

## Olfactory morphology and physiology of elasmobranchs

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### SUMMARY

Elasmobranch fishes are thought to possess greater olfactory sensitivities than teleost fishes due in part to the large amount of epithelial surface area that comprises their olfactory organs; however, direct evidence correlating the size of the olfactory organ to olfactory sensitivity is lacking. This study examined the olfactory morphology and physiology of five distantly related elasmobranch species. Specifically, we quantified the number of lamellae and lamellar surface area (as if it were a flat sheet, not considering secondary lamellae) that comprise their olfactory organs. We also calculated the olfactory thresholds and relative effectiveness of amino acid odorants for each species. The olfactory organs varied in both the number of lamellae and lamellar surface area, which may be related to their general habitat, but neither correlated with olfactory threshold. Thresholds to amino acid odorants, major olfactory stimuli of all fishes, ranged from  $10^{-9.0}$  to  $10^{-6.9}$  mol l<sup>-1</sup>, which indicates that these elasmobranch species demonstrate comparable thresholds with teleosts. In addition, the relative effectiveness of amino acid stimuli to the olfactory organ of elasmobranchs is similar to that previously described in teleosts with neutral amino acids eliciting significantly greater responses than others. Collectively, these results indicate parallels in olfactory physiology between these two groups of fishes.

Key words: EOG, amino acid, olfaction, threshold, olfactory epithelium.

### INTRODUCTION

Elasmobranch fishes (sharks, skates and rays) are widely considered to possess superior olfactory sensitivities compared with bony fishes and are often referred to in the popular media as ‘swimming noses’. This reputation is partly based on anecdotal observations, but is also due to their particularly large olfactory structures, including olfactory epithelial surface area (Schluessel et al., 2008) and olfactory bulb volume (Lisney et al., 2007; Lisney and Collin, 2006). Several studies related the size of their olfactory structures to species ecology, invoking the assumption that differences in the size of olfactory structures correlate with olfactory sensitivity (Lisney et al., 2007; Lisney and Collin, 2006; Northcutt, 1977; Northcutt, 1978; Schluessel et al., 2008; Theisen et al., 1986; Theiss et al., 2009; Yopak et al., 2007). This assumption has been refuted for teleost fishes (Hansen and Zielinski, 2005; Hara, 1994; Yamamoto, 1982) and has yet to be tested for elasmobranchs. To determine whether a correlation exists between olfactory organ (rosette) size and olfactory sensitivity requires a quantitative comparison of the olfactory morphology and response thresholds for multiple elasmobranch species.

Comparative morphological studies demonstrated that elasmobranchs, like teleost fishes, exhibit interspecific differences in the number and surface area of olfactory lamellae (Hansen and Zielinski, 2005; Kajiura et al., 2005; Schluessel et al., 2008; Theiss et al., 2009; Yamamoto, 1982). Whereas the gross morphology and ultrastructure of the elasmobranch olfactory system are well described (Bell, 1993; Bronshtein, 1976; Meng and Yin, 1981; Schluessel et al., 2008; Tester, 1963; Theisen et al., 1986; Zeiske et al., 1986), olfactory thresholds have been assessed for only five elasmobranch species: the nurse shark (Hodgson and Mathewson, 1978); the Atlantic stingray, *Dasyatis sabina* (Silver, 1979); the lemon shark, *Negaprion brevirostris* (Zeiske et al., 1986); the black sea skate, *Raja clavata* (Nikonov et al., 1990); and the scalloped

hammerhead shark, *Sphyrna lewini* (Tricas et al., 2009). Although evidence is limited, the threshold ranges of these species for amino acid stimuli are similar to those estimated for teleosts (Hara, 1994); however, interspecific comparisons of the olfactory capabilities among multiple elasmobranch species are lacking. Only two of these studies integrated olfactory morphology with physiology, each on a single species (Silver, 1979; Zeiske et al., 1986), which precluded the ability to determine whether the size of the olfactory structures correlated with the estimated olfactory thresholds.

This study addresses long-standing assumptions about elasmobranch olfaction by correlating the olfactory morphology and physiology of five phylogenetically diverse elasmobranch species. Specifically, we tested: (1) whether the lamellar surface area of the elasmobranch olfactory organ is correlated with olfactory threshold; (2) whether distantly related species demonstrate differences in olfactory threshold; and (3) whether elasmobranch fishes possess lower olfactory thresholds than teleost fishes.

### MATERIALS AND METHODS

#### Animal collection

The olfactory system was examined in five elasmobranch species: the clearnose skate, *Raja eglanteria* Bosc 1800; the yellow stingray, *Urobatis jamaicensis* (Cuvier 1816); the Atlantic stingray, *Dasyatis sabina* (Lesueur 1824); the lemon shark, *Negaprion brevirostris* (Poey 1868); and the bonnethead shark, *Sphyrna tiburo* (Linnaeus 1758). *Raja eglanteria* were acquired from a captive breeding population at the Mote Marine Laboratory (Sarasota, FL, USA). The other four species were collected from Florida near shore waters using long-lining, seining and hand-netting techniques. All animals used in electrophysiology experiments, except for *S. tiburo*, were transported to the Florida Atlantic University Marine Laboratory at the Gumbo Limbo Environmental Complex (Boca Raton, FL, USA), maintained in tanks with flow-through seawater, and fed a diet of

shrimp and squid daily to satiation. *Sphyrna tiburo* were maintained at Mote Marine Laboratory and fed daily to satiation until tested at that facility. All experiments were conducted in accordance with approved IACUC protocols at both Florida Atlantic University (A08-05) and Mote Marine Laboratory (09-10-SK2).

