Cold acclimation increases cardiac myofilament function and ventricular pressure generation in trout

Jordan M. Klaiman¹, W. Glen Pyle² and Todd E. Gillis¹,*

ABSTRACT
Reducing temperature below the optimum of most vertebrate hearts impairs contractility and reduces organ function. However, a number of fish species, including the rainbow trout, can seasonally acclimate to low temperature. Such ability requires modification of physiological systems to compensate for the thermodynamic effects of temperature on biological processes. The current study tested the hypothesis that rainbow trout compensate for the direct effect of cold temperature by increasing cardiac contractility during cold acclimation. We examined cardiac contractility, following thermal acclimation (4, 11 and 17°C), by measuring the Ca²⁺ sensitivity of force generation by chemically skinned cardiac trabeculae as well as ventricular pressure generation using a modified Langendorff preparation. We demonstrate, for the first time, that the Ca²⁺ sensitivity of force generation was significantly higher in cardiac trabeculae from 4°C-acclimated trout compared with those acclimated to 11 or 17°C, and that this functional change occurred in parallel with a decrease in the level of cardiac troponin T phosphorylation. In addition, we show that the magnitude and rate of ventricular pressure generation was greater in hearts from trout acclimated to 4°C compared with those from animals acclimated to 11 or 17°C. Taken together, these results suggest that enhanced myofilament function, caused by modification of existing contractile proteins, is at least partially responsible for the observed increase in pressure generation after acclimation to 4°C. In addition, by examining the phenotypic plasticity of a comparative model we have identified a strategy, used in vivo, by which the force-generating capacity of cardiac muscle can be increased.

KEY WORDS: Calcium sensitivity, Cardiac contractility, Protein phosphorylation, Thermal acclimation

INTRODUCTION
All physiological processes are sensitive to temperature change due to the thermodynamic effects of temperature on biochemical and biophysical reactions (Hochachka and Somero, 2002). Ectothermic animals are particularly challenged, therefore, by fluctuations in environmental temperature. In spite of these challenges, many temperate fish species including rainbow trout (Oncorhynchus mykiss), Atlantic cod (Gadus morhua) and burbot (Lota lota) remain active despite considerable seasonal variations in temperature. For example, rainbow trout are active in the winter at ~4°C, a temperature that stops the human heart, and can experience temperatures exceeding 20°C in the summer. This broad temperature niche clearly requires thermal compensation of physiological processes, although the mechanisms involved remain poorly understood.

Underlying the influence of temperature on cardiac function are changes in the ability of cardiac myocytes to contract and generate force (Harrison and Bers, 1990). Specifically, an acute decrease in temperature reduces the Ca²⁺ sensitivity of the contractile element and this translates into a reduction in force generation (Churcott et al., 1994; Harrison and Bers, 1990), and limits the capacity of the heart to pump blood around the body. The ability of many temperate fish species to maintain cardiac function at low temperatures following seasonal acclimation provides an excellent opportunity to study mechanisms of thermal acclimation (Klaiman et al., 2011). Previous studies have demonstrated that cold acclimation of rainbow trout results in an increase in the maximal rate of cardiac actomyosin ATPase (AM-ATPase) (Yang et al., 2000; Klaiman et al., 2011) and the speed of twitch kinetics of intact fibers (Aho and Vornanen, 1999), indicating changes to the contractile machinery of the heart. Cold acclimation of trout also affects transcript levels of troponin I (Alderman et al., 2012), troponin C (Genge et al., 2013) and sarcoplasmic reticulum Ca²⁺-ATPase (SERCA) (Korajoki and Vornanen, 2012), all of which are key proteins involved in contraction. Korajoki and Vornanen (Korajoki and Vornanen, 2013) have also found that cold acclimation of burbot causes a fourfold increase in the levels of SERCA in the heart, a modification thought to help maintain Ca²⁺ cycling at low temperatures. The above changes all contribute to cardiac contractility and how it is maintained at low temperatures. Supporting this idea, Graham and Farrell (Graham and Farrell, 1989) demonstrated that maximum cardiac work is similar for rainbow trout acclimated to 5 or 15°C, and Lurman et al. (Lurman et al., 2012) found little difference in the cardiac output of Atlantic cod acclimated to either 10 or 0°C measured at the respective acclimation temperature. Still missing from a full understanding of this cardiac compensatory mechanism is the connection between the necessary molecular- and protein-level changes induced by cold acclimation and the resultant changes in whole heart function. Such knowledge will provide fundamental insight into the adaptive capacity of the vertebrate heart.

The objective of this study was to characterize how thermal acclimation alters the contractility of the trout myocardium and the intact ventricle. Here cardiac contractility is defined as a measure of myocardial function influenced by an altered ability to move Ca²⁺ through the myocyte or to generate force in response to Ca²⁺. It is not related to changes in preload, afterload, or heart rate. These experiments tested the hypothesis that rainbow trout compensate for the direct effect of cold temperature by increasing cardiac contractility during cold acclimation. This hypothesis predicts that cold acclimation will cause an increase in the Ca²⁺ sensitivity of force generation by chemically skinned cardiac trabeculae, as well as increase the pressure generation of the intact ventricle as measured using a Langendorff preparation. Finally, one mechanism by which cardiac contractile function is regulated in vertebrates is via the phosphorylation of a
number of myofilament proteins, including troponin I, and troponin T (Noland and Kuo, 1991; Jideama et al., 1996; Yang et al., 2008). We therefore also examined how thermal acclimation affected the phosphorylation of the myofilament proteins so as to begin identifying a mechanism for any detected functional change.

