels (Arriza et al., 1988; Viengchauren et al., 2007; Faught and Vijayan, 2022).

To further investigate the underlying pathways, I examined the role of TRP channels known to be involved in thermal sensing, focusing in particular on TRPV1 because it is activated by high temperatures (Gau et al., 2013) and was implicated in temperature sensing leading to LOE in rainbow trout (Bard, 2025). Similar to Bard (2025), blocking TRPV1 with the antagonist capsazepine increased CT_{max} relative to sham fish, implicating TRPV1 in the thermal sensing pathways underlying LOE. Bard (2025) also used capsaicin to activate TRPV1, which lowered CT_{max}, but this was not possible in zebrafish because TRPV1 in this species is not responsive to capsaicin (Gau et al., 2013). However, the observation that manipulation of TRPV1 activity altered CT_{max} suggests that changes in thermal sensing may underlie differences in thermal tolerance. In support of this possibility, Melanson et al. (2023) reported that a desensitization of TRPV1 was responsible for the emergence of socially experienced mangrove rivulus (*Kryptolebias marmoratus*) from the water at higher temperatures than that of fish that were socially naive. Thus, cortisol-induced changes in the sensitivity of thermal sensing constitute a possible mechanism for the effects of cortisol treatment on thermal tolerance. As a first step in examining this hypothesis, I asked whether cortisol altered *trpv* paralogue expression. Measurements of *trpv* transcript abundance focused on the gill, which is well known as a site of O_2 and CO_2 sensing in fish (Perry and Gilmour, 2002; Burleson, 2009; Jonz et al., 2015; Perry and Tzaneva, 2016; Florindo et al., 2018; Perry et al., 2023) and could, given the high rate of water flow over gill tissue, also serve as an effective peripheral site of temperature sensing.

Cortisol-treated fish had significantly higher expression of the *trpv4* splice variant 3 in gill tissue, whereas no differences were observed for *trpv1*, as well as *trpv4* splice variants 1

and 2. Similarly, Bard (2025) reported significant effects of cortisol treatment on trpv4 but not *trpv1* in gill tissue. Once cortisol binds to a GR, the cortisol-GR complex enters the nucleus and binds to a glucocorticoid response element (GRE) to regulate transcription (Surjit et al., 2011; Ramamoorthy and Cidlowski, 2016). Thus, an important next step is to determine whether GREs are present in the promoter region of *trpv4*, as was the case for some but not all paralogues of *trpv1* and *trpv4* in rainbow trout (Bard, 2025). It would also be helpful to sample across multiple time points to provide a clearer temporal profile of cortisol-induced transcriptional changes, and to examine TRPV expression in tissues beyond gill, given that TRPV channels are widely expressed (Kumar et al., 2015). Prior studies in mammals suggest that steroid hormones can regulate TRP channels, both by binding GREs to upregulate transcription and by enhancing channel activity (Boychuk et al., 2013; Kumar et al., 2015). It will be critical to explore effects of cortisol on TRPV activity in future studies.

Changes in thermal sensing might also influence thermal preference, and therefore I also explored whether cortisol treatment altered thermal preference measured over 12 h in a shuttlebox system. Whereas CT_{max} reflects the upper thermal limit, thermal preference indicates how the animal typically behaviourally thermoregulates to optimize physiological function (Reynolds, 1977; Reynolds and Casterlin, 1979; Golvanov, 2006; Dillon et al., 2012; Neubauer and Anderson, 2019). Thus, a lowered CT_{max} in response to cortisol treatment does not necessarily mean that preferred temperature will be altered. However, if cortisol-treated fish experience an increase in metabolic rate, then behavioural selection of cooler water could serve to reduce metabolic stress. For example, zebrafish acutely stressed by confinement exhibited a short-term preference for cooler temperatures relative to fish that were not stressed (Jones et al., 2019). By contrast, cortisol-treated trout appeared to prefer warmer temperatures (Bard, 2025). In the present study, cortisol treatment did not significantly affect thermal preference. However, the experimental conditions may not have been optimal for

