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Cardiac remodeling caused by cold acclimation is reversible with rewarming in zebrafish (*Danio rerio*)

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ABSTRACT

Cold acclimation of zebrafish causes changes to the structure and composition of the heart. However, little is known of the consequences of these changes on heart function or if these changes are reversible with rewarming back to the initial temperature. In the current study, zebrafish were acclimated from 27℃ to 20°C, then after 17 weeks, a subset of fish were rewarmed to 27°C and held at that temperature for 7 weeks. The length of this trial, 23 weeks, was chosen to mimic seasonal changes in temperature. Cardiac function was measured in each group at 27°C and 20°C using high frequency ultrasound. It was found that cold acclimation caused a decrease in ventricular cross-sectional area, compact myocardial thickness, and total muscle area. There was also a decrease in end-diastolic area with cold acclimation that reversed upon rewarming to control temperatures. Rewarming caused an increase in the thickness of the compact myocardium, total muscle area, and end-diastolic area back to control levels. This is the first experiment to demonstrate that cardiac remodeling, induced by cold acclimation, is reversible upon re-acclimation to control temperature (27°C). Finally, body condition measurements reveal that fish that had been cold-acclimated and then reacclimated to 27°C, were in poorer condition than the fish that remained at 20°C as well as the control fish at week 23. This suggests that the physiological responses to the multiple changes in temperature had a significant energetic cost to the animal.

Summary statement: The decrease in cardiac muscle density, compact myocardium thickness and diastolic area in zebrafish caused by cold acclimation, was reversed with rewarming to control temperatures.

1. Introduction

An acute decrease in physiological temperature can cause a reduction in the functional capacity of the vertebrate heart (Farrell, 1984; Gillis and Tibbits, 2002). This is due, in part, to a decrease in the level and rate of crossbridge cycling in the contractile element. There are, however, temperate fish species that remain active during the winter despite a decrease in environmental temperature. This suggests that these animals have the capacity to compensate for the effects of temperature on cardiac function (Keen et al., 2017, Gillis et al., 2000; Gillis and TIbbits, 2002, Alderman et al., 2012). Support for this comes from work that characterized the impact of cold acclimation on the structure and function of the rainbow trout (*Oncorhynchus mykiss*) heart. For example, Graham and Farrell (1989) first described an increase in relative heart size in rainbow trout with cold acclimation and this response has since been characterized in multiple studies (Keen et al., 2016, 2018, 2021; Klaiman et al., 2011, 2014). More recent work has reported that cold acclimation of rainbow trout causes an increase in the thickness of the compact myocardium, in the collagen content of the heart, in the Ca^{2+} sensitivity of force activation by the myofilament, and in the contractile function of the isolated heart (Keen et al., 2016; Klaiman et al., 2011, 2014).

A second species of fish that is being used for thermal acclimation studies is the zebrafish (*Danio rerio*) (Johnson et al., 2014; Lee et al., 2016; Little et al., 2013; Little and Seebacher, 2014; Morgan et al., 2022). While zebrafish are considered a tropical species, they are found in environments where water temperatures change seasonally by at least 10°C (Spence et al., 2006; Sundin et al., 2019). Morgan et al. (2022), have recently demonstrated that wild and lab reared zebrafish display physiological plasticity in response to thermal acclimation to compensate for the effects of a decrease in temperature on biochemical and physiological processes. These authors suggest that the response of wild strains of zebrafish to thermal acclimation is stronger than that of lab reared strains (Morgan et al., 2022). Work by Johnson et al. (2014) has

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reported that cold acclimation (to 20°C) of a lab strain of zebrafish caused a decrease in not only cardiac collagen content, but also the relative thickness of the collagen fibers and in the thickness of the compact myocardium. In addition, Lee et al. (2016), using high frequency cardiac ultrasound, found that cold acclimation (to 18°C) of a lab strain of zebrafish caused improved systolic function at low temperatures (18°C) compared to control fish maintained at 28°C. Work by Little et al. (2013) and Little and Seebacher (2014) also demonstrates that cold acclimation of a lab strain of zebrafish caused increased cardiac performance and aerobic scope. Together, these studies suggest that laboratory strains of zebrafish can modify the structure and function of their heart in response to thermal acclimation.

As environmental temperatures change with the seasons over the course of a year, trout and zebrafish experience rewarming in their natural environment on an annual basis. There have been no studies to date that address the response of zebrafish to rewarming following cold acclimation; therefore, it is not known how the heart responds. For example, it is unknown if the changes in function and morphology, caused by cold acclimation, are reversible upon re-acclimation to warm temperatures. To begin addressing this knowledge gap, we utilized histological approaches to characterize changes in heart structure and composition, as well as high frequency cardiac ultrasound to monitor the changes in the function of the zebrafish heart during acclimation from 27°C to 20°C and then from 20°C to 27°C. We predicted that cold acclimation would cause parallel changes to the morphology and function of the heart that result in the maintenance of cardiac output, and that these changes would be reversed with re-acclimation to control temperatures. Cardiac function was also characterized following an acute change in environmental temperature to determine if the ability of the fish to maintain cardiac output under such conditions is affected by cold acclimation.

2. Methods

2.1. Experimental animals and housing

The fish, of mixed sex, used in this study were acquired in May 2021 from Big Al's Pets (Kitchener, ON, Canada) and held at the Hagen Aqualab, University of Guelph, in flow through aquaria at 27°C. It is assumed that these fish had been raised in captivity under controlled conditions for multiple generations. A total of 120 fish were used in this experiment. These animals were held on a 12:12 h light:dark cycle and fed to satiation twice daily with commercial flake food (Tropical Vitality and Colour Flakes, Chorzow, Poland and Nutrafin Max Fish Flakes, Rolf C. Hagen Inc., Baie d'Urfe, Quebec). The flake food was regularly supplemented with frozen chironomid bloodworms (Hikari, Kyorin Co., Ltd., Hyogo, Japan) as well as Gemma Micro 300 (Skretting, Maine, US). All groups were on the same feeding regime, but we did not measure the food intake or activity levels of the different treatment tanks. Ammonia levels in the aquaria were monitored and remained below 0.1 ppm throughout the experimental period. Water hardness and inflow temperature were under the control of the central recirculation system. Water temperature was maintained ±1°C with EHeim Jäger aquarium thermo-control heaters (EHEIM GmbH and Co. KG., Deizisau, Germany) connected to Digital Temperature Controllers (Aqua Logic Inc., San Diego, CA) and water conditions were maintained within recommended levels (Aleström et al., 2020).

2.2. Thermal acclimation

The fish were split into two groups: the control group, composed of 60 fish, and the treatment group composed of 60 fish. The control group remained at 27°C ± 1°C for the duration of the experiment (23 weeks) while the treatment group was acclimated to 20°C ± 1°C by decreasing water temperature 1°C per day (Johnson et al., 2014; Klaiman et al., 2011). These fish were held at that temperature for 17 weeks, and then half of the treatment fish (30 fish) were acclimated back to 27°C by increasing temperature at a rate of 1°C per day. The temperatures of all groups were then held constant for 7 weeks. The temperatures 27°C and 20°C were chosen because the former is a common rearing temperature for zebrafish in the lab and the latter approaches a common winter temperature zebrafish experience in the wild (Spence et al., 2006). Functional imaging of a sample of each group took place at 11, 19, and 23 weeks, and hearts were sampled at 23 weeks. Twenty fish were randomly sampled from the exposure tanks for imaging at each time point, with ~10 being imaged at their acclimation temperature and ~10 being imaged at the opposite treatment temperature. This design allowed us to observe changes in functional parameters over an ecologically relevant timeframe. All procedures were conducted in accordance with the University of Guelph's Animal Care Committee and Canadian Council for Animal Care.

2.3. Ultrasound imaging

A high frequency ultrasound (Vevo 3100 LT ultrasound, FUJIFILM Visual Sonics, Inc., Toronto, ON, Canada) was used to assess the cardiac performance of a sample of the zebrafish at each sampling point (11, 19, and 23 weeks). Imaging took place in a well-lit room in the Hagen Aqualab with an air temperature of ~20 °C. Prior to imaging, fish were anesthetized using Aquacalm (metomidate hydrochloride, 1000 mg/g stock, Syndel Canada) in a water bath held constant at the imaging temperature. Aquacalm activates GABAa receptors in the brain eliciting a hypnotic and immobilizing effect (Ge et al., 2014; Leal-Cardoso et al., 1994). Previous work has demonstrated that this anesthetic has less negative inotropic effect on heart function in fish than does tricaine methanesulfonate (Roberts and Syme, 2016; Hill et al., 2002). This anesthetic has also been used previously in ultrasound studies of cardiac function in fish (Ma et al., 2019). Zebrafish exposed to 6–10 mg/l Aquacalm achieve Stage 3 anesthesia, which is ideal for procedures such as weighing and light epidermal abrasion (Collymore et al., 2014). To determine the most appropriate dose for our experiment, initial experiments were conducted using 8 mg/l and 6 mg/l, and results demonstrated that each rapidly caused stage 3 anesthesia. Doses with lower concentrations did not induce sufficient anesthesia for handling. Preliminary tests with 8 mg/l and 6 mg/l showed that the heart rate of control zebrafish at 27°C were 109 ± 14 bpm and 129 ± 5 bpm, respectively and these values were not statistically different ($p > 0.05$). Importantly, these rates are also similar to that previously measured in unanesthetized zebrafish with light cardiography (125 bpm; Mousavi and Patil, 2020).

