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Digestive enzyme activities are higher in the shortfin mako shark, *Isurus oxyrinchus*, than in ectothermic sharks as a result of visceral endothermy

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Abstract Lamnid sharks are regionally endothermic fishes that maintain visceral temperatures elevated above the ambient water temperature. Visceral endothermy is thought to increase rates of digestion and food processing and allow thermal niche expansion. We tested the hypothesis that, at in vivo temperatures, the endothermic shortfin mako shark, *Isurus oxyrinchus*, has higher specific activities of three digestive enzymes—gastric pepsin and pancreatic trypsin and lipase—than the thresher shark, *Alopias vulpinus*, and the blue shark, *Prionace glauca*, neither of which can maintain elevated visceral temperatures. Homogenized stomach or pancreas tissue obtained from sharks collected by pelagic longline was incubated at both 15 and 25 °C, at saturating substrate concentrations, to quantify tissue enzymatic activity. The mako had significantly higher enzyme activities at 25 °C than did the thresher and blue sharks at 15 °C. This difference was not a simple temperature effect, because at 25 °C the mako had higher trypsin activity than the blue shark and higher activities for all enzymes than the thresher shark. We also hypothesized that the thermal coefficient, or Q_{10} value, would be higher for the mako shark than for the thresher and blue sharks because of its more stable visceral temperature. However, the mako and thresher sharks had similar Q_{10} values for all enzymes, perhaps because of their closer phylogenetic relationship. The higher in vivo digestive enzyme activities in the mako shark should result in higher rates of food processing and may represent a selective advantage of regional visceral endothermy.

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List of symbols

A_{xxx}	Absorbance at a specific wavelength of XXXnm
ANOVA	Analysis of variance
ε	Extinction coefficient
FL	Fork length
Q_{10}	Temperature coefficient = (enzyme activity$_{25\ °C}$/enzyme activity$_{15\ °C}$)

U	Unit of enzyme activity = μmol of substrate converted to product min^{-1}
s.e.m.	Standard error of the mean

Introduction

Although almost all fish species are ectothermic, a small number of regionally endothermic (or spatially heterothermic) fishes can maintain the temperature of specific tissues elevated above ambient water temperature (reviewed in Brill et al. 1994; Dickson and Graham 2004; Goldman et al. 2004). In those fishes, vascular countercurrent heat exchangers, or *retia mirabilia*, allow metabolic heat generated by the tissues to be retained at its site of production instead of being lost to the environment at the gills (Carey and Teal 1966; Carey et al. 1971, 1981, 1984, 1985). The known regionally endothermic fishes (tunas, billfishes, opahs, lamnid sharks, and thresher sharks) are pelagic predators that swim continuously, migrate over long distances, and encounter large, rapid changes in ambient temperature during repeated dives below the thermocline to pursue prey (e.g., Carey and Lawson 1973; Block et al. 2001; Boustany et al. 2002; Bernal and Sepulveda 2005; Runcie et al. 2009; Boustany et al. 2010). Both niche expansion and increased physiological performance of the warmed tissues have been hypothesized to be selective advantages to explain the convergent evolution of regional endothermy in fishes (reviewed in Block and Finnerty 1994; Dickson and Graham 2004), but limited data exist to test these hypotheses.

The present study focuses on a possible physiological benefit resulting from the ability of sharks in the family Lamnidae to maintain elevated and stable visceral temperatures (Carey et al. 1981; Holts and Bedford 1993; Goldman 1997; Goldman et al. 2004; Sepulveda et al. 2004; Stevens et al. 2010). Among elasmobranch fishes, visceral endothermy occurs only in the five lamnid shark species: shortfin mako, *Isurus oxyrinchus* (hereafter referred to as the "mako"), longfin mako, *Isurus paucus*, white, *Carcharodon carcharias*, porbeagle, *Lamna nasus*, and salmon, *Lamna ditropis* (Carey and Teal 1969; Carey et al. 1981, 1985; Goldman 1997; Goldman et al. 2004). The visceral blood supply of lamnid sharks passes through a single large suprahepatic *rete mirabile* (Carey et al. 1981; Fudge and Stevens 1996). Oxygenated blood from the gills travels posteriorly in large pericardial arteries, which penetrate a hepatic venous sinus located on the ventral surface of the esophagus and repeatedly branch until the venous sinus is filled with small arteries, thus forming the suprahepatic *rete*. On the posterior end of the *rete*, the small arteries coalesce into a collecting trunk, which branches off arteries that supply warm blood to the liver, stomach, spleen, and spiral valve intestine (Carey et al. 1981; Fudge and Stevens 1996). The suprahepatic *rete* allows lamnid sharks to retain the heat generated by digestion and assimilation and to maintain visceral temperatures elevated 4–14 °C above the ambient water temperature (Carey et al. 1981; Goldman et al. 2004; Sepulveda et al. 2004). Large adult lamnid sharks are apparently unique among fishes in that they maintain a stable stomach temperature (Carey et al. 1971; Goldman 1997; Goldman et al. 2004).

