Sniffing at the river bottom: Influence of olfactory organ morphology on the life habits of freshwater stingrays (Potamotrygoninae)

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Abstract

The olfaction in batoids have an important role for initial detection of the chemical stimulus produced by prey during the foraging activities. Herein, the morphological and histological description of primary lamellae and secondary folds of olfactory rosettes is given to four species from Rio Negro basin. A simpler structure of olfactory organs in *Paratrygon* sp. does not indicate a primary sensory role during the initial phase of its feeding behavior. In *Potamotrygon wallacei*, the largest surface area of primary lamellae suggests enhanced olfactory sensitivity related to its generalist feeding habits and complex substrate exploration. Histological analysis revealed differences in epithelial cell composition among species, with variations in the secondary folds shape and the distribution of mucous cells. The simplicity of secondary folds in both *Paratrygon* sp. and *Potamotrygon orbignyi* probably is related to their specialized feeding habits, requiring fewer adaptations to detect different types of chemical stimuli. A central muscular layer in primary lamellae was observed only to *P. motoro* and *P. wallacei* and indicates a capacity to expand the olfactory epithelium area. These findings provide insights into the functional morphology of olfactory organs in potamotrygonin stingrays and their ecological implications, evidencing the intricate sensory adaptations crucial for foraging success in diverse freshwater habitats. Additionally, it becomes necessary to take into account the contribution of all sensory systems to understand their foraging behavior. Nonetheless, the generalization of the morphological characteristics of olfactory organ in a potamotrygonin species requires caution, since morphological variations can be found, especially to widespread species.

Introduction

Elasmobranchs use a complex arrangement of sensory systems (gustation, electro and mechanosensorial organs, vision and olfaction) which act jointly to locate prey, potential predators and the presence of conspecifics (McComb and Kajiura, 2008). Studies focusing on olfactory organs in elasmobranchs have seen a notable increase on the recent years (e.g. Cox 2013; Ferrando et al. 2019a; Dymek et al. 2021), driven by the accuracy chemical detection abilities exhibited by apex predator species (Yopak et al. 2015). Elasmobranchs have two different arrangement of multilamellar olfactory structures, oval or elongated shapes (e.g. Theisen et al. 1986; Takami et al. 1994; Ferrando et al. 2019a); however, there are a diversity related to the relative size, number and shape of primary lamellae and secondary folds. Independently of the morphological distinctions, the olfactory organs are mainly used to detect chemical stimulus in the water (Bleckmann and Hoffman, 1999).

As most batoid species, potamotrygonin stingrays depend specially on the ventral sense systems to locate and capture the prey. Evidently, the prominent eyes in most *Potamotrygon* species provide a wide vision of the benthic environment; however, the accuracy of localization of prey is managed by the other sensory organs responsible to detect the prey. Shibuya et al. (2010; 2020) stand that the distribution of lateral line canals and the high density of neuromasts around the mouth in freshwater stingrays are related to the detect the prey, which most of them are formed by benthic species or commonly live buried in the substrate (e.g. Duncan et al. 2016; Shibuya 2022). Even the reduced electrosensory structure in comparison to marine species, the Lorenzini ampullae in short canals concentrated in the dermis near the mouth, the electroreception is likely employed on the final stage of prey strike (Szabo et al. 1972; Szamier and Bennett 1980; Harris et al. 2015).

As the entire sensory systems have integrative actions, the olfaction in may play the role of the initial detection of the chemical stimulus just prior the capture of prey on the foraging activities on the bottom for benthic stingrays. Dymek et al. (2021) analyzed the olfactory organs in five elasmobranch species, including two potamotrygonin stingrays, and showed detailed morphology of the olfactory epithelium. However, the morphological data on the olfactory organs in freshwater stingrays is still scarce, in face of 38 valid species (Fontenelle et al. 2021). An important fact is that the generalization of the morphological characteristics of olfactory organ in a potamotrygonin species requires caution, since environmental factors can vary significantly depending on the river in which they occur. Thus, the aim of the present study is describe the morphological characteristics of lamellar surface of the olfactory organs of freshwater stingrays from Rio Negro basin. The blackwater of Rio Negro presents high acidity (pH: 3.89–6.07), low conductivity (8.8 to 28.6 µS cm^{-1}) and lack of suspended sediments (Küchler et al. 2000). Potamotrygonin stingrays usually are found associated with the substrate, which is covered by of sand and the accumulation leaf litter from the flooded forest (Shibuya 2022). In order to compare the area of lamellae surface, the sensory area and densities of secondary folds were estimated. Histological comparisons can provide new insights related to their foraging and feeding habits. These morphological parameters were related to the habitat use and feeding habits previously investigated (Shibuya et al. 2009; Duncan et al. 2016; Shibuya 2022).

