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ACTIVITIES OF KEY METABOLIC ENZYMES IN THE HEATER ORGANS OF SCOMBROID FISHES

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Summary

Maximal *in vitro* activities of key metabolic enzymes were measured in brain and eye heaters of five species of scombroid fishes. Istiophorid billfishes (blue marlin, striped marlin and Mediterranean spearfish), xiphiid billfishes (Pacific and Mediterranean stocks) and a scombrid fish (butterfly mackerel) were included in the analysis. Our main objectives were (1) to assess the maximum possible substrate flux in heater tissue, and (2) to determine what metabolic substrates could fuel heat production. Heater tissue of all scombroids examined showed extremely high oxidative capacity. Activities of citrate synthase, a commonly measured index of oxidative metabolism, included the highest value ever reported for vertebrate tissue. In most billfishes, citrate synthase activities were similar to or higher than those found for mammalian cardiac and avian flight muscle. Marker enzymes for aerobic carbohydrate metabolism (hexokinase) and fatty acid metabolism (carnitine palmitoyltransferase and 3-hydroxyacyl-CoA dehydrogenase) also displayed extraordinarily high activities. Activities of carnitine palmitoyltransferase measured in heater organs were among the highest reported for vertebrates. These results indicate that heat production could be fueled aerobically by either lipid or carbohydrate metabolism. Inter- and intraspecifically, heater organs of fishes from the colder Mediterranean waters had a higher aerobic capacity and, hence, a greater heat-generating potential, than fishes from the warmer waters of the Pacific. This difference may be attributed to different thermal environments or it may result from allometry, since fishes caught in the Mediterranean were considerably smaller than those caught in the Pacific.

Introduction

The majority of teleost fishes are obligate ectotherms. Thus, variations in environmental temperature profoundly challenge their ability to maintain internal homeostasis. Adaptive responses to this challenge are diverse and include

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behavioral (Crawshaw and Hammel, 1974; Zimmerman *et al.* 1989; Carey and Scharold, 1991) and acclimatory mechanisms (Cossins and Bowler, 1987; Sidell and Moerland, 1989; Crockford and Johnston, 1990). Perhaps the most specialized mode of thermal adaptation occurs in large pelagic fishes of the suborder Scombroidei (mackerels, tunas and billfishes). In these fishes, metabolic heat maintains the temperature of critical tissues considerably warmer than that of the surrounding water (Carey and Teal, 1966; Carey, 1982; Block, 1991).

Two distinct strategies for endothermy have evolved within the scombroid fishes and both utilize muscle as the major source of heat. Endothermy in tunas (Scombridae) and some sharks (Lamnidae) is associated with vascular heat exchangers within the circulation of metabolically active tissues, such as the red muscle, brain, eyes and liver (reviewed in Block, 1991). Tuna have exceptionally high metabolic rates compared to ectothermic fishes (Graham and Laurs, 1982; Brill, 1987). Structural and biochemical studies suggest that the centrally located red muscle that powers sustained swimming has a high aerobic potential and it is presumed to be the source of heat (Bone, 1978; Guppy *et al.* 1979). However, more recent studies demonstrate that the aerobic capacity of the red muscle of many active fish, as measured by metabolite and metabolic enzyme profiles, is similar in tunas and in other scombroid and non-scombroid fishes (Jones and Sidell, 1982; Suarez *et al.* 1986; Moyes *et al.* 1989). The major difference between tunas and billfishes is that tunas have central location of the red muscle mass associated with a vascular heat exchanger (Carey and Teal, 1966). Billfishes, in contrast, have laterally located red muscle, a morphology that correlates with their sub-carangiform, rather than tunniform, mode of locomotion (Hebrank *et al.* 1990). Interestingly, swordfish have a small amount of central red muscle associated with a rudimentary heat exchanger (Carey, 1990).

Regional endothermy involving only a brain and eye heater has evolved in billfishes (Xiphiidae: *Xiphias*; Istiophoridae: *Makaira, Tetrapturus, Istiophorus*) and in a mackerel (Scombridae: *Gasterochisma*). Heat generation in these fishes involves the modification of extraocular muscles into a thermogenic organ (Carey, 1982; Block, 1986). In swordfish, brain temperature may be elevated by as much as 13 °C above ambient water temperature (Carey, 1982; Carey, 1990), allowing these fish to make daily feeding migrations into cold water beneath the thermocline. Without the ability to regulate brain temperature during dives, vision and neural processing might be impaired, severely limiting the ability to capture prey. Heater organs have also been described in a little-known scombrid, the butterfly mackerel *Gasterochisma melampus* (Carey, 1982; Block, 1986). Morphological and mitochondrial DNA sequence data indicate that the brain and eye heater of butterfly mackerel evolved independently of the billfish heater (Block, 1991). In billfishes the heater phenotype is expressed in the superior rectus muscle, whereas in butterfly mackerel it is expressed in the lateral rectus.

The extraocular muscles associated with thermogenesis are composed of modified muscle cells that are structurally and functionally distinct from all other types of muscle in the animal kingdom (Block and Franzini-Armstrong, 1988).

Heater cells lack organized contractile proteins (actin and myosin) and are instead packed with mitochondria separated by stacks of sarcoplasmic reticulum and extensive T-tubules. Mitochondria within blue marlin heater cells occupy over 60 % of the cell volume (Block, 1990). This tight mitochondrial packing, which is among the highest in the animal kingdom, implies that these cells have an extraordinarily high oxidative capacity.

The metabolism of heater organs has received little study owing to the difficulty of obtaining fresh tissue. Mitochondria isolated from istiophorid heater cells can oxidize carbohydrate fuels at maximal rates of 125 nmol O_2 mg^{-1} $protein^{-1}$ min^{-1} (Block, 1991). These studies also demonstrate tight coupling of oxidative phosphorylation and electron transport. Subsequent biochemical and structural studies have suggested that 'excitation–thermogenic' coupling, a calcium-activated thermogenic cycle that is under nervous control, mediates heat generation in heater cells (for a review see Block, 1991). Structural evidence indicates that thermogenesis in heater cells proceeds *via* a signal from the neuromuscular junction which then activates a calcium release mechanism using the same molecular components involved in normal muscle excitation–contraction coupling. Calcium release from the sarcoplasmic reticulum is presumed to activate thermogenesis by one or both of two mechanisms: either (1) by stimulating futile calcium cycling at the sarcoplasmic reticulum at the expense of ATP, or (2) by activating the electron transport chain, through direct stimulation of calcium–proton exchange or *via* calcium-stimulated activation of enzymes involved in electron transport.

Our objectives in the present study were twofold. The first was to quantify the oxidative capacity of heater tissue. We compared maximal activities of citrate synthase, a commonly assayed index of mitochondrial oxidative capacity, from heater organs of five species of scombroid fishes. The second objective was to assess which substrates could potentially fuel heat production. To this end, we measured key enzymes involved in aerobic carbohydrate metabolism (hexokinase), fatty acid metabolism (3-hydroxyacyl-CoA dehydrogenase and carnitine palmitoyltransferase) and glycolysis (pyruvate kinase and lactate dehydrogenase). For comparison, we also measured activity of the above enzymes in the superior rectus and lateral rectus muscles from blue marlin and butterfly mackerel, respectively, and cardiac and red epaxial muscles from swordfish.

