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Plasma non-esterified fatty acids of elasmobranchs: Comparisons of temperate and tropical species and effects of environmental salinity

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Abstract

We investigated the influence of environments with different average temperatures and different salinities on plasma NEFA in elasmobranchs by comparing species from tropical vs. cold temperate marine waters, and tropical freshwater vs. tropical marine waters. The influence of the environment on plasma NEFA is significant, especially with regard to essential fatty acids (EFA) and the *n*-3/*n*-6 ratio. *n*-3/*n*-6 ratios in tropical marine elasmobranchs were lower by two-fold or more compared with temperate marine elasmobranchs, because of higher levels of arachidonic acid (AA, 20:4*n*-6) and docosatetraenoic acid (22:4*n*-6), and less docosahexaenoic acid (DHA, 22:6*n*-3), in the tropical species. These results are similar to those in earlier studies on lipids in teleosts. *n*-3/*n*-6 ratios and levels of EFA were similar between tropical freshwater and tropical marine elasmobranchs. This suggests that the observation in temperate waters that marine fishes have higher levels of *n*-3 fatty acids and *n*-3/*n*-6 ratios than freshwater fishes may not hold true in tropical waters, at least in elasmobranchs. It also suggests that plasma NEFA are little affected by freshwater vs. seawater adaptation in elasmobranchs. Likewise, we found that plasma NEFA composition and levels were not markedly affected by salinity acclimation (2 weeks) in the euryhaline stingray *Himantura signifer*. However, in contrast to our comparisons of freshwater-adapted vs. marine species, the level of *n*-3 fatty acids and the *n*-3/*n*-6 ratio were observed to significantly decrease, indicating a potential role of *n*-3 fatty acids in salinity acclimation in *H. signifer*.

Keywords: Plasma; Non-esterified fatty acids; Lipids; Elasmobranch fish; Salinity acclimation; *n*-3/*n*-6 ratio; Tropical; Temperate

1. Introduction

Elasmobranchs are an ancient group of fishes that have an unusual metabolic organization characterized by heavy utilization of ketone bodies as oxidative fuel in the non-starved state and a low capacity for lipid oxidation in muscle (Speers-Roesch et al., 2006). Probably not coincidentally, elasmobranchs lack an albumin-like protein in the blood (Metcalf and Gemmell, 2005) and possess low levels of plasma non-esterified fatty acids (NEFA) (Ballantyne et al., 1993; Speers-Roesch et al., 2006), which represent a metabolically dynamic fraction of lipid in blood. Studies on teleosts and other ray-finned fishes (actinopterygians) have shown that the levels and composition of plasma NEFA reflect nutritional state and diet and indicate those fatty acids mobilized from extrahepatic or hepatic lipid stores for oxidation and certain anabolic processes in peripheral tissues (Henderson and Tocher, 1987). The physiological relevance of plasma NEFA in actinopterygians is supported by the few studies that have examined the effects on plasma NEFA of environmental factors such as temperature (e.g. Haman et al., 1997) and salinity (e.g. Jarvis and Ballantyne, 2003). Even less is known about how plasma NEFA composition is shaped by the environment in which a fish lives, especially in elasmobranchs. The aim of the present study, therefore, was to shed light on this question, via 1) comparisons of species living in separate ecosystems with different average environmental temperature (cold temperate vs. tropical oceans), 2) comparisons of species adapted to separate ecosystems with different environmental

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salinities (ocean vs. freshwater), and, 3) in relation to goal two, an evaluation of the effect of salinity acclimation on a euryhaline species.

The first goal of the present study was to investigate whether or not plasma NEFA composition varies with latitude in elasmobranchs, by comparison of species inhabiting cold temperate waters with those living in tropical waters. In particular, we were interested in the essential fatty acids (EFA) docosahexaenoic acid (DHA, 22:6*n*-3), eicosapentaenoic acid (EPA, 20:5*n*-3), and arachidonic acid (AA, 20:4*n*-6). These EFA and their elongation products play important physiological roles in fish, including membrane structure and function, reproduction, and eicosanoid metabolism (Tocher, 2003). One of the distinctive features of plasma NEFA (and tissue fatty acid composition) in temperate marine teleosts is a high ratio of *n*-3/*n*-6 fatty acids (4.7 to 14.4) due to elevated levels of DHA and EPA, which are common in cold-water food chains and have important roles in the function of cold-adapted membranes in fishes (Henderson and Tocher, 1987; Hazel and Williams, 1990; Ballantyne et al., 1993). The tissue lipids of tropical marine teleosts, on the other hand, contain less DHA and more AA and its elongation product docosatetraenoic acid (22:4*n*-6), resulting in lower *n*-3/*n*-6 ratios (Sinclair et al., 1983; Gibson et al., 1984; Hansel et al., 1993). In fact, Sinclair et al. (1984) demonstrated that the relative *n*-6 fatty acid content in fishes increased as sampling occurred closer to the equator. Whether or not these trends also exist in lipids from elasmobranchs is questionable, because Ballantyne et al. (1993) found relatively low *n*-3/*n*-6 ratios in plasma NEFA of some temperate marine elasmobranchs. To resolve this issue and provide insight into lipid metabolism of elasmobranchs from different thermal environments, we tested the hypothesis that temperate elasmobranchs possess a higher *n*-3/*n*-6 ratio in plasma NEFA compared with tropical species.

