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Expression of Hsp70, Na^+/K^+ ATP-ase, HIF-1α, IL-1β and TNF-α in captive Pacific bluefin tuna (*Thunnus orientalis*) after chronic warm and cold exposure

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ABSTRACT

Bluefin tuna (*Thunnus* spp.) are recently evolved teleosts with exceptional physiological and morphological specializations, whose aquaculture has become an increasing sector of the bluefin fishery. We studied the molecular expression of target gene transcripts in respect to experimental chronic cold and warm exposure in Pacific bluefin tuna liver, spleen, kidney and gill. The genes of interest were chosen as representative "biomarkers", shown to change in fish exposed to temperature challenges: chaperon molecules (Hsp70); proteins engaged in active transport (Na^+/K^+ ATP-ase); oxygen-dependent genes (HIF-1α); and cytokines (IL-1β, TNF-α). In captive Pacific bluefin tuna, these genes showed constitutive expression in all organs examined. A seven-week chronic exposure to cold temperature resulted in shifts in expression levels of oxygen-dependent HIF transcripts in spleen and gill, and Hsp70 in spleen, while other gene transcripts remain unaffected. The results indicate that a long-term exposure to cold imposes physiological pressure on this endothermic fish species, especially on haemaotopoietic and gas-exchange organs. We suggest that these two genes can be considered as potential biomarkers for thermal shifts in captive bluefin.

1. Introduction

Tunas represent the most valuable fisheries and finfish aquaculture product currently recognized, with more than a half of the total world production concentrated in the Mediterranean Sea. Ranching operations also extend globally to many regions including North America and Australia (Miyake et al., 2003; Fromentin and Powers, 2005). However, wild bluefin tuna fisheries have reached a plateau and efforts to ranch tunas and complete the aquaculture cycle have rapidly expanded. The expansion of bluefin tuna production through aquaculture largely depends upon our ability to maintain captive populations of bluefin tunas for rearing, reproduction and fingerling grow out. To improve the capacity to keep bluefin, reduction of the health risks and environmental influences will have to be defined. Molecular tools for characterizing the captive population health status and its physiology as the response to abiotic factors must be established in the captive environment, bur prior to that we need to detect and quantify appropriate parameters, indicative of fish response.

Gene expression as an indicator of fish health in aquaculture is gaining more value as a reliable molecular tool for the screening of different variables encountered during the rearing cycle (Gornati et al., 2004a). However, the number of studies compared to the number of species already reared is still low (Gornati et al., 2004b; Gornati et al., 2005; Terova et al., 2005), because a scarce number of fish sequences exist, even for species with high economic importance. Only recently, few studies of tunas have addressed genomic level of expression (Castilho et al., 2007; Chini et al., 2008), while in salmon (Rise et al., 2004) and cod (Pérez-Casanova et al., 2008), physiological genomics was used to examine the health and stress of captive and wild fish (Bermejo-Nogales et al., 2007; Fridell et al., 2007; Wang et al., 2008).

Tuna are migratory teleosts with exceptional physiological and morphological specializations that set this group apart from all other teleosts (Block and Stevens, 2001), because of their high metabolic and heart rate, and specific cardiac output (Blank et al., 2004, 2007; Fitzgibbon et al., 2008). Depending upon species, the capacity for heat retention or endothermy is one of their most exceptional characteristics. Tunas use countercurrent heat exchangers that minimize convective heat loss and allow tunas to retain metabolic heat in the swimming muscles, viscera, brain and eyes (Block et al., 2001).

The effect of temperature is manifested at every level of an organism from genes to behavior and changes in temperature over diel or seasonal periods induce shifts in a variety of gene transcripts expression levels that result in numerous metabolic and hormonal adaptations (Hochachka and Somero, 2002). Such plasticity is characteristic for many ectothermic fish (Podrabsky and Somero, 2004) although it is not genetically fixed, but a result of previous acclimation history of the organism (Buckley and Hofmann, 2004).

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Still, little is fully explained about the general shifts in gene expression induced by thermal acclimation, and majority of studies were designed with acute temperature changes, while the understanding of moderate and mild changes that occur daily or seasonally in the animal's habitat, appeared only recently (Fangue et al., 2006; Gracey, 2007; Wang et al., 2007).

