2015

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RESEARCH ARTICLE

Proteomic analysis of cardiac response to thermal acclimation in the eurythermal goby fish *Gillichthys mirabilis*

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ABSTRACT

Cardiac function is thought to play a central role in determining thermal optima and tolerance limits in teleost fishes. Investigating proteomic responses to temperature in cardiac tissues may provide insights into mechanisms supporting the thermal plasticity of cardiac function. Here, we utilized a global proteomic analysis to investigate changes in cardiac protein abundance in response to temperature acclimation (transfer from 13°C to 9, 19 and 26°C) in a eurythermal goby, *Gillichthys mirabilis*. Proteomic data revealed 122 differentially expressed proteins across acclimation groups, 37 of which were identified using tandem mass-spectrometry. These 37 proteins are involved in energy metabolism, mitochondrial regulation, iron homeostasis, cytoprotection against hypoxia, and cytoskeletal organization. Compared with the 9 and 26°C groups, proteins involved in energy metabolism increased in 19°C-acclimated fish, indicating an overall increase in the capacity for ATP production. Creatine kinase abundance increased in 9°C-acclimated fish, suggesting an important role for the phosphocreatine energy shuttle in cold-acclimated hearts. Both 9 and 26°C fish also increased abundance of hexosaminidase, a protein directly involved in post-hypoxia stress cytoprotection of cardiac tissues. Cytoskeletal restructuring appears to occur in all acclimation groups; however, the most prominent effect was detected in 26°C-acclimated fish, which exhibited significantly increased actin levels. Overall, proteomic analysis of cardiac tissue suggests that the capacity to adjust ATP-generating processes is crucial to the thermal plasticity of cardiac function. Furthermore, *G. mirabilis* may optimize cellular functions at temperatures near 19°C, which lies within the species' preferred temperature range.

KEY WORDS: Cardiac function, Goby fish, Heat stress, Proteomics, Temperature acclimation

INTRODUCTION

Temperature has pronounced effects at all levels of biological organization. At the cellular level, temperature alters metabolic rates, perturbs the integrity and structures of proteins, nucleic acids and membranes, and affects a number of other processes including gene expression and protein abundance (Somero, 1995; Hochachka and Somero, 2002; Kültz, 2005; Schulte, 2007; Pörtner and Farrell, 2008; Tomanek, 2011). When challenged by changes in temperature, ectothermic species commonly modify their physiological and biochemical processes through acclimatization or acclimation. The capacity to induce an acclimatization response is thought to play a crucial role in determining the susceptibility of marine ectotherms to changes in global ocean temperature (IPCC, 2013; Somero, 2010). Investigating the underlying sub-cellular mechanisms of acclimation/acclimatization used by teleost fishes is of increasing interest (Lannig et al., 2004; Buckley et al., 2006; Farrell et al., 2009; Logan and Somero, 2010; Jayasundara and Somero, 2013).

The cardiac system is thought to play a central role in determining thermal plasticity in teleosts. With changes in ambient temperature, it is hypothesized that fish hearts have to alter their performance to compensate for the temperature-induced mismatch between oxygen supply and demand (Pörtner, 2001, 2010; Pörtner and Knust, 2007; Pörtner and Farrell, 2008; Farrell et al., 2009). This adjustment in cardiac performance may be challenging because of the anatomical organization of the teleost cardiovascular system. Because most fish hearts lack coronary circulation and receive oxygen only via diffusion from the venous return, this circulatory organization can impose a limitation in sustaining cardiac function at different temperatures (Farrell and Jones, 1992). Considering these temperature-induced challenges, cardiac tissues of eurythermal fishes should be capable of substantial phenotypic plasticity (acclimation capacity) to maintain adequate cardiac function across a range of ambient temperatures.

Based on data gathered from global gene and protein expression analyses, primarily in gill tissues, a number of physiological processes such as induction of the heat shock protein response and oxidative stress response have been proposed to be involved in mediating a thermal stress response in teleosts (Podrabsky and Somero, 2004; Buckley et al., 2006; McLean et al., 2007; Ibarz et al., 2010; Logan and Somero, 2010). However, despite the crucial role played by the cardiac system in determining thermal tolerance, little is known about gene and protein level responses to temperature in fish hearts, apart from a few transcriptomic studies focusing on highly active fish species (Vornanen et al., 2005; Castilho et al., 2009; Jayasundara et al., 2013; Anttila et al., 2014). Recent developments in proteomics technology and the increasing availability of genomic sequence information to facilitate protein identification have allowed for the successful use of proteomic experimental approaches to investigate non-model organisms (Tomanek, 2005, 2011, 2014; Dowd et al., 2008, 2010; Tomanek and Zuzow, 2010; Serafini et al., 2011; Fields et al., 2012).

To further elucidate temperature acclimatory responses in teleost hearts, we examined the cardiac proteomes of differently acclimated groups of a highly eurythermal marine teleost, the longjaw mudsucker *Gillichthys mirabilis* Cooper 1864 (family Gobiidae). *Gillichthys mirabilis* inhabits intertidal regions and estuarine slough environments along the Eastern Pacific coast, from Tomales Bay in Northern California to the Baja California peninsula and the coast of the Gulf of California (Barlow, 1961). In their habitats, these fish encounter a wide range of variation in

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Received 23 December 2014; Accepted 3 March 2015

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List of abbreviations

ANT	adenine nucleotide transporter
CK	creatine kinase
Cr	creatine
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
HEXA	hexosaminidase
HOAD	hydroxyacyl-coenzyme A dehydrogenase
HSP	heat shock protein
ICDH	isocitrate dehydrogenase
LDH A	lactate dehydrogenase A
MDH	malate dehydrogenase
MPT	mitochondrial permeability transition
OCLTT	oxygen and capacity limited thermal tolerance
PCr	phosphocreatine
PRDX 3	thioredoxin-dependent peroxide reductase 3
ROS	reactive oxidative species
SDH	succinate dehydrogenase
SR	sarcoplasmic reticulum
TFA	trifluoroacetic acid
VDAC	voltage-dependent anion channel
WAP-65	warm temperature acclimated protein 65-1

many abiotic factors including widely fluctuating temperatures (between ~5 and ~37°C) (Buckley and Hofmann, 2002, 2004). A number of studies have demonstrated the thermal plasticity of *G. mirabilis* at the whole-organism, physiological, cellular and molecular levels (Sumner and Doudoroff, 1938; Somero and Doyle, 1973; Dietz and Somero, 1992; Buckley and Hofmann, 2002, 2004; Logan and Somero, 2010, 2011). A recent study of cardiac activity in *G. mirabilis* (Jayasundara and Somero, 2013) revealed significant effects of acclimation on the thermal limits of cardiac function, particularly when fish were responding to an acute heat stress. These results indicate that the cardiac system can induce an effective acclimatory response over a range of temperatures and can play a crucial role in supporting aerobic performance and altering thermal limits.

To elucidate cardiac protein abundance changes that might be associated with optimizing or maintaining heart function at different temperatures, we examined changes in global protein levels in fish acclimated for 4 weeks to temperatures ranging both well below (9°C) and above (26°C) the species' preferred range (near 19°C) – an acclimation regime that is in parallel with previous studies (Jayasundara and Somero, 2013). Overall, this study provides new insights into proteomic modifications underlying cardiac phenotypic plasticity with temperature acclimation and shows the potential importance of metabolic reorganization in maintaining adequate energy supplies to support cardiac function under different thermal conditions.

RESULTS

Protein gel image analysis revealed 486 distinct protein spots across all acclimation groups. Of these, 122 proteins (25%) showed significant abundance changes among groups (one-way ANOVA, $P<0.05$ and Bonferroni *post hoc* testing) (Fig. 1). From this set of 122 proteins, we were able to identify 37 proteins using mass spectrometry (supplementary material Table S1). Because post-translational modifications, e.g. phosphorylation and acetylation, can shift the molecular weight or isoelectric point of proteins and may lead to identification of the same protein as two or more different protein spots, one spot alone might not represent the total abundance of a protein. Of the 37 proteins identified, six proteins indicated abundance changes of multiple isoforms or represented a unique spot due to post-translational modifications. Next, we describe these changes in protein abundance based on broad functional categories.

Energy metabolism

The largest fraction of the identified proteins – 17 of 37 proteins (48%) – that exhibited altered abundance were involved in energy metabolism. An enzyme involved in β-oxidation of fatty acids, hydroxyacyl-coenzyme A dehydrogenase (HOAD), increased by ~2-fold in 9°C-acclimated fish compared with all other groups (Fig. 2). Ten distinct protein spots, representing six unique classes of glycolytic proteins, were identified as being different in abundance among acclimation groups (Fig. 3). Three distinct protein spots each were identified for fructose bisphosphate aldolase (two representing aldolase A and one representing aldolase C) (EC 4.1.2.13) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (EC 1.2.1.12). Three of the six proteins, GAPDH, enolase (EC 4.2.1.11) and pyruvate kinase (EC 2.7.1.40), showed a decrease in abundance by over 3-fold in both 9°C- and 26°C-acclimated fish, compared with 19°C-acclimated fish. Aldolase A isoforms showed a similar trend in their abundance profiles, but were highest in 13°C-acclimated fish. Aldolase C, an isoform primarily expressed in brain tissues in mammals and one that has a 5-fold lower K_m for fructose 1,6-bisphosphate compared with aldolase A (Berardini et al., 1997), increased at 9°C by ~5-fold compared with all other groups. Lactate dehydrogenase (LDH) A (EC 1.1.1. 27), another glycolytic protein, abundance decreased with acclimation by over 2-fold in 9°C relative to other treatments.

