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RESEARCH ARTICLE

Physiological plasticity of cardiorespiratory function in a eurythermal marine teleost, the longjaw mudsucker, *Gillichthys mirabilis*

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SUMMARY

An insufficient supply of oxygen under thermal stress is thought to define thermal optima and tolerance limits in teleost fish. When under thermal stress, cardiac function plays a crucial role in sustaining adequate oxygen supply for respiring tissues. Thus, adaptive phenotypic plasticity of cardiac performance may be critical for modifying thermal limits during temperature acclimation. Here we investigated effects of temperature acclimation on oxygen consumption, cardiac function and blood oxygen carrying capacity of a eurythermal goby fish, *Gillichthys mirabilis*, acclimated to 9, 19 and 26°C for 4 weeks. Acclimation did not alter resting metabolic rates or heart rates; no compensation of rates was observed at acclimation temperatures. However, under an acute heat ramp, warm-acclimated fish exhibited greater heat tolerance (CT_{max}=33.3, 37.1 and 38.9°C for 9°C-, 19°C- and 26°C-acclimated fish, respectively) and higher cardiac arrhythmia temperatures compared with 9°C-acclimated fish. Heart rates measured under an acute heat stress every week during 28 days of acclimation suggested that both maximum heart rates and temperature at onset of maximum heart rates changed over time with acclimation. Hemoglobin levels increased with acclimation temperature, from 35 g l^{-1} in 9°C-acclimated fish to 60–80 g l^{-1} in 19°C- and 26°C-acclimated fish. Oxygen consumption rates during recovery from acute heat stress showed post-stress elevation in 26°C-acclimated fish. These data, coupled with elevated resting metabolic rates and heart rates at warm temperatures, suggest a high energetic cost associated with warm acclimation in *G. mirabilis*. Furthermore, acclimatory capacity appears to be optimized at 19°C, a temperature shown by behavioral studies to be close to the species' preferred temperature.

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INTRODUCTION

Changes in cellular temperature have wide-ranging effects on the physiologies of ectothermic species. Metabolic rates change in accord with the Arrhenius relationship (Q_{10} effects) and the structures of biological macromolecules (DNA, proteins) and complex assemblages such as membranes are perturbed (Hochachka and Somero, 2002). In view of these pervasive effects of temperature, ectotherms modify their physiologies through acclimation or acclimatization to cope successfully with changes in temperature during their lifetimes. This physiological reorganization allows an organism to optimize its performance and alter its thermal tolerance. The capacity to acclimate is thought to play a key role in determining the susceptibility of an ectotherm to climate change (Somero, 2010). Therefore, investigating the underlying mechanisms involved in modifying thermal tolerance has recently been of increasing interest (Lannig et al., 2004; Clark et al., 2008a; Nilsson et al., 2009; Eliason et al., 2011; Healy and Schulte, 2012).

The present study extends the analysis of acclimation capacity in the context of temperature effects on physiological systems responsible for oxygen delivery. Here, we focus on a highly eurythermal marine teleost, the longjaw mudsucker, *Gillichthys mirabilis* (Cooper 1864; family Gobiidae) that inhabits intertidal regions and estuarine slough environments along the Eastern Pacific coast, from Tomales Bay in Northern California to the Baja California peninsula and the coast of the Gulf of California (Barlow, 1961). In its habitats, *G. mirabilis* experience temperatures between ~5 and ~37°C, with daily fluctuations of ~12°C being common (Buckley and Hofmann, 2002; Buckley and Hofmann, 2004). A number of studies have demonstrated the thermal plasticity of *G. mirabilis* at different levels of biological organization, with recent studies focusing on changes in protein and gene expression in response to acclimation and acute thermal stress (Sumner and Doudoroff, 1938; Somero and Doyle, 1973; Dietz and Somero, 1992; Buckley and Hofmann, 2002; Buckley and Hofmann, 2004; Logan and Somero, 2010; Logan and Somero, 2011). This species' ability to tolerate a wide range of temperatures and its capacity to induce an acclimatory response at multiple levels of biological organization make it an ideal model for characterizing processes underlying physiological plasticity. In the present study, our focus on capacities for uptake and delivery of oxygen reflects the importance of these physiological activities in establishing thermal optima and tolerance ranges.

Mismatch in oxygen supply and demand due to insufficient provision of oxygen is thought to underlie thermal tolerance in fish, a theory known as 'oxygen and capacity limited thermal tolerance' (Pörtner and Knust, 2007; Pörtner and Farrell, 2008). Oxygen demand rises with an increase in temperature because of Q_{10} effects. Furthermore, oxygen demand also may increase to meet the

increased ATP needs associated with the cellular stress response and restoration of cellular homeostasis during and following thermal stress (Kültz, 2005). This overall increase in oxygen demand commonly leads to a decrease in aerobic scope of the organism (Fry, 1947; Pörtner and Farrell, 2008; Farrell, 2009; Eliason et al., 2011). Decreases in temperature may also lead to limitations in oxygen delivery. The capacity to acclimate and compensate for these temperature effects may be essential if the organism is to maintain physiological performance under changing temperatures (Somero, 2010).

To compensate for these temperature effects and sustain an adequate aerobic metabolism and scope, organisms require an adequate transport of diverse types of solutes, notably O_2, substrates for catabolism and biosynthesis, and humoral messages. To sustain these transport functions, the circulatory system may require adaptive changes. The heart, as the pivotal power supply of the circulatory system, is an important focal point of physiological plasticity (Farrell et al., 2009). Change in temperature significantly affects cardiac function, primarily heart rate, in teleosts (Farrell, 2002; Lillywhite et al., 1999), particularly because cardiac tissues in most teleosts receive blood from venous return with lower oxygen content than that of arterial blood. Consequently, with increasing temperatures, oxygen supply to heart tissues can become limited. Therefore, profound effects of temperature on cardiac performance, systemic oxygen demand and oxygen delivery to tissues (Pörtner and Farrell, 2008) may play important roles in setting thermal limits of organisms (Farrell, 2002; Mark et al., 2002; Gamperl and Farrell, 2004; Lannig et al., 2004; Farrell, 2009; Farrell et al., 2009).

A large number of studies have investigated the mechanisms employed by fishes to adjust oxygen uptake and delivery capacities in the face of thermal stress (Graham and Farrell, 1989; Morita and Tsukuda, 1994; Blank et al., 2004; Steinhausen et al., 2008). In most of these studies, the fish species examined were pelagic species with strong aerobic capacities, e.g. salmonids and tunas. Because of these fishes' active lifestyles, temperature effects on oxygen consumption commonly include direct effects on metabolic rates and gas-transport processes, as well as effects on locomotory activity, unless the organism's activity is somehow restrained. Although research on these active swimmers has provided many insights into thermal relationships in fishes, studies of a sluggish sedentary species with limited aerobic activity, such as *G. mirabilis*, offer a useful and complementary approach that may more be amenable to discerning effects of temperature that are not driven significantly by thermal effects on locomotory activity.

In the present study we tested the capacity of *G. mirabilis* to acclimate and adjust its thermal limits, routine metabolic rates, cardiac performance and oxygen carrying capacity over a wide thermal range that reflected the ambient temperatures experienced by the species. We acclimated *G. mirabilis* to 9, 19 and 26°C, and compared them against a control group of fish acclimated to 13–14°C. Fish from each acclimation group were exposed to a heat ramp to determine how thermal acclimation affected the responses of metabolic rate and heart rate to an acute temperature change, an event that is likely to occur daily in this species' estuarine environment. We examined how metabolic demand during recovery from an acute temperature stress varied with acclimation history, a phenomenon that has received only minimal study in past investigations. To this end, we measured oxygen consumption of quiescent fish before and after an acute heat stress over several time points. To determine whether the changes in oxygen consumption were due to oxygen debt incurred by increased anaerobic metabolism during acute heat stress, we measured blood lactate concentration before and after the stress event (Brooks and Gaesser, 1980). Heart rate measurements were used to develop an index for cardiac performance, as most studies show that an increase in cardiac output in fish during an acute rise in temperature is primarily due to increased heart rates (Farrell, 2002; Gollock et al., 2006; Steinhausen et al., 2008; Sandblom and Axelsson, 2007; Clark et al., 2008a; Casselman et al., 2012). Therefore, we quantified several parameters of heart rate under resting conditions in response to acclimation and acute exposure to temperature changes. We measured temperatures at which fish lost equilibrium as a measure of whole organism's thermal limits (CT_{max} and CT_{min}). Hemoglobin concentration was measured as an indicator of oxygen carrying capacity (Lay and Baldwin, 1999).

