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RESEARCH ARTICLE

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Quantitative comparative analysis of the nasal chemosensory organs of anurans during larval development and metamorphosis highlights the relative importance of chemosensory subsystems in the group

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Abstract

The anuran peripheral olfactory system is composed of a number of subsystems, represented by distinct neuroepithelia. These include the main olfactory epithelium and vomeronasal organ (found in most tetrapods) and three specialized epithelia of anurans: the buccal-exposed olfactory epithelium of larvae, and the olfactory recess and middle chamber epithelium of postmetamorphic animals. To better characterize the developmental changes in these subsystems across the life cycle, morphometric changes of the nasal chemosensory organs during larval development and metamorphosis were analyzed in three different anuran species (*Rhinella arenarum*, *Hypsiboas pulchellus*, and *Xenopus laevis*). We calculated the volume of the nasal chemosensory organs by measuring the neuroepithelial area from serial histological sections at four different stages. In larvae, the vomeronasal organ was relatively reduced in *R. arenarum* compared with the other two species; the buccal-exposed olfactory epithelium was absent in *X. laevis*, and best developed in *H. pulchellus*. In postmetamorphic animals, the olfactory epithelium (air-sensitive organ) was relatively bigger in terrestrial species (*R. arenarum* and *H. pulchellus*), whereas the vomeronasal and the middle chamber epithelia (water-sensitive organs) was best developed in *X. laevis*. A small olfactory recess (likely homologous with the middle chamber epithelium) was found in *R. arenarum* juveniles, but not in *H. pulchellus*. These results support the association of the vomeronasal and middle chamber epithelia with aquatic olfaction, as seen by their enhanced development in the secondarily aquatic juveniles of *X. laevis*. They also support a role for the larval buccal-exposed olfactory epithelium in assessment of oral contents: it was absent in *X. laevis*, an obligate suspension feeder, while present in the two grazing species. These initial quantitative results give, for the first time, insight into the functional importance of the peripheral olfactory subsystems across the anuran life cycle.

KEYWORDS
amphibians, chemical sensing, comparative anatomy, olfactory, vomeronasal organ

1 | INTRODUCTION

Chemosensation of molecules in the external environment plays an essential role in animal behavior (Prasad & Reed, 1999). Most tetrapods possess two bilateral nasal organs for detecting chemicals in their environment: the main olfactory epithelium (OE) and the vomeronasal organ (VNO). These chemosensory epithelia comprise the peripheral components of the olfactory and vomeronasal systems, respectively. These two chemosensory systems have substantial differences at the molecular, morphological and physiological level, which strongly suggests that each olfactory organ serves different behavioral functions (Halpern & Martinez-Marcos, 2003). It has been suggested (e.g., Broman, 1920; Døving & Trotier, 1998) that the VNO is specialized for detection of nonvolatile stimuli, including some pheromones, whereas

the OE participates in the chemosensation of volatile stimuli (although this functional distinction between these two chemosensory systems is still controversial, and does not account for all available data; Baxi, Dorries, & Eisthen, 2006).

Like most tetrapods, anuran amphibians have well developed olfactory and vomeronasal systems. The peripheral components of these two chemosensory systems (i.e., the OE and the VNO) develop early in anurans, at embryonic or early larval stages (Cooper, 1943; Jermakowicz et al., 2004; Jungblut, Pozzi, & Paz, 2011; Nieuwkoop & Faber, 1994; Wang, Zhao, Tai, & Zhang, 2008), and both chemosensory systems appear to be fully functional in anuran larvae (Jungblut, Pozzi, & Paz, 2012; Manzini & Schild, 2010).

Anuran amphibians provide an exceptional model to investigate the structure and function of chemosensory systems, since most anurans have a complex life cycle with an aquatic larval stage that transforms, through metamorphosis, into a terrestrial juvenile form. Moreover, as adults, the degree of association with the aquatic environment varies considerably among species (Duellman & Trueb, 1986). In frogs, the change from an aquatic to a terrestrial environment involves a dramatic transformation of morphology and physiology of the animals, including their chemosensory systems (Reiss & Eisthen, 2008). Yet curiously, for animals that clearly must deal with chemosensation in aquatic and terrestrial environments both during development and as adults, relatively little attention has been paid to functional analysis of the chemosensory systems in this group.

The general anatomical organization of the chemosensory organs is relatively simple in tadpoles: the OE lines the medial and caudal wall of the olfactory cavity, whereas the VNO is an anteriorly located, bean-shaped outpocketing (Jungblut et al., 2012). During metamorphosis, an extreme remodeling occurs, and the adult pattern of the chemosensory organs develops (Reiss & Eisthen, 2008). The postmetamorphic nasal cavity of anurans consists of three interconnected chambers: a principal (superior) chamber (lined with the OE), which connects with the external environment and the buccal cavity through the external and internal nares, respectively; a middle chamber (lined with nonsensory epithelium in most anurans); and an inferior chamber, which is lined with the neuroepithelium of the VNO in its medial recess and a nonsensory epithelium at its lateral recess (Jurgens, 1971).

Development and metamorphosis of the chemosensory systems have been qualitatively studied in a number of anuran species (Benzekri & Reiss, 2011; Cooper, 1943; Hinsberg, 1901; Jermakowicz et al., 2004; Jungblut et al., 2011; Khalil, 1978; Kralovec, Zakova, & Muzakova, 2012; Taniguchi, Toshima, & Saito, 1996; Tsui, 1946; Tsui & Pan, 1946; Wang et al., 2008; Yvroud, 1966). However, comparative studies are quite scarce, and, as far as we are aware, there are no quantitative comparative studies in anurans that analyze shifts in the relative size of the chemosensory subsystems. The goal of the present study was to provide a quantitative comparative analysis of the development and metamorphosis of the olfactory organs in different anuran species. As a first step, we chose to examine three species whose adults have different degree of association with the aquatic environment: the terrestrial Argentine toad, *Rhinella arenarum*, and Montevideo treefrog, *Hypsiboas pulchellus*, and the fully aquatic African clawed frog, *Xenopus laevis*. Larval feeding mode also differs among these species, with *R. arenarum* and *H. pulchellus* being grazers, while *X. laevis* is a midwater suspension feeder. This variation in both larval and adult lifestyle allows some inference about the functional significance of the regional specializations of the peripheral olfactory system.