Six proteins involved in aerobic ATP production were identified (Fig. 4). Four of these proteins – aconitase (EC 4.2.1.3), isocitrate dehydrogenase (EC 1.1.1.42) (ICDH), succinate dehydrogenase (EC 1.3.5.1) (SDH) and malate dehydrogenase (MDH) (EC 1.1.1.37) – are involved in the citric acid cycle (Fig. 4). Additionally, the α and β subunits of mitochondrial ATP synthase, which is involved in oxidative phosphorylation, were identified. Except for aconitase (which decreased only in 9°C hearts by ~3-fold) and SDH (which was equally highly abundant in both 19°C- and 26°C-acclimated hearts), all of the proteins identified here showed an increased abundance (by over 2-fold) in 19°C-acclimated fish. ICDH also showed an increased abundance in 19°C-acclimated fish compared with other treatments, but was also increased in 26°C-acclimated fish compared with 13°C- and 9°C-acclimated fish.

Fig. 5 represents three key proteins involved in maintaining cellular energy homeostasis. Creatine kinase (CK) B, the isoform expressed primarily in brain tissues in mammals, which is involved in phosphocreatine energy shuttle, increased in abundance by ~3-fold in 9°C-acclimated fish (Fig. 5A). Adenylate kinase (EC 2.7.4.3), a protein that is involved in ATP generation, decreased by ~3-fold in 9°C- and 26°C-acclimated fish in comparison to the 13°C- and 19°C-acclimation groups (Fig. 5B). We also identified two isoforms of voltage-dependent anion channel (VDAC 2a and 2b). The two isoforms differ in predicted isoelectric point and molecular weight, but demonstrated sequence homology with the VDAC2 isoform in different fish species. VDAC2a abundance increased ~1.5- to 2-fold in all three acclimation groups compared with fish acclimated to 13°C (Fig. 5C). VDAC2b abundance increased ~2.5- to 3-fold in 26°C-acclimated fish compared with other acclimation groups (Fig. 5D).

Cytoskeletal structure

Five proteins involved in cytoskeletal structure – three isoforms of actin, a tubulin, two isoforms of an actin binding protein, ezrin/moesin and keratin-like protein – were identified (Fig. 6). All three

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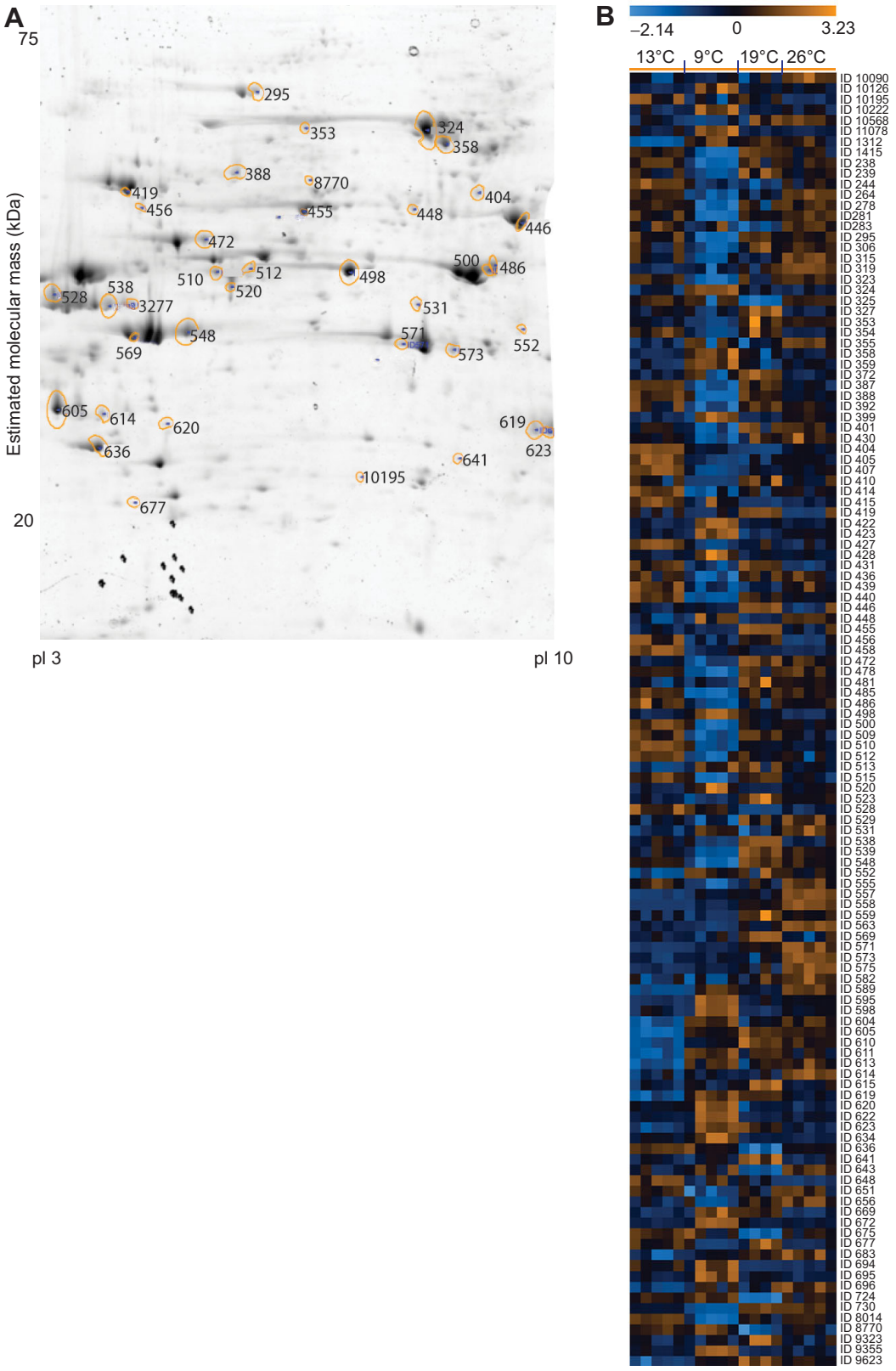

Fig. 1. Protein expression in cardiac tissue from differently acclimated *Gillichthys mirabilis*. (A) A proteome map depicting 486 protein spots from *Gillichthys mirabilis* cardiac tissues from all gels generated from fish acclimated to ambient temperature (*N*=5) or 9°C (*N*=5), 19°C (*N*=4) and 26°C (*N*=4) temperatures. The proteome map represents average pixel volumes for each protein spot. Highlighted spots are those that showed significantly different changes in abundance (one-way ANOVA, $P<0.05$, Bonferroni *post hoc* test) and were identified using tandem mass spectrometry (37 proteins). (B) Heat map depicting expression profiles of 122 proteins that were expressed significantly differently (one-way ANOVA, $P<0.05$, Bonferroni *post hoc* test). The vertical axis represents the protein expression patterns calculated based on normalized spot volumes of each protein. Blue coloring represents down-regulated proteins and orange represents up-regulated proteins. The horizontal axis shows the different acclimation temperature treatments.

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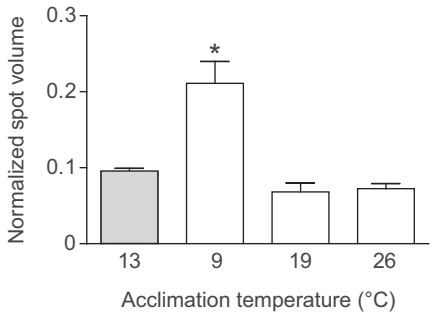

Fig. 2. 3-Hydroxyacyl-COA dehydrogenase (HOAD) protein abundance in heart tissues of fish acclimated to different temperatures. Acclimation temperatures: ambient (13–14°C, gray bar) *N*=5; 9°C, *N*=5; 19°C, *N*=4; and 26°C, *N*=4. Spot volumes were obtained by normalizing against the volume of all proteins and show means±s.e.m. An asterisk indicates a significant difference compared with all other values (one-way ANOVA and Tukey's pairwise comparison, $P<0.05$).

actin isoforms increased with warm acclimation, with 26°C-acclimated fish showing the highest abundance. One of the actin binding protein isoforms and tubulin decreased by ~1.5- to 2-fold in all acclimation groups compared with 13°C-acclimated fish. Ezrin/moesin was markedly lower (~4 fold) in 9°C- and 26°C-acclimated fish, and keratin-like protein was up-regulated (~2- to 3-fold) in all three acclimation groups compared with 13°C-acclimated fish.