Lamnid sharks occupy continental to oceanic habitats in all oceans from tropical to boreal waters and make seasonal horizontal and diel vertical migrations (Carey et al. 1981; Compagno 2001; Boustany et al. 2002; Weng et al. 2005, 2007a, b). Carey et al. (1971) proposed that elevated visceral temperatures would result in higher rates of food processing, which would fuel the high energy demands resulting from the high metabolic rates and activity levels of endothermic fishes. Higher food processing rates would enable lamnid sharks to assimilate prey rapidly and convert food into the lipid reserves necessary for migrations to localized feeding and breeding grounds. With stable visceral temperatures, rates of digestion and assimilation are less affected by rapid changes in ambient temperature during dives. Both effects would contribute to niche expansion because these fishes would be able to exploit colder environments below the thermocline and at higher latitudes (Block and Finnerty 1994; Dickson and Graham 2004; Weng et al. 2005).

In order to isolate visceral endothermy as a variable and test for physiological benefits of visceral endothermy, we compared the mako, *I. oxyrinchus* Rafinesque, to the thresher, *Alopias vulpinus* (Bonnaterre), and blue shark, *Prionace glauca* (Linnaeus), two pelagic species that are related to the lamnids but are unable to maintain elevated visceral temperatures

and that were locally available and possible to sample. The thresher shark is closely related to the lamnid sharks [Order Lamniformes: Family Alopiidae (Compagno 1990; Naylor et al. 1997; Shimada 2005; Velez-Zuazo and Agnarsson 2011)], does not elevate visceral temperatures (C. Sepulveda pers. comm.), displays diel diving behavior (Stevens et al. 2010), and occasionally co-occurs with the mako in the Southern California Bight during the summer (Compagno 1984; Preti et al. 2001; Sepulveda et al. 2005). Because other lamniform sharks were not available, we also sampled the blue shark (Order Carcharhiniformes: Family Carcharhinidae) because the requiem sharks (Order Carcharhiniformes) and the carpet sharks (Order Orectolobiformes) comprise the sister taxon to the Order Lamniformes (Velez-Zuazo and Agnarsson 2011). The blue shark cannot elevate visceral temperatures, occupies the same pelagic habitat as the mako, and makes regular deep dives (Sciarrotta and Nelson 1977; Carey and Scharold 1990; Stevens et al. 2010). These three predators also have similar diets, which, for the mako shark, include jumbo squid, saury, sardine, mackerel, mullet, swordfish, bonito, tuna, yellowtail, market squid, and other sharks (Mannan et al. 1961; Maia et al. 2006; Clark et al. 2010; Preti et al. 2012). The thresher shark feeds upon sardines, anchovies, hake, mackerel, market squid, saury, rockfish, and pelagic crabs (Preti et al. 2001, 2012). The diet of the blue shark includes jumbo squid, armhook squid, pelagic octopuses, jewel squid, herring, salmon, mackerel, lumpfish, dogfish, cod, and, occasionally, seals (McChord and Campana 2003; Preti et al. 2012).

Food processing rates depend upon many factors, including prey acquisition, mastication, gut pH and temperature, digestive enzyme activity, microbial processing, and nutrient absorption (Clements and Raubenheimer 2006). Because we were unable to conduct studies on live sharks, we quantified the activity of three digestive enzymes—gastric pepsin and pancreatic trypsin and lipase—based on the high protein and lipid content of the carnivorous diets of the three shark species studied, and because these enzymes are known to be stable in frozen tissue samples (e.g., Chan et al. 2004). We hypothesized that the mako shark would have higher digestive enzyme activities than the thresher shark and the blue shark at in vivo visceral temperatures. If so, then our results would provide support for the idea that visceral endothermy conveys a potential selective advantage to regionally endothermic sharks by increasing digestive enzyme activities and presumably food processing rates. We compared enzyme activities at 25 °C in the mako with those at 15 °C in the thresher and blue sharks, approximating the average in vivo temperatures of the three species. The mako shark encounters water temperatures of 5–24 °C during dives while maintaining a stable visceral temperature of approximately 25 °C (Carey et al. 1981; Stevens et al. 2010; Abascal et al. 2011). Thresher and blue sharks experience water temperatures of 9–27 and 5–27 °C during dives, respectively (Weng et al. 2005; Cartamil et al. 2010a; Stevens et al. 2010; Patterson et al. 2011). In the northeastern Pacific these two species spend most of their time at ambient temperatures of 14–18 °C (Weng et al. 2005; Cartamil et al. 2010a, b).

