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Anatomy of the olfactory system and potential role for chemical communication in the sound-producing Lusitanian toadfish, *Halobatrachus didactylus*

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Abstract

The current study investigated the structure and function of the olfactory system of the Lusitanian toadfish, *Halobatrachus didactylus*, using histology and electrophysiology (electro-olfactogram [EOG]), respectively. The olfactory system consists of a digitated anterior peduncle, of unknown function, containing the inhalant nostril. This then leads to a U-shaped olfactory chamber with the olfactory epithelium—identified by $G_{\alpha olf}$-immunoreactivity—on the ventral surface. A large lacrimal sac is connected to this tube and is likely involved in generating water movement through the olfactory chamber (this species is largely sedentary). The exhalent nostril lies by the eye and is preceded by a bicuspid valve to ensure one-way flow of water. As do other teleosts, *H. didactylus* had olfactory sensitivity to amino acids and bile acids. Large-amplitude EOG responses were evoked by fluid from the anterior and posterior testicular accessory glands, and bile and intestinal fluids. Anterior gland and intestinal fluids from reproductive males were significantly more potent than those from non-reproductive males. Male urine and skin mucus proved to be the least potent body fluids tested. These results suggest that chemical communication—as well as acoustic communication—may be important in the reproduction of this species and that this may be mediated by the accessory glands and intestinal fluid.

KEYWORDS
accessory glands, electro-olfactogram, intestinal fluid, olfaction, pheromone, reproduction

1 | INTRODUCTION

In the animal kingdom, communication is important for the survival and perpetuation of species, and fishes are no exception. Fishes have a complex and sophisticated array of sensory systems: vision, mechanosensory (hearing and detection of water movement), chemosensory (taste and smell), and, in some species, electro-sensory systems (Montgomery & Carton, 2008). The prevalence and importance of each modality depend strongly on the environmental conditions, life history, and phylogeny of the species in question (Endler, 1992). In

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the marine environment, vision is limited at night, in deep water, and in high turbidity; thus, chemical senses (olfaction and taste) and mechanosenses (hearing and water movements) are, perhaps, the most important senses for communication.

Among the fishes that use acoustic communication, one of the best-studied groups is the Batrachoididae, generally referred to as toadfishes. Toadfishes are well known for their distinctive multiharmonic, male advertisement vocalizations used to attract females to nest sites, and for male–male aggression (Amorim et al., 2008; Bass & McKibben, 2003; Thorson & Fine, 2002). Whereas acoustic communication is well studied in toadfishes, the importance of other senses, such as vision and olfaction, has been mainly investigated in nonvocal species. Apart from one study that suggests that the Gulf toadfish, *Opsanus beta*, uses pulsatile urea excretion for intraspecific communication (Fulton et al., 2017), nothing is known about the role of chemical communication in Batrachoididae. In the aquatic environment, chemical cues can last longer than visual and acoustic cues and can be used in combination with other signals to reinforce or specify the meaning (Hughes, 1996). More broadly, chemosensitivity plays a central role in survival and reproduction in the aquatic environment; fishes use olfaction for food search, avoidance of predators, habitat recognition, reproduction, migration, and conspecific interactions (Kieffer & Colgan, 1992). The use of acoustic intraspecific communication in toadfishes clearly does not exclude other forms of communication.

The Lusitanian toadfish (*Halobatrachus didactylus* [Bloch and Schneider 1801]) is a sound-producing batrachoidid of estuaries and coastal waters living mainly on sand and/or muddy bottoms (Cotter et al., 2013). It is benthic, solitary, and relatively sedentary, being more active from dusk to dawn (Pereira et al., 2021). During the reproductive season (May–July in the Iberian Peninsula), large territorial males (type I) nest under rocks or other hard substrates and produce long advertisement calls (boat whistles) to attract ripe females, and agonistic boat whistles to defend their nest from conspecific nest intruders through the contraction of paired sonic muscles attached to the swim bladder (Modesto & Canário, 2003a; Vasconcelos et al., 2010, 2011). This sophisticated sound repertoire, which includes individual rhythms and distinct chorus patterns (Vieira et al., 2021), is essential for breeding success and is used as an indicator of male condition (Vasconcelos et al., 2012). Smaller (type II) males mimic females morphologically and are thought to adopt sneaking behavior as in other batrachoidids (Brantley & Bass, 1994). In territorial males, two accessory glands are joined to the sperm duct and undergo seasonal changes in weight and fluid production in parallel with the gonado-somatic index. Along the breeding season, type I males increase their investment in accessory glands, and fluids run abundantly from the urogenital pore together with sperm in guarder males (but not in sneakers, whose glands are atrophied) (Modesto & Canário, 2003b). The role of these accessory glands in reproduction is not yet fully understood; however, their fluids contain muco-substances and proteins (Modesto & Canário, 2003b) and a diversity of steroids (Modesto et al., 2015) and can prolong sperm motility, possibly enhancing sperm performance during spermiation or fertilization (Modesto, T., unpublished). In the batrachoid *Porichtys notatus*, these reproductive glands also function in paternal care by inhibiting bacterial growth associated with unhealthy eggs with remarkable specificity (Pepler et al., 2021), and glandular fluids increase sperm velocity in guarder males (Miller et al., 2019). These findings suggest that these glands play important roles in reproduction.

Steroid hormones and/or their derivatives are known to function as reproductive pheromones in some fishes (Stacey, 2014), raising the hypothesis that, in addition to sounds, chemicals such as those released by the accessory glands, or in other biological fluids, could play an important function in *H. didactylus* communication, particularly during reproduction. To evaluate this, the olfactory sensitivity of this species to a range of amino acids, bile acids, and conspecific body fluids (urine, intestinal fluid, bile fluid, mucus, and fluids from the anterior and posterior glands) collected from males from different reproductive status (reproductive and non-reproductive males) was evaluated using electro-olfactogram (EOG). The degree of development and specialization of a given organ is generally indicative of its functional importance. Therefore, a morphological study of the olfactory organ was carried out, not only to assess the importance of olfaction but also because knowing the location of the sensory cells is essential for electrophysiology.

2 | MATERIALS AND METHODS

2.1 | Experimental animals

Adult *H. didactylus*, males and females, were collected in the Ria Formosa lagoon (southern Portugal) using an experimental beam trawler, or by local fishermen using trammel net, under the required permits issued by the Portuguese Agency for Nature Conservancy (ICNF, 7048/2020/DCBN/DAN) and the General Directorate for Marine Resources (DGRM, 1009/2019/DRI). Animal maintenance and experimentation were carried out in certified experimental facilities and following Portuguese national legislation (DL 113/2013) following the guidelines established by the EU Directive 2010/63/EU on "The Protection of Animals Used for Scientific Purposes." The experimental design was previously approved by the CCMAR Ethical Committee for Managing Animal Welfare (ORBEA) and by the Portuguese Veterinary Authority (DGAV) under permit 009272. Fish manipulation was performed by accredited scientists in laboratory animal science by the Portuguese DGAV, equivalent to FELASA category C criteria.

