Enzyme Activities Support the Use of Liver Lipid–Derived Ketone Bodies as Aerobic Fuels in Muscle Tissues of Active Sharks

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ABSTRACT

Few data exist to test the hypothesis that elasmobranchs utilize ketone bodies rather than fatty acids for aerobic metabolism in muscle, especially in continuously swimming, pelagic sharks, which are expected to be more reliant on lipid fuel stores during periods between feeding bouts and due to their high aerobic metabolic rates. Therefore, to provide support for this hypothesis, biochemical indices of lipid metabolism were measured in the slow-twitch, oxidative (red) myotomal muscle, heart, and liver of several active shark species, including the endothermic shortfin mako, *Isurus oxyrinchus*. Tissues were assayed spectrophotometrically for indicator enzymes of fatty acid oxidation (3-hydroxy-o-acyl-CoA dehydrogenase), ketone-body catabolism (3-oxoacid-CoA transferase), and ketogenesis (hydroxy-methylglutaryl-CoA synthase). Red muscle and heart had high capacities for ketone utilization, low capacities for fatty acid oxidation, and undetectable levels of ketogenic enzymes. Liver demonstrated undetectable activities of ketone catabolic enzymes but high capacities for fatty acid oxidation and ketogenesis. Serum concentrations of the ketone β-hydroxybutyrate varied interspecifically (means of 0.128–0.978 μmol mL^{-1}) but were higher than levels previously reported for teleosts. These results are consistent with the hypothesis that aerobic metabolism in muscle tissue of active sharks utilizes ketone bodies, and not fatty acids, derived from liver lipid stores.

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Introduction

Energy metabolism is well understood in most vertebrates. Lipid metabolism in teleost fishes has been well studied, but relatively little is known about lipid dynamics of the Elasmobranchii (sharks, skates, and rays). The literature consistently demonstrates, however, that elasmobranch lipid metabolism is unlike that of teleost fishes, many of which apparently rely on fatty acids released from lipid stores during starvation and to power sustainable activities (reviewed by Ballantyne 1997; however, see Bernard et al. 1999). Elasmobranchs apparently cannot oxidize fatty acids in extrahepatic tissues, possess very low levels of circulating, free fatty acids, store fat in the large liver instead of in adipocytes, and have low levels of fatty acid–binding proteins in the blood (Bone and Roberts 1969; Larsson and Fange 1977; Zammit and Newsholme 1979; Fellows et al. 1980; Fellows and Hird 1981; Driedzic and Hart 1984). Enzyme activity measurements on muscle homogenates and fuel preference studies with isolated mitochondria suggest that ketone bodies and amino acids are the primary fuels involved in elasmobranch aerobic metabolism (Beis et al. 1980; Anderson 1986, 1990; Moyes et al. 1986, 1990; Moon and Mommsen 1987; Singer and Ballantyne 1989; Ballantyne et al. 1992; Chamberlin and Ballantyne 1992). However, almost all of those studies (with the exception of Ballantyne et al. 1992) have utilized relatively inactive benthic or demersal elasmobranch species. Since maintenance metabolism in fishes is generally low, those elasmobranch species may not require high rates of oxidation of liver lipids for fuel. Pelagic sharks that swim continuously to ventilate their gills, forage, and migrate seem to be more likely candidates to demonstrate a reliance on stored lipids for oxidation in the heart and the slow-twitch, oxidative (red) myotomal muscle fibers that are used to power sustained swimming.

Tagging studies performed for shark population assessments verify that some sharks migrate long distances (Casey and Kohler 1992). Not only would they be predicted to use stored lipids as the primary fuel during these long-distance swimming feats, but active sharks would also have to rely on energy stores, most likely fat depots, during periods between meals. Because of the patchy distribution of food within the epipelagic environment (Sund et al. 1981), pelagic species feed sporadically and thus may be more dependent upon liver lipid stores than are less active elasmobranch species. Furthermore, the most active sharks are thought to be the endothermic species of the family Lamnidae [*Lamna nasus* (Bonnaterre 1788), *Lamna ditropis*

Hubbs and Follett 1947, *Isurus oxyrinchus* Rafinesque 1809, *Isurus paucus* Guitart Manday 1966, and *Carcharodon carcharias* (Linneaus 1758)], which use countercurrent heat exchangers to retain heat generated by their continuously active red muscle and thus maintain muscle temperatures above ambient water temperature (Carey and Teal 1969; Carey et al. 1982, 1985). Thus, these species would be expected to be the most dependent on the utilization of lipid stores for aerobic metabolism in oxidative muscle tissues. Therefore, this study focused on several species of pelagic sharks, including the endothermic shortfin mako (*I. oxyrinchus*) and a species suspected of being endothermic, the common thresher [*Alopias vulpinus* (Bonnaterre 1788)].

