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RESEARCH ARTICLE

Cold acclimation alters the connective tissue content of the zebrafish (*Danio rerio*) heart

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ABSTRACT

Thermal acclimation can alter cardiac function and morphology in a number of fish species, but little is known about the regulation of these changes. The purpose of the present study was to determine how cold acclimation affects zebrafish (*Danio rerio*) cardiac morphology, collagen composition and connective tissue regulation. Heart volume, the thickness of the compact myocardium, collagen content and collagen fiber composition were compared between control (27°C) and cold-acclimated (20°C) zebrafish using serially sectioned hearts stained with Picrosirius Red. Collagen content and fiber composition of the pericardial membrane were also examined. Cold acclimation did not affect the volume of the contracted heart; however, there was a significant decrease in the thickness of the compact myocardium. There was also a decrease in the collagen content of the compact myocardium and in the amount of thick collagen fibers throughout the heart. Cold-acclimated zebrafish also increased expression of the gene transcript for matrix metalloproteinase 2, matrix metalloproteinase 9, tissue inhibitor of metalloproteinase 2 and collagen Type I α1. We propose that the reduction in the thickness of the compact myocardium as well as the change in collagen content may help to maintain the compliance of the ventricle as temperatures decrease. Together, these results clearly demonstrate that the zebrafish heart undergoes significant remodeling in response to cold acclimation.

KEY WORDS: Cardiac remodeling, Collagen compostion, Thermal acclimation

INTRODUCTION

Cardiac remodelling in vertebrates can be either pathological or physiological. Pathological remodelling such as left ventricular hypertrophy and cardiac fibrosis occurs in the human heart following myocardial infarction, which leads to diastolic dysfunction and eventual failure (Jalil et al., 1988; Jalil et al., 1989; Nelson et al., 2008; Pauschinger et al., 1999). Physiological remodelling of the heart, including cardiac hypertrophy and increased contractile function, occurs in most vertebrates with exercise (Diffee et al., 2003; Natali et al., 2002; Zaidi et al., 2013) and in some species of fish with cold acclimation (Farrell et al., 1988a; Klaiman et al., 2011; Tsukuda and Kihara, 1989). In cold-acclimated fish hearts, the increase in the amount of contractile machinery is thought to alleviate the loss of contractile strength caused by low temperature as well as to offset the increased load on the heart caused by an increase in blood viscosity (Graham and Farrell, 1989; Klaiman et al., 2011). Cold acclimation has also been found to increase the amount of connective tissue in the heart of male rainbow trout (*Oncorhynchus mykiss*) while warm acclimation caused cardiac atrophy and a decrease in cardiac connective tissue (Klaiman et al., 2011). These findings suggest that trout can reversibly remodel their hearts, and most interestingly, are able to degrade cardiac connective tissue. This ability has not been documented in any other vertebrate under normal physiological conditions.

Increased amounts of cardiac connective tissue are often associated with diastolic dysfunction and other cardiac myopathies, as a result of stiffening of the myocardium (Jalil et al., 1988; Jalil et al., 1989; Nelson et al., 2008; Pauschinger et al., 1999). Such changes have been reported in the hearts of patients suffering from cardiac hypertension, dilated cardiomyopathy and chronic congestive heart failure (Jalil et al., 1988; Jalil et al., 1989; Marijianowski et al., 1995; Nelson et al., 2008; Pauschinger et al., 1999). Changes in the thickness or composition of collagen fibers in the ventricle can also affect myocardial stiffness. For example, an increase in the ratio of Type I:Type III collagen results in increased myocardial stiffness as Type I collagen is less extensible than Type III and has greater tensile strength (Junqueira et al., 1978; Marijianowski et al., 1995; Pauschinger et al., 1999). One logical strategy to improve cardiac function in patients with diastolic dysfunction is to reduce or modify the connective tissue in the heart. By studying the ability of fish species to reversibly remodel their hearts and reduce cardiac connective tissue, we may gain insight into how such a strategy could be engineered.

The relative amount and type of connective tissue present in the cardiac extracellular matrix (ECM) is partially regulated by matrix metalloproteinases (MMPs) (Visse and Nagase, 2003). MMPs are a family of zinc-dependent endopeptidases that are involved in the catabolism of ECM proteins. In fish, MMP13 degrades collagen into gelatin by catalyzing the hydrolysis process (Hillegass et al., 2007). MMP2 and MMP9 then digest this hydrolyzed collagen (gelatin) into waste products that are removed from the body (Li et al., 2002; Kubota et al., 2003). The activity of all MMPs is regulated in part by the tissue inhibitor of metalloproteinase (TIMP), which binds to the MMP proforms and can ultimately reduce the rate of connective tissue degradation (Willenbrock et al., 1993).

One potential model for examining the regulation of cardiac connective tissue is the zebrafish [*Danio rerio* (Hamilton 1822)]. This species is becoming an increasingly useful model for studying cardiac growth and development in vertebrates (Grunwald and Eisen, 2002; Zhen et al., 2012). Zebrafish live in an environment where water temperatures can seasonally vary between 16 and 38°C (Lopez-Olmeda and Sanchez-Vazquez, 2011), but it is not known whether they undergo cardiac remodelling in response to changes in temperature. Recent work has demonstrated that cold acclimation of zebrafish increases the maximal metabolic rate as well as the sustained swimming performance (Little et al., 2013), but does not

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Received 12 December 2013; Accepted 18 February 2014

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affect the resting heart rate (Little and Seebacher, 2013). Together, these studies indicate that zebrafish are effectively compensating for the thermodynamic effects of low temperature on normal physiological processes.

