

# Can social behaviour drive accessory olfactory bulb asymmetries? Sister species of caviomorph rodents as a case in point

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## Abstract

In mammals, the accessory olfactory or vomeronasal system exhibits a wide variety of anatomical arrangements. In caviomorph rodents, the accessory olfactory bulb (AOB) exhibits a dichotomic conformation, in which two subdomains, the anterior (aAOB) and the posterior (pAOB), can be readily distinguished. Interestingly, different species of this group exhibit bias of different sign between the AOB subdomains (aAOB larger than pAOB or vice versa). Such species-specific biases have been related with contrasting differences in the habitat of the different species (e.g. arid vs. humid environments). Aiming to deepen these observations, we performed a morphometric comparison of the AOB subdomains between two sister species of octodontid rodents, *Octodon lunatus* and *Octodon degus*. These species are interesting for comparative purposes, as they inhabit similar landscapes but exhibit contrasting social habits. Previous reports have shown that *O. degus*, a highly social species, exhibits a greatly asymmetric AOB, in which the aAOB has twice the size of the pAOB and features more and larger glomeruli in its glomerular layer (GL). We found that the same as in *O. degus*, the far less social *O. lunatus* also exhibits a bias, albeit less pronounced, to a larger aAOB. In both species, this bias was also evident for the mitral/tufted cells number. But unlike in *O. degus*, in *O. lunatus* this bias was not present at the GL. In comparison with *O. degus*, in *O. lunatus* the aAOB GL was significantly reduced in volume, while the pAOB GL displayed a similar volume. We conclude that these sister species exhibit a very sharp difference in the anatomical conformation of the AOB, namely, the relative size of the GL of the aAOB subdomain, which is larger in *O. degus* than in *O. lunatus*. We discuss these results in the context of the differences in the lifestyle of these species, highlighting the differences in social behaviour as a possible factor driving to distinct AOB morphometries.

**Key words:** accessory olfactory bulb; glomerular layer; octodontid rodents; social behaviour; vomeronasal system.

## Introduction

Among the various olfactory sensory modalities present in mammals, the accessory or vomeronasal olfactory system (VNS) plays a key role in the sensing of pheromone-like semiochemicals, and in the orchestration of a wide variety of neuroendocrine and behavioural responses (Tirindelli

et al. 2009). The sensory surface of the VNS is the vomeronasal organ (VNO), a tubular structure located bilaterally at the base of the nasal septum. In rodents, the VNO neuroepithelium contains two spatially segregated populations of chemosensitive neurons, each co-expressing either V1R receptors and  $G_{\alpha_{i2}}$  protein or V2R receptors and  $G_{\alpha_{o}}$  protein (Jia & Halpern, 1996; Halpern et al. 1998). V1R receptors feature enhanced chemical affinity for small volatile molecules (Inamura et al. 1999; Inamura & Kashiwayanagi, 2000; Leinders-Zufall et al. 2000; Sugai et al. 2006), while V2R receptors display greater affinity for large insoluble molecules (Leinders-Zufall et al. 2004, 2009; Kimoto et al. 2005). In turn, V1R and V2R neurons project separately towards different subdomains of the accessory olfactory bulb (AOB).  $G_{\alpha_{i2}}$ -expressing axons end in glomeruli located in the rostral, or anterior, half of the AOB (aAOB), while  $G_{\alpha_{o}}$ -axons

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end in glomeruli of the caudal, or posterior, half of the AOB (pAOB; Jia & Halpern, 1996; Halpern et al. 1998). From the AOB, secondary projection neurons (mitral and tufted cells) of each subdomain send segregated although partially overlapped projections to multiple forebrain regions associated with neuroendocrine regulation, including the medial amygdala, posteromedial cortical amygdala, the bed nucleus of the stria terminalis and the bed nucleus of the accessory olfactory tract (Scalia & Winans, 1975; von Campenhausen & Mori, 2000; Martínez-Marcos, 2009).

