Supplement to:
Transcriptional responses to thermal acclimation in the eurythermal fish *Gillichthys mirabilis* (Cooper 1864)

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Extended Results and Discussion for online supplemental

*Biological processes with complex expression patterns*

**Cell Cycle**

Under acute heat stress, cell cycle arrest is expected in order to allocate more energy to damage-repair processes characteristic of the cellular stress response (5, 9). The effects of thermal acclimation on cell cycle are likely species-specific, peaking at a thermal optimum where the organism’s growth rate is highest. Therefore, we might expect the 19°C acclimated fish would have the highest rate of cell division given that the thermal preference of *G. mirabilis* ranges between 9°C and 23°C (2). The six genes in this category did not follow an entirely consistent expression pattern, although most genes showed lower expression at 9°C (Fig. S1a). These changes in expression may suggest reduction in cell division at 28°C. Three genes, cyclin-dependent kinase inhibitor 1B (*CDKN1B*), dystonin (*BPAEA*), and transducer of erbB-2 (*TOB1*), were expressed most highly in the 28°C acclimation group and are all thought to promote cell cycle arrest. Three other genes encoding proteins involved in the cell cycle showed
inconsistent trends with acclimation temperature. *SPT5* and *SPT6* encode members of the Septin family, which polymerize into hetero-oligomeric protein complexes that form filaments, and associate with cellular membranes, actin filaments and microtubules (7). Septins are required for normal organization of the actin cytoskeleton and are involved in cytokinesis. The two septin-encoding genes showed different expression profiles; *SPT5* had highest expression at 19°C whereas *SPT6* had highest expression at 28°C. Like *SPT5*, Coronin-1C (*COR1C*), which encodes an actin binding protein thought to be involved in cytokinesis, was expressed most strongly at 19°C.

Based on the expression profiles of *CDKN1B*, *BPAEA* and *TOB1*, there is some indication that cell cycle progression is slowed at 28°C. However, a well-supported hypothesis cannot be drawn due to the multiple functions of these proteins and their inconsistent expression patterns. In addition, the cell cycle is a tightly controlled process governed by several overlapping regulatory mechanisms and therefore positive or negative regulation of cell proliferation may not be apparent at the gene expression level. Follow-up work at the protein or cellular level is required to further investigate what types of changes in cell cycle occur with temperature acclimation.

Carbohydrate and Energy Metabolism

The eight genes that comprised the carbohydrate metabolism category showed two expression profiles (Fig. S1b). Genes encoding glucose-6-phosphatase (G6PC), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and acidic mammalian chitinase (CHIA) were expressed most highly in the 9°C acclimation group. G6PC is a key enzyme in glucose homeostasis and functions both in the generation of glucose from non-carbohydrate carbon substrates (gluconeogenesis) and the breakdown of glycogen into glucose (glycogenolysis). GAPDH plays
an important role in the gluconeogenesis and glycolysis pathways by reversibly catalyzing the oxidation and phosphorylation of G3P to the energy-rich intermediate 1,3BPG. CHIA is involved in the breakdown of chitin, a linear polysaccharide consisting of beta-linked D-glucosamine residues. It is unclear what the function of this protein would be in the gill (10).

Five other genes showed the opposite expression pattern, and were expressed most highly in the 19°C and 28°C acclimation groups (cluster 2). These included genes for three glycolytic enzymes, 6-phosphofructokinase liver-type (PFKL), 6-phosphofructo-2-kinase (PFKFB4), and fructose-bisphosphate aldolase B (ALDOB), as well as genes for two mitochondrial citric acid cycle enzymes, malate dehydrogenase (MDHM) and Acetyl-CoA synthetase 2 (ACS2L). PFKL is a key regulator of glycolysis, catalyzing the important “committed” step of glycolysis. PFKFB4 is an activator of the glycolysis pathway and an inhibitor of the gluconeogenesis pathway. Regulation of PFKFB4 enzyme activity levels is thought to regulate glucose homeostasis. ALDOB is a glycolytic enzyme that catalyzes the reversible conversion of fructose-1,6-bisphosphate to glyceraldehyde 3-phosphate and dihydroxyacetone phosphate. MDHM catalyzes the reversible oxidation of malate to oxaloacetate, utilizing the NAD+/NADH cofactor system in the citric acid cycle. ACS2L is important for energy homeostasis, and converts acetate to acetyl-CoA so that it can be used for oxidation through the citric acid cycle to produce ATP and CO₂. Acetyl-CoA is also a substrate for a variety of protein acetylases that are thought to be of wide-spread significance in metabolic regulation (18, 19) and protein turnover (4). Higher expression of ACS2L in the warmer acclimation groups thus could contribute in two distinct ways to regulating metabolic flux through the citric acid cycle.

Broadly speaking, there appears to be indication of higher expression of glucose-catabolizing genes at higher temperature. This would be consistent with the shifts in fuel preference that have been observed during cold acclimation in other species, with a shift to use
of lipids in the cold and more reliance on glycogen and glucose at higher temperatures (3).