The purpose of this study was to investigate how the morphology and composition of the zebrafish heart is affected by cold acclimation. Male zebrafish were maintained at 27°C (control) or acclimated to 20°C (cold acclimated), and then assessed for changes in cardiac morphology. These temperatures were chosen as 27°C is what the zebrafish are commonly reared at under laboratory conditions (Westerfield, 2007) and 20°C is approaching the minimal temperature at which these fish are found in the natural environment (Lopez-Olmeda and Sanchez-Vazquez, 2011). Cardiac volume and the thickness of the compact myocardium were measured using light microscopy. Collagen content and collagen fiber composition of the compact and spongy myocardium were also quantified. In addition, we examined the connective tissue content of the pericardial membrane, as this structure plays an important role in controlling diastolic function in teleosts (Farrell et al., 1988b). Finally, the expression of gene transcripts for key MMPs (*MMP2*, *MMP9* and *MMP13*), the inhibitor *TIMP2A* and collagen Type I α1 [Collagen, Type I α1 (*COL1A1*) and Collagen, Type I α2 (*COL1A2*)] were characterized in the heart using quantitative PCR. The expression of transcript for collagen Type III was not examined, as the sequence of this gene has not been identified in the zebrafish genome. In fish, TIMP2 is the dominant TIMP inhibitor of metalloproteinases (Hillegass et al., 2007) and *TIMP2A* is the transcript for the protein. This analysis was completed to examine the cellular processes that regulate collagen content.

We tested the hypothesis that cold acclimation of zebrafish would cause significant remodelling of the morphology and collagen content of the heart to compensate for decreased contractile strength and increased blood viscosity. It was predicted that cold acclimation would cause an increase in relative heart size, a decrease in the thickness of the compact myocardium and an increase in the amount of connective tissue throughout the heart.

RESULTS

Morphological characteristics

Table 1 summarizes the effect of cold acclimation on zebrafish body and heart morphology. There was no difference in fish length nor in the average heart volume between the cold-acclimated and control groups ($P>0.05$). It should be mentioned that both formalin fixation and paraffin embedding cause tissue shrinkage. However, this does not affect the comparison as all hearts were treated identically, but the absolute volumes we report are likely underestimates of actual heart size (by ~15–30%) (Carson and Hladik, 2009). The thickness of the compact layer in the cold-acclimated group was significantly thinner (~30%) than that of the control group ($P<0.05$; Table 1, Fig. 1). Cold acclimation also caused the collagen content of the compact myocardium to decrease from 22.8±3.0% to 13.1±2.7% ($P<0.05$). In cross-sections through the middle of the hearts from the control group, compact myocardium equaled 40.3±2.6 μm^2, representing 10.0±0.6% of the total myocardial area. Of this, 9.1±1.8 μm^2 was calculated to be composed of collagen. In the cold-acclimated group, compact myocardium equaled 27.0±1.2 μm^2, representing 7.7±0.4% of the total area of the heart. Collagen was calculated to compose 3.3±0.6 μm^2 of the compact myocardium in the cold-acclimated group. The reduction in the area of the compact myocardium and in the proportion that it makes up of the cross-section was statistically significant ($P<0.05$). The decrease in collagen content caused by cold acclimation, equal to 64%, was statistically significant ($P<0.05$).

Cold acclimation did not affect the proportion or calculated area of the middle cross-section that was composed of spongy myocardium (Table 2). Cold acclimation caused collagen content in the spongy myocardium to decrease by 39% ($P=0.1$; Table 1).

Collagen composition

Cold acclimation significantly affected the collagen fiber composition in both the compact and spongy myocardium. The color of collagen fibers stained with Picrosirius Red and viewed with polarized light depends upon thickness; as fiber thickness or density increases, the color changes from green to yellow to orange to red (Hiss et al., 1988; Junqueira et al., 1982). In the compact myocardium, when collagen fiber type was calculated as a percentage of the total, cold acclimation caused a 31% decrease in the relative proportion of red collagen fibers ($P=0.07$). The proportion of yellow collagen fibers and green collagen fibers increased by 145% ($P<0.05$) and 143% ($P<0.05$), respectively (Fig. 2A). In the spongy myocardium, cold acclimation caused the proportion of the thick red collagen fibers to decrease by 35% ($P=0.06$) and that of the yellow and green collagen fibers to increase by 96% ($P<0.05$) and 86% ($P=0.08$), respectively (Fig. 2A). There was no change in the proportion of the orange collagen fibers in either the spongy or compact myocardium. Finally, Junqueira et al. (Junqueira et al., 1978) suggested that collagen fibers that appear red, orange or yellow are composed of Type I collagen while fibers that appear green are composed of Type III collagen. Therefore, we calculated the ratio of red, orange and yellow fibers to green fibers to estimate the ratio of Type I:Type III collagen. We found that this ratio decreased by 85% ($P<0.05$) in the spongy myocardium and by 81% ($P<0.05$) in the compact myocardium (Table 2). Caution must be taken in interpreting these results as this method may identify an immature, thin Type I fiber as Type III (Rich and Whittaker, 2005); however, these results clearly demonstrate that the proportion of thick fibers are decreasing in the heart with cold acclimation.

