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## **Bigger is not better: Cortisol-induced cardiac growth and dysfunction in salmonids**

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**Key words: myocardial hypertrophy, chronic stress, heart failure, rainbow trout, cardiac performance**

## Summary statement

Here we disclose the first demonstration of corticosteroid-induced heart disease in fish and show that the molecular basis of heart disease is conserved from fish to mammals.

## Abstract:

Stress and elevated cortisol levels are associated with pathological heart growth and cardiovascular disease in humans and other mammals. We recently established a link between heritable variation in post-stress cortisol production and cardiac growth also in salmonid fish. A conserved stimulatory effect of the otherwise catabolic steroid hormone cortisol is likely implied, but has to date not been established experimentally. Furthermore, whereas cardiac growth is associated with failure of the mammalian heart, pathological cardiac hypertrophy has not previously been described in fish. Here we show that rainbow trout (*Oncorhynchus mykiss*) treated with cortisol in the food for 45 days have enlarged hearts with lower maximum stroke volume and cardiac output. In accordance with impaired cardiac performance, overall circulatory oxygen transporting capacity was diminished as indicated by reduced aerobic swimming performance. In contrast to the well-known adaptive/physiological heart growth observed in fish, cortisol-induced growth is maladaptive. Furthermore, the observed heart growth was associated with up-regulated signature genes of mammalian cardiac pathology, suggesting that signaling pathways mediating cortisol-induced cardiac remodeling in fish are conserved from fish to mammals. Altogether, we show that excessive cortisol can induce pathological cardiac remodeling. This is the first study to report and integrate the etiology, physiology and molecular biology of cortisol-induced pathological remodeling in fish.

## Introduction

Cardiovascular disease is the leading cause of death in the western world. In addition to classical risk factors (e.g. hypertension and elevated levels of blood cholesterol) epidemiological evidence and common folklore wisdom suggest an association between stress and coronary heart disease (CHD) risk in humans (Rosengren et al., 1991; Iso et al., 2002; Rosengren et al., 2004). It is also well known that individual variation in physiology and behavior (coping styles, or “personalities”) predicts disease outcome in both animal models and humans (Denollet et al., 1996). Mechanisms underlying stress-induced cardiac pathology risk remain largely unknown; however, increased levels of the steroid hormone cortisol are believed to be important. For example, high cortisol responsiveness to stress is associated with a four-fold increased risk of cardiovascular morbidity and mortality in humans (Denollet, 2000) and clinical use of synthetic cortisol (i.e. cortisone, hydrocortisone, dexamethasone etc.) is associated with abnormal heart growth and other cardiovascular diseases (Souverein et al., 2004). In mammalian experimental models, glucocorticoids like cortisol directly induce cardiomyocyte hypertrophy *in vitro* (Whitehurst et al., 1999; Ren et al., 2012) and *in vivo* (Clark et al., 1982; de Vries et al., 2002; Jensen et al., 2002; Lumbers et al., 2005), indicating a direct role of cortisol in heart remodeling, growth and disease.

Stress - and cortisol-induced cardiac hypertrophy may not be limited to mammals. Cardiac remodeling and deformities are commonly reported in farmed fish (Pope et al., 2007), but a link between stress and development of such diseases has never been established. As in humans, the main glucocorticoid and stress hormone in salmonid fish is cortisol. We recently established a link between cortisol responsiveness to stress and cardiac remodeling in rainbow trout (*Oncorhynchus mykiss*) high (HR) and low (LR) responsive strains and wild-type brown trout (*Salmo trutta*) (Johansen et al., 2011). High post-stress cortisol production is also associated with a range of behavioral and cognitive traits indicating a reactive coping style (Øverli et al., 2005; Øverli et al., 2007; Sørensen et al., 2013), rendering the salmonids a productive model to study proximate physiological mechanisms underlying consistent trait associations (Khan et al., 2016). Regarding cardiac function, individuals responding to stress with high cortisol levels (HR fish) have notably larger ventricles compared to individuals with a low cortisol response (LR fish).

Combined, previous findings indicate a common mechanism behind cortisol associated heart growth in fish and mammals, suggesting either parallel evolution or conservation of important regulatory mechanisms. Of note, although cardiac hypertrophy is associated with failure of the mammalian heart (Lloyd-Jones et al., 2002), the concept of pathological cardiac hypertrophy has not been established in fish. In fact, cardiac growth is a routinely occurring phenomenon in many fishes including salmonids; and is generally considered an adaptive response that enhances myocardial mechanical performance and cardiac pumping capacity (Graham and Farrell, 1989) during e.g. seasonal cold acclimatization (Klaiman et al., 2011) and sexual maturation (Franklin and Davie, 1992). Further, although an association between cortisol exposure and cardiac remodeling has been

observed in fish and mammals, the direct effect of exogenous cortisol administration on cardiac morphology and function has, to our knowledge, not been investigated in fish.

Here, we hypothesized that chronic exposure to high cortisol directly induces cardiac hypertrophy and impairs cardiac performance in rainbow trout. Further, we hypothesized that intracellular signaling pathways mediating cortisol-induced cardiac remodeling in fish are similar to those involved in pathological cardiac hypertrophy in mammals.

## **Materials and methods**

### **Research animals and animal housing**

For 45 and 90 days of cortisol treatment juvenile rainbow trout ( $150 \pm 29$  g, mean  $\pm$  s.d.), obtained from Store Restrup Fiskeri, Nibe, Denmark were used. Experiments were conducted at the Danish Technical University, Institute of Aquatic Resources, Hirtshals, Denmark. Prior to cortisol treatment, fish were kept in rearing tanks (1000 L) supplied with aerated tap water at 17 °C. Lighting was kept at a 14.5/9.5 h light/dark cycle. The fish were subsequently moved and randomly distributed to six 700 L cylinder tanks (45 fish per tank) and allowed to acclimatize for 5 days before the onset of cortisol treatment. During this period and throughout the experiment, the tanks were supplied with aerated recirculating water at 19 °C. Lighting was kept at a 14.5/9.5 h light/dark cycle. Fish were divided into four treatment groups: One tank received control feed for 45 days (n=45), one tank received control feed for 90 days (n=45), two duplicate tanks received cortisol-treated feed for 45 days (n=90) and two duplicate tanks received cortisol-treated feed for 90 days (n=90). Ethical approvals for the experiments were given by the Norwegian and Danish Animal Research Authorities with license numbers 2012/33240 and 2014-15-2934-01041, respectively.

For *in vivo* assessment of effects of cortisol-induced remodeling on cardiovascular performance, juvenile rainbow trout ( $420 \pm 23$  g, mean  $\pm$  s.d. g), obtained from Antens laxodling AB, Alingsås, Sweden, were used. Experiments were conducted at the fish holding facilities at the Department of Biological and Environmental Sciences, University of Gothenburg, Sweden. Fish were kept in tanks supplied with partly recirculating UV-treated and bio-filtered fresh water at 8 °C prior to experimentation. Lighting was kept at a 12/12 h light/dark cycle. The experiments were covered by ethical permit 65-2012 with amendment 169-2013 from the local ethical committee in Gothenburg. For all experiments animals were randomly assigned to either a control or treatment group.

