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JOURNAL OF EXPERIMENTAL ZOOLOGY 306A:1–14 (2006)

Metabolic Organization of the Spotted Ratfish, *Hydrolagus colliei* (Holocephali: Chimaeriformes): Insight Into the Evolution of Energy Metabolism in the Chondrichthyan Fishes

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ABSTRACT The metabolic organization of a holocephalan, the spotted ratfish (*Hydrolagus colliei*), was assessed using measurements of key enzymes of several metabolic pathways in four tissues and plasma concentrations of free amino acids (FAA) and non-esterified fatty acids (NEFA) to ascertain if the Holocephali differ metabolically from the Elasmobranchii since these groups diverged ca. 400 Mya. Activities of carnitine palmitoyl transferase indicate that fatty acid oxidation occurs in liver and kidney but not in heart or white muscle. This result mirrors the well-established absence of lipid oxidation in elasmobranch muscle, and more recent studies showing that elasmobranch kidney possesses a capacity for lipid oxidation. High activities in oxidative tissues of enzymes of ketone body metabolism, including D-β-hydroxybutyrate dehydrogenase, indicate that, like elasmobranchs, ketone bodies are of central importance in spotted ratfish. Like many carnivorous fishes, enzyme activities demonstrate that amino acids are metabolically important, although the concentration of plasma FAA was relatively low. NEFA concentrations are lower than in teleosts, but higher than in most elasmobranchs and similar to that in some ''primitive'' ray-finned fishes. NEFA composition is comparable to other marine temperate fishes, including high levels of *n*-6 and especially *n*-3 polyunsaturated fatty acids. The metabolic organization of the spotted ratfish is similar to that of elasmobranchs: a reduced capacity for lipid oxidation in muscle, lower plasma NEFA levels, and an emphasis on ketone bodies as oxidative fuel. This metabolic strategy was likely present in the common chondrichthyan ancestor, and may be similar to the ancestral metabolic state of fishes. *J. Exp. Zool. 306A:1–14, 2006*.

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Holocephalans, or ratfishes or chimaeras, are a group of mostly deep-water marine cartilaginous fishes that are most closely related to their more notorious relatives, the elasmobranch fishes. The subclasses Holocephali and Elasmobranchii, which together comprise the class Chondrichthyes, diverged around 360 Mya, not long after the appearance of the first chondrichthyans about 420 Mya (Grogan and Lund, 2004). Although this long evolutionary separation has resulted in certain unique anatomical and physiological features, ratfishes, like marine elasmobranchs, are osmoconformers that accumulate high levels of urea (>250 mM) and counteracting methylamines to maintain an internal solute concentration close to that of seawater (Read, '71; Bedford, '83). Ratfishes, however, have less urea and more Na^+ and Cl^- than elasmobranchs (Read, '71), and the principal counteracting solute in at least one species is the methylamine betaine and not trimethylamine oxide, the major counteracting solute in elasmobranchs (Bedford et al., '98).

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There are very few studies on the energy metabolism of holocephalans, involving only limited measurements of plasma lipids and amino acids and tissue activities of certain enzymes. Bedford ('83) analyzed certain free amino acids (FAA) in plasma and red blood cells from *Callorhinchus milii*, but did not measure glutamine, which is particularly important in energy metabolism in elasmobranchs (Ballantyne, '97). Larsson and Fänge ('77) found low levels of non-esterified fatty acids (NEFA) in plasma from the holocephalan *Chimaera monstrosa*, but they used an inaccurate colorimetric technique that probably underestimates NEFA concentration and does not reveal fatty acid composition (see Singer et al., '90). Ritter et al. ('87) measured activities of a limited number of enzymes of intermediary metabolism in liver and brain of the spotted ratfish (*Hydrolagus colliei*), a holocephalan which is common in the northeast Pacific Ocean from close inshore to 1,000 m (Eschmeyer et al., '83).

A detailed examination of the metabolic organization of a holocephalan is of interest considering the unique pattern of aerobic fuel use seen in elasmobranchs: namely, an apparent low or non-existent capacity for extrahepatic lipid oxidation and an increased reliance on ketone bodies and glutamine as oxidative substrates (reviewed by Ballantyne, '97). None of these has been examined in holocephalans. In the present study we ascertained whether, like elasmobranchs, the spotted ratfish, as a representative holocephalan, possesses: (1) reduced capacity for lipid oxidation as indicated by enzyme activities in extrahepatic tissues, (2) increased extrahepatic capacity for ketone body metabolism as indicated by high activity of β-hydroxybutyrate dehydrogenase (β-HBDH), and (3) low levels of NEFA in the blood (using a sensitive method that provides data on NEFA composition). We measured plasma FAA levels to evaluate the importance of FAA (including glutamine) in a holocephalan. Measurements of other enzymes of energy metabolism were made to provide a more comprehensive picture of the metabolic organization of a representative holocephalan.

Similarities between the metabolic organization of holocephalans and elasmobranchs will allow an understanding of the probable metabolic organization of the common chondrichthyan ancestor that swam in the seas over 400 Mya. Commonalities in the pattern of lipid oxidative capacity will be of specific interest in the context of Ballantyne et al.'s ('87) hypothesis that the evolution of urea-based osmoregulation in elasmobranchs led to a de-emphasis on fatty acids as an extrahepatic metabolic fuel because of perturbing effects of high levels of urea on the ability of albumin to transport long-chain fatty acids. Since holocephalans also possess high levels of urea, they would be expected to show a similar metabolic organization to elasmobranchs if this hypothesis is correct.

MATERIALS AND METHODS

Animals

Spotted ratfishes (450–700 g) were captured by otter trawl off northern Vancouver Island, British Columbia, on a Department of Fisheries and Oceans (Canada) groundfish survey aboard CCGS W.E. *Ricker* in May 2004. Depth of capture was 120–200 m and bottom temperature was 6–7°C. Trawls lasted 30–60 min. Only animals still responsive to handling were sampled.

Blood was drawn into heparinized syringes by caudal puncture and centrifuged for 5 min to sediment erythrocytes. Plasma was decanted and immediately frozen in liquid nitrogen, followed by storage at −80°C until use. Animals were then sacrificed by rapidly severing the spinal cord. Liver, heart, kidney, and white muscle were excised within 5 min and frozen in liquid nitrogen for transport back to the University of Guelph where they were stored at −80°C until use.

Tissue preparation

Tissues were thawed on ice and placed in 11 volumes of ice-cold homogenization buffer (50 mM imidazole, 1 mM dipotassium ethylene diaminetetraacetic acid (EDTA), pH 7.4 at 20°C). Homogenization was completed on ice using a Polytron PT1200 (Kinematica, Inc., Newark, NJ) set at high speed (25,000 rpm) for three passes of 10 sec with 30 sec between bursts. Homogenates were centrifuged at 4°C at 500*g* to clear cellular debris and the supernatant was used directly or diluted for enzyme assays. For assays of cytochrome *c* oxidase (CCO), carnitine palmitoyl transferase (CPT), and carnitine octanoyl transferase (COT), Tween 20 was added to the homogenate to make a final concentration of 0.5% and this was mixed slowly on ice for 15 min, then centrifuged as above, and the supernatant used directly for enzyme assays. Tween 20 (0.5%) gave the highest activity of CPT in tissues from *Leucoraja erinacea* and other elasmobranchs when compared with homogenates that were untreated, sonicated, or treated

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with 0.2% Triton-X 100 (TX100) (Speers-Roesch and Ballantyne, unpublished data; J.R. Treberg, unpublished data). Although TX100 gave approximately 50% higher activity of CCO than 0.5% Tween 20, it was not possible to prepare a separate TX100-treated aliquot for CCO assays due to limited tissue availability. In any case, Tween 20 gave higher CCO activity than untreated homogenate and this detergent is widely used in extracting CCO activity (Moyes et al., '97; Hardewig et al., '99; Lucassen et al., 2003).

