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RESEARCH ARTICLE

Bigger is not better: cortisol-induced cardiac growth and dysfunction in salmonids

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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 in salmonid fish too. A conserved stimulatory effect of the otherwise catabolic steroid hormone cortisol is probably 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 diet 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 signalling pathways mediating cortisol-induced cardiac remodelling in fish are conserved from fish to mammals. Altogether, we show that excessive cortisol can induce pathological cardiac remodelling. This is the first study to report and integrate the etiology, physiology and molecular biology of cortisol-induced pathological remodelling in fish.

KEY WORDS: Myocardial hypertrophy, Chronic stress, Heart failure, Rainbow trout, Cardiac performance

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 risk in humans (Rosengren et al., 1991, 2004; Iso et al., 2002). It is also well known that individual variation in physiology and behaviour (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 fourfold 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 remodelling, growth and disease.

Stress- and cortisol-induced cardiac hypertrophy may not be limited to mammals. Cardiac remodelling and deformities are commonly reported in farmed fish (Poppe 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 previously established a link between cortisol responsiveness to stress and cardiac remodelling in rainbow trout (*Oncorhynchus mykiss*) high responsive (HR) and low responsive (LR) strains and wild-type brown trout (*Salmo trutta*) (Johansen et al., 2011). High post-stress cortisol production is also associated with a range of behavioural and cognitive traits indicating a reactive coping style (Øverli et al., 2005, 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 with 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, for example, seasonal cold acclimatization

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List of symbols and abbreviations

ANP	A-type natriuretic peptide
BNP	B-type natriuretic peptide
CSI	cardiosomatic index
f_H	heart rate
Hb	haemoglobin
Hct	haematocrit
M_b	body mass
MLP	muscle LIM protein
MRI	magnetic resonance imaging
M_v	ventricle mass
PCNA	proliferating cell nuclear antigen
P_{DA}	dorsal aortic blood pressure
$P_{DA,dia}$	diastolic pressure
$P_{DA,sys}$	systolic pressure
$\dot{Q}$	cardiac output
RCAN1	regulator of calcineurin 1
RIA	radioimmunoassay
RIN	RNA integrity number
R_{sys}	systemic vascular resistance
SMLC2	slow myosin light chain 2
U_{crit}	critical swimming speed
VMHC	ventricular myosin heavy chain
V_S	cardiac stroke volume

(Klaiman et al., 2011) and sexual maturation (Franklin and Davie, 1992). Further, although an association between cortisol exposure and cardiac remodelling 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 signalling pathways mediating cortisol-induced cardiac remodelling in fish are similar to those involved in pathological cardiac hypertrophy in mammals.

MATERIALS AND METHODS

Research animals and animal housing

Juvenile rainbow trout, *Oncorhynchus mykiss* (Walbaum 1792) (150±29 g, mean±s.d.; obtained from Store Restrup Fiskeri, Nibe, Denmark), were used for 45 and 90 days of cortisol treatment. 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, on a 14.5 h:9.5 h light:dark cycle. The fish were subsequently moved and randomly distributed into 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. A 14.5 h:9.5 h light:dark cycle was maintained. 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 approval for the experiments was given by the Norwegian and Danish Animal Research Authorities with licence numbers 2012/33240 and 2014-15-2934-01041, respectively.

For *in vivo* assessment of effects of cortisol-induced remodelling on cardiovascular performance, juvenile rainbow trout (420±23 g, mean±s.d.), 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 freshwater at 8°C prior to experimentation, on a 12 h: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 a treatment group.