RESULTS

Ca\textsuperscript{2+} activation of cardiac trabeculae

Across the range of experimental Ca\textsuperscript{2+} concentrations, the trabeculae from the 4°C-acclimated trout generated greater forces than those from the 11°C- and 17°C-acclimated trout (Fig. 1A). There was no difference, however, in the maximum Ca\textsuperscript{2+}-activated force (F\textsubscript{max}) or passive force (F\textsubscript{pass}) between trabeculae from the different acclimation groups (Table 1). Temperature acclimation also had no effect on the relative ventricular mass [RVM; (heart mass/body mass)×100] or cardiac collagen content (Table 1). The Ca\textsuperscript{2+} concentration, calculated as pCa (–log[Ca\textsuperscript{2+}]), needed to achieve 50% of maximum Ca\textsuperscript{2+} activated force (pCa\textsubscript{50}) was significantly greater for the trabeculae from the 4°C-acclimated trout than for the trabeculae from the 11°C- and 17°C-acclimated trout (P<0.05; Table 1). It should be noted here that the Ca\textsuperscript{2+} concentrations over which force was generated by the preparations in the present study (pCa 6.2 to pCa 5.2) are higher than in intact trout cardiac myocytes during excitation–contraction coupling (pCa 6.3 to pCa 6.0) at 14°C (Shiels et al., 2002). There are a number of possible explanations for this, including the composition of the buffers used (Gillis and Klaiman, 2011) and potential changes to myofilament lattice spacing that occur during chemical skinnng (Martyn et al., 2004). However, as all preparations in this study were treated identically, the characterized functional differences between groups remain valid.

The rate of isometric tension redevelopment following rapid release–restretch (k\textsubscript{tr}) was used as an estimate of the rate of cross-bridge cycling at each activation pCa (Gillis et al., 2007). When the k\textsubscript{tr} values are plotted against pCa there is a positive sigmoidal relationship between Ca\textsuperscript{2+} concentration and the rate of cross-bridge cycling in the preparations from the 4°C- and 17°C-acclimated fish (Fig. 1B). In contrast, the relationship between k\textsubscript{tr} and pCa was comparatively flat in the preparations from the 11°C-acclimated fish. There were no differences in the mean k\textsubscript{tr} values for the preparations from the three acclimation groups at any of the pCa values (Fig. 1B). This suggests that thermal acclimation did not affect the rate of cross-bridge formation in the cardiac myocardium.

Changes in the level of myofilament protein phosphorylation

The phosphorylation level of cardiac troponin T (cTnT) was ~50% lower in the trabeculae from the 4°C-acclimated trout compared

![Fig. 1. The influence of thermal acclimation on the Ca\textsuperscript{2+} activation of cardiac trabeculae and on the phosphorylation level of the associated contractile proteins.](image)
The influence of thermal acclimation on ventricular pressure generation

The heart mass of the female trout acclimated to 17°C was greater than that of female fish acclimated to 4°C (Table 2). This was the only effect of temperature acclimation on heart mass. Thermal acclimation did not affect RVM or cardiac collagen content in either male or female fish (Table 2). The percentage of the heart composed of compact myocardium was, however, significantly lower in the male fish acclimated to 4°C compared with the male fish acclimated to 17°C (P<0.05, Fig. 2). The Frank–Starling curves obtained using the ventricles from the male and female fish from each of the three acclimation groups are illustrated in Fig. 3A,B. The developed pressure (systolic pressure – diastolic pressure), used as a measure of contractile force, for the ventricles from the male and female fish acclimated to 4°C increased by ~94 and ~96%, respectively, from baseline conditions to maximum balloon volume (+55 μl). For the ventricles from male and female fish acclimated to 17°C these values increased by ~32 and ~25%, respectively. The mean max dP/dt values averaged across all balloon volumes were significantly higher for the ventricles from 4°C-acclimated male and female trout than for the ventricles of all trout from the 11 and 17°C-acclimated animals (P<0.05, Table 3). Finally, the maximal rate of relaxation during diastole (max dP/dt) was significantly greater for the ventricles from the cold-acclimated female fish compared with those from the 11°C- and 17°C-acclimated trout (P<0.05; Fig. 1C, Table 3). In addition, the mean phosphorylation level of troponin I (TnI), slow skeletal troponin T (sSTnT), and myosin regulatory light chain (RLC) was ~24-45% lower in the cardiac trabeculae from the 4°C-acclimated fish than in those from the 11°C- and 17°C-acclimated groups. However, these latter differences were not statistically significant (P=0.32, P=0.36 and P=0.53, respectively).

