EFFECTS OF CHRONIC CORTISOL ELEVATION ON CARDIAC STRUCTURE AND FUNCTION IN ZEBRAFISH *(DANIO RERIO)*

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Abstract

Cardiac deformities are increasingly reported in farmed salmonid fishes. Pathological cardiac hypertrophy is dysfunctional remodelling that includes enlargement of the ventricle and compact myocardium, as well as fibrotic collagen deposition. The current study investigated the effect of chronic cortisol elevation, as a proxy for chronic stress, on zebrafish (*Danio rerio)* heart structure and function. Compact myocardium thickness and heart size increased in wildtype zebrafish exposed to waterborne cortisol treatment. However, pro-hypertrophic molecular markers did not differ in transcript abundance between treatment groups. Measures of cardiac function were not affected by cortisol exposure, nor was swimming performance. Zebrafish lacking functional expression of the glucocorticoid receptor (GR-KO) had hearts that were smaller and with thinner walls relative to body mass than wildtype fish. However, cardiac function and swimming performance in GR-KO fish were comparable to those of matched wildtype fish. Collectively, these data indicate that the zebrafish heart is capable of maintaining function even as morphology changes.

Résumé

Les malformations cardiaques sont de plus en plus fréquentes chez les salmonidés d'élevage. L'hypertrophie cardiaque pathologique est un remodelage dysfonctionnel qui se caractérise par une hypertrophie ventriculaire et un compactage du myocarde, ainsi que par des dépôts de collagène fibrotique. La présente étude a examiné l'effet d'une élévation chronique du taux de cortisol, comme indicateur de stress chronique, sur la structure et la fonction cardiaques du poisson-zèbre (*Danio rerio*). L'épaisseur du myocarde compact et la taille du cœur ont augmenté chez les poissons zèbres de type sauvage exposés à un traitement au cortisol présent dans l'eau, mais les marqueurs moléculaires pro-hypertrophiques n'ont pas changé d'expression entre les groupes traités. Les mesures de la fonction cardiaque n'ont pas été affectées par l'exposition au cortisol, pas plus que les performances de nage. Les poissons zèbres dépourvus d'expression fonctionnelle du récepteur des glucocorticoïdes (GR-KO) avaient un cœur plus petit et des parois plus fines par rapport à leur masse corporelle que les poissons de type sauvage. Les mesures de fonction cardiaque et les performances de nage des poissons GR-KO étant équivalentes à celles des poissons sauvages. Collectivement, ces données indiquent que le cœur du poisson-zèbre est capable de maintenir sa fonction même lorsque sa morphologie change.

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List of Abbreviations

ANOVA	analysis of variance
cDNA	complementary DNA
col1a1	collagen type I alpha 1
col1a2	collagen type I alpha 2
csrp3	cysteine and glycine-rich protein 3
d	day
f_H	heart rate
GR	glucocorticoid receptor
HPI	hypothalamus-pituitary-interrenal
KO	knockout
MR	mineralocorticoid receptor
mWT	matched wildtype
myh7	myosin heavy chain 7
myl2b	myosin light chain 2a

n	sample size
nRT	no reverse transcriptase control
NTC	no-template control
Q	cardiac output
rcan1b	regulator of calcineurin 1
RT-PCR, qPCR	semi-quantitative polymerase chain reaction
SEM	standard error of the mean
SV	stroke volume
U*crit*	maximum sustained swimming speed

Chapter 1: Introduction

1.1 Overview

The research presented in this thesis aimed to investigate the effects of chronic stress on the structure and function of the heart in adult zebrafish (*Danio rerio)*. Recent research indicates that significant structural and functional heart pathologies occur in salmonid fishes raised in aquaculture conditions, and these pathologies have been linked, at least in part, with chronic exposure to conditions that elicit stress responses (Johansen et al., 2017; Poppe et al., 2003). Chronic exposure to stressors often results in the elevation of circulating cortisol levels, and prolonged elevation of cortisol elicits pathological changes in the heart of salmonid fishes (Johansen et al., 2011; Johansen et al., 2017; Nørstrud et al., 2018). Whether chronic stress and/or prolonged exposure to elevated cortisol affects cardiac structure and function in zebrafish or indeed, in other fish groups beyond salmonids, has yet to be determined.

In the current study, zebrafish were exposed to waterborne cortisol and assessed for molecular, structural and functional indicators of pathological cardiac hypertrophy. Cortisol treatment was used to mimic the prolonged elevation of circulating cortisol caused by chronic stress. Compact myocardium thickness was assessed histologically, and transcript abundances of collagen and other markers of cardiac hypertrophy and pathology were measured. Heart rate, stroke volume and cardiac output were measured using high-frequency ultrasound, and swimming performance was evaluated as an indicator of cardiac function in a whole-animal context. Zebrafish lacking functional expression of corticosteroid receptors (knockout lines, KO, generated in our lab using CRISPR/Cas9) were used to further investigate the role of cortisol in pathological cardiac hypertrophy.

I hypothesized that zebrafish experiencing chronically elevated cortisol levels will exhibit pathological cardiac remodelling. Under this hypothesis, I predicted that cortisol-treated fish

would have larger hearts with thicker compact myocardium than sham-treated fish, that transcript abundances associated with cardiac hypertrophy would be elevated in cortisol-treated fish relative to sham fish, and that physiological performance would be impaired in cortisol-treated fish, as indicated by higher heart rate at rest and reduced swimming performance. Below, I review the literature upon which this hypothesis is based, and that provides the rationale for the present project.

1.2 Cardiac abnormalities in farmed salmonids

Overpopulation of fish stocks, disease, and generally poor transport/handling and living conditions are increasingly reported in commercial aquaculture (Johansen et al., 2017; Poppe et al., 2003). Norway, one of the leading producers of Atlantic salmon, reported a rise in salmonid deaths from 41.3 to 52.8 million fish over the years 2015-2019 (Oliveira et al., 2021). Many of these mortalities were attributed to cardiac pathologies and deformities (Oliveira et al., 2021). Diseases such as cardiomyopathy syndrome (Oliveira et al., 2021), heart and skeletal muscle inflammation (Wessel et al., 2020), arteriosclerosis, and myocardial necrosis (Poppe et al., 2007) are commonly reported, as are heart deformities. Farmed fish exhibit heart morphology that is significantly different to that of wild counterparts, with their hearts lacking symmetry and fatty deposits being found within the ventricle (Kristensen et al., 2012; Poppe et al., 2003). Lesions and hypertrophy of the epicardium and cardiac malformations are also observed in the hearts of standard commercial stocks of Atlantic salmon (*Salmo salar*). Under simulated farming conditions i.e., lower water temperatures and an un-natural light cycle, fish developed hearts with asymmetrical morphology, and this factor increased the overall workload of the heart because the enlarged bulbus arteriosus had decreased contractile abilities (Vindas et al., 2024).

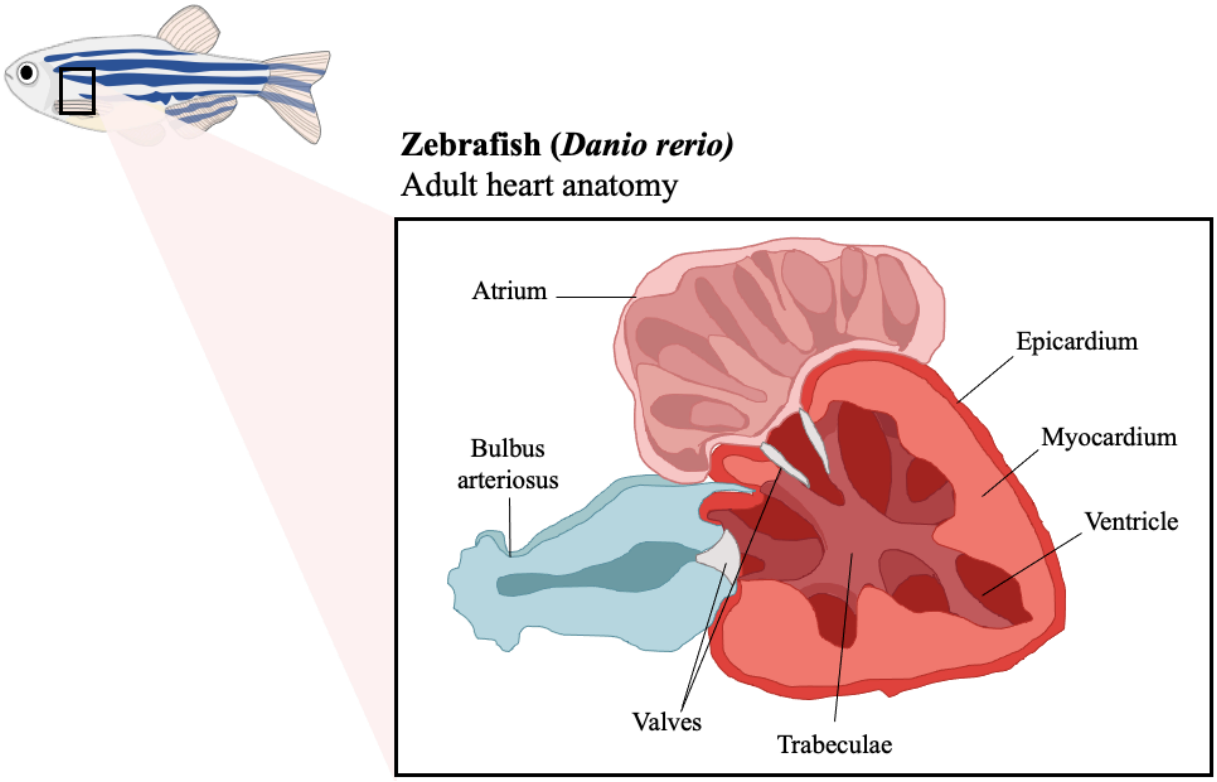

Figure 1.1. Schematic depicting adult zebrafish (*Danio rerio)* heart anatomy.

Fishes, including the zebrafish, have a four-chambered heart in which most of the contractile force is produced by the myocardium, the thick muscular wall of the ventricle (Fig. 1.1). Changes in the structure or stiffness of the ventricle have consequences at the whole animal level owing to the critical role of the ventricle in delivering blood first to the gill, for oxygen uptake, and then to the systemic tissues, for oxygen delivery. Cardiac hypertrophy, characterized by enlargement of the ventricle, is not only observed in farmed salmonid fishes, where it appears to be maladaptive, but is also considered to be an adaptive response to certain physiological stimuli, such as exercise (Palstra et al., 2013). In this type of remodelling, cardiac myocytes increase in number (hyperplasia) and/or grow (increase in cell size, hypertrophy) in response to a stimulus, causing a thickening of the contractile wall of the heart. Adaptive cardiac hypertrophy typically serves to improve cardiac performance, through increases in ventricular size, capacity,

and output, in response to increased oxygen demand (Palstra et al., 2013). Hyperplasia has been observed in the zebrafish heart under hypoxic conditions as an increase in nuclei observed in a given area of the heart, i.e., an increased cell count (Marques et al., 2008). The ability to remodel the heart has been observed in most vertebrate taxa, including snakes during periods of digestion to compensate for the rapid increase in metabolic rate (Martin and Leinwand, 2024), and birds when flying in high-altitude hypoxic conditions (Dunson, 1965). Cardiac hypertrophy is also known to occur in mammals, which includes humans. This is seen adaptively in times of increased exercise and higher biological demand for oxygen such as exercise and pregnancy (Foster et al., 2010; Kaidonis et al., 2021).

Stimuli can also include changes in water temperature. It should be noted that temperature-induced cardiac remodelling produces different compensatory remodelling responses across species. For example, cold-acclimated zebrafish show compact myocardium thickening (Shaftoe et al., 2023), whereas the hearts of rainbow trout (*Oncorhynchus mykiss*) maintain contractile abilities in low temperatures through decreased thickness of the compact myocardium (Farrell et al., 1988; Farrell et al., 1996; Johnson et al., 2014; Vindas et al., 2024). Remodelling also occurs in response to high physiological demand during exercise (Chen et al., 2021; Palstra et al., 2013). Remodelling has also been shown to occur in fishes exposed to low oxygen availability, which is common in some naturally occurring hypoxic environments. For example, when adult zebrafish were exposed to lifelong hypoxia, cardiac remodelling was observed as a 35% increase in compact myocardium thickness, coupled with overall smaller heart sizes (Smith et al., 2025). Zebrafish exposed to chronic hypoxia also displayed remodelling of the ventricle as a reduction of the space within the central cavity as well as an increase in cardiac myocyte densities (Marques et al., 2008).

Cardiac hypertrophy can also be a maladaptive response. Pathological cardiac hypertrophy is characterized by ventricular enlargement, thickening of the compact myocardium, and increased collagen deposition linked with increases in fibrosis and stiffness (Johansen et al., 2011; Johnson et al., 2014; Kim et al., 2023; Oka et al., 2014). Increased expression of markers of collagen deposition such as *col1α1, col1α2* and *col1α3* are typical indicators of fibrotic remodelling (Bard et al., 2021; Johnson et al., 2014). An increased ratio of Type 1 to Type 3 collagen has been shown to be indicative of fibrotic tissue because Type 1 collagen allows for less extension than other types, and thus increased deposition results in a stiffened heart (Shaftoe et al., 2023). Further, ventricular myosin heavy chain (*myh7*) is a pro-hypertrophic marker in fish – it is highly expressed during ventricular growth and is thus strongly upregulated in cases of cardiac hypertrophy (Johansen et al., 2011; Nørstrud et al., 2018). Slow myosin light chain 2 (*smlc2*) is a molecular marker of generalized growth, used as a pro-hypertrophic marker, while upregulation of regulator of calcineurin I (*rcan1*) signals pathological cardiac hypertrophy (Johansen et al., 2011).

