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RESEARCH ARTICLE

It takes time to heal a broken heart: ventricular plasticity improves heart performance after myocardial infarction in rainbow trout, *Oncorhynchus mykiss*

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ABSTRACT

Coronary arteriosclerosis is a common feature of both wild and farmed salmonid fishes and may be linked to stress-induced cardiac pathologies. Yet, the plasticity and capacity for long-term myocardial restructuring and recovery following a restriction in coronary blood supply are unknown. Here, we analyzed the consequences of acute (3 days) and chronic (from 33 to 62 days) coronary occlusion (i.e. coronary artery ligation) on cardiac morphological characteristics and *in vivo* function in juvenile rainbow trout, *Oncorhynchus mykiss*. Acute coronary artery occlusion resulted in elevated resting heart rate and decreased inter-beat variability, which are both markers of autonomic dysfunction following acute myocardial ischemia, along with severely reduced heart rate scope (maximum–resting heart rate) relative to sham-operated trout. We also observed a loss of myocardial interstitial collagen and compact myocardium. Following long-term coronary artery ligation, resting heart rate and heart rate scope normalized relative to sham-operated trout. Moreover, a distinct fibrous collagen layer separating the compact myocardium into two layers had formed. This may contribute to maintain ventricular integrity across the cardiac cycle or, alternatively, demark a region of the compact myocardium that continues to receive oxygen from the luminal venous blood. Taken together, we demonstrate that rainbow trout may cope with the aversive effects caused by coronary artery obstruction through plastic ventricular remodeling, which, at least in part, restores cardiac performance and myocardium oxygenation.

KEY WORDS: Cardiac performance, Cardiac remodeling, Coronary arteriosclerosis, Myocardial infarction

INTRODUCTION

Salmonids are capable of high sustained swimming speeds, and the physiological potential for swimming performance is largely determined by the ability of the cardiorespiratory system for tissue oxygen delivery (Farrell, 2002a). Moreover, recent studies indicate that the ability of fish to withstand environmental perturbations, including hypoxia and warming, is closely linked to cardiac function (Ekström et al., 2017, 2018). Central to this ability to maintain cardiac function under metabolically demanding conditions is an adequate oxygen supply to the myocardium. The hearts of all teleost fishes receive oxygen either entirely or partly (e.g. in salmonids) from the O_2 present in the venous blood that diffuses into the inner spongy myocardial layer of the heart (Farrell et al., 2012; Tota, 1983; Tota et al., 1983). However, some fishes, including salmonids, also have an outer compact ventricular layer, which receives well-oxygenated blood via an arterially derived coronary circulation (Farrell et al., 2012; Tota, 1983; Tota et al., 1983). The relative amount of compact myocardium perfused by the coronary arteries varies from as low as 5% to as high as 70% in teleosts in general, and typically ranges from ∼20% to 50% of ventricular mass in salmonids (Farrell et al., 1988; Santer, 1985; Santer and Walker, 1980).

Both wild and farmed salmonids are predisposed to coronary arteriosclerosis. This involves a thickening of the myointimal layer, causing the inside of the arteries to become narrower, which may compromise coronary flow and myocardial oxygen delivery (Brijs et al., 2020; Farrell, 2002b; Poppe et al., 2002, 2003). In fact, coronary arteriosclerosis has been described as a 'fact of life' in salmonids because of the high prevalence and severity of arteriosclerosis in sexually mature fish (Eaton et al., 1984; Farrell et al., 1990; Mckenzie et al., 1978; Robertson et al., 1961; Van Citters and Watson, 1968). The occurrence of coronary artery lesions is high in first year migrating and spawning fish; however, despite studies suggesting that coronary artery degeneration may be reversible and not cumulative (Maneche et al., 1972; Van Citters and Watson, 1968), others have shown that the occurrence of coronary artery lesions appears to be a progressive condition following the first year of spawning (Kubasch and Rourke, 1990; Saunders and Farrell, 1988). Even so, severe forms of coronary artery lesions in salmonids may cause myocardial ischemia and potentially impair the ability to undertake spawning migrations (Farrell, 2002b), as well as to withstand stressful events (Kongtorp et al., 2004; Poppe et al., 2021) and environmental challenges (Morgenroth et al., 2021).

Experimental studies on rainbow trout and other salmonids have shown that acute coronary artery ligation (∼20–22 h) reduces aerobic scope (Ekström et al., 2018), as well as warming and hypoxia tolerance (Ekström et al., 2017; Ekström et al., 2019; Morgenroth et al., 2021). Moreover, acute blockade of the coronary supply typically impairs cardiac contractility, which is compensated for by increases in resting heart rate (f_H) to sustain routine cardiac output (Agnisola et al., 2003; Ekström et al., 2018; Morgenroth et al., 2021), but this comes at the cost of reduced f_H scope (maximum f_H–resting f_H; Ekström et al., 2018). While acute occlusion of coronary blood flow (i.e. from 24 h up to 5–7 days) did not affect swimming performance in rainbow trout (Daxboeck, 1982; Steffensen et al., 1998), an even shorter occlusion period (i.e. 8 h) resulted in a significant reduction in the maximum

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sustained swimming speed in chinook salmon (*Oncorhynchus tshawytscha*) (Farrell and Steffensen, 1987).

While experimental coronary artery occlusion in fish completely stops blood flow to the entire compact myocardium, it does not impact short-term survival although cardiorespiratory performance is generally impaired (Daxboeck, 1982; Ekström et al., 2018; Morgenroth et al., 2021; Steffensen et al., 1998). A remaining question, however, is to what extent the fish heart can remodel and recover function over time following coronary artery obstruction, and how the temporal dynamics of such potential remodeling manifests with regard to myocardial morphology and composition.

The fish heart is undoubtedly a highly plastic organ and some fish species (e.g. zebrafish) exhibit a remarkable capacity to reconstitute the myocardium with little or no scar tissue formation following mechanical injury (Chablais et al., 2011; González-Rosa and Mercader, 2012). Similarly, the rainbow trout heart is known to be highly phenotypically plastic and remodels across multiple levels of organization in response to environmental stimuli (Gamperl and Farrell, 2004; Johnston and Gillis, 2018; Keen et al., 2016, 2017; Klaiman et al., 2011). For instance, cardiac hypertrophy and increased myocardial interstitial collagen content occurs with cold acclimation in rainbow trout, which probably arises as a compensatory response triggered by the increased hemodynamic stress of pumping cold highly viscous blood, whereas warm acclimation evokes opposite responses (Keen et al., 2017).

In mammals, blockade of regional coronary blood flow (e.g. left anterior descending coronary artery ligation) can lead to significant changes in physiology and regional morphology of the heart (Frangogiannis, 2017). Cardiac repair following abolished coronary supply to the heart typically follows a series of time-dependent events, which are associated with an initial inflammatory reaction and subsequent scar tissue deposition at the site of infarction (Frangogiannis, 2017; Prabhu and Frangogiannis, 2016).

Healing and repair after myocardial infarction is considered a protective mechanism in the long term, yet it is also considered an important determinant of the adverse consequences that occur following myocardial infarction in mammals (Talman and Ruskoaho, 2016). In addition to the severe coronary artery lesions that may compromise myocardial oxygen supply in farmed and wild salmonids, numerous other diseases such as cardiomyopathy syndrome, heart and skeletal muscle inflammation and pancreas disease commonly cause myocardial lesions and injury, in addition to affecting the skeletal muscle, liver and pancreas (Frisk et al., 2020; Poppe et al., 2021; Yousaf et al., 2013).

In the present study, we subjected separate groups of rainbow trout to short-term (3 days, 'acute') and long-term (from 33 to 62 days, 'chronic') experimental coronary artery ligation to test the hypotheses that (i) histo-morphological myocardial alterations are associated with the acute impairment of cardiac function following coronary artery ligation and (ii) trout with ligated coronary arteries are able to remodel their hearts over time (i.e. up to 2 month) so that normal cardiac function is partially or fully restored. To address these hypotheses, we combined analyses of *in vivo* cardiac function including resting and maximum f_H, f_H scope and heart rate variability (HRV), along with detailed analyses of ventricular morphological characteristics to determine compact myocardium density and collagen content at the different time points.

MATERIAL AND METHODS

Experimental animals

Juvenile rainbow trout *Oncorhynchus mykiss* (Walbaum 1792) of both sexes (see Table 1 for biometrics) were obtained from a local fish farm (Vänneåns Fiskodling AB, Norekvarnsvägen, Knäred, Sweden). The fish were maintained in holding tanks supplied with recirculating aerated freshwater (10°C) under a 12 h:12 h photoperiod for at least 4 weeks prior to the initiation of the experiments. Fish were fed twice a week with commercial fish pellets. Ethical permit #165-2015 issued from the regional animal ethics committee in Gothenburg covered all experimental procedures.