**Table 1. Relative ventricular mass, cardiac collagen content and functional parameters of cardiac trabeculae from trout acclimated to 4, 11 or 17°C**

<table>
<thead>
<tr>
<th>Acclimation group</th>
<th>Relative ventricular mass</th>
<th>Collagen (μg mg⁻¹)</th>
<th>F_max (mN mm⁻²)</th>
<th>F_pass (mN mm⁻²)</th>
<th>pCa⁰⁰</th>
<th>Hill coefficient</th>
</tr>
</thead>
<tbody>
<tr>
<td>4°C (N=18)</td>
<td>0.10±0.01</td>
<td>12.5±1.9</td>
<td>22.9±2.3</td>
<td>1.3±0.1</td>
<td>5.69±0.03a</td>
<td>5.8±0.5</td>
</tr>
<tr>
<td>11°C (N=17)</td>
<td>0.11±0.02</td>
<td>11.9±2.1</td>
<td>21.9±2.2</td>
<td>1.1±0.1</td>
<td>5.59±0.02b</td>
<td>5.7±0.5</td>
</tr>
<tr>
<td>17°C (N=32)</td>
<td>0.10±0.01</td>
<td>11.5±0.8</td>
<td>26.9±1.8</td>
<td>1.2±0.2</td>
<td>5.61±0.01b</td>
<td>5.6±0.3</td>
</tr>
</tbody>
</table>

Acclimation temperatures are 4, 11 and 17°C. Values are means ± s.e.m. Different superscript letters denote a significant difference between values in the same column (P<0.05). F_max, maximum Ca²⁺-activated force; F_pass, passive force; pCa⁰⁰, Ca²⁺ concentration at half-maximum force expressed as the −log; Hill coefficients are used as a measure of co-operativity. Relative ventricular mass=[(heart mass/body mass)×100].

Sex-specific differences in ventricle morphology and function

There was no difference in heart mass between male and female fish within each acclimation group (P>0.05, Table 2). However, the RVM was greater for males than females within each acclimation group (P<0.05, Table 2). The only difference in cardiac collagen content between male and female fish was in the compact myocardium of the 11°C acclimation group. Here, the collagen content in female fish was statistically less than that in the compact myocardium of all groups and sexes. N=5–8 hearts for all groups.

**Table 2. Heart mass, relative ventricular mass and percent collagen content of the spongy and compact myocardium in trout acclimated to 4, 11 or 17°C**

<table>
<thead>
<tr>
<th>Acclimation (sex)</th>
<th>Heart mass (g)</th>
<th>Relative ventricular mass</th>
<th>% Collagen spongy</th>
<th>% Collagen compact</th>
</tr>
</thead>
<tbody>
<tr>
<td>4°C (males)</td>
<td>1.14±0.12</td>
<td>0.102±0.01</td>
<td>0.58±0.14</td>
<td>4.91±0.45</td>
</tr>
<tr>
<td>4°C (females)</td>
<td>0.74±0.03*</td>
<td>0.071±0.001*</td>
<td>0.30±0.06</td>
<td>5.63±0.50</td>
</tr>
<tr>
<td>11°C (males)</td>
<td>1.53±0.24</td>
<td>0.104±0.017</td>
<td>0.16±0.07</td>
<td>3.78±0.33</td>
</tr>
<tr>
<td>11°C (females)</td>
<td>1.03±0.09</td>
<td>0.067±0.009*</td>
<td>0.64±0.21</td>
<td>7.00±0.70*</td>
</tr>
<tr>
<td>17°C (males)</td>
<td>1.36±0.15</td>
<td>0.108±0.005</td>
<td>0.29±0.07</td>
<td>5.39±0.44</td>
</tr>
<tr>
<td>17°C (females)</td>
<td>1.45±0.15*</td>
<td>0.081±0.005*</td>
<td>0.50±0.01</td>
<td>3.99±0.52</td>
</tr>
</tbody>
</table>

These parameters are for the hearts used in the Langendorff experiments. Values are means ± s.e.m. Relative ventricular mass=[(heart mass/body mass)×100]. % Collagen: proportion of the tissue composed of collagen determined using histological techniques. There was no effect of thermal acclimation on relative ventricular mass or collagen content (P>0.05). The heart mass of the 17°C-acclimated females was significantly greater than that of 4°C-acclimated females, as indicated by the different superscripts. The interaction value for the % collagen in the compact layer was P=0.057; we indicated that there was an effect of sex within the 11°C-acclimated group.

* Significant effect of sex within an acclimation group. The mean % collagen content in the spongy myocardium of all acclimation groups and sexes was statistically less than that in the compact myocardium of all groups and sexes. N=5–8 hearts for all groups.
The increase in the Ca\textsuperscript{2+} sensitivity of force generation by the skinned muscle preparation with cold acclimation indicates compensation at the level of the cardiac myofilament. Previous studies have shown that cold acclimation of trout increases the activity of AM-ATPase across the physiological range of free Ca\textsuperscript{2+} concentrations without affecting the Ca\textsuperscript{2+} sensitivity of the enzyme (Yang et al., 2000; Klaiman et al., 2011). The present results suggest that the increased AM-ATPase activity translates into increased force-generating capacity of the trabeculae across the range of experimental Ca\textsuperscript{2+} concentrations. The positive sigmoidal relationship between $k_0$ and $p_{Ca}$ in the preparations from the 4 and 17°C groups indicates that the rate of myofilament activation increases with Ca\textsuperscript{2+} concentration. This is the typical response of mammalian trabeculae to increasing Ca\textsuperscript{2+} and is interpreted as an increase in the transition from non-force- to force-generating cross-bridges (Wolff et al., 1995; Adhikari et al., 2004; Gillis et al., 2007). In contrast, the comparatively flat response of the preparations from the 11°C group suggests that the rate of cross-bridge cycling is similar at all activation $p_{Ca}$ values. We do not currently have an explanation for this.