Generally, genes affected by shifts in temperature and revealing major differences in transcriptional responses, include the group of key regulators of cell growth and proliferation pathways, molecular chaperons, enzymes engaged in biosynthesis, those in charge for the membrane integrity (Podrabsky and Somero, 2004; Lund et al., 2006), as well as modulators of both innate and acquired immunity, cytokines in particular (Zou et al., 2000; Prophete et al., 2006; Bowden et al., 2007). In most organisms, Hsps are known to respond to temperature fluctuations, however mechanisms that confer plasticity to the regulation of this ubiquitous response are not well-understood (Lund et al., 2006), moreover in endothermic fish like bluefin. Gill milieu characterized by active uptake and secretion of Na and Cl ions, mediated through Na^+/K^+ ATP-ase, is a prerequisite for adequate tissue oxygenation in oxygen-demanding species, and the effect of temperature on its expression is mainly studied in euryhaline fish species (Kültz and Somero, 1995). HIF function in poikilotherm animals is required for heat acclimation, but except its interaction with a member of Hsp family, the effect of temperature on HIF is presently unknown (Nikinmaa and Rees, 2005). Ambient temperature has a marked influence on immune reactivity in fish as well, the fact used for the planning of immunoprophylaxis in aquaculture, but the effective response vary considerably according to whether the fish is cold, temperate or warm-water species (Manning and Nakanishi, 1996).

The objective of this study was to determine the expression of target genes in Pacific bluefin tuna, experimentally exposed to chronic warm and cold temperatures, in order to identify those genes that might be useful as biomarkers for future thermal assessments. The genes of interest were chosen as representatives of groups that are regularly modified in poikilotherm fish exposed to temperature shock: chaperon molecules (heat shock protein 70 or Hsp70) (Buckley et al., 2006; Lund et al., 2006); proteins engaged in active transport (Na^+/K^+ ATP-ase) (Kültz and Somero, 1995; Metz et al., 2003); oxygen-dependent genes (hypoxia-inducible factor-1α or HIF-1α) (Treinin et al., 2003); and cytokines (interleukin-1β or IL-1β, tumor necrosis factor-α or TNF-α) (Zou et al., 2000; Bowden et al., 2007).

2. Materials and methods

2.1. Fish and tissue sampling

Juvenile Pacific bluefin tuna (*Thunnus orientalis*) were captured by hook and line off San Diego, CA, USA, held on board of a fishing vessel in seawater filled wells, and transported by truck to the Tuna Research and Conservation Center (TRCC) in Pacific Grove, CA, USA. Fish were held in two 109 m^3 circular tanks containing seawater, for an acclimatization period of at least 6 months at 20 °C. The feeding regime was calculated according to the biomass of fish (52 ± 3.6 kg) that were fed a diet of approximately 30 kcal/kg/day. Proximate analysis of total calories and percent content for fat and protein of food items was assessed according Farwell (2001). The feed consisted of squids (*Loligo opalescens*, *Ilex* sp.), anchovy (*Engraulis mordax*), sardine (*Sardinops sagax*), smelt (*Spirinchus starksi*), and a commercially available high-vitamin-content gelatin mix.

Water quality parameters (temperature and oxygen) were monitored daily and the average values are given in Table 1.

Eleven juvenile Pacific bluefin tuna were designated for these experiments and moved to experimental tanks, where they were exposed to a gradual temperature increase or decrease. Seven fish were acclimated to 23 °C (T2 tank), where experimental temperatures were reached in 16 days. Four fish were in a tank at 20 °C (T3) that was gradually reduced to 15 °C over a 14-day period. Fish were maintained in T2 on average for 44.9 days (32–59 days; ± 10.4), whereas in T3 fish were kept on average at 15 °C for 48.8 days (42–56 days; ± 6.4).

Table 1
Mean measures of temperature (°C) and oxygen (mg/L) with standard deviations in warm (T2) and cold water tank (T3), monitored during the experimental period.

	Mean temp. (°C)	St. dev. temp.	Mean O_2 (mg/L)	St. dev. O_2
T2	22.94	0.13	6.46	0.14
T3	15.10	0.16	7.34	0.15

Even though wild bluefin tuna show large thermal tolerance (~1.8–30 °C) (Block and Stevens, 2001; Kitagawa et al., 2007), their preferred temperature is 18 °C (Marcinek et al., 2001). However, previous experimental work with tank bluefin tuna suggested that 15 °C is the minimum temperature at which tuna still feed, reflecting physiological functioning of the fish (see Farwell, 2001). Below that temperature or above 23 °C, acute stressed behavior was noticed with accompanying cessation of the food intake, suggesting impaired physiological functions. The length of the experiment was chosen based on the interaction of different factors, like availability of tanks, scheduled bluefin collection from wild and time considered as a chronic exposure.