### Morphology

The elasmobranch olfactory organs (rosettes) are composed of numerous primary lamellae, which are overlain with an olfactory epithelium and populated with olfactory receptor neurons (ORNs) (Theisen et al., 1986; Zeiske et al., 1986). The number of primary lamellae and total lamellar surface areas were quantified for a minimum of nine individuals for each of the five species. The lamellae were counted from a single olfactory organ from each animal. The number of lamellae was compared among the five species using a one-way analysis of variance (ANOVA) followed by Tukey *post hoc* tests for pair-wise comparisons. We also pooled the lamellar counts for the sharks (benthopelagic) and the batoids (benthic) in order to compare the number of lamellae between habitats using a Mann–Whitney rank sum test (Systat Software, Inc., San Jose, CA, USA).

To quantify the lamellar surface area, a representative subset of 10 lamellae was dissected from one olfactory organ of each animal. This included the first, last and eight intermediate lamellae evenly spaced along the length of the organ. Each lamella was digitally photographed on a micrometer slide to provide scale, and the surface area was measured using the software ImageJ (National Institutes of Health, Bethesda, MA, USA). A quadratic equation was then fit to the 10 surface area measurements, and a Simpson's rule numerical integration was applied in order to approximate the total lamellar surface area for each organ. The total lamellar surface area was then quadrupled to account for the presence of the olfactory epithelium on both sides of each lamella in two organs. The mean total lamellar surface area for each species was compared using an analysis of covariance (ANCOVA) (SPSS Inc., Chicago, IL, USA) with disc width (DW) for the batoids and total length (TL) for the sharks as the covariate, followed by LSD (least significant difference) *post hoc* tests for pair-wise comparisons. Body mass was not used as

the covariate as this measurement was not available for all specimens; thus, surface area comparisons were not made between batoids and sharks due to differing body size metrics.

### Electrophysiology

#### Experimental apparatus

The underwater electro-olfactogram (EOG), an odorant-induced, slow negative potential thought to reflect summated olfactory receptor generator potentials, was measured in the water immediately above the olfactory organ (Caprio, 1995; Silver et al., 1976). The experimental apparatus consisted of an electrically grounded acrylic experimental tank (89 cm × 43 cm × 21 cm) supplied with flow-through seawater, which was mechanically (25 μm polyscreen) and chemically (activated charcoal) filtered (Fig. 1). Seawater was delivered to the tank through three arms of a PVC manifold with the flow for each arm controlled by a ball or gate valve. One arm provided ventilatory water flow over the gills. Seawater flow through the tank was provided *via* a second manifold arm and continuously drained to reduce chemical accumulation. A third arm of the manifold delivered a constant flow of seawater through a flow meter to paired, computer-controlled, three-way solenoid valves. The first valve diverted the water flow to either of two short lengths of tubing (left or right branches) which both connected to the second solenoid valve. This second valve directed the flow from either branch to an odor delivery pipette positioned in the animal's incurrent naris, providing a constant flow of seawater over the olfactory organ. Seawater flowed through only one branch at a time, which enabled the injection of a stimulus into the inactive branch without disturbing the water flow over the olfactory organ. A remotely operated trigger enabled the solenoid valves to divert the water flow to deliver the stimulus bolus to the olfactory organ. This eliminated pressure artifacts in the EOG trace during stimulus injection.

To record an animal's responses to odor stimuli, a non-polarizable Ag–AgCl electrode (E45P-M15NH, Warner Instruments, Hamden, CT, USA) fitted with a seawater/agar-filled capillary tube was positioned just above the olfactory epithelium and a similar reference electrode was placed nearby in contact with the animal's skin. The output from the two electrodes was differentially amplified

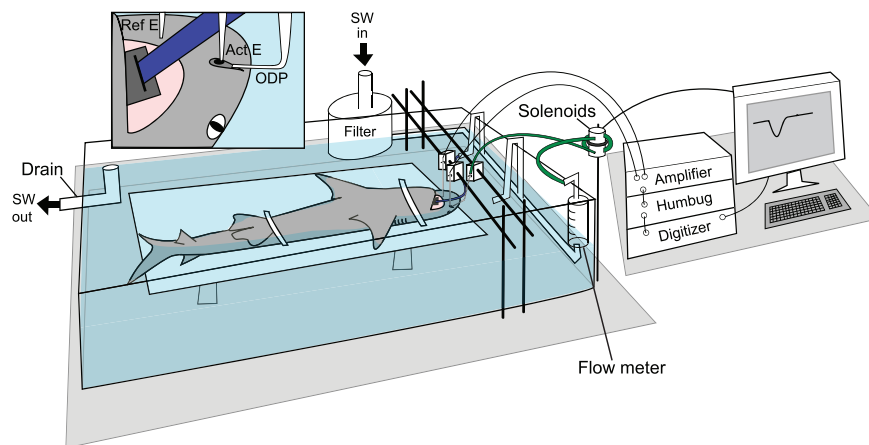


Fig. 1. Experimental apparatus used to record the electro-olfactogram (EOG). Animals were secured onto a platform in an experimental seawater (SW) tank and ventilated with SW. The tank was supplied with mechanically and chemically filtered SW through a PVC manifold. One manifold arm delivered a constant flow of SW through a flow meter; paired, computer-controlled, three-way solenoid valves; and an odor delivery pipette (ODP, inset), which was inserted into the incurrent naris. Amino acid stimuli were delivered through the ODP to the olfactory organ. Paired, non-polarizable, Ag–AgCl electrodes recorded the EOG. The glass tip of the active electrode (Act E, inset) was positioned in the SW immediately above the olfactory epithelium while the glass tip of the reference electrode (Ref E, inset) contacted the skin adjacent to the naris. The output was differentially amplified (1000–10,000×), filtered (high pass 0.1 Hz, low pass 0.1 kHz, 50/60 Hz), digitized (1 kHz) and recorded.