Preparation of cortisol feed

Cortisol treatment refers to oral administration of food pellets coated with hydrocortisone. 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 kilogram of pellets (EFICO Enviro 920, 3 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 (90 kPa) 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 (M_b) each day with commercial pellets (EFICO Enviro 920, 3 mm, Biomar). At the onset of the experiment, the feed was replaced with pellets of the same brand and size that had been coated with either rapeseed oil alone (control feed) or rapeseed oil and hydrocortisone (see above) and the fish were fed 0.8% of their M_b daily, corresponding to a dose of 4 µg cortisol g^{-1} M_b. Following 45 and 90 days of 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^{-1} MS-222 (Sigma-Aldrich) 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 scale, and cardiosomatic index (CSI=ventricle wet mass/M_b) 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 placed in RNAlater® solution (Ambion, Austin, TX, USA) for 24 h at room temperature before being stored at −20°C for later quantitative PCR (qPCR) mRNA analysis, or put in freshly prepared 4% paraformaldehyde for magnetic resonance imaging (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 analysed using radioimmunoassay (RIA) based on the assay by Pottinger and Carrick (2001). In short, steroids were extracted with ethyl acetate (Merck, Kenilworth, NJ, USA; 1:5 plasma:ethyl

acetate) prior to the RIA. A 5–150 µl sample of extract was transferred to 1.5 ml Eppendorf tubes, and 50 µl [1,2,6,7-^{3}H] cortisol (~16,000 cpm; Amersham Pharmacia Biotech, Little Chalfont, UK, 60 Ci mmol^{-1}) 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 analyser (Packard Instrument, Meriden, CT, USA). A 3-parameter hyperbolic function was fitted to the plot of the percentage of [^{3}H]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^{-1}, respectively. For individuals where the plasma cortisol levels were below this limit, the level was set to 0.19 ng ml^{-1}.

MRI of fixed ventricles

Ventricles fixed in 4% paraformaldehyde were thoroughly rinsed with saline water (0.4%) then mounted with cotton and soaked in MRI-compatible perfluoropolyether oil (Fomblin, Sigma-Aldrich) in a 15 mm glass tube. MRI experiments were performed on a pre-clinical 9.4 T MRI system (Agilent Technologies, Palo Alto, CA, USA) equipped with a high-performance gradient coil (inner diameter 60 mm, maximum strength 1000 mT m^{-1}) and a quadrature radio frequency volume coil (19 mm i.d., Rapid Biomedical, Rimpar, Germany). High resolution images were acquired by 3D spoiled gradient echo. The field of view ranged from 12.8×12.8×12.8 mm to 16×16×16 mm depending on the size of the specimen. Other typical parameters were matrix 1024×512×512, repetition time 45 ms, echo time 10 ms, flip angle 45 deg, 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 the area of compact and non-compact myocardium. Compact myocardium area was calculated by subtracting non-compact area (area inside the compact layer in the ventricle) from total area. Compact myocardium area was then divided by non-compact myocardium area to get the ratio of compact to non-compact area.

RNA extraction and qPCR analysis

Ventricles, stored and frozen in RNAlater® (see above), 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) 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) using Superscript III reverse transcriptase (Invitrogen) with oligo $dT_{12–18}$ primers synthesized by Invitrogen. Gene-specific primer sequences for rainbow trout β-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 β-actin was used as a reference gene. qPCR was carried out using a Roche LC480 Light Cycler (Roche Diagnostics, Penzberg, Germany). Reaction volumes were 10 µl and included Light Cycler® 480 SYBR Green I Master (Roche Diagnostics), primers (5 µmol l^{-1}) and cDNA (3 µmol l^{-1}). Cycling conditions were as follows: 10 min at 95°C, 42 cycles of 10 s at 95°C, 10 s at 60°C and 10 s at 72°C, followed by melt curve analysis. All reactions were run in duplicate and controls without DNA template were included to verify the absence of cDNA contamination.

Relative gene expression data were calculated from qPCR raw data as the ratio of expression of the gene of interest (GOI) to that of the internal control (IC, β-actin): E_{IC}^{Cp}/E_{GOI}^{Cp}, where E is priming efficiency and Cp is the crossing point. E-values were calculated for each qPCR 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^{-1} MS-222 (Sigma-Aldrich) buffered with $NaHCO_3$ (400 mg l^{-1}). The fish were weighed and placed on a surgery table covered with wet foam rubber. The gills were continuously irrigated via the mouth with aerated water (~10°C) containing a lower dose of $NaHCO_3$ (100 and 200 mg l^{-1})-buffered MS-222. 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, NY, USA), factory-calibrated to 10°C, was placed around the aorta to measure cardiac output ($\dot{Q}$). The lead from the flow probe was attached 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 a guide (Axelsson and Fritsche, 1994). The catheter was rinsed and filled with heparinized (100 IU ml^{-1}) saline (0.9%) and closed with a pin. The catheter and the flow probe lead were collectively sutured to the skin on the back of the fish with a single 2-0 silk suture. Following surgery, fish were revived in freshwater in their respective holding tank and placed individually into 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, such that the fish was completely unresponsive to physical stimuli at the end of the protocol. The fish was then rapidly transferred to an opaque holding chamber (length: 54 cm, width: 13 cm and depth: 18 cm) with through-flowing water from the same water system and covered