The second goal of the present study was to evaluate how plasma NEFA composition, in particular EFA, differs between tropical elasmobranchs that are adapted to seawater and those that are adapted to life in freshwater. In temperate freshwater teleosts, lower *n*-3/*n*-6 ratios (0.5 to 3.8) are found due to greater input of terrestrial plant-derived fatty acids, which have relatively higher levels of *n*-6 vs. *n*-3 fatty acids (Henderson and Tocher, 1987; Ballantyne et al., 1993). The *n*-3/*n*-6 ratio has therefore been suggested as a distinguishing feature between marine and freshwater teleosts (Henderson and Tocher, 1987). However, there is reason to hypothesize that this general rule does not hold true for comparisons of plasma NEFA or lipid composition between freshwater and marine fishes from tropical waters because, as previously mentioned, tissue lipids of warm-water marine teleosts contain less DHA and more AA and 22:4*n*-6, resulting in low *n*-3/*n*-6 ratios (Sinclair et al., 1983; Gibson et al., 1984; Hansel et al., 1993). We tested this hypothesis and evaluated the influence of adaptation to differing environmental salinity on plasma NEFA by comparing plasma NEFA composition between two tropical marine elasmobranchs and two tropical freshwater elasmobranchs.

To further investigate the effects of salinity on plasma NEFA in elasmobranchs, we measured the effects of a 2 week acclimation to half-strength seawater (15‰) on the levels and composition of plasma NEFA in the tropical freshwater euryhaline stingray *Himantura signifer*. The effects of salinity acclimation on plasma NEFA levels in an elasmobranch have not been previously measured, and are poorly known in other fishes. This experiment allowed us to evaluate whether acute salinity acclimation causes changes in the *n*-3/*n*-6 ratio in plasma NEFA, and whether these changes are similar at all to the patterns seen in lipids of freshwater-adapted vs. marine elasmobranchs. Changes in *n*-3 fatty acids, in particular, have been observed to occur in phospholipids and neutral lipids of some fishes during salinity acclimation although the exact functional role is unclear (e.g. Leray et al., 1984; Cordier et al., 2002; Martinez-Álvarez et al., 2005). Plasma NEFA also have been suggested in sturgeon to be an important fuel source for costly osmoregulatory processes during salinity acclimation (Jarvis and Ballantyne, 2003). Osmoregulatory tissues (e.g. rectal gland, kidney) in elasmobranchs have a high capacity for lipid oxidation (Speers-Roesch et al., 2006), so we hypothesized that levels of total NEFA and individual NEFA important in fatty acid oxidation would increase during salinity acclimation of *H. signifer*.

2. Materials and methods

2.1. Animals, salinity acclimation of H. signifer, and sampling

Spiny dogfish (*Squalus acanthias*) (temperate marine) (1.3 ± 0.1 kg, mean ± SEM) and little skate (*Leucoraja erinacea*) (temperate marine) (657 ± 73 g) were obtained by otter trawl (70–100 m depth) from *R/V W.B. Scott* in Passamaquoddy Bay, New Brunswick, in August, 2004. Fishes were maintained in flow-through seawater aquaria at Huntsman Marine Science Centre (St. Andrews, NB) and sampled within 2 days of capture. The dogfishes were all male; the little skates were of mixed sex. Examination of stomach contents showed all fishes had been feeding. Water temperature could not be measured at trawl depth (70–100 m) but available oceanographic data suggest that the temperature was approximately 8 °C.

Longnose skate (*Raja rhina*) (temperate marine) (7.6 ± 1.5 kg) and *Bathyraja* sp. (temperate marine) (1.2 and 3.4 kg) 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. Animals were of mixed sexes. Responsive animals were sampled upon retrieval of the trawl contents. Examination of stomach contents showed that the animals had been feeding.

Amazonian ocellate river stingray (*Potamotrygon motoro*) (tropical freshwater) (69 ± 4 g) and white-edge whip ray (*H. signifer*) (tropical euryhaline freshwater) (139 ± 36 g) were obtained in July 2004 from a fish farm in Singapore, where they were fed bloodworms (freshwater chironomid larvae); blue-spotted ribbontail stingray (*Taeinura lymma*) (tropical marine) (480 ± 33 g) and brownbanded bamboo shark (*Chiloscyllium punctatum*) (tropical marine) (1988 ± 221 g) were purchased at a live fish market in Singapore about 6 h after being caught in the ocean in July 2004. All species were of mixed sexes. Holding

conditions were as described by Tam et al. (2003) and Speers-Roesch et al. (2006). Briefly, animals were held at 25 °C in plastic aquaria (about 3 animals per aquarium) at the National University of Singapore (NUS) under a 12:12 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 same 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 sampled after 14 days of acclimation. Sampling the control freshwater group at the end of the acclimation period was done in part to obviate possible effects of food deprivation on plasma NEFA. In any case, Zammit and Newsholme (1979) showed minimal effects of long-term starvation on plasma NEFA in an elasmobranch.

Blood was drawn into heparinized syringes by cardiac (*H. signifer*, *L. erinacea*, *P. motoro*, *T. lymma*) or caudal puncture (*Bathyraja* sp., *C. punctatum*, *R. rhina*, *S. acanthias*). 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. All sampling techniques are consistent with accepted procedures for analyses of plasma NEFA in fishes and the small variation in sampling period is unlikely to have had any major effects on plasma NEFA composition.

