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FAST TRACK

Heterologous hybridization to a complementary DNA microarray reveals the effect of thermal acclimation in the endothermic bluefin tuna (*Thunnus orientalis*)

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Abstract

The temperature stress that pelagic fishes experience can induce physiological and behavioural changes that leave a signature in gene expression profiles. We used a functional genomics approach to identify genes that were up- or down-regulated following thermal stress in the Pacific bluefin tuna. Following the acclimation period, 113, 81 and 196 genes were found to be differentially expressed between the control (20 °C) and cold (15°) treatment groups, in ventricle, red muscle and white muscle, respectively. The genes whose expression levels were responsive to thermal acclimation varied according to muscle fibre type, perhaps reflecting the tissue-specific degrees of endothermy characteristic of this species.

Keywords: bluefin tuna, cDNA, cold, microarray, stress, transcriptome

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Introduction

Temperature is a major environmental factor that can govern the depth preferences and biogeographical distributions of pelagic fishes such as bluefin tunas. Their highly migratory behaviour puts bluefin tuna in contact with a wide range of ambient temperatures. Studies employing electronic tags describe bluefin tunas in highly diverse ocean environments, with depths of occurrence to over 1200 m and water temperatures ranging from 0.1 to 32 °C (Block *et al*. 2001; Kitagawa *et al*. 2007).

Physiological adaptations such as high cardiac output and regional endothermy make tunas an interesting model for studies in cardiac and locomotory muscle physiology and thermal stress responses. An elaborate vasculature system employing counter-current heat exchangers (*retia mirabilia*) in the viscera, brain and body musculature provides a means for the conservation of metabolic heat (Carey & Gibson 1983; Block & Carey 1985; Block 1991). The heat generated by muscle contraction and metabolism is thereby partially conserved, and the temperature of the internalized red muscle of bluefin is maintained up to 21 °C above ambient water temperature (Carey & Lawson 1973; Marcinek *et al*. 2001). In contrast, the temperatures of heart and superficial white muscle remain at ambient water temperature. The different ranges of temperatures experienced by the endothermic and ectothermic regions of bluefin tunas raise a number of important questions about the thermal responses of the different types of muscle (red, white and cardiac) in these species.

Differences in thermal responses of these different muscle types are likely to be reflected in variations in patterns of gene expression, as indexed by mRNA populations (transcriptomes) of the cells. Global analysis of gene expression has proven to be a useful means to simultaneously assess the expression of tens of thousands of gene transcripts, providing a resolution and precision of phenotypic characterization not previously possible (Gracey 2007). By using this strategy, multiple genes and pathways can be examined as they work in concert. Using expressed sequence tag (EST) technology, several laboratories have generated complementary DNA (cDNA) microarrays of genes expressed in nonmodel species, including several species of fish (Gracey *et al*. 2001; Podrabsky & Somero 2004; Buckley *et al*. 2006). The use of microarrays for studying fish gene expression has increased in recent years, and arrays have emerged as an important tool for understanding developmental processes as well as basic physiology (Douglas 2006).

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A growing number of studies are utilizing cDNA microarrays generated from one species to examine gene expression in other species — heterologous hybridization — to avoid the expense in time and money that is associated with designing novel microarrays for every new species of interest (Renn *et al*. 2004; Buckley 2007; Kassahn *et al*. 2007). Various factors have been considered that may affect the efficacy of this approach, including the phylogenetic distance between the species involved and the nature and length of DNA probes affixed to the microarray platform. Helpful in overcoming these issues is the choice of experimental design employed, comparing samples from a given species only against other samples from the same species.

In the current study, a cDNA microarray constructed from a Gobiid fish, the long jaw mudsucker *Gillichthys mirabilis* was used to generate gene expression profiles in the muscle tissues of a thermally acclimated Scombrid fish, the Pacific bluefin tuna (*Thunnus orientalis*). The *G. mirabilis* cDNA microarray was constructed from expressed sequence tags from liver, muscle and brain tissues from individuals that had been exposed to either heat or hypoxic stress, as well as from numerous control individuals (for complete descriptions of the cDNA microarray construction, see Gracey 2008). This platform therefore contains much of the stress-responsive genome of this species and has previously been employed in studies measuring the impact of environmental temperature on the *G. mirabilis* transcriptome (Buckley *et al*. 2006). Here, we utilized this genomic tool to characterize the transcriptomic response to thermal acclimation in three types of muscle tissues from bluefin tuna acclimated to either 15 °C (hereafter 'cold acclimated') or to 20 °C (hereafter 'control') to better understand the genomic underpinnings of the thermal physiology of these regionally endothermic fishes.

Materials and methods

Experimental animal collection and thermal acclimation conditions

Specimens were captured on barbless circle hooks off San Diego, California, in waters of 20–21 °C sea surface temperature. Following capture, the tuna were maintained in live wells on board the fishing vessel and transported over land by truck to the Tuna Research and Conservation Center (TRCC) at Stanford University's Hopkins Marine Station in Pacific Grove, California. At the TRCC, fish were held in 109 m^3 circular tanks containing seawater at 20 °C for 3 weeks. Four individuals were then transferred to 15 °C and held for 90 days while a second group of four individuals remained at 20 °C. The tunas were fed three times per week. Mean body size was 74.3 cm fork length (range 70–78 cm) and 8.30 kg (range 7.1–9.4 kg). Ventricle, white muscle and red muscle tissue (~500 mg) from $n = 4$ fishes from each group were sampled and stored at –80 °C until mRNA extraction was performed.

