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RESEARCH ARTICLE

Cortisol response to stress is associated with myocardial remodeling in salmonid fishes

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SUMMARY

Cardiac disease is frequently reported in farmed animals, and stress has been implicated as a factor for myocardial dysfunction in commercial fish rearing. Cortisol is a major stress hormone in teleosts, and this hormone has adverse effects on the myocardium. Strains of rainbow trout (*Oncorhynchus mykiss*) selected for divergent post-stress cortisol levels [high responsive (HR) and low responsive (LR)] have been established as a comparative model to examine how fish with contrasting stress-coping styles differ in their physiological and behavioral profiles. We show that the mean cardiosomatic index (CSI) of adult HR fish was 34% higher than in LR fish, mainly because of hypertrophy of the compact myocardium. To characterize the hypertrophy as physiological or pathological, we investigated specific cardiac markers at the transcriptional level. HR hearts had higher mRNA levels of cortisol receptors (*MR*, *GR1* and *GR2*), increased *RCAN1* levels [suggesting enhanced pro-hypertrophic nuclear factor of activated T-cell (NFAT) signaling] and increased *VEGF* gene expression (reflecting increased angiogenesis). Elevated collagen (*Col1a2*) expression and deposition in HR hearts supported enhanced fibrosis, whereas the heart failure markers *ANP* and *BNP* were not upregulated in HR hearts. To confirm our results outside the selection model, we investigated the effect of acute confinement stress in wild-type European brown trout, *Salmo trutta*. A positive correlation between post-stress cortisol levels and CSI was observed, supporting an association between enhanced cortisol response and myocardial remodeling. In conclusion, post-stress cortisol production correlates with myocardial remodeling, and coincides with several indicators of heart pathology, well-known from mammalian cardiology.

Key words: brown trout, cortisol, hypertrophy, myocardial remodeling, rainbow trout, stress coping style.

INTRODUCTION

The salmonid heart demonstrates a high degree of plasticity, both anatomically and physiologically, in response to environmental changes (Gamperl and Farrell, 2004). It is composed of an inner spongious myocardium, which is supplied with oxygen from venous blood returning to the heart, and an outer compact myocardium that contains coronary blood vessels delivering oxygenated blood from the gills (Pieperhoff et al., 2009). Plastic changes can involve cardiomyocyte hypertrophy and hyperplasia of both compartments (Gamperl and Farrell, 2004), and this is associated with an increased risk of myocardial remodeling and dysfunction, which is an increasing problem in farmed salmonids (Poppe and Taksdal, 2000; Poppe et al., 2002; Poppe et al., 2003; Takle et al., 2006). Myocardial dysfunction is also a problem in other farmed animals, including broiler chickens, where myocardial remodeling and failure is a leading cause of death (Olkowski et al., 1996). The underlying causes of pathological remodeling in fish have not been determined.

Stress can be defined as a condition in which a threat to the biological functions of an organism is perceived by that organism and a set of physiological and behavioral responses are mounted to counteract this challenge. Severe stress is clearly associated with a poor prognosis in individuals with established cardiac pathology and disease, including in humans (Engel, 1971; Meerson, 1994; Maxwell and Robertson, 1998; Brocklebank and Raverty, 2002; Poppe et al., 2007). Still, the mechanism linking stress responsiveness to the development of cardiac disease is poorly understood in fish. In particular, the association between stress and cardiac remodeling has previously not been addressed in salmonid fish, of which approximately two million tons are processed per year in the rapidly developing global aquaculture industry (Food and Agriculture Organization, 2010).

Cortisol is the major steroid stress hormone in salmonid fishes and humans. It has diverse effects on several tissues, including the myocardium. The effects of cortisol in salmonid fish are mediated through both the mineralcorticoid receptor (*MR*) and glucocorticoid receptors 1 and 2 (*GR1* and *GR2*) (Colombe et al., 2000; Bury et al., 2003). Previous work has shown these receptors to be abundantly expressed in the myocardium of teleosts, including the rainbow trout (Greenwood et al., 2003; Sturm et al., 2005). However, a direct relationship between cortisol stress response and myocardial morphology and function has previously not been addressed. In contrast to mammals, where aldosterone is an important hormone

in myocardial remodeling (Funder, 2001; Rocha et al., 2002; Qin et al., 2003), most teleosts, including salmonids, do not produce aldosterone (Bern, 1967; Sangalang and Uthe, 1994), and mineralcorticoid functions are instead mediated by cortisol (Bern and Madsen, 1992; Wendelaar Bonga, 1997). Furthermore, in mammals, glucocorticoids like cortisol directly induce protein synthesis and hypertrophy of cardiomyocytes *in vivo* and *in vitro* (Nichols et al., 1984; Lumbers et al., 2005), and plasma cortisol levels have been found to represent an independent risk factor of cardiac events and death (Yamaji et al., 2009).

Accordingly, to examine the effect of stress and cortisol on myocardial morphology and function, we examined cardiac structure and gene expression in the hearts of two genetically distinct strains of rainbow trout (*Oncorhynchus mykiss* Walbaum 1792) that respond to stress with either a high [high responsive (HR)] or low [low responsive (LR)] cortisol production (Pottinger and Carrick, 1999). We hypothesized that adult HR fish, consistently responding to stress with high serum cortisol, would have bigger hearts than LR fish. However, as cardiac remodeling may either be physiological or pathological in mammals, we also aimed to assess whether stress responsiveness and hence consistently different cortisol exposure throughout life would induce signs of pathology in fish. We were especially interested in genes mediating the response to cortisol, particularly those that are linked to vascularization, fibrosis and cardiac hypertrophy. Finally, we investigated whether a trait correlation of post-stress cortisol response and heart size also existed outside of the selected LR and HR trout lines. To this end, we examined heart size and cortisol responsiveness in wild-type European brown trout (*Salmo trutta* Linnaeus 1758).

