Thyroid Hormone Regulates Cardiac Performance during Cold Acclimation in Zebrafish (Danio rerio)

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SUMMARY

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-diiodothyronine (T\(_2\)) or 3,5,3\(^{\prime}\)-triiodothyronine (T\(_3\)). Supplementation of hypothyroid fish with T\(_2\) or T\(_3\) restored heart rates 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.
INTRODUCTION

The thermal sensitivity of aerobic scope is important in determining vulnerability of organisms to environmental change (Pörtner and Knust 2007; Nilsson et al. 2009; Eliason et al. 2010). At suboptimal temperatures, limitations in oxygen transport reduce the capacity for aerobic metabolism, thereby reducing the energy (adenosine triphosphate, 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 Lefrancois 2007). Hence, remodeling 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 2012a; Korajoki and Vornanen 2012b). 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 activities 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 rates 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 test the hypothesis that TH
regulates cardiac physiology during thermal acclimation in zebrafish (*Danio rerio*).

TH-mediated control of cardiac physiology could explain compensatory changes
in swimming performance during cold 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 (T2) or 3,5,3’-triiodothyronine (T3), would restore heart
rate; ii) that 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 T2 and T3 (+ controls) in zebrafish exposed to different chronic
and acute temperature combinations. We measured resting and maximum heart rates and
stroke volume, metabolic rate of the heart, and SERCA activity and its mRNA transcript
levels to determine whether TH plays a role in cardiac remodeling 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 rates were 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 rates (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 (Fig. S1 and S2).

SERCA activity decreased with hypothyroidism in the cold acclimation treatments, but increased with hypothyroidism in the warm acclimation treatments (acclimation x 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 compared to warm acclimated fish (Fig. 1D; Table 2).

The effects of T2 and T3 supplementation on responses sensitive to hypothyroidism

There were significant differences between cold-acclimated control, hypothyroid, and T2 and T3 supplemented hypothyroid treated fish in resting heart rate (F3,40 = 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 rates (i.e. no significant difference between control and supplemented fish) of hypothyroid fish to control levels (Fig. 2A). Maximal heart rates were 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 (Figs 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 (Figure 3A; $t = 3.038$, df = 14, $p = 0.009$). However, relative stroke volume did not change with the increase in temperature from 18°C to 28°C (Figure 3B; $t = 0.575$, df = 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 FCCP-induced maximal oxygen consumption rates of whole isolated hearts (Fig. 4; resting: $t$-test, $t = 0.055$, df = 18, $p = 0.957$; maximum: $t$-test, $t = 0.070$, df = 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 in the sympathetic and sympathetic/parasympathetic frequency ranges in hypothyroid fish compared to controls (Fig. 5A; sympathetic: $t = 2.343$, df = 14, $p = 0.034$;
sympathetic/parasympathetic: $t = 2.219$, df = 14, $p = 0.044$). However, hypothyroidism did not affect power spectral density within the local or parasympathetic frequency ranges (local: $t = 1.990$, df = 14, $p = 0.066$; parasympathetic: $t = 1.147$, df = 14, $p = 0.270$).

Power spectral densities of maximal heart rates were significantly lower in hypothyroid fish at the local and sympathetic frequency ranges ($t = 3.74$, df = 12, $p = 0.003$ and $t = 3.08$, df = 12, $p = 0.02$, respectively). However, there were no differences between treatment groups in the sympathetic/parasympathetic and parasympathetic frequency ranges ($t = 0.55$, df = 12, $p = 0.59$ and $t = -0.58$, df = 12, $p = 0.57$, respectively; Fig. 5B).

**DISCUSSION**

We found that TH regulates cardiac performance in fish. As predicted, TH increased heart rates 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 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 anesthesia. Unfortunately zebrafish are too small
measure 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 rates is likely to lead to reduced cardiac and aerobic scopes (Eliason et al. 2011). Hypothyroidism affected both resting and maximal heart rates, but not in proportion to each other. In cold acclimated fish, TH reduced both resting and maximal heart rates, 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 et al., 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 T₃ or T₂ 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, and thereby maintaining aerobic scope at low temperatures. Resting heart rates decreased with hypothyroidism, but it is unlikely that SERCA maximal activities are functionally related to resting heart rates 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 AMPK (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 (Dreidzic and Gesser 1994; Moyes 1996). It is therefore possible that underlying changes in fuel types 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 posttranscriptional 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. 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 rates, 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; 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 et al., 2010). Our work shows that TH-mediated increases in sympathetic output to maintain cardiac performance during cold acclimation predates 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 (Portner 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 rates (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 rates in cold acclimated zebrafish are 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 TH sensitivity.