Pathological remodelling can have negative impacts on cardiac function. In a healthy heart, the muscular and compliant ventricle walls of the heart serve to pump oxygenated blood around the body. In a fibrotic heart, the excessive deposition of collagen decreases compliance of the walls (Shaftoe et al., 2023), which decreases the contraction force of the ventricle and reduces the pumping capacity of the heart (Bernardo et al., 2010; Kong et al., 2013). These changes present as diminished cardiac output i.e., a decrease in the volume of blood pumped per unit time. Without the ability to optimally deliver blood and hence oxygen to the tissues, exercise performance is reduced. Fish with lower cardiac output show a reduction in swim performance

(Claireaux et al., 2005). These cardiac abnormalities have stimulated research into the effects of chronic stress and prolonged elevation of cortisol on cardiac structure and function.

1.3 Chronic cortisol elevation and cardiac remodelling

Research suggests that the stressful conditions to which farmed fish are exposed play a role in the prevalence of cardiovascular disease in farmed fishes (Johansen et al., 2011). For example, deformities such as a rounded cardiac ventricle with misaligned bulbus arteriosus (as opposed to the triangular ventricle seen in wild species) were present in Atlantic salmon (*Salmo salar)* exposed to transport stress and overcrowding (Poppe et al., 2003). Similarly, an increase in relative compact myocardium size was observed in European brown trout (*Salmo trutta*) after exposure to confinement stress, leading to overall larger hearts, a finding that suggested that exposure to stressors is associated with cardiac remodelling (Johansen et al., 2011).
Exposure to stressors generally elevates circulating levels of glucocorticoid stress hormones, with cortisol being the main glucocorticoid of teleost fishes (Dinarello et al., 2020; Wendelaar Bonga, 1997). Mammalian and salmonid studies have linked the risk of cardiovascular disease to chronic elevation of cortisol (Johansen et al., 2011; Johansen et al., 2017; Yamaji et al., 2009). For example, in studies on rainbow trout (*Oncorhynchus mykiss*) that were bred to differ in cortisol stress responsiveness, high-responding trout displayed increased ventricular size and thicker compact myocardium as well as greater fibrotic collagen deposition than low-responding trout (Johansen et al., 2011). Confirming a role for cortisol, comparable results were obtained with oral administration of cortisol to trout for 45 d via hydrocortisone-coated feed pellets. Specifically, increased ventricular size and proportion of compact-to-non-compact myocardial volume were observed in the cortisol-treated rainbow trout (Johansen et al.2017). Using a time-

course approach, Nørstrud et al. (2018) established that 3 weeks of oral cortisol administration were sufficient to induce cardiac remodelling in trout, specifically, a 20% increase in ventricular mass. Remodelling is a response to chronic rather than acute cortisol exposure; for example, rainbow trout exposed to 4 d of acute cortisol treatment showed no indication of cardiac hypertrophy (Bard et al., 2021).

Cortisol-induced cardiac remodelling also affected both cardiac function and swimming performance (Johansen et al., 2017). Following 45 d of oral cortisol administration, the difference between resting and maximal cardiac output, i.e. the scope in cardiac output, was significantly reduced in cortisol-treated species (Johansen et al., 2017). Similarly, after 90 d of cortisol treatment, species were subjected to swim respirometry trials in which a reduction in critical swimming speed (*Ucrit*) was exhibited by the cortisol-treated fish (Johansen et al., 2017). Pathological hypertrophy has been linked to prolonged stress and more specifically to cortisol (Lumbers et al., 2005; Severinova et al., 2019).

It should be noted that although adaptive cardiac remodelling has been reported in a range of fish species (Johnson et al., 2014; Palstra et al., 2013; Smith et al., 2025), research to date on pathological cardiac hypertrophy in response to stress has focused on salmonid fishes. Whether prolonged elevation of cortisol causes cardiac hypertrophy in other species is a knowledge gap this thesis attempts to address by focusing on zebrafish. Zebrafish have demonstrated a capacity for exercise-induced cardiac enlargement (Palstra et al., 2013), as well as cardiac remodelling in response to cold acclimation (Johnson et al., 2014) or hypoxia (Smith et al., 2025). A challenge for investigations of cardiac function in zebrafish is the small body size of this species, which does not facilitate the type of experiments or equipment commonly used with much larger bodied fishes, such as salmonids. However, emerging technologies provide

possibilities for the study of zebrafish heart function. For example, high-speed ultrasound has been used to measure *in vivo* heart rates in zebrafish (Evangelisti et al., 2020; Lee et al., 2016; Shaftoe et al., 2023), and ventricle force production was recently measured in this species for the first time (Smith et al., 2025). The zebrafish is particularly interesting for studies of heart function because this species has an endogenous capacity for heart regeneration after mechanical injury (Poss et al., 2002), raising the possibility that this capacity can provide resilience to the deleterious effects of chronic cortisol elevation that have been reported in salmonids. A second advantage of using zebrafish is that it has a single glucocorticoid receptor (GR) rather than the two GR that are more common in fishes (Alsop and Vijayan, 2008), and corticosteroid receptor knockout lines are available for this species (e.g. Faught and Vijayan, 2018).

1.4 Cortisol receptors and the heart

Cortisol is produced as the end-product of activation of the hypothalamic-pituitary-interrenal (HPI) axis (Fig. 1.2). When the fish is exposed to a stressor, the hypothalamus produces corticotropin-releasing factor (CRF), which stimulates the anterior pituitary to release adrenocorticotropic hormone (ACTH). The ACTH is transported via the circulation to the head kidney, where it stimulates the interrenal cells to produce cortisol, with the primary role of this glucocorticoid stress hormone being recovery of homeostasis after exposure to a stressor (Bernier et al., 2009; Faught et al., 2016; Gorissen and Flik, 2016). Cortisol exerts its effect by binding to GR and mineralocorticoid (MR) receptors, both of which are expressed throughout the body and serve as ligand-activated transcription factors (Faught and Vijayan, 2022).

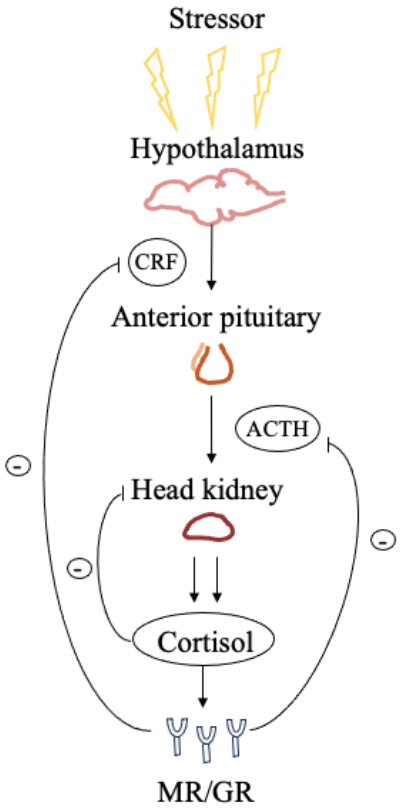

Figure 1.2. Schematic of the hypothalamic-pituitary-interrenal (HPI) axis. The diagram depicts the organs of interest, as well as the hormones produced, which are circled. Capped arrows labelled with a minus symbol indicate negative feedback of cortisol on HPI axis activity. *ACTH*, adrenocorticotropic hormone; CRF, corticotropin releasing factor; *GR*, glucocorticoid receptor; *MR*, mineralocorticoid receptor.

In mammals, cortisol and aldosterone are both ligands to the MR receptors and produce physiological response in ion regulation and homeostasis (Christina Zennaro and Shibata, 2017; Fuller et al., 2019). Fish, which do not produce aldosterone, primarily rely on cortisol for activation of receptor pathways. The GR has a lower affinity for cortisol than the MR, requiring elevated cortisol concentrations to be activated, i.e., those elicited by stressful conditions (Faught and Schaaf, 2023). By contrast, the MR (Griffiths et al., 2012) has an affinity 10-fold greater than that of the GR and is therefore highly sensitive to circulating cortisol levels, being fully occupied by cortisol even at resting or baseline cortisol levels (Faught and Schaaf, 2023). The role of the MR in adult fishes has yet to be fully defined.

In teleost fishes, both GR and MR are expressed in heart tissue. For example, transcripts for two GRs and an MR were reported in the heart of the cichlid *Astatotilapia burtoni* (Greenwood et al., 2003), as well as in the heart of the yellowtail clownfish, *Amphiprion clarkia* (Zhang et al., 2020). Further, these receptors were also present in salmonid cardiac tissue i.e. rainbow trout *Oncorhynchus mykiss* (Bury et al., 2003; Johansen et al., 2011; Sturm et al., 2005), and transcripts for one GR and one MR were reported in the heart of zebrafish (Schaaf et al., 2008; Wilson et al., 2015). In addition, GR protein was measured in the zebrafish heart (Wilson et al., 2015). Also, manipulation of cortisol signalling has been reported to have impacts on the development of the heart. For example, GR knockdown during early development led to smaller hearts and fewer cardiomyocytes in larval zebrafish (Wilson et al., 2015). Also, when cortisol was injected into zebrafish embryos, an increase in the prevalence of cardiac deformities such as pericardial edema and malformation of heart chambers was detected. These developmental defects also resulted in a lowered resting heartbeat after hatching (Nesan and Vijayan, 2012).

The generation of zebrafish lines lacking MR or GR (knockout, KO) using CRISPR/Cas9 technologies has provided additional tools to allow the roles of these receptors to be investigated. For example, MR-KO and GR-KO zebrafish have been used to examine the roles of the receptors in the stress response (Dinarello et al., 2022; Faught and Vijayan, 2018; Faught and Vijayan, 2022; Faught et al., 2016). These studies have revealed a key role for the GR receptor in negative feedback regulation of the HPI axis (Faught and Vijayan, 2018). Zebrafish lacking function expression of GR have significantly elevated baseline circulating plasma cortisol levels in comparison to their wildtype and MR-KO counterparts. It is thought that owing to the absence of negative feedback, which rapidly lowers cortisol levels after a stress response, cortisol remains elevated within the blood of GR-KO zebrafish (Faught and Vijayan, 2018). Similarly,

GR-KO larvae maintained elevated plasma cortisol levels after stress treatment (Castillo-Ramírez et al., 2024). These studies also reported a lower cortisol response (the increase in cortisol above baseline in response to a stressor) in GR-KO fish; however, the mutant zebrafish maintained the ability to mount a cortisol response. The MR-KO mutants exhibited a delayed cortisol response following an acute stressor (Faught and Vijayan, 2018).

Similarly, GR-KO and MR-KO zebrafish provide an opportunity to examine the relative importance of the GR vs the MR in mediating effects of elevated cortisol on cardiac structure and function. Our lab generated GR-KO and MR-KO fish (Hong, 2024) using CRISPR-Cas9 technologies to produce a full gene deletion of the GR (*nr3c1*; NCBI accession number: NP_001018547) or the MR (*nr3c2;* NCBI accession number: NC_007112.7). The founder fish populations were crossed with WT adults to create F1 heterozygous population, and F1 populations were in-crossed to generate F2 homozygous knockout fish as well as matched wild-type fish (mWT). Genotypes were confirmed by Sanger sequencing (Genome Quebec, McGill University, Montreal, QC, CA).

1.5 Hypotheses and predictions

In the current study, I hypothesized that zebrafish experiencing chronically elevated cortisol levels as a result of chronic stress would experience pathological cardiac remodelling. To test this hypothesis, I treated zebrafish with cortisol for 10 d to mimic the elevation of cortisol that would occur during chronic stress and assessed molecular, morphological and functional indicators of pathological cardiac hypertrophy. I also investigated the relative roles of GR and MR in mediating the effects of cortisol in pathological cardiac remodelling, predicting that GR-

KO and MR-KO fish would not experience pathological cardiac remodelling owing to lack of cortisol-binding receptors.

I predicted that myocardium thickness as well as overall heart size, as morphological indices of cardiac hypertrophy, would be elevated in cortisol-treated zebrafish. I also predicted that transcript abundances of markers for hypertrophy (*myl2b* and *csrp3*) and pathological hypertrophy (*myh7, rcan_X1&2*) would be elevated in cortisol-treated individuals. Further, I predicted that markers of fibrosis (*col1α1* and *col1α2)* would be elevated in the hearts of cortisol treated fish. Pathological hypertrophy is predicted to have functional consequences. Fish with a thicker-walled, more fibrotic and therefore stiffer ventricle are likely to experience impaired cardiac performance (Johansen et al., 2017; Kong et al., 2013), which would be manifested as decreased stroke volume in cortisol-treated fish. Cardiac compensation may occur in these individuals; for example, lower stroke volumes could be compensated by higher heart rates for maintenance of cardiac output (Fang et al., 2020). The changes in heart structure would be predicted to impair aerobic swimming performance in cortisol-treated zebrafish.

