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Metabolic organization of freshwater, euryhaline, and marine elasmobranchs: implications for the evolution of energy metabolism in sharks and rays

B. Speers-Roesch[1], Y. K. Ip[2] and J. S. Ballantyne[1,*]

[1]*Department of Integrative Biology, University of Guelph, Guelph, Ontario, NIG 2W1, Canada and* [2]*Department of Biological Science, National University of Singapore, Kent Ridge, Singapore 117543, Republic of Singapore*

*Author for correspondence (e-mail: jballant@uoguelph.ca)

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Summary

To test the hypothesis that the preference for ketone bodies rather than lipids as oxidative fuel in elasmobranchs evolved in response to the appearance of urea-based osmoregulation, we measured total non-esterified fatty acids (NEFA) in plasma as well as maximal activities of enzymes of intermediary metabolism in tissues from marine and freshwater elasmobranchs, including: the river stingray *Potamotrygon motoro* (<1 mmol l^{-1} plasma urea); the marine stingray *Taeniura lymma*, and the marine shark *Chiloscyllium punctatum* (>300 mmol l^{-1} plasma urea); and the euryhaline freshwater stingray *Himantura signifer*, which possesses intermediate levels of urea. *H. signifer* also were acclimated to half-strength seawater (15‰) for 2 weeks to ascertain the metabolic effects of the higher urea level that results from salinity acclimation. Our results do not support the urea hypothesis. Enzyme activities and plasma NEFA in salinity-challenged *H. signifer* were largely unchanged from the freshwater controls, and the freshwater elasmobranchs did not show an enhanced capacity for extrahepatic lipid oxidation relative to the marine species. Importantly, and contrary to previous studies, extrahepatic lipid oxidation does occur in elasmobranchs, based on high carnitine palmitoyl transferase (CPT) activities in kidney and rectal gland. Heart CPT in the stingrays was detectable but low, indicating some capacity for lipid oxidation. CPT was undetectable in red muscle, and almost undetectable in heart, from *C. punctatum* as well as in white muscle from *T. lymma*. We propose a revised model of tissue-specific lipid oxidation in elasmobranchs, with high levels in liver, kidney and rectal gland, low or undetectable levels in heart, and none in red or white muscle. Plasma NEFA levels were low in all species, as previously noted in elasmobranchs. D-β-hydroxybutyrate dehydrogenase (D-β-HBDH) was high in most tissues confirming the importance of ketone bodies in elasmobranchs. However, very low D-β-HBDH in kidney from *T. lymma* indicates that interspecific variability in ketone body utilization occurs. A negative relationship was observed across species between liver glutamate dehydrogenase activity and tissue or plasma urea levels, suggesting that glutamate is preferentially deaminated in freshwater elasmobranchs because it does not need to be shunted to urea production as in marine elasmobranchs.

Key words: elasmobranch, freshwater, marine, salinity, intermediary metabolism, enzyme, ketone body, lipid, non-esterified fatty acid, urea, evolution, *Potamotrygon motoro*, *Himantura signifer*, *Taeniura lymma*, *Chiloscyllium punctatum.*

Introduction

The metabolism of marine elasmobranch fishes differs from teleosts and virtually all other vertebrates in several ways, including a low capacity for extrahepatic lipid oxidation and an increased reliance on ketone bodies (especially β-hydroxybutyrate) as oxidative substrates (Ballantyne, 1997). This situation is reflected by: (1) low or undetectable extrahepatic activities of enzymes involved in fatty acid oxidation (Zammit and Newsholme, 1979; Moon and Mommsen, 1987; Sidell et al., 1987; Moyes et al., 1990; Watson and Dickson, 2001); (2) high extrahepatic activities of enzymes of ketone body catabolism (Zammit and Newsholme, 1979; Beis et al., 1980; Moon and Mommsen, 1987; Watson and Dickson, 2001); (3) low or non-detectable oxidation rates of fatty acids and high oxidation rates of ketone bodies by isolated mitochondria from extrahepatic tissues (Moyes et al., 1990; Ballantyne et al., 1992; Chamberlin and Ballantyne, 1992); and (4) low levels of plasma non-esterified fatty acids (NEFA) (Larsson and Fänge, 1977; Fellows et al., 1980; Ballantyne et al., 1993) which has been attributed to the functional absence of the fatty acid binding protein albumin (Fellows et al., 1980; Fellows and Hird, 1981; Ballantyne, 1997).

It has been suggested that the pattern of extrahepatic energy

metabolism seen in elasmobranchs is a consequence of their osmotic strategy, which involves the retention of high levels of urea as well as methylamines (Ballantyne and Moon, 1986; Ballantyne et al., 1987). Urea disrupts hydrophobic interactions, which are needed for proper structure and function of proteins (Wetlaufer et al., 1964; Yancey et al., 1982). Because hydrophobic interactions are primarily responsible for fatty acid binding to albumin (Peters, Jr, 1996), transport of fatty acids by albumin in elasmobranchs may be compromised. The evolution of urea-based osmoregulation in elasmobranchs may thus have been associated with the development of an extrahepatic metabolic organization that de-emphasized fatty acids in favour of highly soluble ketone bodies and amino acids, which do not require special carriers for transportation around the body (Ballantyne and Moon, 1986; Ballantyne et al., 1987). This proposition will hereafter be referred to as the 'urea hypothesis'.

Freshwater elasmobranchs provide optimal models to test the urea hypothesis because they possess low levels of urea as a result of evolutionary adaptation to a hypotonic environment (Thorson et al., 1967; Tam et al., 2003). If, as posited by the urea hypothesis, high urea levels are a constraint on the ability of marine elasmobranchs to transport and oxidize fatty acids extrahepatically, perhaps freshwater elasmobranchs with their lower urea content have re-emphasized lipids as an extrahepatic metabolic fuel and possibly even consequently reduced the importance of ketone bodies and amino acids.

Two previous studies have touched upon this approach by examining the enzymatic capacity for lipid oxidation in extrahepatic tissues from the obligate freshwater Amazon river stingrays *Potamotrygon* spp., which possess virtually no urea (Thorson et al., 1967; Griffith et al., 1973; Tam et al., 2003). Unfortunately, the findings are contradictory. Driedzic and De Almeida-Val measured high levels of carnitine palmitoyl transferase (CPT), an enzyme essential in facilitating the transport of fatty acids into the mitochondria, in heart from wild-caught adult *Potamotrygon hystrix* (Driedzic and De Almeida-Val, 1996). Previously, however, Singer and Ballantyne detected very low to undetectable levels of enzymes involved in fatty acid oxidation (including CPT) in tissues from captive-bred juvenile *Potamotrygon magdalenae* (Singer and Ballantyne, 1989).

In the present study, we attempt to resolve this disagreement and more thoroughly test the urea hypothesis by examining the metabolic organization of the obligate freshwater Amazonian ocellate river stingray (*Potamotrygon motoro*; Rajiformes: Potamotrygonidae), the euryhaline white-edge whip ray (*Himantura signifer*; Rajiformes: Dasyatidae) found in Southeast Asian rivers and estuaries, the tropical marine blue-spotted ribbontail stingray (*Taeniura lymma*; Rajiformes: Dasyatidae), and the tropical marine brownbanded bamboo shark (*Chiloscyllium punctatum*; Orectolobiformes: Hemiscyllidae). These four species provide particularly good models to evaluate the urea hypothesis because they are phylogenetically diverse and possess similar activity levels and diets while providing a wide interspecific 'urea gradient' that, if the urea hypothesis is correct, might be associated with varying capacities for lipid oxidation. *P. motoro* possesses virtually no urea (Thorson et al., 1967; Griffith et al., 1973; Tam et al., 2003). *H. signifer* in freshwater contain low levels of urea (e.g. plasma=44 mmol l^{-1}), and, whereas *Potamotrygon* spp. have lost the ability to synthesize urea for osmoregulation, *H. signifer* actively synthesizes urea when placed in brackish water (e.g. 20‰) leading to an approximate doubling of urea content (e.g. plasma=83 mmol l^{-1}) (Tam et al., 2003). Finally, *T. lymma* and *C. punctatum* retain much higher levels of urea in plasma (>300 mmol l^{-1}) and tissues (>200 mmol l^{-1}) (Treberg et al., 2006) (Y.K.I., unpublished), a characteristic of marine elasmobranchs. We have exploited this interspecies urea gradient to test the urea hypothesis, by measuring the activities of marker enzymes of fatty acid and ketone body oxidation in tissues from these four species including *H. signifer* in freshwater and acclimated to half-strength seawater. Also, we present the first measurements in freshwater or tropical elasmobranchs of total plasma NEFA, the most metabolically dynamic fraction of lipid in vertebrate blood and an indicator of the importance of lipid as a metabolic fuel (Henderson and Tocher, 1987). To indicate the relative importance of amino acids and carbohydrates as metabolic fuels in comparison with lipids and ketone bodies, we have also measured glutamate dehydrogenase (GDH) and pyruvate kinase (PK). Finally, we measured cytochrome *c* oxidase (CCO), an enzyme of aerobic metabolism, to ensure that major differences in enzyme activities were not simply an artefact of differing aerobic status.