Fish were kept in the Ramalhete Marine Station (CCMAR—University of Algarve), maintained in exterior but shaded 600-L (bottom area: 1.3 m^2) tanks with continuously running natural seawater, aerated under natural photoperiod and temperature, and fed with squid (every day, 3% w/w tank biomass). For environmental enrichment, tanks were provided with shelters plastic (polyvinyl chloride pipes cut in half lengthwise), which were readily accepted.

2.2 | Gross morphology of the olfactory organ

An adult male (total length: 30 cm) was killed with an overdose (5 mL/L) of the anesthetic 2-phenoxyethanol (Sigma-Aldrich, Portugal). The

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localization and external characterization of the nostrils were made using a dissecting microscope (Leica MZ6 StereoZoom Microscope). The olfactory organ was filled through the posterior nostril with molding paste (Express 2 Light Body Flow, 3 M, Douromed, Portugal) using a syringe. The cast was removed after solidification by careful dissection of surrounding tissues, and the images were obtained.

2.3 | Histology

To characterize the general histology of the olfactory organ, two juveniles (12.5 ± 0.9 cm, 38.1 ± 14.95 g) were killed with an overdose of the anesthetic, as described earlier. The anterior part of the head, containing the nasal tubules and olfactory chambers, was dissected and fixed in 4% paraformaldehyde (PFA) overnight at 4°C. Fixed tissues were then washed with sterile phosphate-buffered saline (PBS) and immediately decalcified with 0.5 M ethylenediaminetetraacetic acid (EDTA), pH 8.0, for 7 days. The EDTA solution was renewed every 2 days to accelerate the decalcifying process. Decalcified tissues were stored in 70% ethanol, processed, and embedded in paraffin. Briefly, tissue samples were dehydrated in ethanol (70%, 96%, and 100%), saturated in xylene, and impregnated and embedded in low-melting-point paraffin wax (Histosec, Merck, Sigma-Aldrich). Serial 7-μm sections of each tissue were mounted on poly-L-lysine (Sigma-Aldrich) coated glass slides and stained with Harris hematoxylin–eosin (Bancroft & Layton, 2013), Masson's trichrome (Witten & Hall, 2003), and Alcian blue–periodic acid–Schiff (PAS; Mowry, 2008). Stained histological sections were observed under a microscope (Leica DM2000) coupled to a digital camera (Leica DFC480) linked to a computer for digital image analysis (Leica IM50 software).

2.4 | Immunocytochemistry

A *H. didactylus* individual (26.2 cm; 234 g) was killed as described earlier; the peduncle with the inhalant (anterior) nostril and the anterior part of the olfactory chamber were dissected from the head and fixed in 4% PFA overnight at 4°C. The fixed tissues were washed with 0.1 M PBS and cryoprotected by incubation in an ascending series of sucrose solution (10%, 20%, and 30%). Tissues were mounted in Tissue-Tek optimum cutting temperature (Sakura). Tissue blocks were frozen on dry ice and stored at −20°C. Serial 20-μm transverse sections were obtained using a cryostat (Cryostar NX50, Thermo Scientific) with disposable stainless-steel low-profile blades (MX35 Ultra Low-Profile, Thermo Scientific) and mounted on poly-L-lysine-coated glass slides.

Immunocytochemistry was carried out using the mouse monoclonal anti-$G_{\alpha olf}$ antibody (sc-55545, Santa Cruz, Dallas, TX, USA) raised against G protein alpha olf subunit ($G_{\alpha olf}$). Tissues were washed twice with PBS-T 1× (PBS + Triton) for 5 min, blocked with 3% sheep serum (Sigma-Aldrich) in TCT (Tris buffer + 0.7% carrageenan and 0.5% Triton X-100, pH 7.6) for 2 h at room temperature, and incubated with the primary antibody (1:50) at 4°C overnight. After being washed twice with PBS-T, the sections were incubated with the secondary antibody (1:400 in PBS 1×, Alexa Fluor 546-conjugated anti-mouse IgG, A-11030, Molecular Probes) for 2 h at room temperature. Control staining was performed using PBS without the primary antibody. After being washed with PBS, the tissues were mounted in glycerol gelatine (Sigma-Aldrich, GG1). The sections were observed under a fluorescence microscope (Zeiss Axioimager Z2 coupled to a digital camera Axiocam ICC3).

2.5 | Body fluid sampling

Body fluids—mucus, urine, bile, intestinal fluid, and gland fluids (from anterior and posterior regions)—were collected from type I males in January ($N = 6$, 23.1 ± 0.5 cm, 226.2 ± 13.1 g) and May ($N = 7$, 26.7 ± 1.9 cm, 348.4 ± 85.8 g) 2020, the non-reproductive and reproductive seasons, respectively. Morphotype type I males were easily identified based on size, an elongated urogenital papilla, and secretion of their accessory glands when gently pressed near the vent (Modesto & Canário, 2003b).

For mucous collection, fish were slightly anaesthetized with 2-phenoxyethanol (0.01% in seawater, Sigma-Aldrich) to avoid the stress of manipulation. Clean glass slides were used to carefully remove mucus from the dorsal–lateral region in a front-to-caudal direction: a slide was gently scraped along both sides of the animal twice or thrice, and the skin mucus was carefully collected in a plastic tube. Mucous samples were weighed, and the same weight of seawater was added. The tubes were then vortexed (1 min) and centrifuged, and the supernatant was collected. For the collection of urine, bile fluid, intestinal fluid, and gland fluids, type I males were killed with an overdose of 2-phenoxyethanol, as previously described. They were then dissected, and the gonads and accessory glands were taken out and weighed. Accessory gland fluids were collected by gently slicing the anterior and posterior regions of the accessory glands; the fluids were centrifuged, and the supernatant was collected. Urine and bile fluids were obtained by cutting the urinary bladder and gallbladder, respectively, and draining the fluid into a plastic tube, and intestinal fluid was collected by cutting the posterior extreme of the intestine, near anus, and expelling the contents of the last 10 cm of the intestine into a tube. The intestinal contents were centrifuged, and the supernatant was separated. Equal volumes of each fluid were pooled from either reproductive ($n = 7$) or non-reproductive males ($n = 6$), mixed thoroughly, centrifuged, aliquoted, and frozen at −20°C until used in EOG recording.

2.6 | EOG recording

The EOG was recorded as previously described (Velez et al., 2011) with slight modifications. Briefly, individuals ($n = 6$ females, 20.5 ± 0.5 cm, 154.2 ± 15.3; $n = 7$ males, 24.0 ± 0.6 cm, 190.5 ± 29.4 g) were anaesthetized by immersion in water containing

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2-phenoxyethanol (0.2 mL/L, Sigma-Aldrich) until the response to a tail pinch had stopped. An intramuscular injection of the neuromuscular blocker gallamine tri-ethiodide (Sigma-Aldrich; 10 mg/kg in 0.9% NaCl) was then given. Fish were then placed on a padded surface (with a slight backward tilt), and the gills were flushed with aerated seawater containing 0.1 mL/L of 2-phenoxyethanol, via a plastic tube inserted into the mouth and a submersible pump. The peduncle of the inhalant (anterior) nostril was removed to gain access to the olfactory epithelium. The nostril was constantly irrigated with charcoal-filtered seawater (without anesthetic) under gravity (flow rate: 10 mL/min) via a glass tube. Test solutions were delivered to this tube via a three-way solenoid valve for 4 s. Charcoal-filtered seawater was used to dilute the odorant solutions and to irrigate the olfactory epithelium.

