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Metabolic biochemistry of cardiac muscle in three tuna species (bigeye, *Thunnus obesus*; yellowfin, *T. albacares*; and skipjack, *Katsuwonus pelamis*) with divergent ambient temperature and oxygen tolerances

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Abstract

Bigeye tuna (*Thunnus obesus*) have much greater vertical mobility than yellowfin (*T. albacares*) and skipjack (*Katsuwonus pelamis*) tunas, due to an apparent greater tolerance of the changes in ambient temperature and oxygen occurring with depth. In an attempt to identify physiological processes (e.g., effects of temperature on cardiac function) responsible for these behavioral differences, we examined enzyme activities (at 12 °C, 17 °C, and 25 °C) of cardiac muscle in all three species. Contrary to our expectations, we found few differences and no clear explanatory patterns in maximum enzyme activities (V_{max}) or enzyme activity ratios. For example, citrate synthase (CS) activity was the same in bigeye and skipjack tunas, but ≈ 40% lower in yellowfin tuna, whereas carnitine palmotoyltransferase (CPT) activity in skipjack tuna was approximately double that in the other two species. The ratio of CPT to pyruvate kinase (PK) activity, a measure of the tissues' preference for fatty acids as metabolic substrates, was the same in bigeye and yellowfin tunas, but elevated skipjack tuna. The ratios of lactate dehydrogenase (LDH) to CS activity and of PK to CS activity (anaerobic–aerobic enzyme activity ratios – taken as measures of the tissues' ability to tolerate hypoxia) were both elevated in yellowfin tuna cardiac tissue relative to the other two species. We also found no differences in temperature sensitivity (Q_{10} values) when comparing cardiac enzyme activities across species, nor effects of temperature on the substrate affinity (K_m) of LDH. In sum, our results do not suggest any clear metabolic difference in the cardiac muscle that would explain the apparent greater tolerance of bigeye tuna to acute hypoxia and ambient temperature changes or their substantially greater vertical mobility.

Abbreviations: ATPase – total myofibrillar ATPase activity; CPT – carnitine palmotoyltransferase; CS – citrate synthase; LDH – lactate dehydrogenase; PK – pyruvate kinase; Q_{10} – change in reaction velocities per 10 °C change in temperature; V_{max} – maximum enzyme activities.

Introduction

Yellowfin tuna (*Thunnus albacares*), skipjack tuna (*Katsuwonus pelamis*), and bigeye tuna (*T. obesus*) have overlapping geographical ranges (Sund

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et al. 1981), yet the depth distributions and vertical movement pattern of the latter are dramatically different. Yellowfin and skipjack tunas spend the majority (≈ 60–80%) of their time in the uniform temperature surface layer (water temperatures ≈ 20–28 °C over their range) where oxygen levels are at or near saturation (>6 ml O_2 l^{-1}) (Holland et al. 1990; Cayre and Marsac 1993; Brill et al. 1999; Betraund et al. 2002). Bigeye tuna generally remain in the surface layer at night, and descend at dawn to >500 m depth (Holland et al. 1992; Mohri et al. 1996; Dagorn et al. 2000; Schaefer and Fuller 2002). They thus frequently endure prolonged exposure to ambient temperatures between 3 °C and 10 °C, and oxygen concentrations <1.5 ml O_2 l^{-1} (Hanamoto 1987; Schaefer and Fuller 2002; Musyl et al. 2003). In contrast, water temperatures 8 °C below surface layer temperature and ambient oxygen levels of 3.5 ml O_2 l^{-1} appear to limit the vertical movements of skipjack and yellowfin tunas (Brill 1994; Brill et al. 1999).

Bigeye tuna's ability to access great depths allows them to exploit more effectively deeper-living prey, especially the nektonic organisms of the deep sound-scattering layer (SSL) when they descend at dawn (e.g., Dagorn et al. 2000). Indeed, recent studies of tunas' trophic ecology have (via gut samples) found that bigeye tuna generally select mesopelagic prey from the SSL, while yellowfin tuna feed primarily on epipelagic prey from the mixed layer (Grubbs and Holland 2003).