Total RNA and poly A-RNA extraction for hybridization

Tuna tissues were homogenized with a Teflon pestle and total RNA was extracted according to a standard Trizol protocol (Invitrogen). RNA samples were quality checked by measuring A260/A280 ratios and confirmed by agarose gel electrophoresis. Detailed poly A RNA extraction procedures are available in Supplementary Material, Supporting information.

Microarray preparation

The cDNA microarray prepared for the current study was constructed by arraying polymerase chain reaction (PCR)-amplified cDNA clones reverse transcribed from total RNA isolated from liver, skeletal muscle, and brain of the temperate goby *Gillichthys mirabilis*. The microarray consists of 9206 cloned ESTs. The *G. mirabilis* cDNA microarray construction is described in Gracey (2008).

Microarray hybridization

Samples of 3 μg of mRNA (poly A RNA) from each tissue sampled were reverse transcribed to cDNA, using oligo(dT15) and pdN6 random hexamer primers and amino-allyl UTP. Incubation to 65 °C for 30 min in 0.2 mol/L NaOH and 0.1 mol/L EDTA was performed to remove the RNA template. Single-stranded cDNA was labelled with either Cy3 or Cy5 cyanine monoreactive dye (Amersham) prepared in 100 μL of dimethyl sulfoxide. The cDNA samples were incubated with 5 μL of either Cy3 or Cy5 for 1 h at room temperature and then cleaned through PCR purification columns (QIAGEN) and eluted in 10 μL of Sigma water. Samples were brought to a final volume of 40 μL in 25 mmol/L Hepes, 0.75 mg/mL tRNA (Sigma), 3× SSC and 0.2% SDS. Samples were incubated to 95 °C for 1 min, allowed to cool to room temperature for 5 min, and then applied to microarray slides. All the hybridizations were conducted overnight at 65 °C in hybridization chambers. After the hybridization, the slides were washed by immersion in 0.6× SSC and 0.03% SDS for 2 min to remove unbound dye and then dipped vigorously in 0.6× SSC wash solution. The slides were dried by centrifugation and scanned on an AXON GenePix 400B microarray scanner (Axon Instruments, Molecular Devices). Each individual sample was hybridized once with each dye (dye swap) having always one sample from the control- and one from the cold-acclimation group. The hybridizations performed are listed in Table 1.

Microarray data analysis

The fluorescence ratio of Cy3 to Cy5 was extracted from the 24 arrays used in this experiment using GenePix Pro 4.0 software. This ratio reflects the relative intensity from control to cold-acclimated individuals, and vice versa, on dye swap

Table 1 Array hybridization design used to compare cold stressed and control fishes

Tissue	Array	Dye (fish ID)	Treatment
Ventricle			
	(1252)	Cy3(1) × Cy5(2)	control × cold stressed
	(1243)	Cy5(1) × Cy3(2)	control × cold stressed
	(1250)	Cy3(3) × Cy5(4)	control × cold stressed
	(1246)	Cy5(3) × Cy3(4)	control × cold stressed
	(1232)	Cy3(5) × Cy5(6)	control × cold stressed
	(1235)	Cy5(5) × Cy3(6)	control × cold stressed
	(1283)	Cy3(7) × Cy5(8)	control × cold stressed
	(1267)	Cy5(7) × Cy3(8)	control × cold stressed
Red Muscle			
	(1227)	Cy3(1) × Cy5(2)	control × cold stressed
	(1245)	Cy5(1) × Cy3(2)	control × cold stressed
	(1231)	Cy3(3) × Cy5(4)	control × cold stressed
	(1247)	Cy5(3) × Cy3(4)	control × cold stressed
	(1233)	Cy3(5) × Cy5(6)	control × cold stressed
	(1239)	Cy5(5) × Cy3(6)	control × cold stressed
	(1255)	Cy3(7) × Cy5(8)	control × cold stressed
	(1284)	Cy5(7) × Cy3(8)	control × cold stressed
White muscle			
	(1228)	Cy3(1) × Cy5(2)	control × cold stressed
	(1242)	Cy5(1) × Cy3(2)	control × cold stressed
	(1251)	Cy3(3) × Cy5(4)	control × cold stressed
	(1248)	Cy5(3) × Cy3(4)	control × cold stressed
	(1234)	Cy3(5) × Cy5(6)	control × cold stressed
	(1240)	Cy5(5) × Cy3(6)	control × cold stressed
	(1254)	Cy3(7) × Cy5(8)	control × cold stressed
	(1285)	Cy5(7) × Cy3(8)	control × cold stressed

$n = 4$ individuals were used for both control and cold stressed groups.

arrays. The data were visually inspected, and spots with low-quality areas of the array were flagged and excluded from further analysis. Spatial and intensity-based trends in the data were removed by Lowess normalization (GeneSpring, Agilent). For each spot on the array, the average value of the four control fish (value = control channel Cy5/reference channel Cy3) and their standard deviation were calculated. To test the cold-acclimated treatment, the experimental value (experimental channel Cy5/reference channel Cy3) for each spot on the array was tested if it fell outside two standard deviations of the mean for the control.