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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 $\dot{Q}$ 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 tone was then determined using the pharmacological protocol of Altimiras et al. (1997). Briefly, atropine sulphate (1.2 mg kg^{-1} M_b) was 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, (±)propranolol hydrochloride (3 mg kg^{-1} M_b) was injected to block β-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 (pvb 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.). Data were sampled at 100 Hz using a Power Lab unit (ADInstruments Pty Ltd, Castle Hill, NSW, Australia) connected to a laptop computer running LabChart Pro software (version 7.3, ADInstruments Pty Ltd). From the pulsatile blood pressure signal, mean dorsal aortic blood pressure (P_{DA}), diastolic pressure ($P_{DA,dia}$), systolic pressure ($P_{DA,sys}$), pulse pressure ($P_{DA,pulse}$) and heart rate (f_H) were calculated using the blood pressure module in the LabChart Pro software. $\dot{Q}$ was calculated from the phasic blood flow signal, and based on these primary variables cardiac stroke volume (V_S) was calculated as $V_S=\dot{Q}/f_H$ and systemic vascular resistance (R_{sys}) was calculated as $R_{sys}=P_{DA}/\dot{Q}$, 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 duplicate using microcapillary tubes spun in a Hct centrifuge. Blood haemoglobin (Hb) concentration 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 speed (U_{crit}) was 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 was 19°C.

Statistical analyses

Data are expressed as group means±s.e.m. Differences between cortisol-treated and the respective treatment controls were tested by one-way ANOVA (for differences in plasma cortisol levels), two-way ANOVA (for effects of treatment and sex 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 were normalized to 1, and data are presented as normalized values to treatment control average (fold-change), whereas other data are presented as absolute values. $P<0.05$ was considered statistically significant. For all experiments, a sample size ≥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 remodelling 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 ($P<0.01$). Mean (±s.e.m.) plasma cortisol concentration after 15 days was 27.13±8.2 versus <0.19±0.0 µg l^{-1} (below detection limit) in cortisol-treated ($n=7$) and control ($n=5$) fish, respectively. After 45 days, plasma cortisol concentration equalled 20.38±5.49 versus 8.57±1.99 µg l^{-1} in cortisol-treated ($n=14$) and control fish ($n=14$), respectively.

Consistent with our hypothesis, 45 days of cortisol treatment resulted in a robust 34% increase in CSI compared with controls ($F_{1,52}=80.93$, $P<0.001$; Fig. 1B). Mean (±s.e.m.) CSI was 0.085±0.002 for controls and 0.11±0.002 for cortisol-treated fish. As no sex differences were identified by two-way ANOVA ($F_{1,52}=0.09$, $P=0.77$; Fig. S1), we did not distinguish 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 mass (M_v) and M_b in the two treatment groups following 45 days of treatment to exclude that the elevated CSI was attributed to reduced M_b. Indeed, absolute M_v of cortisol-treated fish (0.22±0.01 g) was higher than that of controls (0.20±0.01 g, $P<0.05$; Fig. 1C), despite absolute M_b being lower in the cortisol-treated fish (198.1±6.2 versus 238.4±10.5 g, $P<0.01$; Fig. 1D). Thus, while cortisol induced substantial cardiac growth, overall somatic growth was stunted by cortisol treatment.