Overall, this study provides new insights into temperature effects on organismal metabolic rates and cardiac function and further elucidates the role of cardiac phenotypic plasticity in setting the thermal optima and limits of fish. These studies can help us to better understand organismal responses to changing temperature and may help predict effects of climate change on ecosystems at the species level (Pörtner, 2010; Somero, 2010).

MATERIALS AND METHODS

Fish collection and acclimation

Gillichthys mirabilis (mean body mass 27.31±8.1 g) were captured using baited wire traps from an estuarine lagoon near the University of California, Santa Barbara (34.39°N, 119.81°W). Fish were then transported to Hopkins Marine Station and held ambient (13–14°C) for 4–5 weeks in four identical flow-through 40 l aquaria. Gender was not determined prior to setting up the acclimation populations because of the difficulty in making this distinction without dissecting the fish. In three of the tanks, temperatures were then adjusted to 9±0.5, 19±0.5 and 26±0.5°C at a rate of 2°C day^{-1}. The fourth tank remained at 13–14°C and this group was used in most analyses as the basis for comparison of the effects of acclimation. Fish were maintained at these four temperatures for 28 days and fed on a commercial fish diet (Bio-Oregon, Warrenton, OR, USA) three times per week. Ammonia and nitrite/nitrate levels (Quick Dip Test Strips, Jungle Laboratories, Cibolo, TX, USA) and dissolved oxygen concentrations (Yellow Springs Instruments YSI52 system, Yellow Springs, OH, USA) were monitored regularly. Acclimation temperatures were based on previous experiments with this species except in the case of the highest acclimation temperature, 26°C. A previous acclimation experiment that examined transcriptional responses in 28°C-acclimated fish suggested that long-term exposure to 28°C was highly stressful (Logan and Somero, 2010). Thus, we decided to use a slightly lower upper acclimation temperature. Metabolic rate and plasma lactate experiments were performed with a different group of fish from those used for heart rate experiments and blood hemoglobin measurements. For heart rate and Hb measurement experiments, fish were sampled every 7 days from the date at which the acclimation temperature was reached, as described in detail below. To minimize post-digestive increase in metabolic rate, individuals were starved 24 h prior to an experiment. The animal care and use protocol for these experiments was approved by the Stanford Institutional Animal Care and Use Committee. Throughout the heart rate measurement experiments, water level was maintained at the top of the tank and a lid covered the tank to preclude air-breathing by the fish when facing oxygen limitations (Todd and Ebeling, 1966).

Determining thermal limits

To assess the effects of acclimation on upper and lower thermal limits, we performed CT_{max} and CT_{min} experiments on each acclimation group (N=5 in all cases). We transferred fish to a smaller tank that was held at the respective acclimation temperature and allowed them to adjust to the new aquarium for 2–3 h. Then, water temperature was increased or decreased at 4°C h^{-1}. CT_{max} and CT_{min} were defined as the maximum and minimum temperatures, respectively, at which loss of equilibrium was detected, based on the fish's loss of ability to maintain dorso-ventral orientation (Kilgour and McCauley, 1986).

Oxygen consumption measurements

To discern effects of acute temperature changes on O_2 consumption and heart rates that were not influenced by activity levels, all rate measurements were recorded using quiescent specimens. During both types of experiments, specimens rarely moved and their O_2 consumption rates thus can be regarded as 'resting' rates (as opposed to 'routine' rates, which include some component of activity-related oxygen consumption). Hereafter, we refer to the rates measured at the respective acclimation temperatures prior to initiation of the thermal ramp as $\dot{M}_{O2initial}$; $T\dot{M}_{O2max}$ refers to the maximum rates observed during the thermal ramp. The difference between $\dot{M}_{O2initial}$ and $T\dot{M}_{O2max}$ is termed as $T\dot{M}_{O2}$ scope.

Oxygen consumption was determined using an intermittent flow-through respirometry technique (Steffensen, 1989). An individual fish (N=6 per group) was placed in an airtight Plexiglas chamber (1 liter volume) that was immersed in a 40 l tank held at the desired temperature. Water from the tank circulated through the chamber and was pumped past an O_2 probe (Oxygen Sensor 4500, Aanderaa Data Instruments, Boston, MA, USA). To measure O_2 consumption, water circulation was shunted away from the chamber holding the fish for 10 min, creating a closed system. The chamber temperature and the decrease in [O_2] during this period were recorded. Then the valve was reopened to flush the system. Following each run, the chamber was washed with ethanol. No background O_2 consumption was observed in chambers lacking fish. Once a fish was placed in the chamber, it was allowed to recover from any handling stress for 2–4 h. Preliminary tests confirmed that 2–4 h were sufficient to recover from handling stress. Once initial O_2 consumption rates were recorded, tank temperature was increased at 4°C h^{-1} to measure O_2 consumption rates during an acute heat stress. Tank temperature was increased up to the CT_{max}, determined as described above. Q_{10} values were calculated based on the resting metabolic rates at the respective acclimation temperatures and at the temperature at which the rates were maximal.

To measure post-heat stress O_2 consumption rates, separate groups of fish acclimated to ambient, 9, 19 and 26°C temperatures were used. Similar to the above experimental setup, fish were exposed to an acute temperature increase (4°C h^{-1}) starting from their respective acclimation temperatures. Tank temperature was increased up to 2°C below the CT_{max}; this was done to avoid potential mortalities that can occur at upper thermal limits. Tank temperature was then rapidly decreased to starting temperatures [13°C (N=3) and 9, 19 and 26°C (N=4)]. Oxygen consumption rates were measured at 2, 4, 6 and 12 h time points. It should be noted that metabolic rates during recovery are measured at respective acclimation temperatures and not at a common temperature.

Heart rate measurements

Heart rates were measured by extracellular recording methods to yield a signal analogous to that of an electrocardiogram (ECG). Individual fish were anesthetized by immersion in ice for 5–25 min (depending on acclimation history). Two 1.2-m-long 33 AWG Teflon-coated stainless steel wires (World Precision Instruments, Sarasota, FL, USA) were inserted ventrally, just anterior to the pelvic girdle, with one wire on each side of the pericardial cavity. A voltage difference was measured between these leads using an AC-coupled amplifier (P55 A.C. Pre-Amplifier, Grass Instruments Company, West Warwick, RI, USA). A PowerLab system (PowerLab/16SP, ADInstruments, Colorado Springs, CO, USA) was used to digitize the ECG signal, which was recorded using LabChart 7.3 software (ADInstruments, Colorado Springs, CO, USA).

After insertion of the leads, fish were placed in a smaller (~16×25×25 cm) temperature-controlled tank (one fish per tank). Total time between removal from ice to being placed in the tank was 5 min or less; a 100% successful recovery rate occurred. A separate wire attached to a wire mesh inserted in the tank was used as a ground. Preliminary experiments showed that *G. mirabilis* reached a stable heart rate within 1 h of handling; this pattern was independent of the acclimation history. Therefore, after inserting the electrodes we allowed 1–2 h recovery time for each specimen prior to increasing tank temperature (4°C h^{-1}) to measure heart rate in response to acute temperature change. To determine the effect of acclimation duration, we measured heart rate response to an acute increase in temperature in fish (N=3) every 7 days from the start date of acclimation. Five to six fish from each temperature group were used for the final time point (28th day of the acclimation). Heart rate increased with an acute increase in temperature until a certain high temperature was reached, after which the rate plateaued (supplementary material Fig. S1). Heart rate recordings were monitored closely to detect cardiac arrhythmia (highly irregular heart rate signal) and each fish was observed carefully to confirm that arrhythmic recordings were not due to movement of the fish. In this experiment, we quantified arrhythmia temperatures (T_A), acute temperature-induced maximum heart rates (f_{Hmax}) and the temperature at onset of maximum heart rates (T_{HRmax}) (supplementary material Fig. S1). To calculate these points we used a segmented linear regression analysis. We also quantified minimum heart rates (f_{Hmin}) and the temperatures at which these occurred (T_{HRmin}) in fish acclimated for 28 days (N=3) during acute decreases in temperature, by cooling the tank at a rate of 4°C h^{-1}. In 19°C- and 26°C-acclimated fish, heart rates decreased to <4 beats min^{-1} and it was difficult to determine arrhythmic patterns. Heart rate values are presented after rounding to the nearest whole number. We also conducted a few preliminary runs by introducing fish to temperatures higher than their acclimation temperatures and exposing them to an acute stress to confirm that the differences detected between 9 and 26°C under acute stress were not due to cumulative effects of thermal stress.