Surgical procedures and experimental protocols

Fish were anesthetized in freshwater (10°C) containing MS-222 (tricaine methanesulfonate, 150 mg l^{-1}) buffered with $NaHCO_3$ (300 mg l^{-1}) and were then positioned on their left lateral side on wet foam on a surgery table. Anesthesia was maintained during surgery by irrigating the gills with 10°C water containing MS-222 (75 mg l^{-1}) and $NaHCO_3$ (150 mg l^{-1}). An incision was made in the isthmus to expose the coronary artery (see Ekström et al., 2017; Farrell and Steffensen, 1987). In one experimental group, the common coronary artery was ligated with a 6-0 silk suture (hereafter coronary-ligated group). A second group was treated identically except that the coronary artery was not ligated (sham-operated group). Electrocardiogram (ECG) electrodes were prepared by soldering 23-gauge hypodermic needles, with the beveled edge blunted, onto two insulated stainless steel wires. Two ECG electrodes were implanted subcutaneously close to the pectoral fins on each side of the heart and sutured in place. A third electrode (ground) was placed in the experimental water tank. In the acute groups, the ligation or sham procedure and the ECG electrode implantation were performed during the same surgery, and after leaving the fish undisturbed for 3 days to recover from surgery, the experiments were initiated. In the chronic group, the fish were returned to their holding tanks (freshwater at 10°C; 12 h:12 h photoperiod) and fed twice a week until ECG implantation, which was performed 30–59 days after the ligation/sham surgical procedure. To identify and distinguish between sham and coronary-ligated individuals in the chronic group, fish were color marked with elastomer tags (Northwest Marine Technology, Inc., Anacortes, WA, USA), which were injected subcutaneously into the periorbital region. After ECG implantation, the fish were held individually in opaque cylindrical plastic tubes (length 380 mm, diameter 90 mm) submerged in a holding tank (105 l) supplied with aerated 10°C freshwater for 3 days. The experiments were then initiated 3 days after ECG implantation. As we had the capacity to run experiments on a maximum of 8 fish per week, ECG data collection for the chronic groups occurred from day 33 to day 62 after the ligation/sham procedure. In all groups, the resting ECG was recorded for at least 4 h on the third day after ECG implantation. Subsequently, fish were placed in a separate circular tank (diameter 600 mm, 24 l) continuously supplied with 10°C freshwater, and were subjected to a chase stress protocol for a maximum of 5 min or until fatigue to elicit a maximum cardiorespiratory response (Clark et al., 2013; Sandblom et al., 2016a). Immediately after the chase protocol, the fish were returned to the holding tubes to record the maximum f_H response. After the experiments, all fish were euthanized by a sharp cranial blow and the heart was dissected out for further histological analysis.

Data acquisition, calculations and power spectral analysis of HRV

Individual ECG signals were amplified using a differential amplifier (Bio Amp FE231, ADInstruments, Sydney, NSW, Australia) and sampled at a sampling rate of 1kHz using LabChart v7.3.7 software

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Table 1. Body size metrics and results from the linear model for body mass, standard length and condition factor in acutely (3 days) and chronically (33–62 days) sham-operated and coronary-ligated rainbow trout (*Oncorhynchus mykiss*)

Body size metric	Treatment	Value	Acute vs chronic	Sham vs ligated	Interaction
Body mass					
Acute	Sham operated, *n*=10	268.6±11.0 g	$F_{1,45}$=7.512, *P*=0.009	$F_{1,45}$=0.960, *P*=0.33	$F_{1,45}$=0.747, *P*=0.40
	Coronary ligated, *n*=10	270.3±13.1 g			
Chronic	Sham operated, *n*=13	226.2±10.8 g			
	Coronary ligated, *n*=16	248.6±13.2 g			
Standard length					
Acute	Sham operated, *n*=10	24.8±0.1 cm	$F_{1,45}$=15.202, *P*<0.001	$F_{1,45}$=2.225, *P*=0.14	$F_{1,45}$=0.091, *P*=0.76
	Coronary ligated, *n*=10	25.1±0.3 cm			
Chronic	Sham operated, *n*=13	25.9±0.3 cm			
	Coronary ligated, *n*=16	26.3±0.3 cm			
Condition factor					
Acute	Sham operated, *n*=10	1.76±0.08	$F_{1,45}$=50.356, *P*<0.001	$F_{1,45}$=0.076, *P*=0.93	$F_{1,45}$=0.719, *P*=0.40
	Coronary ligated, *n*=10	1.71±0.06			
Chronic	Sham operated, *n*=13	1.30±0.03			
	Coronary ligated, *n*=16	1.36±0.05			

The condition factor of the fish was calculated as (100×body mass)/standard length^3.

(ADInstruments). f_H was determined from the R–R wave intervals using the cyclic measurements module in LabChart Pro. Resting f_H was determined from a 600 s period when the f_H was visibly low and stable and did not contain any obvious ectopic beats or artifacts resulting from fish movements, while maximum f_H was determined from the maximum f_H peak following chase. The f_H scope was calculated as maximum f_H−resting f_H (Steinhausen et al., 2008).

In order to evaluate the variability of f_H fluctuations under resting conditions, we analyzed the same 600 s ECG traces selected for obtaining resting f_H values (see above). The LabChart algorithm that detects inflection points in the ECG signal was used to generate beat-to-beat time series with cardiac interval values (i.e. R–R intervals). A frequency domain analysis was then performed on the data using a power spectral density (PSD) estimate of HRV (Akselrod et al., 1981), which is the total power of the spectrum of R–R intervals, using the publicly available CardioSeries software (v2.4, http://www.danielpenteado.com). Specifically, the PSD of the R–R interval series was analyzed by resampling beat-by-beat data points every 500 ms by cubic spline interpolation (2 Hz) containing one interpolated segment of 1024 points. A Hanning window was used to attenuate side effects, and a spectrum was calculated for the segment with a fast Fourier transform algorithm for discrete time series. Subsequently, the whole spectrum was integrated for each experimental treatment group.

Tissue harvest, preparation and histological staining

Excised hearts were immediately immersed in 0.1 mol l^{-1} KCl solution to uniformly arrest all hearts in diastole, followed by fixation in 4% buffered paraformaldehyde (4% in phosphate-buffered saline, PBS) for 24 h at 4°C. Hearts were then dehydrated in a graded series of 70%, 80%, 90% and 3×99.5% ethanol, and cut longitudinally in half; one side of the heart was cleared in xylene and embedded in paraffin wax, and the other half used for determining the compact myocardial proportion. For this, spongy and compact myocardial layers were carefully peeled apart under a dissecting microscope as previously described (Farrell et al., 2007). The heart tissues were dried in an oven at 60°C for 24 h before the dry mass was determined for subsequent calculation of the proportion of compact myocardium. Paraffin wax-embedded samples were sectioned at 7 µm thickness and then prepared for staining. Longitudinal and medial paraffin sections were stained for both Acid Fuchsin Orange G-stain (AFOG) and Picrosirius Red. In AFOG staining, collagen is stained blue, muscle tissue appears orange/brown and fibrin is red/pink. Picrosirius Red staining shows collagen in red on a pale yellow background (muscle tissue). For AFOG staining, sections were deparaffinized in xylene, rehydrated in a graded series of 99.5%, 90%, 80% and 70% ethanol, washed in water and post-fixated in preheated Bouin's fixative (Histolab, Askim, Sweden; 2.5 h at 56°C and 1 h at room temperature). Sections were then washed in tap water, incubated in 1% phosphomolybdic acid (Sigma-Aldrich, St Louis, MO, USA; 5 min), rinsed with distilled water and stained with AFOG staining solution (3 g of Acid Fuchsin, 2 g of Orange G, 1 g of Aniline Blue dissolved in 200 ml of acidified distilled water, pH 1.09, 10 min) (Darehzereshki et al., 2015). Stained sections were rinsed with distilled water (2 min), dehydrated with ethanol and xylene, and mounted. Picrosirius Red histochemical staining was performed in a similar way to AFOG staining, except that samples were stained without the post-fixation procedure.

Quantitative tissue morphology

Longitudinal sections of the middle of the heart stained with AFOG were examined with a Nikon Eclipse E1000 light microscope. The whole slide field was manually scanned at 10× magnification (manual WSI scanning software, Microvisioneer, Esslingen am Neckar, Germany). The publicly available ImageJ analysis software was used for calculating the percentage compact myocardium collagen content, in addition to quantification of interstitial tissue space (termed compact interstitial space) (National Institutes of Health, Bethesda, MD, USA) (Schneider et al., 2012). For this, three longitudinal sections of the middle of the heart stained with Picrosirius Red from each fish were analyzed by using the image threshold tool from ImageJ. For each heart section, five image fields were obtained: two from the base, two from the side and one from the apex of the ventricle (see Fig. S1), in which analysis were performed by using three circular fields of 130 µm diameter per field, yielding a total of 45 fields analyzed per animal. These images were examined at 20× magnification and the results expressed as a percentage (i.e. each image was divided into two classes of pixels for calculating percentage of collagen and interstitial space relative to the whole field). Only collagen from compact myocardium was quantified, thereby avoiding inclusion of epicardium collagen. In addition, any perivascular collagen present in the compact myocardium was also excluded from the analyses. To calculate the percentage of compact ventricular myocardium, we used the

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following equation (Farrell et al., 2007):

$$\frac{M_{\text{compact}}}{M_{\text{ventricle}}} \times 100, \quad (1)$$

where M_{compact} and $M_{\text{ventricle}}$ represent the dry mass of the compact myocardial tissue and the entire ventricle (i.e. combined spongy and compact myocardium), respectively.