When collagen fiber compositions were converted into cross-sectional area, we found that the change in collagen content and fiber composition was primarily due to a decrease in red collagen fibers. In the compact myocardium, the area occupied by red collagen fibers decreased from 4.1±1.4 to 0.8±0.2 μm^2 ($P<0.05$), while in the spongy myocardium the area occupied by red collagen fibers decreased from 29.3±7.9 to 12.3±3.8 μm^2 ($P=0.07$) (Table 2). These represent reductions of 80% and 58%, respectively.

Table 1. Morphological characteristics of zebrafish hearts from fish acclimated to either 27°C (control) or 20°C (cold acclimated)

Group	Fish length (cm)	Heart volume (mm^3)	Compact myocardium thickness (μm)	Compact myocardium collagen (% of tissue)	Spongy myocardium collagen (% of tissue)
Control	3.5±0.1	0.21±0.02	9.1±0.5^a	23.5±2.8^a	15.2±2.6
Cold acclimated	3.3±0.1	0.20±0.01	6.4±0.6^b	14.0±2.6^b	9.9±1.6

Statistical differences between treatment groups are indicated with a different superscript letter ($P<0.05$). Collagen values are expressed as the proportion of tissue area that was occupied by collagen.

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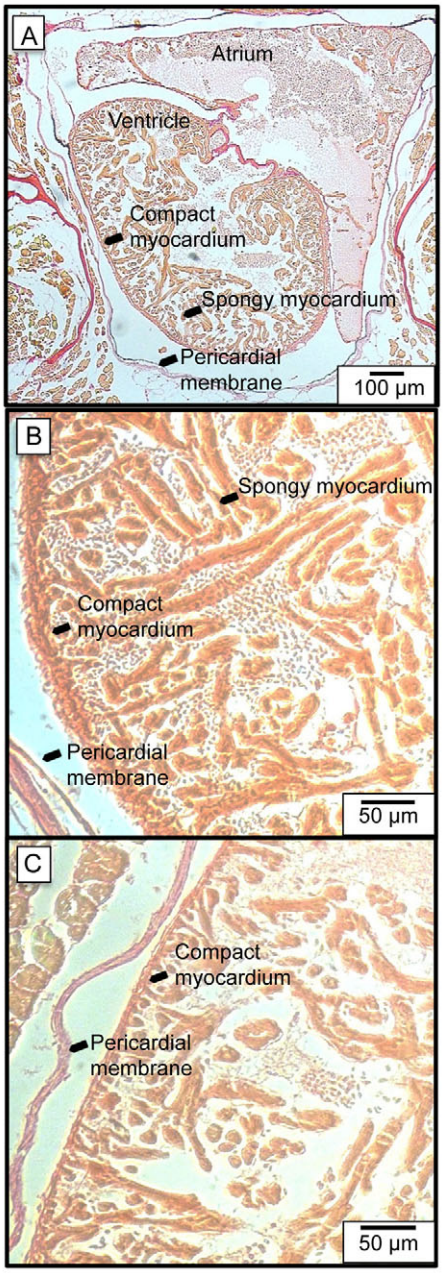

Fig. 1. Representative brightfield micrographs of the zebrafish heart and the compact myocardium in control and cold-acclimated zebrafish. (A) Cross-section of control zebrafish heart at low magnification. (B) Compact myocardium in heart from control zebrafish at high magnification. (C) Compact myocardium in heart from cold-acclimated zebrafish at high magnification.

There was no difference in collagen composition of the pericardial membrane between the control and cold-acclimated fish (61.1±3.1% and 51.6±7.4%, respectively; $P>0.05$). There was also no change in the relative proportion of the different fiber types.

Gene expression

The expression of gene transcripts for *MMP2*, *MMP9*, *COL1A1* and *TIMP2* were significantly upregulated in cold-acclimated fish ($P<0.05$; Fig. 3). These increases were 12.0-fold for *MMP2*, 2.3-fold for *MMP9*, 3.3-fold for *COL1A1* and 1.9-fold for *TIMP2*. The transcript abundances for *COL1A2* and *MMP13* were not affected by cold acclimation ($P>0.05$; Fig. 3C,E). *MMP13* demonstrated the highest overall expression (normalized to *EF1α*) in both control and cold-acclimated groups as compared with other genes, while *COL1A1* had the lowest transcript abundance across all groups. The expression of the housekeeping gene *EF1α* did not change with cold acclimation.

DISCUSSION

Our results indicate that zebrafish remodel cardiac morphology and ECM composition in response to cold acclimation. These changes include a decrease in the thickness of the compact layer, a reduction in collagen content of the compact myocardium, and a decrease in the proportion of thick collagen fibers throughout both myocardial layers. The decrease in collagen content correlated with an increase in the expression of *MMP2* and *MMP9*. We propose that these changes help to maintain the compliance of the heart at low temperatures so that cardiac output can be maintained.

Changes in gross morphology of the heart

The lack of an overall hypertrophic response with cold acclimation was not expected, as other fish that remain active during cold acclimation demonstrate cardiac hypertrophy (Klaiman et al., 2011). We have recently demonstrated that cold-induced cardiac hypertrophy in the trout is due to an increase in the spongy myocardium (Klaiman et al., 2011). Spongy myocardium contains multiple lacunae that fill with blood during diastole. An increase in the spongy myocardium will therefore increase the amount of blood pumped per beat (Klaiman et al., 2011). Previous work suggests that such changes are responsible for cold-acclimated (5°C) trout having a greater, or equal, stroke volume compared with warm-acclimated (15°C) trout (Graham and Farrell, 1989). If heart rate is maintained in zebrafish with cold acclimation, as indicated by Little and Seebacher (Little and Seebacher, 2013), it may not be necessary for these fish to increase stoke volume to maintain cardiac output. This could explain why cardiac hypertrophy was not seen.

