UNIVERSITY OF CALGARY

The Role of Corticotropin-Releasing Hormone Receptor 1 in the Heat Shock-Mediated Behavioural and Metabolic Response in Zebrafish

by

Zachary Skyler Shvartsburd

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE

DEGREE OF MASTER OF SCIENCE

GRADUATE PROGRAM IN BIOLOGICAL SCIENCES

CALGARY, ALBERTA

AUGUST, 2024

Abstract

Activation of the primary stress response, the hypothalamic-pituitary-adrenal (HPA)-axis in mammals or the hypothalamic-pituitary-interrenal (HPI)-axis in fish, requires the binding of corticotropin-releasing hormone (CRH) to CRH receptor 1 (CRHR1), though CRHR1 activity is also implicated in behavioural adjustment and peripheral signalling not associated with the HPI-axis. With global water temperatures rising and daily temperature fluctuations becoming more extreme, the stress response may be a critical mechanism of aquatic species to withstand shifts in temperature. By using a ubiquitous CRHR1 knockout (CRHR1KO) model, I investigated the hypothesis that CRHR1 is involved in the behavioural and metabolic modulation of the developmental and adult life stage stress response during acute heat shock in zebrafish. Following a 1-hour +5°C heat shock, wildtype (WT) fish larvae showed an increase in swimming activity but CRHR1KO larvae failed to respond to the heat shock. Basal oxygen consumption between WT and CRHR1KO larvae were similar, but CRHR1KO had elevated oxygen consumption in non-mitochondrial and ATP-linked processes. Cardiac performance was unchanged between WT and CRHR1KO larvae, indicating differences in behaviour were not due to modulation of heart function. Adult WT zebrafish showed the same increase in swimming activity when exposed to acute heat shock, while CRHR1KO fish had no change in swimming behaviour. The metabolic rate (MO_2) of WT adult fish also increased with heat shock, but this was not associated with increased circulating cortisol, as exposure to metyrapone, an inhibitor of cortisol synthesis, failed to attenuate the heat shock-induced rise in MO_2. The MO_2 of CRHR1KO fish did not increase following heat shock, suggesting CRHR1 action independent of the HPI-axis. Swimming performance was impaired in the CRHR1KO, with a lower aerobic scope and U_{crit} compared to WT. Transcript and metabolite analysis in the muscle revealed potential futile cycling of glycogen

in CRHR1KO fish. In summary, my study shows CRHR1 as a key integrator of the behavioural and metabolic rate response which improves performance following an acute heat shock, implicating CRHR1 as a metabolic thermal sensor for ectotherms.

Acknowledgements

My immediate gratitude goes out to my supervisor, Dr. Matt Vijayan. Your wide range of knowledge relentlessly humbles me, and your endless support for this project always instilled motivation. Your excitement for conducting research is contagious, which never failed to keep me intrigued about the next experiment. I have learned a lot from this opportunity, and I thank you for providing me the chance to work in your lab. I also want to extend my gratitude to my committee members, Dr. Deborah Kurrasch and Dr. Kelsey Lucas, for giving insight and support for my project, and to my external examiner Dr. Kenneth Lukowiak for your time.

All my lab members were quintessential in motivating me to complete this work. Dr. Jithine Rajeswari, your energy is contagious and thank you for all your help. Dr. Analisa Lazaro-Cote, Femila Antomagesh, and Meera Devan, you were paramount in helping develop my skills and supporting me during my studies. A very special thank you to Courtney Smith, an incredible friend and colleague who's support, patience, and kindness show no bounds. Thank you to Rob Hampton who made sure the lab was as happy as the fish and to Dr. Erin Faught, who patiently taught me techniques and pioneered the knockouts and other facets of this lab. Lastly, a monumental thank you to Dr. Andrew Thompson for introducing me to this work and for trusting and training me.

I deeply acknowledge the love and patience from my friends, family, and buddy; everyone always showed their full support and for that I am grateful. A special thank you to my brother, Alex, who always challenged me in perspective and followed up with considerable encouragement to strive for the best, and to my parents and grandparents for raising me in the same way.

Dedication

To Papa, a true role-model

Table of Contents

List of Figures and Tables

List of Symbols, Abbreviations, and Nomenclatures

Symbol	Definition
ACTH	Adrenocorticotropic hormone
ANOVA	Analysis of variance
cAMP	Cyclic adenosine monophosphate
CRH	Corticotropin-releasing hormone
CRHR1	Corticotropin-releasing hormone receptor 1
CRHR2	Corticotropin-releasing hormone receptor 2
CRISPR	Clustered regularly interspaced palindromic repeats
Dpf	Days post-fertilization
ELISA	Enzyme-linked immunosorbent assay
ES	Embryonic stem
GPCR	G protein coupled receptor
GR	Glucocorticoid receptor
GRE	Glucocorticoid responsive element
HOAD	3-Hydroxyacyl-CoA Dehydrogenase
HPA	Hypothalamic pituitary adrenal
HPI	Hypothalamic pituitary interrenal
HS	Heat shock
HSP	Heat shock protein
MMR	Maximum metabolic rate
MC2R	Melanocortin 2 receptor
MR	Mineralocorticoid receptor
MuRF1a	Muscle RING-finger protein 1a
NPY	Neuropeptide Y
OCLTT	Oxygen- and capacity-limited thermal tolerance
PEPCK	Phosphoenolpyruvate carboxykinase
REDD1	Regulated in development and DNA damage-response 1
SMR	Standard metabolic rate

StAR	**Steroidogenic acute regulatory protein**
WT	**Wild type**

Chapter 1: General introduction

1.1 Introduction

The stress response is a complex yet fundamental process for the survival of all vertebrates. Stimuli that initiate this response are diverse and can include physical, chemical, psychological stress that can be sensed from external stimuli or that is internally perceived by the animal, all of which are termed stressors (Wendelaar Bonga, 1997). Stressors can then activate two key physiological responses: the rapid sympathetic adrenergic response inducing the release of catecholamines, or the synthesis of glucocorticoids through either the hypothalamic pituitary adrenal axis in mammals or the hypothalamic pituitary interrenal (HPI)-axis in fish. Generally, these responses are to combat stress, which is defined as a shift from homeostasis due to perception of a stressor (Wendelaar Bonga, 1997), and the physiological stress response acts to return the animal to homeostasis through changes in physiology and behaviour. Catecholamines, once released from storage vesicles in chromaffin cells or sympathetic neuronal cells, act to modulate energy storage and cardiorespiratory function within seconds by increasing glucose production, heartrate, and ventilation (Reid et al., 1998; Fabbri et al., 1998; Kvetnansky et al., 2009). Glucocorticoids are synthesized *de novo* following activation of the HPI-axis, and increase glucose availability through gluconeogenesis in the liver and protein catabolism in the muscle within minutes following the rapid catecholaminergic “fight-or-flight” response (Mommsen et al., 1999; Wendelaar Bonga, 1997).

The primary glucocorticoid in teleosts and humans is cortisol, which binds two highly conserved corticosteroid receptors: the mineralocorticoid receptor (MR) and the glucocorticoid receptor (GR) (Faught et al., 2016a). GR has a low affinity for cortisol, thus cortisol-GR action occurs

solely under stressed conditions when circulating cortisol levels are high (Baker and Katsu, 2017; Faught and Vijayan, 2022a). Conversely, MR is bound by cortisol at basal levels as MR has up to a 10-fold higher affinity for glucocorticoids than GR (Reul et al., 1987; Reul and de Kloet, 1986, 1985). GR is ubiquitously expressed and is the key mediator of cortisol-induced metabolic adjustments; once bound, GR enters the nucleus and homodimerizes to act as a transcription factor by targeting glucocorticoid responsive elements (GREs) (Beato et al., 1996; Mifsud and Reul, 2016). In its unbound state, heat-shock proteins (HSPs) act as co-chaperones alongside other cofactors, such as FK506 binding protein 51 (FKBP5), to modulate endogenous GR and subsequent ligand and DNA binding (Binder, 2009; Cadepond et al., 1994; Kaziales et al., 2020). Activation of GREs facilitate transcriptional changes that propel metabolic adaptation for stress recovery and simultaneously return cortisol to basal levels through negative feedback on the HPI-axis.

HPI-Axis

Perception of a stressor in fish initiates the HPI-axis function through activation of preoptic nuclei in the hypothalamus which releases corticotropin-releasing hormone (CRH) (Figure 1.1; Mommsen et al., 1999; Wendelaar Bonga, 1997). This is analogous to the initiation of the HPA-axis in mammals, where CRH is released from the paraventricular nuclei of the hypothalamus. CRH is a ligand for two G-protein coupled receptors (GPCRs): CRH receptor 1 (CRHR1) and CRH receptor 2 (CRHR2) (Preil et al., 2001). CRHR1 is responsible for propagating the HPI-axis activity by stimulating adenylyl cyclase, which signals the production and cleavage of proopiomelanocortin (POMC) in the anterior pituitary into adrenocorticotropic hormone (ACTH) (Herman et al., 2016). CRHR2, which also binds other peptide hormones of the CRH family, is

suggested to regulate the stress response outside of the HPI-axis through feeding and anxiety-related behaviours (Kishimoto et al., 2000; Sukhareva, 2021). After processing, ACTH is released into the circulation and acts on the melanocortin-2 receptor (MC2R) present on the cells of the adrenal cortex in mammals or on the membrane of the steroidogenic interrenal cells of the head kidney in fish (Faught et al., 2016a). MC2R is another GPCR that activates adenylyl cyclase to increase intracellular cAMP levels, which stimulates steroidogenic acute regulatory protein (StAR) to transport cholesterol from the outer mitochondrial membrane to the inner mitochondrial membrane, a process that is the rate-limiting step for steroid biosynthesis (Alsop and Vijayan, 2008; Faught et al., 2016a). A series of enzymatic reactions converts cholesterol to pregnenolone, 17 α-hydroxypregnenolone, 17 α-hydroxyprogesterone, and finally cortisol (Faught et al., 2016a). Cortisol cannot be stored in intracellular vesicles due to its hydrophobicity, and thus is made *de novo* following activation of the HPI-axis, which contributes to cortisol being a powerful marker of stress in fish (Faught et al., 2016a; Lemos et al., 2023; Mommsen et al., 1999).

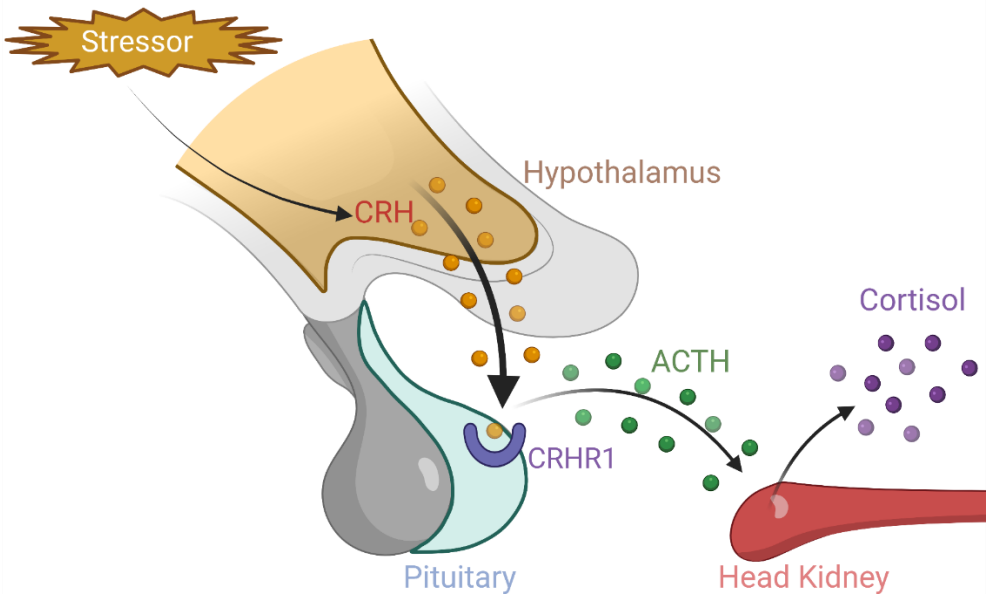


Figure 1.1**.** Schematic of the HPI-axis activation.
The perception of a stressor results in CRH production and release from the hypothalamus, and subsequent CRH-CRHR1 signalling in the pituitary to activate steroidogenesis in the interrenal cells of the head kidney. The final product is cortisol, which is released into circulation. CRH = corticotropin releasing hormone, CRHR1 = corticotropin releasing hormone receptor 1, ACTH = adrenocorticotropic hormone.

Outside of the stress response, cortisol exerts effects on growth, behaviour, reproduction, and osmoregulation (Bernier et al., 2004; Faught and Vijayan, 2016; Mommsen et al., 1999; Sadoul and Vijayan, 2016). Cortisol secretion also follows the circadian rhythm with concentrations peaking in the morning or the onset of the animals most active phase (Cowan et al., 2017; Oliveira et al., 2013). The daily release of cortisol is key in maintaining the biological rhythm of behaviours and processes such as feeding (López-Olmeda and Sánchez-Vázquez, 2010) and reproduction (Oliveria and Sánchez-Vázquez, 2010), predominantly controlled by environmental factors such as light exposure and temperature. Work with zebrafish (*Danio rerio*) shows temperature alters the expression of key transcripts involved in the circadian clock (Lahiri et al., 2005). This enables compensation of changes in biochemical rates due to temperature throughout

the circadian rhythm. Components at all levels of the HPI-axis also show a circadian rhythm with their release, matching that of cortisol (Cowan et al., 2017). Both CRH and CRHR1 transcripts follow rhythmic expression (Azpeleta et al., 2011; López-Olmeda et al., 2013), which suggests the diurnal nature of CRH-CRHR1 signalling is fundamental for the exhibition of cortisol circadian rhythm.

Regulation of CRH receptors and their ligands

CRHR1 and CRHR2 are widely expressed in mammalian and non-mammalian vertebrates alike. A third novel CRH receptor was discovered in catfish, *Ameiurus nebulosus*, and shows 85% homology with mouse CRHR1 and 80% with mouse CRHR2 (Arai et al., 2001); but, discussion of this receptor is limited as its expression is not found in any other species. CRHR1 and CRHR2, on the other hand, share approximately 70% amino acid structure with each other (Grammatopoulos, 2012). Thus, it may not be surprising that these receptors show varying affinity for the CRH-family peptides: namely the frog skin peptide sauvagine (Montecucchi et al., 1980), urotensin I expressed in the urophysis, a specialized caudal neurosecretory structure found in fish (Lederis et al., 1982; Parmentier et al., 2006), and urocortin I (Vaughan et al., 1995), urocortin II (Reyes et al., 2001), and urocortin III (Lewis et al., 2001), which are found in mammals. CRH appears to primarily bind CRHR1 whereas CRHR2 has a higher affinity for urotensin that CRHR1 (Hosono et al., 2015; Lovejoy et al., 2014). Activation of CRHRs by their primary ligands can be reduced through CRH binding protein (CRHBP). CRHBP is a highly conserved glycoprotein, and has high affinity for both CRH and urocortin I, with lower affinity for urocortin II and III (Jahn et al., 2004). Expression of CRHBP in fish is localized to the pituitary, hypothalamus, and preoptic area regions of the brain (Alderman and Bernier, 2007),

and the skin and gills (Mazon et al., 2006), indicating a central and peripheral functionality to the CRH system. It is understood that in humans, approximately 20%-90% of CRH is bound by CRHBP in the brain, depending on the region (Behan et al., 1997).

Expression of CRHRs is widespread in the central nervous system. Though CRHR1 is critical in activating the HPI-axis, it also modulates key neuronal circuits involved in behaviour (Kolber et al., 2010; Kovács, 2013; Müller et al., 2003). This multifaceted role of CRHR1 and its ligands enables a more rapid response than the genomic effects of cortisol-GR, where effects may take up to hours (Harris and Carr, 2016). Glucocorticoids act through negative feedback to inhibit hypothalamic CRH release following acute stress, but extrahypothalamic structures of the brain such as the Barrington's nucleus of the rat micturition center show enhanced CRH production following GR activation (Imaki et al., 1991; Kovács, 2013). Roles in extrahypothalamic signalling indicates that CRH does not solely act on CRHR1 in the pituitary, but in other structures as well, independent of the HPI-axis (Bonfiglio et al., 2011).

Crosstalk between the adrenergic stress response and the HPI-axis is critical in the propagation of the stress response. Noradrenaline stored in the neurons of the locus coeruleus stimulates the stress-induced release of CRH from the hypothalamus, which was observed to enhance HPA-axis activation in rats (Ziegler et al., 1999). Further, immunoreactive CRH neurons are observed to project to the mammalian locus coeruleus and regulate the activity of this brain region through CRHR1 receptors (Berridge and Waterhouse, 2003; Jedema and Grace, 2004). Cortisol also further primes the adrenergic system for responding to stress. For example, elevated cortisol in rainbow trout hepatocytes significantly increases the number of surface β-adrenoceptors per cell by nearly 4-fold to enhance *de novo* glucose synthesis and glycogen breakdown (Reid et al., 1992). Other monoamines, such as serotonin, also intersect with CRH signalling. Serotonin is a

major neuromodulator of the central nervous system that functions in stress, depression, and anxiety-related behaviours (Herculano and Maximino, 2014; Lillesaar, 2011). Immunolabeling of CRH in human brain revealed elevated CRH levels in pontine nuclei containing norepinephrine and serotonin of individuals with major depression (Austin et al., 2003). This crosstalk is even reported in peripheral cell models: enterochromaffin cells of the human colon, which express both CRHR1 and serotonin, have enhanced serotonin release following activation of CRHR1 (Wu et al., 2011). Serotonin-containing neurons also project to the paraventricular nucleus in mammals and directly stimulate CRH-producing cells, whereby serotonergic input can activate the HPA-axis (Hanley and Van de Kar, 2003). Both CRHR1 and CRHR2 control serotonin biosynthesis through modulating the rate-limiting enzyme tryptophan hydroxylase 2 (TPH2) during acute stress in rats, with each receptor showing opposing effects (Donner et al., 2016). In this study, pharmacological blockage of CRHR1 exacerbated TPH2 following an acoustic stressor (Donner et al., 2016), suggesting CRH-CRHR1 acts to reduce serotonergic pathways during stress. In fish, intracerebroventricular (ICV) injection of the CRHR antagonist α-helical-$CRH_{1–41}$ decreased brainstem serotonin levels (Backström et al., 2011), while the serotonin receptor agonist 8-OH-DPAT caused an increase in CRH transcript expression in the hypothalamus and ACTH secretion (Medeiros et al., 2014). These results suggest CRH-CRHR signalling does act as a regulator of serotonin and *vice versa* in fish (Backström and Winberg, 2017).

Peripheral effects in mammals, such as inflammatory, cardiovascular, and gastrointestinal function, are also regulated by CRHR activity (Lewis et al., 2001). Expression of CRHRs in the mouse periphery were first reported in heart and skeletal muscle (Kishimoto et al., 1995). Expression of CRHRs were later discovered in the mouse adrenal gland (Müller et al., 2001).

This work identified that peripheral CRHR1 was required for normal production of glucocorticoids. In mice lacking CRHR1, even administration of ACTH did not promote corticosterone release from the adrenal gland (Müller et al., 2001), which outlines that CRHR1 acts in the stress response outside of its role in the pituitary. CRHR2 function did not appear to enhance ACTH secretion, but its expression was also seen in the mouse adrenal (Müller et al., 2001). Both CRHRs and UCN expression were also observed in the bovine adrenal, suggesting other regulatory components of the peripheral CRH systems (Squillacioti et al., 2011). Further reports suggest CRHR2 and its ligands regulate glucose uptake in skeletal muscle (Roustit et al., 2014) and thermogenesis through skeletal muscle lipid substrate cycling and browning of adipose tissue (Lu et al., 2015; Solinas et al., 2006). Both CRHRs are also differentially expressed in human and mouse cutaneous structures that are involved in a local stress response system, with growth factor-like effects of cell proliferation and differentiation, as well as immune functions (Slominski, 2009; Slominski et al., 2006, 2004, 2013). In fish, the role of peripheral CRHRs is less understood. Though a scarce number of endothermic fish species have a distinct heater organ, thermogenic brown adipose tissue is absent in fish (Block, 1991). CRHRs in zebrafish have a protective effect in cardiac muscle under hypoxia (Williams et al., 2017). CRHR1 expression is also found in the skin and gills of carp (Mazon et al., 2006), tissues that highly interface with the surrounding environment suggesting an iono- or osmoregulatory role of the CRH system here. Thus, our current understanding of the role of CRHRs in fish are as central behaviour modulators, critical propagators of the HPI-axis, as well as signalling to induce protective effects in peripheral tissues during stress.

Glucocorticoids and CRHR1 in stress-related behaviour

Elevated glucocorticoids following HPA/HPI-axis activation during stress is known to elicit behavioural changes. Chronic corticosterone exposure to rats induce anxiety-like and depression-like behaviours (Donner et al., 2012). In fish, cortisol levels may be correlated with key behaviours related to social hierarchy. For example, subordinate fish, those who have "lost" in social dominance, exhibit high cortisol levels following an aggressive interaction with dominant conspecifics (Øverli et al., 2004; Xu et al., 2020). Food consumption is also regulated, with increased glucocorticoids or activation of its receptor reducing appetite and food intake (Blanco et al., 2024; Gregory and Wood, 1999; Nipu et al., 2022). As well, cortisol is known to chronically reduce feed-conversion efficiency, measured as the conversion of food to flesh, and alter other appetite-suppressing neuropeptides such as neuropeptide Y (NPY; (Bernier et al., 2004). CRH is also a known anorexigenic neuropeptide, where ICV injection of CRH results in decreased food consumption (Hill, 2012). Glucocorticoid signalling in early life stages are also shown to modify anxiety-like behaviours in adulthood (Chin et al., 2022)

CRH-expressing neurons in the central nervous system are also critical in behavioural modulation during acute stress. CRH neurons of the mouse paraventricular nucleus, though important for the endocrine stress response, also regulates complex behaviours when activated. For example, conditional limbic CRHR1 deficiency in mice have distinctly reduced anxiety-related behaviours even with functional CRHR1 present in the pituitary (Müller et al., 2003). Administration of CRH directly into the brain modulates behaviours associated with stress: decreased feeding, reduced exploratory and reproductive behaviours, and increased grooming and freezing behaviour (Arborelius et al., 1999). As well, CRH directly modulates the autonomic nervous system by increasing epinephrine and norepinephrine, which stimulates changes in

behaviour alongside heartrate, blood pressure, and other responses. Similarly to glucocorticoids, CRH also plays a role in early-life development of stress-induced behaviours that affect the adult response of mice. For example, lifelong overexpression of forebrain CRH resulted in elevated circulating glucocorticoids and anxiety-like behaviours in mice (Kolber et al., 2010). However, only transient overexpression of CRH during the developmental stages prior to weaning resulted in anxiety- and despair-like behaviours into adulthood, as well as chronic central increase in CRHR1 mRNA (Kolber et al., 2010). Thus, early life regulation of the stress response can impart long-term consequences.

Stress metabolism and CRHR1

Glucocorticoids in the acute stress response for mammals and teleosts alike increase available glucose in circulation to account for elevated metabolism at the tissue level (Faught and Vijayan, 2019, 2016; Mommsen et al., 1999). The attendant rise in glucose following a stressor is largely modulated by GR signalling. GR activation results in elevated hepatic gluconeogenesis, producing glucose from non-carbohydrate substrates including lactate, amino acids and glycerol (Faught and Vijayan, 2016; Mommsen et al., 1999). This is achieved in part through elevated transcription levels of phosphoenolpyruvate carboxykinase (PEPCK), a rate-limiting enzyme in the gluconeogenic pathway (Vijayan et al., 2003). GR signalling also increases muscle proteolysis to provide more gluconeogenic substrates. This is achieved through upregulating players that increase protein catabolism and/or inhibit protein synthesis, primarily regulated in development and DNA damage response 1 (REDD1) and muscle RING finger 1 (MuRF1) (Britto et al., 2014; Waddell et al., 2008). This is a key adaptation during stress, but under chronic scenarios leads to maladaptive muscle atrophy (Britto et al., 2014; Faught and Vijayan,

2019). Glycogenolytic pathways are also enhanced to increase circulating glucose, for which cortisol acts by increasing the adrenergic signalling capacity in the liver (Reid et al., 1992). GR is also a candidate for modulating glucose transport in fish, as zebrafish lacking GR have improved glucose uptake (Faught and Vijayan, 2019). Previous work has shown that impairing CRHR1 expression results in reduced stress-induced glucocorticoid levels (Faught and Vijayan, 2022b; Smith et al., 1998; Timpl et al., 1998), which may impair energy partitioning and transport. However, the direct organismal role of CRHR1 in glucose regulation requires further investigation.

The demand for energy at the cellular level during acute stress can be apparent in endpoints measurable at the whole-animal level. The metabolic rate of the whole animal can be established using respirometry, which is achieved by measuring oxygen loss of the organism's surrounding environment (Brett, 1964). The oxygen uptake of the organism is correlated to the overall metabolic rate. From this, we can determine the basal level of metabolic function, termed as the standard metabolic rate (SMR) in fish, as well as the maximum metabolic rate (MMR), which is the enhanced metabolic function of the animal during stressful conditions (Clark et al., 2013). Respirometry has previously revealed cortisol as an enhancer of the metabolic rate in fish (Pfalzgraff et al., 2022), but how other rapidly signalling components of the stress response such as CRHR1 may directly alter the oxygen uptake of the animal remains to be investigated.

Temperature effects and stress

Climate change poses a threat for all aquatic life as global water temperatures rise above historic averages (Robins et al., 2016). Daily water temperature fluctuation is also expected to increase,

and may lead to an acute thermal shifts affecting aquatic vertebrates (Paaijmans et al., 2013). Other anthropogenic factors also are attributed to changes in water temperature; forest clearcutting and farming, changes in river flow regime, and heated water exhaust from power plants all contribute to thermal alterations of water bodies from acute to chronic changes (Webb et al., 2008). All these factors can induce shifts in temperature that occur in less than a day, which can be physiologically stressful to fish (Goikoetxea et al., 2021). Ectotherms are particularly sensitive to changes in temperature as they lack any internal mechanism to generate heat. Indeed, with a 10°C temperature increase, fish see up to a 3-fold increase in metabolic rate (Clarke and Johnston, 1999; Schulte, 2015; Volkoff and Rønnestad, 2020). Thus, fish operate in a species-specific temperature range, where performance reaches an observed maximum at an optimal temperature (T_{opt}) (Pörtner and Peck, 2010). Performance decreases below and above the T_{opt} until equilibrium is lost at the minimal or maximal critical thermal temperature (CT_{min} and CT_{max}, respectively) (Pörtner and Peck, 2010; Volkoff and Rønnestad, 2020).