The only published study that has measured lipid and ketone-body utilization in an active shark showed that amino acids and ketones are the preferred substrates of mitochondria isolated from red muscle of *I. oxyrinchus* (Ballantyne et al. 1992). Palmitoyl carnitine was not oxidized, indicating that fatty acids were probably not used for fuel in the red muscle of this species. No other studies that we know of have measured mitochondrial enzyme activities or examined other aspects of lipid metabolism or other aerobic tissues in active shark species, with the exception of Dickson et al. (1993), who determined the activity of the Krebs cycle enzyme citrate synthase in the myotomal muscle and heart of several elasmobranchs including the shortfin mako shark.

The objective of this study was to gather additional evidence to support the hypothesis that oxidation of ketones derived from liver lipid stores powers the contraction of red skeletal muscle and heart during sustained swimming in active ectothermic and endothermic species of sharks. Because we could not measure metabolic processes and fuel use during swimming in these active pelagic sharks, we quantified the activities of key enzymes in pathways of lipid catabolism, ketone catabolism, and ketogenesis in liver and aerobic muscle tissues as well as blood-ketone concentrations. If liver-derived ketone bodies are the preferred substrate for oxidation to power sustained swimming in active sharks, red muscle and heart tissue should demonstrate high capacities for ketone utilization, low capacities for fatty acid oxidation, and undetectable levels of ketogenic enzymes. Liver tissue should demonstrate a low capacity for ketone utilization, a high capacity for fatty acid oxidation, and high levels of ketogenic enzymes. Furthermore, we predicted that concentrations of ketones in endothermic shark serum would be high in comparison to species that do not normally utilize ketones.

Material and Methods

Sample Collection

Studies were carried out on 10 shark species: *Isurus oxyrinchus* (shortfin mako), *Alopias vulpinus* (common thresher), *Rhizoprionodon terraenovae* (Richardson 1836) (Atlantic sharpnose), *Carcharhinus acronotus* (Poey 1860) (blacknose), *Negaprion brevirostris* (Poey 1868) (lemon), *Heterodontus francisci* (Girard 1854) (horn), *Prionace glauca* (Linneaus 1758) (blue), *Galeocerdo cuvier* (Peron and LeSueur 1822) (tiger), *Carcharhinus limbatus* (Valenciennes 1839) (blacktip), and *Carcharhinus plumbeus* (Nardo 1827) (sandbar). About one-half of the mako sharks and one blue shark were collected by longline fishing during the National Oceanic and Atmospheric Administration (NOAA) National Marine Fisheries Service (NMFS) Shark Indexing Abundance Program in the Southern California Bight during August 1996. Other makos and blue sharks were collected by rod-and-reel fishing from a small skiff launched out of Dana Point, California. Some makos and the thresher sharks were also collected in this manner by Diego Bernal of Scripps Institution of Oceanography (SIO) in La Jolla, California. Sharpnose, blacknose, and lemon sharks were collected by longline or gill-net fishing in the nearshore waters of the Gulf of Mexico near Panama City, Florida. Dr. John Carlson collected these specimens during the summers of 1997 and 1998 for an ongoing NMFS shark population survey. Tiger, sandbar, and blacktip shark blood samples were collected by Greg Skomal of the Massachusetts Division of Marine Fisheries, Martha's Vineyard Research Station, Martha's Vineyard, Massachusetts, during a 1998 NOAA/NMFS/Northeast Fisheries Science Center spring shark survey. Blood samples from horn sharks were collected during the summer of 1997 from captive specimens maintained at the Southern California Marine Institute in Long Beach, California, and at SIO.

Serum samples were collected from almost all sharks. Venous blood (1.0–2.0 mL) was drawn from live fish via caudal venipuncture with a heparinized 2-mL syringe fitted with an appropriate-size sterile needle. Whole blood was immediately transferred to a microfuge tube containing crystallized heparin and was placed on ice for transport back to California State University—Fullerton (CSUF), where blood was centrifuged for 15 min at maximal speed (16,000 *g*) at 4°C and the serum supernatant was frozen at −80°C until analysis. In some cases, blood collection and centrifugation were performed aboard ship, and serum was subsequently frozen in a −80°C freezer until transport to CSUF.

Tissues were collected from mako, sharpnose, blacknose, thresher, and lemon sharks immediately after specimens had been killed according to procedures approved by the CSUF Institutional Animal Care and Use Committee. Samples of red myotomal muscle, liver, and the entire heart ventricle or a subsample of the ventricle were removed, wrapped in aluminum foil or placed in cryogenic tubes, and immediately frozen on dry ice or in liquid nitrogen. Blood and tissue samples that were not collected near CSUF were shipped to the laboratory packed on dry ice in overnight mail and immediately placed in a −80°C freezer. All tissue samples were stored in a −80°C freezer for 1–24 mo before enzyme assays. Storage for this period did not significantly affect enzyme activity measure-

ments (R. Watson and D. Smith, unpublished data; see also Dickson et al. 1993).