**METHODS AND TECHNIQUES**

**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 ± 0.061 g; 3.968 cm ± 0.036 cm) were purchased from commercial suppliers (Australia; 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 three 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 T₃ (Sigma, Australia), T₂ (Sigma, Australia), or the ethanol vehicle. There were 5 replicate tanks per treatment with 12-15 fish per tank at stocking densities of approximately 1.5 fish l⁻¹. Fish were fed ad libitum with fish flakes (Wardley Tropical Fish Flakes, FL, USA) and maintained in a 12:12 hour light:dark photoperiod. We induced hypothyroidism by maintaining tank water at 0.3 mM propylthiouracil (PTU; Sigma, Castle Hill, Australia), which inhibits TH production at the thyroid gland (Goglia 2005). Hypothyroid groups were also treated with 5µM iopanoic acid (Thermofisher Scientific, Sydney, 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 mm x 400 mm x 220 mm) aerated with a sponge filter. Electrocardiograms (ECGs) were measured with a high gain AC amplifier (BioAmp, AD Instruments, Sydney, Australia) connected to a 4-channel PowerLab (AD Instruments, Australia). The signals were sampled at 1000 hz by Chart software (AD Instruments, Australia), which also calculated heart rates. Electrodes consisted of shielded lead wires (AD Instruments, Australia) with approximately 30 mm of insulation stripped off their distal ends. The bare electrodes were positioned in the water approximately 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 rates for 7-12 individuals per treatment at both 18˚C and 28˚C test temperatures, with at least 24 hours between measures. For measurements of resting heart rates, ECG was measured in fish that were left undisturbed in darkness for at least three hours. During the three-hour 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 rates, we placed fish into a Persepex swimming flume (150 mm length with 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 swam 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 minutes. 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 rates remained near maximal for more than 10 minutes (Priede 1974). Thus heart rate was recorded immediately over a period of 5 minutes 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°C and 28°C with a laser Doppler blood flow meter (ML191, AD Instruments, Australia) connected to a 4-channel PowerLab (AD Instruments, Australia). The fish were anaesthetized in 30 ppm iso-eugenol (AQUI-S; AQUI-S New Zealand Ltd., New Zealand). We used AQUI-S rather than tricaine (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 mm x 60 mm). Anesthesia 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., United Kingdom) 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, Australia). After measurements were completed at 18°C, the fish was heated to 28°C by changing to an anesthetic 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, Australia). 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 heart beat to the baseline immediately preceding the
heartbeat to give a measure of relative stroke volume. We measured stroke volume for 8
individuals from both cold acclimation treatments (hypothyroid and normal thyroid). For
each individual at each temperature, we averaged 50 independent integrals (heart beats)
to calculate the average relative stroke volume.

**Spectral analysis**

We analysed intervals between heartbeats by spectral analysis (Altimiras et al.
1994; 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
(BPM$^2$/hz) at their respective frequencies. Cholinergic (parasympathetic) mechanisms act
at a higher frequency than adrenergic (sympathetic) mechanisms as a result of
neurotransmitter characteristics and post-synaptic 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, Australia) on resting and maximum heart rate data for cold control and cold hypothyroid. For the analyses, we selected 3-5 minutes of uninterrupted ECG data for resting heart rates and 30 seconds 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 minutes 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 7 individuals from each cold acclimation treatment.