Materials and methods

Animals

Potamotrygon motoro (Müller and Henle, 1841) (freshwater; 69±4 g body mass, mean ± s.e.m.) and *Himantura signifer* (Compagno and Roberts, 1982) (euryhaline; 139±36 g) were obtained from a fish farm in Singapore, where they were fed bloodworms (Chironomidae); *Taeniura lymma* (Forskål 1775) (marine; 480±33 g) and *Chiloscyllium punctatum* (Müller and Henle, 1838) (marine; 1988±221 g) were purchased at a live fish market in Singapore about 6 h after being caught in the ocean. All species were of mixed sex. Holding conditions were as described by Tam et al. (Tam et al., 2003). Briefly, animals were held at 25°C in plastic aquaria (about three animals per aquarium) at the National University of Singapore under a 12 h:12 h L:D photoperiod. *T. lymma* and *C. punctatum* were held in full-strength seawater (30–32‰) and *P. motoro* and *H. signifer* were kept in freshwater (0.7‰). Water was changed daily and the animals were not fed. *P. motoro* and *T. lymma* were sampled within 3 days of acquisition; *C. punctatum* were sampled the day they were obtained. Three days after specimen acquisition, a group of *H. signifer* was gradually acclimated to half-strength seawater (15‰) over 2 weeks with the following regime: 0.7‰ (day 1) to 5‰ (day 2) to 10‰ (day 3) to 15‰ (day 4, held until day 14). A control group was kept in freshwater. Both the

control and experimental groups comprised animals in several separate aquaria to avoid tank effects. Animals were not fed for the duration of the experiment. Freshwater and salinity-challenged animals were sacrificed after 14 days of acclimation. The salinity acclimated animals possessed white muscle urea levels that were significantly elevated compared with the freshwater controls (see Treberg et al., 2006).

Tissue sampling and preparation

Animals were sacrificed by rapidly severing the spinal cord and tissues (liver, heart, kidney) were excised within 5 min and frozen in liquid nitrogen for transport back to the University of Guelph, Canada, where the samples were stored at –80°C until use within 6 months of freezing. Rectal gland and white muscle were sampled from *T. lymma* in the same manner, as was rectal gland and red muscle from *C. punctatum*. The white muscle in *T. lymma* was taken at an epaxial location near the spinal column behind the head. Red muscle in *C. punctatum* was taken laterally at the caudal keel.

Tissues were thawed on ice and placed in 10 volumes of ice-cold homogenization buffer [50 mmol l^{-1} imidazole, 1 mmol l^{-1} dipotassium ethylene diaminetetraacetic acid (EDTA), pH 7.4 at 20°C]. Homogenization was completed on ice using a Polytron PT1200 unit set at high speed (25 000 rpm) for three passes of 10 s with 30 s 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 CCO, 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 little skate (*Leucoraja erinacea*) and other elasmobranchs when compared with homogenates that were untreated, sonicated, or treated with 0.2% Triton X-100 (TX100) (B.S.-R. and J.S.B., unpublished; J. R. Treberg, unpublished). This compares well with mammalian studies, which suggest that TX100 is too strong a detergent for use in CPT assays, because it inactivates CPT I; Tween 20 solubilizes CPT II in active form while leaving CPT I membrane-bound and catalytically functional (Woeltje et al., 1987; Zierz et al., 1993). Although TX100 gave higher activity of CCO than 0.5% Tween 20, it was not possible to prepare a separate TX100-treated sample for CCO assays because of the small size of tissue samples. 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., 1997; Lucassen et al., 2003). Notably, TX100 and Tween 20 resulted in near complete loss of D-β-HBDH activity, a result also found in little skate (B.S.-R. and J.S.B., unpublished).

Enzyme assays

Maximal enzyme activities were measured in duplicate using a Cary 300 Bio UV–visible spectrophotometer (Varian Inc., Palo Alto, CA, USA) equipped with a thermostated cell changer maintained at 25°C (±0.1°C) with a Cary Temperature Controller (Varian Inc., Palo Alto, CA, USA). Activities of 3-hydroxyacyl CoA dehydrogenase (HOAD), GDH, PK and D-β-hydroxybutyrate dehydrogenase (D-β-HBDH) were ascertained by measuring the oxidation or reduction of pyridine nucleotides at 340 nm (millimolar extinction coefficient ϵ_{340}, 6.22). CPT and COT were monitored at 412 nm using 5,5′-dithiobis 2-nitrobenzoic acid (DTNB) (millimolar extinction coefficient ϵ_{412}, 13.6). CCO activity was measured at 550 nm (millimolar extinction coefficient ϵ_{550}, 18.5).

Conditions of saturating substrate were used and linearity with protein was ensured. CPT, COT, HOAD and GDH were measured following published procedures (Singer and Ballantyne, 1989); the PK assay was modified from Moon and Mommsen (Moon and Mommsen, 1987) and Driedzic and De Almeida-Val (Driedzic and De Almeida-Val, 1996); CCO was measured using the method of Blier and Guderley (Blier and Guderley, 1988); and D-β-HBDH was measured following LeBlanc and Ballantyne (LeBlanc and Ballantyne, 2000). Conditions were as follows.

Enzyme of aerobic metabolism

Cytochrome *c* oxidase (CCO; E.C. 1.9.3.1): 50 mmol l^{-1} imidazole, pH 8.0, at 20°C, 0.05 mmol l^{-1}; cytochrome *c* (omitted for control).

Enzymes of lipid catabolism

Carnitine palmitoyl transferase (CPT; E.C. 2.3.1.21): 50 mmol l^{-1} imidazole, pH 8.0, at 20°C, 0.2 mmol l^{-1} DTNB, 0.1 mmol l^{-1} palmitoyl CoA; 5 mmol l^{-1} L-carnitine (omitted for control); carnitine octanoyl transferase (COT; E.C. 2.3.1.137): 50 mmol l^{-1} imidazole, pH 8.0, at 20°C, 0.2 mmol l^{-1} DTNB, 0.1 mmol l^{-1} octanoyl CoA, 5 mmol l^{-1}; L-carnitine (omitted for control); 3-hydroxyacyl CoA dehydrogenase (HOAD; E.C. 1.1.1.35): 50 mmol l^{-1} imidazole, pH 8.0, at 20°C, 0.1 mmol l^{-1} NADH, 1 mmol l^{-1} KCN, 0.1 mmol l^{-1} acetoacetyl CoA (omitted for control).

Enzyme of ketone body metabolism

D-β-hydroxybutyrate dehydrogenase (D-β-HBDH; E.C. 1.1.1.30): 50 mmol l^{-1} imidazole, pH 8.0, at 20°C, 11.25 mmol l^{-1} NAD, 50 mmol l^{-1} nicotinamide, 2 mmol l^{-1} dithiothreitol (DTT), 25 mmol l^{-1} D-β-hydroxybutyrate (omitted for control).

Enzyme of glycolysis

Pyruvate kinase (PK; E.C. 2.7.1.40): 50 mmol l^{-1} imidazole, pH 7.4, at 20°C, 0.15 mmol l^{-1} NADH, 5 mmol l^{-1} ADP, 10 mmol l^{-1} $MgCl_2$, 50 mmol l^{-1} KCl, 1 mmol l^{-1} KCN, excess LDH, 5 mmol l^{-1} phosphoenolpyruvate (omitted for control).

Enzyme of amino acid metabolism

Glutamate dehydrogenase (GDH; E.C. 1.4.1.3): 50 mmol l^{-1} imidazole, pH 8.0, at 20°C, 250 mmol l^{-1} ammonium acetate,

0.1 mmol l^{-1} dipotassium EDTA, 0.1 mmol l^{-1} NADH, 1 mmol l^{-1} ADP, 1 mmol l^{-1} KCN, 14 mmol l^{-1} α-ketoglutarate (omitted for control).

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

Measurement of plasma nonesterified fatty acids

Blood was drawn into heparinized syringes by cardiac puncture before sacrificing the fish. Blood was centrifuged at 4°C to obtain plasma. Plasma was frozen in liquid nitrogen for transport back to the University of Guelph, where it was stored at –80°C until use. Plasma nonesterified fatty acids (NEFA) were methylated as described by Singer et al. (Singer et al., 1990). The methyl esters were redissolved in 25 μl of carbon disulfide and 1–3 μl were injected into a gas chromatograph (6890N, Agilent Technologies, Palo Alto, CA, USA) fitted with a flame ionization detector and an automatic injector. Methyl esters were separated on a DB-23 column (J&W Scientific, Folsom, CA, USA). 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. Total plasma NEFA was calculated by summing the absolute amounts of individual fatty acids, which were identified based on retention times of known standards (GLC 463 augmented with 22:5*n*-6 and 23:0, Nu-Check Prep, Elysian, MN, USA) and quantified by comparison to a known amount (15 μg) of an internal standard, heptadecanoic acid (17:0), which was added to each plasma sample prior to methylation. Preliminary analyses showed only trace levels of endogenous 17:0.