Protein turnover

Three proteins involved in protein degradation and transport were identified (Fig. 7A). Selenium binding protein, a protein involved in inter-Golgi apparatus protein transport (Porat et al., 2000), exhibited ~3-fold lower abundance in 19°C-acclimated fish compared with the other treatments. Two other protein isoforms involved in protein degradation were identified as cathepsin B; both forms were higher in 9°C- and 19°C-acclimated fish and only one was higher in 26°C-acclimated specimens when compared with the 13°C-acclimated fish. Cathepsins are lysosomal proteases involved in protein degradation and turnover (Chan et al., 1986). A protein involved in proteasomal activity – proteasome β-3 subunit – was also identified in the current study (Nothwang et al., 1994). This subunit was 3-fold higher in 19°C-acclimated specimens compared with other acclimation temperatures.

Other proteins

Proteins involved in several other cellular processes were also differentially expressed (Fig. 7B–D). Two proteins associated with iron homeostasis – serotransferrin and warm temperature acclimated protein 65-1 (WAP-65) – increased (~2-fold) in 9°C-acclimated fish (Fig. 7B). Thioredoxin-dependent peroxide reductase (PRDX) 3 decreased by ~1.5-fold in both 9°C- and 26°C-acclimated fish compared with 13°C fish (Fig. 7C). Hexosaminidase (HEXA) protein increased in both 9°C- and 26°C-acclimated groups by ~3- to 3.5-fold compared with both ambient and 19°C-acclimated fish (Fig. 7D).

DISCUSSION

The overall analysis revealed significant changes in overall proteome with temperature acclimation. Here, we focus on the 37 proteins that were identified using mass spectrometry. Considering that 48% of the proteins that exhibited altered abundance were involved in energy metabolism, we focus much of our discussion on cellular ATP-generating processes and infer that substantial shifts in energy metabolism are essential for acclimation of heart function. These shifts also reflect use of a different balance of substrates and re-arrangements of metabolons. The 17 proteins involved in energy metabolism and the other 20 proteins identified here are discussed below based on their roles in various cellular processes. It should be noted that future analyses, particularly with advances in genome sequencing efforts, may enable annotation of the 85 protein spots that remained unidentified, providing further insights into temperature acclimation effects on the cardiac proteome of *G. mirabilis*.

Shifts in cardiac energy metabolism and mitochondrial regulation

Lipid metabolism

HOAD enzyme activity is frequently used as a quantitative index for lipid catabolism (Driedzic, 1992). The abundance of this protein was increased in 9°C-acclimated fish (Fig. 2). A similar increase in the expression of genes associated with lipid metabolism was detected in gills of 9°C-acclimated *G. mirabilis* (Logan and Somero, 2010), suggesting an overall increase in lipid catabolism with cold acclimation in this fish. This conjecture is in concert with observations made with other species of fish at low temperatures: fatty acid synthesis and catabolism increase in fish hearts under cold temperatures (Driedzic et al., 1987; Crockett and Sidell, 1990; Driedzic, 1992). These changes may reflect a shift to a more aerobic, lipid-fueled metabolism at low temperatures, where dissolved oxygen is more available, and may also play a role in compensating for Q_{10} effects.

Glucose metabolism

Overall data on glycolytic protein abundance suggest a significant decrease in glycolytic capacity in 9°C-acclimated fish, and to a lesser degree in 26°C-acclimated fish, compared with the 19°C group (Fig. 3). The abundance profiles of 13°C-acclimated specimens appeared to represent a mixture of features of the 19°C- and 9°C-acclimated fish. In the context of the hypothesis known as 'oxygen and capacity limited thermal tolerance' (OCLTT) (Pörtner and Knust, 2007; Pörtner and Farrell, 2008), one interpretation of the elevated abundance of proteins involved in glycolysis in 19°C-acclimated fish is that this is a consequence of limited O_2 supply to the heart, which leads to increased reliance on anaerobic ATP generation. However, if the observed increase in glycolytic enzymes is a reflection of OCLTT effects, we would expect further increases in anaerobic capacity in 26°C-acclimated fish, which was not observed. The concurrent increase in abundance of proteins involved in aerobic metabolism in 19°C-acclimated fish (see below) and low blood lactate levels in 19°C-acclimated fish compared with 9°C-acclimated fish (Jayasundara and Somero, 2013) further suggest that an oxygen limitation interpretation is not likely to be correct. Thus, it appears that 19°C-acclimated fish have increased their capacity for aerobic ATP production, using glucose as the principal metabolic substrate and requiring elevated flux through glycolysis.

Acclimation temperature-specific re-organization of metabolism also is apparent at low temperature. Considering the reduced abundance of glycolytic proteins, particularly LDH, in 9°C-acclimated fish and the marked increase in lipid metabolizing HOAD protein abundance detected at 9°C, we infer that 9°C-acclimated fish hearts may have shifted their metabolic fuel preference from carbohydrates to lipids, as found in other cold-acclimated fish (Driedzic, 1992).

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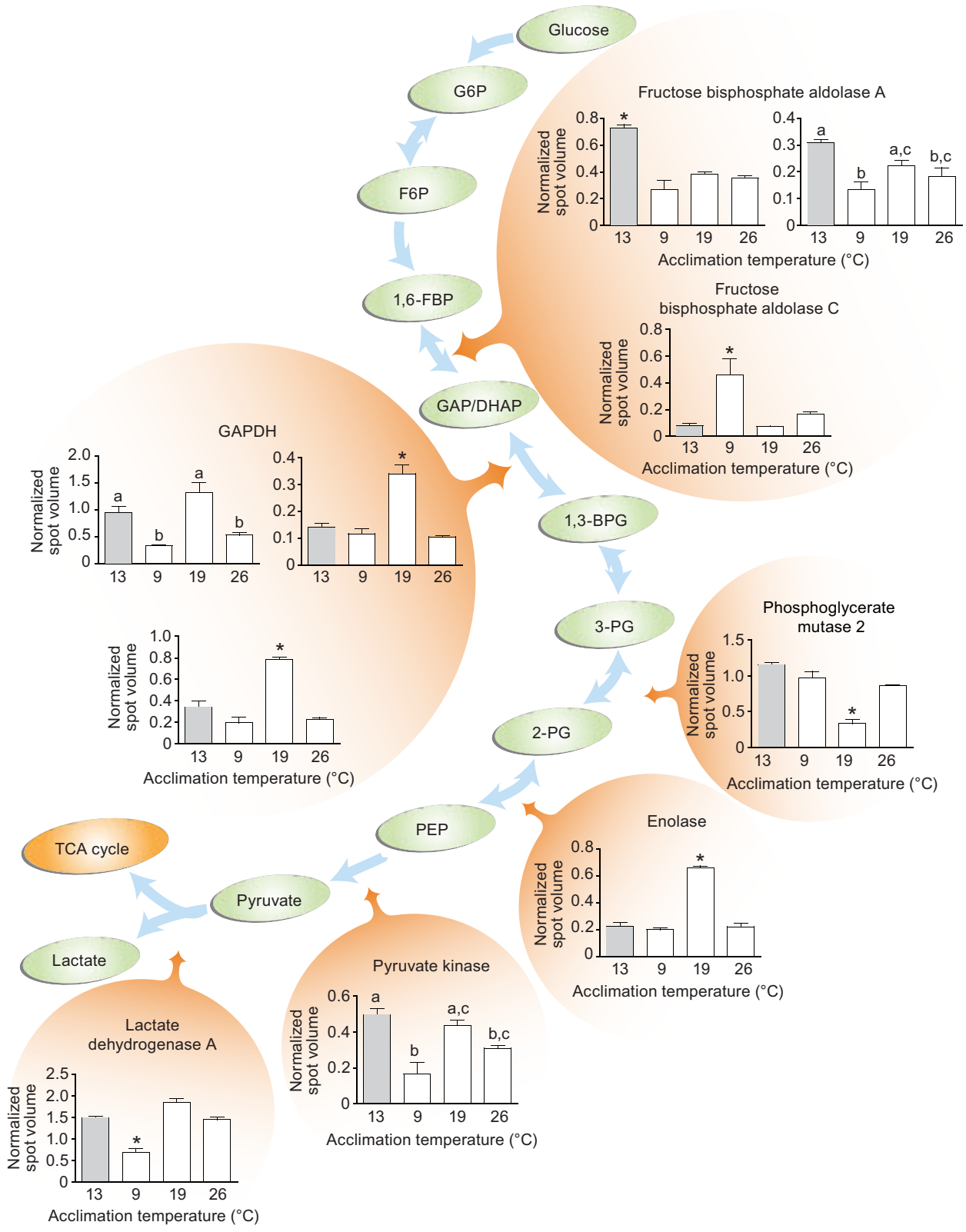

Fig. 3. Glycolytic protein abundance in heart tissues of fish acclimated to different temperatures. Acclimation temperatures: ambient (13–14°C, gray bar), *N*=5; 9°C, *N*=5; 19°C, *N*=4; and 26°C, *N*=4. Spot volumes were obtained by normalizing against the volume of all proteins and show means±s.e.m. An asterisk indicates when only one value is significantly different and all other values are similar to each other. Different letters denote significant differences (same letter denotes no difference) in all values compared with each other (one-way ANOVA and Tukey's pairwise comparison, $P<0.05$). GAPDH, glyceraldehyde 3-phosphate dehydrogenase; G6P, glucose 6-phosphate; F6P, fructose 6-phosphate; 1,6-FBP, fructose 1,6-bisphosphate; GAP/DHAP, glyceraldehyde 3-phosphate/dihydroxyacetone phosphate; 1,3-BPG, 1,3-bisphosphoglyceric acid; 3-PG, 3-phosphoglyceric acid; 2-PG, 2-phosphoglyceric acid; PEP, phosphoenolpyruvate; TCA, tricarboxylic acid cycle.