### **Preparation of cortisol feed**

Cortisol treatment refers to oral administration of prefabricated food pellets coated with hydrocortisone. More precisely, this diet was prepared by dissolving 500 mg (dose modified from Sørensen et al. (2011) cortisol (hydrocortisone powder, Sigma-Aldrich, St. Louis, MO, USA) in 15 g rapeseed oil (First Price, SuperGros A/S, Denmark) per kg pellets (EFICO Enviro 920, 3 mm or 6 mm (depending on fish size) Biomar, Brande, Denmark). This was mixed in a container rebuilt from an 850 W electric cement mixer (174 L) and thorough mixing of the content was ensured by rotation of

this mixer. The container was connected to a vacuum pump and in order for the cortisol to be absorbed into the pellets, a negative pressure of 0.9 Bar was applied before air was slowly let back into the container. Control feed was prepared in the same way but with pure rapeseed oil.

### ***In vivo* cortisol treatment**

During acclimation, all fish were fed 0.8 % of their total body mass each day with commercial pellets (EFICO Enviro 920, 3.0 mm, Biomar, Brande, Denmark). At the onset of the experiment the feed was exchanged with pellets of the same brand and size but that had been coated with either rapeseed oil alone (control feed) or rapeseed oil and hydrocortisone (Sigma-Aldrich, St. Louis, MO, USA) and the fish were fed 0.8 % of their body mass daily, corresponding to a dose of 4 µg cortisol/g BW. Following 45 and 90 days of cortisol treatment cortisol treated fish appeared as healthy as control fish with no internal or external symptoms of pathogen infection.

### **Physiological measurements and sampling**

For blood sampling, fish were lightly anaesthetized with 1 g l<sup>-1</sup> MS-222 before a blood sample was collected from the caudal vein. The blood samples were centrifuged for 5 min at 4 °C, 8000 g and plasma was frozen and stored at -20 °C for later analysis of cortisol levels. Hearts from all experimental fish were surgically excised and the bulbus and atrium removed. The ventricles were blotted dry of blood and weighed on a precision weight scale and cardiosomatic index (CSI=ventricle wet weight/body weight), was calculated. Images of the ventricles were taken using a Canon EOS350 digital camera (Canon, Tokyo, Japan) and processed in Adobe Photoshop CS3 (Adobe Systems Inc., San Jose, CA, USA). Ventricles were either stored on RNAlater® solution (Ambion, Austin, TX, USA) for 24 h at room temperature before they were stored at -20 °C for later qPCR mRNA analysis, put on freshly prepared 4 % Paraformaldehyde for MRI analysis or dried at 70 °C for 24 h for analysis of % water content.

### **Plasma cortisol analysis**

Plasma cortisol was measured in a random selection of individuals treated with cortisol for 15 and 45 days. Plasma cortisol was analyzed using a radioimmunoassay (RIA) based on the assay by Pottinger and Carrick (2001). In short, steroids were extracted with ethyl acetate (Merck, 1:5 plasma, ethyl acetate) prior to the RIA. 5-150 µl of extract was transferred to 1.5 ml eppendorf tubes. 50 µl aliquots containing approximately 16 000 cpm of [1,2,6,7-3H] cortisol (Amersham Pharmacia Biotech, Little Chalfont, UK, 60 Ci mmol<sup>-1</sup>) was added to all samples. The ethyl acetate was evaporated in an exsiccator coupled to a water-jet pump and donkey anti-cortisol antibody (AbD Serotec, Dusseldorf, Germany) was added. After 18 h incubation at 4 °C, dextran-coated charcoal in PBS (1.0 % activated charcoal, Sigma; 0.2 % dextran, Sigma) was added. Supernatant from each tube was transferred to scintillation vials containing 4 ml scintillation fluid (Ultima Gold, Perkin Elmer, Waltham, MA, USA), and counted on a Packard Tri-Carb A1900 TR liquid scintillation analyzer (Packard Instrument, Meriden, CT, USA). A 3-parameter hyperbolic function was fitted to the plot of the percentage of 3H-cortisol bound against a standard curve using SigmaPlot 11 (SPSS Science, Systat Software Inc.,

San Jose, CA, USA). The equation from this function was used to estimate the cortisol concentration in the unknown samples. The lower and upper detection limits of the assay were 0.19 and 655 ng/ml, respectively. For individuals where the plasma cortisol levels were below this limit, the level was set to 0.19 ng/ml.

### **Magnetic resonance imaging (MRI) of fixed ventricles**

Ventricles fixed on 4 % PFA were thoroughly rinsed with saline water (0.4 %) before mounted with cotton and soaked in MRI-compatible perfluoropolyether oil (Fomblin, Sigma-Aldrich, St. Louis, MO, USA) in a 15 mm glass tube. “MRI experiments were performed on a pre-clinical 9.4 T MRI system (Agilent Technologies, Inc., USA) equipped with a high-performance gradient coil (inner diameter 60 mm, max strength 1000 mT/m) and a quadrature RF volume coil (19 mm ID, Rapid Biomedical). High resolution images were acquired by 3D spoiled gradient echo. Field of view ranged from  $12.8 \times 12.8 \times 12.8$  mm to  $16 \times 16 \times 16$  mm depending on the size of specimen. Other typical parameters were matrix  $1024 \times 512 \times 512$ , repetition time TR 45 ms, echo time TE 10 ms, flip angle  $45^\circ$ , 5 averages, acquisition time 16 h 23 min. Fiji image processing software (released under the General Public License) was employed to view the images and measure area of compact and non-compact myocardium. Area of compact myocardium was calculated by subtracting non-compact area (area inside of compact layer in the ventricle) from total area and non-compact area was calculated by subtracting compact area from total area. Area of compact myocardium was then divided by the area of non-compact myocardium to get the ratio of compact to non-compact area.

### **RNA extraction and qPCR analysis**

The hearts, stored and frozen in RNAlater®, were thawed and refrozen in liquid nitrogen before they were freeze-fractured in a BioPulverizer (Biospec Products, Inc., Bartlesville, OK, USA). The pulverized hearts were put into 15 ml plastic tubes and RNA was extracted using TRIzol® reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s protocol. The quality and quantity of the RNA was assessed using a 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA) and a NanoDrop® ND-1000 UV-Vis Spectrophotometer (NanoDrop Technologies, Rockland, DE, USA), respectively. RNA quality was determined from RNA integrity numbers (RINs) calculated by the 2100 Bioanalyzer (range: 1–10). The RIN for the tissue samples ranged from 8.4 to 10.0 with an average of 9.5, confirming excellent RNA quality. First-strand cDNA was synthesized from total RNA treated with 2 ng DNase I (DNA-free™ Kit, Ambion Applied Biosystems) using Superscript III reverse transcriptase (Invitrogen) with oligo dT<sub>12–18</sub> primers synthesized by Invitrogen. Gene specific primer sequences for rainbow trout  $\beta$ -actin, proliferating cell nuclear antigen (*PCNA*), ventricular myosin heavy chain (*VMHC*), slow myosin light chain 2 (*SMLC2*), muscle LIM protein (*MLP*), regulator of calcineurin 1 (*RCAN1*), A-type natriuretic peptide (*ANP*) and B-type natriuretic peptide (*BNP*) were designed and published previously (Johansen et al., 2011). The housekeeping gene  $\beta$ -actin was used as reference gene. Real time PCRs were carried out using a Roche LC480 Light cycler (Roche Diagnostics, Penzberg, Germany). Reaction volumes were 10  $\mu$ l and included Light cycler® 480

SYBR Green I Master (Roche diagnostics GmbH, Mannheim, Germany), primers (5  $\mu$ M) and cDNA (3  $\mu$ M). Cycling conditions were as follows: 10 min at 95 °C, 42 cycles of 10 sec at 95 °C, 10 sec at 60 °C and 10 sec at 72 °C followed by melt curve analysis. All reactions were run in duplicates and controls without DNA template were included to verify the absence of cDNA contamination.