In addition to rodents, the dichotomous segregation of vomeronasal pathways has been described in opossums (Halpern et al. 1995) and rabbits (Villamayor et al. 2018) also, and was initially thought to represent a common feature to all mammals. However, subsequent reports showed that Primates and Laurasiatherians do not show this dichotomous segregation, such that either they possess the V1R pathway only (such as in goats, marmosets, shrews, domestic carnivores, elephants and hippopotami) or no vomeronasal system at all (such as in apes, some bats and aquatic species; Meisami & Bhatnagar, 1998; Takigami et al. 2000, 2004; Ngwenya et al. 2011; Zhao et al. 2011; Kondoh et al. 2017). Moreover, the lesser Tenrec (*Echinops telfairi*), a basal Afrotheria, has a dichotomous but not strictly segregated VNS (Suárez et al. 2009b), while the hyrax (*Procavia capensis*), another basal Afrotheria, possesses the V1R pathway only (Suárez et al. 2011a). Furthermore, not all rodents have a dichotomous VNS; the California ground squirrel (*Otospermophilus beecheyi*), a sciurid rodent, has the V1R pathway only (Suárez et al. 2011a). Thus, the dichotomous organization of the VNS displays a remarkable variability within and between taxa, suggesting that this trait may be more closely related to the species' lifestyles rather than their phylogenetic backgrounds.

These observations have raised the question about the biological rationale behind (the main factors explaining) the existence, conservation or disappearance of these segregated pathways. Evidence from neuronal recording and neural activity markers expression experiments, obtained mostly in rodents, suggest that the V1R pathway mediates male–female attraction, while the V2R pathway seems more related with male–male aggression (Dudley & Moss, 1999; Inamura et al. 1999; Kumar et al. 1999; Matsuoka et al. 1999; Inamura & Kashiwayanagi, 2000; Peele et al. 2003; Sugai et al. 2006; Chamero et al. 2011). In addition, comparative observations across mammals have shown that independently of its phylogenetic position, species with visible sexual dimorphisms show a deterioration of the V2R pathway (Suárez et al. 2011a), a result that is consistent with a sexually related functional segregation of the AOB pathways.

South American hystricognath rodents constitute an interesting model group to study the question posed above. Several recent studies have shown that these wild rodents feature a dichotomous, but asymmetric, organization of the VNS, in which one of the subdomains of the AOB is larger

than the other. More interestingly, both aAOB bias and pAOB bias have been described in different species of this group, in association with conspicuous differences in environmental traits. In *Octodon degus*, a semi-arid inhabitant, the aAOB has twice the overall volume than the pAOB, and contains more and larger glomeruli (Suárez & Mpodozis, 2009a), while in the semi-aquatic capybara (*Hydrochoerus hydrochaeris*), the pAOB has a larger volume and contains more glomeruli than the aAOB (Suárez et al. 2011b).

Given that social interactions in mammals rely to a great extent on pheromones, it could be expected that in addition to sexual and environmental factors, the degree of sociality would also be an important lifestyle trait driving the VNS to different anatomical conformations in different species. Here, we performed a morphometric study of the subdomains of the AOB in *Octodon lunatus* and compared the resulting measurements with that of its sister species, *O. degus*. *Octodon degus* is a diurnal, open field inhabitant, which exhibits an elaborate sociality, including communal nesting, cooperative burrowing, cooperative surveillance and alarm calls display (Ebensperger & Bozinovic, 2000; Ebensperger et al. 2004, 2006; Jesseau et al. 2009, Fulk, 1976; Yáñez, 1976; Cecchi et al. 2003). *Octodon lunatus* is a mainly nocturnal shrub inhabitant that is far less social than *O. degus* (Sobrero et al. 2014). In the field, *O. lunatus* form small groups consisting of two–four adult individuals, which occasionally share resting areas during daytime. Engagement in cooperative behaviours has not been described in this species. We found significant differences between these species, in particular regarding the morphometric bias of the glomerular layer (GL) between AOB subdomains. We conclude that differences in sociality may lead to morphometric AOB differences, even between closely related species.

## Materials and methods

Five adult specimens of *O. lunatus* and six adult specimens of *O. degus* were used in these experiments. These animals were captured in the wild and maintained in an animal facility at the University of Chile. *Octodon lunatus* were captured in Los Molles (Region de Valparaíso, Chile) and *O. degus* were captured in both Rinconada de Maipú (Region Metropolitana, Chile) and Parque Nacional Bosque Fray Jorge (Region de Coquimbo, Chile). All procedures were performed under guidelines established by the Universidad de Chile Bioethical Committee (120731-DB-FSH-UCH) and adhered to Chilean laws [permits 1-130 154.2010 (7989), 1-109.2011 (6749), 1-90.2011 (4731) and 1-95-2012 (4486) by the Servicio Agrícola y Ganadero and 013/2011 by the Corporación Nacional Forestal].