Histological assessment of compact myocardium thickness and heart length was carried out using brightfield microscopy. Real-time RT-PCR was used to measure relative transcript abundances of pro-hypertrophic markers. Heart function was assessed using high frequency ultrasound. Finally, swim performance was evaluated using a Blažka-type swim-tunnel sized for zebrafish.

Chapter 2: Materials and Methods

2.1 Experimental animals

Adult zebrafish were initially purchased from the pet trade (Big Al's, Ottawa, ON, CA) and maintained thereafter at the University of Ottawa aquatics facility. Wild-type (WT) adult zebrafish (total length = 3.8 ± 0.1 cm, mass = 0.452 ± 0.021 g; mean ± SEM, n = 129; male and female fish of 6-12 months old were used) were obtained from the in-house breeding program and held at a density of 4 fish L^{-1} in 3 L or 10 L tanks supplied with flowing, dechloraminated city of Ottawa tap water ("system water"). Standard housing conditions of 14L:10D photoperiod and water temperature of 28 ± 1°C were used. Fish were fed a commercial fish diet (GEMMA 300, Skretting, Maine, USA) until satiation, once daily.

Zebrafish lacking functional expression of either the MR (MR-KO; *nr3c2*$^{-/-}$) or the GR (GR-KO; *nr3c1*$^{-/-}$) together with matched WT fish (mWT) were also used. These fish were generated using CRISPR/Cas9 gene-editing technology (Hong, 2024) and held and maintained as described above. Male fish of 6-12 months old were used. The experiments focused exclusively on male fish because female fish are produced at lower rates than males in the GR-KO line, and therefore were retained for breeding. Although the fish were of similar age, GR-KO fish tended to be longer and heavier (total length = 3.8 ± 0.1 cm, mass = 0.505 ± 0.043 g; mean ± SEM, n = 38) than MR-KO (total length = 3.4 ± 0.1 cm, mass = 0.352 ± 0.031 g; mean ± SEM, n = 25) or mWT fish (total length = 3.8 ± 0.1 cm, mass = 0.452 ± 0.021 g; mean ± SEM, n = 129). Similar differences were noted in previous work on these lines (Hong, 2024) and have been reported for other GR-KO zebrafish lines (Faught and Vijayan, 2019).

All holding and experimental protocols were approved by the University of Ottawa's animal care committee (protocols BL-2118, BL-3675) and were in compliance with the

guidelines of the Canadian Council on Animal Care (CCAC) for the use of animals in teaching and research.

2.2 Cortisol treatment

Zebrafish were treated with cortisol using the waterborne cortisol exposure of Faught and Vijayan (2022). A 10-day treatment period was selected based on previous studies in salmonids in which treatment periods of 7 days resulted in upregulation of pro-hypertrophic molecular markers (Nørstrud et al., 2018), and 45-90 days resulted in remodelling (Johansen et al., 2017). The rainbow trout used in the above studies were held at temperatures of 8-9°C (Nørstrud et al., 2018) whereas zebrafish in the present study were held at 28°C, and the duration of the exposure period was shortened accordingly.

A preliminary trial was conducted to confirm that circulating cortisol levels in treated fish were at the desired level of 50-100 ng mL^{-1} observed in zebrafish exposed to a chronic stressor (Filby et al., 2010; Tea et al., 2019). Zebrafish (n = 6 per treatment group) were exposed to waterborne cortisol (hydrocortisone 21-hemisuccinate sodium salt, Sigma-Aldrich, Oakville, CA) at a concentration of 5 µg mL^{-1} in 0.05% ethanol, to the vehicle alone (sham; 0.05% ethanol) or to control conditions (system water) as described by Faught and Vijayan (2022) for 10 d. Fish were held in 2 L static tanks at a water temperature of 28°C. Full water changes (100%) with renewal of treatment conditions were conducted daily. Daily *ad libidum* feedings were conducted before water changes to reduce bacterial load from waste accumulation. At the end of the treatment period, fish were euthanized in an ice-cold solution of MS-222 (0.42 g L^{-1} tricaine methanesulfonate, Syndel, Nanaimo, BC) buffered using $NaHCO_3$ to a pH of 7, weighed, and total length was measured. Blood was collected by caudal severance using the approach of

Babaei et al. (2013) with 0.5 M ethylenediaminetetraacetic acid (EDTA) as an anti-coagulant. Blood samples were centrifuged for 3 min at 14 000 *g*, and plasma was flash frozen and stored at -80°C for later analysis of cortisol concentrations.

Cortisol concentrations were measured using a commercial enzyme immunoassay (EIA) kit (Neogen, Cat#: 402710, Miami, USA) as described by the manufacturer. Samples were diluted with the 1X extraction buffer provided with the kit, and assayed in duplicate. The intra-assay coefficient of variation was 0.84% and all samples were analyzed in a single assay.

Plasma cortisol concentrations in cortisol-treated fish were significantly higher than those in control or sham-treated zebrafish (Fig. 2.1).

2.3. Morphological assessment of the heart

Fish were treated with cortisol and euthanized using ice cold buffered MS-222 as described above, and mass and total length were measured. To dissect out the heart, the fish was sectioned posterior to the heart in the transverse plane. The thoracic region was opened longitudinally to expose the heart, which was removed, rinsed with saline solution (0.9% NaCl) and soaked in 1 M KCl for 2 min (so that it was fully contracted) as described by Johnson et al. (2014). Hearts were photographed using a dissecting microscope (Zeiss SteREO Discovery, V8-CL 1500 ECO, Ottawa, Ontario, CA), fixed in paraformaldehyde (PFA; 4% in phosphate-buffered saline, ChemCruz, Cat#281692, Dallas,TX, U.S.) overnight at 4°C, and stored in 70% ethanol (EtOH) at 4°C until further analysis.

Fixed hearts were embedded in paraffin wax and sectioned (Leica RM2255 microtome, Deer Park, United States). Sections (5 μm, yielding 3-6 sections per heart) were placed on positively charged microscope slides (Superfrost, ThermoFisher Scientific, Toronto, ON, CA)

and incubated at 65°C for 30 min to allow the paraffin to adhere to the slide. Slides were deparaffinized (3 x 5 min washes with xylene) and rehydrated (2 x 1 min each in 99%, 95% and 80% EtOH). Tissues were stained by incubation in Picrosirius Red (Fisher, Cat# 5030077, Massachusetts, USA) for 1 h at room temperature (21ºC) and rinsed with acetic acid.

Sections were photographed at 10x magnification using brightfield light microscopy (Zeiss AxioImager; North York, ON, CA). Hearts were photographed in their entirety (Appendix 1), ranging from 1 to 3 photos depending on the size of the heart section. An observer who was blind to the treatment group of the fish selected two photographs per section and three sections per heart for analysis of compact myocardium thickness.

Heart length was measured as the longest distance along the length of the ventricle. Compact myocardium thickness was measured in five locations that were selected haphazardly in each photograph, and the mean of all measurements (30 per fish) was used as the compact myocardium thickness for an individual fish. All analysis was carried out by an observer who was blind to the treatment group of the fish. Image analysis was carried out using FIJI (https://fiji.sc). Given the isometric relationship between body mass and heart size in fish (Johansen et al., 2017), heart length and compact myocardium thickness were expressed relative to individual fish mass.

2.4 Assessment of markers of hypertrophy and fibrosis

Transcript abundances of pro-hypertrophic markers and markers of fibrosis (*col1a1, col1a2, myl2b, csrp3, myh7, rcan1b(X1* and *X2*) were measured using real-time RT-PCR. At the end of the treatment period, cortisol- and sham-treated fish were euthanized using ice cold MS-222 as described above, mass and total length was measured, and the heart was dissected out,

flash frozen in lN_2, and stored at -80°C for later analysis of transcript abundances. Total RNA was extracted from samples of three pooled hearts (n = 1) that were homogenized using mortar and pestle on dry ice in TRIzol® reagent (Invitrogen, Burlington, ON, Canada). The RNA pellet was dissolved in nuclease-free water and RNA concentration and purity were measured using a NanoDrop 2000 Spectrophotometer (ThermoFisher Scientific, Waltham, MA, USA).

DNase I treatment (DNAse Amplification Grade kit, Invitrogen) was conducted according to the manufacturer's instructions for the removal of genomic DNA contamination. Reverse transcription of 2µg of DNase-treated RNA into cDNA was carried out according to the manufacturer's instructions using a high-capacity cDNA synthesis kit (Applied Biosystems, Waltham, MA, USA).

A CFX384 Real-Time PCR Detection System (Bio-Rad Laboratories, Mississauga, ON, Canada) was used to assess the relative transcript abundances of the target genes. The PCR cycling conditions consisted of an initial denaturation at 95°C for 3 min, followed by 40 cycles of 30 s at 95°C, annealing for 30 s at 58°C, and 10 s for extension at 72°C. The reactions consisted of 1.5 µL of nuclease free H_2O, 0.5 µL of each forward and reverse primer (Table 2.1), 2.5 µL of cDNA template, and 5 µL of BlasTaq 2XqPCR Master Mix (Applied Biological Material Inc, Richmond, BC, Canada). To assess primer efficiency (Table 2.1), a pooled sample of cDNA was created to generate standard curves for each primer set. The quality controls include no-template controls in which reactions were run without cDNA, and no-reverse transcriptase controls in which the cDNA was synthesized without reverse transcriptase. Melt curves generated after each run were used to confirm the specificity of amplification. Relative transcript abundance was calculated using the delta-delta Ct method ($2^{-\Delta\Delta Ct}$) (Livak and

Schmittgen, 2001) with the housekeeping gene eukaryotic elongation factor, *eefla*, (Table 2.1) for normalization. Values were expressed as fold-change relative to the sham treatment group.

2.4 Assessment of heart function using high-frequency ultrasound

Cardiac imaging was carried out using a Vevo 3100 Ultrasound Imager (FujiFilm VisualSonics, Toronto, Ontario, CA) in association with the MX700 ultrasound probe (29-71 MHz) on cortisol- and sham-treated WT fish, and on (untreated) GR-KO and mWT fish. The experimental approaches used were similar to those described by Shaftoe et al. (2023). Fish were transferred individually into a solution of buffered MS-222 (0.12 g L^{-1}) until the fish lost equilibrium and did not respond to a tactile stimulus. Fish were then transferred to an imaging stage consisting of a glass chamber (~300 mL volume) placed on a platform allowing control of the stage position in the x, y and z directions. The chamber was supplied with flowing, temperature-controlled, aerated anaesthetic solution via a peristaltic pump (Fisher Scientific, Mini-pump Variable Flow). The flowing anaesthetic was directed towards the head to allow for maximal irrigation of the gills. Temperature was maintained at ~25°C during imaging. The chamber contained a sponge with a vertical ~2 cm slit into which the tail of the fish was inserted so that the fish was positioned in dorsal recumbancy with a clear view of the thoracic region.

The probe was lowered into the water and its position was adjusted until the thoracic cavity was displayed, and clear movement of the atrioventricular valves and contraction of the ventricle were visible. The time from anesthetic induction to completion of imaging was <5 min for all fish included in the final data set; fish for which imaging exceeded 5 min were excluded from the data set. This approach was chosen owing to the use of MS-222 for anaesthesia. Previous studies of ultrasound imaging in zebrafish have used Aquacalm (Shaftoe et al. 2023) or

MS-222 in combination with isoflurane (Huang et al., 2010). Although MS-222 has cardio-depressive effects (Denvir et al., 2008), a combination of 65 ppm MS-222 and 65 ppm isoflurane reduced the depressive effects of MS-222 on zebrafish heart rate (Huang et al., 2010). Because I was not able to use isoflurane, imaging was conducted under MS-222 alone and therefore the duration of experiments was minimized to limit its cardio-depressive effects. In addition, the lowest concentration of MS-222 that was sufficient to immobilize the fish was chosen.

Heart rate was calculated from the imaging recordings using the Vevo 3100 image analysis software (VEVO Lab, v. 5.5.1, Fujifilm VisualSonics). Heart rate (f_H) was measured across five peaks using the Doppler wave trace function and averaged across measurements taken at three points in each recording. To determine stroke volume (SV), recordings were taken in B-mode for measurement of the perimeter of contraction. The volume of a single contraction was analyzed. The maximum diastolic area was determined from the frame at which the heart was least contracted, thus having the widest area within the ventricle. The minimum systolic area was measured when the heart appeared to be most contracted, thus having the lowest area within the ventricle. Once the appropriate frames were identified, the ventricle perimeter was traced in each frame, which allowed the software to calculate the stroke volume for a single contraction. Stroke volume was normalized to individual body mass based on the isometric relationship between body mass and heart size in fish (Johansen et al., 2017). Cardiac output (Q) was calculated for each fish as the product of SV and f_H (Sequeira and van der Velden, 2015).

2.5 Assessment of swimming performance

Blazka-type swim tunnels (170 mL; Loligo Systems, Viborg, Denmark) were used to evaluate exercise performance in individual fish. Each swim tunnel was positioned in a Plexiglas

tank containing aerated water at 26-28°C and was emptied and refilled with fresh system water between trials. Calibration of water velocity was carried out using Fluorescent Green Polyethylene Microspheres (1 g; Cospheric, California, US) with a green laser pointer and a Digital Particle Tracking Velocimetry (DPTV) Flow Tracking system (Loligo). The standard curve for each swim tunnel is included in Appendix 2.