At the beginning of an experiment, a zebrafish was transferred to the aerated anesthetic bath and held there until it lost equilibrium and was non-responsive to light tactile stimulus. This typically took ~3 min. The fish was then transferred to the imaging stage using a plastic teaspoon. The stage consisted of a rectangular glass dish (8 cm high by 12 cm long by 8 cm wide), partially immersed in a water bath. The zebrafish was placed in dorsal recumbency in the incision of a non-abrasive kitchen sponge that was submerged in aerated system water containing 6 mg/l Aquacalm. This allowed positioning of the body at ~30° to the probe; resulting in a clear view of the heart (Evangelisti et al., 2020). The stage sat on a platform allowing a minimum of 10 cm maneuvering distance in the x, y, and z axes using fine adjustment knobs. The gills were perfused by inserting a pipette tip into the buccal cavity and recirculating water from the stage using a cassette pump (Manostat Carter Multichannel Cassette Pump, Fisher Scientific Co. L.L.C.). The temperature of the water bath was maintained throughout the experiment using a submersible aquarium heater. An air stone was used to maintain aeration of the water perfusing the gills.

When positioned on the stage, the fish's ventral surface was 2–3 mm from the probe and parallel to the ground. This allowed the heart to be viewed beneath the thoracic musculature. The time from anesthetic induction to the completion of all measurements was <7 min. Once the

Table 1
The effect of thermal acclimation on body morphometrics of zebrafish (*Danio rerio*). Control were zebrafish held at 27°C throughout the experiment; Cold-Acclimated were fish acclimated to 20°C for the entire experiment; Rewarmed were fish acclimated to 20°C until Week 17, then acclimated back to 27°C until Week 23. Statistical comparisons were made between values in the same column for each parameter. Within the same column for each parameter, values indicated by different superscript are significantly different ($p < 0.05$). The number in parentheses following the mean is the n of the measure, with an n being an individual fish. Condition factor, Fulton's condition factor.

		11 Weeks	19 Weeks	23 Weeks
Mass (g)	Control	0.69 ± 0.04^{a} (20)	0.50 ± 0.05^{a} (24)	0.84 ± 0.07^{a} (10)
	Cold-Acclimated	0.62 ± 0.05^{a} (20)	0.62 ± 0.07^{b} (19)	0.69 ± 0.07^{ab} (14)
	Rewarmed	n/a	0.45 ± 0.04^{a} (20)	0.66 ± 0.05^{b} (20)
Length (cm)	Control	3.40 ± 0.06^{a}(20)	3.21 ± 0.06^{a} (24)	3.34 ± 0.07^{a} (10)
	Cold-Acclimated	3.27 ± 0.08^{a} (20)	3.21 ± 0.07^{a} (19)	3.31 ± 0.07^{a} (14)
	Rewarmed	n/a	3.15 ± 0.08^{a} (20)	3.42 ± 0.05^{a} (20)
Condition Factor	Control	1.73 ± 0.10^{a} (20)	1.47 ± 0.14^{a} (24)	2.25 ± 0.15^{a} (10)
	Cold-Acclimated	1.76 ± 0.10^{a} (20)	1.86 ± 0.17^{b} (19)	1.91 ± 0.09^{a} (14)
	Rewarmed	n/a	1.50 ± 0.20^{a} (20)	1.67 ± 0.08^{b} (20)

functional measurements were complete, weight and standard length of the fish was recorded and the fish was then transferred to a beaker containing aerated system water for recovery. To avoid sampling the same fish twice at a given time point, the fish remained in a recovery tank until all fish of their treatment group had been imaged. Fish were randomly sampled from the exposure tanks at weeks 19 and 23. As a result not every fish was characterized at every sampling point.

We used a linear array probe set to a dynamic range of 45–50 dB, using a frequency of 50 MHz and a resolution of 30 μm (Evangelisti et al., 2020). One 200–400 frame B-Mode image was recorded to determine diastolic and systolic area, fractional area change, stroke volume, ejection fraction, and fractional shortening (Evangelisti et al., 2020; Lee et al., 2016). 10–20 s Colour Doppler clips at 200–400 frames, 4 Hz (pulse repetition frequency), and "medium" wall filter were used to quantify ejection time and maximum blood velocity at the atrio-ventricular and bulbo-ventricular valves. The Colour Doppler tracks the displacement of pixels through a virtual "gate". The gate was set to 0.15 mm to reduce noise from tissue translocation surrounding the valve. The gate was positioned overtop of the mitral valve (for atrial velocity) or the bulbo-ventricular valve (for ventricular velocity). We adjusted the view until we could see flashes of yellow that indicate the location of maximum blood velocity, representative of the heart valves (Lee et al., 2016; Evangelisti et al., 2020). This imaging protocol was repeated for all ~20 fish from each treatment group, with ~10 being imaged at their acclimation temperature and ~ 10 being imaged at the opposite treatment temperature, at each sampling point during the experiment.

2.4. Analysis of ultrasound images

All ultarsound data were analyzed using Vevo 3100 image analysis software (VEVO Lab, v. 5.5.1, FUJIFILM Visual Sonics Inc., 2021). Heart rate was calculated both from the Colour Doppler video and from five consecutive heart beats in the Doppler wave trace (average of these is presented, see Fig. S1A for example). Stroke volume, fractional area change, fractional shortening, ejection fraction, end-diastolic area, and end-systolic area were calculated using Vevo Lab's LV Trace Analysis package (FUJIFILM Visual Sonics Inc., 2021, see Fig. S1B for example). LV Trace directly measures chamber area but estimates volume assuming an elliptical chamber geometry. Fractional area change was calculated using the following equation:

$$(DA - SA)/DA = FAC \quad (1)$$

Where FAC is the fractional area change, DA is the maximum diastolic area in mm^2, and SA is the minimum systolic area in mm^2. End-diastolic area was determined by skimming through the B-Mode image until the frame with the largest area was reached before beginning to contract. End-systole was determined by finding the subsequent frame where the area was smallest before beginning to expand.

Peak ejection velocity and ejection time were measured on the Doppler wave traces at the height (blood velocity) and width (ejection time) of peaks. All ejection peak velocities and times were averaged over at least three consecutive beats. Heart rate was extracted from the wave trace by tracing the horizontal distance between five consecutive late atrial peaks (Evangelisti et al., 2020). Functional parameters that include a measure of volume or area were standardized to fish weight to compensate for variation in fish size.

2.5. Heart dissection and histology

At the end of the final measurement of cardiac function at week 23, zebrafish were euthanized by first anesthetizing them in ice cold Aquacalm (40 mg/l) followed by rapid decapitation above the opercula. The whole thorax of the zebrafish was then isolated by amputating the abdomen. Tissues were prepared according to Johnson et al. (2014). Briefly, hearts were rinsed with physiological saline (mM: 94 NaCl, 24 $NaHCO_3$, 5 KCl, 1 $MgSO_4$, 1 Na_2HPO_4, 0.7 $CaCl_2$; buffered to 7.6 pH with HCl at 20°C) and then flushed with 1 M KCl to ensure maximal contraction. Whole thoraxes were fixed for 24 h in 1:15 tissue to 10% neutral buffered formalin (Thermo Fisher, Mississauga, ON, Canada). Fixed tissues were washed in water for 30 min before being decalcified for 1 h in 2 M HCl (15:1 volume:mass). Thereafter, HCl was neutralized by immersing tissue in 1% NH_3 solution for 10 min. A final wash in tap water preceded storage in 70% ethanol. Tissue was embedded in paraffin and sectioned at 5 um (HistoCore AUTOCUT Microtome, Leica Biosystems, Concord, ON, Canada). Sections were separated by 40 um and stained for collagen with picrosirius red according to Junqueira et al. (1979). Muscle stains orange to yellow, while collagen stains a deep red in brightfield (Junqueira et al., 1979). A Nikon Ti Eclipse microscope (Nikon, Melville, NY, USA) was used to take brightfield images of the heart sections. We used brightfield images of the heart and surrounding tissue (5 sections per sample) where the bulbo-ventricular valve was clearly visible (Johnson et al., 2014).

We calculated total muscle content and total collagen content of each section in ImageJ (Schneider et al., 2012, v. 1.53 k). Briefly, a 256-colour brightfield image was transformed into its hue components. A hue value of 1–100 was used for muscle (orange-yellow) and a hue value of 221–255 was used for collagen (red) (modified from Rich and Whittaker, 2005). This allowed for muscle or collagen to be selected while excluding the white-gray background. The number of pixels of each hue were summed and converted to um^2 to find the area of each ventricle composed of collagen and muscle. The values were then averaged across each section per sample. To compare the relative abundance of muscle and collagen in the ventricle, we traced the outer edge of the compact myocardium to find the cross-sectional area of the whole ventricle in each section. The area of muscle and collagen calculated by thresholding was then compared as a percent of that total cross-sectional area. This process was repeated for the spongy myocardium by tracing the inner

Table 2
The effect of thermal acclimation on the cardiac morphology of zebrafish (*Danio rerio*) sampled at 23 weeks. Values are means ± SEM. Values in the same row indicated by a different superscript, are significantly different (ANOVA, $p < 0.05$). The n of these measurements are 10 for Week 11; 10 for Week 19; and 10–16 for Week 23. Muscle area, area of total cross section, or myocardial layer that is composed of muscle; collagen area, area of cross section or specific myocardial layer composed of collagen; Spongy, spongy myocardium; Compact, compact myocardium; n = individual fish.