At first glance, it might appear that all tunas would be only minimally affected by acute temperature changes due to their vascular countercurrent heat exchangers that serve to slow rates of temperature change in red muscle (Neill et al. 1976; Graham and Dickson 2001). However, because their hearts are on the "downstream" side of the vascular heat exchangers, and because the blood supply to the coronary circulation arises directly from the gills, cardiac tissue remains within 1 °C of ambient temperature (Carey et al. 1984). Therefore, while afforded the benefits of retaining metabolically produced heat to elevate swimming muscle temperatures, tunas lack the ability to maintain cardiac muscle temperature significantly above ambient temperature.

Several authors (e.g., Brill et al. 1999; Brill and Bushnell 2001; Blank et al. 2002) have hypothesized that the disparate behavior patterns among yellowfin, skipjack and bigeye tunas stem from differences in the effects of acute temperature reduction on cardiac function, and that cardiac function ultimately determines vertical mobilities and depth distributions. Based on this line of reasoning, the cardiac function of bigeye tuna should be far less impaired by acute reductions in ambient temperature than that of skipjack or yellowfin tunas. The effects of acute 5–10 °C changes in ambient temperature on cardiac function *in vivo* have been studied in skipjack and yellowfin tunas. During such temperature reductions, heart rates and cardiac outputs drop sharply ($Q_{10} \approx 1.5$–2) (Korsmeyer et al. 1997; Blank et al. 2002; Brill, et al. manuscript in preparation). Similar experiments are, however, yet to be conducted on bigeye tuna as they are generally not available in captivity. There are also several lines of direct physiological evidence indicating that bigeye tuna are more tolerant of reductions in ambient oxygen levels than skipjack or yellowfin tunas (Bushnell et al. 1990; Lowe et al. 2000). Based on all these observations, and the well documented differences in depth distributions of bigeye, yellowfin, and skipjack tunas, we hypothesize the existence of biochemical adaptations in bigeye tuna cardiac muscle that are not present in yellowfin and skipjack tuna cardiac muscle.

It is well established that enzyme function is sensitive to temperature (Hochachka and Somero 2002), and enzyme adaptations have been clearly linked to the different geographic and depth distributions among congeneric fish species (e.g., Graves and Somero 1982; Yang et al. 1992). In general, enzyme catalysis halves for every 10 °C drop in ambient temperature (i.e., $Q_{10} = 2$), thereby requiring either a proportional increase in the amount of enzyme for fish acclimated to colder temperatures or expression of different isozymes (Sidell et al. 1987; Sephton et al. 1990; Sephton and Driedzic 1991; Pierce and Crawford 1997). We specifically hypothesize that bigeye tuna should have elevated cardiac enzyme activity levels compared with yellowfin and skipjack tunas, or metabolic isozymes that are less thermally sensitive than those expressed in yellowfin or skipjack tunas. Either of these biochemical

adaptations could contribute to bigeye tuna's greater vertical mobility. To test these hypotheses, we investigated *in vitro* cardiac muscle enzyme activities (V_{max}) in bigeye, yellowfin, and skipjack tunas at 12 °C, 17 °C, and 25 °C.

In summary, we undertook our study to understand how bigeye tuna' cardiac biochemistry might differ from closely related tuna species with different depth distributions, vertical mobility patterns, and tolerances of acute reductions in temperature and oxygen. We specifically seek to understand how bigeye tuna hearts can generate sufficient energy at the extremely cold temperatures and low ambient oxygen conditions routinely encountered by this species. Conversely, we are also attempting to discern how acute temperature changes effect enzymatic activities thereby imposing physiological limits responsible for divergent depth distributions.

Materials and methods

Tissue samples were obtained from yellowfin, skipjack, and bigeye tunas (≈3–10 kg) captured in the central Pacific near the main Hawaiian Islands via longline fishing methods aboard the NOAA research vessel *Townsend Cromwell*. Additional tissue samples from yellowfin and skipjack tunas (≈1–2 kg) were taken from fish held at the Kewalo Research Facility, National Marine Fisheries Service, Southwest Fisheries Science Center, Honolulu Laboratory. (Fish procurement, care, and handling procedures at the Kewalo Research Facility are described in Nakamura (1972)). Animals were sacrificed with either a blow to the head, direct brain destruction (pithing), or an overdose of sodium phenobarbital. Hearts were removed, a sample of ventricular muscle taken, blotted dry, immediately frozen in liquid nitrogen, and stored at −80 °C.