Fish were transferred into the swim tunnel and allowed to acclimate for 20 min at a flow rate of 0 cm s^{-1}. The velocity of water flow was then increased by 0.85 cm s^{-1} every minute to a maximum of 8.47 cm s^{-1}, and fish swam at this velocity until they could no longer hold their position within the swim tunnel (Plaut, 2000). Owing to mechanical issues, the maximum input speed was limited to 8.47 cm s^{-1} and therefore duration of swimming at this velocity was used as an index of swimming performance. Fish of similar lengths between sham- and cortisol-treated fish (3.1 cm± 0.1cm, 3.0 cm ± 0.2 cm ;mean ± SEM, n = 9 & 10, Student's t-test; p = 0.586), and GR-KO and mWT (3.6 cm ± 0.1 cm, 3.5 cm ± 0.1 cm ;mean ± SEM, n = 8, Student's t-test; p = 0.303) fish were selected to minimize differences in the water velocities, and hence swimming effort, experienced by each individual fish. Following a trial, fish were euthanized as described above and mass and total length was recorded, allowing swimming velocities to be expressed in total lengths per second.

2.6 Statistical analyses

All statistical analyses were performed using SigmaPlot v13.0 software (Grafiti LLC, Palo Alto, CA, USA). The significance level (α) was set to 0.05. Data are presented as mean values ± SEM with values for individual fish included in figures. Effects of treatment group on plasma cortisol were analyzed by one-way analysis of variance (ANOVA). Effects of treatment

group and sex on compact myocardium thickness, or treatment group and genotype on compact myocardium thickness, ventricle length, and mass were analyzed by two-way ANOVA. Effects of treatment group on ventricle length, transcript abundances, cardiac output, stroke volume, heart rate and swimming performance were analyzed using Student's *t*-tests, as were effects of genotype on cardiovascular variables and swimming performance. Where data failed the assumptions of normality and equal variance, they were transformed to meet the assumptions, or an equivalent non-parametric test was used.

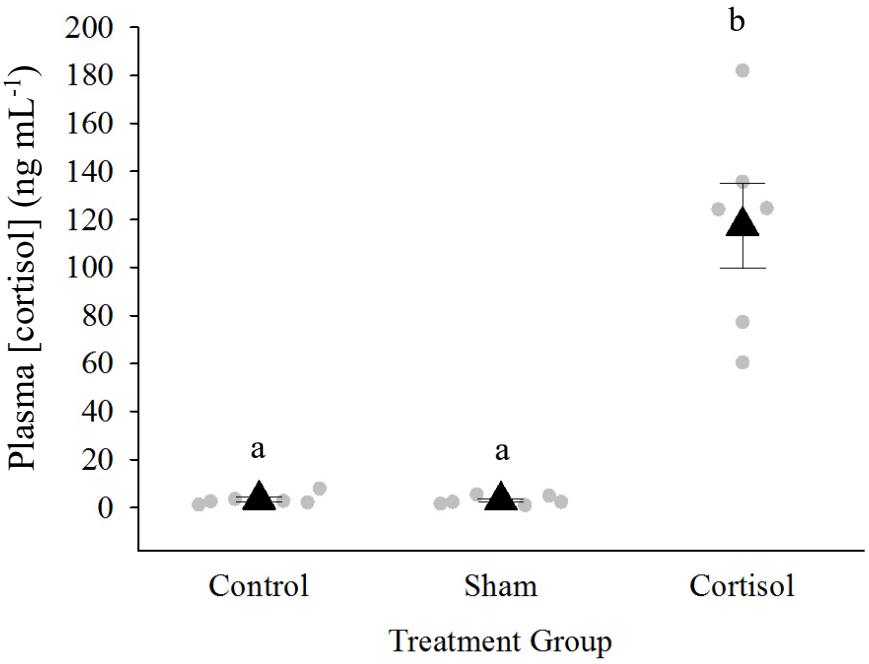

Figure 2. 2. Plasma cortisol concentrations of adult zebrafish, *Danio rerio*, after 10 d of exposure to waterborne cortisol, vehicle alone (sham) or no treatment (control; system water only). Circles present data for individual fish, with the triangles presenting mean values ± SEM (n = 6 fish per treatment). Treatment groups sharing a letter are not significantly different from one another (ANOVA, p = 0.003).

Table 2. 1. Primers used for qPCR

Target	Primer pair	Amplicon size (bp)	Efficiency (%)	Accession number	Reference
col1a1	F: ATGGTATGAATGGCGTGCCT R: CACCAGCAGGTCCCATTTCT	78	107.0	XM_005155976.6	(Johnson et al., 2014)
col1a2	F: ACAAGGAGTCTGCATGTCGG R: TTGGTCCCTTGAGGCCATTG	113	99.1	XM_073930591.1	(Johnson et al., 2014)
myl2b	F: CTTTCTCCCCGTGAGTGATG R: GAGGAGACCATCCTCAATGCC	208	87.6	NM_001040045.2	(Johansen et al., 2011)
csrp3	F: GCGCTTTATTTTCACAAACTCGC R: CTTTACGACAAACCATGCAGATGA	169	101.8	NM_001006026.3	(Johansen et al., 2011)
myh7	F: AACCGAAAGAGGCCGTAAGC R: CAGGCTTGTGTTCTGGGAGT	88	89.5	NM_001112733.1	(Johansen et al., 2011)
rcan1b_X1	F: CGCTCATCGCCTGTAAAGTG R: CAAAGCCTCAAACTGCTCCTTC	71	98.7	XM_073949310.1	(Johansen et al., 2011)
rcan1b_X2	F: GCAGTAATTCCTGTCTGGTGG R: GCCTCAAACTGCTCCCGAAT	79	105.1	XM_073949311.1	(Johansen et al., 2011)
eefla1	F: CTGGAGGCCAGCTCAAACAT R: ATCAAGAAGAGTAGTACCGCTAGCATTAC	87	94.0	NM_131263.1	(Johnson et al., 2014)

F, forward primer 5' → 3'; R, reverse primer 5'→3'.

For full gene names, see List of abbreviations.

Chapter 3: Results

3.1 Compact myocardium thickness and heart size

Compact myocardium thickness was significantly greater in cortisol-treated WT fish than in sham fish (Fig. 3.1). Although a trend for male fish to have greater compact myocardium thickness than female fish was detected, it did not reach statistical significance (Fig. 3.1) and therefore subsequent experiments on WT fish used both male and female fish. Ventricle length also tended to be greater in cortisol-treated fish, with the difference just failing to reach statistical significance (Fig. 3.2). The two trends were not a reflection of significant differences in body mass or total length with sex or treatment group (Table 3.1).

To determine whether the effects of cortisol were mediated by GR or MR, the experiment was repeated using GR-KO, MR-KO and mWT fish. Analysis of compact myocardium thickness in these fish did not detect a significant effect of cortisol treatment (Fig. 3.3A). However, compact myocardium thickness was significantly lower in GR-KO fish than in MR-KO or mWT fish (Fig. 3.3A). This difference appeared to be driven by the significantly greater mass of GR-KO fish relative to mWT or MR-KO fish (Fig. 3.3B). Similarly, ventricle length was significantly lower in GR-KO zebrafish compared to MR-KO or mWT fish (Fig 3.4A). Again, body mass was significantly greater in GR-KO fish compared to both other genotypes (Fig 3.4B), which likely accounted for the lower ventricle length in relation to body mass.

3.2 Gene expression of molecular markers of hypertrophy and fibrosis

Molecular markers associated with cardiac hypertrophy and fibrosis were assessed to investigate the mechanisms underlying the effect of cortisol treatment on compact myocardium thickness. However, no significant differences in the molecular markers of cardiac hypertrophy (Fig 3.5) or fibrosis (Fig 3.6) were detected between treatment groups.

3.3 Cardiac function and swimming performance

To evaluate whether the thicker compact myocardium of cortisol-treated fish affected cardiac or whole-animal performance, metrics of cardiac function were assessed by high frequency ultrasound in resting (anaesthetized) fish, and exercise performance was examined using a swim tunnel. No significant differences in SV, f_H or Q were detected between cortisol- and sham-treated WT fish (Fig. 3.7). To check for potential effects of body length on exercise effort, swimming velocity in body lengths · s^{-1} was compared between treatment groups and found to not vary significantly (Fig 3.8A). No significant differences were detected between treatment groups in the duration of swimming performance at maximum water velocity (Fig. 3.8B).

Measurements of cardiac function and exercise performance were also carried out on GR-KO and mWT fish (in the absence of cortisol treatment), to evaluate the impact of the differences in relative heart size in GR-KO fish. The SV of GR-KO fish tended to be higher than that of mWT fish, and while no significant difference was detected between genotypes in f_H, Q in GR-KO fish was significantly higher than that in mWT fish (Fig. 3.9). The GR-KO and mWT fish used for exercise performance trials were selected to be similar in body length, to avoid issues associated with differences in exercise effort. Evaluation of swimming velocity in body lengths · s^{-1} did not reveal significant differences between GR-KO and mWT fish (Fig 3.10A). No significant differences were detected between genotypes in the duration of swimming performance at maximum water velocity (Fig. 3.10B).

Table 3.1 Summary of mass and total length for WT fish by sex and treatment group.

Experimental group	Sex	*n*	Mass (g)	*P* values	Total length (cm)	*P* values
Sham	Female	6	0.461 ± 0.029		3.7 ± 0.1	
	Male	6	0.416 ± 0.030	$P_{treatment} = 0.523$	3.6 ± 0.1	$P_{treatment} = 1.000$
Cortisol-treated	Female	6	0.455 ± 0.037	$P_{sex} = 0.062$ $P_{sex*treatment} = 0.659$	3.7 ± 0.1	$P_{sex} = 0.268$ $P_{sex*treatment} = 0.748$
	Male	6	0.383 ± 0.020		3.5 ± 0.1	

Values are means ± SEM. Data were analyzed by two-way ANOVA

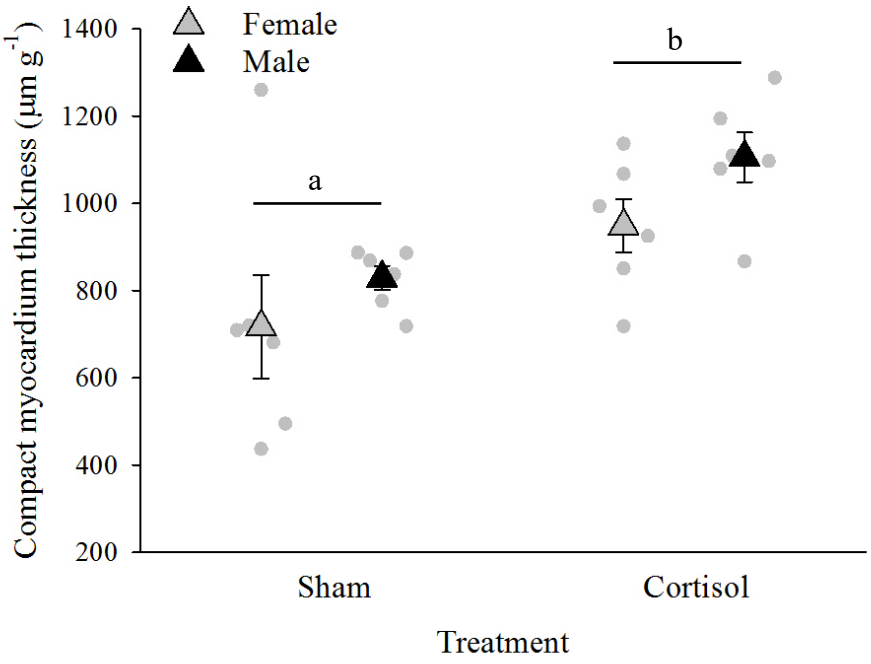

Figure 3. 1. Cortisol treatment increased the compact myocardium thickness of the ventricle in WT zebrafish (*Danio rerio*) regardless of fish sex. Triangles present mean values ± SEM (n = 6 fish per group), with values for individual fish indicated by the circles. Pairs of groups that share a letter are not significantly different from one another (two-way ANOVA; $P_{treatment}$ = 0.003, $\underline{P_{sex}}$ = 0.084, $P_{sex*treatment}$ = 0.766).

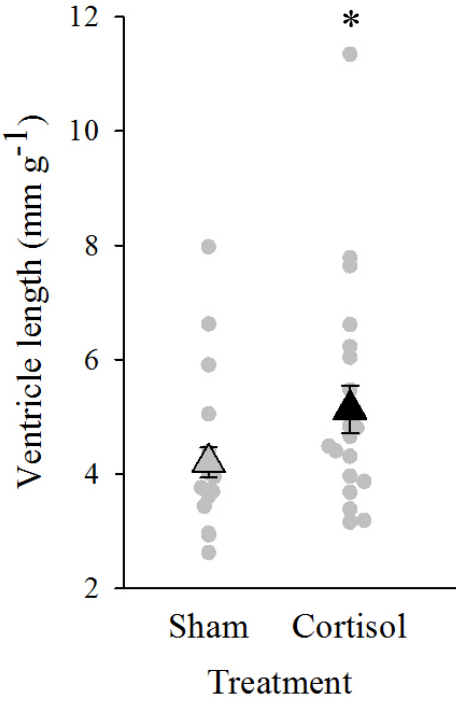

Figure 3.2. Cortisol-treated WT zebrafish (*Danio rerio*) had longer ventricles than sham-treated fish. Triangles are mean values ± SEM (n = 22 fish per group) with values for individual fish indicated by the circles. An asterisk indicates a significant difference from the sham-treated group (Student's t-test, P = 0.05).