detecting an effect of elevated cortisol on thermal preference, because the water in the shuttlebox did not contain cortisol. The recovery experiment of the present study suggested that plasma cortisol levels had returned to baseline values within 48 h of transferring cortisol-treated fish to water lacking cortisol. Thus, the 12 h duration of the thermal preference trial may have been sufficient for plasma cortisol to decrease substantially, making it difficult to detect effects of elevated cortisol on thermal preference. Future measurements of thermal preference should consider cortisol delivery methods that maintain elevated plasma cortisol in the absence of waterborne cortisol.

4.4 Early life stress and adult thermal tolerance:

Developing zebrafish treated with cortisol from 0 to 5 dpf became adult zebrafish with a lower thermal tolerance. Notably, this 'programming' effect of early exposure to cortisol was persistent, with similar reductions in CT_{max} at 3 and 8 months old. The mechanism through which cortisol caused this effect remains to be determined. One possibility is that early exposure to elevated cortisol elicits HPI axis dysregulation that leads to elevated baseline and/or stress-induced cortisol levels in adult fish, which in turn, impacted CT_{max}. Several studies have reported significantly elevated baseline cortisol levels in adult fish that have experienced ELS or cortisol treatment (Hartig et al., 2016; Hall and Tropepe, 2018; Castillo-Ramirez et al., 2019; Fontana et al., 2020; Fontana et al., 2021; Chin et al., 2022). Best et al. (2017) showed that elevating cortisol during embryogenesis increased neurogenesis in certain brain regions but also produced alterations in larval behaviour. In addition, transcript abundances of GR and MR in brain tissue were increased in zebrafish exposed to ELS (Chin et al. 2022), which may make these fish more sensitive to elevated cortisol. Zebrafish exposed to ELS displayed heightened anxiety-like behaviour, characterized by reduced exploration (Chin et al., 2022). The GR co-chaperone FKBP5, which inhibits GR activity,

shows elevated transcript abundances in zebrafish exposed to cortisol during early life (Hartig et al., 2016), possibly as a compensatory response to increased GR abundance (Hartig et al., 2016; Juszczak and Stankiewicz, 2018; Chin et al., 2022). These findings suggest that when fish transition from dependence on maternal cortisol to their own endogenous cortisol production, prior exposure to elevated cortisol levels may prime the HPI axis to over-respond to subsequent challenges.

Interestingly, the ability of ELS to alter adult stress reactivity appears to be time sensitive. For example, stress exposure at 12-16 dpf or 22-26 dpf did not elicit the same anxiety-like behaviours observed in adult fish that were exposed to ELS at 2-6 dpf (Chin et al., 2022). This finding suggests that the embryonic to larval window of up to about 7 dpf represents a critical period in which the stress axis is quite vulnerable to programming effects. The specificity of this window raises questions as to why stress during this stage produces such lasting consequences. During embryogenesis, zebrafish cannot produce their own cortisol (Alsop and Vijayan, 2008). Instead, they rely on cortisol that was maternally deposited into the egg, which supports early developmental processes (Nesan and Vijayan, 2016). Proper regulation of glucocorticoid levels is essential for normal brain development, neurogenesis, and normal behavioural responses because cortisol directly modulates expression of genes involved in the HPI axis and neurogenesis (Nesan and Vijayan, 2016; Best et al., 2017). When maternal stress elevates cortisol deposition, embryos are subjected to elevated glucocorticoid levels, which can disrupt the programming of the HPI axis (Hartig et al., 2016; Nesan and Vijayan, 2016; Best et al., 2017). Exogenous cortisol exposure prior to hatching similarly disrupts HPI axis programming. Thus, the 0-48 hpf window likely represents a developmental point where early stress establishes lasting patterns of HPI activity, leading to downstream effects on fitness and the ability to withstand subsequent stressors (Chin et al., 2022).