	Control	Cold-Acclimated	Rewarmed
Total cross-sectional area (mm^2)	0.711 ± 0.033^a	0.543 ± 0.0214^b	0.622 ± 0.034^{ab}
Total muscle area (mm^2)	0.675 ± 0.031^a	0.449 ± 0.017^b	0.594 ± 0.034^a
Total collagen area (mm^2)	0.0026 ± 0.0009^a	0.0028 ± 0.0004^a	0.0037 ± 0.0008^a
Spongy cross-sectional area (mm^2)	$0.637 \pm .031^a$	0.483 ± 0.019^b	0.565 ± 0.034^{ab}
Spongy muscle area (mm^2)	0.711 ± 0.029^a	0.429 ± 0.017^b	0.538 ± 0.034^{ab}
Spongy collagen area (mm^2)	0.0019 ± 0.0005^a	0.0020 ± 0.0004^a	0.0027 ± 0.0006^a
Compact muscle area (mm^2)	$0.068 \pm .005^a$	0.058 ± 0.004^a	0.056 ± 0.004^a
Compact collagen area (mm^2)	0.0007 ± 0.0004^a	0.0008 ± 0.0001^a	0.0010 ± 0.0003^a
Average compact thickness (μm)	9.9 ± 0.2^a	8.9 ± 0.3^b	10.0 ± 0.3^a

edge of the compact myocardium (excluding the valve) to find the total cross-sectional area of the spongy myocardium. The relative abundance of muscle and collagen in the compact myocardium was the difference between the area values for the whole ventricle and those for the spongy myocardium.

2.6. Body mass and condition

Zebrafish mass and length were measured and used to estimate Fulton's condition factor for males and females throughout the experiment according to the following equation:

$$K = 100^* \left(\frac{M}{L^3}\right) \quad (2)$$

2.7. Statistical analysis

All statistical analysis was completed using RStudio (4.0.3). Differences in functional parameters due to acclimation time and test temperature were assessed using two-way ANOVA to find the main and interactive effects ($p < 0.05$). Differences in histological parameters due to acclimation temperature were assessed using two-way ANOVA to find the main and interactive effects with statistical significance set at $p < 0.05$. Zebrafish were grouped into time points, acclimation group, and test temperature. There was not sufficient sample size to account for the potential effect of sex on the parameters measured; we therefore grouped the sexes for our analyses. Post-hoc Tukey's pairwise test was used to identify pairwise differences between means and to investigate possible interaction effects. All groups were checked for normality and equal variance, and if these were not met, variables were log-transformed prior to testing. Using linear regression of cardiac parameters against time on the stage revealed a significant effect of time on the stage for ventricular ejection velocity, and ventricular ejection time. However, these regressions explained 6% or less of the variation in any parameter; therefore, fish were not excluded from the analyses based on time under anesthetic. Individuals were excluded from analysis if no data could be collected on at least one parameter due to weak signals. Mass, length, and condition factor were similarly compared using ANOVA with acclimation and time, as factors.

3. Results

3.1. Mass and condition factor

At 11 weeks, there was no difference in the mean weight of the cold-acclimated fish and control fish ($p < 0.05$, Table 1). At 19 weeks, the cold-acclimated fish were 24% heavier than the control fish and 38% heavier than the rewarmed fish ($p < 0.05$, Table 1). At 23 weeks, control fish were 22% heavier than the cold-acclimated fish ($p > 0.05$ and 27% heavier than the rewarmed fish, $p < 0.05$, Table 1). Condition factor followed the same pattern as mass with cold-acclimated fish having significantly greater condition at 19 weeks ($p < 0.05$, Table 1) and rewarmed fish having lower condition at 23 weeks ($p < 0.05$, Table 1).

3.2. Cardiac morphology and composition

At 23 weeks, the hearts of the cold-acclimated fish were smaller and contained less collagen than those from the control and rewarmed groups (Table 2, Fig. 1A). The smaller heart size of the cold-acclimated fish is seen in the total cross-sectional area, total muscle area, and cross-sectional area of the spongy myocardium (Table 2). Specifically, the cross-sectional area and total muscle area of the hearts from the cold-acclimated group were 31% lower than that of the control group and 32% lower than that of the rewarmed group; while the cross-sectional area of the spongy myocardium was 31% lower in the cold-acclimated hearts compared to the controls and 34% lower than the hearts from the rewarmed fish (Table 2, Fig. 1A). The thickness of the compact myocardium was also less in the cold-acclimated fish than either the control (24%) or the rewarmed group (33%) (Fig. 2A).

To determine if heart size relative to body size was affected by thermal acclimation, we standardized the cross-sectional area of the ventricle, spongy myocardium, and the compact myocardium to body mass (Fig. 1 B). This analysis demonstrated that there was no difference in the standardized cross-sectional area of the ventricle between the control and cold-acclimated groups but that the standardized cross-sectional area of the ventricle from the rewarmed fish was greater than that of the cold-acclimated group. This response is mirrored in the standardized area of the spongy myocardium (Fig. 1B) but there was no difference in the standardized areas of the compact myocardium from either experimental group or the control group (Fig. 1B). When compact myocardial area was standardized to spongy myocardium, there was also no difference between any of the groups(Fig. 2B).

The lower collagen levels in the cold-acclimated group are reflected in the measurements of total collagen, and the collagen in the spongy myocardium (Fig. 3A). Specifically, total collagen, calculated as μm^2 was lower in the cold-acclimated fish than the control (36%) or rewarmed (47%) group. ($p < 0.05$) (Fig. 3A). Total collagen in the hearts of the control group was also 17% less than the rewarmed group ($p < 0.05$). Similar trends were seen within the spongy myocardium with the cold-acclimated group having less collagen than both the control (42%) and the rewarmed (55%) group($p < 0.05$) (Fig. 3A) and the control group having 23% less collagen than the rewarmed group ($p < 0.05$). Interestingly, there was no difference in the collagen content of the compact myocardium between groups (Fig. 3B).

We found supporting patterns in collagen content even when collagen area was standardized to the area of its respective myocardial layer. Specifically, cold-acclimated spongy myocardium was composed of less collagen (2.0 ± 0.007%) than the control group (2.3 ± 0.01%) and rewarmed group (2.9 ± 0.006%) (Fig. 3C). In addition, the standardized collagen content of the rewarmed group was statistically higher than that of the control group ($p < 0.05$) (Fig. 3C).

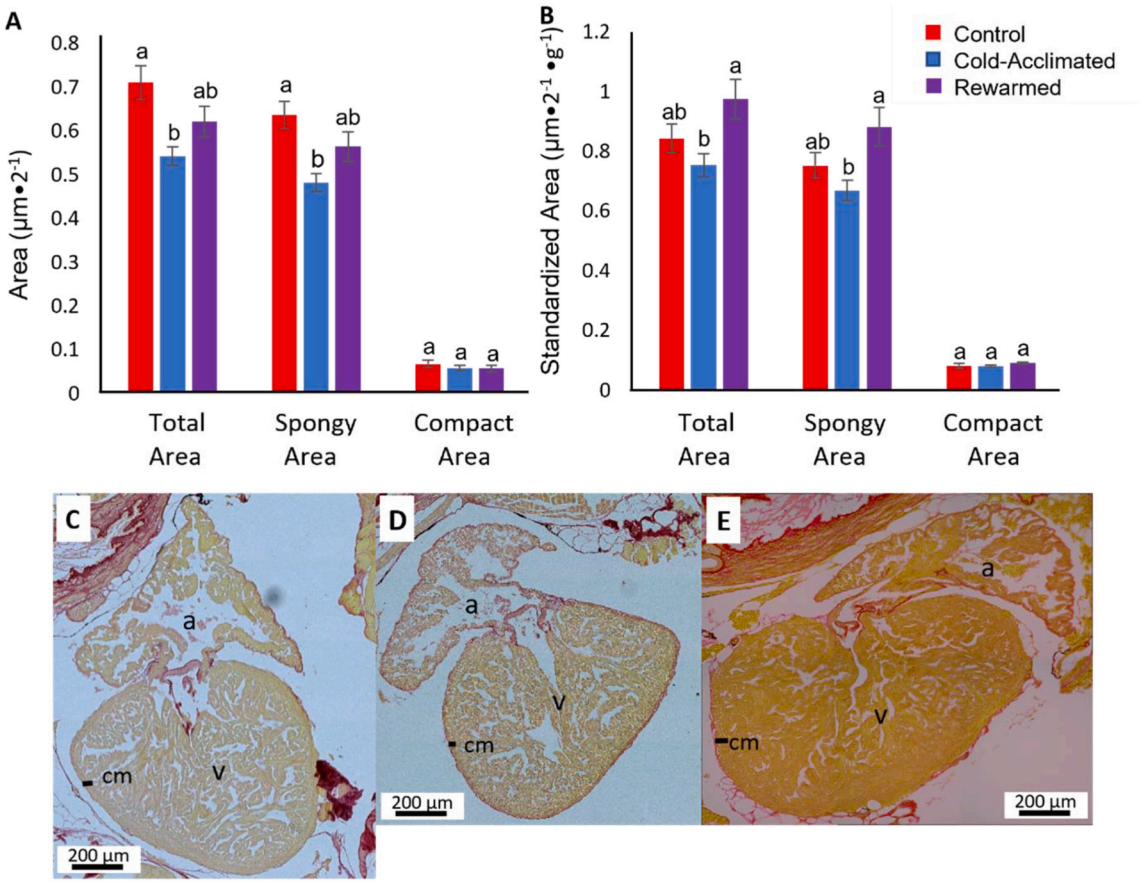

Fig. 1. The effect of thermal acclimation on the cross-sectional area of the zebrafish (*Danio rerio*) ventricle, spongy myocardium, and compact myocardium. (A) Mean (± SEM) area measurements of total cross-sectional area, spongy myocardium, and compact myocardium. (B) Area of total cross-sectional area, spongy myocardium, and compact myocardium standardized to individual fish mass. Measurements were made on samples collected at 23 weeks. Total cross-sectional area includes tissue, collagen, and white space. Total myocardial area is occupied by only cellular material, not collagen or white space. Representative brightfield micrographs of the heart from a control zebrafish (C), cold-acclimated zebrafish (D), and rewarmed zebrafish (E). All images are brightfield at 40× magnification and all values are in mm^2. Tissue area was acquired using the brightness scale in ImageJ to exclude background pixels. Bars with different letters indicate significant differences within a parameter ($p < 0.05$). $n = 10$ for all measurements with n representing the heart from an individual fish. a, atrium; v, ventricle; cm, compact myocardium.