Material and Methods

Samples collections were carried out during the low (dry season) period of hydrologic cycle in 2019–2020 in the middle Rio Negro (Barcelos Municipality, Amazonas State, Brazil). Individuals were collected using a dip net by local fishermen, comprising four species: juveniles of *Paratrygon* sp. (n = 4), *Potamotrygon motoro* (n = 1) and *P. orbignyi* (n = 2) and adults of *P. wallacei* (n = 5). Disc width (DW, mm) of the specimens, as well as length and width of the olfactory rosettes into capsules (mm) were taken prior to dissection (in order to preserve the original shape) to calculate the relative size for each analyzed species. Olfactory capsules were removed, fixed in cold and buffered glutaraldehyde 2.5% solution (pH 7.4) for 24 hours and then preserved in 70% ethanol solution. Specimens were preserved in 10% buffered-formalin solution and stored in 75% ethanol solution. Schematic diagrams of the morphology of primary lamella structures were drawn from dissected specimens with the aid of a stereoscopic microscope (Figs. 1a-d).

The olfactory rosettes were removed from the right side (assuming the bilateral symmetry). The primary lamellae were counted and each of them was dissected, separated, labeled and stored into 0.5 ml microcentrifuge tubes. The secondary folds were counted of one side of contact surface in both anterior and posterior portion of each primary lamella and photographed with a millimetric scale. Width (W_{PL}) and total area (A_{PL}) of primary lamella and morphological measures of the anterior (AP) and posterior (PP) lamellar portions were obtained using ImageJ software (Schneider et al. 2012) (Fig. 1b). The lamellar area was calculated only for the region comprising secondary folds (Fig. 1c) and the W_{PL} and A_{PL} are the sum of measurements of the anterior and posterior portions of the primary lamella. The terminology of olfactory structures followed Ferrando et al. (2017b, 2019a). Calculation of the total lamellar area was based on Ferrando et al. (2019a), with few modifications in order to have an accuracy of the total area. The gross surface area (GSA) was

calculated as the sum of all total area of each primary lamella as following: $GSA_{total} = A_{PL}1 + A_{PL}2 + A_{PL}3 + ... + A_{PL}n$. where: A_{PL} is the total area of each primary lamella. The total area was calculated by the sum of the AP and PP areas, previously taken using ImageJ; *n* is the number of primary lamellae of the olfactory organ.

To characterize the microscopical morphology, the olfactory rosette from the left side of each specimen was initially decalcified in 5% formic acid for 24 h, dehydrated in a graded ethanol solution series (96–100%) and infused in ethanol + resin solution for a minimum of 24 hours. Pieces of the organ were embedded in methacrylate resin (Historesin, Leica) in sagittal and transversal positions and sectioned into 3–5 µm-thickness slices. The slides were stained with Toluidine blue for the delineation of the shape of secondary folds. Additionally, Alcian Blue (AB) counterstained with safranin and Periodic Acid Schiff (PAS) counterstained with Hemotoxilin were applied separately, thus facilitating the discernment of distinct positive reaction of each dye. Since potamotrygonin stingrays have simple secondary folds (compared to most elasmobranchs examined by Ferrando et al. (2019a)), the total surface was not estimated according to the equation recommended by Ferrando et al.'s methodology. Thus, calculation of the total contact area of primary lamellae (A_{TC}) was taken by estimating the area of a 1 x 1 mm square, as follow: $A_{TC} = A_{PL} \times A_{Square}$, where A_{PL} is the total area of primary lamella and A_{Square} is the relative area of 2D perimeter of the silhouette (P_{SIL}) of sagittal slices (in mm^2). The perimeter of the silhouette was taken using subsampled areas of the histological slides (Fig. 1d). Measurements of slices were taken with a histological slide with micrometric scale. The increase rate of total surface of the set of secondary folds is presented in relative percentage (%) of the total linear area of primary lamellae.