2 | MATERIAL AND METHODS

2.1 | Animals

Adults of *Rhinella arenarum* (Hensel, 1867) and *Xenopus laevis* (Daudin, 1802) were obtained from a local supplier, whereas adults of *Hypsiboas pulchellus* (Duméril & Bibron, 1841) (3 males and 4 gravid females) were collected from the wild in the locality of Alberti, Buenos Aires Province, Argentina. Embryos were obtained by *in vitro* fertilization according to methods previously described (Paz et al., 1995). Tadpoles were reared in dechlorinated tap water at a population density of 4–5 animals per liter, under constant photoperiod (12L:12D) and temperature (22 ± 2°C), and fed *ad libitum* for optimal growth. Tadpoles of *R. arenarum* and *H. pulchellus* were staged according to Gosner (1960) (G), whereas *X. laevis* tadpoles were staged according to Nieuwkoop and Faber (1994) (NF). For this study four different developmental stages were analyzed: early larval stage (G27; NF49), mid-larval stage (G31; NF53), pre-climax late larval stage (G39; NF56) and postmetamorphic juveniles (G46; NF66). Tadpoles reaching the desired developmental stage (four subjects for each developmental stage and each species) were randomly selected from the rearing tanks. Their body length (snout-vent length) was measured under a dissecting microscope, and then processed for morphometric analysis (three animals) or immunohistochemical analysis (one animal), as described below. Moreover, we took two additional specimens from the mid-larval stage and juvenile stage from each species to perform a cell density analysis of the olfactory organs (for details see Supporting Information). Thus, a total of twenty animals were examined from each of the three species included in the analysis, giving a total of 60 animals. All experiments were performed in accordance with the principles of laboratory animal care of the Institutional Care and Use Committee of the Facultad de Ciencias Exactas y Naturales, UBA Res CD: 140/00, and the principles of the NIH (publication 8523, revised 1996).

2.2 | Histological procedures

After being anesthetized by immersion in a 0.1% solution of tricaine methanesulfonate (MS222, Sigma-Aldrich, St. Louis, MI), animals were fixed in Bouin's solution for 24 hr at 4°C. They were then dehydrated, cleared in xylene, and embedded in Histoplast (Biopack, Buenos Aires, Argentina). Serial transverse sections, covering the entire nasal region, were cut at 7μm (for immunohistochemistry), 10μm (for measurements of early and mid-larval stages), or 15μm (for measurements of late larval and postmetamorphic stages) and mounted on HiFix glass slides (HF-5001, InProt, Buenos Aires, Argentina). Sections were

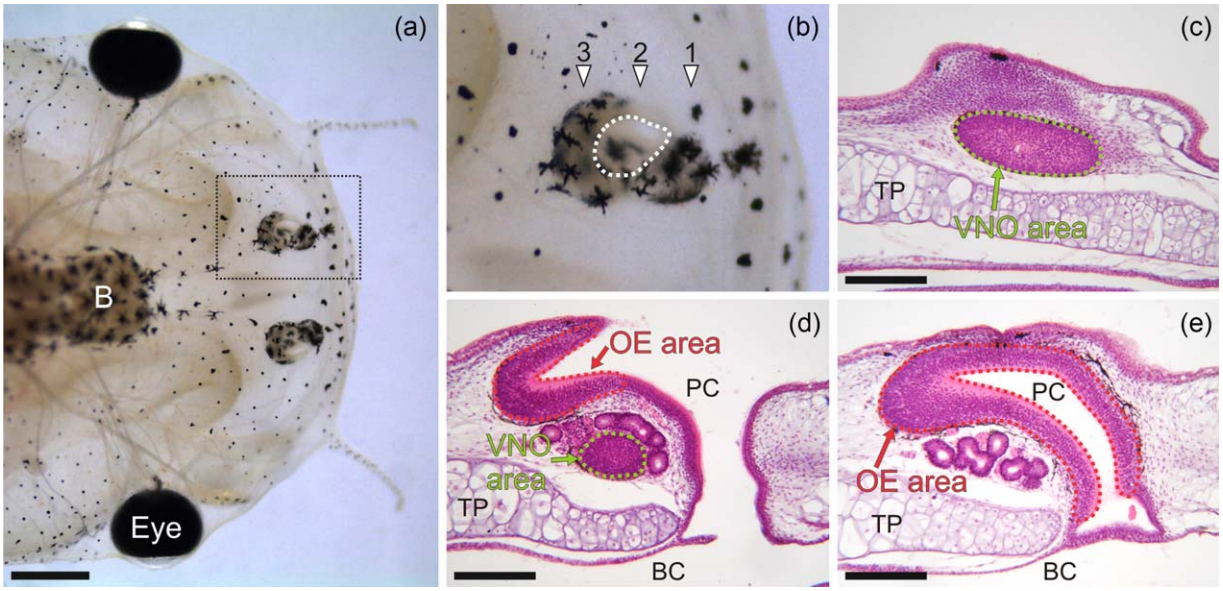

FIGURE 1 Representative images showing: the anatomical region analysed (a, b), and transverse histological sections with the specific area measured delimitated (c–e). a. Dorsal view of the head of a *X. laevis* larva (stage 53). b. Higher magnification of the entire nasal region included in the analysis (boxed area in a). White arrowheads 1, 2, and 3 indicate the estimated planes of the transverse sections shown in c, d, and e, respectively. The white dotted line indicates the external naris. c–e. Transverse histological sections showing examples of the area measured delimitated of the vomeronasal organ (VNO, red) and the olfactory epithelium (OE, green). Top is dorsal and left is medial. B, brain; BC, buccal cavity; PC, principal chamber; TP, trabecular plate. Scale bar, 1,000 μm (a) and 200μm (c,d)

deparaffinated, rehydrated, stained with hematoxylin and eosin, and mounted for conventional light microscopy.

2.3 | Immunohistochemistry

General procedures for immunohistochemistry were followed as in our previous report (Jungblut, Paz, Lopez-Costa, & Pozzi, 2009). The primary antibodies used were rabbit anti-Gαo (sc-387, Santa Cruz; 1:12,000 in PBS), and mouse anti-Neural Cell Adhesion Molecule (NCAM, Dr U. Rutishauser, Developmental Studies Hybridoma Bank, University of Iowa; 1:50 in PBS). After primary antibody incubation (overnight at 4°C), sections were treated with the appropriate biotinylated secondary antibody (Vector Laboratories, Burlingame, CA) followed by avidin-biotin horseradish peroxidase complex (Vectastain ABC Kit, Vector Laboratories). The reaction was developed with the 3,3′-diaminobenzidine tetrahydrochloride (DAB) Staining Kit (Dako, Glostrup, Denmark). All sections were counterstained with hematoxylin. Omission of the primary antiserum (negative control) produced negligible background staining (data not shown).

2.4 | Image analysis, measurements, and statistics

Digital images were taken from each histological section using a Sony Cybershot DSC P-200 camera attached to a Leica Reichhert Polyvar microscope. The total neuroepithelial area of the chemosensory organs (left or right, randomly selected for each animal) were measured in all digital images captured using Image Pro Plus 4.1 software (Media Cybernetics, Silver Spring, MD). Figure 1 shows the anatomical regions included in the analysis, as well as some representative transverse sections with the delimitation of specific epithelial areas analyzed. Volumes were obtained by multiplying the neuroepithelial surface area by the slice thickness (10 or 15μm) and summing over all sections.

Data are expressed as mean ± *SEM*. Statistical comparisons of means were made by one-way ANOVA followed by a Tukey HSD test. The homogeneity of variances within groups was verified with Bartlett's test. As several variables were obtained from each animal we applied the Bonferroni correction. Thus, groups were considered significantly different when $p < 0.0125$.

For details on 3D-reconstruction methods see Supporting Information.

