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RESEARCH ARTICLE

Sensitivity and specificity of the olfactory epithelia of two elasmobranch species to bile salts

Tricia L. Meredith[1,*], John Caprio[2] and Stephen M. Kajiura[1]

[1]Department of Biological Sciences, Florida Atlantic University, Boca Raton, FL 33431, USA and [2]Department of Biological Sciences, Louisiana State University, Baton Rouge, LA 70803, USA

*Author for correspondence at present address: Department of Physiology and Biophysics, University of Miami, Leonard M. Miller School of Medicine, Miami, FL 33136, USA (tmeredith@med.miami.edu)

SUMMARY

Odor detection in vertebrates occurs when odorants enter the nose and bind to molecular olfactory receptors on the cilia or microvilli of olfactory receptor neurons (ORNs). Several vertebrate groups possess multiple, morphologically distinct types of ORNs. In teleost fishes, these different ORN types detect specific classes of biologically relevant odorants, such as amino acids, nucleotides and bile salts. For example, bile salts are reported to be detected exclusively by ciliated ORNs. The olfactory epithelium of elasmobranch fishes (sharks, rays and skates) is comprised of microvillous and crypt ORNs, but lacks ciliated ORNs; thus, it was questioned whether the olfactory system of this group of fishes is capable of detecting bile salts. The present investigation clearly indicates that the olfactory system of representative shark and stingray species does detect and respond to bile salts. Additionally, these species detect glycine-conjugated, taurine-conjugated and non-conjugated bile salts, as do teleosts. These elasmobranchs are less sensitive to the tested bile salts than reported for both agnathans and teleosts, but this may be due to the particular bile salts selected in this study, as elasmobranch-produced bile salts are commercially unavailable. Cross-adaptation experiments indicate further that the responses to bile salts are independent of those to amino acids, a major class of odorant molecules for all tested fishes.

Key words: electro-olfactogram, EOG, bile salt, olfaction, olfactory receptor neuron, ORN, olfactory epithelium.

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INTRODUCTION

The olfactory system functions similarly for most vertebrates and mediates several important life history behaviors, such as feeding, reproduction and predator avoidance (Ache and Young, 2005). Chemicals, either volatized in air for tetrapods or dissolved in water for fishes and aquatic amphibians, enter the nose and bind to molecular G-protein coupled olfactory receptors (ORs) on the cilia or microvilli of olfactory receptor neurons (ORNs) in the olfactory epithelium (Eisthen, 2002). Vertebrate groups, including lampreys, teleost fishes, lungfishes, frogs and mammals, possess two morphologically different types of ORNs coupled to specific G-protein α-subunits: ciliated ORNs utilizing the $G\alpha_{olf}$ transduction cascade, and microvillous ORNs coupled to $G\alpha_o$, $G\alpha_i$ or $G\alpha_q$ (Eisthen, 2004). Lampreys and actinopterygian (but not sarcopterygian) fishes also possess a third type of ORN, the crypt cell, which use the $G\alpha_o$ or $G\alpha_q$ transduction cascades (Ferrando et al., 2006; Hansen et al., 2004; Hansen and Finger, 2000; Laframboise et al., 2007; Zeiske et al., 2003).

The morphologically different types of ORNs are thought to mediate responses to specific odorant classes. The olfactory system of fishes is sensitive to several types of odorants, including amino acids, polyamines, bile salts, prostaglandins, steroids and nucleotides (Hara, 1994; Rolen et al., 2003; Zielinski and Hara, 2006). Amino acids, a feeding stimulant in fishes (Zielinski and Hara, 2006), are detected primarily by microvillous ORNs (Lipschitz and Michel, 2002; Sato and Suzuki, 2001), but also possibly by ciliated and crypt ORNs (Hansen et al., 2004; Vielma et al., 2008). In contrast, several studies demonstrated that for teleosts, bile salts are detected only by ciliated ORNs (Døving et al., 2011; Hansen et al., 2003; Sato and Suzuki, 2001). Additionally, cross-adaptation and mixture experiments confirm that teleosts possess ORs for bile salts that are independent from those for amino acids, prostaglandins, gonadal steroids and polyamines (Laberge and Hara, 2004; Michel and Derbidge, 1997; Zhang and Hara, 2009).

Bile salts, which are produced throughout the vertebrate clade, are biliary steroids created in the liver by the oxidation of cholesterol to facilitate intestinal absorption of lipids and fat-soluble vitamins (Hagey et al., 2010; Haslewood, 1967; Hofmann et al., 2010). Bile salts are typically reabsorbed and reused by the enterohepatic system, but teleost fishes excrete a portion of their bile salts in their urine or feces (Velez et al., 2009; Zhang et al., 2001). These excreted bile salts are potent olfactory stimuli, as several teleost species have demonstrated a high olfactory specificity and sensitivity to these compounds in the nanomolar range and lower (Huertas et al., 2010; Zhang and Hara, 2009). Similarly, sea lampreys, whose olfactory epithelium contains the same three ORN types as teleosts (Laframboise et al., 2007), are extremely sensitive to bile salts (threshold: 10^{-13} mol l^{-1}), employing them as pheromones to attract mates and guide adults to spawning streams (Li et al., 2002; Siefkes and Li, 2004; Sorensen et al., 2005). Teleosts may also use bile salts as pheromones (Hara, 1994; Huertas et al., 2007; Sorensen and Caprio, 1997; Sorensen and Stacey, 2004; Zhang et al., 2001). For example, bile salts released by salmonids were suggested to

mediate the homeward migration of conspecifics to their natal stream (Døving et al., 1980).