Lamellae of the central region of the capsule were used to calculate the depth range at which water can reach the olfactory organ. For this purpose, the mean length of secondary folds was measured of both anterior and posterior lamellar portions of the larger primary lamella: (1) external, that comprise the first small fold; (2) median, which is located in the middle of the lamellae and (3) internal, that is the last fold near the raphe (Fig. 1c). The central region of rosette was considered as the middle one third of the total number of primary lamellae (Fig. 1b). Mean, standard deviation, minimum and maximum of measurements of primary lamellae and secondary folds were presented as mean ± SD and min-max. Voucher specimens were catalogued at Fish Collection of the Instituto Nacional de Pesquisas da Amazônia (INPA), Manaus. Brazil: *Paratrygon* sp., #34959; *Potamotrygon motoro*, # 27091; *P. orbignyi*, # 27088 and *P. wallacei*, # 34960.

Results

Gross Morphology of olfactory rosettes

The olfactory organs of potamotrygonin species comprise a pair of encapsuled rosettes positioned anteriorly to the mouth. The olfactory rosettes are categorized by an elongated shape which have the external edge thinner to the midline and internal edge of the raphe (see Fig. 1b). The water current goes sinuously into the nostrils, flows through the olfactory rosettes (through both anterior and posterior lamellar portions) and drains off posteriorly to the nasal flap. The olfactory organs are covered by a pair of inner folded edge positioned just under the nasal flap, possibly assisting the water flow into the nostrils (Figs. 2a and b). Each olfactory rosette is formed by a central raphe that separated in anterior and posterior lamellar portions. Overall, the anterior portion was larger lamellar side. *Paratrygon* sp. presents roundish lamellae and more concave shape, with the posterior portion slightly short (Fig. 2c), while *Potamotrygon* species have an elongated and curved lamellae (Fig. 2d), with a visibly shorter posterior portion. All species had a pair of process in both anterior and posterior lamellar portions, with most prominent and pointed shape to *Potamotrygon* species (Figs. 2c and d). Morphological measurements of the olfactory structures are presented in the Table 1. High number of primary lamellae was observed to *Potamotrygon motoro* (n = 54), while *Paratrygon* sp. presented the lowest number (min-max: 27–29) among analyzed species. Each primary lamella presents secondary folds positioned parallel to each other in anterior and posterior portions on both sides (see Figs. 2c and d). The length and number of secondary folds varies according to the size of primary lamellae. Higher numbers of secondary folds were found to *P. motoro* and *P. orbignyi* comparing the larger primary lamellae, while the length of the secondary folds of the largest primary lamella was greater for *P. wallacei* (see Table 1).

Table 1

Measures of olfactory organs of potamotrygonin species. The relative size of olfactory organs was calculated using the disc width and rosette width. The to area, number, and length of secondary folds were determined for the largest primary lamella of each examined specimen. AP and PP represent the anterior a posterior portions of primary lamella (PL), respectively. Length-1, Length-2 and Length-3 correspond to specific segments of PL, measured according to th methodology outlined in Fig. 1C.

Species	Disc width (mm)	Olf. rosette width (mm)	Mean and Relative size (%)	Olf. rosette length (mm)	No. PL	Total area of the largest lamella (mm^2)	Secondary folds (largest lamella)							
							No. AP	Length-1	Length-2	Length-3	No. PP	Length-3	Length-2	Len 1
Paratrygon sp. (n = 4)	274–302	7.9–10.5	9.1 (3.2)	3.0-3.6	27–29	9.45 ± 1.64*	24.6 ± 4.1*	0.42 ± 0.04*	1.08 ± 0.05*	1.33 ± 0.08*	20.0 ± 2.0*	1.24 ± 0.08*	1.00 ± 0.1*	0.33 0.03
Potamotrygon motoro (n = 1)	180	11.2	6.2	4.8	52	12.21	36.0	0.32	1.23	1.17	28.0	1.30	1.14	0.33
P. orbignyi (n = 2)	152–241	8.6–13.9	5.7	4.2–6.8	45–48	20.30**	40.0**	0.63**	1.69**	1.42**	26.0**	1.15**	1.58**	0.57
P. wallacei (n = 5)	160–240	11.0-14.9	12.9 (6.6)	4.6–7.7	32–39	24.69 ± 3.86	28.0 ± 1.4	0.53 ± 0.14	1.66 ± 0.23	1.83 ± 0.28	20.4 ± 0.9	1.73 ± 0.22	1.68 ± 0.14	0.55 0.10
* result of three specimens														
** result of one specimen														