Fish were sacrificed by pithing and necropsy was made immediately after the death. Gill tissue was removed by cutting a raker with scissors that were baked in oven and cleaned with RNAase free solutions. The tissues that were removed were immersed in RNAlater stabilization reagent (Quiagen) at room temperature and stored overnight on + 4 °C and − 80 °C until RNA extraction. Liver, spleen and kidney tissues were also removed in the same manner within a few minutes of the fish being sacrificed. Even though during the experiment no mortalities occurred, in order to rule out pathogen influence, gross pathology inspection and parasitological examination was done as described earlier (Mladineo et al., 2008).

2.2. mRNA extraction and cDNA synthesis

Total RNA was extracted following standard protocol from RNeasy Fibrous Tissue Mini Kit (Quiagen), after disruption and homogenization with baked stainless steel beads in Mixer Mill MM 300 (Quiagen). The purity and quantity of total extracted RNA was measured on the Nanodrop in ng/μl. Poly A^+ RNA was purified from total extracted RNA by Oligotex Kit (Quiagen) and again checked for integrity on the Nanodrop.

Total quantity of Poly A^+ RNA was reverse transcribed into cDNA, using iScript™cDNA Synthesis Kit (Bio-Rad), following manufacturer profile. Quantities of cDNA measured on Nanodrop were normalized to 1 μg/μl.

Since EST for blufin tuna lacked sequences of the chosen genes, fragments of genes of interest were amplified in thermo-cycler using primers designed with consensus sequences obtained by comparing Genbank sequences based on human, bird, fish and mammal target genes. Accession numbers of submitted sequences, their bp lengths, primers and annealing temperatures are shown in Table 2. Protocols for amplification included use of 1.75–2.5 mM of $MgSO_4$, 25 mM of dNTP, 1 unit of Platinum Taq High Fidelity (5 U/μl) (Invitrogen) and 1 μg/μl of template per reaction. Products were loaded on a 2–3% agarose gel and visualized by adding SYBR Safe (1%) directly in the gel. Products of adequate size were purified using QIAquick PCR Purification Kit (Qiagen) and cloned in chemically competent TOP10 vector cells using Invitrogen TOPO TA Cloning Kit. Clones were selected and screened for the insert using T3 and T7 primer set. The purified PCR product was sequenced on an ABI 3100 automatic DNA sequencer (Applied Biosystems), using the ABI PRISM BigDye Terminator Cycle Sequencing Kit, in both directions.

Table 2
Degenerated and species-specific primers used in PCR and relative qPCR amplification, profile details (F = forward primer concentration, R = reverse primer concentration, T_m= annealing temperature) and GenBank number of submitted sequences.

	Gene	Bp	Sequence	Profile	GenBank
cDNA	β actin	218	F 5′ ATC GTG GGG CGC CCC AGG CAC C 3′ R GTC ATC TTC TCY CTG TTG GC 3′	T_m = 58 °C	EU30041
	IL-1β	77	F 5′ GGR SAG CGA CAT GGY RCG ATT TCT 3′ G 5′ GGT GCT GAT GTA CCA GTT G 3′	T_m = 52 °C	EU30045
	TNF-α	278	F 5′ CCA GGC RGC CAT CCA TTT AGA AG 3′ R 5′ CCG ACC TCA CCG CGC T 3′	T_m = 64 °C	EU300946
	Na^+/K^+ ATP-ase	284	F 5′ GAG CAY TTC ATC CAC ATC CAT CAC 3′ R 5′ ATC TGG TTG TCR AAC CAC 3′	T_m = 56	EU300940
	Hsp70	574	F 5′ CGA CCA GGG CAA CAG GAC 3′ R 5′ GGT CAG GAT GGA CAC GTC GAA GG 3′	T_m = 60 °C	EU300943 EU300944
	HIF-1α	692	F 5′ CTG GAG CGT AGG AAG GAG AA 3′ R 5′ ACA ATA TGT GAA CTT CAT GTC CA 3′	T_m = 56	EU300942
qPCR	β actin	57	F 5′ CAA GAG AGG TAT CCT GAC C 3′ R 5′ GTA GAA GGT GTG ATG GGA G 3′	T_m = 51 °C F = 200, R = 300	
	IL-1β	68	F 5′ CTC TTC TAC AAA CAG GAC ACT GG 3′ R 5′ AGT TGG GAA AAC GGG CAG 3′	T_m = 53.7 °C F = 400, R = 300	
	TNF-α	72	F 5′ CTG GAG TGG AGA GTT G 3′ R 5′ GGC TGT AGA CCA AGT AG 3′	T_m = 51.5 °C F = 100, R = 500	
	Na^+/K^+ ATP-ase	79	F 5′ GGA TAC GGG TGG CTG GAG 3′ R 5′ GCC ATA CGC TTA GCA GTC AG 3′	T_m = 52.5 °C F = 500, R = 500	
	Hsp70	86	F 5′ GAC ATG AAG CAC TGG C 3′ R 5′ AGG ACC ATG GAG GAG 3′	T_m = 50.5 °C F = 500, R = 300	
	HIF-1α	123	F 5′ TCG GAG GTG TTC TAC GAG C 3′ R 5′ TCC GTT TCC TCT TCT GCC AC 3′	T_m = 61 °C F = 400, R = 300	