Elasmobranch fishes (sharks, rays and skates) possess an olfactory system that is morphologically and physiologically similar to that of teleosts (Hansen et al., 2005; Meredith and Kajiura, 2010; Schluessel et al., 2008; Silver, 1979; Takami et al., 1994; Tricas et al., 2009; Zeiske et al., 1986; Zeiske et al., 1987; Zielinski and Hara, 2006). Like other fishes, elasmobranchs possess microvillous ORNs that are likely coupled to $G\alpha_o$ proteins and crypt ORNs; however, they are unique in their lack of ciliated ORNs and the corresponding $G\alpha_{olf}$ expression present in the olfactory epithelium of most vertebrate taxa (Ferrando et al., 2006; Ferrando et al., 2009; Schluessel et al., 2008). The olfactory epithelium of elasmobranchs is highly sensitive to amino acid odorants (Meredith and Kajiura, 2010; Silver, 1979; Tricas et al., 2009; Zeiske et al., 1986), which is likely because of the presence of microvillous ORNs. Because elasmobranchs lack ciliated ORNs and the associated expression of $G\alpha_{olf}$, it is unknown whether they are able to detect bile salts, though the biological relevance of this odorant class for agnathans and teleosts makes it seem likely. This study tested the hypotheses that the olfactory systems of elasmobranch fishes are able to detect bile salt odorants and that the receptor mechanism for bile salts is independent from that for amino acids.

MATERIALS AND METHODS

Animal collection

We tested bile salts on the olfactory systems of two distantly related elasmobranch species, the bonnethead shark, *Sphyrna tiburo* (Linnaeus 1758) and the Atlantic stingray, *Dasyatis sabina* (Lesueur 1824). Six male and one female *D. sabina* were collected using a seine net, and one male and three female *S. tiburo* were collected using a gill net. All animals were obtained from south Florida nearshore waters and 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. All experiments were conducted in accordance with an approved IACUC protocol from Florida Atlantic University (A08-05).

Electro-olfactogram

The underwater electro-olfactogram (EOG) technique was employed to record the olfactory responses of the two species to bile salt odorants (Fig. 1). Prior to experimentation, a fish was injected (intramuscularly or intravenously) with the paralytic pancuronium bromide ($0.03\,mg\,kg^{-1}$). Immediately upon cessation of active ventilation, the fish was transferred to the experimental tank and secured ventral side up to a submerged platform. The acrylic experimental tank ($89\times43\times21\,cm$) was supplied with flow-through seawater that was mechanically ($25\,\mu m$ polyscreen) and chemically (activated charcoal) filtered. The fish was electrically grounded *via* a silver wire in the tank. Seawater was delivered to the tank through two arms of a PVC manifold with the flow for each arm controlled by a ball valve. One arm provided ventilatory water flow *via* the mouth (*S. tiburo*) or spiracles (*D. sabina*) and over the gills, and a second provided seawater flow through the tank which was continuously drained to reduce chemical accumulation. Either seawater or an adapting amino acid was delivered from one of two large buckets through a flow meter to an odor delivery pipette that provided a constant background flow over the olfactory organ. The odor delivery pipette, mounted in a micromanipulator, was positioned with the tip in the incurrent naris, and the water flow was regulated to $2\,ml\,s^{-1}$ (Tricas et al., 2009).

The active EOG electrode, a non-polarizable Ag-AgCl electrode (E45P-M15NH, Warner Instruments, Hamden, CT, USA) fitted with a seawater/agar-filled glass capillary tube, was positioned in the excurrent naris in the water just above the olfactory epithelium and recorded the fish's responses to odor stimuli (Fig. 1, inset). A similar reference electrode was positioned nearby in contact with the skin (Fig. 1, inset). The output from the two electrodes was differentially amplified 1000× (DP-304, Warner Instruments), filtered (0.1 Hz–0.1 kHz, 50/60 Hz; DP-304, Warner Instruments and Hum Bug, Quest Scientific, North Vancouver, BC, Canada), digitized at 1 kHz (Power Lab 16/30 model ML 880, AD Instruments, Colorado Springs, CO, USA) and recorded (Chart™ Software, AD Instruments).