Enzyme assays

Maximal enzyme activities were measured in duplicate using a Cary 300 Bio UV–Visible spectrophotometer (Varian, Inc., Palo Alto, CA) equipped with a thermostated cell changer maintained at 12°C (±0.1°C) with a Cary Temperature Controller (Varian, Inc.). Activities of most enzymes were ascertained by measuring the oxidation or reduction of pyridine nucleotides at 340 nm (millimolar extinction coefficient ε_{340}, 6.22). CPT, COT, and Citrate synthase (CS) were monitored at 412 nm using 5,5′-dithiobis 2-nitrobenzoic acid (DTNB) (millimolar extinction coefficient å_{412}, 13.6). CCO activity was measured at 550 nm (cytochrome *c* millimolar extinction coefficient ε_{550}, 18.5). Acetoacetyl coenzyme-A thiolase (AcoAT) was monitored at 313 nm (millimolar extinction coefficient ε_{313}, 20.5). Succinyl coenzyme-A ketotransferase (SKT) was measured at 310 nm (millimolar extinction coefficient ε_{310}, 11.9).

Conditions of saturating substrate were used and linearity with protein was ensured. Most enzymes were assayed following the protocols of Singer and Ballantyne ('89). The pyruvate kinase (PK) assay was modified from Moon and Mommsen ('87) and Driedzic and De Almeida-Val ('96. CCO was measured using the method of Blier and Guderley ('88), and D- and L-β-HBDH were measured following LeBlanc and Ballantyne (2000). Conditions were as follows:

Enzymes of aerobic metabolism

Cytochrome *c* oxidase (CCO; E.C. 1.9.3.1): 50 mM imidazole, pH 8.0, at 20°C, 0.05 mM reduced cytochrome *c* (omitted for control).

Citrate synthase (CS; E.C. 4.1.3.7): 50 mM imidazole, pH 8.0, at 20°C, 0.1 mM DTNB, 0.3 mM acetyl CoA, 0.5 mM oxaloacetate (omitted for control).

Enzymes of lipid catabolism

Carnitine palmitoyl transferase (CPT; E.C. 2.3.1.21): 50 mM imidazole, pH 8.0, at 20°C, 0.2 mM DTNB, 0.1 mM palmitoyl CoA, 5 mM L-carnitine (omitted for control).

Carnitine octanoyltransferase (COT; E.C. 2.3.1.137): 50 mM imidazole, pH 8.0, at 20°C, 0.2 mM DTNB, 0.1 mM octanoyl CoA, 5 mM L-carnitine (omitted for control).

3-Hydroxyacyl CoA dehydrogenase (HOAD; E.C. 1.1.1.35): 50 mM imidazole, pH 8.0, at 20°C, 0.1 mM NADH, 1 mM KCN, 0.1 mM acetoacetyl CoA (omitted for control).

Enzyme of lipid synthesis

Malic enzyme (ME; E.C. 1.1.1.40): 50 mM imidazole, pH 7.4, at 20°C, 1 mM $MgCl_2$, 0.4 mM NADP, 1 mM malate (omitted for control).

Enzymes of ketone body metabolism

D-β-hydroxybutyrate dehydrogenase (D-β-HBDH; E.C. 1.1.1.30): 50 mM imidazole, pH 8.0, at 20°C, 11.25 mM NAD, 50 mM nicotinamide, 2 mM dithiothreitol (DTT), 25 mM D-β-hydroxybutyrate (omitted for control).

L-β-hydroxybutyrate dehydrogenase (L-β-HBDH; E.C. 1.1.1.30): 50 mM imidazole, pH 8.0, at 20°C, 11.25 mM NAD, 50 mM nicotinamide, 2 mM DTT, 25 mM L-β-hydroxybutyrate (omitted for control).

Succinyl coenzyme-A ketotransferase (SKT; E.C. 2.8.3.5): 50 mM imidazole, pH 8.0, at 20°C, 5 mM $MgCl_2$, 0.1 mM acetoacetyl CoA, 1 mM succinate (omitted for control).

Acetoacetyl coenzyme-A thiolase (ACoAT; E.C. 2.3.1.9): 50 mM imidazole, pH 8.0, at 20°C, 5 mM $MgCl_2$, 0.1 mM acetoacetyl CoA, 0.2 mM CoA (omitted for control).

Enzymes of glycolysis

Hexokinase (HK; E.C. 2.7.1.1): 50 mM imidazole, pH 7.4, at 20°C, 1 mM glucose, 5 mM $MgCl_2$, 0.16 mM NADP, excess glucose 6-phosphate dehydrogenase, 1 mM ATP (omitted for control).

Pyruvate kinase (PK; E.C. 2.7.1.40): 50 mM imidazole, pH 7.4, at 20°C, 0.15 mM NADH, 5 mM ADP, 10 mM $MgCl_2$, 50 mM KCl, 1 mM KCN, excess lactate dehydrogenase (LDH), 5 mM phosphoenolpyruvate (omitted for control).

Lactate dehydrogenase (LDH; E.C. 1.1.1.27): 50 mM imidazole, pH 7.4, at 20°C, 0.2 mM NADH, 1 mM pyruvate (omitted for control).

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Enzyme of gluconeogenesis

Fructose 1,6-bisphosphatase (FBPase; E.C. 3.1.3.11): 50 mM imidazole, pH 7.4, at 20°C, 15 mM $MgCl_2$, 0.2 mM NADP, excess glucose 6-phosphate dehydrogenase, excess phosphoglucose isomerase, 0.1 mM fructose 1,6-diphosphate (omitted for control).

Enzymes of amino acid metabolism

Glutamate dehydrogenase (GDH; E.C. 1.4.1.3): 50 mM imidazole, pH 8.0, at 20°C, 250 mM ammonium acetate, 0.1 mM dipotassium EDTA, 0.1 mM NADH, 1 mM ADP, 1 mM KCN, 14 mM α-ketoglutarate (omitted for control).

Alanine aminotransferase (AlaAT; E.C. 2.6.1.2): 50 mM imidazole, pH 7.4, at 20°C, 0.2 mM NADH, 10.5 mM α-ketoglutarate, 0.025 mM pyridoxal phosphate, excess LDH, 200 mM L-alanine (omitted for control).

Aspartate aminotransferase (AspAT; E.C. 2.6.1.1): 50 mM imidazole, pH 7.4, at 20°C, 0.2 mM NADH, 7 mM α-ketoglutarate, 0.025 mM pyridoxal phosphate, excess malate dehydrogenase, 40 mM L-aspartate (omitted for control).