## Brain extraction

The animals were deeply anaesthetized using a dose of ketamine/xylazine (2.4 mL kg<sup>-1</sup> and 1.2 mL kg<sup>-1</sup>, respectively) and transcardially perfused with 0.9% saline serum, followed by 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS) solution. The brains were removed, weighed and postfixed for several days. The olfactory bulbs (OBs) were removed and placed in a 30%

sucrose in 0.01 M PBS solution for cryoprotection, until they sank. Then, 40- $\mu\text{m}$  sagittal OB sections were obtained using a freezing-sliding microtome (Leitz Wetzlar 1400, Leitz, Germany). The sections were collected in two series of 40- $\mu\text{m}$  intervals and maintained in a solution of 0.01 M PBS + 0.01% sodium azide. One series was mounted onto gelatin-coated slides, dehydrated and stained using Cresyl violet solution (Fluka; 61123, 0.2% solution, pH 4.3) for stereological quantification.

### Immunohistochemistry

The second series of OB sections was free-floating incubated with 3%  $\text{H}_2\text{O}_2$  + 10% methanol for 10 min, followed by 10 mM sodium citrate solution (pH 6.0), for 30 min at 80 °C. Posteriorly, the sections were incubated in a 5% normal goat serum (NGS) + 0.05% Triton X-100 in 0.01 M PBS (PBST) for 1 h. The slices were incubated with anti- $G_{\alpha_{i2}}$  antibody (1 : 500; cat no. sc-13534, Santa Cruz Biotechnology) with 3% NGS in PBST for 16 h at 5 °C. Subsequently, the sections were rinsed and incubated in biotinylated goat anti-mouse secondary antibody (1 : 500; cat no. BA-9200, Vector Laboratories) for 2 h. In the final steps, the biotinylated sections were incubated with the avidin-biotin complex (ABC Elite Kit; Vector Laboratories) for 1 h, and reacted using 0.25 mg  $\text{mL}^{-1}$  of 3,3'-diaminobenzidine (cat no. D-5905, Sigma-Aldrich) and 0.001%  $\text{H}_2\text{O}_2$  for 3 min.

### Stereological measures

Measurements of AOB volumes and counting of mitral/tufted (M/T) cells were estimated in Cresyl violet-stained serial sections. Stereological measures were made using a Stereoinvestigator 8.0 software (MBF Bioscience, Williston, Vt., USA), a CCD video camera (Microfare, Optometrics<sup>®</sup>) and a bright-field microscope (Eclipse E400, Nikon). AOB samples of each subject were examined every two sections, covering its whole lateral–medial extension. We measured the overall volume and GL volume of both aAOB and pAOB subdomains. For the overall AOB subdomains measurements, the vomeronasal nerve layer (VNL), the GL, the external plexiform layer (EPL) and the mitral/tufted cells layer (M/TL) were considered. The indentation splitting the AOB layers was used to distinguish the aAOB from the pAOB subdomain, as in previous reports (Suárez & Mpodozis, 2009a; Suárez et al. 2011b). The volume measurements were performed using the Cavalieri stereological estimator (CE). In all samples, we used a 30- $\mu\text{m}$  square grid covering the whole AOB area, matched to a 40 $\times$  lens. For CE, the Gundersen coefficients of error ( $m = 1$ ) obtained ranged between 0.009 and 0.018 for *O. lunatus*, and 0.003 and 0.012 for *O. degus*. For comparative purposes, we divided the estimated volume of a given structure by brain volume, taking account for any allometric effects. Brain volume was calculated by dividing the brain mass by fixed brain tissue density (1.036 g  $\text{mm}^{-3}$ ; Iwaniuk & Wylie, 2007; Vega-Zuniga et al. 2013). Thus, volumes are expressed as relative to the brain volume. Cell counts of AOB M/T cells were performed in both species, considering only that restricted to M/TL. The anatomical indentation splitting the M/TL was considered to separate both anterior M/TL and posterior M/TL. The M/T cells number and the M/TL volume of both AOB portions were obtained using an optical fractionator (OF) procedure, matched to a  $\times 100$  oil immersion lens. A 100- $\mu\text{m}$  sampling grid was used, and all 50  $\times$  50  $\mu\text{m}$  frames were counted using a disector height of 15  $\mu\text{m}$  and 7.5  $\mu\text{m}$  guard zones. For OF, the Gundersen coefficients of error ( $m = 1$ ) obtained ranged between 0.03 and 0.07 for *O. lunatus*, and 0.03 and 0.06 for *O. degus*. From these measurements, the density of M/T cells, expressed as cells  $\text{mm}^{-3}$ , of each AOB portion was calculated.