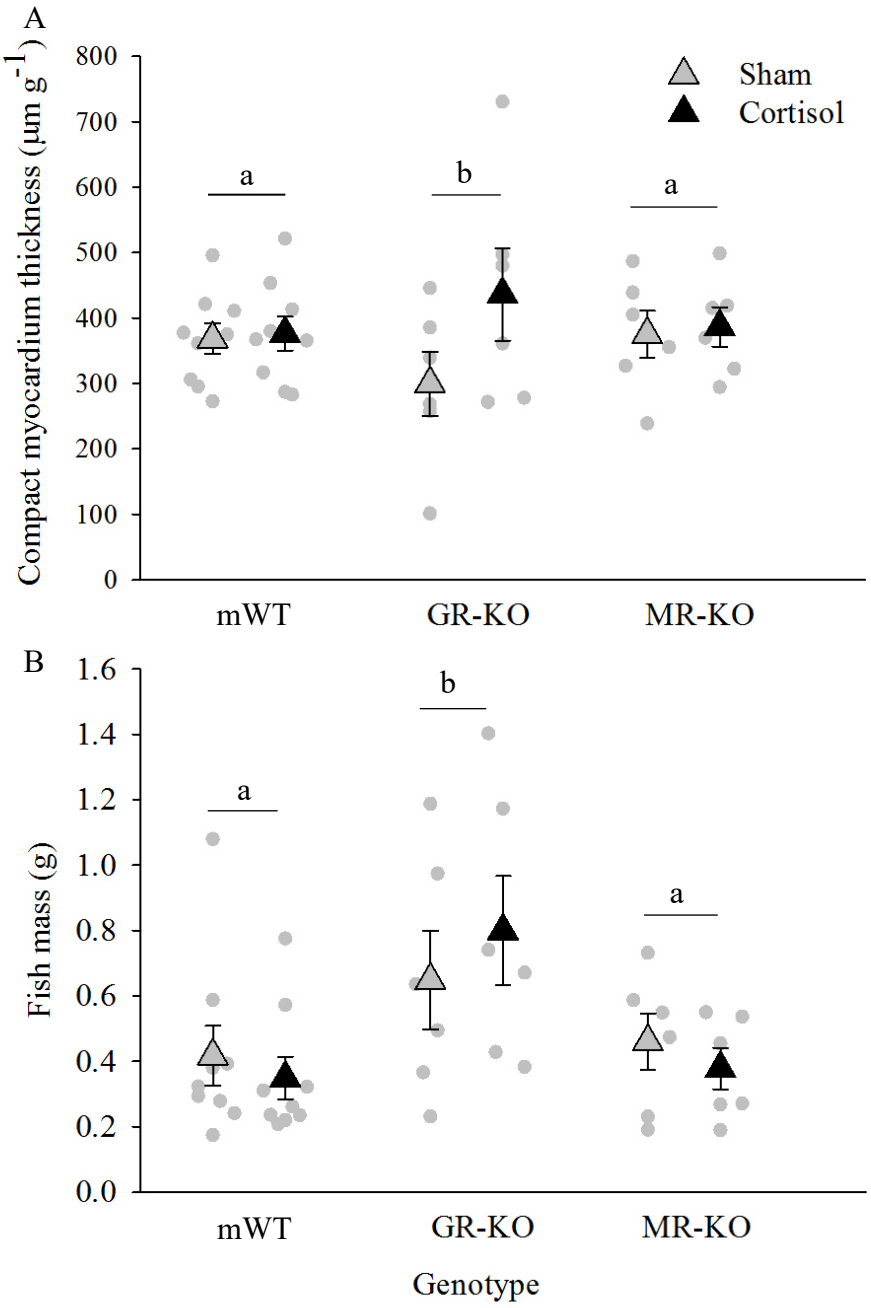

Figure 3.3. Cortisol treatment had no effect on compact myocardium thickness in zebrafish (*Danio rerio*) lacking GR (GR-KO) or MR (MR-KO) receptors, or their matched WT (mWT) fish. Data for compact myocardium thickness (A) and fish mass (B) are presented. In both figures, triangles present mean values ± SEM (n =9, 6 and 6 fish per group for mWT, GR-KO and MR-KO fish, respectively) with values for individual fish indicated by the circles. Pairs of

groups that share a letter are not significantly different from one another (two-way ANOVA; $P_{genotype} < 0.001$, $P_{treatment} = 0.123$, $P_{genotype*treatment} = 0.831$ for panel A; $P_{genotype} < 0.001$, $P_{treatment} = 0.680$, $P_{genotype*treatment} = 0.187$ for panel B).

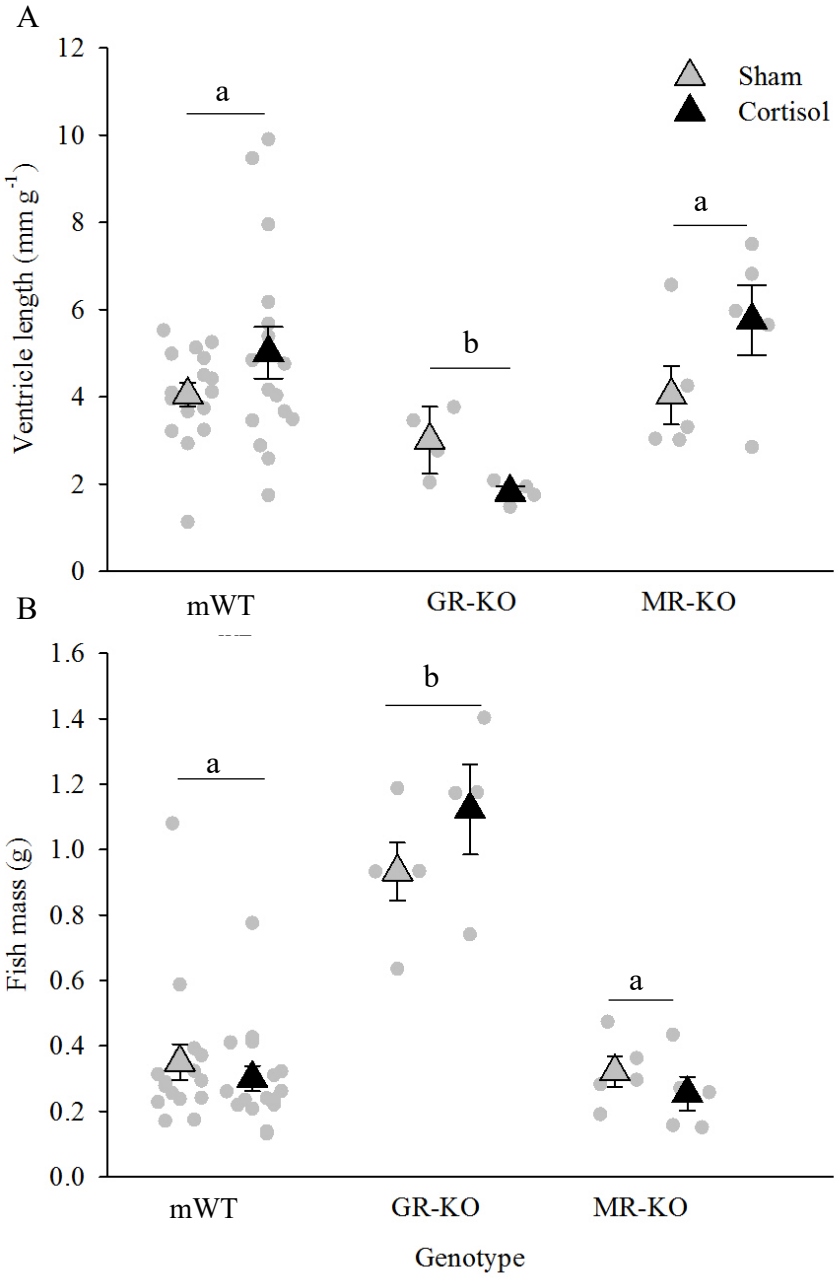

Figure 3.4. Ventricle length in zebrafish (*Danio rerio*) lacking GR (GR-KO) was significantly lower than in fish lacking MR (MR-KO) or matched WT (mWT) fish. Data for ventricle length (A) and fish mass (B) are presented. In both figures, triangles present mean values ± SEM (*n* = 16, 4 and 5 for mWT, GR-KO and MR-KO fish, respectively) with values for individual fish indicated by the circles. Pairs of groups that share a letter are not significantly different from one

another (two-way ANOVA; $P_{genotype} < 0.005$, $P_{treatment} = 0.389$, $P_{genotype*treatment} = 0.177$ for panel A; $P_{genotype} = <0.001$, $P_{treatment} = 0.662$ $P_{genotype*treatment} = 0.225$) for panel B.

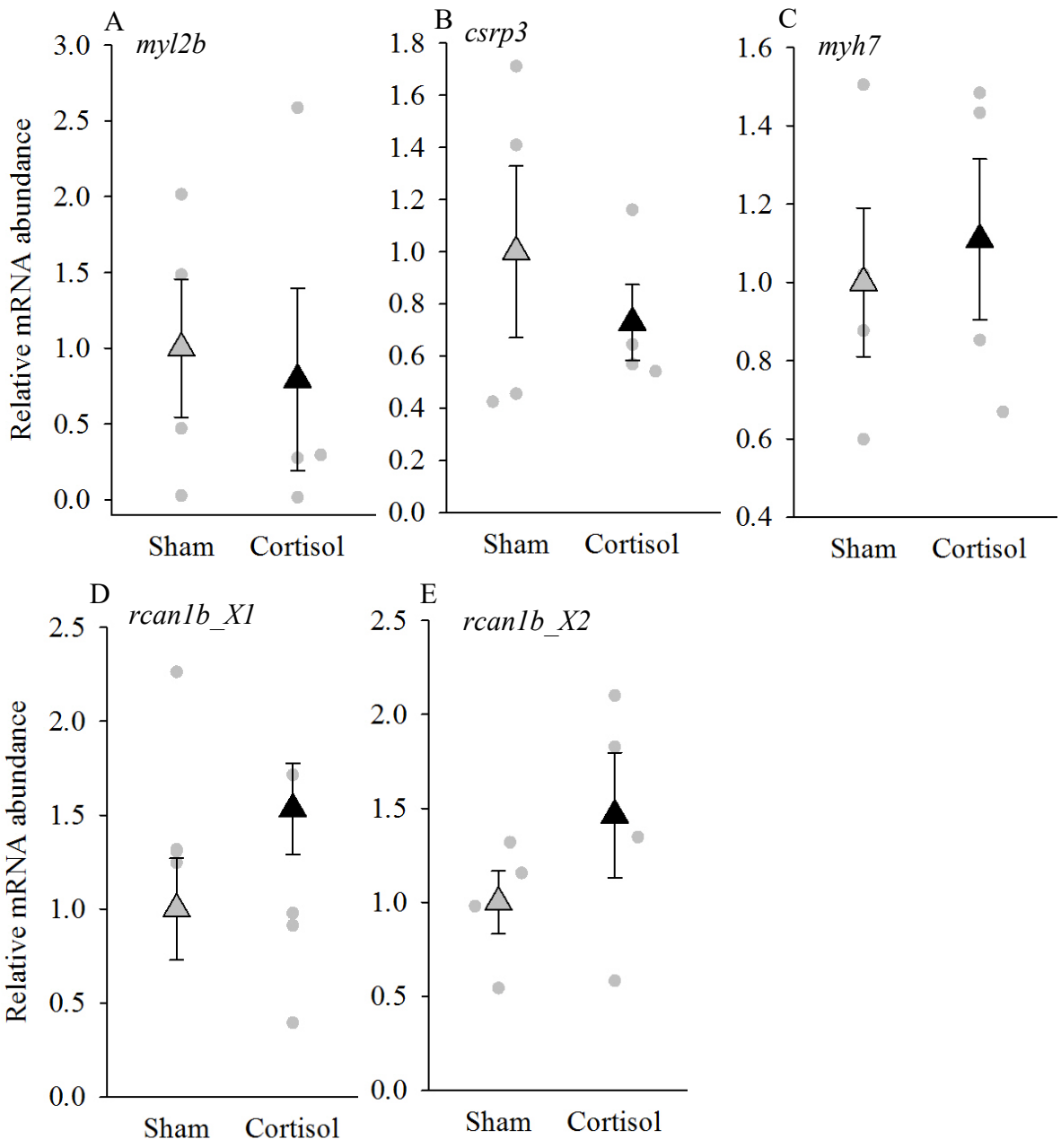

Figure 3.5. Transcript abundances for molecular markers of cardiac hypertrophy, *myl2b* (A), *csrp3* (B), *myh7* (C), *rcan1b_X1* (D) and *rcan1b_X2* (E) in the hearts of sham- vs cortisol-treated WT zebrafish (*Danio rerio*). The triangles present mean values ± SEM (n = 4 fish per group) with values for individual fish indicated by the circles. No differences were detected between

treatment groups (Student's t-tests, $p = 0.794$ for A, $p = 0.479$ for B, $p = 0.709$ for C, $p = 0.194$ for D, and $p = 0.259$ for E).