From each individual fish, we recorded the EOG responses to four commercially available bile salts that are known to be highly stimulatory to the teleost olfactory system (Rolen and Caprio, 2007;

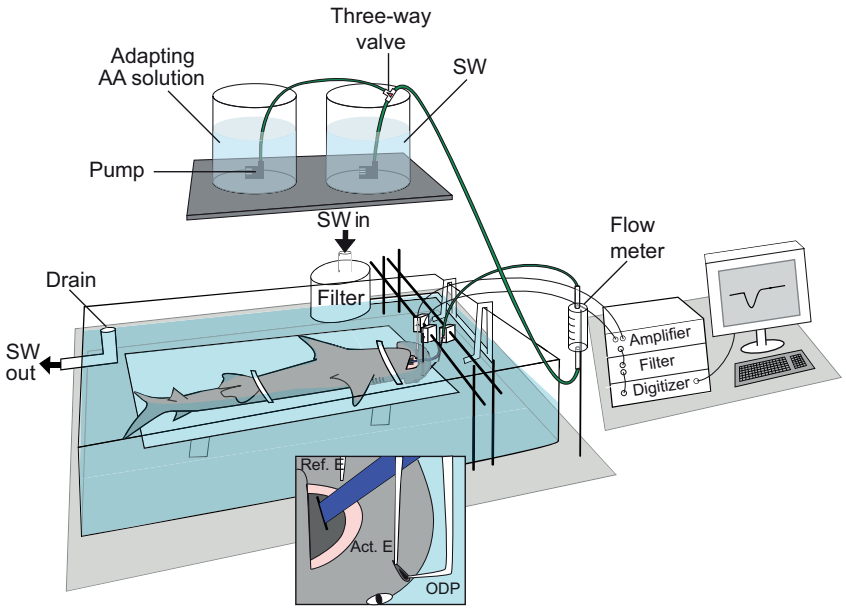

Fig. 1. Experimental apparatus used to record the electro-olfactogram (EOG). See Materials and methods for specifics. AA, amino acid; Act. E, active electrode; ODP, odor delivery pipette; Ref. E, reference electrode; SW seawater.

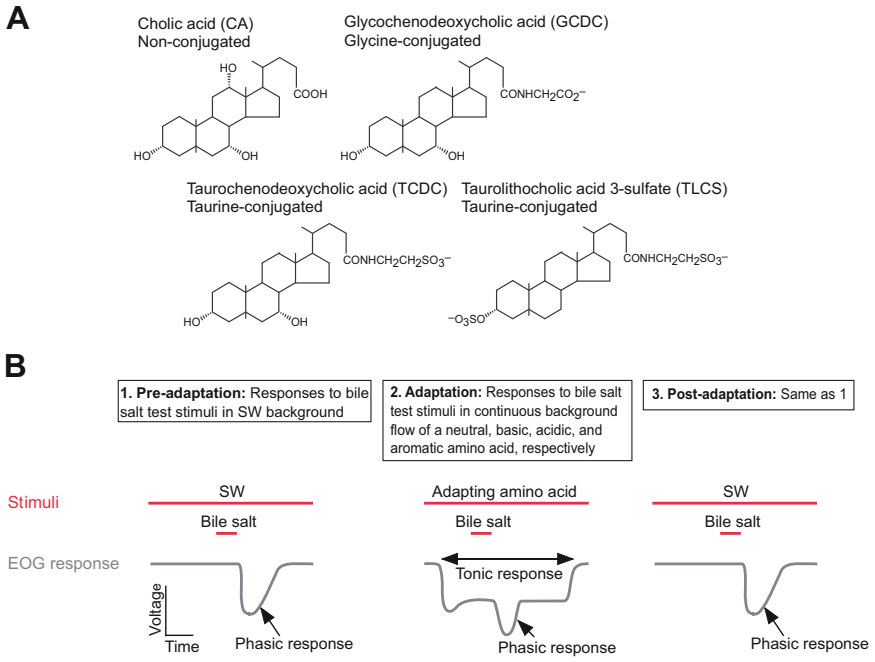

Fig. 2. The molecular structures for each bile salt (A) and the protocol used for the cross-adaptation experiments (B). See Materials and methods for specifics.

Zhang and Hara, 2009). The four bile salts represent three conjugation types: cholic acid (CA), a nonconjugated bile salt; glycochenodeoxycholic acid (GCDC), a glycine-conjugated bile salt; and taurochenodeoxycholic acid (TCDC) and taurolithocholic acid 3-sulfate (TLCS), both taurine-conjugated bile salts (Fig. 2A). The bile salts were applied individually over a background flow of either seawater or an adapting L-amino acid solution. Four L-amino acids, representing four side-chain structural variations, were selected as adapting stimuli: alanine (neutral), glutamic acid (acidic), arginine (basic) and phenylalanine (aromatic) (pH~7.6). All chemicals were purchased from Sigma-Aldrich (St Louis, MO, USA) and were ≥98% pure. Stock solutions of amino acids and bile salts were prepared with filtered seawater daily. For CA and TLCS, 0.1 ml of 100% methanol was the initial solvent. Test solutions were created by diluting the stock solutions to the experimental concentrations with filtered seawater (pH~7.0). The stock solution for CA (10^{-3} mol l^{-1}) was diluted to a working concentration of 10^{-4} mol l^{-1}, and as a result contained a concentration of MeOH solvent diluted to 0.01%.