### Statistical analyses

Non-parametric statistics were performed to analyse the data, as they did not show a normal distribution; the Mann–Whitney *U*-test was chosen to compare between *O. lunatus* and *O. degus* AOB measures, and the Wilcoxon-matched pairs test was used to compare between aAOB and pAOB measures within each of the species. All the statistical analyses were performed using Statistica 6.0 (StatSoft, Tulsa, OK, USA).  $P < 0.05$  or  $P \leq 0.01$  values are related with significant differences in both statistical tests.

### Figure preparation

All the images were obtained using a bright-field microscope and a SPOT camera (Spot Advanced, Diagnostic Instrument). Microphotographs were processed using Adobe Photoshop CS4 (Adobe Systems, San Jose, CA, USA). All the graphs were plotted using SigmaPlot (Systat Software).

## Results

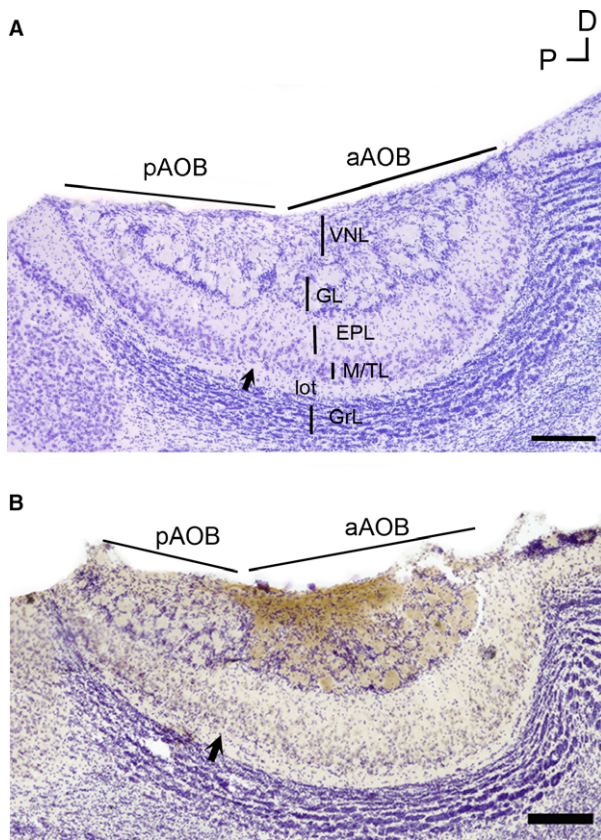
### Anatomical characterization of the *Octodon lunatus* AOB

In *O. lunatus*, the AOB appears as a prominent structure located in the caudal/dorsal region of the main olfactory bulb, with the vomeronasal nerve innervating it from its lateral aspect (as shown by Suárez et al. 2011b; Figs 1a and 2a). The cross-sectional area of the AOB varies across its medial–lateral axis, reaching a maximum value at the middle zone (Fig. 2b). Both the anterior and posterior AOB portions are clearly distinguishable by a glial indentation splitting all layers. At both subdomains, all AOB layers previously reported in other rodent species appear well defined and readily recognizable: VNL, GL, EPL, M/TL, lateral olfactory tract (lot) and granular cells layer (GrL; Fig. 1a). The glomeruli's profiles in both AOB portions are numerous, poorly defined and exhibit different sizes. The soma of M/T cells was mainly located in M/TL, but some scant cells were also observed scattered in the EPL.

The presence of an anatomical split delimiting AOB subdomains coincides with a segregated expression of  $G_{\alpha_{i2}}$ , a protein located exclusively in V1R neurons (Fig. 1b). We observed the  $G_{\alpha_{i2}}$  + immunostaining in both VNL and GL restricted to the aAOB. We did not perform a study of  $G_{\alpha_{o}}$  expression (V2R neurons marker) in this species; however, a conserved, fully segregated pattern of expression, similar to that previously reported in *O. degus*, can be expected to be found in *O. lunatus* (Suárez & Mpodozis, 2009a).

### AOB morphometry of *Octodon lunatus*

We performed a series of morphometric measurements on the AOB of *O. lunatus*. Specifically, we measured the overall volume of each subdomain, the volume of the GL in each subdomain, and the number of M/T cells at M/TL associated



**Fig. 1** Accessory olfactory bulb (AOB) anatomy of *Octodon lunatus*. (a) Microphotograph of a Nissl-stained section through the medial portion of the AOB. All layers of the AOB appear readily identifiable. Note the glial indentation splitting the anterior (aAOB) and posterior (pAOB) subdomains (black arrow). (b) Anti-Gxi2-immunostained, Nissl-counterstained section. The stain (brown) is exclusively restricted to the aAOB portion. EPL, external plexiform layer; GL, glomerular layer; GrL, granular cells layer; lot, lateral olfactory tract; M/TL, mitral/tufted cells layer; VNL, vomeronasal nerve layer. Scale bar: 250  $\mu\text{m}$ .