3.3. Heart rate

At 11 weeks, heart rate was higher when measured at 27°C than at 20°C for the control (1.87×, $p < 0.001$, Fig. 4A) and cold-acclimated groups (1.73×, $p < 0.001$, Fig. 4A). At 19 weeks, heart rate in the rewarmed group was 1.62× higher when measured at 27°C than when measured at 20°C ($p < 0.05$). Heart rate was similarly higher in the cold-acclimated group at 27°C than at 20°C (1.45×, $p = 0.058$). At 23 weeks, there were no differences in heart rate among any groups. It is important to acknowledge that there were differences in the heart rate of the control group measured at weeks 11 and 23 when measured at 27°C. As the same experimental conditions were used on all sampling days, including anesthetic concentration, temperatures, and the use of aerated water to perfuse the gills, this difference is likely due to a change in the response of the fish to the experimental conditions. As we were measuring cardiac function in the same fish at weeks 11, 19, and 23, one possibility is the habituation of the animals to handling prior to anesthesia. Such habituation would reduce the response of the animals to being handled resulting in a lower heart rate. However, the measurements for each group at each experimental time point were made on the same day and the relative relationships between groups are consistent. For example, zebrafish at a given temperature did not differ with the exception of the rewarmed group measured at 27°C for the first time (19 weeks). For these reasons, we will focus the discussion on comparisons between groups made within the sampling days and not between sampling days.

3.4. End-diastolic area, stroke volume, and cardiac output

The values presented in the following sections have been standardized to the mass of the individual fish. The unstandardized values are presented in Table 3. We standardized the functional parameters to compare heart function between groups that varied in mass. At 11 weeks, the standardized end-diastolic area in the control fish measured at 20°C was greater than that measured at 27°C, and in the cold-acclimated fish measured at 20°C (Fig. 4B). At 19 weeks, the end-diastolic area of the control fish was greater than that of the cold-acclimated fish at both experimental temperatures. In addition, the end-diastolic area in the rewarmed fish measured at 27°C was larger than that of the cold-acclimated fish measured at 27°C. At 23 weeks, the end-diastolic area of the rewarmed fish was greater than the control and cold-acclimated fish at both temperatures. Finally, at 23 weeks, the unstandardized end-diastolic area of the cold-acclimated group was significantly less than that of the control and rewarmed groups when measured at both 20°C and 27°C.

Initially, stroke volume was affected by experimental temperature in the control but not in the cold-acclimated fish. Specifically, at 11 weeks, stroke volume, standardized to mass, was halved at 20°C relative to 27°C in the control fish ($p < 0.05$, Fig. 4C) while there was no difference in the stroke volume in the cold-acclimated group when measured at 27°C and 20°C (Fig. 4C). At 19 weeks, stroke volume followed a similar trend to end-diastolic area. Stroke volume was lower in the cold-acclimated fish

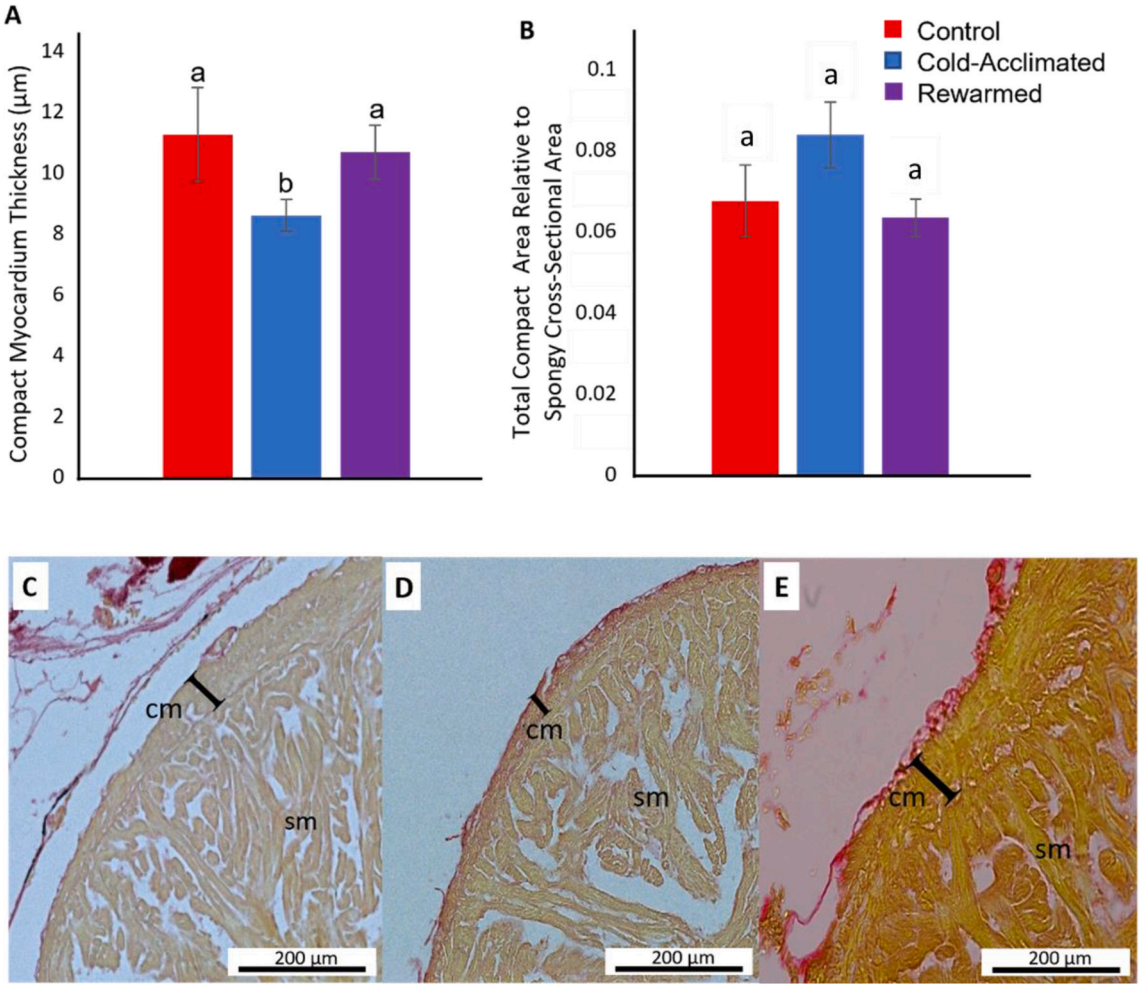

Fig. 2. The effect of cold acclimation on the thickness and relative area of the compact myocardium in zebrafish (*Danio rerio*) hearts. A) Compact myocardial thickness of control, cold-acclimated, and rewarmed zebrafish at 23 weeks measured linearly from its outer edge to its border with the spongy myocardium. B) Compact area standardized to cross-sectional area of spongy myocardium. Representative brightfield micrograph (200× magnification) of the heart from a control zebrafish (C), cold-acclimated zebrafish (D), and rewarmed zebrafish (E). n = 10 for all measurements, where n represents individual fish. Bars represent mean ± SEM. Bars indicated by different letters are statistically different ($p < 0.05$). cm, compact myocardium (see braces); sm, spongy myocardium.

relative to the control fish at both experimental temperatures, and the rewarmed fish measured at 27°C. At 23 weeks, the stroke volume of the rewarmed fish was greater at both temperatures than the cold-acclimated group measured at 20°C (Fig. 4D).

We also evaluated the effect of temperature and acclimation on cardiac output. We present cardiac output standardized to mass. At 19 weeks, standardized cardiac output was greater in the control fish measured at 27°C than in the cold-acclimated group at either temperature (Fig. 4D). Cardiac output was also greater in the rewarmed group than in the cold-acclimated group at 27°C (Fig. 4D). Finally, cardiac output was greater in the rewarmed group at 20°C and 27°C than each other group measured at 20°C (Fig. 4D).

3.5. *Influence of thermal acclimation on the blood flow parameters in the zebrafish heart*

Ventricular filling differed in the late phase (A-wave) at 11 weeks and in the early phase (*E*-wave) at 19 weeks. Specifically, the late atrial ejection velocity (A Velocity) was decreased by 37% in the cold-acclimated group with a decrease in temperature (Table 4). This differed from the effect of temperature at 19 weeks, where the early atrial ejection velocity (E Velocity) was ~50% higher for the control group (Table 4) and for the cold-acclimated group at their physiological temperature (Table 4). Ventricular velocity, which is the maximum velocity at the bulbo-ventricular valve during systole, was maintained across time and treatment (Table 4).