A *t-test* statistical analysis was used to identify genes for which the expression pattern showed a significant effect of the cold treatment. Different individuals ($n = 4$) in each group were treated as replicates. For each spot on the array, the average value for all replicates and their standard deviation were calculated. Outliers were discarded and a new hybridization process was performed for at least two slides (regular and dye swap) for each fish tissue used in each treatment.

Quantitative real-time PCR analysis: verification of microarray results

To confirm the expression levels generated from the microarray analysis, the expression patterns of a candidate gene was analysed using quantitative real-time PCR (RT–PCR) in a 96-well format (Bio-Rad). The Q-PCR assays were performed using iQ SYBR Green Supermix in accordance with manufacturers procedure (Bio-Rad) on an iQCycler platform. The cDNAs were synthesized from 1 μg of ventricle, red muscle or white muscle poly A RNA in a reaction of 25 μL using iScript cDNA Synthesis Kit (Bio-Rad) according to manufacturer's protocol. Template cDNA (1 μg/μL) was diluted 1:10:100 and run in triplicate. Control reactions without cDNA template were performed to check if amplified products were a result of DNA contamination or due to primer-dimer effects. Degenerate bluefin tuna primers were selected to amplify the candidate genes β-actin (EU 30041 — F 5′-CAAGAGAGGTATCCTGACC-3′; R 5′ GTA GAAGGTGTGATGGGAG-3′) and Hsp70 (EU 300943 F 5′ GACATGAAGCACTGGC 3′; R 5′ AGGACCATGGAGGAG 3′). The relative quantification $2^{-\Delta\Delta CT}$ method described in Livak & Schmittgen (2001) was the method used to measure changes in the expression level of the target *Hsp70* gene and control housekeeping gene β-actin. β-actin was considered as a suitable housekeeping gene for normalization since its level of expression was independent of cold acclimation temperature [$\Delta C_{T\ \beta\text{-actin (mean warm temp.} - \text{mean cold temp.)}} = 0$]. One-way ANOVA analysis was performed to identify significant statistical differences in *Hsp70* relative gene expression levels between warm- and cold-acclimated bluefin tuna red muscle, ventricle and white muscle tissues. The Newman–Keuls test was applied a posteriori after the homogeneity of variances had been checked to the 95% confidence level.

Results

Differences in gene expression between control- and cold-acclimated fish were examined using mRNA isolated from ventricle, white muscle and red muscle tissue. Table 2 lists those transcripts determined to be differentially expressed between the two groups that were successfully annotated by comparing their sequences to those in public databases. The number of differentially expressed genes varied among the three tissues. Seventy genes in ventricle, 36 genes in red muscle and 110 genes in white muscle were significantly up-regulated in the cold-acclimated group compared to the control group. Most genes that were up-regulated in ventricle in the cold acclimated group were involved with protein biosynthesis (14%) and transport (14%) (Table 2; Fig. 1a). Among the protein biosynthesis-related genes, the majority of the up-regulated genes encode the 60S ribosomal protein L-type. The up-regulated genes associated with transport encode a group of ferritin proteins, important for iron

Table 2 Selected significantly differentially expressed transcript up- and down-regulated in red muscle, ventricle and white muscle of warm and cold acclimated BFT ($P < 0.05$)