Statistical analysis

Activities of each enzyme were compared between species for each tissue and between tissues of each species using one-way ANOVA with Tukey's test. Concentrations of total NEFA were compared between species using one-way ANOVA with Tukey's test. Data was log transformed prior to ANOVA if unequal variances were found (Zar, 1999). Additionally, comparisons of enzyme activities and total NEFA concentration were made between *H. signifer* in freshwater and *H. signifer* in half-strength seawater using a Student's *t*-test because the acclimation to seawater can be treated as a separate experiment among the between-species comparison. Levels of COT and CPT were compared within tissues using a Student's *t*-test.

During the course of the study it became apparent that there was a consistent relationship between GDH activity in liver and urea content in plasma, liver or white muscle across species. Thus, mean and individual GDH activities in liver were regressed against mean and individual liver and white muscle urea contents of the animals used in the present study (from Treberg et al., 2006).

All analyses were run on SigmaStat (SPSS Inc, Chicago, IL, USA). Significance was accepted at $P<0.05$.

Results

The levels of key enzymes of several metabolic pathways indicated the relative importance and capacities for utilization of different metabolic fuels in marine, euryhaline and freshwater elasmobranchs (Table 1, Table 2). For species and tissue comparisons, focus will be directed at significant twofold or greater differences and comparisons most germane to testing the urea hypothesis; full statistical and enzyme results are presented in Table 1 and Table 2.

CCO as a control for oxidative capacity

CCO, an indicator of the potential for aerobic metabolism, provided a control for possible differences in aerobic status between tissues and, especially, between species when comparing the activities of mitochondrial enzymes such as CPT. We found that in the stingrays, tissue-specific CCO activities did not vary greatly between species (Tables 1 and 2), so relative aerobic status probably has little effect on our interpretations of other aerobic enzymes. CCO was higher in each tissue from *C. punctatum* compared with the stingrays. We have noted below where CCO levels may help explain enzyme data in certain species.

Lipid metabolism: evidence for extrahepatic lipid oxidation and transport

Our measurements of CPT, a mitochondrial enzyme that catalyzes the rate-limiting step in carnitine-dependent oxidation of long-chain fatty acids (McGarry and Brown, 1997), show that the capacity for fatty acid oxidation exists in certain extrahepatic tissues, particularly kidney and rectal gland, of elasmobranchs. CPT was detected in all tissues from all species with the exception of white muscle in *T. lymma* (marine) and red muscle in *C. punctatum* (marine) (Fig. 1). In *P. motoro* (freshwater) and *H. signifer* (both salinities), the highest levels of CPT (0.27–0.77 U g^{-1} wet mass) were found in liver, relatively high levels (0.13–0.17 U g^{-1} wet mass) were found in kidney, and significantly lower but readily detectable levels were found in heart (0.03–0.09 U g^{-1} wet mass) (Fig. 1; Table 1). The same was true in *C. punctatum*, except heart CPT was at the limit of detection and in some individuals was undetectable. CPT levels in *T. lymma* were similar to the other rays, but its kidney had significantly higher activity than in the other species or tissues: 8–10 times greater than that found in kidney of the other species and over twofold greater than that found in liver of *T. lymma* (Fig. 1). This was not due to higher aerobic status as CCO activity was similar to that of the other species (Table 1). Rectal gland from *T. lymma* and *C. punctatum* had high CPT activity (0.30–0.49 U g^{-1} wet mass) (Fig. 1; Table 2) that was relatively similar to levels in kidney (except that of *T. lymma*) when controlled for the higher CCO levels (data not shown) in rectal gland (Table 2). The existence of extrahepatic lipid oxidation in elasmobranchs, and the

Table 1. *Activities of enzymes of intermediary metabolism in liver, kidney and heart from the freshwater ocellate river stingray (Potamotrygon motoro), white-edge whip ray (Himantura signifer) in freshwater, H. signifer in half-strength seawater, the marine blue-spotted ribbontail stingray (Taeniura lymma), and the seawater brownbanded bamboo shark (Chiloscyllium punctatum)*

	P. motoro (FW)	*H. signifer* (FW)	*H. signifer* (½ SW)	*T. lymma* (SW)	*C. punctatum* (SW)
Liver					
CCO	3.21±0.42^{e,KH}	4.32±0.28 (8)^{e,KH}	4.81±0.45 (7)^{e,KH}	5.54±0.86^{e}	17.0±1.6 (4)^{abcd,H}
CPT	0.27±0.03^{bcde,KH}	0.77±0.07 (9)^{a,KH}	0.63±0.05 (7)^{a,KH}	0.62±0.04^{a,KH}	1.09±0.28 (3)^{a,H}
COT	0.26±0.04^{bc,KH}	1.26±0.26 (9)^{ad,KH}	0.71±0.14 (7)^{ad,KH}	0.18±0.04^{bce,KH}	0.64±0.13 (3)^{d,H}
HOAD	2.55±0.43^{bc,KH}	6.90±0.57 (9)^{ade,KH}	7.40±1.01 (7)^{ade,KH}	4.98±0.35^{bc,KH}	2.53±0.13 (4)^{bc,KH}
D-β-HBDH	6.03±0.99^{e,K}	8.47±0.66^{de,KH}	7.81±0.93^{de,K}	3.77±0.39^{bc,KH}	2.08±0.37(4)^{abc,H}
PK	16.3±0.7^{de,KH}	15.5±1.2^{de,KH}	14.9±0.7^{de,KH}	21.9±1.1^{abce,KH}	7.31±1.05 (4)^{abcd,KH}
GDH	36.0±5.4^{de,KH}	31.7±3.4 (9)^{de,KH}	25.7±2.6 (7)^{de,KH}	6.33±0.45^{abc,K}	9.62±0.40 (4)^{abc,KH}
Kidney					
CCO	9.53±0.97^{e,L}	14.2±1.6^{e,L}	15.4±0.85^{L}	10.2±1.6 (5)^{e}	21.7±2.3^{abd,H}
CPT	0.13±0.01^{de,LH}	0.17±0.02^{de,LH}	0.15±0.01^{de,L}	1.31±0.06^{abce,LH}	0.53±0.04^{abcd,H}
COT	0.082±0.021^{bcde,L}	0.20±0.01^{ade,L}	0.20±0.01^{ade,L}	0.29±0.04 ^{abce,LH}	0.53±0.07^{abcd,H}
HOAD	0.55±0.04^{de,L}	0.82±0.09^{d,L}	0.83±0.04^{d,L}	1.71±0.17^{abce,LH}	0.98±0.10^{ad,L}
D-β-HBDH	2.28±0.28^{de,L}	2.21±0.12^{de,LH}	1.90±0.09^{de,LH}	0.04±0.02^{abce,LH}	0.92±0.12^{abcd}
PK	81.1±4.3^{bcde,L}	54.9±1.3^{ae,LH}	55.6±1.9^{ae,LH}	58.4±1.4^{ae,LH}	27.0±2.7^{abcd,LH}
GDH	13.7±1.4^{de,LK}	9.62±1.04^{de,L}	12.6±0.8^{de,LH,*}	53.4±3.3^{abce,LH}	26.5±2.7^{abcd,LH}
Heart					
CCO	12.3±0.7 (4)^{de,L}	12.9±2.0 (5)^{de,L}	14.4±1.4 (5)^{de,L}	5.87±0.95 (4)^{abce}	39.9±2.7^{abcd,LK}
CPT	0.033±0.010^{bcd,LK}	0.072±0.003^{ae,LK}	0.088±0.014^{ae,L}	0.085±0.008^{ae,LK}	0.010±0.004^{bcd,LK}
COT	0.060±0.017^{bc,L,†}	0.199±0.016^{ade,L,†}	0.182±0.016^{ade,L,†}	0.060±0.019^{bc,LK}	0.066±0.019^{bc,LK,†}
HOAD	0.37±0.03 (5)^{bcde,L}	0.72±0.07^{ae,L}	0.70±0.08^{ae,L}	0.78±0.09^{ae,LK}	1.23±0.10^{abcd,L}
D-β-HBDH	3.82±0.20 (5)^{bce}	10.9±0.6^{ade,LK}	9.50±0.90^{ade,K}	2.48±0.43^{bc,LK}	0.87±0.24^{abc,L}
PK	83.9±5.7 (5)^{L}	77.5±4.1^{LK}	85.0±7.4^{LK}	104.1±4.7^{LK}	84.0±16.4^{LK}
GDH	2.66±0.21 (5)^{b,LH}	4.60±0.32^{a,L}	4.19±0.30^{LK}	3.31±0.67^{K}	4.24±0.30^{LK}

FW, freshwater; SW, sea water; ½ SW, half-strength (15‰) seawater.

Values are mean ± s.e.m.; *N*=6 except where noted in parentheses.

Activities were measured at 25°C. ND, not detectable. See List of abbreviations for enzyme names.

Activities are expressed as μmol min^{-1} g^{-1} wet mass. Lowercase letters in superscript indicate a significant difference (one-way ANOVA, $P<0.05$) from the same enzyme in the same tissue: ^{a}*P. motoro*, ^{b}*H. signifer* (FW), ^{c}*H. signifer* (½ SW), ^{d}*T. lymma*, ^{e}*C. punctatum*. Uppercase letters in superscript indicate a significant difference (one-way ANOVA, $P<0.05$) from the same enzyme activity in the same species in: ^{L}liver, ^{K}kidney, ^{H}heart; *significant difference between *H. signifer* (FW) and *H. signifer* (1/2SW) (Student's *t*-test, $P<0.05$); †COT activity is significantly different from CPT activity within the same tissue (Student's *t*-test, $P<0.05$). ND activities were omitted from statistical tests.

similarity of freshwater and marine forms, is evidence against the urea hypothesis.