Total lamellar area

The gross surface area was considered as the sum of the area of anterior and posterior portions of both side of primary lamellae. *Paratrygon* sp. presents the smallest total area (Table 2). Despite the very few differences of total area among *Potamotrygon* species, the result of *P. orbignyi* should be considered carefully, in order to the damage of primary lamellae located on the edges of olfactory rosettes, thus, the area might be underestimated. The largest lamellar surface was calculated to *P. wallacei*, even the low number of primary lamellae compared to *P. motoro*. Table 2 shows differences between the gross lamellar area (linear area) and the contact surface area, considering the perimeter of secondary folds of each species (Figs. 2e-h). *Paratrygon* sp. presents slightly waved secondary folds (Fig. 2e), with an increase of 9.0% of total area of primary lamellae. Despite *Potamotrygon* species have similar morphology of the primary lamellae, their secondary folds have distinct forms. *Potamotrygon motoro* presents a triangular shape of secondary folds (Fig. 2f), which increases 42.5% of total surface area of olfactory lamellae. Likewise *Paratrygon* sp., *P. orbignyi* has wave-shaped secondary folds (Fig. 2g) that provide a smaller increase of lamellar contact surface (5.7%). Conversely, the wide and flattened surface of secondary folds of *P. wallacei* (Fig. 2h) has a largest expansion of lamellar contact comparing to the other examined species (115.4%).

Table 2

Measures of the gross surface area of primary lamellae, defined as the sum of areas of all lamellae within one rosette, considering a 1 x 1 mm square linear area. Ratio values represent the increase in surface area using the perimeter of the silhouette of the secondary folds. The estimated area and its percentage were calculated based on the total area of all primary lamellae within one olfactory rosette.

Species	Gross surface area	Ratio	Estimated total area
Paratrygon sp.	163.10 ± 2.38*	1.09 ± 0.04 [a]	177.78 (9.0%)
Potamotrygon motoro	244.23**	1.42 ± 0.08[b]	348.03 (42.5%)
P. orbignyi	525.51***	1.06 ± 0.02[a]	555.46 (5.7%)
P. wallacei	548.94 ± 5.51	2.15 ± 0.24[c]	1,182.42 (115.4%)
* result of three specimens			
** result of one specimen			
*** sum of surface area of 34 lamellae of one specimen.			
[a] based on one specimen			
[b] based on three specimens			
[c] based on four specimens			

Histological analyses of secondary folds

The primary lamellae are characterized by having secondary folds on both sides. These secondary folds exhibit morphological differences among the examined species, both in terms of shape and composition of epithelial cells. The epithelium is a sensory region covered by ciliary supporting cells placed on the sensory receptor cells. The apical zone of secondary folds is a non-sensory region presenting mucous cells and non-ciliary cells (Figs. 3a-h). The wavy-shaped secondary folds of *Paratrygon* sp. (Fig. 3a) has a group of PAS-positive mucus cells (Fig. 3b), while *Potamotrygon* species have goblet cells secreting AB-positive and PAS-positive substances. Goblet cells are distributed among non-ciliary supporting cells which is covered by a mucus granules, except to *P. motoro* that has goblet cells in the sensory region (Fig. 3d). In all examined species, the middle zone of secondary folds is filling by connective tissue that originated from the center of primary lamella. Secondary folds in *Paratrygon* sp., *Potamotrygon motoro* and *P. orbignyi* have similar size along the primary lamellae. In contrast, distinct width and size of secondary folds are found on the primary lamellae of *P. wallacei* (Fig. 3f). The connective tissue in the middle of larger secondary folds of *P. wallacei* expands under the support and basal cells of epithelium in both sensory and non-sensory regions. The wall of basal portion among primary lamellae presents a PAS-positive mucus cells apically positioned on the epithelium and extending to near the secondary folds (Fig. 3e). The primary lamellae of *Paratrygon* sp. and *Potamotrygon orbignyi* have a thick connective tissue that support secondary folds on both sides. *Potamotrygon motoro* and *P. wallacei* present thin layer of connective tissue in both sides of primary lamellae. In these species, the central portion of primary lamella is filled by smooth muscle longitudinally arranged, which is thicker in *P. wallacei* (Figs. 3c and f).

