Am J Physiol Regul Integr Comp Physiol 305: R1010–R1020, 2013.
First published September 4, 2013; doi:10.1152/ajpregu.00254.2013.

Effects of temperature acclimation on Pacific bluefin tuna (*Thunnus orientalis*) cardiac transcriptome

Nishad Jayasundara, Luke D. Gardner, and Barbara A. Block

Stanford University, Hopkins Marine Station, Pacific Grove, California

Submitted 21 May 2013; accepted in final form 1 September 2013

Jayasundara N, Gardner LD, Block BA. Effects of temperature acclimation on Pacific bluefin tuna (*Thunnus orientalis*) cardiac transcriptome. *Am J Physiol Regul Integr Comp Physiol* 305: R1010–R1020, 2013. First published September 4, 2013; doi:10.1152/ajpregu.00254.2013.— Little is known about the mechanisms underpinning thermal plasticity of vertebrate hearts. Bluefin tuna hearts offer a unique model to investigate processes underlying thermal acclimation. Their hearts, while supporting an endothermic physiology, operate at ambient temperature, and are presented with a thermal challenge when migrating to different thermal regimes. Here, we examined the molecular responses in atrial and ventricular tissues of Pacific bluefin tuna acclimated to 14°C, 20°C, and 25°C. Quantitative PCR studies showed an increase in sarcoplasmic reticulum Ca^{2+} ATPase gene expression with cold acclimation and an induction of Na^{+}/Ca^{2+}-exchanger gene at both cold and warm temperatures. These data provide evidence for thermal plasticity of excitation-contraction coupling gene expression in bluefin tunas and indicate an increased capacity for internal Ca^{2+} storage in cardiac myocytes at 14°C. Transcriptomic analysis showed profound changes in cardiac tissues with acclimation. A principal component analysis revealed that temperature effect was greatest on gene expression in warm-acclimated atrium. Overall data showed an increase in cardiac energy metabolism at 14°C, potentially compensating for cold temperature to optimize bluefin tuna performance in colder oceans. In contrast, metabolic enzyme activity and gene expression data suggest a decrease in ATP production at 25°C. Expression of genes involved in protein turnover and molecular chaperones was also decreased at 25°C. Expression of genes involved in oxidative stress response and programmed cell death suggest an increase in oxidative damage and apoptosis at 25°C, particularly in the atrium. These findings provide insights into molecular processes that may characterize cardiac phenotypes at upper thermal limits of teleosts.

bluefin tuna; cardiac function; excitation contraction coupling; temperature acclimation; transcriptomics

BLUEFIN TUNAS (FAMILY SCOMBRIDAE) are migratory apex predators. They are renowned for their endothermic physiology, supported by counter-current heat exchangers and elevated metabolic rates (8). Juvenile Pacific bluefin tunas (PBFT), *Thunnus orientalis,* sustain minimal metabolic rates of 220–235 $mg \cdot kg^{-1} \cdot h^{-1}$ at 20°C, a rate ~1.3-fold higher than that of yellowfin tunas at the same temperature (5), and about 1.5–4-fold higher than routine metabolic rates of salmonids (26). This high aerobic demand of bluefin tunas (BFT) is sustained by an enhanced cardiac physiology that maintains performance across large thermal gradients experienced by these fish (8). However, in contrast to their skeletal muscle, visceral organs, eyes, and brain that are buffered from the environmental temperature variations by conserving heat, BFT hearts operate at ambient temperature. They lack counter-current heat exchangers, and ventricles receive blood through coronary circulation directly from the gills. Therefore, BFT hearts have to support an endothermic physiology and maintain elevated cardiac performance at ambient temperature; hence, cardiac limitations likely define thermal boundaries for these organisms (6, 8, 19, 22, 39, 57).

PBFT predominantly migrate across sea surface temperatures ranging from 10°C to 25°C and dive to waters as cold as 5–6°C in search of prey (7). As juveniles, they migrate from the warm waters of western Pacific into the cold California current off North America's western shores, and return to the Pacific Ocean to spawn in waters near Japan. Blank et al. (6) showed that PBFT have the capacity to retain cardiac function across a large temperature range (2°C–25°C) compared with tropical tunas, but also indicated that cardiovascular performance in PBFT is strongly temperature dependent, leading to cardiac bradycardia at cold temperatures. Biochemical studies demonstrated that Ca^{2+} stored in sarcoplasmic reticulum (SR) significantly contributes to maintaining excitation-contraction (EC) coupling in PBFT compared with other tunas and is thought to be crucial for sustaining cardiac performance in the cold for this species (22, 39, 57).

Under chronic temperature changes, vertebrate hearts, including teleosts, are shown to modulate their transcriptome and proteome to restore cellular homeostasis and adjust their cardiac phenotype to shift to a new thermal optimum (2, 10, 23, 57, 61). Although PBFT inhabit a wide thermal range, metabolic rate studies indicated that metabolic thermal-minimum zone of juvenile PBFT is around 16°C–18°C (5). Therefore, during seasonal migrations, PBFT might alter their phenotype (acclimatization) to compensate for temperature effects and optimize performance at different temperatures (27). Previous studies demonstrated that PBFT hearts increase the intracellular Ca^{2+} cycling through the SR during cold acclimation (57) and modify the role of K^{+} channels (21) with temperature acclimation. Castilho et al. (10) showed changes in gene expression patterns in PBFT ventricle at 15°C and 20°C, based on a heterologous hybridization with a *Gillichthys mirabilis*-specific microarray. However, this study did not examine PBFT at warmer acclimation temperatures. The first study to determine cardiac molecular responses to temperature acclimation utilizing a microarray approach in an active teleost—rainbow trout—examined acclimation effects at 4°C and 18°C (61). This study indicated changes in expression of genes involved in a number of biological pathways, including glycolysis, protein biosynthesis, and cytoskeletal structure, which might underlie cardiac hypertrophy detected in cold-acclimated (4°C) trout ventricle. Although several studies have provided insights into teleost transcriptomic responses to temperature (10, 42, 52, 61), very little is known about gene expression

Address for reprint requests and other correspondence: N. Jayasundara, Nicholas School of the Environment, Duke University, Box 90328, Durham, NC (e-mail: nishad.jayasundara@duke.edu).

changes in different cardiac tissue types (atrial, compact, and spongy tissues), at temperatures near the upper thermal limit of active pelagic fishes.

To further investigate the thermal plasticity of PBFT heart, in this study, we investigated gene expression and metabolic enzyme activity changes in cardiac tissues in fish acclimated to 14°C, 20°C, and 25°C. To examine the gene level regulation of EC coupling proteins that results in cardiac phenotype described by Shiels et al. (57), we quantified the gene expressions of sarcoplasmic reticulum Ca^{2+} ATPase (SERCA), ryanodine receptor (RyR), and Na^{+}/Ca^{2+}-exchanger (NCX) using gene-specific primers. Large-scale transcriptomic studies provide a powerful tool to examine species' responses to environmental stress. Therefore, we utilized a PBFT-specific oligonucleotide microarray (24) to investigate molecular responses to cellular perturbations caused by long-term temperature changes in PBFT. To determine effects of acclimation on PBFT cardiac energy metabolism, we coupled our metabolic gene expression data with enzyme assay studies. We focused our studies on atrial (AT), ventricular compact (VC), and ventricular spongy (VS) tissues of the tuna heart, considering the anatomical differences and temperature and oxygen variations experienced by these tissues (8).

This study is the first to investigate broad-scale molecular mechanisms underpinning thermal limits of a large pelagic fish. Understanding these mechanisms in bluefin tunas has important ecological and economic implications and can be used as a tool to predict shifts in thermal niche of these species in response to global ocean temperature changes.

MATERIALS AND METHODS

Fish Origin, Acclimation, and Tissue Collection

PBFT were captured in waters off of Baja, Mexico, by hook and line and transported to the Tuna Research and Conservation Center at Hopkins Marine Station of Stanford University. They were held at 20°C under a 12:12-h light-dark photoperiod, as previously described (8). Upon 3 mo of acclimation to captive conditions, a total of 10 fish (mean mass: 11.5 ± 0.1 kg, mean fork length: 81.7 cm) were transferred to two identical tanks, while five fish remained at 20°C. Fish in each tank were held at either 14°C ($n = 5$) or 25°C ($n = 5$) for 4 wk. These three acclimation temperatures were decided on the basis of previous assessments of the optimal lower and upper temperatures for maintaining PBFT in captivity. The 4 wk time period was determined on the basis of the number of previous teleost acclimation studies, including studies in our laboratory (42, 52, 57, 61). Throughout the acclimation period, we did not detect any changes in overall activity level or feeding behavior in these specimens. Fish were killed by pithing; then atrial, ventricular compact, and spongy tissues were isolated from each fish. Part of the tissues were stored in RNAlater (Applied Biosystems, Carlsbad, CA) overnight at 4°C and then transferred to −20°C for RNA extraction. Separate sets of tissues from each sample were freeze-clamped and stored in −80°C for enzyme activity measurements. The animal care and use protocol for these experiments was in accordance with the Stanford University Institutional Animal Care and Use Committee requirements, and all of the relevant protocols in this study were approved by this committee.