Materials and methods

Animals

Swordfish (*Xiphias gladius*, 8–14 kg) and striped marlin (*Tetrapturus audax*, 54 kg) were caught on longline gear north of the Hawaiian Islands during January, 1991. Mediterranean swordfish (*Xiphias gladius*, 1–10 kg) and Mediterranean spearfish (*Tetrapturus belone*, 2–4 kg) were caught on longlines in the Gulf of Taranto in southern Italy during October, 1990. Blue marlin (*Makaira nigricans*, 35–250 kg) were caught on hook and line off the Kona coast of Hawaii during July

and August, 1988 and 1989. Tissues from billfishes were removed from the fish within 5–30 min *post mortem* and small pieces of tissue were freeze-clamped with copper tongs precooled in liquid nitrogen. Two adult butterfly mackerel (*Gasterochisma melampus*, 140 cm and 142 cm snout to fork length, weights not available) were caught on longline off the Tasmanian coast (47°S; 146°E) and placed in a blast freezer on shipboard (−60°C) within a short time after capture.

Tissue preparation

All tissues were stored at −80°C. Small pieces of tissue (0.20–0.30 g wet mass) were removed from the freezer and care was taken to remove all other tissue types prior to weighing. Ten percent weight/volume homogenates were prepared by mincing samples on an ice-cold stage, adding the appropriate volume of extraction medium, and homogenizing with a ground-glass homogenizer. Extraction medium consisted of 75 mmol l^{-1} Tris–HCl, 1 mmol l^{-1} EDTA, 2 mmol l^{-1} $MgCl_2$, pH 7.4 at 25°C. Extraction media for hexokinase and pyruvate kinase also contained 1 mmol l^{-1} DTT (dithiothreitol). After initial homogenization, samples were sonicated in two 15 s bursts, separated by a 15 s cooling interval, at either 35 % maximal power (Artek-Sonic 300 Dismembrator) or 100 % maximal power (Tekmar Sonic Disruptor TMS-50). Both sonication regimes yielded identical results.

Determination of enzyme activities

Enzyme activities were monitored with either a Perkin-Elmer Lambda 4 or Lambda 6 spectrophotometer. All assays were conducted at 25°C. Assays were performed under saturating substrate conditions according to protocols optimized for fish muscles (Sidell *et al.* 1987). Enzyme units are defined below.

Hexokinase (HK; EC 2.7.1.1). Phosphorylation of glucose to glucose 6-phosphate by HK was determined by monitoring the rate of $NADP^+$ reduction by exogenous glucose-6-phosphate dehydrogenase at 340 nm. The reaction mixture consisted of 7.5 mmol l^{-1} $MgCl_2$, 0.8 mmol l^{-1} EDTA, 1.5 mmol l^{-1} KCl, 0.4 mmol l^{-1} $NADP^+$, 2.5 mmol l^{-1} ATP, 1.0 mmol l^{-1} D-glucose, 10.0 mmol l^{-1} creatine phosphate, 0.9 units ml^{-1} creatine phosphokinase, 0.7 units ml^{-1} glucose-6-phosphate dehydrogenase, 75 mmol l^{-1} Tris–HCl, pH 7.2 at 25°C. Reactions were initiated by the addition of glucose.

Pyruvate kinase (PK; EC 2.7.1.40). The reaction rate was monitored by measuring NADH oxidation at 340 nm during the reduction of pyruvate to lactate. The reaction mixture consisted of 150 mmol l^{-1} KCl, 1 mmol l^{-1} KCN, 10 mmol l^{-1} $MgSO_4$, 0.15 mmol l^{-1} NADH, 5 mmol l^{-1} ADP, 0.02 mmol l^{-1} fructose 1,6-bisphosphate, 2.5 mmol l^{-1} phosphoenolpyruvate, 10 units ml^{-1} lactate dehydrogenase, 50 mmol l^{-1} imidazole, pH 6.7 at 25°C. Reactions were initiated by the addition of phosphoenolpyruvate.

Lactate dehydrogenase (LDH; EC 1.1.1.27). The reaction rate was monitored by measuring the oxidation of NADH at 340 nm during the reduction of pyruvate

to lactate. The reaction mixture consisted of 0.15 mmol l^{-1} NADH, 1 mmol l^{-1} KCN, 50 mmol l^{-1} imidazole, 2.5 mmol l^{-1} pyruvate, pH 7.2 at 25 °C.

Citrate synthase (CS; EC 4.1.3.7). The reduction of DTNB [5,5′-dithiobis(2-nitrobenzoic acid)] by free coenzyme A was monitored at 412 nm. The reaction mixture consisted of 0.5 mmol l^{-1} oxaloacetic acid, 0.25 mmol l^{-1} DTNB, 0.4 mmol l^{-1} acetyl-CoA, 75 mmol l^{-1} Tris–HCl, pH 7.8 at 25 °C. Reactions were initiated by adding oxaloacetic acid. In some of the tissue types, background deacylase activity prior to initiation was significant and was subtracted from the activity following addition of oxaloacetic acid.

Carnitine palmitoyltransferase (CPT; EC 2.3.1.21). Most studies that have assayed CPT activity have used palmitoyl-CoA as the substrate. However, some studies indicate that the substrate yielding the highest estimates of activity is an unsaturated form of this 16-carbon fatty acid, palmitoleolyl-CoA (Eggington, 1986; B. D. Sidell, E. L. Crockett and W. R. Driedzic, in preparation). We therefore performed independent measurements using each substrate. In both cases, reduction of DTNB by released CoA–SH was monitored at 412 nm. The reaction mixture consisted of 1.5 mmol l^{-1} EDTA, 0.25 mmol l^{-1} DTNB, 1.25 mmol l^{-1} carnitine HCl, 0.035 mmol l^{-1} palmitoyl-CoA or 0.035 mmol l^{-1} palmitoleolyl-CoA, 75 mmol l^{-1} Tris–HCl, pH 7.8 at 25 °C. Reactions were initiated by adding carnitine. Background deacylase activity prior to initiation was subtracted from total activity. CPT activities measured using palmitoyl-CoA as the substrate will be designated CPT [16:0] and those using palmitoleolyl-CoA as the substrate will be designated as CPT [16:1].

3-Hydroxyacyl-CoA dehydrogenase (3-HOAD; EC 1.1.1.35). Oxidation of NADH was monitored at 340 nm during the reduction of 3-ketoacyl-CoA to 3-hydroxyacyl-CoA. Reaction media contained 1 mmol l^{-1} EDTA, 1 mmol l^{-1} KCN, 0.15 mmol l^{-1} NADH, 0.1 mmol l^{-1} acetoacetyl-CoA, 50 mmol l^{-1} imidazole, pH 7.3 at 25 °C. Reactions were initiated by adding acetoacetyl-CoA. In some tissues, background activity prior to initiation was significant and was subtracted from total activity following initiation.

Statistical analysis

Differences in enzyme activities among species were assessed using analysis of variance followed by a Fisher PLSD test. Within each species, differences in measured CPT activities using as substrate either palmitoyl-CoA or palmitoleoyl-CoA were assessed with a paired Student's *t*-test. In all cases $P<0.05$ was judged to be significant.

Only two individual samples of suitably frozen heater tissue were obtained from butterfly mackerel and Mediterranean spearfish, and only one sample was obtained from striped marlin. The difficulty of obtaining tissues from all species warranted inclusion of these small sample sizes in the present study. Major points will be emphasized below, stressing enzyme activities from those species with larger sample sizes. Owing to the difficulty of obtaining specimens, a systematic study of factors such as seasonality, body size or age was not attempted.