A reduction in temperature increases the passive viscoelastic properties of muscle, leading to an increase in muscle stiffness (Mutungi and Ranatunga, 1998). In the heart, such an effect has the potential to decrease ventricle compliance and cause a reduction in diastolic function. One feature of the zebrafish heart that may play a role in compensating for the influence of low temperature on ventricle compliance is the compact myocardium. The compact myocardium is a layer of densely packed cells on the outside of the ventricle that helps to provide biomechanical support to the spongy myocardium (Hu et al., 2001; Pieperhoff et al., 2009). In this position it has the ability to influence the extension of the ventricle. We propose that the reduction in the thickness of the compact layer in zebrafish with cold acclimation is to help increase ventricle compliance so that stroke volume can be maintained at low physiological temperature. The decrease in the thickness of the compact myocardium in the present study corresponds with previous research that found the same response in cold-acclimated trout (Farrell et al., 1988a; Klaiman et al., 2011). This suggests that such a change to the thickness of the compact myocardium may be a common response in fish that seasonally acclimate to low temperatures.

Collagen

A change in myocardial collagen content alters the biomechanical characteristics of the muscle (Jalil et al., 1988; Jalil et al., 1989). For example, an increase in collagen content increases the stiffness of the muscle (Jalil et al., 1989). The decrease in the collagen content of the compact myocardium with cold acclimation may, therefore, be another compensation to help maintain ventricle compliance at

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Table 2. Area occupied by compact myocardium, spongy myocardium and connective tissue in cross-section through the middle of cardiac ventricles from control (27°C) and cold-acclimated (20°C) zebrafish

	Cross-sectional area (μm^2)	Percentage of total cross-sectional area	Area composed of connective tissue (μm^2)	Percentage of cross-sectional area composed of collagen	Type I:Type III collagen
Control compact	40.3±2.6^a	10.0±0.6^a	9.1±1.8^a	22.8±3.0^a	43.8±12.6^a
Cold-acclimated compact	27.0±1.2^b	7.7±0.4^b	3.3±0.6^b	13.1±2.7^b	8.2±2.7^b
Control spongy	368.6±22.5	90.0±0.5	51.9±9.7	14.0±2.6	27.4±12.4^a
Cold-acclimated spongy	332.1±19.6	92.3±0.3	31.3±6.4	9.6±1.7	4.1±1.6^b

Area is the calculated area of cross-section of the ventricle; percentage of total cross-sectional area is the percentage of cross-section made up of either spongy or compact myocardium; area composed of connective tissue is the area of cross-section of compact or spongy myocardium composed of connective tissue; and the Type I:Type III collagen ratio was calculated by summing the proportional values of fibers that appeared red, orange and yellow and dividing this by the proportion of fibers stained green. Values in the same column with different superscript letters are significantly different from each other (P<0.05). n=9 for all measurements.

low physiological temperatures. Furthermore, the 39% decrease in total collagen content that we observed in the spongy myocardium, while not statistically significant (P=0.1), suggests that a common strategy is used in both myocardial layers. The decrease in collagen content in the zebrafish heart with cold acclimation in the present study contradicts findings in rainbow trout (Klaiman et al., 2011). In trout, however, the increase in connective tissue content with cold acclimation is accompanied by cardiac hypertrophy, a strategy thought to help increase stroke volume. While these responses differ, it is clear that trout and zebrafish use phenotypically flexible hearts to remain active in habitats that vary considerably in temperature throughout the year (Klaiman et al., 2011; Lopez-Olmeda and Sanchez-Vazquez, 2011).

In addition to changes in overall collagen content, we found that the hearts of cold-acclimated trout had significantly thinner collagen fibers and a decreased ratio of Type I:Type III collagen. In human hearts, increased collagen fiber thickness and increased Type I:Type III collagen are associated with stiffening of the myocardium (Jalil et al., 1988; Jalil et al., 1989; Nelson et al., 2008; Pauschinger et al., 1999). Therefore, the changes observed in collagen fiber composition in the trout heart should decrease ventricle stiffness and, as a result, help compensate for the effect of low temperature on ventricle compliance. However, future studies are required to determine whether the change in collagen content and composition in the zebrafish heart leads to changes in the passive properties of the heart.

We observed no collagen remodelling of the pericardial membrane in response to cold acclimation. This membrane was examined as it assists with atrial filling in teleost fish and acts to limit distension of the ventricle during diastole (Farrell et al., 1988b). It was thought that a change in collagen content could be a strategy to reduce membrane stiffness and, as a result, enable a greater stroke volume. However, this does not appear to be the case.

Gene expression

The increased expression of the gene transcripts for *MMP2* and *MMP9* after 4 weeks of cold acclimation suggests that the heart is increasing its capacity to remove collagen from the ECM. The observed decrease in cardiac collagen content with cold acclimation supports this idea. Long et al. (Long et al., 2013) demonstrated that the expression of *MMP13* is highly upregulated in zebrafish hearts in the first 24 h of cold stress. As hearts were sampled in the present study at 4 weeks, it is not surprising that we did not observe a similar increase. However, the endogenous levels of the transcript for MMP13 were greater than that for either MMP2 or MMP9, suggesting that the heart maintains high levels of this protease.