(DP-304, Warner Instruments) at 1000–10,000 $\times$ , filtered (0.1 Hz–0.1 kHz, 50/60 Hz) (DP-304, Warner Instruments & Hum Bug, Quest Scientific, North Vancouver, BC, Canada), digitized at 1 kHz using a Power Lab<sup>®</sup> 16/30 model ML 880 (AD Instruments, Colorado Springs, CO, USA) and recorded using Chart<sup>™</sup> Software (AD Instruments).

#### Experimental protocol

Our experimental procedures closely followed previous EOG studies with elasmobranchs (Silver, 1979; Tricas et al., 2009; Zeiske et al., 1986). Prior to experimentation, an animal was injected (intramuscularly or intravenously) with the paralytic, pancuronium bromide (0.03 mg kg<sup>-1</sup>). Immediately upon cessation of active ventilation the animal was transferred to the experimental tank, secured ventral side up with Velcro<sup>®</sup> straps to a submerged platform, and ventilated with seawater *via* the mouth (sharks) or spiracles (batoids). For the batoids, a small sponge was fitted into the mouth to direct water flow from the spiracles over the gills. The odor delivery pipette was mounted in a micromanipulator, and the pipette tip was positioned in the incurrent naris with water flow regulated to 2 ml s<sup>-1</sup> (Tricas et al., 2009). A test dye solution was delivered through the odor delivery pipette to confirm that seawater flowed over the olfactory epithelium and exited the excurrent naris. The EOG electrodes were mounted in micromanipulators with the recording electrode positioned into the excurrent naris just above the olfactory epithelium and the reference electrode positioned nearby in contact with the skin. Appropriate placement of the recording electrode in each animal was confirmed by observing an adequately sized (minimum of 30  $\mu$ V) response to a 1.0 ml injection of 10<sup>-3</sup> mol l<sup>-1</sup> alanine (standard). The heart beat was monitored with an electro-cardiogram (EKG) (sharks) or visually (batoids) throughout the experiments.

The olfactory responses to 20 L-amino acids applied individually were recorded from each animal. Amino acid stock solutions (10<sup>-1</sup> mol l<sup>-1</sup>) were prepared with filtered seawater weekly and stored at 4°C (pH 7.1–8.4). Dilutions were made daily from the stock solutions and incubated throughout an experiment in a water bath in the experimental tank. 1 ml of seawater was removed from one branch of solenoid tubing and replaced with 1 ml of amino acid stimulus to maintain constant volume. The solenoid valves were then triggered, which directed the water flow through the branch that contained the amino acid. The bolus of amino acid was transported through the odor delivery pipette, to the olfactory epithelium of the animal. Successive amino acid stimuli were administered two minutes after the EOG trace returned to approximately baseline level.

Amino acid stimuli became diluted during transport from the injection site to the tip of the odor delivery pipette. To quantify the dilution factor, 1.0 ml of dye solution was injected into one of the solenoid branches in place of an amino acid stimulus and samples were collected from the odor delivery pipette at 1 s intervals. The absorbance of the samples was measured with a spectrophotometer, and the dilution factor was calculated using the ratio of absorbance of the most concentrated sample solution to the stock dye solution. Stimuli delivered to the olfactory epithelium of the fish were diluted to 6% of their injected concentration. Therefore, injecting a 10<sup>-3</sup> mol l<sup>-1</sup> solution would present a 10<sup>-4.2</sup> mol l<sup>-1</sup> stimulus to the olfactory epithelium.

The relative effectiveness of amino acid stimuli was tested by quantifying EOG responses to each of the 20 amino acids at an injection concentration of 10<sup>-3</sup> mol l<sup>-1</sup>. To determine the concentration–response relationships and olfactory threshold, five

or six highly stimulatory amino acids were subsequently tested at increasing injection concentration from 10<sup>-7</sup> to 10<sup>-3</sup> mol l<sup>-1</sup> for each animal. A 10<sup>-3</sup> mol l<sup>-1</sup> alanine standard was administered after approximately every fifth amino acid to determine the relative responsiveness of the tested amino acids throughout the experiment. The response to a 1.0 ml injection of seawater, the control, was also recorded periodically throughout the experiment.

#### Analysis

To compare the relative effectiveness of the 20 amino acid stimuli for each fish, the response magnitude of each test amino acid was expressed as a percentage of the alanine standard (Silver, 1979). The response to alanine was recorded periodically throughout each experiment, as the magnitude of the response to the standard could change over experimental time. Thus, responses to amino acid test stimuli were taken as a percentage of a calculated response to the alanine standard. This calculated response to the standard was obtained by determining the response magnitude at the exact time of the test stimulus using a regression of the alanine responses (response magnitude *vs* time) preceding and following the test stimulus. Relative responses for each of the 20 amino acids were averaged for each species, and those averages were compared among amino acids within each species using a one-way ANOVA. Bonferroni *t*-tests were used for *post hoc* comparisons to account for the large number of pair-wise comparisons among the 20 amino acids (Systat Software, Inc.) (Zar, 1999).

Concentration–response relationships of five or six of the most stimulatory amino acids were determined for each species. Olfactory thresholds were calculated by regressing the amino acid concentration–response curve for each of the amino acids to its intersection with the control response to seawater for each individual animal. We employed Pearson product moment correlation tests to ascertain whether threshold to each amino acid varied with body size for each species. Olfactory thresholds to alanine, phenylalanine and serine were calculated for all five species and consequently were each compared among species using a one-way Kruskal–Wallis ANOVA on ranks. Threshold data for the three amino acids were pooled both for each species and for each amino acid and were compared using one-way Kruskal–Wallis ANOVAs on ranks.