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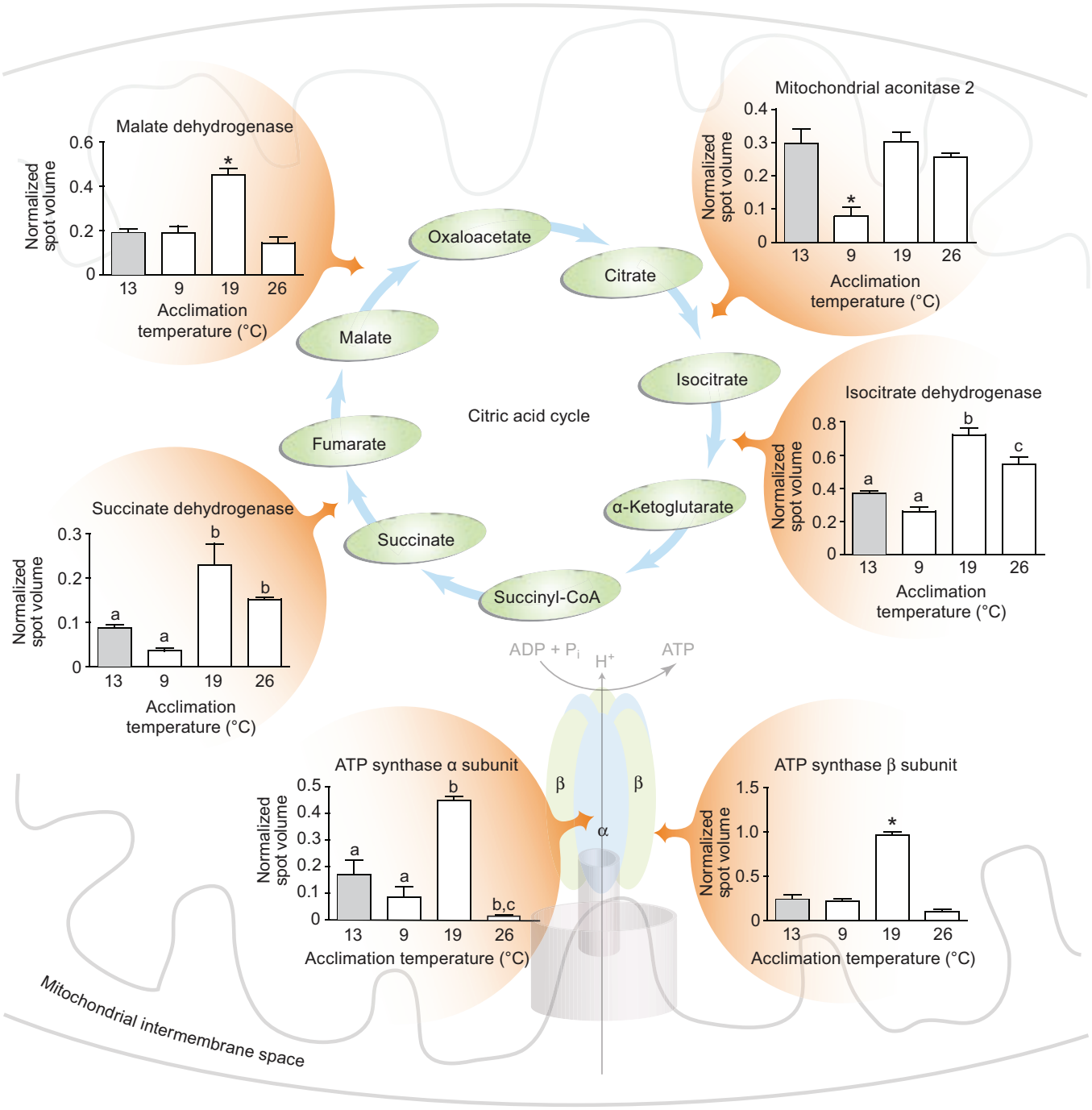

Fig. 4. Abundance levels of proteins involved in the citric acid cycle and oxidative phosphorylation in heart tissues of fish acclimated to different temperatures. Acclimation temperatures: ambient (13–14°C, gray bar), *N*=5; 9°C, *N*=5; 19°C, *N*=4; and 26°C, *N*=4. Spot volumes were obtained by normalizing against the volume of all proteins and show means±s.e.m. An asterisk indicates when only one value is significantly different and all other values are similar to each other. Different letters denote significant differences (same letter denotes no difference) in all values compared with each other (one-way ANOVA and Tukey's pairwise comparison, $P<0.05$).

Citric acid cycle and oxidative phosphorylation

Overall, the relative abundance of proteins involved in aerobic ATP production was highest in 19°C-acclimated fish and lowest in 9°C- and 13°C-acclimated fish. The higher protein abundance for aconitase, ICDH, SDH, MDH and ATP synthase in 19°C-acclimated fish suggests an increased capacity for cardiac ATP synthesis in this group compared with other acclimation groups (Fig. 4).

The decrease in the abundance of some of the proteins involved in the citric acid cycle in 26°C-acclimated fish might be viewed as thermal compensation by the heart to a higher acclimation temperature, i.e. a means for offsetting Q_{10} effects that otherwise would significantly elevate metabolic rate. Considering that the protein abundance of SDH, MDH and ICDH in 26°C-acclimated fish is only decreased by ~30–60% compared with that of 19°C-acclimated fish, and assuming a Q_{10} value of 2, metabolic reaction rates may be similar in 26°C- and 19°C-acclimated fish at their respective acclimation temperatures. However, the marked decrease in two constituents of the ATP synthase complex,

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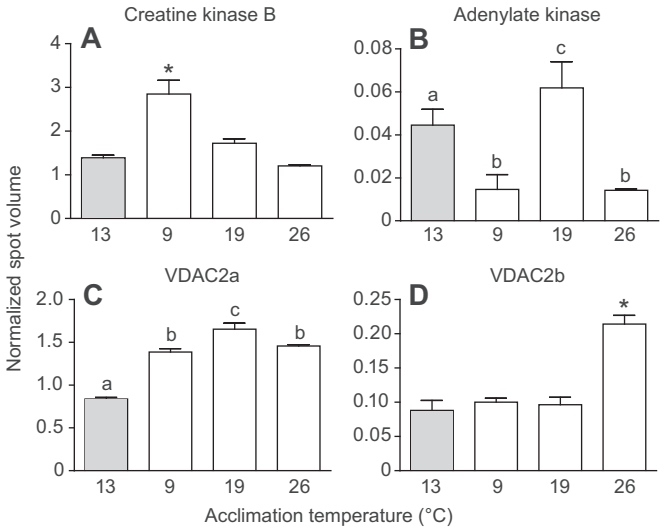

Fig. 5. Abundance levels of proteins involved in cellular energy homeostasis in heart tissues of fish acclimated to different temperatures. (A) Creatine kinase B, (B) adenylate kinase (C,D) voltage-dependent anion channel protein (VDAC2a and 2b). Acclimation temperatures: ambient (13–14°C, gray bar), *N*=5; 9°C, *N*=5; 19°C, *N*=4; and 26°C, *N*=4. Spot volumes were obtained by normalizing against the volume of all proteins and show means±s.e.m. An asterisk indicates when only one value is significantly different and all other values are similar to each other. Different letters denote significant differences (same letter denotes no difference) in all values compared with each other (one-way ANOVA and Tukey's pairwise comparison, $P<0.05$).

particularly a 90% decrease in ATP synthase α-subunit, suggests that the overall capacity for oxidative phosphorylation in 26°C-acclimated fish hearts is likely to be lower than in 19°C-acclimated fish. Notably, Jayasundara and Somero (2013) showed no thermal compensation in resting heart rates with acclimation – that is, resting heart rate did not decrease over time to counteract Q_{10} effects on heart rates – in 26°C-acclimated fish and remained ∼35 beats min^{-1} higher than in the 19°C-acclimated group. This implies an elevated cardiac muscle activity in 26°C-acclimated fish at rest. The current data on the abundance of enzymes involved in aerobic metabolism in 26°C-acclimated fish suggest that sustaining increased muscle activity is not supported by an increase in ATP-generating capacity. It is possible that other processes, such as an increase in the efficiency of cardiac excitation–contraction coupling mechanisms or an increased concentration of primary structural proteins, e.g. actin (see 'Cytoskeletal reorganization', below) and myosin in the heart muscles, may account for at least a portion of the altered performance of hearts of 26°C-acclimated fish (Bers, 2002; Vornanen et al., 2002). Interestingly, 26°C-acclimated fish hearts also showed an improved acute thermal response with acclimation; maximum heart rate in response to an acute increase in temperature increased by ∼35 beats min^{-1} over the 4 week acclimation period in this group. In addition, compared with 19°C-acclimated fish, both maximum heart rate and temperature at the onset of maximum heart rate were increased, by ∼15 beats min^{-1} and 1.5°C, respectively, in 26°C-acclimated fish (Jayasundara and Somero, 2013), suggesting an enhanced acute thermal stress response in heart tissues in these fish. However, considering the protein abundance changes discussed earlier, this increase in acute response appears to be decoupled from aerobic ATP-generating capacity.