Frozen aliquots of amino acids (10^{-2} M in seawater) and pooled samples of each body fluid from reproductive and non-reproductive males were diluted in charcoal-filtered seawater (35 ppt) immediately prior to use. The amino acids used were L-cysteine (sulfur containing), L-arginine (basic), L-glutamate (acidic), and L-leucine (aliphatic, neutral). The two common fish bile acids, taurocholic acid (TCA) and taurolithocholic acid (TLC), were dissolved in methanol (10^{-2} M) and diluted in seawater immediately prior to use. The order in which odorants were given was randomized, but each odorant was always given from the lowest to the highest concentration. The recording electrode was placed at a position that resulted in the largest response to the "standard" stimulus (10^{-3} M L-cysteine). The reference electrode was placed lightly on the skin of the head nearby. The fish was ground with an Ag/AgCl pellet electrode placed under the head. The d.c. signal was amplified using a NeuroLog head-stage NL100 and NL109 bridge amplifier passed through an NL125 filter with low pass set at 30 Hz (Digitimer Ltd., https://www.digitimer.com/). The signal was digitized (Digidata 1440A, Molecular Devices, https://www.moleculardevices.com/) and stored on a personal computer running AxoScope software (version 10.6, Molecular Devices). Response amplitudes were normalized to the amplitude of response to 10^{-3} M L-cysteine (the "standard"). Responses to the standard were recorded regularly at the beginning and end of each group of samples (every three to five samples) throughout the recording session.

2.7 | Data treatment and statistical analysis

The amplitude of the peak of the EOG was measured in millivolts. This was blank subtracted (amplitude of EOG in response to water treated in the same way as stimulus solutions but without the addition of odorant), and the amplitudes were normalized to the amplitude of response to the standard stimulus (10^{-3} M L-cysteine). To compare the effects of concentration and fluid source (reproductive and non-reproductive males), a two-way repeated-measures ANOVA was performed on data for all fluids with reproductive status and dilution as the two factors and a Fisher's least significant difference post hoc test. A *p*-value of <0.05 was taken as statistically significant.

3 | RESULTS

3.1 | Gross morphology of the olfactory organ

The paired olfactory organs are situated in the dorsal region of the head, rostral to the eye (Figure 1). The anterior (inhalant) nostril is located in a tube-shaped structure (peduncle) that has small finger-like projections at the distal end. The posterior (exhalant) nostril near the eye is simple and rounded (Figure 1a).

The cast showed that the internal structure of each olfactory organ consists of an elongated tubiform chamber that starts at the base of the peduncle and a single accessory nasal sac (lacrimal sac)

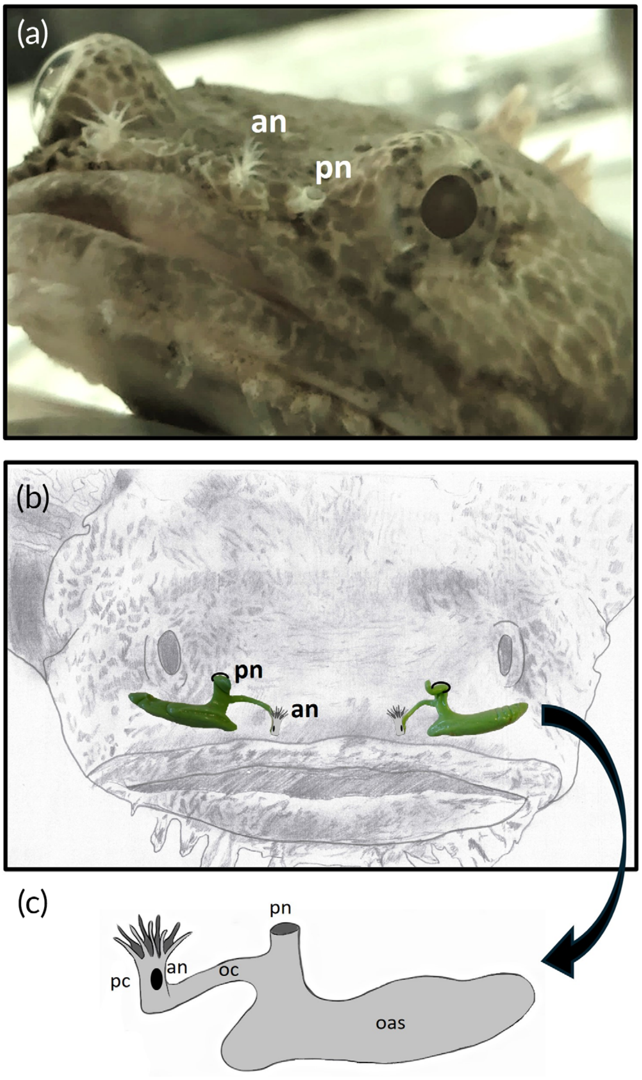

FIGURE 1 Structures of the olfactory organ of *Halobatrachus didactylus*. (a) Image of the head of an adult showing the anterior nostril (an) and the posterior nostril (pn). (b) Illustration of the head with an image of the right and left casts of the internal structure of the olfactory organ (green) to illustrate its localization within the fish head. (c) Schematic representation of the olfactory organ showing the peduncle (pc), the anterior nostril (an), the olfactory chamber (oc), the posterior nostril (pn), and the olfactory accessory sac (oas).

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that expands ventrally under the eye (Figure 1b,c). Histological studies showed that the olfactory epithelium is located in the tubiform chamber (see Figure 2), hereafter olfactory chamber (oc).

3.2 | Histological characterization of the olfactory organ

Figure 2 shows the internal morphology of the olfactory organ where the sections sampled for this study are labeled with different letters (a–f). The initial part of the anterior nostril, below the bottom of the finger-like projections, is lined by a nonsensory stratified cuboidal epithelium with some mucous cells (Figure 2a). At the base of the peduncle, in the transition to the tubiform chamber (olfactory chamber) the epithelium lining the inner cavity is histologically different in the ventral and dorsal sides; the ventral side is composed of pseudostratified epithelium (sensory epithelium [SE]) and the dorsal side of stratified cuboidal epithelium rich in mucous cells (nonsensory epithelium [NSE]) (Figure 2b). This histological structure was found throughout the olfactory chamber (Figure 2c,d). The olfactory accessory sac (Figure 2e) is lined by stratified cuboid epithelium composed of two or three cell layers. In the connection between the accessory sac and the base of the posterior nostril, there is a valve in the form of two thin lips (Figure 2e, black arrows). The posterior nostril is lined with a stratified cuboidal epithelium that is abundant in mucous cells (Figure 2f).