Discussion

The investigation of olfactory organ revealed a morphological diversity in potamotrygonin stingrays. However, the delicate structure of the olfactory epithelium and the small size of the organs turned a challenge in maintaining adequate fixation to prevent degradation of lamellar structures. Additionally, the difficult handling of olfactory rosettes during the dissection of primary lamellae (often damaged during the individualization process) impeded a complete examination and the quantification of secondary lamellae. Nonetheless, the current results provide relevant information that, combined with knowledge of their life habits (Shibuya et al. 2009; Duncan et al. 2016; Shibuya et al. 2016; Shibuya 2022), can elucidate the functional role of the olfactory system. The bottom of the river may present different types of obstacles such as litter and tree trunks alongside sandy substrate, making the benthic zone an excellent refugee for insects larvae and crustaceans (e.g. Goulding and Ferreira 1983; Nessimian et al. 1998). Therefore, the behavior of stirring up the substrate becomes essential for stingrays to uncover the hidden prey (Garrone-Neto and Sazima 2009; Shibuya et al. 2012).

Only young individuals of *Paratrygon* sp. were examined, and during this life stage, this species is associated with sandy beaches, feeding on small fish (Shibuya et al., 2009). Comparing to the *Potamotrygon* species, *Paratrygon* sp. exhibited smaller nostril size, lower number of total lamellae, and reduced lamellar area, even considering the silhouette of secondary folds. *Paratrygon* sp. is a large-sized species, reaching up to 93.0 cm DW in the Rio Negro (Sánchez-Duarte et al. 2013), and the morphological simplicity of its olfactory organs, compared to *Potamotrygon* species, may indicate lower olfactory efficiency. However, it is necessary to consider two important factors. Firstly, *Paratrygon* sp. inhabit river channels as adults (e.g. Shibuya 2022). The high water flow in such environments may hinder the detection of chemical stimuli released by their prey. Furthermore, fish living in river channel are not small in size (compared to those living on the beaches) (Shibuya et al. 2009) and live on the substrate, indicating that *Paratrygon* sp. does not primarily rely on chemical cues for foraging. Additionally, this species exhibits a widespread distribution of lateral line canals and a high number of pores on the dorsal surface that may play a primary role in locating mechanical stimuli generated by prey near ray's body (Shibuya et al. 2010, 2020). Thus, it becomes necessary to take into account the contribution of all sensory systems to understand how this species obtains its food.

Despite examining few specimens, *P. motoro* exhibited a larger lamellar area than *P. orbignyi*; however, the opposite was observed when considering the silhouette of the secondary folds. Although both species have high numbers of primary lamellae and secondary folds, the difference in the increment of lamellar surface area may be related to their respective life habits. *Potamotrygon motoro* is an active species that constantly explores different types of habitats, often found associated with various types of substrates in search of prey (Shibuya 2022). This species primarily consumes crabs and small fish (Shibuya et al., 2009). In contrast, *P. orbignyi* has a specialist feeding habit, consuming aquatic insects in sandy beaches of all studied populations. Considering the need to explore a broader range of habitats and consuming a diverse types of prey, *P. motoro* requires an olfactory system capable of detecting a variety of chemical stimuli released by its prey. These ecological characteristics can explain the increase of lamellar surface given by the secondary folds' silhouette. *Potamotrygon wallacei* stands out having high values of total surface area, especially considering the silhouette of secondary folds. The estimate of the total contact area exceeded 100% for *P. wallacei*, highlighting the morphological importance of secondary folds for enhancing the sensitivity of olfactory organs. The generalist feeding habits of this species (see Shibuya et al. 2009), along with its ability to explore complex substrate of flooded forest (Duncan et al. 2016) for locating prey, underscore the necessity of *P. wallacei* to identify chemical stimuli, including those released by smaller prey hidden in leaf litter, such as small shrimps and insects larvae.