Statistical analysis

Statistical analyses were performed using R software v. 1.1.383 (http://www.R-project.org/). Linear models were fitted by using the 'lm' function from the stats package in R to assess the effect of sham operation versus coronary artery ligation within groups (i.e. acute and chronic) on body characteristics (body mass, standard length and condition factor), compact dry mass proportion, compact collagen content and compact interstitial space. For all the above variables, an additional analysis was performed by using linear models to investigate the influence of time since sham/ligation surgery (i.e. from 33 to 62 days) among fish within the chronic group.

The same procedure was performed to assess the effect of sham operation versus coronary artery ligation within acute and chronic groups on resting f_H, maximum f_H, f_H scope and HRV. Additional analyses were performed by including time since sham/ligation surgery as a co-variate to test for the effect of variation in time since surgery among fish within the chronic group for resting f_H and HRV. Maximum f_H and f_H scope were analyzed by including chase time as a co-variate to test for differences in time until exhaustion during the chase; as chase time did not have an effect, it was excluded from the final models. Likewise, when time since sham/ligation surgery had no effect on the investigated variable, the final analysis was performed by excluding it as a covariate from the models. To correct for potential body mass differences, body mass was initially included as a covariate in all models. However, because body mass only showed significant effects for collagen content, the final data analysis excluded body mass as a covariate from all remaining models.

All values are presented as means±s.e.m. Statistical significance was accepted at $P \leq 0.05$. When significant effects were found in linear models, these were further explored with pairwise comparisons within acute and chronic groups. Normality of the residuals were visually inspected by using histograms and boxplots and when necessary, appropriate transformations were performed. Homogeneity of variances for each model was tested using Levene's test.

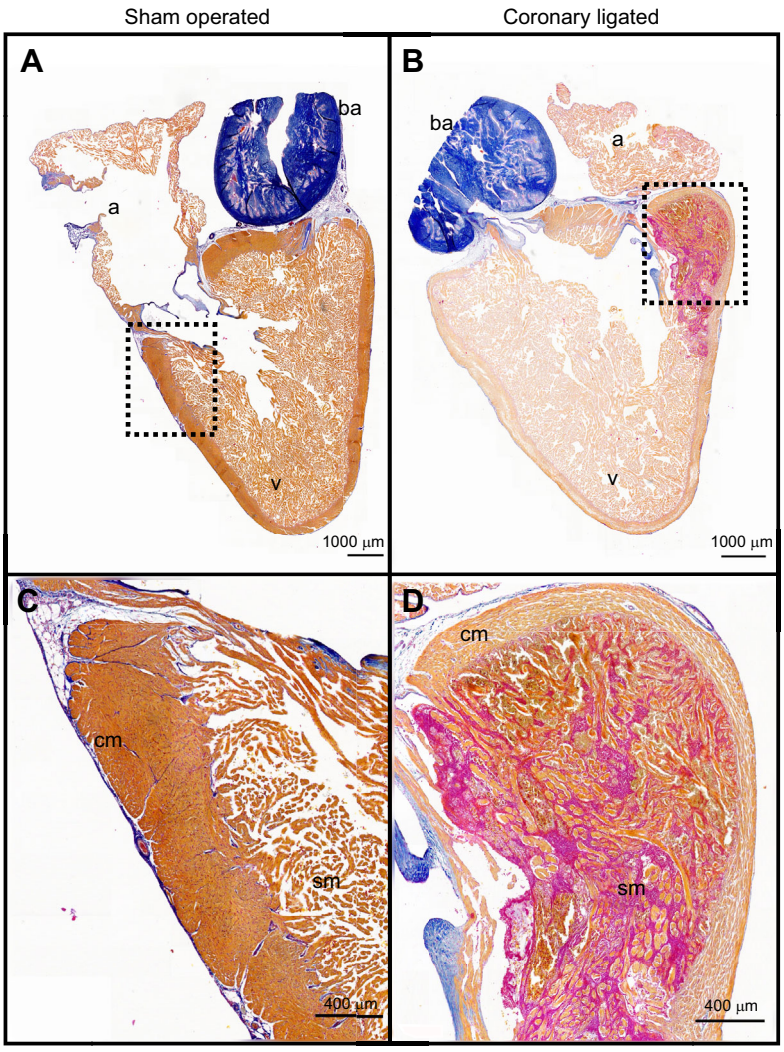

Fig. 1. Effects of acute coronary artery ligation on heart morphology in rainbow trout (*Oncorhynchus mykiss*). Histological sections of sham-operated (A,C) and coronary-ligated (B,D) rainbow trout hearts examined 3 days following surgery. C and D show magnified pictures of the boxed region in A and C, respectively. Bright-field images stained with Acid Fuchsin Orange G (AFOG) depict cardiac muscle in yellow/brown, collagen in blue (e.g. bulbus arteriosus) and fibrin in red/pink. The fibrin-based provisional matrix serves as a scaffold for cell migration and proliferation and represents the initial inflammatory response following coronary artery ligation. Note that the compact myocardium in coronary-ligated fish (D) exhibits apparently separated individual cardiomyocytes (i.e. compact myocardium interstitial space). cm, compact myocardium; sm, spongy myocardium; a, atrium; v, ventricle; ba, bulbus arteriosus.

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RESULTS

Body characteristics of experimental groups

In the acute group, a total of 10 fish were sham operated and 10 fish were coronary ligated, while in the chronic group, a total of 13 fish were sham operated and 16 fish were coronary ligated. All fish survived the surgical procedures. Body size metrics are summarized in Table 1. There were no morphometric differences between sham-operated and coronary-ligated fish within treatment groups. However, the overall body mass was significantly lower, while standard length was higher in the chronic group, resulting in a significantly lower condition factor in the chronic group relative to the acute group.

Ventricular myocardial remodeling after coronary artery ligation

The hearts of acutely coronary-ligated fish exhibited markedly increased interstitial spacing (Fig. 1B,D; see also Fig. 4 and Fig. S1 for Picrosirius Red staining), as well as an inflammatory response at the base of the heart, as indicated by the fibrin-based provisional matrix stained in pink/red and infiltration of inflammatory cells (Fig. 1B,D; Fig. S2). These alterations were not found in acutely sham-operated fish, which had hearts with normal histological appearance (Fig. 1A,C). Some acutely ligated individuals exhibited a large part of the spongy myocardium, immediately adjacent to the compact myocardium, that was occupied by fibrin (fibrin-based provisional matrix serves as a scaffold for migration of inflammatory cells and fibroblasts; Frangogiannis, 2015; Fig. 1D), while others exhibited much less pronounced signs of fibrin deposition in this region. In contrast, in the chronic coronary-ligated group, only one fish exhibited a clear presence of fibrin-based provisional matrix 40 days after the coronary artery ligation surgery (Fig. 2B,E).

Coronary artery ligation significantly reduced the proportion of compact myocardium relative to that in sham-operated trout in both the acute (20.9±0.8% versus 26.3±1.7%; t_{43}=−2.842, P=0.007; Fig. 3) and chronic experimental groups (24.0±1.0% versus 31.51±1.1%; t_{43}=−4.986, P<0.0001; Fig. 3). Acutely