2.2. Measurement of plasma non-esterified fatty acids (NEFA)

Plasma NEFA were methylated as described in 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. Fatty acids were identified by comparing their retention times to those of known standards (GLC 463 augmented with 22:5*n*-6 and 23:0, Nu-Check Prep, Elysian, MN, USA). Absolute amounts of fatty acids were calculated by adding a known amount 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.

2.3. Statistical analysis

Between species comparisons of the NEFA parameters of interest were carried out using one-way ANOVA with Tukey's test. Data was log transformed prior to ANOVA if unequal variances were found (Zar, 1999). For multi-species comparisons, data for *Bathyraja* sp. was omitted due to the small sample size for that species; also, data for *H. signifer* in brackish water was not included in these analyses. Comparisons of total and individual NEFA 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.

Table 1
Percentages (mean±SEM) of individual non-esterified fatty acids (NEFA) and total NEFA (mean±SEM) (nmol mL^{-1}) in plasma of tropical marine (SW) and freshwater (FW) elasmobranchs: ocellate river stingray (*Potamotrygon motoro*) (FW), blue-spotted ribbontail stingray (*Taeniura lymma*) (SW), and brownbanded bamboo shark (*Chiloscyllium punctatum*) (SW)

	P. motoro	*T. lymma*	*C. punctatum*
	Tropical FW	Tropical SW	Tropical SW
	(*n*=7)	(*n*=4)	(*n*=5)
Fatty acid	Percentages (% by mol)	Percentages (% by mol)	Percentages (% by mol)
14:0	2.11±0.37	1.99±0.32	1.15±0.07
14:1	1.37±0.22	0.89±0.07	0.24±0.20
16:0	21.2±2.7	24.5±0.6	21.5±1.6
16:1	5.00±0.47	4.71±0.52	2.30±0.25
18:0	8.51±0.95	13.0±0.1	10.3±0.9
18:1	20.1±0.7	12.1±0.4	16.5±0.7
18:2*n*-6	7.17±1.22	1.30±0.07	0.39±0.11
18:3*n*-3	4.69±0.78	ND	0.08±0.08
18:4*n*-3	ND	ND	ND
20:0	1.04±0.49	ND	0.68±0.15
20:1	2.42±1.50	2.39±0.28	0.85±0.30
20:2*n*-6	0.34±0.26	0.92±0.07	0.47±0.21
20:3*n*-6	0.30±0.17	0.25±0.18	0.24±0.11
20:4*n*-6	7.70±0.86	10.2±0.8	14.0±0.6
20:3*n*-3	0.26±0.26	ND	0.07±0.07
20:4*n*-3	1.76±1.61	0.25±0.17	0.16±0.11
20:5*n*-3	2.88±0.51	3.78±0.20	2.38±0.63
22:0	2.31±0.90	0.56±0.23	0.09±0.06
22:1	2.17±0.51	1.25±0.10	1.28±0.26
23:0	1.04±0.42	0.48±0.12	ND
22:2*n*-6	0.84±0.74	ND	ND
22:4*n*-6	1.21±0.76	7.02±0.39	7.15±0.67
22:5*n*-6	1.23±0.82	2.95±0.29	1.73±0.20
22:5*n*-3	1.07±0.60	2.59±0.07	3.38±0.19
22:6*n*-3	1.64±0.25	8.92±0.72	15.1±2.6
24:0	1.62±0.69	ND	ND
24:1	ND	ND	ND
Total (% by mol)	100	100	100
Total (nmol·mL^{-1})	105.7±20.2 [a]	215.7±27.6 [a]	171.6±32.0 [a]
Total saturates (% by mol)	37.9±2.7	40.5±0.9	33.7±2.3
Total monoenes (% by mol)	31.0±1.2	21.3±0.7	21.2±1.0
Total polyenes (% by mol)	31.1±2.8	38.1±1.4	45.1±2.7
n-3 polyenes (% by mol)	12.3±1.3	15.6±0.9	21.1±3.3
n-6 polyenes (% by mol)	18.8±2.1	22.6±1.0	23.9±0.7
n-3/*n*-6	0.68±0.08	0.70±0.06	0.89±0.16
Monoenes/polyenes	1.06±0.12	0.57±0.05	0.48±0.04
Unsaturation index [b]	141.9±10.4	196.3±7.2	237.0±16.7
Mean chain length [c]	18.24±0.22	18.61±0.09	18.99±0.13

ND = not detectable.

[a] Total NEFA concentration from Speers-Roesch et al. (2006).

[b] $\Sigma m_i n_i$, where m_i is the mole percentage and n_i is the number of carbon–carbon double bonds in the fatty acid $_i$.

[c] $\Sigma f_i c_i$, where f_i is the mole fraction and c_i is the number of carbon atoms of fatty acid $_i$.

Table 2
Percentages (mean±SEM) of individual non-esterified fatty acids (NEFA) and total NEFA (mean±SEM) (nmol mL^{-1}) in plasma of temperate marine (SW) elasmobranchs: spiny dogfish (*Squalus acanthias*), little skate (*Leucoraja erinacea*), longnose skate (*Raja rhina*), and *Bathyraja* sp.