While cold acclimation increased gene transcripts for gelatinases in the zebrafish heart, this result may not be entirely predictive of MMP activity. The increase in the expression of gene transcripts for *TIMP2* indicates that there may also be an attempt to regulate MMP activity. This idea is supported by Li et al. (Li et al., 2002), who suggested that an increase in the production of MMPs, without a counterbalancing increase in TIMPs, leads to functional defects. It was also interesting that the transcript for *COL1A1* (but not *COL1A2*) was upregulated in the cold-acclimated fish, despite the observation that collagen content decreased overall. This increase may be associated with the active maintenance of collagen content in the heart. To tease apart the timing of events in the remodelling of collagen content in the heart, future studies need to quantify the expression of these genes at multiple stages during the acclimation period.

In the control fish, the compact myocardium made up ~10% of the total area and ~7% in the cold-acclimated group. By isolating mRNA from the entire heart, it is likely that a majority of the transcripts that were amplified in the qPCR protocols were from the spongy layer. However, the histological changes that were characterized in the spongy layer (decrease in red collagen fibers and in Type I:Type III collagen) were also seen in the compact layer. In addition, although the decrease in collagen content was significant in the compact myocardium, there was also a 39% reduction in the mean value in the spongy myocardium. Therefore, it is likely that the changes in transcript levels characterized in the entire heart were reflective of what was occurring at the molecular level in both myocardial layers.

Conclusions and perspectives

The present study indicates that the morphology and composition of the zebrafish heart changes in response to cold acclimation. Specifically, these changes include a reduction in the thickness of the compact myocardium, as well as a reduction in the collagen content and in the relative proportion of thick collagen fibers (Type I) throughout the heart. Together these changes may help to compensate for the direct effect of low temperature on the compliance of the ventricle, and therefore allow cardiac output to be maintained. This study also indicates that zebrafish are a good model to study the molecular basis of cold-induced cardiac remodelling in fish. This remarkable ability has the potential to increase our understanding of the regulation of collagen deposition in the human heart. Being able to reduce collagen content in the diseased heart, specifically Type I fibers, as zebrafish are able to with cold acclimation, would be of significant benefit.

MATERIALS AND METHODS

Experimental animals

Adult male zebrafish (*D. rerio*), maintained in a colony at the Hagen Aqualab, University of Guelph, Ontario, were transferred into two 30 l

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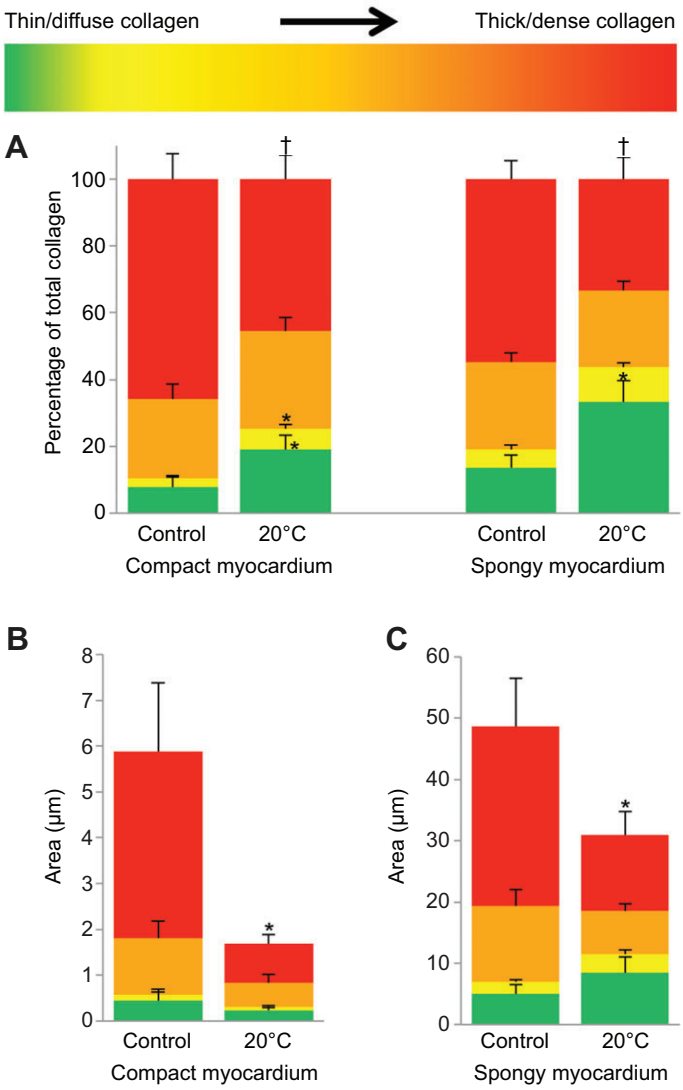