MATERIALS AND METHODS

HR and LR strains of rainbow trout

The selection regime initiated at Windermere Laboratory, NERC Institute of Freshwater Technology, UK, generating the two lines of rainbow trout (LR and HR), has been described in detail previously (Pottinger and Carrick, 1999). In short, the parental generation of HR and LR fish was established on the basis of consistent divergence in plasma cortisol responses following repeated stress testing (3 h of confinement stress once a month for five consecutive months). The crosses between the selected parents were carried out and the F1 generation hatched in 1997. The F4 generation of LR and HR rainbow trout was hatched in spring 2006 and transported to the Norwegian Institute for Water Research Marine Research Station (Solbergstrand, Norway) in December 2007. The fish were then mixed and reared in two 1000 l fiberglass tanks until sampled as fully adult sexually mature individuals 2 years later (January 2010). At the time of sampling, all LR and HR trout were 40 months old and had a mean (±s.d.) body length of 47.5±5.3 and 49.2±4.8 cm, respectively. Only mature females were available for this study. The transport and study procedures of the experimental animals were reviewed and approved by the Norwegian Food Safety Authority (www.mattilsynet.no) and the Norwegian Animal Research Authority (http://www.fdu.no/fdu/), respectively.

Confinement stress

Female European brown trout hatched at Aqua Center Boračko Lake, Konjic, Bosnia and Herzegovina, were used for these experiments. The parent generation consisted of wild adult endemic brown trout caught in the River Neretva drainage. At the onset of the experiment, the female fish were 21 months old and had a mean body length of 25.9±1.7 cm. Brown trout were transferred to 50 l aquaria and overhead windows provided light. Light tubes situated 40 cm above each aquarium were turned on 15 min after sunrise every day to provide additional light for behavioral observations. The artificial light was turned off 15 min prior to sunset. During the experimental period (10 March–25 June), photoperiod increased by 10 min per day at the experimental location (Boračko Lake). The fish were hand fed commercial food pellets daily. On day 16 the experimental fish were subjected to standardized confinement stress essentially as described by Øverli et al. (Øverli et al., 2006). In brief, fish were placed in pierced 1.5 or 1.9 l plastic boxes (Gefrier Box Frosty, PLAST TEAM GmbH, Flensburg, Germany) adjusted to the size of the fish (1.5 l boxes for fish ranging from 20 to 25 cm and 1.9 l boxes for fish ranging from 25 to 30 cm). The boxes were submerged in water in the aquarium for 2 h. The experimental model was approved by the State Veterinary Office of Bosnia and Herzegovina (http://www.vet.gov.ba).

Sampling and imaging of trout hearts

HR and LR trout were anaesthetized in benzocaine (1.5 ml l^{-1} Benzoak®, A.C.D. SA, Braine-l'Alleud, Belgium) and body length was measured (cm) before the fish were killed by decapitation. Three LR and three HR hearts were surgically excised, the bulbus and atrium removed, and the ventricles placed in 4% paraformaldehyde (PFA) (Electron Microscopy Sciences, Hatfield, PA, USA) for histochemistry analysis. Six LR and six HR hearts were sampled for gene expression analysis and the ventricle was weighed before being cut into smaller pieces, which were put on RNAlater® (Ambion, Austin, TX, USA) and stored at –20°C.

Following confinement stress, 27 brown trout were anaesthetized in benzocaine (1.5 ml l^{-1}) and body length was measured (cm) before the fish were killed by decapitation. A blood sample was taken and the blood was immediately spun (3 min at 13,000 ***g***). Plasma was frozen and stored at –20°C for subsequent cortisol analysis. The hearts were excised and the atrium and bulbus removed before the ventricles were blotted dry and weighed (g). The cardiosomatic index (CSI) was determined by calculating the ratio of ventricle mass to fish length (g cm^{-1}). Body length, rather than body mass, was used to calculate CSI because several fish had running eggs upon netting from the holding tank. 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).

Cortisol analysis

Plasma cortisol levels from the brown trout were analyzed using a specific radioimmunoassay (RIA) essentially as described previously (Pottinger and Carrick, 2001). In short, ethyl acetate (Merck Chemicals, Darmstadt, Germany) was used for cortisol extraction. Before the donkey anti-cortisol antibody was added (1:6000 dilution, AbD Serotec, Dusseldorf, Germany), 60 Ci mmol l^{-1} [1,2,6,7–^{3}H] cortisol (Amersham Pharmacia Biotech, Little Chalfront, UK) was added to all samples, standards and controls. Samples were tested against a standard curve made with inert cortisol (Sigma Aldrich, St Louis, MO, USA) in scintillation fluid (Ultima Gold, Perkin Elmer, Waltham, MA, USA) on a Packard Tri-Carb A1900 TR liquid scintillation analyzer (Packard Instrument, Meriden, CT, USA). Cortisol concentrations were calculated from the equation of a three-parameter hyperbolic function fitted to a plot of the percentage of [^{3}H] cortisol bound against inert cortisol using Sigmaplot 11 (SPSS Science, Systat Software Inc., San Jose, CA, USA).

Histochemical AFOG staining and imaging

Rainbow trout hearts stored in 4% PFA were cut into thick slices using a razor blade. These slices were further fixed in fresh 4% PFA at 4°C before embedding in paraffin. Dewaxed sections of 5 μm

thickness were subjected to Acid-Fuchsin-Orange G (AFOG) staining. AFOG sections were scanned or micrographed using an Axio Scope with a 5× objective (Zeiss, Jena, Germany), and images were processed in Adobe Photoshop CS3.

Calculation of compact myocardium area

Scanned AFOG-stained ventricular sections from rainbow trout hearts were processed using ImageJ (NIH, Bethesda, MD, USA). In brief, color images were converted into 8-bit grayscale, pixels scaled to mm, and brightness and contrast adjusted before edge detection was applied, allowing area calculation in mm^2 of the two muscular layers – the compact and spongious myocardium. The area of compact myocardium was divided by total area, giving the relative area of compact myocardium.