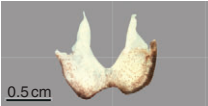




## RESULTS

### Morphology

Olfactory organs were dissected from five elasmobranch species ( $N \geq 9$ ), and the mean number of lamellae and total lamellar surface areas were quantified (Table 1). Lamellae from all five species were largest in the center of the organ and tapered in size towards the medial and lateral ends. Epithelial pigmentation differed among species; the three batoid species exhibited a predominantly white/tan epithelium whereas the epithelium of the two shark species was characterized by a black/brown pigmentation. All five species differed significantly from each other in the number of lamellae that comprise their olfactory organs (ANOVA,  $F_{4,49}=694.258$ ,  $P<0.001$ ; Tukey test,  $P<0.001$  for all comparisons). *Raja eglanteria* possessed the fewest lamellae (28.8 $\pm$ 0.63) and *S. tiburo* the most (68.6 $\pm$ 3.35). The two shark species had significantly more lamellae than the three batoid species (Mann–Whitney,  $U=140.00$ ,  $P<0.001$ ).

Total lamellar surface area scaled positively as a power function with body size (DW or TL) for all species (Fig. 2). Total lamellar surface area differed significantly within the batoids (ANCOVA,  $F_{2,27}=41.662$ ,  $P<0.001$ ); *R. eglanteria* possessed a significantly smaller mean total lamellar surface area than both *U. jamaicensis*

Table 1. The species used in this study represent five elasmobranch families

Species	# of lamellae	<i>n</i>	Lamella
Rajidae Clearnose skate <i>Raja eglanteria</i>	28.8±0.63	10	
Urolophidae Yellow stingray <i>Urobatis jamaicensis</i>	36.3±1.01	11	
Dasyatidae Atlantic stingray <i>Dasyatis sabina</i>	50.2±1.32	10	
Carcharhinidae Lemon shark <i>Negaprion brevirostris</i>	43.6±1.65	14	
Sphyrnidae Bonnethead shark <i>Sphyrna tiburo</i>	68.6±3.35	9	

The third column indicates the mean number of lamellae  $\pm$  s.d. that comprises each rosette. All five species differed significantly from each other in the number of lamellae. The far right column illustrates a representative lamella dissected from an olfactory rosette for each species. Line drawings of batoids are modified from McEachran and de Carvalho (2002), and line drawings of sharks are modified from Compagno (2002).

and *D. sabina* (LSD test,  $P < 0.001$  for both comparisons). Within the sharks, *N. brevirostris* had a significantly smaller mean total lamellar surface area than *S. tiburo* (ANCOVA,  $F_{1,20} = 65.023$ ,  $P < 0.001$ ).

### Electrophysiology

#### Relative effectiveness of amino acids

EOG responses were recorded from the olfactory epithelium of five elasmobranch species ( $n \geq 6$  per species) to 20 common amino acids. A typical EOG response is characterized by a rapid negative potential followed by a slower recovery back to baseline. The larger the response magnitude, the longer the trace took to rebound to baseline

level. The magnitude of the EOG response to the same stimulus was approximately three times greater in the two shark species compared with the three batoid species. For each of the five species, mean EOG response magnitudes to the 20 amino acids varied significantly (ANOVA,  $P < 0.001$  for all 5 spp.), with alanine and serine being particularly stimulatory. Pair-wise comparisons among amino acids within a species are illustrated in Fig. 3.

#### Concentration–response relationships and olfactory threshold

The concentration–response relationships for five or six highly stimulatory amino acids were very similar within each species (Fig. 4). The logarithm of the EOG response increased with a logarithmic increase in stimulus concentration. Olfactory thresholds to these amino acids were estimated for all five species (Fig. 5). The olfactory thresholds to amino acids did not vary with body size of teleost fishes (Hara, 1994) nor for any of our species (Pearson product moment correlation,  $P > 0.05$  for all); therefore, body size was not used as a covariate during our analyses. Mean thresholds to the most stimulatory amino acids ranged between  $10^{-9.0}$  and  $10^{-6.9}$  mol l<sup>-1</sup> for the five species (Table 2). Olfactory thresholds to alanine, phenylalanine and serine did not differ significantly among species (alanine: Kruskal–Wallis,  $H_4 = 4.035$ ,  $P = 0.401$ ; phenylalanine: Kruskal–Wallis,  $H_4 = 1.909$ ,  $P = 0.753$ ; serine: Kruskal–Wallis,  $H_4 = 9.051$ ,  $P = 0.060$ ). As a result, the thresholds for all five species were pooled for each of the three amino acids; the median thresholds to alanine ( $10^{-7.8}$ ), phenylalanine ( $10^{-7.9}$ ) and serine ( $10^{-7.2}$ ) were significantly different (Kruskal–Wallis,  $H_2 = 6.808$ ,  $P = 0.033$ ); however, the Dunn's method *post hoc* test did not detect any significant differences in the pair-wise comparisons. Olfactory thresholds to the three amino acids were also pooled for each species, and again, there were no significant differences in olfactory threshold among species (Kruskal–Wallis,  $H_4 = 6.365$ ,  $P = 0.174$ ).