Activities are presented as units · gram wet weight^{-1} (units g ww^{-1}) where one unit equals 1 μmol substrate converted to product per minute. Protein was measured using the Bio-Rad standard assay (Bio-Rad, Hercules, CA). All chemicals were obtained from Sigma Chemical Co. (St. Louis, MO).

Measurement of plasma NEFA

Plasma NEFA were methylated as described in Singer et al. ('90). The methyl esters were redissolved in 25 μl of carbon disulfide and 1 μl were injected into a gas chromatograph (6890N, Agilent Technologies, Palo Alto, CA) fitted with a flame ionization detector and an automatic injector. Methyl esters were separated on a DB-23 column (J&W Scientific, Folsom, CA). The column temperature was initially 50°C, increased to 180°C over 10 min, held at 180°C for 5 min, and then increased over 5 min to 240°C where it was held for 5 min. Fatty acids were identified by comparing their retention times (RTs) to those of known standards (GLC 463 augmented with 22:5*n*-6 and 23:0, Nu-Check Prep, Elysian, MN). This method allows the detection of fatty acids ranging from 4 to 24 carbon chain lengths. Absolute amounts of fatty acids were calculated by adding a known amount (15 μg) of an internal standard, heptadecanoic acid (17:0), to the plasma samples prior to methylation. Preliminary analyses showed only trace amounts of endogenous 17:0.

Measurement of plasma FAA

FAA in plasma were measured using a commercially available EZ faast kit (Phenomenex, Torrence, CA) run on a Waters Micromass Quattro Micro API tandem mass spectrometer (Waters, Milford, MA) equipped with an electrospray ionization interface, a nitrogen generator (Peak Scientific, Bedford, MA), and a Waters 2695 separation module (Waters). Separation of amino acids was achieved using methanol and water each with 10 mM ammonium formate at a flow rate of 0.25 ml min^{-1}. The separation uses 83% methanol and 17% water for the initial 11 min, and 68% methanol and 32% water for an additional 2 min. The column (Phenomenex EZ faast 4 μm AAA-MS 250 × 2.0 mm) was re-equilibrated for 8 min between runs and run at 35°C.

The mass spectrometer was operated in the positive-ion mode using the following conditions: nitrogen was used for desolving at a flow rate of 150 L hr^{-1} and cone gas was supplied at a flow rate of 100 L hr^{-1}. Argon was used as the collision gas. Capillary voltage was set to 3.0 kV. Cone and collision voltages were optimized for individual amino acid groups and their representative internal standard as specified in the kit. Data were acquired in MRM mode for parent–daughter ion combinations. Amino acids in plasma samples were identified based on RT and the mass-to-charge ratio (m/z) of the parent–daughter ion combinations and quantified using a five-point calibration of 2, 5, 10, 50, 100 nmol for each amino acid.

HPLC-grade methanol and water were obtained from Fisher (Ottawa, Ont., Canada); all other chemicals were obtained from Sigma Chemical Co.
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Statistical analysis

Activities of each enzyme were compared between tissues using one-way ANOVA with Tukey's test. Data were log-transformed prior to ANOVA if unequal variances were found (Zar, '99). Levels of COT vs. CPT as well as levels of D-β-HBDH vs. L-β-HBDH were compared within liver and kidney with a Student's t-test. All analyses were run on SigmaStat (SPSS Inc., Chicago, IL). Significance was accepted at $P < 0.05$.

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RESULTS

The levels of key enzymes of several metabolic pathways in liver, kidney, heart, and white muscle (Table 1) provided a quantitative and qualitative view of the metabolic organization of the spotted ratfish.

Oxidative metabolism

Activities of CCO, the terminal step in the electron transport chain and as such an indicator of the potential for aerobic metabolism, showed that the most aerobic tissues were the heart and kidney and the least the white muscle. Based on activities of CS, a component enzyme of the Krebs cycle also indicative of aerobic metabolism, the most aerobic tissues were the heart and kidney and the least the liver.

Lipid metabolism

Activity of HOAD, an enzyme involved in β-oxidation of fatty acids, was detected in all tissues with the highest activity in the kidney and heart, and lowest in the white muscle. CPT, a mitochondrial enzyme that catalyzes the rate-limiting step in carnitine-dependent oxidation of long-chain fatty acids (McGarry and Brown, '97), was not detected in heart or white muscle. Kidney and liver contained similar levels of CPT. COT, a peroxisomal enzyme that exports medium-chain fatty acids produced by peroxisomal β-oxidation for further mitochondrial oxidation (Ramsay, '99), was detectable in all tissues although activities were low in liver, heart, and white muscle. In kidney, COT activity was significantly higher than in other tissues and four-fold, and significantly, greater than CPT activity; in liver the two enzymes had similar activities. ME, an enzyme

TABLE 1. Activities (mean ± SEM) of enzymes of intermediary metabolism in tissues from the spotted ratfish (Hydrolagus colliei) (n = 6, except where noted in parantheses)

	Liver	Kidney	Heart	White muscle
Aerobic metabolism				
CCO	1.66 ± 0.11^{bc}	6.29 ± 0.48 (5) ^{ad}	7.02 ± 0.70^{ad}	0.92 ± 0.05^{bc}
CS	0.075 ± 0.011^{bcd}	7.01 ± 0.47 (5)^{ad}	11.6 ± 1.5^{ad}	0.73 ± 0.05^{abc}
Lipid catabolism				
CPT	0.066 ± 0.012^{cd}	0.076 ± 0.012 (5)^{cd}	ND^{ab}	ND^{ab}
COT	0.054 ± 0.012^{bd}	0.30 ± 0.04 (5)^{acd,*}	0.018 ± 0.004^{b}	0.010 ± 0.003^{ab}
HOAD	0.18 ± 0.02^{bcd}	0.35 ± 0.03 (5)^{ad}	0.34 ± 0.02^{ad}	0.021 ± 0.005^{abc}
Lipid synthesis				
ME	0.010 ± 0.003^{c}	0.40 ± 0.01^{c}	2.63 ± 0.34^{abd}	0.041 ± 0.010^{c}
Ketone body metabolism				
D-β-HBDH	0.61 ± 0.08^{d}	1.51 ± 0.24 (5)^{d}	1.23 ± 0.14^{d}	0.043 ± 0.013^{abc}
L-β-HBDH	$0.022 \pm 0.014^{\psi}$	$0.014 \pm 0.008^{\psi}$	ND	ND
SKT	0.083 ± 0.013^{c}	0.66 ± 0.05^{c}	3.05 ± 0.38^{abd}	0.12 ± 0.02^{c}
ACoAT	1.78 ± 0.17^{bcd}	3.08 ± 0.08^{acd}	1.31 ± 0.08^{abd}	0.097 ± 0.014^{abc}
Glycolysis				
HK	0.076 ± 0.030^{bc}	1.73 ± 0.05^{acd}	5.47 ± 0.45^{abd}	0.029 ± 0.003^{bc}
PK	0.31 ± 0.04 (5)^{bcd}	6.71 ± 0.31^{ad}	9.11 ± 1.15^{ad}	37.4 ± 3.7^{abc}
LDH	2.35 ± 0.31^{bcd}	19.5 ± 1.1^{ac}	70.7 ± 3.1^{abd}	20.2 ± 2.8^{ac}
Gluconeogenesis				
FBPase	0.056 ± 0.010^{bd}	0.12 ± 0.01^{acd}	0.036 ± 0.003^{bd}	0.021 ± 0.005^{ab}
Amino acid metabolism				
GDH	1.25 ± 0.12^{bd}	10.8 ± 0.4 (5)^{acd}	1.62 ± 0.12^{bd}	0.033 ± 0.005^{abc}
AlaAT	1.69 ± 0.18^{bd}	14.4 ± 1.0 (4)^{acd}	1.95 ± 0.22 (3)^{bd}	0.22 ± 0.02^{b}
AspAT	7.62 ± 0.24^{cd}	7.02 ± 0.94 (4)^{cd}	25.4 ± 1.9 (3)^{abd}	2.65 ± 0.11^{abc}

Activities were measured at 12°C. ND = not detectable.