Relative gene expression data was calculated from real-time PCR raw data using the formula:

$${}_IC E^{Cp/GOI} E^{Cp} = \text{Expression of GOI in ratio to IC}$$

where IC is internal control ( $\beta$ -actin), GOI is gene of interest, E is priming efficiency, and Cp is crossing point. E values were calculated for each real-time RT PCR reaction using LinRegPCR software (version 11.30.0) (Ruijter et al., 2009).

### **Surgical instrumentation for *in vivo* cardiovascular measurements**

Individual fish were netted from the holding tanks and anaesthetized in aerated water containing 200 mg l<sup>-1</sup> of tricaine methane sulphonate (MS-222; Sigma-Aldrich, Sweden) buffered with NaHCO<sub>3</sub> (400 mg l<sup>-1</sup>). The fish were weighed and placed on a surgery table covered with wet rubber foam. The gills were continuously irrigated via the mouth with aerated water (~10 °C) containing a lower dose of NaHCO<sub>3</sub> buffered MS-222 (100 mg l<sup>-1</sup> and 200 mg l<sup>-1</sup>, respectively). The right operculum was carefully retracted and the ventral aorta was surgically exposed taking care not to damage the pericardium and adjacent small blood vessels and nerves. A 4-0 silk suture was placed under the vessel, which was carefully lifted and a 2.5PSL or 2.5PSB Transonic perivascular blood flow probe (Transonic Systems Inc, Ithaca, New York, USA), factory-calibrated to 10 °C, was placed around the aorta to measure cardiac output (CO). The lead from the flow probe was sutured to the skin with several 2-0 silk sutures close to the opercular cavity. The dorsal aorta was then cannulated via the roof of the buccal cavity with a PE-50 catheter using a sharpened steel wire as guide (Axelsson and Fritsche, 1994). The catheter was rinsed and filled with heparinized (100 IU ml<sup>-1</sup>) saline (0.9 %) and closed with a pin. The catheter and the flow probe lead were collectively sutured to the skin at the back of the fish with a single 2-0 silk suture. Following surgery, fish were revived in fresh water in their respective holding tank and placed individually in a plastic tube floating in the tank. They were left to recover from surgery for approximately 24 h before experimentation commenced.

### **Experimental protocol for *in vivo* cardiovascular measurements**

The fish was removed from the holding tube and transferred using a water-filled plastic bag to a round tank (diameter: 655 mm, volume: 75 l) supplied with through flowing water (8 °C). To trigger a maximal cardiovascular response, fish were subjected to a manual chase protocol where vigorous escape behaviours were encouraged by repeatedly touching the body of the fish for 10 min, making the fish completely unresponsive to physical stimuli at the end of the protocol. The fish was then rapidly transferred to an opaque holding chamber (length: 54, width: 13 and depth: 18 cm) with through flowing water from the same water system and covered to avoid external visual stimuli. A blood

sample (0.5 ml) was quickly withdrawn into a heparinized syringe from the dorsal aortic cannula to determine maximal haematological status after stress and cardiovascular variables (maximal) were then recorded for 2-3 h after the chase protocol. The maximum cardiovascular response was defined as the period when cardiac output peaked after the exhaustive chase protocol. Typically, the maximum response occurred within 15 min and reported data represent the means from a 2 min period taken during the maximum response.

The following day, i.e. when the fish had recovered from the preceding exercise protocol for ~24 h, basal cardiovascular variables were recorded for several hours. When low and stable cardiovascular variables had been confirmed another blood sample (0.5 ml) was withdrawn into a heparinized syringe from the catheter to determine basal haematological status. Cardiac autonomic tones were then determined using the pharmacological protocol of Altimiras et al. (1997). Briefly, atropine sulphate [ $1.2 \text{ mg kg body mass } (M_b)^{-1}$ ] was first injected via the catheter to block muscarinic receptors and cardiovascular variables were allowed to stabilize for 30-60 min before a recording was made. Finally, ( $\pm$ ) propranolol hydrochloride ( $3 \text{ mg kg } M_b^{-1}$ ) was injected to block  $\beta$ -adrenergic receptors, and cardiovascular variables were allowed to stabilize for at least 30 min before a final recording was made.

#### **Acquisition of cardiovascular variables and analytical procedures**

The dorsal aortic catheter was connected to a DPT-6100 blood pressure transducer (pvt Medizintechnik, Kirchseeon, Germany) that was calibrated against a static column of water and referenced to the fluid in the holding tubes. A 4ChAmp pre-amplifier (Somedic AB, Hörby, Sweden) was used to amplify the signal from the transducer. The blood flow probe was connected to a three channel 400 series Transonic blood flow meter (Transonic Systems Inc, Ithaca, New York, USA). Data was sampled at 100 Hz using a Power Lab unit (ADInstruments Pty Ltd, Castle Hill, Australia) connected to a laptop computer running LabChart Pro software (version 7.3; ADInstruments Pty Ltd, Castle Hill, Australia). From the pulsatile blood pressure signal mean dorsal aortic blood pressure ( $P_{DA}$ ), diastolic pressure ( $P_{DA \text{ DIA}}$ ), systolic pressure ( $P_{DA \text{ SYS}}$ ), pulse pressure ( $P_{DA \text{ PULSE}}$ ) and heart rate ( $f_H$ ) were calculated using the blood pressure module in the LabChart Pro software. Cardiac output ( $CO$ ) was calculated from the phasic blood flow signal and based on these primary variables cardiac stroke volume ( $V_S$ ) was calculated as  $V_S = Q/f_H$  and systemic vascular resistance ( $R_{SYS}$ ) was calculated as  $R_{SYS} = P_{DA}/CO$  assuming that central venous blood pressure is close to zero and that changes in venous pressure are negligible in these calculations (Sandblom and Axelsson, 2007).

Blood haematocrit (Hct) was determined in duplicates using micro capillary tubes spun in a hematocrit centrifuge. Blood hemoglobin concentration (Hb) was determined using a Hemocue Hb 201+ unit (Hemocue® AB, Ängelholm, Sweden) with values corrected for salmonid fish blood according to Clark et al. (2008).

## Experimental protocol for assessing swimming performance

Critical swimming speeds were determined using a swimming respirometer as described previously (Skov et al., 2011). The swimming trials were conducted at the Danish Technical University, Institute of Aquatic Resources, Hirtshals, Denmark and the temperature during trials were 19 C.

## Statistical analyses

Data are expressed as group mean  $\pm$  s.e.m. Differences between cortisol-treated and respective treatment controls were tested by one-way ANOVA (for differences in plasma cortisol levels), two-way ANOVA (for effects of treatment and gender on CSI) or unpaired t-tests with Welch's correction for unequal variance when relevant. Average CSI, relative compact to non-compact tissue and mRNA levels of control fish was normalized to 1, and data are presented as normalized values to treatment control average (fold change), whereas other data are presented as absolute values. P-values  $<0.05$  were considered statistically significant. For all experiments, a sample size  $\geq 8$  was used to ensure satisfactory statistical power. All statistical analyses were performed in GraphPad Prism 6 (GraphPad Software, San Diego, CA, USA).