Up-regulated genes Red muscle	Ventricle	White muscle
60S acidic ribosomal protein P0	Glutamine/Q-rich-associated protein	(NC_003424) putative tata binding protein interacting protein
60S ribosomal protein L19	(NM_021245) myozenin 1; myozenin; calsarcin-2	(NC_004331) hypothetical protein
6-phosphofructokinase, liver type	26S protease regulatory subunit 6B (TAT-binding protein-7)	26S proteasome non-ATPase regulatory subunit 1
Alpha-parvin	26S proteasome non-ATPase regulatory subunit 1	40S ribosomal protein S11
Amyloid protein-binding protein 1	60S acidic ribosomal protein P0	40S ribosomal protein S16
ATP-binding cassette, subfamily B, member 7,	65 kDa Yes-associated protein	40S ribosomal protein S19
Cholesteryl ester transfer protein precursor	Aldehyde oxidase	60S acidic ribosomal protein P1
Dystrobrevin binding protein 1 (Dysbindin)	ATP-dependent helicase DDX39	60S ribosomal protein L10
Glucosamine — fructose-6-phosphate aminotransferase	BB282193	60S ribosomal protein L24
Homo sapiens forkhead box P2	Calmodulin (CaM)	60S ribosomal protein L27
Lipin 1	CCAAT/enhancer binding protein zeta	60S ribosomal protein L28
L-lactate dehydrogenase A chain	Coagulation factor IX precursor	60S ribosomal protein L34
Next to BRCA1 gene 1 protein	Coronin-6 (Clipin E)	60S ribosomal protein L35a
NK-tumour recognition protein	Cullin homologue 3 (CUL-3)	60S ribosomal protein L37
Nucleolar transcription factor 1	Cyclin G1 (Cyclin G)	60S ribosomal protein L8
Phosphatidylinositol transfer protein beta isoform	Cysteine dioxygenase type I	65 kDa Yes-associated protein
Phosphofurin acidic cluster sorting protein 1	Cytochrome P450 24A1, mitochondrial precursor	6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 4
Phosphoribosyl pyrophosphate synthetase-associated protein	Desmoplakin (DP) 250/210 kDa paraneoplastic pemphigus	Aldehyde oxidase
Probable cation-transporting ATPase 13A3	Dihydroxyacetone phosphate acyltransferase	Alpha-actinin 1 (Alpha-actinin cytoskeletal isoform)
Probable mannose-1-phosphate guanyltransferase	Elongation factor 1-alpha (EF-1-alpha)	ATP-dependent Clp protease ATP-binding subunit ClpX-like
Protein C20orf175	Enigma homologue (Enigma-like PDZ and LIM domains protein)	Beta-galactoside-binding lectin (14 kDa lectin)
Putative adenosylhomocysteinase 2	Eukaryotic translation initiation factor 3 subunit 4	Betaine — homocysteine S-methyltransferase
Putative polypeptide	Eukaryotic translation initiation factor 3 subunit 7	Cathepsin F precursor
Retinol-binding protein IV, cellular	Ferritin, middle subunit (Ferritin M)	CC003931
Ubiquitin	Ferritin, middle subunit (Ferritin M)	PIR | T02995 | T02995 unspecific monooxygenase-
Ubiquitin carboxyl-terminal hydrolase isozyme L5	Forkhead box protein P4 (Fork head-related protein-like A)	Cell cycle control protein cwf5
	Growth arrest and DNA-damage-inducible protein GADD45	CG8947-PA (*Drosophila melanogaster*)
	GTP cyclohydrolase I feedback regulatory protein (GFRP)	Chromosome 6 open reading frame 209 (*Homo sapiens*)
	GTP:AMP phosphotransferase mitochondrial	Creatine kinase, M chain
	Guanine nucleotide-binding protein G(I)/G(S)/G(T) beta	CTD small phosphatase-like protein (CTDSP-like)
	Heat shock cognate 71 kDa protein	Cytochrome P450 24A1, mitochondrial precursor
	Histone H2B	db55e12. × 1 Blackshear/Soares normalized *Xenopus* egg library *Xenopus*
	Hook homologue 2 (h-hook2)	Density-regulated protein (DRP)
	Hypothetical protein BC016816 (*Homo sapiens*	Dipeptidyl-peptidase III (DPP III)
	KIAA1040 protein (*Homo sapiens*)	Elongation factor 1-alpha (EF-1-alpha)
	Mid1 interacting protein 1 (Gastrulation specific G12-like)	Elongation factor 1-alpha, somatic form (EF-1-alpha-S)
	Mitochondrial carrier triple repeat 1	Eukaryotic translation initiation factor 3 subunit 1
	Myosin regulatory light chain 2, smooth muscle minor	Eukaryotic translation initiation factor 3 subunit 7
	Nucleolar transcription factor 1 (Upstream binding)	Eukaryotic translation initiation factor 4 gamma 2
	Probable dolichyl pyrophosphate Glc1Man9GlcNAc2	Ferritin, middle subunit (Ferritin M)
	Probable RNA-dependent helicase p68	fk94c05.y1 Zebrafish Research Genetics C32 fin Danio rerio cDNA 5′

Table 2 *Continued*

Up-regulated genes Red muscle	Ventricle	White muscle
	Procollagen-lysine,2-oxoglutarate 5-dioxygenase 1	Gelsolin precursor (Actin-depolymerizing factor) (ADF)
	Proteasome subunit beta type 1-	Heat shock 70 kDa protein (HSP70)
	Protein FAM49B	Heat shock cognate 71 kDa protein (Hsc70.1)
	Protein KIAA1173	Heat shock protein HSP 90-alpha
	Ras-related protein Rab-6B	Heme oxygenase
	Similar to transcription factor UBF (*Danio rerio*)	hypothetical protein MGC63493 (*Danio rerio*)
	Tubulin beta chain (Beta tubulin)	insulin-like growth factor binding protein 1 (*Danio rerio*)
	Tubulin beta chain	Jumonji/ARID domain-containing protein 1D (SmcY protein)
	Tyrosine-protein phosphatase, nonreceptor type 6	Kelch-like protein 13 (BTB and kelch domain containing
	Ubiquitin	Kelch-like protein 21
		KIAA0261 (*Homo sapiens*)
		Maleylacetoacetate isomerase
		Mid1 interacting protein 1 (Gastrulation specific G12-like
		NipSnap2 protein
		Organic cation transporter protein
		Ornithine decarboxylase antizyme, long isoform
		Parvalbumin alpha (A1)
		peptidylglycine alpha-amidating monooxygenase COOH-terminal
		Probable cysteine desulphurase, mitochondrial precursor
		Probable general secretion pathway protein E
		Probable mitochondrial import receptor subunit TOM40
		Proteasome subunit alpha type 3
		Proteasome-associated protein ECM29 homologue
		Protein C14orf103 homologue
		Protein expressed in T-cells and eosinophils in atopic dermatitis
		Ras-like protein 3 (Roughened protein)
		Ras-related protein Rab-10
		Rho-related GTP-binding protein RhoG
		Sarcalumenin precursor
		Serine – pyruvate aminotransferase, mitochondrial precursor
		Suppressor of G2 allele of SKP1 homologue
		TC178346
		T-complex protein 1, zeta subunit
		T-complex protein 1, zeta subunit
		Tob1 protein
		Translocon-associated protein gamma subunit (TRAP-gamma)
		Ubiquitin
		Ubiquitin carboxyl-terminal hydrolase isozyme L5
		Ubiquitin-conjugating enzyme E2 variant 2
		VANGL2 | vang-like 2 (van gogh, *Drosophila*),
		mRNA (cDNA clone MGC:76845)
		X box binding protein-1 (XBP-1)
		zgc:92423 (*Danio rerio*)