One milliliter of bile salt solution was injected into the tubing immediately above the odor delivery pipette and transported *via* the background flow to the olfactory epithelium, during which time it became further diluted. To quantify the dilution factor of the injected stimuli, 1.0 ml of fast green dye solution was injected into the tubing in place of a test stimulus and samples were collected every 2 s at the tip of the odor delivery pipette. The spectrophotometric absorbance of the most concentrated sample was measured and its concentration was calculated to determine the dilution factor. Stimuli delivered to the olfactory epithelium of the fish were diluted to approximately 9% of their injected concentration. Therefore, injecting a 10^{-4} mol l^{-1} solution would present a ~10^{-5} mol l^{-1} stimulus to the olfactory epithelium. Recordings lasted variable amounts of time (3–10 h) and continued until a fish's responses were no longer reliable.

Relative effectiveness

The relative effectiveness of the bile salt stimuli was tested by quantifying the EOG responses of each fish to the four bile salt solutions delivered in random order at an injection concentration of 10^{-4} mol l^{-1}. Each bile salt was tested at least twice, and the mean response was calculated for each fish to each bile salt. A 10^{-3} mol l^{-1} alanine standard was administered throughout each experiment after approximately every fifth bile salt stimulus. Occasionally, the magnitude of the alanine response changed by more than 30%, in which case the preceding set of responses were not included in analyses. To compare the relative effectiveness of the four bile salt stimuli among multiple fish, each fish's response to each bile salt was expressed as a percentage of the most recently measured response to the alanine standard. The mean relative responses of all fish to each of the four bile salts were log transformed when necessary to achieve normality and compared within each species using a two-way ANOVA. Holm–Sidak tests were used for *post hoc* comparisons (Systat Software, San Jose, CA, USA). The responses to seawater and 0.1% methanol dissolved in seawater were also recorded periodically throughout the experiment to serve as control stimuli.

Concentration–response relationships

Concentration–response relationships were determined for each species by recording each fish's EOG responses to the four bile salts delivered at increasing injection concentrations from 10^{-6} to 10^{-3} mol l^{-1}. Each bile salt was tested at least twice and the mean response was taken. Olfactory thresholds were calculated for each fish by regressing each bile salt concentration–response curve to its intersection with the control response to seawater. Olfactory thresholds to the four bile salts were log transformed when necessary to achieve normality and compared within each species using a two-way ANOVA. Threshold data were then pooled for each species and compared using a Mann–Whitney rank sum test (Systat Software).

Cross-adaptation

Cross-adaptation experiments were employed to determine whether bile salt and amino acid odorants bind to independent or overlapping molecular olfactory receptors. The experimental procedures for the cross-adaptation experiments paralleled those that were previously

described for teleosts (Caprio and Byrd, 1984; Zhang and Hara, 2009). The four bile salts represented non-conjugated, glycine-conjugated and taurine-conjugated groups (Fig. 2A). The concentrations of the bile salts were chosen as the nearest whole log concentration that elicited similar EOG response magnitudes as 10^{-3} mol l^{-1} CA in order to reduce response variation due to differences in stimulatory effectiveness (Caprio and Byrd, 1984). The chosen concentrations were consistent for all individuals of both species: 10^{-3} mol l^{-1} CA, 10^{-4} mol l^{-1} GCDC, 10^{-3} mol l^{-1} TCDC and 10^{-4} mol l^{-1} TLCS. After establishing the appropriate bile salt concentrations, we recorded from each fish the EOG responses to the four bile salts administered at least twice each in random order at approximately 3 min intervals in a background flow of seawater. The background flow was then switched to one of four randomly chosen adapting L-amino acids, each representing a side-chain structural group. The adapting amino acid was presented continuously over the olfactory epithelium for a minimum of 10 min, resulting in a tonic EOG response during which phasic responses to the four bile salts were recorded at least twice each (Fig. 2B). This phasic response is seen only if the molecular ORs for the test stimulus are independent of those for the adapting stimulus. Once responses to all of the bile salts were recorded during a particular adapting regime, the background flow was returned to seawater for a minimum of 30 min to allow the olfactory receptors to de-adapt, or recover. The EOG responses to the bile salts delivered in random order in a background flow of seawater were once again recorded to confirm that the fish was still responding at the pre-adaptation level. This procedure was repeated for each of the three remaining adapting regimes.

To determine the magnitude of depression in the bile salt responses during amino acid adaptation, the EOG responses to the bile salts during adaptation were taken as a percentage of the responses during the unadapted state (PUR), after subtracting the control responses (Caprio and Byrd, 1984):

$$\mathrm{PUR} = \frac{(\text{adapted response} - \text{control A})}{(\text{unadapted response} - \text{SW})} \times 100 , \qquad (1)$$

where Control A represents the magnitude of the EOG response to an amino acid during adaptation with that same amino acid and SW is the response to an injection of seawater during the unadapted state (background flow of seawater).