The temperature a fish is adapted to, and its capacity to avoid or acclimate to any shifts in environmental temperature all determine T_{opt} and CT limits. Endotherms have established autonomous regulation of internal temperature through brown fat and muscle shivering thermogenesis, but ectotherms lack these adaptations. Instead, the sole mechanism to combat thermal intolerance for most ectotherms is the avoidance of suboptimal temperatures (Prosser and Nelson, 1981). This behaviour is evident when observing wild population distributions in specific habitats. Marine species, such as Atlantic cod (*Gadus morhua*), pollack (*Pollachius pollachius*), and ballan wrasse (*Labrus bergylta*), were observed to adjust vertical distribution to cope with fluctuating environmental temperatures (Freitas et al., 2021). This behaviour is not limited to marine fish, as freshwater species display thermoregulatory movements due to shifts in

habitat temperatures, including trout (Biro, 1998; Hitt et al., 2017) and other salmonids during river migration (Armstrong et al., 2016; Goniea et al., 2006; Ritter et al., 2020). Both freshwater and marine fish even respond to anthropogenic alterations in water temperature. Power plant discharge water generate a warm zone within an aquatic environment which can affect species distribution (Encina et al., 2008; Füstös et al., 2023). Cooke and colleagues (2004) observed smallmouth bass (*Micropterus dolomieu*) inhabiting a thermal discharge canal during winter; fishes will inhabit/avoid these heated regions independent of the access to food, further indicating the priority of behavioural avoidance of suboptimal temperatures (Neill and Magnuson, 1974). Even social behaviours are altered by water temperatures, exemplified with a decrease in sociability, or an increased distance from conspecifics, in three-spined stickleback (*Gasterosteus aculeatus*) (Pilakouta et al., 2023).

Though most fish lack the ability to generate heat, other internal mechanisms can adapt cells for changes in water temperature to preserve function. Temperature stress will impair cell membrane fluidity, for which fish can combat this through homeoviscous adaptation, a cellular response that alters the membrane lipid and cholesterol composition during thermal stress (Malekar et al., 2018). Cortisol has been shown to have nongenomic effects by modulating the membrane fluidity of trout hepatocytes to regulate stress signalling (Dindia et al., 2012), which suggests it may also be used in heat stress homeoviscous adaptation. As well, the cellular stress response functions to protect cell machinery from denaturation due to internal environmental changes. This is primarily managed by HSPs, which are cochaperones that are rapidly transcribed and synthesized to prevent misfolding of proteins (Iwama et al., 1999, 1998). HSPs are conserved across all organisms, and function in protein folding and translocation. HSP90 and HSP70, specifically, are essential chaperones for GR by regulating cortisol binding, nuclear

translocation, and disassembly, indicating a connection between the cellular heat shock response and HPI-axis function (Kirschke et al., 2014; Pratt et al., 2006).

Metabolic rate is strongly associated with temperature (Clarke and Johnston, 1999). If a fish is outside its T_{opt}, then its performance will be limited due to a change in the SMR, as well as the MMR the organism may achieve. This results in constraints on the aerobic capacity, or aerobic scope, an animal has for maintenance functions as well as processes required for additional activity (Brett, 1971, 1964). For example, as temperature decreases, the activity of key aerobic enzymes such as citrate synthase are reduced (Ressel et al., 2022). This limits cellular metabolism, resulting in impaired muscle function and energy utilization of other key tissues. Higher temperatures will increase the metabolic rate primarily due to faster reaction rates, resulting in an elevated basal energy consumption and limited capacity to increase metabolic demands when required (Ressel et al., 2022). Due to the high correlation between temperature and metabolic rate in ectotherms, temperature must be accounted for in all related studies, particularly with respirometry (Chabot et al., 2016; Schulte, 2015). How an ectotherm can tolerate temperature-dependent metabolic demand is postulated to also be governed by oxygen utilization and supply (Pörtner et al., 2017). However, defining the mechanisms behind heat stress and integrating it with the thermal constraints of the whole organism remains incomplete. The oxygen- and capacity-limited thermal tolerance (OCLTT) concept theorizes temperature can limit performance by constraining oxygen supply while increasing oxygen demand (Pörtner et al., 2017). This concept incorporates several physiological components with metabolic rate and oxygen availability to understand how species react to shifts in temperature. Key components outlined by Pörtner and colleagues (2017) include aerobic scope, cardiac performance, CO_2 production, and mitochondrial function among others. However, these adjustments are largely

based around aerobic metabolism and do not account for anaerobic capacities (Pörtner et al., 2017). Furthermore, OCLTT focuses on thermal tolerances and limits at stable and routine maintenances of the organism, which limits its applicability when discussing maximal tissue and whole-animal performances during short-term acute temperature fluctuation (Pörtner et al., 2017). Even regarding this, OCLTT exemplifies the complex nature of how ectotherms may acclimate to shifts in temperature through multiple physiological adjustments and reiterates the lack of knowledge surrounding the mechanisms behind thermal tolerance.

Model organism, *Danio rerio*, and the CRHR1 knockout

Due to the ease of genetic manipulation, zebrafish have become an excellent model organism in stress physiology, including biomedicine and developmental biology. In biomedical research alone, the number of yearly publications including zebrafish have multiplied nearly 25-fold from the years 2000 to 2017 (Teame et al., 2019). This can be attributed to the ease of maintaining a laboratory stock of zebrafish, reducing time and cost compared to other models. Zebrafish are a fecund species, with females releasing 100's of viable eggs per week. The developing embryos are visible due to a transparent chorion, from which they hatch by 2-3 days post fertilization (DPF). Furthermore, the embryo and subsequent larvae develop rapidly, with fish reaching sexual maturity before 4 months. Larval and adult zebrafish behaviour is well documented (Kalueff et al., 2013). Adult zebrafish have also been used for metabolic and performance-based studies (Gilbert et al., 2014; Messerli et al., 2020; Wakamatsu et al., 2019).

Zebrafish are designated as eurytherms, which are ectotherms that can tolerate and adapt to a relatively large range of temperatures (López-Olmeda and Sánchez-Vázquez, 2011). CT limit

studies reveal that zebrafish can survive in water temperatures ranging from a minimum of 6.7°C and a maximum of 41.7°C (Cortemeglia and Beitinger, 2005; Schaefer and Ryan, 2006). As well, zebrafish can withstand and acclimate to seasonal temperature shifts between 14.2-33°C and daily fluctuations of over 5°C (López-Olmeda and Sánchez-Vázquez, 2011). As zebrafish are not severely endangered by acute changes in water temperature, they are a prime candidate for investigating physiological changes associated to temperature change without inducing long-term harm. Thus, zebrafish have been used in previous studies for investigating performance during thermal stress (Scott and Johnston, 2012; Wakamatsu et al., 2019) and early-life temperature sensing (Gau et al., 2013).

With the discovery of the CRISPR/cas9 system, zebrafish have become an excellent model for understanding the role of certain genes at the organismal level (Li et al., 2016). Previous works have outlined the power of using zebrafish over mammalian models, where generation time of the KO in zebrafish is quicker due to shorter maturation times and high fecundity (Liu et al., 2019). Our lab has generated KO models that are not viable in mammals (Faught and Vijayan, 2022a, 2019). For example, due to improper lung development, ubiquitous GRKO mice are not viable and are thus constrained to tissue-specific KOs (Tronche et al., 1998), whereas GRKO zebrafish can survive and produce offspring for several generations (Faught and Vijayan, 2019). These KO models enable a systematic tool to investigate specific facets of the stress response to understand effects at the whole-organism level. To this end, a CRHR1KO zebrafish line has been generated in our lab to assess CRHR1 function. Mammalian KO models have been used to investigate CRHR1: to our knowledge, two groups simultaneously published the first articles on a ubiquitous CRHR1KO model using mice. Timpl and colleagues (1998) used homologous recombination in mouse embryonic stem (ES) cells, replacing the *crhr1* fragment encoding

transmembrane domains 4-7 with a neomycin cassette. These mutant ES cells were used to generate chimeric mice which carried the mutation in the germ line to produce a heterozygous F1 generation. The F1 were intercrossed to generate the homozygous CRHR1KO F2 generation used in their work. Here, the basal levels and attendant rise in corticosterone following a stressor were impaired in the CRHR1KO mice. Smith et al. (1998) also used homologous recombination in ES cells but instead replaced the last 12 amino acids of the first extracellular domain to the fourth transmembrane domain with a neomycin cassette. Circulating plasma corticosterone levels were also markedly lower, with the typical diurnal rise of glucocorticoid not occurring in the CRHR1 mutant mice. However, these homozygous mutants reportedly faced challenges not described in CRHR1KO generated by Timpl et al. (1998); a 15% mortality of male homozygotes was noted, and all progeny of homozygous mutants died within 48 hours after birth, attributed to impaired lung development due to insufficient maternal corticosterone (Smith et al., 1998). This limits the study of the CRHR1KO mouse development to one generation of homozygous mutants without external supplementation of glucocorticoid. To our knowledge, our lab has generated the first ubiquitous CHR1KO zebrafish model, a stable KO line for which larval behaviour and HPI-axis function has already been reported (Faught and Vijayan, 2022b). Stressed levels of cortisol in CRHR1KO larvae were significantly lower than WT and failed to increase cortisol production following a 1-hour treatment of CRH. With an established CRHR1KO model, further roles of CRHR1 in the stress response can be studied.

1.2 Hypothesis and Objectives

The main goal of this thesis is to identify the role of CRHR1 during acute temperature stress. The activation of the HPI-axis to elicit the stress-response is well-studied in fish. However, this is

primarily through investigation of the principal glucocorticoid, cortisol, and its role in modulating stress through its receptors, GR and MR (Faught and Vijayan, 2022b, 2022a, 2019). Our novel CRHR1KO zebrafish line can be used to investigate the role of CRHR1 during acute temperature stress. Currently, it is not fully elucidated whether components of the HPI-axis have direct effects in the control of energy expenditure in metabolically active tissues like muscle alongside regulating cortisol biosynthesis. Though central CRHR1 activation has been linked to depression and anxiety-like behaviours in mammalian models (Müller et al., 2003; Sun et al., 2023; Timpl et al., 1998), to our knowledge there is no study that has investigated its systems-level role of the whole-animal metabolic rate, or its effect on behaviour in an adult fish model. Previous work in our lab has revealed CRHR1 as a critical component of the behavioural and cortisol response to an acute physical stressor in larval zebrafish (Faught and Vijayan, 2022b), but how CRHR1 may modulate different stressors, or how the adult model may respond, required investigation. This thesis aimed to employ the CRHR1KO zebrafish model to understand how CRH-CRHR1 signalling may regulate the behavioural response, metabolic rate, and swimming performance of these fish when exposed to elevated temperature. Further, this work will develop our understanding in the integration of the stress response during heat shock, which is ecologically relevant in the context of climate change.

The **hypothesis** of this thesis is that CRHR1 is involved in the behavioural and metabolic modulation of the developmental and adult life stage stress response during acute heat shock in zebrafish. Specifically, the objectives of this study were to:

1. Determine behavioural modulation and metabolic rate adjustment during acute heat shock in zebrafish larvae using high throughput methods. (Chapter 2).

2. Observe acute behavioural, metabolic rate, and peripheral transcriptional adjustments in adult CRHR1KO zebrafish following acute heat stress (Chapter 3).

Chapter 2: CRHR1 activation modulates the behaviour and bioenergetics of developing zebrafish during acute temperature elevation

2.1 Introduction

Early life regulation of stress hormones is critical for the development and survival of vertebrates. De novo synthesis of cortisol, the primary stress hormone in fish, occurs after hatching in zebrafish (Alsop and Vijayan, 2008). Early life modulation of cortisol functions in stress programming that is persistent into adulthood (Reyes-Contreras et al., 2019). As well, key components of the hypothalamic-pituitary-adrenal (HPA)-axis, or the analogous hypothalamic-pituitary-interrenal (HPI)-axis in teleosts, also play a role in early life stress development (Ivy et al., 2010). The HPA/HPI-axis is a highly conserved physiological stress response that acts to recover homeostasis in the whole organism. Upon perception of a stressor, corticotropin-releasing hormone (CRH) is released from the hypothalamus and binds its primary receptor in the pituitary, CRH receptor 1 (CRHR1). Following CRH-CRHR1 binding in the pituitary, proopiomelanocortin (POMC) is synthesized and cleaved into adrenocorticotropic hormone (ACTH), which is released into circulation and activates melanocortin-2 receptor (MC2R) in the steroidogenic interrenal cells within the head kidney of fish, which is analogous to the adrenal gland in mammals (Faught et al., 2016a; Mommsen et al., 1999). This leads to the de novo synthesis and release of cortisol, the primary glucocorticoid in teleosts, which facilitates energy substrate mobilization, including glucose and amino acids, to cope with the increased energy demand that is associated with re-establishing homeostasis post-stressor (Faught et al., 2016a; Mommsen et al., 1999). However, other components of the HPI-axis may modulate the animal's response to stress independently of cortisol action. For example, the coordinated action of rapid

CRH-CRHR1 signalling, followed by cortisol activity have been shown to modulate the acute stress-related behavioural activity of zebrafish larvae (Faught and Vijayan, 2022b). However, as stress is energy demanding, the bioenergetics of the acute HPI axis activation as part of the stress response remains to be investigated.

The bioenergetics of the animal, which is its ability to store and release energy through work (Skulachev et al., 2012), can be largely understood through its mitochondrial function. Oxidative phosphorylation within the mitochondria is the primary source of ATP to the cell and dysfunction of the electron transport chain (ETC) is known to result in a multitude of defects related to energy metabolism, acidosis, and aging (Nicolson, 2014). Mitochondria are the primary contributors to aerobic metabolism of an organism; thus, oxygen consumption, which is a non-invasive and physiologically relevant measurement can also be linked to the bioenergetics of the mitochondria. Oxygen consumption of cells and tissues are a prospective tool for mitochondrial disease screening prior to the use of muscle biopsy or other invasive techniques (Westerlund et al., 2018). Zebrafish embryos and larvae have also become potential targets for bioenergetic profiling when modeling different pathologies that impair the metabolic rate (Dogra et al., 2023; Stackley et al., 2011).

An increase in metabolic rate will require an increased level of oxygen transport to tissues. This adjustment can be made through an increase in cardiac output, whereby the heartrate can elevate to improve circulation of oxygenated blood. Stress steroids can modulate the cardiovascular performance of fish. Glucocorticoids act in remodelling piscine heart output through hyperplasia and hypertrophy (Johansen et al., 2011, 2017), and is a known potentiator of cardiac disease and deformation in humans (Souverein et al., 2004) and mammalian models (de Vries et al., 2002; Lumbers et al., 2005), all of which appears to be maladaptive. However, these effects are all in

chronic scenarios or with lifelong high cortisol stimulation, whereas the cardiac response to acute HPI axis activity is less studied. Both GR and MR, as well as CRHR1 are highly expressed in the myocardium of teleosts (Faught and Vijayan, 2022b), suggesting that the activation of the HPI-axis can have a rapid effect on cardiac performance. However, the literature shows contradictory effects of acute stress on heartrate in fish (Bloecher et al., 2024; Svendsen et al., 2021). These differences may be attributed to the type of acute stressor used in the study, which lends itself to further study.

Previous work to identify the systems-level role of CRHR1 revealed extrapituitary function in the adrenal gland to regulate cortisol secretion in mice (Müller et al., 2001). However, investigating the role of CRHR1 during early development in mammals is challenging as the development of mutant offspring is severely impaired. Progeny of homozygous mutant CRHR1-deficient mice failed to survive beyond 48 h after birth due to improper lung development (Smith et al., 1998). In contrast, CRHR1KO zebrafish are a robust model that can produce viable progeny through multiple generations (Faught and Vijayan, 2022), enabling us to view the role of CRHR1 during the critical developmental window without exogenous perturbation. Previously, this zebrafish CRHR1KO model revealed CRH acts in modulating stress-related hyperactivity (Faught and Vijayan, 2022b), but the underlying regulations, particularly the bioenergetic response during acute stress, begs further investigation.

Here, we investigate the role of CRHR1 in early life behaviour and metabolic rate regulation using the CRHR1KO zebrafish model. We have previously used the light-dark behavioural paradigm to test for anxiety-related behaviours and activity of zebrafish larvae at 4-6 days post-fertilization (dpf) (Faught and Vijayan, 2022b). Changes in stress-related behavioural activity may imply shifts in energy utilization, so we utilized a Seahorse Bioenergetics analyzer (Agilent

Technologies, California) to investigate mitochondrial function (Dogra et al., 2023) in WT and CRHR1KO zebrafish larvae in response to heat stress. Also, heartrate was analyzed to determine whether CRHR1 regulates cardiac performance during acute heat stress to accommodate behavioural and bioenergetic changes. This work aims to elucidate the bioenergetics of CRH-CRHR1 signalling, and how it modulates the behavioural and metabolic responses to an acute heat shock in zebrafish larvae.

2.2 Materials and Methods

Zebrafish maintenance

Zebrafish (Tupfel long fin strain) were held in recirculating systems (Tecniplast, Italy) on a 14 h:10 h light:dark cycle (light on 09:00). Water was maintained at 28.5°C, 800 μS conductivity, and a pH of 7.5. Fish were fed twice daily with Gemma Micro (500) adult zebrafish food in the morning (Skretting, Canada) and live Artemia (Brine Shrimp Direct, Utah) in the afternoon. CRHR1KO zebrafish were generated using CRISPR-cas9 in our lab as described previously (Faught and Vijayan, 2022b). CRHR1KO fish used in this study were all homozygous mutants. For breeding, fish were set up 30 min following the afternoon feed in breeding traps, and embryos were collected the following day before the morning feed. Embryos were maintained in E3 embryo medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM CaCl2, 0.33 mM MgSO4, and 0.1 ppm methylene blue as antifungal) in 10-cm petri dishes (60–200 embryos per dish) stored in a temperature-controlled incubator also on a 14 h light:10 h dark cycle set at 28.5°C. Embryo media were refreshed daily (approx. 50%) and all dead embryos/larvae were removed. This maintenance continued until embryos or larvae were required for experiments at 4 days post-

fertilization (dpf). All procedures were performed under the approval of the Animal Care Committee (AC17-0079 and AC21-0061) and under the standards set by the Canadian Council of Animal Care.

Behaviour

To measure the behaviour of the larvae, 6 dpf WT and CRHR1KO were transferred into a 24 well plate containing 2.5mL of E3 media per well either maintained at 28°C or 33°C. Following 1 hour after transferring the larvae, the plate was loaded into a Noldus Danio Vision Observation Chamber (Noldus, the Netherlands) and a light-dark paradigm was employed (Schnörr et al., 2012), where fish were exposed to 6 min of intense light followed by a 4 min dark period to assess swimming activity. Automated tracking through Ethovision (Noldus, the Netherlands) was used to measure total distance swam in both the light and the dark phase.

Seahorse Bioenergetics

At 4dpf, zebrafish larvae were transferred into 24-well Seahorse XF24 Islet Capture Microplates (Agilent Technologies, California) to determine the oxygen consumption rate (OCR) as described previously (Dogra et al., 2023). Zebrafish were exposed to either 700uL of E3 media containing vehicle (0.05% DMSO) or 5ug/mL cortisol for 16 h (Faught and Vijayan, 2022) with an islet capture screen placed above to hold the larvae in place. Sensors were hydrated and calibrated per the manufacturers specifications, and OCR measurements were obtained using the Seahorse XF24 Extracellular Flux Analyzer (Agilent Technologies, California). Bioenergetic measurements were taken as previously described (Dogra et al., 2023). Briefly, 7 oxygen reads

were taken initially to obtain basal OCR, 7 reads following oligomycin treatment, 6 reads following carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP) treatment, and 7 reads following antimycin A treatment, with each read lasting 6 mins (Dogra et al., 2023). OCR following FCCP treatment remained lower than basal OCR, and thus were removed from analysis. The mean of the two lowest consecutive OCR measurements following each drug treatment was used to determine OCR for each larva.

Heartrate

Six WT or CRHR1KO zebrafish larvae aged 4 dpf were transferred to a petri dish containing 25mL of E3 media with either 0.05% DMSO (vehicle) or 0.05% DMSO and 5ug/mL cortisol and left for 16 h overnight at 28°C. Following the exposure, the petri dishes were either stored for 1 h at 28°C or 33°C, after which MS-222 was added to a final concentration of 50 mg/L which acts as a light anaesthetic to slow swimming while not impairing the heart beat frequency (Craig et al., 2006). Once larvae movement was slowed (approximately 2 min after MS-222 addition), each larva was video recorded for 10-30 s using a Leica M165 microscope equipped with a Leica DL 550 camera (Leica Microsystems, Germany). Epinephrine (EPI) was immediately added after recording to a final concentration of 100 nM and video recording was repeated after 5 min of exposure.

To determine whether heartrate modulation during elevated temperature was through activation of adrenergic receptors, 6 WT and 6 CRHR1KO larvae aged 4 dpf were transferred to a petri dish containing 25 mL of E3 media. The following morning, when larvae were aged 5 dpf, propranolol (PROP), a known β-adrenergic receptor antagonist, was added to each petri dish to a

final concentration of 100µM. Following 30 min of treatment, larvae were then subjected to either 28°C or 33°C for 1 h, then treated with 50 mg/L MS-222. Heartrate was assessed in the same way as above. EPI was added to a final concentration of 100 nM and video recording was repeated after 5 mins of treatment to confirm β-adrenergic receptor blockage by PROP. All videos were analyzed in VLC Media Player (VideoLAN, France) as previously described (Thompson et al., 2022a). Briefly, heartrate was determined by manually counting heartbeats over a 5-15 s.

Statistics

All statistical analyses were carried out and graphs were plotted using GraphPad Prism 8 (GraphPad Software, CA, USA). All data are shown as mean ± SEM. Behaviour and OCR data was analyzed by two-way ANOVA ($\alpha = 0.05$, Holm-Sidak post hoc), while heartrate was analyzed via three-way ANOVA ($\alpha = 0.05$, Holm-Sidak post hoc). If interactions were observed from the three-way ANOVA, the non-significant experimental groups were consolidated, and a two-way ANOVA was used to further analyse the two-way interaction. Data that failed the assumptions of normality or homoscedasticity were log-transformed, while untransformed data is shown in the figures.

2.3 Results

Behaviour

WT larvae were significantly more active in the light phase following HS (701.60 ± 22.94 mm) compared to ambient (552.28 ± 24.07 mm, $p < 0.001$, Figure 2.1B). CRHR1KO zebrafish

showed similar swimming activity to WT at ambient (499.94 ± 16.77 mm) but were significantly less active than all other groups following HS (414.12 ± 26.84 mm, $p < 0.05$). The total distance travelled by WT zebrafish larvae showed no significant difference between 28°C (527.72 ± 16.31 mm) and 33°C (560.82 ± 16.02 mm) in the dark phase of the behaviour paradigm ($p > 0.05$, Figure 2.1A). Distance travelled in the dark by CRHR1KO zebrafish at 28°C was comparable to WT at both temperatures (553.41 ± 16.76 mm, $p > 0.05$), but after 33°C treatment, CRHR1KO larvae were less mobile (456.35 ± 21.00 mm) than both WT treatment groups and CRHR1KO at 28°C ($p < 0.05$).

Seahorse bioenergetics

Basal OCR measurements at 28°C were not discernable between WT (323.04 ± 12.38 pmol O_2/min) and CRHR1KO larvae (348.40 ± 13.57 pmol O_2/min, $p > 0.05$). OCRs following HS were also not significant between WT (379.11 ± 7.85 pmol O_2/min) and CRHR1KO (414.18 ± 10.40 pmol O_2/min, $p > 0.05$); however, there was a significant main effect showing OCR increases with temperature ($p < 0.001$, Figure 2.2A) and is overall greater in the CRHR1KO larvae compared to the WT larvae ($p = 0.012$). Mitochondrial respiration appeared unchanged in WT after HS ($p > 0.05$, Figure 2.2B), but CRHR1KO larvae had a significantly greater OCR following HS compared to all other groups ($p < 0.05$). Non-mitochondrial respiration was elevated in both WT and CRHR1KO following HS compared to WT at ambient water temperature, but CRHR1KO larvae at 28°C also had an elevated non-mitochondrial OCR comparable to HS groups ($p < 0.05$, Figure 2.2C). ATP-linked OCR also increased with temperature in both WT and CRHR1KO larvae, but OCR of the CRHR1KO larvae were significantly higher than the WT in response to the HS ($p < 0.05$, Figure 2.2D). OCR associated

with proton leak in WT was significantly reduced at 33°C ($p < 0.05$, Figure 2.2E), which was not seen in the CRHR1KO larvae ($p > 0.05$).

Following 16 h cortisol exposure, basal OCR remained similar between WT and CRHR1KO ($p > 0.05$), and a main temperature effect was observed, with increased OCR in HS groups ($p = 0.005$, Figure 2.3A). Cortisol treated larvae showed similar mitochondrial OCR between WT and CRHR1KO fish independent of temperature ($p > 0.05$, Figure 2.3B). A significant main effect showed an increase in non-mitochondrial OCR following 33°C HS independent of genotype ($p < 0.001$, Figure 2.3C). ATP-linked OCR, after the 16 h cortisol treatment was similar between the genotypes and the HS treatments ($p > 0.05$, Figure 2.3D). The cortisol-treated WT showed no difference in OCR due to proton leak between water temperatures ($p > 0.05$, Figure 2.3E). CRHR1KO larvae had a significantly higher proton leak-related OCR at ambient temperature compared to WT ($p = 0.039$, Figure 2.3E) CRHR1KO larvae at 28°C also had a higher proton leak-related OCR than WT at 33°C and CRHR1KO at 33°C ($p < 0.05$).

Heartrate

Heartbeat frequency of WT and CRHR1KO remained comparable to each other in all treatments ($p > 0.05$, Figure 2.4A). A temperature main effect was observed, with elevated temperature increasing heartrate ($p < 0.001$). EPI also significantly increased the heartrate independent of temperature or genotype ($p < 0.001$). A two-way temperature x EPI interaction was observed from the three-way ANOVA ($P < 0.001$), so the genotype factor was consolidated. A significant difference in heartrate was observed between both 28°C treated larvae (non-EPI and EPI-treated) and EPI 33°C larvae and both 33°C treated larvae (non-EPI and EPI-treated) ($P < 0.05$). There

was also a significant difference in heartrate between the non-EPI 28°C larvae and EPI-treated 28°C larvae ($P < 0.05$). Following the 16 h cortisol treatment prior to the heartrate measurement, temperature and EPI treatment significantly increased the heartrate ($p < 0.001$, Figure 2.4B), with no differences observed between the genotypes and no interactions were observed between factors. Larvae treated with PROP 90 mins before heartrate measurement showed a significant EPI main effect ($P < 0.001$) and a temperature x EPI interaction ($p = 0.0315$). No differences were observed between genotypes, thus genotype data was consolidated to investigate the two-way interaction, where the heartrate of larvae at 28°C showed a lower trend compared to larvae at 33°C ($p = 0.060$) when not treated with EPI. Following PROP treatment, larvae at 33°C had a significantly higher heartrate than larvae at 33°C after EPI treatment ($p = 0.003$, Figure 2.4C).