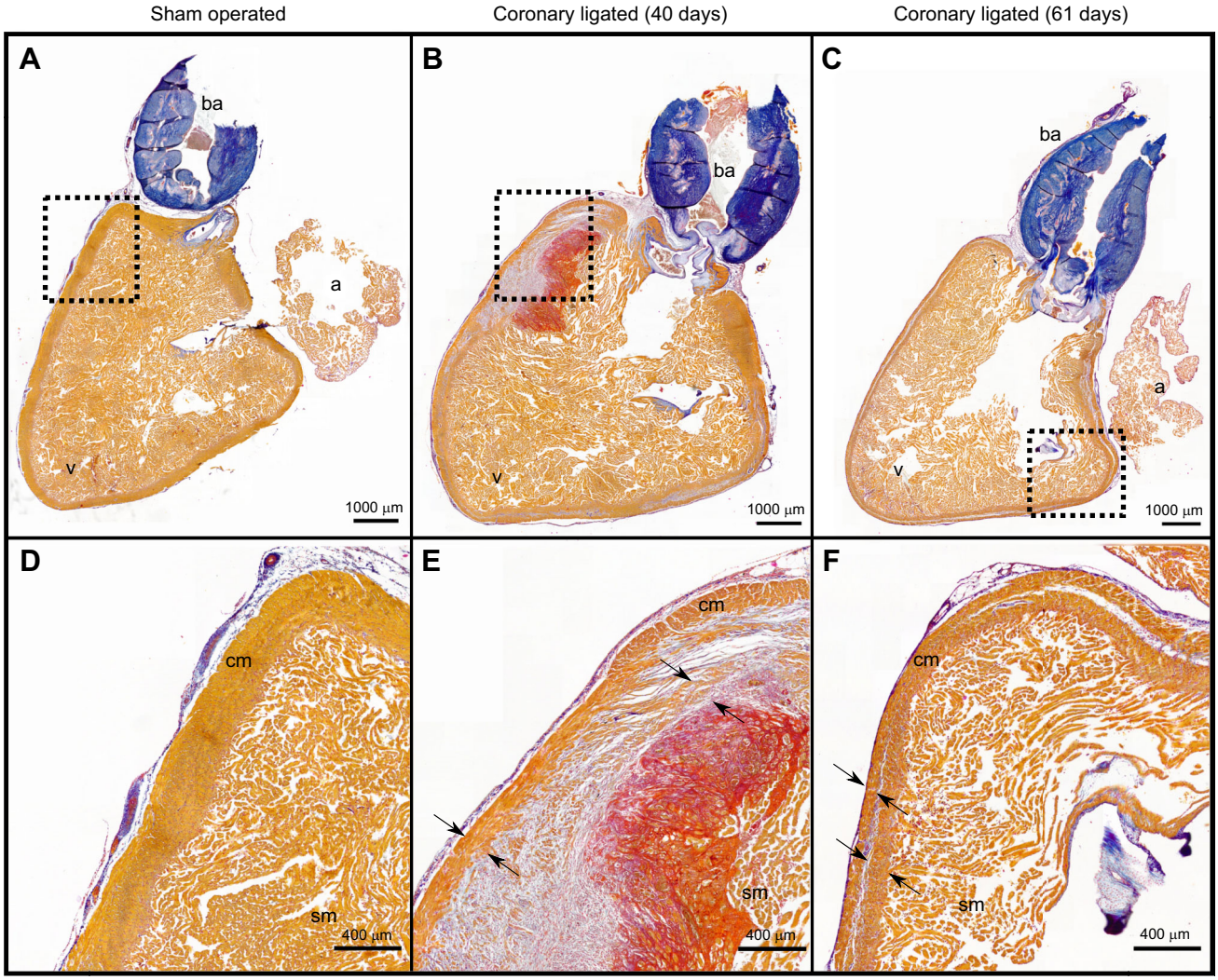

Fig. 2. Effects of chronic coronary artery ligation on heart morphology in rainbow trout. Histological sections of sham-operated (A,D; examined 34 days following surgery) and coronary-ligated (B,C,E,F) rainbow trout hearts examined 33–62 days following surgery. D–F show magnified pictures of the boxed region in A–C, respectively. The bright-field images stained with AFOG depict cardiac muscle in yellow/brown, collagen in blue (e.g. bulbus arteriosus) and fibrin in red/pink. The fibrin-based provisional matrix serves as a scaffold for cell migration and proliferation and represents the inflammatory response following coronary artery ligation. Note the transition from an inflammatory phase (stained in red) to collagen deposition (stained in light blue) in coronary-ligated fish (B,C,E,F). In the chronically coronary-ligated fish, the compact myocardium has remodeled into two distinct layers (black arrows in E and F) separated by a layer of collagen (stained in light blue, E and F). cm, compact myocardium; sm, spongy myocardium; a, atrium; v, ventricle; ba, bulbus arteriosus.

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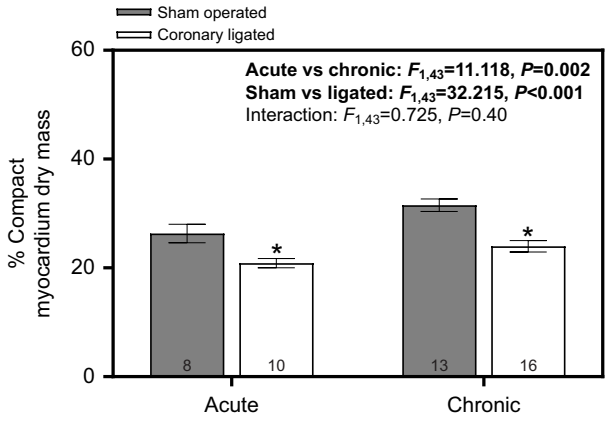

Fig. 3. Effects of coronary artery ligation on the proportions of compact myocardium in rainbow trout heart. The proportions of compact myocardium (dry mass) 3 days (acute) and 33–62 days (chronic) after surgical coronary artery ligation in rainbow trout. Results from the linear model for the respective variables are shown; statistically significant (P≤0.05) findings are in bold. Asterisks indicate a significant difference between sham-operated and coronary-ligated fish within acute and chronic groups. Numbers within bars denote sample sizes. Data are means±s.e.m.

coronary-ligated trout exhibited a ∼4-fold increase in interstitial space that was mainly found between cardiomyocytes in the compact myocardium relative to sham-operated fish (12.0±1.2% versus 2.9±0.1%; t_{44}=6.568, P<0.0001; Fig. 4A,B,E; Fig. S1). In addition, acutely coronary-ligated fish exhibited a significantly lower collagen content relative to sham-operated trout (6.7±0.3% versus 8.4±0.6%; t_{44}=−2.846, P=0.007; Fig. 4F).

Interestingly, chronically ligated fish exhibited a 2.5-fold higher compact collagen content relative to sham-operated trout (20.6±1.2% versus 8.1±0.5%, respectively; t_{44}=13.004, P<0.001; Fig. 4F). This was mainly deposited in the compact myocardial tissue, thereby splitting it into two muscle layers separated by a distinct layer of loosely organized collagen fibers (Figs 2C,F and 4D; see also Fig. S1). In addition, chronically ligated fish exhibited a ∼3-fold increase in compact interstitial space, which was mainly found between the loosely organized collagen fibers in the mid-myocardial layer (2.2±0.4% versus 6.5±1.0%; t_{45}=6.148, P<0.0001; Fig. 4C–E; Fig. S1).

Among all ventricular morphological characteristics, body mass emerged as a significant predictor only for collagen content when treatment groups were combined (β=0.0009, P=0.001). As collagen content increased significantly with the chronic ligation, we explored further the relationship between body mass and collagen content for both sham-operated and coronary-ligated groups and found that body mass was positively correlated with the amount of collagen deposited in the compact myocardium, only in ligated fish (r^2=0.28, P=0.03; Fig. S3A).

Effects of acute and chronic coronary artery ligation on f_H performance and HRV

Acutely coronary-ligated trout exhibited increased resting f_H relative to sham-operated fish (49.2±3.7 versus 36.7±2.3 beats min^{-1}; t_{41}=2.452, P=0.02; Fig. 5A), while the maximum f_H was not affected (65.5±2.0 versus 63.0±2.0 beats min^{-1}; Fig. 5B). This meant that the scope for f_H was significantly lower in acutely coronary-ligated fish (15.3±3.0 versus 26.8±2.8 beats min^{-1}; t_{35}=−2.930, P=0.006; Fig. 5C). However, the increase in resting f_H was attenuated over time and, thus, resting f_H (30.9±1.9 versus 31.1±2.6 beats min^{-1}; Fig. 5A), maximum f_H (64.6±1.3 versus 66.6±0.9 beats min^{-1}; Fig. 5B), as well as f_H scope (35.2±1.6 versus 37.3±2.4 beats min^{-1}, Fig. 5C) did not differ between sham-operated and coronary-ligated fish in the chronic groups.

The elevated resting f_H after acute coronary artery ligation was associated with a significantly reduced HRV relative to sham-operated trout (18,367±7399 versus 54,853±14,281 ms^2; t_{41}=−3.672, P=0.0007; Fig. 6). In contrast, in the chronically ligated trout, HRV did not differ from that of the corresponding sham group (37,020±5866 versus 65,439±14,347 ms^2; t_{41}=−1.436, P=0.16), suggesting recovery (i.e. increase) of HRV following the initial decline with acute ligation (Fig. 6B,C). Additionally, among all *in vivo* f_H performance measures, time since sham/ligation surgery within the chronic group emerged as a significant predictor only for HRV (β=−0.03774, P=0.047). This means that although HRV showed signs of recovery following the initial decline with acute coronary artery ligation, there was a trend for a subsequent reduction in HRV between 33 and 62 days after coronary artery ligation, indicating that the partial recovery of HRV may be temporary (r^2=0.23, P=0.06; Fig. S3B).

DISCUSSION

In the present study, we integrated morphological and physiological measures to understand the temporal dynamics of plastic changes in ventricular morphology and cardiac performance of a salmonid fish following myocardial infarction, which we experimentally induced by surgical blockade of the coronary artery. We characterized the time course of myocardial remodeling following the coronary artery obstruction, which is critical for understanding the capacity for myocardial remodeling that may aid in sustaining cardiovascular function and survival in salmonid fish. Specifically, we demonstrate a series of structural changes in the myocardium that largely resemble the patterns observed with mammalian heart infarction. Yet, several features suggest adaptive cardiac repair in rainbow trout, resulting in long-term (up to ∼2 months) restoration of cardiac function.