**The role of protein phosphorylation in altering contractility**

As all cellular membranes had been dissolved using a detergent, the involvement of sarcomemal Ca\textsuperscript{2+} handling proteins was eliminated and so the increased contractile function of the trabeculae, caused by acclimation to 4°C, was due to changes in the contractile element. In mammals, the phosphorylation of cTnT in the heart causes a decrease in Ca\textsuperscript{2+} sensitivity, AM-ATPase activity and maximal tension generation (Noland and Kuo, 1991; Jideama et al., 1996), while the dephosphorylation of the cardiac contractile proteins, including cTnT, has been shown to have the opposite effect (Yang et al., 2008). For example, Yang et al. (Yang et al., 2008) demonstrated that the treatment of isolated mammalian myofilament proteins with protein phosphatase 1 caused an increase in AM-ATPase activity and Ca\textsuperscript{2+} sensitivity. These changes occurred in conjunction with a decrease in the phosphorylation of cTnI, cTnT, RLC and cMyBP-C (Yang et al., 2008). We also demonstrated that the phosphorylation of trout cardiac trabeculae by protein kinase A caused a decrease in maximum Ca\textsuperscript{2+}-activated force and $k_0$ at submaximal Ca\textsuperscript{2+} levels, indicating that the function of the trout myocardium is influenced by the phosphorylation of the contractile proteins (Gillis and Klaiman, 2011). Therefore, we propose that the decrease in the phosphorylation state of cTnT contributes to the increased contractility of the cardiac trabeculae with cold acclimation.

**DISCUSSION**

The goal of this study was to determine how thermal acclimation of rainbow trout alters cardiac contractility. We specifically examined how acclimation to 4, 11 and 17°C influenced the Ca\textsuperscript{2+} sensitivity of force generation by cardiac trabeculae and the function of the intact ventricle using a modified Langendorff perfusion. The main findings were that: (1) acclimation to 4°C increases the Ca\textsuperscript{2+} sensitivity of force generation, but not maximum force development, (2) this increased calcium sensitivity with cold acclimation occurred in parallel with a decrease in the level of cTnT phosphorylation, and (3) the magnitude and rate of ventricular pressure generation was greater in hearts from trout acclimated to 4°C compared with those from trout acclimated to 11 or 17°C. Together these results indicate that it is modification to the myofilaments that contributes to the improved force-generating capacity of the trout heart with cold acclimation. Below we discuss these findings and the insight they provide into the functional plasticity of the vertebrate heart.

**The influence of thermal acclimation on the Ca\textsuperscript{2+} activation of force generation**

There was no difference in the developed pressure for the ventricles from males and females within any acclimation group; however, the diastolic pressure was higher in the ventricles from the male fish in the 11 and 17°C groups compared with the hearts from the females in the same group (Fig. 2).

The influence of thermal acclimation on the relative proportion of compact myocardium in male and female trout. (A) Proportion of compact myocardium in ventricles from male and female fish acclimated to 4, 11 and 17°C. Values are means ± s.e.m. The $N$ for each plotted data point is 5–8 hearts. Lines with an asterisk indicate a significant difference between sexes within an acclimation group. Different letters within bars indicate significant differences between acclimation temperatures when each sex is analysed separately ($P$≤0.05). Panels B–D are low magnification images of picrosirius red-stained cross-sections of ventricles. (B) 4°C-acclimated, (C) 11°C-acclimated and (D) 17°C-acclimated rainbow trout. Panels E–G are high magnification images. (E) 4°C-acclimated, (F) 11°C-acclimated and (G) 17°C-acclimated rainbow trout. On the micrographs yellow/orange indicates muscle, red/pink indicates collagen, cm indicates the compact myocardium, and sm the spongy myocardium.
Fig. 3. The influence of thermal acclimation on the magnitude and rate of ventricular pressure development. (A,B) Ventricular pressure curves for 4°C-(blue symbols), 11°C-(black symbols) and 17°C-acclimated (red symbols) male (A) and female (B) rainbow trout. Squares indicate ventricular developed pressures while circles indicate diastolic pressures. (C,D) Maximum rates (max dP/dt_{sys}; squares) and minimum rates (max dP/dt_{dia}; circles) of pressure development in ventricles from 4°C-, 11°C- and 17°C-acclimated male (C) and female (D) rainbow trout over a range of balloon volumes. Diastolic and developed pressure (systolic pressure – diastolic pressure) are measured in mmHg; max dP/dt_{sys} and max dP/dt_{dia} are measured in mmHg s\(^{-1}\). In C and D, positive values are max dP/dt_{sys} and negative values are max dP/dt_{dia}. The balloon volume of 0 is the volume at baseline conditions. Values are means ± s.e.m. The N for each plotted data point is 5–8. Significant results from the two-way ANOVA are indicated. In each parameter tested there was a significant effect of balloon volume (P<0.05). There was no significant interaction between acclimation×sex and balloon volume in any parameter tested (P>0.05).