Enzyme analyses

All enzyme activity assays and K_m determinations were performed in temperature-controlled cuvettes at 12, 17, and 25 (±0.2) °C in a Shimadzu Bio-Spec spectrophotometer (model 1601, Shimadzu Scientific Instruments Inc., Columbia, Maryland, USA) equipped with UVPC and UV Probe Spectroscopy software.

Unless stated otherwise, enzyme activities were measured by following the change in NADH concentration at 340 nm. Assays were run in duplicate or triplicate for each sample. Enzyme activities were expressed as μmol substrate converted to product per minute per gram of wet tissue weight (U g^{-1}). Expression of enzymatic activity per unit wet weight (as opposed to per unit dry weight) is preferred as this expression indicates the metabolic activity per unit of living flesh (Childress and Somero 1979). All reagents were obtained from Sigma Chemical Company, St. Louis, MO, USA.

Total myofibrillar ATPase

Our methods were slightly modified from those described in Sidell et al. (1987). Tissue samples (≈200 mg) were homogenized (using Tissuemizer Model SDT-1810, Tekmar, OH, USA) in 3 ml of ice cold extraction medium consisting of 75 mM imidazole, 1 mM EDTA (ethylene diamine tetraacetic acid), and 1 mM DTT (dithiothreitol), pH = 7.5 at 25 °C. An amount of 20 μl of homogenate was added to 1 ml assay buffer consisting of 50 mM imidazole, 50 mM KCl, 3.5 mM $MgCl_2$, 0.1 mM $CaCl_2$, oligomycin 5 μg per ml^{-1}, pH = 7.5 at 25 °C. The final reaction mixture, made fresh daily using the assay buffer, contained 5 mM ATP, 0.2 mM NADH, 2 mM PEP (phosphoenolpyruvate), 5 U ml^{-1} pyruvate kinase, 3 U ml^{-1} lactate dehydrogenase.

PK, CS, and CPT

Tissue samples (≈200 mg) were powdered under liquid nitrogen and homogenized in 11 volumes of ice cold extraction medium consisting of 20 mM hepes, 1 mM EDTA, and 0.1% Triton X-100, pH = 7.4 at 25 °C. The assay mixture for LDH contained 50 mM hepes, 0.20 mM pyruvate, and 0.15 mM NADH, pH = 7.0 at 25 °C; and the assay mixture for PK contained 50 mM hepes, 5 mM ADP, 100 mM KCl, 10 mM $MgCl_2$, 0.15 mM NADH, 5 U LDH, and 5 mM PEP, pH = 7.4 at 25 °C. For both PK and CS activity measurements, the absorbance was monitored following the addition of 50 μl diluted homogenate to 1.0 ml of the assay medium. The assay mixture

for CS contained 20 mM Tris, 0.05% Triton X-100, 0.10 mM DTNB [5,5-dithio-bis(2-nitrobenzoic)] acid, 0.1 mM acetyl CoA, and 0.5 mM OAA (oxaloacetate), pH = 8.0 at 25 °C; and 25 μl of homogenate added to 1.0 ml of the assay medium. Background activity in absence of OAA was subtracted from the total activity. Production of oxidized coenzyme A was monitored with DTNB by following the increase in absorbance at 412 nm. The assay mixture for CPT contained 20 mM Tris, 0.20 mM DTNB, 0.10 mM palmitoyl CoA, and 5 mM L-carnitine, pH = 8.0 at 25 °C; and 50 μl diluted homogenate to 1.0 ml of the assay medium. Background activity in absence of carnitine was subtracted from the total activity. Liberation of free CoA was monitored with DTNB by following the increase in absorbance at 412 nm.

LDH

Tissue samples (≈200 mg) were homogenized in 11 volumes of ice-cold imidazole-HCl extraction buffer containing EDTA (80 mM, pH 6.6 at 20 °C). The homogenate was centrifuged in an Eppendorf microcentrifuge for 5 min and the supernatant retained. LDH activity was then determined as a function of nine pyruvate concentrations (0.01–2.0 mM) at three different temperatures (12 °C, 17 °C, and 25 °C). LDH K_m activities were calculated from Lineweaver–Burk plots.