The period of late ventricular filling (diastole), defined by the late atrial ejection time (A Time), was affected by temperature and acclimation at 11 weeks. The A Time was faster at 27°C than at 20°C in both the control fish (24%, $p < 0.05$) and the cold-acclimation (22%, $p < 0.05$). The A Time of the controls at 27°C was also 9% shorter than that of the cold-acclimated group at 27°C ($p < 0.05$, Table 5). At 19 weeks, A Time of the rewarmed group was 18% longer at 20°C relative to 27°C ($p < 0.05$, Table 5).

Ventricular ejection time represents the phase of systole when the ventricle ejects blood into the bulbous arteriosus and is measured as the time interval between the opening and closing of the bulbo-ventricular valve. At 11 weeks, ventricular ejection time was 37% ($p < 0.05$) and 80% ($p < 0.05$) longer at 20°C than at 27°C for control fish and the cold-acclimated fish, respectively (Table 5). An effect of experimental temperature on this parameter was also observed at 19 weeks, where it was 48% longer in the control fish at 20°C than at 27°C and 32% longer in cold-acclimated fish at 20°C than at 27°C (Table 5).

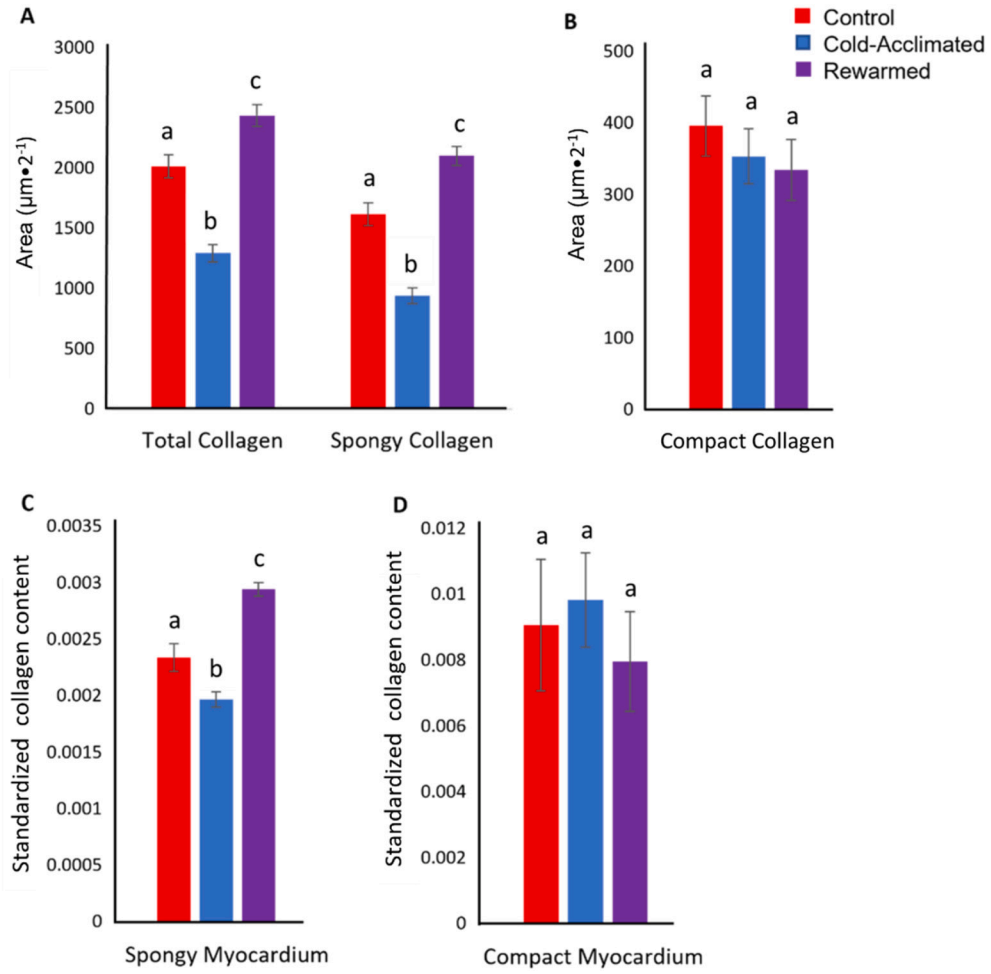

Fig. 3. The effect of thermal acclimation on collagen content in ventricular myocardium of zebrafish (*Danio rerio*) A) Total collagen content of the whole ventricle and of the spongy myocardium. B) Collagen content of the compact myocardium. C) Collagen content of the spongy myocardium standardized to measured area of spongy myocardium. D) Collagen content of the compact myocardium standardized to area of the compact myocardium. Bars represent mean ± SEM. Bars with different letters indicate significant differences within a parameter ($p < 0.05$). n = 10 for all measurements with n representing an individual fish.

3.6. Isovolumetric relaxation time, isovolumetric contraction time, and myocardial performance index

Isovolumetric relaxation time (IVRT) and isovolumetric contraction time (IVCT) indicate diastolic and systolic function, respectively. IVRT is defined as the time interval between the closure of the bulbo-ventricular valve and the opening of the atrio-ventricular valve. IVCT is the time interval between the closure of the atrio-ventricular valve and the opening of the bulbo-ventricular valve. Myocardial performance index (MPI) integrates these parameters with ventricular ejection time to relate relaxation and contraction. At 11 weeks, IVRT was lower at 27°C than at 20°C for the control (28%, $p < 0.01$) but not cold-acclimated groups (Table 6). At the same sampling day IVCT was lower at 27°C than at 20°C for the control (36%, $p < 0.01$, Table 6) and cold-acclimated groups (36%, $p < 0.01$, Table 6). MPI was higher at 27°C than at 20°C for the cold-acclimated group at 11 weeks (26%, $p < 0.001$, Table 6) but at 23 weeks, MPI did not differ between the rewarmed, cold-acclimated, or control groups at either test temperature.

4. Discussion

The results of this study demonstrate that the effect of cold acclimation on the structure and function of the zebrafish heart can be remediated by subsequent re-acclimation to control temperatures. Compared to the control fish, the changes caused by cold acclimation include a lower total heart cross-sectional area, compact myocardium thickness, end-diastolic area, and cardiac output. The lower body condition of the fish that were acclimated to 20°C and then acclimated back to 27°C, relative to the control fish, suggests that the physiological responses induced by these multiple changes in physiological temperature have a negative consequence on energy reserves.

4.1. Influence of thermal acclimation on the structure and composition of the zebrafish heart

The lower total cross-sectional area of the ventricle and muscle cross-sectional area of the spongy myocardium, indicate that the hearts of cold-acclimated fish were smaller than those of control fish. The lack of difference in the standardized cross-sectional area of the hearts from these two groups however suggests that the relative proportion of heart tissue to biomass is the same, and that the difference in absolute heart size is due to the growth of the hearts in the control fish, rather than atrophy of the heart in the cold-acclimated fish. A previous study that looked at thermal acclimation of zebrafish did not see a change in heart size with cold acclimation (Johnson et al., 2014). However, in that study, histological measurements were made following 5 weeks of cold acclimation. The longer cold exposure in the current study would provide more time for any effects to manifest. This idea is supported by a previous study of three-spine stickleback (*Gasterosteus aculeatus*), where the hearts of fish that were cold-acclimated for 23 weeks were smaller than those of control fish (Ressel et al., 2022).

One response of the heart to thermal acclimation that is consistent between trout and zebrafish is the decrease in the thickness of the compact myocardium with cold acclimation and the increase with warm acclimation (Johnson et al., 2014; Klaiman et al., 2011; Keen 2016). The compact myocardium, a comparatively dense layer of muscle