**Influence of temperature acclimation on ventricle function**

Previous *in situ* studies have shown that hearts from cold-acclimated trout maintain the same power output per gram, but had an overall increase in absolute power output (Graham and Farrell, 1989; Graham and Farrell, 1990). This difference has been attributed to an increase in heart size with cold acclimation (Graham and Farrell, 1989; Graham and Farrell, 1990). In this study we report that there was an increase in the pressure-generating capacity of the heart, but we did not observe cardiac hypertrophy. We propose, therefore, that the increased Ca\(^{2+}\) sensitivity of the myocardium seen in the skinned muscle is translated into an increase in the pressure-generating capacity of the whole heart.}

**Table 3. Function of Langendorff perfused hearts from thermally acclimated trout, tested at 15°C**

<table>
<thead>
<tr>
<th>Acclimation (sex)</th>
<th>Baseline</th>
<th>Averaged</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>diastolic</td>
<td>diastolic</td>
</tr>
</tbody>
</table>
| 4°C (males)       | 0.4±0.2  | 5.1±0.4\(^a\) | 35.0±4.4 | 52.8±1.3\(^a\) | 242.1±20.7\(^a,b\) | 336.5±10.9\(^a\) | −203.5±31.2 | −231.7±7.8\(^a\)
| 4°C (females)     | 0.2±0.2  | 5.9±0.3\(^a,b,c\) | 35.1±1.1 | 52.1±1.1\(^a\) | 321.2±49.5\(^b\) | 396.9±3.3\(^a\) | −212.8±14.0 | −280.5±6.6\(^b\)
| 11°C (males)      | 0.6±0.2  | 6.8±0.4\(^a\) | 31.7±2.8 | 46.6±1.2\(^c\) | 213.4±16.6\(^b\) | 261.3±10.1\(^c\) | −174.7±9.3 | −211.9±7.2\(^c\)
| 11°C (females)    | 0.6±0.1  | 5.3±0.3\(^a\) | 27.2±1.9 | 40.4±1.0\(^d\) | 180.6±16.5\(^b\) | 254.5±4.4\(^b\) | −152.9±10.9 | −210.9±6.0\(^d\)
| 17°C (males)      | 0.6±0.1  | 5.6±0.3\(^a\) | 29.9±2.1 | 37.0±1.0\(^e\) | 207.6±19.8\(^b\) | 243.9±8.3\(^c\) | −187.8±16.8 | −222.8±5.9\(^e\)
| 17°C (females)    | 0.5±0.1  | 4.7±0.4\(^a\) | 27.9±2.2 | 34.7±1.2\(^d\) | 171.1±14.4\(^b\) | 201.4±8.8\(^b\) | −161.5±18.2 | −206.5±7.0\(^d\)

Values are means ± s.e.m. Pressure is measured in mmHg; max dP/dt_{sys}, maximal rate of pressure development during systole; max dP/dt_{dia}, minimum rate of pressure development during diastole; max dP/dt_{sys} and max dP/dt_{dia} are measured in mmHg s\(^{-1}\). Baseline measurements were made at a balloon volume at which the ventricular end diastolic pressure was between 0 and 1 mmHg, and developed pressure was greater than 20 mmHg. Averaged Langendorff parameters are the least square means ± s.e.m., averaged over all balloon volumes. Different superscript letters denote a significant difference between values in the same column (P<0.05). N=5–8 hearts for all groups.
trabeculae from the trout acclimated to 4°C translates into the increased pressure-generating capacity of the trout ventricle with cold acclimation. The higher rates of pressure development suggest that cold acclimation has a positive inotropic effect on the heart.

The results of the whole ventricle experiments indicate that acclimation to 4°C increases the rate of ventricular pressure generation. However, we did not see an increase in the rate of cross-bridge cycling in the trabeculae studies. This suggests that the increased rate of pressure generation is due to changes in Ca$^{2+}$ handling by the myocytes. We hypothesize that the contractile kinetics of intact fibers is determined by the rate of cross-bridge cycling and the rate of Ca$^{2+}$ cycling. Evidence for our hypothesis is that cold acclimation increases Ca$^{2+}$ release and uptake from the sarcoplasmic reticulum (SR), as well as the twitch kinetics of intact trout cardiac fibers (Aho and Vornanen, 1998; Aho and Vornanen, 1999). These changes in function have been shown to occur in conjunction with a proliferation of sarcoplasmic reticulum (SR) content and an increase in the amount of SERCA in the heart with cold acclimation in cold-active species (Keen et al., 1994; Tiitu and Vornanen, 2002; Shiels et al., 2011; Korajoki and Vornanen, 2012; Korajoki and Vornanen, 2013). This increased roll of the SR with cold acclimation would also complement the increase in the Ca$^{2+}$ sensitivity of the myofilament characterized in the current study.

**Variation in morphological remodelling**

Thermal acclimation did not affect the RVM of either male or female fish (Tables 1 and 3). This indicates that acclimation to 4°C did not cause cardiac hypertrophy. Although several studies describe hypertrophy in trout cardiac muscle in response to cold acclimation (Graham and Farrell, 1989; Klaiman et al., 2011; Vornanen et al., 2005), this phenomenon is not universally reported (Driedzic et al., 1996; Gamperl and Farrell, 2004). It has been proposed that other factors may be involved in triggering the hypertrophic response, including seasonal changes in photoperiod and thyroid hormones (Tiitu and Vornanen, 2003; Gamperl and Farrell, 2004). In the current study we used the same acclimation protocol as Klaiman et al. (Klaiman et al., 2011) where acclimation to 4°C induced cardiac hypertrophy in the male trout. The one difference between the current study and our previous study is the supplier of the rainbow trout. It is therefore possible that there were differences in the genetic background, and/or husbandry of the fish in the two studies, and that this translated to the variation in the hypertrophic response. Identifying the mechanism(s) underlying the difference in the hypertrophic response represents an interesting avenue for future studies.