Nevertheless, carbohydrate metabolism is regulated at many different levels (enzyme kinetics, production and activity) under several controls (transcription, translation, and post-translational modification). If a process is primarily regulated at the metabolic level, there may be no quantitative relationship between mRNA levels and function. For example, it has been shown that mRNA levels do not correlate with glycolytic enzyme activities in at least three species (8). Recent efforts in the field of systems biology have integrated metabolomic data with transcriptomic data to identify ‘reporter reactions’ that may soon provide clues on whether regulatory control for a given reaction is at the metabolic level or at the transcriptional level (8), which would allow one to determine which metabolic reactions can be largely interpreted by mRNA levels.

Cell Death

The cell death gene ontology category includes apoptosis (programmed cell death), and necrosis/cytolysis (death due to rupture of cell membranes and the loss of cytoplasm). Induction of apoptotic genes is expected at extreme temperatures in order to rid tissues of permanently stress-damaged cells (9). Under acclimation to non-stressful temperatures, expression differences in apoptotic genes could occur for homeostatic purposes or tissue remodeling. Apoptosis is involved in homeostasis by balancing the rate of cell division in the tissue with the rate of cell death (15). Tissue remodeling is known to occur in the gills of other fish species in response to higher acclimation temperatures, presumably to increase respiratory surface area to match increased oxygen needs (13).

Three of the four genes in the cell death category were associated with apoptosis, but did not display a consistent pattern (Fig. S1c). However, one of these genes, Caspase recruitment
domain-containing protein 7 (NLRP1), encodes a protein that is a key mediator of apoptosis and NLRP1 was expressed most strongly in the 28°C acclimated group. NLRP1 forms cytoplasmic structures called death effector filaments and enhances cytochrome c-dependent activation of pro-caspase-9 and apoptosis. It also stimulates apoptosis through activation of caspase-3, caspase-1 and caspase-5 (17). Overexpression of NLRP1 has been shown to induce apoptosis in human cells (12). The remaining three genes encode proteins that are implicated in cell death but primarily function in other processes. RNA-binding protein TIA-1 (TIA1), expressed most highly in the 9°C and 19°C acclimation groups, encodes an RNA-binding protein that possesses nucleolytic activity against cytotoxic lymphocyte target cells and may be involved in apoptosis via cytochrome c (6, 16). DNase gamma (DNSL3), which also showed highest expression levels at 9°C and 19°C, is a member of the DNase family and can mediate breakdown of DNA during apoptosis (14). Complement component 7 (CO7) is a constituent of the membrane attack complex, important in both the innate and adaptive immune response by forming pores in the plasma membrane of target cells and may be involved in cytolysis (18).

Based upon NLRP1 expression, we hypothesize that 28°C may foster increased apoptosis in this species. This could be due to a homeostatic response rather than a stress-related response because gill tissue turnover is known to be high (11), there is no evidence of the classic heat shock response, and gill remodeling is likely complete after one month (13). Interestingly, scanning electron micrography did reveal that the gill lamellae of all four arches of the 28°C acclimated fish seemed to be longer than the lamellae of the 9°C and 19°C groups (B. Lee and C. Logan, unpublished observations). Further investigation is required to determine whether 28°C fosters increased apoptosis in this species, especially given the disparate expression profiles of the other genes in the cell death category. If increased apoptosis is occurring, then the higher
rates of translation predicted at high temperatures would find an additional explanation along with increased protein turnover in cells not undergoing apoptosis.

Transcription Factors

Genes encoding ten different transcription factors exhibited significant differences in expression between acclimation groups (Fig. S1d). Transcriptional regulation is highly complex and, as expected, disparate patterns of gene expression linked to transcription show no uniform response to temperature. Instead, they suggest that the overall differences in transcription found in differently acclimated fish may be mediated by complex shifts in the abundance and activities of many transcription factors.

Six of these genes were expressed most highly at 28°C, including CCAAT/enhancer binding protein delta (CEBPD), Signal transducer and activator of transcription 3 (STAT3), cAMP-dependent transcription factor ATF-4 (ATF4), Protein flightless-1 homolog (FLII), zinc finger protein 143 (ZN143), and Defective in cullin neddylation 1 (DCNL2). CEBPD was up-regulated more highly than any other gene in the dataset. CEBPD is a transcriptional activator of genes involved in immune and inflammatory responses. Both CEBPD and STAT3 are induced during acute heat stress in G. mirabilis as well (1). Two other transcription factors were expressed most highly at 9°C, including zinc finger protein 653 (ZN653) and Xenopus nuclear factor 7 (XNF7). Another transcription factor, Myocyte-specific enhancer factor 2C (MEF2C), was expressed most highly at 9°C and 28°C.

REFERENCES


16. Tian Q, Streuli M, Saito H, Schlossman SF, Anderson P. A polyadenylate binding protein localized to the granules of cytolytic lymphocytes induces DNA fragmentation in target