## Results

### Chronic cortisol exposure induces cardiac hypertrophy in rainbow trout

To test whether chronic cortisol exposure directly induces cardiac remodeling in salmonid fish, rainbow trout were fed cortisol-containing feed for 45 days. Increased plasma cortisol levels were confirmed after 15 and 45 days of cortisol treatment by one-way ANOVA ( $p < 0.01$ ). Plasma cortisol concentration after 15 days were  $27.13 \pm 8.2 \mu\text{g/l}$  vs.  $>0.19 \pm 0.0 \mu\text{g/l}$  (below detection limit) in cortisol treated ( $n = 7$ ) and controls ( $n = 5$ ) respectively. After 45 days, plasma cortisol concentration equaled  $20.38 \pm \text{s.e.m.} \mu\text{g/l}$  vs  $8.57 \pm 1.99 \mu\text{g/l}$  in cortisol treated ( $n = 14$ ) and controls ( $n = 14$ ) respectively.

Consistent with our hypothesis, 45 days of cortisol treatment resulted in a robust 34 % increase in CSI compared to controls ( $F_{(1,52)}=80.93$ ),  $p < 0.001$ , Fig. IB). Mean CSI  $\pm$  s.e.m. was  $0.085 \pm 0.002$  for controls and  $0.11 \pm 0.002$  for cortisol treated fish. Since no gender differences were identified by the Two-way ANOVA ( $F_{(1,52)}=0.09$ ,  $p=0.77$ , Fig. S1), we did not separate between sexes in subsequent analyses.

While visual inspection of ventricles gave a clear indication that these were larger in cortisol treated fish (illustrated in Fig. 1A), we also compared absolute ventricular weights and body weights in the two treatment groups following 45 days of treatment to exclude that the elevated CSI was attributed to reduced body weight. Indeed, absolute ventricular weights of cortisol-treated fish ( $0.22 \pm 0.01 \text{ g}$ ) were higher than in controls ( $0.20 \pm 0.01 \text{ g}$ ;  $p < 0.05$ , Fig. 1C), despite absolute body weights being lower in the cortisol treated fish ( $198.1 \pm 6.2$  versus  $238.4 \pm 10.5 \text{ g}$ ,  $p < 0.01$ , Fig. 1D). Thus, while cortisol induced substantial cardiac growth, overall somatic growth was stunted by the cortisol treatment.

The salmonid heart consists of two separate layers of myocardium, i.e. spongy and compact myocardium. The inner spongy myocardium is supplied with blood from the venous circulation, while the outer compact myocardium resembles the mammalian myocardium and is supplied with blood from coronary arteries (Pieperhoff et al., 2009). The compact myocardium has a greater force-generating capacity than the spongy myocardium. We therefore assessed the relative proportion of compact to non-compact volume in the two treatment groups by Magnetic resonance imaging (MRI) of the ventricles (see Fig. 1E). Interestingly, cortisol-treated fish had a higher proportion of compact to non-compact myocardial volume compared to controls ( $p < 0.05$ , Fig. 1F), indicating that the cortisol-induced cardiac growth is primarily due to growth of the compact myocardium. There was no significant difference in myocardial water content between controls and cortisol-treated fish, consistent with the increase in CSIs not resulting from myocardial tissue edema (Fig. 1G).

To indicate if the observed heart growth was due to hypertrophy or hyperplasia (i.e. cell proliferation) cardiac mRNA levels of the cell proliferation marker PCNA were measured. PCNA levels were not significantly increased by the cortisol treatment suggesting that cortisol-induced heart growth was driven mainly by hypertrophy (Fig. 1H).

### **Hypertrophic remodeling is associated with increased expression signature molecules of cardiac pathology**

In mammals, including humans, pathological stimuli induce hypertrophic growth and remodeling of the heart characterized by up-regulation of specific molecular markers. We investigated the expression levels of SMLC2, VMHC, MLP, RCAN1, ANP and BNP in ventricles of rainbow trout treated with cortisol for 45 days. SMLC2 mRNA levels were doubled ( $p < 0.05$ , Fig. 2A) in cortisol-treated fish whereas a trend towards increased VMHC was observed ( $p = 0.14$ , Fig. 2B).

The mammalian stress-sensor muscle LIM protein (MLP) was similarly upregulated ( $p < 0.05$ , Fig. 2C) by the cortisol-treatment. MLP is necessary for stress-induced, pro-hypertrophic nuclear factor of activated T-cell (NFAT) signaling in mammals (Heineke et al., 2003). Calcineurin–NFAT signaling is a major pathway involved in mammalian pathological hypertrophy and remodeling and we assessed its activation by measuring mRNA levels of a direct downstream target gene of NFAT, RCAN1. Interestingly, RCAN1 mRNA levels were 102 % higher than in respective controls following cortisol treatment ( $p < 0.05$ , Fig. 2D). This indicates increased activation of pro-hypertrophic NFAT-signaling in rainbow trout hearts exposed to chronically elevated cortisol levels.

Natriuretic peptides are well-known signature molecules of heart failure progression in mammalian cardiac pathology (Lerman et al., 1993; Maisel et al., 2002). In line with a pathological transcriptional program profile of cardiac remodeling in cortisol treated rainbow trout, mRNA levels of A-type natriuretic peptide (ANP) was upregulated (Fig. 2E). mRNA levels of B-type natriuretic peptide (BNP) were not significantly changed (Fig. 2F).

## Chronic cortisol exposure impairs cardiovascular function and aerobic swimming performance in rainbow trout

Upregulation of signature molecules of cardiac pathology, indicate that cortisol-induced heart growth is not functionally adaptive. To test this, cardiovascular scopes (i.e. the difference between maximum and resting values) were assessed *in vivo* in a separate cohort of rainbow trout treated with cortisol for 45 days. To trigger a maximal cardiovascular response, fish were subjected to a period of exhaustive exercise. Maximum cardiovascular performance was assessed when cardiac output (CO) was maximal immediately after the exhaustive exercise. Resting cardiovascular values were then assessed following a 24 h recovery period.

Despite having larger ventricles (mean CSI  $\pm$  s.e.m. was  $0.12 \pm 0.009$  vs.  $0.085 \pm 0.003$  in cortisol treated (n=13) and control (n=20) fish,  $p < 0.001$ ) with more compact myocardium, maximum CO ( $p < 0.05$ ) was lower in cortisol-treated fish. Resting CO was not significantly changed but CO scope ( $p < 0.05$ ) was also lower in the cortisol group (Fig. 3A). Similarly, maximum but not resting stroke volume ( $V_s$ ) were lower in cortisol treated fish, and there was a clear trend ( $p = 0.55$ ) for a reduced  $V_s$  scope (Fig. 3B). There was no significant difference in maximum heart rate ( $f_H$ ) between treatment groups, but resting  $f_H$  was higher in cortisol-treated fish ( $p < 0.05$ , Fig. 3C), which meant that the  $f_{H \text{ scope}}$  was reduced ( $p < 0.05$ ) in this group (Fig. 3C).