The ventricles of male fish acclimated to 4°C had less compact myocardium than those of male fish acclimated to 17°C. Previous studies have reported similar results in trout and zebrafish (Farrell et al., 1988; Klaiman et al., 2011; Johnson et al., 2014), and we have suggested that such a decrease in compact myocardium helps to maintain cardiac compliance at low temperatures (Johnson et al., 2014). Such an effect should translate into the heart generating lower diastolic pressures compared with a heart with more compact myocardium when measured at the same temperature, yet we observed no difference in the mean diastolic pressures between the ventricles from the 4°C- and 17°C-acclimated males.

Finally, cold acclimation has been shown to cause an increase in cardiac connective tissue content in male trout and this response is thought to occur in parallel with the cardiac hypertrophic response (Klaiman et al., 2011). The lack of a hypertrophic response in the current study is probably why we did not find an increase in connective tissue content in male trout following acclimation to 4°C.

**Sex-specific differences in cardiac morphology and function**

The higher RVM in the males in each thermal acclimation group means that there is more heart muscle per gram of body mass compared with females. This would potentially allow more power per gram of body mass to be developed by the heart, enabling oxygen to be delivered to the tissues faster. This result is supported by previous work by Franklin and Davie (Franklin and Davie, 1992), who reported that mature male rainbow trout had a higher RVM than mature females, and that juvenile males had a higher RVM than juvenile females.

The higher max $\frac{dP}{dt}_{\text{sys}}$ for the ventricles from the cold-acclimated females compared with all other fish indicates a faster rate of relaxation between contractions. There was, however, no unique morphological characteristic of the ventricles from the cold-acclimated females. There was also no difference in the passive tension of the trabeculae from the trout acclimated to 4°C compared with those from the other acclimation groups (all female). This suggests that it is differences in the Ca$^{2+}$ handling properties of the myocytes in the intact ventricles that are responsible for this difference in diastolic function. For example, Aho and Vornanen (Aho and Vornanen, 1998) demonstrated that cold acclimation of trout increases the rate of Ca$^{2+}$ uptake by the sarcoplasmic reticulum. This would cause cytosolic Ca$^{2+}$ concentrations to decrease at a faster rate between beats, and as a result, allow the heart to relax at a faster rate. An increase in the rate of relaxation coupled with the increase in max $\frac{dP}{dt}_{\text{sys}}$, discussed above, probably plays a role in enabling the higher basal heart rates previously reported for cold-acclimated trout (Aho and Vornanen, 2001).

The higher diastolic pressure in the ventricles from the male fish acclimated to 11 and 17°C compared with females in the same acclimation groups probably reflects the greater proportion of compact myocardium in the male hearts. These findings support the hypothesis, mentioned above, that the thickness of the compact myocardium plays a role in regulating the compliance of the ventricle. It has also been suggested that an increase in collagen content in the compact myocardium plays a role in regulating ventricular compliance (Johnson et al., 2014). However, while there was significantly more collagen in the compact myocardium of the female fish acclimated to 11°C than in males from the same acclimation group, the diastolic pressure was higher in the males.

**Conclusions and perspectives**

The results of this study demonstrate that the ventricles from the 4°C-acclimated trout generate more pressure at a faster rate than those from the 11°C- and 17°C-acclimated fish. The increased levels of ventricular developed pressure with acclimation to 4°C occurred without a hypertrophic response, indicating that it is alterations to existing myofilaments that are responsible, at least in part, for these functional improvements. These changes in function can be explained by the increased Ca$^{2+}$ sensitivity of the skinned trabeculae from the 4°C-acclimated fish and caused by the decrease in the phosphorylation level of cTnT detected in the myocardium from these fish. Such a simple strategy to increase contractile function could be a low energetic cost alternative to a hypertrophic response where new muscle components are generated. In addition, these changes to contractile function can be rapidly reversed when physiological conditions change. The ability of the trout to adjust cardiac contractile function in response to a change in environmental temperature enables this species to remain active over a range of seasonal temperatures and to also exploit a range of thermal niches. Finally, by studying how trout maintain cardiac function during cold
acclimation we have identified a strategy used in vivo by which cardiac contractile function can be improved.

**Materials and Methods**

**Thermal acclimation**

The rainbow trout used in this study (*Onchorhyncus mykiss* Walbaum 1792) were obtained from Alma aquaculture research station (Alma, Ontario, Canada) and kept for a minimum of 2 weeks at 11°C in 2000-liter environmentally controlled recirculation systems at the University of Guelph before any temperature manipulation. All fish were adults and mean body mass was 1497±75 g. There was no difference in body mass between male and female fish at the start of the experiment (P>0.05). The acclimation protocol was as described by Klaiman et al. (Klaiman et al., 2011) and fish were held at their acclimation temperature for a minimum of 8 weeks prior to experimentation. The specific acclimation temperatures (4, 11 and 17°C) to experimentation. The specific acclimation temperatures (4, 11 and 17°C) were used as they are within the natural range of rainbow trout (Klaiman et al., 2011). All fish were killed with a blow to the head and severing of the cerebral spinal column. The heart was then removed, rinsed in ice-cold physiological saline (mmol l⁻¹: 94 NaCl, 24 NaHCO₃, 5 KCl, 1 MgSO₄, 1 Na₂HPO₄, 0.7 CaCl₂) and then utilized as required. The University of Guelph Animal Care Committee approved all protocols.