3 | RESULTS

In all three species examined, the chemosensory epithelia of the OE and the VNO were both pseudostratified, and clearly discernible using classical histological staining. Moreover, the immunohistochemical analysis for NCAM and $G_{\alpha o}$ proteins, two specific markers that have been previously used to identify chemosensory neurons in amphibians and other vertebrates (Gonzalez, Morona, Lopez, Moreno, & Northcutt, 2011; Jungblut et al., 2011, 2012), were effective in staining the nasal chemosensory organs in the three species analyzed (Figures 2 and 3). This confirmed the sensory nature of all epithelial structure included in the quantitative analysis. Immunostaining was particularly helpful in establishing boundaries between sensory and nonsensory epithelia, which allowed accurate measurements in the histological sections.

Analysis of neuroepithelial cell density revealed minimal variation across the different chemosensory organs and species included in the

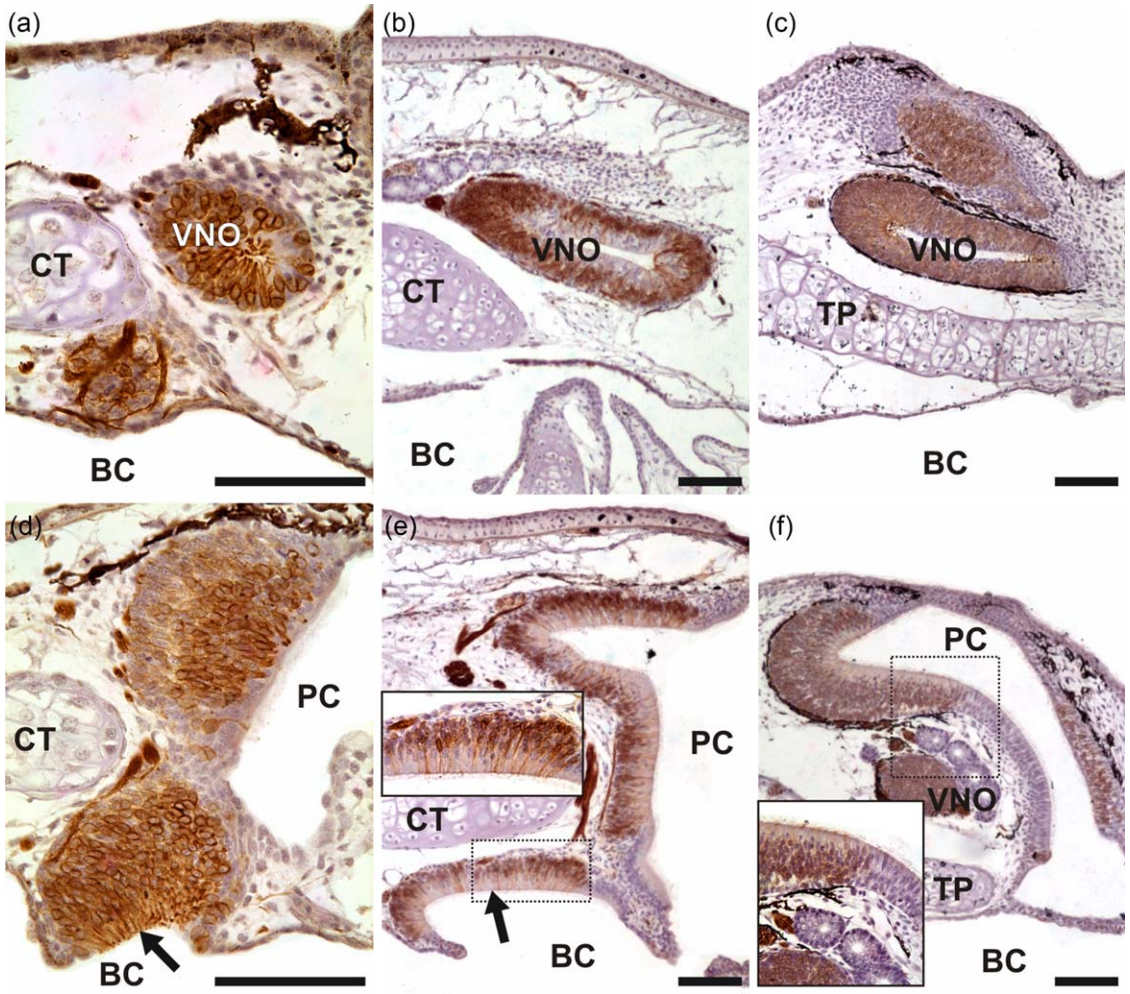

FIGURE 2 Immunohistochemical localization of the Gαo protein in the chemosensory organs (only left side) of *R. arenarum* (a and d), *H. pulchellus* (b and e), and *X. laevis* (c and f) at mid-larval stages. a–c. Representative transverse sections at the level of the vomeronasal organ (VNO). d–e. Transverse sections in a more caudal region than in a–c, where the olfactory epithelium (OE) is observed in the olfactory principal chamber (PC). Arrows in d and e point to the buccal exposed OE (bexOE) of *R. arenarum* and *H. pulchellus*, respectively. Insets in e and f shows higher magnification of the respective boxed areas. Top is dorsal and left is medial. BC, buccal cavity; CT, cornua trabeculae; TP, trabecular plate. Scale bar, 100μm

analysis (see Supporting Information). This validates the assumption that the overall volume (both absolute and relative) is informative with respect to the degree of development of the different chemosensory epithelia analyzed.

3.1 *Rhinella arenarum*

The general morphology of the olfactory and vomeronasal organs during larval development and metamorphosis of the toad *R. arenarum* was described in detail in a previous report (Jungblut et al., 2011). During the larval phase the OE is divided into two branches, previously identified in *R. arenarum* and other species as the dorsal and ventral OE (Jermakowicz et al., 2004; Jungblut et al., 2011). Synonyms for these two branches of the OE can also be found in earlier literature. The dorsal branch corresponds to the neuroepithelium of the 'upper sac' (*Hauptlumen* of Rowedder, 1937; and *Sac Supérieur* of Yvroud, 1966) and the ventral branch corresponds to the neuroepithelium of the 'posterior lower sac' (*Choanengang* of Rowedder, 1937; and *Sac Choanal* of Yvroud, 1966). The anterior portion of the ventral OE is directly exposed to the buccal cavity. Since it could have a different functional significance, given that this chemosensory epithelium is exposed to a different environment (the buccal cavity), we measured this portion of the OE separately from the rest of the OE (exposed to the olfactory cavity), and we refer here to this particular section of the ventral OE as the buccal-exposed olfactory epithelium (bexOE).

As the animals were growing during the larval phase (Figure 4a), the nasal chemosensory organs increased their size as well (Figure 4b). The VNO and the OE increased their size about 12 fold from early (G27) to late (G39) larval stages (Figure 4b and Table 1). By contrast, the bexOE increased its total volume by only about 3.5 fold, from G27 to G39 (Figure 4b and Table 1). For a comprehensive interpretation of the morphological organization of the chemosensory organs during the larval phase of *R. arenarum* see the 3D-reconstruction in Supporting Information Supp-movie 1 and Supporting Information Supp-Figure 1.

