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RESEARCH ARTICLE

Thyroid hormone regulates cardiac performance during cold acclimation in zebrafish (*Danio rerio*)

Alexander G. Little and Frank Seebacher*

ABSTRACT

Limitations to oxygen transport reduce aerobic scope and thereby activity at thermal extremes. Oxygen transport in fish is facilitated to a large extent by cardiac function so that climate variability may reduce fitness by constraining the performance of the heart. In zebrafish (*Danio rerio*), thyroid hormone (TH) regulates skeletal muscle function and metabolism in response to thermal acclimation. Here, we aimed to determine whether TH also regulates cardiac function during acclimation. We used propylthiouracil and iopanoic acid to induce hypothyroidism in zebrafish over a 3 week acclimation period to either 18 or 28°C. We found that cold-acclimated fish had higher maximum heart rates and sarco-endoplasmic reticulum Ca^{2+}-ATPase (SERCA) activity than warm-acclimated fish. Hypothyroid treatment significantly decreased these responses in the cold-acclimated fish, but it did not affect the warm-acclimated fish. TH did not influence SERCA gene transcription, nor did it increase metabolic rate, of isolated whole hearts. To verify that physiological changes following hypothyroid treatment were in fact due to the action of TH, we supplemented hypothyroid fish with 3,5-diiodothryronine (T_2) or 3,5,3′-triiodothyronine (T_3). Supplementation of hypothyroid fish with T_2 or T_3 restored heart rate and SERCA activity to control levels. We also show that, in zebrafish, changes in cardiac output in response to warming are primarily mediated by heart rate, rather than by stroke volume. Thus, changes in heart rate are important for the overall aerobic capacity of the fish. In addition to its local effects on heart phenotype, we show that TH increases sympathetic tone on the heart at rest and during maximum exercise. Our findings reveal a new pathway through which fish can mitigate the limiting effects of temperature variability on oxygen transport to maintain aerobic scope and promote thermal tolerance.

KEY WORDS: Heart rate, Sympathetic tone, SERCA, Cardiac muscle, Temperature

INTRODUCTION

The thermal sensitivity of aerobic scope is important in determining the vulnerability of organisms to environmental change (Pörtner and Knust, 2007; Nilsson et al., 2009; Eliason et al., 2011). At suboptimal temperatures, limitations in oxygen transport reduce the capacity for aerobic metabolism, thereby reducing the energy (ATP) available for fitness-related activities like locomotion, feeding, growth and reproduction (Fry, 1971; Pörtner and Knust, 2007). As scope decreases and maximal rates approach resting rates, fitness is compromised because there is insufficient ATP to sustain activity (Pörtner and Knust, 2007). Cardiac scope, that is the difference between maximum and resting cardiac output, is one of the main factors limiting aerobic scope, and therefore thermal tolerance in fish (Claireaux and Lefrançois, 2007). Hence, remodelling cardiac physiology to maintain function in variable thermal environments is among the most critical responses to sustain fitness-related performance.

Individuals can remodel cardiac physiology in response to thermal variation during their lifetime. Many fish species alter calcium cycling, heart rate, heart morphology and sympathetic outflow to optimize oxygen delivery across a temperature range (Aho and Vornanen, 1998; Aho and Vornanen, 1999; Shiels et al., 2002; Klaiman et al., 2011; Shiels et al., 2011; Korajoki and Vornanen, 2013; Korajoki and Vornanen, 2012). For instance, sarcoplasmic reticulum Ca^{2+} uptake via the sarco-endoplasmic reticulum Ca^{2+}-ATPase (SERCA), which plays an important role in muscle force production and fatigue resistance, increases in the heart of cold-active rainbow trout during cold exposure (Aho and Vornanen, 1999). This increase is accompanied by higher heart rates and faster contraction velocities (Aho and Vornanen, 1999), both of which are typical cold acclimation responses in fish (Tiitu and Vornanen, 2003a). The regulatory mechanisms that mediate these reversible changes in cardiac physiology are poorly understood, but are of major importance because they are likely to be mechanistically linked to thermal tolerance.

Thyroid hormone (TH) is a critical regulator of thermal acclimation in fish (Little et al., 2013). During chronic cold exposure of zebrafish, TH mediates increases in gene expression of SERCA 1 and upregulates SERCA activity in skeletal muscle. These responses are associated with increased tail beat frequencies and locomotor performance (Little and Seebacher, 2013). Additionally, TH upregulates a number of genes that regulate aerobic metabolism, and increases metabolic scope (Little et al., 2013). Whether TH also regulates cardiac physiology during thermal acclimation in fish is not known. However, TH does determine cardiac function in mammals. For instance, in mammals TH increases the expression and activity of proteins involved in calcium cycling through both genomic (transcriptional) and non-genomic pathways (Kahaly and Dillmann, 2005; Ketzer et al., 2009). TH is a transcriptional regulator of SERCA and its inhibitor phospholamban (Carr and Kranias, 2002). It mediates the relative proportions of activated (dephosphorylated) phospholamban and SERCA, thereby controlling the contractile properties of the heart (Carr and Kranias, 2002). TH also regulates sympathetic outflow to increase heart rate in mammals (Carr and Kranias, 2002). It is intriguing that the effects of TH on skeletal muscle and energy metabolism in mammals parallel its effects during cold acclimation in fish. If the effects of TH are evolutionarily conserved, it is also likely that TH regulates heart function in fish. We therefore tested the hypothesis that TH regulates cardiac physiology during thermal acclimation in zebrafish, *Danio rerio* (Hamilton 1822).