Tissue Preparation

Approximately 200 mg of each tissue was dissected from frozen samples and homogenized in nine volumes of homogenization buffer (80 mM imidazole, 2 mM EDTA, 400 mM urea, 200 mM trimethylamine n-oxide [TMAO], 150 mM KCl, 3 mM $MgCl_2$, 10 mM NaCl, pH 7.0, at 20°C, 1% w/v Triton X-100) in a Kontes Duall ground-glass homogenizer maintained at 4°C. Preliminary studies showed that enzyme activities were higher when Triton X-100 was used in the homogenization buffer. Following homogenization, red muscle and heart samples were sonicated with three 10-s bursts of a cell disrupter (Heat Systems/Ultrasonics, model W-375) set at maximum power. Liver samples were not sonicated in order to facilitate the separation of the lipid component upon centrifugation. Crude homogenates were centrifuged at 10,000 *g* for 15 min at 4°C to separate cell debris, and the clear supernatant was pipetted from between the pellet and the surface lipid layer and kept on ice until use in enzyme assays.

Enzyme Assays

Assays were performed in 2.0 mL total volume at 20° ± 1°C utilizing a Hewlett Packard 8452A diode-array spectrophotometer equipped with a water-jacketed cuvette holder connected to a circulating water bath. An assay temperature of 20°C was chosen because it approximates the mean water temperature at capture for all species studied. All assays were performed under saturating substrate concentrations to provide optimal rates of enzyme activity. Activities for all enzymes are reported as international units (IU or μmol substrate converted to product min^{-1}) per gram tissue wet weight. Biochemicals were purchased from Sigma Chemical Company, St. Louis.

Tissues were analyzed for 3-hydroxy-o-acyl-CoA dehydrogenase (HOAD, E.C. 1.1.1.35) activity to obtain an indication of the potential for fatty acid oxidation. HOAD, a key step of β-oxidation, catalyzes the conversion of 3-L-hydroxyacyl-CoA and NAD^+ to β-ketoacyl-CoA and NADH. Tissue homogenate was dispersed in a solution of 0.15 mM NAD^+, 400 mM urea, 200 mM TMAO, 150 mM KCl, 3 mM $MgCl_2$, 10 mM NaCl, and 80 mM Tris base, pH 8.0, at 20°C. Changes in absorbance at 340 nm (A_{340}) were recorded as background rates before initiation of the reaction with 0.5 mM β-L-hydroxybutyryl-CoA. HOAD activity was assayed in the physiological direction of β-ketoacyl-CoA formation, unlike most previous research on HOAD in fishes (e.g., Moon and Mommsen 1987; Tullis et al. 1991), because we found in studies of other fishes (teleosts) that, at the same ratio of substrate to tissue homogenate, HOAD activities were usually higher when run in this direction relative to the reverse direction. Furthermore, when run in the reverse direction, HOAD activity decreased as the substrate : enzyme ratio increased, suggesting that a competing reaction or inhibition was occurring. This did not happen when the reaction was run in the forward direction; HOAD activity increased with an increase in substrate : enzyme ratio, up to saturation at approximately 0.4 mM β-L-hydroxybutyryl-CoA (R. Herrick and K. Dickson, unpublished data).

The capacity of tissues to oxidize ketone bodies was assessed by measuring the specific activity of an enzyme in the pathway that transforms ketone bodies into the acetyl-CoA that ultimately ends up in the Krebs tricarboxylic acid cycle. The enzyme 3-ketoacyl-CoA transferase (also referred to as 3-oxoacid-CoA transferase [OAT, E.C. 2.8.3.5]) catalyzes the conversion of acetoacetate and succinyl-CoA to acetoacetyl-CoA and succinate. The activity of 3-ketoacyl-CoA transferase was assayed spectrophotometrically in the direction of acetoacetate formation by following the decrease in A_{303} caused by the removal of acetoacetyl-CoA (Beis et al. 1980). This procedure provides an estimate of ketone-body utilization in the tissue, even though the reaction proceeds backward during analysis (Sugden and Newsholme 1973). The OAT assays were performed following the procedures of Williamson et al. (1971) and Sugden and Newsholme (1973), using a millimolar extinction coefficient of 20.5. The assay solution consisted of 7 mM $MgCl_2$, 5 mM iodoacetamide, 0.1 mM acetoacetyl-CoA, 400 mM urea, 200 mM TMAO, 150 mM KCl, 3 mM $MgCl_2$, 10 mM NaCl, and 80 mM Tris base, pH 8.0, at 20°C. The nonspecific hydrolysis of acetoacetyl-CoA was measured before initiation of the reaction with a final concentration of 50 mM sodium succinate.