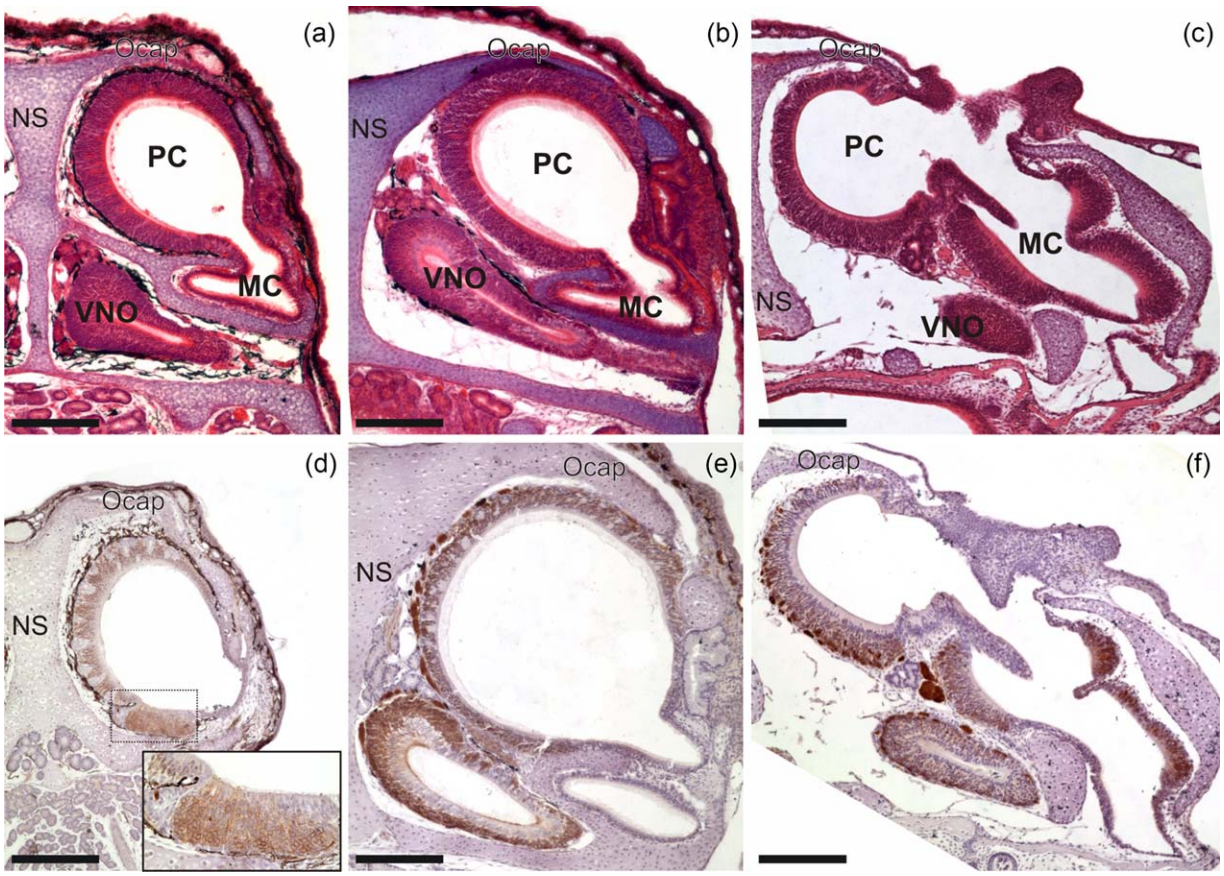

FIGURE 3 Anatomical organization of the chemosensory organs at postmetamorphic stages in *R. arenarum* (a and d), *H. pulchellus* (b and e), and *X. laevis* (c and f). Only the left side is shown. a–c. Representative histological sections in the transverse plane at a level in which the three nasal chambers could be observed: the principal chamber (PC), the midle chamber (MC), and the vomeronasal organ (VNO) located in the inferior chamber. d–f. Immunohistochemical detection of the neuronal marker NCAM in the chemosensory organs. d. A section anterior to that in a. The olfactory recess (OR) is seen in the boxed area (inset is a higher magnification). e and f. Sections caudal to those in b and c, respectively. NS, nasal septum; Ocap, olfactory capsule. Scale bar, 200μm

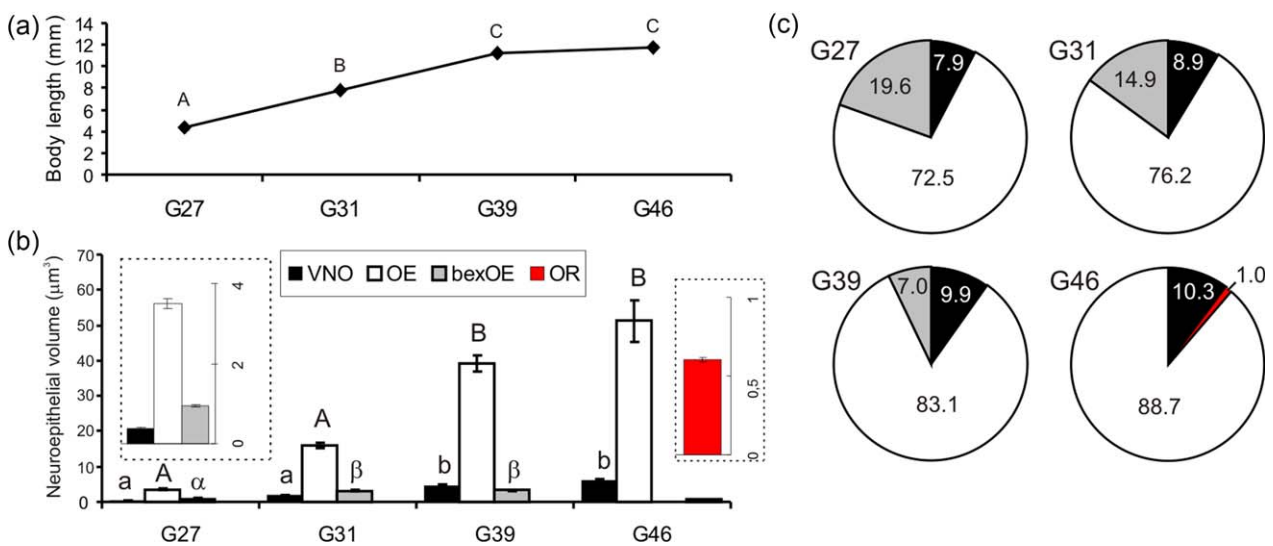

FIGURE 4 Morphometric analysis of the nasal chemosensory organs of *R. arenarum* during larval development and postmetamorphic stages. a. Body length. b. Neuroepithelial volume of the nasal chemosensory organs: vomeronasal organ (VNO, black), olfactory epithelium (OE, white), buccal exposed olfactory epithelium (bexOE, gray), and olfactory recess (OR, red). Insets in the graph show the bars below with a diferent scaling on the *Y* axis. Different letters indicate significant statistical differences ($p < 0.0125$). **C.** Relative size of the different chemosensory organs compared to the total sensory volume (i.e., VNO + OE + bexOE + OR)