2.4 Discussion

CRH-CRHR1 signalling is known to play a key role in early life development of anxiogenic and depressive behaviours (Kolber et al., 2010). Here, we show that zebrafish lacking CRHR1 fail to behaviourally respond to an acute HS, which follows similarly to previous reports of impaired swimming activity in the CRHR1KO zebrafish model following a swirling stressor (Faught and Vijayan, 2022b). CRHR1 may be critical in sensing changes in temperature as a stressor by regulating the behavioural response through central neuronal signalling. Not only do CRH neurons in the hypothalamus increase in number following exposure to a stressor (Berg-Maurer et al., 2016), but CRH neuronal populations show differing responsiveness to specific stressors (Lovett-Barron et al., 2020). For instance, Lovett-Barron et al. (2020) observed 40.5% of medial *crh*-expressing neurons of zebrafish larvae responded solely to either heat, salinity, or acidity stress. How different stressors may activate certain neuronal clusters is not only specific to CRH

neurons, but is also seen in hypothalamic arginine vasopressin and oxytocin neurons (Lovett-Barron et al., 2020). This stressor-specific response may impact the central behavioural response to stress (Müller et al., 2003), but also the extent of the HPI-activation by pituitary CRHR1 (Faught et al., 2016a). Together, the behavioural response during heat shock may be modulated by central extrapituitary CRH-CRHR1 signalling coordinated with HPI-axis activation.

The non-mitochondrial OCR of CRHR1KO zebrafish larvae is lower than WT at 28°C, while the CRHR1KO OCR associated with mitochondrial function, particularly ATP synthesis, increases to a greater degree than WT following HS. This suggests that CRHR1 under the unstressed basal condition regulates oxygen consumed for functions beyond oxidative phosphorylation. Cellular functions that consume oxygen outside of the mitochondria include protein folding, DNA demethylation, lipid synthesis, pro-oxidant, and proinflammatory processes (Banh et al., 2016; Chacko et al., 2014). During heat stress, CRHR1 signalling may limit the organism's ATP-synthetic capacity as seen in the increased ATP-linked OCR in CRHR1KO fish. This may be a protective effect, as unchecked ATP synthesis may not be sustainable, as protein synthetic pathways and survival of neuronal cells rely on non-mitochondrial energy mobilization (Pontes et al., 2015).

Increasing temperature alters the bioenergetics of the mitochondria. During chronic exposure to heat, oxygen consumption associated with metabolism and proton leak both typically increase (Sokolova, 2023). Strikingly, OCR associated with the proton leak significantly decreased in HS-exposed WT. This may indicate a protective system during acute HS that limits proton leak-attributed oxygen consumption to conserve oxygen for aerobic metabolism used during the escape response (Sokolova, 2021). High OCR following oligomycin treatment, and the subsequent inhibition of ATP synthesis, can be attributed to several functions such as

mitochondrial maintenance of the proton motive force (Sokolova, 2021), adenosine nucleotide translocase (ANT) (Kreiter et al., 2021), and uncoupling protein (UCP) function (Nicholls, 2021). It appears that WT fish can attenuate the oxygen used for these processes during HS and rather focuses on ATP-linked oxygen utilization. ANT facilitates ADP-ATP exchange across the inner mitochondrial matrix, maximizing available ADP in the mitochondrial matrix and ATP in the cytosol (Bround et al., 2020). UCPs in mammals are a component of thermogenesis (Nicholls, 2021), but the role of UCPs in ectotherms, who do not thermoregulate beyond avoidance of suboptimal environmental temperatures, is unclear. Production of reactive oxygen species (ROS) due to high electron flux in the ETC must also be regulated. Increased proton leak is also implicated in suppressing the formation of ROS (Hirschenson et al., 2022), for which our results suggest CRHR1 signalling induces mitochondria to focus on ATP-synthesis rather than limit ROS production during acute HS. Exactly how these systems are regulated by CRHR1 or cortisol during HS in ectotherms begs further investigation. Recent work suggests that a high proportion of oxidative phosphorylation compared to proton leak respiration (i.e. lower proton leak OCR) during exposure to elevated temperatures indicates high heat tolerance (Chung and Schulte, 2020). With a higher ATP-linked respiration and similar proton leak compered to WT, it appears the mitochondria of CRHR1KO fish may be more tolerant to acute HS. Overall, we see HS increases the basal OCR, but CRHR1 signalling may limit the capacity of oxidative phosphorylation for non-mitochondrial processes.

We show temperature acts to increase heartrate but appears predominantly modulated by the adrenergic system rather than the HPI-axis. CRHR1KO fish show no difference in heartrate with the WT in all treatment groups, indicating CRHR1 may not regulate early life cardiac performance during acute stress and suggests heartrate is not representative of the metabolic

performance or CRHR1 activation during acute HS. As well, our results show CRHR1 does not control the acute action of the adrenergic system on heartrate. Interestingly, previous work showed CRH infusion increased heartrate in rats (Fisher et al., 1982), though whether it was CRHR1 that specifically modulated this was not identified. There is reportedly direct crosstalk with the CRH system and the adrenergic system, as CRH increases norepinephrine release in the central nervous system (Jedema and Grace, 2004), but interactions with cardiac performance still require further investigation. There are contradictory effects of acute stress on heartrate in fish reported in the literature (Bloecher et al., 2024; Svendsen et al., 2021). These differences may be attributed to the type of acute stressor used in the study, or potential changes in other parameters of cardiac performance, such as the stroke volume of the heart, which can also control cardiovascular output alongside increased heartrate to accommodate elevated tissue oxygen requirements (Thorarensen et al., 1996). Pink salmon (*Oncorhynchus gorbuscha*) show elevated aerobic scope when exposed to increasing water temperature, but reduced cardiac stroke volume (Clark et al., 2011), indicating that tissue metabolism may outpace oxygenated blood transport and limit the animal's ability to cope with increased metabolic demands. When chronically treated with cortisol, rainbow trout develop cardiac hypertrophy which impairs cardiac performance by limiting total stroke volume (Johansen et al., 2017). How an impaired HPI-axis may interfere with cardiac development in zebrafish has yet to be determined, and whether CRHR1 plays a direct role in the adult function of the heart.

Taken together, CRHR1 has a critical role in the early life behavioural response to HS which may be regulated by changes in the mitochondrial bioenergetic profile. Chronic cortisol exposure modulates CRHR1 signalling as seen in differences between WT and CRHR1KO fish bioenergetics following the 16-hour cortisol exposure. Though CRHR1 does not appear to

modulate the heartrate, the effects on the development of the heart and cardiac remodelling remain unknown, and other cardiac performance markers should be further investigated. In conclusion, CRHR1 is a key player in the behavioural response to acute HS, which appears predominantly controlled through bioenergetic regulation.

2.5 Acknowledgements

This study was supported by the Natural Sciences and Engineering Research Council (NSERC) of Canada Discovery grant to MMV. I am grateful for the assistance by Dr. Jithine Rajeswari in collection of the larval behaviour data.

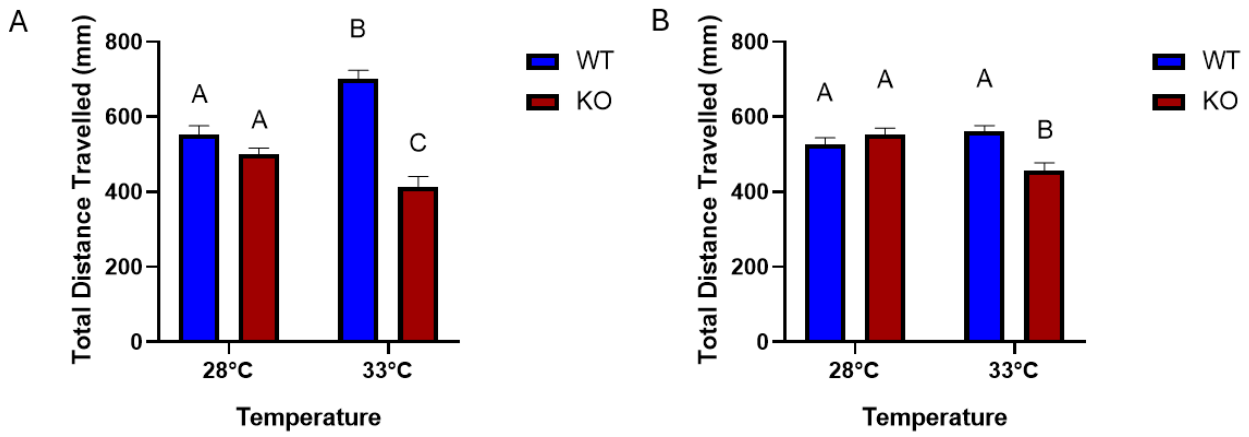


Figure 2.1 CRHR1 regulates the behavioural response following heat shock
The total distance travelled by 6 dpf WT and CRHR1KO zebrafish larvae exposed to 28°C or 33°C water temperatures for 1 hour, measured for 6 min in the light (A) or 4 min in the dark (B). Zebrafish were placed individually in a 24-well plate and incubated at either 28°C or 33°C for 1 hour prior to behavioural tracking. Light and dark was controlled and the larvae activity tracked using Noldus Ethovision software in a Daniovision system. Bars represent mean ± SEM (n = 149-237 larvae). Bars with different letters denote significant difference (ANOVA, Holm-Sidak *post-hoc* test, $P < 0.05$). Abbreviations: wildtype (WT), corticotropin-releasing hormone receptor 1 knockout (KO).

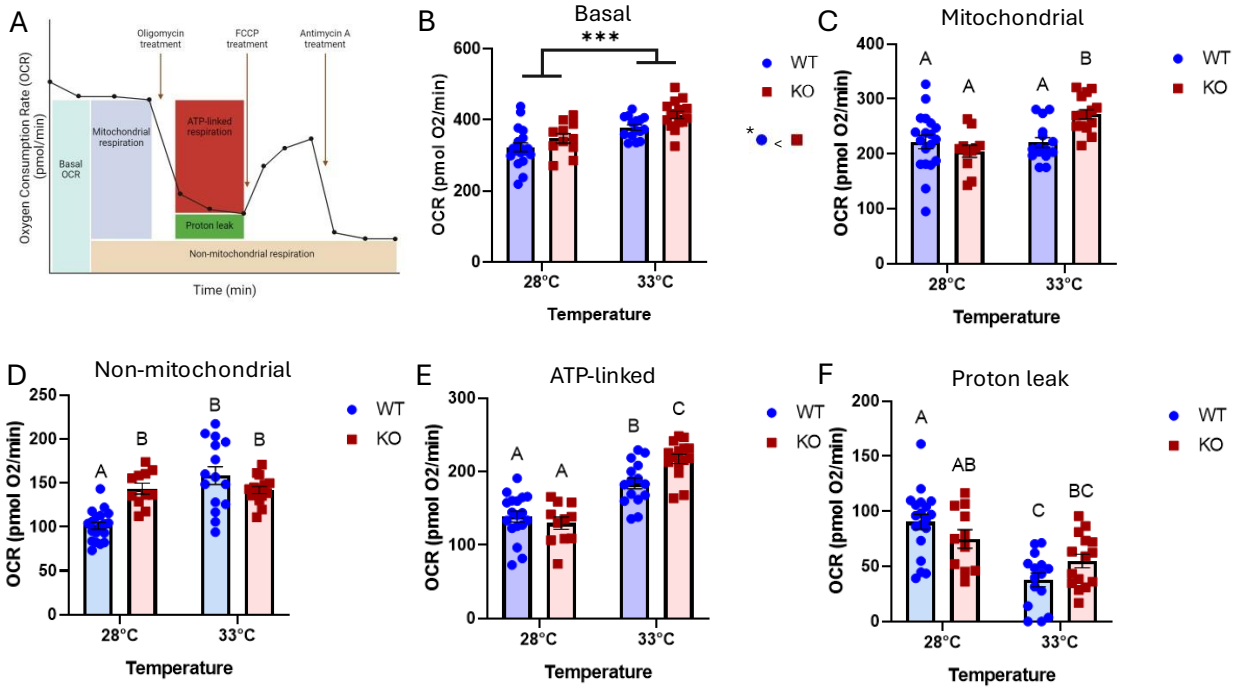


Figure 2.2 CRHR1 regulates mitochondrial bioenergetics

(A) Representative graph depicting the workflow of the Seahorse Bioanalyzer and how each respiratory value is calculated. Basal oxygen consumption rate (OCR) is the initial OCR measured without pharmacological treatment. Oligomycin treatment blocks ATP-linked OCR by inhibiting ATP synthase. FCCP treatment increases the overall OCR of mitochondria, but it did not produce a maximal OCR in this experiment. Antimycin A treatment inhibits complex II of the electron transport chain and blocks mitochondrial OCR. (B) Basal, (C) mitochondrial, (D) non-mitochondrial, (E) ATP-linked, and (F) proton leak-associated OCR of 5 dpf WT and CRHR1KO zebrafish larvae following 1-hour exposure to 28°C or 33°C water temperature. OCR was measured using the Agilent Seahorse XF24 Extracellular Flux Analyzer as detailed in the methods. Bars represent mean ± SEM (n=11-19). Bars with different letters denote significant difference (ANOVA, Holm-Sidak *post-hoc* test, $P < 0.05$). Abbreviations: wildtype (WT), corticotropin-releasing hormone receptor 1 knockout (KO), oxygen consumption rate (OCR), carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP).

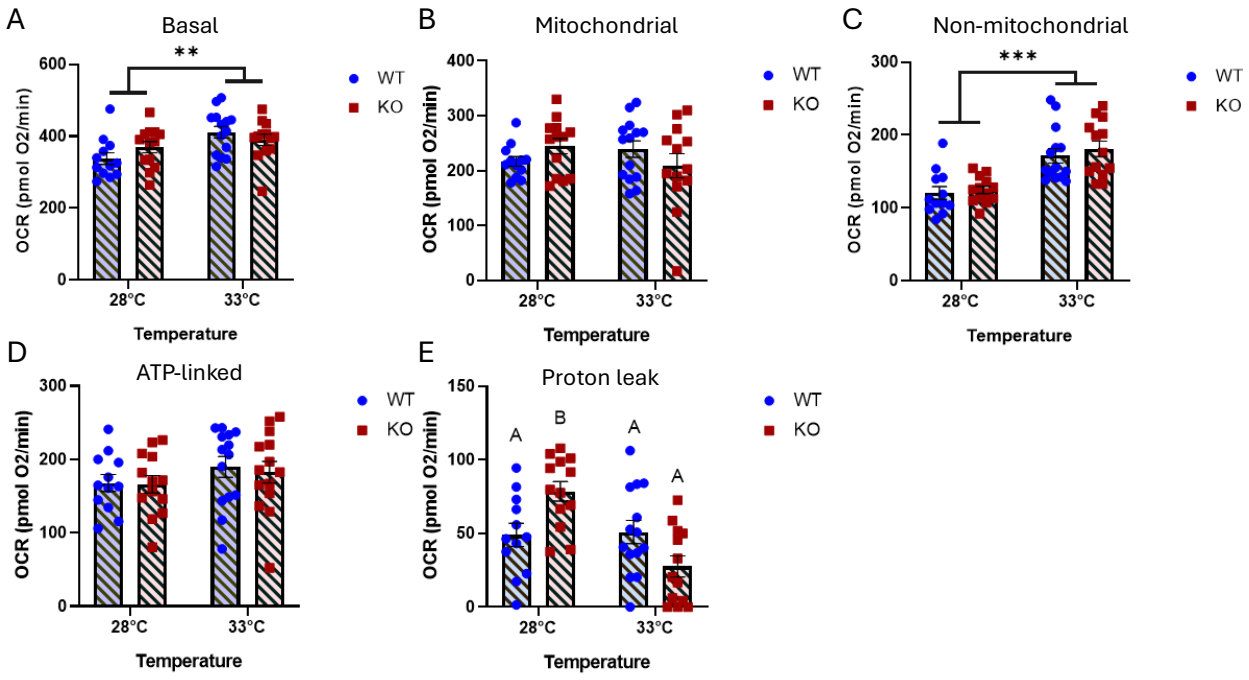
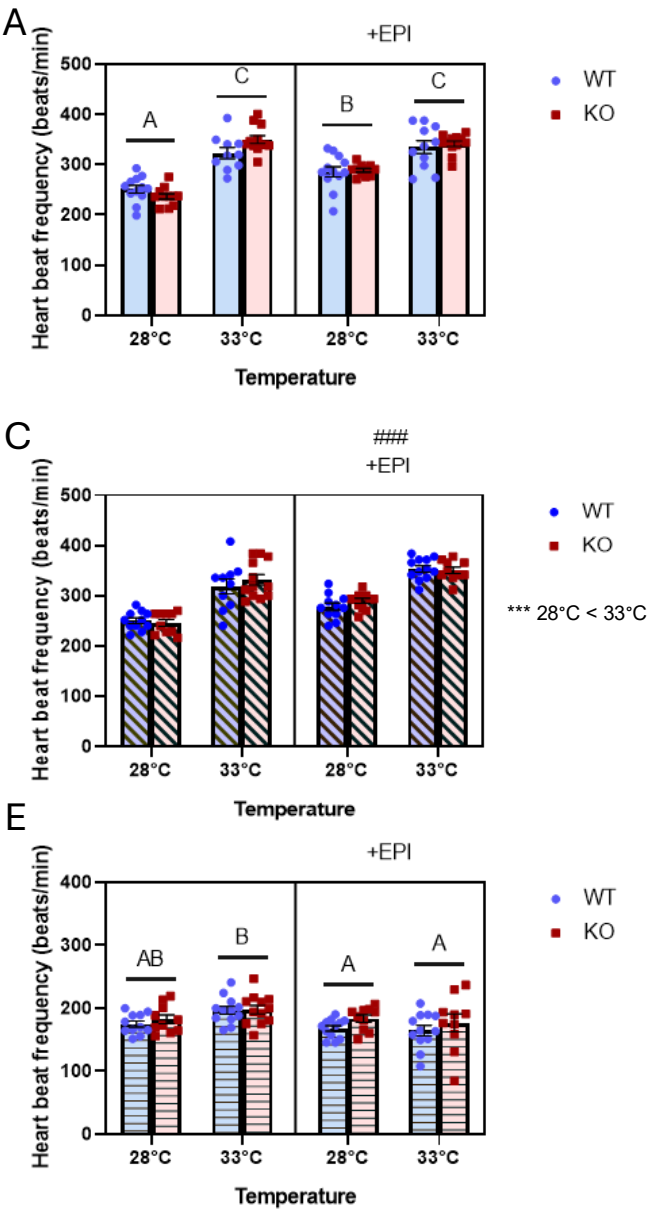


Figure 2.3 Mitochondrial bioenergetics following chronic cortisol treatment
(A) Basal, (B) mitochondrial, (C) non-mitochondrial, (D) ATP-linked, and (E) proton leak-associated oxygen consumption of 5 dpf WT and CRHR1KO zebrafish larvae following 16-hour treatment of cortisol and 1-hour exposure to 28°C or 33°C water temperature. OCR was measured using the Agilent Seahorse XF24 Extracellular Flux Analyzer as detailed in the methods. Bars represent mean ± SEM (n=11-19). (A,C) Asterisks denote significant main effect (Two-way ANOVA, ** $P < 0.01$, *** $P < 0.001$). Bars with different letters denote significant difference due to the interaction (ANOVA, Holm-Sidak *post-hoc* test, $P < 0.05$). Abbreviations:

wildtype (WT), corticotropin-releasing hormone receptor 1 knockout (KO), oxygen consumption rate (OCR) carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP).



B

Source of Variation	P value
Temperature	<0.0001
Epi Treatment	0.0003
Genotype	0.3500
Temperature x Epi Treatment	0.0008
Temperature x Genotype	0.0648
Epi Treatment x Genotype	0.9044
Temperature x Epi Treatment x Genotype	0.1165

D

Source of Variation	P value
Temperature	<0.0001
Epi Treatment	<0.0001
Genotype	0.4648
Temperature x Epi Treatment	0.4454
Temperature x Genotype	0.9207
Epi Treatment x Genotype	0.9938
Temperature x Epi Treatment x Genotype	0.1746

F

Source of Variation	P value
Temperature	0.2138
Epi Treatment	0.0067
Genotype	0.0900
Temperature x Epi Treatment	0.0315
Temperature x Genotype	0.6305
Epi Treatment x Genotype	0.3823
Temperature x Epi Treatment x Genotype	0.8837

Figure 2.4 CRHR1 does not modulate cardiac performance during acute heat shock. Heartbeat frequency of 6 dpf WT and CRHR1KO larvae before and following EPI treatment in the absence of cortisol (A), after 16-hour cortisol treatment (B), and following 90-min exposure to PROP (C). Heartrate was determined by counting the rate of ventricular ejection of blood in lightly anaesthetized larvae over a 5-15 s video recording. The p-value tables of each three-way ANOVA (genotype, temperature, and EPI treatment) for the heartrate data in A, C and E are shown in B, D and F, respectively. Bars represent mean ± SEM (n=10-12). (A,E) Genotype

factor was consolidated to investigate the two-way Temperature x EPI interaction. Different letters denote significant difference due to the interaction (ANOVA, Holm-Sidak *post-hoc* test, $P < 0.05$). (C) Symbols denote significant main effects between treatment groups (Three-way ANOVA, ### indicates EPI effect, *** indicates temperature effect, $P < 0.001$). Abbreviations: wildtype (WT), corticotropin-releasing hormone receptor 1 knockout (KO), epinephrine (EPI), propranolol (PROP).

Chapter 3: CRHR1 integrates the heat shock-mediated behavioural activity and metabolic rate changes in adult zebrafish

3.1 Introduction

The ability to cope with environmental challenges is a primary objective of the stress response. The corticosteroid stress axis activity is a highly conserved and critical part of the adaptive stress response. For instance, upon stressor perception, the hypothalamus-pituitary-adrenal (HPA) axis in mammals and the hypothalamus-pituitary-interrenal (HPI) axis in teleosts is activated leading to the synthesis and release of corticosteroids, which assists in re-establishing homeostasis (Faught et al., 2016a; Mommsen et al., 1999). Corticotropin-releasing hormone (CRH), released from the hypothalamus, is the proximal neuroendocrine response that stimulates the pituitary gland to release adrenocorticotropic hormone (ACTH), a post-translationally formed biologically active peptide from proopiomelanocortin (Faught et al., 2016a). Although the action of CRH is mediated by activating two receptors, the CRH receptor 1 (CRHR1) and CRH receptor 2 (CRHR2) (Sukhareva, 2021), the primary receptor involved in the synthesis and release of ACTH is CRHR1. Once ACTH is released into the circulation, it binds to the melanocortin 2 receptor (MC2R) in the adrenal cortex (or interrenal tissue in teleosts) to stimulate corticosteroid biosynthesis. The primary stress-induced corticosteroid released into the circulation is cortisol in humans and teleosts (Mommsen et al., 1999), and this glucocorticoid via activation of either the glucocorticoid receptor (GR) and/or the mineralocorticoid receptor (MR) is essential for stress coping (Faught et al., 2016a; Faught and Vijayan, 2022a).

While most studies have focused on the role of glucocorticoids in adjusting animals to stress (Jimeno and Verhulst, 2023), recently we demonstrated a coordinated role for CRH along with

cortisol in the temporal behavioural changes seen in response to an acute stress in zebrafish (Faught and Vijayan, 2022). The CRHR1 played an important role in the acute behavioural changes seen after an acute stress, but maintenance of this behavioural response over longer periods required the activation of GR and MR in zebrafish (Faught and Vijayan, 2022). It has been shown that cortisol increases the metabolic rate in fish, but most of these studies were after longer exposure to this steroid as the cortisol action required the activation of GR (Pfalzgraff et al., 2022), a ligand bound transcription factor that leads to genomic signalling and response spanning several hours (Faught et al., 2016a). However, it is unknown whether the activation of the HPI-axis and the associated neuropeptides released prior to the secretion of cortisol have any role in the acute metabolic rate changes that are seen in fish after an acute stressor exposure (Barton and Schreck, 1987).

Acute temperature changes have been shown to increase the activity levels and metabolic rate in fish (Biro et al., 2009; Schulte, 2015). This change in metabolic rate usually involves increased muscle activity and acute changes in protein synthesis that are essential for cellular protection against heat shock (Iwama et al., 1999; Pörtner et al., 2017), as well as the mobilization of energy substrates to provide fuel for the enhanced energy demand (Clarke and Fraser, 2004). Indeed, heat shock leads to transcriptional and translational activation of critical proteins involved in maintaining the cellular protein homeostasis (Kültz, 2005), including the upregulation of the highly conserved heat shock proteins (HSPs) (Iwama et al., 1999). Heat shock also activates the organismal stress response, and the associated increase in the corticosteroid release also leads to an increase in the metabolic rate as an adaptive response to cope with stress (Jimeno and Verhulst, 2023; Mommsen et al., 1999). While studies have examined the changes in metabolic rate associated with chronic cortisol elevation (Pfalzgraff et

al., 2022), it is unclear whether the activation of the HPI axis and the associated neuropeptide release also leads to an increase in metabolic rate independent of cortisol action. If that is the case, it would suggest that the HPI-axis may be priming the animal to respond to a stressor even prior to the release of corticosteroids, but this has not been tested yet.

Sensing a stressor generally elevates the metabolic rate of an animal (Christensen et al., 2019; Schwieterman et al., 2019; Sloman et al., 2000), which increases from the standard metabolic rate (SMR) up to the deemed maximum metabolic rate (MMR) the animal can produce. The difference between the MMR and SMR is known as the aerobic scope, which is indicative of the aerobic capacity of the animal available above basal maintenance metabolism and has been correlated with fitness-related traits (Clark et al., 2013). For instance, sockeye salmon (Oncorhynchus nerka) populations that require a further migration upriver to spawn, and thus rely on their swim performance, have a larger aerobic scope compared to populations with a shorter migration (Farrell et al., 2008). Chronic cortisol exposure elevates both the standard metabolic rate (SMR) and the maximum metabolic rate (MMR) in rainbow trout (Oncorhynchus mykiss) and improves recovery from exhaustive swimming (Pfalzgraff et al., 2022), but the acute response needs investigation. SMR, MMR and, thus, aerobic scope can all be shifted by a change in temperature, previously observed in westslope cutthroat trout (Oncorhynchus clarkii lewisi) and rainbow trout (Enders and Durhack, 2022) and among tropical species (Johansen and Jones, 2011; Nilsson et al., 2009; Rummer et al., 2014), but how components of the stress response interact with the response to temperature is not fully clear.