Activities are expressed as $\mu mol\, min^{-1}\, g\, ww^{-1}$. Letters indicate a significant difference (one-way ANOVA, $P < 0.05$) from the activity of the same enzyme in, a: liver; b: kidney; c: heart; d: white muscle. *Signifies that COT activity is significantly different from CPT activity within the same tissue (Student's *t*-test, $P < 0.05$). $^{\psi}$denotes that L-β-HBDH activity is significantly different from D-β-HBDH activity within the same tissue (Student's *t*-test, $P < 0.05$). Enzyme abbreviations are as follows: cytochrome *c* oxidase (CCO); citrate synthase (CS); carnitine palmitoyl transferase (CPT); carnitine octanoyl transferase (COT); 3-hydroxyacyl CoA dehydrogenase (HOAD); malic enzyme (ME); D-β-hydroxybutyrate dehydrogenase (D-β-HBDH); L-β-hydroxybutyrate dehydrogenase (L-β-HBDH); succinyl coenzyme-A ketotransferase (SKT); acetoacetyl coenzyme-A thiolase (AcoAT); hexokinase (HK); pyruvate kinase (PK); lactate dehydrogenase (LDH); fructose 1,6-bisphosphatase (FBPase); glutamate dehydrogenase (GDH); alanine aminotransferase (AlaAT); aspartate aminotransferase (AspAT).

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usually associated with lipid synthesis, was highest in heart and lowest in white muscle and liver.

The absolute concentrations (nmol ml^{-1}) and percentages (by mol) of individual NEFA in the plasma of *H. colliei* are presented in Table 2. Total mean NEFA concentration was 652.3 nmol ml^{-1}.

TABLE 2. Absolute amounts (mean ± SEM) and percentages (mean ± SEM) of total and individual non-esterified fatty acids in plasma of spotted ratfish (Hydrolagus colliei) (n = 8)

Fatty acid	Concentration (nmol ml^{-1})	Percentage (% by moles)
14:0	7.95 ± 1.08	1.21 ± 0.09
14:1	1.88 ± 0.45	0.40 ± 0.19
*iso*16	42.3 ± 10.4	5.85 ± 1.34
16:0	104.9 ± 11.1	16.1 ± 0.7
16:1	39.3 ± 4.3	5.97 ± 0.23
18:0	30.7 ± 3.0	4.76 ± 0.17
18:1	141.4 ± 18.0	21.5 ± 1.6
18:2*n*-6	9.68 ± 0.84	1.56 ± 0.15
18:3*n*-3	3.75 ± 3.00	0.54 ± 0.43
18:4*n*-3	108.5 ± 11.9	17.3 ± 1.7
20:0	1.52 ± 0.40	0.21 ± 0.05
20:1	29.7 ± 3.4	4.51 ± 0.17
20:2*n*-6	3.02 ± 0.80	0.42 ± 0.10
20:3*n*-6	ND	ND
20:4*n*-6	20.4 ± 2.1	3.14 ± 0.19
20:3*n*-3	0.08 ± 0.08	0.01 ± 0.01
20:4*n*-3	1.97 ± 0.46	0.28 ± 0.06
20:5*n*-3	49.1 ± 5.8	7.59 ± 0.52
22:0	1.20 ± 0.48	0.17 ± 0.07
22:1	8.01 ± 1.19	1.21 ± 0.11
23:0	0.86 ± 0.27	0.12 ± 0.04
22:2*n*-6	ND	ND
22:4*n*-6	2.50 ± 0.59	0.36 ± 0.09
22:5*n*-6	0.60 ± 0.18	0.09 ± 0.03
22:5*n*-3	6.33 ± 0.55	1.02 ± 0.09
22:6*n*-3	35.3 ± 4.0	5.34 ± 0.22
24:0	ND	ND
24:1	2.77 ± 0.71	0.38 ± 0.09
Total	652.3 ± 63.8	100
Total saturates	189.5 ± 22.4	28.4 ± 1.0
Total monoenes	223.0 ± 24.6	33.9 ± 1.4
Total polyenes	241.2 ± 21.5	37.6 ± 1.7
n-3 Polyenes	205.0 ± 18.3	32.1 ± 1.6
n-6 Polyenes	36.3 ± 3.5	5.57 ± 0.29
n-3/*n*-6	5.83 ± 0.33	
Monoenes/polyenes	0.93 ± 0.08	
Unsaturation Index[1]	199.3 ± 5.9	
Mean chain length[2]	18.1 ± 0.01	

ND = not detectable.
[1] $\sum m_i n_i$, where m_i is the mole percentage and n_i is the number of carbon–carbon double bonds in the fatty acid$_i$.
[2] $\sum f_i c_i$, where f_i is the mole fraction and c_i is the number of carbon atoms of fatty acid$_i$.

Aside from two individual measurements of trace amounts of 13:1, NEFA shorter than C:14 were not detected. Polyenes were the dominant fatty acids detected, with stearidonic acid (18:4*n*-3) representing 45% of total polyenes and 17% of total NEFA. Eicosapentaenoic acid (EPA, 20:5*n*-3), docosahexaenoic acid (DHA, 22:6*n*-3), and arachidonic acid (AA, 20:4*n*-6) also were present in high concentrations, representing 20%, 15%, and 8.5% of total polyenes, respectively. Total monoenes were present at similar levels to total polyenes, and consisted principally of oleic acid (18:1), which was 63% of total monoenes and also the most abundant individual fatty acid overall (21.5% of total NEFA). Saturates were less abundant than unsaturates; the major saturate was palmitic acid (16:0), representing 55% of total saturates. The mean *n*-3/*n*-6 ratio was 5.83.