The observed increase in resting heart rate could be caused by either altered cholinergic or adrenergic autonomic nervous input to the heart, or possibly by a cortisol-induced resetting of the cardiac pacemaker (i.e. the spontaneous intrinsic heart rate). Pharmacological agents were used to block muscarinic and  $\beta$ -adrenergic receptors to evaluate cholinergic and adrenergic tones and intrinsic heart rate ( $f_{H \text{ intr}}$ ) following complete autonomic blockade (Altimiras et al., 1997). Indeed, cholinergic tone was decreased in cortisol-treated fish (Fig. 4A), whereas adrenergic tone and  $f_{H \text{ intr}}$  was not significantly altered (Fig. 4B). Thus, increased resting  $f_H$  in cortisol-treated fish originated from decreased cholinergic inhibition.

It is well known that factors such as blood viscosity and vascular resistance affect the workload of the heart. Elevated cardiac workload could possibly mediate the cardiac enlargement observed following cortisol treatment. Therefore, blood hematological variables such as hematocrit (Hct) and hemoglobin (Hb) content as well as hemodynamical variables such as systemic blood pressure and vascular resistance ( $R_{SYS}$ ) were investigated. Neither resting (Fig. 4C-D) nor maximum (Fig. 4E-F) Hct and Hb levels differed between control and cortisol treated fish (Fig. 4C-F) indicating that blood viscosity was similar in the two groups.

Neither resting nor maximum systolic, diastolic or mean dorsal aortic blood pressures differed significantly between treatment groups (Fig. S2), but maximum  $R_{SYS}$  was higher in cortisol treated fish (Fig. 4H). Resting  $R_{SYS}$  was similar in both groups (Fig. 4G).

Since cardiovascular oxygen transporting capacity is a strong determinant of maximum aerobic swimming capacity in fish (Claireaux et al., 2005), we also investigated if the impaired cardiac

pumping capacity observed affected swimming performance. This was tested in a separate cohort of fish treated with cortisol for 90 days (mean CSI  $\pm$  s.e.m. was  $0.0782 \pm 0.003$  vs.  $0.105 \pm 0.003$  in control (n=6) and cortisol treated (n=10) fish,  $p < 0.0001$ ). Indeed, maximum swimming performance (Ucrit) was reduced in the cortisol-treated fish indicating that impaired cardiac function translates to a reduced overall physical performance (Fig 4I).

## Discussion

In the present work, we showed that exogenous cortisol administration in rainbow trout induced substantial ventricular growth, likely through hypertrophy of the compact myocardium. These larger hearts generated lower maximum cardiac output (CO) and stroke volume ( $V_s$ ) with reduced CO scope. At the molecular level, cortisol-induced hypertrophic remodeling was associated with up-regulated signature genes of mammalian pathological hypertrophy, indicating that intracellular signaling pathways mediating cortisol-induced cardiac remodeling in fish are similar to those involved in pathological cardiac hypertrophy in mammals. Moreover, the observed cardiovascular changes were linked to reduced aerobic swimming capacity. Combined, our results are consistent with the hypothesis that elevated cortisol causes non-adaptive pathological cardiac hypertrophy, remodeling and dysfunction in rainbow trout.

The current findings are in line with our previous finding that endogenous stress-induced cortisol production correlates with heart size and remodeling in rainbow trout (Johansen et al., 2011). Our previous investigations were performed on 40 months old (sexually mature) adults that had spent their entire lives under rearing conditions. It is therefore reasonable to assume that stress-induced cortisol production is sufficient to induce myocardial growth and remodeling in fish. Chronic stress has been shown to induce increases in plasma cortisol levels, comparable to those seen in the current study in both magnitude and duration (Barton and Iwama, 1991; Wendelaar Bonga, 1997). Thus, we believe that the current experimental protocol can be extrapolated to for example intensive aquaculture conditions.

To our knowledge, only one previous study has investigated the effect of chronic cortisol administration on cardiac performance in fish. In line with our findings, Nesan and Vijayan (2012) reported that embryo exposure to chronically increased cortisol levels leads to cardiac performance dysfunction in Zebrafish (*Danio rerio*). In mammals, excessive glucocorticoids induce cardiomyocyte hypertrophy both *in vitro* (Ren et al., 2012) and *in vivo* (de Vries et al., 2002; Ahmed, 2013). Further, glucocorticoid-induced cardiac hypertrophy in rat myocytes and myocardium is associated with an increase in hypertrophy markers such as ANP and SMLC2 (De et al., 2011; Ren et al., 2012).

In accordance with this, we found a consistent gene expression up-regulation of signature molecules of mammalian hypertrophy (i.e. ANP, SMLC2, MLP and RCAN1) in cortisol-treated rainbow trout ventricles. Several of these hypertrophy markers serve as specific molecular markers of pathological hypertrophy in humans. For example, ANP and SMLC2 are part of the fetal gene

program, which is reinitiated during pathological cardiac hypertrophy and remodeling. Both genes are upregulated in pressure overload-induced hypertrophy in rats (Schiaffino et al., 1989) and human cardiac hypertrophy (Swynghedauw, 1999). Further, the cardiac stress sensor MLP is upregulated in several models of heart disease in rodents (Boateng et al., 2007) and necessary for stress-induced NFAT signaling (Heineke et al., 2003).

In mammals, extensive evidence exists showing that calcineurin-NFAT signaling is essential for and activated in pathological hypertrophy only (Wilkins et al., 2004). Since NFAT is a transcription factor, its activity can be measured by the mRNA expression of target genes, e.g. RCAN1 (Rothermel et al., 2001). In the current study, cortisol treatment induced an increase in RCAN1 mRNA expression, which is in line with previous findings of increased RCAN1 expression in rainbow trout with endogenously high plasma cortisol levels (Johansen et al., 2011). Combined, these data indicate pro-hypertrophic NFAT-signaling in the rainbow trout heart in response to chronic elevation of cortisol, and that NFAT signaling in pathological cardiac hypertrophy is conserved from fish to mammals.

It is reasonable to speculate that cortisol can be involved in adaptive cardiac hypertrophy in salmonids given the right circumstances. Upon salmonid sexual maturation and spawning migration, plasma levels of corticosteroids peak (Schmidt and Idler, 1962). In fact, plasma levels of cortisol can be exceptionally high during spawning migration (chronically up to 640 ng/ml), similar to those occurring during chronic stress (Carruth et al., 2000). Interestingly, this hyper-activation of both the hypothalamus- pituitary-interrenal (HPI) - and gonadal (HPG) axes during migration coincides with massive cardiac growth. In contrast to the cardiac hypertrophy observed in the current study though, cardiac growth in migrating fish is associated with improved mechanical performance and cardiac pumping capacity (Franklin and Davie, 1992). One explanation could be that cortisol serves as a pro-hypertrophic stimulus (Ren et al., 2012), but that other factors (e.g. anabolic sex steroids) are necessary for the hypertrophy to be accompanied by adaptive changes such as increased force generating capacity. Assuming such a role for cortisol, large increases in cortisol under different circumstances (such as for example during chronic stress), could potentially remodel the heart in a non-adaptive manner similar to the current observations. The exact combination of circumstances and exposure levels promoting adaptive vs maladaptive cardiac hypertrophy clearly deserves further scrutiny.