### DISCUSSION

Olfaction plays an integral role for elasmobranchs in food localization (Parker, 1913; Sheldon, 1909; Sheldon, 1911) and also

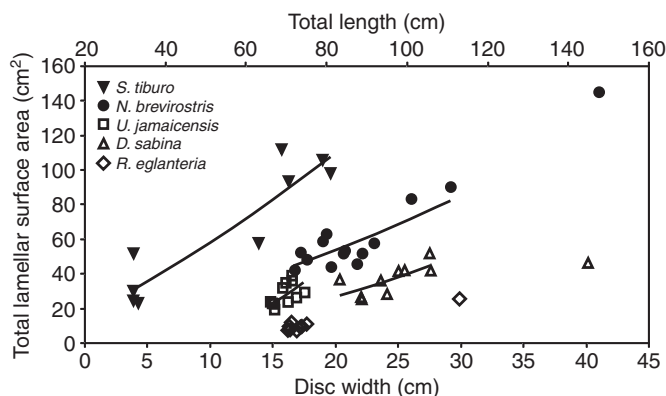


Fig. 2. Total lamellar surface area for the five elasmobranch species tested scaled positively as a power function with body size (disc width or total length). Regression lines are indicated for *R. eglanteria*:  $y = 0.0402x^{1.8988}$ ; *U. jamaicensis*:  $y = 0.0297x^{2.4689}$ ; *D. sabina*:  $y = 0.242x^{1.5725}$ ; *N. brevirostris*:  $y = 0.0947x^{1.4371}$ ; *S. tiburo*:  $y = 0.3052x^{1.3342}$ . Outliers were excluded from the regressions for *R. eglanteria*, *D. sabina* and *N. brevirostris*. *Raja eglanteria* had a significantly smaller mean total lamellar surface area than *U. jamaicensis* and *D. sabina*. *Negaprion brevirostris* had a significantly smaller mean total lamellar surface area than *S. tiburo*.



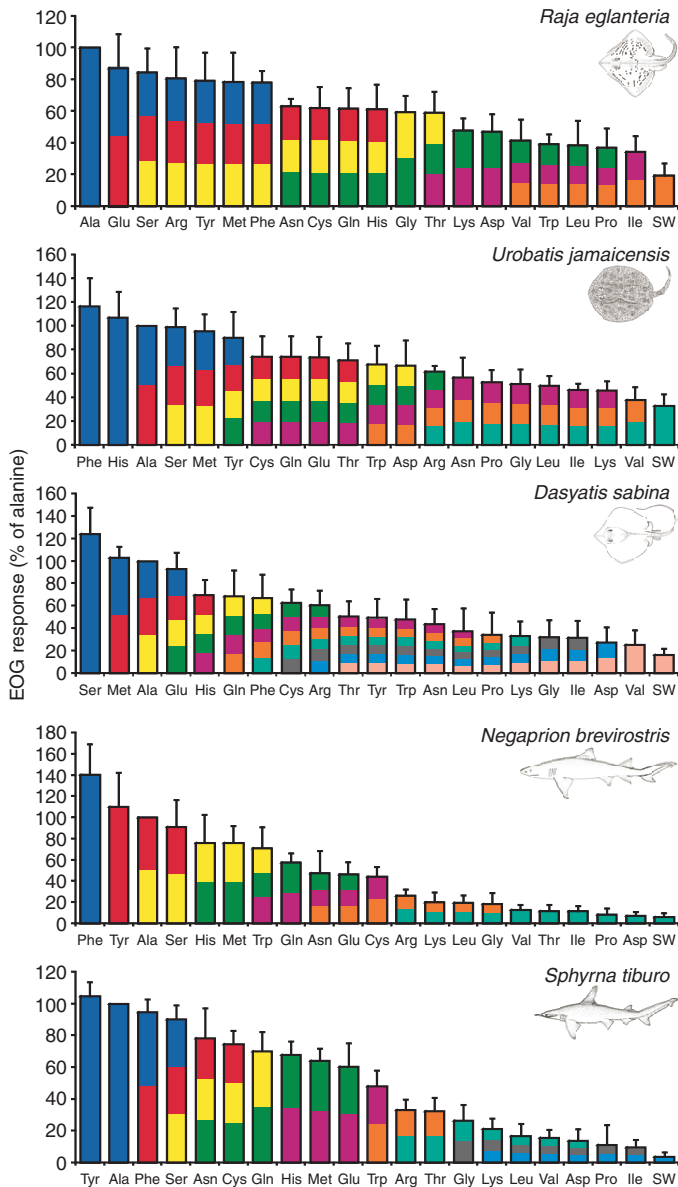


Fig. 3. Mean electro-olfactogram (EOG) responses ( $\pm$ s.d.) for five elasmobranch species ( $n \geq 6$ ) to 20 amino acids and the seawater (SW) control. Response magnitudes are expressed as a percentage of the response to the standard ( $10^{-3} \text{ mol l}^{-1}$  alanine). Colors represent the results of pair-wise comparisons using Bonferroni  $t$ -tests. For each species, amino acids that share a bar color do not differ significantly from each other. Ala, alanine; Arg, arginine; Asn, asparagine; Asp, aspartic acid; Cys, cysteine; Gln, glutamine; Glu, glutamic acid; Gly, glycine; His, histidine; Ile, isoleucine; Leu, leucine; Lys, lysine; Met, methionine; Phe, phenylalanine; Pro, proline; Ser, serine; Thr, threonine; Trp, tryptophan; Tyr, tyrosine; Val, valine; SW, seawater. Line drawings of batoids are modified from McEachran and de Carvalho (McEachran and de Carvalho, 2002), and line drawings of sharks are modified from Compagno (Compagno, 2002).