We also hypothesized that the Q_{10} value, or the ratio between the enzyme activities at 25 and 15 °C, would be higher for the mako shark than for the thresher shark or the blue shark. We predicted that, if the digestive enzymes of endothermic fishes are modified to operate over a narrow range of temperatures, then reducing the assay temperature by 10 °C to a temperature outside of the in vivo temperature range of the mako would alter the enzyme activities by a greater degree than in the two ectothermic species, resulting in higher Q_{10} values (Angilletta 2009).

Methods

Experimental procedures

Tissue samples were collected from individual mako, thresher, and blue sharks during annual abundance surveys conducted from 2008 to 2011 in the Southern California Bight by National Marine Fisheries Service personnel of the Southwest Fisheries Science Center, La Jolla, CA. Samples ($\sim$5 g) of stomach, pancreas, and intestine (the latter used as a negative control) from each individual [mako ($N = 16$, 90–193 cm FL, 9–85 kg), blue ($N = 16$, 72–197 cm FL, 2–44 kg), and thresher ($N = 6$, 80–177 cm FL, 9–92 kg)] were dissected from sharks that were dead for less than 4 h, frozen in liquid nitrogen, then transported to California State University Fullerton on dry ice, and stored at −80 °C for up to 36 months. Subsamples ($\sim$0.5 g) of each tissue were homogenized in a tenfold dilution of

50 mM Tris–HCl buffer (pH 7.8 at 25 °C) using a chilled Kontes Duall ground-glass homogenizer. The homogenate was centrifuged at 0 °C for 10 min at 12,000 *g*, after which an aliquot (100 μL) of the supernatant was stored at −80 °C for later determination of soluble protein concentration. The remaining supernatant was stored on ice until the appropriate assay for pepsin, trypsin, or lipase was run. The extracted enzymes were activated, if necessary, and then incubated in triplicate at 15 and 25 °C with the appropriate substrate at saturating concentrations (as determined by preliminary assays). Digestive enzyme activities are reported in μmol of substrate converted to product per minute (international units, *U*) per gram of tissue wet mass, or $U\ g^{-1}$ tissue.

Pepsin Assay [modified from Anson (1938)]

Pepsin is a gastric proteolytic enzyme that cleaves peptide bonds between hydrophobic and aromatic amino acids within proteins. In this endpoint assay, pepsin from shark stomach tissue cleaves tyrosine and tryptophan from the hemoglobin substrate. The tyrosine released was quantified by measuring absorbance at 280 nm (A_{280}) in a diode-array spectrophotometer (Hewlett-Packard 8452A, Palo Alto, CA, USA). A mixture of 100 μL of homogenized tissue supernatant and 100 μL of 2 % horse hemoglobin (0.155 mM final concentration) in 60 mM HCl (pH 2) was incubated at either 15 or 25 °C for 10 min. The reaction was stopped by adding 1000 μL of 5 % trichloroacetic acid, after which the mixture was centrifuged at 6000 *g* for 6 min at 4 °C, and A_{280} of the supernatant measured. The change in A_{280} of the sample (relative to a negative control with 50 mM Tris–HCl homogenization buffer substituted for tissue supernatant) during the 10-min incubation period, along with the extinction coefficient of the end product tyrosine ($\varepsilon_{tyrosine}$) of 1.25 $cm^2\ \mu mol^{-1}$ at 280 nm, was used to calculate the units of pepsin activity. One unit (*U*) of pepsin activity = one μmol of tyrosine liberated min^{-1}.

Trypsin Assay [modified from Preiser et al. (1975) and German et al. (2004)]

Trypsin is a pancreatic serine protease that cleaves peptide bonds after arginine and lysine residues. In this endpoint assay, trypsin from shark pancreas tissue cleaves p-nitroaniline from the substrate N-alpha-benzoyl-L-arginine p-nitroanilide, releasing p-nitroaniline. The assay sensitivity was increased using the Bratton Marshall reaction where a naphthyl group binds to the p-nitroaniline, producing p-nitroaniline-N-1-naphthyl which absorbs light at 550 nm. Trypsin is stored intracellularly in an inactive zymogen form. Therefore, it was first activated by incubating 300 μL of homogenized pancreas supernatant with 45 μL of enterokinase (4 U mL^{-1} in 40 mM succinate buffer, pH 5.6) for 15 min at room temperature. Next, 25 μL of the activated trypsin supernatant was mixed with 175 μL of 2 mM N-alpha-benzoyl-L-arginine p-nitroanilide (1.75 mM final concentration) suspended in 100 mM Tris buffer (pH 7.8 at 25 °C) and incubated at either 15 or 25 °C for 60 min. The reaction was stopped by adding 200 μL of 0.2 N HCl to the mixture, and then, the following three reagents were added at 3-min intervals: 50 μL of 0.1 % sodium nitrite, 50 μL of 0.5 % ammonium sulfamate, and 50 μL of 0.05 % N-1-naphthylethylenediamine in 95 % ethanol. The reaction proceeded at room temperature for 5 min, after which 100 μL of the supernatant was added to a Falcon #3075 flat-bottom 96-well microplate to measure the change in absorbance of the sample (relative to a negative control using 50 mM Tris–HCl homogenization buffer substituted for tissue supernatant) in a SPECTRAmax 190 microplate spectrophotometer (Molecular Devices, Sunnyvale, CA, USA). We determined that the change in A_{550} over time was linear over the entire 60-min reaction period by incubating the substrate (1.75 mM final concentration) with 25 μL of activated pancreas supernatant and stopping the reaction at 10-min intervals for 60 min. The extinction coefficient ($\varepsilon_{p\text{-nitroaniline-N-1-naphthyl}}$) × pathlength value of 3.86 mM^{-1} was calculated from a standard curve and used to convert the change in A_{550} during the 60-min incubation period to units of trypsin activity. One unit of trypsin activity = one μmol of p-nitroaniline liberated min^{-1}.