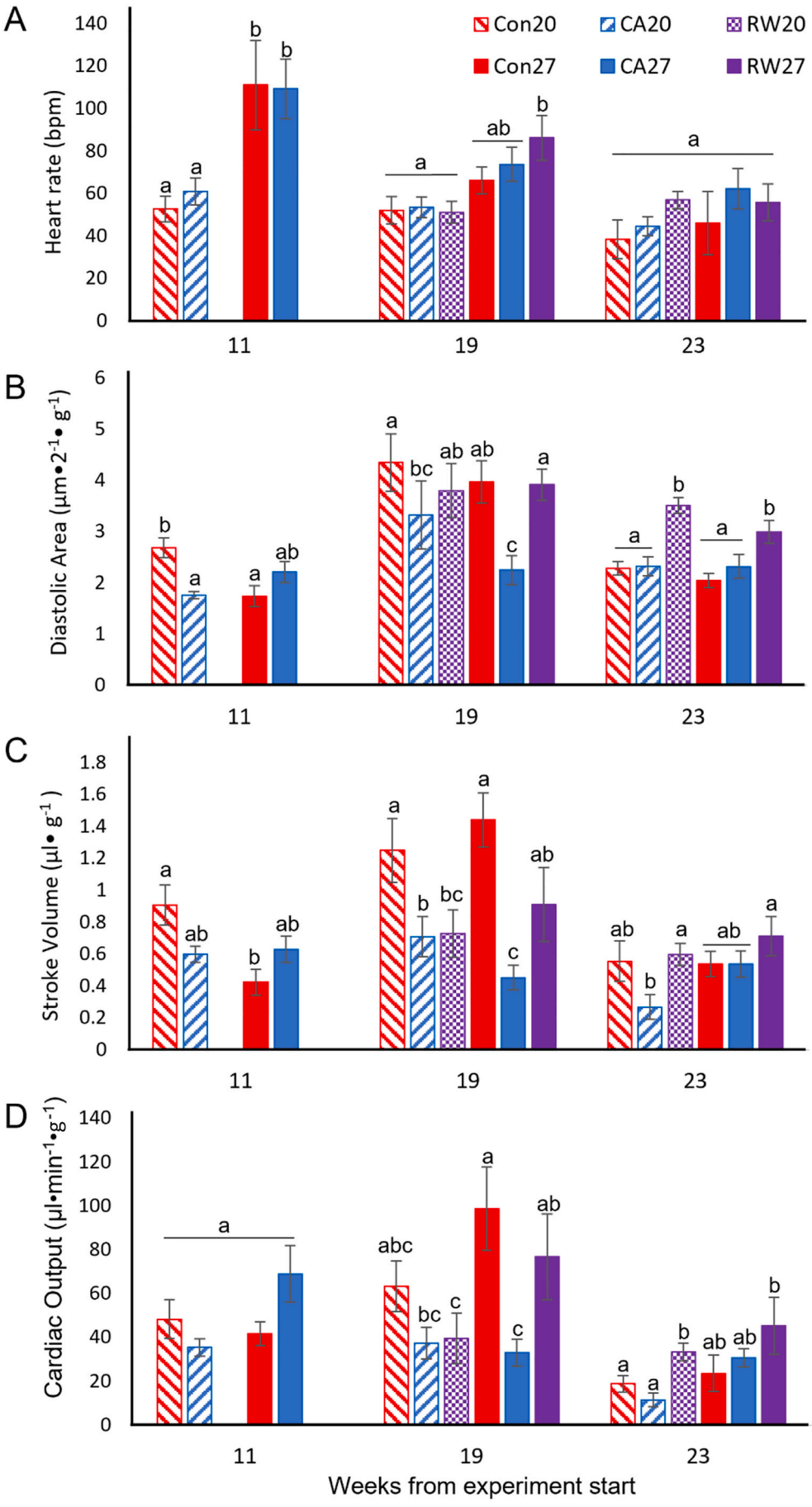

Fig. 4. The effect of thermal acclimation on the functional parameters of the zebrafish (*Danio rerio*) heart measured using cardiac ultrasound. A) Heart rate; B) Diastolic area; C) Stroke volume and D) Cardiac output. Measurements were made at either 27°C or 20°C. Numbers in legend represent the temperature, in ℃, at which the measurement was made. Con are control zebrafish held at 27°C throughout the experiment; CA are fish acclimated to 20°C for the entire experiment; RW are fish acclimated to 20°C until Week 17, then acclimated back to 27°C until Week 23. The values presented in panels B, C, and D have been standardized to fish mass. Bars represent mean ± SEM. Bars annotated with different letters at the same time point are different from each other (ANOVA $p < 0.05$). Samples sizes for each measurement are 10 for Week 11; 10 for Week 19; and 10–16 for Week 23. n represents an individual fish.

Table 3
The effect of thermal acclimation on the unstandardized functional parameters of the zebrafish (*Danio rerio*) heart measured using cardiac ultrasound. Con were zebrafish held at 27°C throughout the experiment; CA were fish acclimated to 20°C for the entire experiment; RW were fish acclimated to 20°C until Week 17, then acclimated back to 27°C until Week 23. Numbers in column headings represent the temperature in ℃ at which the measurement was made. Values are means ± SEM. Values in the same row indicated by a different superscript, are significantly different (ANOVA, $p < 0.05$). The n of these measurements are 10 for Week 11; 10 for Week 19; and 10–16 for Week 23. n = individual fish. w, week.

	Con20	CA20	RW20	Con27	CA27	RW27
End-Diastolic Area ($mm•2^{-1}$)						
w11	1.41 ± 0.12^{b}	1.15 ± 0.06^{ab}	n/a	1.20 ± 0.08^{a}	1.30 ± 0.15^{ab}	n/a
w19	1.05 ± 0.07^{a}	1.21 ± 0.05^{b}	1.16 ± 0.07^{ab}	1.35 ± 0.08^{ab}	1.28 ± 0.08^{ab}	1.46 ± 0.06^{ab}
w23	1.78 ± 0.14^{a}	1.40 ± 0.07^{b}	1.69 ± 0.05^{a}	1.72 ± 0.18^{a}	1.46 ± 0.11^{b}	1.57 ± 0.06^{a}
Stroke Volume (uL)						
w11	0.45 ± 0.07^{b}	0.36 ± 0.04^{b}	n/a	0.36 ± 0.04^{a}	0.45 ± 0.10^{b}	n/a
w19	0.29 ± 0.03^{ab}	0.35 ± 0.02^{ab}	0.37 ± 0.05^{ab}	0.43 ± 0.06^{a}	0.37 ± 0.04^{b}	0.45 ± 0.05^{ab}
w23	0.65 ± 0.09^{a}	0.36 ± 0.08^{a}	0.47 ± 0.03^{a}	0.68 ± 0.16^{a}	0.24 ± 0.04^{a}	0.32 ± 0.07^{a}
Cardiac Output ($ul•min^{-1}$)						
w11	39.43 ± 6.92^{a}	22.16 ± 2.70^{a}	n/a	48.99 ± 5.33^{a}	53.59 ± 9.10^{a}	n/a
w19	30.17 ± 3.37^{a}	41.70 ± 7.40^{a}	22.47 ± 3.26^{a}	28.90 ± 2.83^{a}	37.20 ± 6.75^{a}	37.20 ± 6.75^{a}
w23	40.08 ± 6.21^{a}	19.67 ± 4.11^{a}	23.94 ± 3.38^{a}	47.86 ± 15.59^{a}	19.87 ± 3.83^{a}	16.10 ± 3.49^{a}

encapsulating the heart, is also thought to influence diastolic function (Keen et al., 2017). It has been proposed that a decrease in the thickness of the compact myocardium with cold acclimation helps to maintain proper diastolic filling by decreasing the stiffness of the heart (Johnson et al., 2014; Klaiman et al., 2011). The observed changes in the thickness of this myocardial layer in the current study may, therefore, be related to the regulation of diastolic function with thermal acclimation. In the current study, when the cross-sectional area of the compact myocardium was standardized to the cross-sectional area of the spongy myocardium, there was no difference between any of the three experimental groups. This also suggests that the relative proportion of this myocardial layer is being regulated.

Rewarming of the fish following cold acclimation appears to stimulate cardiac growth and collagen deposition. This is supported by the histological measurements of muscle cross-sectional area, compact myocardium thickness, and ultrasound determination of end-diastolic area of the rewarmed group at week 23 compared to the cold-acclimated group. The standardization of the functional end-diastolic area measurements to body mass, completed at 23 weeks, suggests that the size of the heart, as a ratio of body size, is the same between the control and cold-acclimated groups but is larger in rewarmed fish. This increase in relative proportion may be a consequence of cardiac hypertrophy that occurred along with a decrease in body weight. The average body weight of the rewarmed fish was 4% less than that of the cold-acclimated fish and 18% less than that of the control fish. The proportionally larger heart of the rewarmed fish would, however, be more effective at circulating blood through the animal. Determining how rewarming stimulated cardiac growth in these fish would be an interesting next step.

The results of the current study align with a previous study that found that the hearts of cold-acclimated zebrafish contained less collagen than those of control fish (Johnson et al., 2014). These results are the opposite to what has been reported multiple times for rainbow trout where there was more collagen in the hearts of cold-acclimated fish (4°C); and the hearts of warm-acclimated fish (17°C) had less collagen than those of the control fish (12°C) (Klaiman et al., 2011; Keen et al., 2016; Johnston and Gillis, 2022). The relative amount of collagen in the myocardium of the rewarmed hearts is also greater than that in the cold-acclimated group, suggesting that increased structural support is required for the larger cross-sectional area of the myocardium we observed in rewarmed zebrafish. Interestingly, the increase in collagen that occurred with rewarming occurred exclusively in the spongy myocardium. This myocardial layer represents approximately 90% of the total myocardium of the heart (Table 2) and expands during diastole as blood fills the lacunae between the myocardial trabeculae. The cross-sectional area of spongy myocardial muscle was higher in the rewarmed group than in the cold-acclimated group, suggesting that the growth of the spongy myocardium is also due to hypertrophy of the muscle. Regulation of the passive biomechanical properties of the tissue by changing collagen content and muscle mass has significant potential, therefore, to affect both diastolic and systolic function (Klaiman et al., 2011; Gamperl and Farrell, 2004; Johnston and Gillis, 2022).

Like zebrafish, trout remain active across a range of seasonal temperatures (Klaiman et al., 2011); however, as mentioned above, cold acclimation of these fish causes cardiac hypertrophy and an increase in connective tissue, while warm acclimation causes atrophy of the heart and a decrease in connective tissue (Keen et al., 2016, 2018; Klaiman et al., 2011). The opposite responses of trout and zebrafish to thermal acclimation, therefore, suggest that there are different strategies to compensate for the effect of a seasonal temperature change on cardiac function. One hypothesis to explain this differential response is related to the difference in blood pressure between zebrafish and trout (Keen et al., 2017). The end diastolic pressure of adult zebrafish, that weigh between 0.3 and 1.0 g, is ~0.5 mmHg (Hu et al., 2001), while the end diastolic pressure of ~750 g adult trout is ~25–45 mmHg (Clark and Rodnick, 1999). This indicates that the zebrafish heart is under less pressure than that of trout and that there is less pressure available to inflate the zebrafish heart during diastole. An increase in the stiffness of the zebrafish myocardium, caused by an acute decrease in temperature, could therefore make it more difficult for the zebrafish heart to fill with blood during diastole. A reduction in connective tissue, as well as a lower relative ventricular muscle mass, as found in the current study, would help compensate for an increase in tissue stiffness and, therefore, help maintain diastolic function in these very small fish. Finally, one difference between zebrafish and rainbow trout is that the temperature at which cardiac remodeling has been observed differs by 16°C (20°C and 4°C, respectively). While it is generally thought that it is the net change in physiological temperature that is responsible for inducing cardiac remodeling in these fish species (Keen et al., 2017), it is interesting to consider that there may be some consequence of the proximity of the temperature range to 0°C on the response of the two species. Such an effect would however, be difficult to test for.