Because of the low sample size of responses for each bile salt in each adapting regime ($N \geq 2$ animals each), data were pooled for each adapting regime and the responses to the bile salts in the unadapted state were compared with those during the adapted state using paired *t*-tests (Systat Software).

RESULTS

Relative effectiveness

EOG responses to four bile salts were recorded from the olfactory epithelium of *D. sabina* (N=5–6 individuals) and *S. tiburo* (N=4 individuals). The mean EOG response magnitudes to the four 10^{-4} mol l^{-1} bile salts varied significantly for both species (two-way ANOVA, $P<0.001$ for both species) and ranged from 7 to 18% of the response to the 10^{-3} mol l^{-1} alanine standard (Fig. 3). The responses of *D. sabina* to the bile salts were consistently greater than those of *S. tiburo*. TLCS (18% of Ala) and GCDC (13% of Ala) were most stimulatory to *D. sabina* and *S. tiburo*, respectively.

Concentration–response relationships

EOG responses of the two elasmobranch species to the four bile salts were tested at increasing injection concentrations from 10^{-6} to 10^{-3} mol l^{-1}. The general shapes of the concentration–response relationships for the four bile salts were similar within each species (Fig. 4). As expected, the EOG response increased with increasing bile salt concentration. CA was the least effective bile salt for *D. sabina* compared with the other bile salts tested over multiple log steps of concentration. Mean olfactory thresholds to the four bile salts estimated for both species ranged between $10^{-7.2}$ and $10^{-5.5}$ mol l^{-1} ($N \geq 3$ individuals for each bile salt; Table 1). Olfactory thresholds to the four bile salts did not differ significantly within each species (two-way ANOVA, P=0.873 for *D. sabina*, P=0.070 for *S. tiburo*); therefore, the mean olfactory thresholds were pooled for each species for interspecific comparison. The pooled group of olfactory thresholds was significantly lower for *D. sabina* than for *S. tiburo* (Mann–Whitney, U=190.00, P=0.020).

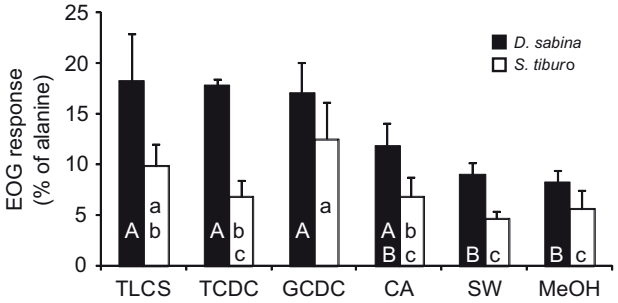

Fig. 3. Mean (+s.e.m.) EOG responses of *Dasyatis sabina* (N=5–6 individuals) and *Sphyrna tiburo* (N=4 individuals) to four bile salts at an injection concentration of 10^{-4} mol l^{-1} and to two controls, seawater (SW) and 0.1% MeOH. Response magnitudes are expressed as percentages of the responses to the standard (10^{-3} mol l^{-1} alanine). Letters within each bar represent the results of pair-wise comparisons using Holm–Sidak tests. For each species, bars not sharing a letter are significantly different. The mean EOG response magnitudes to the four bile salts varied significantly for both species and ranged from 7 to 18% of the response to the standard. CA, cholic acid; GCDC, glycochenodeoxycholic acid; TCDC, taurochenodeoxycholic acid; TLCS, taurolithocholic acid 3-sulfate.

Cross-adaptation

The cross-adaptation protocol was employed to test whether bile salt and amino acid odorants interact with independent or overlapping molecular olfactory receptors. The mean of the PURs to the four bile salts tested during adaptation with each of four L-amino acids were 89.7±7.1% for *D. sabina* and 102.5±6.5% for *S. tiburo* ($N \geq 2$ individuals for each bile salt in each adapting regime; Fig. 5). Once the responses were pooled for each adapting regime, the responses to the bile salts during the adapted state did not differ significantly from those during the unadapted state for either species ($P>0.05$ for all tests). These results indicate that bile salts bind to molecular olfactory receptors different from those that bind amino acids. The mean responses of both *D. sabina* and *S. tiburo* to 10^{-3} mol l^{-1} CA were significantly greater than the control responses to both seawater and 0.1% MeOH ($P<0.05$).