Lipase Assay [modified from Iijima et al. (1998) and German et al. (2004)]

Bile-salt-activated lipase is a carboxylic esterase that cleaves ester bonds to produce an alcohol and a carboxylic acid anion. In this endpoint assay, lipase from shark pancreas tissue cleaves p-nitrophenol from the synthetic lipid substrate p-nitrophenol-myristate.

Bile salts must be present to act as a cofactor to emulsify the substrate. Therefore, 20 μL of homogenized pancreas supernatant was added to a solution of 5.3 mM sodium cholate and 0.25 mM 2-methoxyethanol in 250 mM Tris buffer (pH 9.0 at 25 °C) and the mixture was incubated at either 15 or 25 °C for 15 min. Next, p-nitrophenol-myristate (0.54 mM final concentration) was added and incubated for 30 min. The reaction was stopped by adding 5:2 v/v acetone/heptane, and the mixture was centrifuged at 5350 *g* for 2 min. The lower aqueous layer was placed into a Falcon #3075 flat-bottom 96-well microplate, and the absorbance at 405 nm was measured in a SPECTRAmax 190 microplate spectrophotometer. The extinction coefficient ($\varepsilon_{\text{p-nitrophenol}}$) × pathlength value of 2.02 mM^{-1} was calculated from a standard curve and used to convert the change in A_{405} of the sample (relative to a negative control using 50 mM Tris–HCl homogenization buffer substituted for tissue supernatant) over the 15-min incubation period to units of lipase activity. One unit of lipase activity = one μmol of p-nitrophenol liberated min^{-1}.

Q_{10} measurements

In order to quantify the effects of temperature on the activity of each digestive enzyme in each species, the Q_{10} for each of the three enzymes was calculated for the temperature range of 15–25 °C using the equation $Q_{10} = (R_{25\,°C}/R_{15\,°C})$, where R = enzyme activity at the indicated temperature. Because the enzyme activities at the two temperatures were not independent, a Q_{10} value was obtained for each enzyme in each individual shark, and then, species means were calculated.

Protein assay

The protein concentration was determined for each tissue sample (Bradford method, Bio-Rad kit # 500-0001, Hercules, CA, USA) and used to test for correlations between digestive enzyme activity and the amount of protein in the supernatant, which would indicate inconsistent tissue sample quality or errors in sample preparation or enzyme assay procedures. Tissue supernatant was serially diluted up to 100-fold in 50 mM Tris buffer (pH 7.8 at 25 °C). Then, 10 μL of each diluted homogenate was mixed with 200 μL of Bio-Rad dye reagent, incubated at room temperature for 5 min, and A_{595} was recorded using a SPECTRAmax 190 microplate spectrophotometer. Only the absorbance values that fell within the linear range (from 0 to 1.0 mg ml^{-1}) of the bovine gamma globulin standard curve were used to determine the sample protein concentration (mg protein g^{-1} tissue).

Statistical analysis

In order to meet the assumptions of normality and homoscedasticity, data were log-, inverse-, or reciprocal-transformed for the statistical tests. We used Pearson's correlation coefficients to test for significant correlations between the digestive enzyme activity and protein concentration in each tissue type of each species, and between digestive enzyme activity and shark mass for each species. Because no significant size or protein concentration effects were found within species, we tested for effects of species, temperature, and their interaction for each of the three enzyme activities ($U\ g^{-1}$) using a two-way mixed-model analysis of variance (ANOVA) and Student's *t* test post hoc tests using a Bonferroni correction in SAS (v. 9.1.3; Cary, NC, USA). Mean Q_{10} values for each enzyme were tested for interspecific differences using ANOVA and Student's *t* test post hoc tests with a Bonferroni correction. Differences were considered significant if $P < 0.05$ for ANOVA tests, $P < 0.0033$ for enzyme activity post hoc tests, and $P < 0.025$ for Q_{10} post hoc tests.