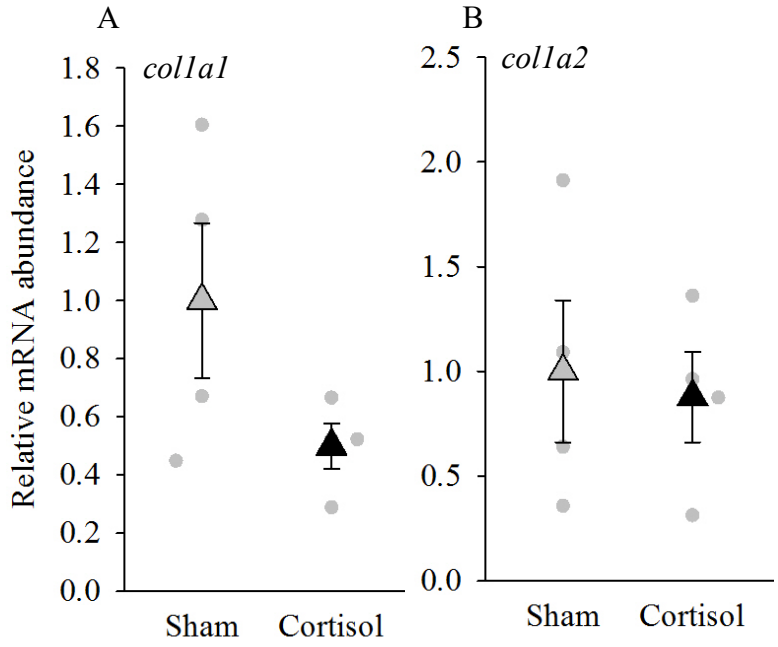

Figure 3.6. Transcript abundance for molecular markers of fibrosis, *col1a1* (A) and *col1a2* (B) in the hearts of sham- vs cortisol-treated WT zebrafish, *Danio rerio*. The triangles present mean values ± SEM (n = 4 fish per group) with values for individual fish indicated by the circles. No differences were detected between treatment groups (Student's t-tests, $p = 0.259$ for A, $p = 0.772$ for B).

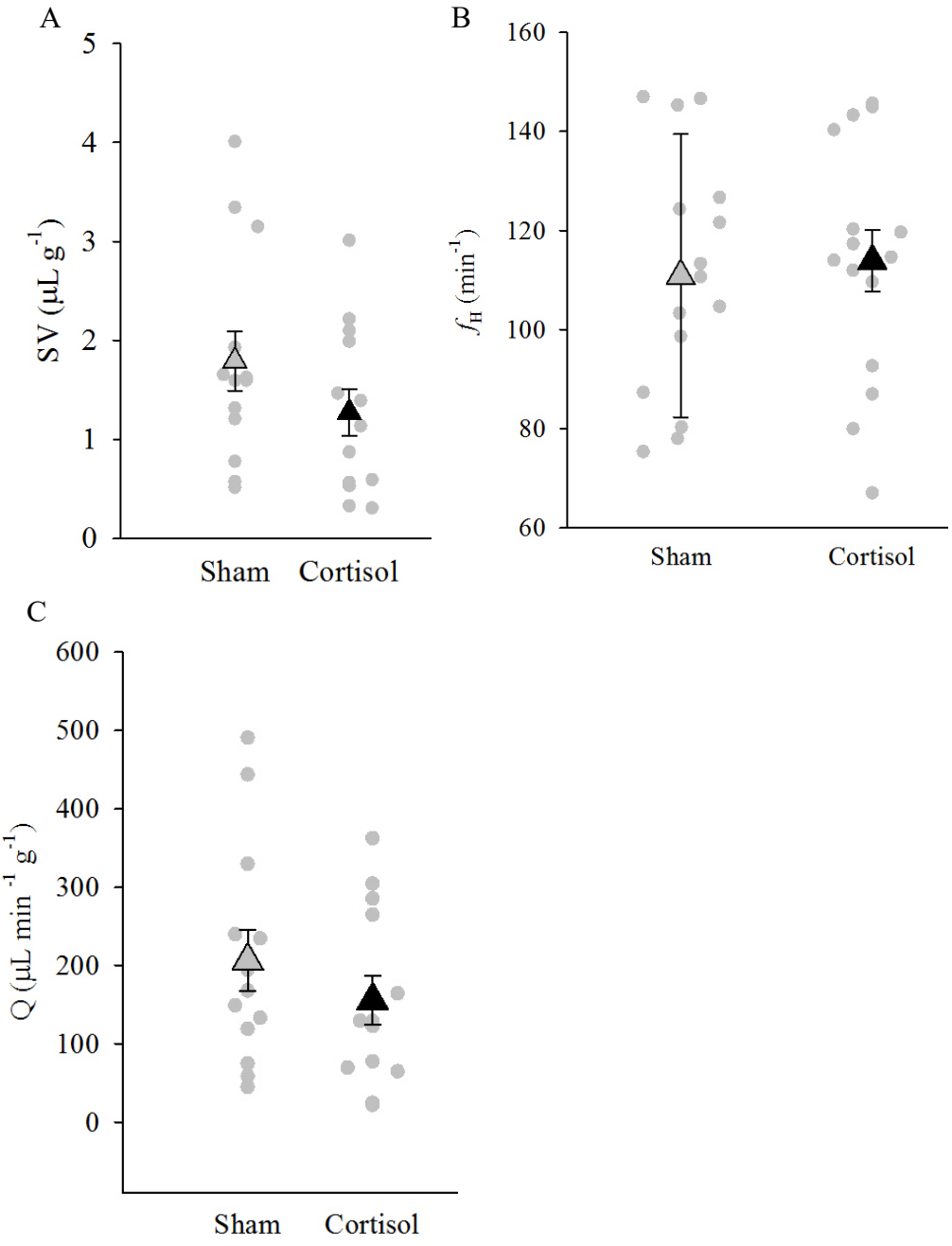

Figure 3.7. Cortisol treatment did not significantly alter heart function in WT zebrafish (*Danio rerio*). High frequency ultrasound was used to measure stroke volume (SV; A) and heart rate (f_H; B), with cardiac output (Q; C) being calculated from these values. Triangles present mean values ± SEM (n = 13, 15 and 13 fish per group for panels A to C, respectively) with values for

individual fish indicated by the circles. No significant differences were detected (Student's *t*-tests, $P = 0.184$, 0.713 and 0.356 for panels A to C, respectively).

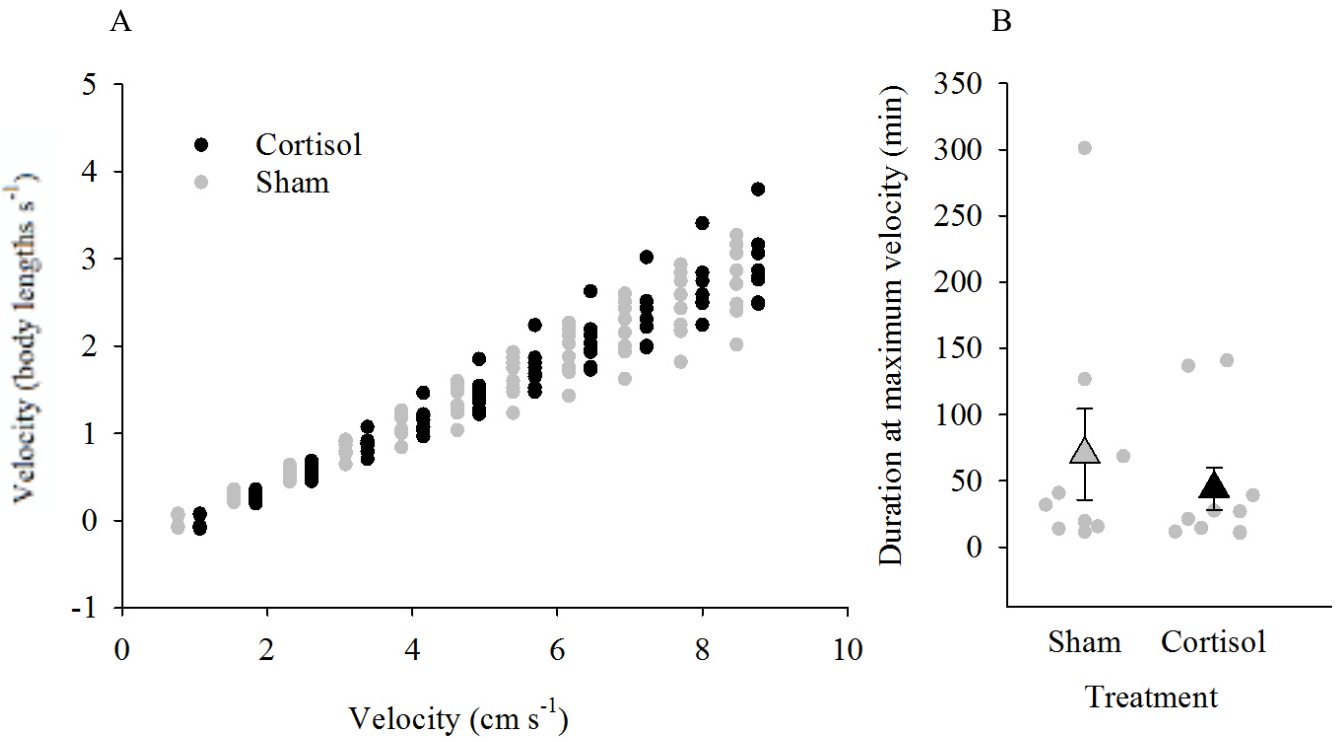

Figure 3. 8. Exercise performance was not altered by cortisol treatment in WT zebrafish, *Danio rerio*. Panel A presents velocity in body lengths · s^{-1} as a function of absolute water velocity in cm s^{-1} to allow relative swimming effort of the sham- and cortisol-treated fish to be compared. Comparison of relative velocity at an absolute velocity of 8.47 cm s^{-1} did not reveal a significant difference between treatment groups (Student's *t*-test, $p = 0.374$). Panel B presents the duration of swimming at the maximum velocity. Triangles present mean values ± SEM ($n = 9$ and 10 in sham- and cortisol-treated groups, respectively) with values for individual fish indicated by the circles. No significant differences in exercise duration were detected (Student's *t*-test, $p = 0.488$).

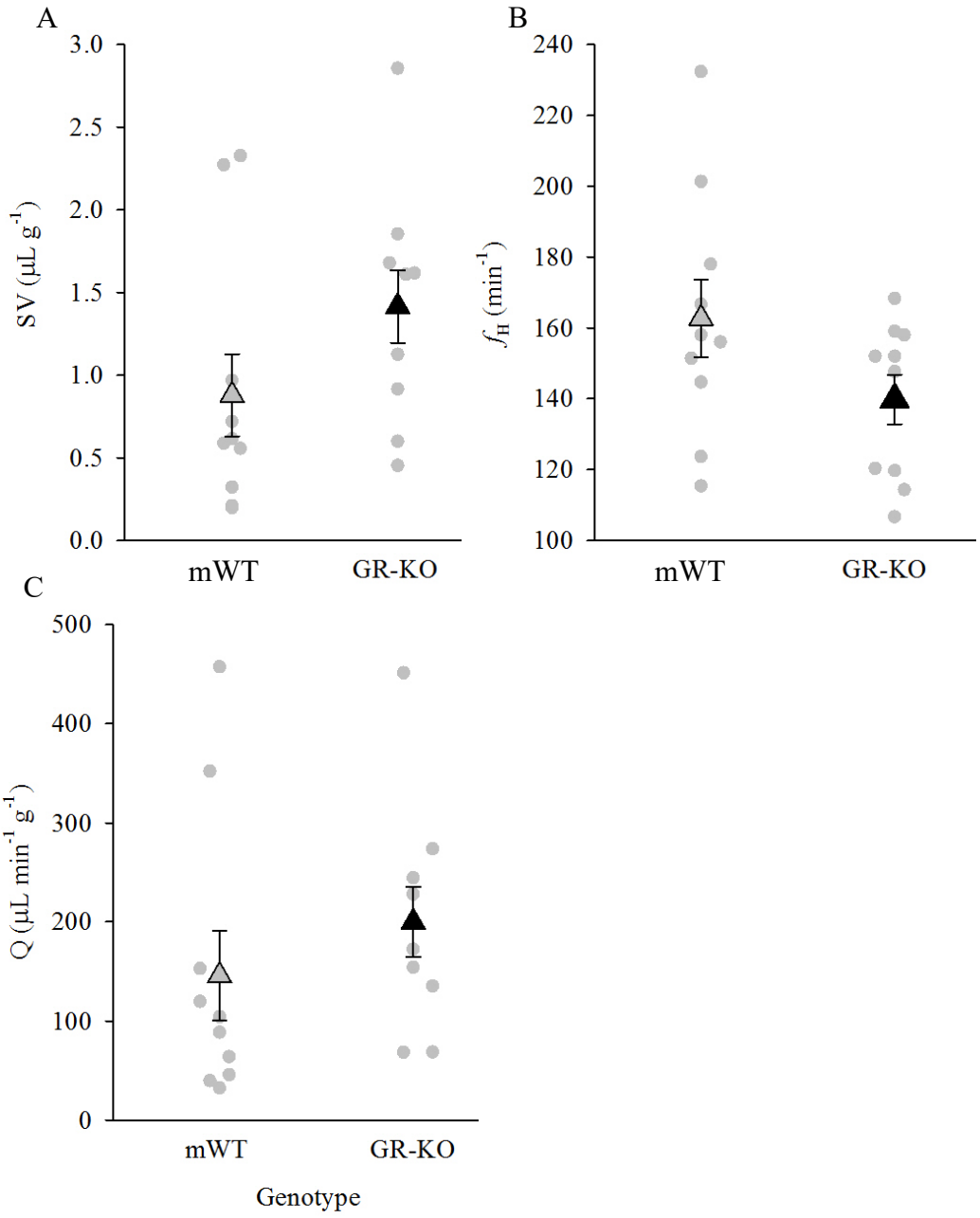

Figure 3. 9. Cardiac function was not significantly altered in zebrafish (*Danio rerio*) lacking the GR (GR-KO) relative to matched WT (mWT) fish. High frequency ultrasound was used to measure stroke volume (SV; A) and heart rate (f_H; B), with cardiac output (Q; C) being calculated from these values. Triangles present mean values ± SEM (n = 10 fish per group) with values for individual fish indicated by the circles. No significant differences between mWT and

GR-KO fish were detected (Student's *t*-tests, $p = 0.089$, 0.0954, and 0.394 for panels A to C, respectively).