Measurement of blood parameters: hemoglobin and plasma lactate

Fish were euthanized for blood collection by cervical transection immediately after anesthetizing by immersion in ice for 15 min. Blood was drawn from the caudal vein and hemoglobin concentrations ([Hb]) were determined using HemoCue 201+, a handheld hemoglobin analyzer (Ängelholm, Sweden). Measurements were made in triplicate and calibrated for fish blood as described by Clark and colleagues (Clark et al., 2008b). [Hb] was measured every 7 days during the acclimation period (N=3) and a final measurement was made on day 28 (N=6).

For hematocrit (Hct) measurements, blood was drawn into heparinized capillary tubes and then centrifuged for 5 min in a

hematocrit centrifuge (LWS M24 Combo Centrifuge, LW Scientific, Lawrenceville, GA, USA). A digital caliper was used to measure the percentage of red blood cells in whole blood; measurements were performed in triplicate. Hct was measured in fish acclimated for 28 days (N=3) only. Mean cell hemoglobin concentration was calculated based on [Hb]/(Hct/100) (Clark and Farrell, 2011). Hct and blood [Hb] were measured in different individuals due to the limited amount of blood that could be drawn from a single individual. Considering the number of confounding factors that can affect Hct and [Hb] content, we were particularly careful to subject the fish to minimal handling stress and collected blood samples immediately after euthanizing each specimen (Gallaugher and Farrell, 1992).

For the plasma lactate analysis, blood was drawn from the caudal vein and mixed with 3.2% sodium citrate solution (ratio of 1:5 sodium citrate volume:blood volume) to prevent clotting. Whole blood samples were centrifuged at 5300 ***g*** for 10 min at 4°C and plasma was collected and stored at −80°C. Lactate concentrations were measured using a commercial lactate kit according to the manufacturer's instructions (BioVision Research Products, Mountain View, CA USA). Plasma samples were diluted 1:50 and were analyzed in a SpectraMax microplate reader (Bio-Rad Laboratories, Hercules, CA, USA) at 450 nm in comparison to a manufacturer-provided lactate standard. Measurements were made in duplicate.

Plasma lactate was measured in fish acclimated for 28 days to ambient temperature and 9, 19 and 26°C, following an acute exposure to a heat stress and during recovery from this stress exposure at 2 and 8 h time points (N=3–4 for all time points). It should be noted that plasma lactate measurements were made in a third group of fish that were caught in Santa Clara and Santa Barbara, CA. We performed preliminary tests to confirm that there was no difference between the two populations in plasma lactate levels. However, fish were placed into acclimation tanks randomly to account for any potential population specific responses.

Data analysis and statistics

To test for statistically significant effects, data from the different acclimation groups were analyzed using a two-tailed t-test (specified when used) or one-way ANOVA. Most of the comparisons used the values obtained in experiments with the ambient temperature (13–14°C) group as a basis of comparison because the other three acclimation groups were removed from this temperature to aquaria where either cold acclimation (9°C) or warm acclimation (19 and 26°C) occurred. Values across time points in heart rate and metabolic rate experiments were analyzed using one-way repeated-measures ANOVA within an acclimation group and two-way repeated-measures (mixed-model) ANOVA across acclimation groups. The statistical significance in temperature-induced maximum and minimum heart rates and their onset temperatures at each time point were determined separately for each parameter using one-way repeated-measures ANOVA. A Bonferroni *post hoc* test was applied in all analyses. Results were considered significant at P<0.05. All results are presented as means ± s.e.m. All statistical tests were conducted using GraphPad Prism version 4.00 (GraphPad Software, La Jolla, CA USA).

RESULTS

Effect of acclimation on thermal limits

CT_{max} increased with increasing acclimation temperature (Table 1) and was 5.6°C higher in 26°C-acclimated fish compared with 9°C-acclimated fish (two-tailed t-test, P<0.05). CT_{min} differed between 26°C-acclimated fish and 9°C-acclimated fish by ~2°C; however, except for 9°C-acclimated fish, none of the CT_{min} values differed significantly compared with fish held at ambient temperature.

Effect of acclimation on metabolic rates

$\dot{M}_{O2initial}$ values measured at temperatures of acclimation were significantly higher in 19°C- (1.9-fold) and 26°C-acclimated (~2.6-fold) fish compared with fish held at ambient temperature (Fig. 1). These differences show that complete metabolic compensation (equal rates at acclimation temperatures) did not occur in the warm-acclimated groups. Q_{10} values calculated based on $\dot{M}_{O2initial}$ values between 9°C- and 19°C-acclimated fish and 9°C- and 26°C-acclimated fish were 2.7 and 1.6, respectively. In contrast to the effects found in the two warm-acclimation groups, the $\dot{M}_{O2initial}$ of 9°C-acclimated fish was not different from the rate of the ambient temperature group, a result consistent with full compensation in $\dot{M}_{O2initial}$ to this 4–5°C difference in acclimation temperature.

As expected on the basis of Q_{10} effects, metabolic rates increased with an acute increase in water temperature in all acclimation groups (Fig. 1). However, the rate of increase was different across different acclimation groups. Fish acclimated to 19°C fish showed the lowest Q_{10} value (1.71) compared with 9°C- (1.94) and 26°C-acclimated (1.86) groups (Fig. 1). T$\dot{M}_{O2max}$ in 26°C-acclimated fish was significantly higher (by 67.3 mg kg^{-1} h^{-1}) than that of the ambient temperature group (Fig. 1). T$\dot{M}_{O2max}$ values in 9°C- and 19°C-acclimated fish were significantly different from each other (two-

Table 1. Critical thermal maximum (CT_{max}; N=5) and minimum (CT_{min}; N=5), temperature at onset of cardiac arrhythmia (T_A), minimum heart rate (f_{Hmin}; N=3), temperature at onset of minimum heart rate (T_{HRmin}), hematocrit (Hct; N=3) and mean cell hemoglobin content (MCHC; N=3) in control fish (at ambient temperature ambient temperature, 13°C) and fish acclimated to 9, 19 and 26°C for 28 days

Acclimation temperature	CT_{max} (°C)	CT_{min} (°C)	T_A (°C)	f_{Hmin} (beats min^{-1})	T_{HRmin} (°C)	Hct (%)	MCHC (g l^{-1})
Control	35.1±0.4	1.7±0.1	33.4	7±0.5	1.8 ± 0.3	43±2	99.7±3.1
9°C[1]	33.3±0.1*	0*	31.4*	11±1*	0±0*	34±1[a]	112.3±4.2
19°C	37.1±0.2*	2.1±0.1	36.2*	6	2.3±0.1	26±2[a]	247.1±1.1[a]
26°C	38.9±0.1*	2.1±0.3	38.7*	3*	2.2±0.6	52±3[a]	137.2±1.7[a]

[1]9°C-acclimated fish only started showing signs of loss of equilibrium after ~20 min at 0°C and were responding to stimuli by swimming away. Our cooling devices were not able to lower the water temperature beyond 0°C as ice crystals were forming in the tank and around the coolant tubing. MCHC was calculated based on blood [Hb]/(Hct/100).