TH-mediated control of cardiac physiology could explain compensatory changes in swimming performance during cold

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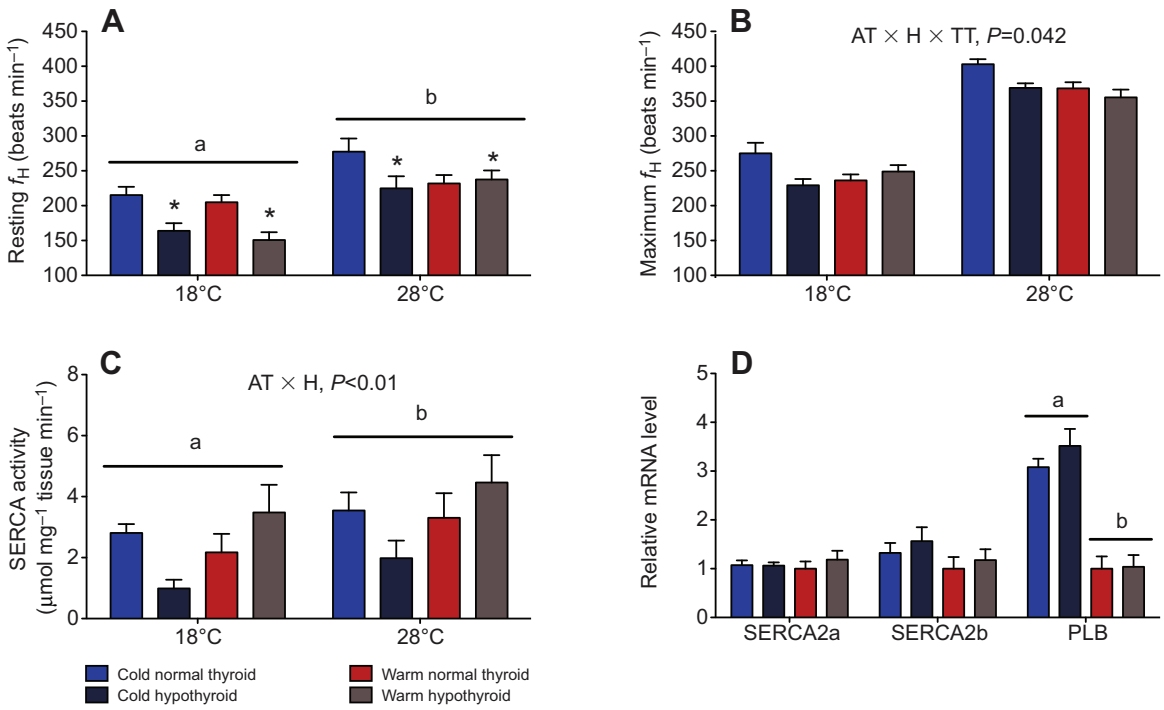

Fig. 1. The effects of thermal acclimation and hypothyroidism. The panels show data for (A) resting heart rate (f_H), (B) maximum heart rate, (C) sarco-endoplasmic reticulum Ca^{2+} ATPase (SERCA) activity and (D) relative mRNA transcript levels for SERCA2a, SERCA2b and phospholamban (PLB) in cold-acclimated normal thyroid (blue), cold-acclimated hypothyroid (navy), warm-acclimated normal thyroid (red) and warm-acclimated hypothyroid (brown) fish. Responses were measured at different test temperatures (18 and 28°C), and different letters indicate a significant main effect of test temperature; an asterisk indicates a significant main effect of hypothyroidism, and significant interactions involving hypothyroidism are indicated. AT, acclimation treatment; H, hypothyroid treatment; TT, test temperature; N=7–12, except for mRNA where N=5–6 per treatment group.

acclimation (Little et al., 2013; Little and Seebacher, 2013). We therefore aimed to determine whether TH regulates cardiac scope in response to cold exposure, and whether it acts locally at the level of the heart, or centrally to alter autonomic tone. We hypothesized that: (i) hypothyroid treatment reduces heart rate in cold-acclimated fish, and that supplementation with active forms of thyroid hormone, 3,5-diiodothyronine (T_2) or 3,5,3′-triiodothyronine (T_3), would restore heart rate; (ii) TH increases heart rate by upregulating cardiac SERCA expression and activity; and (iii) TH increases adrenergic tone on the heart during cold acclimation. We used a multifactorial experimental design in which we induced hypothyroidism, followed by supplementation with T_2 and T_3 (plus controls) in zebrafish exposed to different chronic and acute temperature combinations. We measured resting and maximum heart rate, stroke volume, metabolic rate of the heart, and SERCA activity and its mRNA transcript levels to determine whether TH plays a role in cardiac remodelling during cold exposure. We used spectral analyses on heart rate data to determine whether TH regulates cardiac function by modulating the autonomic tone on the heart.

RESULTS

Thermal acclimation and hypothyroidism

Hypothyroidism decreased resting heart rate, and resting heart rate was higher at 28°C than at 18°C (Fig. 1A, Table 1). There was a three-way interaction between acclimation treatment, hypothyroidism and test temperature in their effect on maximum heart rate (Table 1). Hypothyroidism decreased maximum heart rate in the cold acclimation treatments at both test temperatures (Fig. 1B). Hypothyroidism had a relatively small effect in the warm acclimation treatments, where it increased maximum heart rate at the cold test temperature, but decreased it at the warm test temperature (Fig. 1B). Examples of raw heart rate traces for all treatments are given in supplementary material Figs S1 and S2.

Table 1. Results of a three-way PERMANOVA testing for the effects of acclimation temperature (AT), hypothyroid treatment (H) and test temperature (TT) on resting heart rate (f_H), maximum heart rate and sarco-endoplasmic reticulum (SERCA) activity

	Resting f_H		Maximum f_H		SERCA activity	
	$F_{1,79}$	P	$F_{1,78}$	P	$F_{1,62}$	P
AT	1.51	0.22	4.40	0.039	1.80	0.15
H	17.69	<0.001	6.86	0.012	1.75	0.16
TT	37.67	<0.001	311.59	<0.001	3.11	0.048
AT×H	1.47	0.23	8.40	0.005	8.73	0.002
AT×TT	0.26	0.66	0.66	0.41	0.00	0.99
H×TT	5.50	0.020	0.47	0.50	0.48	0.62
AT×H×TT	2.03	0.16	4.20	0.042	0.64	0.54

PERMANOVA, permutational analysis of variance.