The presence of a pair of lamellar processes on primary lamellae may serve an important function in guiding water flow along the olfactory organ, due to their prominent and curved shape. In addition to freshwater stingrays, the lamellar process has also been observed in marine batoid species such as *Aetobatus narinari*, *Aptychotrema rostrata*, and *Neotrygon kuhlii* (Schluessel et al. 2008) and may indicate its role in maintaining water flow over the primary lamella to identify chemical cues from prey buried in the bottom (Kyne and Bennett 2002; Schluessel et al. 2010; Jacobsen and Bennett 2011). The increase in surface area provided by the secondary folds is notable for enhancing olfactory sensitivity. Although potamotrygonin stingrays do not exhibit the complex branching of secondary folds seen in marine elasmobranchs (Ferrando et al. 2017a, 2019a, b; Dymek et al. 2021), morphological diversity may indicates an increase of sensitivity surface area. Dymek et al. (2021) previously described the morphology of olfactory organs in two *Potamotrogon* species, showing a different morphology to *P. motoro* when compared to that observed in the present study. Despite Dymek et al. (2021) having used specimens from the ornamental market, these morphological differences corroborate the importance of considering the populations being examined. Widely distributed species such as *P. motoro* may exhibit ecological and morphological distinctions, so information regarding the origin of the specimens and the characteristics of the habitat where these stingrays used to live are essential for inferring the functional role of the lamellar structure and can indicate adaptations and pressures to their particular habitats (Schluessel et al., 2008). Taxonomic revisions that have been carried out on widely distributed species of potamotrygonines (e.g.

Loboda and Carvalho, 2013; Silva and Carvalho, 2015; Loboda et al. 2021), emphasize the need to distinguish populations in ecological and functional morphology studies.

In contrast to observations by Dymek et al. (2021), the goblet cells of *P. motoro* and *P. wallacei* were AB and PAS-positive, producing mucus in the non-sensory lamellar region. Therefore, mucus granules were also found on both sensory and non-sensory surface of *Paratrygon* sp., which were only PAS positive, indicating an assistance to cilia movement. However, the presence of goblet cells on the top of secondary folds can be considered to have a protective function. The benthic-associated life habits of freshwater stingrays mean that olfactory lamellae are in direct contact with river bottom elements, resulting in constant friction with sand and leaf-litter fragments. Thus, the presence of goblet cells and mucus granules on the lamellar surface may serve as important protection against potential friction damage and infection to the olfactory epithelium. The reduced or absence of goblet cells likely mean a lack of mucus-propel function, a characteristic observed in many elasmobranch species (Cox 2013). Instead, the cilia movement may be driven by water flow, playing a significant hydrodynamic role in guiding chemical stimuli to sensory cells. Nonetheless, a minimal contribution of ciliary propulsion may exist, especially in *P. motoro*, which exhibits a few goblet cells in sensory region.

According to the classification of primary lamella morphology by Ferrando et al. (2019a), the secondary folds of all examined species was classified as non-branched and of short size. The presence of a central layer of smooth muscle into the primary lamellae was observed in both *P. motoro* and *P. wallacei*, but neither observed in *Paratrygon* sp. nor *P. orbignyi*, nor described in previously studied freshwater stingrays (Dymek et al. 2021). The longitudinal arrangement of smooth muscle may be related to the process of expanding the primary lamellae, optimizing the contact of the olfactory epithelium with the water current. This characteristic supports the relationship between increased surface area of the olfactory epithelium and the foraging capabilities of *P. motoro* and *P. wallacei* in various types of habitats (Duncan et al. 2016; Shibuya 2022), possibly regulating the expansion of primary lamellae based on the need for chemosensitivity accuracy.