Seven unknown peaks were detected in all samples. One peak eluted immediately after 15:1 (RT = 15.18 min), one peak eluted immediately after 16:0 (RT = 15.64), one peak eluted between 16:1 and 17:0 (RT = 16.08), and the remaining four eluted consecutively between 17:1 and 18:0 (RT = 17.18, 17.43, 17.55, 17.73 min). Methyl ester standards of the branched-chain fatty acids phytanic acid, 16-methylheptadecanoic acid, and 14-methylpentadecanoic acid (Ultra Scientific, North Kingstown, RI), as well as pristanic acid (Sigma) that we methylated, were analyzed to determine if they explained any of the unknown peaks. None of the unknown peaks was phytanic acid, pristanic acid, or 16-methylheptadecanoic acid, but the peak at RT = 15.18 was identified based on RT to be 14-methylpentadecanoic acid (*iso*16:0). Its concentration was 42.3 ± 10.4 nmol ml^{-1} (5.85 ± 1.34% of identified fatty acids) (Table 2). The remaining unknown peaks accounted for a total of 21.0 ± 1.8% of the known plus unknown fatty acids by weight. The percentages given for identified fatty acids in the preceding paragraph do not take into account these unknown peaks.

Ketone body metabolism

The capacity for ketone body oxidation, as measured by the activity of D-β-HBDH which oxidizes D-β-hydroxybutyrate (D-β-HB) to acetoacetate in the mitochondria, was found in all tissues. Kidney, heart, and liver showed the highest levels of D-β-HBDH whereas white muscle possessed the lowest level. Activity of L-β-HBDH, which is specific for the laevo-rotary enantiomer of β-HB (L-β-HB), was undetectable in heart and

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white muscle and was low in liver and kidney, representing only 4% and 1% of the activity of D-β-HBDH, respectively. SKT, an enzyme that catalyzes the formation of acetoacetyl coenzyme A via transfer of coenzyme A from succinyl coenzyme A to acetoacetate, was found in all tissues with heart showing the highest level and other tissues showing similar low levels. ACoAT, an enzyme that catalyzes the cleavage of acetoacetyl coenzyme A to form acetyl coenzyme A, was detected in all tissues; kidney possessed the highest activity, liver and heart also had relatively high levels, and white muscle showed the lowest activity.

Glycolysis

Heart and kidney showed the highest levels of HK, an enzyme that phosphorylates exogenous glucose for glycolysis or glycogen synthesis, whereas liver and white muscle had low levels. PK, which catalyzes the conversion of phosphoenolpyruvate to pyruvate in the final step of glycolysis, was highest in white muscle and lowest in liver. LDH, the enzyme involved in the interconversion of pyruvate and lactate, was highest in heart and lowest in liver.

Gluconeogenesis

Gluconeogenic capacity as assessed by activity of FBPase, a gluconeogenic enzyme responsible for the conversion of glucose-6-phosphate to glucose, was highest in kidney and lowest in white muscle and heart.

Amino acid metabolism

GDH, an enzyme that catalyzes the oxidative deamination of glutamate to α-ketoglutarate, was most active in kidney and least active in white muscle. AlaAT, an enzyme that transaminates alanine to form pyruvate, also was highest in kidney and lowest in white muscle. AspAT, an enzyme that transaminates aspartate to produce oxaloacetate, had highest activity in heart and lowest in white muscle.

The levels of amino acids in plasma of spotted ratfish are provided in Table 3. The concentration of total plasma FAA was 719.7 nmol ml^{-1}. Essential and non-essential amino acids comprised 65.2% and 34.8% of the total FAA, respectively. The most common amino acids were valine (21.9% of total), isoleucine (13.1%), leucine (11.1%), and lysine (8.9%). Glutamine was detectable, but at low levels (2.94%).

TABLE 3. Absolute amounts (mean ± SEM) and percentages (mean ± SEM) of free amino acids in plasma from the spotted ratfish (Hydrolagus colliei) (n = 6)

Amino acid	Concentration (nmol ml^{-1})	Percentage (% by moles)
Aspartate	1.72 ± 0.33	0.23 ± 0.04
Glutamate	4.53 ± 0.85	0.60 ± 0.09
Asparagine	0.21 ± 0.05	0.03 ± 0.01
Serine	50.4 ± 9.8	6.28 ± 0.66
Glutamine	20.9 ± 1.1	2.94 ± 0.38
Glycine	13.7 ± 1.7	1.93 ± 0.27
Histidine	12.0 ± 1.8	1.82 ± 0.44
Threonine	23.1 ± 2.5	3.14 ± 0.29
Alanine	35.2 ± 6.9	4.55 ± 0.51
Arginine	42.1 ± 4.2	5.65 ± 0.37
Tyrosine	1.46 ± 0.46	0.19 ± 0.05
Valine	165.7 ± 15.9	22.0 ± 0.4
Methionine	14.7 ± 3.1	1.91 ± 0.25
Tryptophan	7.88 ± 1.05	1.06 ± 0.10
Phenylalanine	16.6 ± 1.0	2.28 ± 0.17
Isoleucine	101.7 ± 14.1	13.1 ± 0.7
Leucine	85.6 ± 11.3	11.1 ± 0.4
Lysine	65.1 ± 4.6	8.86 ± 0.63
Proline	14.5 ± 2.6	1.84 ± 0.17
Ornithine	40.9 ± 6.8	5.22 ± 0.38
Citrulline	1.13 ± 0.10	0.17 ± 0.03
GABA	39.7 ± 6.4	5.08 ± 0.34
Cystine	0.46 ± 0.06	0.06 ± 0.00
Total	719.7 ± 74.5	100
Total essential	492.4 ± 47.7	65.2 ± 0.5
Total non-essential	227.3 ± 27.3	34.8 ± 0.5

DISCUSSION

Lipid metabolism

Our salient finding is that spotted ratfish show the same pattern of lipid oxidation as elasmobranchs—namely, a low or non-existent reliance on lipid as fuel in muscle. CPT activity was non-detectable in heart, as in bowfin and some elasmobranchs but not teleosts (Sidell et al., '87; Singer and Ballantyne, '91). Other data show low CPT activities in heart from some elasmobranchs, suggesting some variability in lipid use by elasmobranch heart (Driedzic and De Almeida-Val, '96; J. Berges and J.S. Ballantyne, unpublished data, given in Ballantyne ('97); Speers-Roesch (2005)). Undetectable or very low CPT levels are normal in white muscle even for teleosts (Moyes et al., '89; Crockett and Sidell, '90). Spotted ratfish apparently do not possess significant discrete lateral line red muscle, which is not surprising given their sluggish life style, so we were unable to determine

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if this tissue lacks CPT activity like in elasmobranchs (Zammit and Newsholme, '79; Moyes et al., '90). The presence of detectable CPT in kidney from spotted ratfish indicates its capacity for lipid oxidation, as in bowfin and Arctic char (*Salvelinus alpinus*) (Singer and Ballantyne, '91; Bystriansky, 2005). Our finding is at odds with Singer and Ballantyne ('89), whose results suggested that elasmobranchs lack CPT in kidney (as do lake sturgeon (Singer et al., '90)). However, more recent work shows high CPT levels in kidney from little skate (J. Berges and J.S. Ballantyne, unpublished data given in Ballantyne ('97)), several tropical stingrays, and brownbanded bamboo shark (*Chiloscyllium punctatum*) (Speers-Roesch, 2005). The emerging picture is that elasmobranchs do not lack lipid oxidation in extrahepatic tissues per se, but rather do not rely on lipids as a metabolic fuel in muscle only. Our results for the spotted ratfish extend this model to the Chondrichthyes as a whole.