Immunocytochemistry showed $G_{\alpha olf}$ immunopositive cells distributed at the surface of the pseudostratified epithelium (Figure 3e,f), confirming that pseudostratified epithelium is the sensory olfactory epithelium (SE). In the SE we could identify, at the apical region, the nuclei of the supporting cells (SC), in the central region the nuclei of the olfactory receptor neurons (ORNs), and at the bottom the nuclei of the basal cells (BC) (Figure 3a). The NSE, found along the dorsal region of the olfactory chamber, is a stratified cuboidal epithelium rich in mucous cells (Figure 3b–d). These round mucous cells stained positively with Alcian blue/PAS (Figure 3b) but not with hematoxylin–eosin and Masson's trichrome staining (Figure 3c,d). Most mucous cells stained deep blue (more acidic); occasionally there were some mucous cells that stained pink/purple (more neutral).

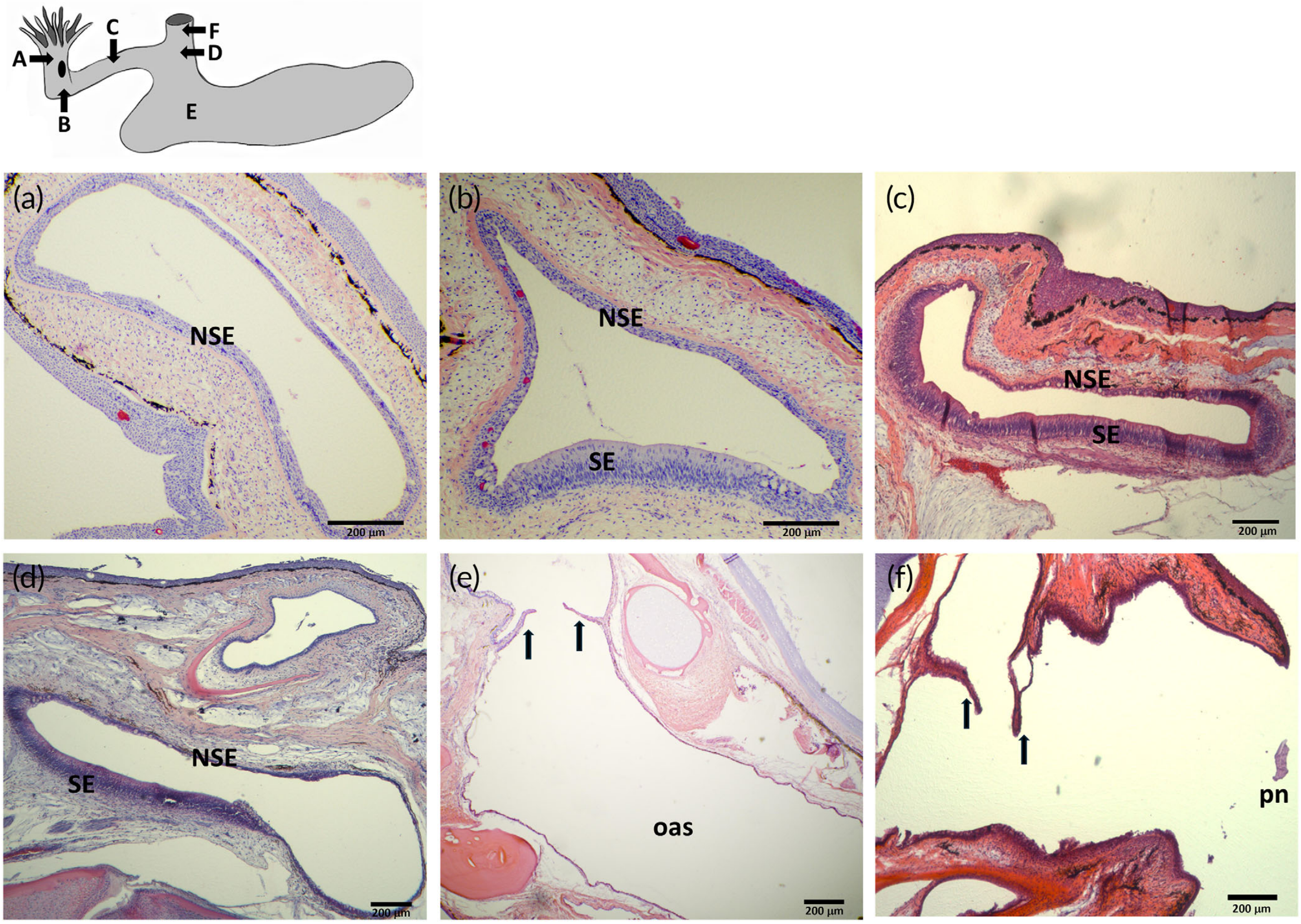

FIGURE 2 Histology of different regions of the olfactory organ of *Halobatrachus didactylus*, represented by letters (a–e) in the scheme (top). (a) Peduncle, (b) transition between the peduncle and the olfactory chamber, (c) olfactory chamber, (d) transition between the olfactory chamber and the posterior nostril, (e) olfactory accessory sac (oas) with lip-like valve (black arrows), and (f) posterior nostril. NSE, nonsensory epithelium; SE, sensory epithelium. Scale bars represent 200 µm.

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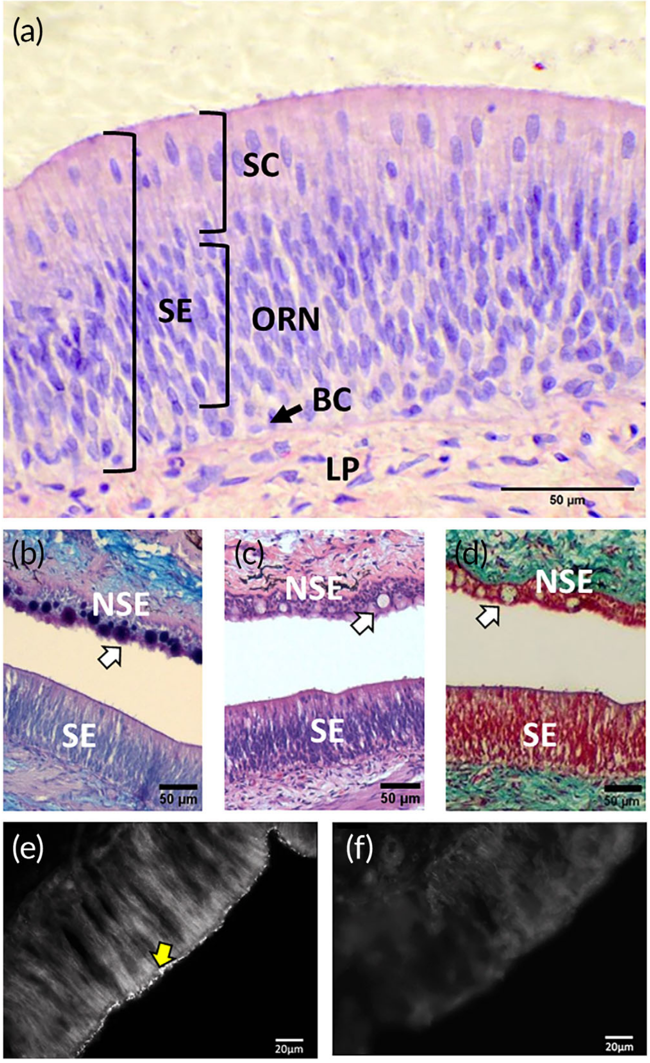