	S. acanthias	*L. erinacea*	*R. rhina*	*Bathyraja* sp.
	Temperate SW	Temperate SW	Temperate SW	Temperate SW
	(*n*=5)	(*n*=7)	(*n*=5)	(*n*=2)
Fatty acid	Percentages (% by mol)	Percentages (% by mol)	Percentages (% by mol)	Percentages (% by mol)
14:0	1.83±0.17	1.52±0.18	1.53±0.17	1.20±0.06
14:1	0.59±0.21	0.19±0.10	0.80±0.15	0.53±0.02
16:0	14.3±0.3	18.9±1.0	22.5±0.6	20.0±1.9
16:1	3.15±0.25	4.69±0.34	5.34±0.67	5.70±0.66
18:0	2.76±0.13	3.21±0.24	4.24±0.19	4.40±0.02
18:1	14.4±0.6	19.2±1.0	24.3±0.5	23.2±1.3
18:2*n*-6	2.64±0.61	1.67±0.14	1.19±0.10	1.05±0.03
18:3*n*-3	0.13±0.08	0.26±0.05	1.08±0.61	ND
18:4*n*-3	1.26±0.20	2.21±0.83	0.28±0.17	0.44±0.03
20:0	0.21±0.09	0.12±0.05	0.31±0.15	0.23±0.23
20:1	8.61±0.43	4.13±0.94	1.84±0.51	0.75±0.29
20:2*n*-6	0.11±0.07	0.44±0.05	ND	ND
20:3*n*-6	0.05±0.05	0.10±0.05	ND	ND
20:4*n*-6	3.01±0.27	3.18±0.23	4.60±0.43	5.96±0.99
20:3*n*-3	0.04±0.04	0.19±0.09	ND	ND
20:4*n*-3	8.71±4.75	0.44±0.05	0.06±0.06	0.38±0.01
20:5*n*-3	17.0±2.2	18.9±3.2	15.0±1.6	18.7±0.8
22:0	1.45±1.45	0.59±0.28	ND	ND
22:1	5.20±1.55	2.54±1.04	0.94±0.08	0.79±0.25
23:0	0.22±0.09	0.69±0.11	0.05±0.05	ND
22:2*n*-6	ND	ND	ND	ND
22:4*n*-6	0.32±0.13	0.59±0.10	0.21±0.21	0.64±0.07
22:5*n*-6	0.21±0.09	0.41±0.09	0.07±0.07	0.39±0.01
22:5*n*-3	1.87±0.24	1.73±0.14	1.52±0.17	1.50±0.12
22:6*n*-3	11.5±0.5	14.0±1.3	14.2±1.1	14.1±0.2
24:0	ND	ND	ND	ND
24:1	0.39±0.21	0.03±0.03	ND	ND
Total (% by mol)	100	100	100	100
Total (nmol·mL^{-1})	415.5±74.1	572.6±66.8	167.0±20.4	251.8±76.3
Total saturates	20.8±1.5	25.0±0.7	28.6±0.6	25.9±2.2
Total monoenes	32.4±1.3	30.8±2.5	33.2±0.7	31.0±2.1
Total polyenes	46.8±1.7	44.2±3.0	38.2±1.1	43.2±0.2
n-3 polyenes	40.5±2.5	37.8±3.0	32.1±1.0	35.1±0.9
n-6 polyenes	6.34±0.91	6.39±0.40	6.07±0.36	8.04±1.03
n-3/*n*-6	7.24±1.51	6.04±0.59	5.37±0.39	4.46±0.68
Monoenes/polyenes	0.70±0.05	0.75±0.12	0.88±0.04	0.72±0.05
Unsaturation index [a]	256.0±5.9	251.9±11.6	227.4±4.0	250.4±1.6
Mean chain length [b]	19.16±0.06	18.84±0.05	18.47±0.04	18.63±0.02

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

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

3. Results

Total plasma NEFA in the study species ranged from 105.7 nmol mL^{-1} in *P. motoro* to 572.6 nmol mL^{-1} in *L. erinacea* (Tables 1, 2 and 3). The amount of plasma NEFA was statistically similar between most species, though *L. erinacea* had higher levels than all other species except for *S. acanthias*.

The fatty acid composition of plasma NEFA is presented in Tables 1, 2, and 3. NEFA shorter than C14 were not detected. The composition of most fatty acids was relatively similar between species and showed no consistent correlation with the

Table 3
Percentages (mean±SEM) of individual non-esterified fatty acids (NEFA) and total NEFA (mean±SEM) (nmol mL^{-1}) in plasma of the tropical white-edge whip ray (*Himantura signifer*) in freshwater (FW) and acclimated to half-strength (15‰) seawater (1/2SW) for 2 weeks