Enzyme activities are expressed in units of activity (micromoles of product produced per minute per gram wet mass of tissue) (units g^{-1} tissue) to facilitate comparison of cellular physiologies between the different species.

Electron microscopy

Tissue was fixed in 2.5 % glutaraldehyde, 0.1 mol l^{-1} cacodylate, pH 7.2, post-fixed in 1 % osmium tetroxide, *en bloc* stained in 2 % aqueous uranyl acetate, and embedded in Spurr. Thin sections were cut on an RMC MT6000 ultramicrotome, stained with uranyl acetate and lead citrate, and viewed with an electron microscope (model CM10; Philips Electronic Instruments, Inc., Mahwah, NJ).

Results

Enzyme activities of heater organs

All heater organs displayed exceptionally high aerobic capacities, as indicated by the very high citrate synthase (CS) activity relative to those of swordfish red epaxial and cardiac muscle (Table 1; Fig. 1). Intraspecifically, CS activities were higher in heater organs from Mediterranean swordfish than in those from Pacific swordfish ($P<0.001$). This difference extended interspecifically as well. Citrate

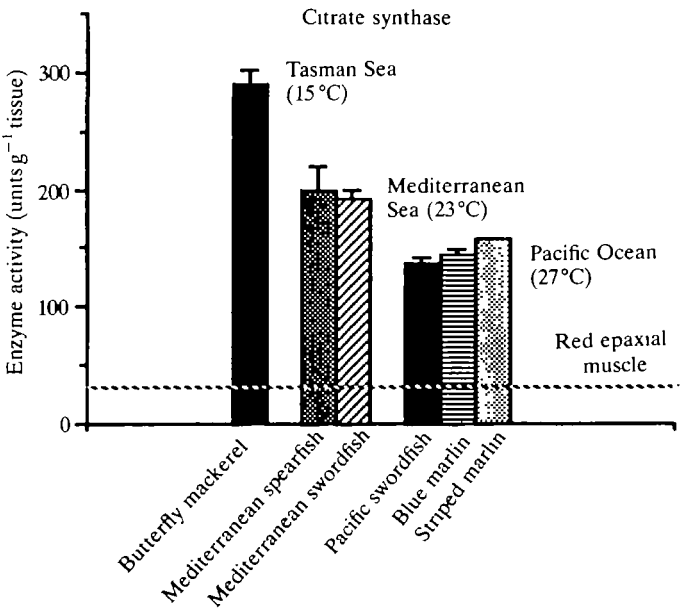

Fig. 1. Citrate synthase (CS) activity of heater organs from four species of billfishes (swordfish, *Xiphias gladias*; Mediterranean spearfish, *Tetrapturus belone*; blue marlin, *Makaira nigricans*; and striped marlin, *Tetrapturus audax*) and one species of scombrid (butterfly mackerel, *Gasterochisma melampus*). The locations of capture and the corresponding average water surface temperatures are also indicated. For comparison, CS activity of swordfish red epaxial muscle obtained in the present study is shown by a dashed line. Error bars represent standard errors. For values of N, see Table 1.

Table 1. *Enzyme activities in heater organs, red ocular, red epaxial and cardiac muscle from four species of billfishes and the scombrid* Gasterochisma melampus

	Species	Tissue	CS	CPT[16:0]	CPT[16:1]	3-HOAD	HK	PK	LDH
A	Mediterranean swordfish	Heater organ	193.19±5.37 [7]	1.86±0.12 [7]	2.83±0.14 [7]	39.4±6.52 [6]	24.98±1.06 [7]	96.11±3.43 [7]	256.35±11.87 [7]
B	Pacific swordfish	Heater organ	136.26±6.57 [4]	1.91±0.19 [4]	3.53±0.32 [4]	29.62±2.51 [4]	27.61±0.86 [4]	80.22±5.66 [4]	293.41±10.11 [4]
C	Blue marlin	Heater organ	144.06±4.92 [5]	1.23±0.15 [5]	2.27±0.15 [5]	37±46±2.93 [5]	20.95±1.13 [6]	238.8±15.19 [6]	293.75±12.54 [5]
D	Striped marlin	Heater organ	158.44 [1]	1.91 [1]	3.09 [1]	23.90 [1]	44.01 [1]	177.49 [1]	321.54 [1]
E	Mediterranean spearfish	Heater organ	199.76±19.36 [2]	2.42±0.36 [2]	2.98±0.33 [2]	52.09±14.28 [2]	30.90±6.55 [2]	126.36±6.76 [2]	311.90±16.08 [2]
F	Butterfly mackerel	Heater organ	290.08±11.40 [2]	2.94±0.00 [2]	3.75±0.37 [2]	72.02±14.8 [2]	23.96±3.06 [2]	216.72±26.37 [2]	379.42±45.02 [2]
G	Blue marlin	Red ocular muscle	6.50±1.41 [4]	ND	ND	1.75±0.45 [4]	2.45±0.25 [4]	514.57±42.86 [4]	1278.14±140.66 [4]
	Butterfly mackerel	Red ocular muscle	42.65 [1]	0.22 [1]	0.37 [1]	31 49 [1]	4.02 [1]	700.96 [1]	1479.10 [1]
	Swordfish	Red epaxial muscle	27.75±3.29 [2]	0.44 [1]	0.59 [1]	8.48 [1]	1.79 [1]	194.53 [1]	128.62 [1]
	Swordfish	Cardiac muscle	40.94±3.03 [2]	0.38±0.095 [2]	0.64±0.095 [2]	12.44±2.44 [2]	21.50±0.20 [2]	112.86±27.98 [2]	425.84±152.94 [2]
			A=E; B=C	A=B=E; E=F	A=C=E; B=E=F	A=B=C; A=C=E E=F	A=B=F; B=E	A=B=E; C=F	A=B=C=D=E=F

All activities are measured at 25°C; values are means±S.E.; enzyme activities are expressed in units g^{-1} tissue; sample sizes are in parentheses; ND, not detectable.

Last row shows species that are not significantly different ($P<0.05$: Fisher's PLSD, after ANOVA). CS, citrate synthase; CPT, carnitine palmitoyltransferase; 3-HOAD, 3-hydroxyacyl-CoA dehydrogenase; HK, hexokinase; PK, pyruvate kinase; LDH, lactate dehydrogenase.