The mechanism by which cortisol and glucocorticoids induce cardiac hypertrophy remains controversial. In mammals, cortisol can induce cardiac hypertrophy signaling by directly binding to nuclear receptors (ligand-inducible transcription factors) in the cardiomyocyte. Ren et al. (2012) showed that as many as 75 genes in the cardiac hypertrophy signaling pathway were altered by *in vitro* glucocorticoid treatment. Cortisol can, however, also act systemically by altering variables such as blood pressure, blood viscosity and/or vascular resistance and thereby increase the workload of the

heart (Barton et al., 1987; Ahmed, 2013). We did not find any indications of increased blood viscosity since neither hematocrit nor hemoglobin were altered by cortisol treatment.

In rodents, excessive glucocorticoids have been shown to induce hypertension (Ahmed, 2013) which is a major risk factor for pathological cardiac hypertrophy in mammals (Lloyd-Jones et al., 2002). We did not see an increase in dorsal aortic blood pressure. However, systemic resistance was increased in cortisol treated fish. Ventral aortic blood pressure and branchial resistance are more direct measures of cardiac afterload, but increased systemic resistance has been shown to increase ventral and dorsal aortic blood pressure in trout (Conklin et al., 1997). Thus, our findings suggest that the observed increase in systemic resistance plays a role for mediating cardiac hypertrophy by elevating cardiac workload. In addition, increased systemic resistance can partly explain the reduced CO observed. Interestingly, cortisol treated fish appeared to compensate for the reduction in CO by increasing heart rate.

The increased heart rate could reflect increased catecholaminergic innervation or decreased cholinergic tone on the heart. Reid et al. (1996) showed that cortisol administration in rainbow trout altered the secretion of catecholamines which are well known for their inotropic effects on the heart. By pharmacologically blocking both adrenergic and cholinergic receptors, we found that the increased heart rate in cortisol-treated fish was likely due to a reduction in cholinergic tone rather than an increase in adrenergic signaling.

In summary, our results suggest a causative mechanism for the previously observed association between endogenous stress responsiveness and cardiac hypertrophy. This is the first study to report and integrate the etiology, physiology and molecular biology of cortisol-induced pathological remodeling in fish. Our data indicate cortisol and probably also stress as contributors to cardiac pathology in teleosts. Further, our data indicate that gene activation in pathological cardiac hypertrophy is evolutionarily conserved between fish and mammals. This in turn actualizes the question of why and how an apparently maladaptive trophic effect of an otherwise catabolic hormone is preserved in both piscine and tetrapod trajectories of vertebrate development.

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## **Competing interests:**

None declared.

## **Author contributions**

IBJ initiated and provided the overall direction of the study with input from ØØ. and ES. IBJ performed *in vivo* cortisol treatment, qPCR and plasma cortisol analyses. ES, AG, AE and IBJ performed *in vivo* cardiovascular measurements. IBJ performed physiological measurements, sampling and analyses with help from MAV, IGL, EH, MF and ØØ. PVS, MF and IBJ assessed swimming performance. LZ performed magnetic resonance imaging. GEN, ØØ, IS, EH, and ES provided financing and infrastructure for running experiments. IBJ wrote the manuscript with particular input from GEN, IGL, ES, AG, MF and ØØ.

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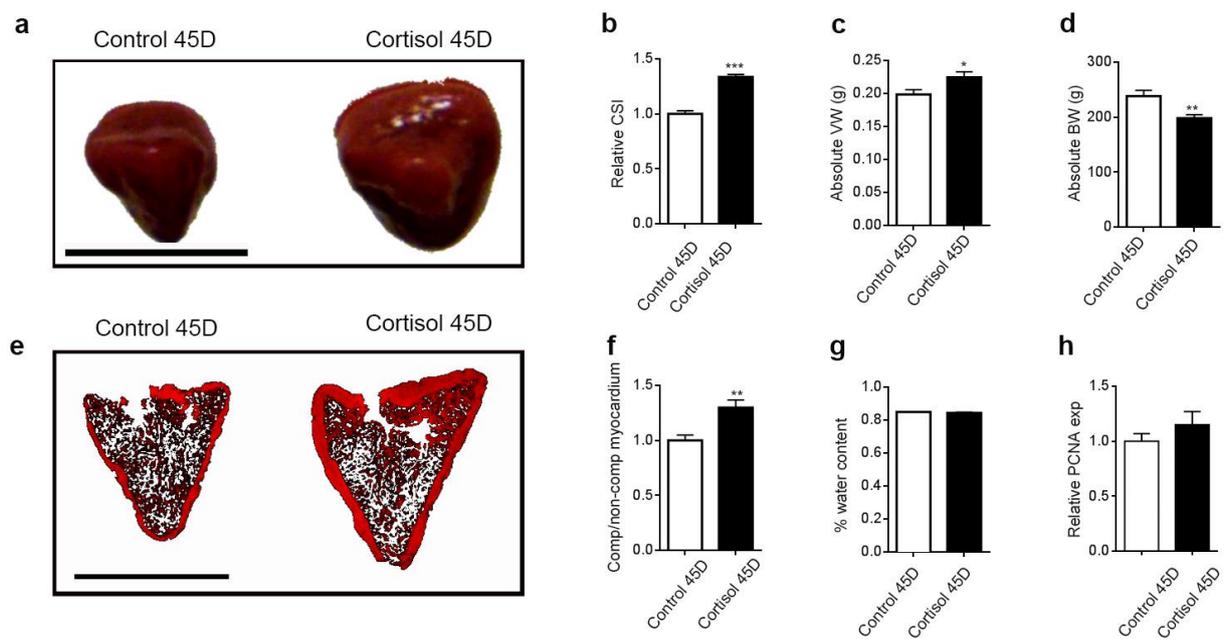
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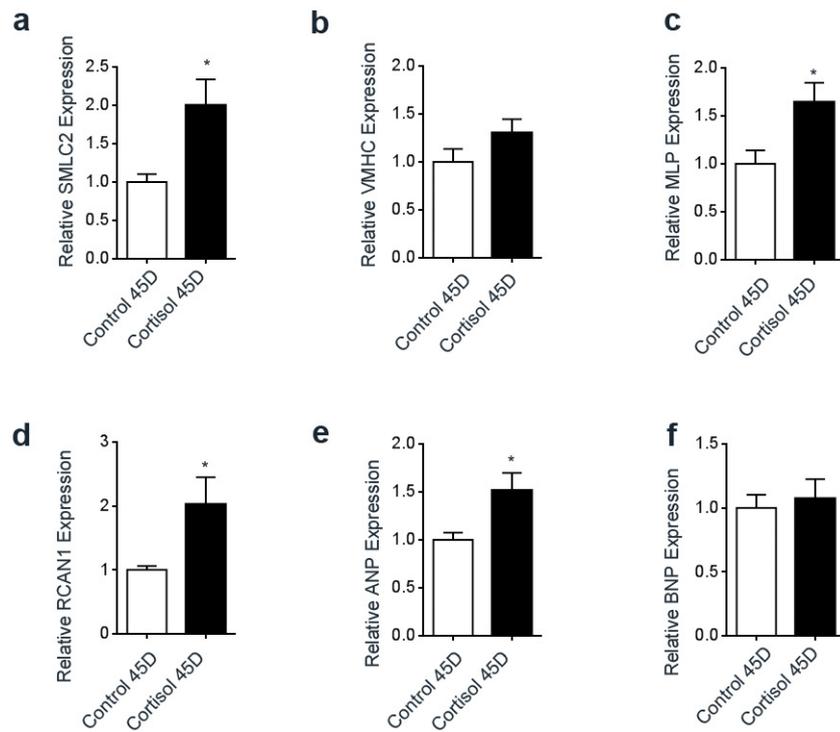
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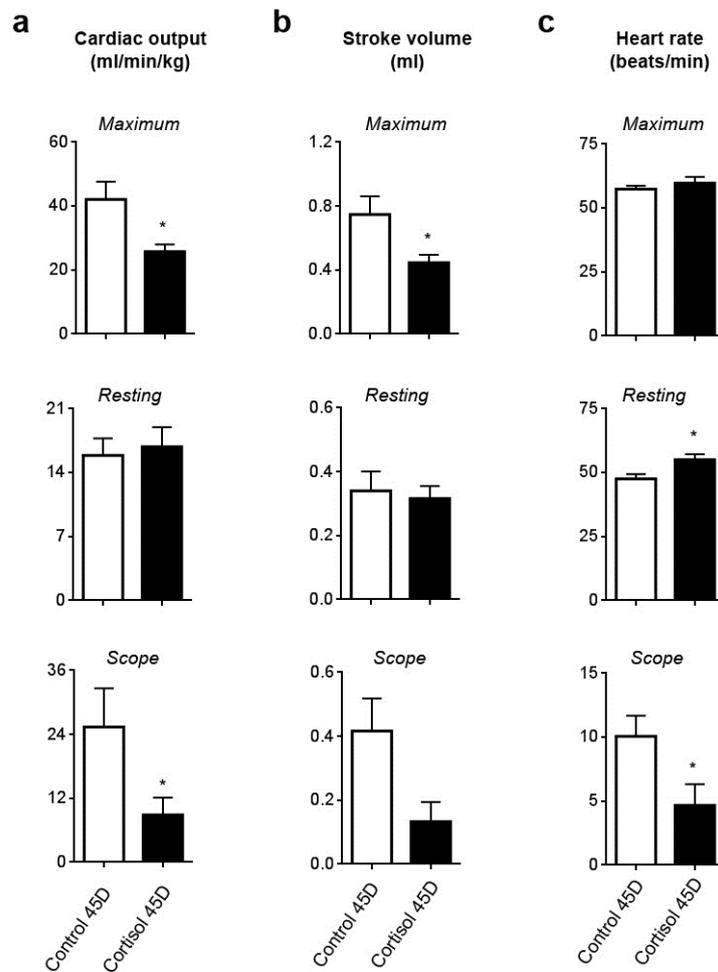
## Figures