FIGURE 3 Histological and immunohistological characterization of the olfactory epithelium of *H. didactylus*. (a) Histology of the sensory epithelium stained with hematoxylin–eosin and (e) immunocytochemistry for G_{aolf} protein and (f) respective negative control. (b–d) Nonsensory epithelium (NSE) stained with Alcian blue–periodic acid–Schiff, hematoxylin–eosin, and Masson's trichrome, respectively. BC, nucleus of basal cells located closed to the lamina propria (LP); NSE, nonsensory epithelium; ORN, nuclei of olfactory receptor neurons; SC, nuclei of supporting cells; SE, sensory epithelium. Immunopositive cells (yellow arrow). Mucous cells (open arrows). Scale bars represent 50 μm (a–d) and 20 μm (e and f).

3.3 | Electrophysiology

H. didactylus was olfactory sensitive to all amino acids tested (Figure 4). Of the four, the amino acid that produced the largest EOG responses was L-cysteine (Figure 4a), and the amino acid that produced the smallest EOG responses was L-glutamate (Figure 4d).

H. didactylus showed olfactory sensitivity to both bile acids tested (Figure 5). Although the amplitude of responses at the highest concentration tested was similar (Figure 5a,b), the concentration–response curve to TLC fitted best a non-linear sigmoidal regression, and the response to TCA fitted best a non-linear polynomial regression.

Male and female *H. didactylus* had olfactory sensitivity to all body fluids tested (Figures 6 and 7). For both sexes, the most olfactory potent stimulus was the anterior gland fluid, and the least potent was the mucus (Figure 6a,f). Statistical analysis showed that, in females, there were significant differences between olfactory sensitivity to fluid from the anterior gland ($p = 0.009$) and intestinal fluid ($p = 0.027$) collected from reproductive and non-reproductive males (Figure 6). Females were more sensitive to fluids collected from the anterior gland and intestinal fluid collected from reproductive males.

Similarly, there were significant differences between the olfactory sensitivity of males to anterior gland fluid ($p = 0.031$) and intestinal fluid ($p = 0.005$) from reproductive and non-reproductive males (Figure 7). Males were more sensitive to fluids collected from the anterior gland of reproductive males at dilution 1:10,000 ($p = 0.06$) and 1:1000 ($p < 0.001$). Sensitivity to intestinal fluid was higher when this fluid was collected from reproductive males at dilution 1:100,000 ($p = 0.003$), 1:10,000 ($p < 0.001$), and 1:1000 ($p < 0.001$). There were no statistical differences between the sexes for olfactory sensitivity to posterior gland fluid, bile, urine, and mucus. It is noteworthy, again, that urine and mucus were the least potent stimuli.

4 | DISCUSSION

To the best of the authors' knowledge this is the first comprehensive study of the anatomy and function of the olfactory system of a batrachoidid fish. Palazón-Fernández and Sarasquete (2015) previously described the basic morphology and identified the presence of an SE, without lamellae, on the inner wall of the nasal cavity in *H. didactylus*. The present study goes further and characterizes the entire olfactory system, externally and internally, and tests the olfactory capacity of this species to different odors and biological fluids from conspecifics.

H. didactylus has a well-developed olfactory system, consisting of a pair of structures located rostrally: an inhalant nostril located in a digitated peduncle and a rounded exhalant nostril with a valve, a tubiform olfactory chamber with a unilamellar SE covering the ventral surface, and a prominent accessory nasal sac. There is great variability in the structure of the teleost olfactory system; this depends on the ecology, phylogeny, life history, and hydrodynamics of the species (Cox, 2008; Hansen & Zielinski, 2005). In many teleosts, the olfactory epithelium lines a multilamellar olfactory rosette (Hansen & Zielinski, 2005), but *H. didactylus* has an elongated olfactory tube, and there is no multilamellar rosette. The lamellar folding increases the surface available for olfactory sensory activity; however, some species have a flat olfactory epithelium. The round goby, *Neogobius melanostomus*, lacks a multilamellar olfactory structure; however, olfaction is known to mediate reproductive behavior (Belanger et al., 2003). The presence of an elongated tubular peduncle containing the anterior nostril is found in inactive species with a bottom-dwelling lifestyle,

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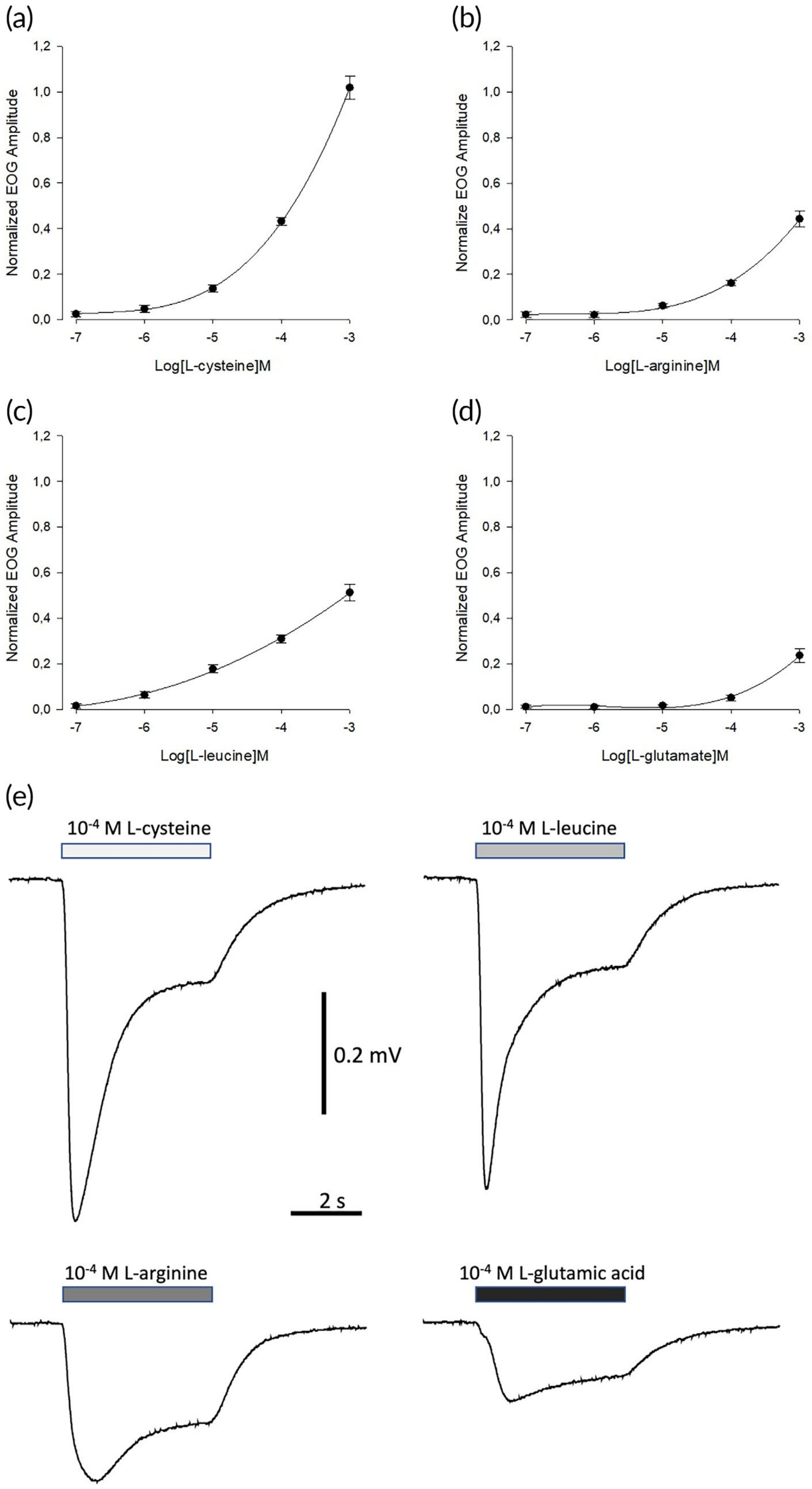