Fig. 2. Influence of cold acclimation on collagen composition in the compact and spongy myocardium of zebrafish, *Danio rerio*. (A) The relative proportion of collagen fiber colors in the compact myocardium and spongy myocardium of control (27°C) and cold-acclimated (20°C) zebrafish. Red denotes the thickest/densest fibers, while green denotes the thinnest. Proportions were calculated using measurements made in five cross-sections from each heart. (B) Area, calculated as μm^2, occupied by each of the four collagen fiber types in the compact myocardium within the middle cross-section of hearts from control and cold-acclimated zebrafish. (C) As B, but for the spongy myocardium. *Values of the same fiber type in the same myocardial layer are significantly different between treatment groups ($P<0.05$); †the P-value between the two mean values for this fiber type is <0.07. $n=9$ for all measurements.

recirculating tanks (each holding 40 fish) in an environmental chamber. The water in these tanks was held at 27°C using emersion heaters connected to a computer-controlled environmental control system. Fish were held at 27°C for 4 weeks prior to experimentation. The control tank was maintained at 27°C for the duration of the experiment. The temperature of the second tank (cold-acclimated fish) was decreased 1°C per day from 27 to 20°C using the environmental control system (Klaiman et al., 2011; McClelland et al., 2006), and then held at 20°C for 4 weeks prior to sampling. Fish were fed *ad libitum* once per day for the duration of the experiment. After the acclimation period, zebrafish were euthanized using buffered tricaine methanesulfonate (45 mg l^{-1}). The thoracic cavity was immediately opened and the heart was rinsed with physiological saline (in mmol l^{-1}: 94 NaCl, 24 $NaCO_3$, 5 KCl, 1 $MgSO_4$, 1 Na_2HPO_4, 0.7 $CaCl_2$, pH 7.6 at 15°C) and then bathed in 1 mol l^{-1} KCl to cause maximal contraction prior to fixation. Only males were used in this study as Klaiman et al. (Klaiman et al., 2011) demonstrated that cold acclimation did not cause cardiac hypertrophy or induce an increase in connective content in female trout. The University of Guelph's Animal Care Committee approved care and use of all experimental animals, as per the principles of the Canadian Council for Animal Care.

Histology

The entire thorax region of each fish, containing the heart, was fixed in 2% buffered formalin (24 h, 4°C), decalcified (1 h, 20°C; Surgipath Decalcifier II, Winnipeg, MB, Canada) and stored in 70% ethanol. Each thorax was embedded in paraffin wax and 5 μm transverse sections were made from the entire tissue (Chablais et al., 2011; Klaiman et al., 2011). Every fifth section was mounted on a glass slide, resulting in a mean (±s.e.m.) of 33.3±1.2 sections per heart. Sections were stained for collagen with Picrosirius Red (Electron Microscopy Sciences, Hatfield, PA, USA) (Junqueira et al., 1979a; Junqueira et al., 1979b; Rich and Whittaker, 2005). A Nikon Ti microscope (Nikon, Melville, NY, USA) was used to take brightfield and polarized light images of the ventricle sections and surrounding pericardial membrane. Brightfield images were used to measure heart size (Fig. 1A) and compact layer thickness (Fig. 1B,C). The polarized images were used to analyze collagen content and fiber thickness in the spongy myocardium, compact myocardium and pericardial membrane (Rich and Whittaker, 2005) (Fig. 4). As the thickness of fibers imaged in this manner increases, the color changes from green to yellow to orange to red (Hiss et al., 1988; Junqueira et al., 1982). The cross-sectional area of each heart section was quantified using ImageJ (National Institutes of Health, Bethesda, MD, USA), and these area measurements were used to calculate the heart volume using a trapezoidal estimation equation (Rosen and Harry, 1990):

$$\text{Volume} = \sum_{i=1}^{n-1} \frac{(y_i + y_{i+1})}{2}(t + d)\,, \quad (1)$$

where n is the total number of sections, y_i is the cross-sectional area of the ith section through the heart, t is the section thickness (=5 μm) and d is the distance between sections (=20 μm).

The average thickness of the compact layer was determined using ImageJ software at four random locations from five sections per heart (Klaiman et al., 2011). The amount of collagen in the spongy myocardium, compact myocardium and pericardial membrane was determined from the same five sections per heart as described by Rich and Whittaker (Rich and Whittaker, 2005). Briefly, using ImageJ, 256-color polarized images were transformed into their hue component and the color of each pixel was automatically determined using the histogram function. The percentage of collagen in each layer of the heart or membrane was calculated by dividing the total number of collagen-colored pixels (defined by Rich and Whittaker, 2005) by the total number of pixels in that region of the heart. Lastly, the proportion of each collagen fiber color (red, orange, yellow and green fibers) in the compact and spongy layers was determined by dividing the number of pixels of that fiber color by the total number of collagen-colored pixels in each layer.

Calculation of area and connective tissue content of spongy and compact myocardium

The area of the spongy myocardium was measured using ImageJ in a cross-section through the middle of the heart (a section in which the atrial ventricular valve was clearly visible). This value was then subtracted from the total cross-sectional area of the section to determine the area of the compact myocardium. The area of the spongy and compact myocardium that was composed of connective tissue was calculated using the measured percentage value from the polarized light images for each myocardium type.