Sequences were aligned with consensus sequences stored in GenBank by Clustal X, implemented in the MEGA 3.1 software, using default parameters and submitted in GenBank. Characterization of genes full-length sequences as well as the assessment of the phylogenetic relationships between genes from this research and those already in GenBank, is a part of another study.

2.3. Relative real-time PCR quantification

Real-time PCR assays were performed using iQ SYBR Green Supermix in accordance with manufacturers' procedure (Bio-Rad) on an iQCycler platform. Template cDNA was diluted 1:10 and run in triplicate. Control reactions without cDNA template (no-template controls) including RNA samples were performed to check that amplified products were not a result of DNA contamination or due to primer–dimer effects. Primer concentration was optimized for each target and housekeeping gene in order to obtain the concentration that yields the lowest C_T values (Table 1). During that run, after 95 °C was reached, raw data from a melting curve were collected and the products were subjected to different temperature increments, enabling the ruling out of eventual primer–dimer formation (Nolan, 2004).

The threshold was calculated using maximum curvature approach, with per-well baseline cycles determined automatically. Data analysis window was set at 95.00% of a cycle, centered at the end of the cycle. Weighted mean digital filtering has been applied, with the global filtering off. The products of the reaction with the single-peak melting curve were visualized after loading on 2% agarose gel.

β-actin, GAPDH (Glyceraldehyde-3-phosphate dehydrogenase) and 18ssu rDNA were evaluated as housekeeping genes for the thermal effect. β-actin and 18ssu rDNA were considered as suitable housekeeping gene for normalization since its level of expression were independent of acclimation temperature ($\Delta C_{T\,\beta\text{-actin (mean warm temp.} - \text{mean cold temp.)}} = 0$) (Livak and Schmittgen, 2001). Even though both genes were found to be not ideal internal control for normalization in some studies indicating dependence upon physiological state and tissue type (see Small et al., 2008), they showed no shifts in expression under our experimental conditions. The use of β-actin was favored over 18ssu rDNA, since its broad use in fish studies make it widely comparable with other experiments (Jorgensen et al., 2006). Amplification efficiency between target and standard (β-actin) was evaluated comparing their serial dilutions; ΔC_T ($C_{T,\ target} - C_{T,\beta\text{-actin}}$) was calculated for each cDNA dilution ($N = 5$) and data were fit using least-squares linear regression analysis (Livak and Schmittgen, 2001).

The $2^{-\Delta\Delta C}{}_T$ method was used to calculate relative changes in target gene expression compared to housekeeping gene (Livak and Schmittgen, 2001). After obtaining average values of $\Delta\Delta C_T$ for each sample and its standard deviation, data were transformed using logarithmic transformation to correct non-homogeneous variance, and this for, graphs showing expression levels have negative values (Krebs, 1998). The difference in gene expression levels between warm and cold acclimated animals was calculated by two-tailed independent sample Student t-test, while the difference between same types of tissue and all sampled tissues on two experimental temperatures was calculated by multi-factor ANOVA, using STATISTICA 6 software.