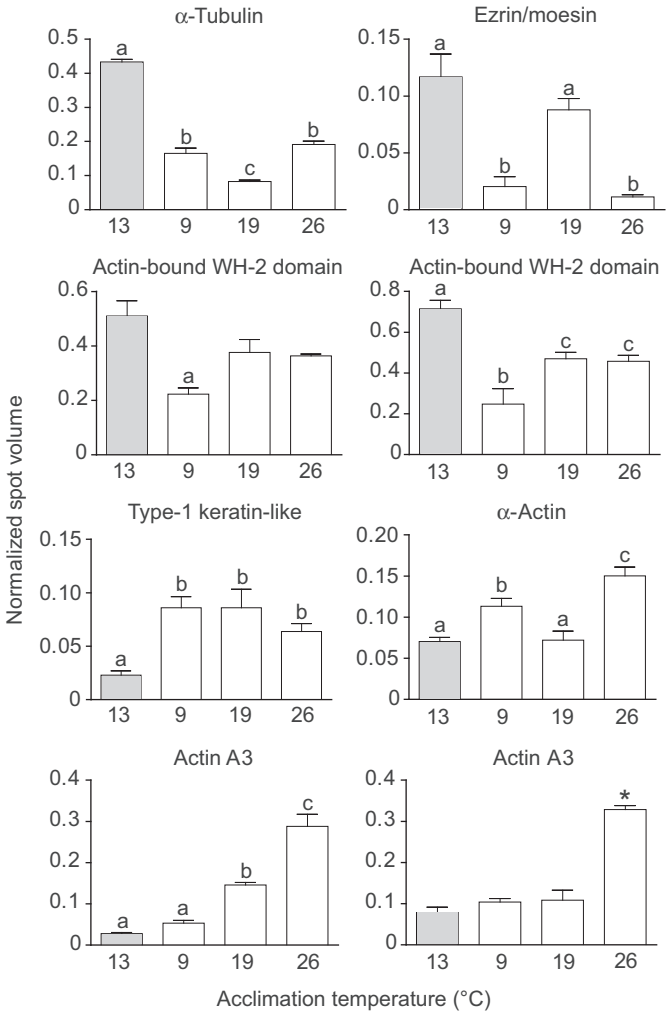

Fig. 6. Abundance levels of proteins involved in cytoskeletal reorganization in heart tissues of fish acclimated to different temperatures. Acclimation temperatures: ambient (13–14°C, gray bar), *N*=5; 9°C, *N*=5; 19°C, *N*=4; and 26°C, *N*=4. Spot volumes were obtained by normalizing against the volume of all proteins and show means±s.e.m. An asterisk indicates when only one value is significantly different and all other values are similar to each other. Different letters denote significant differences (same letter denotes no difference) in all values compared with each other (one-way ANOVA and Tukey's pairwise comparison, $P<0.05$).

Regulation of energy metabolism through the phosphocreatine energy circuit

Metabolic rates in cardiac muscles are in part regulated by the rearrangement of macromolecular assemblies and tight coupling of ATP-producing (e.g. mitochondrion) and -consuming processes [e.g. myosin activation, sarcoplasmic reticulum (SR) Ca^{2+}-ATPase] through the phosphocreatine circuit (Guzun et al., 2012; Wallimann et al., 2007). CK, which catalyzes the reaction of ATP + creatine (Cr) to phosphocreatine (PCr), plays a role in this circuit in several ways. First, PCr has a greater diffusion rate than ATP (Vendelin et al., 2004). Second, Cr and PCr are metabolically inert in comparison to ADP and ATP, as no enzymes other than CK bind them, which means that PCr only gets depleted where CK is localized. Third, because the Gibbs free energy of ATP hydrolysis is dependent on the ratio of ATP to ADP, and CK coupled (co-localized or compartmentalized) to ATPases can quickly replenish ATP from phosphocreatine, ΔG_{ATP} is always maximal in the vicinity of ATPases (Dzeja and Terzic, 2003). To further emphasize

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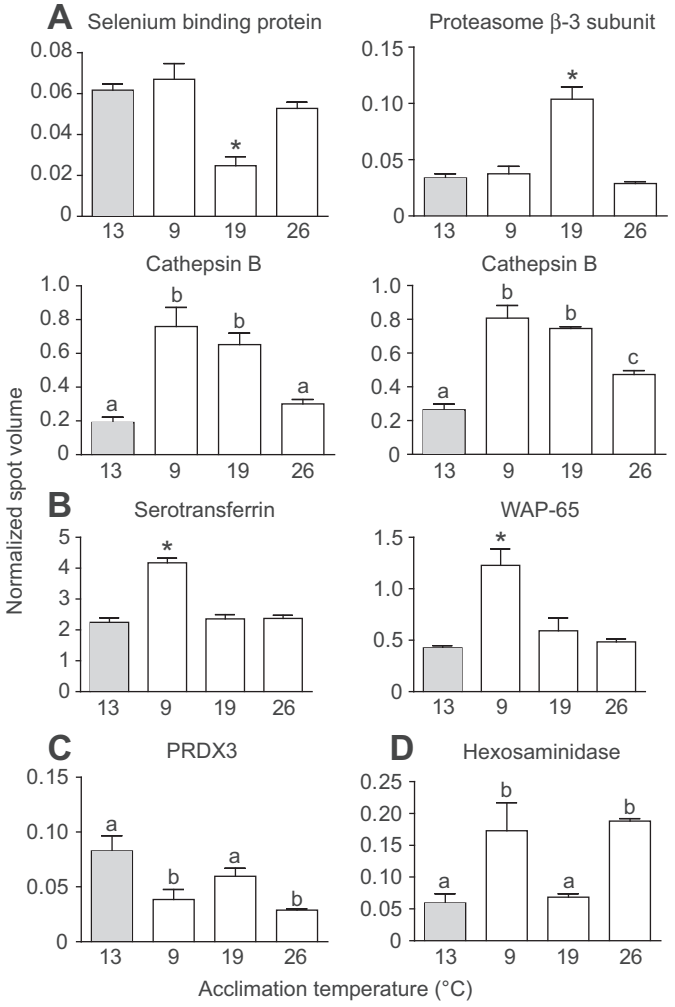

Fig. 7. Abundance levels of proteins involved in other cellular processes that were differentially expressed in heart tissues of fish acclimated to different temperatures. (A) Protein degradation and transport, (B) iron homeostasis, (C) thioredoxin-dependent peroxide reductase (PRDX) and (D) hexosaminidase. Acclimation temperatures: ambient (13–14°C, gray bar), *N*=5; 9°C, *N*=5; 19°C, *N*=4; and 26°C, *N*=4. Spot volumes were obtained by normalizing against the volume of all proteins and show means±s.e.m. An asterisk indicates when only one value is significantly different and all other values are similar to each other. Different letters denote significant differences (same letter denotes no difference) in all values compared with each other (one-way ANOVA and Tukey's pairwise comparison, $P<0.05$).

the importance of this factor, $\Delta G^{2+}_{Ca\ transport}$ for Ca^{2+} uptake (+51 kJ mol^{-1}) is only slightly less than the energy released by ATP hydrolysis (55 kJ mol^{-1}) under physiological conditions, highlighting the need for a locally high ATP:ADP ratio (Wallimann and Hemmer, 1994). Fourth, tight coupling between CK and SR Ca^{2+}-ATPase leads to CK-PCr-derived ATP and the dissociation of ADP from the Ca^{2+}-ATPase, thereby lowering the apparent K_m for ATP by the Ca^{2+}-ATPase (Wallimann et al., 2007).