The four-fold higher activity of COT compared with CPT in the kidney, and the presence of COT and the absence of CPT in heart and white muscle may indicate a preference for medium-chain fatty acids during lipid oxidation in the spotted ratfish. These results suggest active peroxisomal β-oxidation and export to the mitochondria of carnitine esters of medium-chain fatty acids by COT in these tissues. Accordingly, heart mitochondria from *Squalus acanthias* oxidized octanoyl carnitine ester but not palmitoyl carnitine ester under isosmotic conditions (Moyes et al., '90). On the other hand, peroxisomal β-oxidation was not detectable in heart and red muscle from *H. colliei*, *S. acanthias*, or rainbow trout (Moyes et al., '90). Further studies are needed to examine the role of peroxisomal β-oxidation and COT in lipid metabolism in the Chondrichthyes.

The occurrence of appreciable levels of HOAD in the heart, while CPT is undetectable and COT is very low, is a paradox also seen in elasmobranchs. It is apparently not attributable to carnitine-independent fatty acid oxidation or peroxisomal β-oxidation (Moyes et al., '90). An alternate role of HOAD may be a possible explanation. Molecular studies of the sequence and expression of HOAD in muscle and other tissues from chondrichthyans may prove informative.

ME activities were nearly identical to those measured in little skate (Moon and Mommsen, '87). The high activity recorded from heart is probably attributable to the role of ME in amino acid metabolism rather than lipid synthesis (Chamberlin et al., '91).

Total plasma NEFA concentration in *H. colliei* is higher than in *Chimaera monstrosa* (Larsson and Fänge, '77). These authors used a colorimetric technique that probably underestimates NEFA concentration (Singer et al., '90). Our measurements indicate plasma NEFA concentration in spotted ratfish is $\geqslant 60\%$ higher than any elasmobranch examined by Ballantyne et al. ('93), but similar to that in little skate ($572.6 \pm 66.8\,\mathrm{nmol\,ml^{-1}}$) (Speers-Roesch, 2005). Plasma NEFA levels in spotted ratfish are about half of the lowest levels reported from marine teleosts by Ballantyne et al. ('93). Because spotted ratfish heart and white muscle show no indication of lipid oxidation, the NEFA levels likely suffice for the needs of the kidney. Supporting this contention, the level of plasma NEFA in spotted ratfish is similar to that found in the Florida gar (*Lepisosteus platyrhincus*) (N.T. Frick, J.S. Bystriansky, and J.S. Ballantyne, unpublished data) and bowfin (Singer and Ballantyne, '91), both "primitive" non-teleost actinopterygians that also show a limited capacity for lipid oxidation in extrahepatic tissues (see below).

The plasma NEFA concentration in spotted ratfish exceeds the solubility of fatty acids in water (Windholz, '83), indicating the presence of a plasma fatty acid carrier. Considering the similarity to elasmobranchs in enzyme and NEFA measurements, holocephalans may also lack an albumin capable of carrying NEFA. The absence of the more soluble and albumin-independent short- and medium-chain NEFA in plasma indicates that this is not an alternate form of lipid transport in spotted ratfish. Lipoproteins should be investigated as possible NEFA carriers in holocephalans, as occurs in spiny dogfish (Lauter et al., '67). Q2

Composition of plasma NEFA in spotted ratfish is similar to that reported in other marine fishes, with high levels of 18:1, 16:0, 16:1, 18:0, and long chain polyunsaturated fatty acids (EPA, 20:5*n*-3, DHA, 22:6*n*-3, and AA, 20:4*n*-6) (Ballantyne et al., '93). The predominant fatty acid was oleic acid (18:1), reflecting the high amounts of 18:1 in triglycerides and diacyl glyceryl ethers in liver and muscle of *Hydrolagus barbouri* and *H. novaezealandiae* (Hayashi and Takagi, '80; Hayashi et al., '83). High levels of 18:1 also characterize plasma NEFA of teleosts and elasmobranchs (Ballantyne et al., '93), bowfin (Singer and Ballantyne, '91), and sturgeon (Singer et al., '90), confirming its

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metabolic importance in fishes. One peculiarity of spotted ratfish NEFA is the very high levels of stearidonic acid (18:4*n*-3) (18% of total identified plasma NEFA). In elasmobranchs and other fishes, 18:4*n*-3 constitutes <3% of plasma NEFA (Singer et al., '90; Ballantyne et al., '93; Speers-Roesch, 2005). High levels of 18:4*n*-3 in plasma NEFA of spotted ratfish suggest substantial feeding within a food chain with dinoflagellates or haptophyte algae at its base, since 18:4*n*-3 is typical of these phytoplankters (Mansour et al., '99). The high levels of 20:5*n*-3, 22:6*n*-3, and other *n*-3 fatty acids and the resultant high *n*-3/*n*-6 ratio in spotted ratfish is similar to other carnivorous temperate marine fishes, which obtain high levels of *n*-3 fatty acids in their diet (Ballantyne et al., '93; Speers-Roesch, 2005).

The unidentified peaks seen in the NEFA analysis of spotted ratfish plasma deserve further attention. Our identification of one of the unknowns as 14-methylpentadecanoic acid (*iso*16:0) appears to be the first record of a branched-chain fatty acids in plasma NEFA from a fish. Branched-chain fatty acids, including *iso*16:0, are known from fish oils (Ackman, '89). *Iso*16:0 has been found to comprise 1.7% of fatty acids in alkyl glyceryl ethers in livers from holocephalans (Hayashi and Takagi, '80; Hayashi et al., '83), but the high level of *iso*16:0 in plasma NEFA from spotted ratfish is nonetheless unusual and should be verified by GC-MS as well as investigated further. The other unknowns in *H. colliei* plasma NEFA may represent other branched-chain fatty acids or C16 polyenic fatty acids (Ackman, '89).

Ketone body metabolism

An important finding of the present study is the high capacity for ketone body metabolism in aerobic tissues of spotted ratfish, as found in elasmobranchs but not other fishes (Ballantyne, '97). In particular, the high activities of D-β-HBDH contrast with the low activities of this enzyme in sturgeon, bowfin, and, especially, teleosts (Singer et al., '90; Singer and Ballantyne, '91; LeBlanc and Ballantyne, '93). Although levels of D-β-HBDH are higher in heart and kidney than previously reported for elasmobranchs (Moon and Mommsen, '87; Singer and Ballantyne, '89; Battersby et al., '96; Treberg et al., 2003), this may be due to the usage of D/L-β-HB in two of these studies, which may have resulted in inhibition by L-β-HB (Stuart and Ballantyne, '97). Zammit and Newsholme ('79), using D-β-HB, found D-β-HBDH activities in elasmobranch hearts that are very similar to that found in heart from spotted ratfish. SKT and ACoAT levels were similar to those in elasmobranchs and ACoAT was similar to levels in teleosts, bowfin, and sturgeon (Zammit and Newsholme, '79; Beis et al., '80; Moon and Mommsen, '87; Singer et al., '90; Singer and Ballantyne, '91; J. Berges and J.S. Ballantyne, unpublished data presented in Ballantyne, '97). These similarities in activities of SKT and ACoAT suggest that the role of acetoacetate as an oxidative fuel is fairly conserved among fishes; it is the high activities of D-β-HBDH that makes ketone body metabolism in elasmobranchs, and holocephalans, so unique. Supporting this contention, acetoacetate levels in plasma from Atlantic salmon (*Salmo salar*) are similar to levels found in spiny dogfish and *Scyliorhinus canicula* whereas plasma β-HB is higher in the elasmobranchs (Conlon et al., '94; Soengas et al., '96; Richards et al., 2003). Levels of ketone bodies in plasma of holocephalans should be measured to ascertain if their profile matches that seen in elasmobranchs.