The activity of HOAD, an enzyme involved in β-oxidation of fatty acids, was readily detectable in all tissues, with the highest activity in liver in all species (Fig. 2). Lower activities were seen in kidney, heart and rectal gland; however, *T. lymma* (marine) possessed twofold higher kidney HOAD levels than in the other species (Fig. 2; Table 1). Despite a lack of CPT, *T. lymma* white muscle had low HOAD activity and HOAD activity in *C. punctatum* (marine) red muscle was similar to that in its heart and kidney (Fig. 2), indicating the paradoxical presence of β-oxidation apparently without the capacity to transport long chain fatty acids into the mitochondria. The similar HOAD levels among freshwater and marine species suggests β-oxidation is unconstrained by urea levels, in contrast to the prediction of the urea hypothesis.

COT, a peroxisomal enzyme that exports medium chain fatty acids produced by peroxisomal β-oxidation for further mitochondrial oxidation (Ramsay, 1999), was detected in all tissues from all species with the exception of *T. lymma* white muscle (Tables 1 and 2). High levels of COT were found in liver and kidney. Lower levels were measured in cardiac and white muscles, although COT activity was significantly greater than that of CPT in heart of most species. The level of COT in rectal gland from *C. punctatum* was tenfold higher than in rectal gland from *T. lymma*. Our observation of similar COT levels across species show that utilization of medium chain fatty acids is not enhanced in freshwater *versus* marine elasmobranchs.

Total plasma NEFA ranged from 106 nmol ml^{-1} in *P. motoro* (freshwater) to 216 nmol ml^{-1} in *T. lymma* (marine) (Table 3). Intermediate levels were found in *H. signifer* (123 nmol ml^{-1} in freshwater and 154 nmol ml^{-1} in half-

Table 2. *Activities of enzymes of intermediary metabolism in rectal gland and white muscle from the blue-spotted ribbontail stingray (Taeniura lymma) and rectal gland and red muscle from the brownbanded bamboo shark (Chiloscyllium punctatum)*

	T. lymma		*C. punctatum*	
	Rectal gland	White muscle	Rectal gland	Red muscle
CCO	25.2±2.5^{LKHM}	1.10±0.30 (5)^{KR}	40.4±5.5^{*,LK}	41.2±7.1^{LK}
CPT	0.30±0.03^{LKHM}	ND	0.49±0.10^{HM}	ND
COT	0.053±0.012^{LK,†}	ND	0.63±0.14^{*,HM}	0.044±0.013^{LKR}
HOAD	1.43±0.05^{LM}	0.24±0.03^{LKR}	1.73±0.12^{*,LKHM}	0.90±0.14^{LR}
D-β-HBDH	4.95±0.34^{KHM}	0.32±0.05^{LHR}	2.04±0.46^{*,H}	1.36±0.08
PK	97.9±5.2^{LKM}	522±17^{LKHR}	61.1±5.9^{*,LKM}	221.1±22.3^{LKHR}
GDH	3.53±0.30^{K}	1.32±0.18^{K}	4.60±0.31^{*,LK}	5.41±0.34^{LK}

Activities were measured at 25°C. ND=not detectable. See List of abbreviations for enzyme names.

Activities are expressed as μmol min^{-1} g^{-1} wet mass (mean ± s.e.m., N=6 for *T. lymma* except where noted in parentheses; N=4 for *C. punctatum*); *enzyme activity in rectal gland from *C. punctatum* is significantly different from the activity of the same enzyme in rectal gland from *T. lymma* (Student's *t*-test, P<0.05); uppercase letters in superscript indicate significant difference (one-way ANOVA, P<0.05) from the same enzyme activity within species in: ^{L}liver, ^{K}kidney, ^{H}heart, ^{R}rectal gland, ^{M}skeletal muscle (*T. lymma*) or red muscle (*C. punctatum*) (data other than for rectal gland, white muscle and red muscle are in Table 1); †COT activity is significantly different from CPT activity within the same tissue (Student's *t*-test, P<0.05). ND activities were omitted from statistical tests.

strength seawater) and *C. punctatum* (marine) (171.6 nmol ml^{-1}) (Table 3). These data show that peripheral transport of NEFA is not enhanced in freshwater elasmobranchs compared with marine species. Total NEFA concentrations did not differ significantly between the species. Data for individual NEFA amounts are not provided in the present study, but it is notable that NEFA of less than C14 were not detected.

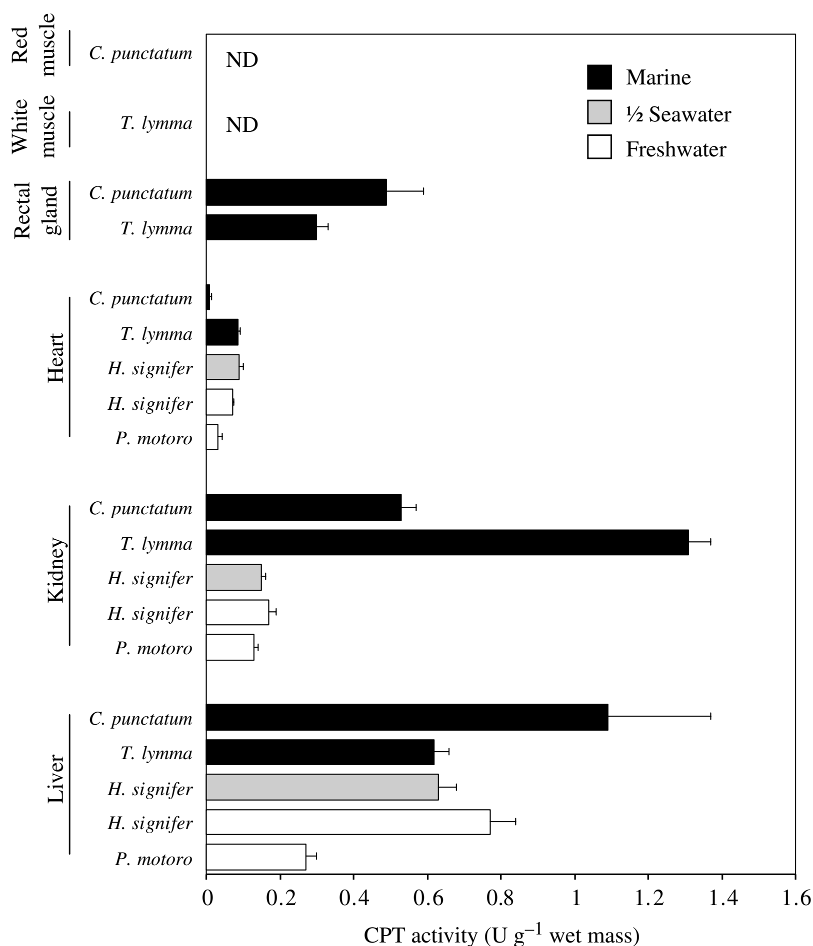

Fig. 1. Carnitine palmitoyl transferase (CPT) activities (mean ± s.e.m.) in liver and extrahepatic tissues of freshwater, euryhaline and marine elasmobranchs with varying levels of urea: *Potamotrygon motoro* (freshwater) (no urea), *Himantura signifer* in freshwater (low urea), *H. signifer* acclimated to half-strength seawater (½ Seawater) (increased urea), *Taeniura lymma* (marine) (high urea), and *Chiloscyllium punctatum* (marine) (high urea). See text or Fig. 4 for specific urea levels. Sample sizes and the results of statistical comparisons between tissues and species are given in Tables 1 and 2. The salinity (and thus urea level) for each species or acclimation group is indicated by the shading of the bars. Enzyme measurements were made at 25°C. ND, not detectable.