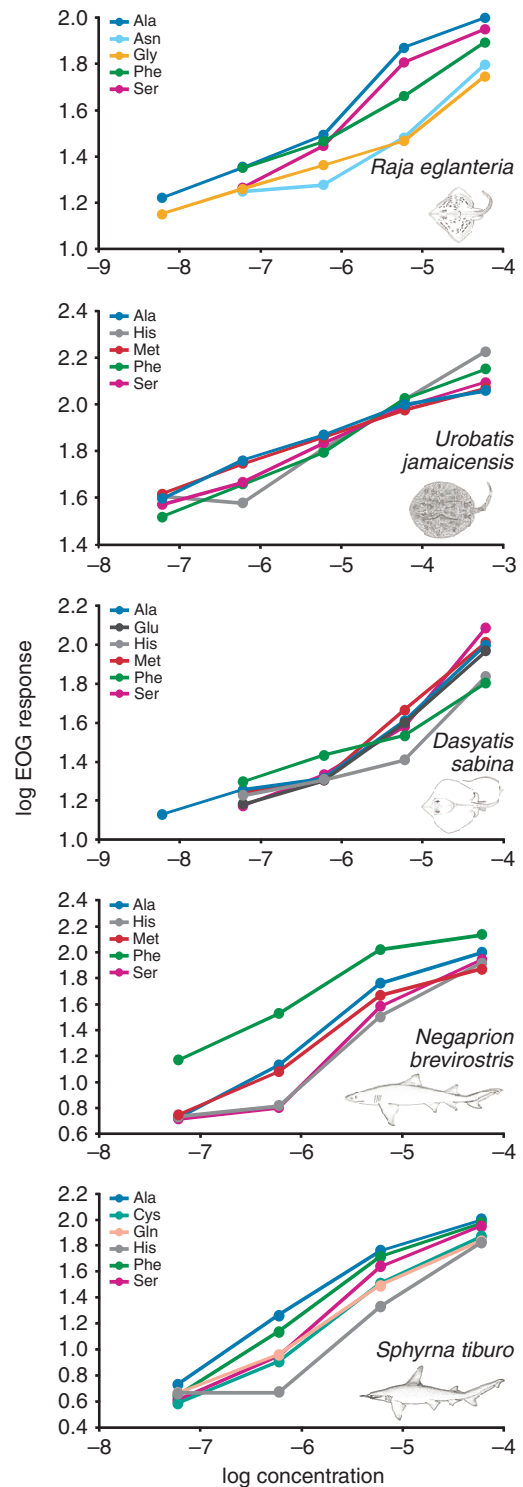


Fig. 4. Concentration–response relationships of five or six highly stimulatory amino acids for each species. Response magnitudes represent the mean response for each amino acid at each concentration ( $10^x \text{ mol l}^{-1}$ ) and are expressed as a percentage of the standard ( $10^{-3} \text{ mol l}^{-1}$  alanine). Refer to Fig. 3 for statistical differences.

probably in mating (Johnson and Nelson, 1978; Kajiura et al., 2000), predator detection (Rasmussen and Schmidt, 1992), and in homing and navigation (Edrén and Gruber, 2005). Olfaction is considered especially important as a distant sense because chemical signals can become entrained in currents and transported much farther in the

marine environment than mechanical or electrical signals (Hueter et al., 2004). This enables elasmobranchs to detect chemical cues emanating from distant sources in their expansive environment. Elasmobranchs are often reputed to possess greater olfactory sensitivity than teleost fishes, although sensitivity has been estimated

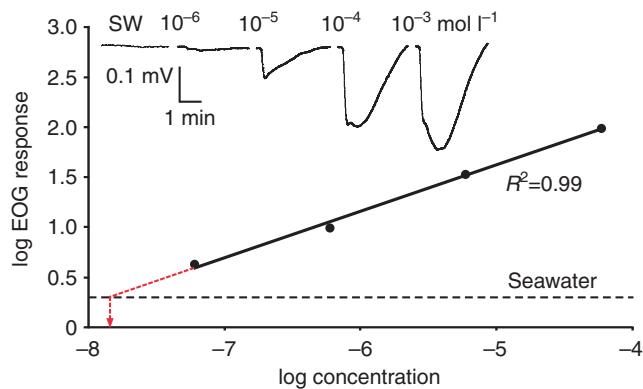


Fig. 5. Representative electro-olfactogram (EOG) concentration–response curve for a *S. tiburo* individual. The magnitude of the log EOG response (a percentage of the  $10^{-3} \text{ mol l}^{-1}$  alanine standard) is linearly related to the log amino acid stimulus concentration ( $10^x \text{ mol l}^{-1}$ ). The horizontal dashed black line indicates the averaged response to the seawater (SW) control. The olfactory threshold is calculated as the point where the regression line for the best-fit line of the response intersects the averaged response to the SW control. The inset shows representative EOG responses to the SW control and to increasing log concentrations of L-alanine. Based on absorbance calculations of diluted dye, all stimuli were diluted to 6% of their injected concentration at the entrance to the incurrent naris. Plotted are the estimated diluted stimulus concentrations at arrival to the olfactory organ.

in only five elasmobranch species (Nikonov et al., 1990; Silver, 1979; Tricas et al., 2009; Zeiske et al., 1986). Also, several recent morphological studies assumed that interspecific differences in the size of elasmobranch olfactory structures confer differences in olfactory sensitivity (Kajjura et al., 2005; Schluessel et al., 2008; Theiss et al., 2009). This study is the first to address these assumptions by comparing the olfactory morphology and physiology of five phylogenetically diverse elasmobranch species. We found that elasmobranchs and teleost fishes have comparable amino acid thresholds and that gross epithelial surface area (i.e. not taking into account secondary lamellae) is not a good predictor of olfactory sensitivity.