TABLE 1 Specimens examined and morphometric data

		Specimens examined				Morphometric data[a]					
Species	Dev. Stage	A	B	C	Total	BL	OE	VNO	bexpOE	MCE	OR
Rhinella arenarum	Early larva	1	3		4	4.33 ± 0.21	3.49 ± 0.13	0.38 ± 0.15	0.94 ± .03	-	-
	Mid larva	1	3	2	6	7.80 ± 0.12	15.95 ± 0.65	1.87 ± 0.15	3.12 ± 0.17	-	-
	Late Larva	1	3		4	11.25 ± 0.14	39.16 ± 2.40	4.68 ± 0.15	3.28 ± 0.25	-	-
	Juvenile	1	3	2	6	11.81 ± 0.27	51.25 ± 5.94	5.95 ± 0.47	-	-	0.60 ± .01
Hypsiboas pulchellus	Early larva	1	3		4	4.87 ± 0.12	10.34 ± 0.14	3.84 ± 0.21	4.54 ± 0.25	-	-
	Mid larva	1	3	2	6	10.00 ± 0.20	25.99 ± 2.31	8.70 ± 0.23	9.72 ± 0.59	-	-
	Late Larva	1	3		4	12.12 ± 0.12	65.29 ± 3.87	11.07 ± 1.01	17.35 ± 1.07	-	-
	Juvenile	1	3	2	6	16.92 ± 0.32	73.45 ± 7.07	11.52 ± 1.12	-	-	-
Xenopus laevis	Early larva	1	3		4	6.87 ± 0.42	2.05 ± .07	0.43 ± .05	-	-	-
	Mid larva	1	3	2	6	12.25 ± 0.14	27.69 ± 0.49	9.36 ± 0.21	-	-	-
	Late Larva	1	3		4	14.25 ± 0.14	40.77 ± 2.84	14.49 ± 0.83	-	2.66 ± 0.11	-
	Juvenile	1	3	2	6	12.87 ± 0.24	52.77 ± 3.27	14.79 ± 0.11	-	17.52 ± 1.94	-

Capital letters correspond to the number of specimens assigned for: Immunohistochemistry (A), morphometric analysis of the body length (BL) and chemosensory organs (B), and cell density analysis of the chemosensory epithelia (C).
[a]Values represent mean ± *SEM*. Units are in mm for BL and μm^3 for the different chemosensory organs.

At the end of metamorphosis (G46) there was no significant change in body size (Figure 4a) or in the volume of the VNO and the OE (Figure 4b), compared to the latest larval stages analyzed; although there was a slight increase in the volume of the OE. Interestingly, the larval bexOE was completely absent in postmetamorphic animals and a novel sensory epithelium (the olfactory recess, OR) was developed in the floor of the principal chamber (Figures 3d and 4b). See also 3D-reconstruction of the chemosensory organs at juvenile stage in Supporting Information Supp-movie 2 and Supporting Information Supp-Figure 2.

When the mean relative sizes of the nasal chemosensory organs were calculated, as a percentage of the total sensory volume (TSV: OE + VNO + bexOE + OR), we observed that the bexOE was gradually reduced, whereas the OE gradually increased in size throughout larval development and metamorphosis (Figure 4c). On the other hand, the VNO slightly increased its relative size from about 8 to 10% of the TSV, whereas the newly formed OR represented only about the 1% of the TSV in postmetamorphic animals (Figure 4c).

3.2 *Hypsiboas pulchellus*

The body length and the nasal chemosensory organs increased their size throughout larval development in *H. pulchellus* (Figure 5a,b). As observed in *R. arenarum* there was a branch of the OE directly exposed to the buccal cavity (Figure 2e). For a comprehensive interpretation of the morphological organization of the chemosensory organs during the larval phase of *H. pulchellus* see the 3D-reconstruction in Supporting Information Supp-movie 3 and Supporting Information Supp-Figure 3.

At early larval stages (G27) the nasal chemosensory organs, especially the VNO and the bexOE, were bigger in this species compared to sizes measured in *R. arenarum*. The volume of the VNO increased by about threefold from early larval stages (G27) to late larval stages (G39; Figure 5b and Table 1). On the other hand, the volume of OE increased about 6.5 fold from G27 to G39; whereas the volume of the bexOE increased about fourfold during larval development (Figure 5b and Table 1). When metamorphosis was complete (G46) there was a significant increase in the body size in postmetamorphic animals, compared with the last larval stages analyzed (Figure 5a). As observed in *R. arenarum*, there were no significant changes in either the OE or VNO size after metamorphosis, whereas the bexOE was completely absent at G46 (Figure 5b). There was no OR in the floor of the principal cavity of postmetamorphic animals of *H. pulchellus*. See also 3D-reconstruction of the chemosensory organs at the juvenile stage in Supporting Information Supp-movie 4 and Supporting Information Supp-Figure 4.

The mean relative size of each nasal chemosensory organ compared to the TSV revealed that the bexOE and the VNO have a great relative importancce in *H. pulchellus* during larval stages (mainly at early and mid-larval stages), representing together almost 50% of the TSV (Figure 5c). The general trend of the relative sizes of the chemosensory organ in *H. pulchellus* was similar to that observed in *R. arenarum*. The bexOE was gradually reduced, whereas the OE was gradually increased throughout larval development and metamorphosis (Figure 5c). However, the VNO showed a gradual reduction of its relative size during the larval phase and a slight increase after metamophosis.

3.3 *Xenopus laevis*

Body length grows throughout larval development in *X. laevis*, but it was slightly reduced in postmetamorphic juveniles, compared to the latest larval stages examined (Figure 6a). As observed in the other two species analyzed, the nasal chemosensory organs increased their total size throughout larval development in *X. laevis* (Figure 6b). The VNO