Statistical analyses

Statistical comparison of maximal cardiac enzyme activities, activity ratios, and K_m values among the three species of fish were performed using one-way analysis of variance (ANOVA, Holm Sidak method). Cardiac enzyme temperature sensitivity (Q_{10} values) between the two temperature ranges (17–12 °C and 25–17 °C) were compared using paired *t*-tests. Pair-wise comparisons of Q_{10} values (both within cardiac enzymes and across species, and within species and across cardiac enzymes) were performed using a two-way ANOVA (Holm Sidak method).

All procedures were done using Sigma Stat for Windows, version 3 (SPSS Inc., Chicago, Illinois, USA). Statistical significance is reported when $P < 0.05$.

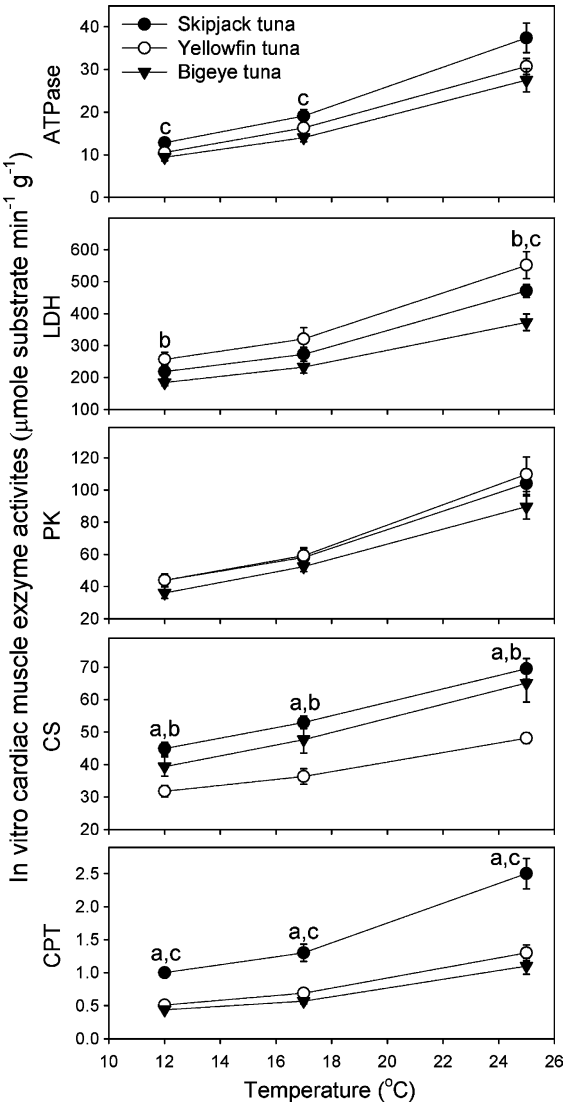

Figure 1. In vitro cardiac muscle enzyme activities (V_{max}) in bigeye, yellowfin, and skipjack tunas measured at 12 °C, 17 °C, and 25 °C. Significant differences in pairwise comparisons are indicated by the lower case letters: "a" = skipjack tuna versus yellowfin tuna, "b" = yellowfin tuna versus bigeye tuna, "c" = bigeye tuna versus skipjack tuna.

Results

Results of cardiac muscle enzyme activities at 12 °C, 17 °C, and 25 °C are summarized in Figure 1. There were few significant differences between species. The largest differences were in CS and CPT activities. The former was elevated in both skipjack and bigeye tunas (by ≈ 40%) compared to yellowfin tuna. CPT activity in