RNA Extraction, cDNA Synthesis, Sequencing, and Primer Design

Total RNA was purified from all tissue samples using TRIzol reagent, as recommended by the manufacturer (Invitrogen Life Technologies, Carlsbad, CA). Concentration and purity of the RNA were determined using a NanoDrop spectrophotometer (ND-1000, NanoDrop Technologies, Wilmington, DE) with 230-, 260-, and 280-nm readings. Two micrograms of each sample of total RNA were reverse transcribed to single-stranded cDNA with SuperScript II reverse transcriptase (Invitrogen) using oligo(dT) as primer. Partial cDNA products encoding SERCA, RyR, and NCX genes were initially amplified by PCR from heart cDNA employing degenerate primers designed on the basis of highly conserved regions of these genes published previously. Following gel purification (MinElute Gel Extraction, Qiagen, Valencia, CA) and dideoxynucleotide sequencing (Geneway Research, Hayward, CA), partial cDNA sequences were obtained and confirmed by BLAST analysis as SERCA, RyR, and NCX genes. Gene-specific primers were designed using Primer Premier software (Premier Biosoft International, Palo Alto, CA). These primers only yielded one PCR product per each gene of interest.

Quantitative PCR Analysis

Quantitative PCR assays were performed on the basis of gene-specific primers using iQ SYBR Green Supermix (Bio-Rad, Hercules, CA), according to the manufacturer's protocol, at primer-specific annealing temperatures on a Bio-Rad iQCycler platform (Bio-Rad, Hercules, CA). After the last amplification cycle, the temperature was increased to 95°C for 1 min and then decreased to 55°C to run 82 cycles, increasing by 0.5°C per cycle, to obtain melting curves, which confirmed the absence of nonspecific PCR products and primer dimers. mRNA expression levels were normalized to total RNA content by using triplicate 1-μl aliquots of each 20-μl cDNA reaction mixture that was produced with 2 μg of total RNA. cDNA in each qPCR incubation was, thus, derived from 0.1 μg of total RNA. One reference atrial RNA sample served as the basis for a standard dilution series, demonstrating a linear relationship between threshold cycle (Ct) and $\log_{10}$ of template availability and was used as the basis for calculating relative abundance values in the remaining samples. β-actin was used as a house-keeping gene (46) (data not shown). β-actin expression was influenced by temperature. However, the expression differences between different acclimation groups for each tissue type remained less than 1.3-fold.

A one-way ANOVA statistical analysis with a Bonferroni post hoc test was conducted across temperature groups for a given tissue to compare expression values for each gene in each tissue type. Statistical significance was accepted if $P < 0.05$.

Enzyme Assays

Freeze-clamped atrial, ventricular, compact, and spongy tissues were homogenized using metal beads and a Tissue Lyser (Qiagen, Valencia, CA), with a homogenization buffer (50 mM KPO_4, pH 6.8 at 4°C) resulting in a 9:1 dilution/1 g of tissue. Twenty-five microliters of this homogenate was used per each reaction for each enzyme assay. Enzyme activities were measured for each tissue type from every acclimation temperature. Each measurement was made in triplicate, and average activity per gram of fresh tissues was calculated. All activity measurements were made in a thermostatted Shimadzu BioSpec UV-visible spectrophotometer at 25°C.

Citrate synthase (CS) (EC. 4.1.3.7), lactate dehydrogenase (LDH) (EC 1.1.1.27), and 3-hydroxyacyl CoA dehydrogenase (HOAD) (EC 1.1.1.35) assays were performed as previously described (15, 32) on atrial, VC, and VS tissue homogenates. Statistical significance was accepted if $P < 0.05$ based on a one-way ANOVA with a Bonferroni post hoc test, tested across temperature groups for a given tissue.

Array Platform, RNA Processing, and Hybridization

As described by Gardner et al. (24), the array platform (BFT 4 × 44K array) consisted of 7078 unique sequences derived from Atlantic bluefin tuna. Three probes were designed to represent each

unique sequence. The microarray design (GPL 15374) and data (GSE36886) are publically accessible at the Gene Expression Omnibus repository.

A total of 36 extracted RNA samples from atrial, ventricular compact, and spongy tissues from fish acclimated to 14°C ($n = 4$), 20°C ($n = 4$), and 25°C ($n = 4$) were used in this study. One microgram of RNA from each tissue sample was reverse transcribed and amplified with the Ambion AminoAllyl MessageAmp II kit (Applied Biosystems, Carlsbad, CA) following manufacturers' instructions. To accurately compare across different samples with a minimal number of hybridizations, we employed a reference design. The reference RNA sample consisted of 1 μg of pooled RNA from every experimental sample. RNA samples were reverse transcribed with T7 Oligo (dT) primer and Arrayscript reverse transcriptase, followed by second-strand cDNA synthesis to generate amplified RNA (aRNA) from every experimental and reference sample. Twenty micrograms of purified aRNAs from reference and experimental samples were separately labeled with Cy3 or Cy5 *N*-Hydroxysuccinimide ester dyes followed by column purification. Dye incorporation was assessed with a NanoDrop spectrophotometer (ND-1000, NanoDrop Technologies, Wilmington, DE). Hybridization steps were performed on the basis of the hybridization protocol of the two-color microarray-based gene expression analysis protocol with reagents from the gene expression hybridization kit (Agilent Technologies, Santa Clara, CA). Then, 825 ng of each Cy3- and Cy5-labeled aRNA was competitively hybridized to the array. Array slides were hybridized at 65°C for 17 h in a rotating hybridization oven. All washing steps were also performed on the basis of the two-color microarray-based gene expression analysis protocol (Agilent Technologies). Each array on 4 × 44K slides was scanned separately using an Axon GenePix 4000B microarray scanner (Axon Instruments, Molecular Devices, Sunnyvale, CA).

Data Extraction, Normalization, and Analysis

Array images were analyzed using Feature Extraction 4.0 software (Agilent Technologies) Spots that Feature Extraction software identified as "good" among all samples were included in further analysis. Lowess normalization was performed to correct for dye biases. Log_2-ratios were calculated on the basis of experimental and reference samples and were analyzed using GeneSpring GX 11.0 software (Agilent Technologies). Log_2 ratio values for each sample were averaged across experimental replicates. The statistical significance was determined using a one-way ANOVA test for each tissue type independently across each temperature group ($P < 0.001$). *P* values were adjusted for false discovery rate (FDR), with a Benjamini-Hochberg multiple-testing correction followed by a Tukey's post hoc analysis. Entities with a minimum of three-fold expression difference (determined using a volcano plot) when compared against two conditions from the same tissue type (e.g., 14°C atrium vs. 25°C atrium) were selected. Since there were three probes representing each gene, if any one of the probes showed opposite directionality in expression, all three probes were removed from the analysis. The resulting list of microarray entities were hierarchically clustered with the Euclidean distance metric and visualized as a heat map plot. Using the same entity list, we performed a principal component analysis (PCA) on experimental conditions. PCA can determine overall variation among transcriptomic profiles from each condition; conditions with similar scores for one or more PCA components can be considered similar in their transcriptomic expression profile. Statistically significant genes were assigned to a functional group based on Blast2GO software (16) or previously published literature. Differentially expressed probes from each functional category are presented as heat maps with each row representing a unique probe and each column representing a tissue type acclimated to a particular temperature.

RESULTS AND DISCUSSION

SERCA, NCX, and RyR Gene Expression

Excitation-contraction coupling proteins, such as SERCA, RyR, and NCX, play a vital role in regulating cytosolic Ca^{2+} concentration in PBFT myocytes (11, 39, 57, 59). In the current study, SERCA gene expression in atrial and ventricular tissues was significantly increased with cold acclimation (CA) (Fig. 1*A*). Compared with 25°C fish, cold-acclimated atrial tissues showed a 5.9-fold increase, and VC and VS tissues showed a 5.4- and 4-fold increase in SERCA gene expression, respectively. Compared with 20°C, NCX gene expression increased at both 14°C and 25°C in atrial and ventricular tissues (Fig. 1*B*). Warm acclimation (WA) had a profound effect on NCX gene expression particularly on ventricular tissues, showing a three-fold difference in expression compared with 20°C. Temperature acclimation had little or no effect on RyR gene expression, except in warm-acclimated VS tissues, where RyR expression increased by 1.8-fold compared with other acclimation groups (Fig. 1*C*).