The salmonid heart consists of two separate layers of myocardium, i.e. spongious and compact myocardium. The inner spongious myocardium is supplied with blood from the venous circulation, while the outer compact myocardium resembles the mammalian myocardium and is supplied with blood from the coronary arteries (Pieperhoff et al., 2009). The compact myocardium has a greater force-generating capacity than the spongious myocardium. We therefore assessed the relative proportion of compact to non-compact volume in the two treatment groups by MRI of the ventricles (see Fig. 1E). Interestingly, cortisol-treated fish had a higher proportion of compact to non-compact myocardial volume compared with 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 CSI not resulting from myocardial tissue oedema (Fig. 1G).

To indicate whether 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,

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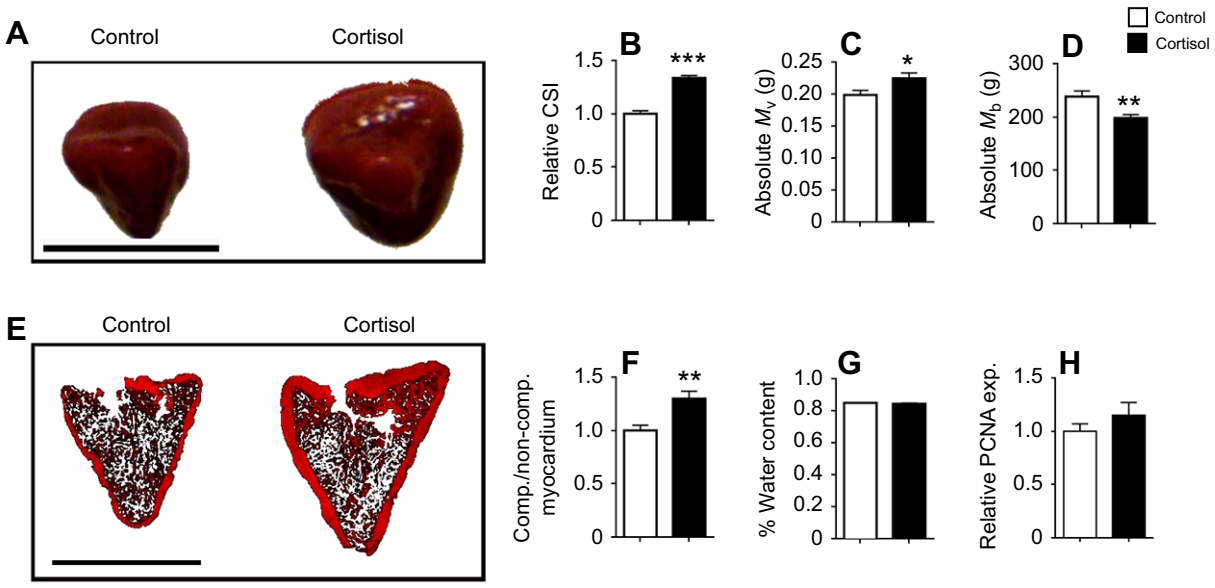

Fig. 1. Chronic cortisol exposure increases ventricular size in rainbow trout. (A) Image of ventricles of fish treated with cortisol for 45 days (244 g M_b and 0.29 g M_v, right) and the corresponding control fish [325 g body mass (M_b) and 0.18 g ventricle mass (M_v), left]. Scale bar, 1 cm. (B) Relative cardiosomatic index (CSI; wet mass/M_b) following 45 days of cortisol treatment (n=28/group). (C) Absolute M_v and (D) absolute M_b following 45 days of cortisol treatment (n=28/group). (E) Magnetic resonance image of cross-sections of ventricles of fish treated with cortisol for 45 days (700 g M_b and 0.86 g M_v, right) and control fish (600 g M_b and 0.44 g M_v, left). Scale bar, 1 cm. (F) Relative proportion of compact to non-compact myocardium following 45 days of cortisol treatment (n=21/group). (G) Water content (g water/g M_v×100) in ventricles following 45 days of cortisol treatment ($n_{control}$=8, $n_{cortisol}$=7). (H) mRNA abundance of proliferating cell nuclear antigen (PCNA) relative to the standard gene β-actin following 45 days of cortisol treatment ($n_{control}$=9, $n_{cortisol}$=10). Data are either means±s.e.m. (C,D,G) or means±s.e.m. relative to treatment control (B,F,H). The mean CSI, ratio of compact to non-compact 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 versus control; **P<0.01 versus control; ***P<0.001 versus control.

suggesting that cortisol-induced heart growth was driven mainly by hypertrophy (Fig. 1H).