Note: T_A for each fish was determined during an acute exposure to a heat ramp starting from respective acclimation temperatures. Statistical significance when compared with the ambient temperature group is denoted by an asterisk (one-way ANOVA, P<0.05). f_{Hmin} and T_{HRmin} were recorded during an acute cold ramp.

Statistical significance when compared with the control group is denoted by superscripted 'a' (one-way ANOVA, P<0.05). [Hb] and Hct measurements were made in two different groups of fish (see Materials and methods).

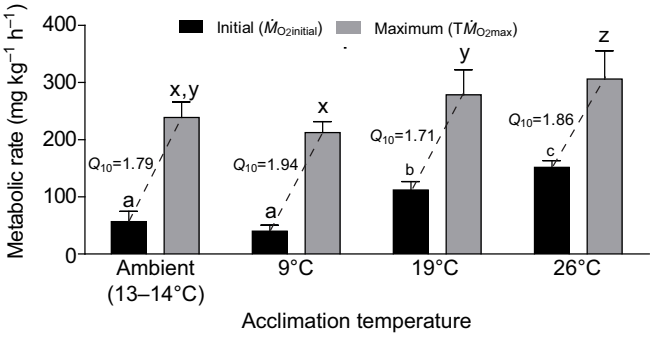

Fig. 1. Initial ($\dot{M}_{O2initial}$) and maximum metabolic rates ($T\dot{M}_{O2max}$) during acute exposure to increasing temperature in fish acclimated to ambient temperature (13–14°C) and to 9, 19 and 26°C for 28 days (N=6 per group). $\dot{M}_{O2initial}$ was measured at the respective acclimation temperatures prior to the heat ramp. $T\dot{M}_{O2max}$ is the maximum oxygen consumption rate recorded during the heat ramp. Q_{10} values are calculated based on $\dot{M}_{O2initial}$ measured at acclimation temperatures and temperatures at which $T\dot{M}_{O2max}$ was recorded. The difference between $\dot{M}_{O2initial}$ and $T\dot{M}_{O2max}$ ($T\dot{M}_{O2}$ scope) is ~180 mg kg^{-1} h^{-1} in all acclimation groups. Different letters denote statistically significant differences in $\dot{M}_{O2initial}$ (a, b and c) and $T\dot{M}_{O2max}$ (x, y and z) across acclimation groups (one-way repeated-measures ANOVA, P<0.05).

tailed t-test, P<0.05), suggesting a general trend of an increase in temperature-induced maximum metabolic rate with warm acclimation. The difference between $\dot{M}_{O2initial}$ and $T\dot{M}_{O2max}$, the temperature-induced metabolic scope ($T\dot{M}_{O2}$ scope), remained within 180–200 mg kg^{-1} h^{-1} across all acclimation groups.

During recovery from a heat stress, i.e. after tank temperature was decreased to the initial (acclimation) temperature, metabolic rates decreased within 2–4 h to values similar to $\dot{M}_{O2initial}$ in all acclimation groups except for 26°C-acclimated fish (Fig. 2). For 26°C-acclimated fish, metabolic rates remained between 250 and 300 mg kg^{-1} h^{-1} (approximately twofold higher than initial rates) for over 4 h. Data across all acclimation groups, particularly in 26°C-acclimated fish, showed a high individual variability in recovery metabolic rates.

Effect of acclimation on heart rates

Initial heart rates measured at acclimation temperature for 9°C-, 19°C- and 26°C-acclimated fish were 28±1.2, 56±1.1 and 90±0.8 beats min^{-1} after 7 days of acclimation, respectively; rates did not change significantly with continued acclimation out to 28 days (Fig. 3). Temperatures at onset of arrhythmia (T_A) changed significantly with acclimation temperature (Table 1). Compared with the fish held at ambient temperature, T_A decreased by 2°C after 28 days of acclimation to 9°C. In both 19°C- and 26°C-acclimated fish, T_A increased over the acclimation period, with 26°C-acclimated specimens showing the highest T_A (38.7±0.16°C).

Maximum heart rates induced during exposure to acute temperature increase (f_{Hmax}) were 92±1.7, 120±0.5 and 135±0.6 beats min^{-1} for 9°C-, 19°C- and 26°C-acclimated fish in 28 day-acclimated specimens, respectively (Fig. 4, supplementary material Table S1). In 26°C-acclimated fish, f_{Hmax} was 104±0.8 beats min^{-1} after 7 days of acclimation and increased by 31 beats min^{-1} after 28 days. In contrast, f_{Hmax} did not change significantly in 19°C-acclimated fish over the acclimation period; however, the temperature at onset of maximum heart rate (T_{HRmax}) increased over 28 days from 27.8±0.92 to 34.2±0.06°C. Fish acclimated to 9°C showed a different acclimatory response compared with 19°C- and 26°C-acclimated specimens. In 9°C-acclimated fish, f_{Hmax} (92 beats min^{-1}) and T_{HRmax} (24°C) were similar after 7 and 28 days of acclimation. However, during week 2, f_{Hmax} decreased to 76 beats min^{-1} and T_{HRmax} decreased to 19.9±0.71°C; T_{HRmax} then increased gradually to 24.1±0.01°C over the third week of acclimation, followed by an increase in f_{Hmax}. The differences between initial heart rates and maximum heart rates induced by

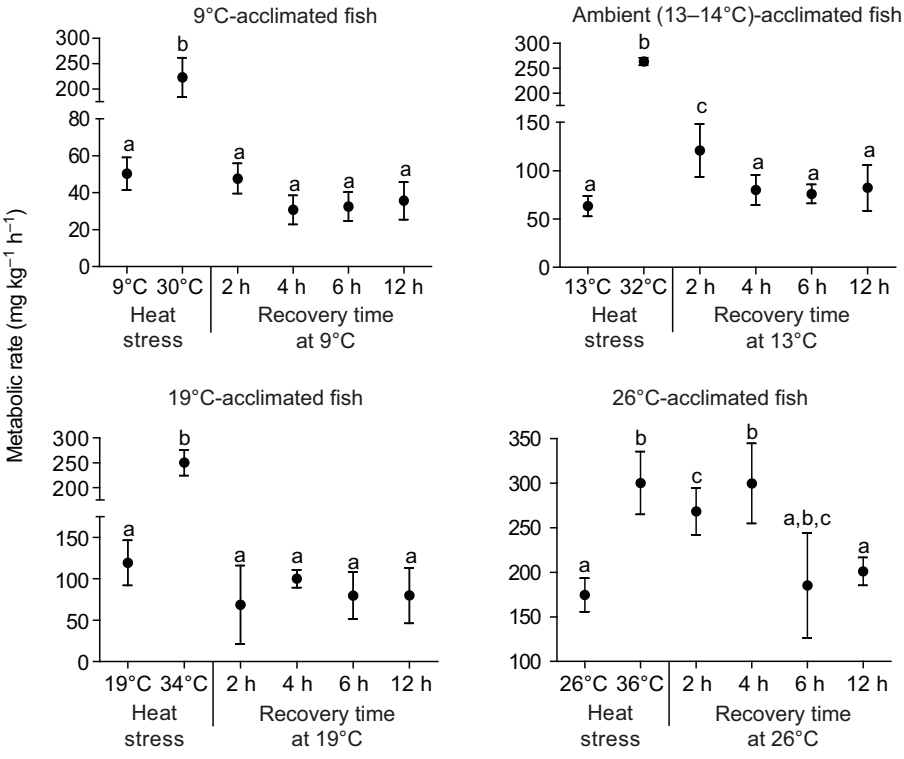

Fig. 2. Metabolic rates at the acclimation temperature, the temperature of maximal O_2 consumption, and during recovery from heat stress (2, 6, 8 and 12 h) in fish acclimated to ambient temperature and to 9, 19 and 26°C for 28 days (N=3–4 per time point in each group). It should be noted that metabolic rates during recovery are measured at acclimation temperatures and not at a common temperature. Different letters denote statistically significant differences at different time points within each acclimation group (one-way repeated-measures ANOVA, P<0.05).