It is unclear whether the CK protein detected here (Fig. 5A) is the cytosolic or the mitochondrial isoform; a close examination of the CK peptide sequences identified suggests that it is more likely to be the cytosolic isoform. Based on the role of the cytosolic isoform of CK within the PCr circuit, it is possible that the increase in the abundance of this protein in cardiac muscle of cold-acclimated *G. mirabilis* contributes to maintaining higher ATP:ADP ratios during periods of high ATP turnover (Dzeja and Terzic, 2003) and, thereby, compensates for reduced reaction rates in cold temperatures (Christensen et al., 1994). Its invertebrate analog, arginine kinase, has been shown to play an important role during temperature acclimation in the cheliped myofibril tissue of porcelain crabs (Garland et al., 2015).

Adenylate kinase plays a crucial role in maintaining cellular energy homeostasis by converting two ADPs to ATP and AMP (Fischer, 2013), thereby generating ATP for ATPases and AMP as a substrate for oxidative phosphorylation. Its contribution to ATP generation might be coupled to CK or not, depending on ATP turnover rates (Dzeja and Terzic, 2003; Dzeja et al., 1996).

The mitochondrial isoform of CK also forms complexes with VDAC and the adenine nucleotide transporter (ANT) (Brdiczka, 2007). VDACs are generally involved in the transport of organic anions, mitochondrial metabolites, (phospho-) creatine, ATP, ADP and inorganic phosphate (P_i) across the outer mitochondrial membrane, which raises the possibility that the closure of these channels regulates the overall activity of the mitochondrion (Lemasters and Holmuhamedov, 2006; Lemasters et al., 2012). ANTs are antiporters that exchange one ATP for one ADP. Octameric CK forms complexes with both VDACs on the outer mitochondrial membrane and with cardiolipin patches that surround the ANT (Wallimann et al., 2007). The formation of the ANT–CK–VDAC complex allows CK to convert the ATP transported by the ANT across the inner membrane into PCr, which can be transported across the outer membrane by VDAC, affecting channeling of the ATP substrate and the PCr product. Importantly, the ADP from the CK reaction can be immediately transported back to serve as a substrate for oxidative phosphorylation. The low ATP:ADP ratio of the matrix that results from this phosphoryl transfer reaction supports the synthesis of ATP by ATP synthase. Thus, an increased abundance of CK could facilitate quick recycling of ADP from ATP and therefore maintain low ATP:ADP ratios at the site of ATP production (mitochondrion) or the sustained supply of ATP through PCr at the site of high ATP turnover, e.g. ATPases. Because it is more likely that the CK isoform we identified is cytosolic, we posit that CK plays a greater role in the latter of the two processes in this context, i.e. greater supply of ATP at the site of high ATP turnover.

In addition to the complex with CK, different VDAC isoforms and their conformations form alternative complexes with e.g. hexokinase, which affects the phosphorylation of glucose and thus the rate of glycolysis (Brdiczka, 2007). Thus, through the formation of complexes with ANT, CK and hexokinase, VDAC links oxidative phosphorylation with glycolysis. Whereas complex formation with hexokinase seems to be specific to VDAC1 (Blachly-Dyson et al., 1993), the present data set showed that VDAC2a mirrors the patterns in the glycolytic enzymes (highest abundance at 19°C), suggesting a potential link between this isoform and glycolytic processes. However, VDAC2b increased in 26°C-acclimated fish and might therefore have a distinct role from VDAC2a. VDACs are also part of the mitochondrial permeability transition (MPT) pore, which might play a role during the mitochondrial pathway of apoptosis (Brdiczka, 2007). VDAC2, possibly as part of the MPT pore, can inhibit the activation of mitochondrial apoptosis (Cheng et al., 2003). Thus, the increase in VDAC2, particularly VDAC2b at the highest acclimation temperature, might be an anti-apoptotic measure. Furthermore, VDAC2 associates with the ryanodine receptor 2 (RyR2) at the junction between the SR and the mitochondrion and might affect Ca^{2+} uptake in cardiac muscles (Min et al., 2012). It should be noted that the importance of internalized Ca^{2+} stores, and thus the roles of SR Ca^{2+}-ATPase and RyR2, in excitation–contraction coupling of

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G. mirabilis hearts remains to be studied. Finally, VDAC might also play a role in maintaining high ATP turnover rates while other metabolic enzymes decreased their abundance in 26°C-acclimated fish, especially as CK levels stayed the same between 19°C- and 26°C-acclimated fish.

Temperature and oxygen availability have several effects on mitochondrial respiration rates and membrane structure (Lemasters, and Holmuhamedov, 2006), and thermal acclimation can alter the fluidity and composition of mitochondrial membranes (Dahlhoff et al., 1991; Hochachka and Somero, 2002). Teleost cardiac tissues, when experiencing changes in ambient temperature, particularly at high temperatures, may experience reduced oxygen levels as a result of decreased blood oxygen content in the venous return (Lannig et al., 2004; Farrell et al., 2009). The combined effects of high temperature and low oxygen availability in fish cardiac myocytes may require profound changes in mitochondrial function and regulation. VDAC2 may play a role in this regulation during acclimation to a new temperature. Differential abundance profiles detected in the two VDAC2 proteins suggest temperature-specific roles for each protein. The increased expression of VDAC2b in 26°C-acclimated fish suggests that this protein might be crucial to sustaining cardiac mitochondrial activity at higher temperatures. Given the physiological evidence that heart rate is sustained at higher levels in 26°C-acclimated *G. mirabilis* (Jayasundara and Somero, 2013), it is likely that a new steady-state condition is also established in cardiac tissues of *G. mirabilis* and that both VDAC2a and VDAC2b, as global controllers of mitochondria, play a crucial role in this process.

In summary, overall data on proteins involved in cellular energy metabolism and mitochondrial function indicate an increased capacity for ATP production in fish acclimated to 19°C compared with 9°C- and 26°C-acclimated fish. Only two enzymes showed higher abundances with cold acclimation, thus showing cold compensation: aldolase C and CK. Given the role of CK in the PCr shuttle of phosphoryl groups and thus the maintenance of high rates of ATP production and hydrolysis in tissues such as cardiac muscle that require high ATP turnover, the increase might be beneficial in overall energy metabolism in the cold without having to increase the abundance of other metabolic enzymes. Interestingly, both of these proteins were also identified as the brain-specific isoform based on sequence homology. Considering the high energetic demand and ATP-turnover rates in brain tissues, it is possible that these isoforms contribute to compensate for reduced metabolic reaction rates in cold-acclimated fish.

In the 13°C-acclimated group, the abundance of metabolic enzymes remained similar to that of one or both 19°C- and 9°C-acclimated groups, except for one of the aldolase A and VDAC isoforms. This mixed response parallels the cardiac performance data for temperature-acclimated *G. mirabilis* (Jayasundara and Somero, 2013). In that study, maximum heart rate and other physiological parameters were decreased in 9°C-acclimated fish and increased in 19°C-acclimated fish compared with the 13°C group. However, this trend was not consistent across the proteome as discussed below for proteins involved in cytoskeletal structure. The somewhat contradictory patterns of protein abundance for fish held at 13°C may be the result of a longer period of acclimation at a constant temperature (9 weeks) compared with the other groups (4 weeks).

Cytoskeletal reorganization

Changes in the abundance of cytoskeletal proteins suggest significant reorganization of the cytoskeleton with acclimation (Fig. 6). Several studies on marine teleosts and invertebrates have implicated temperature-induced cytoskeletal reorganization at both the transcriptome and the proteome level (Podrabsky and Somero, 2004; Vornanen et al., 2005; Buckley et al., 2006; Lockwood et al., 2010; Tomanek and Zuzow, 2010). These cytoskeletal changes have been discussed in light of small heat shock protein (HSP) chaperoning activity; small HSPs are triggered by cytoskeletal perturbations and are thought to play a crucial role in stabilizing the cytoskeleton (Derham and Harding, 2002; Haslbeck et al., 2005). In the current study, we did not detect any changes in any molecular chaperoning molecules, suggesting that *G. mirabilis* may have attained a steady-state cytoskeletal structure with acclimation.

As discussed above, 26°C-acclimated *G. mirabilis* maintained elevated resting heart rate compared with other groups (Jayasundara and Somero, 2013). Earlier discussion on the aerobic capacity of cardiac muscles suggested that this elevated muscle activity is not associated entirely with increased aerobic ATP generation. It is possible that the increase in actin abundance detected in 26°C-acclimated fish may play a role in modifying cardiac contractile machinery to maintain higher resting heart rates and also improve the acute thermal response compared with 19°C-acclimated fish. Increased contractility is associated with elevated α-skeletal actin content in the mouse heart (Hewett et al., 1994). However, a direct relationship between temperature and actin abundance in improving contractility has not been shown. These data, coupled with differences detected in abundance of other cytoskeletal proteins in all acclimation groups compared with the 13°C-acclimated fish, suggest that cytoskeletal reorganization plays an important role in the cardiac temperature acclimation response.