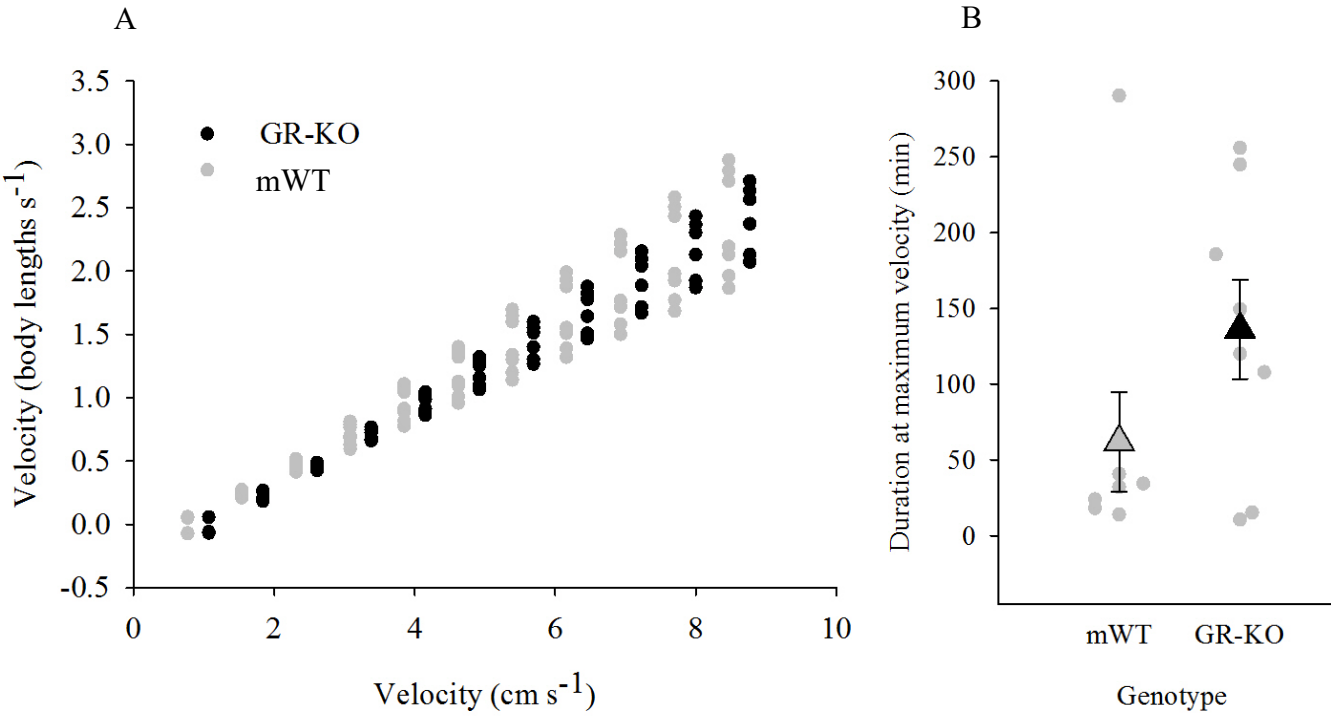

Figure 3.10. Swim performance did not differ between zebrafish (*Danio rerio*) lacking the GR (GR-KO) and their matched WT (mWT) fish. Data for water velocity (A) and swimming duration at the top water velocity (B) are presented. Panel A presents velocity in body lengths · s^{-1} as a function of absolute water velocity in cm s^{-1} to allow relative swimming effort between genotypes to be compared. Comparison of relative velocity at an absolute velocity of 8.47 cm s^{-1} did not reveal a significant difference between genotypes (Student's *t*-test, $p = 0.574$). Triangles are mean values ± SEM ($n = 8$ fish per group) with values for individual fish indicated by the circles. No significant difference in duration of swimming was detected (Panel B, Student's *t*-tests, $p = 0.072$).

Chapter 4: Discussion

4.1 Effects of cortisol treatment on cardiac structure and function

Cortisol treatment increased compact myocardium thickness and ventricle length in WT zebrafish, providing evidence of hypertrophic remodelling. These responses are similar to those observed in salmonid fishes, where exposure to stress or cortisol treatment resulted in larger (heavier) ventricles with a higher percentage of compact myocardium (Johansen et al., 2011; Johansen et al., 2017; Nørstrud et al., 2018). In salmonid fishes, ventricular hypertrophy was accompanied by increased deposition of collagen within the ventricle walls, detected using histological techniques and measurement of *col1a* transcript abundances (Johansen et al., 2011). Elevated collagen content is indicative of a more fibrotic heart, which in turn increases the risk of dysfunction. By contrast, transcript abundances of *col1a1* and *col1a2* were not elevated in cortisol-treated WT zebrafish relative to sham-treated fish, although future research should use histological approaches to visualise collagen deposition in zebrafish heart sections because morphological changes may be more persistent than changes in gene expression.

Ventricular hypertrophy in salmonid fishes was also accompanied by elevated expression of molecular markers for cardiac growth, including myosin light chain 2a (*myl2b*), ventricular myosin heavy chain 7 (*myh7*) and cysteine and glycine-rich protein 3 (*csrp3*), as well as those indicative of pathological hypertrophy, including regulator of calcineurin (*rcan1*), which is a downstream target of nuclear factor of activated T-cells (NFAT) signalling (Johansen et al., 2011; Johansen et al., 2017; Nørstrud et al., 2018). Cardiac growth and pathological hypertrophy can be distinguished by the activation of different signalling pathways. In the case of cardiac growth, pro-hypertrophic markers such as *csrp3* and *myl2b* are activated to promote generalized muscle growth, maintain cardiomyocyte structure of the heart wall, and play a role in the contractile function (Keen et al., 2016; Keen et al., 2018). In contrast, genes such as *rcan1* are

typically upregulated in individuals exhibiting pathological growth (Keen et al., 2016). Unlike the effects of stress or cortisol in salmonid fishes, cortisol treatment had no significant impact on transcript abundances of markers for cardiac growth or pathological hypertrophy in the zebrafish of the present study. However, low sample size likely limited the capacity to detect subtle differences. The apparent absence of pathological hypertrophy in zebrafish despite cortisol-induced cardiac growth may reflect the capacity of the zebrafish heart to regenerate (Poss et al., 2002). For example, full regeneration of cardiomyocyte number was attained within 30 days of a partial cryoinjury (Bertozzi et al., 2020). Also using a cryoinjury approach, Rodius et al. (2016) observed that fibrotic tissue began to be deposited 1-3 days post-injury and nearly full regeneration was achieved by 7 days post-injury. The regeneration process includes the repair of fibrotic tissue through oxidation of collagen deposition at the site of injury (Akam-Baxter et al., 2024). This capacity for regeneration may counter the negative impacts of cortisol treatment on cardiac structure in the zebrafish.

It is also possible that the heart was still at an early stage of collagen deposition after 10 d of cortisol exposure in zebrafish. In salmonids, pathological cardiac hypertrophy was observed in fish exposed to 45-90 d of oral cortisol treatment (Johansen et al., 2017), although molecular markers of cardiac growth and pathological hypertrophy were elevated by 7-21 d (Nørstrud et al., 2018). The 10 d treatment period for zebrafish was chosen based on the higher water temperature at which they were held relative to salmonid fishes, but it is possible that a longer exposure period is necessary to observe the full effects of cortisol treatment. Future work would benefit from using a time-course approach.

To identify the receptor that mediates the effects of cortisol on cardiac morphology, the effects of cortisol treatment were examined in zebrafish lacking functional expression of GR or

MR. Unexpectedly, however, cortisol treatment did not affect compact myocardium thickness or ventricle length in mWT zebrafish, making it difficult to interpret the results of this experiment. The lack of impact of cortisol treatment in mWT fish remains unexplained. However, this experiment yielded an interesting observation in GR-KO fish, which have elevated baseline cortisol levels (in the absence of cortisol treatment) owing to the loss of negative feedback regulation of the HPI axis that controls cortisol biosynthesis (Faught and Vijayan, 2018). Expressed relative to body mass, compact myocardium thickness and ventricle length in GR-KO fish were significantly lower than in MR-KO and mWT fish. This observation is discussed below.

Heart function, assessed by high-frequency ultrasound in anaesthetized fish, was not altered by cortisol treatment in WT fish. That is, no significant differences were detected between cortisol- and sham-treated WT fish in SV, f_H or Q. Despite the thickening of the compact myocardium, which could lower ventricular volume, there was no significant reduction in stroke volume. Ventricle length and compact myocardium thickness increased by 89% and 75%, respectively, between treatment groups. It is possible that the increase in ventricle length was sufficient to compensate for any reduction in ventricular volume caused by thickening of the compact myocardium. Alternatively, the increase in compact myocardium may have allowed for a more forceful contraction to maintain stroke volume. A reduction in stroke volume was detected in cortisol-treated rainbow trout, but only following exhaustive exercise, and was attributed to the increased fibrosis of the ventricle, which would be expected to increase stiffness (Johansen et al. 2017). Measurements of cardiac function following exhaustive exercise were not possible in zebrafish because of the need to anaesthetize the fish to carry out ultrasound; in trout,

the fish were instrumented with a flow probe to measure cardiac function in real time in active animals (Johansen et al. 2017).

The anaesthetic used for ultrasound was MS-222, which has been reported to have cardio-depressive effects (Denvir et al., 2008; Huang et al., 2010; Topic Popovic et al., 2012). Previous ultrasound measurements in zebrafish have used a combination of MS-222 and isoflurane, which alleviates the cardio-depressive effects of MS-222 (Huang et al., 2010; Lee et al., 2016). Alternatively, metomidate hydrochloride (Aquacalm) has been used as an anaesthetic (Shaftoe et al., 2023). However, Aquacalm is not currently available in Canada and isoflurane could not be used in the current study owing to safety considerations. Therefore, to minimize the cardio-depressive effects of MS-222, measurements were made within 5 min of initiating anaesthesia. Heart rate in adult zebrafish is reported to be 140–180 min^{-1} (Huang et al., 2010), although values of 100-120 min^{-1} have also been reported (Shaftoe et al., 2023); heart rates measured in the current study averaged 112 ± 4 min^{-1}. Future studies would benefit from the use of a different anaesthetic to be able to observe subtle differences in heart function which may have been difficult to detect in the current study.

Given that cardiac function was unaffected by cortisol treatment, it was not surprising that swimming performance also did not differ between treatment groups. Exercise capacity is directly influenced by cardiac function because the higher tissue O_2 demand during exercise is met by increasing cardiac output, largely through increases in heart rate (Claireaux et al., 2005). Thus, impairment of cardiac function would be expected to reduce exercise performance. For example, cortisol-treated rainbow trout exhibited reduced cardiac output following exhaustive exercise and had a lower critical swimming speed (U_{crit}; Johansen et al. 2017). Measurement of U_{crit}, which is the maximal speed at which a fish can sustain swimming, is a standard approach

for evaluating exercise performance (Wakamatsu et al., 2020), although variations on this approach have been reported in the literature (e.g. Bamford and Seebacher, 2024; Perry et al., 2012). By contrast, the current study measured the duration over which swimming could be sustained at a set water velocity. This method tests endurance at a single speed rather than determining the maximal speed at which a fish can sustain swimming. However, more validation of the approach is needed to determine whether it is as effective as a standard U_{crit} trial in assessing swimming performance.

4.2 Effects of GR knockout on cardiac morphology and function

Relative to their body mass, the hearts of GR-KO fish had thinner compact myocardium and shorter ventricle length than the hearts of MR-KO or mWT fish. Heart morphology is often expressed relative to body mass in fishes owing to the isometric relationship between body mass and heart size (Johansen et al., 2017). Although compact myocardium thickness in absolute units did not differ across genotypes, ventricle length was significantly greater in GR-KO fish (Appendix 3). Despite having longer ventricles in absolute terms, ventricle length expressed relative to body size was significantly lower in GR-KO fish than in the other genotypes, indicating that the significantly larger body mass of GR-KO fish was the main factor driving the apparent differences in relative size across genotypes. For a given age, the GR-KO fish used in the present study were larger than the corresponding MR-KO or mWT fish (Hong, 2024). Similarly large body size was reported for the GR-KO mutant line developed by Faught and Vijayan (2019) and was linked to differences in metabolism. Larger hearts in the larger GR-KO individuals were anticipated due to the predicted isometric relationship, however hearts are unexpectedly small for the observed body masses. To my knowledge, however, the current study

is the first to report disproportionately small heart size in GR-KO fish. Although exposure of zebrafish embryos to elevated cortisol was found to increase heart deformities and lower resting heart rate post-hatch (Nesan and Vijayan, 2012), data on heart development in the absence of GR signalling are sparse (Wilson et al., 2015). Evaluation of heart size and morphology across the development of GR-KO fish would be helpful in determining at what point heart size fails to remain in proportion to body mass. Similarly, measurements of the expression of key cardiac genes might provide insight into the cardiac structure of GR-KO fish (e.g. Nesan and Vijayan 2012).