Hypertrophic remodelling is associated with increased expression of signature molecules of cardiac pathology

In mammals, including humans, pathological stimuli induce hypertrophic growth and remodelling 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 *VHHC* was observed (P=0.14; Fig. 2B).

The mammalian stress-sensor *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) signalling in mammals (Heineke et al., 2003). Calcineurin–NFAT signalling is a major pathway involved in mammalian pathological hypertrophy and remodelling 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 signalling 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 programme profile of cardiac remodelling in cortisol-treated rainbow trout, mRNA levels of *ANP* were upregulated (Fig. 2E). mRNA levels of *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 scope (i.e. the difference between maximum

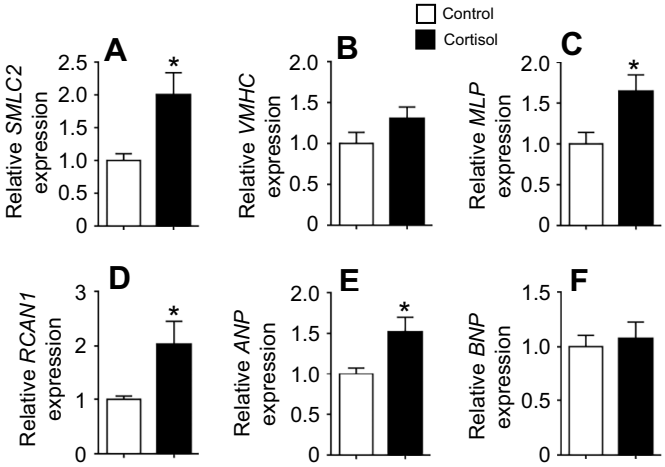

Fig. 2. Cortisol-induced hypertrophic remodelling is associated with increased expression of signature molecules of cardiac pathology in rainbow trout. mRNA abundance of (A) slow myosin light chain 2 (*SMLC2*), (B) ventricular myosin heavy chain (*VMHC*), (C) muscle LIM protein (*MLP*), (D) regulator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

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and resting values) was 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 $\dot{Q}$ was maximal immediately after the exhaustive exercise. Resting cardiovascular values were then assessed following a 24 h recovery period.

Despite having larger ventricles [mean±s.e.m. CSI was 0.12±0.009 versus 0.085±0.003 in cortisol-treated (n=13) and control (n=20) fish, P<0.001] with more compact myocardium, maximum $\dot{Q}$ (P<0.05) was lower in cortisol-treated fish. Resting $\dot{Q}$ was not significantly changed but $\dot{Q}$ scope (P<0.05) was also lower in the cortisol group (Fig. 3A). Similarly, maximum but not resting V_S was 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 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 scope was reduced (P<0.05) in this group (Fig. 3C).

The observed increase in resting f_H could be caused by either altered cholinergic or altered adrenergic autonomic nervous input to the heart, or possibly by a cortisol-induced resetting of the cardiac pacemaker (i.e. the spontaneous intrinsic f_H). Pharmacological agents were used to block muscarinic and β-adrenergic receptors to evaluate cholinergic and adrenergic tone and intrinsic f_H following complete autonomic blockade (Altimiras et al., 1997). Indeed, cholinergic tone was decreased in cortisol-treated fish (Fig. 4A), whereas adrenergic tone and intrinsic f_H were 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 haematological variables such as Hct and Hb content as well as haemodynamical 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, indicating that blood viscosity was similar in the two groups.

Neither resting nor maximum $P_{DA,sys}$, $P_{DA,dia}$ or mean P_{DA} 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 the two groups (Fig. 4G).