With the advent of genetic knockout (KO) models, the functional role of different components of the HPI-axis may be investigated in a highly targeted manner. For instance, previous work with ubiquitous CRHR1KO mice have showed impaired glucocorticoid synthesis capacity and altered

stress-related behaviour (Timpl et al., 1998). Recently we showed that ubiquitous CRHR1KO zebrafish larvae showed no stressor-induced elevation in cortisol levels and activity (Faught and Vijayan, 2022a). However, whether these changes affect the metabolic rate of the animal is currently unknown, as acute stress invariably increases the MR in animals (Barton and Schreck, 1987; Kuti et al., 2022). CRH treatment elevates the respiration rate and thermogenesis of mouse muscle tissue (Solinas et al., 2006), however the role of the CRHR's in this remain untested. Currently, it is known that CRHR1 is critical for the acute stress-induced hyperactivity of zebrafish larvae (Faught and Vijayan, 2022a), but it is unknown whether this is life-stage specific, or its function in metabolic rate adjustment. To test the metabolic rate we used temperature shock, as this abiotic stressor is a bona-fide acute stimulator of metabolic rate in animals (Schulte, 2015). Also, this will provide us an ecological context, given the predicted increases in environmental temperature and the impact that may have on ectothermic organisms (Beitinger et al., 2000; Brett, 1971; Comte et al., 2013; Farrell, 2009; Nilsson et al., 2009; Perry et al., 2005; Rummer et al., 2014; Schulte, 2015). We hypothesize that CRHR1 is a key receptor that also links temperature changes to behavioural and metabolic response, thereby integrating heat shock to the organismal stress response. To this end, we exposed WT and CRHR1KO adult zebrafish to an acute heat shock (HS), which consisted of a +5°C increase in ambient water temperature (28°C) and assessed the metabolic rate and swimming performance. We also determined whether the acute HS-induced cortisol release was responsible for the higher metabolic rate by inhibiting cortisol production using metyrapone, a cortisol biosynthesis inhibitor. Our results indicate that CRHR1 is essential for an acute HS-induced increase in metabolic rate in adult zebrafish and this occurs prior to the stress-induced cortisol elevation in zebrafish.

3.2 Materials and Methods

Animal Husbandry

Adult zebrafish (Tupfel long fin strain) were held in a recirculating system (Tecniplast, Italy) on a 14 hours:10 hours light:dark cycle (light on 09:00; light off 23:00). Water was maintained at 28°C, 800 μS conductivity, and a pH of 7.5. Fish were fed twice daily with Gemma Micro (500) adult zebrafish food in the morning (Skretting, Canada) and live Artemia (Brine Shrimp Direct, Utah) in the afternoon. The CRHR1KO zebrafish was generated using CRISPR/Cas9 as described previously (Faught and Vijayan, 2022b) and all offspring were produced from homozygous mutant parents. Fish were last fed at 16:00 the previous day of all experiments. All procedures were performed under the approval of the Animal Care Committee (AC17-0079 and AC21-0061) and under the standards set by the Canadian Council of Animal Care.

Experimental Design

For all studies, fish were exposed to either 28°C (ambient) or a 33°C heat shock (HS) water in an acute way adapted for each study design. In the behavioural study, data was collected upon immediate exposure of WT and CRHR1KO zebrafish to ambient or HS. For the remaining studies (resting intermittent-flow respirometry study, swim-tunnel respirometry study, and the novel tank exposure study), all fish were exposed to ambient or HS temperatures for an hour prior to data collection.

Behavioural Study

Changes in behaviour was assessed in zebrafish following immediate exposure to a novel environment to identify CRHR1-mediated responses to acute HS. Zebrafish underwent the vertical swim assessment as previously described (Thompson et al., 2022b) to determine swimming activity at ambient or HS temperatures. The vertical swim assessment evaluates overall distance swam as well as thigmotaxis in a novel tank environment by determining "boldness" of the animal, which is associated with time spent near the top of the tank (Egan et al., 2009; Maximino et al., 2010; Thompson et al., 2022b). Zebrafish were individually transferred with minimal handling from the housing system to a novel 2L 15cm x 15cm x 20cm system tank with a white background containing system water at either ambient or HS in a temperature-controlled room. A Sony Handycam HDR-CX550 (Tokyo, Japan) was placed approximately 50cm from the novel tank and recording began immediately after the fish was placed into the tank. After 5 mins, recording was stopped, and the fish was returned to the housing system. Water in the novel tank was changed between all trials. Fish were randomly chosen from the system for the trial and the sex was noted. Behaviour was analysed using Noldus Ethovision XT 14 (Wageningen, the Netherlands). Hyperactivity, representative of anxiety-like behaviours (Maximino et al., 2010), were defined as cumulative time spent above 90% mean swimming speed. Total swimming distance for the duration of the recording was also assessed.

Metabolic rate

To assess basal rates of aerobic metabolism, zebrafish were weighed (Supplemental Table 6.1) and placed in resting intermittent-flow respirometers consisting of 60mm long chambers each containing a total volume of 20 mL submerged in a temperature-controlled water bath (Loligo Systems, Viborg, Denmark) at 28°C. Fish were weighed and immediately placed in the respirometer chamber at 11:00. Following a 1h acclimation, which has previously been shown to enable accurate SMR measurements in zebrafish (Little et al., 2013), oxygen consumption (MO_2) was recorded using AutoResp (Loligo Systems, Viborg, Denmark) following a 60s flush, 60s wait, 120s measure cycle repeated for 8 cycles over 30 min, then fish were returned to their housing system. Following at least a week, the same fish were returned to the respirometer at 33°C (HS) and SMR measurements were repeated in the same way. The 3 lowest consecutive MO_2 readings were averaged to obtain SMR (Thompson et al., 2022b). A total of 4 respirometer chambers were ran simultaneously, with background oxygen consumption calculated from a single, empty chamber, while zebrafish were placed in the remaining 3 chambers for each trial. Immediately after obtaining SMR at 33°C, fish were euthanized using 0.5 mg/L of MS-222 (buffered 1:2 with sodium bicarbonate) and brain and whole body were sampled and stored at -80°C.

Metyrapone treatment

Elevated temperature acts as a stressor and thus activates cortisol biosynthesis through the HPI-axis (Wendelaar Bonga, 1997). To test whether cortisol was regulating the SMR, cortisol synthesis was blocked with a known 11β-hydroxylase inhibitor, metyrapone. WT zebrafish were

placed into static 2L tanks containing either system water or 325.4uM metyrapone (Vera-Chang et al., 2018) dissolved in system water held at 33°C. Half of the water was exchanged daily and replenished with the appropriate treatment. Morning and afternoon feeding were maintained for 4 days of exposure, after which fish were placed into a resting intermittent flow respirometer at 33°C and metabolic rate was measured as described above. Fish were sampled immediately after and whole body was homogenized for cortisol quantification.

Swim performance

Zebrafish were weighed (Supplemental Table 6.1) and immediately placed into a 170mL swim tunnel respirometer (Loligo Systems, Viborg, Denmark) between 11:00 and 18:00 to assess MMR and swim performance. Water flow was manually controlled and calibrated using neutrally buoyant fluorophores tracked and analysed by DPTV 2 (Loligo Systems, Viborg, Denmark). Fish were acclimated for 1h at a flow rate of 4.5cm/s and the oxygen consumption was measured using a 60s flush, 60s wait, 180s measure cycle. Following acclimation, flow rate was increased by 4.5cm/s every 10 min, allowing 2 full MO_2 measurement cycles at each swimming speed. Swimming was stopped once the fish rested against the honeycomb grate located at the back of the tunnel for longer than 5s. Aerobic scope was determined by subtracting the lowest MO_2 from the highest MO_2 during the recording period. Ucrit was calculated using the formula $U_{crit} = U + U_f \times (T_f/T)$, derived from Brett (1964), where U is the water velocity of the last completed swim cycle in cm/s, U_f is the water velocity of the final swim cycle the fish failed on in cm/s, T_f is the time spent successfully swimming during the final swim cycle in s, and T is the total duration of one swim cycle in s.

Novel tank exposure and tissue sampling

Housed zebrafish were placed individually into static 2L tanks containing system water held in a temperature-controlled room set at either 28°C or 33°C. After 1 hour, fish were removed and euthanized in 0.5 mg/L of MS-222 (buffered 1:2 with sodium bicarbonate). Brain, liver, muscle, and the remaining whole body were sampled and stored in -80°C for further analysis.

Cortisol quantification

Whole body was sampled from fish taken from the system, from the resting intermittent-flow respirometer study, and from fish in the 1-hour novel tank exposure and stored at -80°C until analysis. Tissue was ground and homogenized in 50mM Tris buffer, pH 7.5, with protease inhibitors (Roche Diagnostics, Canada) using a handheld tip sonicator. Steroids were extracted from whole body homogenate using a diethyl ether extraction protocol as previously described (Faught et al., 2016b), after which an in-house cortisol ELISA previously validated for zebrafish was used (Faught et al., 2016b).

Muscle metabolites

To determine effects on metabolites following HS, epaxial muscle posterior to the dorsal fin was sampled and stored at -80°C from the 1-hour novel tank exposure. Approximately 3-5mg of tissue was homogenized in the same way as the whole body. Muscle homogenate was spun down at 13 000 x G for 2 minutes and the supernatant was separated and used for all metabolite

analyses. Protein content was measured using a commercially available kit (Pierce BCA kit 23223/23224, VWR, Canada). Muscle glycogen was determined by measuring glucose in the homogenate before and after treatment with amyloglucosidase (Sigma-Aldrich), and tissue glucose was measured through enzymatic determination with hexokinase (Sigma-Aldrich) as previously described (Faught and Vijayan, 2022a).

Enzyme activities

Enzyme activities were quantified as described previously (Ings et al., 2011). Muscle homogenate held in 50% glycerol buffer (50% glycerol, 21 mM Na_2HPO_4, 0.5 mM EDTA, 0.2% BSA, and 5 mM β-mercaptoethanol, pH 7.5) was kept at -20°C until analysis. The kinetics of the following enzymes were measured in 50 mM imidazole (pH 7.4) at 340 nm using a microplate reader (SpectraMax 190, Molecular Devices, California):

Hexokinase (EC 2.7.1.1): 1 mM glucose, 5 mM $MgCl_2$, 10 mM KCl, 0.25 mM NADH, 2.5 mM phosphoenolpyruvate (PEP), 5 U/ml lactate dehydrogenase (LDH), and 2.5 U/ml pyruvate kinase. Reaction started with 1 mM ATP.

3-Hydroxyacyl-CoA dehydrogenase enzyme (HOAD; EC 1.1.1.35): 0.1 mM NADH; reaction was started with 0.1 mM acetoacetyl-CoA.

Pyruvate Kinase (EC 2.7.1.40): 3 mM KCl, 10 mM $MgCl_2$, 0.12 mM NADH, 2.5 mM ADP, 20 U/ml LDH. Reaction started with 2.5 mM PEP.

Lactate dehydrogenase (EC 1.1.1.27): 0.12 mM NADH. Reaction started with 1 mM Pyruvate.

RNA extraction and quantitative polymerase chain reaction

Brain tissue of resting intermittent-flow respirometer-exposed fish was dissected and stored at -80°C. Epaxial muscle from fish treated with the 1-hour novel tank exposure was sampled and stored in the same way. Tissue was homogenized in Trizol (VWR, Canada) and RNA was extracted according to the manufacturer's protocol. RNA was quantified using a Spectradrop Micro-Volume Microplate (Spectramax 190, Molecular Devices, CA, USA) and 1µg of total RNA was treated with DNase I (Thermo Scientific, Waltham, MA, USA), followed by cDNA synthesis using the high-capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA, USA). qPCR was performed with Sso Advanced Universal SYBR Green Supermix (BioRad, Canada) on the Applied Biosystems QuantStudio 3 Real-Time PCR System (Applied Biosystems, USA). Samples were run with the following cycling conditions: 94°C for 2 min, 40 cycles of 95°C for 30s, and 30s at 60°C, and a final extension step at 72°C for 10 min. Primers used for qPCR analysis are listed in Table 1. Melting curve analysis was done to confirm amplicon specificity. All transcript abundances were normalized to β-actin and analyzed using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001), with untreated WT groups standardized to a value of 1.

Statistics

All statistical analyses were carried out and graphs were plotted using GraphPad Prism 8 (GraphPad Software, CA, USA). All data are shown as mean ± SEM. Two treatment comparisons were analyzed by unpaired *t*-test ($\alpha = 0.05$), while all multi-treatment comparisons, in the cases of comparing behaviour, metabolic rate, and swim performance of WT and CRHR1KO at ambient or HS, were analyzed using two-way ANOVA ($\alpha = 0.05$, Holm-Sidak

post hoc). Data that failed the assumption or normality or homoscedasticity were log-transformed with all data presented as untransformed in the figures.

3.3 Results

Zebrafish behaviour

WT fish show a marked increase in swimming activity from 28°C (2249.4±172.9cm) to 33°C (4522.3±676.8cm) ($p < 0.001$; Figure 3.1A) and an increase in burst swimming from 28°C (5.8± 0.9s) to 33°C (50.4±14.2s) ($p < 0.001$; Figure 3.1D) which is absent in CRHR1KO ($p > 0.05$). Elevated activity of the HS WT can be seen for all time points throughout recording ($P < 0.0001$, Figure 3.1B). Heat map visualization of swimming activity suggests WT was more exploratory of the novel environment compared to CRHR1KO, which remained near the bottom of the tank (Figure 3.1C).

Metabolic rate and swimming performance

WT SMR measured in the resting intermittent-flow respirometer increased with temperature, from 541.0±63.0 $mgO_2/kg/h$ to 789.1±61.5 $mgO_2/kg/h$ ($p = 0.0089$, Figure 3.2C). This increase was not observed in the CRHR1KO ($P > 0.05$, Figure 3.2C). Temperature failed to influence the Ucrit in WT and CRHR1KO fish ($p < 0.05$), but a significant main effect in the Ucrit was seen between genotypes ($P = 0.0251$, Figure 3.3D). SMR measured in the swim tunnel respirometer showed a significant increase with temperature ($P < 0.0001$, Figure 3.3A), but no difference between genotypes ($P > 0.05$). MMR also did not change with an increase in temperature in both WT and CRHR1KO ($p < 0.05$), but MMR was significantly reduced in CRHR1KO compared to

WT at both temperatures ($P < 0.0001$, Figure 3.3B). Aerobic scope did not change with temperature in WT or CRHR1KO ($p < 0.05$, Figure 3.3C), but was overall reduced in CRHR1KO ($p = 0.0002$).

Whole-body cortisol content

Whole-body cortisol was assessed from unexposed fish at 28°C and fish from the respirometer at 33°C to verify HPI-axis activation. Cortisol values were significantly different from fish sampled from their home systems at 28°C between WT (37.05±11.41 ng/g tissue) and CRHR1KO (4.222±1.786 ng/g tissue) ($P = 0.0399$, Figure 3.2A). Cortisol in fish sampled from the resting intermittent-flow respirometer at 33°C were also significantly higher in WT (98.96±ng/g wet weight) compared to CRHR1KO (17.44±5.755 ng/g wet weight) ($P = 0.0019$, Figure 3.2B).

Metyrapone treatment

To investigate if the absence of cortisol in HS CRHR1KO resulted in the reduced metabolic rate observed, MO_2 was measured in 4-day metyrapone-treated and untreated WT fish at 33°C. Metyrapone treatment had no effect on the MO_2 ($p > 0.05$; Figure 3.2D). Fish were sampled and whole-body cortisol confirmed that the metyrapone treatment reduced cortisol biosynthesis ($p = 0.03$; Figure 3.2C).

Stress axis transcript quantification

Muscle *hsp70* (Figure 3.4A), *hsp90.1* (Figure 3.4B), and *hsp90.2* (Figure 3.4C) were all significantly increased in zebrafish exposed to HS independent of genotype ($P < 0.0001$). Muscle transcripts of *gr* did not change in WT zebrafish when exposed to HS ($p > 0.05$, Figure 3.5D). Muscle transcripts of *gr* increased significantly in CRHR1KO after HS treatment ($P = 0.0448$). GR-responsive genes *redd1*, *murf1a*, and *fkbp5* failed to change with temperature in WT and CRHR1KO fish, and no difference between genotype was detected ($p > 0.05$, Figure 3.5E-G). Muscle *mr* transcripts were unchanged with HS in WT zebrafish ($p > 0.05$, Figure 3.5H), but were significantly elevated in HS CRHR1KO compared to HS WT ($P = 0.0207$). Transcripts for *lpl* and *pparγ* did not change with temperature in WT and CRHR1KO fish, and expression was similar between genotypes ($p > 0.05$, Figure 3.5I,J). Enzyme activity of HOAD was not significantly affected by temperature or genotype ($p > 0.05$, Figure 3.5K).

Energy metabolism

Glucose content of the muscle was not significantly affected by temperature or genotype ($p > 0.05$, Figure 3.6A). Glycogen content of the muscle appears elevated in WT compared to CRHR1KO at ambient temperature, but this was not deemed significant ($P = 0.0506$, Figure 3.6B). *Gys1* transcript abundance did not change in WT during HS ($p < 0.05$, Figure 3.6C) but was significantly increased in HS CRHR1KO compared to ambient ($P = 0.0036$). WT fish showed no change in *pyg1* transcript abundance ($p > 0.05$, Figure 3.6D), but was significantly increased with HS in CRHR1KO ($p = 0.0275$). Lactate dehydrogenase enzyme activity was also significantly increased with temperature ($P = 0.0266$, Figure 3.6G) and in CRHR1KO compared

to WT (P = 0.0080). *Pklr* transcript abundance, and enzyme activities of hexokinase and pyruvate kinase showed no significant differences (P > 0.05, Figure 3.6D-F).

3.4 Discussion

Our results demonstrate for the first time that CRHR1, a key receptor involved in the downstream stimulation of corticosteroid production, is essential for the acute HS-induced enhanced behavioural activity and metabolic rate in zebrafish. Previous studies have shown that elevated cortisol levels related to stressor exposure increases the metabolic rate in fishes (Mommsen et al., 1999; Pfalzgraff et al., 2022), but this study underscores a key role for CRHR1 in integrating the temperature change with metabolic changes independent of cortisol release. Suboptimal temperatures impair the performance of the animal, particularly ectotherms who cannot thermoregulate (Wakamatsu et al., 2019). The molecular mechanisms that protect the animal from the harmful effects of HS require energy, ultimately limiting the maximal swimming performance from the animal (Pörtner et al., 2017). The CRHR1KO zebrafish model reveals a systems-level role of CRHR1 in integrating the metabolic requirements of the behavioural avoidance of HS. Here, we propose CRHR1 as a metabolic sensor of HS that increases metabolic rate to support the behavioural response both through the canonical activation of the HPI-axis and independently.

The CRHR1KO adult zebrafish have a clear lack of circulating cortisol, which was previously observed in the larvae (Faught and Vijayan, 2022b). The impaired HPI-axis activation likely abolishes the stress-induced de novo synthesis of cortisol. The work with CRHR1KO larvae elucidated the coordinated roles of CRH-CRHR1 signalling and cortisol in maintaining the

stress-related behavioural response (Faught and Vijayan, 2022b). It was postulated that the rapid CRHR1 activation initiated the hyperactive behavioural response to the stressor, while the delayed cortisol worked in concert to sustain this behaviour (Faught and Vijayan, 2022b). Our work also showed that the increase in MO_2 during HS is also abolished in CRHR1KO fish (Figure 3.2C), which suggests that CRHR1 signalling drives energy flux to support the metabolic demand of the behavioural response. In zebrafish larvae, blocking cortisol biosynthesis with metyrapone exposure inhibited the stress-mediated hyperactivity 60 min and 240 min post-stressor, but did not alter the hyperactivity at 15 min post-stressor (Faught and Vijayan, 2022b). In the present study, we show that metyrapone treatment does not abolish the HS-induced MO_2 increase (Figure 3.2E). Though more work is required to understand the role of CRHR1 in sustaining the hyperactivity in adult zebrafish, we propose that CRHR1 also functions in prolonging the behavioural response by driving an increase in MO_2 and acting as a metabolic sensor during stress.

Though CRHR1 is known to regulate behaviour and the cortisol synthesis in response to a stressor, for the first time we show that this receptor plays a key role in modulating the metabolic rate independent of glucocorticoid action. CRHR1 dysfunction is linked to several behavioural disorders in mammals, including anxiety and depression (Hernández-Díaz et al., 2021; Reul and Holsboer, 2002). A lack of CRHR1 in zebrafish larvae has also been shown to impair the behavioural and endocrine stress response by impaired cortisol biosynthesis (Faught and Vijayan, 2022b). Mammalian studies reveal the widespread distribution of CRHR1 in the central nervous system and its role in modulating the stress-induced behavioural response outside of the HPI axis (Donner et al., 2016; Müller et al., 2003; Sun et al., 2023); however, studies focusing on CRHR1 in fish are limited. The impaired rapid behavioural response to HS with fish lacking

CRHR1 suggests it may employ simultaneous central neuronal and endocrine regulation of the stress response. In the periphery, expression of CRHR1 has been found in the ovary of African cichlid fish *Astatotilapia burtoni* (Chen and Fernald, 2008), Japanese pufferfish *Fugu rubripes* (Cardoso et al., 2003), olive flounder *Paralichthys olivaceus* (Zhou et al., 2019), and zebrafish (Zhou et al., 2021), and ovary, heart, gills, and skeletal muscle of chum salmon *Oncorhynchus keta* (Pohl et al., 2001). Though CRHR1 expression is observed in the periphery, our understanding of its role outside of central activation is lacking. CRHR1 in peripheral tissues can be activated by circulating CRH or urotensins which may integrate metabolic changes independent of the HPI-axis (Lovejoy et al., 2014), but further work needs to be done to understand the regulation of peripheral CRHR1. The current study reveals CRHR1 may act as a metabolic sensor to prime the whole animal metabolism to respond to stress before and in concert with cortisol action.

Temperature sensing in fish have yet to be fully described, though here we propose CRHR1 acts as a temperature sensor to elicit behavioural modification beneficial for survival. Fish avoid water with temperatures that are outside their optimal temperature range, a behavioural stress response that allows the animal to move to a favourable environment by increasing locomotion (Brett, 1971; Schurmann and Christiansen, 1994). The lack of temperature sensing is evident from the lack of behavioural activity and metabolic rate changes which underscores the role of CRHR1 as an integrator of heat sensing to allow the animal to cope with temperature shock. This suggests that CRHR1 is required for escape behaviours critical for avoiding suboptimal water temperatures (Prosser and Nelson, 1981), though the mechanism remains to be elucidated. Indeed, other GPCRs have been shown to act as environmental sensors. For instance, calcium-sensing receptor (CaSR), a GPCR, acts as a temperature sensor by adjusting frequency and

oscillations of cytosolic Ca^{2+} (Brennan et al., 2023). Also, the conformation of GPCRs can be modified by temperature. This was shown using chromatography with purified β_2-adrenergic receptor, which presented two incomplete chromatographic peaks when treated with a ephedrine and pseudoephedrine conformational probe mixture at 35°C, but the peaks merged at 38°C and retention timing decreased, suggesting a conformational change due to an increase in temperature (Gao et al., 2017). This could enable GPCRs to alter their binding affinity and subsequently enhance or inhibit their signalling cascade due to a shift in environmental temperature. Although, we don't know the mechanism by which HS modulates CRHR1 activation and downstream activity changes, we propose this may include either receptor structural changes and/or downstream changes in the signalling pathways. CRHR1 itself has been shown to act as a sensor of other environmental factors. In mouse pituitary and nonpituitary cells, the proton-sensitive histidine residues in CRHR1 that form hydrogen bonds with other residues to arrange the tertiary structure can be disrupted by acidic conditions, ultimately resulting in activation, deactivation or a change its ligand sensitivity (Kameda et al., 2018). This suggests CRHR1 can act as an environmental sensor as it appears capable of directly integrating external stimuli with a physiological response.

Elevated cortisol levels following HPI-axis activation will bind GR in key tissues to liberate glucose from available stores and enhance proteolysis. Redundant glycogen metabolism appeared in the CRHR1KO as *gys1* and *pyg1* were upregulated in CRHR1KO fish with increased temperature exposure (Figure 3.6C,D). Also, the trend of increased glycogen content of the muscle in CRHR1KO exposed to HS may indicate reduced glycogen utilization in CRHR1KO (Figure 3.6B), though this study focuses on an acute response following 1 hour after exposure to elevated temperature and more time may have been required to detect changes in muscle

glycogen content following HS. The impaired Ucrit (Figure 2B), MMR and aerobic scope of CRHR1KO (Figure 3.2D,E) suggests aerobic metabolism relies on proper CRHR1 signalling. As well, there appears to be a greater reliance on anaerobic metabolism as LDH activity is significantly upregulated in CRHR1KO fish (Figure 3.6G). Here, it appears CRHR1 acts to prime the animal for the energetic cost of acute stress by increasing the oxidative capacity of the animal. CRHR1 may be a critical component in modulating an aquatic ectotherm's oxygen and capacity limited thermal tolerance (OCLTT), which is a hypothesis that integrates the whole-animal's physiology into its aerobic capacity throughout a thermal range (Pörtner et al., 2017). OCLTT is a complex characterization of an animals metabolic capacity within a thermal range, thus CRHR1 crosstalk with other metabolic pathways may also prepare the animal for the metabolic demand of temperature stress by enhancing energy substrate availability. However, constraints of the OCLTT concept limit the integration of anaerobic metabolism, as well as acute shifts in temperature rather than longer-term stable maintenances of the animal (Pörtner et al., 2017).Typically, a chronic exposure to temperature nearing the CT_{max} will result in a lowered aerobic scope (Johansen and Jones, 2011; Nilsson et al., 2009; Rummer et al., 2014), for which CRHR1 may have a protective effect in the short-term and act to supply tissues with oxygen to enable a greater aerobic scope, swim performance, and a higher capacity to cope with elevated temperature. Aerobic scope is also highly correlated with the cardiac performance of fish (Farrell, 2009), which has been linked to the collapse of the aerobic scope seen with exposure to temperatures approaching an animal's critical thermal maxima (CT_{max}), thus the role of CRHR1 in cardiac performance should be investigated. CRHR2 expression is seen in mammalian heart tissue, and activation has been shown to reduce arterial pressure and regulate contraction and heartrate while centrally-administered CRH controls arterial pressure in humans through CRHR1

(Takefuji and Murohara, 2019). Opposing function of CRH receptors in the cardiovascular system may act in concert to modulate the aerobic capacity of an animal during environmental stressors such as temperature.

This work both elucidates potential tissue-specific roles for CRHR1 outside of the HPI-axis as well as build upon the mechanistic view of how ectotherms can tolerate and respond to rising water temperatures. During short-term temperature exposure, CRHR1 is required for a normal behavioural response and metabolic rate adjustment which may be required for the survival of wild populations. Short-term temperature fluctuation is expected to increase with climate change (Meehl and Tebaldi, 2004), thus it is imperative to understand how animals can physiologically adapt to acute heat shock. Further work is required to identify the role of CRHR1 and other proteins involved in the stress response in chronic temperature elevation, especially in the context of global water temperature increases (Meehl and Tebaldi, 2004). Overall, this work reveals the systems-level role of CRHR1 in enhancing an organism's capacity to respond to acute rises in environmental temperature.