Heart function did not differ significantly between mWT and GR-KO zebrafish. Despite having relatively smaller hearts, the stroke volume of GR-KO fish tended to be larger than that of mWT fish, whereas cardiac output and heart rate of the GR-KO fish did not significantly differ from those of mWT fish. How GR-KO individuals achieve higher stroke volume despite relatively smaller heart size remains unclear. More detailed visualization of ventricle contractions and/or measurement of force production by ventricular myocytes would be useful in identifying the underlying mechanisms. No difference in swimming performance was detected between genotypes. However, it is possible that the GR-KO fish were unable to elevate cardiac output during exercise to the same extent as mWT fish, i.e. that the scope for increasing cardiac output was lower in GR-KO fish. This possibility requires further study.

4.3 Experimental limitations and conclusions

The current study used waterborne delivery of cortisol dissolved in ethanol as described in previous work on zebrafish (Faught and Vijayan, 2022) to elevate circulating cortisol concentrations. Initial efforts focused on the use of chronic stressors to increase endogenous

cortisol as has been successfully reported for zebrafish in the literature (Chakravarty et al., 2013; Faught and Vijayan, 2018; Pavlidis et al., 2013). However, in preliminary trials, these methods were not effective in achieving prolonged elevation of cortisol. Research on salmonids used cortisol delivery via food (Johansen et al., 2017), but previous work in our lab with this approach revealed a rapid return of cortisol to baseline levels within a few hours of feeding (KM Gilmour, unpublished data). Although waterborne delivery of cortisol was effective in elevating cortisol levels, it required daily water renewal and the inclusion of ethanol in the water of sham-treated animals tended to leave the water cloudy. Although it was not apparent in plasma cortisol levels measured at the end of 10 d, these factors may have induced stress in sham-treated fish during the exposure period.

Overall, the present study provided evidence of cardiac remodelling in cortisol-treated WT zebrafish, consisting of increased compact myocardium thickness as well as increased ventricle length. Whether these changes in morphology were accompanied by increases in fibrosis remains to be confirmed, although no evidence of increased transcription of collagen was detected. Despite this morphological remodelling, there did not seem to be any functional impacts on cardiac or swimming performance, an outcome that differed from the consequences of cortisol treatment on cardiac function in salmonid fishes. This apparent difference between species may reflect the high capacity for regeneration and repair of heart tissue in zebrafish (Bertozzi et al., 2020; Poss et al., 2002). Although trout have also shown a capacity for heart repair, with remodelling and repair occurring after coronary artery ligation (Zena et al., 2021), regeneration in zebrafish includes loss of the fibrotic scar (Long et al., 2025), which may provide protection against fibrosis.

An unexpected finding of the current study was the observation in GR-KO fish, which are of larger body mass than WT zebrafish, of heart length and compact myocardium thickness that were disproportionately lower than in WT fish. Despite their relatively smaller heart, GR-KO had a trend for larger stroke volume. Consequently, the relatively smaller heart did not have functional consequences for swimming performance in GR-KO fish. This interesting observation warrants further study.

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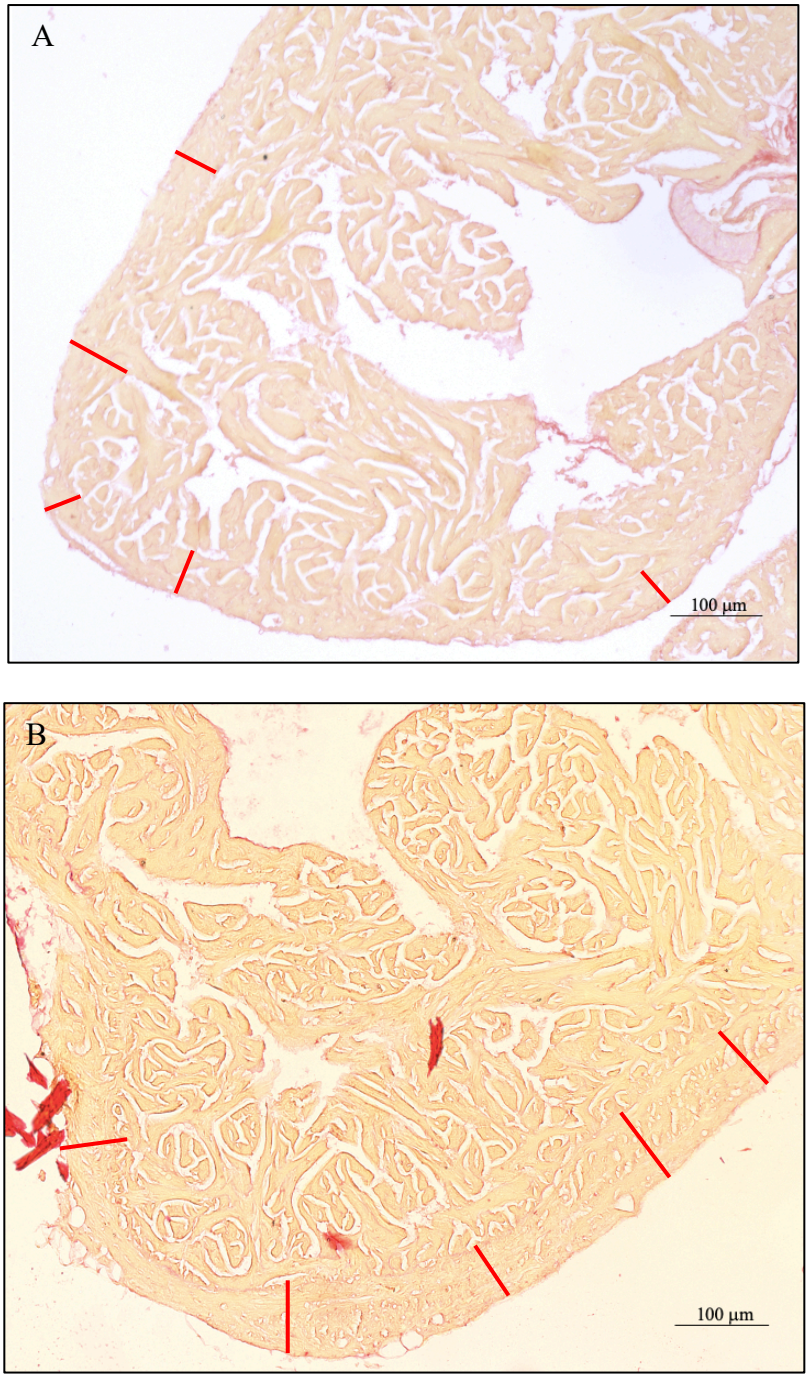
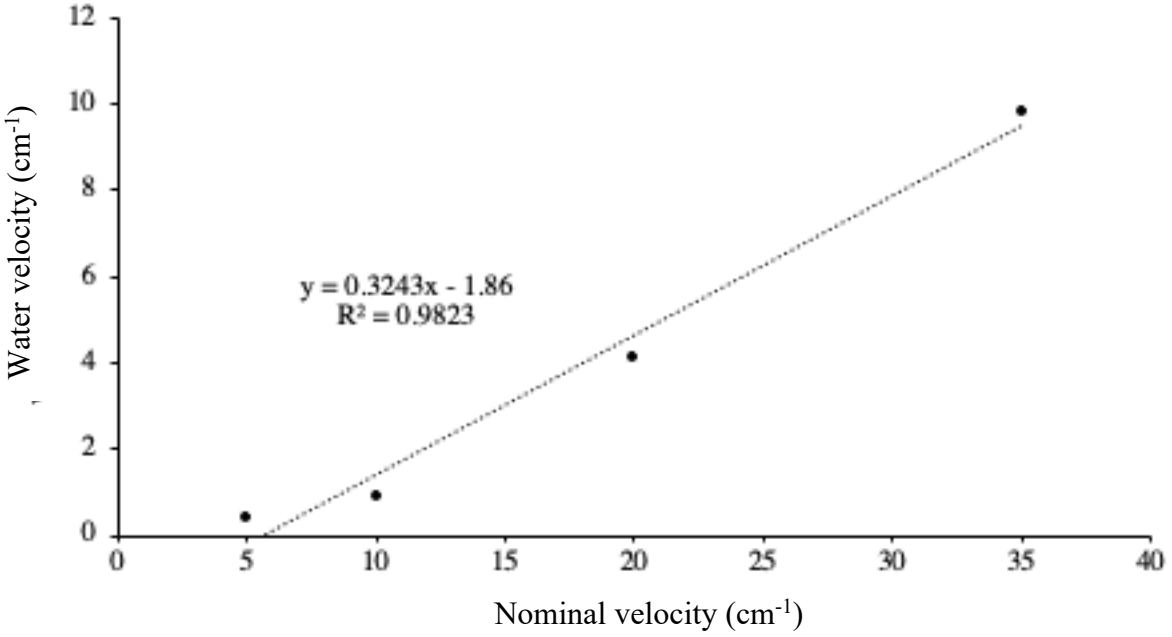
Appendices

Appendix 1. Sample images of Picrosirius red stained ventricle tissues used to measure compact myocardium thickness of sham- (A) and cortisol-treated (B) zebrafish. Images were taken using bright field microscopy at a 10X magnification. Red lines indicate measurements of compact myocardium thickness.

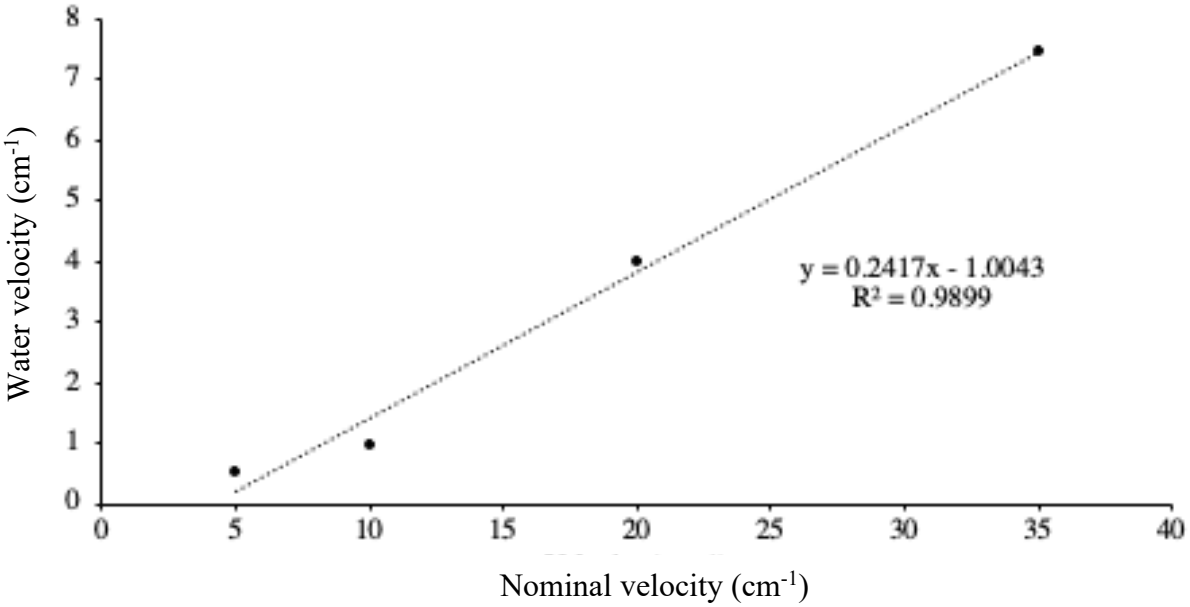


Appendix 2: Standard curves for calibration of water velocities of swim tunnels 1 (A) & 2 (B).

A



B



Appendix 3: Summary of compact myocardium thickness in absolute values across genotypes.

Genotype	Treatment	*n*	Compact myocardium thickness (μm)	*P* values	*n*	Ventricle length (mm)	*P* values
mWT	Sham	9	368.4 ± 23.4	$P_{treatment}$ = 0.951	16	1.33 ± 0.09	$P_{treatment}$ = 0.055
	Cortisol	9	376.3 ± 26.1		16	1.39 ± 0.08	
GR-KO	Sham	6	299.7 ± 49.1	$P_{genotype}$ = 0.951	4	2.65 ± 0.19	$P_{genotype}$ = <0.001
	Cortisol	6	436.4 ± 70.7	$P_{genotype*treatment}$ = 0.204	4	2.07 ± 0.33	$P_{genotype*treatment}$ = 0.105
MR-KO	Sham	6	375.4 ± 36.0		5	1.19 ± 0.08	
	Cortisol	6	386.6 ± 30.2		5	1.30 ± 0.10	

Values are means ± SEM. Data were analyzed by two-way ANOVA.