Tissue capacity for ketogenesis was assessed by measuring the specific activity of hydroxy-methylglutaryl-CoA synthase (HMG-CoA synthase, E.C. 4.1.3.5), an enzyme that condenses acetoacetyl-CoA with acetyl-CoA to form 3-hydroxy-3-methylglutaryl-CoA (Voet and Voet 1995), which is suspected to be the rate-limiting step of ketogenesis (Clinkenbeard et al. 1975). HMG-CoA synthase activity was assayed spectrophotometrically according to Clinkenbeard et al. (1975) by monitoring the acetyl-CoA-dependent decrease in A_{300} due to acetoacetyl-CoA consumption. The assay mixture contained 0.1 mM EDTA, 0.1 mM acetoacetyl-CoA, 400 mM urea, 200 mM TMAO, 150 mM KCl, 3 mM $MgCl_2$, 10 mM NaCl, and 80 mM Tris base, pH 8.0, at 20°C. The assay mixture with homogenate was allowed to incubate for 2 min before the background rate was measured at 300 nm. Addition of 0.2 mM acetyl-CoA initiated the reaction. The millimolar extinction coefficient for acetoacetyl-CoA for this assay (7.91) was determined as the slope of the best-fit linear regression of acetoacetyl-CoA concentrations made up in the assay mixture versus A_{300}.

Temperature Effects on Mako Red Muscle Enzymes

As an endothermic shark, the shortfin mako is able to maintain its red muscle temperature significantly above ambient water

temperature (Carey and Teal 1969; Carey et al. 1985). Carey et al. (1985) reported an average mako red muscle temperature of 24°C, elevated an average of 4°C above ambient temperature. Therefore, all three enzyme assays for a subset of mako shark red muscle samples ($n = 3$) were measured at both 20° and 25°C to determine the effects of temperature on the three enzyme activities in this tissue. For these samples, HOAD, OAT, and HMG-CoA synthase specific activities were measured at the two temperatures using one red muscle homogenate prepared for each individual shark.

Serum Ketone Body Analysis

Serum β-hydroxybutyrate (β-HBA) concentrations were measured with Sigma Diagnostics kit 310-UV in which β-hydroxybutyrate dehydrogenase catalyzes the conversion of β-HBA + NAD^+ to acetoacetate + NADH. Although acetoacetate, acetone, and β-HBA together make up "ketone bodies" and are all transported via the bloodstream, acetoacetate easily decarboxylates into β-HBA and acetone (Hadley 1985), thus making accurate measurements of serum acetoacetate difficult. Most studies that have measured ketone bodies in fish blood have reported β-HBA concentrations only, presumably because β-HBA is the predominant ketone body in the blood and acetoacetate is ultimately transformed into β-HBA before oxidation in the target tissue (Zammit 1981). Furthermore, studies that have measured both β-HBA and acetoacetate in elasmobranch blood show that β-HBA concentrations exceed those of acetoacetate (deRoos 1994). Thus, only β-HBA was quantified in the shark blood samples that were collected in this study as a measure of ketone-body concentration.

Data Analysis

The data for each shark species were first tested for any significant relationship between fish length and the activity of each enzyme. Because mass was not recorded for most sharks, size-scaling relationships were examined using total length (TL) of all individuals. Fork length was converted to TL if TL data were not available, using the species-specific equations described in Kohler et al. (1995). The linear relationship between shark TL and enzyme activity for the three enzymes and between shark TL and serum ketone concentration was assessed to determine whether the correlation coefficient (r) was significant at $\alpha = 0.05$. The only significant effect of size was a negative relationship between TL and red muscle HOAD activity in the shortfin mako, and the red muscle HOAD activities for all individuals of the other species studied fell within the range found for the mako. Therefore, interspecific differences among shark species in the mean activity of each of the three enzymes in red muscle, heart, and liver and in mean serum ketone concentration were assessed using a one-way ANOVA. A Student's paired t-test was used to test for statistical differences in the capacity to oxidize fatty acids versus ketones (HOAD vs. OAT) in each tissue for each species. A one-way ANOVA was used to test for differences among the three tissues for all three enzymes. If ANOVAs showed significant interspecific differences, a Tukey-Kramer a posteriori test was performed. To test for significant temperature effects in the mako red muscle, a Student's paired t-test was used. All statistical analyses were performed at a significance level of $\alpha = 0.05$ using Minitab Statistical Software (Minitab, State College, Pa.).