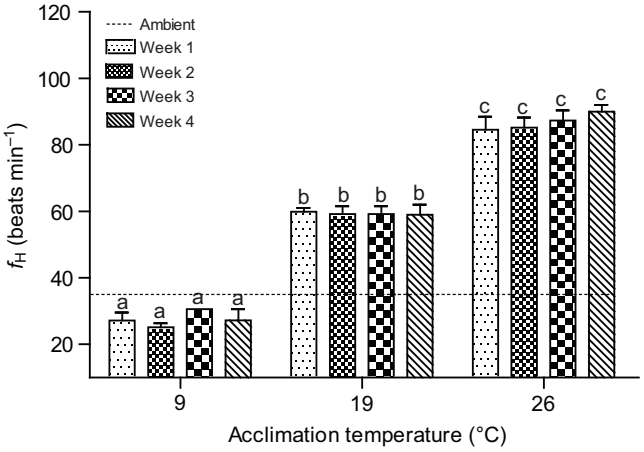

Fig. 3. Resting heart rates (f_H) in fish acclimated to 9, 19 and 26°C for 7 (N=3), 14 (N=3), 21 (N=3) and 28 days (N=5–6). The dotted line represents resting heart rate of the ambient temperature (13°C; N=6) group. Resting heart rates of all acclimation groups at all time points were significantly different from the ambient temperature group (one-way ANOVA, P<0.05). No statistically significant differences were detected across time points within an acclimation group.

temperature (Tf_H scope) were 64, 64 and 45 beats min^{-1} in 9°C-, 19°C- and 26°C-acclimated fish, respectively.

Cold tolerance of cardiac function increased with acclimation to 9°C: 9°C-acclimated fish sustained cardiac function at significantly lower temperatures (T_{HRmin}) (~0°C) than 19°C- and 26°C-acclimated fish (2.2 and 2.3°C, respectively) (Table 1). Heart rate values at T_{HRmin} for 9°C-acclimated, control, 19°C- and 26°C-acclimated specimens were 11, 7, 6 and 3 beats min^{-1}, respectively (Table 1).

Blood parameters

Acclimation had a significant effect on blood Hb levels (Fig. 5). In 19°C- and 26°C-acclimated specimens blood [Hb] increased by 1.6-fold and 2-fold, respectively, over 28 days. This increase occurred gradually over the acclimation period for 19°C-acclimated fish, while 26°C fish markedly increased [Hb] within 7 days. Hematocrit levels in 26°C-acclimated fish were twice those of 19°C-acclimated fish (two-tailed t-test, P<0.001; Table 1). Mean cell hemoglobin content (MCHC) was highest in 19°C-acclimated fish; 26°C and 9°C-acclimated fish had statistically significant increases in MCHC compared with the ambient temperature fish. MCHC data showed that 19°C-acclimated fish had the highest concentration of Hb molecules per blood cell (2.5-fold), potentially indicating an ability to optimize O_2 carrying capacity without elevating Hct levels.

Plasma lactate concentration decreased significantly with 28 days of warm acclimation (Fig. 6). Fish acclimated to 9°C had the highest lactate concentrations (14.5 mmol l^{-1}), whereas only 4.5 mmol l^{-1} lactate was detected in 26°C-acclimated fish. With an acute increase in temperature, lactate levels increased by approximately twofold in all acclimation groups except for 19°C-acclimated fish. During recovery, the rates of decrease in lactate concentration varied among groups, but in all cases lactate levels returned to values not significantly different from pre-heat stress values by 2 h of recovery.

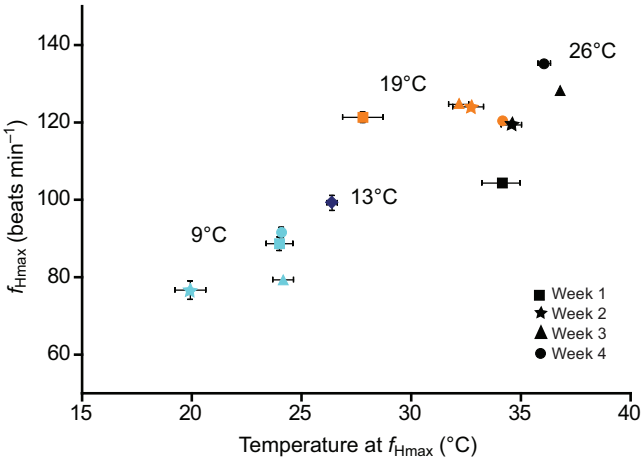

Fig. 4. Maximum heart rates (f_{Hmax}) and temperature at onset of maximum heart rate (T_{HRmax}) in fish acclimated to 9°C (light blue), 19°C (orange) and 26°C (black) for 7 (N=3; squares), 14 (N=3; stars), 21 (N=3; triangles) and 28 days (N=5–6; circles) and the ambient temperature (13°C; N=6; dark blue diamond). See supplementary material Table S1 for exact values and statistical differences.

DISCUSSION

Upper and lower thermal limits

The present study revealed significant changes in the heat tolerance of *G. mirabilis* with acclimation. Similar to previous findings (de Vlaming, 1971; Logan and Somero, 2010), warm acclimation increased upper thermal limits by more than 5°C, with 26°C-acclimated fish having a CT_{max} of 38.9°C and 9°C-acclimated fish a CT_{max} of 33.9°C (Table 1). However, the highest CT_{max} value recorded in this study was 0.8°C lower than that reported previously for this species (Logan and Somero, 2010). The difference may be due to the lower warm acclimation temperature used in the present study (26°C compared with 28°C). Temperatures at onset of cardiac arrhythmia (T_A) similarly increased with acclimation temperature; however, this increase was marginal between 19°C- and 26°C-acclimated fish (Table 1). Arrhythmia temperatures only differed by ~1°C from CT_{max} temperatures (Table 1), suggesting that cardiac dysfunction at these temperatures may be associated with loss of equilibrium.

Lower thermal limits of cardiac function were altered significantly with acclimation. Fish acclimated to 9°C were able to maintain a heart rate of 11±1 beats min^{-1} at ~0°C. This ability suggests a marked inducible cold tolerance in cardiac performance (beats min^{-1}) and lower thermal limits (T_{HRmin}) with cold acclimation (Table 1). Lack of statistically significant differences between CT_{min} values of 13–14°C-, 19°C- and 26°C-acclimated fish suggests that acclimating to warmer temperatures does not affect whole-organism cold tolerance under acute conditions. However, the differences detected in f_{Hmin} values across acclimation groups show that warm acclimation affects heart rates at low temperature (Table 1), and thus the ability to circulate adequate oxygen at these temperatures.

Metabolic rate during an acute thermal stress

Oxygen consumption rates measured at the different groups' respective acclimation temperatures under initial resting conditions ($\dot{M}_{O2initial}$) did not exhibit strong temperature compensation in 19°C- and 26°C-acclimated fish. In contrast to the two warm-acclimated groups, comparisons of the 9°C-acclimated and ambient temperature groups showed that $\dot{M}_{O2initial}$ was not different, i.e. full compensation of metabolism occurred. Q_{10} values calculated based on $\dot{M}_{O2initial}$ values between 9 and 19°C (2.7) and 9 and 26°C (1.6) demonstrate this lack of thermal compensation when acclimated to warm temperatures. Although the Q_{10} between 9 and 26°C (1.6) suggests partial compensation at 26°C, the elevated $\dot{M}_{O2initial}$ detected

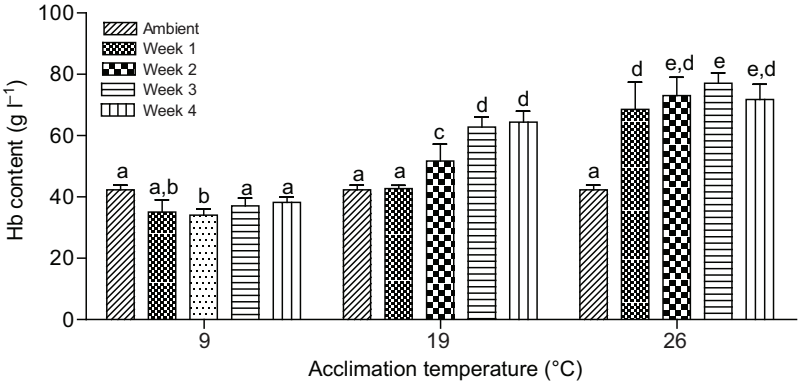

Fig. 5. Blood hemoglobin content in fish acclimated to 9, 19 and 26°C for 7 (*N*=3), 14 (*N*=3), 21 (*N*=3) and 28 days (*N*=5–6) compared with the ambient temperature (13°C) group (*N*=6). Different letters denote statistically significant differences at different time points within and across each acclimation group (two-way ANOVA, $P<0.05$).

at the latter temperature infers an overall reduction in aerobic scope in 26°C-acclimated fish, as discussed below. It is also possible that reduced Q_{10} effects in 26°C-acclimated fish are a result of compromised biological functions due to high temperatures, which lead to reduced ability to increase $\dot{M}_{O2initial}$.