FIGURE 4 Semilogarithmic plots of mean ± SEM normalized EOG (electro-olfactogram) responses of *Halobatrachus didactylus* to (a) L-cysteine (*N* = 10), (b) L-arginine (*N* = 8), (c) L-leucine (*N* = 8), and (d) L-glutamate (*N* = 8). Data from both sexes have been pooled. (e) Typical EOG responses to the four amino acids.

burrowing in the substrate or shelters, apparently facilitating olfactory search and direct assessment of the surrounding enabling suction of water into the olfactory chamber (Kasumyan, 2004).

Diverse species actively regulate water flow through the olfactory chamber using one or two accessory nasal sacs. If accessory nasal sacs develop in the vicinity of bones, they are termed "lachrymal sacs"; if

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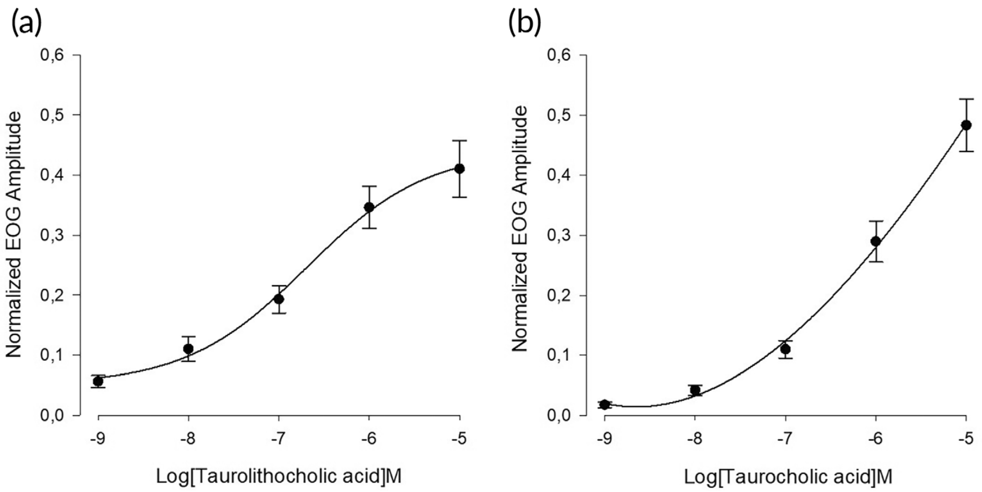

FIGURE 5 Semilogarithmic plots of mean ± SEM normalized EOG (electro-olfactogram) responses of *Halobatrachus didactylus* to (a) taurolithocholic acid ($N = 10$) and (b) taurocholic acid ($N = 9$). Data from both sexes have been pooled.

they are located at the median of the ethmoid, they are termed "ethmoidal sacs" (Kuciel, 2013). These structures are found in many species, most often in sedentary bottom-living fish, and are primarily used to assist water circulation through the olfactory chamber and may be necessary for unidirectional water flow (Kapoor & Ojha, 1972). *H. didactylus* has a single accessory lachrymal sac; in the connection between the lachrymal sac and the base of the posterior nostril, there is a valve in the form of two thin lips. In most teleosts that have a single accessory nasal sac, water may be actively drawn into the olfactory chamber, circulated inside, and expelled from the olfactory chamber by pumping action caused by expansion and compression of the accessory sac during ventilation and foraging (Nevitt, 1991). Compression of the accessory nasal sacs can also be due to buccal contractions (Kuciel, 2013). The function of the valve located between the posterior nostril and the accessory nasal sack is likely to ensure one-way water flow through the chamber (Cox, 2008); indeed, preliminary observations with dye solutions support this. Overall, the occurrence of a projected anterior nostril, an elongated canal-like olfactory chamber, a large accessory nasal sac, and a valve in the posterior nostril may be considered a significant architecture to help facilitate suction and an efficient water circulation through the olfactory organ and evidence for adaptation to a sedentary bottom-dwelling life history (Sarkar et al., 2013). The occurrence of a large and specialized olfactory organ suggests that olfaction plays an important role in this species.

The presence of the olfactory epithelium was confirmed by the occurrence of $G_{\alpha olf}$ immunopositive cells distributed on the surface of the pseudostratified epithelium; in other species, $G_{\alpha olf}$ is expressed in ciliated ORNs (Belanger et al., 2003; Hansen et al., 2004); the uniform distribution of $G_{\alpha olf}$ immunopositive cells in the olfactory epithelium of *H. didactylus* suggests that this species has mostly ciliated ORNs, but this awaits experimental confirmation. The olfactory epithelium itself has the structure previously described in other teleosts (Hansen & Zielinski, 2005), a pseudostratified epithelium composed of neuronal cells, BCs, and SCs. The olfactory epithelium lies on the ventral side of the olfactory chamber; the dorsal region is lined by NSE. Mucous cells are present only in the NSE; the mucus produced by these cells may reduce friction force for water ventilation, protect cells against bacteria and physical particles, and/or participate in osmoregulation and ion exchange (Shephard, 1994). The mucous cells tested positive for Alcian blue/PAS but negative for hematoxylin–eosin and Masson's trichrome staining, which strongly suggests that they are rich in acidic mucins, as found in other species (Kim et al., 2018; Kim & Park, 2016). Mucin cells probably help the smooth flow of water through the olfactory chamber by binding microscopic debris that is ejected through the posterior nostril and probably forms a suitable medium for diffusion of odorants (Ghosh & Chakrabarti, 2014).