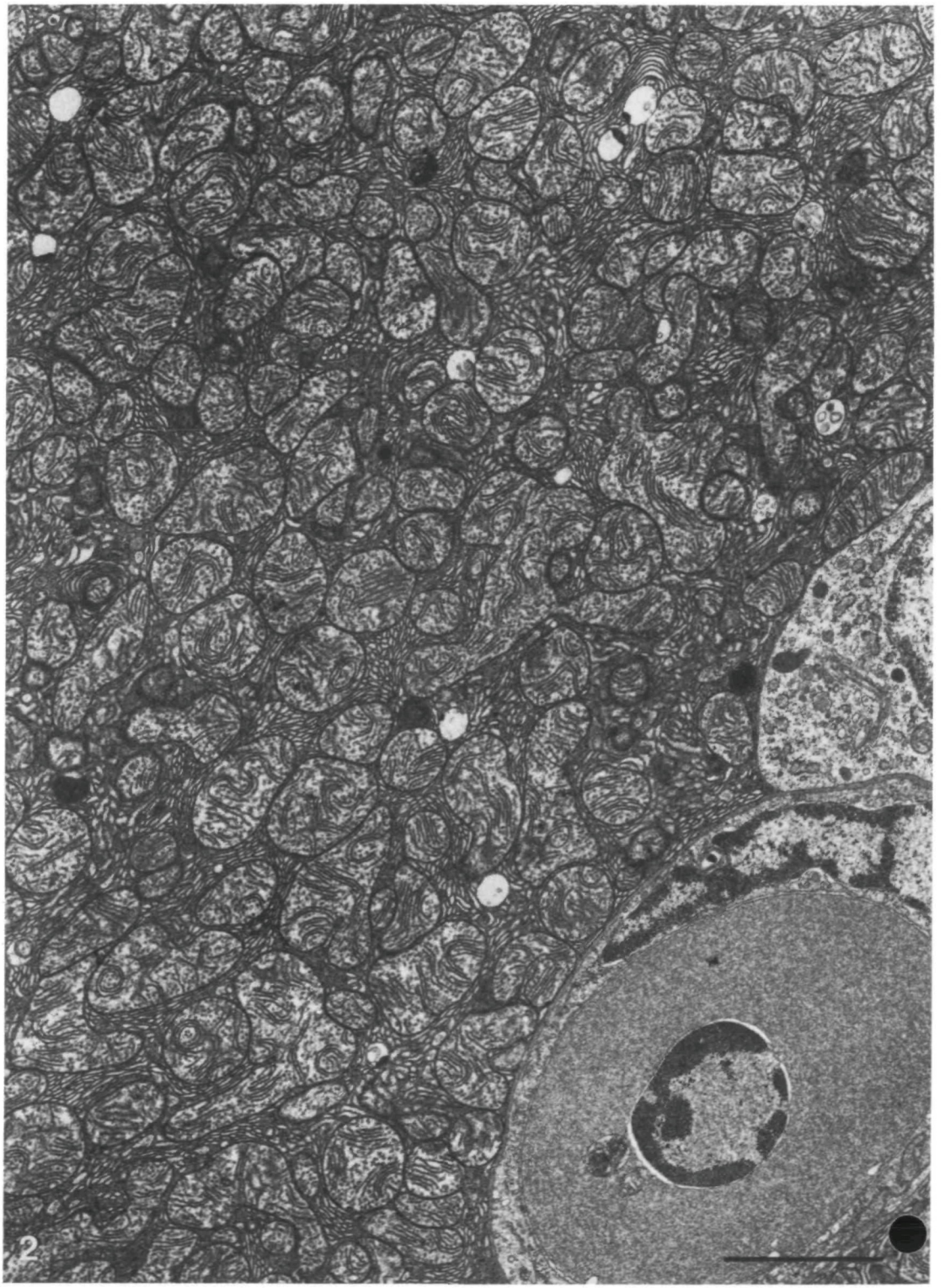
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Fig. 2. Electron micrograph of the heater cell of *Xiphias gladias*. Preliminary results with Mediterranean swordfish heater organs indicate mitochondrial volumes of 68±3%. The network of membranes between the mitochondria constitute the sarcoplasmic reticulum and transverse tubules, which regulate calcium release and re-uptake in normal muscle cells. Scale bar, 2 μm.

synthase activities from Mediterranean istiophorid heater organs (Mediterranean spearfish) were substantially higher than those of Pacific istiophorids (blue marlin) ($P<0.001$). Within Pacific billfishes (blue marlin and swordfish) and within Mediterranean billfishes (Mediterranean spearfish and swordfish), CS activities did not differ significantly. Citrate synthase activity of the striped marlin heater organ fell within the 95% confidence intervals (CI) for Pacific billfishes. High CS activity is reflected in the heater cell morphology. Mitochondria dominate the pictures of all heater cells (Figs 2 and 3). Stacks of smooth membranes, composed of sarcoplasmic reticulum and transverse tubules, are situated between the mitochondria.

Heater tissue from all species examined displayed a substantial capacity for aerobic metabolism of fatty acids, as indicated by high activities of 3-HOAD and CPT relative to those of red epaxial and cardiac muscle (Table 1; Figs 4A,B). 3-HOAD exhibited significantly higher activity in butterfly mackerel heater organs when compared to both groups of swordfish and blue marlin. Activity of 3-HOAD was not significantly different between Mediterranean and Pacific swordfish and blue marlin. In all heater organs studied, activities of CPT were higher when palmitoleolyl-CoA [16:1] was provided as the substrate than when palmitoyl-CoA [16:0] was used (Table 1; $P<0.05$). Thus, the latter measurements more closely represent the maximal activity of this enzyme. With palmitoleoyl-CoA [16:1] as the substrate, CPT activity was not significantly different between heater organs of Mediterranean spearfish and Mediterranean swordfish, or between Mediterranean spearfish and Pacific swordfish. CPT[16:1] activity was similar in blue marlin and Mediterranean swordfish heater organs and less than that found in butterfly mackerel and Pacific swordfish.

As with enzymes of fatty acid and oxidative metabolism, the marker enzyme for aerobic carbohydrate metabolism, hexokinase (HK), displayed considerable activity in all heater organs relative to those of swordfish red epaxial and cardiac muscle (Table 1; Fig. 4C). Activities of this enzyme were similar in heater organs of both groups of swordfish and butterfly mackerel. Mediterranean spearfish heater organs exhibited significantly higher HK activities than heater organs from all other species except Pacific swordfish. Activity of HK was lowest in blue marlin heater organs. Hexokinase activity of the striped marlin heater organ fell above the 95% CI for all other species examined.

The glycolytic enzymes, lactate dehydrogenase (LDH) and pyruvate kinase (PK) had low activities in heater organs of all species examined when compared to blue marlin white epaxial muscle (Table 1; Fig. 5A,B; values for blue marlin white epaxial muscle are from Suarez *et al.* 1986). Activities of LDH, a marker enzyme