4.2. *Influence of thermal acclimation on the active and passive properties of the zebrafish heart*

The lower standardized end-diastolic volume, stroke volume, and cardiac output of the cold-acclimated group measured at 27°C compared to the control group at 19 weeks suggests that the hearts of the cold-acclimated animals are less functional at this experimental temperature. However, there was no difference in standardized end-diastolic volume, stroke volume, or cardiac output between the hearts of the rewarmed fish and control fish. This result suggests that rewarming can make the function of the heart of the cold-acclimated fish more similar

Table 4
The effect of thermal acclimation on blood flow velocity during ventricular filing and evacuation of zebrafish (*Danio rerio*). Values are means ± SEM. Values in the same row indicated by a different superscript, are significantly different (ANOVA, $p < 0.05$). The n of these measurements are 10 for Week 11; 10 for Week 19; and 10–16 for Week 23. n = individual fish. Ejection fraction (Stroke volume/Diastolic volume*100), calculation includes tissue in volume estimates; w, week. Con are zebrafish held at 27°C throughout the experiment; CA are fish acclimated to 20°C for the entire experiment; RW are fish acclimated to 20°C until Week 17, then acclimated back to 27°C until Week 23. Numbers in column headings represent the temperature in °C at which the measurement was made. n = individual fish. w, week; E Velocity, velocity of passive blood flow from atrium to ventricle; A Velocity, velocity of blood flow into ventricle powered by contraction of the atrium.

	Con20	CA20	RW20	Con27	CA27	RW27
Ejection Fraction (%)						
w11	44.65 ± 2.90[a]	44.77 ± 2.90[a]	n/a	38.56 ± 3.17[a]	43.81 ± 3.10[a]	n/a
w19	29.69 ± 2.28[a]	27.66 ± 2.75 [a]	23.04 ± 3.22[a]	37.46 ± 3.09[a]	23.03 ± 3.39[a]	26.86 ± 4.33[a]
w23	25.41 ± 4.50[a]	13.63 ± 1.98[a]	19.36 ± 2.28[a]	26.07 ± 3.27[a]	27.81 ± 2.79[a]	25.58 ± 3.90[a]
E Velocity ($mm \bullet 2^{-1}$)						
w11	52.54 ± 5.71[a]	54.09 ± 6.81[a]	n/a	56.27 ± 6.98[a]	35.68 ± 7.19[a]	n/a
w19	47.25 ± 14.81[a]	42.35 ± 8.09[a]	79.29 ± 21.09[b]	71.59 ± 25.32[b]	28.83 ± 5.26[c]	53.17 ± 11.83[b]
w23	43.89 ± 7.98[a]	73.62 ± 15.66[a]	72.11 ± 17.33 [a]	118.53 ± 34.93[a]	41.85 ± 11.63[a]	50.80 ± 60.94 [a]
A Velocity ($mm \bullet 2^{-1}$)						
w11	122.55 ± 24.58[ab]	108.13 ± 24.29[a]	n/a	129.11 ± 23.08[ab]	166.44 ± 23.22[b]	n/a
w19	157.71 ± 30.45[a]	168.43 ± 32.48[a]	109.02 ± 19.45[a]	150.48 ± 18.87[a]	81.68 ± 5.86[a]	201.67 ± 39.82 [a]
w23	45.36 ± 6.23[a]	70.89 ± 20.81[a]	104.02 ± 20.05[a]	83.37 ± 24.68[a]	132.67 ± 34.12[a]	141.34 ± 28.63[a]
Ventricular Velocity ($mm \bullet 2^{-1}$)						
w11	219.96 ± 34.22[a]	195.43 ± 31.68[a]	n/a	236.95 ± 29.26[a]	236.67 ± 27.69[a]	n/a
w19	142.86 ± 18.63[a]	133.84 ± 14.61[a]	170.75 ± 25.66[a]	138.71 ± 19.62[a]	140.89 ± 19.94[a]	151.79 ± 22.27[a]
w23	74.20 ± 12.93[a]	78.22 ± 13.05[a]	141.40 ± 22.82[a]	171.19 ± 36.05[a]	88.00 ± 16.37[a]	149.65 ± 22.64[a]

to that of the hearts from the control group at 27°C.

Together, the histological characterization and measurements of end-diastolic area suggest that the structure and composition of the zebrafish heart is regulated in response to thermal acclimation. These two characteristics, as well as the capacity for active force generation, determine the effectiveness of the heart to contract and relax. In the current study, these functional parameters were estimated using measurements of isovolumetric contraction time (IVCT) and isovolumetric relaxation time (IVRT). The IVCT in the hearts of the cold-acclimated fish were shorter (i.e. faster) at 27°C than at 20°C at weeks 11 and 23, illustrating the impact of a decrease in temperature on contractile function. The shorter IVCT in the cold-acclimated fish compared to the control fish at week 11 when measured at 27°C, and in the rewarmed fish compared to controls at week 19 when measured at 27°C, suggest that there is a greater capacity to contract at the same temperature. This could be caused by changes to the Ca^{2+} sensitivity of the myofilament, as has been demonstrated to occur in the trout heart (Klaiman et al., 2014), or changes in expression of calcium sensitive proteins as seen in zebrafish (Genge et al., 2013) with cold acclimation.

One factor that needs to be considered when interpreting the functional results of this study is that the use of an anesthetic could influence the measured changes in the active properties of the heart caused by thermal acclimation. Cold acclimation alters cardiac function, in part, by affecting autonomic tone (Little and Seebacher, 2014). The anesthetic used in the current study has been reported to not affect the active properties of the fish heart (Roberts and Syme, 2016; Hill et al., 2002), but as a $GABA_A$ receptor agonist, it has the potential to reduce cardiac function. However, as mentioned above, the higher IVCT of cold-acclimated fish compared to control fish at week 11 indicates an increase in contractile function. This difference in function would not be caused by the anesthetic.

Compared to the IVCT measurements, which is a measure of the active properties of the heart, there is less variation between groups in the IVRT. The only detected difference was at 11 weeks between the hearts of cold-acclimated fish measured at 20°C and those from the control fish measured at 27°C. This suggests that the ability of the hearts to relax following contraction are not significantly different between groups. Diastolic function of the heart is regulated in part by the rate that Ca^{2+} is transported back into the sarcoplasmic reticulum (SR) at the end of systole, and by the passive properties of the myocardium. For example, cardiac fibrosis can lead to diastolic dysfunction by affecting the ability of the heart to relax between beats. The significant changes to the collagen content of the zebrafish heart with cold-acclimation and subsequent re-acclimation to control temperature suggests that there is active regulation of the passive stiffness of the heart and, therefore, diastolic function. The passive stiffness of the heart can also be regulated on a short time scale by the phosphorylation of titin, a large sarcomeric protein (Patrick et al., 2010), as well as by the phosphorylation of phospholamban, a protein that regulates the activity of sarcoendoplasmic reticulum Ca^{2+} ATPase (SERCA) (Gorski et al., 2015). It is not known if the phosphorylation of titin or SERCA is involved in the regulation of diastolic function in zebrafish during thermal acclimation. However, Little and Seebacher (2014) report that cold-acclimated zebrafish had higher SERCA activity than warm-acclimated fish.

The measurements of blood velocity indicate that both the active and passive components of the heart are modulated during cold-acclimation and rewarming and are also affected by experimental temperature. The early filling of the ventricle is dependent on the passive properties of the myocardium, and the early atrial ejection velocity (E Velocity) and the early ventricular filling period (E Time) are used as indicators of this capacity. For example, an effect of an acute change in temperature is the shorter ventricular ejection time at 27°C than at 20°C for control and cold-acclimated fish at 11 and 19 weeks. There was additionally a trend for the E Velocity to be faster in the hearts of cold-acclimated and control fish when measured at their respective physiological temperature. For example, at week 19, the E Velocity of the cold-acclimated fish was higher at 20°C than at 27°C, while the E Velocity of the control fish was higher at 27°C than at 20°C. As in the cold-acclimated fish, the E Velocity in the rewarmed fish at weeks 19 and 23 trended toward being higher at 20°C than at 27°C, although this difference was not statistically significant. While the blood was moving faster at one experimental temperature in the hearts of the cold-acclimated and control during early ventricular filling, the lack of difference in the early ventricular filling period (E Time) suggests that this does not lead to a change in how long it takes to fill the ventricle at the start of diastole. This is further evidence that there is effective compensation for the effects of a change in temperature on the passive properties of the ventricle. This also supports the hypothesis that changes in the structure and composition of the myocardium with thermal acclimation are utilized to modulate myocardial stiffness. It is important to acknowledge that acute regulation of sarcomeric proteins, such as titin via phosphorylation, can also play a role in altering the passive biomechanical properties of the myocardium (Koser et al., 2019; Krysiak et al., 2018; Shiels and White, 2008). However, there has been no work to date that has examined this possibility in fish during acute changes in temperature.