to each subdomain (for more details, see Materials and methods). We found that the overall aAOB volume was significantly larger than the pAOB volume (aAOB:  $0.689 \pm 0.04 \text{ mm}^3$ ; pAOB:  $0.507 \pm 0.03 \text{ mm}^3$ ;  $Z = 2.02$ ,  $P = 0.04$ ,  $n = 5$ ; Fig. 3a). Concordantly, the number of M/T cells counted in the aAOB M/TL was double the number of cells counted in the pAOB M/TL (aAOB:  $10\,341 \pm 1022$ ; pAOB:  $5227 \pm 297$ ;  $Z = 2.02$ ,  $P = 0.04$ ,  $n = 5$ ; Fig. 3b). There was also not a significant difference in M/T cells density between both AOB subdomains (aAOB:  $183\,751 \pm 82\,176 \text{ cells mm}^{-3}$ ; pAOB:  $184\,253 \pm 82\,400 \text{ cells mm}^{-3}$ ;  $Z = 0.13$ ,  $P = 0.8$ ,  $n = 5$ ). In contrast, the volume of the GL did not show a significant difference between both AOB subdomains (aAOB:  $0.069 \pm 0.008 \text{ mm}^3$ ; pAOB:  $0.064 \pm 0.007 \text{ mm}^3$ ;  $Z = 1.21$ ,  $P = 0.2$ ,  $n = 5$ ; Fig. 3c). Thus, both the overall volume of the AOB subdomains and the number of M/TL cells showed a significant bias towards the aAOB, while for the GL no noticeable bias was found.

### AOB morphometric comparison between *Octodon lunatus* and *Octodon degus*

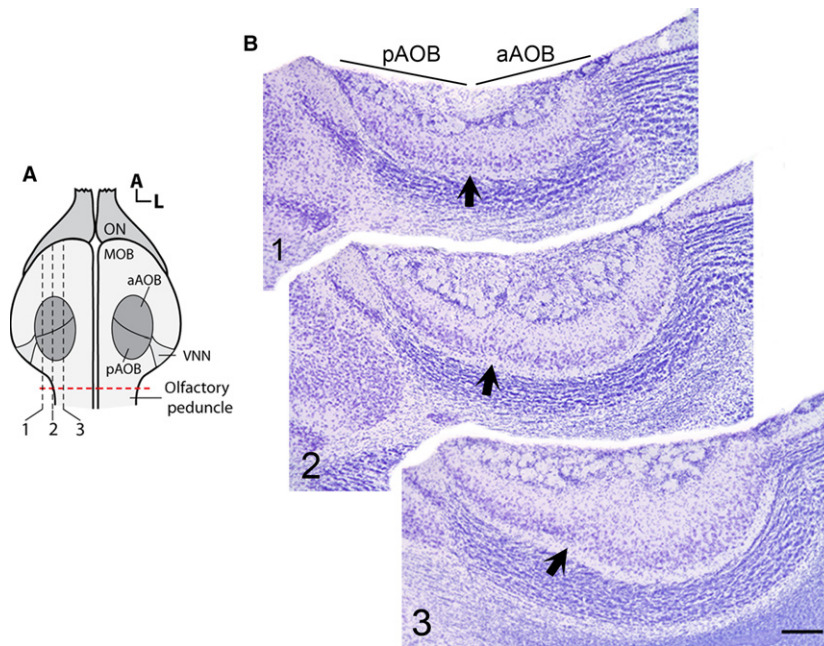
In agreement with previous reports (Suárez & Mpodozis, 2009a), we found that the AOB of *O. degus* shows a marked bias to a larger aAOB, regarding its overall volume (aAOB:  $0.616 \pm 0.06 \text{ mm}^3$ ; pAOB:  $0.363 \pm 0.03 \text{ mm}^3$ ;  $Z = 2.2$ ,  $P = 0.03$ ,  $n = 6$ ; Fig. 4a), number of neurons in the M/TL (aAOB:  $14\,219 \pm 2522$ ; pAOB:  $6883 \pm 1030$ ;  $Z = 2.02$ ,  $P = 0.04$ ,  $n = 6$ ; Fig. 4b) and GL volume (aAOB:  $0.1 \pm 0.01 \text{ mm}^3$ ; pAOB:  $0.061 \pm 0.005 \text{ mm}^3$ ;  $Z = 2.2$ ,  $P = 0.03$ ,  $n = 6$ ; Fig. 4c). There was not a significant difference in M/T cells density between both AOB subdomains (aAOB:  $110\,145 \pm 9112 \text{ cells mm}^{-3}$ ; pAOB:  $99\,125 \pm 11\,543 \text{ cells mm}^{-3}$ ;  $Z = 0.67$ ,  $P = 0.6$ ,  $n = 5$ ). Based on these data, we performed a series of inter-species comparisons, as described below.