Results

Enzyme data at 20°C were collected for 10–22 mako sharks (87–167 cm TL), 4–10 Atlantic sharpnose sharks (42–86 cm TL), two blacknose sharks (56 and 82 cm TL), one lemon shark (68 cm TL), and one or two common thresher sharks (135 and 262 cm TL). Specific activities of HOAD, OAT, and HMG-CoA synthase were measured in vitro for red myotomal muscle, heart ventricle, and liver of all species except for blacktip shark red muscle (Table 1). Correlation analysis revealed that the only variable that scaled significantly with fish length was red muscle HOAD activity in the mako ($r = -0.61$; $P < 0.05$). However, because the red muscle HOAD activities for all individuals of the other species studied fell within the range found for the mako and those that were similar in size (the two thresher sharks) had very similar activities as did makos of similar sizes, interspecific comparisons were made on the basis of mean activities.

The capacity for ketone oxidation (OAT activity) was high compared to that for fatty acid oxidation (HOAD) in red muscle and heart, but the opposite was true for liver, in which OAT activity could not be detected (Table 1). Capacities for fatty acid oxidation and ketogenesis were high in liver tissue (Table 1). For all shark species, HOAD and HMG-CoA synthase values were significantly higher in liver than in red muscle or heart ventricle (ANOVA, $P < 0.05$). OAT values were significantly higher in heart and red muscle when compared to liver for all sharks (ANOVA, $P < 0.05$). The HMG-CoA synthase activity, an index of ketogenic capacity, was low or undetectable in red muscle and heart tissue of all sharks but was present at measurable levels in the liver of the mako, Atlantic sharpnose, and thresher sharks (Table 1). The liver of the lemon and blacknose sharks did not have a high ketogenic capacity concurrent with low ketone-oxidation capacity (OAT), as did the other shark species (Table 1). This may be an artifact of the small sample size, or those sharks may not have been poised for high rates of ketogenesis at the time of capture (e.g., if they had recently been feeding consistently).

The endothermic mako sharks did not have elevated enzyme activities compared to the ectothermic sharks, with the exception of heart OAT (ketone-oxidation capacity), for which mako shark enzyme activity was significantly higher ($P < 0.05$; Table 1). Because the red muscle of mako sharks may operate at

Table 1: Activities of the enzymes HOAD, OAT, and HMG at 20°C in red myotomal muscle, heart ventricle, and liver of five species of sharks

Species and Common Name	Red Muscle			Heart			Liver		
	HOAD	OAT	HMG	HOAD	OAT	HMG	HOAD	OAT	HMG
Isurus oxyrinchus									
(shortfin mako shark)	1.50 ± .60	6.81 ± 2.50	.02 ± .06	.26 ± .11	6.02 ± 1.43	.01 ± .017	13.87 ± 13.29	0 ± 0	.45 ± .36
	.21–2.70	.51–11.64	0–0.24	.08–.45	3.25–8.47	0–.08	2.65–37.46	0	0–.98
	(21)	(22)	(22)	(21)	(21)	(21)	(10)	(12)	(11)
Rhizoprionodon terraenovae									
(Atlantic sharpnose shark)	2.07 ± .47	5.99 ± 1.00	.03 ± .06	.75 ± .38	1.48 ± 1.21	.03 ± .09	10.13 ± 4.62	0 ± 0	.35 ± .23
	1.49–2.50	4.84–7.27	0–.13	.07–1.24	0–3.12	0–.28	3.84–17.78	0	0–.62
	(4)	(4)	(4)	(9)	(10)	(10)	(10)	(10)	(10)
Carcharhinus acronotus									
(blacknose shark)	NM	NM	NM	.91	2.95	0	16.71	0	.04
	…	…	…	.78–1.04	2.51–3.38	0	14.86–18.54	0	.02–.06
	…	…	…	(2)	(2)	(2)	(2)	(2)	(2)
Alopias vulpinus									
(common thresher shark)	1.21	6.15	0	.20	3.14	0	25.80	0	.75
	1.14–1.28	5.81–6.49	0	…	…	…	25.02–26.57	0	.56–.93
	(2)	(2)	(2)	(1)	(1)	(1)	(2)	(2)	(2)
Negaprion brevirostris									
(lemon shark)	1.51	2.71	0	1.23	2.06	0	3.20	0	0
	(1)	(1)	(1)	(1)	(1)	(1)	(1)	(1)	(1)

Note. Activities are expressed in International Units (IU, μmol substrate converted to product min^{-1}) per gram tissue wet weight. For each species, the first line gives the mean ± 1 SEM, with the range of values below, followed by the number of individuals in parentheses; no SEM values given if $n = 1$ or 2. A value of 0 indicates undetectable activity, and NM = not measured. HOAD = β-hydroxy-o-acyl-CoA dehydrogenase, OAT = 3-oxoacid-CoA transferase, and HMG = 3-hydroxy-methylglutaryl-CoA synthase.