While acute coronary artery occlusion resulted in elevated resting f_H and decreased HRV, along with reduced f_H scope, clear changes in ventricle structure and cardiac performance occurred with the more prolonged chronic coronary artery ligation. This included consistent changes in the compact myocardium, which was split into two layers separated by a fibrous collagen layer. An intriguing possibility is that this splitting of the compact myocardium may have created a region in the mid-compact myocardium that continued to receive oxygen via diffusion from the luminal blood. Alternatively, this peculiar myocardial remodeling may be an adaptive response serving to maintain structural stability and integrity during the cardiac cycle.

Effects of acute coronary artery ligation on cardiac morphology and myocardial organization

Acute abolishment of coronary blood flow to the trout heart for 3 days led to increased extracellular matrix breakdown (i.e. reduced collagen content), and probably cardiomyocyte loss, as suggested by the apparent reduction in compact myocardial mass. Thus, our observations in trout follow the same general patterns as observed in mammals following cardiac ischemia and consequent infarction, in which degradation of the interstitial cardiac matrix occurs (Dobaczewski et al., 2006; Liehn et al., 2011), and is followed by clearing of the damaged tissue and replacement with collagen in areas devoid of cardiomyocytes (Frangogiannis, 2015; Rog-Zielinska et al., 2016). An inflammatory process was also evident in the luminal space at the base of the heart in coronary-ligated trout.

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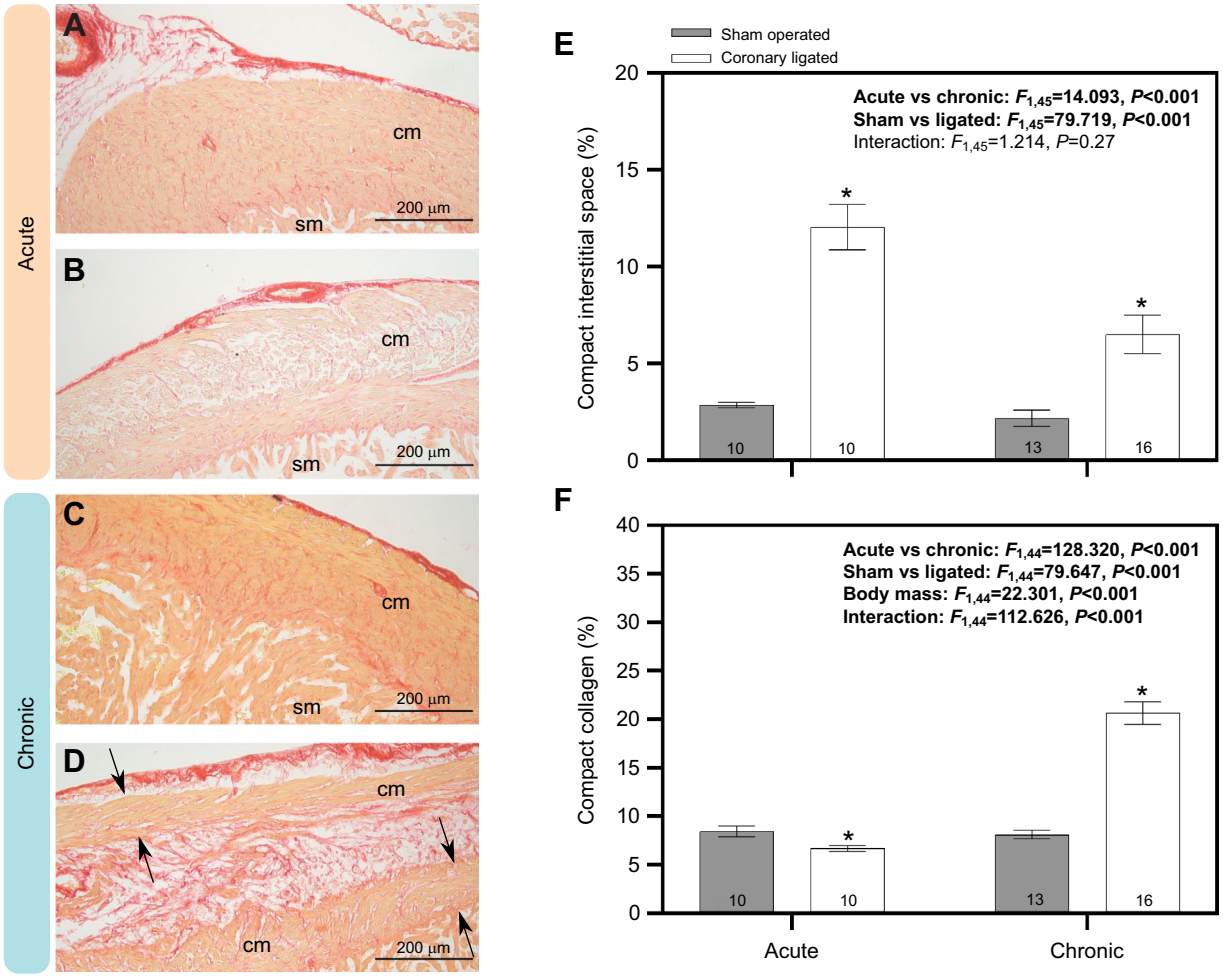

Fig. 4. Effects of acute and chronic coronary artery ligation on heart morphology in rainbow trout. (A–D) Representative bright-field images of acute (3 days post-surgery; A,B) and chronic sham-operated (34 days post-surgery; C) and coronary-ligated (31 days post-surgery; D) rainbow trout stained with Picrosirius Red, allowing quantification of compact collagen content and compact interstitial space. Note the apparent separation of individual cardiomyocytes in the compact myocardium in B. Also, note that the compact myocardium in D is divided into an outer and inner layer (black arrows) with a layer of collagen in between. cm, compact myocardium; sm, spongy myocardium. (E,F) Results from the linear model for the respective variables; statistically significant (P≤0.05) findings are in bold. Asterisks indicate significant differences between sham-operated and coronary-ligated fish within acute and chronic groups. Body mass exhibited a significant effect only on collagen content. Numbers within bars denote sample sizes. Data are means±s.e.m.

This area is bordered the compact myocardium, forming a fibrin-based provisional matrix (stained in pink/red by AFOG), which may serve as a scaffold for migration and proliferation of inflammatory cells into the compact myocardium. In the mammalian heart, an increase in vascular permeability leads to extensive extravasation of plasma proteins that form a fibrin-based provisional matrix network (Dobaczewski et al., 2006). However, in the complete absence of coronary arterial perfusion, this fibrin-based provisional matrix must originate from the luminal venous blood in the rainbow trout heart, which may set the stage for compact tissue repair in the acute stages of the infarct (Frangogiannis, 2017).

Long-term cardiac remodeling and repair following chronic coronary artery ligation

Chronically ligated trout had a nearly 2.5-fold greater fibrotic collagen content in the compact myocardium compared with the sham group. An even more remarkable finding, however, relates to the anatomical arrangement of the collagen deposits within the compact myocardium. Instead of involving the full thickness of the myocardial wall, i.e. a transmural infarct as typically seen in mammalian infarcts (Lutgens et al., 1999; Mathieu et al., 2012), all chronically ligated fish exhibited a clearly visible layer of collagen fibers located in a median position in the compact myocardium, which seemed to split the compact myocardium into an outer and inner layer (Fig. 2; Fig. S1). This arrangement means that the compact interstitial space was primarily associated with spaces between collagen fibers, and contrasts with the typical dense compact fibrosis commonly seen in post-infarction mammalian myocardium (Rog-Zielinska et al., 2016). In fact, infarct scar tissue (i.e. dense collagen) in mammals exhibits heightened diffusion properties for small molecules such as oxygen when compared with uninjured heart tissue (Davis et al., 2012). Thus, as oxygen supply to the compact myocardium via the coronary arteries was completely abolished after coronary artery ligation, the normal compact myocardium to luminal oxygen gradient may be reversed, and it is possible that the mid-myocardial fibrous layer demarks a region of the compact myocardium (i.e. the inner layer) that continued to receive oxygen from the luminal venous blood. However, how the more distant cardiomyocytes located in the outer compact layer are supplied with oxygen following coronary artery ligation remains unknown. It might be provided by some diffusion from the venous blood, as oxygen diffusion may be facilitated by the