**Fig. 1. Chronic cortisol exposure increases ventricular size in rainbow trout.** (A) Image of ventricles of control (325g body weight and 0.18 g ventricle weight, left) and fish treated with cortisol for 45 days (244 g body weight and 0.29 g ventricle weight, right). Scale bar, 1 cm. (B) Relative cardiosomatic index (CSI) (wet weight/body weight) following 45 days (45D) of cortisol treatment (n=28/group). (C) Absolute ventricle weight and (D) absolute body weight following 45 days of cortisol treatment (n=28/group). (E) Magnetic Resonance (MR)-image of cross sections of ventricles of control (600 g body weight and 0.44 g ventricle weight, left) and fish treated with cortisol for 45 days (700 g body weight and 0.86 g ventricle weight, right). Scale bar, 1 cm. (F) Relative proportion of compact (comp) to non-compact (non-comp) myocardium following 45 days of cortisol treatment (n=21/group). (G) Percent (%) water content (g water/g wet weight\*100) in ventricles following 45 days of cortisol treatment (n<sub>control</sub>=8, n<sub>cortisol</sub>=7). (H) mRNA abundance of Proliferating cell nuclear antigen (PCNA) relative to the standard gene  $\beta$ -actin following 45 (0) days of cortisol treatment (n<sub>control</sub>=9, n<sub>cortisol</sub>=10. Data are either means  $\pm$  s.e.m. (C, D and G) or means  $\pm$  s.e.m relative to treatment control (B, F and H). The mean CSI, ratio of comp to non-comp myocardium and PCNA expression of control fish were normalized to 1. Statistical differences were tested by Two-way ANOVA (for CSI) or unpaired two-tailed t-tests. \*p<0.05 vs. control.

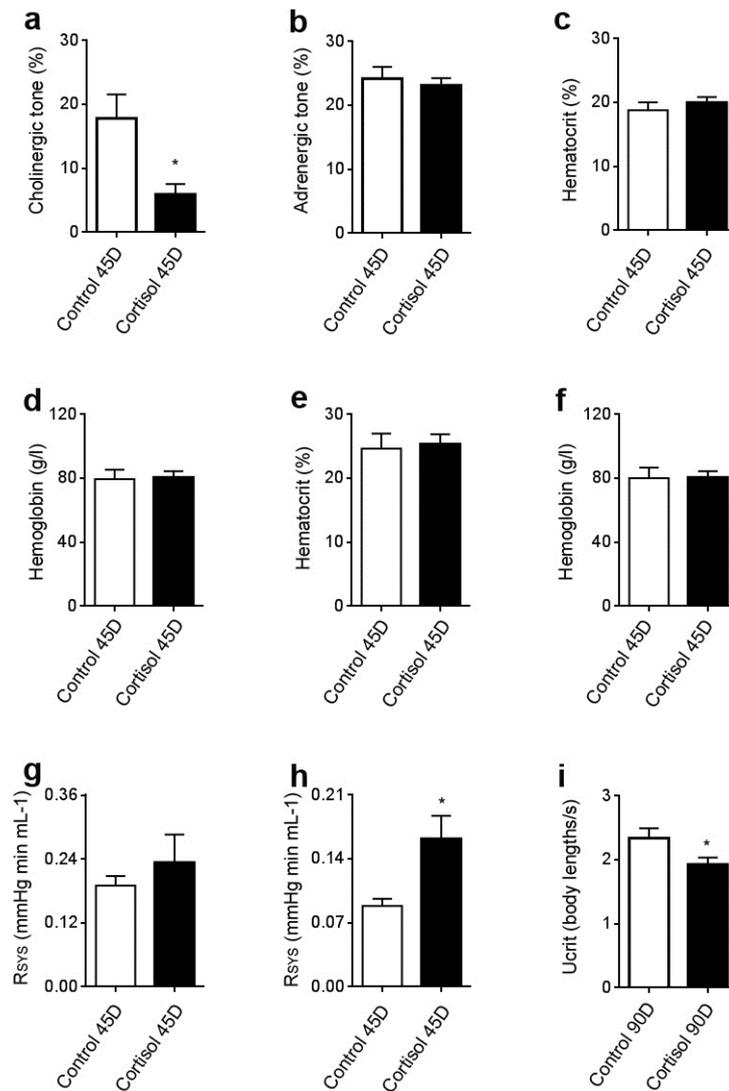


**Figure 2. Cortisol-induced hypertrophic remodeling is associated with increased expression levels of signature molecules of cardiac pathology in rainbow trout.** mRNA abundance of (A) slow myosin heavy chain 2 (SMLC2), (B) ventricular myosin heavy chain (VMHC), (C) muscle Lim Protein (MLP), (D) regulator of calcineurin 1 (RCAN1), (E) A-type natriuretic peptide (ANP) and (F) B-type natriuretic peptide relative to the standard gene  $\beta$ -actin following 45 days (45D) of cortisol treatment ( $n_{\text{control}}=9$ ,  $n_{\text{cortisol}}=10$ ). Data are means  $\pm$  s.e.m relative to control. Statistical differences were tested by unpaired two-tailed t-tests. \* $p < 0.05$  vs. control.