Results

Enzyme activities ($U\ g^{-1}$) and Q_{10} values are summarized in Fig. 1 and Table 1, respectively. The values of digestive enzyme activities for each species at its estimated average in vivo temperature are indicated by solid bars (Fig. 1). Enzyme activity in each tissue, for each species, was independent of shark mass for the size range of sharks used in this study. Enzyme activity did not correlate with protein concentration for any tissue or species. Intestine tissue was used as a negative control in all digestive enzyme assays and yielded no detectable activities (data not shown).

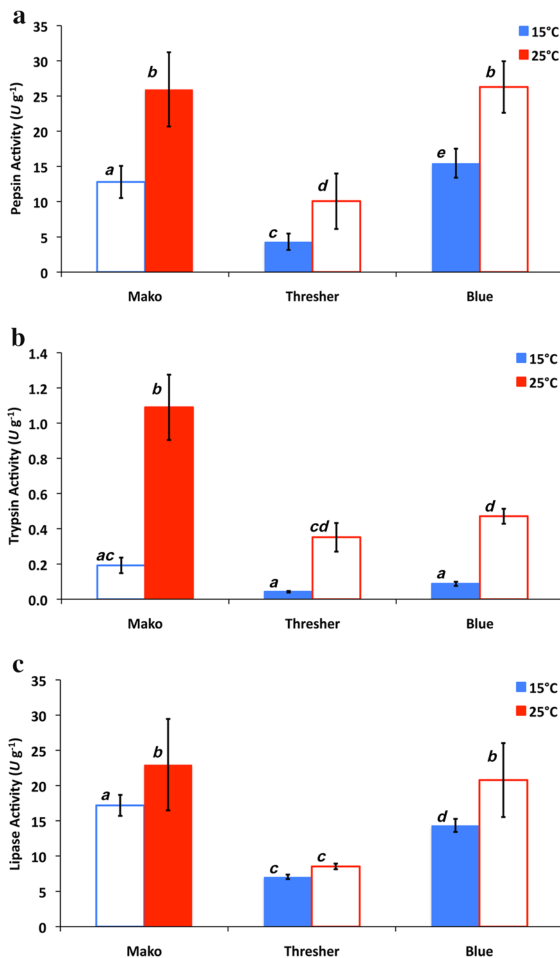

Fig. 1 Mean (± s.e.m.) pepsin (**a**), trypsin (**b**), and lipase (**c**) activity (units per gram wet tissue weight) in the shortfin mako shark ($N = 16$), the thresher shark ($N = 6$), and the blue shark ($N = 16$) at 15 °C (*blue*) and 25 °C (*red*). Solid bars indicate activity at the estimated mean in vivo temperatures of the three species, and letters *a–d* denote means that differ significantly for each enzyme (Student's *t* test post hoc tests on log-transformed data with a Bonferroni correction, $P < 0.0033$). (Color figure online)

Pepsin

The effect of temperature on pepsin activity differed among the three species studied based on the significant interaction between temperature and species ($F_{1,2} = 8.18$, $P = 0.0012$). Pepsin activity in all three species studied was significantly greater at 25 °C than at 15 °C ($F_1 = 646.57$, $P < 0.0001$). Pepsin activities in the mako and blue shark were significantly greater than in the thresher shark at both 25 °C ($t_2 = 21.25$, $P < 0.0001$, and $t_2 = 23.29$, $P < 0.0001$, respectively) and 15 °C ($t_2 = 21.49$, $P < 0.0001$, and $t_2 = 27.38$, $P < 0.0001$, respectively). Pepsin activity at 15 °C was significantly greater in the blue shark than in the mako shark ($t_2 = 7.98$, $P < 0.0001$). At in vivo temperatures, pepsin activity (Fig. 1a) in the mako at 25 °C was significantly greater than that in the thresher at 15 °C ($t_2 = 36.22$, $P < 0.0001$) and in the blue shark at 15 °C ($t_2 = 11.96$, $P < 0.0001$). The pepsin Q_{10} value (Table 1) for the mako did not differ significantly from that of the thresher shark, but in both species it was significantly greater than in the blue shark ($t_2 = 3.64$, $P = 0.0005$ and $t_2 = 3.10$, $P = 0.0027$, respectively).