Table 2 *Continued*

Down-regulated genes Red muscle	Ventricle	White muscle
26S proteasome non-ATPase regulatory subunit 3	(NC_002758) hypothetical protein (*Staphylococcus aureus* ssp.)	72 kDa type IV collagenase precursor
Actin, alpha skeletal muscle (Alpha-actin 1)	40S ribosomal protein S20	Acidic leucine-rich nuclear phosphoprotein 32 family
ADP-ribosylation factor GTPase-activating protein 3	Alkaline phytoceramidase	Actin, alpha skeletal muscle (Alpha-actin 1)
Alpha-1 catenin (102 kDa cadherin-associated protein)	ATP synthase alpha chain, mitochondrial precursor	Similar to SP|P07222|NPM_XENLA Nucleophosmin
Apolipoprotein A-IV precursor	BETA3 protein	Aldehyde dehydrogenase family 7 member A1
Betaine – homocysteine S-methyltransferase	BJ047360 NIBB Mochii normalized Xenopus neurula library CC162912	Alpha enolase (2-phospho-D-glycerate)
Brain-type organic cation transporter		Annexin A6 (Annexin VI) (Lipocortin VI)
Cytochrome P450 2J1	CCAAT/enhancer binding protein delta (C/EBP delta)	Apolipoprotein A-IV precursor (Apo-AIV)
Danio rerio lipoprotein lipase mRNA	Creatine kinase, M chain	ATP synthase alpha chain, mitochondrial precursor
Danio rerio runt-related transcription factor b (runxb), mRNA	F-box only protein 32 (Muscle atrophy F-box protein)	ATP synthase beta chain, mitochondrial precursor
Desmoplakin (DP) 250/210 kDa paraneoplastic pemphigus	fj04h03.y1 Zebrafish adult olfactory Danio rerio cDNA 5′, mRNA	Chitotriosidase 1 precursor
Dihydroceramide delta(4-desaturase	FK506-binding protein 1B	Chymotrypsin B
Fructose-bisphosphate aldolase A	Fructose-bisphosphate aldolase A	Citrate synthase, mitochondrial precursor
Haloacid dehalogenase-like hydrolase domain containing	Heme oxygenase	Cullin homologue 5 (CUL-5) (Vasopressin-activated)
Hypothetical protein MGC33302 (*Homo sapiens*)	Insulin-like growth factor binding protein 1 (*Danio rerio*)	DNA-directed RNA polymerase II largest subunit
Inhibitor of nuclear factor kappa-B kinase alpha subunit	Intermediate filament protein ON3	Enigma homologue (Enigma-like PDZ and LIM domains protein)
Microsomal signal peptidase 18 kDa subunit	Lipoprotein lipase precursor	Enolase
N-acetylglucosamine-6-sulphatase precursor	Myosin heavy chain, fast skeletal muscle	FK506-binding protein 1B
Orphan nuclear receptor TR2	Protein C20orf149	Fructose-1,6-bisphosphatase
Oxysterol-binding protein 1	Protein C20orf45 (CGI-107)	Gastrulation specific protein G12
Pecanex-like 3 (*Mus musculus*)	Protein kinase C and casein kinase substrate in neurones	Glucose-6-phosphate isomerase
Polypyrimidine tract-binding protein 1 (PTB)	Serotransferrin I precursor (Siderophilin I) (STF I)	GLutamate dehydrogenase
Probable aminotransferase T01B11.2	Suppressor of cytokine signalling 3 (SOCS-3)	Glyceraldehyde-3-phosphate dehydrogenase
Protein kinase C, eta type	Thrombospondin-1 precursor	Glycogen phosphorylase, muscle form
Proto-oncogene protein c-fos (Cellular oncogene fos)	Tob1 protein (Transducer of erbB-2 1)	Heat-shock protein beta-1 (HspB1)
Receptor-interacting serine/threonine-protein kinase 2	TPR repeat containing protein KIAA1043	Importin beta-3 (Karyopherin beta-3
SH3 domain GRB2-like protein B2 (Endophilin B2)	Tropomodulin-3 (Ubiquitous tropomodulin) (U-Tmod)	Intrinsic factor precursor (IF) (INF)
Similar to hypothetical protein (*Rattus norvegicus*)		Keratin, type II cytoskeletal 8 (Cytokeratin 8)
Similar to hypothetical protein FLJ37794 (*Homo sapiens*)		KIAA0930 protein (*Homo sapiens*)
Solute carrier family 2, facilitated glucose transporter,		L-lactate dehydrogenase A chain
Thrombospondin-1 precursor		Myosin heavy chain, fast skeletal muscle
Troponin C, slow skeletal and cardiac muscles		Myosin light chain 3, skeletal muscle
Tyrosine-protein phosphatase, nonreceptor type 2		Nicotinamide riboside kinase 2
		Nucleolysin TIA-1 (RNA-binding protein TIA-1)