A lack of temperature compensation of metabolism at higher temperatures (19 and 26°C) may reflect elevated energetic costs associated with acclimating at warm temperatures, especially at high temperatures near 26°C. Thus, non-thermally compensated rates of metabolism may reflect costs arising from more rapid transport of ions across membranes and temperature-dependent effects on the weak-bonded structures of proteins and other large molecules. For example, Somero and Doyle (Somero and Doyle, 1973) showed increased protein degradation in gill tissues of warm-acclimated *G. mirabilis*. This finding suggests that warm-acclimated fish may have higher energetic costs in order to sustain protein homeostasis, in at least some tissues. Transcriptomic data on steady-state-acclimated *G. mirabilis* are consistent with this hypothesis (Logan and Somero, 2010). Gene expression changes in gill tissues suggested higher energetic costs for fish acclimated to 19 and 28°C compared with fish at 9°C for maintaining increased macromolecular turnover and transport at warm temperatures (Logan and Somero, 2010). When steady-state-acclimated fish are exposed to an acute heat stress, a complex cellular stress response, including induction of heat shock proteins in gill and muscle tissues, is manifested in *G. mirabilis* (Buckley and Hofmann, 2004; Buckley et al., 2006; Logan and Somero, 2011). These experiments showed the highest fold-change differences in induction of stress-related proteins in 28°C-acclimated fish compared with other acclimation groups. Dietz and Somero (Dietz and Somero, 1992) showed that the threshold temperatures at which heat shock proteins are induced vary with prior thermal history, suggesting that ATP demand to produce these molecular chaperones may vary depending on acclimation history. Overall, these findings support the conjecture that maintaining elevated, i.e. non-temperature-compensated, metabolic rates at higher acclimation temperatures allows the organism to provide sufficient energy to support both the cellular stress response (Kültz, 2005) and subsequent cellular homeostatic response. These responses are needed to repair or replace damaged macromolecules and to facilitate restoration of cellular homeostasis at different acclimation temperatures and when exposed to an acute heat stress. However, sustaining an elevated $\dot{M}_{O2initial}$ at high temperatures to support

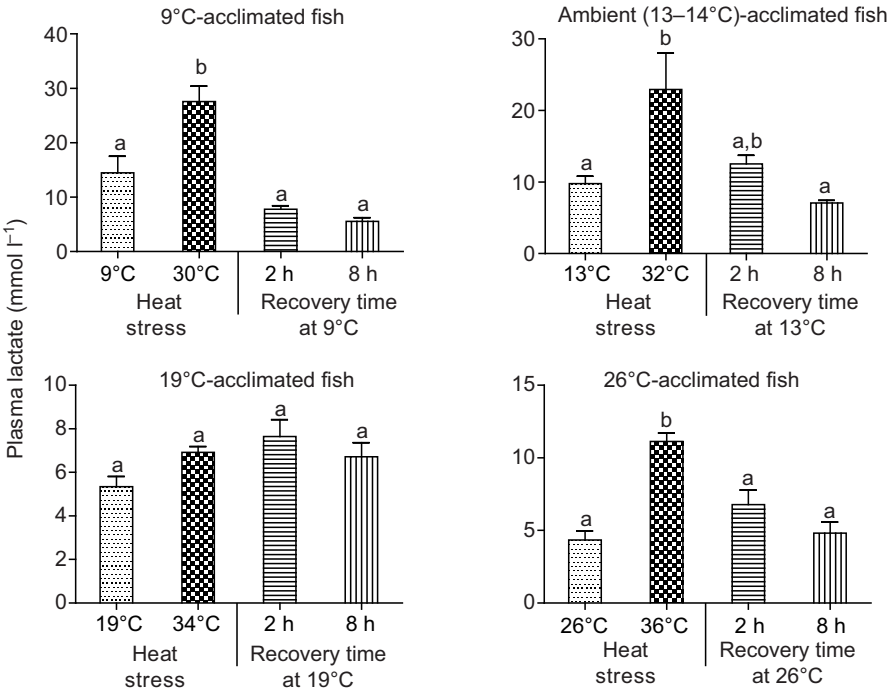

Fig. 6. Plasma lactate concentrations in fish acclimated to ambient temperature and to 9, 19 and 26°C for 28 days when exposed to an acute heat stress and 2 and 8 h after recovering from it (*N*=3 per time point). It should be noted that recovery occurred at the acclimation temperatures and not at a common temperature. Different letters denote statistically significant differences at each time point within an acclimation group (one-way ANOVA, $P<0.05$).

cellular stress responses may have consequences for the fitness of the organism, if an increased fraction of metabolic output is directed towards repair of cellular damage and away from anabolic processes such as biosynthesis and growth.

Temperature-induced maximum metabolic rates ($T\dot{M}_{O2max}$) also increased with warm acclimation in *G. mirabilis* (Fig. 1). It should be noted that maximum metabolic rates measured here occurred due to effects of temperature alone and not from changes in activity, and that $T\dot{M}_{O2max}$ occurred at different temperatures for each group of fish. Nonetheless, the increase in $T\dot{M}_{O2max}$ induced by warm acclimation is rather a surprising finding, when considering Fry's aerobic performance curves (Fry, 1947; Farrell, 2009). As discussed by Farrell (Farrell, 2009), with acclimation to warm temperatures, fish are expected to lower rates of oxygen consumption to maintain maximum aerobic scope for activity. Therefore, according to this view, in *G. mirabilis* $\dot{M}_{O2initial}$ and $T\dot{M}_{O2max}$ rates should be lowered with warm acclimation to sustain maximum capacity for aerobic activity. However, the observed increase in $T\dot{M}_{O2max}$ coupled with increased $\dot{M}_{O2initial}$ in *G. mirabilis* with warm acclimation will in fact reduce the aerobic potential available for any other activity, i.e. an escape response, especially at extreme temperatures. Future studies on *G. mirabilis* involving maximum metabolic rates due to exercise would provide more insights into the interplay between energetic demands of thermal stress versus demands associated with aerobically powered locomotory activity.

Despite the disadvantages of increased $T\dot{M}_{O2max}$ during an acute exposure to rising temperature, this resulted in a nearly constant $T\dot{M}_{O2}$ scope (180–200 $mg\,kg^{-1}\,h^{-1}$) across all acclimation groups (Fig. 1). Although we did not conduct conventional thermal performance curves (Fry, 1947; Farrell, 2009) in this study, an advantage of the current approach is the ability to infer that the increase in oxygen consumption with acute heat stress detected here is solely due to effects of high temperature on metabolic processes, exclusive of effects due to locomotory activity. Therefore, the constant $T\dot{M}_{O2}$ scope detected in *G. mirabilis* might reflect a compensatory response to obviate energetic limitations when inducing a cellular stress response during an acute heat stress, as discussed earlier. This capacity to maintain a constant $T\dot{M}_{O2}$ scope with acclimation might be a unique characteristic of eurythermy and may underlie inducible thermal tolerance in this fish.