This study compared the number of lamellae and lamellar surface area of five phylogenetically diverse elasmobranch species from

similar near-shore habitats. Whereas lamellar surface area increases with body size of an elasmobranch, the number of lamellae does not; therefore, body size was not included as a covariate during analyses (Fishelson and Baranes, 1997; Schluessel et al., 2010). Our results corroborate those of previous studies, which found interspecific differences in the number of lamellae and surface area for elasmobranchs (Kajjura et al., 2005; Schluessel et al., 2008; Theiss et al., 2009). A recent study (Schluessel et al., 2008) quantified the number of lamellae and epithelial surface area for 21 elasmobranch species and concluded that those factors did not correlate with phylogeny but did with habitat (i.e. benthic-pelagic species possessed more lamellae and greater epithelial surface area than benthic species). When grouped by habitat, the two shark species in this study (benthic-pelagic) possessed significantly more lamellae than the three batoid species (benthic), supporting the results of Schluessel et al. (Schluessel et al., 2008).

Teleost fishes also exhibit a wide diversity in the surface area and arrangement of the olfactory epithelium (Hansen and Zielinski, 2005; Kleerekoper, 1969; Yamamoto, 1982). These variations in teleost olfactory organ morphology were suggested to correlate with either ecology or phylogeny (Yamamoto, 1982; Zielinski and Hara, 2001). In their survey of olfactory morphology of over 70 species of teleosts, Hansen and Zielinski found that number of lamellae did not correlate with phylogeny or ecology in teleosts and they could not provide an alternative explanation for the wide variation in lamellar configuration (Hansen and Zielinski, 2005).

Studies on elasmobranch olfactory morphology have attempted to correlate differences in lamellar surface area with olfactory threshold (Kajjura et al., 2005; Schluessel et al., 2008; Theiss et al., 2009) even though the lack of correlation was previously demonstrated in teleost species (Hara, 1994). The rationale behind this proposed correlation is that a species with a greater lamellar surface area should possess a greater number of ORNs and molecular olfactory receptors, which would increase the probability of odorant binding, and thus the ability to detect odorants at a lower concentration than species with less lamellar surface area. Although previous studies found interspecific differences in gross lamellar surface area (Kajjura et al., 2005; Schluessel et al., 2008; Theiss et al., 2009), the number and density of ORNs, extent to which secondary lamellar folding increased surface area, and olfactory thresholds were not quantified. In this study, we tested the hypothesis that lamellar surface area positively correlates with olfactory

Table 2. Mean olfactory thresholds ( $10^x \text{ mol l}^{-1}$ ) of five elasmobranch species to highly stimulatory amino acids (AA) after compensation for stimulus dilution

AA	<i>R. eglanteria</i>		<i>U. jamaicensis</i>		<i>D. sabina</i>		<i>N. brevirostris</i>		<i>S. tiburo</i>	
	Threshold	<i>n</i>	Threshold	<i>n</i>	Threshold	<i>n</i>	Threshold	<i>n</i>	Threshold	<i>n</i>
Ala	-7.85	7	-8.56	6	-7.63	6	-7.44	7	-8.36	6
Phe	-8.10	6	-7.85	4	-7.61	5	-8.97	5	-7.65	6
Ser	-7.81	5	-8.18	6	-6.93	6	-7.01	6	-7.49	6
His	–	–	-7.51	6	-7.28	6	-7.18	6	-7.35	6
Met	–	–	-8.48	6	-7.42	6	-7.25	7	–	–
Asn	-7.29	6	–	–	–	–	–	–	–	–
Gly	-7.64	6	–	–	–	–	–	–	–	–
Glu	–	–	–	–	-7.00	6	–	–	–	–
Gln	–	–	–	–	–	–	–	–	-7.51	6
Cys	–	–	–	–	–	–	–	–	-7.32	6

Olfactory thresholds fell between  $10^{-9.0}$  and  $10^{-6.9} \text{ mol l}^{-1}$ , and did not differ significantly among species per amino acid (alanine, phenylalanine and serine) nor among species when thresholds were pooled. When the thresholds were pooled for each amino acid, the median thresholds to alanine, phenylalanine and serine were significantly different; however, the Dunn's method *post hoc* test did not detect significant differences in any of the pair-wise comparisons. Ala, alanine; Asn, asparagine; Cys, cysteine; Gln, glutamine; Glu, glutamic acid; Gly, glycine; His, histidine; Met, methionine; Phe, phenylalanine; Ser, serine.

sensitivity. We found that although interspecific variation in olfactory lamellar surface area occurred, olfactory thresholds to amino acid odorants did not differ significantly among the five species, with all species demonstrating similar thresholds of between  $10^{-9.0} \text{ mol l}^{-1}$  and  $10^{-6.9} \text{ mol l}^{-1}$  (Table 2). Even though olfactory organs of elasmobranchs are characterized by secondary lamellar folding that greatly increases the actual surface area of the olfactory epithelium, these organs are not more sensitive to amino acid stimuli than the smaller organs of teleost fishes that do not possess secondary lamellae (Hansen and Zielinski, 2005; Hara, 1994; Yamamoto, 1982). Also, olfactory threshold did not correlate significantly with body size for any species; individuals within a species were all selected to be of a similar size, which may obscure any potential size effects.

Greater lamellar surface area may not confer greater olfactory sensitivity because it does not necessarily translate to a greater number or density of ORNs; also ORN quantity is only one of several factors that affect sensitivity. First, the background level of odorants in the environment was shown to affect the olfactory threshold of fishes. An increase in the background level of amino acids would cause the animal's olfactory receptors to become adapted to that amino acid concentration. This would cause an increase in the amino acid olfactory threshold to the concentration of the adapting stimulus (Caprio, 1982). Second, sensitivity is also determined by the number of ORNs converging onto a specific glomerulus in the olfactory bulb, where the axons of ORNs synapse with the dendrites of mitral/tufted neurons (Hamdani and Doving, 2007). With a high convergence ratio of ORNs, glomeruli in the bulb would have a greater chance of being stimulated at low odor concentrations. Third, olfactory receptors also possess various binding affinities for different odorants, as is the case with fish olfactory receptors and amino acids (Bruch and Rulli, 1988; Cagan and Zeiger, 1978). If a given receptor has a high binding affinity for a particular odorant and occurs in sufficient numbers in the olfactory epithelium, the animal would experience a high sensitivity to that odorant because a relatively low concentration of the odorant is probably adequate to maximally occupy the receptor binding site and trigger a physiological response. Finally, we only determined the olfactory responses of elasmobranchs to amino acids; it is possible that their olfactory thresholds to other types of odorants may be different.