RNA extraction and qRT-PCR analysis

The hearts stored 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 heart was thoroughly mixed before 280 mg were put into a 15 ml plastic tube and stored at –80°C for a maximum of 4 days. RNA was extracted from heart tissue 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). RINs for the heart samples ranged from 9.50 to 9.90 with a mean (±s.d.) of 9.7±0.03, 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 $dT_{12–18}$ primers synthesized by Invitrogen.

The selected cardiac marker genes used in this study are presented in Table 1. Gene-specific primers 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*, also called *CRP*), regulator of calcineurin 1 (*RCAN1*, also called *MCIP*), mineralcorticoid receptor (*MR*), glucocorticoid receptor 1 and 2 (*GR1* and *GR2*), vascular endothelial growth factor (*VEGF*), collagen alpha 2 (1) (*COL1a2*), collagen alpha 1 (1) (*COL1a1*), A-type natriuretic peptide (*ANP*) and B-type natriuretic peptide (*BNP*) were designed using the web-based Primer3 program (http://frodo.wi.mit.edu/primer3/) and synthesized by Invitrogen. The housekeeping gene β-actin was chosen as the internal control gene. GenBank accession numbers for the genes whose sequences were retrieved from NCBI (www.ncbi.nlm.nih.gov/) are listed in Table 1. Rainbow trout *RCAN1* was retrieved at the Dana-Farber Cancer Institute database (http://compbio.dfci.harvard.edu) based on a BLAST with *Danio rerio RCAN1* found in the NCBI database (GenBank accession no. BC076439.1). A minimum of five primer

Table 1. Specific marker genes with primers used for quantitative real-time PCR

Gene	Primer pair	GenBank accession number	Function/marker
β-actin	F: AGCCCTCCTTCCTCGGTAT R: AGAGGTGATCTCCTTGTGCATC	NM001124235.1	Housekeeping gene
PCNA	F: AGCAATGTGGACAAGGAGGA R: GGGCTATCTTGTACTCCACCA	EZ763721.1	Cardiomyocyte hyperplasia
VMHC	F: TGCTGATGCAATCAAAGGAA R: GGAACTTGCCCAGATGGTT	AY009126.1	Cardiomyocyte hypertrophy
SMLC2	F: TCTCAGGCGGACAAGTTCA R: CGTAGCACAGGTTCTTGTAGTCC	NM001124678.1	Cardiomyocyte hypertrophy
MLP	F: AGTTCGGGGACTCGGATAAG R: CGCCATCTTTCTCTGTCTGG	NM001124725.1	Cardiomyocyte hypertrophy
RCAN1	F: AGTTTCCGGCGTGTGAGA R: GGGGACTGCCTATGAGGAC	BC076439.1 (*Danio rerio*)*	NFAT-activity/pathological cardiomyocyte hypertrophy
MR	F: CAGCGTTTGAGGAGATGAGA R: CCACCTTCAGAGCCTGAGAC	AY495581.1	Cortisol sensitivity
GR1	F: AGGTTGTCTCAGCCGTCAAA R: GCAGCTTCATCCTCTCATCAT	NM001124730.1	Cortisol sensitivity
GR2	F: ACTCCATGCACGAGATGGTT R: CGGTAGCACCACACAGTCAT	NM001124482.1	Cortisol sensitivity
VEGF	F: AGTGTGTCCCCACGGAAA R: TGCTTTAACTTCTGGCTTTGG	AJ717301.1	Angiogenesis
COL1a2	F: GGTTCGGCGAGACCATTA R: GTTGTGTGGCCATGCTCTG	NM001124207.1	Fibrosis
COL1a1	F: CGCTTCACATACAGCGTCAC R: AATGCCAAATTCCTGATTGG	NM001124177.1	Fibrosis
ANP	F: CCACAGAGGCTCTCAGACG R: ATGCGGTCCATCCTAGCTC	NM001124211.1	Heart failure
BNP	F: TGGCCTTGTTCTCCTGTTCT R: GGAGACTCGCTCAACCTCAC	NM001124226.1	Heart failure

F, forward primer 5′→3′; R, reverse primer 5′→3′.
For full gene names, see List of symbols and abbreviations.
*The rainbow trout *RCAN1* sequence was found by BLAST with *Danio rerio RCAN1* (see Materials and methods for details).

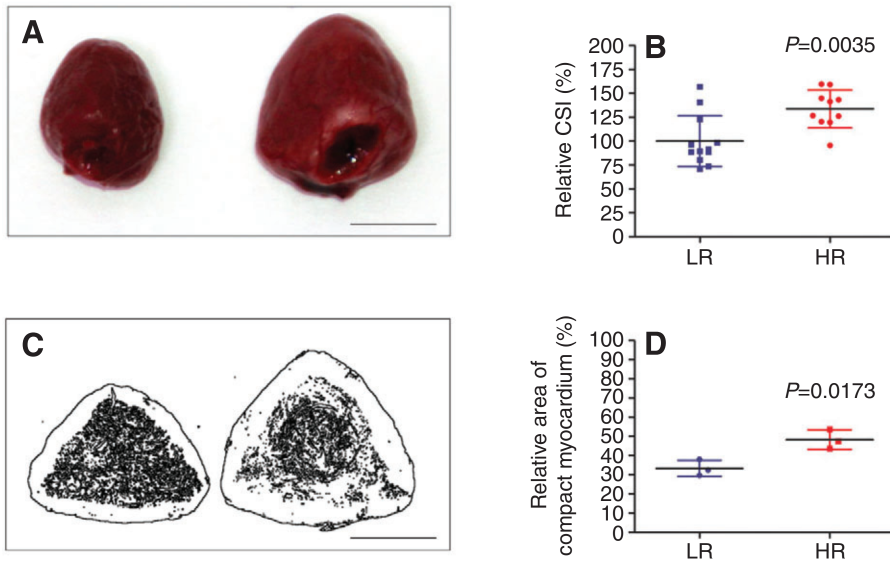

Fig. 1. High responsive (HR) fish have bigger ventricles and more compact myocardium than low responsive (LR) fish. (A) Representative image of ventricles of size-matched (47 cm) LR (left) and HR (right) rainbow trout. Scale bar, 1 cm. (B) Relative cardiosomatic index (CSI) (g cm^{-1}) of LR (N=12) and HR (N=10) fish, shown as one graphical point per fish and as means ± s.e.m. (% relative to LR). (C) Representative image of ventricular sections of size-matched (47 cm) LR (left) and HR (right) fish converted to black-and-white, showing the outer compact and the inner spongious myocardium. Scale bar, 1 cm. (D) Area of compact myocardium relative to spongious myocardium (%) in LR and HR ventricle sections shown as one graphical point per fish and as means ± s.e.m. (N=3). Statistical differences were tested using an unpaired t-test and P-values are indicated.