Table 2 *Continued*

Down-regulated genes Red muscle	Ventricle	White muscle
		Nucleoside diphosphate kinase NBR-B
		Pepsin A precursor
		Phosphatidylinositol 3,4,5-trisphosphate-dependent
		Phosphoglycerate mutase 2
		Phosphorylase b kinase beta regulatory chain
		Plasma protease C1 inhibitor precursor
		Probable RNA-dependent helicase p68
		Protein C14orf103
		Protein C22orf19 (NF2/meningioma region protein pK1.3)
		Protein FAM3A precursor
		Protein FAM49B
		Protein kinase C and casein kinase substrate in neurones
		Proto-oncogene protein c-fos (Cellular oncogene fos)
		Rabphilin-3 A (Exophilin 1)
		Ribulose bisphosphate carboxylase large chain precursor
		RNA-binding protein 5 (RNA binding motif protein 5)
		Sarcoplasmic/endoplasmic reticulum calcium ATPase 3
		Serotransferrin I precursor (Siderophilin I) (STF I)
		T-cell activation protein phosphatase 2C (*Homo sapiens*)
		Thrombospondin-1 precursor
		Triosephosphate isomerase
		Tropomyosin 1 alpha chain (Alpha-tropomyosin)
		Troponin C, skeletal muscle (TNC)
		Troponin T, fast skeletal muscle isoforms
		Tumor differentially expressed protein 2 (Tumor)
		Tyrosine aminotransferase
		Unknown (protein for MGC:66340); wu:fb73e11 (*Danio rerio*)

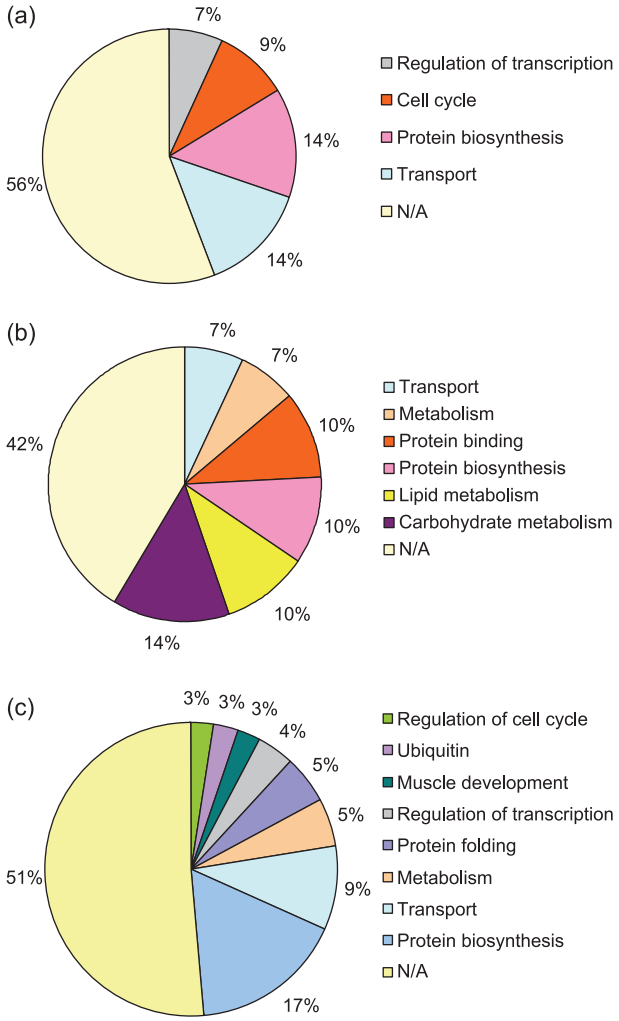

Fig. 1 Percentage of total up-regulated genes identified in the Gene Ontologies (Biological Process) of ventricle (a), red muscle (b) and white muscle (c).