SERCA protein actively transports cytoplasmic Ca^{2+} into the SR, facilitating relaxation, which plays a key role in maintaining PBFT cardiac function. Previous studies show that compared with other tunas, PBFT cardiac myocytes have a large Ca^{2+} release transient (22), the highest rates of SR Ca^{2+} uptake across a range of temperatures (11), and an increased SERCA protein expression (39). Given the similarities in the temperature sensitivity of SERCA protein in enzyme assays across tuna species (11), the species-specific Ca^{2+} uptake

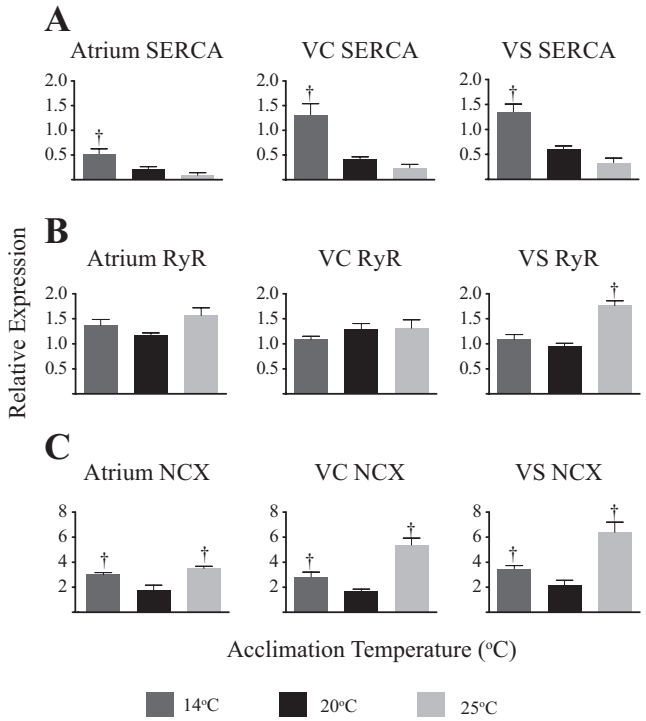


Fig. 1. Relative mRNA expression of sarcoplasmic reticulum Ca^{2+} ATPase (SERCA; *A*), ryanodine receptor (RyR; *B*), and Na^+/Ca^{2+}-exchanger (NCX; *C*) based on quantitative PCR analysis in 14°C, 20°C, and 25°C acclimated PBFT atrial and ventricular compact (VC) and ventricular spongy (VS) tissues. Values are expressed as means ± SD ($n = 5$). †Significant difference compared with 14°C within a tissue ($P < 0.05$).

differences detected in previous studies are considered to be a result of varying SERCA protein expression in the SR. Two previous studies on acclimated PBFT demonstrated the overall thermal plasticity of PBFT cardiac contractile mechanism (21, 57). In addition to bluefin tunas, several other teleosts have also demonstrated increases in SR function in the cold (1, 34, 36, 37, 62). Increased internal Ca^{2+} regulation by SR in rainbow trout in the cold is also associated with elevated SERCA gene and protein expression (36). The current data on increased SERCA mRNA expression in PBFT hearts complement these findings and suggest that upregulation of SERCA gene during CA may underlie the increase in Ca^{2+} uptake by SR in the cold.

In contrast to cold-acclimated tissues, the lack of a SERCA transcriptional response at 25°C in all tissues implies a reduced role for this protein under WA. This could be due to faster rates of Ca^{2+} ion diffusion with increased temperatures offsetting the need for elevated SR Ca^{2+} uptake and loading capacity. It is also possible that this decrease in SERCA expression might be a thermal compensatory strategy to counter accelerated SERCA enzyme activity under warm temperatures. However, Shiels et al. (57) demonstrated that inhibition of SR in 24°C acclimated myocytes had little or no impact on amplitude or decay of Ca^{2+} current, confirming that warm temperatures reduce the need for Ca^{2+} cycling through the SR in PBFT cardiac myocytes. SERCA mRNA expression data under WA from the current study suggest that this reduced role of SR at warm temperatures is potentially regulated at the transcriptional level. Overall, these data suggest that changes in SERCA gene expression contribute to thermal malleability of Ca^{2+} uptake to the SR in the bluefin tuna cardiomyocytes.

Ryanodine receptors function as SR Ca^{2+} release channels (4). RyR mRNA expression showed little or no effect of temperature acclimation in PBFT, except for in VS at 25°C. Previous studies on trout also showed no effect of temperature acclimation on RyR protein expression (4), suggesting very little regulation of RyR activity at the transcriptional level in teleosts during thermal acclimation. The marginal increase in RyR expression in 25°C VS tissues suggests that these tissues may exhibit increased RyR activity under warm temperatures, potentially leading to more efficient Ca^{2+} release from the SR. Further studies are necessary to better understand the role of RyR under thermal acclimation in teleosts.

NCX is primarily involved in Ca^{2+} efflux, but also mediates Ca^{2+} influx under certain conditions (59). Previous studies indicate that NCX plays an important role in maintaining cardiac contractility in fish as they experience large ambient temperature gradients (22, 30, 63). In trout atrium, Ca^{2+} influx via reverse-mode NCX appears to trigger SR Ca^{2+} release (29, 30) and shows a lower temperature sensitivity compared with mammals. Galli et al. (22) showed that in bonito, NCX plays a more pivotal role in Ca^{2+} entry into cardiomyocytes compared with related scombrid fish. While the precise mechanisms require further investigation, these existing studies imply that NCX is a key protein in maintaining cardiac contractility in fish, particularly when they are exposed to temperatures outside of their normal thermal range. The increase in NCX expression detected in both cold- and warm-acclimated fish in the present study supports this hypothesis. Furthermore, the increased NCX gene expression at 14°C compared with 20°C may also possibly offset Q_{10} effects of temperature to maintain efficient Ca^{2+} cycling from the cytoplasm. The elevated NCX gene expression at 25°C may potentially contribute to elevated sarcolemmal Ca^{2+} flux, to compensate for reduced SR Ca^{2+} involvement under 25°C, as discussed previously. Further studies involving NCX functional studies are necessary to better understand the role of this protein in PBFT hearts.

It should be noted that quantitative PCR data presented here may indicate expression profiles of only one isoform or multiple isoforms of each of the EC coupling genes (SERCA, NCX, and RyR) in PBFT. A recent study on eurythermal crucian carp showed two cardiac isoforms of SERCA proteins, while burbot and trout appear to have only one SERCA isoform (36, 37). Previous studies characterizing SERCA protein in PBFT using Western blots identified only one protein for this species (39). Furthermore, only one cardiac isoform for RyR has been previously described in fish (48). Thus, PBFT may only express one isoform of SERCA and RyR genes, as characterized in our current study. However, alternative splicing is thought to generate multiple variants of RyR (60), as well as NCX (54) in mammalian hearts. In addition, four genes coding for NCX are described in teleosts (50). Therefore, it is possible that in fish, including in tunas, different isoforms and splice variants of EC coupling genes are potentially expressed, particularly during changes in environmental factors, such as temperature.

Global Transcriptomic Analysis

Overall transcriptomic data showed an increase in energy metabolism and protein turnover in CA hearts. In contrast, during WA, a decrease in expression of genes involved in molecular chaperoning activity and an increase in genes associated with oxidative damage and programmed cell death is apparent, particularly in the atrium. Here, we discuss these findings and overall changes in gene expression patterns in detail.

Tissue-Specific Gene Expression Variation

The 4,730 probes representing 3,250 unique genes (46% of all genes) showed a 3-fold or greater expression change (ANOVA, FDR corrected, $P < 0.001$ for temperature effect) in PBFT atrial, VC, and VS tissues at 14°C, 20°C, and 25°C. They are hierarchically clustered and presented as a heat map in Fig. 2*A*. It is possible that some of the transcriptomic changes detected here result from cold-induced cardiac hypertrophy detected in teleosts (61). However, it should be noted that, although cardiac cell volume increased with CA, as demonstrated on the basis of histological analysis by Shiels et al. (57), overall cardiac mass remained constant across acclimation temperatures in the current study and in previous PBFT acclimation experiments (57). Additionally, the greatest transcriptomic changes—both upregulation and downregulation of genes—were detected in warm-acclimated atrial tissues. Therefore, considering hypertrophy is primarily detected in teleost ventricular tissues, it is unlikely that overall transcriptomic responses detected here are simply due to changes in cell size or volume.