The undetectable or low activities of L-β-HBDH compared with the high activities of D-β-HBDH indicate that in ratfish the D-stereoisomer of β-HB is preferred, as is the case in mammals (Webber and Edmond, '77) and elasmobranchs (Speers-Roesch, 2005).

Amino acid metabolism

Although the total amount of FAA in plasma (719.7 nmol ml^{-1}) was lower than in *Callorhinchus milii*, elasmobranchs, and teleosts (~2,000–3,500 nmol ml^{-1}; Bedford, '83; Gutierrez et al., '87; Barton et al., '95), the high activities of GDH, AlaAT, and AspAT indicate that amino acids are utilized extensively as oxidative substrates in spotted ratfish. The levels of these enzymes were similar to those found in elasmobranchs, for which amino acids are thought to be important oxidative fuels due to a reduced reliance on lipids (Ballantyne, '97). The same appears to be the case in the spotted ratfish.

Although recently it has been suggested that low plasma glutamine levels are not a universal trait of elasmobranchs (Ballantyne, 2001), glutamine levels in spotted ratfish were similarly low as levels in spiny dogfish (Chamberlin and Ballantyne, '92) and little skate (Boyd et al., '77). Whether, as in elasmobranchs (Ballantyne, '97), glutamine is important in holocephalans as an oxidative substrate needs to be determined. The

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proportions of ornithine and citrulline, two main intermediates in the ornithine–urea cycle, as well as the major constituents of plasma FAA were similar to that found in *Callorhinchus milii* and elasmobranchs (Boyd et al., '77; Bedford, '83; Gutierrez et al., '87). Data on plasma levels of γ-aminobutryic acid in fishes are sparse, but the levels in spotted ratfish are similar to that found in Pacific hagfish (*Eptatretus stouti*) (Fincham et al., '90).

Other metabolic pathways

The levels of enzymes of aerobic metabolism (CS and CCO) and gluconeogenesis (FBPase) were similar to those measured previously in other fishes including *H. colliei*, elasmobranchs, and actinopterygians (Moon and Mommsen, '87; Ritter et al., '87; Blier and Guderley, '88; Singer et al., '90; Dickson et al., '93; Battersby et al., '96; Treberg et al., 2003). Comparisons of HK activity between different studies are complicated by this enzyme's freeze unstability (Sidell et al., '87). However, several fold higher HK activity in kidney and heart of spotted ratfish compared with freeze-thawed samples from bowfin, lake sturgeon, and Arctic char, as well as fresh little skate tissues (Moon and Mommsen, '87; Singer et al., '90; Singer and Ballantyne, '91; Bystriansky, 2005) suggest that in this species these tissues rely more on glucose as a metabolic fuel than in other fishes.

Spotted ratfish showed lower PK in all tissues compared with teleosts (Sidell et al., '87; Blier and Guderley, '88) and shallow-water elasmobranchs (Moon and Mommsen, '87; Dickson et al., '93; Treberg et al., 2003); the levels were similar to that found in the deep-sea squaloid *Centroscyllium fabricii* (Treberg et al., 2003). LDH in white muscle was five–eight-fold lower than in little skate or spiny dogfish, but similar to *Centroscyllium fabricii* (Moon and Mommsen, '87; Treberg et al., 2003). These reduced enzyme activities may reflect adaptation to a deep-sea environment. Although, unlike many holocephalans, the spotted ratfish is not exclusively a deep-sea inhabitant, it is commonly found to depths of 900 m and is characteristically sluggish (Eschmeyer et al., '83). It is well known that deep-sea fishes show adaptations in white muscle biochemistry that reflect their nutrient-poor environment and reduced locomotory capacities, including reduced PK and LDH activities (Somero, '82). On the other hand, Childress and Somero ('79) argued that depth-related influences on heart LDH in fishes are minimal and the similarity in heart LDH between spotted ratfish (present study), *S. acanthias*, and *Centroscyllium fabricii* (Treberg et al., 2003) support this contention. The similarity noted above of white muscle CS (and CCO) activities with that of shallow-water elasmobranchs and teleosts matches the lack of a consistent decrease in white muscle CS with increasing depth in teleosts (Somero, '82).

Evolutionary considerations

The spotted ratfish, a representative holocephalan, possesses a metabolic organization similar to that of an elasmobranch. The absence of substantial lipid oxidation in muscle and a high reliance on ketone bodies as metabolic fuel can now be considered a general metabolic attribute of the Chondrichthyes. From an evolutionary perspective, this is a fascinating discovery because it strongly suggests that this metabolic organization was present in the last common ancestor of all chondrichthyan fishes, which swam in the oceans over 360 Mya but is poorly known from the fossil record (Grogan and Lund, 2004). It also indicates that this pattern goes back almost to the origin of the jawed vertebrates.

The presence of a substantial capacity for lipid oxidation in the kidney—an extrahepatic tissue—is not consistent with the urea hypothesis. However, lipid delivery to this tissue presumably can be served by the available plasma NEFA and these may be carried by a non-albumin fraction as appears to be the case in elasmobranchs, which show a similar capacity for lipid oxidation in the kidney (Speers-Roesch, 2005). The lack of a capacity for lipid oxidation in heart in spotted ratfish, and low or non-existent lipid oxidation in heart and red muscle from elasmobranchs, may reflect urea's perturbing effects on fatty acid transport by albumin. Whether or not the urea hypothesis is true, the question of why fatty acid oxidation was attenuated in muscle and not also in other extrahepatic tissues deserves attention. Investigations of the lipid metabolism of other urea-retaining vertebrates, including the coelacanth (*Latimeria chalumnae*) and crab-eating frog (*Rana cancrivora*), may be useful in further testing the urea hypothesis.