pairs were designed at exon junctions for each gene and the primers showing the lowest crossing point values, a single peak melting curve and amplification of the right amplicon were chosen (Table 1). The quantitative real-time (qRT) PCR products were also sequenced to verify that the primers amplified the right cDNA.

Statistical analysis

Data are expressed at single observation points or as group means ± s.e.m. The mean CSI of LR fish was normalized to 100%. mRNA levels and differences in CSI and area of compact myocardium between the HR and LR groups were examined using the Student's t-test, and the association between CSI and post-stress plasma cortisol was assessed by linear regression analysis (least-squares method) with Pearson's product-moment correlation coefficient as a measure of the resulting linear relationship. mRNA expression levels are presented as normalized values to LR mean (fold change), and differences were tested using an unpaired t-test. P-values <0.05 were considered statistically significant. All statistical analyses were performed in GraphPad Prism 5 (GraphPad Software, San Diego, CA, USA).

RESULTS

HR fish have larger ventricles and compact myocardium than LR fish

To examine the relationship between selection for post-stress plasma cortisol levels and heart size, freshly excised ventricles from adult HR and LR fish were weighed and CSI was calculated. The CSI of HR fish was 33.70±6.26% higher than that of LR fish (100.00±7.67%, P=0.0035; Fig. 1A,B). To evaluate the structural basis for the increased relative heart size of HR fish, sections of the ventricles were converted into grayscale images to distinguish the compact and spongious muscular layers. The relative area of compact myocardium in HR ventricles was 48.40±2.95% whereas that of LR fish was 33.40±2.44% (P=0.0173; Fig. 1C,D).

Markers of cardiomyocyte hypertrophy are upregulated in ventricles of HR fish

To evaluate whether the increased heart size in the HR fish was a result of myocyte hypertrophy or hyperplasia, markers of the two processes were measured by qRT-PCR. *PCNA* expression was 1.39±0.15-fold higher in HR ventricles compared with LR ventricles

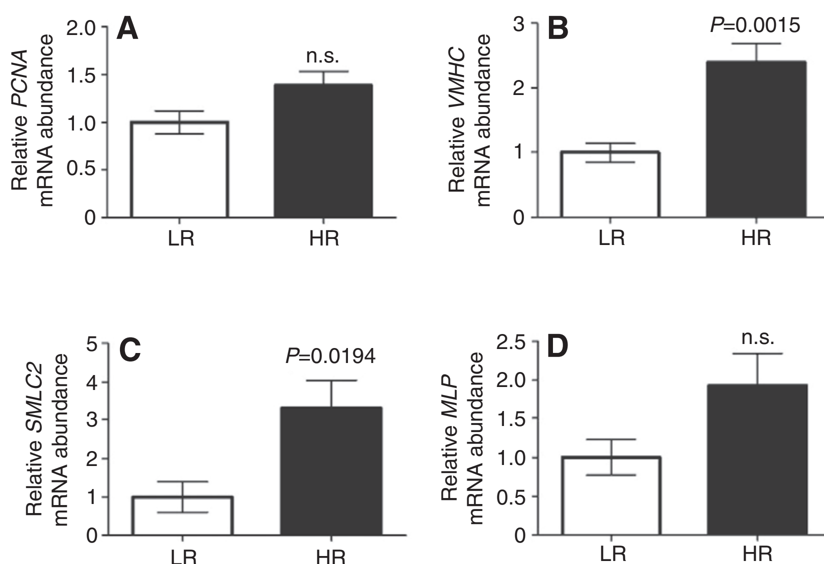

Fig. 2. Markers of hypertrophy, but not of hyperplasia, are upregulated in ventricles of HR rainbow trout. Relative mRNA expression levels of (A) proliferating cell nuclear antigen (*PCNA*) and the hypertrophic markers (B) ventricular myosin heavy chain (*VMHC*), (C) slow myosin light chain 2 (*SMLC2*) and (D) muscle LIM protein (MLP) relative to the standard gene β-actin in HR and LR ventricles. Data are means ± s.e.m. relative to LR expression (N=6). Statistical differences were tested using an unpaired t-test and P-values are indicated. n.s., not significant.

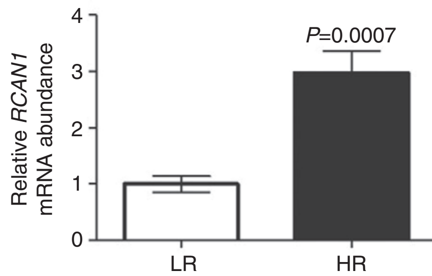

Fig. 3. Pro-hypertrophic nuclear factor of activated T-cell (NFAT) signaling is upregulated in the HR rainbow trout ventricle. mRNA expression level of NFAT-regulated regulator of calcineurin 1 (*RCAN1*) gene relative to the standard gene β-actin in HR and LR ventricles. Data are means ± s.e.m. relative to LR expression (*N*=6). Statistical difference was tested using an unpaired *t*-test and the *P*-value is indicated.

(1.00±0.12); however, this result was not statistically significant (*P*=0.07; Fig. 2A). In contrast, there was a 2.40±0.29-fold higher expression of *VMHC* in HR ventricles compared with LR ventricles (1.00±0.14, *P*=0.0015) and a 3.31±0.73-fold higher expression of *SMLC2* in HR ventricles compared with LR ventricles (1.00±0.40, *P*=0.0194; Fig. 2B,C). MLP expression was also 1.93±0.41-fold higher in HR ventricles than in LR ventricles (1.00±0.23); however, this finding was not significant (*P*=0.0763; Fig. 2D).