DISCUSSION

Elasmobranchs possess microvillous and crypt ORNs in their olfactory epithelium, but they lack the ciliated ORNs found in the olfactory epithelium of teleosts and most other vertebrates (Eisthen, 2004; Schluessel et al., 2008). Teleosts use ciliated ORNs to detect bile salt odorants, which serve as important olfactory cues (Døving et al., 1980; Hansen et al., 2003; Hansen and Zielinski, 2005; Sato

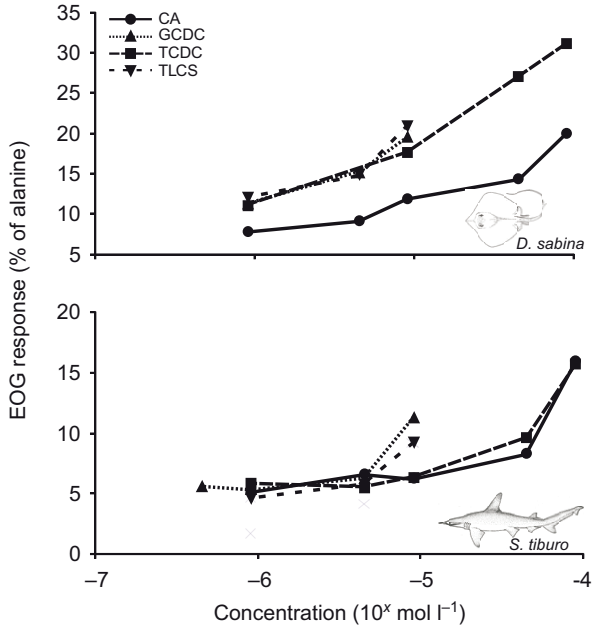

Fig. 4. Concentration–response relationships of four bile salts for *D. sabina* and *S. tiburo*. Response magnitudes represent the mean response for each bile salt at each tested concentration and are expressed as a percentage of the standard (10^{-3} mol l^{-1} alanine). The EOG response increased with increasing bile salt concentration. Thresholds estimated for the two species to each bile salt are given in Table 1. CA, cholic acid; GCDC, glycochenodeoxycholic acid; TCDC, taurochenodeoxycholic acid; TLCS, taurolithocholic acid 3-sulfate.

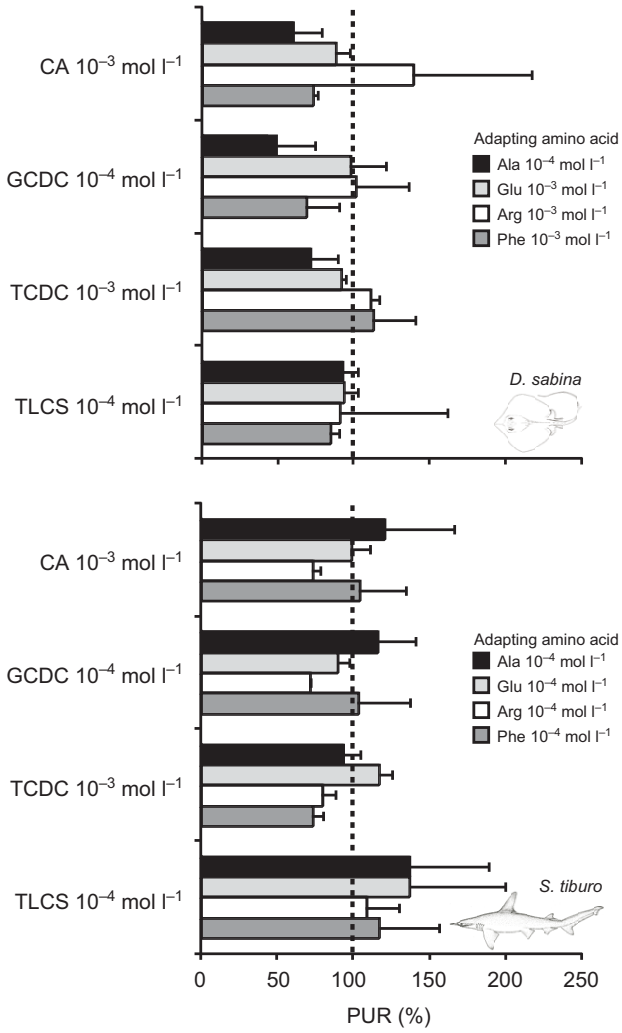

Fig. 5. Mean (+s.e.m.) percentage unadapted responses (PURs) of *D. sabina* and *S. tiburo* to four bile salts presented individually during each of four L-amino acid adapting regimes. Once pooled for each adapting regime, the responses to the bile salts during the adapted state did not differ significantly from those during the unadapted state for either species. The dashed line indicates 100% PUR. CA, cholic acid; GCDC, glycochenodeoxycholic acid; TCDC, taurochenodeoxycholic acid; TLCS, taurolithocholic acid 3-sulfate.

and Suzuki, 2001). This study investigated whether elasmobranchs, which lack ciliated ORNs, are able to detect bile salt odorants. The two elasmobranch species tested responded to bile salts, despite lacking ciliated ORNs, though they were less sensitive to the particular bile salts used in this study than previously documented for teleosts and agnathans. In addition, elasmobranchs, like teleosts, possess molecular ORs sensitive to bile salts that are relatively independent of those that detect amino acids, which are food-related odorants; this demonstrated that even though they employ only two ORN morphotypes in their olfactory epithelium, neither of which are ciliated ORNs, elasmobranchs are still able to distinguish bile salts from other odorants with a high degree of specificity.