Although the urea hypothesis remains an attractive explanation for the metabolic organization of chondrichthyans, the lessened role of lipids as a metabolic fuel in muscle in chondrichthyans may reflect an ancestral metabolic characteristic

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among fishes. Supporting this view, the concentrations of plasma NEFA in spotted ratfish ($652.3 \pm 63.8\,nmol\,ml^{-1}$) and little skate ($572.6 \pm 66.8\,nmol\,ml^{-1}$; Speers-Roesch, 2005) are similar to that found in primitive non-urea-retaining fishes, including the ancient, jawless hagfish (*Myxine glutinosa*) ($550.5\,nmol\,ml^{-1}$; P.J. LeBlanc, C. Hyndman, and J.S. Ballantyne, unpublished data) as well as the ''primitive'' ray-finned fishes the bowfin ($758 \pm 110\,nmol\,ml^{-1}$; Singer and Ballantyne, '91) and Florida gar ($606.6 \pm 27.7\,nmol\,ml^{-1}$; N.T. Frick, J.S. Bystriansky, and J.S. Ballantyne, unpublished data), all of which have levels lower than in teleosts ($\geqslant 1{,}183\,nmol\,ml^{-1}$; Ballantyne et al., '93). Bowfin and Florida gar, as well as lake sturgeon (Singer et al., '90), also possess little or no CPT in heart, red muscle, and kidney when compared with teleosts (Singer and Ballantyne, '91; N.T. Frick, J.S. Bystriansky, and J.S. Ballantyne, unpublished data). The ratio of CPT activity to PK activity indicates the relative importance of lipid catabo-

TABLE 4. Ratios of CPT/PK in liver, kidney, and heart of representative chondrichthyans, teleosts, a neopterygian (a ''primitive'' ray-finned fish), and a myxine (a ''primitive'' jawless fish)

	Liver	Kidney	Heart
Chondrichthyes			
Hydrolagus colliei[1]	0.212 ± 0.060	0.011 ± 0.001	0 ± 0
Potamotrygon motoro[2]	0.017	0.002	0.00036
Himantura signifer[2]	0.049	0.003	0.00095
Taeniura lymma[2]	0.028	0.022	0.00083
Chiloscyllium punctatum[2]	0.149	0.020	0.00012
Potamotrygon hystrix[3]	—	—	0.008
Leucoraja erinacea[4]	0.020	0.016	0.002, 0[5]
Squalus acanthias[5]	—	—	0
Mean ± SEM	0.079 ± 0.033	0.012 ± 0.004	0.0014 ± 0.0009
Teleostei			
Lophius piscatorius[5]	—	—	0.013
Scomber scombrus[5]	—	—	0.008
Gaidropsarus vulgaris[5]	—	—	0.006
Morone saxatilis[5]	—	—	0.017
Dicentrarchus labrax[5]	—	—	0.013
Zoarces americanus[6]	—	—	0.008
Makaira nigricans[7]	—	—	0.004
Salvelinus alpinus[8]	0.070	0.011	0.011
Gadus morhua[9]	—	—	0.007
Mean ± SEM	0.070	0.011	0.0096 ± 0.0014
Neopterygii			
Lepisosteus platyrhinchus[10]	0.013	—	0.004
Myxini			
Myxine glutinosa	0.091[11]	—	0.002[9]
Mean teleostei/mean chondrichthyes	0.9	0.9	7.1
Neopterygii/mean chondrichthyes	0.2	—	3
Myxini/mean chondrichthyes	1.2	—	1.5

Per gram wet weight activities were used in ratio calculations. A zero indicates that non-detectable CPT activity was found. For mean calculation multiple values for one tissue in a species were first averaged. References for data are marked with letters and provided below with temperature at which measurements were made. Sample sizes for *H. colliei* are six for all tissues except for kidney ($n = 5$).

[1]Present study, 12°C.
[2]Speers-Roesch (2005), 25°C.
[3]Driedzic and De Almeida-Val (1996), 25°C.
[4]PK values: Moon and Mommsen (1987); CPT values: J. Berges and J.S. Ballantyne, unpublished data (see Ballantyne, '97), both 10°C.
[5]Sidell et al. ('87), 15°C.
[6]Driedzic and Stewart ('82), 10°C.
[7]Suarez et al. ('86), 25°C.
[8]Bystriansky (2005), 10°C.
[9]Hansen and Sidell ('83), 15°C.
[10]N.T. Frick, J.S. Bystriansky, and J.S. Ballantyne, unpublished data, 25°C.
[11]Leary et al. ('97), 10°C.

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lism and glycolysis for energy metabolism. The ratio of CPT to PK in heart of chondrichthyans is similar to that in hagfish, about three times lower than in the Florida gar (a ''primitive'' ray-finned fish), and seven-fold lower than in teleosts (Table 4). A similar pattern emerges when comparing mean heart CPT activities from chondrichthyans (including those species listed in Table 4) with those of: representative teleosts (including those species listed in Table 4); ''primitive'' ray-finned fishes (bowfin (Singer and Ballantyne, '91), lake sturgeon (Singer et al., '90), and Florida gar); and hagfishes (Pacific hagfish (Moyes et al., '90) and *M. glutinosa*). Chondrichthyans and hagfish possess similar levels of heart CPT, teleosts possess approximately nine-fold higher levels, and ''primitive'' ray-finned fishes possess intermediate levels (Speers-Roesch, 2005). These comparisons of CPT/PK ratios and absolute CPT levels in heart suggest that the capacity for lipid oxidation in heart of chondrichthyans is more similar to the ancient hagfishes than to the derived teleosts, and support Singer and Ballantyne's ('91) contention that certain ''primitive'' ray-finned fishes show a capacity for fatty acid oxidation that is intermediate between chondrichthyans and teleosts. In kidney, however, the CPT/PK ratio is similar between chondrichthyans and Arctic char, a teleost, and in liver there are no clear relationships to evolutionary position (Table 4), suggesting that major phylogenetic differences in lipid oxidation among fishes are restricted to muscle. Heart of ''primitive'' fishes may rely more heavily on carbohydrate-based metabolism (Sidell, '83), and the same may be true for red muscle. The ratios of CPT/PK and the CPT values provided in the present study support this idea. It is worth noting that the lampreys may be an exception to this general idea, as they rely extensively on fatty acid oxidation and possess high levels of CPT in muscle (Power et al., '93; LeBlanc et al., '95). However, this emphasis on fatty acid oxidation in muscle may have evolved independently as an adaptation to their complex migratory life history.

Although the lower utilization of fatty acids as oxidative fuels in chondrichthyan muscle may in part reflect an ancestral characteristic of the fishes, there is a clear preference for ketone bodies rather than lipids as oxidative substrates. Singer and Ballantyne ('91) suggested that ketone body usage is a primitive feature in fishes. The capability to use ketone bodies among fishes certainly is ancient, as muscle mitochondria from lamprey readily oxidize β-HB (LeBlanc et al., '95), but no group has relied so heavily on it as have the chondrichthyans. An emphasis on ketone bodies as a major source of acetyl CoA in skeletal muscle and heart was probably needed, in the absence of significant lipid oxidation, to enable the active predatory lifestyle for which many chondrichthyans are notorious. Teleosts, on the other hand, adopted lipids as a high-energy oxidative fuel. Although it is possible that the emphasis on ketone bodies in chondrichthyans appeared in response to reduced fatty acid oxidation in muscle due to impaired plasma fatty acid transport (as postulated by the urea hypothesis), it may be that the implementation of ketone bodies as a major fuel in chondrichthyan muscle preceded and then caused the de-emphasis on fatty acid oxidation. Our shows that this metabolic reorganization must have occurred early in chondrichthyan evolution and close to the origin of jawed vertebrates.

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Q3	Beis et al., '80 not found in ref list. Pl. check.	
Q4	Fellows et al., 1980 not cited in the text. Pl. check.	
Q5	Henderson and Tocher, 1987 is not cited in the text. Pl. check.	
Q6	Suarez and Mommsen 1987 is not cited in the text. Pl. check.	
Q7	West et al., 1999 is not cited in the text. Pl. check.	