First, we compared the morphometric measurement obtained for each subdomain in each of the species. For the aAOB we did not observe significant inter-species differences, in the overall volume ( $U = 8$ ,  $Z = 1.19$ ,  $P = 0.2$ ,  $n_{\text{deg}} = 6$ ,  $n_{\text{lun}} = 5$ ; Fig. 5a) or in the number of M/T cells ( $U = 5$ ,  $Z = 1.46$ ,  $P = 0.14$ ,  $n_{\text{deg}} = 5$ ,  $n_{\text{lun}} = 5$ ; Fig. 5b). However, the GL volume of aAOB was significantly smaller in *O. lunatus* than in *O. degus* ( $U = 1$ ,  $Z = 2.46$ ,  $P = 0.01$ ,  $n_{\text{deg}} = 6$ ,  $n_{\text{lun}} = 5$ ; Fig. 5c). For the pAOB we did not find significant inter-species differences for any morphometric measurements: overall volume ( $U = 14$ ,  $Z = 0.09$ ,  $n_{\text{deg}} = 6$ ,  $n_{\text{lun}} = 5$ ; Fig. 5a), number of M/T cells ( $U = 6$ ,  $Z = 1.25$ ,  $P = 0.21$ ,  $n_{\text{deg}} = 5$ ,  $n_{\text{lun}} = 5$ ; Fig. 5b) and GL volume ( $U = 4$ ,  $Z = 1.92$ ,  $P = 0.06$ ,  $n_{\text{deg}} = 6$ ,  $n_{\text{lun}} = 5$ ; Fig. 5c).

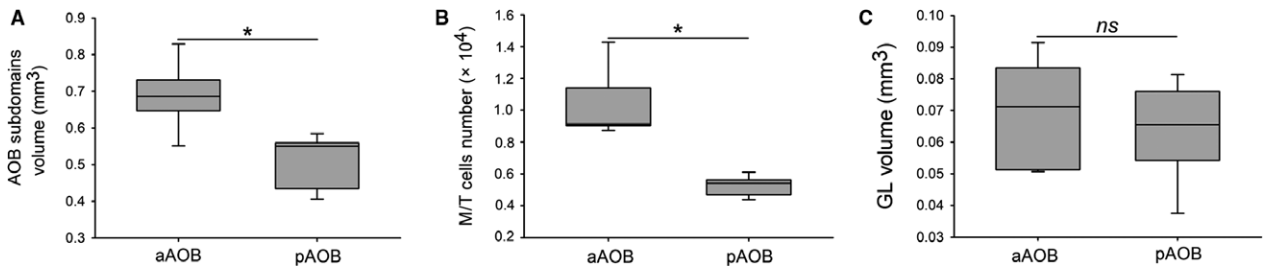
Moreover, we performed a comparison of the ratio aAOB/pAOB for each of the morphometric measurements performed on each species. We did not find significant differences between these species in the ratio of overall volume of the subdomains (aAOB/pAOB:  $U = 4$ ,  $Z = -1.92$ ,  $P = 0.06$ ,  $n_{\text{deg}} = 6$ ,  $n_{\text{lun}} = 5$ ; Fig. 5d) or in the ratio of the number of M/T cells per subdomain (aAOB/pAOB:  $U = 5$ ,  $Z = 1.46$ ,  $P = 0.1$ ,  $n_{\text{deg}} = 6$ ,  $n_{\text{lun}} = 5$ ; Fig. 5e). However, for the GL, *O. lunatus* exhibited a significantly smaller volumetric ratio than *O. degus* (aAOB/pAOB:  $U = 0$ ,  $Z = -2.64$ ,  $P = 0.008$ ,  $n_{\text{deg}} = 6$ ,  $n_{\text{lun}} = 5$ ; Fig. 5f).