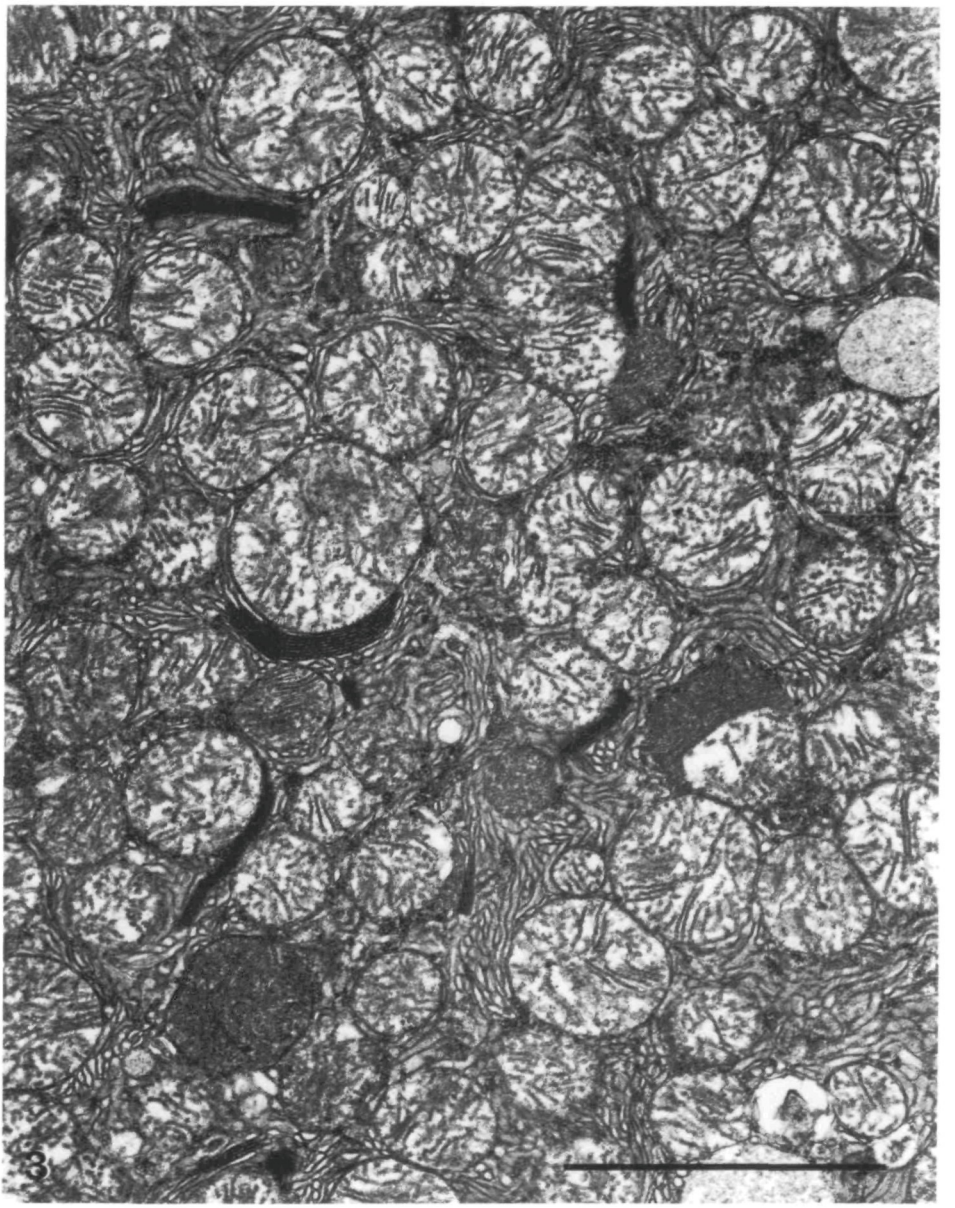

Fig. 3. Electron micrograph of the heater cell of *Makaira nigricans*. In this species, mitochondria occupy 63 % of the cell volume (Block, 1990). As in swordfish (Fig. 2), the membranes surrounding the mitochondria constitute the sarcoplasmic reticulum and transverse tubules. Scale bar, 2 μm.

for anaerobic flux, showed no significant difference among heater organs from all species examined. Activities of PK, another measure of anaerobic capacity (Gesser and Poupa, 1974; Hansen and Sidell, 1984), were similar and lower in

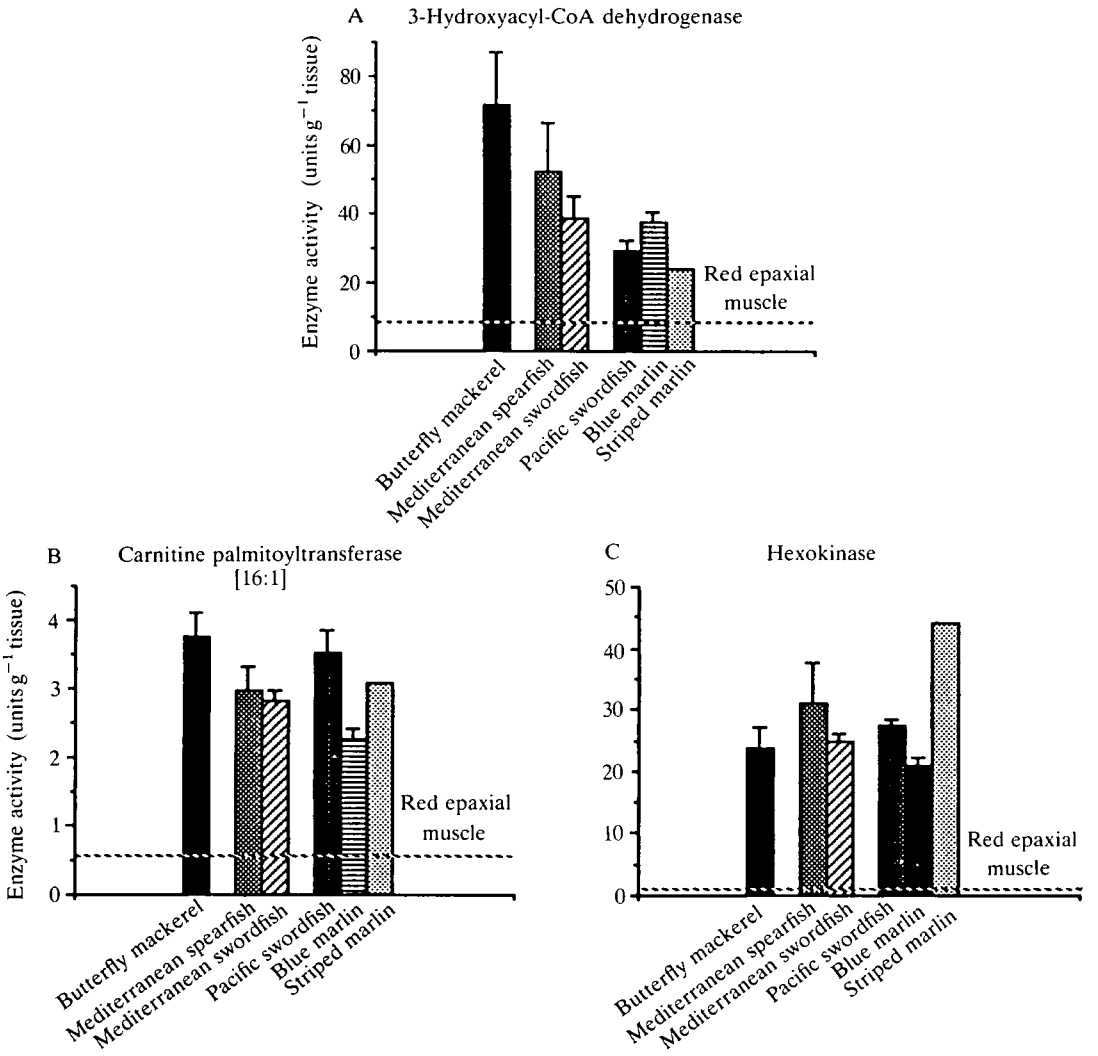

Fig. 4. Activities of enzymes involved in fatty acid metabolism. (A) 3-hydroxyacyl-CoA dehydrogenase, and (B) carnitine palmitoyltransferase, and aerobic carbohydrate metabolism, (C) hexokinase, of heater organs from four species of billfishes and one species of scombrid (see Fig. 1 legend for species names). Activities of these enzymes from swordfish red epaxial muscle obtained in the present study are indicated by dashed lines. Error bars represent standard errors. For values of *N*, see Table 1.

heater organs from both stocks of swordfish and Mediterranean spearfish when compared to butterfly mackerel and blue marlin.