Overall trends in transcriptomic profiles across treatments, visualized using PCA (Fig. 2*B*), illustrated that the variation in gene expression was primarily due to effects of temperature; 46.92% of the variation, explained by principal component 1

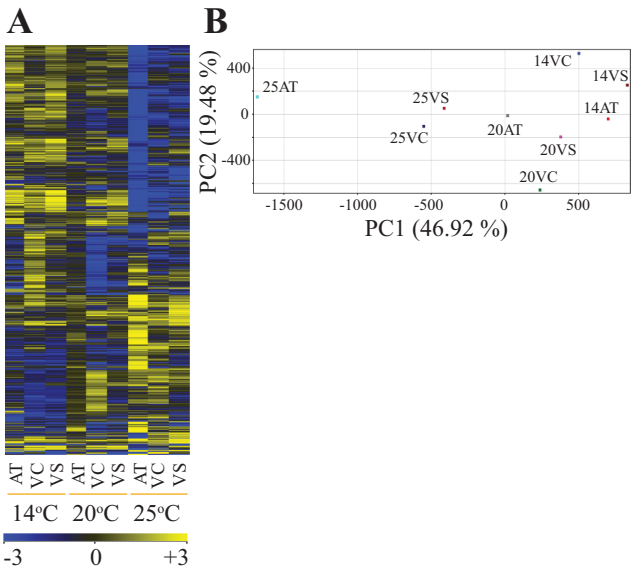


Fig. 2. *A*: heat map of the normalized expression (log_2-ratio) of the 4,730 probes (3,250 unique genes) that showed statistically significant difference at least in one tissue type (ANOVA, false discovery rate corrected, $P < 0.001$ for temperature effect) with a three-fold expression difference when compared against two acclimation conditions in cardiac tissues of fish exposed to 14°C, 20°C, and 25°C. Genes are ordered in rows and each cluster represents each tissue type. The color scale bar indicates three-fold or greater change in expression, with blue down-regulation, yellow up-regulation, and black for no change in expression. *B*: principal component loading plot of principal component 1 (PC1) vs. principal component 2 (PC2). AT, atrium; VC, ventricular compact; VS, ventricular spongy.

(PC1), appears to be driven by the difference in warm-acclimated and cold-acclimated tissues. In particular, gene expression pattern differs considerably in the atrium at 25°C compared with other tissues. In contrast, the 19.48% of the expression variation explained by principal component 2 appears to result from gene expression differences in ventricular tissues, primarily defined by the VC layer. VS layer demonstrated the least temperature-induced gene expression variation under temperature acclimation. PCA also showed that variation in gene expression was increased among tissues under WA, whereas expression patterns were similar across atrial and ventricular tissues at 14°C.

In PBFT hearts, the compact layer is perfused by oxygen-rich coronary circulation. The VS tissues are not as prominently perfused by coronary vessels, and they also receive blood with a reduced oxygen content from venous blood returning to the heart (17). Consequently, ventricular tissues—across the compact and spongy layers—are potentially exposed to an oxygen gradient and a temperature gradient depending on the ambient temperature. This may lead to differences in thermal response across the ventricle, potentially explaining the expression pattern variation detected across VC and VS tissues. Nonetheless, molecular responses of these two tissues share more similar gene expression patterns compared with atrial tissues. Importantly, the PCA analysis indicates that atrial tissues are more severely impacted by the warm temperatures. Unlike ventricular tissues, teleost atrium lacks extensive coronary circulation and may be subjected to oxygen limitations with increasing temperatures (17). This difference in tissue oxygenation may lead to the marked gene expression differences detected between WA atrial and ventricular tissues. These significantly differentially expressed genes from the transcriptomic analysis were assigned to different functional categories and are discussed below in detail.

Effect of Acclimation on Different Biological Processes

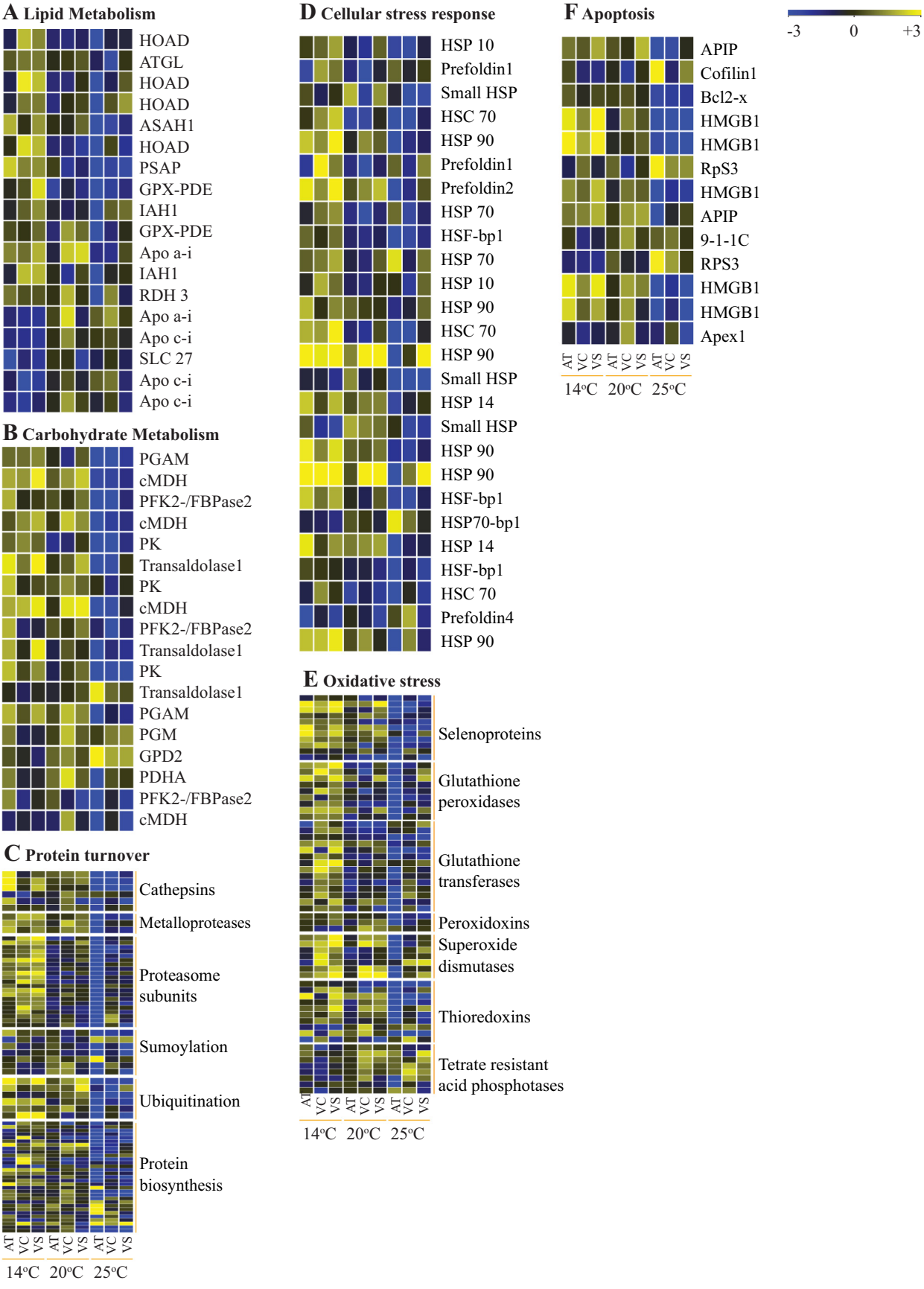
Cardiac energy metabolism. To sustain an elevated cardiac performance and to maintain cellular homeostasis (38) across a thermal gradient, PBFT have to sustain an adequate energy production. Transcriptomic analysis of PBFT hearts revealed significant changes in expression of 52 genes involved in cellular metabolism in cold- and warm-acclimated tissues (Supplemental Fig. S1 and S2). Here, coupled with metabolic enzyme activity data, we discuss eight genes involved in lipid metabolism (Fig. 3*A*) and six genes pertaining to carbohydrate metabolism (Fig. 3*B*). These 14 genes were selected from the 52 genes due to their direct involvement with carbohydrate and lipid metabolism. Overall trends indicated a global downregulation of genes involved in lipid and carbohydrate metabolism under WA, particularly in the atrium.

Lipid metabolism. Genes involved in lipid metabolism are upregulated in cold-acclimated PBFT hearts (Fig. 3*A*). The gene coding for HOAD, a key enzyme involved in β-oxidation of fatty acids that is frequently used as a quantitative index for lipid metabolism (18) is upregulated in both VC and VS tissues at 14°C. Genes coding for fatty acid and sphingolipid-metabolizing enzymes—isoamyl acetate-hydrolyzing esterase (20), glycerophosphodiester phosphodiesterase (EC 3.1.4.46), *n*-acylsphingosine amidohydrolase (EC 3.5.1.23), and proactivator polypeptide precursor (EC 3.2.1.45)—were also upregulated under CA. These data provide strong evidence for temperature-induced changes in membrane lipid composition and a metabolic shift toward lipid synthesis during CA.

Increase in lipid metabolism under CA is further demonstrated by enzyme assay studies based on HOAD enzyme activity (Fig. 4*A*). Both atrial and VS tissues showed a 1.5-fold increase in HOAD activity at 14°C compared with 25°C. However, in contrast to gene expression analysis, VC tissue showed no difference with acclimation, demonstrating the capacity to metabolize lipids at 25°C in this tissue. Overall, our results are indicative of an increase in lipid metabolism with CA in PBFT hearts.