The integration of all sensory systems in freshwater stingrays is indeed an important characteristic for the success of this group in a diversity of freshwater habitats, such as lakes, streams, shallow and deep waters such as beaches and river channels (Duncan et al. 2015; Shibuya 2022). The synchronized performance of different sensory modalities plays an essential role in feeding behavior, with each sensory element having a primary function in different phases of foraging (search, location, and the phase prior to prey apprehension). Differences of the morphology of olfactory organs in potamotrygonin stingrays may determine the level of accuracy in prey detect as specialized adaptations corresponding to the life habits of each species. Further examination of other species may reveal whether morphological diversity of olfactory organs have phylogenetic inferences for the Potamotrygoninae subfamily.

Declarations

The authors have no conflict of interest to declare.

Ethics approval

The specimens were collected with permission of Instituto Chico Mendes de Conservação da Biodiversidade – ICMBio (ICMBio, Brazilian Environmental Agency, license #15068–6). All protocols involving the handling of animals were previously approved by the Ethics Committee for Animal Experimentation/Federal University of Amazonas (CEUA/UFAM, protocol nº 007/2019) in accordance with the guidelines of the Brazilian Committee for the Control of Animal Experimentation.

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Figures

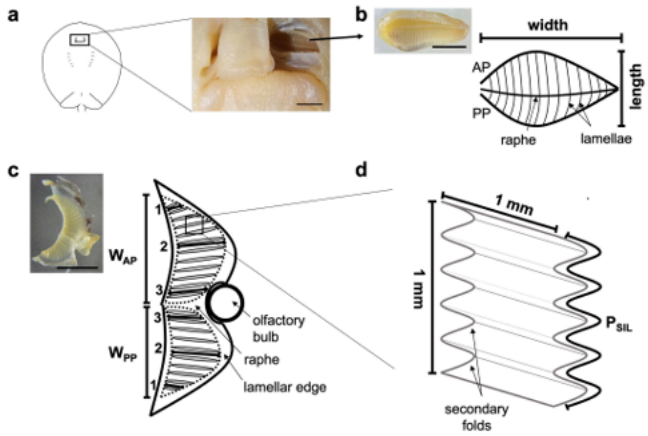

Figure 1

Diagram illustrating the measurements taken from the olfactory organ in Potamotrygoninae species. (a) The olfactory system consists of paired organs located on the ventral surface, positioned anteriorly to the mouth. (b) Width and length of each rosette were measured within the capsule before dissection to prevent disruption of the original shape. The olfactory rosette is composed of a set of primary lamellae, divided into anterior (AP) and posterior (PP) portions by a central raphe (detail of a rosette of the left side). (c) Width of each portion (W_{AP} and W_{PP}) for each primary lamella was measured. The secondary folds (in gray) were counted and lengths were taken from external (1), medial (2) and internal (3) folds. Surface areas of W_{AP} and W_{PP} were measured, considering only the region containing secondary folds (delimited by dashed lines). (d) To estimate the total sensory area, the perimeter of the silhouette of secondary folds (P_{SIL}) was measured from histological slides in 1.0 mm^2 and extrapolated to the total area of primary lamellae. Scale bar: 0.5 cm.