	H. signifier	*H. signifier*
	Tropical FW	Tropical 1/2SW
	(*n*=9)	(*n*=6)
Fatty acid	Percentages (% by mol)	Percentages (% by mol)
14:0	1.51±0.41	1.54±0.53
14:1	1.93±0.61	1.64±0.54
16:0	16.3±1.4	17.9±1.4
16:1	4.21±0.56	5.32±0.66
18:0	5.61±0.66	4.92±0.99
18:1	17.0±1.0	20.3±1.9
18:2*n*-6	1.90±0.29	1.43±0.39
18:3*n*-3	0.86±0.45	0.59±0.24
18:4*n*-3	ND	ND
20:0	0.80±0.32	0.30±0.20
20:1	1.17±1.00	0.47±0.47
20:2*n*-6	1.43±0.52	2.94±1.08
20:3*n*-6	0.87±0.65	ND
20:4*n*-6	19.0±2.2	18.8±1.2
20:3*n*-3	ND	ND
20:4*n*-3	1.35±0.63	0.16±0.16
20:5*n*-3	6.84±1.10	5.73±1.03
22:0	0.84±0.40	1.18±0.79
22:1	2.71±0.78	2.00±0.71
23:0	0.83±0.50	0.75±0.50
22:2*n*-6	0.21±0.13	0.40±0.20
22:4*n*-6	3.96±0.76	4.51±0.87
22:5*n*-6	2.74±0.61	3.63±0.64
22:5*n*-3	2.54±0.31	2.64±0.29
22:6*n*-3	5.47±1.09	3.28±0.36
24:0	ND	ND
24:1	ND	ND
Total (% by mol)	100	100
Total (nmol·mL^{-1})	122.8±40.9 [a]	154.1±46.0 [a]
Total saturates (% by mol)	25.8±1.8	26.6±2.3
Total monoenes (% by mol)	26.9±2.3	29.8±2.2
Total polyenes (% by mol)	47.2±3.6	44.1±3.2
n-3 polyenes (% by mol)	17.2±1.5	12.4±1.3 *
n-6 polyenes (% by mol)	30.0±2.5	31.8±2.5
n-3/*n*-6	0.60±0.06	0.42±0.04 *
Monoenes/polyenes	0.66±0.15	0.74±0.12
Unsaturation index [b]	230.2±15.1	209.7±12.3
Mean Chain Length [c]	18.87±0.10	18.67±0.11

ND = not detectable.
[a] Total NEFA concentration from Speers-Roesch et al. (2006).
[b] $\Sigma m_i n_i$, where m_i is the mole percentage and n_i is the number of carbon–carbon double bonds in the fatty acid $_i$.
[c] $\Sigma f_i c_i$, where f_i is the mole fraction and c_i is the number of carbon atoms of fatty acid $_i$.
* Denotes a significant difference between *H. signifer* (FW) and *H. signifer* (1/2SW) (Student's *t*-test, $p<0.05$).

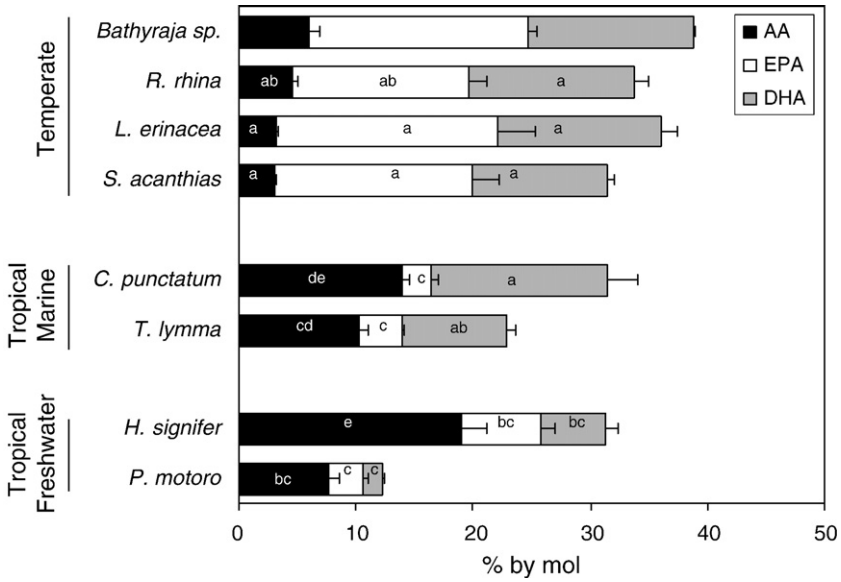

Fig. 1. Percentages (mean±SEM) of the essential fatty acids arachidonic acid (AA, 20:4*n*-6), eicosapentaenoic acid (EPA, 20:5*n*-3), and docosahexaenoic acid (DHA, 22:6*n*-3) in plasma non-esterified fatty acids of elasmobranchs from temperate marine, tropical marine, and tropical freshwater environments. See captions for Tables 1, 2, and 3 for full species names and sample sizes. For each fatty acid, values with the same letter are not statistically different between species (one-way ANOVA with Tukey's, $p<0.05$). *Bathyraja* sp. was omitted from statistical analyses due to small sample size.

species' habitat (i.e. tropical vs. cold water or freshwater vs. seawater). The most striking exceptions were the EFA, and the resultant *n*-3/*n*-6 ratio. Whereas the percentages of total saturates, monoenes, and polyenes were not associated with environment (Tables 1, 2, and 3), the relative amounts of EFA were. DHA was at least two-fold higher in the marine species, and there was no difference between the tropical vs. temperate marine species (Fig. 1). EPA was several-fold higher in the cold-water species vs. both marine and freshwater tropical species (the tropical species had similar levels to one another) (Fig. 1). (Although EPA levels in *R. rhina* vs. *H. signifer* were not statistically different, the *p* value was nearly significant (0.06).) AA was lower in the temperate species vs. the tropical species, and was not consistently different between fresh- and seawater species (Fig. 1). (The means of AA in *P. motoro* and *R. rhina* were nearly significantly different, $p=0.08$.) Largely due to these major differences in EFA composition, a striking difference was observed in the *n*-3/*n*-6 ratios between tropical and cold-water elasmobranchs, with the latter having much higher values (Fig. 2). Fresh- and seawater tropical elasmobranchs