All vertebrate classes produce bile salts that differ structurally from clade to clade. Within fishes, an evolutionary progression occurs from 5α bile alcohols produced by agnathans to 5β bile alcohols of chondrichthyans and C_{24} bile acids of actinopterygians (Hagey et al., 2010). Specifically, sea lampreys produce C_{24} and C_{27} bile alcohols and hagfish produce 5α-myxinol disulfate, a C_{27} 5α bile alcohol disulfate. Elasmobranch fishes primarily produce a C_{27} bile alcohol, 5β-scymnol 27-sulfate (Bridgwater et al., 1962; Hagey et al., 2010; Hofmann et al., 2010). The most common bile salts produced by ray-finned fishes are C_{24} bile acids, such as CA (used in this study) (Hagey et al., 2010; Haslewood, 1967). Teleosts also produce a variety of other C_{24} bile salts, including taurine and glycine conjugates, and possess a high olfactory sensitivity to all of these compounds (Denton et al., 1974; Michel and Lubomudrov, 1995; Rolen and Caprio, 2007; Zhang and Hara, 2009; Zhang et al., 2001).

Because teleost olfactory responses to bile salts are mediated by ciliated ORNs, which elasmobranchs lack, elasmobranchs must use either microvillous or crypt ORNs for bile salt detection, a strategy

Table 1. Olfactory thresholds (10^x mol l^{-1}) of *Dasyatis sabina* and *Sphyrna tiburo* to four bile salts after compensation for stimulus dilution

	D. sabina		*S. tiburo*	
Bile salt	Threshold	*N*	Threshold	*N*
CA	–6.4±0.49	4	–5.5±0.31	4
GCDC	–6.6±0.59	3	–6.2±0.31	4
TCDC	–6.8±0.29	6	–5.7±0.22	4
TLCS	–7.2±0.92	3	–6.5±0.59	4

CA, cholic acid; GCDC, glycochenodeoxycholic acid; TCDC, taurochenodeoxycholic acid; TLCS, taurolithocholic acid 3-sulfate.
Data are means ± s.e.m. (*N*, number of individuals).

thus far undocumented in fishes. It is currently unknown which receptor types agnathans use to detect particular odorant classes, as it was only recently discovered that lampreys possess three ORN types similar to those in the teleost olfactory epithelium (Laframboise et al., 2007). In teleosts, ORN type and function are correlated (Hansen et al., 2003), so it is puzzling that elasmobranchs detect a comparable suite of odorants with fewer types of ORNs. One possible explanation is that elasmobranchs possess multiple sub-types of microvillous and crypt ORNs with at least somewhat distinct odorant specificities. A study on the G-protein associations of different ORN morphotypes in teleosts reported the presence of three sub-classes of microvillous ORNs, each coupled to a specific G-protein α-subunit (Hansen and Zielinski, 2005). It is possible that multiple sub-classes of microvillous ORNs exist in the elasmobranch olfactory epithelium and that at least one type mediates responses to bile salt odorants.

The two elasmobranch species tested demonstrated smaller relative EOG responses (Fig. 3) and less sensitivity (Table 1) to bile salts compared with teleosts and agnathans. For *D. sabina* and *S. tiburo*, the olfactory thresholds to the four bile salts ranged from $10^{-7.2}$ to $10^{-5.5}$ mol l^{-1}. These thresholds occur at the high end of the range for teleost fishes, which exhibit olfactory thresholds to bile salts between 10^{-12} to 10^{-6} mol l^{-1} (Hara, 1994; Huertas et al., 2010; Michel and Lubomudrov, 1995; Zhang and Hara, 2009), and are notably higher than thresholds estimated for agnathans, which reach the sub-picomolar range (Li et al., 1995; Siefkes and Li, 2004; Sorensen et al., 2005). With respect to bile salts being biologically relevant to fishes, it is rather interesting to note that bile salts are also detected by the taste system of fishes, with an estimated threshold for rainbow trout to taurocholic acid in the picomolar range (Yamashita et al., 2006) and an estimated threshold for the channel catfish to bile salts in the 10–100 pmol range (Rolen and Caprio, 2008). Unfortunately, little is currently known concerning the physiology of the gustatory system in any elasmobranch. A potential reason for the relatively small response magnitudes and limited sensitivities reported here for the olfactory system of elasmobranchs is that the bile salts tested are produced by teleosts (Zhang and Hara, 2009). CA is one of the most commonly produced bile salts in teleost fishes (Hagey et al., 2010; Haslewood, 1967). If elasmobranchs use C_{24} bile salts to localize teleost prey, one would predict a high olfactory sensitivity to these compounds, such as CA; our results do not support this. Teleosts are thought to potentially use bile salts produced by conspecifics as pheromones (Sorensen and Caprio, 1997; Sorensen and Stacey, 2004; Zhang et al., 2001). If this occurs in elasmobranchs as well, then bile salts that are produced by elasmobranchs, such as 5β-scymnol 27-sulfate, might prove more potent as olfactory stimuli. Unfortunately, these elasmobranch-produced bile salts are currently commercially unavailable, so they must either be isolated or synthesized in order to be used in future olfactory studies.