Ketone body metabolism: important in freshwater, euryhaline and marine elasmobranchs

The capacity for ketone body oxidation, as measured by the activity of D-β-HBDH which oxidizes D-β-hydroxybutyrate to acetoacetate in the mitochondria, was detected in all tissues from all species and no evidence for decreased ketone body utilization was noted in freshwater forms (Fig. 3; Tables 1 and 2). D-β-HBDH activity was similarly high in all tissues (including tissues with readily detectable CPT) of all species, with the notable exception of white muscle and kidney from *T. lymma* (marine) which both showed significantly lower activities compared with its

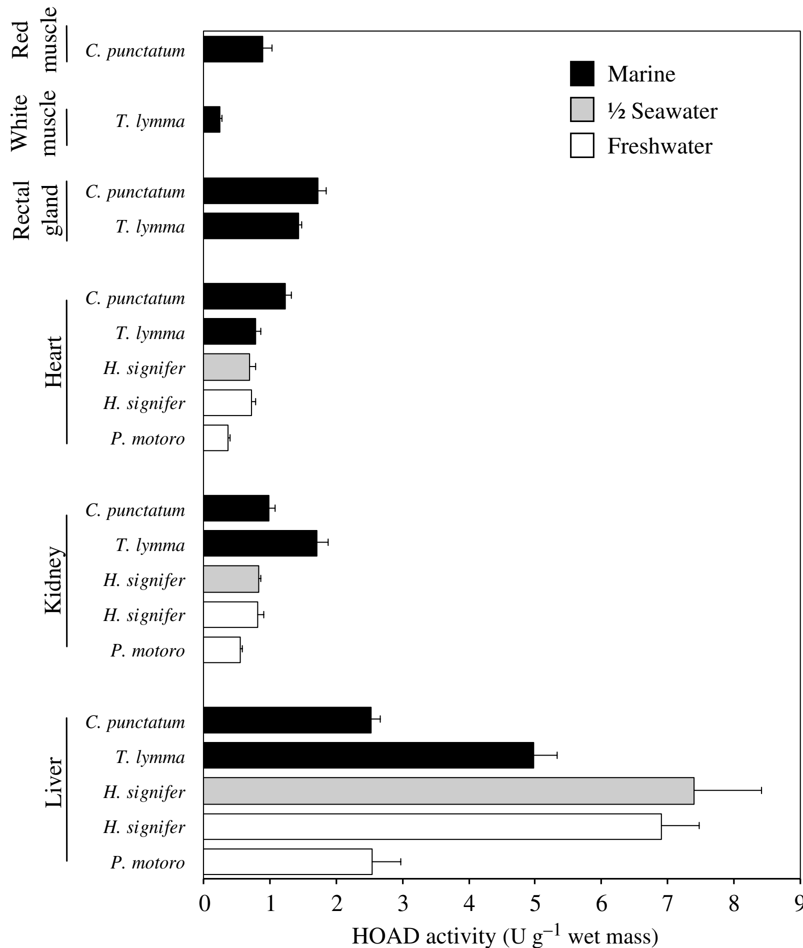

Fig. 2. 3-Hydroxyacyl CoA dehydrogenase (HOAD) activities (mean ± s.e.m.) in liver and extrahepatic tissues of freshwater, euryhaline and marine elasmobranchs with varying levels of urea: *Potamotrygon motoro* (freshwater) (no urea), *Himantura signifer* in freshwater (low urea), *H. signifer* acclimated to half-strength seawater (½ Seawater) (increased urea), *Taeniura lymma* (marine) (high urea) and *Chiloscyllium punctatum* (marine) (high urea). See text or Fig. 4 for specific urea levels. Sample sizes and the results of statistical comparisons between tissues and species are given in Tables 1 and 2. The salinity (and thus urea level) for each species or acclimation group is indicated by the shading of the bars. Enzyme measurements were made at 25°C. ND, not detectable.

other tissues (Fig. 3; Tables 1 and 2). Kidney D-β-HBDH in *T. lymma* was significantly lower than that in other species. Preliminary measurements indicated very low activities of L-β-HBDH in all species and tissues (~0.5–4% of D-β-HBDH activity; data not shown).

Table 3. *Total nonesterified fatty acids in plasma of the freshwater ocellate river stingray (Potamotrygon motoro), white-edge whip ray (Himantura signifer) in freshwater and half-strength seawater, the marine blue-spotted ribbontail stingray (Taeniura lymma), and the brownbanded bamboo shark (Chiloscyllium punctatum)*

	Type of water	N	Total NEFA (nmol ml^{-1})
P. motoro	FW	7	105.7±20.2
H. signifer	FW	9	122.8±40.9
H. signifer	½ SW	6	154.1±46.0
T. lymma	SW	4	215.7±27.6
C. punctatum	SW	5	171.6±32.0

Values are mean ± s.e.m. FW, freshwater; SW, sea water; ½ SW, half-strength (15‰) seawater.

There were no significant differences between total nonesterified fatty acids (NEFA) concentration in each species (one-way ANOVA, P>0.05) nor between *H. signifer* (FW) and *H. signifer* (½ SW) (Student's *t*-test, P>0.05).

Other metabolic pathways: glycolysis and amino acid metabolism

Glycolytic capacity, as indicated by PK, provided information on the importance of carbohydrates as metabolic fuel. PK was highest in muscle (heart, red, and white muscle) and lowest in liver in all species. PK levels did not vary greatly between species; significant differences typically involved less than a 1.5-fold difference in activity (Tables 1 and 2).

GDH, which catalyzes the oxidative deamination of glutamate to α-ketoglutarate, indicated the importance of amino acid as oxidative substrates. GDH activities were highest in liver and kidney. Liver GDH was lowest in the marine species and highest in the freshwater species (see below); in other tissues GDH was relatively similar between species, except kidney in *T. lymma* (marine), which had higher levels than other species (Tables 1 and 2).

Effects of salinity acclimation on H. signifer metabolic organization

GDH in kidney increased following salinity acclimation of *H. signifer*; it was the only enzyme to change significantly (Table 1). Protein content g^{-1} wet mass was unchanged in tissues of salinity-acclimated *H. signifer* (data not shown, Student's *t*-test, P>0.05), suggesting no significant tissue dehydration that could confound mass specific enzyme activities. The lack of changes in enzymes of fatty acid oxidation and total plasma NEFA upon salinity acclimation in *H. signifer* (Table 3), suggest urea has little short-term effect on lipid metabolism.

Relationship of GDH in liver and urea content

A significant negative correlation between liver GDH activity and white muscle or liver urea content was observed across species (both r=0.99, P<0.002, linear regression ANOVA). When previously published values for liver GDH

and urea in white muscle from three other elasmobranchs (freshwater *P. magdalenae*, little skate, and spiny dogfish; see figure legends for references) were included the relationship remained highly significant (Fig. 4) (r=0.93, P<0.001, linear regression ANOVA). This significant negative relationship also held when GDH was expressed as mg^{-1} protein; when individual values for GDH activity and white muscle urea were used instead of means; when liver GDH was standardized against liver CCO; and when previously measured values for plasma urea in *T. lymma* (marine), *P. motoro* (freshwater), and *H. signifer* in freshwater and brackish water (Tam et al., 2003) (Y.K.I., unpublished) as well as spiny dogfish (Bedford, 1983) and little skate (Goldstein and Forster, 1971) were used.

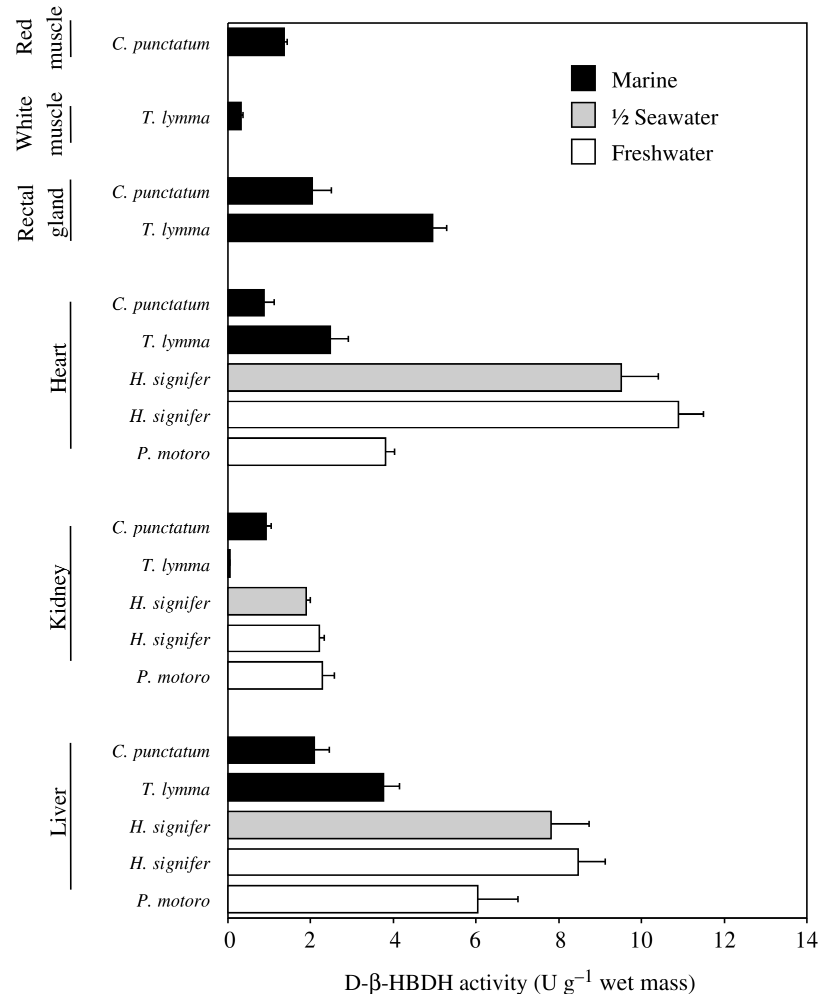

Fig. 3. D-β-hydroxybutyrate dehydrogenase (D-β-HBDH) activities (mean ± s.e.m.) in liver and extrahepatic tissues of freshwater, euryhaline and marine elasmobranchs with varying levels of urea: *Potamotrygon motoro* (freshwater) (no urea), *Himantura signifer* in freshwater (low urea), *H. signifer* acclimated to half-strength seawater (½ Seawater) (increased urea), *Taeniura lymma* (marine) (high urea) and *Chiloscyllium punctatum* (marine) (high urea). See text or Fig. 4 for specific urea levels. Sample sizes and the results of statistical comparisons between tissues and species are given in Tables 1 and 2. The salinity (and thus urea level) for each species or acclimation group is indicated by the shading of the bars. Enzyme measurements were made at 25°C. ND, not detectable.