Genes encoding three proteins involved in fatty acid transport-solute carrier family 27 (SLC 27), apolipoprotein a-i and c-i (Apo a-i and c-i) were downregulated under CA. These proteins participate in the reverse transport of cholesterol from tissues to the liver for excretion, by promoting cholesterol efflux from tissues (13). Decrease in gene expression of such triglyceride-carrying proteins at 14°C suggests that cold-acclimated cardiac tissues may reduce lipid efflux to utilize them as local energy stores.

Carbohydrate metabolism. Warm acclimation resulted in significant downregulation of genes involved in glycolysis and associated pathways (Fig. 3*B*). Decrease in expression of genes encoding glycolytic enzymes—pyruvate kinase (PK) (EC: 2.7.1.40), pyruvate dehydrogenase (PDHA) (EC 1.2.4.1), and phosphoglycerate mutase (PGAM) (EC: 5.4.2.1)—suggests an overall depression of glycolysis under WA. Downregulation of the gene for transaldolase 1 (EC:2.2.1.2), an enzyme linking the pentose phosphate pathway to glycolysis, at 25°C further


A Lipid Metabolism
HOAD
ATGL
HOAD
HOAD
ASAH1
HOAD
PSAP
GPX-PDE
IAH1
GPX-PDE
Apo a-i
IAH1
RDH 3
Apo a-i
Apo c-i
SLC 27
Apo c-i
Apo c-i
B Carbohydrate Metabolism
PGAM
cMDH
PFK2-/FBPase2
cMDH
PK
Transaldolase1
PK
cMDH
PFK2-/FBPase2
Transaldolase1
PK
Transaldolase1
PGAM
PGM
GPD2
PDHA
PFK2-/FBPase2
cMDH
C Protein turnover
Cathepsins
Metalloproteases
Proteasome subunits
Sumoylation
Ubiquitination
Protein biosynthesis
AT VC VS AT VC VS AT VC VS
14°C 20°C 25°C
D Cellular stress response
HSP 10
Prefoldin1
Small HSP
HSC 70
HSP 90
Prefoldin1
Prefoldin2
HSP 70
HSF-bp1
HSP 70
HSP 10
HSP 90
HSC 70
HSP 90
Small HSP
HSP 14
Small HSP
HSP 90
HSP 90
HSF-bp1
HSP70-bp1
HSP 14
HSF-bp1
HSC 70
Prefoldin4
HSP 90
E Oxidative stress
Selenoproteins
Glutathione peroxidases
Glutathione transferases
Peroxidoxins
Superoxide dismutases
Thioredoxins
Tetrate resistant acid phosphotases
AT VC VS AT VC VS AT VC VS
14°C 20°C 25°C
F Apoptosis
APIP
Cofilin1
Bcl2-x
HMGB1
HMGB1
RpS3
HMGB1
APIP
9-1-1C
RPS3
HMGB1
HMGB1
Apex1
AT VC VS AT VC VS AT VC VS
14°C 20°C 25°C
-3 0 +3

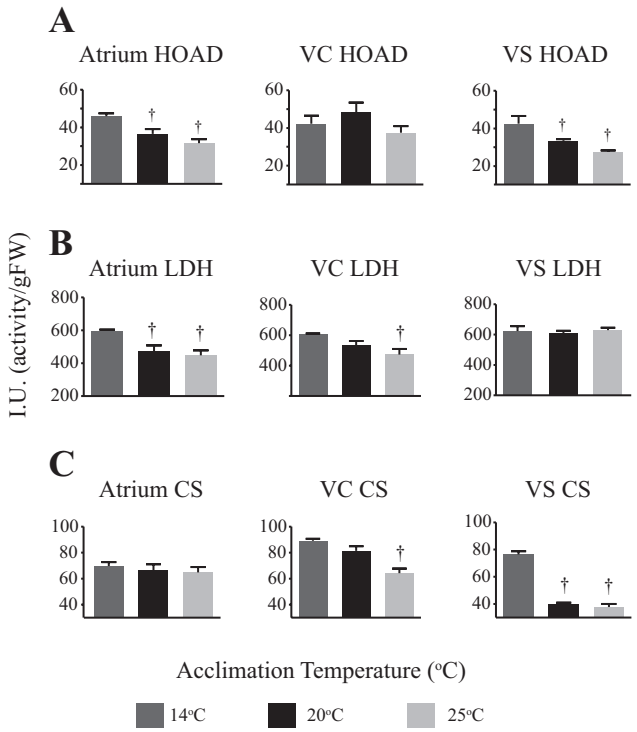


Fig. 4. Maximum enzyme activity rates of 3-hydroxyacyl CoA dehydrogenase (HOAD; *A*), lactate dehydrogenase (LDH; *B*), and citrate synthase (CS; *C*), measured as international units per gram fresh weight of tissue in atrial, ventricular compact, and spongy tissues from fish acclimated to 14°C, 20°C, and 25°C. Values are presented as means ± SE ($n = 5$). †Significant difference compared with 14°C within a tissue ($P < 0.05$).

implies a decrease in glycolysis during WA. Another downregulated gene during WA encodes 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (PFK-2/FBPase-2). This enzyme catalyzes the synthesis and degradation of fructose 2,6-bisphosphate, the most potent stimulator of 6-phosphofructo-1-kinase (49). Marsin et al. (44) showed that the heart type 6-phosphofructo-1-kinase isoform stimulates glycolysis under energy-deprived conditions in monocytes, suggesting that the decrease in PFK-2/FBPase-2 at 25°C in PBFT might result in diminished ability to stimulate glycolysis under WA.

Because of perfusion limitations that occur during increases in temperature (17), cardiac cells may rely more on anaerobic metabolism to meet the increased ATP demand. However, during severe cardiac ischemia, glycolysis is arrested (47). It is possible that the decrease in glycolytic gene expression in warm-acclimated PBFT hearts is to reduce glycolysis, thus decreasing accumulation of glycolytic products (lactate, NADH, and H^+ ions) (28) in myocytes as a result of low-perfusion capacity (oxygenation). Alternatively, given the high glycolytic capacity of cardiac muscle tissues, it is possible that a decrease in glycolytic gene expression at 25°C might be a thermal compensatory mechanism by PBFT hearts.

LDH enzyme activity was measured to further examine effects of acclimation on anaerobic metabolism. LDH activity was increased (~1.5-fold) in the atrial and VC tissues at 14°C compared with 25°C (Fig. 4*B*). Results from both the atrial and VC LDH enzyme activity are consistent with the observed gene expression patterns, validating the conjecture that reduced glycolysis and anaerobic metabolism occurs in these tissues at 25°C. Cardiac cells also use plasma lactate as a metabolic fuel to generate pyruvate, particularly during exercise (18). Thus, although LDH is an indicator of anaerobic metabolism, the increase in in vitro LDH activity at 14°C may suggest that lactate oxidation is also potentially increased with elevated LDH activity. The VS layer showed no effect of acclimation on LDH enzyme activity and was elevated under both CA and WA compared with VC tissues, suggesting that VS tissues may depend more on glycolysis for ATP production. Considering the potential differences in tissue oxygenation of VC and VS layers, it is possible that VS myocytes have evolved better mechanisms to sequester glycolytic products resulting from increased glycolysis. This hypothesis is supported by Gemelli et al. (25) describing a higher lactate accumulation and a greater capacity to oxidize and incorporate lactate into the total cardiac lipids by VS compared with VC tissues in PBFT. The relative increase in glycolytic gene expression and LDH activity at 14°C in all tissues suggests an increased glycolytic capacity at this temperature. This might be a result of increased glycogen stores in cardiac myocytes, as previously shown in cold-acclimated PBFT (57) to maintain an adequate energy supply at cold temperatures (18).

Overall aerobic metabolism. We quantified CS, an enzyme involved in tricarboxylic acid cycle activity, to measure overall aerobic metabolic capacity in PBFT hearts (Fig. 4*C*). Acclimation temperature had no effect on CS activity in the atrium, but CS activity decreased at 25°C in both VC (1.3-fold) and VS tissues (2-fold) compared with cold-acclimated hearts. These changes in CS activity could be a thermal compensatory response to offset Q_{10} effects. However, the marked decrease in CS activity in warm-acclimated VS tissues suggests an overall decrease in aerobic metabolic capacity and reduced ATP production in these tissues. Furthermore, the gene for cytosolic malate dehydrogenase (E.C. 1.1.1.37), an oxidoreductase enzyme that interconverts malate and oxaloacetate is also downregulated during warm acclimation (Fig. 3*B*). These data and the increase in LDH activity in warm-acclimated VS tissues further suggest a potential oxygen delivery limitation to the VS layer compared with VC layer. The overall increase in mitochondrial density in cold-acclimated PBFT demonstrated by Shiels et al. (57) might contribute to this increase in aerobic metabolism.