3.5 Acknowledgements

This study was supported by the Natural Sciences and Engineering Research Council (NSERC) of Canada Discovery grant to MMV. I am grateful for the assistance by Dr. Jithine Rajeswari for tissue collection and analyzing brain *hsp* qPCR data.

Table 3.1 Forward and reverse primers of target genes used for quantitative real-time PCR.

Target	Forward primer (5` - 3`)	Reverse primer (5` - 3`)
gr	GTAGAACTACGAATGTCTTA	GCAACAGACAGCCAGACAGCTCACT
mr	CCCATTGAGGACCAAATCAC	AGTAGAGCATTTGGGCGTTG
dnajb1a	CGGAGCAGAGGATAAATTCAAAG	AAAGTGTAGCTAGGGCCATTG
dnajb1b	AGCCGAGGAAAAGTTCAAGG	GAGCACCTCCCTTCAGTC
hsp70	ACTTACTCCGACAACCAGCC	AAGGTCACTTCGATCTGCGG
hsp90.1	CAAACTCGGACTTGGCATCG	CCCTCCAGAACGGGCATATC
hsp90.2	TCAAACTGGGCTTAGGCATTGA	CTCCTCTATCGGGGCAGAAC
gys1	CACAACATGCTGGAGGATAG	CCATCGGTAAGAGAGGAGAA
pyg1	TGTAAAGTCCTCGCGCACA	ACATCCCCGAGTCCTGCTAT
pklr	CCAGTTTAACACGCGCGGC	GGGAAGTGTCCTTTGGCTGT
redd1	TGGACTCTGACTCCGACAACC	ACCACTTCTTTACACAACGCCTC
murf1a	GGAAGAAAACTGCCAGGCACAG	CTGGGTGATCTGCTCCAGAAGATG
fkbp5	GTGTTCGTCCACTACACC	TCTCCTCACGATCCCACC
lpl	ACAATTGACCCAACTGCTGA	GGTTCTTCGAGGGTCCGAAA
ppary	GGTTTCATTACGGCGTTCAC	TGGTTCACGTCACTGGAGAA
dgat2	CCATACTTGCTGCATATTCC	ATGTCATGATAAACTGCAGC
elovl2	CACTGGACGAAGTTGGTGAA	TTGAGGACACACCACCAGA
fas	GGAGCAGGCTGCCTCTGTGC	TTGCGGCCTGTCCCACTCCT
β-actin	TGTCCCTGTATGCCTCTGGT	AAGTCCAGACGGAGGATG

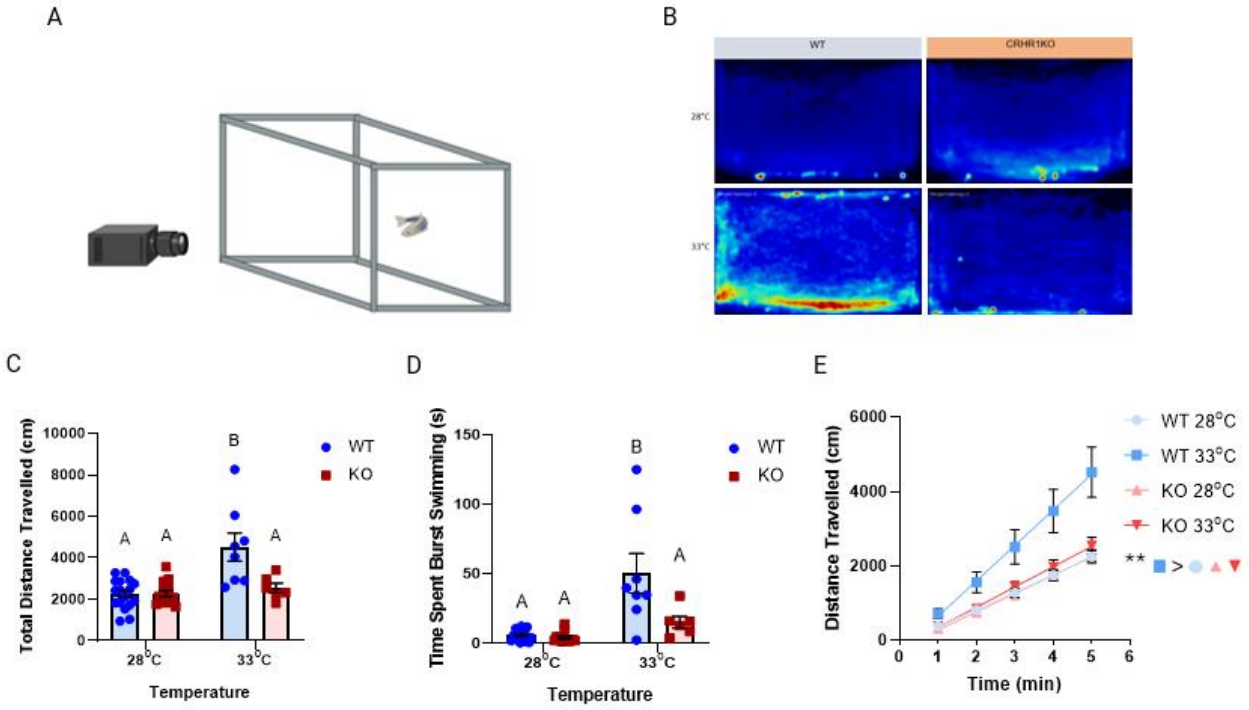


Figure 3.1 CRHR1 is essential for the acute heat shock-induced activity
(A) Diagram of the experimental setup for the vertical swim assessment. The recording camera was positioned to record from the side of the novel tank with the zebrafish. (B) Representative heatmap of the mean position of each treatment in the novel tank environment during the 5 min recording period, as observed by the camera field of view shown in (A). The light blue (less activity), yellow (more activity) and red (most activity) regions of the heatmap represent the overall activity of the fish in the tanks. (C) Total distance travelled by WT and CRHR1KO zebrafish exposed to a novel tank environment at 28°C or 33°C for 5 mins. (D) Total time zebrafish spent burst swimming, defined as swimming speeds greater than 90% of the average speed of each fish during the 5 min recording period. (E) Distance travelled by zebrafish per minute over the 5 min period. Adult zebrafish were transferred from their housing system to a novel tank containing either 28°C or 33°C system water. Video recording started immediately after placement of fish and lasted for 5 mins. Behaviour was analysed using Noldus Ethovision XT 14. Bars with different letters denote significant difference (ANOVA, Holm-Sidak *post-hoc* test, $P < 0.05$). (E) Asterisks denote significant difference between treatment groups (linear regression, ** $P < 0.01$). Abbreviations: wildtype (WT), corticotropin-releasing hormone receptor 1 knockout (KO).

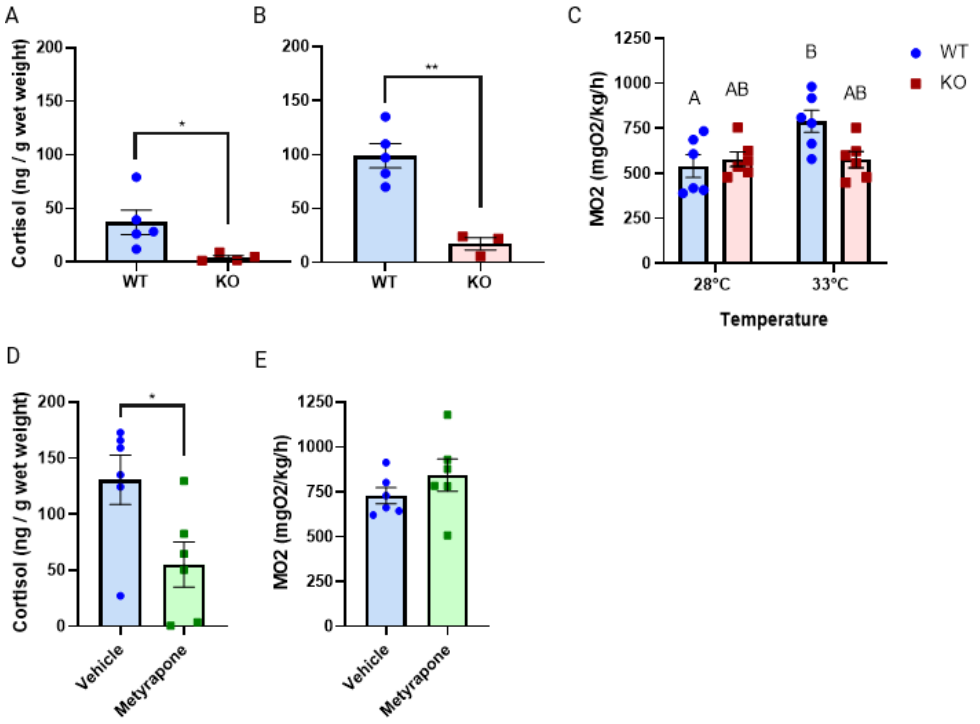


Figure 3.2 CRHR1 is required for the heat shock-induced increase in metabolic rate
(A) Whole-body cortisol concentration of WT and CRHR1KO zebrafish sampled from the housing system at 28°C or (B) after 90 min exposure to the intermittent flow respirometer at 33°C using an in-house cortisol ELISA. (C) Metabolic rate of WT and CRHR1KO fish at 28°C and 33°C after 60 min of acclimation in the resting intermittent-flow respirometer. MO_2 was calculated as the mean of the three lowest consecutive measurements from each individual. (D) Whole-body cortisol concentration of WT zebrafish exposed for 4 days to 325.4 µM metyrapone and sampled immediately after 90 min exposure to the resting intermittent-flow respirometer at 33°C. (E) Metabolic rate of untreated and metyrapone-treated WT fish at 33°C after 60 min of acclimation in the resting intermittent-flow respirometer. MO_2 was calculated as in (C). Asterisks indicate significant difference (student's T-test; * $P < 0.05$, ** $P < 0.01$). Bars with different letters denote significant difference (ANOVA, Holm-Sidak *post-hoc* test, $P < 0.05$). Abbreviations: wildtype (WT), corticotropin-releasing hormone receptor 1 knockout (KO), metabolic rate (MO_2), enzyme-linked immunosorbent assay (ELISA).

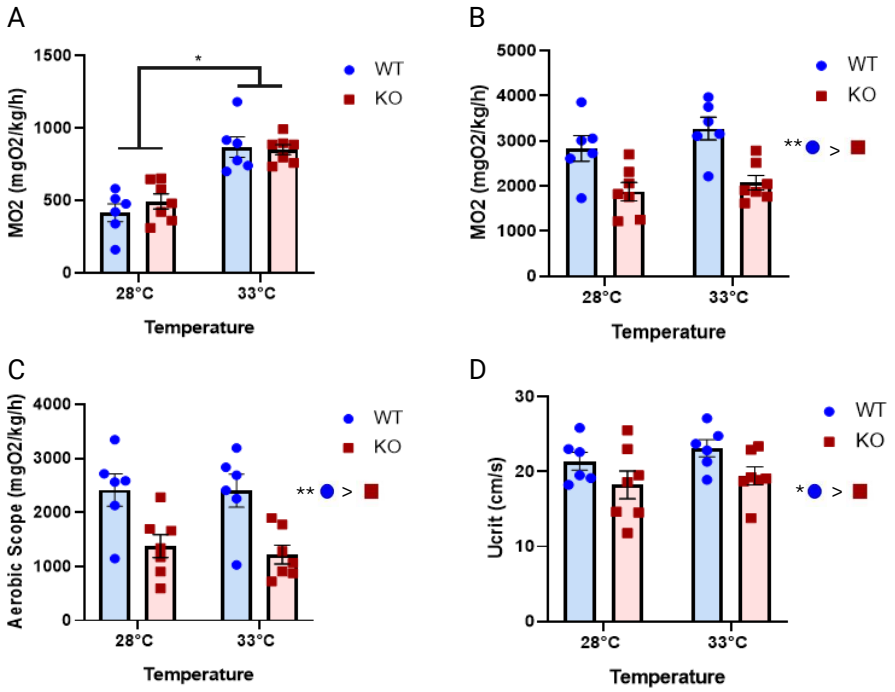


Figure 3.3 CRHR1 is essential for maintaining the heat-induced aerobic scope and swimming performance

(A) The SMR of WT and CRHR1KO fish measured in a swim tunnel respirometer during the 1-hour acclimation at either 28°C or 33°C prior to the forced swim. This value was taken as the single lowest MO_2 measurement during acclimation at a water flow speed of 4.5cm/s. (B) The MMR of fish taken as the single largest MO_2 value measured during the forced swim test. (C) Aerobic scope of fish calculated as the difference between the MMR and SMR (MMR – SMR). (D) U_{crit} of fish following the forced swim test, where water flow was slowly increased by 4.5cm/s every 10 min until the fish collapsed against the back grate of the swim tunnel. Asterisks indicate significant main effects (Two-way ANOVA, * $P < 0.05$, ** $P < 0.01$). Abbreviations: standard metabolic rate (SMR), wildtype (WT), corticotropin-releasing hormone receptor 1 knockout (KO), maximum metabolic rate (MMR), metabolic rate (MO_2), critical swimming speed (U_{crit}).

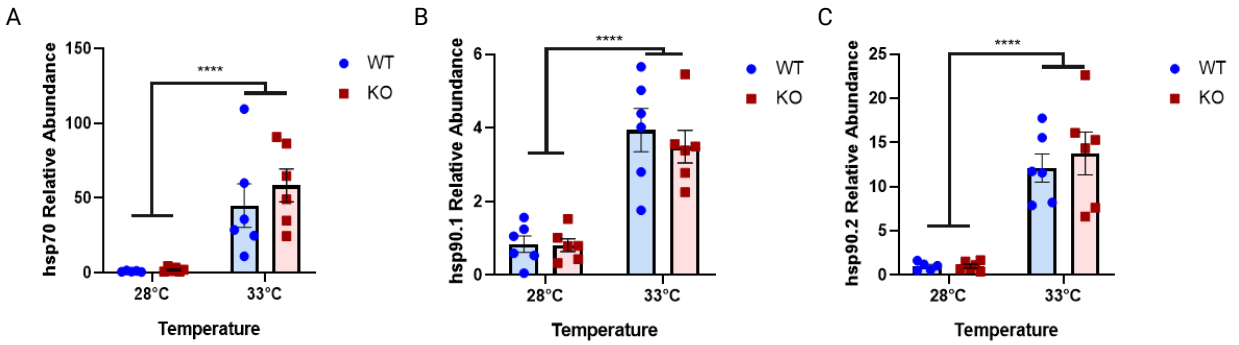


Figure 3.4 CRHR1 does not modulate the acute cellular heat shock response
(A) Transcript abundance of *hsp70*, (B) *hsp 90.1*, and (C) *hsp 90.2* in the epaxial muscle of WT and CRHR1KO zebrafish exposed to a novel tank environment containing 28°C or 33°C water for 1 hour. Transcripts were normalized to β-actin expression and the WT 28°C fish were standardized to 1 using the $2^{-\Delta\Delta Ct}$ method. Asterisks indicate significant main effect (Two-way ANOVA, **** $P < 0.0001$). Abbreviations: wildtype (WT), corticotropin-releasing hormone receptor 1 knockout (KO), heat shock protein 70 (*hsp70*), heat shock protein 90.1 (*hsp90.1*), heat shock protein 90.2 (*hsp90.2*).

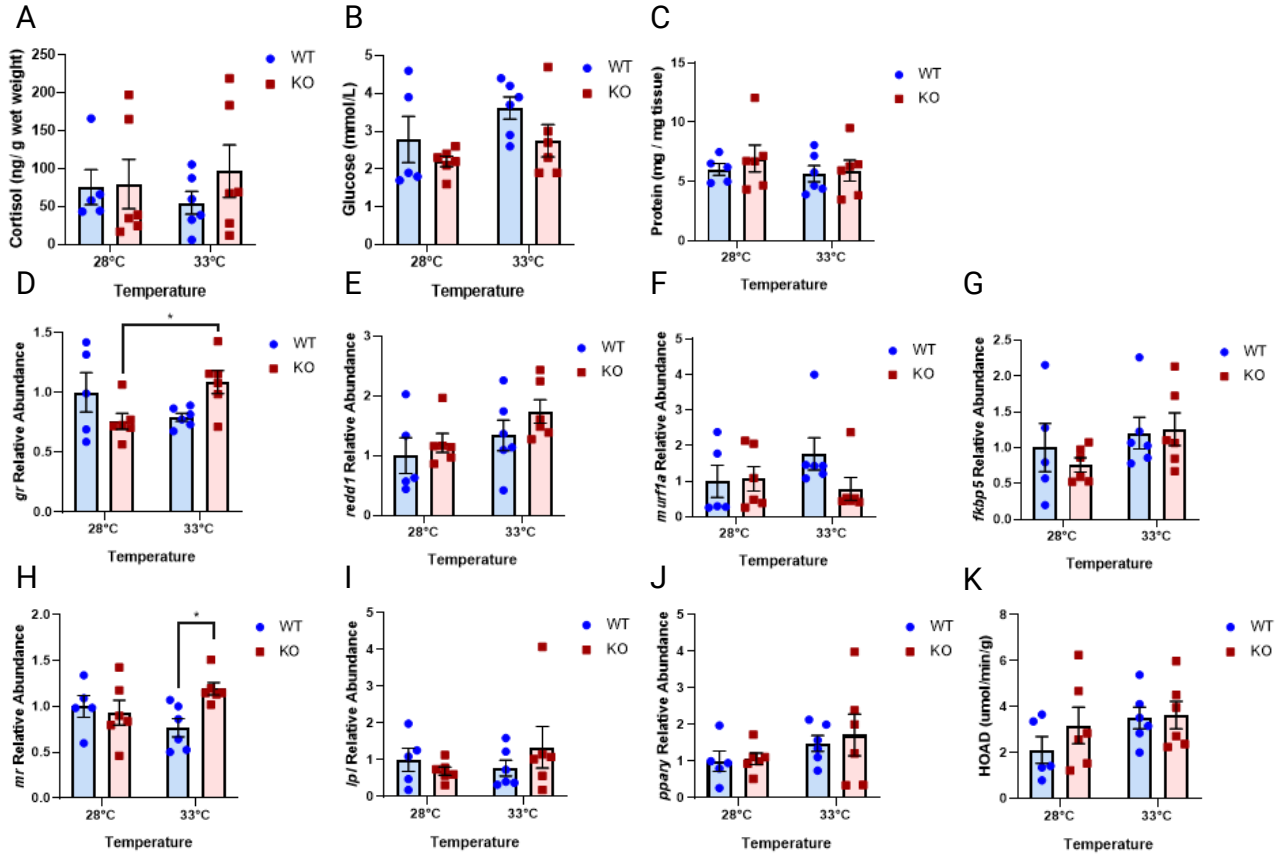


Figure 3.5 The acute heat shock did not affect cortisol signalling

(A) Whole-body cortisol, (B) blood glucose, and (C) whole-body protein concentration of WT and CRHR1KO zebrafish exposed to 28°C or 33°C novel tank environment for 1 hour. (D) Transcript abundance of *gr*, (E) *redd1*, (F) *murf1a*, (G) *fkbp5*, (H) *mr*, (I) *lpl* and (J) *ppary*, and (K) enzyme activity of HOAD measured in the epaxial muscle. Transcripts were normalized to β-actin expression and WT 28°C fish were standardized to 1 using the $2^{-\Delta\Delta Ct}$ method. Asterisks indicate a significant difference between treatment interaction (two-way ANOVA, Holm-Sidak *post-hoc*, * $P < 0.05$). Abbreviations: wildtype (WT), corticotropin-releasing hormone receptor 1 knockout (KO), glucocorticoid receptor (*gr*), regulated in development and DNA damage response 1 (*redd1*), muscle ring-finger protein 1 (*murf1a*), FK506 binding protein 5 (*fkbp5*), mineralocorticoid receptor (*mr*), lipoprotein lipase (*lpl*), peroxisome proliferator-activated receptor γ (*pparγ*), 3-hydroxyacyl-COA dehydrogenase (HOAD).

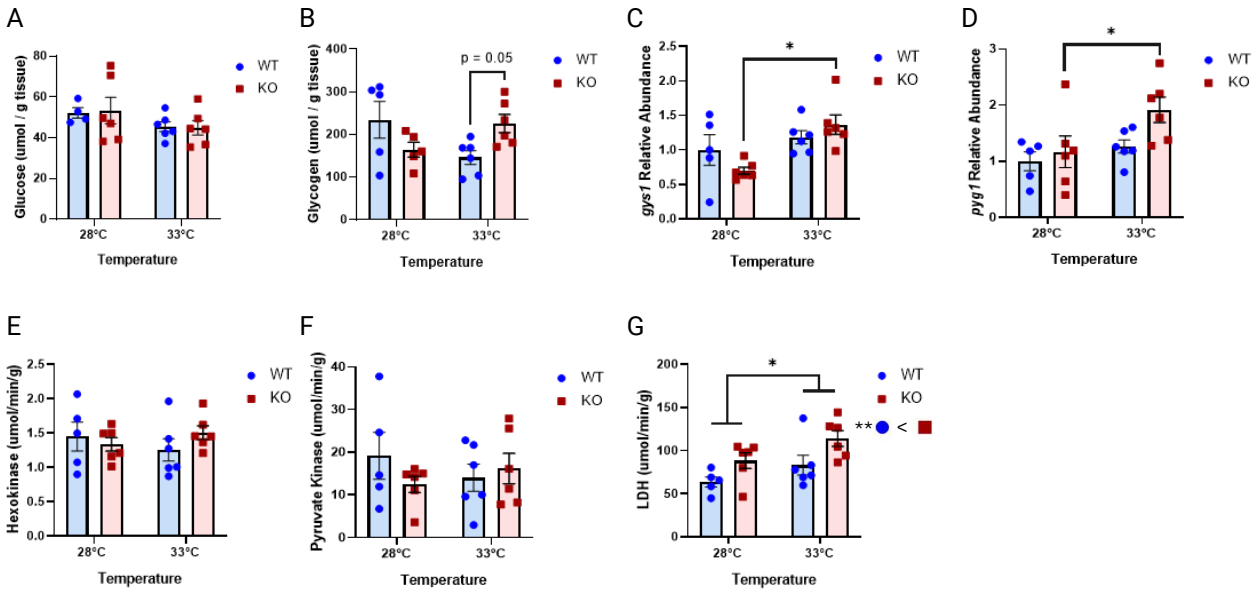


Figure 3.6 CRHR1 affects acute heat shock-mediated muscle carbohydrate and lactate metabolism

(A) Glucose concentration and (B) glycogen concentration measured in WT and CRHR1KO zebrafish epaxial muscle exposed to the novel tank test for 1 hour at either 28°C or 33°C. (C) Transcript abundance of muscle *gys1* and (D) *pyg1*, and (E) enzyme activity of hexokinase, (F) pyruvate kinase, and (G) lactate dehydrogenase measured in WT and CRHR1KO zebrafish epaxial muscle exposed to the novel tank test for 1 hour at either 28°C or 33°C. Asterisks indicate a significant difference between treatment interaction (two-way ANOVA, Holm-Sidak *post-hoc*, * $P < 0.05$) or (G) main effects (Two-way ANOVA, * $P < 0.05$, ** $P < 0.01$). Abbreviations: wildtype (WT), corticotropin-releasing hormone receptor 1 knockout (KO), glycogen synthase 1 (*gys1*), glycogen phosphorylase 1 (*pyg1*), lactate dehydrogenase (LDH).

Chapter 4: General conclusion

4.1 Findings

CRHR1 has a clear role in modulating the physiological response to acute stress in both mammals and teleosts through HPA/HPI-axis regulation (Faught et al., 2016a; Faught and Vijayan, 2022b), and independent central and peripheral effects (Kolber et al., 2010; Lewis et al., 2001). The fundamental endpoint of HPI-axis activation and subsequent cortisol action is to mobilize energy substrates to respond and recover from stress (Faught et al., 2016a; Mommsen et al., 1999; Wendelaar Bonga, 1997). Cortisol and direct central CRHR1 signalling simultaneously modifies the behaviour of the organism to respond to the stressor (Müller et al., 2001; Oliveira et al., 2013). However, the nature of the stress response and CRHR1 activity can depend on the type of stressor. (Lovejoy et al., 2014; Lovett-Barron et al., 2020). This may also affect which ligands of the CRH family are synthesized, where ligand-specific interactions with CRHR1 and downstream signalling may occur (Lovejoy et al., 2014; Lovett-Barron et al., 2020). Arguably one of the most disruptive environmental stressors for an aquatic ectotherm is a shift in temperature (Goikoetxea et al., 2021), and climate change may result in rising daily temperature fluctuations that cause stress (Paaijmans et al., 2013). This thesis aims to broaden our understanding of the systems-level function CRHR1 plays during the acute stress response. Specifically, I investigated how CRHR1 regulates the stress response to acute heat shock (HS). To this end, global CRHR1 signalling in fish has recently become easier to investigate through a ubiquitous knockout of CRHR1 in a zebrafish model (Faught and Vijayan, 2022b), which I employed for this work. Previous work alongside this thesis confirms the importance of CRH-CRHR1 signalling in behavioural regulation during stress (Kolber et al., 2010; Kovács, 2013;

Müller et al., 2003). The metabolic adjustment to respond to these stressors require further study, but this thesis sought to identify behavioural phenotypes which may correlate with changes in the metabolic rate and performance of the animal, both at the early developmental stage and in adulthood.

The work in chapter 2 investigated the HS effects on the established CRHR1KO zebrafish larvae, and further clarified how bioenergetic changes and adrenergic signalling may be regulating the response to this stressor. The behaviour following HS reflects what was previously reported with a swirling stressor (Figure 2.1; Faught and Vijayan, 2022), which supports that the HS activates the stress response. The increase in swimming activity is expected, which may be related to avoidance behaviour and/or increased reaction rates due to the increase in water temperature (Alfonso et al., 2021; Schulte, 2015). CRHR1KO fish also failed to respond to the HS, linking CRHR1 to the temperature-induced behavioural modulation. Investigating the mitochondrial bioenergetics of WT and CHRHR1KO fish during HS revealed CRHR1 as a modulator of oxygen consumption related to ATP synthesis, proton leak, and non-mitochondrial function (Figure 2.2). Suboptimal energy partitioning may correlate with the differences in behaviour observed in the CRHR1KO fish, as increasing the rate of energy flux is implicated in a rise of fitness of the animal (Sokolova, 2021). As the CRHR1KO larvae have impaired mitochondrial bioenergetic function when exposed to elevated temperature, we conclude that CRHR1 is critical in modulating the bioenergetic response to HS, which may cause the impaired behavioural response and result in reduced fitness of the animal (Kovács, 2013; Sokolova, 2021).