As cardiovascular oxygen-transporting capacity is a strong determinant of maximum aerobic swimming capacity in fish (Claireaux et al., 2005), we also investigated whether 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±s.e.m. CSI was 0.0782±0.003 versus 0.105±0.003 in control (n=6) and cortisol-treated (n=10) fish, P<0.0001]. U_{crit} 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

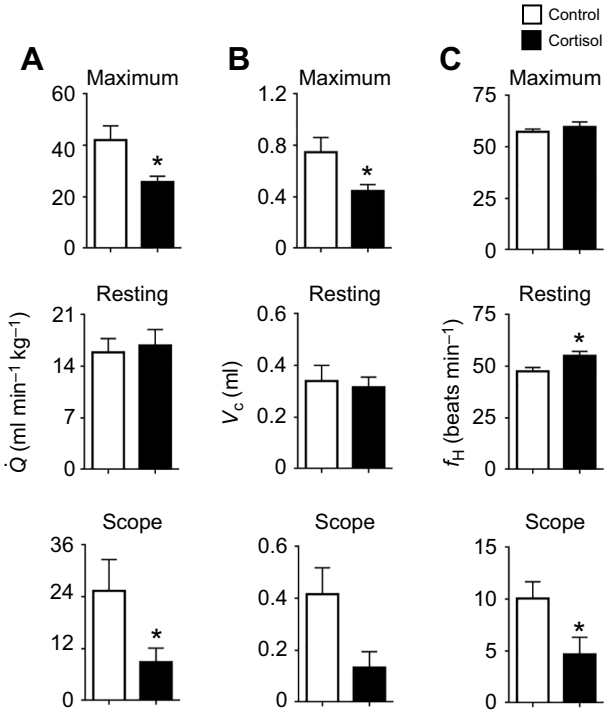

Fig. 3. Chronic cortisol exposure impairs cardiovascular function in rainbow trout. (A) Maximum cardiac output ($\dot{Q}$; upper panel, $n_{control}$=8, $n_{cortisol}$=9), resting $\dot{Q}$ (middle panel, $n_{control}$=5, $n_{cortisol}$=9) and $\dot{Q}$ scope (lower panel, $n_{control}$=5, $n_{cortisol}$=9). (B) Maximum stroke volume (V_S; upper panel, $n_{control}$=8, $n_{cortisol}$=9), resting V_S (middle panel, $n_{control}$=5, $n_{cortisol}$=9) and V_S scope (lower panel, $n_{control}$=5, $n_{cortisol}$=9). (C) Maximum heart rate (f_H; upper panel, $n_{control}$=12, $n_{cortisol}$=10), resting f_H (middle panel, $n_{control}$=9, $n_{cortisol}$=10) and f_H scope (lower panel, $n_{control}$=9, $n_{cortisol}$=10). Data are for control fish and fish treated with cortisol for 45 days. Statistical differences were tested by unpaired two-tailed *t*-tests. *P<0.05 versus control.