At 11 weeks, the faster A Velocity measured in the cold-acclimated

Table 5
The effect of thermal acclimation on ventricular filling and evacuation of the zebrafish (*Danio rerio*) heart. Con were zebrafish held at 27°C throughout the experiment; CA were fish acclimated to 20°C for the entire experiment; RW were fish acclimated to 20°C until Week 17, then acclimated back to 27°C until Week 23. Numbers in column headings represent the temperature in °C at which the measurement was made. Values are means ± SEM. Values in the same row indicated by a different superscript, are significantly different (ANOVA, $p < 0.05$). The n of these measurements are 10 for Week 11; 10 for Week 19; and 10–16 for Week 23. n = individual fish. ms, milliseconds; w, week; E Time, early atrial ejection time; A Time, late atrial ejection time.

	Con20	CA20	RW20	Con27	CA27	RW27
E Time (ms)						
w11	508.91 ± 29.86 [a]	464.44 ± 30.35 [a]	n/a	329.79 ± 35.63 [a]	300.32 ± 39.78 [a]	n/a
w19	331.76 ± 23.70 [a]	355.76 ± 36.70 [a]	342.33 ± 25.88 [a]	292.81 ± 21.14 [a]	274.48 ± 26.05 [a]	320.80 ± 37.25 [a]
w23	262.67 ± 22.46 [a]	378.12 ± 29.09 [a]	341.83 ± 25.53[a]	334.60 ± 50.83 [a]	345.19 ± 36.19[a]	373.67 ± 21.59[a]
A Time (ms)						
w11	55.34 ± 1.90[a]	58.55 ± 1.66[b]	n/a	41.83 ± 1.68[c]	45.76 ± 2.07[d]	n/a
w19	59.01 ± 3.39 [a]	56.84 ± 3.61 [a]	57.31 ± 1.09[a]	56.12 ± 1.42[a]	51.10 ± 1.43[a]	51.76 ± 1.29[a]
w23	58.33 ± 1.90 [ab]	54.35 ± 3.18[a]	64.59 ± 2.14[b]	52.30 ± 2.77[a]	53.49 ± 1.99[a]	51.83 ± 1.91[a]
Ventricular Ejection Time (ms)						
w11	192.89 ± 10.71[a]	182.08 ± 10.39[a]	n/a	140.60 ± 9.03[b]	100.88 ± 8.35[b]	n/a
w19	245.66 ± 17.63[a]	227.68 ± 10.10[a]	201.18 ± 10.26[b]	165.98 ± 10.77[b]	172.05 ± 13.06[b]	191.42 ± 8.72[b]
w23	191.73 ± 16.26[a]	181.67 ± 13.91[a]	187.78 ± 5.56[a]	213.3 ± 26.79[a]	190.79 ± 15.63[a]	211.08 ± 14.56[a]

hearts at 27°C compared to at 20°C weeks is suggestive of greater contractile function in the atrium at the higher experimental temperature. This higher rate of contraction likely translates into the shorter A Time at 27°C for this same group compared to 20°C. The shorter A Time at 27°C than at 20°C for the control group at 11 weeks also suggests greater contractile function at the higher experimental temperature. The longer ventricular ejection time seen in the control and cold-acclimated groups at 20°C than at 27°C at week 11 and 19 likely helps to maximize the blood flow at each contraction. Such a response would help to compensate for the lower heart rate seen in the control and cold-acclimated fish at 20°C than at 27°C at week 11. The maintenance of ventricular blood velocity during systole across the experimental temperatures for control, cold-acclimated, and rewarmed groups suggests that there is maintenance of the contractile capacity of the ventricle during acute temperature exposure as well as during temperature acclimation in these zebrafish.

4.3. The cost of remodeling

The functional and anatomical consequences of cold acclimation were reversed when the cold-acclimated fish were acclimated back to 27°C. Stroke volume, end-diastolic area, and cardiac output returned to control levels at 19 weeks. These results suggest that reversible cardiac remodeling can occur in fishes living in a natural environment where temperatures change twice per year. The changes to condition factor monitored throughout our experiment also suggest, however, that exposing zebrafish to cold acclimation followed by acclimation back to 27°C has an energetic cost. It has been previously demonstrated that there is an energetic cost in killifish for maintaining physiological function in suboptimal conditions (Borowiec et al., 2015). Our results align with these conclusions. Since the condition factor and mass of the rewarmed fish were lower than those of the control, but there was no difference in these factors between the control and cold-acclimated fish, suggests that it is the rewarming, and not low temperature, that is responsible. The lower mass of the rewarmed fish may be due to a reduction in feeding due to stress, a decrease in the efficiency of nutrient assimilation, or an increase in energy utilization. As we did not measure food intake or metabolic rate it is not possible to delineate these, but it is apparent that rewarming has consequence on growth. Relating body condition to the results of our ultrasound experiments suggests that changes to heart function are reversible even if that plasticity comes at a cost. For example, zebrafish reared under a fluctuating temperature regime grow at a slower rate (Schaefer and Ryan, 2006). Our results may also reflect this higher energetic cost of compensating for changes in environmental temperature when reversing the cold-acclimated phenotype. The rewarmed group had the lowest body condition, and the decrease in body condition that we characterized was driven by a change in mass. One final thought regarding this result is that a lab reared strain of zebrafish was used in this experiment, and as suggested by Morgan et al. (2022), it is possible that this strain may have less capacity to respond to prolonged thermal acclimation as would a wild strain. Such under-compensation could translate into higher energetic costs for the maintenance of physiological function as environmental temperature changes compared to wild strains of zebrafish. Future studies of such differences would be useful to understand how domestication alters the ability of animals to respond to changes in their environment.

4.4. Perspectives

Zebrafish are a remarkably resilient species that can survive a range of environmental conditions. The results of this study demonstrate that zebrafish can reversibly remodel their hearts in response to changes in temperature. However, there does appear to be a detrimental consequence of the thermal acclimation experiment, as indicated by the decrease in body condition in fish that were cold-acclimated and then acclimated back to 27°C. In addition, our results indicate that the cardiac phenotype continues to change during cold acclimation and that this response is complex and dynamic. This suggests that it is important to make multiple measurements over time to accurately evaluate the capacity of fish to respond to a change in environmental temperature.

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CRediT authorship contribution statement

Jared B. Shaftoe: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Data curation, Writing – original draft, Writing – review & editing. **Elizabeth A. Manchester:** Methodology, Validation, Formal analysis, Investigation, Data curation, Writing – review & editing. **Todd E. Gillis:** Conceptualization, Methodology, Resources, Writing – review & editing, Funding acquisition.

Table 6
The effect of thermal acclimation on the systolic and diastolic functional indicators of the zebrafish (*Danio rerio*) heart. Isovolumetric Relaxation Time (IVRT), time it takes for; Isovolumetric contraction time (IVCT), Myocardial performance index (IVRT+IVCT/ventricular ejection time), estimates diastolic and systolic function combined; w, week. Con are zebrafish held at 27°C throughout the experiment; CA are fish acclimated to 20°C for the entire experiment; RW are fish acclimated to 20°C until Week 17, then acclimated back to 27°C until Week 23. Numbers in column headings represent the temperature in °C at which the measurement was made. Values are means ± SEM. Values in the same row indicated by a different superscript, are significantly different (ANOVA, $p < 0.05$). The n of these measurements are 10 for Week 11; 10 for Week 19; and 10–16 for Week 23. n = individual fish.

	Con20	CA20	RW20	Con27	CA27	RW27
Isovolumetric Relaxation Time (ms)						
w11	85.72 ± 5.04 ^{ab}	87.64 ± 3.90^{a}	n/a	61.78 ± 4.21^{b}	74.84 ± 3.21 ^{ab}	n/a
w19	73.92 ± 8.48^{a}	67.76 ± 6.34 ^{a}	70.72 ± 4.54 ^{a}	72.00 ± 4.87^{a}	62.49 ± 6.46 ^{a}	70.59 ± 7.35^{a}
w23	58.86 ± 9.54^{a}	55.71 ± 4.49^{a}	67.82 ± 5.81^{a}	57.00 ± 3.17^{a}	60.02 ± 3.39^{a}	65.40 ± 6.18^{a}
Isovolumetric Contraction Time (ms)						
w11	58.22 ± 4.19^{a}	47.28 ± 1.36^{a}	n/a	37.81 ± 1.12^{b}	30.07 ± 1.35^{c}	n/a
w19	87.01 ± 7.80^{a}	69.03 ± 9.09^{a}	65.41 ± 4.54^{a}	95.95 ± 20.23^{a}	55.20 ± 6.46^{ab}	50.13 ± 2.17^{b}
w23	58.11 ± 6.76^{ab}	66.50 ± 4.88^{a}	63.99 ± 4.32^{ab}	51.13 ± 2.96^{ab}	41.40 ± 2.33^{b}	62.07 ± 8.93^{ab}
Myocardial Performance Index (a.u.)						
w11	0.78 ± 0.07 ^{ab}	0.75 ± 0.03^{a}	n/a	0.85 ± 0.07^{ab}	1.06 ± 0.04^{b}	n/a
w19	0.68 ± 0.07^{a}	0.58 ± 0.04^{a}	0.71 ± 0.07^{a}	1.09 ± 0.17^{a}	0.72 ± 0.09^{a}	0.63 ± 0.04^{a}
w23	0.65 ± 0.13^{a}	0.72 ± 0.04^{a}	0.70 ± 0.04^{a}	0.52 ± 0.07^{a}	0.55 ± 0.04^{a}	0.60 ± 0.06^{a}

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

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