The adult zebrafish CRHR1KO offers a powerful model for investigating CRH-CRHR1 signalling at the systems level. The purpose of chapter 3 was to identify differences the adult CRHR1KO zebrafish had from WT in behavioural, metabolic and performance adaptations to

HS. Behaviour of the CRHR1KO fish mirrored that seen in the larvae: increased activity was observed in WT following HS, but CRHR1KO failed to respond (Figure 3.1). The role of CRHR1 in central modulation of stress behaviours has been investigated (Faught and Vijayan, 2022b; Reul and Holsboer, 2002; Sukhareva, 2021), but here we explored a novel perspective that CRHR1 may regulate the metabolic rate which enables the increased swimming activity following HS. Impairment in the metabolic rate response of CRHR1KO fish following HS reflects the lack of the behavioural response (Figure 3.2). Further, attenuation of cortisol biosynthesis in WT fish by a 4-day metyrapone exposure did not impair the heat-induced metabolic rate increase (Figure 3.2E), implicating a role for CRHR1 in the stress response independent of cortisol. This suggests CRHR1 is a fundamental modulator in the metabolic rate response to HS which is critical in the avoidance behaviour mechanism to escape suboptimal temperature (Harris and Carr, 2016; Prosser and Nelson, 1981; Schulte, 2015). Further testing via swim tunnel respirometry revealed CRHR1KO fish have impaired maximum swimming speed (U_{crit}) and aerobic scope compared to WT (Figure 3.3). This may be due to limited metabolite availability, as bioenergetic modulation by CRHR1 is also seen through changes in glycogen metabolism gene expression and a trend of reduced total muscle glycogen content is observed in the CRHR1KO fish (Figure 3.5; Figure 3.6). Impaired regulation of metabolites may divert cellular resources away from the key processes involved in the stress response, such as ATP-synthesis and anaerobic metabolism. Notably observed in chapter 2, mitochondrial and non-mitochondrial bioenergetics were altered in the CRHR1KO fish (Figure 2.2), which suggests CRHR1 is essential in directing energy resources during stress. This in conjunction with observations in chapter 3 support the role of CRHR1-signalling in adjusting the glycolytic capacity and energy substrate availability of the muscle to prime the animal for the acute stress

response. As energy must be consumed to facilitate an increase in swimming activity to support the behavioural response during heat stress (Skulachev et al., 2012; Sokolova, 2021), CRHR1 may also regulate the behavioural response by increasing available energy substrates. Taken together, we propose CRHR1 as a key modulator of energy substrates critical in supplying energy for the behavioural response and aerobic performance essential in coping with an acute stressor (Faught et al., 2016a).

The work in this thesis suggests that CRHR1 acts as a critical regulator of the behavioural stress response and works independently of the HPI-axis to facilitate bioenergetic regulation of metabolically active tissues. CRHR1KO larvae present an altered bioenergetic profile from WT larvae (Figure 2.2), which parallels the adult CRHR1KO fish's impaired metabolic rate response (Figure 3.2) and swim performance (Figure 3.3) during acute HS. Both life stages of the CRHR1KO failed to respond to HS, indicating CRHR1 as a potential sensor of elevated water temperature by modulating the behavioural and metabolic rate response. The results suggest that CRHR1 is key in integrating the metabolic changes required to support the energy demand of increased aerobic capacity and behavioural adaptation during acute stress in the whole zebrafish.

4.2 Significance of the study

Though the effects of cortisol in response to different stressors has been established (Faught and Vijayan, 2022b; Johansen et al., 2011; Mommsen et al., 1999), the role of other components of the HPI-axis, especially during heat stress, has not been fully elucidated. This work is the first to identify the whole-animal role for CRHR1 in the acute HS response in a teleost model. This work suggests CRHR1 integrates the behavioural stress response through bioenergetic

modulation. Though CRH brain signalling is involved in anxiety- and depression-related behaviour (Arborelius et al., 1999; Müller et al., 2003), the data from this thesis uniquely integrates the behavioural response and swimming performance to the metabolic capacity of the animal. CRHR1 signalling appears fundamental in simultaneously controlling the metabolic rate in zebrafish, which correlates to the behavioural response during HS. We observed cortisol can regulate the bioenergetics of the mitochondria of the zebrafish, while CRHR1 appears to regulate the metabolic rate during acute HS independently of cortisol action. This uniquely implicates CRHR1 as a potential sensor to temperature modulation key in adapting the animal to HS. Taken together, this work provides perspective of how CRHR1 acts globally to enable a response to the consequences of climate change, and its impact on the early-life development in fish.

4.3 Future work

In the context of this thesis, only the effects of acute HS were tested. However, the role of CRHR1 during chronic stress scenarios can be investigated using this CRHR1KO zebrafish model as well. Extended exposure to suboptimal temperature remains a threat for aquatic life, where fish can cope for short periods but cannot survive indefinitely (Pörtner, 2010). The extent the CRH-CRHR1 system is involved in prolonged heat exposure is unknown. The next steps for this work would be testing the role of CRHR1 during chronic exposure to elevated temperature. This would elucidate how CRHR1 may modulate the effects of temperature on development into adulthood (Scott and Johnston, 2012). Furthermore, our work is limited to lab reared fish that are adapted to a constant temperature. This is proven to increase the sensitivity of the cellular HS response, and impair thermal plasticity in the metabolic rate and swimming performance compared to wild zebrafish (Morgan et al., 2022). As well, an aquatic environment has an

oscillating temperature imperative for the biological clock, and benefits larval hatching, growth, and survival (Villamizar et al., 2012). Whether these thermocycling conditions during the early developmental stages alter the stress response remain to be investigated. Thus, future work should focus on oscillating temperatures that mimic predicted climate change-induced temperature fluctuations, and how CRHR1 regulates the response to chronic exposure and improves the thermal plasticity of the zebrafish.

Though CRH shows binding affinity for both CRHRs (Hosono et al., 2015; Lovejoy et al., 2014), the value of CRHR2 in the stress response still remains unclear. Sukhareva (2021) postulates two potential roles for CRHR2 in the CRH-mediated stress response: it signals the return to homeostasis that opposes CRHR1 action or CRHR2 activates alternate behaviours to that of CRHR1, such as passive, depression-like behaviours rather than active defensive behaviours attributed to CRHR1 in rats (Maier and Watkins, 2005). Knowledge of CRHR2 in fish is limited, though its expression is wide both centrally and peripherally in ovary, cardiac, and skeletal muscle tissue (Pohl et al., 2001; Zhou et al., 2019). Here, the CRHR1KO model can be used to identify CRH family peptide action on CRHR2 without interference from HPI-axis activation through CRHR1. Further work can use the CRHR1KO fish to characterize CRHR2 by testing the behavioural response to acute stress following exposure to a selective CRHR2 antagonist such as astressin$_2$-B to clarify the organismal role of the CRHRs (Wang et al., 2011). As well, generating a CRHR2KO and CRHR1/CRHR2 double KO zebrafish model, previously done in mice (Preil et al., 2001), should be the next steps in developing a system for understanding the role of the CRHRs in the control of stress-related behaviours and metabolic modulation at the whole-organism level.

The data from this thesis proposes that CRHR1 may act as a metabolic sensor during HS. Both CRHR1 and β-adrenergic receptors show proton sensitive histidine residues which enhance receptor action in an acidic environment (Ghanouni et al., 2000; Kameda et al., 2018). The capacity of these GPCRs to adjust based on environmental stimuli underpins their potential to act as temperature sensors. Indeed, recent work proposes GPCRs have the capacity to adapt to differing temperatures in *Caenorhabditis elegans* (Ohnishi et al., 2024). Here, the GPCR SRH-40 acts alongside the classical thermosensor TRP channels in central sensory neurons for thermal acclimation, suggesting either a temperature-sensitive independent or dual TRP-GPCR signalling mechanism (Ohnishi et al., 2024). Whether CRHR1 acts alongside TRP channels in thermosensation remains to be identified, though CRH family signalling and TRPA1 channels have been suggested to regulate stress adaptation (Konkoly et al., 2022). TRPA1 channels also act in cold and heat perception (Oda et al., 2016; Saito and Shingai, 2006), and are simultaneously expressed with UCN1 in the murine Edinger-Westphal nucleus (Konkoly et al., 2022). How TRP channels function in the absence of CRHR1 signalling should be investigated as the stress-TRP interplay is far from clear. TRP agonists and antagonists can be employed to directly activate or deactivate TRPs for investigating interactions with the CRH-CRHR1 system (Vay et al., 2012).

An organism's response to stress is diverse and varies by the type of stressor, which physiological pathways are activated, and environmental conditions, among others (Lovejoy et al., 2014; Lovett-Barron et al., 2020; Sokolova, 2021). Here, we used a ubiquitous CRHR1KO model for the first time to investigate the acute HS response. This thesis concludes that CRHR1, the critical receptor in activating the HPI-axis, also modulates several other facets of the stress response. We show that during HS, CRHR1 plays a fundamental role in behavioural adjustment

which coincides with a shift in the metabolic rate in zebrafish. Using the ubiquitous CRHR1KO zebrafish, disturbances of the CRH-CRHR1 system can be investigated across different life stages. This revealed that even in the early developmental stages, CRHR1 modulates heat-induced behaviour and energy flux, which is reflected in adult CRHR1KO fish having an impaired behavioural and metabolic rate response during HS. In conclusion, CRHR1 appears to respond to HS, not only by activating the HPI-axis, but also working independently of cortisol to prime the animal for the response to heat stress.

Literature Cited

Alderman, S.L., Bernier, N.J., 2007. Localization of corticotropin-releasing factor, urotensin I, and CRF-binding protein gene expression in the brain of the zebrafish, Danio rerio. J. Comp. Neurol. 502, 783–793. https://doi.org/10.1002/cne.21332

Alfonso, S., Gesto, M., Sadoul, B., 2021. Temperature increase and its effects on fish stress physiology in the context of global warming. J. Fish Biol. 98, 1496–1508. https://doi.org/10.1111/jfb.14599

Alsop, D., Vijayan, M.M., 2008. Development of the corticosteroid stress axis and receptor expression in zebrafish. Am. J. Physiol.-Regul. Integr. Comp. Physiol. 294, R711–R719. https://doi.org/10.1152/ajpregu.00671.2007

Arai, M., Assil, I.Q., Abou-Samra, A.B., 2001. Characterization of Three Corticotropin-Releasing Factor Receptors in Catfish: A Novel Third Receptor Is Predominantly Expressed in Pituitary and Urophysis*†*The nucleotide sequences reported in this paper have been submitted to the GenBank database with accession numbers cfCRF-R1, AF229359; cfCRF-R2, AF229360; and cfCRF-R3, AF229361.†This work was supported by NIDDK Grant 45020–04A1 from the NIH. Endocrinology 142, 446–454. https://doi.org/10.1210/endo.142.1.7879

Arborelius, L., Owens, M.J., Plotsky, P.M., Nemeroff, C.B., 1999. The role of corticotropin-releasing factor in depression and anxiety disorders. J. Endocrinol. 160, 1–12. https://doi.org/10.1677/joe.0.1600001

Armstrong, J.B., Ward, E.J., Schindler, D.E., Lisi, P.J., 2016. Adaptive capacity at the northern front: sockeye salmon behaviourally thermoregulate during novel exposure to warm temperatures. Conserv. Physiol. 4, cow039. https://doi.org/10.1093/conphys/cow039

Austin, M.C., Janosky, J.E., Murphy, H.A., 2003. Increased corticotropin-releasing hormone immunoreactivity in monoamine-containing pontine nuclei of depressed suicide men. Mol. Psychiatry 8, 324–332. https://doi.org/10.1038/sj.mp.4001250

Azpeleta, C., Sánchez-Bretaño, A., Isorna, E., Nisembaum, L., Velarde, E., Pedro, N., 2011. Understanding the circadian system as a net of clocks: Daily expression of clock genes in the hypothalamus-pituitary-interrenal axis in Carassius Auratus. Presented at the Avances en Endocrinología Comparada, pp. 37–40.

Backström, T., Pettersson, A., Johansson, V., Winberg, S., 2011. CRF and urotensin I effects on aggression and anxiety-like behavior in rainbow trout. J. Exp. Biol. 214, 907–914. https://doi.org/10.1242/jeb.045070

Backström, T., Winberg, S., 2017. Serotonin Coordinates Responses to Social Stress—What We Can Learn from Fish. Front. Neurosci. 11, 595. https://doi.org/10.3389/fnins.2017.00595

Baker, M.E., Katsu, Y., 2017. 30 YEARS OF THE MINERALOCORTICOID RECEPTOR: Evolution of the mineralocorticoid receptor: sequence, structure and function. J. Endocrinol. 234, T1–T16. https://doi.org/10.1530/JOE-16-0661

Banh, R.S., Iorio, C., Marcotte, R., Xu, Y., Cojocari, D., Rahman, A.A., Pawling, J., Zhang, W., Sinha, A., Rose, C.M., Isasa, M., Zhang, S., Wu, R., Virtanen, C., Hitomi, T., Habu, T., Sidhu, S.S., Koizumi, A., Wilkins, S.E., Kislinger, T., Gygi, S.P., Schofield, C.J., Dennis, J.W., Wouters, B.G., Neel, B.G., 2016. PTP1B controls non-mitochondrial oxygen consumption by regulating RNF213 to promote tumour survival during hypoxia. Nat. Cell Biol. 18, 803–813. https://doi.org/10.1038/ncb3376

Barton, B.A., Schreck, C.B., 1987. Metabolic Cost of Acute Physical Stress in Juvenile Steelhead. Trans. Am. Fish. Soc. 116, 257–263. https://doi.org/10.1577/1548-8659(1987)116<257:MCOAPS>2.0.CO;2

Beato, M., Truss, M., Chávez, S., 1996. Control of Transcription by Steroid Hormones. Ann. N. Y. Acad. Sci. 784, 93–123. https://doi.org/10.1111/j.1749-6632.1996.tb16231.x

Behan, D.P., Khongsaly, O., Owens, M.J., Chung, H.D., Nemeroff, C.B., De Souza, E.B., 1997. Corticotropin-Releasing Factor (CRF), CRF-Binding Protein (CRF-BP), and CRF/CRF-BP Complex in Alzheimer's Disease and Control Postmortem Human Brain. J. Neurochem. 68, 2053–2060. https://doi.org/10.1046/j.1471-4159.1997.68052053.x

Berg-Maurer, C.M. vom, Trivedi, C.A., Bollmann, J.H., Marco, R.J.D., Ryu, S., 2016. The Severity of Acute Stress Is Represented by Increased Synchronous Activity and Recruitment of Hypothalamic CRH Neurons. J. Neurosci. 36, 3350–3362. https://doi.org/10.1523/JNEUROSCI.3390-15.2016

Bernier, N.J., Bedard, N., Peter, R.E., 2004. Effects of cortisol on food intake, growth, and forebrain neuropeptide Y and corticotropin-releasing factor gene expression in goldfish. Gen. Comp. Endocrinol. 135, 230–240. https://doi.org/10.1016/j.ygcen.2003.09.016

Berridge, C.W., Waterhouse, B.D., 2003. The locus coeruleus–noradrenergic system: modulation of behavioral state and state-dependent cognitive processes. Brain Res. Rev. 42, 33–84. https://doi.org/10.1016/S0165-0173(03)00143-7

Binder, E.B., 2009. The role of FKBP5, a co-chaperone of the glucocorticoid receptor in the pathogenesis and therapy of affective and anxiety disorders. Psychoneuroendocrinology, NEUROACTIVE STEROIDS: EFFECTS AND MECHANISMS OF ACTION 34, S186–S195. https://doi.org/10.1016/j.psyneuen.2009.05.021

Biro, P.A., 1998. Staying Cool: Behavioral Thermoregulation during Summer by Young-of-Year Brook Trout in a Lake. Trans. Am. Fish. Soc. 127, 212–222. https://doi.org/10.1577/1548-8659(1998)127<0212:SCBTDS>2.0.CO;2

Biro, P.A., Beckmann, C., Stamps, J.A., 2009. Small within-day increases in temperature affects boldness and alters personality in coral reef fish. Proc. R. Soc. B Biol. Sci. 277, 71–77. https://doi.org/10.1098/rspb.2009.1346

Blanco, A.M., Antomagesh, F., Comesaña, S., Soengas, J.L., Vijayan, M.M., 2024. Chronic cortisol stimulation enhances hypothalamus-specific enrichment of metabolites in the rainbow

trout brain. Am. J. Physiol.-Endocrinol. Metab. 326, E382–E397. https://doi.org/10.1152/ajpendo.00410.2023

Block, B.A., 1991. Chapter 11 - Endothermy in fish: thermogenesis, ecology and evolution, in: Hochachka, P.W., Mommsen, T.P. (Eds.), Biochemistry and Molecular Biology of Fishes, Phylogenetic and Biochemical Perspectives. Elsevier, pp. 269–311. https://doi.org/10.1016/B978-0-444-89124-2.50015-4

Bloecher, N., Hedger, R., Finstad, B., Olsen, R.E., Økland, F., Svendsen, E., Rosten, C., Føre, M., 2024. Assessment of activity and heart rate as indicators for acute stress in Atlantic salmon. Aquac. Int. https://doi.org/10.1007/s10499-024-01409-3

Bonfiglio, J.J., Inda, C., Refojo, D., Holsboer, F., Arzt, E., Silberstein, S., 2011. The Corticotropin-Releasing Hormone Network and the Hypothalamic-Pituitary-Adrenal Axis: Molecular and Cellular Mechanisms Involved. Neuroendocrinology 94, 12–20. https://doi.org/10.1159/000328226

Brennan, S.C., Mun, H.-C., Delbridge, L., Kuchel, P.W., Conigrave, A.D., 2023. Temperature sensing by the calcium-sensing receptor. Front. Physiol. 14, 1117352. https://doi.org/10.3389/fphys.2023.1117352

Brett, J.R., 1971. Energetic Responses of Salmon to Temperature. A Study of Some Thermal Relations in the Physiology and Freshwater Ecology of Sockeye Salmon (Oncorhynchus nerkd). Am. Zool. 11, 99–113. https://doi.org/10.1093/icb/11.1.99

Brett, J.R., 1964. The Respiratory Metabolism and Swimming Performance of Young Sockeye Salmon. J. Fish. Res. Board Can. 21, 1183–1226. https://doi.org/10.1139/f64-103

Britto, F.A., Begue, G., Rossano, B., Docquier, A., Vernus, B., Sar, C., Ferry, A., Bonnieu, A., Ollendorff, V., Favier, F.B., 2014. REDD1 deletion prevents dexamethasone-induced skeletal muscle atrophy. Am. J. Physiol.-Endocrinol. Metab. 307, E983–E993. https://doi.org/10.1152/ajpendo.00234.2014

Bround, M.J., Bers, D.M., Molkentin, J.D., 2020. A 20/20 view of ANT function in mitochondrial biology and necrotic cell death. J. Mol. Cell. Cardiol. 144, A3–A13. https://doi.org/10.1016/j.yjmcc.2020.05.012

Cadepond, F., Jibard, N., Binart, N., Schweizer-Groyer, G., Segard-Maurel, I., Baulieu, E.E., 1994. Selective deletions in the 90 kDa heat shock protein (hsp90) impede hetero-oligomeric complex formation with the glucocorticosteroid receptor (GR) or hormone binding by GR. J. Steroid Biochem. Mol. Biol. 48, 361–367. https://doi.org/10.1016/0960-0760(94)90076-0

Cardoso, J.C.R., Power, D.M., Elgar, G., Clark, M.S., 2003. Isolation and Characterisation of the Corticotropin Releasing Factor Receptor 1 (CRFR1) Gene in a Teleost Fish, Fugu rubripes. DNA Seq. 14, 215–218. https://doi.org/10.1080/1042517031000112624

Chabot, D., Steffensen, J.F., Farrell, A.P., 2016. The determination of standard metabolic rate in fishes. J. Fish Biol. 88, 81–121. https://doi.org/10.1111/jfb.12845

Chacko, B.K., Kramer, P.A., Ravi, S., Benavides, G.A., Mitchell, T., Dranka, B.P., Ferrick, D., Singal, A.K., Ballinger, S.W., Bailey, S.M., Hardy, R.W., Zhang, J., Zhi, D., Darley-Usmar, V.M., 2014. The Bioenergetic Health Index: a new concept in mitochondrial translational research. Clin. Sci. 127, 367–373. https://doi.org/10.1042/CS20140101

Chen, C.-C., Fernald, R.D., 2008. Sequences, expression patterns and regulation of the corticotropin-releasing factor system in a teleost. Gen. Comp. Endocrinol. 157, 148–155. https://doi.org/10.1016/j.ygcen.2008.04.003

Chin, J.S.R., Phan, T.-A.N., Albert, L.T., Keene, A.C., Duboué, E.R., 2022. Long lasting anxiety following early life stress is dependent on glucocorticoid signaling in zebrafish. Sci. Rep. 12, 12826. https://doi.org/10.1038/s41598-022-16257-5

Chung, D.J., Schulte, P.M., 2020. Mitochondria and the thermal limits of ectotherms. J. Exp. Biol. 223, jeb227801. https://doi.org/10.1242/jeb.227801

Clark, T.D., Jeffries, K.M., Hinch, S.G., Farrell, A.P., 2011. Exceptional aerobic scope and cardiovascular performance of pink salmon (Oncorhynchus gorbuscha) may underlie resilience in a warming climate. J. Exp. Biol. 214, 3074–3081. https://doi.org/10.1242/jeb.060517

Clark, T.D., Sandblom, E., Jutfelt, F., 2013. Aerobic scope measurements of fishes in an era of climate change: respirometry, relevance and recommendations. J. Exp. Biol. 216, 2771–2782. https://doi.org/10.1242/jeb.084251

Clarke, A., Fraser, K.P.P., 2004. Why does metabolism scale with temperature? Funct. Ecol. 18, 243–251. https://doi.org/10.1111/j.0269-8463.2004.00841.x

Clarke, A., Johnston, N.M., 1999. Scaling of metabolic rate with body mass and temperature in teleost fish. J. Anim. Ecol. 68, 893–905. https://doi.org/10.1046/j.1365-2656.1999.00337.x

Cooke, S.J., Bunt, C.M., Schreer, J.F., 2004. Understanding Fish Behavior, Distribution, and Survival in Thermal Effluents Using Fixed Telemetry Arrays: A Case Study of Smallmouth Bass in a Discharge Canal During Winter. Environ. Manage. 33, 140–150. https://doi.org/10.1007/s00267-003-0175-2

Cortemeglia, C., Beitinger, T.L., 2005. Temperature Tolerances of Wild-Type and Red Transgenic Zebra Danios. Trans. Am. Fish. Soc. 134, 1431–1437. https://doi.org/10.1577/T04-197.1

Cowan, M., Azpeleta, C., López-Olmeda, J.F., 2017. Rhythms in the endocrine system of fish: a review. J. Comp. Physiol. B 187, 1057–1089. https://doi.org/10.1007/s00360-017-1094-5

Craig, M.P., Gilday, S.D., Hove, J.R., 2006. Dose-dependent effects of chemical immobilization on the heart rate of embryonic zebrafish. Lab Anim. 35, 41–47. https://doi.org/10.1038/laban1006-41

de Vries, W.B., van der Leij, F.R., Bakker, J.M., Kamphuis, P.J.G.H., van Oosterhout, M.F.M., Schipper, M.E.I., Smid, G.B., Bartelds, B., van Bel, F., 2002. Alterations in Adult Rat Heart after

Neonatal Dexamethasone Therapy. Pediatr. Res. 52, 900–906. https://doi.org/10.1203/00006450-200212000-00015

Dindia, L., Murray, J., Faught, E., Davis, T.L., Leonenko, Z., Vijayan, M.M., 2012. Novel Nongenomic Signaling by Glucocorticoid May Involve Changes to Liver Membrane Order in Rainbow Trout. PLOS ONE 7, e46859. https://doi.org/10.1371/journal.pone.0046859

Dogra, D., Meza-Santoscoy, P.L., Gavrilovici, C., Rehak, R., de la Hoz, C.L.R., Ibhazehiebo, K., Rho, J.M., Kurrasch, D.M., 2023. kcna1a mutant zebrafish model episodic ataxia type 1 (EA1) with epilepsy and show response to first-line therapy carbamazepine. Epilepsia 64, 2186–2199. https://doi.org/10.1111/epi.17659

Donner, N.C., Montoya, C.D., Lukkes, J.L., Lowry, C.A., 2012. Chronic non-invasive corticosterone administration abolishes the diurnal pattern of *tph2* expression. Psychoneuroendocrinology 37, 645–661. https://doi.org/10.1016/j.psyneuen.2011.08.008

Donner, N.C., Siebler, P.H., Johnson, D.T., Villarreal, M.D., Mani, S., Matti, A.J., Lowry, C.A., 2016. Serotonergic systems in the balance: CRHR1 and CRHR2 differentially control stress-induced serotonin synthesis. Psychoneuroendocrinology 63, 178–190. https://doi.org/10.1016/j.psyneuen.2015.09.024

Egan, R.J., Bergner, C.L., Hart, P.C., Cachat, J.M., Canavello, P.R., Elegante, M.F., Elkhayat, S.I., Bartels, B.K., Tien, A.K., Tien, D.H., Mohnot, S., Beeson, E., Glasgow, E., Amri, H., Zukowska, Z., Kalueff, A.V., 2009. Understanding behavioral and physiological phenotypes of stress and anxiety in zebrafish. Behav. Brain Res. 205, 38–44. https://doi.org/10.1016/j.bbr.2009.06.022

Encina, L., Rodríguez-Ruiz, A., Granado-Lorencio, C., 2008. Distribution of common carp in a Spanish reservoir in relation to thermal loading from a nuclear power plant. J. Therm. Biol. 33, 444–450. https://doi.org/10.1016/j.jtherbio.2008.08.002

Fabbri, E., Capuzzo, A., Moon, T.W., 1998. The role of circulating catecholamines in the regulation of fish metabolism: an overview. Comp. Biochem. Physiol. C Pharmacol. Toxicol. Endocrinol. 120, 177–192. https://doi.org/10.1016/s0742-8413(98)10017-8

Farrell, A.P., 2009. Environment, antecedents and climate change: lessons from the study of temperature physiology and river migration of salmonids. J. Exp. Biol. 212, 3771–3780. https://doi.org/10.1242/jeb.023671

Faught, E., Aluru, N., Vijayan, M.M., 2016a. The Molecular Stress Response, in: Schreck, C.B., Tort, L., Farrell, A.P., Brauner, C.J. (Eds.), Fish Physiology. Academic Press, pp. 113–166.