**Heart metabolic rate**

Cold acclimated euthyroid and cold acclimated hypothyroid zebrafish were euthanized by immersion in a buffered MS222 (tricaine methane sulphonate) solution (0.4 g MS222/l + 0.8 g Na₂HCO₃/l) and intact hearts were immediately dissected, weighed and placed in fish Ringer’s solution (115 mM NaCl, 5.6 mM NaHCO₃, 2.7 mM...
KCl, 8.4 mM Na pyruvate, 1.2 mM MgCl₂, 0.64 mM NaHPO₄, 0.97 mM HEPES, 3.2 mM HEPES Na salt, 2.1 mM CaCl₂, pH 7.0) in separate wells of an XF24 islet capture plate (part #101122-100; Seahorse Bioscience, MA, USA). Islet plates are proprietary well plates designed to keep non-adhesive cells and tissues cells in the bottom chamber of the well, while permitting free exchange of media and the diffusion of compounds. These plates are suitable for other non-adherent cell types, 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 mg ± 0.095) and cold acclimated hypothyroid fish (heart mass = 1.34 mg ± 0.102) 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 #101122-100, Seahorse Bioscience, MA, USA), 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 a 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 three minute mixing period, a two minute delay, and a two minute measurement period. After the first four loops, 5 µM of carbonyl-cyanid p-[trifluoromethoxy]-phenyl-hydrazone (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 euthanized 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°C 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 mM sucrose, 5 mM EDTA, 20 mM imidazole, pH 7.2). The homogenate was then incubated in assay buffer (25 mM imidazole, 0.2 mM CaCl₂, 80 mM KCl, 5 mM MgCl₂) in the presence and absence of 10 µM thapsigargin, which is a specific inhibitor of SERCA. The reaction was started by adding 30 mM ATP to the assay, and stopped by adding an equal volume of 0.8N 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 euthanized as above, and hearts (n = 5-6 per treatment) for 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, Sydney, Australia). A 0.7-1.0 µg aliquot of total RNA from each sample was treated with DNase I (Sigma, Castle Hill, Australia) and reverse-transcribed using RNAse HMMLV reverse transcriptase (Bioscript; Bioline, Sydney, Australia) and random hexamer primers (Bioline).

Quantitative RT-PCR was performed on an Applied Biosystems 7500 qRT-PCR machine (Applied Biosystems, Sydney, Australia) 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, Australia), 4.5 mM MgCl₂, 50–900 nM 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 target genes to elongation factor 1-alpha (EF1α) (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 analyzed 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 permutational ANOVAs to analyze datasets with acclimation temperature, hypothyroid treatment, and test temperature as factors. We used two-way permutational ANOVAs to analyze mRNA levels in warm and cold acclimated, hypothyroid and euthyroid fish. We used a one-way permutational ANOVAs to compare control, hypothyroid, and T2, and T3 supplemented cold acclimated fish. Following permutational ANOVAs, 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 rates, 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 does 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°C 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 temperatures, 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.

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.

COMPETING INTERESTS

No competing interests declared.

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FIGURE CAPTIONS

Figure 1 The effects of thermal acclimation and hypothyroidism on resting heart rate (A), maximum heart rate (B), SERCA activity (C) and relative mRNA transcript levels for SERCA2a, SERCA2b and phospholamban (PLB; D) 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 labeled in enclosed boxes. AT, acclimation treatment; H, hypothyroid treatment; TT, test temperature; n = 7-12, except for mRNA where n = 5-6 per treatment group.

Figure 2 The effects of supplementation with T2 and T3 on TH-sensitive responses in cold-acclimated fish. Resting heart rate (A), maximum heart rate (B) and SERCA activity are shown for cold-acclimated normal thyroid (blue), cold-acclimated hypothyroid (navy), and cold acclimated hypothyroid fish supplemented with either T2 (teal) or T3 (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.
Figure 3 The relative roles of heart rate and stroke volume during increased cardiac demand. Heart rate (A) and relative stroke volume (B) measured at 18°C and 28°C in anesthetized 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.

Figure 4 The effect of hypothyroidism on resting and maximal metabolic rates in isolated hearts. Resting and maximal oxygen consumption of hearts isolated from cold acclimated normal thyroid (blue) and cold acclimated hypothyroid fish (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.