3. Results

We were able to successfully amplify and measure constitutive gene expression of IL-1β, TNF-α, HIF-1α, Hsp70 and Na^+/K^+ATP-ase in all four organs. When total expression values for all organs were summed together and compared between two temperatures, statistically significant differences were only found in HIF-1α expression ($p = 0.000263$), upregulated in cold. Hsp70 showed tendency for upregulation as well, but at the edge of statistical significance ($p = 0.06$). Compared to other target genes, TNF-α showed the widest range of transcripts oscillations.

Fig. 1 depicts variation in transcripts levels for each organ after warm and cold exposure. HIF-1α had the most abundant transcripts in liver, whereas the lowest expression occurred in spleen and gill from the warm tank. After cold acclimation, gill and spleen transcript abundance increased 5.37 and 6.645 folds respectively. These were found to be statistically significant at a level of $p<0.05$ (gill $p = 0.004833$, spleen $p = 0.000065$). Hsp70 was constitutively most abundant in liver (0.47 fold in relation to β-actin), in both warm and cold acclimated fish. In general, there was no statistical difference between Hsp70 tissue expressions in warm and cold, except in spleen ($p = 0.033$).

At warm water temperature (23 °C) for each target gene, expressions were compared between four organs (Fig. 2). Statistical difference between tissues was observed for Hsp70 in liver compared to kidney, spleen, gills ($p = 0.0000076$; 0.021; 0.0198), where the gene was upregulated, as well as for Na^+/K^+ATP-ase between the highest expression in kidney and lowest in spleen ($p = 0.007$).

In cold acclimation (15 °C), organs did not show statistical variation in target expressions, except between Na^+/K^+ATP-ase levels in spleen and gills ($p = 0.008$) (Fig. 3). Again, the highest transcripts levels were observed for TNF, which was the most expressed this time in liver, as well as Hsp70. IL-1β and HIF were the most expressed in gills, while Na^+/K^+ATP-ase was in kidney.

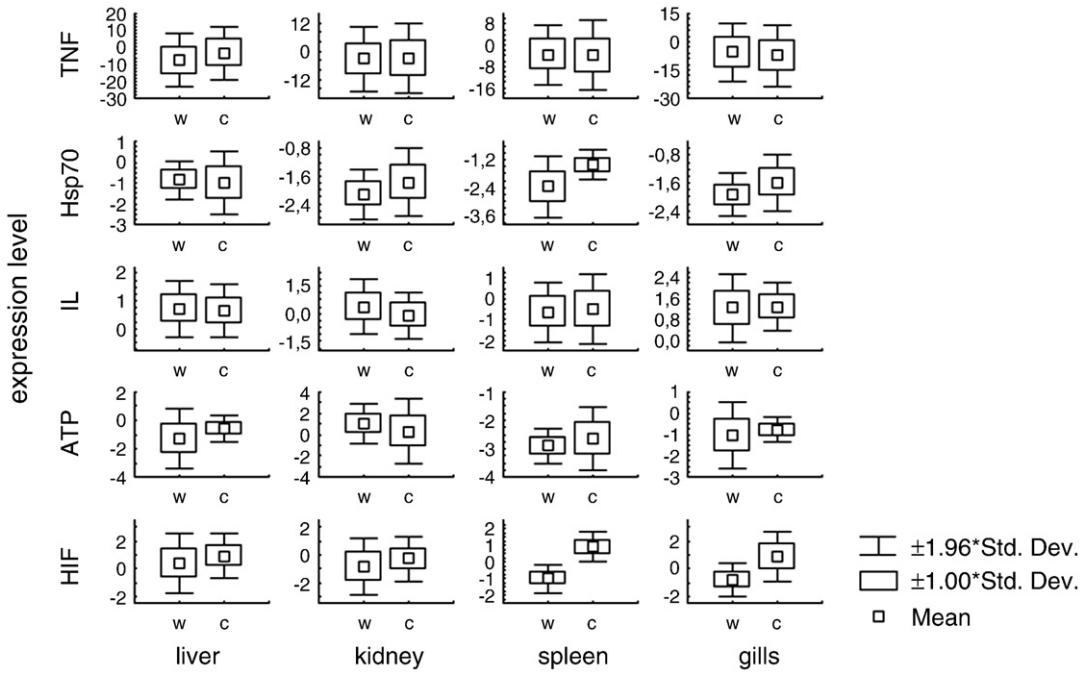

Fig. 1. Differences in organ expression of target genes analyzed in warm water (w) and cold water tank (c). Statistically significant level of difference ($p<0.05$) is observed for Hsp70 expression in spleen ($p=0.033166$), and for HIF in spleen ($p=0.000065$) and gills ($p=0.004833$).