Faught, E., Best, C., Vijayan, M.M., 2016b. Maternal stress-associated cortisol stimulation may protect embryos from cortisol excess in zebrafish. R. Soc. Open Sci. 3, 160032. https://doi.org/10.1098/rsos.160032

Faught, E., Vijayan, M.M., 2022a. The Mineralocorticoid Receptor Functions as a Key Glucose Regulator in the Skeletal Muscle of Zebrafish. Endocrinology 163, bqac149. https://doi.org/10.1210/endocr/bqac149

Faught, E., Vijayan, M.M., 2022b. Coordinated Action of Corticotropin-Releasing Hormone and Cortisol Shapes the Acute Stress-Induced Behavioural Response in Zebrafish. Neuroendocrinology 112, 74–87. https://doi.org/10.1159/000514778

Faught, E., Vijayan, M.M., 2019. Loss of the glucocorticoid receptor in zebrafish improves muscle glucose availability and increases growth. Am. J. Physiol.-Endocrinol. Metab. 316, E1093–E1104. https://doi.org/10.1152/ajpendo.00045.2019

Faught, E., Vijayan, M.M., 2016. Mechanisms of cortisol action in fish hepatocytes. Comp. Biochem. Physiol. B Biochem. Mol. Biol., Dr. Thomas W. Moon 199, 136–145. https://doi.org/10.1016/j.cbpb.2016.06.012

Fisher, L.A., Jrivier, E., Rivier, C., Spiess, J., Vale, W., Brown, M.R., 1982. Corticotropin-releasing factor (CRF): central effects on mean arterial pressure and heart rate in rats. Endocrinology 110, 2222–2224. https://doi.org/10.1210/endo-110-6-2222

Freitas, C., Villegas-Ríos, D., Moland, E., Olsen, E.M., 2021. Sea temperature effects on depth use and habitat selection in a marine fish community. J. Anim. Ecol. 90, 1787–1800. https://doi.org/10.1111/1365-2656.13497

Füstös, V., Sály, P., Szalóky, Z., Tóth, B., Vitál, Z., Specziár, A., Fleit, G., Baranya, S., Józsa, J., Erős, T., 2023. Effects of a nuclear power plant warmwater outflow on environmental conditions and fish assemblages in a very large river (the Danube, Hungary). Ecohydrology 16, e2512. https://doi.org/10.1002/eco.2512

Gao, X., Yang, L., Li, Q., An, Y., Liao, S., Gao, H., Zhao, X., Bian, L., Zheng, X., 2017. Investigation on temperature-induce conformational change of immobilized β2 adrenergic receptor. Biochem. Biophys. Res. Commun. 494, 634–640. https://doi.org/10.1016/j.bbrc.2017.08.105

Gau, P., Poon, J., Ufret-Vincenty, C., Snelson, C.D., Gordon, S.E., Raible, D.W., Dhaka, A., 2013. The Zebrafish Ortholog of TRPV1 Is Required for Heat-Induced Locomotion. J. Neurosci. 33, 5249–5260. https://doi.org/10.1523/JNEUROSCI.5403-12.2013

Ghanouni, P., Schambye, H., Seifert, R., Lee, T.W., Rasmussen, S.G.F., Gether, U., Kobilka, B.K., 2000. The Effect of pH on β2 Adrenoceptor Function. J. Biol. Chem. 275, 3121–3127. https://doi.org/10.1074/jbc.275.5.3121

Gilbert, M.J.H., Zerulla, T.C., Tierney, K.B., 2014. Zebrafish (Danio rerio) as a model for the study of aging and exercise: Physical ability and trainability decrease with age. Exp. Gerontol. 50, 106–113. https://doi.org/10.1016/j.exger.2013.11.013

Goikoetxea, A., Sadoul, B., Blondeau-Bidet, E., Aerts, J., Blanc, M.-O., Parrinello, H., Barrachina, C., Pratlong, M., Geffroy, B., 2021. Genetic pathways underpinning hormonal stress

responses in fish exposed to short- and long-term warm ocean temperatures. Ecol. Indic. 120, 106937. https://doi.org/10.1016/j.ecolind.2020.106937

Goniea, T.M., Keefer, M.L., Bjornn, T.C., Peery, C.A., Bennett, D.H., Stuehrenberg, L.C., 2006. Behavioral Thermoregulation and Slowed Migration by Adult Fall Chinook Salmon in Response to High Columbia River Water Temperatures. Trans. Am. Fish. Soc. 135, 408–419. https://doi.org/10.1577/T04-113.1

Grammatopoulos, D.K., 2012. Insights into mechanisms of corticotropin-releasing hormone receptor signal transduction. Br. J. Pharmacol. 166, 85–97. https://doi.org/10.1111/j.1476-5381.2011.01631.x

Gregory, T.R., Wood, C.M., 1999. The Effects of Chronic Plasma Cortisol Elevation on the Feeding Behaviour, Growth, Competitive Ability, and Swimming Performance of Juvenile Rainbow Trout. Physiol. Biochem. Zool. https://doi.org/10.1086/316673

Hanley, N.R., Van de Kar, L.D., 2003. Serotonin and the neuroendocrine regulation of the hypothalamic--pituitary-adrenal axis in health and disease. Vitam. Horm. 66, 189–255. https://doi.org/10.1016/s0083-6729(03)01006-9

Harris, B.N., Carr, J.A., 2016. The role of the hypothalamus-pituitary-adrenal/interrenal axis in mediating predator-avoidance trade-offs. Gen. Comp. Endocrinol. 230–231, 110–142. https://doi.org/10.1016/j.ygcen.2016.04.006

Herculano, A.M., Maximino, C., 2014. Serotonergic modulation of zebrafish behavior: Towards a paradox. Prog. Neuropsychopharmacol. Biol. Psychiatry, Special Issue: Zebrafish models of brain disorders 55, 50–66. https://doi.org/10.1016/j.pnpbp.2014.03.008

Herman, J.P., McKlveen, J.M., Ghosal, S., Kopp, B., Wulsin, A., Makinson, R., Scheimann, J., Myers, B., 2016. Regulation of the hypothalamic-pituitary-adrenocortical stress response. Compr. Physiol. 6, 603–621. https://doi.org/10.1002/cphy.c150015

Hernández-Díaz, Y., González-Castro, T.B., Juárez-Rojop, I.E., Tovilla-Zárate, C.A., López-Narváez, M.L., Genis-Mendoza, A.D., Fresan, A., Nicolini, H., 2021. The role of rs242941, rs1876828, rs242939 and rs110402 polymorphisms of CRHR1 gene and the depression: systematic review and meta-analysis. Genes Genomics 43, 1339–1349. https://doi.org/10.1007/s13258-021-01133-9

Hill, J.W., 2012. PVN pathways controlling energy homeostasis. Indian J. Endocrinol. Metab. 16, S627. https://doi.org/10.4103/2230-8210.105581

Hirschenson, J., Melgar-Bermudez, E., Mailloux, R.J., 2022. The Uncoupling Proteins: A Systematic Review on the Mechanism Used in the Prevention of Oxidative Stress. Antioxidants 11, 322. https://doi.org/10.3390/antiox11020322

Hitt, N.P., Snook, E.L., Massie, D.L., 2017. Brook trout use of thermal refugia and foraging habitat influenced by brown trout. Can. J. Fish. Aquat. Sci. 74, 406–418. https://doi.org/10.1139/cjfas-2016-0255

Hosono, K., Kikuchi, Y., Miyanishi, H., Hiraki-Kajiyama, T., Takeuchi, A., Nakasone, K., Maehiro, S., Okubo, K., 2015. Teleocortin: A Novel Member of the CRH Family in Teleost Fish. Endocrinology 156, 2949–2957. https://doi.org/10.1210/en.2015-1042

Imaki, T., Nahan, J., Rivier, C., Sawchenko, P., Vale, W., 1991. Differential regulation of corticotropin-releasing factor mRNA in rat brain regions by glucocorticoids and stress. J. Neurosci. 11, 585–599. https://doi.org/10.1523/JNEUROSCI.11-03-00585.1991

Ings, J.S., Servos, M.R., Vijayan, M.M., 2011. Exposure to municipal wastewater effluent impacts stress performance in rainbow trout. Aquat. Toxicol. 103, 85–91. https://doi.org/10.1016/j.aquatox.2011.02.013

Ivy, A.S., Rex, C.S., Chen, Y., Dubé, C., Maras, P.M., Grigoriadis, D.E., Gall, C.M., Lynch, G., Baram, T.Z., 2010. Hippocampal Dysfunction and Cognitive Impairments Provoked by Chronic Early-Life Stress Involve Excessive Activation of CRH Receptors. J. Neurosci. 30, 13005–13015. https://doi.org/10.1523/JNEUROSCI.1784-10.2010

Iwama, G.K., Thomas, P.T., Forsyth, R.B., Vijayan, M.M., 1998. Heat shock protein expression in fish. Rev. Fish Biol. Fish. 8, 35–56. https://doi.org/10.1023/A:1008812500650

Iwama, G.K., Vijayan, M.M., Forsyth, R.B., Ackerman, P.A., 1999. Heat Shock Proteins and Physiological Stress in Fish. Am. Zool. 39, 901–909. https://doi.org/10.1093/icb/39.6.901

Jahn, O., Tezval, H., van Werven, L., Eckart, K., Spiess, J., 2004. Three-amino acid motifs of urocortin II and III determine their CRF receptor subtype selectivity. Neuropharmacology 47, 233–242. https://doi.org/10.1016/j.neuropharm.2004.03.018

Jedema, H.P., Grace, A.A., 2004. Corticotropin-Releasing Hormone Directly Activates Noradrenergic Neurons of the Locus Ceruleus Recorded In Vitro. J. Neurosci. 24, 9703–9713. https://doi.org/10.1523/JNEUROSCI.2830-04.2004

Jimeno, B., Verhulst, S., 2023. Do glucocorticoids indicate stress? Meta-analysis reveals a tight association between glucocorticoids and metabolic rate. eLife 12. https://doi.org/10.7554/eLife.88205

Johansen, I.B., Lunde, I.G., Røsjø, H., Christensen, G., Nilsson, G.E., Bakken, M., Øverli, Ø., 2011. Cortisol response to stress is associated with myocardial remodeling in salmonid fishes. J. Exp. Biol. 214, 1313–1321. https://doi.org/10.1242/jeb.053058

Johansen, I.B., Sandblom, E., Skov, P.V., Gräns, A., Ekström, A., Lunde, I.G., Vindas, M.A., Zhang, L., Höglund, E., Frisk, M., Sjaastad, I., Nilsson, G.E., Øverli, Ø., 2017. Bigger is not better: cortisol-induced cardiac growth and dysfunction in salmonids. J. Exp. Biol. 220, 2545–2553. https://doi.org/10.1242/jeb.135046

Johansen, J.L., Jones, G.P., 2011. Increasing ocean temperature reduces the metabolic performance and swimming ability of coral reef damselfishes. Glob. Change Biol. 17, 2971–2979. https://doi.org/10.1111/j.1365-2486.2011.02436.x

Kalueff, A.V., Gebhardt, M., Stewart, A.M., Cachat, J.M., Brimmer, M., Chawla, J.S., Craddock, C., Kyzar, E.J., Roth, A., Landsman, S., Gaikwad, S., Robinson, K., Baatrup, E., Tierney, K., Shamchuk, A., Norton, W., Miller, N., Nicolson, T., Braubach, O., Gilman, C.P., Pittman, J., Rosemberg, D.B., Gerlai, R., Echevarria, D., Lamb, E., Neuhauss, S.C.F., Weng, W., Bally-Cuif, L., Schneider, H., 2013. Towards a Comprehensive Catalog of Zebrafish Behavior 1.0 and Beyond. Zebrafish 10, 70–86. https://doi.org/10.1089/zeb.2012.0861

Kameda, H., Yamamoto, M., Tone, Y., Tone, M., Melmed, S., 2018. Proton Sensitivity of Corticotropin-Releasing Hormone Receptor 1 Signaling to Proopiomelanocortin in Male Mice. Endocrinology 160, 276–291. https://doi.org/10.1210/en.2018-00920

Kaziales, A., Barkovits, K., Marcus, K., Richter, K., 2020. Glucocorticoid receptor complexes form cooperatively with the Hsp90 co-chaperones Pp5 and FKBPs. Sci. Rep. 10, 10733. https://doi.org/10.1038/s41598-020-67645-8

Kirschke, E., Goswami, D., Southworth, D., Griffin, P.R., Agard, D., 2014. Glucocorticoid Receptor Function Regulated by Coordinated Action of the Hsp90 and Hsp70 Chaperone Cycles. Cell 157, 1685–1697. https://doi.org/10.1016/j.cell.2014.04.038

Kishimoto, T., Pearse, R.V., Lin, C.R., Rosenfeld, M.G., 1995. A sauvagine/corticotropin-releasing factor receptor expressed in heart and skeletal muscle. Proc. Natl. Acad. Sci. 92, 1108–1112. https://doi.org/10.1073/pnas.92.4.1108

Kishimoto, T., Radulovic, J., Radulovic, M., Lin, C.R., Schrick, C., Hooshmand, F., Hermanson, O., Rosenfeld, M.G., Spiess, J., 2000. Deletion of Crhr2 reveals an anxiolytic role for corticotropin-releasing hormone receptor-2. Nat. Genet. 24, 415–419. https://doi.org/10.1038/74271

Kolber, B.J., Boyle, M.P., Wieczorek, L., Kelley, C.L., Onwuzurike, C.C., Nettles, S.A., Vogt, S.K., Muglia, L.J., 2010. Transient Early-Life Forebrain Corticotropin-Releasing Hormone Elevation Causes Long-Lasting Anxiogenic and Despair-Like Changes in Mice. J. Neurosci. 30, 2571–2581. https://doi.org/10.1523/JNEUROSCI.4470-09.2010

Konkoly, J., Kormos, V., Gaszner, B., Correia, P., Berta, G., Biró-Sütő, T., Zelena, D., Pintér, E., 2022. Transient receptor potential ankyrin 1 ion channel expressed by the Edinger-Westphal nucleus contributes to stress adaptation in murine model of posttraumatic stress disorder. Front. Cell Dev. Biol. 10, 1059073. https://doi.org/10.3389/fcell.2022.1059073

Kovács, K.J., 2013. CRH: the link between hormonal-, metabolic- and behavioral responses to stress. J. Chem. Neuroanat. 54, 25–33. https://doi.org/10.1016/j.jchemneu.2013.05.003

Kreiter, J., Rupprecht, A., Škulj, S., Brkljača, Z., Žuna, K., Knyazev, D.G., Bardakji, S., Vazdar, M., Pohl, E.E., 2021. ANT1 Activation and Inhibition Patterns Support the Fatty Acid Cycling Mechanism for Proton Transport. Int. J. Mol. Sci. 22, 2490. https://doi.org/10.3390/ijms22052490

Kültz, D., 2005. Molecular and evolutionary basis of the cellular stress response. Annu. Rev. Physiol. 67, 225–257. https://doi.org/10.1146/annurev.physiol.67.040403.103635

Kvetnansky, R., Sabban, E.L., Palkovits, M., 2009. Catecholaminergic Systems in Stress: Structural and Molecular Genetic Approaches. Physiol. Rev. 89, 535–606. https://doi.org/10.1152/physrev.00042.2006

Lahiri, K., Vallone, D., Gondi, S.B., Santoriello, C., Dickmeis, T., Foulkes, N.S., 2005. Temperature Regulates Transcription in the Zebrafish Circadian Clock. PLOS Biol. 3, e351. https://doi.org/10.1371/journal.pbio.0030351

Lederis, K., Letter, A., McMaster, D., Moore, G., Schlesinger, D., 1982. Complete Amino Acid Sequence of Urotensin I, a Hypotensive and Corticotropin-Releasing Neuropeptide from Catostomus. Science 218, 162–165. https://doi.org/10.1126/science.6981844

Lemos, L.S., Angarica, L.M., Hauser-Davis, R.A., Quinete, N., 2023. Cortisol as a Stress Indicator in Fish: Sampling Methods, Analytical Techniques, and Organic Pollutant Exposure Assessments. Int. J. Environ. Res. Public. Health 20, 6237. https://doi.org/10.3390/ijerph20136237

Lewis, K., Li, C., Perrin, M.H., Blount, A., Kunitake, K., Donaldson, C., Vaughan, J., Reyes, T.M., Gulyas, J., Fischer, W., Bilezikjian, L., Rivier, J., Sawchenko, P.E., Vale, W.W., 2001. Identification of urocortin III, an additional member of the corticotropin-releasing factor (CRF) family with high affinity for the CRF2 receptor. Proc. Natl. Acad. Sci. 98, 7570–7575. https://doi.org/10.1073/pnas.121165198

Li, M., Zhao, L., Page-McCaw, P., Chen, W., 2016. Zebrafish genome engineering using the CRISPR-Cas9 system. Trends Genet. TIG 32, 815–827. https://doi.org/10.1016/j.tig.2016.10.005

Lillesaar, C., 2011. The serotonergic system in fish. J. Chem. Neuroanat., Functional Neuroanatomy of the Serotonin System 41, 294–308. https://doi.org/10.1016/j.jchemneu.2011.05.009

Little, A.G., Kunisue, T., Kannan, K., Seebacher, F., 2013. Thyroid hormone actions are temperature-specific and regulate thermal acclimation in zebrafish (Danio rerio). BMC Biol. 11, 26. https://doi.org/10.1186/1741-7007-11-26

Liu, K., Petree, C., Requena, T., Varshney, P., Varshney, G.K., 2019. Expanding the CRISPR Toolbox in Zebrafish for Studying Development and Disease. Front. Cell Dev. Biol. 7. https://doi.org/10.3389/fcell.2019.00013

Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods San Diego Calif 25, 402–408. https://doi.org/10.1006/meth.2001.1262

López-Olmeda, J.F., Blanco-Vives, B., Pujante, I.M., Wunderink, Y.S., Mancera, J.M., Sánchez-Vázquez, F.J., 2013. Daily Rhythms in the Hypothalamus-Pituitary-Interrenal Axis and Acute Stress Responses in a Teleost Flatfish, Solea senegalensis. Chronobiol. Int. 30, 530–539. https://doi.org/10.3109/07420528.2012.754448

López-Olmeda, J.F., Sánchez-Vázquez, F.J., 2011. Thermal biology of zebrafish (*Danio rerio*). J. Therm. Biol. 36, 91–104. https://doi.org/10.1016/j.jtherbio.2010.12.005

López-Olmeda, J.F., Sánchez-Vázquez, F.J., 2010. Feeding Rhythms in Fish: From Behavioral to Molecular Approach, in: Biological Clock in Fish. CRC Press.

Lovejoy, D.A., Chang, B.S.W., Lovejoy, N.R., Del Castillo, J., 2014. Molecular Evolution of GPCRS: CRH/CRH receptors. J. Mol. Endocrinol. 52, T43–T60. https://doi.org/10.1530/JME-13-0238

Lovett-Barron, M., Chen, R., Bradbury, S., Andalman, A.S., Wagle, M., Guo, S., Deisseroth, K., 2020. Multiple convergent hypothalamus–brainstem circuits drive defensive behavior. Nat. Neurosci. 23, 959–967. https://doi.org/10.1038/s41593-020-0655-1

Lu, B., Diz-Chaves, Y., Markovic, D., Contarino, A., Penicaud, L., Fanelli, F., Clark, S., Lehnert, H., Cota, D., Grammatopoulos, D.K., Tabarin, A., 2015. The corticotrophin-releasing factor/urocortin system regulates white fat browning in mice through paracrine mechanisms. Int. J. Obes. 2005 39, 408–417. https://doi.org/10.1038/ijo.2014.164

Lumbers, E.R., Boyce, A.C., Joulianos, G., Kumarasamy, V., Barner, E., Segar, J.L., Burrell, J.H., 2005. Effects of cortisol on cardiac myocytes and on expression of cardiac genes in fetal sheep. Am. J. Physiol.-Regul. Integr. Comp. Physiol. 288, R567–R574. https://doi.org/10.1152/ajpregu.00556.2004

Maier, S.F., Watkins, L.R., 2005. Stressor controllability and learned helplessness: The roles of the dorsal raphe nucleus, serotonin, and corticotropin-releasing factor. Neurosci. Biobehav. Rev., Animal Models of Depression and Antidepressant Activity 29, 829–841. https://doi.org/10.1016/j.neubiorev.2005.03.021

Malekar, V.C., Morton, J.D., Hider, R.N., Cruickshank, R.H., Hodge, S., Metcalf, V.J., 2018. Effect of elevated temperature on membrane lipid saturation in Antarctic notothenioid fish. PeerJ 6, e4765. https://doi.org/10.7717/peerj.4765

Maximino, C., de Brito, T.M., da Silva Batista, A.W., Herculano, A.M., Morato, S., Gouveia, A., 2010. Measuring anxiety in zebrafish: A critical review. Behav. Brain Res. 214, 157–171. https://doi.org/10.1016/j.bbr.2010.05.031

Mazon, A.F., Verburg-van Kemenade, B.M.L., Flik, G., Huising, M.O., 2006. Corticotropin-releasing hormone-receptor 1 (CRH-R1) and CRH-binding protein (CRH-BP) are expressed in the gills and skin of common carp Cyprinus carpio L. and respond to acute stress and infection. J. Exp. Biol. 209, 510–517. https://doi.org/10.1242/jeb.01973

Medeiros, L.R., Cartolano, M.C., McDonald, M.D., 2014. Crowding stress inhibits serotonin 1A receptor-mediated increases in corticotropin-releasing factor mRNA expression and adrenocorticotropin hormone secretion in the Gulf toadfish. J. Comp. Physiol. B 184, 259–271. https://doi.org/10.1007/s00360-013-0793-9

Meehl, G.A., Tebaldi, C., 2004. More Intense, More Frequent, and Longer Lasting Heat Waves in the 21st Century. Science 305, 994–997. https://doi.org/10.1126/science.1098704

Messerli, M., Aaldijk, D., Haberthür, D., Röss, H., García-Poyatos, C., Sande-Melón, M., Khoma, O.-Z., Wieland, F.A.M., Fark, S., Djonov, V., 2020. Adaptation mechanism of the adult zebrafish respiratory organ to endurance training. PLoS ONE 15, e0228333. https://doi.org/10.1371/journal.pone.0228333

Mifsud, K.R., Reul, J.M.H.M., 2016. Acute stress enhances heterodimerization and binding of corticosteroid receptors at glucocorticoid target genes in the hippocampus. Proc. Natl. Acad. Sci. 113, 11336–11341. https://doi.org/10.1073/pnas.1605246113

Mommsen, T.P., Vijayan, M.M., Moon, T.W., 1999. Cortisol in teleosts: dynamics, mechanisms of action, and metabolic regulation. Rev. Fish Biol. Fish. 9, 211–268. https://doi.org/10.1023/A:1008924418720

Montecucchi, P.C., Anastasi, A., de CASTIGLIONE, R., Erspamer, V., 1980. Isolation and Amino Acid Composition of Sauvagine. Int. J. Pept. Protein Res. 16, 191–199. https://doi.org/10.1111/j.1399-3011.1980.tb02952.x

Morgan, R., Andreassen, A.H., Åsheim, E.R., Finnøen, M.H., Dresler, G., Brembu, T., Loh, A., Miest, J.J., Jutfelt, F., 2022. Reduced physiological plasticity in a fish adapted to stable temperatures. Proc. Natl. Acad. Sci. 119, e2201919119. https://doi.org/10.1073/pnas.2201919119

Müller, M.B., Preil, J., Renner, U., Zimmermann, S., Kresse, A.E., Stalla, G.K., Keck, M.E., Holsboer, F., Wurst, W., 2001. Expression of CRHR1 and CRHR2 in Mouse Pituitary and Adrenal Gland: Implications for HPA System Regulation. Endocrinology 142, 4150–4153. https://doi.org/10.1210/endo.142.9.8491

Müller, M.B., Zimmermann, S., Sillaber, I., Hagemeyer, T.P., Deussing, J.M., Timpl, P., Kormann, M.S.D., Droste, S.K., Kühn, R., Reul, J.M.H.M., Holsboer, F., Wurst, W., 2003. Limbic corticotropin-releasing hormone receptor 1 mediates anxiety-related behavior and hormonal adaptation to stress. Nat. Neurosci. 6, 1100–1107. https://doi.org/10.1038/nn1123

Neill, W.H., Magnuson, J.J., 1974. Distributional Ecology and Behavioral Thermoregulation of Fishes in Relation to Heated Effluent from a Power Plant at Lake Monona, Wisconsin. Trans. Am. Fish. Soc. 103, 663–710. https://doi.org/10.1577/1548-8659(1974)103<663:DEABTO>2.0.CO;2

Nicholls, D.G., 2021. Mitochondrial proton leaks and uncoupling proteins. Biochim. Biophys. Acta BBA - Bioenerg. 1862, 148428. https://doi.org/10.1016/j.bbabio.2021.148428

Nicolson, G.L., 2014. Mitochondrial Dysfunction and Chronic Disease: Treatment With Natural Supplements. Integr. Med. Clin. J. 13, 35–43.

Nilsson, G.E., Crawley, N., Lunde, I.G., Munday, P.L., 2009. Elevated temperature reduces the respiratory scope of coral reef fishes. Glob. Change Biol. 15, 1405–1412. https://doi.org/10.1111/j.1365-2486.2008.01767.x

Nipu, N., Antomagesh, F., Faught, E., Vijayan, M.M., 2022. Glucocorticoid receptor activation reduces food intake independent of hyperglycemia in zebrafish. Sci. Rep. 12, 15677. https://doi.org/10.1038/s41598-022-19572-z

Oda, M., Kurogi, M., Kubo, Y., Saitoh, O., 2016. Sensitivities of Two Zebrafish TRPA1 Paralogs to Chemical and Thermal Stimuli Analyzed in Heterologous Expression Systems. Chem. Senses 41, 261–272. https://doi.org/10.1093/chemse/bjv091

Ohnishi, K., Sokabe, T., Miura, T., Tominaga, M., Ohta, A., Kuhara, A., 2024. G protein-coupled receptor-based thermosensation determines temperature acclimatization of Caenorhabditis elegans. Nat. Commun. 15, 1660. https://doi.org/10.1038/s41467-024-46042-z

Oliveira, C.C.V., Aparício, R., Blanco-Vives, B., Chereguini, O., Martín, I., Javier Sánchez-Vazquez, F., 2013. Endocrine (plasma cortisol and glucose) and behavioral (locomotor and self-feeding activity) circadian rhythms in Senegalese sole (Solea senegalensis Kaup 1858) exposed to light/dark cycles or constant light. Fish Physiol. Biochem. 39, 479–487. https://doi.org/10.1007/s10695-012-9713-2

Oliveria, C., Sánchez-Vázquez, F.J., 2010. Reproduction Rhythms in Fish, in: Biological Clock in Fish. CRC Press.