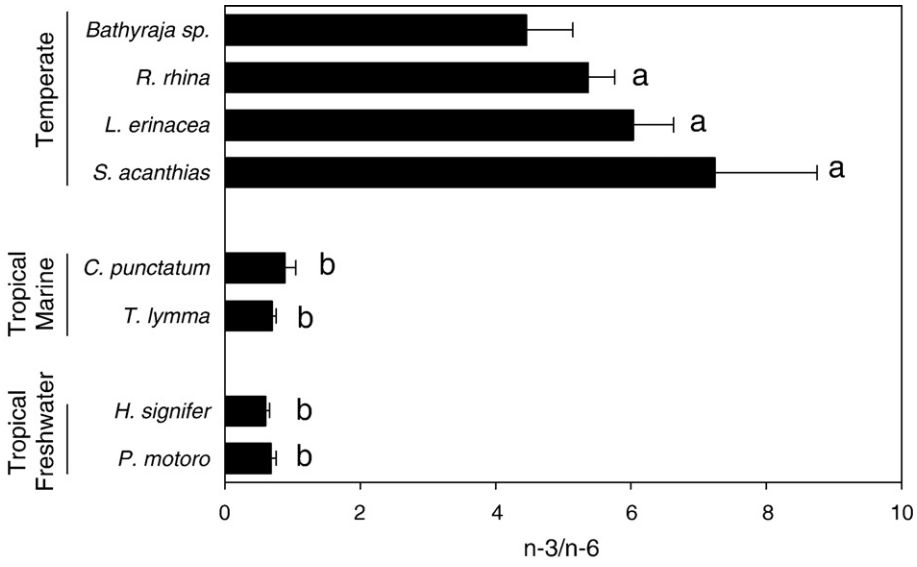

Fig. 2. *n*-3/*n*-6 ratio (mean±SEM) of plasma non-esterified fatty acids of elasmobranchs from temperate marine, tropical marine, and tropical freshwater environments. See captions for Tables 1, 2, and 3 for full species names and sample sizes. Values with the same letter are not statistically different (one-way ANOVA with Tukey's, $p<0.05$). *Bathyraja* sp. was omitted from statistical analyses due to small sample size.

had similar *n*-3/*n*-6 ratios. Correspondingly, levels of total *n*-3 fatty acids were significantly higher in all temperate species compared with tropical species with the exception of *R. rhina* vs. *C. punctatum* (this comparison was significant if critical value is $p<0.10$) (Tables 1, 2, and 3). Likewise, levels of *n*-6 fatty acids were significantly lower in all tropical species compared with any of the temperate species (Tables 1, 2, and 3). Aside from AA, 22:5*n*-6 and especially 22:4*n*-6 were significant contributors to the higher percentage of *n*-6 fatty acids in tropical elasmobranch plasma NEFA, whereas levels of these fatty acids were low in the temperate species (Tables 1, 2, and 3).

Neither total plasma NEFA nor concentrations or percentages of individual fatty acid were significantly different between freshwater *H. signifer* and those in brackish water (Table 3). The percentage of *n*-3 fatty acids and the ratio of *n*-3/*n*-6 fatty acids decreased significantly in the salinity acclimated *H. signifer* compared with the animals in freshwater (Table 3).

4. Discussion

4.1. Levels of plasma NEFA in elasmobranchs

The measurements of plasma NEFA in the present study are the first made on freshwater or tropical marine elasmobranchs. Total plasma NEFA concentrations were similar to the levels seen in temperate marine elasmobranchs, including the species from the present study as well as those surveyed by Ballantyne et al. (1993) (193–399 nmol mL^{-1}). Our measurements of plasma NEFA in spiny dogfish are similar to those reported by Ballantyne et al. (1993) for the same species. The level of total plasma NEFA we observed in little skate is the highest yet reported for an elasmobranch, but it is still about half the amount usually seen in teleosts (Ballantyne et al., 1993). Our results confirm Ballantyne et al.'s (1993) finding that circulating levels of NEFA in elasmobranchs are several-fold lower than in teleosts.

Although plasma NEFA levels are low in elasmobranchs, they still exceed the solubility of the fatty acids in water (Windholz, 1983). Recently it has been conclusively shown that marine elasmobranchs lack albumin (Metcalf and Gemmell, 2005), so it is likely that NEFA are carried entirely by lipoproteins (see Lauter et al., 1968). A lack of albumin is not a likely explanation for why elasmobranchs possess relatively low levels of plasma NEFA, as a number of teleosts (and perhaps most) also lack albumin and utilize lipoproteins to transport NEFA (De Smet et al., 1998; Metcalf et al., 1999a; Metcalf et al., 1999b; Metcalf and Gemmell, 2005). Rather, it is possibly a consequence of the fact that, unlike teleosts, elasmobranchs do not rely on fatty acid oxidation in skeletal muscle, and possess a reduced or non-existent capacity for the same in heart (Speers-Roesch et al., 2006), thus lessening the requirement for plasma NEFA.