Fishes, in general, have high olfactory sensitivity to small-molecular-weight molecules such as amino acids and bile acids (Hara, 1994); *H. didactylus* is no exception, showing sensitivity to all amino acids and bile acids tested. Furthermore, this species was also sensitive to all conspecific body fluids tested; both males and females produced stronger EOG responses to anterior gland and intestinal fluids collected from reproductive than those from non-reproductive males. In fishes, the chemical cues can be released through a range of body fluids, including intestinal fluid (Hubbard, 2003, 2017; Miranda et al., 2005; Velez et al., 2009), urine (Miranda et al., 2005; Vermeirssen & Scott, 2001), and skin mucus (Buchinger et al., 2014; Huertas et al., 2007). However, in the current study, the body fluids that evoked the lowest olfactory responses were the urine and the mucus. Freshwater species, such as the goldfish and the Mozambique tilapia (Keller-Costa et al., 2014) and masu salmon (Yambe et al., 2006), use urine as the vehicle for sex pheromones. However, for osmoregulatory reasons, marine teleosts produce much less urine than freshwater fishes. It is likely, therefore, that marine teleosts use other routes of release for pheromones, and this may explain the weak EOG responses to conspecific urine (Appelt & Sorensen, 2007; Sorensen & Sato, 2005).

H. didactylus is a scaleless, slimy fish; its skin is covered by a film of abundant mucus, raising the hypothesis that the mucus could play a role in intraspecific communication, as proposed for the European

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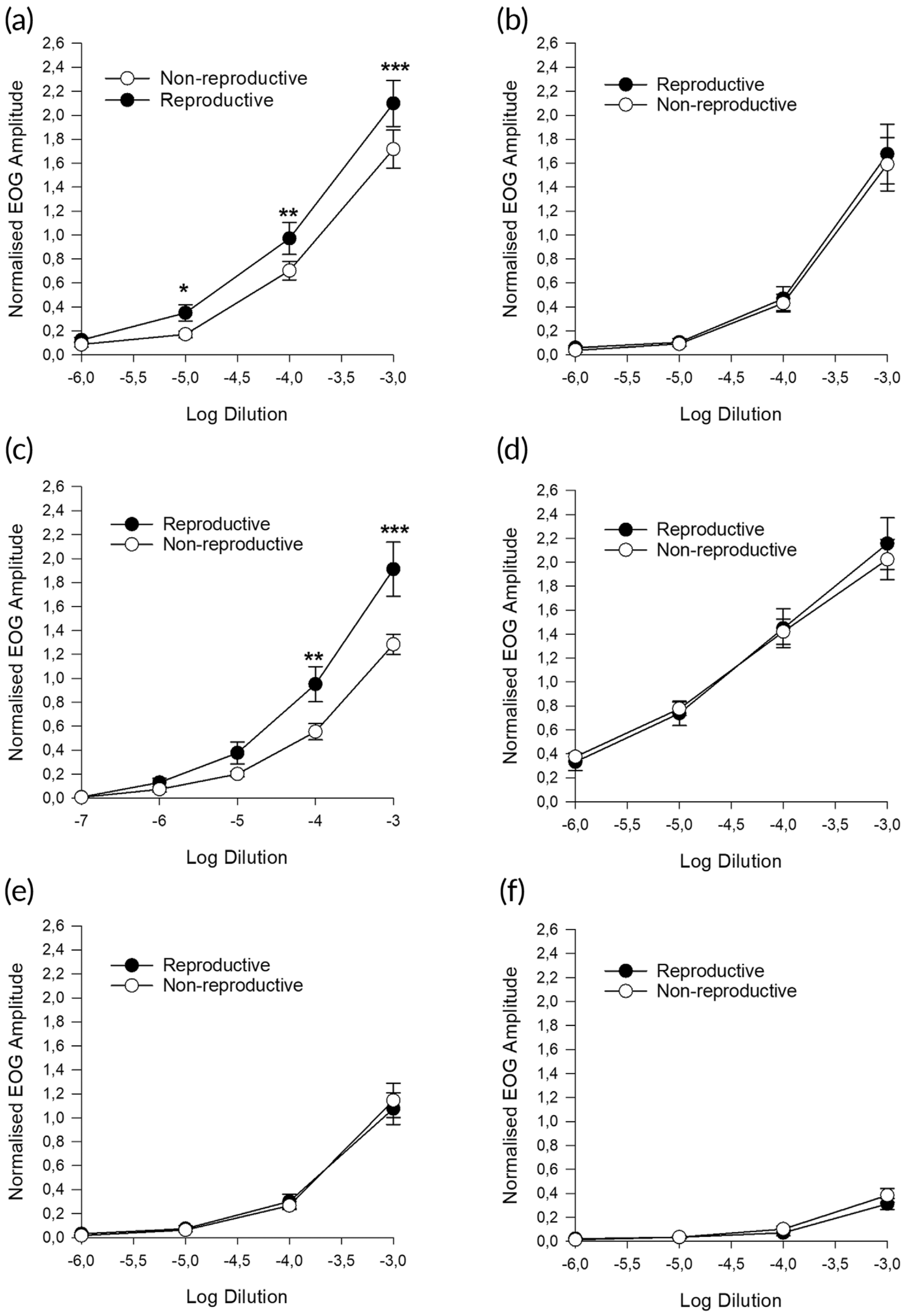

FIGURE 6 Semilogarithmic plots of mean ± SEM normalized EOG (electro-olfactogram) responses of female *Halobatrachus didactylus* to (a) anterior gland fluid ($N = 6$), (b) posterior gland fluid ($N = 6$), (c) intestinal fluid ($N = 6$), (d) bile fluid ($N = 6$), (e) urine ($N = 6$), and (f) mucus ($N = 6$) collected from reproductive (black circles) and non-reproductive (white circles) males. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

eel *Anguilla anguilla* (Huertas et al., 2007). However, in *H. didactylus*, responsiveness to skin mucus was much lower than the other fluids, suggesting that mucus is not involved in chemical communication.

Olfactory sensitivity to bile acids is widespread among fishes (Buchinger et al., 2014). *H. didactylus* is sensitive to bile acids and conspecific bile fluid (a concentrated source of bile acids). In eels, it was suggested that bile acids may be used as an indicator of the reproductive status and/or sex (Huertas et al., 2010). In *H. didactylus* we found no differences in olfactory potency of bile fluid from reproductive and non-reproductive males; however, this does not exclude the use of bile acids in intraspecific communication. Indeed, the olfactory potency of the intestinal fluid collected from reproductive males was significantly higher than that from non-reproductive males. As the olfactory potency of the intestinal fluid is due, in part, to bile acids (Velez et al., 2009), it is possible that the bile acids released in the feces of reproductive and non-reproductive males are different; this hypothesis needs further investigation. In the Mozambique tilapia, for example, reproductive females release much larger quantities of bile acids in their feces than either males or non-reproductively active females (Ashouri et al., 2023).