Summing all target transcripts levels from both warm and cold exposure, the highest expression was observed in liver, followed by gill and kidney, with the lowest in spleen.

4. Discussion

Seven-week chronic exposure of Pacific bluefin tuna to cold temperature resulted in significant shifts in expression levels of oxygen-dependent HIF transcripts in spleen and gill, and the molecular chaperone Hsp70 in spleen. This is in accordance with previous data gathered from Northern blot and cDNA microarray analysis in poikilotherm fish (Buckley et al., 2006; Place et al., 2004; Podrabsky and Somero, 2004; Lund et al., 2006). Fish that routinely experience rapid temperature fluctuations are able to alter gene expression in relatively short time (Podrabsky and Somero, 2004), but the length of time that elapses in order to restore cellular homeostasis during a long-

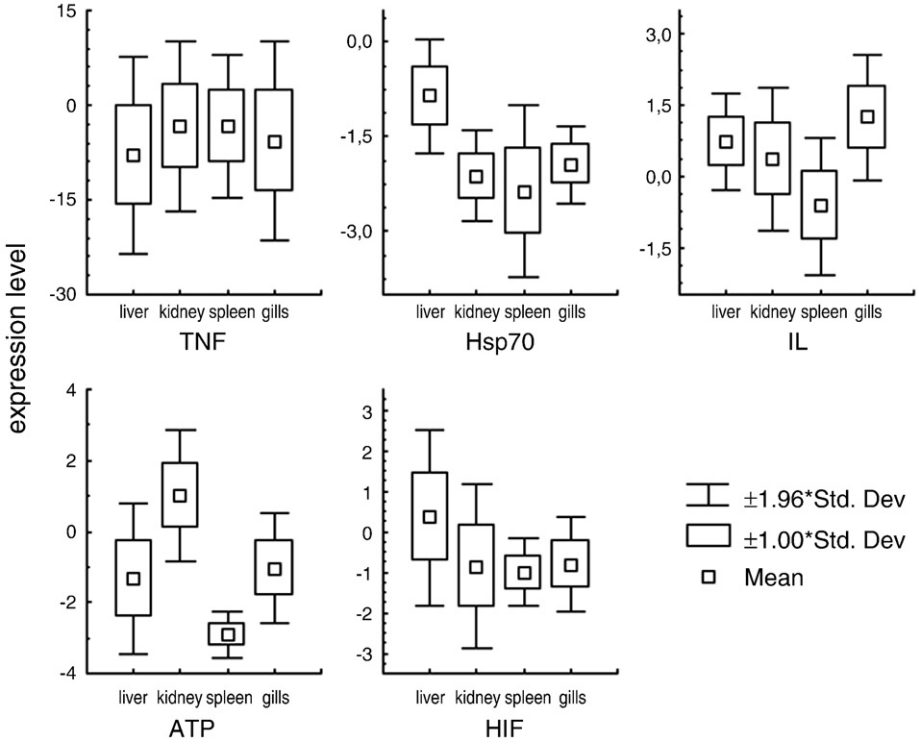

Fig. 2. Abundance of transcripts in sampled organs from warm water tank (T2). Statistical difference between tissues was observed for Hsp70 in liver compared to kidney, spleen, gills ($p=0.0000076$; 0.021; 0.0198), as well as for Na^+/K^+ATP-ase between expression in kidney and spleen ($p=0.007$).