Enzyme activities of ocular, epaxial and cardiac muscle

Enzyme activities of the superior rectus of blue marlin and the lateral rectus of butterfly mackerel were measured because these muscles developmentally give

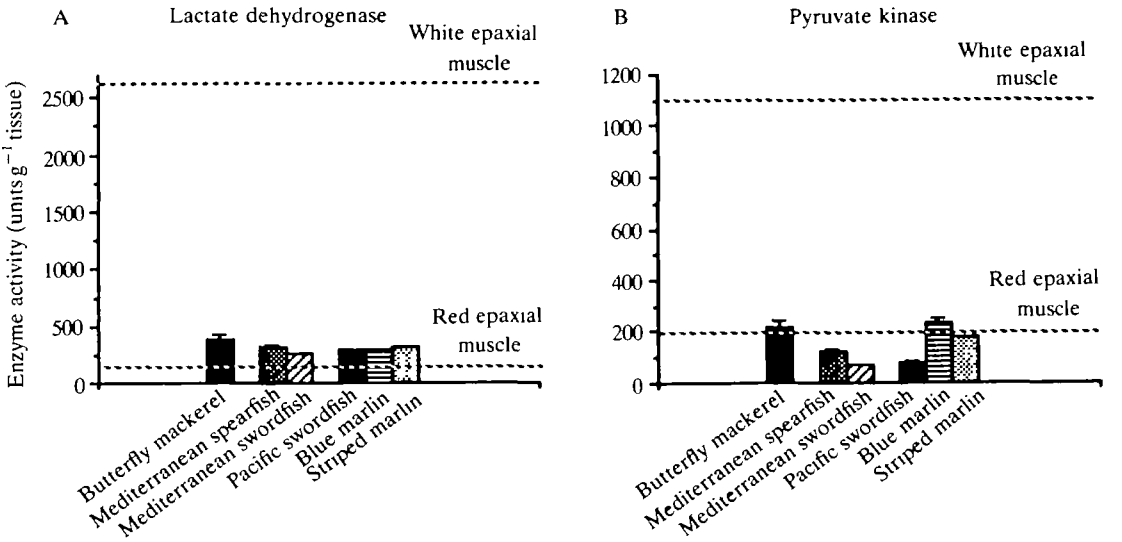

Fig. 5. Activities of the glycolytic enzymes (A) lactate dehydrogenase (LDH) and (B) pyruvate kinase (PK) from four species of billfishes and one species of scombrid (See Fig. 1 legend for species names). Activities of these enzymes from swordfish red epaxial muscle obtained in the present study are indicated by dashed lines. Activities of LDH and PK from blue marlin white epaxial muscle (Suarez *et al.* 1986) are also indicated. Error bars represent standard errors. For values of *N*, see Table 1.

rise to the heater organs of istiophorids and scombrids, respectively (Block, 1986). Ocular muscles are among the most complex in the vertebrate body and are composed of many different fiber types, with a predominance of red fibers (both slow oxidative and fast oxidative glycolytic). Thus, ocular muscle should display metabolic capacities not specific for any one fiber type.

Activities of CS, 3-HOAD and HK, all indicators of aerobic capacity, were substantially lower in blue marlin ocular muscle compared to heater organs of all species tested (Table 1). Activities of the glycolytic enzymes, PK and LDH, in contrast, were significantly higher than those measured in heater organs. There was no detectable CPT activity in blue marlin ocular muscle using either palmitoyl-CoA or palmitoleoyl-CoA as substrate.

The lateral rectus of butterfly mackerel was more oxidative than the superior rectus of blue marlin but substantially less oxidative than heater organs (Table 1). Activities of CS, 3-HOAD, HK and PK from butterfly mackerel ocular muscle fell above the 95% CIs for those of marlin ocular muscle. Lactate dehydrogenase activity of butterfly mackerel ocular muscle fell within the 95% CI for marlin ocular muscle. Unlike marlin ocular muscle, however, butterfly mackerel ocular muscle had measurable activities of CPT using both substrates.

Activities of CS, CPT and 3-HOAD were much higher in swordfish heater organs than in swordfish red epaxial or cardiac muscle (Table 1). Cardiac HK activity was similar to that found for heater tissue, whereas red epaxial muscle HK

activity was, on average, about 15 times lower than that of heater tissue. Pyruvate kinase activity of swordfish cardiac muscle was similar to the lowest PK values found for heater organs. In contrast, PK activities of swordfish red epaxial muscle were more similar to the highest heater organ PK values. Finally, heater organ LDH activity was higher than that found for red epaxial muscle and lower than that found for cardiac muscle.

Discussion

Oxidative potential of heater tissue

Our results demonstrate that heater tissue is extraordinarily oxidative, as shown by extremely high activities of CS, a marker enzyme for Krebs cycle activity. Activities of this enzyme were 4–14 times higher in heater organs than in red epaxial and/or cardiac muscles of various scombroids, including swordfish (this study), billfish (Suarez *et al.* 1986), tuna (Guppy *et al.* 1979) and mackerel (Sidell *et al.* 1987). Citrate synthase activity of heater organs far exceeded that for slow oxidative swimming muscle of non-scombroid fishes (Crockett and Sidell, 1990; Moyes *et al.* 1989; Kleckner and Sidell, 1985; Jones and Sidell, 1982).

Comparison with aerobic muscle tissue from other vertebrate groups further emphasizes the extraordinary oxidative capacity of billfish heater tissue (Table 2). To facilitate comparison of CS activities obtained from the literature, we have assumed a Q_{10} of 2 to adjust activities to 25 °C. Under this assumption, the aerobic potential of heater tissue was higher than that of cardiac muscle from a variety of mammals (Dreidzic *et al.* 1987; Alp *et al.* 1982). Moreover, the oxidative capacity of heater tissue was greater than that of avian flight muscle, one of the most aerobic tissues known (Alp *et al.* 1982). Citrate synthase activities of heater organs from Mediterranean swordfish, Mediterranean spearfish and butterfly mackerel even exceeded that reported for hummingbird flight muscle (Suarez *et al.* 1990).

To our knowledge, only the aerobic capacity of insect flight muscle surpasses that of heater tissue (Table 2; Alp *et al.* 1982). The extraordinarily high aerobic demand of insect flight muscle is due in part to the high contraction frequencies associated with flight and the large ATP demands of both the myosin-ATPase and Ca^{2+}-ATPase. When compared to oxidative vertebrate muscle, insect flight muscle has very high CS activity per unit mitochondrial volume. It is likely that the direct supply of oxygen to the tissues *via* tracheae is partially responsible for this deviation from the vertebrate pattern.