Table 2: Activities and Q_{10} values between 20° and 25°C of HOAD, OAT, and HMG in red myotomal muscle of the shortfin mako shark (*Isurus oxyrinchus*)

Red Muscle Assay Temperature	HOAD (Fatty Acid Oxidation)	OAT (Ketone Oxidation)	HMG (Ketogenesis)
20°C	1.97 ± .44	10.22 ± 1.80	0
25°C	2.70 ± .62	10.42 ± 1.57	0
Q_{10}	1.88	1.04	…

Note. Activities are expressed as μmol substrate converted to product min^{-1} per gram tissue wet weight. Values are means ± 1 SD for three individuals. A value of 0 indicates undetectable activity. HOAD = β-hydroxy-o-acyl-CoA dehydrogenase, OAT = 3-oxoacid-CoA transferase, and HMG = 3-hydroxy-methylglutaryl-CoA synthase. The activity of HOAD increased significantly between 20° and 25°C ($P = 0.02$, paired *t*-test), but there was no significant effect of temperature on OAT or HMG activity.

temperatures over 4°C above ambient at environmental temperatures of about 20°C (Carey et al. 1985), enzyme activities in some mako red muscle samples were compared at 20° and 25°C (Table 2). The activity of HOAD (fatty acid oxidation) was significantly greater at 25° than at 20°C in mako shark red muscle (Student's paired *t*-test, $P = 0.02$), but OAT (ketone oxidation) activity was similar at both 20° and 25°C and HMG-CoA synthesis activity (ketogenesis) was undetectable at both temperatures. Although red muscle HOAD activity at 25°C in the mako (Table 2) is greater than that at 20°C for the other shark species studied (Table 1), it is much lower than that of the liver at 20°C (Table 1).

Shark serum ketone concentrations varied among the species studied, ranging from undetectable levels in the one lemon shark (*Negaprion brevirostris*) to a mean of 0.978 ± 0.89 μmol β-hydroxybutyrate mL^{-1} in the mako shark (*Isurus oxyrinchus*; Table 3). The only significant interspecific difference in mean blood ketone concentrations was that the mako was significantly greater than the sandbar shark (*Carcharhinus plumbeus*; one-way ANOVA and post hoc Tukey-Kramer test, $P < 0.05$; lemon shark data omitted).

Discussion

The red myotomal muscle and heart ventricle of all of the shark species tested in this study had an elevated capacity for ketone oxidation compared to fatty acid oxidation and little potential for ketogenesis (Table 1). The sharks also possessed detectable amounts of blood-borne ketones, with the exception of the one lemon shark (Table 3). Finally, liver tissue had a high capacity for fatty acid oxidation and possessed significant ketogenic activity (Table 1). Taken together, the results are consistent with the hypothesis that, in active sharks, fatty acids from the lipids stored in the liver (in the form of di- and triacylglycerols; Ballantyne 1997) are broken down into acetyl-CoA via β-oxidation within the liver. This is reflected in the high HOAD activities in the liver tissue that were measured in this study. The acetyl-CoA would subsequently undergo ketogenesis in the

Table 3: Serum β-hydroxybutyrate concentrations and ranges for the eight species of sharks studied

Species and Common Name	*n*	β-Hydroxybutyrate Concentration	Range
Carcharhinus limbatus (blacktip shark)	2	.128 ± .05	.09–.17
Carcharhinus plumbeus (sandbar shark)	25	.206 ± .24	0–.66
Galeocerdo cuvier (tiger shark)	2	.171 ± .19	.03–.31
Negaprion brevirostris (lemon shark)	1	0	0
Prionace glauca (blue shark)	6	.262 ± .15	.08–.45
Rhizoprionodon terraenovae (Atlantic sharpnose shark)	25	.513 ± .37	.09–1.10
Isurus oxyrinchus (shortfin mako shark)	9	.978 ± .89	.08–2.89
Heterodontus francisci (horn shark)	8	.594 ± .47	.06–1.33

Note. Concentrations are in μmols mL^{-1} and are expressed as mean ± 1 SD.