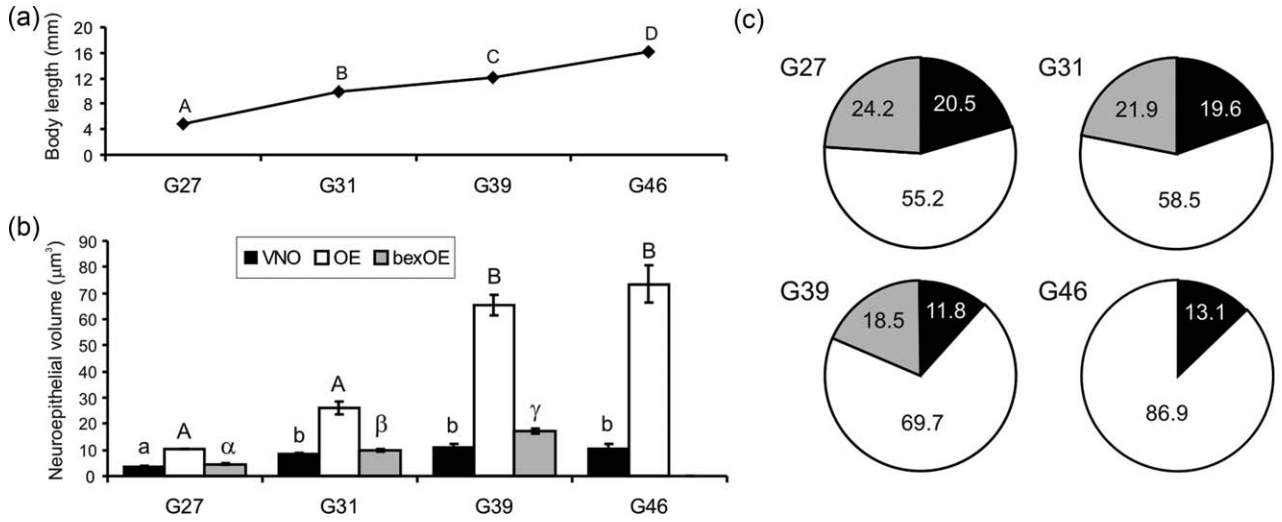

FIGURE 5 Morphometric analysis of the nasal chemosensory organs of *H. pulchellus* during larval development and postmetamorphic stages. a. Body length. b. Neuroepithelial volume of the nasal chemosensory organs: vomeronasal organ (VNO, black), olfactory epithelium (OE, white), and buccal exposed olfactory epithelium (bexOE, gray). Different letters indicate significant statistical differences ($p < 0.0125$). c. Relative size of the different chemosensory organs compared to the total sensory volume (i.e., VNO + OE + bexOE)

expands its total volume by about 32 fold from early (NF 49) to late (NF 56) larval stages, whereas, the OE expands its volume by about 20 fold in the same time period (Figure 6b and Table 1). There was no bexOE in *X. laevis* larvae. However, at late larval stages (NF 56) the neuroepithelium of the incipient developing middle chamber (MCE) was already present. For a comprehensive interpretation of the morphological organization of the chemosensory organs during the larval phase of *X. laevis* see Supporting Information Supp-movie 5 and Supporting Information Supp-Figure 5.

After metamorphosis, the size of the VNO seems not to be modified, whereas the OE showed a slight increase compared to the latest larval stages analyzed. The most notable modification occurred in the MCE which increased its total size by about 6.5 fold during metamorphic climax, expanding considerably from the late larval stage (NF 56) to the juvenile stage (NF 66; Figure 6b and Table 1). See also Supporting Information Supp-movie 6 and Supporting Information Supp-Figure 6 for a comprehensive overview of the morphological organization at the juvenile stage.

When the relative sizes of the nasal chemosensory organs were calculated in *X. laevis*, we observed a different pattern from that observed in *R. arenarum* and *H. pulchellus*. The relative size of the OE was gradually reduced throughout larval development and metamorphosis from about 83 to 62% of the TSV (Figure 6c); whereas, the VNO increased from about 17 to 25% during larval development and

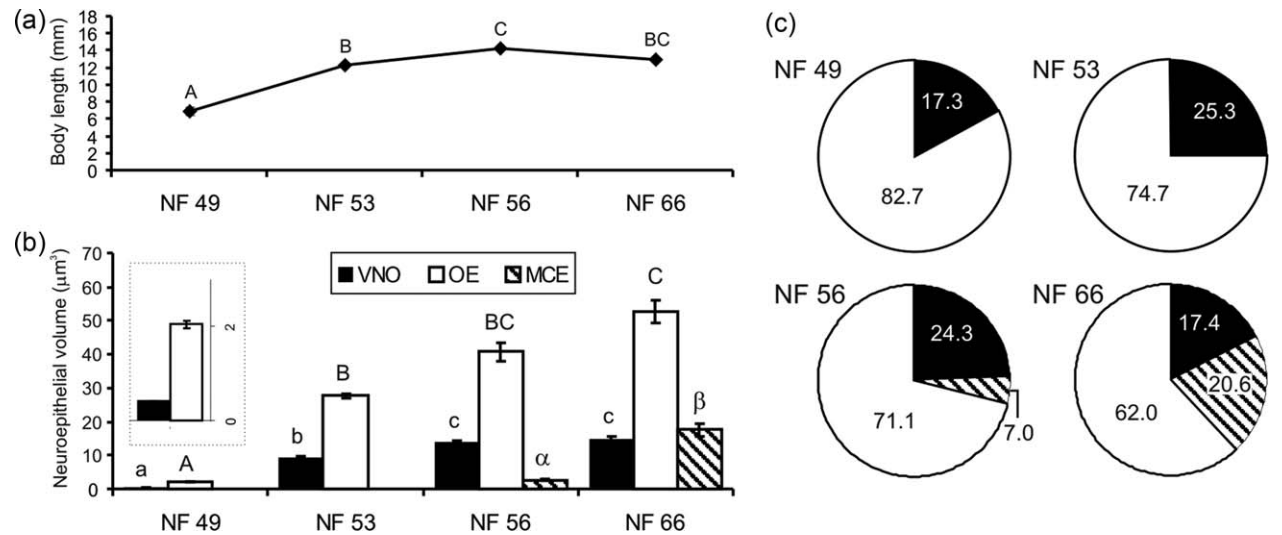

FIGURE 6 Morphometric analysis of the nasal chemosensory organs of *X. laevis* during larval development and postmetamorphic stages. a. Body length. b. Neuroepithelial volume of the nasal chemosensory organs: vomeronasal organ (VNO, black), olfactory epithelium in the principal chamber (OE, white), and neuroepithelium in the middle chamber (MCE, striped). Insets in the graph shows the bars below with a diferent scaling on the *Y* axis. Different letters indicate significant statistical differences ($p < 0.0125$). c. Relative size of the different chemosensory organs compared to the total sensory volume (i.e., VNO + OE + MCE)

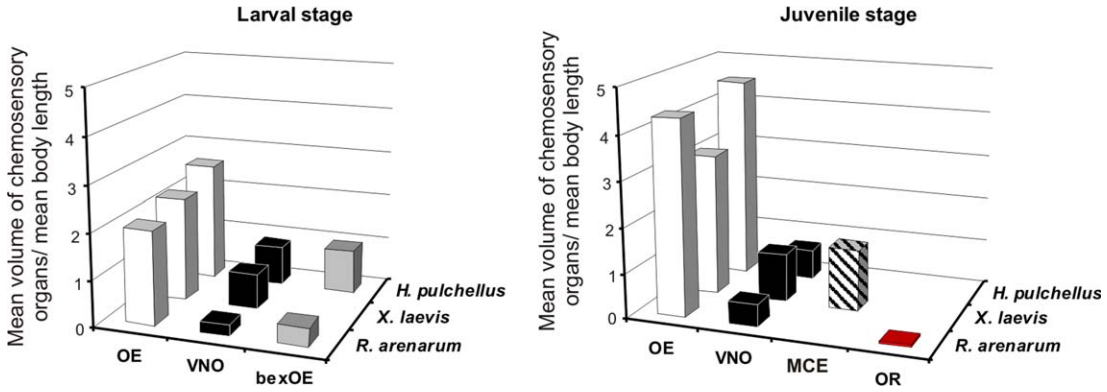

FIGURE 7 Cross-species comparison of relativized volume of nasal chemosensory organs at mid-larval (left) and juvenile stages (right). bexOE, buccal exposed olfactory epithelium. MCE, neuroepithelium of the middle chamber. OE, olfactory epithelium of the principal chamber. OR, olfactory recess. VNO, vomeronasal organ. Scale of vertical axis is $\mu m^3/mm$

remained above 17% after metamorphosis (Figure 6c). On the other hand, the MCE was about the 20% of the TSV after completion of metamorphosis.