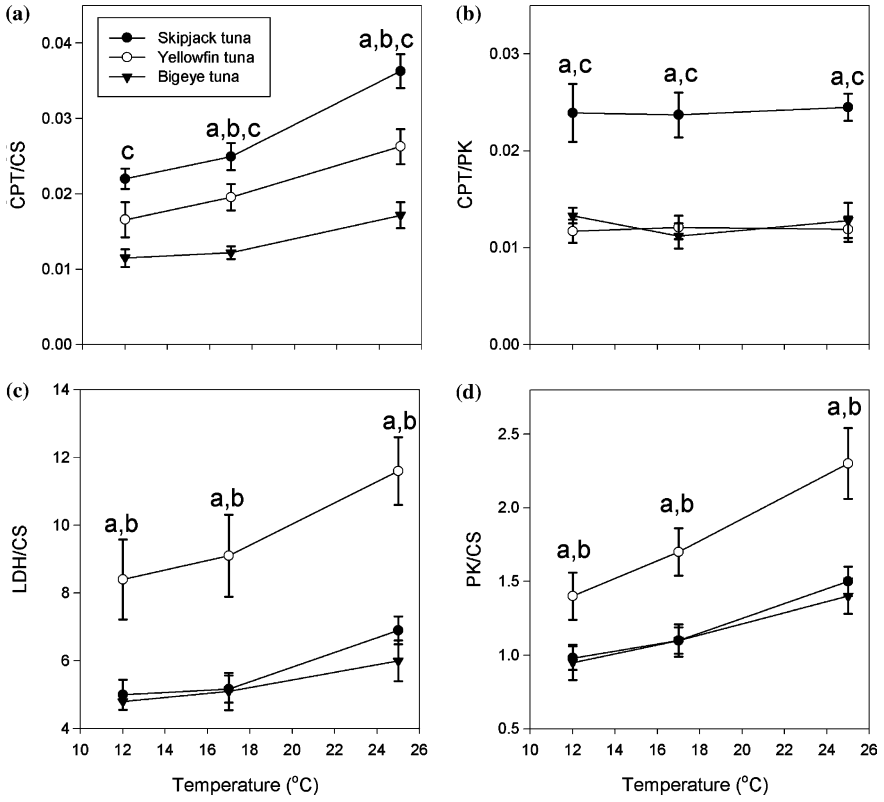

Figure 2. *In vitro* cardiac muscle enzyme activity ratios in bigeye, yellowfin, and skipjack tunas measured at 12 °C, 17 °C, and 25 °C. Significant differences in pairwise comparisons are indicated by the lower case letters: "a" = skipjack tuna versus yellowfin tuna, "b" = yellowfin tuna versus bigeye tuna, "c" = bigeye tuna versus skipjack tuna.

skipjack tuna cardiac muscle was approximately double that in yellowfin and bigeye tuna cardiac muscle. In contrast, there were no interspecific differences in PK activities. Because of the lower CS activity in yellowfin cardiac muscle, the LDH/CS and PK/CS activity ratios were approximately 1.5 times those in skipjack and bigeye tuna cardiac muscle at all three temperatures (Figure 2). The ratios of CPT to PK and CPT to CS activities were both elevated in skipjack tuna cardiac muscle and imply that skipjack tuna cardiac muscle is most adapted to use fatty acids as metabolic substrates.

We found only one significant difference when comparing temperature sensitivity (Q_{10} values) over the 12–17 °C and 17–25 °C temperature ranges for all cardiac enzymes. The influence of temperature on PK activity from skipjack tuna cardiac tissue (mean Q_{10} ± sem) was 1.77 ± 0.12 over the 12–17 °C temperature change, but significantly greater (Q_{10} = 2.11 ± 0.07) over 17–25 °C.

Table 1. Mean (± SEM) Q_{10} values for maximal *in vitro* activities of metabolic enzymes and total myofibrillar ATPase in cardiac tissue from three species of tunas measured over the temperature interval 12–25 °C

	Skipjack tuna	Yellowfin tuna	Bigeye tuna
ATPase	2.29 ± 0.11	2.30 ± 0.09	2.28 ± 0.07
LDH	1.86 ± 0.13	1.82 ± 0.8	1.83 ± 0.09
PK	1.97 ± 0.07	2.02 ± 0.05	2.07 ± 0.17
CS	1.40 ± 0.03	1.38 ± 0.04	1.47 ± 0.05
CPT	2.09 ± 0.13	2.05 ± 0.15	2.00 ± 0.24

All other enzyme activities showed a "linear" response to temperature. We therefore present only the Q_{10} values over the temperature range of 12–25 °C in Table 1.