Metabolic rate during recovery from an acute thermal stress

Many studies have characterized whole organismal or cellular metabolic activity during recovery from hypoxia or exercise in terms of payment of an oxygen debt incurred under these conditions (Brooks and Gaesser, 1980; Hochachka, 1986; Lee et al., 2003; Hallman et al., 2008; Steinhausen et al., 2008). However, as discussed previously, multiple sources of stress-related energy costs may be present, and very little is known about the absolute or relative costs due to these different stress-related activities. Data from the present study, to our knowledge, are the first to report oxygen consumption rates associated with recovering from an acute increase in temperature, without confounding effects of exercise, in fish acclimated over a wide range of temperatures. Considering the potential oxygen debt that can arise due to inadequate oxygen delivery to tissues, coupled with reduced dissolved oxygen concentrations at elevated temperatures, increased oxygen consumption rates were expected during recovery from a heat stress. However, only 26°C-acclimated fish maintained elevated oxygen consumption rates for over 4 h during recovery (Fig. 2), suggesting that only this group of fish incurred an oxygen debt with an acute stress. Fish from the 9°C and 19°C acclimation groups appear to compensate for any metabolic cost of an acute stress in less than 2 h. It is also possible that, as mentioned above, high energetic costs due to increased needs for repairing heat-induced damage at extreme temperatures (Logan and Somero, 2011) may have resulted in elevated metabolic rates during recovery in 26°C-acclimated fish.

The increase in plasma lactate levels in ambient temperature, 9°C- and 26°C-acclimated fish during the acute heat exposure suggests an elevated anaerobic metabolism during this period (Fig. 6). However, these results did not provide conclusive evidence for increased lactate-based oxygen debt in 26°C-acclimated fish compared with other groups. In all acclimation groups, plasma lactate levels decreased rapidly when temperature was lowered to their respective acclimation temperatures, indicating that accumulated lactate was rapidly utilized as a metabolic fuel or gluconeogenic substrate during recovery. Both ambient temperature fish and 26°C-acclimated fish showed a similar decrease in lactate concentrations after 2 h of returning to their respective acclimation temperatures. Considering that only 26°C-acclimated fish showed increased metabolic rates during recovery (Fig. 2), it is possible that this increase in oxygen consumption in 26°C-acclimated specimens is not to repay oxygen debt arising from lactate generation. Alternatively, it is possible that cellular anaerobiosis was in fact increased during acute heat shock in 26°C-acclimated fish, but the resulting glycolytic products were accumulated in skeletal muscles and were not released to the blood stream, and/or were used as an intermediate in cellular metabolic processes *in situ* (Gladden, 2004). Warm acclimation may have induced efficient mechanisms to sequester and synthesize anaerobic products within skeletal muscles, i.e. increased lactic acid oxidation (Grad et al., 2005), leading to reduced plasma lactate level concentrations in 26°C-acclimated fish. High plasma lactate levels measured under initial resting conditions in 9°C-acclimated fish and during acute stress compared with warm-acclimated specimens further supports this conjecture. Studies on other fish did not show an effect of acclimation temperature on plasma or muscle lactate levels [in rainbow trout (Kieffer et al., 1994) and common killifish (Healy and Schulte, 2012)], and plasma lactate returned to initial levels post-acute heat stress after 24 h in chinook salmon *Oncorhynchus tshawytscha* (Mesa et al., 2002), suggesting species-specific responses in lactate production and removal.

Plasma lactate levels in 19°C-acclimated fish are different from those of all other acclimation groups (Fig. 6). The only increase (1.4-fold) was detected at 2 h during recovery and was not in fact statistically significant. This suggests that acclimation at 19°C may optimize oxygen circulation at rest and during an acute heat stress and may have induced better mechanisms to sequester and metabolize ensuing anaerobic products (Gladden, 2004).

Heart rate and oxygen carrying capacity after 4 weeks of acclimation

As mentioned earlier, initial metabolic rates in warm-acclimated *G. mirabilis* did not show metabolic compensation to temperature. The higher oxygen consumption demands in warm-acclimated fish require an increased workload from the cardiovascular system to support an elevated capacity to provide oxygen to the cells. A number of studies have demonstrated a strong relationship between changing metabolic rates and heart rates (Brodeur, 2001; Farrell, 2002; Gollock et al., 2006; Sandblom and Axelsson, 2007; Clark et al., 2008a; Steinhausen et al., 2008; Casselman et al., 2012). The current heart rate data show a similar relationship with metabolic rates and are discussed in detail below.

The present study, the first to characterize cardiac function in *G. mirabilis*, showed significant changes in heart rates with acute and acclimatory temperature exposure. The most notable effects of acclimation occurred during an acute exposure to a heat stress. In particular, the temperature at which onset of cardiac arrhythmia occurred was significantly influenced by cold and warm acclimation: a 7°C difference in T_A was found between 9°C- and 26°C-acclimated fish. These T_A values were not significantly different from their respective CT_{max} values, suggesting a tight relationship between arrhythmia and loss of equilibrium.

Considering the exceptional acclimatory capacity evident in *G. mirabilis*, the lack of acclimation effect on resting heart rates was unexpected. As described by Lillywhite and colleagues (Lillywhite et al., 1999), with acclimation to high temperatures, heart rates should reset to lower resting values to maintain cardiac scope for adequate oxygen supply during acute changes in temperature. However, initial resting heart rates in *G. mirabilis* remained elevated with warm acclimation (Fig. 3). Given the similar lack of temperature compensation in $T\dot{M}_{O2initial}$, this lack of compensation in resting heart rates with warm acclimation is most likely a reflection of the need to support a higher level of metabolism at elevated temperatures that results in part from increased costs of the cellular stress response. Maximum heart rates induced by acute temperature increase measured during an acute heat ramp were also increased with warm acclimation (Fig. 4), but did not lead to a constant Tf_H scope across acclimation groups. Tf_H scope data indicate a reduced cardiac scope with warm acclimation in this species.

In addition to cardiac performance, hematological parameters play an important role in modifying oxygen delivery. Blood [Hb] has been used as an index for oxygen carrying capacity in fish and other organisms (Lay and Baldwin, 1999). The increase in [Hb] with warm acclimation demonstrates an increased capacity to circulate oxygen at warm temperatures (Fig. 5). However, this increase was only ~7 g l^{-1} between 19°C- and 26°C-acclimated fish as opposed to an ~30 g l^{-1} difference detected between 9°C- and 19°C-accimated fish. This infers that capacity to elevate blood [Hb] decreases rapidly above 19°C, suggesting that oxygen delivery to tissues is maximized in 19°C-acclimated fish compared with other acclimation groups. Furthermore, the 19°C-acclimated fish had the highest MCHC of any acclimation group, and their Hct (26±2%) was only one-half that of the 26°C-acclimated specimens (52±3%; Table 1). A number of previous studies have shown changes in Hct and [Hb] with temperature acclimation, with Houston and Cyr (Houston and Cyr, 1974) demonstrating an increase in both these factors with warm acclimation in trout and goldfish. However, the magnitude of this increase was significantly smaller than values measured in the present study for temperature-acclimated *G. mirabilis*. Furthermore, a later study on trout and other fish only demonstrated relatively smaller increases in Hct and Hb values with temperature acclimation (Gallaugher and Farrell, 1992).

Although the Hct and [Hb] values reported here are within the range of values previously demonstrated for teleosts, Hct values as high as ~50% are typically measured in active, aerobically swimming teleosts or in fish exposed to hypoxic conditions (Fänge, 1992; Gallaugher and Farrell, 1992; Brauner and Val, 2005). Considering that a number of factors including swelling of red blood cells and splenic contraction resulting from handling stress can influence Hct values (Gallaugher and Farrell, 1992), the rather high blood Hct measured in 26°C-acclimated fish warrants further investigation. It is possible that acclimation to temperatures as high as 26°C or to temperatures below the optimum temperature range (e.g. near 9°C) for this species may exacerbate stress responses while the fish is being removed from water and may lead to higher Hct values as measured in 9°C- and 26°C-acclimated fish. Despite these confounding factors and the lack of previous Hct and [Hb] data available for sluggish eurythermal teleosts for further comparison, the high MCHC calculated for 19°C-acclimated fish suggests an especially efficient oxygen transport in these specimens at this temperature. These data indicate that optimizing oxygen transport at temperatures near the species' thermal optimum thus may entail elevating the amount of Hb per cell and reducing Hct, thereby improving the flow properties of the blood. However, it should be noted that Hct measurements and [Hb] measurements were made in two separate batches of fish. Therefore, further studies are necessary to better understand the effects of temperature acclimation on erythropoiesis of this species.