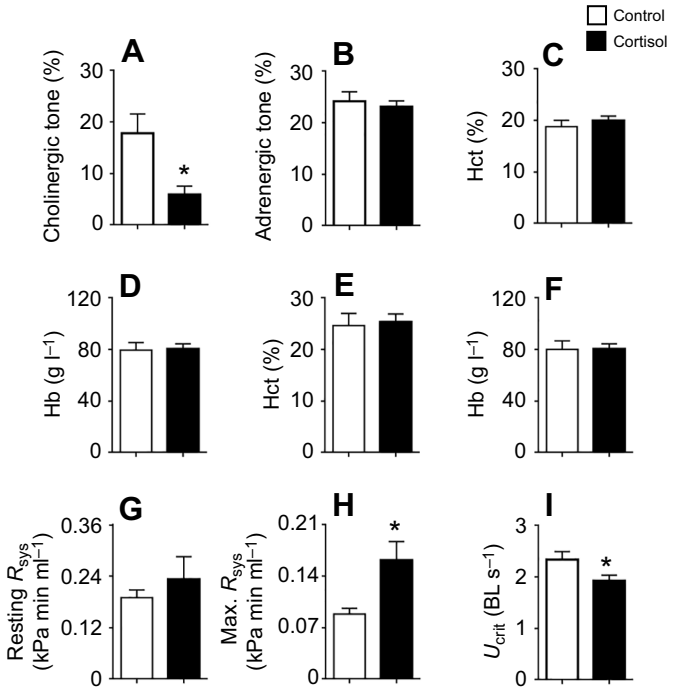

Fig. 4. Haemodynamic and haematological variables and swimming performance following chronic cortisol exposure in rainbow trout. (A) Cholinergic and (B) adrenergic tone (n=9/group). (C–F) Resting haematocrit (Hct; C) and haemoglobin (Hb; D) (n=9/group), and maximum Hct (E) and Hb (F) (n=9/group). (G) Resting systemic resistance (R_{sys}) ($n_{control}$=5, $n_{cortisol}$=9) and (H) maximum R_{sys} ($n_{control}$=7, $n_{cortisol}$=9). Data are for control fish and fish treated with cortisol for 45 days. (I) Swimming performance (U_{crit}; BL, body lengths) following 90 days of cortisol treatment ($n_{control}$=6, $n_{cortisol}$=10). Statistical differences were tested by unpaired two-tailed *t*-tests. *P<0.05 versus control.

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growth, probably through hypertrophy of the compact myocardium. These larger hearts generated lower maximum $\dot{Q}$ and V_S with reduced $\dot{Q}$ scope. At the molecular level, cortisol-induced hypertrophic remodelling was associated with up-regulated signature genes of mammalian pathological hypertrophy, indicating that intracellular signalling pathways mediating cortisol-induced cardiac remodelling 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, remodelling 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 remodelling in rainbow trout (Johansen et al., 2011). Our previous investigations were performed on 40 month 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 remodelling 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 programme, which is reinitiated during pathological cardiac hypertrophy and remodelling. 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 is necessary for stress-induced NFAT signalling (Heineke et al., 2003).

In mammals, extensive evidence exists that calcineurin–NFAT signalling is essential for and activated in pathological hypertrophy only (Wilkins et al., 2004). As 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 signalling in the rainbow trout heart in response to chronic elevation of cortisol, and that NFAT signalling in pathological cardiac hypertrophy is conserved from fish to mammals.

It is reasonable to speculate that cortisol may be involved in adaptive cardiac hypertrophy in salmonids under particular 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^{-1}), similar to those occurring during chronic stress (Carruth et al., 2000). Interestingly, this hyper-activation of both the hypothalamus–pituitary–interrenal (HPI) and hypothalamus–pituitary–gonadal (HPG) axes during migration coincides with massive cardiac growth. In contrast to the cardiac hypertrophy observed in the current study, however, 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 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 versus 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 signalling 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 signalling pathway were altered by *in vitro* glucocorticoid treatment. However, cortisol can 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 as neither Hct nor Hb was 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 P_{DA}. However, R_{sys} was increased in cortisol-treated fish. Ventral aortic blood pressure and branchial resistance are more direct measures of cardiac afterload, but increased R_{sys} 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 R_{sys} plays a role in mediating cardiac hypertrophy by elevating cardiac workload. In addition, increased R_{sys} can partly explain the reduced $\dot{Q}$ observed. Interestingly, cortisol-treated fish appeared to compensate for the reduction in $\dot{Q}$ by increasing f_H.

The increased f_H 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 f_H in cortisol-treated fish was probably due to a reduction in cholinergic tone rather than an increase in adrenergic signalling.

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 remodelling in fish. Our data

indicate that cortisol and probably also stress contribute 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
The authors declare no competing or financial interests.

Author contributions
Conceptualization: I.B.J., E.S., I.G.L., Ø.Ø.; Methodology: I.B.J., E.S., P.V.S., L.Z., I.S.; Validation: I.B.J.; Formal analysis: I.B.J., E.S., P.V.S., A.G., L.Z., Ø.Ø.; Investigation: I.B.J., E.S., P.V.S., A.G., A.E., I.G.L., M.A.V., L.Z., E.H., M.F., Ø.Ø.; Resources: E.S., P.V.S., E.H., I.S., G.E.N., Ø.Ø.; Writing - original draft: I.B.J.; Writing - review & editing: I.B.J., E.S., A.G., A.E., I.G.L., M.A.V., E.H., M.F., G.E.N., Ø.Ø.; Visualization: I.B.J., I.G.L., M.F.; Supervision: E.H., I.S., G.E.N., Ø.Ø.; Project administration: I.B.J., E.S.; Funding acquisition: I.B.J., E.S., I.S., G.E.N., Ø.Ø.