Pro-hypertrophic NFAT signaling is upregulated in the HR fish ventricle

To further examine the cardiac hypertrophy of the HR fish, we assessed whether the calcineurin–NFAT pathway was activated, as this signaling pathway is one of the major ones involved in mammalian pathological hypertrophy. The mRNA expression level of the NFAT target gene *RCAN1* was 2.96±0.38-fold higher in HR ventricles compared with LR ventricles (1.00±0.14, *P*=0.0007; Fig. 3).

Vascularization is increased in the HR fish ventricle

Visual investigation of surface vessels of HR and LR ventricles clearly revealed a higher degree of vascularization of HR ventricles (representative image in Fig. 4A), but this difference was not quantified. In line with this observation, *VEGF* expression was 2.66±0.19-fold greater in HR ventricles compared with LR ventricles (1.00±0.14, *P*<0.0001; Fig. 4B).

Increased collagen deposition and expression indicates enhanced fibrosis in the ventricles of HR fish

Excessive fibrosis is a hallmark of pathological myocardial remodeling in mammals, and to examine fibrosis in the trout hearts we stained ventricular sections histochemically with a marker for collagen depositions (AFOG) and measured Col 1a2 and Col 1a1 mRNA expression levels. There was moderate staining for collagen in all examined ventricles of HR fish (*N*=3), while no staining was observed in the LR ventricles (*N*=3; Fig. 5A left panel). Magnification of the HR ventricle revealed areas with (Fig. 5A, inset 1) or without (Fig. 5A, inset 2) collagen depositions, indicating focal fibrosis and disruption of muscle structure in the HR heart. Further supporting enhanced fibrosis in HR hearts, *COL1a2* mRNA levels were 2.16±0.36-fold higher in HR ventricles compared with LR ventricles (1.00±0.21, *P*=0.0207; Fig. 5B). We also observed a 1.84±0.39-fold higher expression of *COL1a1* in HR ventricles compared with LR ventricles (1.00±0.17), but this was not statistically significant (*P*=0.0770; Fig. 5C).

ANP and *BNP* mRNA levels are not increased in ventricles of HR fish

To assess further signs of remodeling in HR hearts, we measured expression of *ANP* and *BNP*, which are sensitive markers of heart failure in mammals. We found no significant differences in either *ANP* (1.35±0.26 HR *vs* 1.00±0.30 LR, *P*=0.4019; Fig. 6A) or *BNP* mRNA expression levels (0.90±0.10 HR *vs* 1.00±0.17 LR, *P*=0.6401; Fig. 6B).

Myocardial cortisol receptors are increased in the ventricles of HR fish

To assess whether differences in post-stress cortisol levels are accompanied by differences in the expression of myocardial cortisol receptors, we measured *MR*, *GR1* and *GR2* mRNA levels in ventricles of HR and LR fish. HR fish, which consistently respond to stress with high plasma cortisol (Pottinger and Carrick, 1999; Pottinger and Carrick, 2001; Trenzado et al., 2003; Schjolden et al., 2006), displayed higher levels of all three receptors in the ventricle compared with LR fish (Fig. 7A–C). Specifically, the expression level of *MR* was 2.08±0.24-fold higher in HR ventricles than in LR ventricles (1.00±0.05, *P*=0.0012; Fig. 7A), that of *GR1* was 2.36±0.24-fold higher (*vs* 1.00±0.09, *P*=0.0003; Fig. 7B) and that of *GR2* was 2.19±0.10-fold higher (*vs* 1.00±0.08, *P*<0.0001; Fig. 7C).

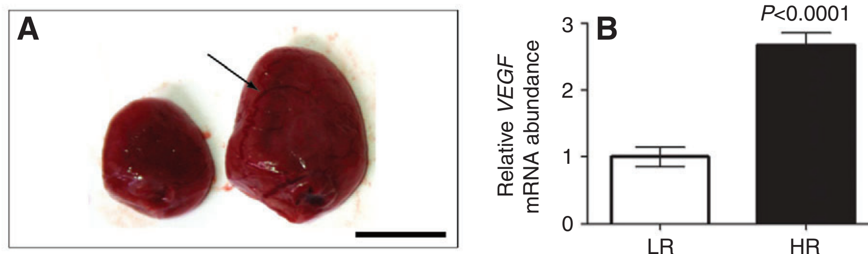

Fig. 4. Vascularization is increased in the HR rainbow trout ventricle. (A) Representative image of ventricles of size-matched (47 cm) LR (left; *N*=12) and HR (right; *N*=10) fish. Arrow indicates extensive vascularization of the HR ventricle. Scale bar, 1 cm. (B) mRNA expression level of the angiogenic marker vascular endothelial growth factor (*VEGF*) relative to the standard gene β-actin in HR and LR ventricles, presented as means ± s.e.m. relative to LR expression (*N*=6). Statistical difference was tested using an unpaired *t*-test and the *P*-value is indicated.