Figure 5 The effect of hypothyroidism on the autonomic regulation of heart rate. Power spectral densities for local, sympathetic, sympathetic/parasympathetic, and parasympathetic frequency ranges (0-0.02hz, 0.02-0.07hz, 0.07-0.19hz and 0.19-0.7hz, respectively) of heart rates of cold acclimated normal thyroid (blue) and cold acclimated hypothyroid (navy) fish during rest (A) and maximal activity (B) at 28°C. An asterisk indicates a significant effect of hypothyroidism; n = 8 (rest), n=7 (active).
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 (Resting HR), maximum heart rate (Maximal HR) and SERCA activity.

<table>
<thead>
<tr>
<th></th>
<th>Resting HR</th>
<th></th>
<th>Maximum HR</th>
<th></th>
<th>SERCA activity</th>
<th></th>
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</thead>
<tbody>
<tr>
<td></td>
<td>F&lt;sub&gt;1,79&lt;/sub&gt;</td>
<td>p</td>
<td>F&lt;sub&gt;1,78&lt;/sub&gt;</td>
<td>p</td>
<td>F&lt;sub&gt;1,62&lt;/sub&gt;</td>
<td>p</td>
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<tr>
<td>AT</td>
<td>1.51</td>
<td>0.22</td>
<td>4.40</td>
<td>0.039</td>
<td>1.80</td>
<td>0.15</td>
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<tr>
<td>H</td>
<td>17.69</td>
<td>&lt;0.001</td>
<td>6.86</td>
<td>0.012</td>
<td>1.75</td>
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<tr>
<td>TT</td>
<td>37.67</td>
<td>&lt;0.001</td>
<td>311.59</td>
<td>&lt;0.001</td>
<td>3.11</td>
<td>0.048</td>
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<tr>
<td>ATxH</td>
<td>1.47</td>
<td>0.23</td>
<td>8.40</td>
<td>0.005</td>
<td>8.73</td>
<td>0.002</td>
</tr>
<tr>
<td>ATxTT</td>
<td>0.26</td>
<td>0.66</td>
<td>0.66</td>
<td>0.41</td>
<td>0.00</td>
<td>0.99</td>
</tr>
<tr>
<td>HxTT</td>
<td>5.50</td>
<td>0.020</td>
<td>0.47</td>
<td>0.50</td>
<td>0.48</td>
<td>0.62</td>
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<tr>
<td>ATxHxTT</td>
<td>2.03</td>
<td>0.16</td>
<td>4.20</td>
<td>0.042</td>
<td>0.64</td>
<td>0.54</td>
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</table>
Table 2 Results of a two-way PERMANOVA testing for the effects of acclimation temperature (AT) and hypothyroid treatment (H) on mRNA concentrations of the sarcoplasmic reticulum Ca\(^{2+}\)-ATPase isoforms 2a and 2b (SERCA2a and SERCA2b, respectively) and phospholamban (PLB) in the heart of zebrafish.

<table>
<thead>
<tr>
<th></th>
<th>SERCA2a</th>
<th></th>
<th>SERCA2b</th>
<th></th>
<th>PLB</th>
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<tr>
<td></td>
<td>F(_{1,20})</td>
<td>p</td>
<td>F(_{1,20})</td>
<td>p</td>
<td>F(_{1,20})</td>
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<tr>
<td>ACC</td>
<td>0.19</td>
<td>0.80</td>
<td>1.87</td>
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<td>TH</td>
<td>0.36</td>
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<tr>
<td>ACCxTH</td>
<td>0.36</td>
<td>0.62</td>
<td>0.25</td>
<td>0.83</td>
<td>0.26</td>
</tr>
</tbody>
</table>
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A

Heart Rate (BPM)

18°C 28°C

Cold Normal Thyroid

B

Relative Stroke Volume

18°C 28°C

*
The figure shows the comparison of oxygen consumption in nmol gram tissue $^{-1}$ min $^{-1}$ between Cold Normal Thyroid and Cold Hypothyroid conditions at resting and maximum levels. At resting, Cold Normal Thyroid has a lower oxygen consumption compared to Cold Hypothyroid. At maximum levels, both conditions show a higher oxygen consumption, with Cold Normal Thyroid slightly lower than Cold Hypothyroid.