The concentration–response relationships for the five or six highly stimulatory amino acids tested were very similar within each species (Fig. 4). There appears to be differences in relative effectiveness at lower concentrations compared with that at  $10^{-4.2} \text{ mol l}^{-1}$  (Fig. 3); however, due to the similarity in relative effectiveness for these amino acids, these differences were not significant. The responses of each species to the five or six highly stimulatory amino acids tested at multiple concentrations increased predictably with increase in stimulus concentration. Some interspecific variability in the relative effectiveness of the 20 amino acids tested at  $10^{-4.2} \text{ mol l}^{-1}$  was observed but the neutral amino acids, such as alanine, serine and methionine, were generally highly stimulatory (Table 3). Neutral amino acids were previously demonstrated to be especially potent stimuli for a few elasmobranch species and for teleost fishes (Caprio and Byrd, 1984; Silver, 1979; Tricas et al., 2009; Zeiske et al., 1986). Tricas et al. (Tricas et al., 2009) found that the response of scalloped hammerhead sharks to cysteine was about twice that for alanine; however, cysteine was a less effective stimulus for the five species we tested (44–75% of alanine), which included another sphyrid species. Valine, proline and isoleucine were some of the least effective stimuli for the species we tested and for scalloped hammerhead sharks and teleosts as well.

Table 3. Ranked mean electro-olfactogram (EOG) responses of five elasmobranch species ( $n \geq 6$ ) to 20 amino acids

AA	<i>R. eglanteria</i>	<i>U. jamaicensis</i>	<i>D. sabina</i>	<i>N. brevirostris</i>	<i>S. tiburo</i>
Ala	1	3	3	3	2
Arg	4	13	9	12	13
Asn	8	14	13	9	5
Asp	15	12	19	20	18
Cys	9	7	8	11	6
Gln	10	8	6	8	7
Glu	2	9	4	10	9
Gly	12	16	17	15	14
His	11	2	5	5	8
Ile	20	18	18	18	20
Leu	18	17	14	14	16
Lys	14	19	16	13	15
Met	6	5	2	6	10
Phe	7	1	7	1	3
Pro	19	15	15	19	19
Ser	3	4	1	4	4
Thr	13	10	10	17	12
Trp	17	11	12	7	11
Tyr	5	6	11	2	1
Val	16	20	20	16	17

Each amino acid has been ranked from most stimulatory (1) to least stimulatory (20) for each species. Highlighted bars indicate amino acids that were consistently most (light gray) or least (dark gray) stimulatory for all five species. Ala, alanine; Arg, arginine; Asn, asparagine; Asp, aspartic acid; Cys, cysteine; Gln, glutamine; Glu, glutamic acid; Gly, glycine; His, histidine; Ile, isoleucine; Leu, leucine; Lys, lysine; Met, methionine; Phe, phenylalanine; Pro, proline; Ser, serine; Thr, threonine; Trp, tryptophan; Tyr, tyrosine; Val, valine.

All three are neutral, non-polar, hydrophobic amino acids, which are characteristics shared by alanine, a highly stimulatory amino acid; however, valine and isoleucine have branched side-chains in contrast to alanine's short side chain. Also, proline is considered an imino acid due to the presence of a secondary amine group. Although the molecular characteristics of the amino acids may not be good predictors of relative effectiveness as olfactory stimuli, our results on the relative effectiveness of amino acids support those of the previous elasmobranch olfactory physiology studies (Silver, 1979; Tricas et al., 2009; Zeiske et al., 1986).

Amino acid thresholds for over 30 species of teleosts range from  $\sim 10^{-9}$  to  $10^{-7} \text{ mol l}^{-1}$  (Hara, 1994). This amino acid concentration range approximates the level of free amino acids in seawater (Hara, 1994; Kuznetsova et al., 2004; Pocklington, 1971). As the olfactory threshold of a fish varies depending on the background level of odorants (Caprio, 1982), and many fishes are subject to similar environmental amino acid levels, both teleosts and elasmobranchs converged on similar amino acid sensitivities. As a result, we cannot support the claim that elasmobranchs demonstrate superior olfactory sensitivities compared with teleost fishes. This finding is contrary to assumptions that persist in the scientific literature, popular science media and culturally. Elasmobranchs are thought to have a particularly acute sense of smell, and while their ability to detect odors at  $10^{-9} \text{ mol l}^{-1}$  is remarkable, they are no more sensitive than teleosts.

This study examined the olfactory morphology and physiology of five elasmobranch species. We found that elasmobranchs, like teleost fishes, exhibit interspecific differences in the olfactory organs; however, these differences did not correlate with differences in amino acid threshold. Although elasmobranchs are reputed to possess greater olfactory sensitivities than bony fishes, they

demonstrate comparable amino acid thresholds as teleosts, further highlighting the olfactory system parallels between these two groups. Future studies should test the responses of elasmobranchs to other biologically relevant odorants, such as bile salts, and use cross-adaptation techniques to determine whether elasmobranchs possess similar amino acid receptors as teleost fishes.

#### LIST OF ABBREVIATIONS

DW	disc width
EKG	electro-cardiogram
EOG	electro-olfactogram
LSD	least significant difference
ORN	olfactory receptor neuron
TL	total length

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