liver mitochondria (based on the measurable values of HMG-CoA synthase), and then the ketones would be exported via the blood for use as an oxidative fuel in other tissues, including red locomotor muscle and heart. In mammals, the ketone-oxidation enzyme, OAT, is not expressed in the liver so that ketones can be exported and not consumed (Zammit et al. 1979; Voet and Voet 1995). Likewise, in sharks, the liver does not appear to utilize its synthesized ketones because OAT activity in the liver was consistently undetectable (Table 1). There was also an elevated capacity for ketone-body oxidation and generally low potentials for fatty acid catabolism and for ketogenesis in red myotomal muscle and heart ventricle (Table 1). This is consistent with the idea that the blood supplies the red muscle and heart with ketones synthesized from liver lipid stores since neither tissue is ketogenic. In addition, initial studies using techniques from Zammit and Newsholme (1979) and Tullis et al. (1991) consistently found carnitine palmitoyl transferase activity (CPT, E.C. 2.3.1.21) to be undetectable in shark red myotomal muscle and heart ventricle samples. Because CPT is required for transport of fatty acids into the mitochondria, CPT activity is another indicator of fatty acid catabolism in tissues. Thus, the data support the hypothesis that active, obligate swimming sharks, including the endothermic shortfin mako, mobilize liver lipids in the form of ketones, rather than fatty acids, to provide energy for aerobic tissues such as red muscle and heart.

Tissue enzyme activities varied significantly among individuals of the same species (Table 1). Some of this can be explained by effects of fish size. For mako red muscle HOAD, a significant correlation with fish TL was found ($r^2 = 0.38$). Although no other significant correlations with fish length were found, these r^2 values ranged from 0 to 0.26, indicating that up to 26% of the within-species variation can be attributed to effects of fish size. All tissue samples were frozen immediately after fish capture; however, because many of the tissue samples were collected by others, there may have been differences in how the samples were handled, even though we did give the same detailed handling instructions to all collectors. Yet, we did not detect any consistent trends among samples that would point to this as a factor. There may also be unknown differences related to season, state of sexual maturity, sex, or recent feeding history, but we were unable to assess the influence of these factors in this study.

The ketone concentrations measured in this study were also quite variable (Table 3). Serum ketone concentrations are known to vary with degree of starvation in other vertebrates, including other sharks. *Squalus acanthias* starved for 3–8 d and 20–29 d possessed mean blood ketone concentrations of 7.20 and 13.47 μmol mL^{-1}, respectively (deRoos 1994), which are values that are well above the maximum ketone concentrations detected in this study. Since the stomach contents of the fish collected for this study were not examined, it is not possible to determine how much of the variability in the data can be attributed to differences in the time elapsed since each shark's last meal. Despite the variability, the concentrations of ketones measured in shark blood are considerably higher than those found in teleosts (< 0.001 μmol mL^{-1} for all species tested by Zammit and Newsholme [1979]). Based on substrate preferences for mitochondrial oxidation and tissue enzyme activities, teleosts are able to use fatty acids for aerobic metabolism in extrahepatic tissues and are not known to oxidize ketones at high levels (reviewed by Ballantyne 1997). Thus, although serum concentrations cannot be used to determine turnover rates for ketones (which are not known for either teleosts or elasmobranchs), finding high ketone concentrations in the blood of active sharks relative to teleosts does fit in with the hypothesized mobilization of ketones from the liver for use as aerobic fuels in the muscle tissues.

The endothermic shortfin mako shark did not demonstrate elevated activities of enzymes compared to the other species, even when assayed at physiological temperatures, except for the OAT activity in the heart ventricle and the HOAD activity at 25°C in red muscle (Tables 1, 2). The mako and the endothermic lamnids in general have larger, more muscular and more vascularized hearts than do other sharks (Emery et al. 1985). In addition, they have high hemoglobin and hematocrit values and a greater blood pressure than do ectothermic sharks, presumably because they possess a greater metabolic rate and a higher aerobic scope (Emery 1986; Lai et al. 1997). Therefore, the observed elevated OAT activity (ketone-oxidation capacity) in the mako shark heart may correlate with higher cardiac demands associated with the continuous swimming activity of endothermic sharks. However, published values of OAT at 10°C in the heart of three less active shark species [11.5 IU g^{-1} tissue wet weight in *Mustelus asterias* Cloquet 1821, 9.9 IU g^{-1} in *Scyliorhinus canicula* (Linneaus 1758), and 9.4 IU g^{-1} in *Squalus acanthias* Linneaus (Zammit and Newsholme 1979)] are higher than the values measured in this study.

The higher HOAD activity at physiological temperatures in mako red muscle relative to that in other shark species (and the little skate, *Raja erinacea*; Moon and Mommsen 1987) may indicate an elevated capacity for β-oxidation. This could be used to turn over short-chain fatty acids within the muscle tissue, rather than lipids supplied exogenously from the liver. Yet, the mako red muscle and heart HOAD activities are lower than those previously measured in a range of marine teleost fishes, in which CPT activity is also detectable (reviewed by Dickson 1995). This may be related to the greater use of fatty acids as aerobic fuels by the muscle mitochondria in teleosts relative to elasmobranchs.