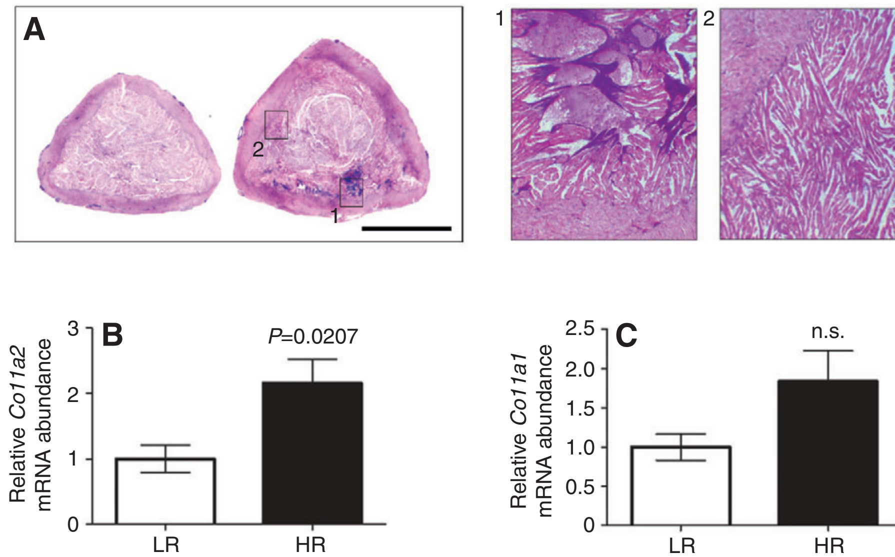

Fig. 5. Increased collagen deposition and expression in ventricles of HR rainbow trout. (A) Representative image of Acid-Fuchsin-Orange G (AFOG)-stained ventricular sections of size-matched (47 cm) LR (left) and HR (right) fish (N=3). AFOG staining is highly sensitive for collagen depositions (red/purple, fibrin; blue, collagen). Scale bar, 1 cm. Outlined areas in the HR ventricle with (1) or without (2) collagen depositions are magnified. mRNA expression level of the fibrotic markers (B) collagen alpha 2(1) (*COL1a2*) and (C) collagen alpha 1(1) (*COL1a1*) relative to the standard gene β-actin in HR and LR ventricles. Data are means ± s.e.m. relative to LR expression (N=6). Statistical differences were tested using an unpaired *t*-test and *P*-values are indicated. n.s., not significant.

Post-stress cortisol response is positively correlated to CSI in wild-type brown trout

Finally, to validate our results from the HR–LR selection model in wild-type trout, plasma cortisol levels were measured and CSI was calculated in a European brown trout population using an established stress model (acute confinement stress). There was a positive correlation between CSI and post-confinement plasma cortisol in these wild-type fish (R^2=0.347, P=0.0012, N=27; Fig. 8), supporting our finding of an association between stress responsiveness and myocardial remodeling in HR and LR fish.

DISCUSSION

Our results show that fish responding to stress with high post-stress plasma cortisol levels had significantly bigger hearts than fish responding with low cortisol levels, and this phenomenon was observed in both a genetic selection model of rainbow trout and in wild-type brown trout. Immunohistochemical studies suggested that this was a result of growth of the compact myocardium, as this area, relative to the spongious myocardium, was bigger in HR ventricles compared with LR ventricles. Such an increase in compact myocardium is also seen during ontogenetic growth in salmonid fishes, during which the compact layer increases in both amount and thickness (Poupa et al., 1974; Farrell et al., 1988).

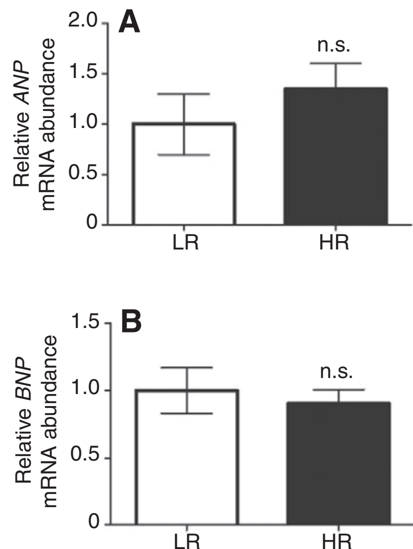

Fig. 6. No increase in the heart failure biomarkers *ANP* and *BNP* in ventricles from HR rainbow trout. mRNA expression level of the heart failure markers (A) A-type natriuretic peptide (*ANP*) and (B) B-type natriuretic peptide (*BNP*) relative to the standard gene β-actin in HR and LR ventricles. Data are means ± s.e.m. relative to LR expression (N=6). Statistical differences were tested using an unpaired *t*-test. n.s., not significant.

In mammals, under certain physiological (i.e. training or pregnancy) or pathological (i.e. hypertension or myocardial infarction) conditions, cardiomyocytes may undergo hypertrophy, which leads to increased muscular mass. Although recent findings suggest that cardiomyocytes within the diseased adult human heart can proliferate (Beltrami et al., 2001), most evidence to date indicates that myocyte proliferation is not a significant component of the mammalian cardiac growth response (Pasumarthi and Field, 2002). In adult teleostean hearts, including in rainbow trout, previous studies have shown that ventricular growth occurs through both hyperplasia and hypertrophy (Farrell et al., 1988; Clark and Rodnick, 1998; Poss et al., 2002). In the current study, there was a consistent upregulation of genes involved in the hypertrophic gene program. For instance, mRNA levels of commonly used hypertrophy markers in mammals (*RCAN1*, *SMLC2* and the fish homologue of β-MHC, *VMHC*) (Swynghedauw, 1999; Lim et al., 2001) were significantly higher in HR ventricles compared with LR ventricles. Furthermore, although the hypertrophy marker *MLP* did not differ significantly between LR and HR ventricles, it displayed a trend towards being higher in the HR ventricles. We also found a trend towards a higher expression of the proliferation marker *PCNA* (Yu et al., 1992; Köhler et al., 2005), indicating increased cell proliferation in the HR ventricles compared with LR ventricles. Nevertheless, based on the combined gene expression profile of the current study, we believe that the bigger relative heart size in HR fish is primarily due to hypertrophy of existing compact cardiomyocytes. In cold-acclimated rainbow trout, Vornanen et al. observed an increase in heart size of 33% (Vornanen et al., 2005) that was, similar to the results of our study, associated with increased expression of *VMHC* and *SMLC2*. Furthermore, they reported a fivefold increase in the expression of *MLP* mRNA. The authors interpreted these results as an activation of genes associated with adaptive cardiac hypertrophy without the