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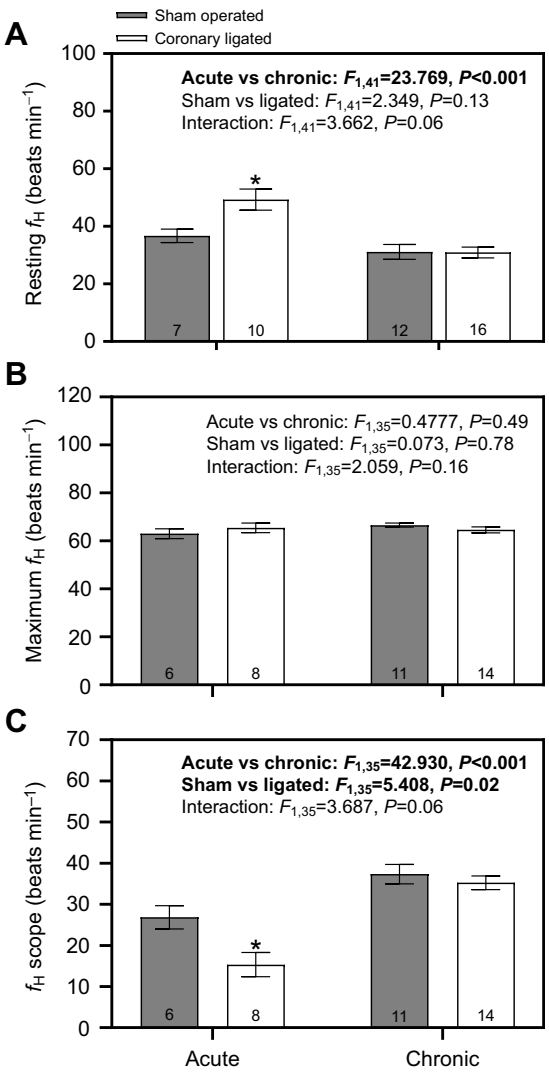

Fig. 5. Effects of acute and chronic coronary artery ligation on cardiac performance of rainbow trout. Resting heart rate (f_H; A), maximum f_H following chase to exhaustion (B) and f_H scope, calculated as the difference between maximum and resting f_H (C) in acute and chronically sham-operated and coronary-ligated rainbow trout. Results from the linear model for the respective variables are shown; statistically significant ($P \leq 0.05$) findings are in bold. Asterisks indicate a significant difference between sham-operated and coronary-ligated fish within acute and chronic groups. Numbers within bars denote sample sizes. Data are means±s.e.m.

high diffusive properties of the fibrous collagen layer. Alternatively, some superficial coronary vessels may regrow and provide oxygen to the outer compact myocardium while the inner layer continues to receive oxygen from the venous blood. While these hypotheses remain to be examined, the rearrangement of compact myocardium discovered here is, as far as we are aware, a completely novel finding.

Another remaining question is how cardiac oxygen demand is met before the compact myocardium has remodeled into the two separate layers. As a pronounced opening of interstitial spaces in the compact layer was observed in acutely ligated trout, it is possible that this acute tissue response allows luminal venous blood to reach more distant cardiomyocytes in the outer myocardial layer.

Post-experimental examination revealed that the suture was tied around the coronary artery in all acutely ligated fish; however, in the chronic group, it was not always possible to locate the silk suture. This may indicate that the suture was encapsulated by fibrous connective tissue or even expelled, and that coronary blood flow may have been restored sometime during the experimental period. Thus, all fish may not have been chronically ligated throughout the entire experimental period. However, this does not bias our results as the ligation per se resulted in distinct morphological alterations in the compact myocardium that were consistently found in all chronically coronary-ligated fish, but never found in any of the chronically sham-operated fish.

Cardiac remodeling following myocardial injury is a continuous and dynamic process that starts early and may last for several months depending on the species (e.g. fishes and amphibians; Godwin et al., 2017; Grivas et al., 2014; Lafontant et al., 2012; Liao et al., 2017). Thus, we cannot rule out that further changes in cardiac morphology and function would manifest beyond the time scale evaluated here. For example, maturing fibrotic scar tissue may underlie changes in cardiac geometry and function, in which an excessive scar tissue deposition may cause excessive ventricular wall stiffening, and thereby compromise diastolic relaxation and ventricular filling (Christia et al., 2013; Frangogiannis, 2014). As body mass was positively correlated with the amount of collagen deposited in the compact myocardium in chronically ligated juvenile trout, and the relative amount of compact myocardium increases with body mass in salmonids (Brijs et al., 2017; Farrell et al., 1988; Poupa et al., 1974), this may suggest that cardiac function could be progressively and more severely affected by a restricted coronary supply as fish grow larger. Moreover, the extent to which the coronary artery may regrow and re-vascularize the compact myocardium, and whether scar tissue may regress following even longer abolishment of the coronary supply to the heart, is currently unknown and warrants further study.

Effects of acute and chronic coronary artery ligation on cardiac performance

The increase in resting f_H following acute coronary artery ligation is consistent with earlier findings in rainbow trout (Ekström et al., 2017, 2018, 2019), and seems to persist for at least 7 days after ligation (Ekström et al., 2018). This probably reflects a compensatory response to maintain cardiac output when ventricular contractility and thus stroke volume is compromised (Farrell, 2007; Morgenroth et al., 2021; Steffensen et al., 1998). The reduced compact myocardium mass and interstitial collagen content found in the acute group may underlie such impaired ventricular contractility. Conversely, as mentioned above, the reduced compact myocardium mass and the formation of a layer of mid-myocardial fibrosis oriented around the ventricle in the chronic group may have stabilized wall stress during the cardiac cycle, and therefore, contributed to normalization of cardiac function.

It was recently shown that the elevated resting f_H in acutely coronary-ligated fish is probably due to a withdrawal of inhibitory cardiac vagal tone, while sympathetic tone is unchanged (Ekström et al., 2019). As vagal tone on the heart seems to be the main contributor to HRV in rainbow trout (Le Mével et al., 2002), the considerable reduction in total HRV observed here in the acutely ligated trout probably reflected a reduced vagal tone. It is worth mentioning that rainbow trout modulate f_H mainly by altering vagal tone during various cardiorespiratory challenges, e.g. a withdrawal of vagal tone elicits tachycardia during moderate exercise and increasing vagal tone elicits bradycardia during hypoxia (Perry and Desforges, 2006; Randall, 1982). In addition, baroreflex regulation of f_H is mainly modulated by augmenting or withdrawing the vagal tone on the heart, thereby safeguarding tissue perfusion pressure

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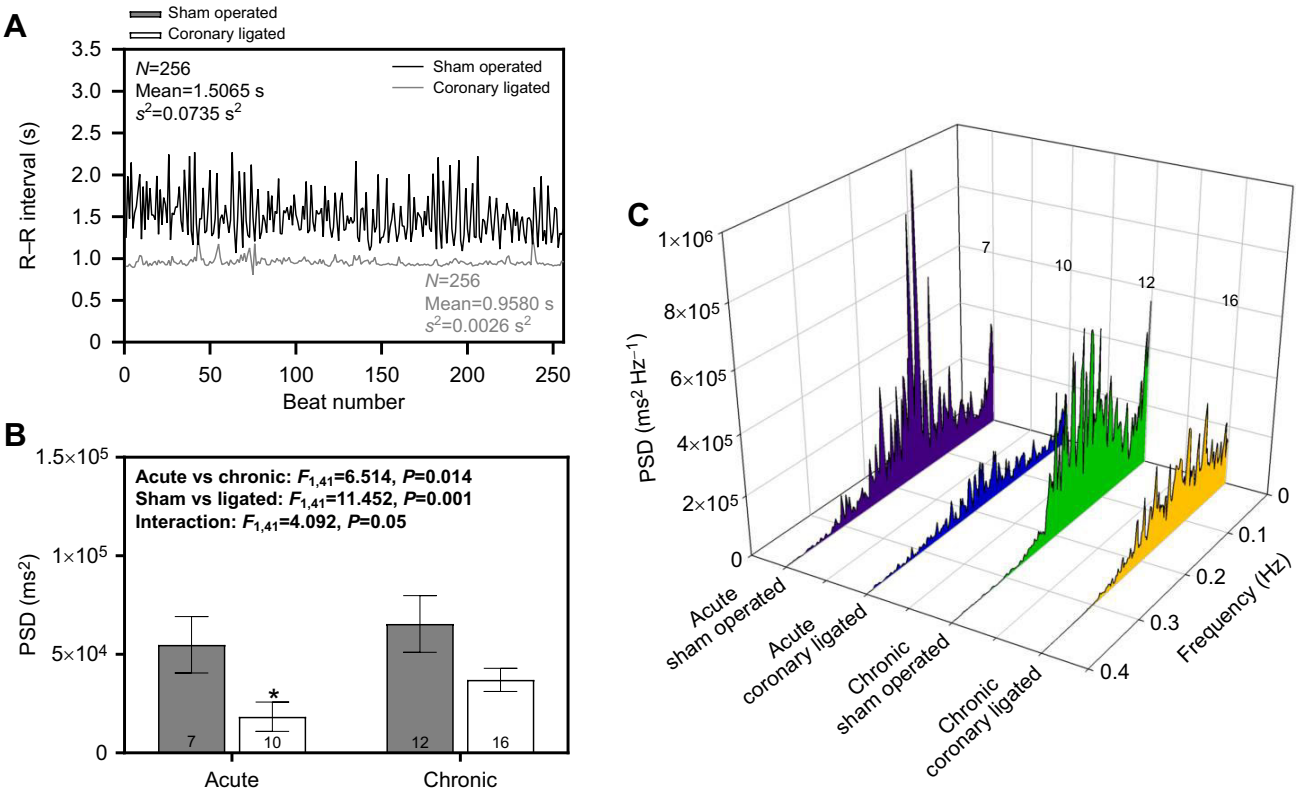