Trypsin

There was a significant interaction between temperature and species on trypsin activity ($F_{1,2} = 7.63$, $P = 0.0018$). Trypsin activity in the mako, thresher, and blue sharks was significantly greater at 25 °C than at 15 °C ($F_1 = 115.26$, $P < 0.0001$). Trypsin activity was significantly greater in the mako than in the thresher and blue sharks at 25 °C ($t_2 = 6.22$, $P < 0.0001$ and $t_2 = 6.66$, $P < 0.0001$, respectively), but did not differ significantly among the three species at 15 °C. At in vivo temperatures (Fig. 1b), trypsin activity in the mako at 25 °C was significantly greater than in the thresher shark at 15 °C ($t_{15} = 10.31$, $P < 0.0001$) and the blue shark at 15 °C ($t_{15} = 13.06$, $P < 0.0001$). The Q_{10} value for trypsin in the mako did not differ from that in the thresher or blue sharks, but Q_{10} in the thresher was significantly greater than in the blue shark ($t_2 = 3.19$, $P = 0.0021$; Table 1). The reciprocal-transformed trypsin Q_{10} values failed Levene's test ($P = 0.0011$), but passed Welch's test for homogeneity of variance ($F_2 = 3.30$, $P = 0.0521$). Because all three species had very low trypsin activities at 15 °C, all trypsin Q_{10} values were high (Table 1).

Lipase

There was no significant interaction between temperature and species on lipase activity. Lipase activity in the mako and blue sharks, but not the thresher shark, was significantly greater at 25 °C than at 15 °C ($F_1 = 91.62$, $P < 0.0001$). There was a significant effect of species on lipase activity ($F_2 = 287.00$,

Table 1 Temperature coefficient (Q_{10}) mean ± s.e.m. values, for pepsin, trypsin, and lipase in mako ($N = 16$), thresher ($N = 6$), and blue ($N = 16$) sharks

Shark species	Pepsin Q_{10}	Trypsin Q_{10}	Lipase Q_{10}
Mako	2.03 (−0.060, +0.062)^{x}	4.78 (−0.91, +1.46)$^{x,\ y}$	1.28 (−0.053, +0.058)^{x}
Thresher	2.08 (−0.196, +0.206)^{x}	7.45 (−1.12, +1.60)^{x}	1.21 (−0.038, +0.040)^{x}
Blue	1.69 (−0.046, +0.047)^{y}	5.56 (−0.23, +0.25)^{y}	1.39 (−0.054, +0.059)^{y}

Because the data had to be transformed (square root for pepsin and reciprocal for trypsin and lipase) to meet the assumptions of the statistical analysis, the mean and s.e.m. values presented are based on the transformed data, after reverse-transforming them. For each enzyme, significant interspecific differences in Q_{10} values (ANOVA, $P < 0.025$) are indicated by letters (x, y)

$P < 0.0001$). Lipase activity at 25 °C did not differ significantly between the mako and blue shark, but in both species it was significantly greater than in the thresher shark ($t_2 = 17.23$, $P < 0.0001$ and $t_2 = 16.03$, $P < 0.0001$, respectively). Lipase activity at 15 °C was significantly greater in the mako than in the blue shark ($t_2 = 3.66$, $P = 0.0008$), which was significantly greater than in the thresher shark ($t_2 = 13.12$, $P < 0.0001$). Post hoc tests indicated that lipase activity in the mako was higher than in the thresher and blue sharks at in vivo temperatures (Fig. 1c). Lipase activity was significantly greater in the mako at 25 °C than in the thresher shark at 15 °C ($t_{15} = 20.88$, $P < 0.0001$) and the blue shark at 15 °C ($t_{15} = 10.51$, $P < 0.0001$). There was a significant interspecific difference in the lipase Q_{10} values ($F_2 = 37.46$, $P < 0.0001$; Table 1). The lipase Q_{10} did not differ between the lamniform mako and thresher sharks, but those values were significantly lower than in the blue shark ($t_2 = 7.86$, $P < 0.0001$; and $t_2 = 8.21$, $P < 0.0001$, respectively).

Discussion

Interspecific comparisons of digestive enzyme activities

This is the first study to test for physiological benefits of visceral endothermy in elasmobranchs by comparing closely related species that can and cannot elevate visceral temperatures above ambient water temperature. At in vivo temperatures, the mako shark had significantly higher activities of all three digestive enzymes studied than did the thresher and blue sharks, supporting the hypothesis that regional endothermy increases the physiological performance of the warmed visceral organs in this species (Carey and Teal 1966; Carey et al. 1981, 1982, 1984; Stevens and McLeese 1984; Block and Carey 1985; Block and Finnerty 1994; Goldman 1997). At the estimated in vivo temperatures, mean pepsin activity in the mako shark was 6 times higher than in the thresher and 1.7 times higher than in the blue shark; trypsin activity in the mako was 27 times higher than in the thresher and 12 times higher than in the blue shark; and lipase activity in the mako was 3.2 times higher than in the thresher and 1.6 times higher than in the blue shark. All enzyme activities for a given species were higher at 25 °C than at 15 °C, except lipase in the thresher shark, possibly due to the small sample size for thresher sharks in this study. The within-species temperature effects were expected, given that increasing temperature increases molecular motion resulting in higher reaction rates. Nevertheless, when compared at 25 °C, all enzyme activities in the mako were significantly higher than in the thresher shark, and trypsin activity was higher in the mako than in the blue shark, which indicates that the high digestive enzyme activities in the mako shark result from more than a simple temperature effect.