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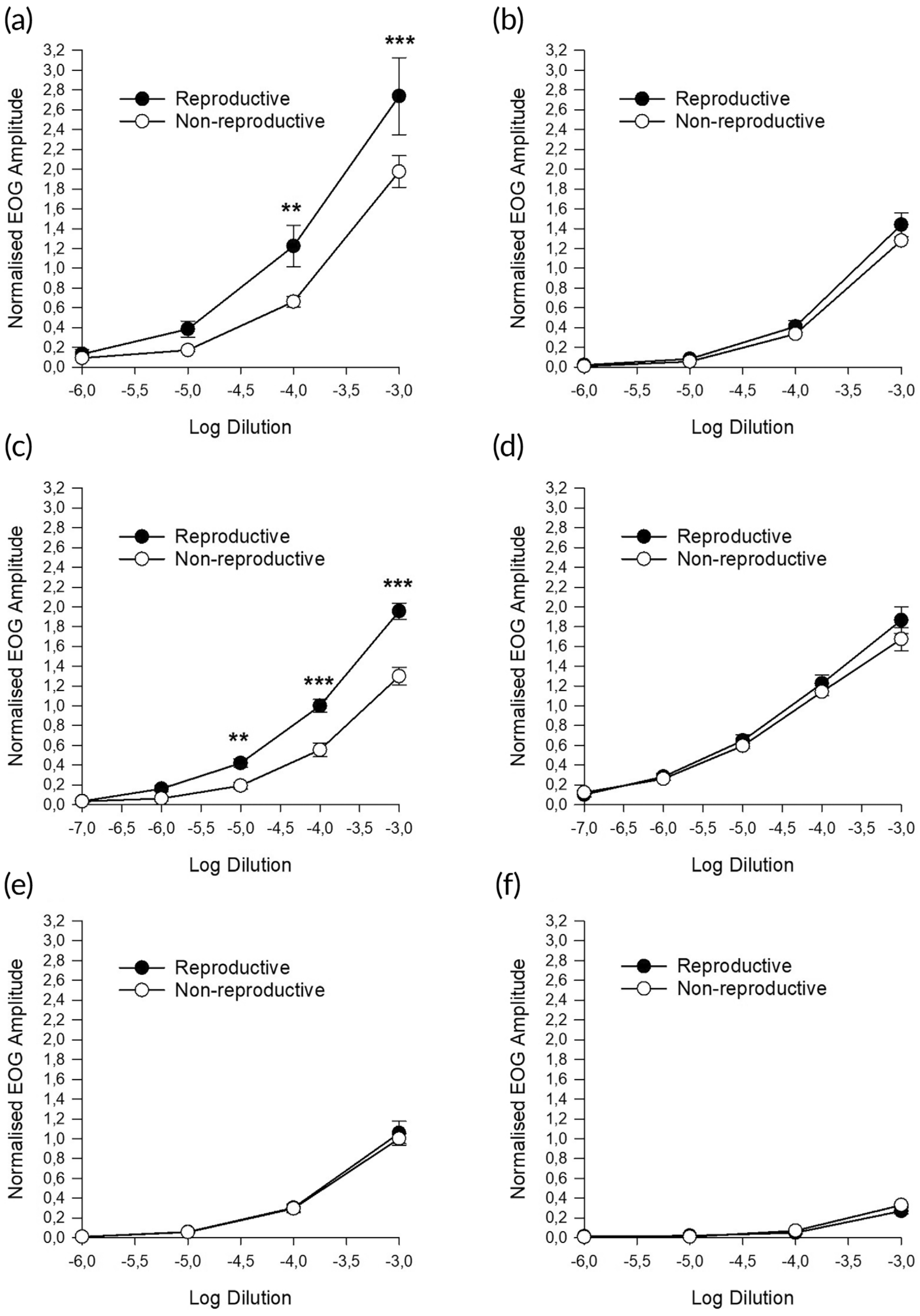

FIGURE 7 Semilogarithmic plots of mean ± SEM normalized EOG (electro-olfactogram) responses of male *Halobatrachus didactylus* to (a) anterior gland fluid ($N = 6$), (b) posterior gland fluid ($N = 4$), (c) intestinal fluid ($N = 6$), (d) bile fluid ($N = 6$), (e) urine ($N = 5$), and (f) mucus ($N = 5$) collected from reproductive (black circles) and non-reproductive (white circles) males. $^{**}p < 0.01$ and $^{***}p < 0.001$.

The most potent body fluid was that collected from the anterior testicular gland, the potency of which was greater from reproductive males than that from non-reproductive males. In some fishes, the accessory glands are thought to play a role in mate attraction by secreting steroid pheromones (Arbuckle et al., 2005; Hong et al., 2006; Jasra et al., 2007; Locatello et al., 2002; Resink et al., 1989; Serrano et al., 2008). In the black goby, type I males attract spawning females by releasing a conjugated steroid pheromone produced in one of their accessory glands (Colombo et al., 1980). In contrast, in black goby sneaker or type II males, mesorchial glands are reduced, and their ejaculates are pheromonally inconspicuous, thereby avoiding detection by type I males (Locatello et al., 2002). The posterior testicular gland fluid of *H. didactylus* evoked high olfactory responses but did not show any significant differences between reproductive seasons, suggesting that its role is distinct from that of the anterior gland fluid. The differential effect of action of the two glands was also evident in their steroidogenic capacity (Modesto et al., 2015), which suggests a specific role of each gland in the reproductive process. Both glands have in vitro steroidogenic capacity (Modesto et al., 2015); many sex pheromones identified to

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date in fish are steroidal, raising the hypothesis that fluids from the accessory glands may act as pheromones.

5 | CONCLUSIONS

H. didactylus has a large and well-developed olfactory organ, which indicates that olfaction plays an important role. Its morphology, however, is markedly different from that of most other teleosts. In addition, this fish has olfactory sensitivity to all odorants and odors tested (bile acids, amino acids, and body fluids). The intestinal fluid and the anterior gland fluid collected from reproductive males evoked larger-amplitude responses than those from non-reproductive males, suggesting a role in intraspecific communication. In contrast to freshwater teleosts, urine proved to be a weak olfactory stimulus. Further studies are needed to fully understand the role of olfaction in *H. didactylus*. However, our results strongly suggest that, besides producing sound, this species uses olfaction for communication, supporting the hypothesis that this may be multimodal, befitting the ecology of this sedentary and nocturnal fish, and aiding interaction in conditions in which visual cues are ineffectual.

AUTHOR CONTRIBUTIONS

Teresa Modesto, Peter C. Hubbard, and Zélia Velez conceived the study, analysed the data, and wrote the manuscript. Peter C. Hubbard and Gonçalo Marcelino carried out electrophysiology. Beatriz Neves Gregório carried out the histological techniques and contributed to the writing of the manuscript. Nathalie Marquet carried out immunocytochemistry. Rita A. Costa was involved in student supervision and histology. Pedro M. Guerreiro provided animals and resources and critical insights to the manuscript. All authors contributed with revisions to the manuscript and gave approval for publication.

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