Quantitative real-time PCR

The transcript abundance of six genes associated with connective tissue regulation (*MMP2*, *MMP9*, *MMP13*, *COL1A1*, *COL1A2* and *TIMP2*) were quantified in the hearts of the fish in the control and cold-acclimated groups (n=3–5; 5 pooled hearts per n). Total RNA was extracted from tissue homogenized in Trizol (Life Technologies, Grand Island, NY, USA) according to the manufacturer's instructions, and quantified using a

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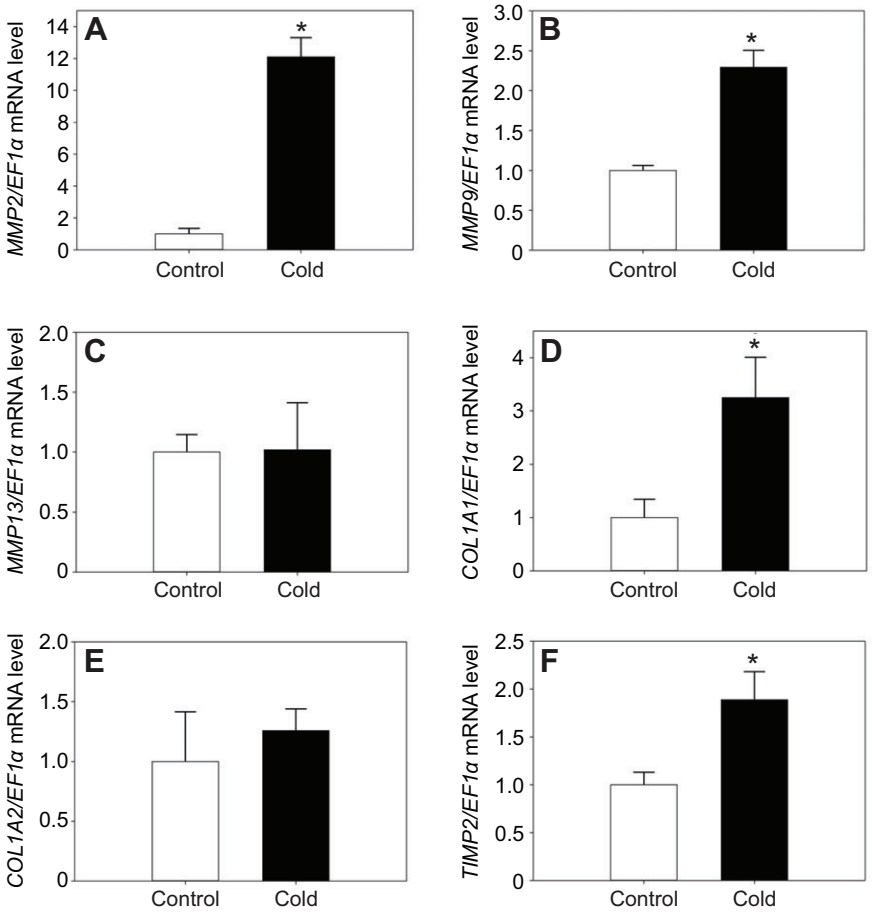

Fig. 3. The influence of cold acclimation on the expression of gene transcripts. (A) Matrix metalloproteinase 2 (*MMP2*); (B) matrix metalloproteinase 9 (*MMP9*); (C) matrix metalloproteinase 13 (*MMP13*); (D) tissue inhibitor of metalloproteinase 2 (*TIMP2*); (E) collagen Type I, α1 (*COL1A1*); and (F) collagen Type I, α2 (*COL1A2*). The expression of each transcript in the cold-acclimated (20°C) samples is relative to the amount of that transcript in the control group (27°C). This value is set to 1 in each panel. Isoform transcript abundance was normalized to the mRNA abundance of *elongation factor 1α* (*EF1α*). Asterisks indicate a significant effect of cold acclimation on gene expression (P<0.05). n=3–5 for each measurement; five pooled hearts per n.

Nanodrop 8000 (ThermoFisher Scientific, Ottawa, ON, Canada). One microgram of total RNA was treated with DNase I (Sigma-Aldrich) and used to synthesize cDNA with the High Capacity cDNA Synthesis Kit (Life Technologies) following the manufacturers' instructions. Duplicate cDNA reactions in which the Multiscribe RT enzyme was omitted were included for 10% of total samples, chosen randomly, to verify the efficacy of the DNase treatment. Transcript abundances were measured in duplicate reactions on a StepOne Plus (Life Technologies) using default cycling