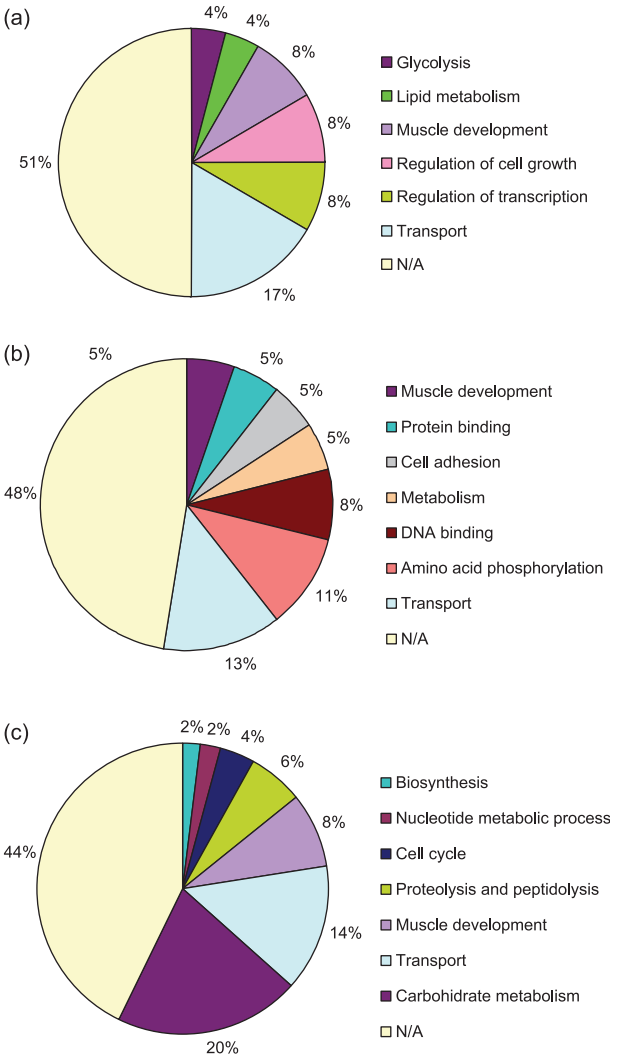

Fig. 2 Percentage of total down-regulated genes identified in the Gene Ontologies (Biological Process) of ventricle (a), red muscle (b) and white muscle (c).

transport and homeostasis. An increase in the expression of a 71-kDa heat shock protein and cytochrome P450 were also observed. In the red muscle, genes encoding carbohydrate metabolism (14%), lipid metabolism (10%) and protein biosynthesis (10%) were also generally up-regulated (Fig. 1b). L-lactate dehydrogenase-A (LDH-A) and lipin 1, which are involved in carbohydrate and lipid metabolism, respectively, were both more highly expressed in the cold-acclimated group. As in the ventricle, an increase in the expression of genes encoding 60S ribosomal proteins was observed. Genes encoding products involved in protein biosynthesis (17%), protein folding (5%) and transport (9%) were up-regulated in white muscle from the cold-acclimation group (Fig. 1c). Several ribosomal proteins were up-regulated, including 40S and 60S ribosomal proteins (Table 2). Also up-regulated were heat shock genes encoding *Hsp70, Hsp71, Hsp90*.

Similar to the up-regulated genes, the number of differentially down-regulated genes varied among the three tissues tested. Following the cold-acclimation, 43 genes in ventricle tissue, 45 genes in red muscle tissue and 86 genes in white muscle were significantly down-regulated compared to the control group (Table 2). The most striking constituency of transcripts down-regulated in ventricle were those involved in transport (17%), regulation of transcription (8%), muscle development (8%) and regulation of cell growth (8%) (Fig. 2a). Genes encoding myosin heavy chain, lipase precursor and protein kinase C were among those that showed lower levels of expression in ventricle of cold-acclimated fish compared to control. Transcripts down-regulated in red muscle of cold-acclimated individuals were those involved in transport (13%), protein amino acid phosphorylation (11%) and DNA binding (8%) (Fig. 2b). Protein kinase C and

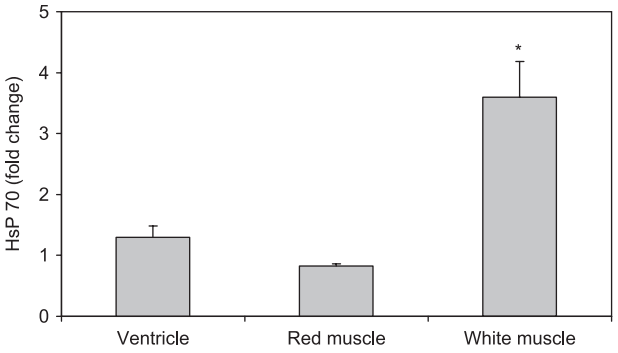

Fig. 3 Relative gene expression level of candidate gene *Hsp70* in cold-stressed Thunnus orientalis ($n = 7$) determined by quantitative real-time PCR. Expression levels were normalized to the housekeeping gene β-actin. (*) Significant values between means.

cytochrome P450 were down-regulated in red muscle. Genes involved in carbohydrate metabolism (20%), transport (14%) and muscle development (8%) were down-regulated in white muscle (Fig. 2c). L-lactate dehydrogenase A was most prominently down-regulated among the genes involved in carbohydrate metabolism. The stress protein *Hsp70* was up-regulated in the cold-acclimated group only in the white muscle. This result was confirmed by an independent method, quantitative real-time PCR. The relative expression of *Hsp70* (normalized to β-actin) was significantly up-regulated in the cold-acclimated group only in white muscle, paralleling the results obtained via microarray analysis (Fig. 3).

Discussion

This study examined the effect of thermal acclimation on broad-scale patterns of gene expression in endothermic and ectothermic muscle tissues of juvenile Pacific bluefin tuna. Each type of muscle tissue exhibited a distinct effect of acclimation on global gene expression, perhaps reflecting tissue-specific differences in thermal physiology and plasticity of response to variable environmental temperatures.