Table 2. Results of a two-way PERMANOVA for the effects of acclimation temperature (AT) and hypothyroid treatment (H) on mRNA concentrations of SERCA isoforms 2a and 2b (SERCA2a and SERCA2b, respectively) and phospholamban (PLB) in the zebrafish heart

	SERCA2a		SERCA2b		PLB	
	$F_{1,20}$	P	$F_{1,20}$	P	$F_{1,20}$	P
ACC	0.19	0.80	1.87	0.16	38.15	<0.001
TH	0.36	0.61	0.70	0.46	0.12	0.85
ACC×TH	0.36	0.62	0.25	0.83	0.26	0.71

SERCA activity decreased with hypothyroidism in the cold acclimation treatments, but increased with hypothyroidism in the warm acclimation treatments (acclimation × thyroid treatment interaction; Fig. 1C, Table 1). SERCA activity increased with increasing test temperature (Fig. 1C, Table 1). There was no effect of hypothyroidism on the mRNA transcript levels of SERCA2a, SERCA2b or phospholamban (Fig. 1D, Table 2). We did not detect SERCA1 mRNA. Phospholamban mRNA transcript levels were significantly higher in cold-acclimated fish than in warm-acclimated fish (Fig. 1D, Table 2).

The effects of T_2 and T_3 supplementation on responses sensitive to hypothyroidism

There were significant differences between cold-acclimated control, hypothyroid and T_2 and T_3-supplemented hypothyroid fish in resting heart rate ($F_{3,40}$=4.10, P<0.01), maximal heart rate ($F_{3,35}$=6.69, P<0.001) and SERCA activity ($F_{3,32}$=3.12, P<0.01). *Post hoc* Monte Carlo comparisons showed that supplementation with T_2 restored resting heart rate (i.e. no significant difference between control and supplemented fish) of hypothyroid fish to control levels (Fig. 2A). Maximal heart rate was restored to control levels by supplementation with T_3 (Fig. 2B). Both T_2 and T_3 restored maximum SERCA activity of hypothyroid fish to control levels (Fig. 2C).

Heart rate and stroke volume in anaesthetized fish

There was a significant increase in resting heart rate in the anaesthetized fish when warmed from 18°C to 28°C (Fig. 3A; t=3.038, d.f.=14, P=0.009). However, relative stroke volume did not change with the increase in temperature from 18°C to 28°C (Fig. 3B; t=0.575, d.f.=14, P=0.583). Examples of raw data traces are given in supplementary material Fig. S3.

Heart oxygen consumption

There was no effect of hypothyroidism on resting or carbonyl-cyanid *p*-[trifluoromethoxy]-phenyl-hydrazone (FCCP)-induced maximal oxygen consumption rates of whole isolated hearts (Fig. 4; resting: t-test, t=0.055, d.f.=18, P=0.957; maximum: t-test, t=0.070, d.f.=18, P=0.945).

Autonomic tone on the heart rate during hypothyroidism

In zebrafish at rest, there was a significant decrease in power spectral density (PSD) in the sympathetic and sympathetic/parasympathetic frequency ranges in hypothyroid fish compared with controls (Fig. 5A; sympathetic: t=2.343, d.f.=14, P=0.034; sympathetic/parasympathetic: t=2.219, d.f.=14, P=0.044). However, hypothyroidism did not affect PSD within the local or parasympathetic frequency ranges (local: t=1.990, d.f.=14, P=0.066; parasympathetic: t=1.147, d.f.=14, P=0.270).

PSD of maximal heart rate was significantly lower in hypothyroid fish at the local and sympathetic frequency ranges (t=3.74, d.f.=12, P=0.003 and t=3.08, d.f.=12, P=0.02, respectively). However, there were no differences between treatment groups in the sympathetic/parasympathetic and parasympathetic frequency ranges (t=0.55, d.f.=12, P=0.59 and t=–0.58, d.f.=12, P=0.57, respectively; Fig. 5B).

DISCUSSION

We found that TH regulates cardiac performance in fish. As predicted, TH increased heart rate in zebrafish through both local and central regulatory pathways. Thermal acclimation of whole-animal performance relies on the coordination of skeletal muscle function, energy metabolism and oxygen delivery to compensate for the thermodynamic effects of changing temperatures. Here, we show that in addition to its influence on skeletal muscle and metabolism

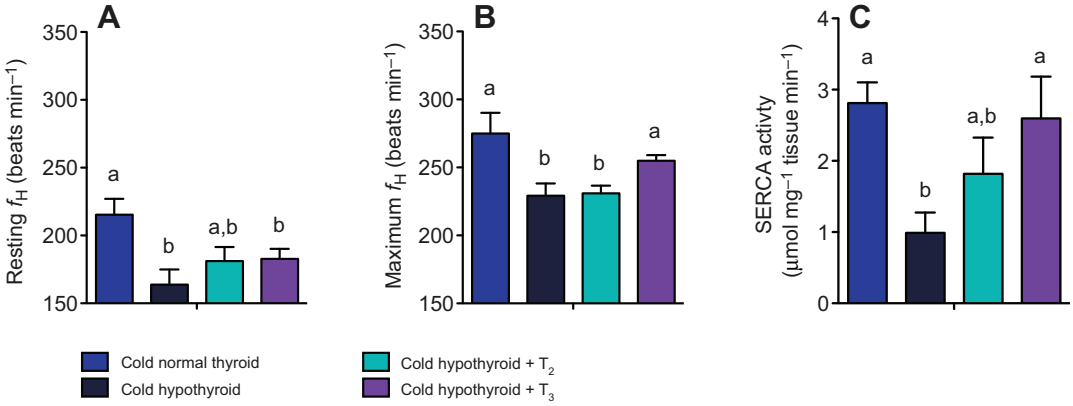

Fig. 2. The effects of supplementation with T_2 and T_3 on TH-sensitive responses in cold-acclimated fish. (A) Resting heart rate (f_H), (B) maximum heart rate and (C) SERCA activity are shown for cold-acclimated normal thyroid (blue), cold-acclimated hypothyroid (navy) and cold-acclimated hypothyroid fish supplemented with either 3,5-diiodothryronine (T_2, teal) or 3,5,3′-triiodothyronine (T_3, purple). All responses were measured at 18°C because hypothyroidism did not interact with test temperature in the cold-acclimated fish. Different letters indicate significant differences between treatment groups as determined by *post hoc* tests; N=7–12 per treatment group.