Fig. 6. Changes in heart rate variability (HRV) following acute and chronic coronary artery ligation in rainbow trout. (A) Representative tachograms of 256 consecutive R–R interval values of one representative acute (3 days) sham-operated rainbow trout and one representative acute coronary-ligated rainbow trout. Note the high inter-beat variability (R–R interval) for the sham-operated fish compared with the nearly complete lack of variability in the coronary-ligated trout. Mean values, variance (s^2) and the number (N) of analyzed R–R intervals are indicated. (B) Power spectral density (PSD) estimate of HRV as determined from the total power of the R–R interval spectrums in C. Results from the linear model for the respective variables are shown; statistically significant ($P \leq 0.05$) findings are in bold. Asterisks indicate a significant difference between sham-operated and coronary-ligated fish within the acute group. Numbers within bars denote sample sizes. Data are means±s.e.m. (C) Spectrums of the R–R interval showing reduced variability 3 days after acute coronary artery ligation relative to sham-operated fish, which appears to partially recover 33–62 days after surgery (chronic coronary ligated). For clarity, the s.e.m. for the pooled data have been removed. Numbers by each spectrum denote sample sizes.

during short-term imbalances in blood pressure (Sandblom and Axelsson, 2005, 2011; Sandblom et al., 2016b).

Autonomic dysfunction with impaired vagal modulation of the heart following insufficient coronary blood supply could, therefore, compromise the ability of fish to maintain normal cardiovascular reflex regulation. Indeed, a reduced baroreflex sensitivity and HRV are recognized as significant predictors of mortality after myocardial infarction in humans (La Rovere et al., 1998; Ponikowski et al., 1997), which may also be the case in fish. Thus, assessment of HRV using standard electrocardiogram techniques may be a useful scanning tool for cardiac disease in farmed fish.

Consistent with previous findings, rainbow trout increased f_H following exhaustive swimming activity (maximum f_H) (Farrell, 2002a). However, the coronary-ligated fish had a markedly reduced f_H scope, which was attributed to an elevated resting f_H and an inability to compensate with further increases in maximum f_H. Interestingly, the subsequent recovery of f_H scope in the chronically ligated trout was mainly attributed to reduced resting f_H while maximum f_H remained unaltered. Again, the lowering of resting f_H following chronic ligation can probably be attributed to a gradual recovery of cardiac contractility and resting stroke volume.

Conclusions and future perspectives

Although acute abolishment of the coronary O_2 supply to the heart limits the ability of fish to cope with metabolically demanding stressors (Ekström et al., 2018, 2019; Morgenroth et al., 2021; Steffensen et al., 1998), it is still unclear whether the high prevalence and severity of coronary arteriosclerosis in salmonids can be considered a risk factor and explain the high mortality rates in farmed salmonids (10–20%) (Poppe et al., 2007; Sommerset et al., 2021). Coronary arteriosclerosis appears to be an ongoing condition in frequent spawning species, such as steelhead trout and Atlantic salmon (Kubasch and Rourke, 1990; Saunders and Farrell, 1988), and the time course required for salmonids to recover from a potential myocardial injury may be critical for fish regularly exposed to stressful conditions, such as those applied in aquaculture (e.g. grading, transportation and various treatments for disease and parasites; Nilsson et al., 2019; Overton et al., 2019; Poppe et al., 2007; Sviland Walde et al., 2021).

Our data show that juvenile rainbow trout can cope with impaired coronary blood supply by remodeling of the ventricle, which may lead to restored cardiovascular performance over time. Importantly, despite a reduced cardiac function after acute obstruction of coronary blood flow (i.e. reduced scope for f_H and HRV), coronary-ligated fish showed a resetting of resting f_H and restored scope for f_H in the long term (i.e. up to 2 months). Future emphasis should be directed towards exploring whether the ventricular remodeling and associated improvements of cardiac function are adaptive in the longer term (i.e. months to years), or are simply signs of a pathological process ultimately leading to progression of cardiac functional deterioration.

Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: L.A.Z., A.E., A.G., E.S.; Methodology: L.A.Z., A.E., A.G., C.O., M.A., H.S., E.S.; Validation: L.A.Z.; Formal analysis: L.A.Z.; Investigation: L.A.Z., A.E.; Resources: L.A.Z., C.O., M.A., H.S., E.S.; Data curation: L.A.Z.; Writing - original draft: L.A.Z., A.E., A.G., E.S.; Writing - review & editing: A.E., A.G., C.O., M.A., H.S., E.S.; Visualization: L.A.Z.; Supervision: E.S.; Project administration: E.S.; Funding acquisition: L.A.Z., E.S.

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Data availability
Data are available from the Dryad digital repository (Zena et al., 2021): https://doi.org/10.5061/dryad.xd2547dj1.

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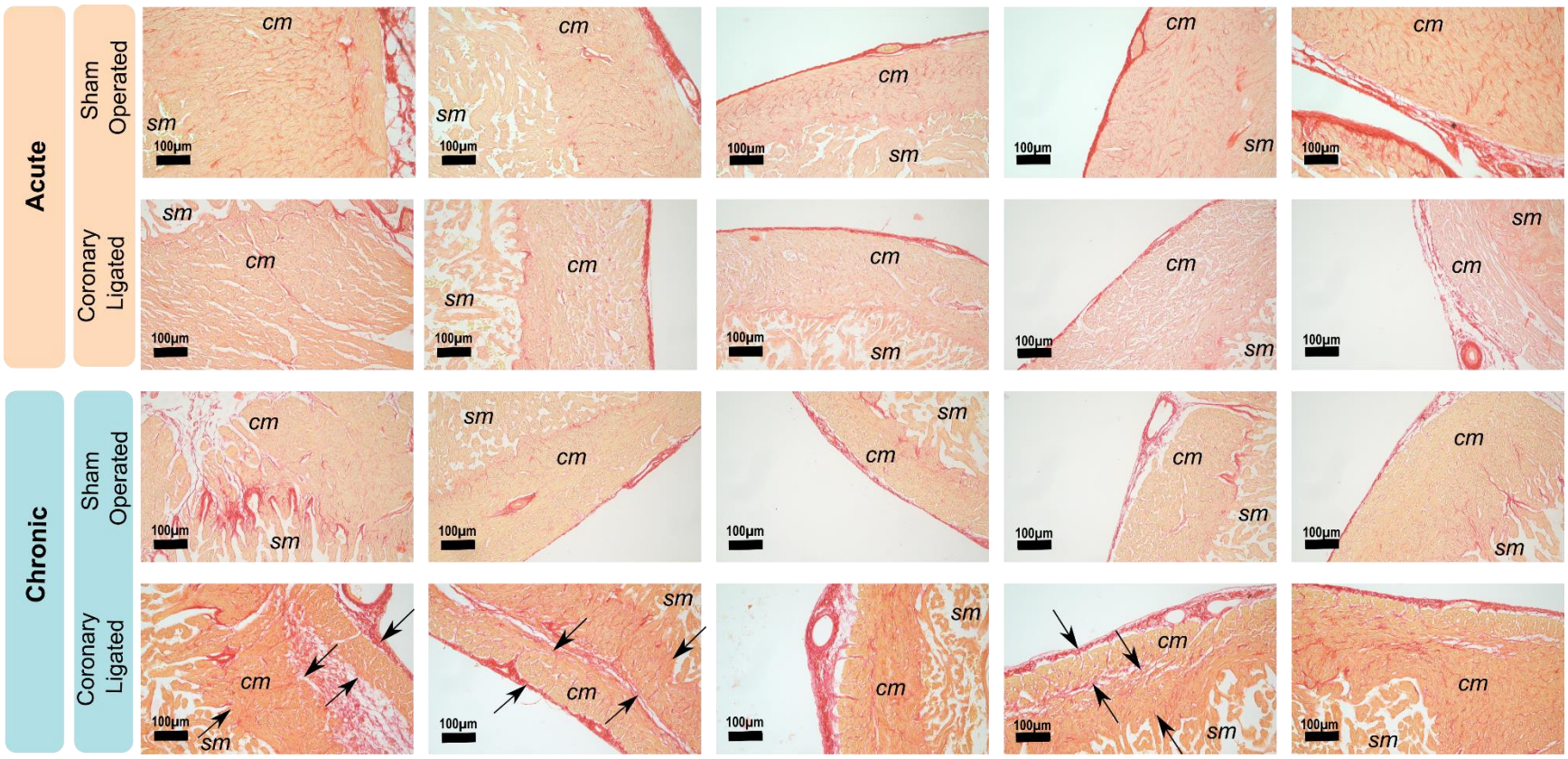

Fig. S1. Effects of acute and chronic coronary ligation on heart morphology in rainbow trout (*Oncorhynchus mykiss*). Histological sections of acute (3 days post-surgery) and chronic (33-62 days post-surgery) sham-operated and coronary-ligated rainbow trout hearts. Examples are from five image fields (*i.e.*, two from the base, two from the side and one from the apex of the ventricle) from one ventricle section stained with picrosirius red that was used to calculate percent compact myocardium and percent interstitial space in all samples analyzed (see Material and Methods – Quantitative tissue morphology). Note an increase in interstitial space with apparently separated individual cardiomyocytes in the compact myocardium of one acute coronary ligated fish (imagens in the second row). Also, note that the compact myocardium in one chronic ligated fish (imagens in the fourth row) is divided into an outer and inner layer (arrows) with a layer of collagen in-between. The abbreviations are: *cm*, compact myocardium; *sm*, spongy myocardium.