Finally, to facilitate comparisons between the three species analyzed, we plotted the mean volume of the chemosensory organs relativized to the mean body length in a representative larval stage (the mid larval stage) and juvenile stage (Figure 7a,b, respectively) for each species. When viewed in this way, the overall volume of the OE during the larval stage did not vary greatly among species, but there was considerable variation in the volume of additional sensory epithelia such as the VNO and bexOE. On the other hand, the juvenile stage shares a common pattern in terrestrial species (*R. arenarum* and *H. pulchellus*), in which the relative size of the OE is much greater than that of the VNO, whereas in *X. laevis* (a fully aquatic frog), the relative size of the water-sensitive structures (i.e., MCE and VNO) is much greater, and that of the OE comparatively smaller.

4 | DISCUSSION

Among animals, there is much variation in the size of chemosensory systems used for the detection of environmental stimuli. A larger size of the peripheral and/or central chemosensory structures implies a larger number of sensory neurons sensing the environment, which is thought to be correlated with improved performance at particular behavioral tasks (e.g., Corfield et al., 2015; Green et al., 2012; Schubert, Houck, Feldhoff, Feldhoff, & Woodley, 2008; Smith, Laitman, & Bhatnaghar, 2014; Van Valkenburgh et al., 2011; Woodley, 2007; Yopak, Lisney, & Collin, 2015). Although less elaborated than in some other vertebrates, such as some fishes and mammals (Van Valkenburgh, Smith, & Craven, 2014), anuran amphibians have well developed chemosensory organs (Reiss & Eisthen, 2008). Moreover, the presence of specialized structures in key anatomical areas that allow the animals to detect both air-borne and water-borne stimuli or to maximize the contact of chemosensory neurons with the external environment, as we showed here, highlights the relevance of this sensory modality in anurans.

In the present study, we demonstrated that the relative size of the peripheral components of the chemosensory systems of anurans varies during development and metamorphosis, and that this variation differs among the species analyzed. Interestingly, species whose postmetamorphic stages are more associated with the aquatic environment showed a larger relative size of the VNO and other "water-sensitive structures"; whereas those species with more terrestrial adults showed a larger relative size of the OE at postmetamorphic stages. In addition, we quantitatively described specialized structures, that is, branches of OE exposed to the buccal cavity (bexOE), the neuroepithelium in the MC, and the neuroepithelium of the OR, that were not present in all species included in the analysis; some of these structures were only present at the larval stages whereas others were specializations of postmetamorphic stages. In the remaining part of the discussion, we attempt to contextualize the biological significance that these findings could have in light of known ecological, behavioral, and physiological data on olfactory and vomeronasal function across the anuran life cycle.

4.1 | Larval stages

Tadpoles use chemical cues for mediating a wide variety of behaviors, including foraging behavior (Crossland & Shine, 2011; Veeranagoudar, Shanbhag, & Saidapur, 2004), predator avoidance (Fraker, 2009; Gonzalo, Lopez, & Martin, 2009; Kiesecker, Chivers, & Blaustein, 1996), alarm cue detection (Fraker et al., 2009; Hews, 1988; Hews & Blaustein, 1985; Kiesecker et al., 1999; Mirza, Ferrari, Kiesecker, & Chivers, 2006), and kin recognition (Villinger & Waldman, 2008; Waldman, 2005). Unfortunately, there are no studies we know of that distinguish which chemosensory organ (olfactory or vomeronasal) is responsible for the detection of the chemical cues that trigger the aforementioned behaviors.

An interesting study was recently published by Sansone et al. (2015). The authors found that sulfated steroids are detected by olfactory and vomeronasal neurons in tadpoles of *X. laevis*. As far we are aware, this is the first stimulus identified that activates vomeronasal neurons in anuran larvae. Sulfated steroids function as migratory pheromones in lamprey (Sorensen et al., 2005), and have been reported as natural vomeronasal stimuli in rodents (Nodari et al., 2008), where they

are thought to transmit social information, although a clear behavioral output has not been identified yet. Sansone et al. (2015) also found that sulfated steroids are naturally excreted by tadpoles (and adults) into the breeding water, suggesting that they might be involved, in some way, in *Xenopus* intraspecific communication. However, it is not yet clear whether sulfated steroid detection in fact triggers behavioral responses in larvae (and adults) of *Xenopus*.

The present study showed two interesting differences in peripheral olfactory anatomy among larvae of the studied species. First, there were differences in the relative size of the VNO. In particular, tadpoles of the hylid *H. pulchellus* and the pipid *X. laevis* had a relatively larger VNO than those of the bufonid *R. arenarum* (Figure 7). The vomeronasal system appears to be fully functional in tadpoles (Jungblut et al., 2012). Although its precise role in sensing stimuli in aquatic environment remains unresolved, in most tetrapods the VNO mediates the detection of pheromones or molecules related to intraspecific communication (Halpern and Martinez-Marcos, 2003; but see Baxi et al., 2006). There are two well-known social behaviors mediated by chemical cues in tadpoles: response to conspecific alarm pheromones and kin recognition.

Curiously, even if the use of chemical stimuli to recognize siblings (kin recognition) and for alarm pheromones is widespread in anuran larvae (Fraker et al., 2009; Waldman, 2005), both of these social behaviors are particularly well known in bufonid tadpoles (e.g., Gramapurohit, Veeranagoudar, Mulkeegoudra, Shanbhag, & Saidapur, 2006; Hagman & Shine, 2008; Hews & Blaustein, 1985; O'Hara & Blaustein, 1982). This ability of the bufonid tadpoles to detect putative vomeronasal stimuli, even with a relative small VNO, highlights the fact that differences in the relative size of a particular chemosensory organ between different species must be carefully interpreted in the light of its potential function. In our study, we found that cell density of the chemosensory organs were quite similar between the species included in the analysis (see Supporting Information). Therefore, it can be assumed that a more voluminous organ has more chemosensory neurons that contribute to the detection of external stimuli. However, there are some others factors that could be taken into account, such as the repertoire of chemosensory receptors (olfactory or vomeronasal) that chemosensory organs of different species express. In the same way, there is another crucial point to consider in amphibians: it has been found that V2R vomeronasal receptors are predominately, but not exclusively, expressed in the VNO in *Xenopus* tadpoles (Syed, Sansone, Nadler, Manzini, & Korsching, 2013), suggesting that a functional overlap between the VNO and the OE could exist in anuran larvae.

In tadpoles of *X. laevis* chemodetection of peptides derived from the major histocompatibility complex (MHC) serves the animals as a mechanism to discriminate among familiar full siblings (Villinger & Waldman, 2008), and peptides also appear to be functioning as alarm pheromones in tadpoles of *Rana sylvatica* (Fraker et al., 2009). The chemosensory organ involved in the detection of MHC derived peptides was not identified in *X. laevis* tadpoles. However, in rodents, MHC derived peptides, which mediate social interaction, are detected by neurons expressing V2R vomeronasal receptors in the VNO (Leinders-Zufall et al., 2004).