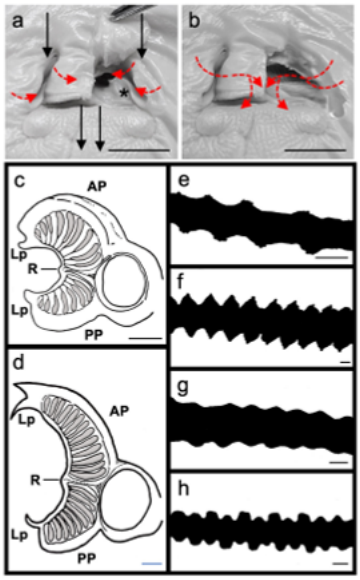

Figure 2

Water flow in the nostrils of potamotrygonin species. (a) the entrance occurs laterally to the nasal flaps (red dashed arrows), when the water current under the disc is directed posteriorly (black arrows). The water flows into the rosettes with the aid of the inner folded edge (in asterisk) (b) The water runs through the olfactory rosettes and drain off under the posterior region of nasal flap (red dashed arrows). Sagittal view of theprimary lamella of *Paratrygon* sp. (c) shows a roundish shape, while *Potamotrygon* species (d) have similar shape of primary lamella. The silhouette of the secondary folds is slightly waved in *Paratrygon* sp. (e). Despite the similar shape of primary lamellae, the silhouettes of secondary folds are visibly distinct among *Potamotrygon motoro* (f), *P. orbignyi* (g) and *P. wallacei* (h). Silhouettes of secondary folds on the primary lamella to each species are based on the histological slices in longitudinal position of the olfactory rosettes. AP, anterior lamellar portion; Lp, lamellar process; PP, posterior lamellar portion; R, raphe. Scales: a and b (1.0 cm); c and d (2.0 mm); e-h (100 µm).

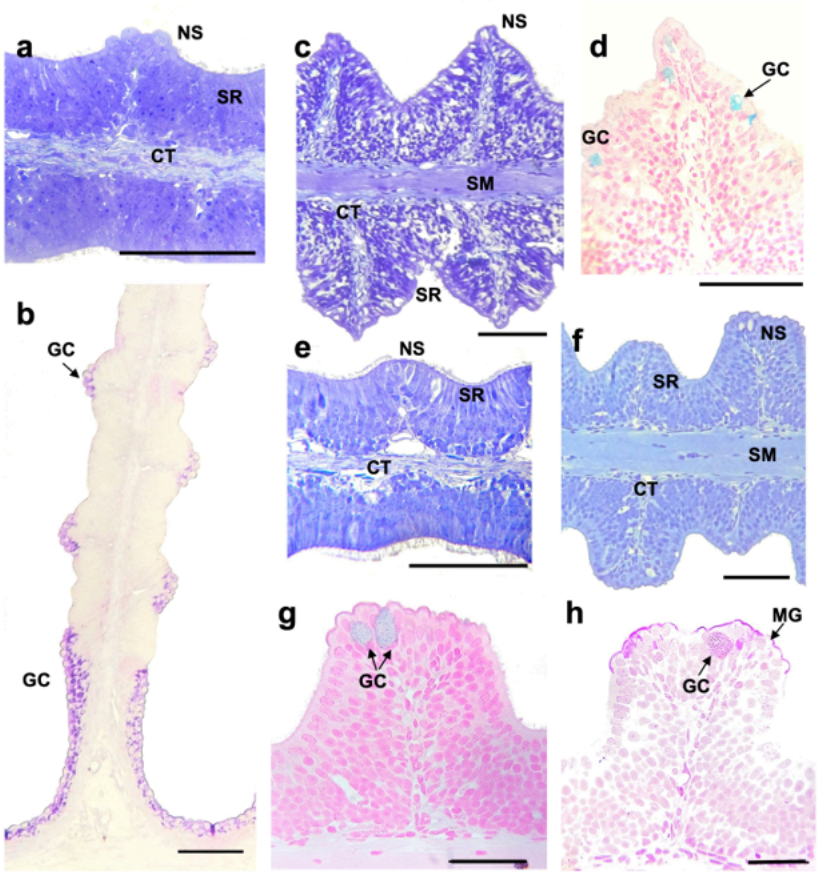

Figure 3

Histological structure of the olfactory lamellae of potamotrygonin species. Transversal sections through one primary lamellae show the morphology of secondary folds. (a) The slightly wavy secondary fold of *Paratrygon* sp. presents the apical zone with PAS-positive goblet cells (in b). The triangular secondary folds of *Potamotrygon motoro*(c) have AB- positive goblet cells (in d). (e) *Potamotrygon orbignyi* has a dense short ciliary sensory region. (f) Different size and shape of secondary folds were found in *P. wallacei* and goblet cells are AB and PAS positives (g and h, respectively). Note the presence of central smooth muscles in primary lamellae of *P. motoro* and *P. wallacei*. a, c, e and f are stained with Toluidine blue; b, in PAS; d and g, in Alcian blue counter staining with Safranin; h, in Periodic Acid Schiff counter staining with Hematoxilin. CT, connective tissue; SM, smooth muscles; GC, goblet cells; MG, mucus granules; SR, sensory region; NS, non-sensory region. Scale bars: 100 µm (A-F) and 50 µm (G and H).