### Discussion

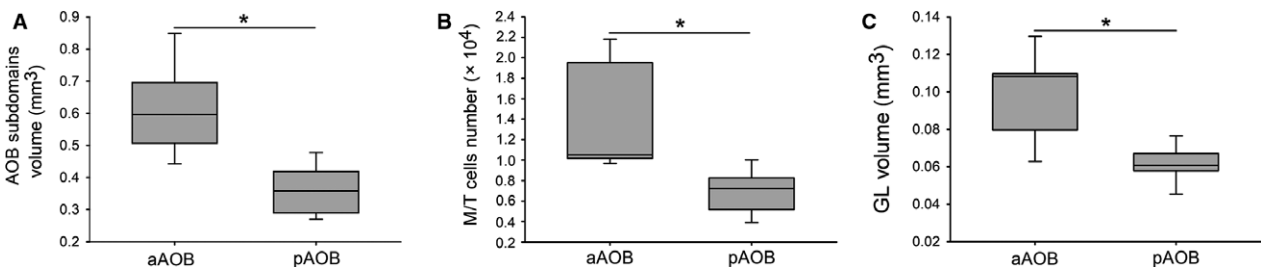
Our morphometric measurements show that *O. lunatus* has an overall volumetric bias favouring the aAOB subdomain. Interestingly, such bias is of the same sign, albeit less pronounced, than the AOB bias exhibited by its sister species, *O. degus*. Just as in *O. degus*, in *O. lunatus* we found that this bias was readily evident at the M/TL. But, unlike in *O. degus*, in *O. lunatus* this bias was not present at the GL. In comparison with *O. degus*, in *O. lunatus* the aAOB GL was significantly reduced in volume, while the pAOB GL displayed a similar volume. Thus, we conclude that between



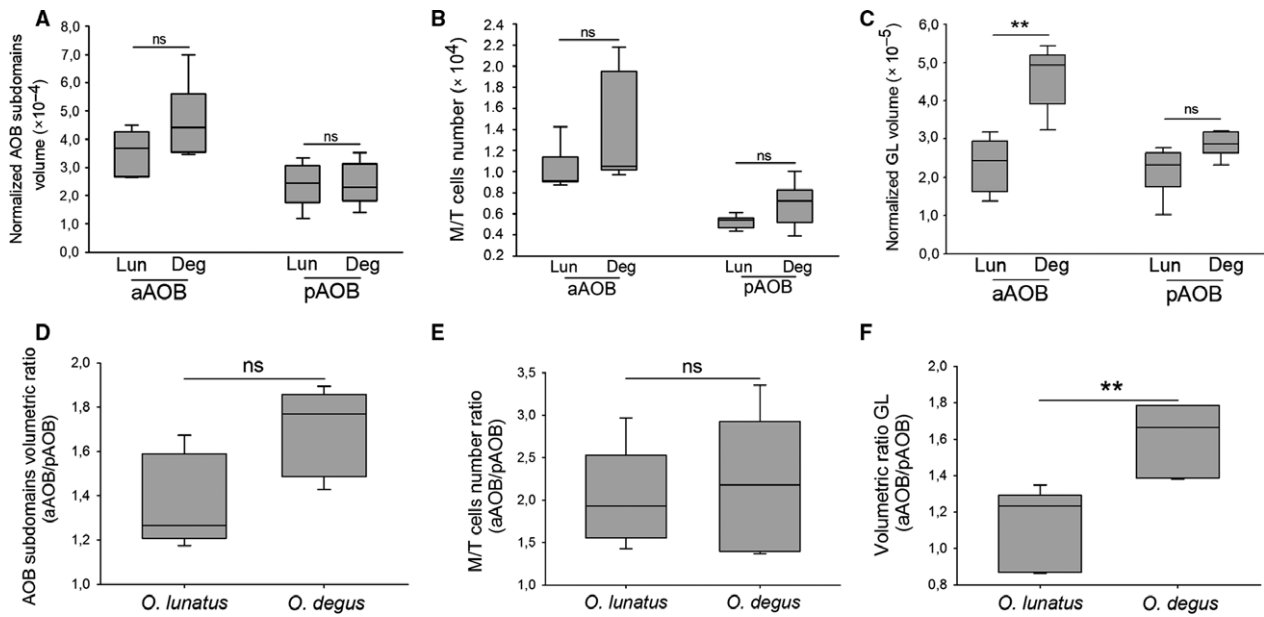
**Fig. 2** Sagittal series of accessory olfactory bulb (AOB) profiles in *Octodon lunatus*. (a) Schema (dorsal view) of the olfactory bulb illustrating the relative position of the AOB subdomains. The red dotted line in (a) indicates the position of the cut made to separate the olfactory bulbs (OBs) from the brain. Dashed lines and numbers indicate the approximate level of each of the sections depicted in (b). (b) Microphotographs of the corresponding series of sagittal sections, stained with Nissl. The glial indentation dividing the AOB subdomains is appreciable at all levels (dark arrows). Note the variation of the cross-sectional area of the AOB. MOB, main olfactory bulb; ON, olfactory nerve; VNN, vomeronasal nerve. Other abbreviations as in Fig. 1. Scale bar: 250  $\mu$ m.