Enzyme-specific activity is the product of the substrate turnover rate of a single enzyme molecule and the number of molecules present. If the three-dimensional structure and substrate turnover rates for each of the three enzymes are conserved among species, then differences in enzyme activity at a given temperature should represent differences in the amount of enzyme present in the digestive tract at the time of sampling. The consistently low pepsin, trypsin, and lipase activities in the thresher shark

would then mean that this species had fewer digestive enzymes per gram of tissue than did the mako and blue sharks sampled. The quantity of pepsin, trypsin, and lipase in the tissues at the time of sampling is a function of how much enzyme is released during feeding and the rates of enzyme replenishment within the digestive tissues, which are unknown in sharks (Secor 2009). If an individual shark had fed just prior to sampling, it is likely that the digestive enzyme activities measured would be lower than in a shark that had not recently fed. All of the sharks used in this study were caught on baited hooks, implying that their stomachs were not full prior to capture. For the few individual sharks for which stomach fullness data were also obtained (A. Preti pers. comm.), there was no correlation between enzyme activity and stomach fullness. The measured digestive enzyme activities may also have been influenced by the recent diet of individual sharks, if the sharks adjust the relative amounts of digestive enzymes that they synthesize and secrete to digest a particular prey species most effectively (Matus de la Parra et al. 2007; Secor 2009). Such differences among individuals may explain the large variation in enzyme activities within species found in this and other studies of fish digestive enzyme activities (e.g., Chan et al. 2004; Matus de la Parra et al. 2007).

It is possible that the pepsin and lipase activities are similar in the mako and blue sharks, but differ in the thresher shark, because the feeding ecology of these three species has a greater influence on digestive enzyme activity than their phylogenetic relationship. The consistently lower digestive enzyme activities in the thresher shark could be explained if prey species of the thresher were easier to digest than prey of the mako and blue sharks, for example because of lower lipid content or lower amount of indigestible material such as bone. Stomach content data for sharks collected during the longline surveys in which the individuals used in this study were collected indicate that: The local thresher shark population eats primarily anchovy and sardine, the blue shark eats mostly cephalopods, and the mako shark consumes nearly equal amounts of cephalopods and pelagic teleosts (Preti et al. 2001, 2012). Clark et al. (2010) determined that market squid, *Loligo opalescens*, has a lower nutritional content (~2 % lipid, 12 % protein, 1.5 % ash) and is easier to digest than Pacific sardine, *Sardinops sagax* (~15 % lipid, 17 % protein, 2.5 % ash). More information is needed on other factors that contribute to food processing to assess the potential interacting effects of temperature, diet, absorption rate, and assimilation efficiency in these sharks.

We could find no previous data for the activities of pepsin, trypsin, or lipase in any elasmobranch species with which to compare the enzyme activities measured in this study, but those values are within an order of magnitude of values measured in several teleost fishes using similar methods (e.g., Chan et al. 2004; German et al. 2004; Neumann 2009; Odedeyi and Fagbenro 2010). However, digestive enzyme activities reported for the Pacific bluefin tuna, *Thunnus orientalis,* a teleost that has evolved visceral endothermy, are two to three orders of magnitude higher than measured in the sharks, but increase similarly with temperature (Matus de la Parra et al. 2007). Stevens and McLeese (1984) found that trypsin and chymotrypsin activities increased threefold over the range of visceral temperatures (10–30 °C) measured in the endothermic Atlantic bluefin tuna, *Thunnus thynnus*. In albacore, *Thunnus alalunga*, and yellowfin, *T. albacares*, tunas, eastern Pacific bonito, *Sarda chiliensis*, and chub mackerel, *Scomber japonicus*, Neumann (2009) measured higher pepsin, trypsin, and lipase activities at higher temperatures (15–25 °C). However, unlike the sharks in the present study, the activities for the albacore, a viscerally endothermic species, were not always higher than those for the ectothermic scombrid species at in vivo temperatures.

Although the ectothermic sharks cannot elevate visceral temperatures above ambient temperature, they could increase food processing rates by moving into warmer waters and thermoregulating behaviorally. Based on the results of the present study, the blue shark could achieve the same pepsin and lipase activities as the mako shark if it remained in 25 °C water, but its trypsin activity would still be less than half that of the mako shark. The thresher could achieve activities similar to the mako shark only by remaining in 25 °C water and synthesizing 2.5 times as many enzymes within the digestive tract. Acoustic tracking data show that thresher and blue sharks spend up to 15 % of their time in waters below 10 °C, whereas endothermic white, porbeagle, and salmon sharks spend up to 30, 80, and 90 % of their time, respectively, in waters below 10 °C (Carey and Scharold 1990; Weng et al. 2005, 2007a, b; Nasby-Lucas et al. 2009; Pade et al. 2009; Cartamil et al. 2010a).