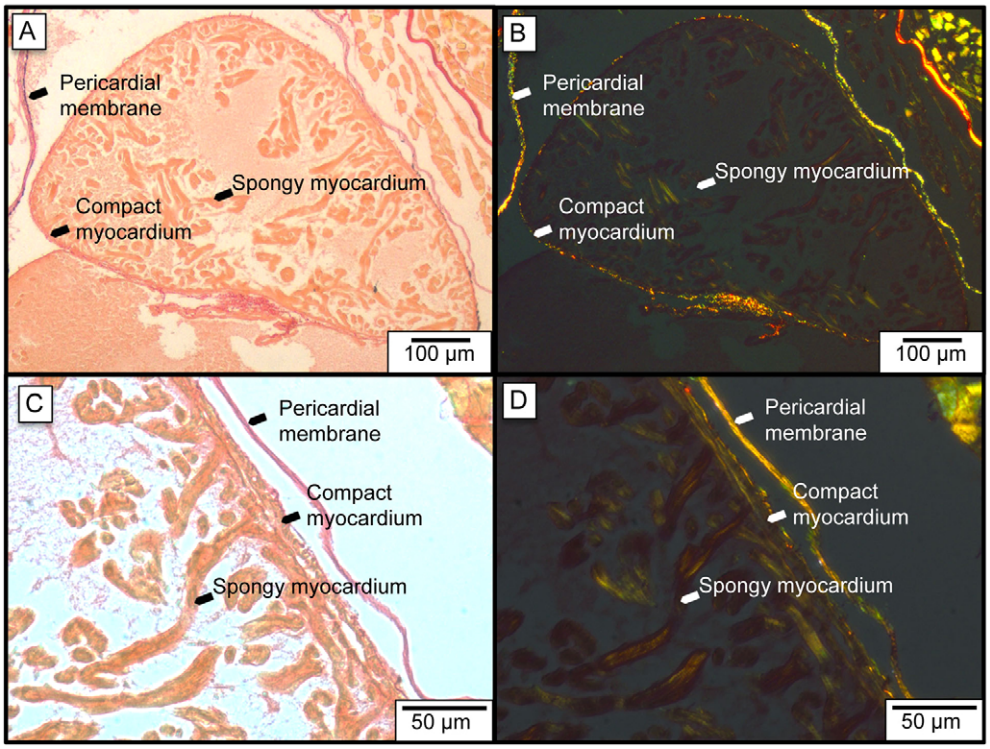

Fig. 4. Histological detection of collagen deposition in heart ventricles of control zebrafish. Tissue sections were stained using Picrosirius Red to detect collagen. Representative low magnification brightfield (A), low magnification polarized (B), high magnification brightfield (C) and high magnification polarized (D) images are presented. Compact myocardium, spongy myocardium and pericardial membrane are labeled on the figures. In the polarized images, collagen fibers were red, orange, yellow or green. With this technique, thick/dense fibers are detected as red, intermediate fiber diameters are detected as orange and yellow, and thin fibers are detected as green.

Table 3. Forward and reverse primer sequences used to amplify transcripts for matrix metalloproteinase 2 (*MMP2*), *MMP9*, *MMP13*, the tissue inhibitor of metalloproteinase (*TIMP2*), collagen Type I, α1 (*COL1A1*), and collagen Type I, α2 (*COL1A2*) using quantitative PCR

Gene	Sequence	Amplicon size (bp)	GenBank accession number
MMP2	F: GCCTTAATGGTGATGGTCACA R: GGTCTGTCGATGTTCAGCAG	132	NM_198067
MMP9	F: TGGGCACCTGCTCGTTGA R: TTGGAGATGACCGCCTGC	172	NM_213123.1
MMP13	F: ATGGTGCAAGGCTATCCCAAGAGT R: GCCTGTTGTTGGAGCCAAACTCAA	289	AF506756.1
TIMP2	F: ATGGGGTGTGACTGCAAGAT R: AGGCGTAGTGGTCAGACTGG	130	NM_182874.1
COL1A1	F: GGCTTCCAGTTCGAGTATGG R: ATGCAATGCTGTTCTTGCAG	129	BC161663.1
COL1A2	F: GGCTGCAGTAGACACACTGG R: CAATGTCCAAAGGTGCAATG	103	NM_182968.2

The amplicon size amplified by each primer set, as well as the GenBank accession numbers are provided for each isoform. F, forward primer; R, reverse primer.

conditions and a dissociation cycle. Each 15 µl reaction contained 1× PerfeCta Fast SYBR Green Master Mix (Quanta BioSciences), 200 nmol l^{-1} of each gene-specific primer (Table 3) and 1:15 vol:vol cDNA. Custom oligos for *EF1α*, *COL1A1*, *TIMP2*, *COL1A2*, *COL1A3* and *MMP2* were designed using Primer 3. Gene-specific primers for *MMP13* and *MMP9* were adopted from Hillegass et al. (Hillegass et al., 2007) and Wu et al. (Wu et al., 2010), respectively. All reactions generated a single-peaked dissociation curve at the predicted amplicon melting temperature. The mRNA abundance of each gene was quantified by fitting the threshold cycle to the antilog of standard curves prepared from serially diluted cDNA. Isoform transcript abundance was normalized to the mRNA abundance of *elongation factor 1α* (*EF1α*; GenBank accession number: AY422992.1; forward primer: TCTCAGGCTGACTGTGCTGT; reverse primer: GGTCTGTCCGTTCTTGGAGA). All non-reverse transcribed control samples failed to amplify.

Statistical analysis

Heart volume, area and volume of compact and spongy myocardium, area and volume of connective tissue in spongy and compact myocardium, compact layer thickness, percentage of collagen in each layer, and proportion of collagen fiber types in each layer were each compared between control and cold-acclimated fish with Student's *t*-tests. For each gene, a Student's two-tailed *t*-test was used to compare differences in transcript abundance between control and cold-acclimated fish. A Shapiro–Wilk test was used to determine whether data were normally distributed, and any group that did not fit the assumption of normality was log-transformed prior to analysis. A critical α=0.05 was used throughout. All data are presented as means ± s.e.m.

Acknowledgements

The authors thank Dr A. L. Alderman for comments on an earlier version of the manuscript.

Competing interests

The authors declare no competing financial interests.

Author contributions

A.C.J., A.J.T., J.M.K., E.F.J. and T.E.G. designed the study; A.C.J., A.J.T. and E.F.J. completed the experiments; and A.C.J., A.J.T., J.M.K., E.F.J. and T.E.G. wrote the paper.

Funding

This work was supported by a Discovery Grant from the Natural Sciences and Engineering Research Council (NSERC) to T.E.G. and NSERC Canadian Graduate Scholarships to J.M.K. and A.J.T.

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