Protein turnover

Temperature stress is known to challenge proteostasis, leading to protein damage. Abnormally folded proteins or proteins that are not repaired are selectively degraded by the ubiquitin–proteasomal pathway or via lysosomal proteolysis (Kültz, 2005). Changes in the abundance of cathepsins, proteasome β-3 subunit and selenium binding protein (Fig. 7A) in 19°C-acclimated fish hearts suggest that they may have the highest protein degradation rates compared with other groups. Considering that this temperature has been shown by physiological studies to be within the preferred temperature range of the organism (Jayasundara and Somero, 2013), it is conceivable that maintaining a high capacity for protein turnover in cardiac tissues might be beneficial to sustaining cellular homeostasis. However, if maintaining an increased cathepsin level is beneficial, it is difficult to interpret the decreased abundance of these proteins in hearts of 13°C- and 26°C-acclimated fish, particularly in the warmest treatment, as higher temperatures can lead to increased protein degradation. Similar to the current study, a transcriptomic study by Logan and Somero (2010) showed that warm-acclimated gill tissues had an overall decrease in expression of genes coding for cathepsins and proteasomal degradation pathways. However, a subset of genes coding for ubiquitin-dependent proteolysis were up-regulated. Direct measurements of protein degradation rates in *G. mirabilis* gill tissues also showed an increase in the rate of proteolytic activity in warm-acclimated fish (Somero and Doyle, 1973). This discrepancy between increased proteolytic activity and decreased abundance of proteins involved in protein degradation might potentially be due to differences in tissues – gills versus hearts – tested in the two studies. Furthermore, the proteasome complex is a large, multimeric structure composed of numerous

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subunits, making interpretation of changes in expression of a few subunits difficult. These findings warrant further research to better understand tissue-specific protein biosynthesis and degradation with temperature acclimation.

Reversibly damaged proteins are refolded by molecular chaperones such as HSPs. Several studies investigating vertebrate hearts including those of teleosts have shown an induction of cytoprotective networks including HSP expression and other molecular chaperones (Vornanen et al., 2005; Horowitz, 2010). One surprising finding – or lack thereof – in the current study is the absence of induction of a molecular chaperoning response. Logan and Somero (2010) also showed that mRNA for HSPs did not vary among acclimation groups in cold- and warm-acclimated gill tissues of *G. mirabilis*. The lack of induction of such a molecular chaperoning network may be due to experimental limitations resulting from the challenges of measuring chaperone levels in muscle tissues (Carberry et al., 2014). However, it is also possible that *G. mirabilis* hearts were able to restore cellular homeostasis and reach a 'steady-state' cardiac phenotype with acclimation, leading to similar expression profiles of molecular chaperones across treatments.

Iron homeostasis, antioxidant response and hypoxic injury

The increase in abundance of serotransferrin and WAP-65 proteins in 9°C-acclimated fish suggests that iron homeostasis might be perturbed during cold acclimation. Transferrins are iron-binding blood plasma glycoproteins that play an important role in iron homeostasis by transporting iron from sites of absorption and heme degradation to those of storage and utilization (Crichton and Charloteaux-Wauters, 1987). WAP-65 also showed abundance changes similar to those of serotransferrin (Fig. 7B). This protein is thought to be involved in the warm temperature acclimatory response (Kikuchi et al., 1995) and the heat stress response in fish (Picard and Schulte, 2004; Pierre et al., 2010; Choi, 2010). In contrast to previous studies, WAP-65 increased with 9°C acclimation in *G. mirabilis*, suggesting that this protein may play a role in thermal acclimation regardless of the temperature and that its role might vary depending on the organism. Kikuchi and colleagues (1995) found that WAP-65 has a sequence similar to that of hemopexins, a group of proteins involved in scavenging free heme groups and acting as a major vehicle for heme transport in the plasma, thereby preventing heme-mediated oxidative stress and heme-bound iron loss (Tolosano and Altruda, 2002). Considering the similar abundance changes detected in serotransferrin and WAP-65, it is possible that these proteins play an important role in iron homeostasis in cold-acclimated fish. However, further interpretation of the differences in abundance of these proteins and their role in cold-acclimated cardiac tissues is confounded by potential differences in blood plasma lactate levels across acclimation groups.

Another protein that showed changes in abundance is PRDX3. This protein belongs to the peroxiredoxin protein family and is involved in scavenging peroxides in the mitochondrion. PRDX reduces H_2O_2 and alkyl hydroperoxides with the use of hydrogens provided by thioredoxin, thioredoxin reductase and NADPH (Chae et al., 1994; Murphy, 2012). Studies show that PRDX3-transfected cells can induce a marked resistance to hypoxia-induced peroxide formation and apoptosis (Nonn et al., 2003). Furthermore, PRDX3 is shown to protect mammalian cardiac tissues against hypoxia-induced oxidative stress and apoptosis (Kumar et al., 2009; Zhao et al., 2009). As described by Ishii and Yanagawa (2007), the increased presence of heme and Fe^{2+} is shown to enhance the expression and activity of PRDXs. Based on the previous discussion on iron homeostasis, it is possible that PRDX plays an important role in protection against heme- and Fe^{2+}-induced reactive oxygen species (ROS) in 19°C- and 13°C-acclimated *G. mirabilis* compared with fish exposed to the 9 and 26°C treatments.

HEXA protein was increased in both 9°C- and 26°C-acclimated groups (Fig. 7D). This protein is involved in metabolic signaling by removing N-acetylglucosamine (O-GlcNAc) from proteins. The role of HEXA in regulating O-GlcNAc levels is directly associated with cytoprotection against hypoxia and ischemic injury in mammalian cardiac tissues (Liu et al., 2006, 2007a,b; Champattanachai et al., 2007). However, the precise mechanism of this protective role is not completely understood. The increase in this protein in *G. mirabilis* hearts suggests that it may play a similar role, protecting cardiac cells from hypoxic injury, in 9°C- and 26°C-acclimated fish hearts. Additionally, this indicates some capacity for *G. mirabilis* at 9 and 26°C to induce necessary cytoprotective mechanisms to re-establish cellular homeostasis in cardiac myocytes when acclimating to a temperature outside their preferred temperature range. An increase in HEXA gene expression was also detected in 9°C-acclimated gill tissues of *G. mirabilis* (Logan and Somero, 2010). In that study, the increase was hypothesized to be related to changes in lipid biosynthesis, and we cannot rule out a tissue- and temperature-specific role for HEXA.

Conclusions

Cardiac tissues of cold- and warm-acclimated *G. mirabilis* demonstrated changes in abundance of proteins involved in several physiological processes, including protein turnover, cytoskeletal reorganization, iron homeostasis and antioxidant response. The most prominent abundance changes, however, were detected in proteins associated with cardiac energy metabolic pathways. Abundance data of proteins representing glycolysis, the citric acid cycle and oxidative phosphorylation suggest that 19°C-acclimated fish have a greater abundance of proteins required for ATP production compared with other treatments. In contrast, 26°C-acclimated fish showed a decrease in proteins associated with glycolysis, the citric acid cycle and, especially, oxidative phosphorylation, suggesting an overall decrease in levels of proteins supporting ATP production. While this decrease might to some extent be explained by compensation for higher reaction rates at a warmer acclimation temperature, there is no increase in anaerobic capacity, as predicted by previous studies showing an increase in anaerobiosis at elevated temperatures for both invertebrate (Sommer et al., 1997) and vertebrate species (Clark et al., 2008; Lannig et al., 2004). Also, our proteomic data contradict several studies investigating metabolic rates in teleosts, including *G. mirabilis*, that have shown that aerobic scope is not reduced at temperatures near the thermal limits of these organisms (Healy and Schulte, 2012; Jayasundara and Somero, 2013; Gräns et al., 2014). Considering the remarkable hypoxia tolerance demonstrated by *G. mirabilis* in its habitat and the lack of a protein abundance profile that reflects a cardiac hypoxia response at the transcriptomic level (Gracey et al., 2001), it is possible that metabolic shifts detected in the current study were due to direct temperature effects on cellular ATP-generating processes including overall mitochondrial integrity. Importantly, increases in CK at the coldest acclimation temperature suggest that adjustments in the PCr energy circuit might be key to maintaining high ATP turnover rates at low temperatures. Overall, our data suggest that the capacity to generate adequate levels of ATP and to

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compensate for temperature effects on energy metabolic processes might be crucial in determining thermal plasticity of cardiac function. Future identification of additional proteins, including the 85 protein spots that remained uncharacterized from this study, may reveal other mechanisms underlying thermal acclimation in *G. mirabilis* hearts and enhance the interpretations given to the changes identified here.

The current data, coupled with findings from Jayasundara and Somero (2013), indicate that *G. mirabilis* cardiac function, and, therefore, aerobic performance and fitness, is potentially optimized near 19°C. However, the data also show that this species can adjust its cardio-respiratory function at the physiological, cellular and molecular levels to compensate for effects of temperature well beyond 19°C; thereby, this organism has the capacity to 'resist' thermal effects for a significant period of time at constant temperatures as low as 9°C and as high as 26°C, a trait that might be a consequence of the ability of this fish to inhabit intertidal and estuarine environments that vary greatly in temperature.