**Ca²⁺ activation of cardiac trabeculae**

Only female fish were used for the trabeculae work as cold acclimation can increase the relative amount of connective tissue in the heart of male rainbow trout (Klaiman et al., 2011), and as a result, affect the amount of contractile machinery per unit area of muscle. Experiments were conducted on permeabilized ventricular trabecular preparations as described by Gillis and Klaiman (Gillis and Klaiman, 2011). After the hearts were rinsed in saline, they were placed on a chilled dissection stage containing ice-cold relaxing solution [mmol l⁻¹: 100 KCl, 10 3-(N-morpholino)propanesulfonic acid (MOPS), 5 dipotassium EGTA, 9 MgCl₂, 4 NaATP, 20 2,3-butanedione monoxime (BDM); pH 7.0 at 4°C]. Trabeculae sheets (4.0 mm×2.0 mm) were dissected, skinned overnight and then further dissected to produce preparations that were ~2.0 mm×0.35 mm (Gillis and Klaiman, 2011).

**Mechanics instrument**

The mechanical measurements were made using an instrument from Aurora Scientific (Aurora, Ontario, Canada) mounted on the stage of an inverted microscope (model Eclipse TE 2000U, Nikon, Japan) as described by Gillis and Klaiman (Gillis and Klaiman, 2011). The instrument was composed of a permeabilized fiber test system (model 802B, Aurora Scientific), a force transducer (model 400A, Aurora Scientific) and a servo-motor (model 306C, Aurora Scientific). The temperature of the well plate is regulated via three thermonlectric coolers, and well temperature was maintained at 15±1°C throughout the experiment. Sarcomere length (SL) was measured by fast Fourier transform (FFT) analysis using the high-speed video sarcomere length system (model 901A, Aurora Scientific).

**Solutions for mechanical measurements**

Ca²⁺ activation of the trabeculae was measured at 15°C as we, and others, have previously measured Ca²⁺ activation of cardiac trabeculae from a variety of species at this temperature. Solution composition was determined using a computer program that calculates the equilibrium concentration of ligands and ions as in Gillis and Klaiman (Gillis and Klaiman, 2011). The activation solution contained (mmol l⁻¹): 15 phosphate, 15 EGTA, at least 40 MOPS, 1 free Mg²⁺, 135 Na⁺ + K⁺, 5 mmol l⁻¹ NaATP and 250 units ml⁻¹ creatine phosphokinase (CPK; Sigma, St Louis, MO, USA) at pH 7.0. Ionic strength was 0.17 mol l⁻¹. The Ca²⁺ level (expressed as pCa=−log[Ca²⁺]) was varied between pCa 9.0 and pCa 5.0 by adjusting CaCl₂.

**Experimental protocol**

The protocol was as previously described (Gillis and Klaiman, 2011). In brief, the muscle preparations were incubated in relaxation buffer without BDM at ~4°C and then mounted onto the apparatus using aluminium T-clips wrapped on the ends of the muscle preparation. The temperature of the mounting well was 15°C and the experimental preparation was held here for at least 10 min before any measurements. SL was set to 2.2 μm while the trabeculae was in relaxing solution. The passive force at pCa 9.0 was measured using a release–restretch protocol and the trabeculae were activated over a range of free [Ca²⁺] to measure steady-state isometric force and the rate of isometric tension redevelopment (kₚ) following rapid (≈4L₂ s⁻¹) release–restretch (15% Lₛ, where Lₛ represents trabeculae length). If a consistent SL pattern was not obtained throughout the preparation, the preparation was discarded. Only trabeculae that maintained >85% maximal force throughout the experimental protocol were analyzed for force–pCa, muscle kinetics, and phosphorylation detection.

**Phosphorylation detection and collagen measurement**

Phosphorylation of the contractile proteins in the cardiac trabeculae used in the experiments was measured using Pro-Q Phosphoprotein stain (Molecular Probes, Eugene, OR, USA) as described by Gillis and Klaiman (Gillis and Klaiman, 2011). The level of phosphorylation of each protein was compared between the thermally acclimated samples via the ratio of the density of the Pro-Q stained bands/actin using ImageJ. We have previously identified cTnI, ssTnT, RLC and TnI using western blotting or tandem mass spectroscopy (Klaiman et al., 2011; Alderman et al., 2012). The TnI isoform is not specified as trout heart contains multiple isoforms and these run together in the identified band (Alderman et al., 2012). Protein loading between lanes was assessed by measuring the density of actin bands visible after SYPRO staining. Total collagen of the spongy myocardium was measured as hydroxyproline by amino acid analysis (Kafiennah and Sims, 2004).