Fig. 3. Annotated heat maps showing the differentially expressed probes at 14°C, 20°C, and 25°C in each tissue type, representing unique genes from each functional category (GO database, UniProt Knowledge database and published literature search): lipid metabolism (*A*), carbohydrate metabolism (*B*), protein turnover (*C*), cellular stress response (*D*), oxidative stress (*E*), and apoptosis (*F*). Each row represents a unique probe and each column represents a tissue type at a given temperature. The color scale bar indicates three-fold or greater change in expression, with blue denoting downregulation, yellow denoting upregulation, and black denoting no change in expression. Individual probe names for genes involved in these categories are available in the electronic supplemental material (Supplemental Figs. S1–S6).

Protein turnover. Transcriptomic analysis revealed 60 genes involved in protein turnover, and these genes were grouped into proteolysis (cathepsin, metalloproteases, proteins involved in ubiquitination, and sumoylation) and protein biosynthesis (Fig. 3*C*) (Supplemental Fig. S3, *A* and *B*). The most striking pattern in this gene set is the downregulation of genes involved in protein biosynthesis and proteolysis under WA, particularly in the atrium and their marked increase under CA.

Temperature stress is known to cause protein damage (27). Unrepaired, damaged proteins are degraded and removed from the cell through proteolysis, and new proteins are synthesized to maintain adequate protein levels. Current data infer an increase in total protein turnover during CA. Increase in protein biosynthesis was also previously detected in cold-acclimated PBFT ventricles (10) and rainbow trout hearts (61), which was attributed to cold-induced cardiac hypertrophy. These data suggest that PBFT hearts are able to induce the necessary genes to sustain an apparently higher level of protein turnover than hearts of warm-acclimated specimens.

Decreased expression of genes involved in protein turnover machinery at 25°C is rather surprising, considering protein denaturation is likely to be exacerbated at warm temperatures. Previous studies also documented a similar decrease in protein degradation in two highly eurythermal species, *Gillichthys mirabilis* and *Austrofundulus limnaeus* acclimated to warm temperatures, but also showed a concurrent increase in protein synthesis (42, 52). Podrabsky and Somero (52) described this as an attempt by the organisms to maintain an adequate protein level at the warm temperatures that these fish experience in highly fluctuating intertidal environments. Considering PBFT experience temperatures as high as 25°C only for a period of time during their migrations to the western Pacific Ocean, it is possible that these fish do not have the capacity to upregulate genes involved in protein turnover machinery during constant exposure to 25°C.

Molecular chaperones and oxidative stress response. Cellular response to temperature includes an increase in expression of molecular chaperones to repair and refold damaged proteins (38). Fifty-four genes involved in overall cellular stress response showed significantly different expression patterns across treatments (Supplemental Fig. S4). Fifteen of these genes are directly involved in molecular chaperoning activity (Fig. 3*D*) and are discussed below in detail.

Heat shock proteins (HSP) play a critical role as chaperones during stress-induced protein unfolding (38) and are involved in protective mechanisms against apoptosis (3) and oxidative stress (33). Genes coding for HSP 10, 14, 70, 90, and the constitutive form of HSP (HSC70) were decreased at 25°C and increased at 14°C. HSP10 is shown to protect cardiac myocytes against apoptosis during ischemia/reperfusion injury (40). In contrast to other HSP genes, gene expression of HSP 70 binding protein (HSP-70-bp 1), which inhibits ATP-binding domain of HSP 70 interfering with its function (56), was increased at 25°C, potentially resulting in further repression of HSP70 chaperoning activity under WA.

The increase in HSP gene expression at 14°C implies a protective role played by these proteins during cold stress. The downregulation of HSPs in PBFT cardiac tissues at 25°C, particularly in the atrium, is surprising, considering the crucial role of HSPs as a repair protein during temperature stress. However, goby fish *Gillichthys mirabilis* also showed a lack of pronounced HSP gene expression with WA (42), inferring that eurythermal species can overcome acute temperature stress effects through acclimation. Similarly the decrease in HSP gene expression in warm-acclimated PBFT could also be due to the efficiency with which PBFT heart muscles were able to acclimate to warm temperatures and restore its cellular homeostasis. However, increased expression of HSP70-bp-1 and significant decrease in expression of constitutive heat shock proteins (HSC70) that are involved in protection against oxidative stress and apoptosis under WA, suggests decreased capacity for molecular chaperoning activity in PBFT at 25°C (38).

Eukaryotic cells constantly cope with reactive oxygen species (ROS). The imbalance between ROS production and elimination results in oxidative stress and can cause damage to cellular macromolecules. Cold and warm temperatures induce oxidative stress in various organisms, including fish (40). Transcriptomic data recognized 18 differentially expressed genes that are involved in cellular redox signaling and control (Fig. 3*E*; Supplemental Fig. S5). Eight isoforms of genes coding for tartrate-resistant acid phosphatase (TRAP), a protein catalyzed by formation of ROS (55), were detected in the current study. Most of these isoforms (six in atrial tissues and five in ventricular tissues) were increased during WA. Eight genes coding for proteins that are involved in cellular protection against oxidative stress—peroxiredoxins, glutathione peroxidases, superoxide dismutase, glutathione transferases, thioredoxin, and several other selenoproteins, (45, 58) are downregulated under WA in all cardiac tissues. The atrium, in particular, showed a universal downregulation of these enzymes at 25°C. In contrast, these genes are upregulated or remain constant in expression at 14°C.

These data indicate that during CA, PBFT hearts are able to upregulate genes involving antioxidant mechanisms to protect against cold-induced oxidative stress. However, under WA, particularly in the atrium, ROS generation is potentially increased with the increase in TRAP gene expression and the protective mechanisms against oxidative damage, including HSP response, appear to be decreased.

Apoptosis. Apoptosis is a complex cell-death program that is inherent in multicellular organisms. Gene ontology analysis across treatments identified 16 significantly differentially expressed genes involved in apoptotic pathways (Fig. 3*F*; Supplemental Fig. S6). Five of those genes showed an increase in expression under WA. Ribosomal protein 4S (RpS3), which activates caspase-8/caspase-3 to induce apoptosis (31), was significantly upregulated in all three tissues types at 25°C. Genes coding for cofilin 1, an initiator or apoptosis, and rad9-hus1-rad1 complex (9-1-1C), involved in triggering apoptosis (35, 51), were upregulated in atrial and VS tissues under WA.

High-mobility-group-box 1 (HMGB1) gene was downregulated under WA and upregulated in the cold. Podrabsky and Somero (52) described this protein as a potential global gene expression temperature sensor. Increased expression of HMGB1 inhibits apoptosis via several mechanisms (41). Two other genes (*APEX1* and *APIP*) that are downregulated at 25°C and upregulated at 14°C are involved in positive regulation of antiapoptotic pathways (9, 64). *Bcl2-x*, which is involved in prevention of hypoxia-induced apoptosis (14), is also downregulated under WA. These data suggest that tuna hearts, particularly the atrium, are upregulating genes involved in inducing apoptosis at 25°C. Inhibition of glycolysis—as evident in warm-acclimated PBFT tis-

sues—is also shown to result in induction of apoptosis (53). Increase in programmed cell death can contribute to the development and progression of cardiac dysfunction in PBFT, as demonstrated in mammalian hearts (12).

Summary. Overall qPCR data elucidate how cold-acclimated PBFT alters EC coupling gene expression levels. In particular, SERCA gene expression is increased at 14°C, potentially optimizing the role of SR in cardiac myocytes. The metabolic enzyme activity data and transcriptomic data in 14°C-acclimated PBFT showed an increase in overall ATP production and an induction of genes involved in compensating for effects of cold temperatures. These changes are potentially associated with increased cell size detected in cold-acclimated PBFT myocytes (57). In contrast, transcriptomic data and metabolic enzyme activity data at 25°C, particularly in the atrium, indicated a decrease in overall ATP production; reduced expression of genes involved in molecular chaperones and oxidative stress responses; induction of genes potentially leading to increased ROS production; and an increase in expression of apoptotic genes. The interplay between apoptosis and cellular stress response is thought to prevent further damage or facilitate recovery to maintain cell survival (2). Assayag et al. (2) demonstrated an antiapoptotic cardiac phenotype to heat stress developed by long-term temperature acclimation in mammals. Induction of molecular chaperoning genes was demonstrated to be critical in maintaining this phenotype. Therefore, on the basis of our current data, we hypothesize that diminished cellular stress response and increased oxidative damage may mediate apoptosis in PBFT cardiac cells, particularly in the atrium. This is likely due to perfusion limitations that may occur with increasing temperatures (17) and may lead to reduced cardiac performance in bluefin tunas during long-term exposure to warm temperatures.