Reliance on ketones as a fuel source other than during periods of starvation is unusual among vertebrates (Robinson and Williamson 1980; Sheridan 1988; Chamberlin and Ballantyne 1992; Ballantyne 1997). Energetically, a fatty acid that undergoes hepatic ketogenesis and then subsequent oxidation in the muscle yields as much ATP as one that undergoes complete

oxidation (Voet and Voet 1995). The only energetic "cost" of using ketones is the free energy of one GTP hydrolysis per molecule of acetoacetate and the synthesis of the ketogenic enzymes themselves. It is therefore unclear if there is a significant energetic advantage of ketone oxidation over fatty acid oxidation or vice versa.

With the exception of the freshwater stingrays (Potamotrygonidae), elasmobranchs have very low or undetectable amounts of serum albumin, which binds fatty acids and allows their transport in the blood (reviewed by Ballantyne 1997). If fatty acids were released from the liver di- and triacylglycerol stores, a lack of functioning serum albumin would prevent their transport to other tissues for oxidation. Ballantyne (1997) suggested that the absence of albumin in most elasmobranchs may be related to the counteracting solute strategy (urea and trimethylamine oxide [TMAO]) that is used by elasmobranchs to osmoconform in seawater (Yancey et al. 1982). Urea, which has strongly perturbing effects on hydrophobic interactions (including those that maintain the tertiary conformation of proteins), may affect albumin formation or binding to fatty acids in a way that is not counteracted by TMAO (Yancey et al. 1982; Ballantyne and Moon 1986*a*, 1986*b*; Ballantyne et al. 1987).

It is also possible that fatty acids are not transported in the blood simply because they are insoluble due to the high blood osmolarity in sharks. Additional solutes, such as albumin and fatty acids, may not be tolerated in high enough concentrations in elasmobranch blood to be transported in sufficient quantities to meet the energetic needs of the extrahepatic tissues. Ketones are more soluble, do not require a transport protein, and thus may provide an effective alternative, as appears to be true in pulmonate snails (Stuart et al. 1998). However, none of these ideas has been tested, and effects of urea, methylamines, and other osmoregulatory solutes on albumin and enzyme structure and function with respect to lipid metabolism warrants further investigation.

This study has provided additional evidence, from a range of active sharks, to support the idea that elasmobranchs mobilize liver lipid stores in the form of ketones for oxidation by active muscle tissues. Thus, liver lipids may be an important fuel source for aerobic metabolism in elasmobranchs but only after conversion to ketones within the liver. Because we measured only the tissues' potential for the different metabolic pathways, more direct studies of the conversion of liver lipids to ketones, their export into the blood, and their uptake and use as fuels by the aerobic muscle tissues in live sharks are needed to confirm this hypothesis.

In addition to liver lipids, both carbohydrates and proteins may also be used as aerobic fuel sources. However, in sharks, carbohydrates apparently do not serve as a significant aerobic fuel, based on low levels of carbohydrates in the diet and low plasma glucose concentrations (Patent 1970; Ballantyne 1997). Elasmobranchs do have significant glycogen stores in muscle and liver (reviewed by Ballantyne 1997), but these may be reserved for anaerobic function. Protein catabolism and amino acid oxidation may be an important energy source in shark extrahepatic tissues, but there is even less published research on amino acid metabolism than on lipid metabolism in sharks. Studies of mitochondria isolated from spiny dogfish (*S. acanthias*) and shortfin mako sharks show that glutamine, in particular, appears to be an important energy source in red muscle; oxidation rates were similar to those of ketones (Ballantyne et al. 1992; Chamberlin and Ballantyne 1992). The mitochondria of the mako shark also oxidized glutamate and proline at high rates (Ballantyne et al. 1992).

Because predatory sharks have a diet rich in protein (Love 1996), amino acids may be an immediate source of energy for oxidative metabolism during the absorptive and postabsorptive states. Mobilization of liver lipid stores as ketones for energy may occur secondarily, once short-term fasting begins. Sharks have low, but detectable, levels of amino acids in their blood compared to mammals, and amino acids are soluble in blood (Garrett and Grisham 1995). Thus, amino acids may be transported to muscle tissues to be oxidized (Chamberlin and Ballantyne 1992). Oxidation of amino acids by sharks also has the added benefit of supplying nitrogen for urea synthesis (Ballantyne 1997). Additional studies, preferably on live sharks, will be needed to demonstrate the relative importance of amino acid versus ketone-oxidative metabolism for continuous swimming in sharks during times of feeding and fasting.

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