Fueling heat production

To meet the large aerobic potential of heater cells, the Krebs cycle must be supplied with two-carbon compounds from carbohydrate and/or lipid metabolism. Our results indicate that heater cells have a high capacity to fuel oxidative metabolism with either class of substrate. Marker enzymes for fatty acid metabolism, 3-HOAD and CPT, showed much higher activities in heater organs than in either red epaxial or cardiac muscle from other scombroids (this study;

Table 2. *Mitochondrial volumes and citrate synthase activities of aerobic muscles*

Cell type	% Mitochondrial volume	CS activity at 25 °C (units g^{-1} tissue)*
Hummingbird flight muscle (*Selasphorus rufus*)	35[m]	170[l]
Pigeon pectoralis muscle (*Columba livia*)	29[i]	115[a]
Frog calling muscle (*Hyla versicolor*)	21[k]	102[k]
Laboratory rat cardiac muscle	42[e]	96[a]
Antarctic fish red pectoral muscle (*Gobionotothen gibberifrons*)	25[j]	124[f]
Skipjack tuna deep red muscle (*Katsuwonus pelamis*)	34[c]	21[h]–61[g]
Bumblebee flight muscle (*Bombus* sp.)	43[d]	366[a]
Heater tissue	63–>70[b]	136–290

* Values obtained at assay temperatures other than 25 °C were corrected to this temperature assuming a Q_{10} of 2: (a) Alp *et al.* (1982), bumblebee value was averaged for males and workers; (b) Block (1986, and unpublished observations); (c) C. Moyes, personal communication; (d) Casey and Ellington (1989); (e) Kayer *et al.* (1985); (f) Crockett and Sidell (1990); (g) K. A. Dickson (in preparation); (h) Hochachka *et al.* (1978); (i) James and Meek (1979); (j) Londraville and Sidell (1990); (k) Marsh and Taigen (1978), averaged for laryngeal and trunk muscles; (l) Suarez *et al.* (1990), pectoralis and supracoracoideus muscles; (m) Suarez *et al.* (1991), averaged for pectoralis and supracoracoideus muscles.

Suarez *et al.* 1986; Guppy *et al.* 1979; Sidell *et al.* 1987). CPT activities of heater organs were, in fact, among the highest reported for any vertebrate tissue, including mammalian cardiac muscle (Driedzic *et al.* 1987).

As indicated by HK activity, the capacity of heater tissue for aerobic glucose metabolism is also high. Heater tissue HK activities were 10 times higher than red epaxial muscle activities and comparable to cardiac muscle activities from tuna (Guppy *et al.* 1979) and billfish (this study). Hexokinase activity is sufficient to meet the maximal energetic demand of cardiac muscle from many fishes (Sidell *et al.* 1987). Thus, comparison with cardiac muscle underscores the heater tissue's high capacity for aerobic glucose metabolism.

Our results demonstrate that heater tissue has a limited capacity for anaerobic metabolism. Heater organ LDH activities were only 5–10 % of the activity found in scombroid white muscle (Fig. 5A) (Guppy *et al.* 1979; Suarez *et al.* 1986), and fell in the middle range of values reported for scombroid red epaxial muscle and cardiac muscle (this study; Sidell *et al.* 1987; Guppy *et al.* 1979). A low anaerobic capacity is not surprising, considering the predominantly aerobic poise of heater tissue. Large amounts of myoglobin and a rich supply of well-oxygenated blood

(Block, 1991) make it unlikely that heater cells would encounter a hypoxic state severe enough to require a large capacity for anaerobic metabolism. Moderate anaerobic potential may indicate the ability of heater cells to fuel heat production partially *via* lactate metabolism. Heater tissue could potentially convert blood-borne lactate to glycogen for later use in carbohydrate metabolism. Alternatively, heater cells could convert lactate to pyruvate for immediate shunting into the Krebs cycle. Tuna are known to possess a high lactate turnover rate, which is due both to lactate transport by the blood to other organs and to lactate metabolism *in situ* (Weber *et al.* 1986).

Enzyme activities of red ocular muscle fell between those reported for white and red epaxial muscles from other scombrids (Guppy *et al.* 1979; Suarez *et al.* 1986), suggesting that this muscle, unlike red epaxial muscle, is not composed solely of slow oxidative fibers. Histochemical studies indicate that the superior rectus muscle of marlin is composed of at least three fiber types; fast oxidative glycolytic, slow oxidative and fast glycolytic fibres (Block, 1986).

Owing to the pelagic nature of the fishes used in this study, we were forced to perform all of our measurements on tissues that had first been frozen in liquid nitrogen. In a variety of fishes, PK, LDH, CS, CPT and 3-HOAD are stable in frozen tissue, while the stability of HK is variable (Sidell *et al.* 1987). However, Hochachka *et al.* (1978) have shown that freezing skipjack tuna red epaxial muscle decreases CS activity by about 30 % and PK and LDH activities by about 50 %. If similar decreases occurred during the freezing of heater tissue, CS activity would increase from approximately 150 to 210 units g^{-1} tissue in Pacific billfishes and from 200 to 260 units g^{-1} tissue in Mediterranean billfishes.

Block (1991) calculated that swordfish heater tissue must have the capacity to generate at least 50–100 W kg^{-1} to maintain the cranial–water temperature gradients measured by acoustic telemetry. This estimate is based on several assumptions, including a 100 % efficient carotid heat exchanger. Based on the enzyme activities reported in the present study, would heater organs have the capacity to produce this much heat? Crabtree and Newsholme (1972*a*,*b*) have presented evidence that flux through carbohydrate and lipid pathways correlates with maximal activities of HK and CPT, respectively. Maximal catalytic activities of these two enzymes yield an estimate of the theoretical thermogenic capacity of heater tissue during the complete oxidation of fatty acids and/or glucose. Experimental evidence suggests that *in vivo* glucose 6-phosphate inhibits 80 % of the maximal HK activity (England and Randle, 1967). Furthermore, there are two populations of CPT, one on the cytoplasmic side of the outer mitochondrial membrane and one on the matrix side of the inner mitochondrial membrane. Assuming an equal distribution of activity between these populations, calculations of the flux through fatty acid metabolism should be based on half the measured CPT activity (Zammit and Newsholme, 1979; Bremer, 1983). Taking these factors into account, calculations for swordfish heater tissue indicate that complete oxidation of either glucose or palmitoleoyl-CoA could yield approximately 250 W kg^{-1} (Table 3). Hence, heater organs could maintain a substantial thermal

Table 3. *Estimates of thermogenic capacity of heater organs based on enzyme activity*

Enzyme	Substrate	Potential heat production ($W\,kg^{-1}$)
HK	Glucose	246
CPT[16:0]	Palmitoyl-CoA	171
CPT[16:1]	Palmitoleoyl-CoA	253

Potential heat production was calculated assuming complete oxidation of the substrate. Complete oxidation of glucose yields 686 kcal mol^{-1} and complete oxidation of palmitoyl- and palmitoleoyl-CoA yields 2340 kcal mol^{-1} (Lehninger, 1975).

Activity for each enzyme was averaged for all species having sample sizes of at least 2. Average hexokinase (HK) activity was 25.7 units g^{-1} tissue, for CPT[16:0], 2.1 units g^{-1} tissue, and for CPT[16:1], 3.1 units g^{-1} tissue.

Estimates of heat production for HK reflect 20 % of the calculated value and those for CPT represent 50 % of the calculated value based on maximal enzyme activities. See text for explanation.

gradient between the brain and surrounding water even if thermal conductivity through the system is 2–3 times greater than the values assumed by Block (1991). Although the above calculations are based on several assumptions, they nonetheless underscore the capacity of heater tissue to fuel heat production using either carbohydrate or lipid substrates.