Overall, the two elasmobranch species in this study demonstrated comparable EOG responses to the bile salts; however, some interspecific differences were evident. The EOG responses of *D. sabina* to the four bile salts were consistently of greater relative magnitude than those of *S. tiburo* (Fig. 3). This is contrary to a trend we previously reported where the EOG responses of two shark species to amino acids were approximately three times greater than those of three batoid species (skates and rays), suggesting that this trend is odorant dependent (Meredith and Kajiura, 2010). Significantly lower olfactory thresholds ($10^{-7.2}$ and $10^{-6.9}$ mol l^{-1}) to the four tested bile salt odorants were observed for *D. sabina* than for *S. tiburo* ($10^{-5.5}$ to $10^{-6.5}$ mol l^{-1}; Table 1). The bile salt olfactory thresholds reported here are notably higher than those reported for the same two species to amino acids ($10^{-8.1}$ to $10^{-7.3}$ mol l^{-1} thresholds for *D. sabina* and $10^{-8.4}$ to $10^{-7.3}$ mol l^{-1} thresholds for *S. tiburo*) (Meredith and Kajiura, 2010). Because bile salts may be used in intraspecific communication, it is possible that differences exist between the sexes in the responsiveness to these stimuli. Our uneven sampling of each sex for both species and a small sample size for each sex preclude statistical comparisons, although a study with teleosts did not find differences in the bile salt responses between sexes for goldfish or Mozambique tilapia (Huertas et al., 2010).

Although the relative magnitude of the responses of both species to all four bile salts were within 11% of each other, CA was the least stimulatory with the highest olfactory threshold for both species. In addition, the olfactory responsivity for *D. sabina* to CA across multiple stimulus concentrations is less than that for the other three bile salts (Fig. 4). This may reflect the differences in amidation between CA and the other three bile salts; free bile salts (non-conjugated) also elicited smaller maximum responses from the olfactory system of lake char (*Salvelinus namaycush*) when compared with amidated bile salts (conjugated with either taurine or glycine) (Zhang and Hara, 2009). The distinct relative effectiveness and concentration–response curves between free and amidated bile salts seen here may suggest that elasmobranchs detect them using distinct molecular OR types. Teleosts and agnathans distinguish among bile salts depending on amidation and the type and position of the conjugating group (Li and Sorensen, 1997; Michel and Derbidge, 1997; Rolen and Caprio, 2007; Zhang and Hara, 2009). Between three and six bile salt ORs were characterized for teleosts out of the ~100 molecular ORs which have been identified (Ngai and Alioto, 2008). Cross-adaptation or mixture experiments that test bile salts from different groups against each other would elucidate the likelihood of this possibility.

Although we cannot conclusively confirm whether elasmobranchs possess multiple OR types that distinguish among bile salt odorants, our cross-adaptation data (Fig. 5) demonstrated that amino acid and bile salt odorants interact with independent molecular ORs in the olfactory epithelium of both elasmobranch species. Though the mean PURs were occasionally >100% because of the small sample size and inter-individual variability, overall they were near 100% under all amino acid adapting regimes (89% for *D. sabina* and 102% for *S. tiburo*). Thus, the continuous presence of an amino acid at the olfactory epithelium had little effect on the responses to bile salts, indicating that these two groups of odorants bind to relatively independent molecular ORs. Teleost fishes also possess independent OR populations that distinguish between bile salt and amino acid odorants (Michel and Derbidge, 1997; Zhang and Hara, 2009), though it is currently unknown whether this also occurs in agnathans. Amino acids and bile salts have distinct molecular structures that activate different ORs in both elasmobranch and teleost fishes. Even though they lack the seemingly necessary ciliated ORN type in their olfactory epithelium, elasmobranchs are able to distinguish bile salts from other odorants with a high degree of specificity. For most vertebrates, including teleosts, the axons of ORNs expressing different classes of ORs converge onto separate regions in the olfactory bulb (Friedrich and Korsching, 1998; Hamdani and Døving, 2007; Nikonov and Caprio, 2001; Xu et al., 2000). It is unknown whether elasmobranchs, a phylogenetically basal vertebrate group of fishes, possess a similar chemotopic olfactory

bulb map. Future studies should test the responses of elasmobranchs to elasmobranch-produced bile salts, investigate which ORN type(s) expresses molecular ORs that detect bile salts, and determine whether the responses to bile salt stimuli are chemotopically mapped onto the elasmobranch olfactory bulb.

LIST OF ABBREVIATIONS

CA	cholic acid
EOG	electro-olfactogram
GCDC	glycochenodeoxycholic acid
OR	olfactory receptor
ORN	olfactory receptor neuron
TCDC	taurochenodeoxycholic acid
TLCS	taurolithocholic acid 3-sulfate

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