Perspectives and Significance

The current study provides insights into molecular physiological underpinnings of cardiac thermal responses in PBFT. The cold acclimation response detected here suggests that PBFT has the capacity to maintain or improve cardiac performance at cold temperatures; this perhaps underlies this species' ability to expand its vertical and horizontal thermal niche and migrate to colder oceans. In contrast, warm acclimation responses indicate a reduced capacity to restore cellular homeostasis at warm temperatures. This may affect cardiac performance and overall fitness of PBFT when inhabiting warm ocean habitats. To further investigate this hypothesis, experiments are currently under way to quantify blood Po_2 levels, as well as several cardiac parameters and aerobic scope in PBFT acclimated to cold and warm temperatures. With increasing ocean temperatures, thermal effects on cardiac function, as described in the current study, particularly during spawning seasons in subtropical seas, may lead to reduced reproductive fitness or shifts in PBFT spawning grounds.

ACKNOWLEDGMENTS

We highly appreciate all of the contributions from late Dr. David Towle in microarray EST (express sequence tags) assembly. We thank the Tuna Research and Conservation Center team and the Monterey Bay Aquarium staff, *F/V Shogun* crew for helping with collecting and maintaining the Pacific bluefin tuna. We especially thank Luis Rodriguez for his help during fish acclimation.

Present address: Nicholas School of the Environment, Duke University, Durham, NC 22708.

GRANTS

Grants from the National Oceanic and Atmospheric Administration to B. A. Block, as well as the Monterey Bay Aquarium Foundation, supported this study. We thank the Government of Mexico for permitting our team to collect tunas for research purposes.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

Author contributions: N.J. and B.A.B. conception and design of research; N.J. and L.D.G. performed experiments; N.J. analyzed data; N.J. and L.D.G. interpreted results of experiments; N.J. prepared figures; N.J. drafted manuscript; N.J., L.D.G., and B.A.B. edited and revised manuscript; N.J., L.D.G., and B.A.B. approved final version of manuscript.

REFERENCES

1. **Aho E, Vornanen M.** Ca^{2+}-ATPase activity and Ca^{2+} uptake by sarcoplasmic reticulum in fish heart: effects of thermal acclimation. *J Exp Biol* 201: 525–532, 1998.
2. **Assayag M, Gerstenblith G, Stern MD, Horowitz M.** Long- but not short-term heat acclimation produces an apoptosis-resistant cardiac phenotype: a lesson from heat stress and ischemic/reperfusion insults. *Cell Stress Chaperones* 15: 651–664, 2010.
3. **Beere HM.** "The stress of dying": the role of heat shock proteins in the regulation of apoptosis. *J Cell Sci* 117: 2641–2651, 2004.
4. **Birkedal R, Christopher J, Thistlethwaite A, Shiels HA.** Temperature acclimation has no effect on ryanodine receptor expression or subcellular localization in rainbow trout heart. *J Comp Physiol B* 179: 961–969, 2009.
5. **Blank JM, Farwell CJ, Morrissette JM, Schallert RJ, Block BA.** Influence of swimming speed on metabolic rates of juvenile pacific bluefin tuna and yellowfin tuna. *Physiol Biochem Zool* 80: 167–177, 2007.
6. **Blank JM, Morrissette JM, Landeira-Fernandez AM, Blackwell SB, Williams TD, Block BA.** In situ cardiac performance of Pacific bluefin tuna hearts in response to acute temperature change. *J Exp Biol* 207: 881–890, 2004.
7. **Block BA, Jonsen ID, Jorgensen SJ, Winship a. J, Shaffer SA, Bograd SJ, Hazen EL, Foley DG, Breed GA, Harrison AL, Ganong JE, Swithenbank A, Castleton M, Dewar H, Mate BR, Shillinger GL, Schaefer KM, Benson SR, Weise MJ, Henry RW, Costa DP.** Tracking apex marine predator movements in a dynamic ocean. *Nature* 475: 86–90, 2011.
8. **Block BA, Stevens ED.** *Tuna: Physiology, Ecology and Evolution*. San Diego, CA: Academic, 2001.
9. **Cao G, Xiao M, Sun F, Xiao X, Pei W, Li J, Graham SH, Simon RP, Chen J.** Cloning of a novel Apaf-1-interacting protein: a potent suppressor of apoptosis and ischemic neuronal cell death. *J Neurosci* 24: 6189–6201, 2004.
10. **Castilho PC, Buckley BA, Somero GN, Block BA.** Heterologous hybridization to a complementary DNA microarray reveals the effect of thermal acclimation in the endothermic Bluefin tuna (*Thunnus orientalis*). *Mol Ecol* 18: 2092–2102, 2009.
11. **Castilho PC, Landeira-Fernandez AM, Morrissette J, Block BA.** Elevated Ca^{2+} ATPase (SERCA2) activity in Tuna hearts: Comparative aspects of temperature dependence. *Comp Biochem Physiol A* 148: 124–132, 2007.
12. **Cesselli D, Jakoniuk I, Barlucchi L, Beltrami AP, Hintze TH, Nadal-Ginard B, Kajstura J, Leri A, Anversa P.** Oxidative stress-mediated cardiac cell death is a major determinant of ventricular dysfunction and failure in dog dilated cardiomyopathy. *Circ Res* 89: 279–286, 2001.
13. **Chen J, Shi YH, Hu HQ, Niu H, Li MY.** Apolipoprotein A-I, a hyperosmotic adaptation-related protein in Ayu (*Plecoglossus altivelis*). *Comp Biochem Physiol B* 152: 196–201, 2009.
14. **Cheng EH, Levine B, Boise LH, Thompson CB, Hardwick JM.** Bax-independent inhibition of apoptosis by Bcl-Xl. *Nature* 379: 554–556, 1996.