Øverli, Ø., Korzan, W.J., Höglund, E., Winberg, S., Bollig, H., Watt, M., Forster, G.L., Barton, B.A., Øverli, E., Renner, K.J., Summers, C.H., 2004. Stress coping style predicts aggression and social dominance in rainbow trout. Horm. Behav. 45, 235–241. https://doi.org/10.1016/j.yhbeh.2003.12.002

Paaijmans, K.P., Heinig, R.L., Seliga, R.A., Blanford, J.I., Blanford, S., Murdock, C.C., Thomas, M.B., 2013. Temperature variation makes ectotherms more sensitive to climate change. Glob. Change Biol. 19, 2373–2380. https://doi.org/10.1111/gcb.12240

Parmentier, C., Taxi, J., Balment, R., Nicolas, G., Calas, A., 2006. Caudal neurosecretory system of the zebrafish: ultrastructural organization and immunocytochemical detection of urotensins. Cell Tissue Res. 325, 111–124. https://doi.org/10.1007/s00441-006-0180-x

Pfalzgraff, T., Lund, I., Skov, P.V., 2022. Prolonged cortisol elevation alters whole body and tissue metabolism in rainbow trout (Oncorhynchus mykiss). Comp. Biochem. Physiol. A. Mol. Integr. Physiol. 263, 111098. https://doi.org/10.1016/j.cbpa.2021.111098

Pilakouta, N., O'Donnell, P.J., Crespel, A., Levet, M., Claireaux, M., Humble, J.L., Kristjánsson, B.K., Skúlason, S., Lindström, J., Metcalfe, N.B., Killen, S.S., Parsons, K.J., 2023. A warmer environment can reduce sociability in an ectotherm. Glob. Change Biol. 29, 206–214. https://doi.org/10.1111/gcb.16451

Pohl, S., Darlison, M.G., Clarke, W.C., Lederis, K., Richter, D., 2001. Cloning and functional pharmacology of two corticotropin-releasing factor receptors from a teleost fish. Eur. J. Pharmacol. 430, 193–202. https://doi.org/10.1016/S0014-2999(01)01391-7

Pontes, M.H., Sevostyanova, A., Groisman, E.A., 2015. When too much ATP is bad for protein synthesis. J. Mol. Biol. 427, 2586–2594. https://doi.org/10.1016/j.jmb.2015.06.021

Pörtner, H.-O., 2010. Oxygen- and capacity-limitation of thermal tolerance: a matrix for integrating climate-related stressor effects in marine ecosystems. J. Exp. Biol. 213, 881–893. https://doi.org/10.1242/jeb.037523

Pörtner, H.-O., Bock, C., Mark, F.C., 2017. Oxygen- and capacity-limited thermal tolerance: bridging ecology and physiology. J. Exp. Biol. 220, 2685–2696. https://doi.org/10.1242/jeb.134585

Pörtner, H.O., Peck, M.A., 2010. Climate change effects on fishes and fisheries: towards a cause-and-effect understanding. J. Fish Biol. 77, 1745–1779. https://doi.org/10.1111/j.1095-8649.2010.02783.x

Pratt, W.B., Morishima, Y., Murphy, M., Harrell, M., 2006. Chaperoning of glucocorticoid receptors. Handb. Exp. Pharmacol. 111–138. https://doi.org/10.1007/3-540-29717-0_5

Preil, J., Müller, M.B., Gesing, A., Reul, J.M.H.M., Sillaber, I., van Gaalen, M.M., Landgrebe, J., Holsboer, F., Stenzel-Poore, M., Wurst, W., 2001. Regulation of the Hypothalamic-Pituitary-Adrenocortical System in Mice Deficient for CRH Receptors 1 and 2. Endocrinology 142, 4946–4955. https://doi.org/10.1210/endo.142.11.8507

Prosser, C.L., Nelson, D.O., 1981. The role of nervous systems in temperature adaptation of poikilotherms. Annu. Rev. Physiol. 43, 281–300. https://doi.org/10.1146/annurev.ph.43.030181.001433

Reid, S.D., Moon, T.W., Perry, S.F., 1992. Rainbow trout hepatocyte beta-adrenoceptors, catecholamine responsiveness, and effects of cortisol. Am. J. Physiol.-Regul. Integr. Comp. Physiol. 262, R794–R799. https://doi.org/10.1152/ajpregu.1992.262.5.R794

Reid, S.G., Bernier, N.J., Perry, S.F., 1998. The adrenergic stress response in fish: control of catecholamine storage and release. Comp. Biochem. Physiol. C Pharmacol. Toxicol. Endocrinol. 120, 1–27. https://doi.org/10.1016/S0742-8413(98)00037-1

Ressel, K.N., Cominassi, L., Sarrimanolis, J., O'Brien, K.M., 2022. Aerobic scope is not maintained at low temperature and is associated with cardiac aerobic capacity in the threespine stickleback Gasterosteus aculeatus. J. Fish Biol. 100, 444–453. https://doi.org/10.1111/jfb.14955

Reul, J.M.H.M., Bosch, F.R. van den, Kloet, E.R. de, 1987. Relative occupation of type-I and type-II corticosteroid receptors in rat brain following stress and dexamethasone treatment: functional implications. J. Endocrinol. 115, 459–467. https://doi.org/10.1677/joe.0.1150459

Reul, J.M.H.M., de Kloet, E.R., 1986. Anatomical resolution of two types of corticosterone receptor sites in rat brain with *in vitro* autoradiography and computerized image analysis. J. Steroid Biochem. 24, 269–272. https://doi.org/10.1016/0022-4731(86)90063-4

Reul, J.M.H.M., de Kloet, E.R., 1985. Two Receptor Systems for Corticosterone in Rat Brain: Microdistribution and Differential Occupation. Endocrinology 117, 2505–2511. https://doi.org/10.1210/endo-117-6-2505

Reul, J.M.H.M., Holsboer, F., 2002. On the role of corticotropin-releasing hormone receptors in anxiety and depression. Dialogues Clin. Neurosci. 4, 31–46.

Reyes, T.M., Lewis, K., Perrin, M.H., Kunitake, K.S., Vaughan, J., Arias, C.A., Hogenesch, J.B., Gulyas, J., Rivier, J., Vale, W.W., Sawchenko, P.E., 2001. Urocortin II: A member of the corticotropin-releasing factor (CRF) neuropeptide family that is selectively bound by type 2 CRF receptors. Proc. Natl. Acad. Sci. 98, 2843–2848. https://doi.org/10.1073/pnas.051626398

Reyes-Contreras, M., Glauser, G., Rennison, D.J., Taborsky, B., 2019. Early-life manipulation of cortisol and its receptor alters stress axis programming and social competence. Philos. Trans. R. Soc. B Biol. Sci. 374, 20180119. https://doi.org/10.1098/rstb.2018.0119

Ritter, T.D., Zale, A.V., Grisak, G., Lance, M.J., 2020. Groundwater Upwelling Regulates Thermal Hydrodynamics and Salmonid Movements during High-Temperature Events at a Montane Tributary Confluence. Trans. Am. Fish. Soc. 149, 600–619. https://doi.org/10.1002/tafs.10259

Robins, P.E., Skov, M.W., Lewis, M.J., Giménez, L., Davies, A.G., Malham, S.K., Neill, S.P., McDonald, J.E., Whitton, T.A., Jackson, S.E., Jago, C.F., 2016. Impact of climate change on UK estuaries: A review of past trends and potential projections. Estuar. Coast. Shelf Sci. 169, 119–135. https://doi.org/10.1016/j.ecss.2015.12.016

Roustit, M.M., Vaughan, J.M., Jamieson, P.M., Cleasby, M.E., 2014. Urocortin 3 activates AMPK and AKT pathways and enhances glucose disposal in rat skeletal muscle. J. Endocrinol. 223, 143–154. https://doi.org/10.1530/JOE-14-0181

Rummer, J.L., Couturier, C.S., Stecyk, J.A.W., Gardiner, N.M., Kinch, J.P., Nilsson, G.E., Munday, P.L., 2014. Life on the edge: thermal optima for aerobic scope of equatorial reef fishes are close to current day temperatures. Glob. Change Biol. 20, 1055–1066. https://doi.org/10.1111/gcb.12455

Sadoul, B., Vijayan, M.M., 2016. Stress and Growth, in: Schreck, C.B., Tort, L., Farrell, A.P., Brauner, C.J. (Eds.), Fish Physiology, Biology of Stress in Fish. Academic Press, pp. 167–205. https://doi.org/10.1016/B978-0-12-802728-8.00005-9

Saito, S., Shingai, R., 2006. Evolution of thermoTRP ion channel homologs in vertebrates. Physiol. Genomics 27, 219–230. https://doi.org/10.1152/physiolgenomics.00322.2005

Schaefer, J., Ryan, A., 2006. Developmental plasticity in the thermal tolerance of zebrafish Danio rerio. J. Fish Biol. 69, 722–734. https://doi.org/10.1111/j.1095-8649.2006.01145.x

Schnörr, S.J., Steenbergen, P.J., Richardson, M.K., Champagne, D.L., 2012. Measuring thigmotaxis in larval zebrafish. Behav. Brain Res. 228, 367–374. https://doi.org/10.1016/j.bbr.2011.12.016

Schulte, P.M., 2015. The effects of temperature on aerobic metabolism: towards a mechanistic understanding of the responses of ectotherms to a changing environment. J. Exp. Biol. 218, 1856–1866. https://doi.org/10.1242/jeb.118851

Schurmann, H., Christiansen, J.S., 1994. Behavioral thermoregulation and swimming activity of two arctic teleosts (subfamily gadinae)—the polar cod (Boreogadus saida) and the navaga (Eleginus navaga). J. Therm. Biol. 19, 207–212. https://doi.org/10.1016/0306-4565(94)90032-9

Scott, G.R., Johnston, I.A., 2012. Temperature during embryonic development has persistent effects on thermal acclimation capacity in zebrafish. Proc. Natl. Acad. Sci. U. S. A. 109, 14247–14252. https://doi.org/10.1073/pnas.1205012109

Skulachev, V.P., Bogachev, A.V., Kasparinsky, F.O., 2012. Principles of Bioenergetics. Springer Science & Business Media.

Slominski, A., 2009. On the role of the corticotropin-releasing hormone signalling system in the aetiology of inflammatory skin disorders. Br. J. Dermatol. 160, 229–232. https://doi.org/10.1111/j.1365-2133.2008.08958.x

Slominski, A., Pisarchik, A., Tobin, D.J., Mazurkiewicz, J.E., Wortsman, J., 2004. Differential Expression of a Cutaneous Corticotropin-Releasing Hormone System. Endocrinology 145, 941–950. https://doi.org/10.1210/en.2003-0851

Slominski, A., Zbytek, B., Pisarchik, A., Slominski, R. m., Zmijewski, M. a., Wortsman, J., 2006. CRH functions as a growth factor/cytokine in the skin. J. Cell. Physiol. 206, 780–791. https://doi.org/10.1002/jcp.20530

Slominski, A.T., Zmijewski, M.A., Zbytek, B., Tobin, D.J., Theoharides, T.C., Rivier, J., 2013. Key Role of CRF in the Skin Stress Response System. Endocr. Rev. 34, 827–884. https://doi.org/10.1210/er.2012-1092

Smith, G.W., Aubry, J.-M., Dellu, F., Contarino, A., Bilezikjian, L.M., Gold, L.H., Chen, R., Marchuk, Y., Hauser, C., Bentley, C.A., Sawchenko, P.E., Koob, G.F., Vale, W., Lee, K.-F., 1998. Corticotropin Releasing Factor Receptor 1–Deficient Mice Display Decreased Anxiety, Impaired Stress Response, and Aberrant Neuroendocrine Development. Neuron 20, 1093–1102. https://doi.org/10.1016/S0896-6273(00)80491-2

Sokolova, I.M., 2021. Bioenergetics in environmental adaptation and stress tolerance of aquatic ectotherms: linking physiology and ecology in a multi-stressor landscape. J. Exp. Biol. 224, jeb236802. https://doi.org/10.1242/jeb.236802

Sokolova, I.M., 2023. Ectotherm mitochondrial economy and responses to global warming. Acta Physiol. 237, e13950. https://doi.org/10.1111/apha.13950

Solinas, G., Summermatter, S., Mainieri, D., Gubler, M., Montani, J.P., Seydoux, J., Smith, S.R., Dulloo, A.G., 2006. Corticotropin-Releasing Hormone Directly Stimulates Thermogenesis in Skeletal Muscle Possibly through Substrate Cycling between de Novo Lipogenesis and Lipid Oxidation. Endocrinology 147, 31–38. https://doi.org/10.1210/en.2005-1033

Souverein, P.C., Berard, A., Staa, T.P.V., Cooper, C., Egberts, A.C.G., Leufkens, H.G.M., Walker, B.R., 2004. Use of oral glucocorticoids and risk of cardiovascular and cerebrovascular disease in a population based case–control study. Heart 90, 859–865. https://doi.org/10.1136/hrt.2003.020180

Squillacioti, C., De Luca, A., Liguori, G., Paino, S., Mirabella, N., 2011. Expression of urocortin and corticotropin-releasing hormone receptors in the bovine adrenal gland. Gen. Comp. Endocrinol. 172, 416–422. https://doi.org/10.1016/j.ygcen.2011.04.004

Stackley, K.D., Beeson, C.C., Rahn, J.J., Chan, S.S.L., 2011. Bioenergetic Profiling of Zebrafish Embryonic Development. PLOS ONE 6, e25652. https://doi.org/10.1371/journal.pone.0025652

Sukhareva, E.V., 2021. The role of the corticotropin-releasing hormone and its receptors in the regulation of stress response. Vavilov J. Genet. Breed. 25, 216–223. https://doi.org/10.18699/VJ21.025

Sun, J., Qiu, L., Zhang, H., Zhou, Z., Ju, L., Yang, J., 2023. CRHR1 antagonist alleviates LPS-induced depression-like behaviour in mice. BMC Psychiatry 23, 17. https://doi.org/10.1186/s12888-023-04519-z

Svendsen, E., Føre, M., Økland, F., Gräns, A., Hedger, R.D., Alfredsen, J.A., Uglem, I., Rosten, C.M., Frank, K., Erikson, U., Finstad, B., 2021. Heart rate and swimming activity as stress indicators for Atlantic salmon (Salmo salar). Aquaculture 531, 735804. https://doi.org/10.1016/j.aquaculture.2020.735804

Takefuji, M., Murohara, T., 2019. Corticotropin-Releasing Hormone Family and Their Receptors in the Cardiovascular System. Circ. J. 83, 261–266. https://doi.org/10.1253/circj.CJ-18-0428

Teame, T., Zhang, Z., Ran, C., Zhang, H., Yang, Y., Ding, Q., Xie, M., Gao, C., Ye, Y., Duan, M., Zhou, Z., 2019. The use of zebrafish (Danio rerio) as biomedical models. Anim. Front. Rev. Mag. Anim. Agric. 9, 68–77. https://doi.org/10.1093/af/vfz020

Thompson, W.A., Shvartsburd, Z., Vijayan, M.M., 2022a. The antidepressant venlafaxine perturbs cardiac development and function in larval zebrafish. Aquat. Toxicol. 242, 106041. https://doi.org/10.1016/j.aquatox.2021.106041

Thompson, W.A., Shvartsburd, Z., Vijayan, M.M., 2022b. Sex-Specific and Long-Term Impacts of Early-Life Venlafaxine Exposure in Zebrafish. Biology 11, 250. https://doi.org/10.3390/biology11020250

Thorarensen, H., Gallaugher, P.E., Farrell, A.P., 1996. The limitations of heart rate as a predictor of metabolic rate in fish. J. Fish Biol. 49, 226–236. https://doi.org/10.1111/j.1095-8649.1996.tb00019.x

Timpl, P., Spanagel, R., Sillaber, I., Kresse, A., Reul, J.M., Stalla, G.K., Blanquet, V., Steckler, T., Holsboer, F., Wurst, W., 1998. Impaired stress response and reduced anxiety in mice lacking a functional corticotropin-releasing hormone receptor 1. Nat. Genet. 19, 162–166. https://doi.org/10.1038/520

Tronche, F., Kellendonk, C., Reichardt, H.M., Schütz, G., 1998. Genetic dissection of glucocorticoid receptor function in mice. Curr. Opin. Genet. Dev. 8, 532–538. https://doi.org/10.1016/S0959-437X(98)80007-5

Vaughan, J., Donaldson, C., Bittencourt, J., Perrin, M.H., Lewis, K., Sutton, S., Chan, R., Turnbull, A.V., Lovejoy, D., Rivier, C., Rivier, J., Sawchenko, P.E., Vale, W., 1995. Urocortin, a mammalian neuropeptide related to fish urotensin I and to corticotropin-releasing factor. Nature 378, 287–292. https://doi.org/10.1038/378287a0

Vay, L., Gu, C., McNaughton, P.A., 2012. The thermo-TRP ion channel family: properties and therapeutic implications. Br. J. Pharmacol. 165, 787–801. https://doi.org/10.1111/j.1476-5381.2011.01601.x

Vera-Chang, M.N., St-Jacques, A.D., Gagné, R., Martyniuk, C.J., Yauk, C.L., Moon, T.W., Trudeau, V.L., 2018. Transgenerational hypocortisolism and behavioral disruption are induced by the antidepressant fluoxetine in male zebrafish Danio rerio. Proc. Natl. Acad. Sci. U. S. A. 115, E12435–E12442. https://doi.org/10.1073/pnas.1811695115

Vijayan, M.M., Raptis, S., Sathiyaa, R., 2003. Cortisol treatment affects glucocorticoid receptor and glucocorticoid-responsive genes in the liver of rainbow trout. Gen. Comp. Endocrinol. 132, 256–263. https://doi.org/10.1016/S0016-6480(03)00092-3

Villamizar, N., Ribas, L., Piferrer, F., Vera, L.M., Sánchez-Vázquez, F.J., 2012. Impact of Daily Thermocycles on Hatching Rhythms, Larval Performance and Sex Differentiation of Zebrafish. PLOS ONE 7, e52153. https://doi.org/10.1371/journal.pone.0052153

Volkoff, H., Rønnestad, I., 2020. Effects of temperature on feeding and digestive processes in fish. Temp. Multidiscip. Biomed. J. 7, 307–320. https://doi.org/10.1080/23328940.2020.1765950

Waddell, D.S., Baehr, L.M., van den Brandt, J., Johnsen, S.A., Reichardt, H.M., Furlow, J.D., Bodine, S.C., 2008. The glucocorticoid receptor and FOXO1 synergistically activate the skeletal muscle atrophy-associated MuRF1 gene. Am. J. Physiol.-Endocrinol. Metab. 295, E785–E797. https://doi.org/10.1152/ajpendo.00646.2007

Wakamatsu, Y., Ogino, K., Hirata, H., 2019. Swimming capability of zebrafish is governed by water temperature, caudal fin length and genetic background. Sci. Rep. 9, 16307. https://doi.org/10.1038/s41598-019-52592-w

Wang, L., Million, M., Rivier, J., Rivier, C., Craft, N., Stenzel-Poore, M.P., Taché, Y., 2011. CRF Receptor Antagonist Astressin-B Reverses and Prevents Alopecia in CRF Over-Expressing Mice. PLoS ONE 6, e16377. https://doi.org/10.1371/journal.pone.0016377

Webb, B.W., Hannah, D.M., Moore, R.D., Brown, L.E., Nobilis, F., 2008. Recent advances in stream and river temperature research. Hydrol. Process. 22, 902–918. https://doi.org/10.1002/hyp.6994

Wendelaar Bonga, S.E., 1997. The stress response in fish. Physiol. Rev. 77, 591–625. https://doi.org/10.1152/physrev.1997.77.3.591

Westerlund, E., Marelsson, S.E., Ehinger, J.K., Sjövall, F., Morota, S., Åsander Frostner, E., Oldfors, A., Darin, N., Lundgren, J., Hansson, M.J., Fellman, V., Elmér, E., 2018. Oxygen consumption in platelets as an adjunct diagnostic method for pediatric mitochondrial disease. Pediatr. Res. 83, 455–465. https://doi.org/10.1038/pr.2017.250

Williams, T.A., Bergstrome, J.C., Scott, J., Bernier, N.J., 2017. CRF and urocortin 3 protect the heart from hypoxia/reoxygenation-induced apoptosis in zebrafish. Am. J. Physiol. Regul. Integr. Comp. Physiol. 313, R91–R100. https://doi.org/10.1152/ajpregu.00045.2017

Wu, S.V., Yuan, P.-Q., Lai, J., Wong, K., Chen, M.C., Ohning, G.V., Taché, Y., 2011. Activation of Type 1 CRH Receptor Isoforms Induces Serotonin Release from Human Carcinoid BON-1N Cells: An Enterochromaffin Cell Model. Endocrinology 152, 126–137. https://doi.org/10.1210/en.2010-0997

Xu, X., Zhang, Z., Guo, H., Qin, J., Zhang, X., 2020. Changes in Aggressive Behavior, Cortisol and Brain Monoamines during the Formation of Social Hierarchy in Black Rockfish (Sebastes schlegelii). Anim. Open Access J. MDPI 10, 2357. https://doi.org/10.3390/ani10122357

Zhou, H., Chen, A., Lu, W., 2021. Corticotropin-releasing hormone reduces basal estradiol production in zebrafish follicular cells. Mol. Cell. Endocrinol. 527, 111222. https://doi.org/10.1016/j.mce.2021.111222

Zhou, H., Ge, C., Chen, A., Lu, W., 2019. Dynamic Expression and Regulation of Urotensin I and Corticotropin-Releasing Hormone Receptors in Ovary of Olive Flounder Paralichthys olivaceus. Front. Physiol. 10.

Ziegler, Cass, Herman, 1999. Excitatory Influence of the Locus Coeruleus in Hypothalamic-Pituitary-Adrenocortical Axis Responses to Stress. J. Neuroendocrinol. 11, 361–369. https://doi.org/10.1046/j.1365-2826.1999.00337.x

Appendix

6.1 Chapter 2 Supplemental methods and figures

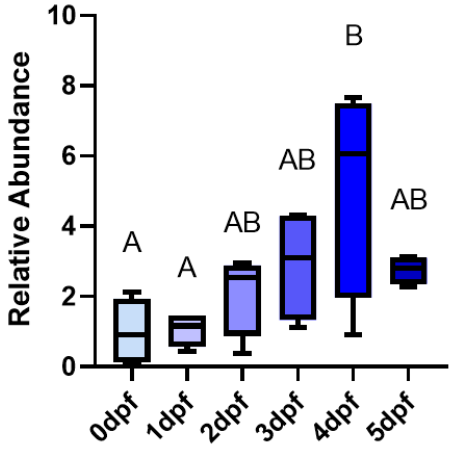
Zebrafish embryo and larvae sampling

Zebrafish embryos/larvae were treated with an MS-222 overdose until unresponsive (~1 min). Water was removed and embryos/larvae were flash frozen on dry ice in microcentrifuge tubes containing groups of 20 (0-2 dpf) or 12 (3-5 dpf) larvae and stored at -80°C.

RNA extraction and qPCR

Tissue was homogenized in Trizol (VWR, Canada) and RNA was extracted according to the manufacturer's protocol. RNA was quantified using a Spectradrop Micro-Volume Microplate (Spectramax 190, Molecular Devices, CA, USA) and 1µg of total RNA was treated with DNase I (Thermo Scientific, Waltham, MA, USA), followed by cDNA synthesis using the high-capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA, USA). qPCR was performed with Sso Advanced Universal SYBR Green Supermix (BioRad, Canada) on the Applied Biosystems QuantStudio 3 Real-Time PCR System (Applied Biosystems, USA). Samples were run with the following cycling conditions: 94°C for 2 min, 40 cycles of 95°C for 30s, and 30s at 60°C, and a final extension step at 72°C for 10 min. Melting curve analysis was done to confirm amplicon specificity. All transcript abundances were normalized to β-actin and analyzed using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001), with untreated WT groups standardized to a value of 1.

Chapter 2 supplemental figures



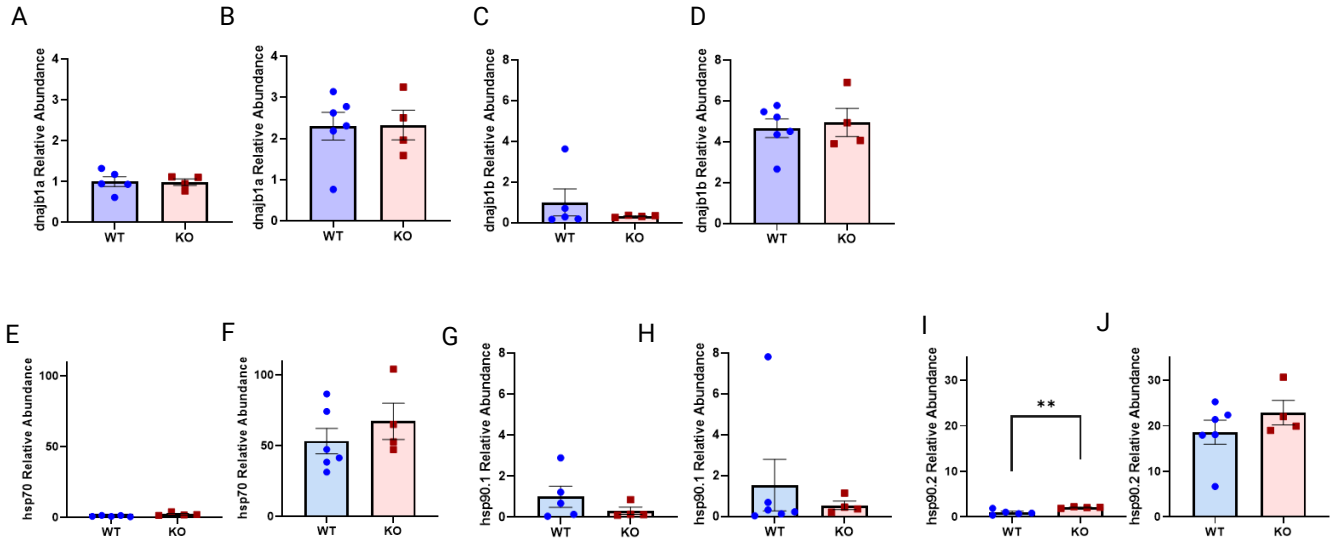
Supplemental Figure 6.1
Transcript abundance of *crhr1* at different ages of WT zebrafish embryos/larvae. Transcripts were normalized to β-actin expression and 0dpf embryo transcript abundance was standardized to 1 using the $2^{-\Delta\Delta Ct}$ method. Each sample was N = 4. Bars represent mean ± SEM. Abbreviations: days post-fertilization (dpf), corticotropin-releasing hormone receptor 1 (*crhr1*).

6.2 Chapter 3 supplemental figures and tables

Supplemental Table 6.1

Mean weight (mg ± SEM) of fish used for adult metabolic rate experiments. Weights were taken immediately prior to placing fish in the respective respirometers.

	WT		CRHR1KO	
	28°C	33°C	28°C	33°C
Resting respirometer	290.0 ± 31.2	285.0 ± 35.1	516.7 ± 35.0	513.3 ± 43.1
Swim tunnel respirometer	626.7 ± 30.6	609.8 ± 28.6	492.3 ± 21.8	516.5 ± 25.0



Supplemental Figure 6.2

Transcript abundance of brain *hsp*'s sampled from either WT or CRHR1KO adult zebrafish (A,C,E,G,I) taken from their holding tanks at 28°C or (B,D,F,H) from the respirometer at 33°C. Transcripts were normalized to β-actin expression and WT 28°C fish were standardized to 1 using the $2^{-\Delta\Delta Ct}$ method. Bars represent mean ± SEM. Asterisks indicate a significant difference between treatment groups (student's T-test, ** $P < 0.01$). Abbreviations: wildtype (WT), corticotropin-releasing hormone receptor 1 knockout (KO).