Discussion

The present study provides evidence against the urea hypothesis. The presence of CPT, and therefore fatty acid oxidation, in extrahepatic tissue (kidney and rectal gland) of elasmobranchs shows that one of the main tenets of the urea hypothesis – that extrahepatic fatty acid oxidation does not occur in elasmobranchs – is false. The prediction of the urea hypothesis that freshwater elasmobranchs with low urea levels should have an elevated capacity for extrahepatic fatty acid oxidation relative to marine elasmobranchs with high urea levels is false. All the species examined possess a similar metabolic organization as indicated by enzyme activities, notably a high reliance on ketone bodies as oxidative fuel and a similar capacity to oxidize lipids in kidney, rectal gland, liver, and, to a lesser extent, heart. In fact, the freshwater *P. motoro* generally had the lowest CPT activities, and the marine *T. lymma* and *C. punctatum* had the highest CPT levels in kidney as well as high CPT activities in rectal gland (Fig. 1). All species had similar levels of plasma NEFA. Tissue CCO levels were largely similar between species indicating that our comparisons are not markedly influenced by aerobic status. These results, as well as the absence of significant modifications to lipid metabolism associated with increased urea levels during salinity acclimation in *H. signifer*, suggest that urea has had minimal effects on the evolution of the pattern of lipid or ketone body oxidation in elasmobranchs.

Metabolic organization of freshwater, euryhaline, and marine elasmobranchs

A novel and important finding of the present study is the discovery of high activities of CPT in kidney from *P. motoro* (freshwater), *H. signifer* (euryhaline), *T. lymma* (marine) and *C. punctatum* (marine), as well as rectal gland from *T. lymma* and *C. punctatum*. The levels of CPT in kidney of the species we examined were comparable to those found in kidney of Arctic char (*Salvelinus alpinus*), a teleost (Table 4). Kidney from *T. lymma* possessed very high CPT levels coinciding with higher HOAD activity than in the other species, implying prominent lipid oxidation. In fact, CPT and HOAD in kidney correlated significantly using values from individual animals of all the current study species, supporting a role for fatty acid oxidation in this tissue in elasmobranchs (data not shown; r=0.87, P<0.001, linear regression ANOVA). Readily detectable levels of CPT were measured in heart from the

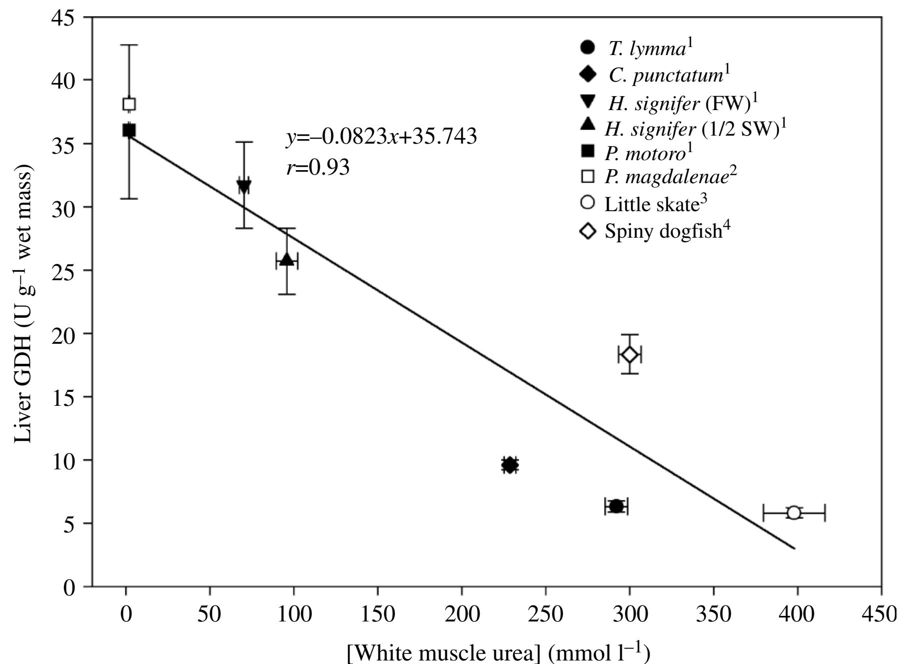

Fig. 4. Relationship between white muscle urea concentration and liver glutamate dehydrogenase (GDH) activity in elasmobranchs. Values are mean ± s.e.m. See text for full names of species. Error bars for GDH in *Potamotrygon motoro* and *P. magdalenae* are asymmetrical to avoid overlapping bars. [1]GDH values are from the present study (see Table 1 for sample sizes) and urea values are from the same animals (N=5–8, taken from various studies) (Treberg et al., 2006). [2]GDH value (Singer and Ballantyne, 1989); urea value was assumed to be the same as in *P. motoro* (Treberg et al., 2006). [3]GDH value (Moon and Mommsen, 1987); urea value (Forster and Goldstein, 1976). [4]GDH value (Battersby et al., 1996); urea value (Treberg and Driedzic, 2002). GDH activities for spiny dogfish and little skate were adjusted to 25°C using Q_{10}=2. The regression is significant (r=0.93, P<0.001, linear regression ANOVA).

stingrays, although they are about five- to eight-fold lower than found in teleost hearts (Table 4). These findings, especially in kidney and rectal gland, contradict the view that elasmobranchs possess greatly reduced or non-existent lipid oxidation in extrahepatic tissues. However, previously published measurements of CPT in kidney from elasmobranchs are restricted to one record of non-detectable CPT in kidney from *P. magdalenae* (Singer and Ballantyne, 1989). We consider our more comprehensive measurements to be more reliable. In fact, CPT values for little skate kidney [J. Berges and J.S.B., unpublished; (see Ballantyne, 1997)] match those measured in the present study (Table 4). No published data exist for CPT levels in elasmobranch rectal gland, but the present study suggests that lipid oxidation is high in this tissue, even when placed in the context of the higher CCO levels. A report of undetectable CPT in rectal gland from little skate should be reassessed [J. Berges and J.S.B., unpublished; (see Ballantyne, 1997)].

The question of the presence or absence of CPT in heart from elasmobranchs is more complex. Various authors (Sidell et al., 1987; Moyes et al., 1990; Zammit and Newsholme, 1979) reported undetectable levels of CPT in heart from little skate and spiny dogfish, and we show in the present study that *C. punctatum* heart possesses barely detectable CPT despite the presence of higher CCO levels compared with our other study species (Table 4). On the other hand, Driedzic and De Almeida-Val found teleost-like levels of CPT in heart from *P. hystrix* (Driedzic and De Almeida-Val, 1996), and the present study shows low levels of CPT are also present in three tropical stingrays (Table 4). Similarly low levels of CPT are reported even in little skate heart (see Ballantyne, 1997) (B.S.-R. and J.S.B., unpublished) (Table 4). Overall, however, elasmobranchs possess much lower (eight- to 13-fold) CPT activities in heart compared with teleosts (Table 4). Considering this as well as the high levels of D-β-HBDH in elasmobranch hearts, and the superior performance of perfused little skate heart in the presence of acetoacetate rather than palmitate (Driedzic and Hart, 1984), we agree with the contention that ketone bodies are much more important than lipids as a fuel source for elasmobranch hearts. However, low levels of CPT in some species suggest that lipids may be oxidized to some extent in the hearts of some elasmobranchs. Notably, CPT and HOAD correlated significantly in heart from the stingrays in the present study (*C. punctatum* data was omitted since CPT was barely detectable), supporting a role for lipid oxidation in this tissue in some species (data not shown; values for individual animals were used; r=0.45, P<0.05, linear regression ANOVA). The absence of CPT in red muscle of *C. punctatum* (despite a high aerobic capacity as indicated by CCO) and white muscle of *T. lymma,* corresponds well with many studies showing that red and white muscle from elasmobranchs lack CPT (Table 4), do not oxidize fatty acids, and do not rely on fatty acids for recovery from exercise (Zammit and Newsholme, 1979; Moyes et al., 1990; Ballantyne et al., 1992; Richards et al., 2003).

Based on the findings for CPT in the present study, we propose a revised model of extrahepatic lipid oxidation in elasmobranchs: kidney and rectal gland have a high capacity to oxidize lipids which is comparable to that in liver, heart possesses a non-detectable or minor capability for lipid oxidation, and skeletal muscle, including red and white muscle, does not use lipids as oxidative fuel. More simply, muscle in elasmobranchs does not use lipids as a major oxidative fuel, unlike in teleosts where red muscle and heart heavily rely on fatty acid oxidation. Molecular studies may uncover tissue-specific promoters or isoforms of the CPT I gene (McGarry and Brown, 1997) that could explain this demarcation.