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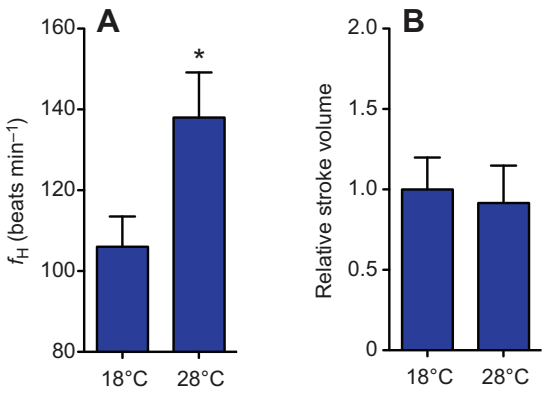

Fig. 3. The relative roles of heart rate and stroke volume during increased cardiac demand. (A) Heart rate (f_H) and (B) relative stroke volume measured at 18 and 28°C in anaesthetized cold-acclimated normal thyroid fish. An asterisk indicates a significant effect of temperature, which was used as the stimulus to increase cardiac demand; *N*=7–8 per treatment group.

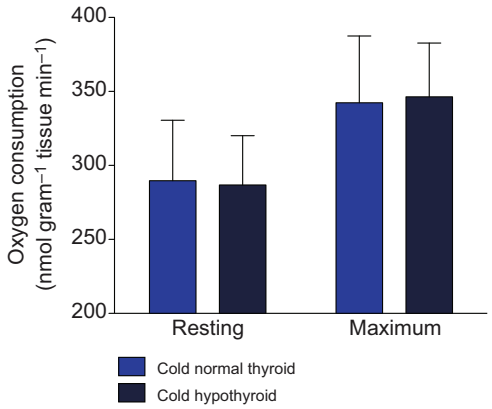

Fig. 4. The effect of hypothyroidism on resting and maximal metabolic rate in isolated hearts. Resting and maximal oxygen consumption of hearts isolated from cold-acclimated normal thyroid (blue) and cold-acclimated hypothyroid (navy) fish. Maximum metabolic rate was measured by treating the hearts with FCCP to stimulate maximal mitochondrial flux. All measurements were made at 18°C; *N*=9 per treatment group.

in zebrafish (Little et al., 2013; Little and Seebacher, 2013), TH regulates cardiac performance in a temperature-specific manner. We show that cardiac output in zebrafish is modulated primarily by changes in heart rate, rather than by stroke volume. Hence, changes in heart rate are a principal modulator of blood flow and oxygen delivery. A caveat to this conclusion is that both heart rate and stroke volume are influenced by anaesthesia. Unfortunately, zebrafish are too small to enable measurement of these parameters by *in situ* instrumentation when the fish are active. However, regardless of the relative roles of stroke volume and heart rate, the effect of TH on heart rate is likely to have important functional consequences for the overall aerobic capacity of the fish. A reduction in maximal heart rate is likely to lead to reduced cardiac and aerobic scopes (Eliason et al., 2011). Hypothyroidism affected both resting and maximal heart rate, but not in proportion to each other. In cold-acclimated fish, TH reduced both resting and maximal heart rate, so that scope would be relatively unaffected. However, in warm-acclimated fish at the warm test temperature, resting heart rate increased and maximal heart rate decreased with hypothyroidism, with the net result that scope would be reduced. Hence, thyroid hormone would affect thermal performance breadth of the heart, particularly at warmer temperatures.

In the cold-acclimated fish, increased maximum heart rate was paralleled by increases in SERCA activity and sympathetic outflow, both of which are typical cold acclimation responses in fish (Aho and Vornanen, 1998; Aho and Vornanen, 1999; Shiels et al., 2002; Klaiman et al., 2011; Shiels et al., 2011; Korajoki and Vornanen, 2012). The SERCA activity levels reported in our fish are comparable to those previously measured in cold-acclimated carp and trout (Aho and Vornanen, 1998), but both our zebrafish and the carp and trout activities are approximately two orders of magnitude larger than those measured in a recent multispecies comparison (Landeira-Fernandez et al., 2012). The lower maximum heart rate of cold-acclimated hypothyroid fish was mirrored by decreased SERCA activity and reduced sympathetic outflow. Supplementation of cold-acclimated hypothyroid fish with T_3 or T_2 acted to restore maximum heart rate and SERCA activity. This supplementation treatment verified the principal role of TH in regulating these responses. This regulatory role is likely to enhance whole-animal cold tolerance by increasing cardiac output to increase oxygen transport, thereby maintaining aerobic scope at low temperatures. Resting heart rate decreased with hypothyroidism, but it is unlikely that SERCA maximal activities are functionally related to resting heart rate, which would not require high rates of sarcoplasmic reticulum Ca^{2+} replenishment.

An increase in SERCA activity during cold acclimation would require a higher energy turnover. However, we found that both the resting and maximal metabolic rates of the heart in cold-acclimated fish were unchanged by TH status. This finding is in contrast to the effect of TH on skeletal muscle during cold acclimation, where it upregulates aerobic metabolism in parallel with SERCA activity (Little et al., 2013). It is possible that a different regulatory pathway, such as sympathetic outflow or low-energy activation of AMP-activated protein kinase (Seebacher, 2009), simultaneously upregulates aerobic metabolism in the heart independently from TH status. At the same time, mitochondrial fuel types can vary with changes in metabolic demand, such as those associated with exercise or temperature (Driedzic and Gesser, 1994; Moyes, 1996). It is therefore possible that underlying changes in fuel type may alter energy metabolism in ways not detected by oxygen consumption rates.