As is the case for most fishes, there is a general lack of genome sequence information for bluefin tuna. Therefore, we used a heterologous hybridization analysis using cDNA microarray generated from another fish species. The primary technical challenge presented by heterologous hybridization is the problem of sequence divergence between the species for which the microarray was constructed and the species providing the cDNA against which it was hybridized (Buckley 2007). The hybridization strategy we employed followed that of an earlier study designed to demonstrate the efficacy of heterologous hybridization. Renn *et al*. (2004) showed consistent expression profiles using a cDNA microarray that was generated from an African cichlid fish, *Astatotilapia burtoni*, to study the transcriptomes of seven other fish species from closely related taxa to a very distantly related species.

The current study avoids the problem of phylogenetic distance between *G. mirabilis* and *T. orientalis* because, in every case, samples from only one species (*T. orientalis*) were competitively hybridized against one another. Thus, the effect of phylogenetic distance between the target and probe cDNAs on hybridization kinetics affected each sample equally. The results of the present study support the ability of heterologous hybridization to reveal biologically relevant patterns of gene expression across considerable taxonomic distance in fishes. The fact that many transcripts in the differentially acclimated bluefin tuna were of unknown function demonstrates that, for nonmodel organisms, considerable potential exists for new environmentally sensitive genes to be discovered. It should be noted that another caveat associated with heterologous hybridization is that in some cases multiple or alternate members of a gene family will bind to a given feature on the array and so it is possible that discerning specifically which member of that gene family was detected by cross-species hybridization can potentially be an issue. Nevertheless, the grouping of differentially expressed genes into overall Gene Ontology categories does provide valuable information on the functional classes represented by these genes.

Differential gene expression in cardiac muscle following thermal acclimation

Unlike tropical tuna species, bluefin tuna expanded their niche by migrating to higher latitudes where they experience sustained exposures to low temperatures (Block *et al*. 1998; Block *et al*. 2001). During migrations, bluefin tuna can spend long periods in 15 °C and lower water temperatures (Kitagawa *et al*. 2007). Adaptations to cold include elevated levels of endothermy and enhanced cardiac power output. Blank *et al*. (2004) demonstrated experimentally that bluefin tuna hearts maintain function at 2 °C. In a study comparing various tuna species, Castilho *et al*. (2007) demonstrated that the activity of SERCA2, a key cardiac enzyme, is highest in bluefin tuna when measured at 5 °C. While some fish species such as rainbow trout increase heart mass in response to cold-acclimation (Vornanen *et al*. 2005), relative heart mass of bluefin tuna does not change in response to cold (data not shown). Apparently, the bluefin tuna heart is able to adjust to low temperatures without having to increase the quantity of tissue or levels of cardiac enzymes. In accordance with this, cDNA microarray analyses in the current study indicate that none of the cardiac hypertrophy markers were up-regulated during cold acclimation.

Differential gene expression in red muscle following thermal acclimation

Tunas have a higher proportion of slow-twitch, relative to fast twitch, myotomal muscle than other teleosts (Graham

et al. 1983). In tunas, as in all other fishes, slow sustained swimming is powered by slow-twitch aerobic red muscle, while fast-twitch glycolytic white muscle is recruited as swimming speed increases (Altringham & Johnston 1990). Evidence for adaptation to a higher operating temperature in deeper muscle tissues is reflected in the ability of deep tuna muscle to function at 30 °C, developing almost 40% more power than at 25 °C (Altringham & Block 1997).

Bluefin tuna red muscle comprises only a small portion of the total body mass but has a high fat content, high capillarity, high mitochondrial protein concentration and high myoglobin content, which results in the dark red colour (Korsmeyer *et al*. 1996). The microarray analysis indicated that bluefin tuna aerobic red muscle tissue increases expression of genes involved in carbohydrate and lipid metabolism in response to cold acclimation, possibly a process similar to the fattening observed during bluefin tuna migrations to oceanic cold water masses (Nottestad *et al*. 1999; Mourete *et al*. 2002).

In the current study, deep red muscle showed the lowest number of thermally responsive genes, possibly due to the temperature stability of this tissue, which works relatively independent of the external ambient temperatures.

Differential gene expression in white muscle following thermal acclimation

The white muscle, which comprises 46–55% of a bluefin tuna's body weight, and is known to accumulate unusually large amounts of lactate during burst activity (Dickson 1996). Acidification is avoided by rapidly removing the lactate after burst swimming (Guppy *et al*. 1979; Bernal *et al*. 2001). In response to cold acclimation, genes related to mitochondrial function and carbohydrate metabolism were down-regulated in white muscle (Table 2). However, the most dramatic effect of thermal acclimation on white muscle transcripts involved genes related to protein biosynthesis. The overall up-regulation of genes coding for ribosomal proteins suggests that the protein synthesis machinery of tuna white muscle is enhanced in the cold. Temperature related down-regulation of gene transcripts of carbohydrate metabolism suggests that while capacity for glycolytic energy production from carbohydrates might be decreased, mitochondrial function is not compensated.

In summary, gene expression patterns following thermal acclimation in bluefin tuna varied according to muscle fibre type, perhaps reflecting the tissue-specific degrees of endothermy characteristic of this species. The baseline data collected here may allow future studies to assess the thermal histories of wild populations of bluefin tuna that are acclimatized to different ranges of temperatures.

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