15. **Colowick SP, Kaplan NO (Editors).** *Methods in Enzymology*. New York: Academic, 1955.
16. **Conesa A, Götz S, García-Gómez JM, Terol J, Talón M, Robles M.** Blast2GO: a universal tool for annotation, visualization and analysis in functional genomics research. *Bioinformatics* 21: 3674–3676, 2005.
17. **Davie PS, Farrell AP.** The coronary and luminal circulations of the myocardium of fishes. *Can J Zool* 69: 1993–2001, 1991.
18. **Driedzic WR.** Cardiac energy metabolism. In: *Fish Physiology,* vol. 12, part 12A, edited by Hoar WS, Randall D, and Farrell AP. San Diego, CA: Academic, 1992, p. 219–258.
19. **Farrell AP.** Features heightening cardiovascular performance in fishes, with special reference to tunas. *Comp Biochem Physiol* 113A: 61–67, 1996.
20. **Fukuda K, Kiyokawa Y, Yanagiuchi T, Wakai Y, Kitamoto K, Inoue Y, Kimura A.** Purification and characterization of isoamyl acetate-hydrolyzing esterase encoded by the IAH1 gene of *Saccharomyces cerevisiae* from a recombinant *Escherichia coli*. *Appl Microbiol Biotechnol* 53: 596–600, 2000.
21. **Galli GLJ, Lipnick MS, Block BA.** Effect of thermal acclimation on action potentials and sarcolemmal K^+ channels from Pacific bluefin tuna cardiomyocytes. *Am J Physiol Regul Integr Comp Physiol* 297: R502–R509, 2009.
22. **Galli GLJ, Lipnick MS, Shiels HA, Block BA.** Temperature effects on Ca^{2+} cycling in Scombrid cardiomyocytes: a phylogenetic comparison. *J Exp Biol* 214: 1068–1076, 2011.
23. **Gamperl AK, Farrell AP.** Cardiac plasticity in fishes: environmental influences and intraspecific differences. *J Exp Biol* 207: 2539–2550, 2004.
24. **Gardner LD, Jayasundara N, Castilho PC, Block BA.** Microarray gene expression profiles from mature gonad tissues of Atlantic bluefin tuna, *Thunnus thynnus* in the Gulf of Mexico. *BMC Genomics* 13: 530, 2012.
25. **Gemelli L, Martino G, Tota B.** Oxidation of lactate in the compact and spongy myocardium of tuna fish (*Thunnus thynuss thynnus L.*). *Comp Biochem Physiol B* 658: 321–326, 1980.
26. **Groot C, Margolis L, Clarke WG.** *Physiological Ecology of Pacific Salmon.* Vancouver, Canada: UBC Press, 1995.
27. **Hochachka PW, Somero GN.** *Biochemical Adaptation: Mechanism and process in Physiological Evolution.* New York: Oxford University Press, 2002.
28. **Hochachka PW.** Defense strategies against hypoxia and hypothermia. *Science* 231: 234–241, 1986.
29. **Hove-Madsen L, Llach A, Tort L.** Quantification of calcium release from the sarcoplasmic reticulum in rainbow trout atrial myocytes. *Euro J Physiol* 438: 545–552, 1999.
30. **Hove-Madsen L, Llach A, Tibbits GF, Tort L.** Triggering of sarcoplasmic reticulum Ca^{2+} release and contraction by reverse mode Na^+/Ca^{2+} exchange in trout atrial myocytes. *Am J Physiol Regul Integr Comp Physiol* 284: R1330–R1339, 2003.
31. **Jang CY, Lee JY, Kim J.** RpS3, a DNA repair endonuclease and ribosomal protein, is involved in apoptosis. *FEBS Lett* 560: 81–85, 2004.
32. **Jayasundara N, Healy TM, Somero GN.** Effects of temperature acclimation on cardiorespiratory performance of the Antarctic notothenioid *Trematomus bernacchii. Polar Biol* 36: 1047–1057, 2013.
33. **Kalmar B, Greensmith L.** Induction of heat shock proteins for protection against oxidative stress. *Adv Drug Deliv Rev* 61: 310–318, 2009.
34. **Keen JE, Vianzon D, Farrell AP, Tibbits GF.** Effect of temperature and temperature acclimation on the ryanodine sensitivity of the trout myocardium. *J Comp Physiol B* 164: 438–443, 1994.
35. **Klamt F, Zdanov S, Levine RL, Pariser A, Zhang Y, Zhang B, Yu LR, Veenstra TD, Shacter E.** Oxidant-induced apoptosis is mediated by oxidation of the actin-regulatory protein cofilin. *Nat Cell Biol* 11: 1241–1246, 2009.
36. **Korajoki H, Vornanen M.** Expression of SERCA and phospholamban in rainbow trout (*Oncorhynchus mykiss*) heart: comparison of atrial and ventricular tissue and effects of thermal acclimation. *J Exp Biol* 215: 1162–1169, 2012.
37. **Korajoki H, Vornanen M.** Temperature dependence of sarco(endo)plasmic reticulum Ca^{2+} ATPase expression in fish hearts. *J Comp Physiol B* 183: 467–476, 2013.
38. **Kültz D.** Molecular and evolutionary basis of the cellular stress response. *Annu Rev Physiol* 67: 225–257, 2005.
39. **Landeira-fernandez AM, Morrissette JM, Blank JM, Block BA.** Temperature dependence of the Ca^{2+}-ATPase (SERCA2) in the ventricles of tuna and mackerel. *Am J Physiol Regul Integr Comp Physiol* 286: R398–R404, 2004.
40. **Lin KM, Lin B, Lian IY, Mestril R, Scheffler IE, Dillmann WH.** Combined and individual mitochondrial HSP60 and HSP10 expression in cardiac myocytes protects mitochondrial function and prevents apoptotic cell deaths induced by simulated ischemia-reoxygenation. *Circulation* 103: 1787–1792, 2001.
41. **Liu G, Wang J, Park Y, Tsuruta Y, Lorne EF, Zhao X, Abraham E.** High-mobility group protien-1 inhibits phagocytosis of apoptotic neutrophils through binding to phosphatidylserine. *J Immunol* 181: 4240–4246, 2008.
42. **Logan CA, Somero GN.** Transcriptional responses to thermal acclimation in the eurythermal fish *Gillichthys mirabilis* (Cooper 1864). *Am J Physiol Regul Integr Comp Physiol* 299: R843–R852, 2010.
43. **Lushchak VI.** Environmentally induced oxidative stress in aquatic animals. *Aquat Toxicol (Amst)* 101: 13–30, 2011.
44. **Marsin AS, Bouzin C, Bertrand L, Hue L.** The stimulation of glycolysis by hypoxia in activated monocytes is mediated by AMP-activated protein kinase and inducible 6-phosphofructo-2-kinase. *J Biol Chem* 277: 30778–30783, 2002.
45. **Michiels C, Raes M, Toussaint O, Remacle J.** Importance of Se-glutathione peroxidase, catalase, and Cu/Zn-SOD for cell survival against oxidative stress. *Free Radic Biol Med* 17: 235–248, 1994.
46. **Mladineo I, Block BA.** Expression of Hsp70, Na^+/K^+ ATP-ase, HIF-1α, IL-1β, and TNF-α in captive Pacific bluefin tuna (*Thunnus orientalis*) after chronic warm and cold exposure. *J Exp Mar Biol Ecol* 374: 51–57, 2009.
47. **Neely JR, Grotyohann LW.** Role of glycolytic products in damage to ischemic myocardium. Dissociation of adenosine triphosphate levels and recovery of function of reperfused ischemic hearts. *Circ Res* 55: 816–824, 1984.
48. **O'Brien J, Meissner G, Block BA.** The fastest contracting muscles of nonmammalian vertebrates express only one isoform of the ryanodine receptor. *Biophys J* 65: 2418–2427, 1993.
49. **Okar DA, Manzano A, Navarro-Sabatè A, Riera L, Bartrons R, Lange AJ.** PFK-2/FBPase-2: maker and breaker of the essential biofactor fructose-2,6-bisphosphate. *Trends Biochem Sci* 26: 30–35, 2001.
50. **On C, Marshall CR, Chen N, Moyes CD, Tibbits GF.** Gene structure evolution of the Na^+-Ca^{2+} exchanger (NCX) family. *BMC Evol Biol* 8: 127, 2008.
51. **Parrilla-Castellar ER, Arlander SJH, Karnitz L.** Dial 9-1-1 for DNA damage: the Rad9-Hus1-Rad1 (9-1-1) clamp complex. *DNA Repair* 3: 1009–1014, 2004.
52. **Podrabsky JE, Somero GN.** Changes in gene expression associated with acclimation to constant temperatures and fluctuating daily temperatures in an annual killifish *Austrofundulus limnaeus. J Exp Biol* 207: 2237–2254, 2004.
53. **Pradelli LA, Bénéteau M, Chauvin C, Jacquin MA, Marchetti S, Muñoz-Pinedo C, Auberger P, Pende M, Ricci JE.** Glycolysis inhibition sensitizes tumor cells to death receptors-induced apoptosis by AMP kinase activation leading to Mcl-1 block in translation. *Oncogene* 29: 1641–1652, 2010.
54. **Quednau BD, Nicoll DA, Philipson KD.** Tissue specificity and alternative splicing of the Na^+/Ca^{2+} exchanger isoforms NCX1, NCX2, and NCX3 in rat. *Am J Physiol Cell Physiol* 272: C1250–C1261, 1997.
55. **Räisänen SR, Alatalo SL, Ylipahkala H, Halleen JM, Cassady AI, Hume DA, Väänänen HK.** Macrophages overexpressing tartrate-resistant acid phosphatase show altered profile of free radical production and enhanced capacity of bacterial killing. *Biochem Biophys Res Commun* 331: 120–126, 2005.
56. **Raynes DA, Guerriero V Jr.** Inhibition of Hsp70 ATPase activity and protein renaturation by a novel Hsp70-binding protein. *J Biol Chem* 273: 32883–32888, 1998.
57. **Shiels HA, Di Maio A, Thompson S, Block BA.** Warm fish with cold hearts: thermal plasticity of excitation-contraction coupling in bluefin tuna. *Proc Biol Sci* 278: 18–27, 2011.
58. **Steinbrenner H, Sies H.** Protection against reactive oxygen species by selenoproteins. *Biochim Biophys Acta* 1790: 1478–1485, 2009.
59. **Tibbits GF, Moyes CD, Hove-Madsen L.** Excitation-contraction coupling in teleost heart. In: *Fish Physiology,* vol. 12, part 12A, edited by Hoar WS, Randal DJ, Farrell AP. San Diego, CA: Academic, 1992, p. 267–304.

60. **Tunwell RE, Wickenden C, Bertrand BM, Shevchenko VI, Walsh MB, Allen PD, Lai FA.** The human cardiac muscle ryanodine receptor-calcium release channel: identification, primary structure and topological analysis. *Biochem J* 318: 477–487, 1996.
61. **Vornanen M, Hassinen M, Koskinen H, Krasnov A.** Steady-state effects of temperature acclimation on the transcriptome of the rainbow trout heart. *Am J Physiol Regul Integr Comp Physiol* 289: R1177–R1184, 2005.
62. **Vornanen M, Shiels HA, Farrell AP.** Plasticity of excitation-contraction coupling in fish cardiac myocytes. *Comp Biochem Physiol A* 132: 827–846, 2002.
63. **Vornanen M.** Na^+/Ca^{2+}-exchanger current in ventricular myocytes of fish heart: contribution to sarcolemmal Ca^{2+} influx. *J Exp Biol* 202: 1763–1775, 1999.
64. **Walton M, Lawlor P, Sirimanne E, Williams C, Gluckman P, Dragunow M.** Loss of Ref-1 protein expression precedes DNA fragmentation in apoptotic neurons. *Mol Brain Res* 44: 167–170, 1997.