TH-mediated increases in gene transcription of SERCA isoforms and SERCA activity enhance skeletal muscle performance in cold-acclimated fish (Little and Seebacher, 2013). However, there are pronounced differences in the effects of TH between skeletal and cardiac muscle. Here, we show that SERCA transcript levels in the heart do not depend on TH. In mammals, TH regulates phospholamban gene expression and thereby SERCA activity (Carr and Kranias, 2002). In contrast, phospholamban transcript levels in our zebrafish were not sensitive to TH. These results indicate that TH influences SERCA activity in zebrafish via post-transcriptional mechanisms, possibly through the allosteric regulation of phospholamban. In the mammalian heart, TH is known to increase the fraction of phosphorylated (non-inhibitory) relative to non-phosphorylated (inhibitory) phospholamban (Carr and Kranias, 2002; Ketzer et al., 2009), in addition to controlling the transcriptional expression of phospholamban. Thus, our working hypothesis here is that TH regulates the phosphorylation state of phospholamban to increase SERCA activity in the absence of changes to overall SERCA and phospholamban mRNA levels.

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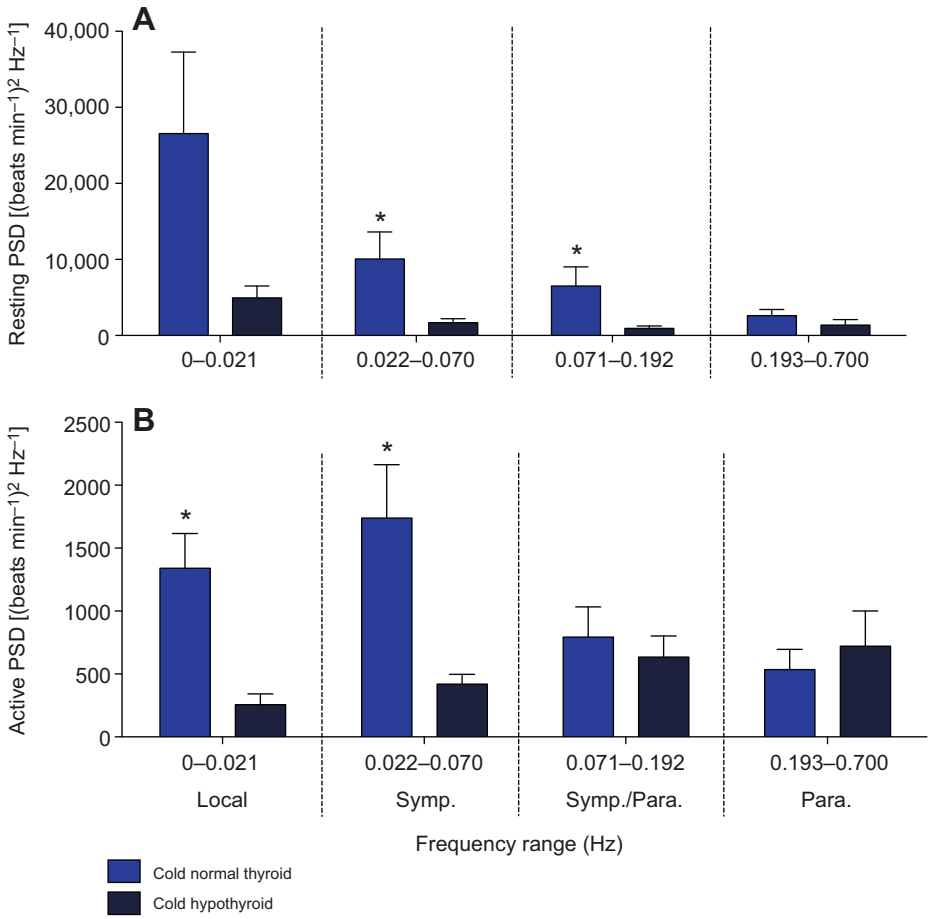

Fig. 5. The effect of hypothyroidism on the autonomic regulation of heart rate. Power spectral density (PSD) for local, sympathetic, sympathetic/parasympathetic and parasympathetic frequency ranges (0–0.02, 0.02–0.07, 0.07–0.19 and 0.19–0.7 Hz, respectively) of heart rate (f_H) of cold-acclimated normal thyroid (blue) and cold-acclimated hypothyroid (navy) fish during (A) rest and (B) maximal activity at 28°C. An asterisk indicates a significant effect of hypothyroidism; *N*=8 (rest), *N*=7 (active).

Unfortunately, the small size of the zebrafish heart (<2 mg) and the lack of a specific antibody make it difficult to determine the ratio of non-phosphorylated to phosphorylated phospholamban experimentally through western blot analysis.

Increases in SERCA activity increase the potential for high maximum heart rate, but that potential can be realized only with simultaneous increases of other regulatory mechanisms such as dihydropyridine and ryanodine receptor densities and the intrinsic function of pacemaker cells (Tiitu and Vornanen, 2003b; Haverinen and Vornanen, 2007). In addition to muscle-specific mechanisms, autonomic tone is a principal regulator of heart rate in vertebrates (Altimiras et al., 1994; Altimiras et al., 1995; Altimiras, 1999). We found that TH enhances the sympathetic tone on the heart both at rest and during exercise. Cold acclimation can enhance the sensitivity to adrenaline in fish (Aho and Vornanen, 2001), which may be mediated by increasing β-adrenergic receptor densities on the heart surface (Keen et al., 1993). Hence, in addition to increased tone on the heart, the heart can also become more sensitive to sympathetic stimulation. Interestingly, these responses are similar in mammals, where TH acts directly on the dorsomedial hypothalamus to control heart rate (Warner and Mittag, 2012), and also increases β-adrenergic receptor densities on the heart surface (Kahaly and Dillmann, 2005). We have shown that TH upregulates sympathetic output during cold acclimation, but it is not known whether its actions are mediated through the hypothalamus or by changes in β-adrenergic receptor densities. One or both of these pathways could explain how TH regulates cardiac performance during cold acclimation. This is interesting because recent work in rats argues that mammalian thermogenesis is primarily regulated through the central actions of TH on the hypothalamus, rather than its peripheral effects on thermogenic tissues (Cannon and Nedergaard, 2010). Our work shows that TH-mediated increases in sympathetic output to maintain cardiac performance during cold acclimation predate the evolution of endothermy; this suggests that the regulatory role of TH in thermogenesis of mammals may be evolutionarily derived from the role of TH in early ectothermic vertebrates.