We found no differences in Q_{10} values when pair-wise comparisons were made within enzymes and across species (Table 1). We did find some differences when pairwise comparisons were made within species and across enzymes, but

Table 2. Pairwise comparisons of Q_{10} values made within species and across enzymes

	ATPase	LDH	PK	CS	CPT
ATPase	–	SJ, YF, BE		SJ, YF, BE	
LDH		–		SJ	YF
PK			–	YE, BE	BE
CS				–	
CPT					–

Significant differences in enzyme temperature sensitivities within species are indicated by the letters "SJ", "YF", and "BE", referring to skipjack, yellowfin and bigeye tuna, respectively. LDH and CS were less temperature sensitive than total myofibrillar ATPase activity (ATPase) in all three tuna species, and CS was generally less temperature sensitive than the other enzymes.

there was no apparent pattern suggestive of differences in the thermal tolerances among the three tuna species (Table 2). For example, LDH and CS were less temperature sensitive than total myofibrillar ATPase activity (ATPase) in all three tuna species, and CS was consistently the least temperature sensitive.

Data on K_m of LDH for pyruvate in cardiac muscle at three temperatures are shown in Table 3. There were no differences in the affinity of LDH for pyruvate at all experimental temperatures.

Discussion

Our results approximate the cardiac enzyme activity levels previously reported for skipjack and yellowfin tunas (Hochachka et al. 1978; Guppy et al. 1979; Moyes et al. 1992; Dickson 1995). The mass-specific enzyme activities we found were also generally comparable to those of other teleosts (Driedzic 1992). These data are consistent with the idea that there are upper limits to mass-specific enzyme activity levels in tissue required to produce constant work (i.e., function aerobically), and that tunas achieve their exceptionally high cardiac energy outputs primarily by ventricular hypertrophy (Brill and Bushnell 1991, 2001).

Our original hypothesis was that cardiac enzyme activities from bigeye tuna would differ from both skipjack and yellowfin tunas, reflecting bigeye tuna's greater tolerance of reductions in ambient temperature that accompany their extensive daily vertical migrations. Neither our data on cardiac enzyme activities (Figure 1), nor temperature sensitivity (Table 1) supported this idea. Our results, however, agree with the temperature sensitivities of cardiac enzymes reported for other teleosts (e.g., Sephton and Driedzic 1991).

Similarly, LDH affinity for pyruvate (K_m values) were not different at any given temperature suggesting a conservation of catalytic properties among the tuna congeners (Table 3). This was also an unexpected result. Differences in the LDH affinity for pyruvate have been demonstrated in a range of eurythermal and stenothermal fishes (Coppes and Somero 1990; Hochachka and Somero 2002), and differences in habitat temperature of 2–8 °C have been shown to result in measurable differences in the affinity of swimming muscle LDH for pyruvate (i.e., K_m) (Graves and Somero 1982; Graves et al. 1983). Somero et al. (1996) reviewed the extensive literature on the effects of temperature for a broad range of terrestrial and aquatic heteroterms and concluded that "it is clear that the degree of eurythermy or stenothermy found at the whole organism level is mirrored in... the formation of enzyme–ligand (substrate and cofactor) complexes." It seems clear to us that bigeye tuna can be considered "eurythermal" and yellowfin and skipjack tunas "stenothermal".

Previous studies (Driedzic and Hart 1984; Sidell et al. 1987) have shown temperature acclimation can alter carbohydrate versus fatty acid metabolism in fish hearts. Hearts isolated from cold-acclimated fish, and supplied with fatty acids, show increased oxygen consumption and power output compared to those provided with glucose (Sephton et al. 1990; Sephton and

Table 3. LDH affinity for pyruvate (mean K_m values ± SEM, μM) in cardiac tissue from skipjack, yellowfin, and bigeye tunas measured at 12 °C, 17 °C, and 25 °C

Temperature (°C)	Skipjack tuna	Yellowfin tuna	Bigeye tuna
12	59 ± 13 $n = 6$	50 ± 13 $n = 5$	52 ± 10 $n = 6$
17	65 ± 12 $n = 6$	83 ± 29 $n = 5$	70 ± 20 $n = 6$
25	82 ± 14 $n = 6$	81 ± 23 $n = 5$	86 ± 24 $n = 7$