Therefore, visceral endothermy should allow lamnid sharks to maintain high food processing rates to a greater degree than possible by behavioral thermoregulation alone and thus to spend more time in cold waters without compromising digestive system function.

Thermal sensitivity of digestive enzymes

We expected that, among the three species studied, the endothermic mako shark would have the highest Q_{10} values because its digestive enzymes should be optimized to operate at a stable temperature. Lower Q_{10} values were expected in the two ectothermic sharks because their enzymes should be adapted to operate effectively over a wider range of temperatures (Angilletta 2009). Instead, we found that the mako and thresher sharks have higher Q_{10} values than the blue shark for pepsin and trypsin and that these two lamniform species have lower Q_{10} values than the blue shark for lipase. The similar Q_{10} values in the mako and thresher sharks, which differ from those in the blue shark, could reflect the phylogenetic relationships among the three species (Velez-Zuazo and Agnarsson 2011). The larger genetic distance between the blue shark and the two lamniform sharks may be reflected in the blue shark having digestive enzymes with a different amino acid sequence, conferring a different thermal sensitivity, than the two lamniform species. Alternatively, the 10 °C temperature range over which Q_{10} values were estimated in this study may not be large enough to reveal the predicted interspecific effects.

Benefits of visceral endothermy

The results of this study support the hypothesis that visceral endothermy enhances physiological performance of the digestive tract in lamnid sharks. Proteases such as pepsin, trypsin, and chymotrypsin hydrolyze the peptide bonds within proteins, and this process is thought to be the rate-limiting step in protein degradation and absorption during digestion (reviewed in Secor 2009). Therefore, by increasing the activity of digestive enzymes, particularly trypsin which had the highest Q_{10} value, visceral endothermy should increase the food processing rate in the mako shark without incurring the additional costs associated with synthesizing more enzymes or remaining in warm waters. Higher digestive enzyme activities would allow the mako shark, and presumably other lamnid sharks, to digest more proteins and lipids per day than can individuals from closely related species (e.g., the thresher shark) or sympatric ectothermic species (e.g., the blue shark). Consequently, lamnid sharks could have more nutrients available to fuel their high metabolic rates, and excess nutrients could be assimilated into somatic tissues, lipid reserves for migrations, or yolk for oophagous pups in pregnant females (Carey et al. 1981; Gilmore 1993). A similar benefit has been proposed for the Pacific bluefin tuna, which, like other endothermic tunas, has a higher standard metabolic rate than do similarly sized ectothermic species at the same water temperature (Sepulveda and Dickson 2000; Sepulveda et al. 2003; Blank et al. 2007). Although no metabolic rate data exist for thresher or blue sharks, the standard metabolic rate of the mako shark is greater than that of any other shark measured to date, including ectothermic lemon, *Negaprion brevirostris*, scalloped hammerhead, *Sphyrna lewini*, and leopard sharks (Graham et al. 1990; reviewed in Bernal et al. 2001; Sepulveda et al. 2007).

It is possible that regional endothermy was important for the evolution and radiation of the ancestral lamnid sharks during the Miocene epoch approximately 25 million years ago (Purdy 1996; Dickson and Graham 2004). During that time, the Tethys Sea, a large tropical habitat that had existed for approximately 70 million years, was greatly reduced, and oceanic cooling at high latitudes created a latitudinal temperature gradient, causing a shift in wind patterns and the formation of oceanic gyres (Purdy 1996; Martin 2003; Lyle et al. 2008). The upwelling of cold nutrient-rich water into warm habitats resulted in localized areas of high primary productivity (Purdy 1996; Martin 2003; Lyle et al. 2008). The reduction in tropical habitat and ensuing shifts in prey availability may have forced the ancestral lamnid sharks that had evolved in tropical waters (e.g., *Carcharodon megalodon* and *Isurus hastalis*) to pursue their prey into temperate waters and either adapt to decreased temperatures or use physiological and behavioral strategies to modulate changes in tissue temperatures (Purdy 1996; Dickson and Graham 2004; Lyle et al. 2008). In sharks with the ability to retain heat within the viscera, changes in the temperature and physiological function of the digestive tract would have been

minimized during extended excursions into cold water, while a competing ectothermic shark that was reliant upon behavioral thermoregulation would have had to return to warmer water to increase its rate of digestion. Thus, visceral endothermy would have freed ancestral lamnid sharks from the constraint of returning to warm water to digest prey rapidly after foraging, allowing them to exploit cold habitats and expand their thermal niche.

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Conflict of interest The authors declare that they have no conflict of interest.

Human and animal rights statement All applicable international, national, and institutional guidelines for the care and use of animals were followed. All procedures performed in this study were approved by the CSUF Institutional Animal Care and Use Committee.

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