TH is a central regulator of thermal acclimation in fish, where it simultaneously regulates skeletal muscle function, metabolism and cardiac performance to maintain whole-animal performance in response to temperature variation. It has been suggested that whole-animal performance at thermal extremes is constrained by limitations in oxygen availability and transport, which reduce aerobic scope (Pörtner and Knust, 2007). Evolutionary and plastic changes in heart phenotypes of fish that experience variable thermal environments suggest that cardiac performance is an important component underlying these limitations in oxygen transport (Aho and Vornanen, 1998; Aho and Vornanen, 1999; Shiels et al., 2002; Eliason et al., 2011; Klaiman et al., 2011; Shiels et al., 2011; Korajoki and Vornanen, 2012). The ability to maintain cardiac scope, and thereby aerobic scope, by increasing maximum heart rate (and presumably oxygen transport) during cold acclimation is therefore likely to have important ecological consequences for species that periodically encounter thermal gradients over their lifetimes. For instance, increased maximal heart rate in cold-acclimated zebrafish is paralleled by higher sustained swimming speeds at cold temperatures (Little et al., 2013; Little and Seebacher, 2013). The overall selective advantage of these responses would be

a decoupling of physiology and performance from thermal variability (Claireaux and Lefrançois, 2007). Importantly, TH may play a regulatory role in maintaining aerobic scope by alleviating limitations in oxygen transport. This finding adds a new mechanistic dimension to understanding thermal tolerance, and TH may be a central pathway that mitigates the constraints of whole-animal functions at thermal extremes. Interestingly, several studies have shown individual- and population-level differences in thermal tolerance and acclimation capacity (Meffe et al., 1995; Eliason et al., 2011; Seebacher et al., 2012). An interesting future direction of study will be to determine whether these differences are correlated with individual- or population-level patterns of TH sensitivity.

MATERIALS AND METHODS

Animals and treatments

All experiments were carried out with the approval of the University of Sydney Animal Ethics Committee (approval number L04/8-2012/1/5803). Zebrafish (0.561±61 g, 3.968±0.036 cm) were purchased from commercial suppliers (Livefish, Bundaberg, QLD, Australia). Fish were split into two temperature treatments, a cold acclimation group at 18°C and a warm acclimation group at 28°C, and held at these temperatures (±0.5°C) for 3 weeks. Within acclimation groups, fish were separated into control and hypothyroid treatment groups. Within the cold-acclimated hypothyroid group, fish were further divided into three treatment groups: fish supplemented daily with T_3 (Sigma, Castle Hill, NSW, Australia), T_2 (Sigma) or the ethanol vehicle. There were five replicate tanks per treatment with 12–15 fish per tank at stocking densities of ~1.5 fish l^{-1}. Fish were fed *ad libitum* with fish flakes (Wardley Tropical Fish Flakes, The Hartz Mountain Corporation, Secaucus, NJ, USA) and maintained on a 12 h:12 h light:dark photoperiod. We induced hypothyroidism by maintaining tank water with 0.3 mmol l^{-1} propylthiouracil (PTU; Sigma), which inhibits TH production at the thyroid gland (Goglia, 2005). Hypothyroid groups were also treated with 5 µmol l^{-1} iopanoic acid (Thermo Fisher Scientific, Sydney, NSW, Australia) daily to inhibit deiodinase activity (Goglia, 2005). Gravid females were excluded from the experiments.

Heart rate

For heart rate measurements, fish were placed in circular rubber containers (70 mm diameter, 35 mm height) with a removable stainless steel mesh lid, and submerged in a plastic bin (650×400×220 mm) aerated with a sponge filter. Electrocardiograms (ECGs) were measured with a high gain AC amplifier (BioAmp, AD Instruments, Sydney, NSW, Australia) connected to a 4-channel PowerLab (AD Instruments). The signals were sampled at 1000 Hz by Chart software (AD Instruments), which also calculated heart rate. Electrodes consisted of shielded lead wires (AD Instruments) with ~30 mm of insulation stripped off their distal ends. The bare electrodes were positioned in the water ~20 mm from either side of the fish. With the software running, the electrodes were manipulated in space to optimize the ECG signal. We took resting and maximum heart rate for 7–12 individuals per treatment at both 18 and 28°C test temperatures, with at least 24 h between measurements. For measurements of resting heart rate, ECG was measured in fish that were left undisturbed in darkness for at least 3 h. During the 3 h period, we determined the three intervals of 10–15 beats with the lowest heart rates and averaged them to determine resting heart rate. For measurements of maximum heart rate, we placed fish into a Perspex swimming flume (150 mm length, 26 mm diameter) tightly fitted to an inline submersible pump (12 V, iL500, Rule, Miami, FL, USA). A variable DC power source (MP3090, Powertech, Osborne Park, WA, Australia) was used to adjust the flow speed through the flume, and bundles of hollow straws were positioned at each end of the flume to separate it from the pump and promote laminar flow. Fish were swum in the flume at maximum capacity, which was defined as when the fish visibly struggled to maintain its position in the water column, for a period of 5 min. The fish were then removed from the swim flume and placed immediately into the ECG chambers described above. Work in trout showed that during recovery from critical swim speeds at 15°C, heart rate remained near maximal for more than 10 min (Priede, 1974). Thus, heart rate was recorded immediately over a period of 5 min and the maximum heart rate was calculated by averaging three periods of 10–15 beats with the highest heart rates.