Insights into the process of acclimation

The present study showed that both temperature-induced maximum heart rates (f_{Hmax}) and the temperature at onset of maximum heart rates (T_{HRmax}) are altered with acclimation, inducing a dual-faceted cardiac acclimatory response. The data on 19°C-acclimated fish showed that acclimation at this temperature had no effect on maximum heart rates (Fig. 4). The 120 beats min^{-1} f_{Hmax} detected in these fish is similar to what are suggested to be maximum heart rates for most teleosts (Farrell, 1991). The increase in T_{HRmax} in 19°C-acclimated fish suggests a decrease in temperature dependency of biological reaction rates (Q_{10} effects) with acclimation in this group. This capacity to alter T_{HRmax} while maintaining f_{Hmax} – thus maintaining cardiac scope at higher temperatures – might be the optimum compensatory response, given that 19°C is within the preferred temperature range of *G. mirabilis*.

The response at 26°C was the opposite of that at 19°C. The lack of acclimation effect on T_{HRmax} suggests that cardiac tissues were not able to compensate for acute increase in temperature similar to 19°C-acclimated fish. f_{Hmax} after 1 week at 26°C showed that Tf_H scope is significantly reduced and hearts were not able to compensate for effects of temperature in a short period of time. However, the increase in f_{Hmax} with longer acclimation time at 26°C suggests that certain compensatory changes were occurring in the heart to increase Tf_H scope. The differences in the effect of temperature detected in the first week in each acclimation group indicate that only 19°C fish were able to maintain Tf_H scope while inducing compensatory responses over time. The marked decrease in Tf_H scope after 1 week in 26°C-acclimated fish suggests that cardiac tissues were not able to compensate immediately for pervasive effects of temperature. However, the change in Tf_H scope over the acclimation period at 26°C shows a remarkable capacity to restore myocyte function and any other processes that were affected by temperature and to regain performance. The finding that T_{HRmax} did not increase beyond 36°C in both 19°C- and 26°C-acclimated fish suggests an absolute thermal limit for modifying cardiac function.

Overall, it appears that there may be two separate processes that are altered with acclimation: one process that sets the absolute maximum heart rate and associated Tf_H scope, and the other regulating temperature dependency of heart rate. In 9°C-acclimated fish, both of these mechanisms appear to play a role during weeks 2 and 3 to return f_{Hmax} and T_{HRmax} to values similar to week 1 after 28 days of acclimation. A combination of intrinsic cardiac or environmental factors and interplay between them governs resting and maximal heart rates in lower vertebrates (Lillywhite et al., 1999). A number of processes including force–frequency relationships, excitation–contraction coupling, cardiac anatomy, myocardial oxygen supply, cardiac energy metabolism and sensitivity to

hormonal modulation can be altered with acclimation to modify temperature dependency of maximum heart rates (Farrell and Jones, 1992; Farrell et al., 1996; Lillywhite et al., 1999). Furthermore, changes detected in blood-oxygen carrying capacity may have played a role in modulating f_{Hmax} and T_{HRmax} with acclimation. A previous study on rainbow trout showed a strong relationship with anemia and cardiac workload and cardiac remodeling in an acclimation-temperature-dependent manner (Simonot and Farrell, 2007). This suggests the potential for alterations in cardiac parameters and cardiac workload in *G. mirabilis* with changes in blood-oxygen carrying capacity. Consequently, it is possible that the increase in T_{HRmax} detected over the course of acclimation in 19°C-acclimated specimens resulted from a reduced cardiac workload due to high MCHC values measured in these fish after 28 days of acclimation. However, this relationship appears to be complex, as initial resting heart rates or f_{Hmax} did not show a direct relationship with changes in [Hb] and Hct measurements.

Based on current evidence, it is difficult to conjecture exactly what mechanisms were altered to induce the acclimatory effects detected in heart rates of 9°C-, 19°C- and 26°C-acclimated *G. mirabilis*. Further studies are required to better understand how the processes discussed above vary with acclimation time and temperature. Nonetheless, both heart rate data (T_{HRmax} and f_{Hmax}) and the changes in blood [Hb] demonstrate that inducing a plastic response is a complex, highly dynamic process that changes constantly with acclimation.

Conclusions

In the present study we provide evidence for a remarkable capacity for *G. mirabilis* to adjust its upper and lower thermal tolerance with steady-state temperature acclimation. This shift in thermal limits appears to be associated with the capacity to acclimate cardiac function and blood circulation. The effects of acclimation became especially evident when fish were exposed to an acute heat stress. When acclimating to a temperature, an organism may induce compensatory responses to offset Q_{10} effects on metabolic pathways to alter overall routine oxygen consumption. However, lowering metabolic rates, particularly at warm temperatures, may lead to an inadequate energy supply to support a sufficient cellular stress response. This might be disadvantageous for an organism that inhabits a highly fluctuating thermal environment, where the organism is continuously faced with temperature-induced cellular perturbations. Therefore, eurythermal sedentary species such as *G. mirabilis* may not induce mechanisms with acclimation to adjust their resting metabolic rates and heart rates. As shown by Healy and Schulte (Healy and Schulte, 2012), resting metabolic rates of another eurythermal teleost, *Fundulus heteroclitus*, did not show a thermal compensatory response with acclimation, and only showed a narrow range of temperatures where acclimation had an effect on aerobic scope. Furthermore, the work of Fry (Fry, 1947) demonstrates that an increase in routine metabolic rates at warmer temperatures and a decrease in maximum metabolic rates at colder temperatures lead to reduced aerobic scope in fish. However, for a sedentary species without much aerobically powered swimming activity, this reduction in capacity for aerobic performance may be less critical than for an actively swimming pelagic species; rather, maintaining adequate oxygen consumption rates supported by improved cardiac responses to induce and sustain cellular stress responses might be more important for their survival in a highly variable thermal environment.

Considering the thermal variability of estuarine environments, it is difficult to predict whether *G. mirabilis* in its native habitat would induce a similar acclimation response as observed in the present study. In its environment, *G. mirabilis* may only induce short-term acclimatory responses that may be similar to effects observed after 7 days of acclimation in this study. The cardiac limitations detected in 26°C-acclimated fish after 7 days of acclimation and in 9°C-acclimated fish after 14 days illustrate the importance of investigating the effects of duration and magnitude of a thermal stress when determining thermal limits. In conclusion, the present study demonstrates that temperature acclimation is a complex and dynamic process, and that thermal responses may be optimized at organisms' preferred temperatures, even for a eurythermal organism such as *G. mirabilis*.

LIST OF SYMBOLS AND ABBREVIATIONS

CT_{max}	critical thermal maximum
CT_{min}	critical thermal minimum
f_{Hmax}	temperature-induced maximum heart rate
f_{Hmin}	temperature-induced minimum heart rate
Hb	hemoglobin
Hct	hematocrit
$\dot{M}_{O2initial}$	metabolic rate measured at respective acclimation temperatures
T_A	temperature at onset of arrhythmia
Tf_h scope	difference between initial heart rate and temperature-induced f_{Hmax}
T_{HRmax}	temperature at onset of maximum heart rate
T_{HRmin}	temperature at onset of minimum heart rate
T$\dot{M}_{O_2}$ scope	difference between T$\dot{M}_{O2max}$ and $\dot{M}_{O2initial}$
T$\dot{M}_{O2max}$	temperature-induced maximum metabolic rate

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AUTHOR CONTRIBUTIONS

N.J. was involved in conception and experimental design and conducted the study, interpreted the findings, and drafted and revised the article. G.N.S was involved in conception, experimental design and revising the article.

COMPETING INTERESTS

No competing interests declared.

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