The relative activities of COT and CPT suggest that both

Table 4. *Mean carnitine palmitoyl transferase activity in liver, kidney, heart, and red muscle of representative elasmobranchs and teleosts*

	CPT activity (μmol min^{-1} g^{-1} wet mass)			
	Liver	Kidney	Heart	Red muscle
Elasmobranchs				
Potamotrygon motoro[a]	0.27	0.13	0.033	–
Himantura signifer (FW)[a]	0.77	0.17	0.072	–
Taeniura lymma[a]	0.62	1.31	0.085	–
Chiloscyllium punctatum[a]	1.09	0.53	0.010	ND
Potamotrygon hystrix[b]	–	–	0.17	–
Potamotrygon magdalenae[c]	0.002	ND	ND	–
Leucoraja erinacea	0.09[d]	0.24[d]	0.10[d], ND[e]	0.02[d]
Squalus acanthias	0.09[f]	–	<0.01[fg], ND[e]	<0.01[fg]
Raja clavata[g]	–	–	<0.01	<0.01
Scyliorhinus canicula[g]	–	–	<0.01	<0.01
Mustelus asterias[g]	–	–	<0.01	<0.01
Mean ± s.e.m.	0.41±0.16	0.40±0.20	0.038±0.016	0.0033±0.0033
Teleosts				
Lophius piscatorius[e]	–	–	0.23	–
Scomber scombrus[e]	–	–	0.28	–
Gaidropsarus vulgaris[e]	–	–	0.41	–
Morone saxatilis[e]	–	–	0.52	–
Hemitripterus americanus[e]	–	–	0.16	–
Zoarces americanus[h]	–	–	0.21	–
Makaira nigricans[i]	–	–	0.39	0.29
Gadus morhua[j]	–	–	0.44	–
Cyprinus carpio[f]	0.39	–	0.17	0.32
Oncorhynchus mykiss[f]	0.30	–	0.28	0.51
Salvelinus alpinus[k]	0.20	0.17	0.29	0.21
Mean ± s.e.m.	0.30±0.05	0.17	0.31±0.04	0.33±0.06
Mean teleost/mean elasmobranch	0.7	0.4	8.0	100
Mean teleost/mean elasmobranch (Q_{10}=2, adjusted to 25°C)	1.4	1.0	13.3	67

Letters indicate references for data, which are provided below with the temperature at which measurements were made. ND, not detectable; –, not measured.

For mean calculations multiple values for one tissue in a species were first averaged; ND and <0.01 were taken as zero. The ratio of mean teleost carnitine palmitoyl transferase (CPT) to mean elasmobranch CPT is given using the CPT values at the original assay temperature and CPT values adjusted to 25°C (Q_{10}=2).

[a]Present study, 25°C; [b]25°C (Driedzic and De Almeida-Val, 1996); [c]25°C (Singer and Ballantyne, 1989); [d]J. Berges and J.S.B., unpublished, 10°C (see Ballantyne, 1997); [e]15°C (Sidell et al., 1987); [f]15°C (Moyes et al., 1990); [g]10°C (Zammit and Newsholme, 1979); [h]10°C (Driedzic and Stewart, 1982); [i]25°C (Suarez et al., 1986); [j]15°C (Hansen and Sidell, 1983); [k]J. S. Bystriansky and J.S.B., unpublished, 10°C.

medium- and long-chain fatty acids are important metabolic substrates in liver and kidney of elasmobranchs. COT levels were very different in rectal gland between *C. punctatum* and *T. lymma* implying phylogenetic variability in medium-chain fatty acid utilization in this tissue. In heart from all species, except *T. lymma*, COT activity was higher than CPT activity, suggesting that medium-chain fatty acids may be more important in mitochondrial oxidation in this tissue in elasmobranchs; medium-chain fatty acids exported from peroxisomes by COT can enter mitochondria independently of CPT (McGarry and Foster, 1980). This finding is consistent with the low levels of mitochondrial oxidation of octanoyl carnitine ester but not palmitoyl carnitine ester found in heart from *S. acanthias* (Moyes et al., 1990). However, the high levels of COT observed in the present study also suggest active peroxisomal β-oxidation, which Moyes et al. showed to be non-detectable in heart and red muscle from *S. acanthias* and other fishes (Moyes et al., 1990). Further study on peroxisomal β-oxidation and COT in elasmobranchs is warranted.

Despite the absence of detectable CPT, HOAD was detectable in white muscle from *T. lymma*. Heart and red muscle from *C. punctatum* possessed even higher levels of HOAD (perhaps because of these tissues' higher aerobic status as indicated by CCO), despite non-detectable or barely detectable CPT activities and low COT levels. This paradox is seen in other elasmobranchs and is not apparently attributable

to carnitine-independent fatty acid oxidation or peroxisomal β-oxidation (Moyes et al., 1990). Studies characterizing the elasmobranch HOAD are needed to ascertain this enzyme's true role, if any.

The measurements of total plasma NEFA in the present study are the first for freshwater or tropical marine elasmobranchs. As in temperate marine elasmobranchs, total plasma NEFA concentrations were several-fold lower than that found in teleosts (Ballantyne et al., 1993). *T. lymma* and *C. punctatum* possessed levels similar to temperate marine elasmobranchs (Ballantyne et al., 1993). *P. motoro* and *H. signifer* possessed the lowest levels of plasma NEFA yet recorded for an elasmobranch, indicating that adaptation to freshwater and low urea has not enhanced the availability of lipids as oxidative fuel. Evidently, the low levels of plasma NEFA in the elasmobranchs from the present study adequately supply the high capacity for lipid oxidation in kidney (especially in *T. lymma*) and rectal gland, as well as the low levels of lipid oxidation in heart. In teleosts, higher plasma NEFA levels probably occur due to a greater reliance on lipid oxidation in heart as well as skeletal muscle.

The elasmobranchs in the present study must possess a lipid carrier in their blood because their plasma NEFA concentrations exceed the solubility of the fatty acids in water (Windholz, 1983). Other elasmobranchs appear to lack albumin capable of carrying NEFA (Fellows et al., 1980) and the low NEFA levels suggests this also is the case in our study species. Lipoproteins may be the major NEFA carrier in elasmobranchs. Utilization of short- and medium chain NEFA, which have increased solubility and subsequently do not rely on special carriers such as albumin (Moyes et al., 1990), as an alternative method to deliver lipid extrahepatically does not appear to occur in elasmobranchs as these fatty acids were absent in plasma of all species.

High levels of D-β-HBDH in tissues from *P. motoro* and *H. signifer* suggest that ketone bodies are important oxidative fuels in freshwater and euryhaline elasmobranchs, as is the case in *C. punctatum* and *T. lymma* and other marine elasmobranchs studied to date (Ballantyne, 1997). This indicates that urea has little effect on the importance of ketone body oxidation in elasmobranchs. Unlike other elasmobranchs, though, *T. lymma* possesses a very low level of D-β-HBDH in its kidney, similar to that found in teleost kidney (LeBlanc and Ballantyne, 1993). This result makes it probable that *T. lymma* kidney does not appreciably utilize ketone bodies, and the concomitant presence of very high levels of CPT suggests that kidney in *T. lymma* favours lipids over ketone bodies as oxidative fuel – an unprecedented finding in an elasmobranch and an illustration of the importance of taking a wide phylogenetic scope when investigating the metabolic capacities of larger taxonomic groupings. Interestingly, when activities of CPT and D-β-HBDH in kidney from all of the species in the current study are regressed, a highly significant negative relationship is found (Fig. 5A). This suggests a trade-off in the utilization of lipids *versus* ketone bodies in kidney of elasmobranchs, supporting the concept that in elasmobranchs ketone bodies are