The second significant difference observed during larval development was the presence of a branch of the OE directly exposed to the buccal cavity (bexOE). The bexOE was more highly developed in *H. pulchellus* than *R. arenarum* tadpoles, and it was completely absent in *X. laevis*. Patches of OE exposed to the buccal cavity have been described in tadpoles of a number of species (e.g., *Anaxyrus* [*Bufo*] *americanus*) (Jermakowicz, et al., 2004; *Ascaphus truei*, Benzekri & Reiss, 2011). At the ultrastructural level, the bexOE is similar, but not identical, to the main OE (Benzekri & Reiss, 2011), hinting at possible functional differences.

Our quantitative, comparative, and ontogenetic analysis allowed us to infer the relative importance of this chemosensory structure during development. The bexOE is clearly a specialized larval structure. When present, its relative importance was highest during early larval stages and gradually declined during ontogeny until disappearing completely after metamorphosis, although this decline was much greater in *R. arenarum* than in *H. pulchellus* (Figures 5 and 6).

Tadpoles of *H. pulchellus* and *R. arenarum* are active feeders that scrape material from submerged substrates, while *X. laevis* tadpoles are obligate suspension feeders that filter organic material from the water column (Seale, 1982). It appears likely that neurons of the bexOE allow active grazers to obtain rapid information about the nature and quality of the organic material that they are feeding on, complementing the role of oral gustatory receptors.

4.2 | Postmetamorphic stages

As in larvae, the postmetamorphic chemosensory organs play a role in a wide range of behaviors, ranging from food acquisition (Shinn & Dole, 1978, 1979), to orientation and homing (Forester & Wisnieski, 1991; Grubb, 1975; Sinsch, 1990), to mate detection and selection (Poth, Wollenberg, Vences, & Schulz, 2012; Starnberger et al., 2013; Wabnitz, Bowie, Tyler, Wallace, & Smith, 1999; Woodley, 2014).

The present data show greater variation in the chemosensory organs across postmetamorphic animals of the three species analyzed (Figure 7). Interestingly, besides differences that could be found during larval stages, the two species whose juveniles are more associated with terrestrial environments (*H. pulchellus* and *R. arenarum*), seem to share a common pattern in the relative importance of the different chemosensory organs after metamorphosis (showing greater relative size of air-sensitive structures, i.e., the OE); whereas an inverted pattern is observed in the fully aquatic frog *X. laevis* (showing greater relative importance of water sensitive structures, i.e., the VNO and MCE).

In *H. pulchellus* and *R. arenarum*, the relative size of the OE (in terms of the total chemosensory volume calculated for each species) increases throughout development and becomes greater at postmetamorphic stages (Figures 4c, 5c, and 7), whereas in *X. laevis* the relative importance of the OE decreases throughout development and is somewhat reduced, compared with the other two species, at the end of metamorphosis (Figures 6c and 7). Similar results have been described in comparative studies in mammals with different degree of association with the aquatic environment (Van Valkenburgh et al., 2011). In this study, authors estimated, from high-resolution CT scans of dry skulls, the surface area of respiratory and olfactory turbinates in a variety of

terrestrial, freshwater, and marine carnivorans. Relative to body mass or skull length, aquatic species showed significantly less olfactory surface area than terrestrial species, which is probably associated with a decreased reliance on olfaction when foraging under water (Van Valkenburgh et al., 2011).

Contrasting with the pattern observed in the relative size of the OE of juveniles, the VNO is larger (both in total and relative size) in the secondarily aquatic *X. laevis* than in the other two species analyzed (Figure 7 and Table 1). This finding reinforces the idea that the VNO is specialized for response to nonvolatile, aquatic stimuli (Broman, 1920; Døving & Trotier, 1998). Similar results have been described in caecilians, in which the VNO seems to be more developed in aquatic species (Schmidt & Wake, 1990). However, it is important to note that the VNO is still fairly well developed in juveniles of *R. arenarum* and *H. pulchellus*, since there is no relevant decline or increase in absolute size of the organ during metamorphosis. This result is probably related to the fact that most anurans, including *R. arenarum* and *H. pulchellus*, return to water to breed. Unfortunately, as in tadpoles, there is little information available on the functional role of the vomeronasal organ in postmetamorphic animals, making interpretation of these differences difficult. The aquatic-borne peptide pheromone splendipherin (Wabnitz et al., 1999) is likely detected by the VNO, as is known to be the case for peptide pheromones of newts (Iwata et al., 2013). However, there are no studies that confirm that this aquatic pheromone activates neurons in the VNO of the frogs.

Another crucial difference among the three species analyzed is in the presence of two additional sensory epithelia, the olfactory recess (OR) found in *R. arenarum* and the neuroepithelium of the middle chamber (MCE) found in *X. laevis*. Comparative analysis suggests that these two epithelia are at least partly homologous with each other, and are specialized for detection of water-borne odorants (Benzekri & Reiss, 2011; Helling, 1938; Reiss & Eisthen, 2008). At the anatomical level, the ultrastructure of the MCE resembles the larval OE, rather than the postmetamorphic OE (Hansen, Reiss, Gentry, & Burd, 1998). Analysis of odorant and vomeronasal receptor gene expression has shown that the postmetamorphic MCE expresses both "aquatic" odorant receptors (Freitag, Krieger, Strotmann, & Breer, 1995), as well as V1R (Date-Ito, Ohara, Ichikawa, Mori, & Hagino-Yamagishi, 2008) and V2R (Hagino-Yamagishi et al., 2004; Syed, Sansone, Nadler, Manzini, & Korsching, 2015) vomeronasal receptors, again resembling the larval main OE. As discussed above for larval stages, the presence of vomeronasal receptors in the MCE suggests potential overlapping function between the VNO and MCE (and probably the OR) which should be taken into account in future morpho-functional studies.

The presence of a small OR in *R. arenarum* may be associated with the return to water for breeding in this species (Bionda, Lajmanovich, Salas, Martino, & di Tada, 2011), but this makes its absence in *H. pulchellus* difficult to interpret, since, as we mentioned before, this species likewise returns to water to breed (Solé & Pelz, 2007). In the only broad survey of the presence and size of the OR across anuran species, Helling (1938) found a partial correlation with aquatic habits, such that it was significantly enlarged in the highly aquatic *Telmatobius hauthali*, but also found it absent in a number of species with aquatic breeding.

This initial quantitative survey of the development of the peripheral olfactory system across three anuran species with differing larval and adult lifestyles highlights the significant variation in this system, which appears to be correlated with functional and ecological differences among the species. The emphasis in the literature on anuran visual and auditory systems has perhaps led to the neglect of the olfactory sense, which nevertheless is known to be important in a variety of contexts, as detailed above. Further work should help us to better understand the diversity within anurans. Moreover, the comparative analysis of the functional morphology of the chemosensory organs in anurans provides a vital window to understanding the relative function of the olfactory and vomeronasal systems in aquatic and terrestrial environments.

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AUTHOR CONTRIBUTIONS

LDJ, DAP, and AGP designed the comparative study. AGP supervised the project. LDJ performed the observations, analysed data and performed three-dimensional reconstructions. LDJ and JOR interpreted the results and wrote the paper, but all authors discussed the results and implications and commented on the manuscript at all stages.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

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