Stroke volume

Stroke volume is the volume of blood that the heart pumps in a single heartbeat. We measured stroke volume to determine whether cardiac output changes as a result of changes in heart rate, stroke volume or both. Understanding these relationships is necessary to interpret the functional relevance of changes in heart rate. We used acute temperature change as a mechanism to alter heart rate and oxygen demand. Relative stroke volume was measured in anaesthetized cold-acclimated euthyroid fish at 18 and 28°C with a laser Doppler blood flow meter (ML191, AD Instruments) connected to a 4-channel PowerLab (AD Instruments). The fish were anaesthetized in 30 ppm iso-eugenol (AQUI-S; AQUI-S New Zealand Ltd, Lower Hutt, New Zealand). We used AQUI-S rather than tricaine melthanesulphonate (MS222) because it does not interfere with vagal nerve transmission (Hill et al., 2002) or elevate resting cortisol levels (Iversen et al., 2003), both of which could affect stroke volume. Fish were rested ventral side up along a shallow groove cut into a rectangular foam platform (30×60 mm). Anaesthesia was maintained by pumping 17 ppm AQUI-S solution (at 18°C) continuously through the oral cavity of the fish and across its gills with a peristaltic pump. An OxyFlo Needle Probe (MNP110XP, Oxford Optronix Ltd, Milton, Abingdon, UK) was positioned with a stereotaxic apparatus on the ventral surface of the fish, just anterior to the heart. Once a clear signal of flow was established, neither the fish nor the probe was disturbed until measurements at both temperatures were complete. The signals were sampled at 1000 Hz by Chart software (AD Instruments). After measurements were completed at 18°C, the fish was heated to 28°C by changing to an anaesthetic solution kept at 28°C. A thermocouple was positioned on the skin directly lateral to the heart to measure temperature in real time. Heartbeats are easily identified on the blood flow curve by cyclic increases in flow as a result of contraction. Heart rate could therefore be measured simultaneously using the Chart software (AD Instruments). We calculated stroke volume as the integral of flow during a single heartbeat. Variation in the position of the probe on each individual alters the blood flow reading. We therefore normalized the integral of flow during a heartbeat to the baseline immediately preceding the heartbeat to give a measure of relative stroke volume. We measured stroke volume for eight individuals from both cold acclimation treatments (hypothyroid and normal thyroid). For each individual at each temperature, we averaged 50 independent integrals (heartbeats) to calculate the average relative stroke volume.

Spectral analysis

We analysed intervals between heartbeats by spectral analysis (Altimiras et al., 1994; Altimiras et al., 1995; Altimiras, 1999) to assess whether hypothyroidism alters autonomic control of heart rate in cold-acclimated zebrafish. By transforming heart rate data into a frequency domain, periodic processes can be analysed by peaks in spectral density [(beats $min^{-1})^2$ Hz^{-1}] at their respective frequencies. Cholinergic (parasympathetic) mechanisms act at a higher frequency than adrenergic (sympathetic) mechanisms as a result of neurotransmitter characteristics and postsynaptic transmission (Altimiras et al., 1994). Hence, variation at lower frequencies in the power spectrum can be attributed to sympathetic responses, whereas high frequency peaks result from parasympathetic responses; peaks in the mid-frequency range may be either sympathetic or parasympathetic, and peaks at very low frequencies relate to changes in vasomotor tone brought about by local or blood-borne mediators such as angiotensin, for example (Akselrod et al., 1981). Based on previous work on autonomic control of heart rate in ectothermic vertebrates (Seebacher and Franklin, 2004), spectra were divided into four components: ultra low (0.000–0.021 Hz), very low (0.022–0.070 Hz), low (0.071–0.192 Hz) and high (0.193–0.700 Hz), relating to local regulation, sympathetic regulation, sympathetic and/or parasympathetic regulation and parasympathetic regulation, respectively. Spectral analyses were conducted in Chart software (AD Instruments) on resting and maximum heart rate data for cold control and

cold hypothyroid fish. For the analyses, we selected 3–5 min of uninterrupted ECG data for resting heart rates and 30 s of ECG data for maximum heart rates to transform into a power spectrum. Spectral analyses for maximum heart rates were performed on ECGs recorded at 18°C. However, spectral analyses for resting heart rates were performed on ECGs recorded at 28°C because the signal at 18°C was too weak to get 3–5 min of uninterrupted data. We used a fast Fourier transform of 32k, and implemented a Cosine–Bell data window with 50% overlap. The average power spectrum density was calculated at the ultra low, very low, low and high frequency ranges for seven individuals from each cold acclimation treatment.

Heart metabolic rate

Cold-acclimated euthyroid and cold-acclimated hypothyroid zebrafish were killed by immersion in a buffered MS222 solution (0.4 g MS222 l^{-1} + 0.8 g Na_2HCO_3 l^{-1}) and intact hearts were immediately dissected, weighed and placed in fish Ringer's solution (115 mmol l^{-1} NaCl, 5.6 mmol l^{-1} $NaHCO_3$, 2.7 mmol l^{-1} KCl, 8.4 mmol l^{-1} sodium pyruvate, 1.2 mmol l^{-1} $MgCl_2$, 0.64 mmol l^{-1} $NaHPO_4$, 0.97 mmol l^{-1} Hepes, 3.2 mmol l^{-1} Hepes sodium salt, 2.1 mmol l^{-1} $CaCl_2$, pH 7.0) in separate wells of an XF24 islet capture plate (part no. 101122-100; Seahorse Bioscience, North Billerica, MA, USA). Islet plates are proprietary 24-well plates designed to keep non-adhesive cells and tissue cells in the bottom chamber of the well, while permitting the free exchange of media and the diffusion of compounds. These plates are suitable for other non-adherent cell types and small intact tissues, and have even been used to assay whole zebrafish embryos (Stackley et al., 2011). Here, hearts for cold-acclimated control fish (heart mass 1.19±0.095 mg) and cold-acclimated hypothyroid fish (heart mass 1.34±0.102 mg) were confined to the bottom of the well with the islet capture screens, and Ringer's solution was refreshed immediately before analysis of oxygen consumption. Oxygen consumption rate was measured at 18°C with a Seahorse XF24 analyzer (part no. 101122-100, Seahorse Bioscience), which measures dissolved oxygen with solid-state sensor probes, according to the manufacturer's instructions. The assay protocol programmed into the Seahorse Analyzer consisted of repeated cycles (loops) of fluid mixing in the wells, followed by a delay period and a measurement period. The chamber is sealed only during the measurement period to allow measurement of decreasing oxygen concentration resulting from tissue respiration. We ran pilot experiments to optimize the program parameters to ensure that the media was adequately mixed and the chamber did not become hypoxic. In each experimental run, we measured four loops to ensure that there was a stable baseline; each loop consisted of a 3 min mixing period, a 2 min delay and a 2 min measurement period.