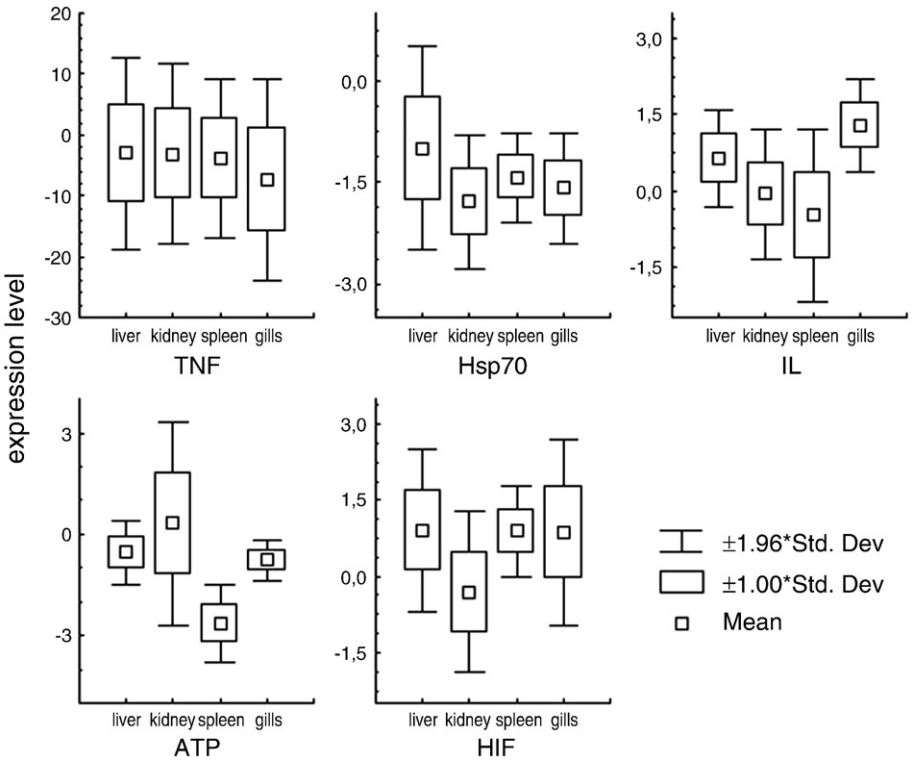

Fig. 3. Abundance of transcripts in sampled organs from cold-water tank (T3), showing no statistically significant level of difference ($p<0.05$) between sampled tissues.

term temperature exposure has only recently been investigated (Fangue et al., 2006; Wang et al., 2007). A group of the hallmark molecules used as a thermal stress marker are the members of the heat shock protein family (Hsp). Being intronless in a large part, these genes are able to shift in gene expression levels in a matter of minutes, allowing thus immediate message translation into a new protein (Iwama et al., 1998). Among them, Hsp70 interacts with misfolded proteins, preventing them from forming aggregates (Fink, 1999) that consequently can become cytotoxic (Bucciantini et al.; 2002). Assessing the general cellular response to heat stress in the goby (*Gillichthys mirabilis*), Buckley et al. (2006) have found that molecular chaperons were transcriptionally the most responsive genes, displaying tissue-specific response. Tissue-specificity to Hsp70 expression was shown in bluefin tuna herein, indicating that a long-term exposure to cold imposes exceptional pressure on this endothermic teleost species, especially on haemaotopoietic and gas-exchange organs, evidencing in turn, the site of considerable tissue-specific protein damage. The highest abundance of Hsp70 in spleen may also be a functional result reflecting the need for protein chaperoning, given the spleen is a haematopoietic organ; a site for the germination of new and the destruction of old erythrocytes (Fänge and Nilsson, 1985).

Protein-degradation process in tuna spleen indicated by Hsp70 upregulation is oxygen-dependent, evidenced by the measured upregulation of HIF transcripts in the same organ. Interestingly, *in vitro* studies have demonstrated that Hsp90 interacts with HIF-1α, affecting its function and stabilizing influence of the protein (Katschinski et al., 2004; Minet et al., 1999). HIF has a central role in regulation of gene expression during hypoxia and regulation of erythropoietin in mammals; although it is made continuously, it is rapidly degraded during normoxia. The onset of hypoxia leads to essentially instantaneous stabilization and accumulation of HIF-1α subunit that travels to the nucleus in order to bind to hypoxia response elements (HREs) in the promoter or enhancer region of hypoxia-inducible genes (Nikinmaa and Rees, 2005). Even though aquatic hypoxia is frequently associated with changes in temperature, food availability or pollutant exposure, temperature effects on HIF function have been neglected most of the time. It is known that in the nematode *Caenorhabditis elegans* HIF is required for heat acclimation (Treinin et al., 2003), but nothing has been done in respect to fish response. Fish respond to hypoxia by increase of hematocrit and swelling of erythrocytes, which are both HIF-influenced through erythropoietin-regulation. At this moment, we can only suggest that in cold acclimated tuna, upregulation of HIF in spleen can be an indirect result of tissue and protein damage regulated through HSP, or a measure to increase the hematocrit during hypoxic conditions induced by temperature. Upregulation of HIF gene transcripts in gill is not that clear, but may be related to the fact that this region is primarily associated with oxygen uptake. Given the oxygen partial pressure or saturation is high in the cold, the upregulation would suggest that fish response is related to the diffusional effects of the cold imparted by a slowing of the heart, evidenced in *in vitro* and whole animal performance studies (Blank et al., 2004). However, we have recently shown that bluefin tuna increases metabolic rate in response to cold (Blank et al., 2007) suggesting that indeed cold induces a need for increased metabolic activity. Thus, in cold challenged tuna, elevated oxygen consumption and the gill upregulation of HIF may be related to the whole animal responses observed, suggestive of diffusional limitations when cold exposed. What remains unknown is how the increased need for oxygen at the whole animal level is aided by the HIF upregulation, indicating that the response of bluefin tunas to hypoxia both in the wild and captivity is most likely a complex process.