MATERIALS AND METHODS

Fish collection and experimental design

Gillichthys mirabilis (mean body mass 27.31±8.1 g) were captured from an estuarine lagoon in Santa Barbara, CA, USA (34.39°N, 119.81°W), using baited traps. Fish were transported to Hopkins Marine Station and held at ambient temperature (13–14°C) for 2 weeks in a flow-through aquarium. They were then randomly divided into four identical flow-through 40 l aquariums (30–35 fish per tank), and held at ambient temperature for an additional 3–4 weeks. Subsequently, in three of the tanks, temperatures were adjusted to 9±0.5, 19±0.5 or 26±0.5°C at a rate of 2°C per day, allowing fish to slowly adjust to their acclimation temperatures; the other aquarium remained at ambient temperature. Acclimation temperatures were chosen for consistency with previous experiments conducted in the same laboratory (Jayasundara and Somero, 2013). Fish were maintained at these four temperatures for 28 days and were sampled for heart tissues. Fish held at ambient temperature were used as the common starting point and basis for comparison of effects of cold (9°C) and warm (19°C and 26°C) acclimation. For tissue sampling, fish were killed by cervical transection immediately after immersion in ice for 15–20 min. Tissues were snap frozen in liquid N_2, and stored at −80°C. Atrial and ventricular tissues were not separated. None of the tissues were subsampled, and each experimental replicate in the study represents an individual fish. Sex of the specimens was not determined. Throughout the experiment, ammonia and nitrite/nitrate levels (Quick Dip Test Strips, Jungle Laboratories, Cibolo, TX, USA) and dissolved oxygen concentrations (YSI52, Yellow Spring, OH, USA) were monitored regularly. Fish were fed *ad libitum* on a commercial fish diet (Bio-Oregon, Warrenton, OR, USA) three times per week.

The animal care and use permits for all the experiments and protocols in this study were approved by Stanford Institutional Animal Care and Use Committee.

Proteomic analysis

Protein extraction

Protein extraction was conducted based on Valkova and Kültz (2006) and Dowd and colleagues (2008). Briefly, heart tissue samples were homogenized in glass homogenizers kept on ice, using 4 volumes of ND-RIPA buffer (Dowd et al., 2008). Supernatant was collected after centrifugation for 20 min at 19,000 ***g*** at 4°C. Aliquots of the supernatant were stored in 2 ml siliconized microcentrifuge tubes at −80°C. Protein concentrations were measured based on the BCA protein assay (Pierce, Rockford, IL, USA) using a Spectra Max microplate reader (Molecular Devices, Sunnyvale, CA, USA) at an absorbance of 485 nm: 1 mg of protein from each sample was precipitated by adding 3 volumes of ice-cold acetone with 10% trichloroacetic acid and incubating for 2 h at −20°C; precipitated proteins were collected by centrifugation for 5 min at 18,000 ***g*** and 4°C; protein pellets were washed with ice-cold acetone, centrifuged again, and then air-dried for 5 min; protein samples were then resuspended in 380 µl of IPG rehydration buffer as described by Dowd and colleagues (2008).

Two-dimensional gel electrophoresis

The 2D gel electrophoresis method used here is adapted from Valkova and Kültz (2006). Immediately after resuspension, samples were added to IPG strips (18 cm, pH range 3–10 non-linear; Bio-Rad, Hercules, CA, USA) using passive rehydration overnight at 4°C. All 24 IPG strips were then processed in two batches by isoelectric focusing in a Protean IEF Cell (Bio-Rad), covered in 2 ml of mineral oil, at 250 V for 15 min, 10,000 V slow ramping for 3 h, followed by 10,000 V for 47,000 Vh. IPG strips were then frozen at −80°C. IPG strips were randomly selected from different treatment groups to minimize experimentally induced variation and the second-dimension run was completed as described by Dowd and colleagues (2008) using 12% Tris-glycine polyacrylamide/SDS gels (Jule Inc., Milford, CT, USA). Eight second-dimension gels (16×16×0.15 cm) were electrophoresed simultaneously by SDS-PAGE at 30 mA per gel (constant current) and 14°C using four Protean Xii units (Bio-Rad). Electrophoresis was stopped once the Bromophenol Blue dye front had reached the bottom of the gel. Gels were stained immediately with colloidal Coomassie Blue for 24 h in the dark and then destained by repeatedly washing with Milli-Q water for 48 h. Gels were imaged as 16-bit TIFF files with 600 dpi resolution using an Epson 1680 transparency scanner.

Spot identification and statistical analysis

Delta 2D gel analysis software (version 3.6, Decodon, Greifswald, Germany) (Berth et al., 2007) was used for image analysis and spot identification. From each acclimation group only the highest quality gels (gels with minimal horizontal or vertical streaking determined based on visual examination) were used for further analysis. Five of the six gels for 13°C- and 9°C-acclimated groups, and four of the six gels for 19°C- and 26°C-acclimated fish were deemed to be of high quality.

Protein spots on all 2D gels were detected and warped to a reference gel from each treatment group that appeared to have the most spots. Subsequently, the reference gels from 9°C-, 19°C- and 26°C-acclimated fish were warped to a reference gel from the 13°C-acclimated fish. By fusing all the gel images, a master gel was then created. This image contains all spots present on any gel and each spot at its maximal intensity. Using a spot detection tool, spots on the master gel were detected and possible artifacts were removed after visual inspection. We then generated a proteome map, which represents mean volumes for each spot (Fig. 1A). Spot boundaries from the master gel were then exported to all individual gels, thereby eliminating variability in spot quantification and ensuring close to 100% spot matching. After background subtraction, protein spot volumes were normalized against the total spot volume of all proteins in a gel image. Normalized spot volumes were analyzed within Delta2D by using one-way ANOVA across all four acclimation groups ($\alpha<0.05$), followed by standard Bonferroni *post hoc* testing.

Mass spectrometry

Proteins that exhibited significantly different expression among treatments were identified using mass spectrometry. Sample preparation for the mass spectrometer was conducted as described by Tomanek and Zuzow (2010). Briefly, protein spots were excised and destained twice with 25 mmol l^{-1} ammonium bicarbonate in 50% acetonitrile and dehydrated with 100% acetonitrile. Then, proteins were digested with 11 ng μl^{-1} of trypsin (Promega, Madison, WI, USA) overnight at 37°C; 0.1% trifluoroacetic acid (TFA) and acetonitrile (v:v 2:1) were used to extract digested proteins. Individual digested protein samples were mixed with 5 µl of matrix solution (0.2 mg ml^{-1} α-hydroxycyano cinnamic acid in acetonitrile) and spotted on an Anchorchip™ target plate (Bruker Daltonics Inc., Billerica, MA, USA). The spotted proteins were washed with 0.1% TFA and recrystallized using a mixture of acetone/ethanol/0.1% TFA (ratio of 6:3:1). A matrix-assisted laser desorption ionization tandem time-of-flight (MALDI-TOF-TOF) mass spectrometer (Ultraflex II; Bruker Daltonics Inc.) was used to obtain peptide mass fingerprints. A

combination of peptide mass fingerprint (PMF or MS) and tandem mass spectra (MS/MS) data were searched against two databases using Mascot (version 3.1; Matrix Science Inc., Boston, MA, USA) to identify proteins. One database is an EST library that contains 35,718 *G. mirabilis* sequences (Gracey, 2008). For PMFs that did not match with any ESTs in the *G. mirabilis* database, a general search against the NCBInr metazoan sequence database was conducted. We allowed for one missed cleavage during trypsin digestion. For tandem MS we set the precursor ion mass tolerance to 0.6 Da, as recommended for Mascot. The molecular weight search (MOWSE) score that indicated a significant hit was set for >24, and the threshold *P*-value was set for <0.05 to confirm that the probability of a match was not a random event. Combined PMF and MS/MS spectra were also searched against a decoy database to confirm that the false discovery rate was 5%.

Normalized spot volumes of identified proteins were tested for statistical significance by one-way ANOVA followed by a Tukey's pairwise comparison test ($P<0.05$).

Acknowledgements
We thank Mr Marcus Zuzow for his support throughout the protein identification process including mass spectrometry. We also thank Ms Laurie Kost for helping with fish maintenance and Mr Scot Simon, UC Santa Barbara, CA, USA, for his help in fish collection.

Competing interests
The authors declare no competing or financial interests.

Author contributions
N.J. and G.N.S. conceived and designed the experiments; L.T. and W.W.D. developed proteomics methods; N.J. executed the experiments, interpreted the findings and drafted the manuscript; G.N.S., L.T. and W.W.D. interpreted the findings and revised the article.

Funding
National Science Foundation grants EF-1041227 (to L.T.) and IOS-0718734 (to G.N.S.) supported this project.

Supplementary material
Supplementary material available online at
http://jeb.biologists.org/lookup/suppl/doi:10.1242/jeb.118760/-/DC1

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