The capacity for heat production by heater tissue depends on the availability of substrates from both endogenous and blood-borne pools. Preliminary studies indicate that heater cells from adult Pacific swordfish contain small lipid droplets (B. A. Block, unpublished data). These lipid deposits were rarely seen in juvenile swordfish or istiophorid heater cells (Fig. 2). The small amount of lipid stores in adult swordfish may supplement blood-borne substrates in fueling heat production during the extensive dives into cold water beneath the thermocline. Istiophorids do not make prolonged dives into cold water (B. A. Block, D. T. Booth and F. C. Carey, in preparation; Holland *et al.* 1990; Holts and Bedford, 1990; Yuen *et al.* 1974) and could potentially fuel heat production primarily with carbohydrates. Interestingly, juvenile swordfish (1–2 kg) have fewer intracellular lipid stores than do adult swordfish. Indeed, heater tissues from juvenile swordfish and adult istiophorids are histologically very similar. This similarity could relate to common lifestyle features between young swordfish and adult istiophorids (B. A. Block, unpublished observations). Although these differences in tissue composition subtly correlate with the animals' thermal ecologies, the relatively small amounts of intracellular fuel in heater cells of billfishes (Block and Franzini-Armstrong, 1988) suggest that heat production is supported primarily by blood-borne fuels, either glucose or fatty acids.

Biochemical, morphological and ecological correlates

Acoustic telemetry studies reveal that swordfish diurnally make very deep (500–600 m) dives into cold waters beneath the thermocline for as long as 12 h. During these dives, swordfish can maintain cranial temperatures of 29°C while water temperature may range from 8 to 26°C (Carey and Robison, 1981; Carey, 1990). Similar studies on istiophorid billfish indicate that these fish make shallower dives (maximum of 210 m for blue marlin; B. A. Block, D. T. Booth and F. C. Carey, in preparation;) and spend significantly less time at depth than do swordfish. Telemetry studies indicate that blue marlin tracked in Hawaiian waters encounter water temperatures ranging from 17 to 27°C. Although no telemetered measurements of brain and eye temperatures have been made on free-swimming istiophorids, freshly caught blue marlin maintain a cranial temperature 2–6°C above surface water temperature (Block, 1991).

Swordfish have significantly larger heater organs than similarly sized istiophorids. This observation led Block (1990, 1991) to hypothesize that expression of the heater phenotype within billfish eye muscles correlates with the thermal biology of the fishes. Differences in oxidative potential observed between the fishes assayed in the present study also appear to correlate with thermal ecology. Both inter- and intraspecifically, heater organs of billfish caught in the Mediterranean had higher CS activity than those caught in the Pacific (Table 1; Fig. 1). It would seem reasonable to attribute this difference to differences in water temperature. Bathythermographic data from the Pacific swordfish cruise indicate that surface water temperatures were 26°C during the longlining operations (B. A. Block, unpublished data). Surface water temperatures in August ranged from 26 to 27°C for the istiophorids caught off the Kona coast of Hawaii. Mediterranean surface water temperatures during October were 23°C (B. A. Block, unpublished data). The mixed layer of relatively warm surface water in the Mediterranean (50 m in depth) is shallower than that in Hawaii (70–100 m in depth). Colder water temperatures may necessitate a higher thermogenic capacity of the heater tissue from Mediterranean billfishes and could explain the greater potential for heat generation observed in these species relative to Pacific stocks.

Although there are relatively few thermal or behavioral data available, it is tempting to speculate on the extraordinarily high oxidative capacity of butterfly mackerel heater organs (Table 1). To our knowledge, the CS value reported here is higher than that found for any other vertebrate tissue. There are at least three factors that could contribute to this exceptional aerobic potential. First, butterfly mackerel may require heater organs with a very high oxidative capacity in order to maintain an adequate cranial–water temperature gradient. These fish inhabit the cold Tasman Sea off the coasts of New Zealand and Australia, which may necessitate a high capacity for heat generation relative to billfishes that can bask in the comparatively warm surface waters of the Pacific and Mediterranean. Sea surface temperatures obtained by fisheries observers aboard Japanese longliners during the time of capture of the *Gasterochisma* used in this study ranged from 13.8 to 16.1°C (C. Proctor, personal communication). In addition, the counter-

current heat exchanger associated with the heater organ of butterfly mackerel has a different vascular origin and, thus, a different morphology from that of billfishes (J. Finnerty and B. A. Block, personal communication). This morphological difference could increase convective heat loss, thereby requiring greater heat production. Finally, our results indicate that the muscle giving rise to butterfly mackerel heater organs (the lateral rectus) is more oxidative than the muscle giving rise to billfish heater organs (the superior rectus) (Table 1). Regardless of the underlying reason for this difference, heater organs of butterfly mackerel could simply be predisposed to having a relatively greater aerobic potential.

Because of their skeletal muscle origin, heater organs are not composed only of heater cells but also contain small amounts of muscle fibers, connective tissue and blood vessels. Histological sections demonstrate that swordfish have more of the superior rectus muscle expressing the heater phenotype than do istiophorids (Block, 1986, 1990). Contractile muscle fibers are scarce in histological preparations of swordfish heater tissue relative to istiophorid preparations. Interspecific differences in expression of the heater phenotype may explain some of the interspecific differences found in enzyme activity. For example, greater proportions of fast oxidative glycolytic fibers in heater tissue might increase the relative activity of glycolytic enzymes and decrease the activity of oxidative enzymes. We tested this possibility by assaying the heater organ homogenates used in our enzyme studies on SDS–polyacrylamide gels to assess qualitatively the amount of myosin present in heater organs of the different species. Because heater cells express very little myosin, variation in the quantity of this protein would probably reflect differing amounts of contaminating contractile fibers. The results (not shown) indicated no major interspecific differences in the amount of myosin from any of the heater organs examined. Thus, differences in enzyme activities between species are not necessarily due to variation in muscle fiber contamination. An alternative explanation for the differences in aerobic capacity of heater organs relates to interspecific variation in the muscle fiber type developmentally giving rise to heater cells. If the ontogenetic trajectory giving rise to the heater cell within a species is from an oxidative fiber type, the heater cell may be predisposed to have a greater oxidative capacity than one derived from a more glycolytic fiber.

Allometry

Along with thermal environment, body size could also contribute to intra- and interspecific differences in enzyme activities between billfish heater organs. Intraspecifically, Mediterranean swordfish were substantially smaller and exhibited greater CS activities than did Pacific swordfish (1–10 kg *versus* 8–49 kg, respectively). This relationship holds interspecifically as well; among istiophorids, CS activity of heater organs from Mediterranean spearfish (2–4 kg) was higher than those of both blue marlin (35–250 kg) and striped marlin (54 kg). Because of the scaling of surface area to volume, smaller fish certainly experience greater rates of heat loss than do larger fish. Thus, all else being equal, heater organs of

smaller fish would need to generate more heat to maintain an equivalent thermal gradient to those of larger fish. Heater tissue of juvenile swordfish has a softer texture than does that of mature swordfish and marlin (A. Tullis, personal observation), suggesting that less connective tissue is associated with the vascular heat exchanger. Histological sections through heater tissue of juvenile and adult swordfish corroborate this observation. In juvenile swordfish the heat exchanger is very poorly developed, consisting of single pairs of arteries and veins dispersed throughout the tissue. This morphology is dramatically different from that of adult swordfish, which have large clusters of arteries and veins coursing through the tissue. The lack of a prominent heat exchanger in the juvenile swordfish would further increase conductive heat loss, and, therefore, necessitate greater heat production.

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