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Supplementary information
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Figure S1

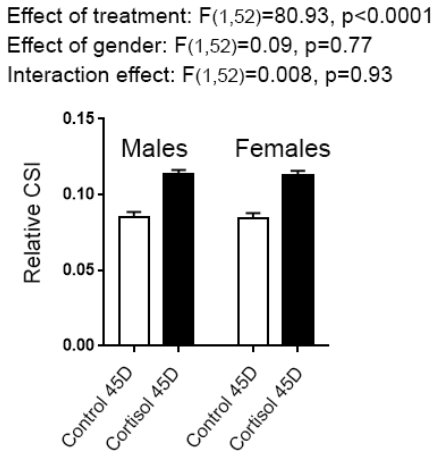

Fig. S1. Effects of chronic cortisol administration on cardiosomatic index in male and female rainbow trout. Relative cardiosomatic index (CSI) (wet weight/body weight) following 45 days (45D) of cortisol treatment in male ($n_{control}$=16, $n_{cortisol}$=17) and female ($n_{control}$=12, $n_{cortisol}$=11) rainbow trout. Data are means ± s.e.m. Statistical differences were tested by two-way ANOVA.

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Figure S2

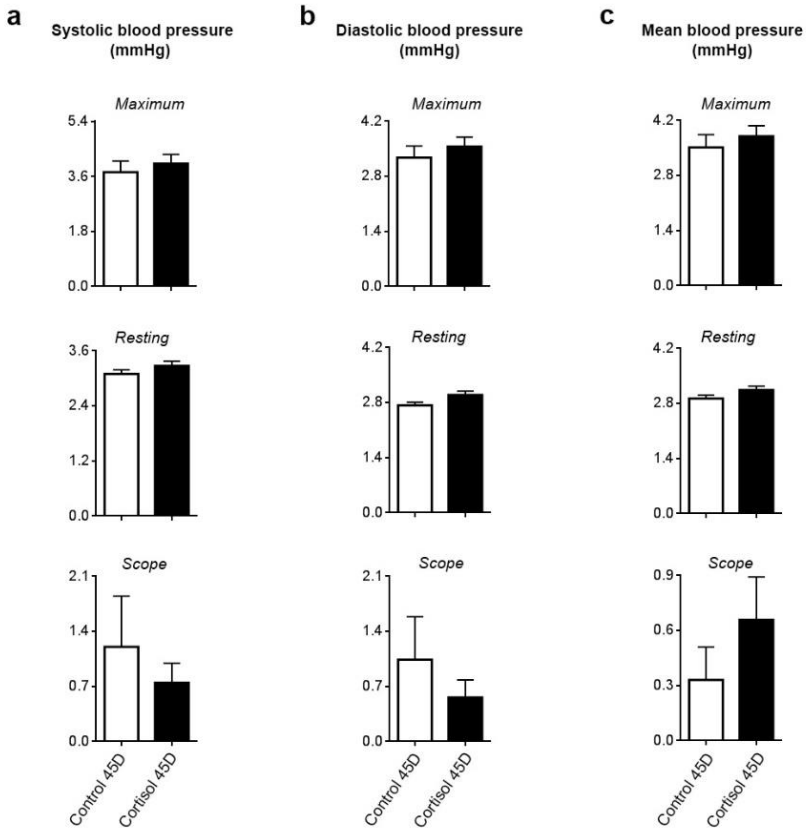

Fig. S2. Effects of chronic cortisol administration on blood pressure in rainbow trout. Maximum (A, upper panel, ncontrol=11, ncortisol=10) and resting (A, middle panel, ncontrol=9, ncortisol=10) systolic blood pressure, systolic blood pressure scope (A, lower panel, ncontrol=9, ncortisol=10), maximum (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 ± s.e.m. Statistical differences were tested by unpaired t-tests.

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