After the first four loops, 5 µmol l^{-1} of FCCP was injected and oxygen consumption was measured for a further four loops. FCCP perforates the mitochondrial membrane and thereby elicits maximal substrate oxidation rates. We used the values measured in the last two loops of normal and FCCP-excited cardiac respiration for analysis. Oxygen consumption rates were measured in hearts from 10 fish from each treatment, and normalized to heart mass.

SERCA assay

Zebrafish were killed as above, and hearts for enzymes assays were extracted and transferred immediately to liquid nitrogen and stored at –80°C for later analysis. We measured the maximal activity of SERCA at 18 and 28°C to assess how acclimation temperature and thyroid status affect maximal rates of ATP hydrolysis. Maximal SERCA activity was determined in eight zebrafish per treatment according to published protocols (James et al., 2011). In brief, hearts were homogenized in homogenization buffer (250 mmol l^{-1} sucrose, 5 mmol l^{-1} EDTA, 20 mmol l^{-1} imidazole, pH 7.2). The homogenate was then incubated in assay buffer (25 mmol l^{-1} imidazole, 0.2 mmol l^{-1} $CaCl_2$, 80 mmol l^{-1} KCl, 5 mmol l^{-1} $MgCl_2$) in the presence and absence of 10 µmol l^{-1} thapsigargin, which is a specific inhibitor of SERCA. The reaction was started by adding 30 mmol l^{-1} ATP to the assay, and stopped by adding an equal volume of 0.8 mol l^{-1} percholoric acid. The relative increase in inorganic phosphate was determined using a Molybdenum Blue colorimetric assay against a standard curve. SERCA activity was expressed as the difference in inorganic phosphate liberated in the presence and absence of thapsigargin.

mRNA concentrations

Zebrafish were killed as above, and hearts (N=5–6 per treatment) for quantitative real-time PCR (qPCR) analysis were extracted and stored in RNAlater (Ambion, Austin, TX, USA) at −20°C. RNA was extracted from samples using TRIreagent (Molecular Research Centre, Cincinnati, OH, USA), following the manufacturer's instructions. RNA concentration and quality were verified using a NanoDrop (Thermo Fisher Scientific). A 0.7–1.0 µg aliquot of total RNA from each sample was treated with DNase I (Sigma) and reverse-transcribed using RNase HMMLV reverse transcriptase (Bioscript; Bioline, Sydney, Australia) and random hexamer primers (Bioline).

qPCR was performed on an Applied Biosystems 7500 qRT-PCR machine (Applied Biosystems) according to published protocols (Seebacher and Walter, 2012). In short, primers for SERCA1, SERCA2a, SERCA2b and phospholamban, were adopted from published work (McCurley and Callard, 2008; Little and Seebacher, 2013) or designed according to their respective GenBank sequences. Real-time PCR reactions contained 1×SensiMix SYBR (Bioline), 4.5 mmol l^{-1} $MgCl_2$, 50–900 nmol l^{-1} primer and ~100 ng cDNA. The cycle consisted of 95°C for 7 min, 40 cycles of 95°C for 20 s, 58°C for 1 min. Dissociation curve analysis was performed after the amplification step to verify the presence of only a single PCR product. We measured mRNA expression levels of the target genes in hearts from 5–6 individuals from each treatment group, and normalized values to elongation factor 1-alpha (EF1α) expression (McCurley and Callard, 2008). mRNA levels were expressed relative to the warm-acclimated control treatment for the warm/cold hypothyroid experiment.

Statistical analysis and data presentation

Data are presented as means ± s.e.m. Datasets were analysed by permutational analysis of variance (PERMANOVA; Primer 6 PRIMER-E Ltd, Plymouth, UK). We were particularly interested in whether the action of TH is temperature specific, and we therefore included all interactions between acclimation and test temperatures and thyroid treatment in the statistical models. We used three-way PERMANOVA to analyse datasets with acclimation temperature, hypothyroid treatment and test temperature as factors. We used two-way PERMANOVA to analyse mRNA levels in warm- and cold-acclimated hypothyroid and euthyroid fish. We used a one-way PERMANOVA to compare control, hypothyroid, and T_2- and T_3-supplemented cold-acclimated fish. Following PERMANOVA, means were compared with Monte Carlo planned pairwise comparisons in PERMANOVA software. In fish from the supplementation treatments, we analysed only those responses that changed with the hypothyroid treatment (i.e. resting and maximal heart rate and SERCA activity). We chose a permutational analysis in preference to frequentist statistical tests because it uses the data per se for statistical inferences rather than making assumptions about underlying frequency distributions of the data. This approach is preferable for relatively small datasets, particularly when comparing physiological treatments (Drummond and Vowler, 2012).

Permutational analyses test the null hypothesis that the data values are randomly distributed across all treatments, which would be the case if there were no treatment effects. The statistical results therefore reflect the actual populations in the experiments and do not have recourse to an assumed known distribution of values as in frequentist probability tests (Drummond and Vowler, 2012).

Means of heart rate and relative stroke volume in cold-acclimated fish were compared between 18 and 28°C test temperatures using a paired one-tailed t-test and a paired two-tailed t-test, respectively. We used a one-tailed t-test for heart rate because heart rate is known to increase with increasing body temperature, whereas the relationship between stroke volume and temperature is less resolved. We used independent two-tailed t-tests to compare means between the cold-acclimated euthyroid and hypothyroid fish for heart oxygen consumption rates, and spectral densities at the ultra low, very low, low and high frequency ranges.

Competing interests

The authors declare no competing financial interests.

Author contributions

A.G.L. and F.S. conceived and designed the experiments, A.G.L. conducted the experiments, and A.G.L. and F.S. wrote the manuscript.

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Supplementary material

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