4.2. NEFA composition and environment

The contributions of saturated, monounsaturated, and polyunsaturated fatty acids to total NEFA as well as the monoenes/polyenes ratio were similar between the tropical elasmobranchs and the temperate marine elasmobranchs. In all species the individual fatty acids comprising the bulk of plasma NEFA included palmitic acid (16:0), palmitoleic acid (16:1), stearic acid (18:0), and oleic acid (18:1), which are preferentially oxidized in fishes (Tocher, 2003). The physiologically essential *n*-3 and *n*-6 polyunsaturated fatty acids such as docosahexaenoic acid (DHA, 22:6*n*-3), eicosapentaenoic acid (EPA, 20:5*n*-3), and arachidonic acid (AA, 20:4*n*-6) were also well represented in all species. However, while the relative contributions of the saturated and monoenoic C16 and C18 fatty acids were similar between the tropical elasmobranchs and the temperate marine elasmobranchs, the tropical elasmobranchs possessed more AA and less DHA (Fig. 1). The tropical elasmobranchs, especially the marine *C. punctatum* and *T. lymma*, also had relatively high levels of docosatetraenoic acid (22:4*n*-6), the elongation product of AA, whereas in temperate marine elasmobranchs this fatty acid is either absent or present in low levels (<1%) in plasma NEFA (present study; Ballantyne et al., 1993). These results are consistent with previous work done on tropical marine teleosts, which showed that their lipids also include less DHA, and more AA and 22:4*n*-6 than temperate species (Sinclair et al., 1983; Gibson et al., 1984; Hansel et al., 1993). In elasmobranchs, high levels of 22:4*n*-6 in plasma NEFA, along with a high AA/DHA ratio, could be considered a marker of a tropical species. Further studies should address this possibility.

An important consequence of the lower DHA and greater AA and 22:4*n*-6 levels found in the tropical elasmobranchs is that, as we hypothesized, plasma NEFA *n*-3/*n*-6 ratios in the tropical elasmobranchs were lower by two-fold or more compared with those in the temperate marine elasmobranchs (present study; Ballantyne et al., 1993) (Fig. 2). Similarly low *n*-3/*n*-6 ratios due in part to higher AA and lower DHA levels also have been observed in serum NEFA of a tropical stingray from the Caribbean (*Dasyatis americana*) (Semeniuk et al., 2007). Lipids of tropical marine teleosts (possessing less DHA and more AA and 22:4*n*-6) also have low *n*-3/*n*-6 ratios relative to cold-water teleosts (Sinclair et al., 1983; Gibson et al., 1984; Hansel et al., 1993). Sinclair et al. (1984) demonstrated that the relative *n*-6 fatty acid content in fishes increased as sampling occurred closer to the equator, suggesting that *n*-6 fatty acids (especially AA and its essential precursor 18:2*n*-6) are more common in inshore tropical marine vs. cold-water marine food webs. The results of the present study support this contention, although 18:2*n*-6 levels were not different between the tropical and temperate elasmobranchs. The accumulation of *n*-3 fatty acids in cold temperate fishes may also be related to their role in maintaining proper membrane fluidity in cold-water ectothermic marine fishes (Hazel and Williams, 1990).

Interestingly, the *n*-3/*n*-6 ratio was as low (<1) in the tropical marine *T. lymma* and *C. punctatum* as in the tropical freshwater *P. motoro* and *H. signifer*, whereas a commonly cited difference between freshwater fishes and marine fishes (from high latitudes) is that the former possess low *n*-3/*n*-6 ratios (0.5 to 3.8) and the latter possess high *n*-3/*n*-6 ratios (4.7 to 14.4) in their lipids due to differences in terrestrial vs. marine input of plant-derived fatty acids (Henderson and Tocher, 1987). Our results support the hypothesis that this rule probably cannot be applied to elasmobranchs from tropical latitudes. Instead, our results suggest that DHA alone may be useful as a marker for marine vs. freshwater

elasmobranchs as it was higher in all the marine species. This finding is also of interest because it suggests that adaptation to freshwater vs. seawater has no major effect on most aspects of plasma NEFA composition in elasmobranchs, including AA and EPA levels and the *n*-3/*n*-6 ratio. Whether DHA levels are higher in marine elasmobranchs for any reason other than dietary input remains unknown.

In most cases, interspecies NEFA composition did not vary greatly. Of possible metabolic interest were the lower amounts of EPA and DHA and greater amount of their precursor 18:3*n*-3 in plasma from *P. motoro* relative to the other species. 18:2*n*-6, a precursor of AA, also was much higher in *P. motoro*. This may indicate a lessened synthesis of polyunsaturated fatty acids in this species. The high levels of AA in *H. signifer* may indicate an important role of this fatty acid in metabolism of this species, whereas saturates are lower in plasma NEFA and may be less important. *P. motoro* and *H. signifer* were fed the same food at the fish farm (freshwater bloodworms) so these differences in plasma NEFA composition probably reflect real differences in lipid metabolism rather than diet.