Driedzic 1991; Bailey and Driedzic 1993). We therefore expected bigeye tuna hearts to have the highest cardiac CPT activity, but this was not the case. Rather, skipjack tuna cardiac muscle had the highest levels of CPT activity; as well as the highest CPT to CS and CPT to PK activity ratios (Figure 2, Panels A and B). These data suggest that fat oxidation contributes relatively less to the energy demands in bigeye and yellowfin tuna hearts, than skipjack tuna hearts.

Bigeye tuna are also apparently more tolerant of acute reductions in ambient oxygen occurring with depth than are skipjack and yellowfin tunas (Lowe et al. 2000). Studies of crustaceans living in the oxygen minimum zone have shown that they are able to do so due to a suite of physiological and biochemical adaptations (e.g., Childress 1971; Belman and Childress 1975; Sanders and Childress 1990). Equivalent studies of cardiac and swimming muscle enzyme activities (and activity ratios) in *Sebastolobus* spp. (family Scorpaenidae) have likewise demonstrated physiological and biochemical adaptations (Yang et al. 1992), such as elevated anaerobic to aerobic cardiac enzyme activity ratios (PK/CS and LDH/CS) indicative of greater hypoxia tolerance in deeper living species. We found no such enzyme activity differences suggestive of a greater tolerance to hypoxia in bigeye tuna hearts (Figure 2, Panels c and d).

Effects acute temperature changes on teleost cardiac function

Bailey et al. (1991) examined the effects of acute temperature change on the performance and metabolism of eel (*Anguilla anguilla*) and pickerel (*Esox niger*) hearts. The latter remain active at cold temperatures (e.g., 5 °C), whereas the former become quiescent. These differences were reflected in the chronotropic and inotropic responses to acute 10 °C reductions in temperature of both perfused hearts and ventricular strips. In results that parallel ours, Bailey et al. (1991) found that cardiac enzyme activities from both pickerel and eel hearts showed approximately equal temperature sensitivities. The impairment of eel heart function with decreasing temperature was not to due to an impaired ability to metabolize ATP, nor to generate it through aerobic pathways, but rather due to a slowing of calcium extrusion from the myocyte resulting in a prolonged relaxation period.

As we have stated previously, there is no direct evidence that the chronotropic or inotropic effects of acute temperature reductions in bigeye tuna hearts are less than those observed in skipjack or yellowfin tuna hearts, because the requisite experiments on bigeye tuna are yet to be conducted. If differences in tuna cardiac responses to acute temperature change are eventually found, we hypothesize that differences in the effect of temperature on calcium dynamics (e.g., excitation–contraction coupling, EC coupling) will be the underlying explanation. The myocardium from skipjack, yellowfin and Pacific bluefin (*T. orientalis*) tuna has been shown to be more dependent on sarcoplasmic calcium release for EC coupling than are other teleosts (Keen et al. 1992; Shiels et al. 1999, 2004), and it is probable that bigeye tuna hearts are likewise. Similar to bigeye tuna, and unlike yellowfin or skipjack tunas, Atlantic (*T. thynnus*) and Pacific bluefin tunas subject themselves to up 13 °C changes in ambient temperature during their daily vertical movements (Marcinek et al. 2001; Wilson et al. 2005). *In situ* perfused bluefin tuna hearts also show decreases in heart rate, cardiac output, and power with decreasing temperature, but retain cardiac function at temperatures below those tolerated by perfused yellowfin tuna heats (Blank et al. 2002, 2004). These differences appear to be explained by the observations that bluefin tuna ventricles have faster calcium inactivation kinetics than other teleosts (Shiels et al. 2004), due to high rates of calcium uptake by the sarcoplasmic reticulum and elevated levels of Ca^{2+}-activated ATPase (Landeira-Fernandez et al. 2004). It is also possible that species specific differences could lie in the calcium binding properties of cardiac troponins (which initiate contraction through the activation of myofibrillar ATPase). The available evidence is, however, against this idea (Yang et al. 2000).

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