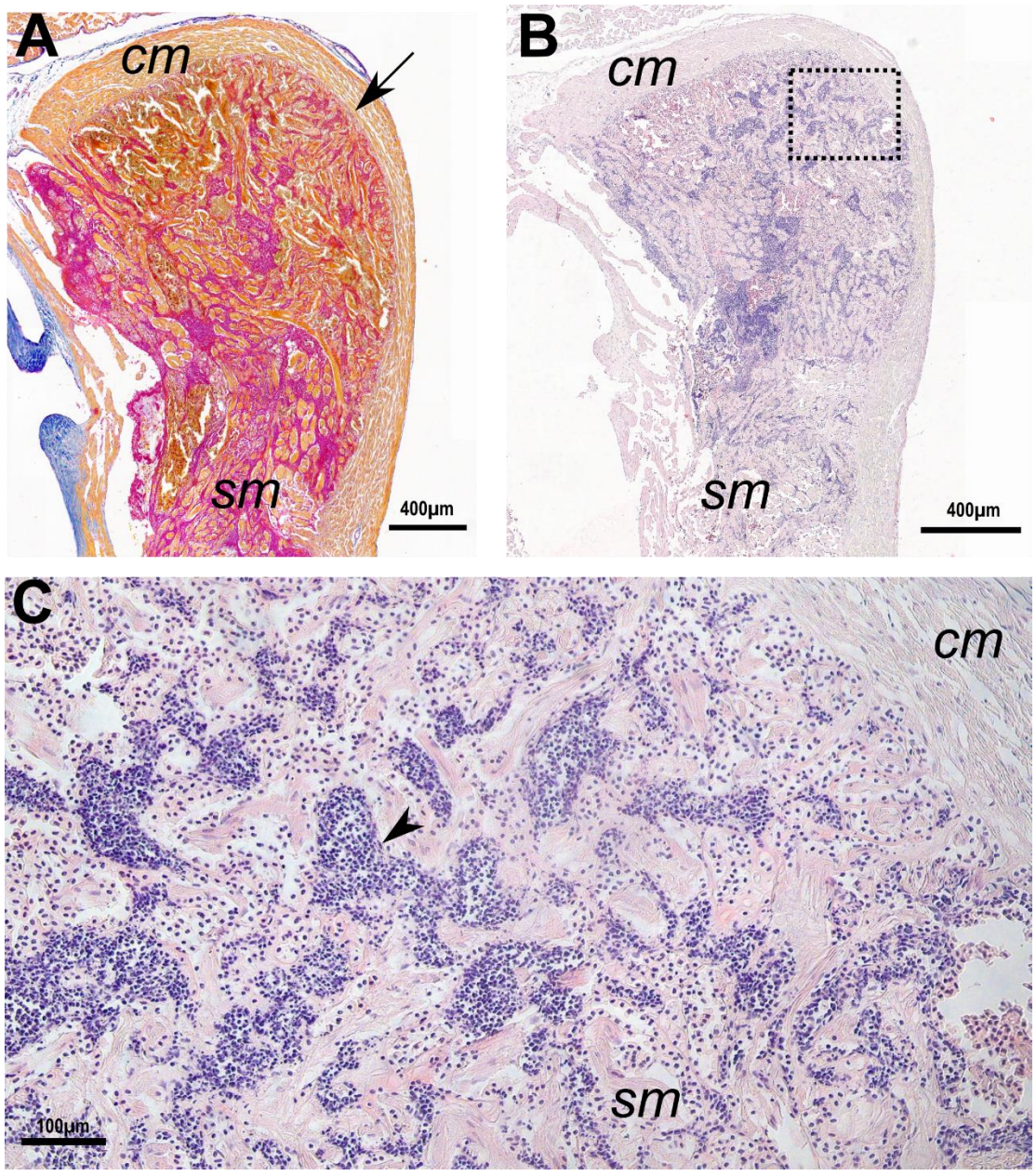

Fig. S2. Heart inflammatory response in acute coronary-ligated rainbow trout (*Oncorhynchus mykiss*). Histological sections of one coronary-ligated rainbow trout heart examined 3 days following surgery. Bright-field image stained with acid fuchsin orange G in A (AFOG) depicts cardiac muscle in yellow/brown, collagen in blue, and fibrin in red/pink. Bright-field images stained with hematoxylin and eosin showing large amounts of inflammatory cells in the spongy myocardium in B and C (arrowhead). Magnified picture in C of the respective area within the hatched square in B. Note that fibrin-based provisional matrix overlaps with large amounts of inflamatory cells (A and B). Also, note an increase in interstitial space in the compact myocardium with apparently separated individual cardiomyocytes in A, B and C. For comparisons with sham-operated fish see Figure 1 and Supplementary Material S1. Abbreviations: cm, compact myocardium; sm, spongy myocardium.

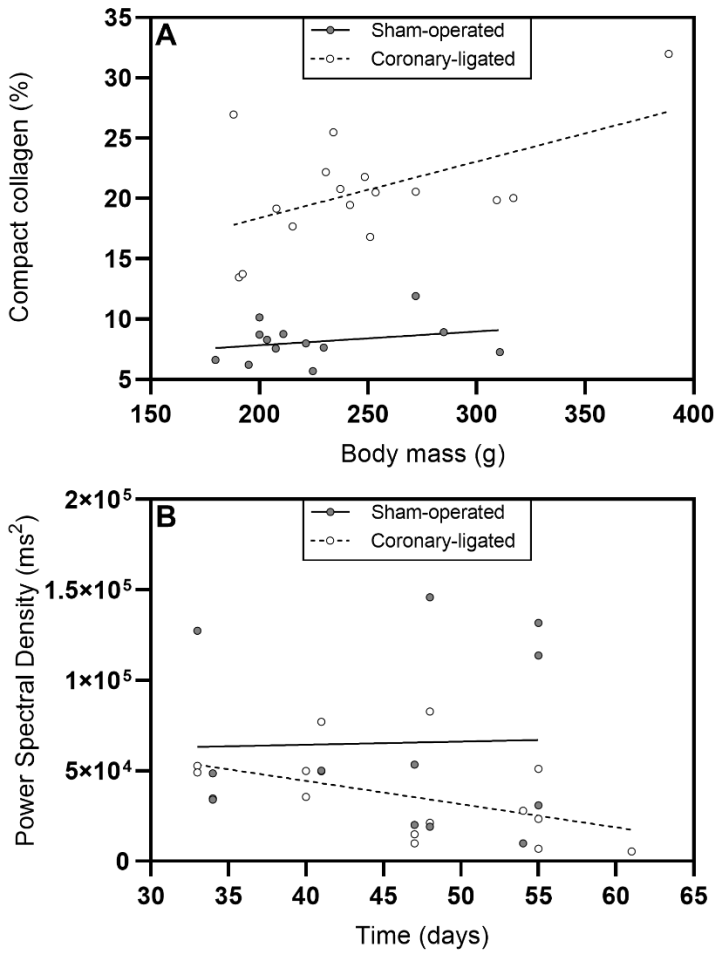

Fig. S3. Relationships in rainbow trout between the percentage of collagen content in the compact myocardium and body mass (A), and between heart rate variability and time since chronic coronary ligation surgery (B). Panel A shows linear regressions between percentage compact collagen and body mass in sham-operated trout (solid line and filled circles, n = 13; regression analysis; Y = 0.01139x + 5.553, r^2 = 0.07, P = 0.37) and coronary-ligated trout (dashed line and open circles; n = 16; Y = 0.04682x + 9.011, r^2=0.28, P=0.03). Panel B shows linear regressions between heart rate variability and time since ligation surgery in sham-operated trout (solid line and filled circles, n = 12; regression analysis; Y = 174.3x + 57,435; r^2 = 0.001, P = 0.92) and coronary-ligated trout (dashed line and open circles; n = 16; Y = -1,284x + 95,763, r^2=0.23, P=0.06).