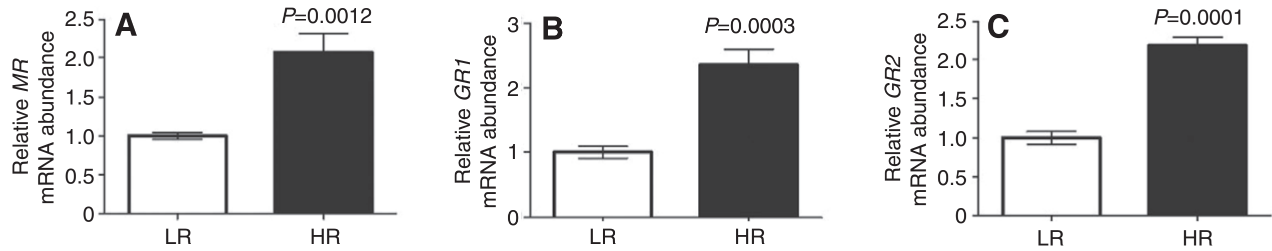

Fig. 7. Cortisol receptors are upregulated in ventricles of HR rainbow trout. mRNA expression level of (A) mineralcorticoid receptor (*MR*), (B) glucocorticoid receptor 1 (*GR1*) and glucocorticoid receptor 2 (GR2) relative to the standard gene β-actin in HR and LR ventricles. Data are means ± s.e.m. relative to LR expression (*N*=6). Statistical differences were tested using an unpaired *t*-test and *P*-values are indicated.

pathological features that impair the function of the hypertrophic mammalian heart. Fish hearts can undergo hypertrophy (and hyperplasia) as a routine remodeling mechanism (Farrell et al., 1988). Therefore, as opposed to mammals, where cardiac hypertrophy is often associated with a failing heart (Lloyd-Jones et al., 2002), a bigger fish heart is not necessarily detrimental. For example, an increased ventricular mass in cold-acclimated rainbow trout serves to offset the reduction in cardiac power output observed with acute decreases in temperature, and also enhances stroke volume (Graham and Farrell, 1989). Also, in the growing eel (*Anguilla anguilla* L.), structural remodeling of the heart, such as growth of the compact myocardium, is associated with enhanced mechanical performance (Cerra et al., 2004).

The calcium/calcineurin-regulated *NFATc* family is thought to have arisen following recombination approximately 500 million years ago, producing a new group of signaling and transcription factors (the *NFATc* genes) found only in genomes of vertebrates. It has been proposed that recombination enabled Ca^{2+} signals to be redirected to a new transcriptional program, which provided part of the groundwork for vertebrate morphogenesis and organogenesis, including the cardiovascular system (Wu et al., 2007). Calcineurin–NFAT signaling was first shown by Molkentin et al. (Molkentin et al., 1998) to be important in development of cardiac hypertrophy, and today extensive evidence exists showing that this intracellular signaling pathway is essential and is activated in pathological hypertrophy only (Wilkins et al., 2004). As the *NFATc* proteins are transcription factors, their activity can be measured by

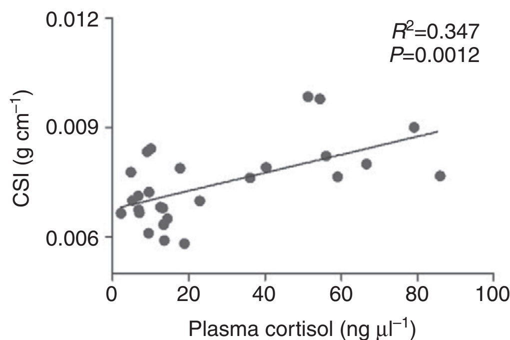

Fig. 8. Post-stress cortisol response is positively correlated to cardiosomatic index in wild-type brown trout. Post-stress plasma cortisol (ng µl^{-1}) was measured after confinement of wild-type brown trout (*N*=27) and was correlated to the cardiosomatic index (CSI) (heart mass/body length, g cm^{-1}) for each individual fish (one point represents one fish). The statistical analysis was performed using linear regression analysis (least-squares method) with Pearson's product-moment correlation coefficient as a measure of the resulting linear relationship.

expression of target genes. *RCAN1* is known to be a direct *NFATc* target in the heart (Rothermel et al., 2003; Oh et al., 2010). In this study we showed that HR hearts, which are bigger than LR hearts mainly because of increased hypertrophy of the compact myocardium, had almost threefold higher *RCAN1* expression. Such an increase in *RCAN1* expression indicates increased NFAT signaling and hence that the growth of HR hearts has a pathological character. Alternatively, NFAT activation in fish may serve a more physiological role given their adaptive hypertrophic potential. To our knowledge, this is the first time NFAT activation, well known in the hypertrophic mammalian heart, has been shown to occur in fish. Our results thus imply that NFAT activation in cardiac hypertrophy is evolutionarily conserved between mammals and fish, indicating that fish can serve as models with biomedical relevance.

In mammals, catecholamines (CAs) are important mediators of stress-related cardiac remodeling as they induce myocardial hypertrophy and cardiomyocyte death. In fish, though, the effects of CAs on the myocardium seem less pronounced (Tota et al., 2010). As it has been shown that LR fish respond to stress with higher plasma levels of the CA epinephrine than HR fish (Schjolden and Winberg, 2007), and the HR hearts were bigger than LR despite lower post-stress plasma CA, we propose that cortisol constitutes the main stress-induced influence on the trout heart.

We found a higher expression of the angiogenic gene *VEGF* in the HR ventricles compared with the LR ventricles, which likely reflects the increased vascularization necessary to support the thicker layer of compact myocardium (Cerra et al., 2004), as this is the muscular layer vascularized by coronary vessels. Increased vascularization was indeed extensive when HR ventricles were visually examined.