4.3. Plasma NEFA and salinity acclimation

A significant decrease in the percentage of *n*-3 fatty acids was observed in the plasma NEFA of salinity acclimated *H. signifer*, which resulted in a significant decrease in the *n*-3/*n*-6 ratio. This suggests that either: 1) *n*-3 fatty acids may be important during salinity acclimation and are utilized faster than they are mobilized, or, 2) *n*-3 fatty acids are not helpful during salinity acclimation and their mobilization from the liver is decreased. No change in the *n*-3/*n*-6 ratio was found in plasma NEFA from shortnose sturgeon or Arctic char during salinity acclimation (Jarvis and Ballantyne, 2003; Bystriansky et al., 2007). Studies on membrane phospholipid composition in tissues of salinity acclimated fishes show that concentrations of *n*-3 fatty acids can change, although in varying directions. During salinity acclimation, 22:6*n*-3 in tissue phospholipids decreases in some teleosts (Takeuchi et al., 1989; Cordier et al., 2002), but in rainbow trout intestinal brush border membrane phospholipids it increases (Leray et al., 1984) and total *n*-3 fatty acid content has been noted to increase in phospholipids from guppies (*Poecilia reticulata*) during salinity exposure (Daikoku et al., 1982). In the Adriatic sturgeon (*Acipenser naccarii*), total gill phospholipids also show increases in 22:6*n*-3 content, total *n*-3 content, and the *n*-3/*n*-6 ratio upon seawater acclimation (Martinez-Álvarez et al., 2005). A role for *n*-3 fatty acids in enhancing ionoregulation in tissues such as gill has been suggested (Di Costanzo et al., 1983; Martinez-Álvarez et al., 2005). If this is the case in *H. signifer* it might explain the decreases in *n*-3 fatty acids and *n*-3/*n*-6 ratio upon salinity acclimation. Alternatively, an argument could be made that *n*-3 fatty acids should be preferentially excluded from membrane phospholipids and mobilization decreased during salinity acclimation in *H. signifer*, because at this time the animal is actively synthesizing urea (Tam et al., 2003). A low *n*-3/*n*-6 ratio in mitochondrial membranes of elasmobranchs has been proposed to be an adaptation to urea's fluidizing effects on biological membranes (Glemet and Ballantyne, 1996). Fatty acid analyses of membrane phospholipid composition of tissues from salinity acclimated *H. signifer* are needed. Finally, our results for the salinity acclimation do not appear to support a functional importance for the lower *n*-3/*n*-6 ratio seen in freshwater-adapted vs. marine fishes. In fact we observed a decrease in this ratio with increased salinity. The lower ratio in freshwater fishes largely may be due to dietary input. Further studies are clearly needed, however, especially considering the variable results from previous studies looking at *n*-3 fatty acids in salinity acclimation of fish.

The few studies that have examined plasma NEFA during salinity acclimation in fishes indicate that the importance of mobilized lipids during salinity acclimation likely varies among fishes. In shortnose sturgeon (*Acipenser brevirostrum*), a substantial 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 significant change in total plasma NEFA concentration or fatty acid composition after 96 h of seawater acclimation (Bystriansky et al., 2007). With the exception of *n*-3 fatty acids and the *n*-3/*n*-6 ratio, and contrary to our hypothesis, no significant changes in the percentages or absolute concentrations (data not shown for individual NEFA) of total or individual plasma NEFA were seen in salinity acclimated *H. signifer* in the present study. This suggests that lipids, although a rich energy source that apparently can be oxidized in osmoregulatory tissues (e.g. kidney, rectal gland) of elasmobranchs (Speers-Roesch et al., 2006), may not serve a major role in meeting any increased energetic costs associated with salinity acclimation in *H. signifer*. However, it is worth noting when considering these reports, as well as the present study, that changes in NEFA flux can occur without changes in concentration (Haman et al., 1997). Further studies, including measurements of NEFA flux, are needed to assess the importance of lipids in salinity acclimation in fishes.

The influence of the environment on plasma NEFA in elasmobranchs is significant, especially with regard to EFA and the *n*-3/*n*-6 ratio. Our results demonstrate that *n*-3/*n*-6 ratios in tropical marine elasmobranchs are lower by two-fold or more compared with temperate marine elasmobranchs, because of higher levels of AA and 22:4*n*-6, and less DHA, in the tropical species. These results are similar to earlier studies on lipids in teleosts, and may largely be due to dietary input. *n*-3/*n*-6 ratios and levels of EFA were similar between tropical freshwater and tropical marine elasmobranchs. This suggests that the observation in cold temperate waters that marine fishes have much higher levels of *n*-3 fatty acids and *n*-3/*n*-6 ratios than freshwater fishes may not hold true in warm tropical waters, at least in elasmobranchs. It also suggests that plasma NEFA are little affected by freshwater vs. seawater adaptation in elasmobranchs. Likewise, during salinity acclimation we found that NEFA composition and levels are not markedly affected by salinity acclimation in the euryhaline *H. signifer*. However, in contrast to our comparisons of freshwater-adapted vs. marine species, the level of *n*-3 fatty acids, and thus the *n*-3/*n*-6 ratio, was observed to significantly decrease, indicating a potential role of *n*-3 fatty acids in salinity acclimation in *H. signifer*. Further studies are needed to evaluate the mechanistic and functional basis of our observations.

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