The sodium pump (Na^+/K^+ ATP-ase) coordinates active transport of Na^+ ions out of the cell and K^+ ions into the cell, fueled by the hydrolysis of a molecule of ATP. The pump enables the cell to maintain osmotic balance and membrane potential, backing up as well the secondary active transport of glucose and amino acids. In gill epithelia, mitochondria-rich cells represent the most important key in osmoregulation, controlled by multiple hormones (Kültz and Somero, 1995; Metz et al., 2003). In tuna,

the long-term cold acclimation did not affect gill or other organs Na^+/K^+ ATP-ase expression, suggesting that gill and Na^+/K^+ ATP-ase of tunas that function at ambient temperature, are not affected by chronic temperature shifts. This would be consistent with the need for the bluefin tuna gill to operate over a wide range of ambient temperatures. In respect to blufin internal organs, apart from the heart, gills have and function at ambient temperature, and metabolic heat is not able to stabilize its thermal oscillation. This for, target gene expression in blufin gills has a similar trend as in other poikilothermic fish.

Ambient temperature has a marked influence on immune reactivity in fish, however some components are more sensitive than the others (Bowden et al., 2007). The temperature range at which optimal immune response is obtained is termed permissive, while the temperature below these, but still within the physiological range, tends to be immunosuppressive (Manning and Nakanishi, 1996). In tuna, long-term cold acclimation did not have a significant impact on both cytokines expression levels. TNF-α shows to be constitutively expressed in all sampled organs, as demonstrated in other fish (Laing et al., 2001; Garcia-Castillo et al., 2002), but to our knowledge there are no *in vivo* studies of temperature-dependent regulation of TNF-α in fish. Interestingly, most of previous studies have found constitutive expression of IL-1β in fish only after the antigen or lipopolysacharide (LPS) stimulation of immune competent cells (Pleguezuelos et al., 2000; Zou et al., 2000; Pelegrin et al., 2004; Scapigliati et al., 2001). The exceptions of this fact, where its constitutive expression without a prior stimulation was demonstrated, were reported in salmon, common carp (Engelsma et al., 2001) and now in this study. Anadromous and landlocked Atlantic salmon (*Salmo salar*) showed the highest IL-1β levels in gill (Ingerslev et al., 2006) in accordance with observation in bluefin tuna, suggesting the key role of gill as a first barrier to outside challenges. Other studies have drawn variable conclusions in respect to temperature effect on other cytokines production (Hardie et al., 1995; Bly and Clem, 1992; Metz et al., 2006; Zou et al., 2000), which suggests that cytokine expression is tissue- and species-specific, and in tuna, the endothermy must play a significant role in steady levels of cytokines.

In summary, we have examined expressions of Hsp70, Na^+/K^+ ATP-ase, HIF-1α, IL-1β and TNF-α genes in liver, kidney, gill and spleen of an endothermic teleost exposed to long-term exposure to warm and cold temperature. Tunas are unusual given their capacity to have temperature of internal organs, except the heart, elevated above ambient, while external organs such as gills are more exposed to the environment, thus ectothermic. Seven-week cold acclimation period in Pacific bluefin tuna was marked by upregulation of HIF-1α in gills and spleen, as well as Hsp70 upregulation in spleen. Even though other genes of interest did not show shifts in expression, it still remains to clarify if tuna is able to balance their expression to constitutive levels in a shorter period of time, or indeed their usefulness as temperature-dependent stress biomarkers should be ruled out.

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