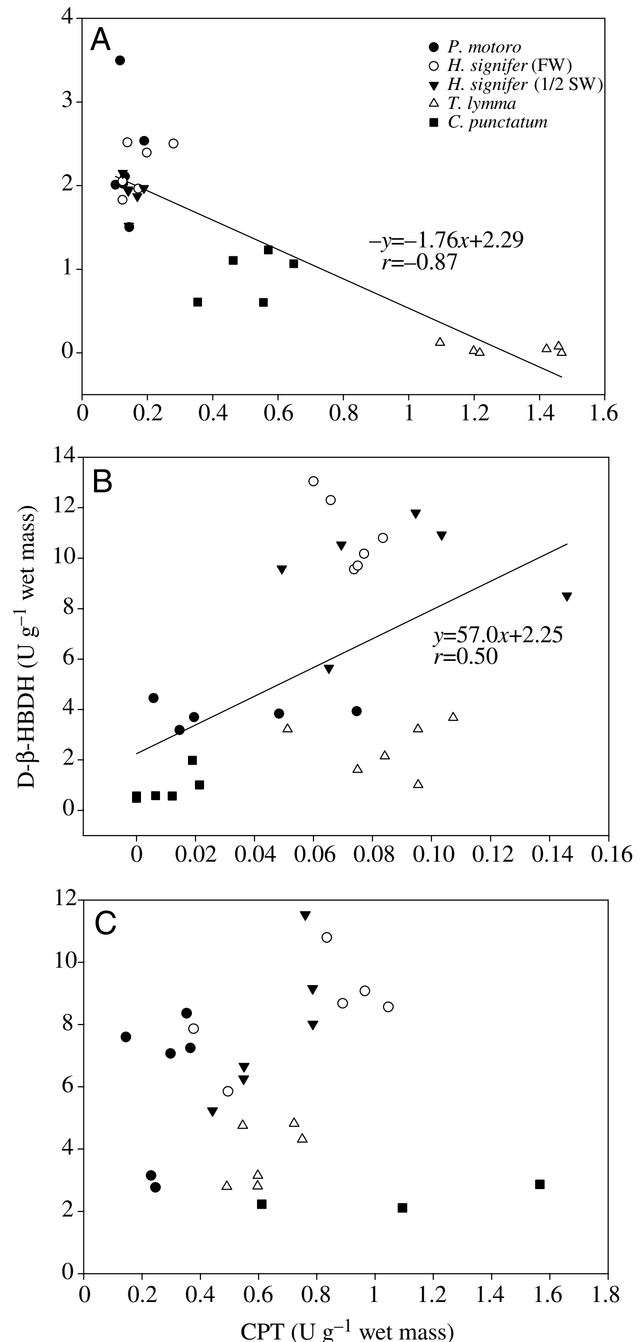

Fig. 5. Relationships between activities of carnitine palmitoyl tranferase (CPT) and D-β-hydroxybutyrate dehydrogenase (D-β-HBDH) in kidney (A), heart (B) and liver (C) of *Potamotrygon motoro*, *Himantura signifer* in freshwater (FW), *H. signifer* in half-strength (15‰) seawater (½ SW), *Taeniura lymma*, and *Chiloscyllium punctatum*. Each point refers to an individual animal. The regressions are significant for A ($P<0.001$, linear regression ANOVA) and B ($P<0.01$, linear regression ANOVA).

an alternate to lipid as a delivery source of acetyl CoA to extrahepatic tissues (Ballantyne, 1997). When CPT and D-β-HBDH in heart are regressed across species, though, a significant positive relationship is found (Fig. 5B). Tissue-

specific differences in the interrelationships of lipolytic and ketolytic pathways thus are apparent. The absence of a significant, positive relationship between CPT and D-β-HBDH in liver (Fig. 5C) is perplexing because of CPT's role in transporting fatty acids into mitochondria for ketogenesis (Ballantyne, 1997). These observations demonstrate the need for further studies to establish the relationships between lipid and ketone body metabolism in various tissues of elasmobranchs.

In tissues where lipids are not oxidized the possibility exists that other fuels such as carbohydrates or amino acids could substitute as oxidative substrates. Our measurements of PK and GDH activities indicate the relative importance of glycolysis and amino acid catabolism, respectively, in energy metabolism of our study species. PK and GDH activities corroborate that levels of glycolysis and amino acid catabolism in all tissues are generally similar to that of teleosts, where lipid oxidation is ubiquitous (Sidell et al., 1987; Dickson, 1995; Ballantyne, 2001). Ketone bodies and not carbohydrates or amino acids appear to be the preferred oxidative substitute for lipids in elasmobranchs, although the relatively high GDH activity in *T. lymma* kidney may indicate an increased reliance on amino acids in the apparent near absence of ketone body oxidation. Aside from this single observation and GDH in liver (see below), GDH and PK activities were similar across species in all tissues. This suggests little effect of urea on the relative importance of amino acids and carbohydrates as metabolic fuels in elasmobranchs.

Effects of salinity acclimation on metabolic organization of H. signifer

The effects of salinity acclimation on elasmobranch energy metabolism have not been previously studied. Higher GDH in kidney of salinity challenged *H. signifer* may reflect an increased oxidative reliance on amino acids in this tissue to help offset increased osmoregulatory costs (Ballantyne, 2001). Although the absence of significant changes in other enzyme activities in salinity acclimated *H. signifer* may be due in part to transitory modifications of energy metabolism, the unchanged CPT, COT or HOAD activities also suggest that increasing urea levels have no effect on enzymes of fatty acid oxidation.

No significant change in the concentration of total plasma NEFA was seen in salinity acclimated *H. signifer*, in contrast to the prediction of the urea hypothesis that this parameter should decrease. There are few studies on plasma NEFA dynamics during salinity acclimation in fishes. In freshwater shortnose sturgeon (*Acipenser brevirostrum*), mobilization of fatty acids leading to increased plasma NEFA was observed after 2 weeks acclimation to brackish water (20‰) (Jarvis and Ballantyne, 2003), but in Arctic char there was no change in total plasma NEFA concentration after 96 h of seawater acclimation (J. S. Bystriansky and J.S.B., unpublished). The importance of lipids during salinity acclimation of fishes probably varies on a temporal scale and between species.

Relationship of GDH in liver and urea content

We observed a negative relationship between liver GDH activities and urea levels in white muscle or liver (or plasma) in our study species; i.e. liver GDH activity is high in freshwater elasmobranchs and low in marine species. This relationship appears robust, remaining significant when data for other elasmobranchs from previous studies were included (Fig. 4).

The relationship between liver GDH and urea content in elasmobranchs may relate to the contrasting roles of glutamate in hepatic energy production and ureogenesis. Glutamate can be deaminated to α-ketoglutarate by GDH for energy production (Ballantyne, 1997), or it can be converted to glutamine by glutamine synthetase (GS) in order to shuttle nitrogen to the ornithine-urea cycle (Anderson, 1991). Assuming the ratios of the aminating to deaminating directions for GDH are the same in all species examined, we postulate that in marine elasmobranchs, with high levels of urea, the importance of the glutamate-glutamine-urea pathway results in de-emphasis on glutamate catabolism; hence, the lower hepatic GDH levels. Freshwater elasmobranchs, however, have a lessened or non-existent urea synthesis (Tam et al., 2003); available glutamate can be preferentially deaminated for energy production. The consequent greater flux through this pathway would lead to higher hepatic GDH activities in freshwater *Potamotrygon* spp. and *H. signifer*. In salinity-challenged *H. signifer* the trend towards lower liver GDH with increased urea also supports our explanation. Our hypothesis is further corroborated by the observation of a positive relationship between hepatic GS activities and urea content in freshwater, euryhaline, and marine elasmobranchs (Webb and Brown, Jr, 1980; Tam et al., 2003). This trade-off between energy- and urea-production in elasmobranchs living in different salinities is a good example of how environment can influence both the long-term (evolutionary) and short-term (acclimatory) metabolic organization of an animal.

Evolutionary considerations: the urea hypothesis

Our findings that, (1) extrahepatic lipid oxidation does occur in elasmobranchs, (2) the capacity for lipid oxidation and transport is not enhanced in freshwater elasmobranchs with low urea, (3) salinity acclimation and associated urea content increases in a euryhaline stingray do not cause reorganization of metabolic pathways related to fatty acid oxidation, provide evidence against the urea hypothesis. It might be argued that *P. motoro* and *H. signifer* have simply retained the metabolic organization of their marine ancestors (which possessed high levels of urea) by common phylogeny and thus the resulting similarity cannot be used as evidence against the urea hypothesis. Even if this is the case, our results show that the decrease in urea content in elasmobranchs that invaded freshwater was not a selective force sufficiently powerful to cause the adoption of a teleost-like metabolic organization that emphasizes lipids over ketone bodies as oxidative fuel for muscles. This conclusion contrasts with what is predicted by the urea hypothesis. Our counter-evidence becomes more

convincing when we consider the long-term freshwater adaptation (at least 15–20 mya) of potamotrygonid stingrays (Lovejoy, 1997), and the possibility that the earliest gnathostomes, from which the teleosts evolved, may have been ureosmotic [(Griffith, 1991); i.e. freshwater adaptation in elasmobranchs mirrors the evolution of hypoosmoregulation in teleosts). Based on our results, and in the absence of data from an unrelated ureosmotic fish (i.e. coelacanth), alternatives to the urea hypothesis should be entertained, including the possibility that a lower reliance on lipids as oxidative fuels in muscle is ancestral among the fishes.

In summary, the present study provides evidence against the urea hypothesis because the freshwater elasmobranchs did not show an enhanced capacity for extrahepatic lipid oxidation relative to the marine species. We also demonstrated that extrahepatic lipid oxidation occurs in elasmobranchs, disproving one of the urea hypothesis' central tenets. Based on our measurements of CPT, we propose a revised model of tissue-specific lipid oxidation in elasmobranchs, with high levels in liver, kidney, and rectal gland, low to very low levels in heart, and non-detectable levels in skeletal muscle. The importance of ketone bodies as oxidative fuel in elasmobranchs is confirmed, but the unprecedented, very low levels seen in kidney from *T. lymma* indicate that variability in ketone body utilization occurs among elasmobranchs.

List of abbreviations

CCO	cytochrome *c* oxidase
COT	carnitine octanoyl transferase
CPT	carnitine palmitoyl transferase
D-β-HBDH	D-β-hydroxybutyrate dehydrogenase
DTNB	5,5′-dithiobis 2-nitrobenzoic acid
GDH	glutamate dehydrogenase
HOAD	3-hydroxyacyl CoA dehydrogenase
NEFA	non-esterified fatty acid
PK	pyruvate kinase
TX100	Triton X-100

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