A second possibility is that elevated cortisol levels during early life act directly on thermosensors and/or neural circuitry involved in thermoregulation to program thermal tolerance. For example, exposure to cortisol during early development may act on brain centres processing thermal information to change thermoregulatory set points inducing thermal stress related behaviours at lower temperatures. Thermal tolerance shows a high degree of plasticity to thermal acclimation in adult fishes (Moyano et al., 2017; McKenzie et al., 2021). However, whether similar effects occur as a result of rearing differences in developing fish remains unclear at present (Ruthsatz et al., 2024). Some studies have reported strong or limited effects of embryonic incubation temperature on thermal tolerance whereas others showed no significant effects of developmental temperature on CT_{max} (Lechner et al., 2024; Blanchard et al., 2025; Gavarikar and Craig, 2025).

Further research is needed to examine the interacting effects of thermal and other stressors to better understand thermoregulation in developing fish under environmental strain. Much work remains to identify the mechanisms underlying the programming effects of early life cortisol exposure on thermal tolerance.

4.5 Further considerations and implications of this study

The finding that elevated plasma cortisol compromises thermal tolerance in both adult and developing fish is a concerning outcome in a rapidly warming world. Freshwater ectotherms, which are often confined to restricted habitats, have few options to escape warming. They can either disperse, which is limited, or rely on physiological acclimation and long-term evolutionary change (Chevin et al., 2010). Acclimation provides short-term resilience by adjusting physiological rates and maintaining performance across fluctuating environments (Gunderson and Stillman, 2015). Yet, this mechanism is limited as heat stress disrupts membrane fluidity, protein structure, and reaction kinetics, imposing a biochemical ceiling on

plasticity that cannot be surpassed (Morgan et al., 2020; Ern et al., 2023) In addition, the current rate of climate warming may outpace the ability of fish to maintain metabolic stability, undermining fitness despite acclimatory adjustments (Seebacher et al., 2014; Gunderson and Stillman, 2015).

The remaining strategy is adaptive evolution, in which individuals with higher upper thermal limits are favoured and gradually shift thermal tolerance of populations (Hoffmann et al., 2013). However, upper thermal tolerance evolves slowly and is limited by a physiological ceiling, meaning that increases in thermal tolerance will not outpace the rate of climate warming (Morgan et al., 2020). Moreover, individuals already near their upper thermal limit are less phenotypically flexible and more susceptible to extreme events than those with lower limits (Stillman, 2003). As a result, both genetic adaptation and phenotypic plasticity appear insufficient to match the unprecedented pace of anthropogenic warming (Huey et al., 2012).

Although the present study used cortisol treatment as a proxy for chronic stress, fish in the wild may experience a range of anthropogenic factors and natural stressors that may influence one another synergistically, additively or even in an antagonistic manner (Jackson et al., 2016; Fong et al., 2017; Guttierez et al., 2025). For example, Todgham et al., (2005) demonstrated that when osmotic stress immediately followed thermal stress in tidepool sculpins (*Oligocottus maculosus*), there was a synergistic interaction. However, when the osmotic shock occurred 8 h after the thermal stress and was 12°C above the baseline temperature, an antagonistic cross-tolerance effect emerged, reducing mortality. Interestingly, they also found that a 15°C heat shock resulted in higher mortality under osmotic shock. These observations highlight how timing and stress intensity can affect physiological outcomes. Importantly, there may be windows of reprieve in natural habitats where fish are not stressed, allowing a return to homeostasis. As my work has shown a robust ability to recover CT_{max} in adult fish when cortisol levels fall, future work should investigate how

temporal variation and stressor intensity interact to shape an animal's thermal tolerance. This information could provide further insight into whether stressor combinations increase thermal vulnerability or may provide thermal resilience.

Collectively, the findings of the present study highlight the vulnerability of freshwater fishes to ongoing environmental stressors in combination with climate warming.

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