**Figure 3. Chronic cortisol exposure impairs cardiovascular function in rainbow trout.**

Maximum (A, upper panel,  $n_{\text{control}}=8$ ,  $n_{\text{cortisol}}=9$ ) and resting (A, middle panel,  $n_{\text{control}}=5$ ,  $n_{\text{cortisol}}=9$ ) cardiac output (CO), CO scope (A, lower panel,  $n_{\text{control}}=5$ ,  $n_{\text{cortisol}}=9$ ), maximum (B, upper panel,  $n_{\text{control}}=8$ ,  $n_{\text{cortisol}}=9$ ) and resting (B, middle panel,  $n_{\text{control}}=5$ ,  $n_{\text{cortisol}}=9$ ) stroke volume ( $V_S$ ),  $V_S$  scope (B, lower panel,  $n_{\text{control}}=5$ ,  $n_{\text{cortisol}}=9$ ), maximum (C, upper panel,  $n_{\text{control}}=12$ ,  $n_{\text{cortisol}}=10$ ) and resting (C, middle panel,  $n_{\text{control}}=9$ ,  $n_{\text{cortisol}}=10$ ) heart rate ( $f_H$ ) and  $f_H$  scope (C, lower panel,  $n_{\text{control}}=9$ ,  $n_{\text{cortisol}}=10$ ) following 45 days (45D) of cortisol treatment is shown. Statistical differences were tested by unpaired two-tailed t-tests. \* $p < 0.05$ , vs. control.



**Figure 4. Hemodynamic and hematological variables and swimming performance following chronic cortisol exposure in rainbow trout.** (A) Cholinergic and (B) adrenergic tone ( $n=9/\text{group}$ ), resting (C) hematocrit and (D) hemoglobin ( $n=9/\text{group}$ ), maximum (E) hematocrit and (F) hemoglobin ( $n=9/\text{group}$ ) and (G) resting ( $n_{\text{control}}=5$ ,  $n_{\text{cortisol}}=9$ ) and (H) maximum ( $n_{\text{control}}=7$ ,  $n_{\text{cortisol}}=9$ ) systemic resistance ( $R_{\text{sys}}$ ) following 45 days (45D) of cortisol treatment. (I) Swimming performance ( $U_{\text{crit}}$ ) following 90 days (90D) of cortisol treatment ( $n_{\text{control}}=6$ ,  $n_{\text{cortisol}}=10$ ). Statistical differences were tested by unpaired two-tailed t-tests. \* $p<0.05$ , vs. control.

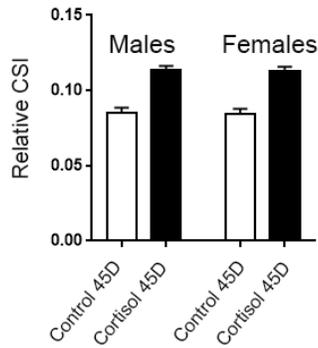
## Supporting Figures

Figure S1

Effect of treatment:  $F(1,52)=80.93$ ,  $p<0.0001$

Effect of gender:  $F(1,52)=0.09$ ,  $p=0.77$

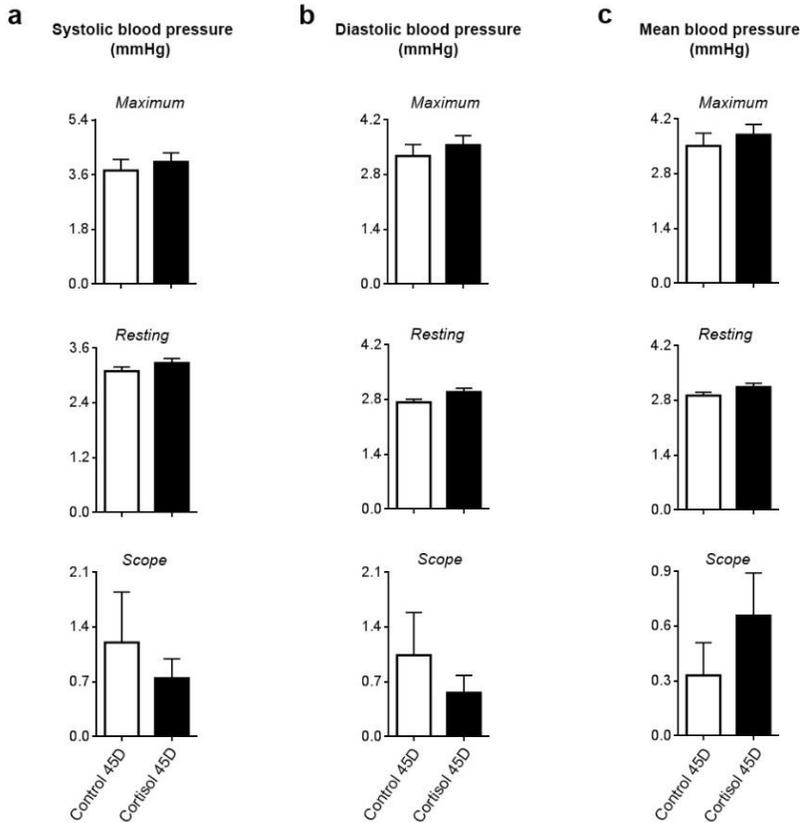
Interaction effect:  $F(1,52)=0.008$ ,  $p=0.93$



**Fig. S1. Effects of chronic cortisol administration on cardiosomatic index in male and female rainbow trout.**

Relative cardiosomatic index (CSI) (wet weight/body weight) following 45 days (45D) of cortisol treatment in male ( $n_{\text{control}}=16$ ,  $n_{\text{cortisol}}=17$ ) and female ( $n_{\text{control}}=12$ ,  $n_{\text{cortisol}}=11$ ) rainbow trout. Data are means  $\pm$  s.e.m. Statistical differences were tested by two-way ANOVA.

Figure S2



**Fig. S2. Effects of chronic cortisol administration on blood pressure in rainbow trout.** Maximum (A, upper panel, ncontrol=11, ncortisol=10) and resting (A, middle panel, ncontrol=9, ncortisol=10) systolic blood pressure, systolic blood pressure scope (A, lower panel, ncontrol=9, ncortisol=10), maximum (B, upper panel, ncontrol=11, ncortisol=10) and resting (B, middle panel, ncontrol=9, ncortisol=10) diastolic blood pressure, diastolic blood pressure scope (B, lower panel, ncontrol=9, ncortisol=10), maximum (C, upper panel, ncontrol=11, ncortisol=10) and resting (C, middle panel, ncontrol=9, ncortisol=10) mean blood pressure and mean blood pressure scope (C, lower panel, ncontrol=9, ncortisol=10) following 45 days (45D) of cortisol treatment. Data are means  $\pm$  s.e.m. Statistical differences were tested by unpaired t-tests.