The fibrillar collagen network serves as an elastic element in the heart. Structural remodeling of this network is, however, associated with pathological conditions such as fibrosis (Abrahams et al., 1987; Caulfield and Bittner, 1988). Fibrosis is the dominant histological reaction to injury in the mammalian heart and it adversely increases tissue stiffness and impairs normal cardiac function (Schnitt et al., 1993; Weber et al., 1994). In fish, though, collagen remodeling has been postulated to play an important role in the maintenance of mechanical cardiac performance (Cerra et al., 2004; Icardo et al., 2005). We also found enhanced collagen deposition in the HR ventricles compared with the LR ventricles, as evaluated by AFOG staining. Still, as the collagen depositions were non-homogenously distributed, revealing fibrosis and disruption of muscle structure in certain areas (see Fig. 5A), we believe this may still represent pathological collagen production, similar to what is found during pathological focal fibrosis in mammals. In adaptive cardiac hypertrophy in trout, however, collagen gene expression was reduced approximately fivefold (Vornanen et al., 2005). Thus, a higher

expression of *COL1a2* and a trend towards higher *COL1a1* in the HR hearts compared with the LR hearts, as observed in our study, indicates a different response than in adaptive cold-acclimation, and could be interpreted as related to pathological fibrosis.

Because expression of genes regulating hypertrophy and fibrosis could indicate pathological myocardial remodeling in the HR trout, we wanted to investigate the expression of *ANP* and *BNP*, which are commonly used markers of heart failure in mammals (Lerman et al., 1993; Maisel et al., 2002). Generally, little data is available concerning secretion of natriuretic peptides associated with cardiac growth and remodeling in non-mammalian species (Tota et al., 2010), and the regulation and/or function of *ANP* and *BNP* can be different in fish and mammals (Loretz and Pollina, 2000). For instance, the interplay between CA and natriuretic peptides seems to differ. As LR fish respond to stress with higher plasma CA (Schjolden and Winberg, 2007), this could have predicted differences in *ANP* and *BNP* production. Yet, the expression of *ANP* and *BNP* did not differ between LR and HR ventricles. Alternatively, assuming an upregulation of natriuretic peptides with heart failure in fish similar to that in mammals, our results indicate that the HR fish had not developed heart failure despite the indications of pathological remodeling.

A limiting factor determining the sensitivity of a cell to glucocorticoids is the intracellular concentration of glucocorticoid receptors (Vanderbilt et al., 1987; Dong et al., 1989). Hence, altering the expression of these receptors renders a tissue capable of adjusting the biological response according to the requirements of the environment. In rainbow trout, cortisol actions are mediated through three different glucocorticoid receptors, *GR1*, *GR2* and *MR* (Colombe et al., 2000; Bury et al., 2003). In the HR ventricles, expression of all three receptors was significantly higher than in the smaller LR ventricles. Thus, it is probable that increased cortisol receptor expression in HR fish makes them more sensitive to the actions of cortisol and that cortisol-dependent intracellular signaling is increased. In addition, these fish experience higher post-stress cortisol levels regularly throughout life, at least in stressful environments such as intensive aquaculture. This assumption implies a role for cortisol not only in cardiac hypertrophy of fish, but also as one of the causative factors of pathological heart conditions in the fish farming industry.

In mammals, the mineralcorticoid aldosterone has been shown to induce fibrosis (Funder, 2001). Also, mineralcorticoid-induced cardiac hypertrophy is associated with increased NFAT activity through increased calcineurin activity (Takeda et al., 2002; Tong et al., 2004). Salmonid fish, however, lack aldosterone (Sangalang and Uthe, 1994) and the main corticosteroid is thought to be cortisol, acting as both a mineralcorticoid and a glucocorticoid hormone (Bern and Madsen, 1992; Wendelaar Bonga, 1997). Thus, cortisol in fish might mimic the effect of aldosterone on the mammalian heart and induce fibrosis and increased NFAT activity, as reflected by increased *RCAN1* expression in the current study.

In our study, the HR–LR selection model provided a tool to study the effects of transiently elevated cortisol levels. However, the HR and LR rainbow trout were bred for several generations during the selection regime. Thus, founder effects, unique mutations or random genetic drift could in fact account for the observed divergence in CSI. Nevertheless, a positive correlation between cortisol responsiveness following stress and relative heart size was also revealed in wild-type European brown trout. This finding suggests an evolutionary conserved correlation between heart size and cortisol responsiveness and that this trait correlation is not an incidental artifact of the HR–LR selection regime. Indeed, a link between high cortisol responsiveness to stress and increased cardiovascular morbidity and mortality has also been reported in humans (Pedersen and Denollet, 2003).

In conclusion, high post-stress cortisol levels seem to be associated with cardiac remodeling and altered gene expression in salmonid fish. Although the significance of cortisol-related cardiac remodeling for the fish remains speculative, both hypertrophy (Lloyd-Jones et al., 2002) and high serum cortisol (Yamaji et al., 2009) have been identified as independent risk factors for adverse cardiac events in humans. Thus, a follow-up study should involve experiments to determine whether HR fish have impaired cardiac function and are more prone to stress-induced mortality. In aquaculture there is increasing worry about stress-induced mortality of fish carrying cardiac anomalies (Brocklebank and Raverty, 2002; Poppe et al., 2007); we suggest that cortisol-induced cardiac myocardial remodeling may be one of the explanatory factors.

LIST OF SYMBOLS AND ABBREVIATIONS

AFOG	acid-fuchsin-orange G
ANP	A-type natriuretic peptide
BNP	B-type natriuretic peptide
CA	catecholamine
COL1a1	collagen alpha 1(1)
COL1a2	collagen alpha 2(1)
CRP	cysteine rich protein
CSI	cardiosomatic index
GR1	glucocorticoid receptor 1
GR2	glucocorticoid receptor 2
HR	high responder
LR	low responder
MCIP	modulatory calcineurin-interacting protein 1
MLP	muscle LIM protein
MR	mineralcorticoid receptor
NFAT	nuclear factor of activated T-cell
PCNA	proliferating cell nuclear antigen
PFA	paraformaldehyde
qRT-PCR	quantitative real-time PCR
RCAN1	regulator of calcineurin 1
RIA	Radioimmunoassay
SMLC2	slow myosin light chain 2
VEGF	vascular endothelial growth factor
VMHC	ventricular myosin heavy chain
β-MHC	β-myosin heavy chain

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