**Measurement of ventricular pressure generation using Langendorff perfusion**

Male and female rainbow trout were utilized in this experiment to identify any sex-specific responses to thermal acclimation. Following acclimation the fish were killed as described above and the whole hearts were continuously perfused with a modified Krebs-Henseleit buffer containing (mmol l⁻¹): 150 NaCl, 5.4 KCl, 1.5 MgSO₄, 10 glucose, 2.5 CaCl₂, 10 TES; pH 7.8, oxygenated with 100% O₂ at 15°C (Keen et al., 1994; Patrick et al., 2010) at a pressure of 5 mmHg via a 22-gauge cannula inserted into the atrium. This temperature was chosen to match the trabeculae study. A small fluid-filled balloon connected to a pressure transducer (PowerLab 4/30, ADInstruments, Dunedin, New Zealand) was inserted into the ventricle of the heart via the bulbus (Fig. 4). The balloon and pressure transducer system was used that described by Yang and Pyle (Yang and Pyle, 2012). Platinum electrodes were placed on the atrium to pace the heart at 32 beats min⁻¹. Hearts were allowed to stabilize for 20 min before baseline function was recorded. The balloon volume was then increased in 5 μl increments and the hearts were left to stabilize for 5 min before readings were taken to generate ventricular pressure–volume curves (Frank–Starling curves). Preliminary work established that 5 min was sufficient for ventricular pressures to stabilize after an increase in balloon volume. The net change in balloon volume from baseline systolic pressure to maximum systolic pressure was 55 μl. Above this, pressure generation became highly variable, indicating a loss of functional integrity. Data were collected using a PowerLab data acquisition system and analysed using Chart software (ADInstruments). Using this preparation we measured ventricular pressure (in mmHg) at both diastole and systole, and calculated the change in pressure with time (dP/dt,
Fig. 4. Diagram of the Langendorff preparation used to characterize the ventricular pressure generation in the heart. The atrium is attached to a needle for constant perfusion and a balloon, connected to a pressure transducer, is inserted into the ventricle through the bulbus. Platinum electrodes were placed on the atrium to enable pacing of the heart.

in mmHg s\(^{-1}\)) during systole and diastole. Maximal dP/dt\(_{90}\) and maximal dP/dt\(_{max}\) at baseline conditions are summarized in Table 3.

The pacing rate (32 beats min\(^{-1}\)) utilized is within the normal resting range for trout at 15°C (Altimiras and Larsen, 2000). For baseline function the balloon was inflated to give a ventricular end diastolic pressure between 0 and 1 mmHg and developed pressure of greater than 20 mmHg. Previous studies utilizing in situ cardiac preparations for studies of teleost fish set input pressure close to 0 for baseline conditions (Graham and Farrell, 1989; Farrell et al., 1996; Mendonça et al., 2007). It should be noted that systolic pressure (output pressure) was not set at a constant as in previous in situ work (Graham and Farrell, 1990; Farrell et al., 1996; Lurman et al., 2012), rather the diastolic and systolic pressures were measured inside the ventricle after changes in balloon volumes. The systolic pressures that were measured at baseline conditions were within the range of those that were used in previous in situ studies that controlled output pressures. For example, in rainbow trout tested at 15°C, Graham and Farrell (1990) started experiments by setting output pressure to 29 mmHg; and in Farrell et al. (Farrell et al., 1996) output pressure was regulated between 30 and 60 mmHg. Moreover, the systolic pressures measured were similar to those used in studies on other fish species including the Atlantic cod, in which output pressures were set to between 15 and 60 mmHg during testing (Lurman et al., 2012).

Inserting a balloon inside a ventricle with a restricted lumen, due to the presence of spongy myocardium, has the potential to impair contractile function. However, it was thought that the balloon would assume the shape of the lumen as its volume was increased, causing forces to be uniformly distributed to the lumen walls.

Histology

The hearts from the Langendorff studies were arrested using 1 mol l\(^{-1}\) KCl and then fixed in 10% neutral buffered formalin for a minimum of 2 weeks before transfer to 70% ethanol for storage then histological processing. The fixed ventricles were cut into three pieces along the horizontal axis using a razor. The middle third of each heart was used for the histological studies. Before transfer to 70% ethanol for storage then histological processing. The low magnification brightfield images of the ventricle sections. The low magnification images were used to quantify the percentage of collagen in the myocardium of the heart of rainbow trout (Oncorhynchus mykiss). The high magnification images were used to calculate the relative amount of muscle in the compact and spongy myocardium. The high magnification images were used to calculate the percentage of collagen in the myocardium (Fig. 2D–F). The per cent collagen content in each myocardial layer was analysed using an ImageJ (National Institutes of Health, Bethesda, MD, USA) batch macro as described by Hadi et al. (Hadi et al., 2011).

Statistical analysis

A one-way analysis of variance (ANOVA) with a Tukey post hoc test was used to compare the F\(_{max}\) passive force, pCa\(_{30}\) and Hill coefficients, as well as differences in the phosphorylation state of the contractile regulatory proteins, between the three thermally acclimated groups. The mean k\(_{b}\) values for each acclimation group were compared at each pCa using a one-way ANOVA followed by Holm–Šidák tests. A Bonferroni correction was used in the analysis of the k\(_{b}\) data set as multiple comparisons were made and significance was thus determined at P<0.007. A two-way ANOVA followed by Holm–Šidák tests for multiple comparisons was used to assess the effect of acclimation temperature and sex on heart mass, RVM, the proportion of compact myocardium and the percentage collagen content in the spongy and compact myocardium. All percentage data were logit transformed before analysis. For comparisons between groups (acclimation and sex combined as one factor), absolute changes in the Langendorff parameters were analysed by a two-way ANOVA followed by Holm–Šidák tests for multiple comparisons between treatment (acclimation\(\times\)sex) and preload (balloon volume); see Angelone et al. (Angelone et al., 2012) and Ludbrook (Ludbrook, 1994). Any data set that failed to conform to the assumption of homogeneity of variance was log transformed prior to analysis. Differences were considered to be significant at P<0.05, unless otherwise noted. All statistical analysis was completed using Sigmamap version 13 (Systat Software Inc., San Jose, CA, USA).

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Competing interests

The authors declare no competing financial interests.

Author contributions

J.M.K., W.G.P. and T.E.G. designed the study. J.M.K. completed the experiments. J.M.K., W.G.P. and T.E.G. analysed the data and wrote the paper.

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