**Fig. 3** *Octodon lunatus* exhibits an emphasized anterior accessory olfactory bulb (aAOB), with exception of the glomerular layer (GL). Box plot graphs showing the measures for overall volume (a), mitral/tufted (M/T) cells number (b) and GL volume (c), corresponding to aAOB and posterior (pAOB). The graphs show the two central percentiles and the median, with error bars. \* $P < 0.05$ , significant differences obtained running Wilcoxon-matched pair tests. ns, not significant.



**Fig. 4** *Octodon degus* exhibits a full-bias to a larger anterior accessory olfactory bulb (aAOB). Box plot graphs showing the measures for overall volume (a), mitral/tufted (M/T) cells number (b) and glomerular layer (GL) volume (c), corresponding to aAOB and posterior (pAOB). The graphs show the two central percentiles and the median, with error bars. \* $P < 0.05$ , significant differences obtained running Wilcoxon-matched pair tests. ns, not significant.



**Fig. 5** (a–c) *Octodon lunatus* and *O. degus* exhibit similar accessory olfactory bulb (AOB) measures, with the exception of the anterior (aAOB) glomerular layer (GL). Box plot graphs showing the measures for normalized overall volume (a), mitral/tufted (M/T) cells number (b), and normalized GL volume (c), corresponding to aAOB and posterior (pAOB). The graphs show the two central percentiles and the median, with error bars. \*\* $P \leq 0.01$ , significant difference obtained running Mann–Whitney  $U$ -tests. ns, not significant. (d–f) *Octodon lunatus* and *O. degus* exhibit similar AOB bias extent, with the exception of the GL. aAOB/pAOB ratio for overall volume (d), M/T cells number (e) and GL volume (f). Box plot graphs showing the two central percentiles and median, with error bars. \*\* $P \leq 0.01$ , significant differences obtained running Mann–Whitney  $U$ -tests. ns, not significant.

these sister species there is a very specific difference in the anatomical conformation of the AOB, namely, the relative size of the GL of the aAOB subdomain, which is larger in *O. degus* than in *O. lunatus*. In what follows we discuss these results in the context of differences in the life mode in these species.

#### ***Octodon lunatus* vs *Octodon degus* AOB differences: A matter of physical environmental factors?**

It has been suggested that salient cues of the species habitat could be a key underlying factor explaining the asymmetries observed in the relative size of anterior and posterior portions of the AOB in caviomorph rodents. A bias to a more prominent aAOB has been reported in *O. degus*, which lives in a semi-arid habitat, and performs scent-marking behaviours (such as dustbathing) that likely involve the usage of volatile pheromones (Fulk, 1976; Ebensperger & Caiozzi, 2002). This scent-marking mode implies the preferential use of the V1R pathway, which would explain the greater development of the aAOB observed in this species (Suárez & Mpodozis, 2009a). In clear contrast, a bias to a more prominent pAOB has been reported in the capybara (*H. hydrochaeris*), a semi-aquatic wetland inhabitant. The capybara engages in scent-marking behaviours using oily, non-volatile molecules secreted by two kinds of scent glands: anal glands and the morrillo gland (located at the top of the snout; MacDonald et al. 1984; Herrera &

MacDonald, 1994). Such scent-marking mode would be associated with the preferential usage of the V2R pathway and, hence, with an anatomical bias favouring the pAOB (Suárez et al. 2011b).

Unlike *O. degus*, which have a preference to live in open areas, or savannas, of the mediterranean region of central Chile, *O. lunatus* is a shrub inhabitant, more frequently observed in the hills of the coastal range of this same region (Glanz & Meserve, 1982; Sobrero et al. 2014). Both species come to coexist, however, inhabiting different patches in xeric or sclerophic landscapes affected by the same climatic regime. Even when at a microscale level the habitat of *O. lunatus* may be slightly more humid than that of *O. degus*, this difference in humidity is far from comparable to the one that exists between the wetlands of the capybaras and the savanna of the degus. In agreement, and unlike the capybara, *O. lunatus* lack specialized glands producing non-volatile pheromones, and do not present an anatomical bias towards the pAOB. Thus, a preferred usage of non-volatile cues in this species is very unlikely. Furthermore, the overall volume of the pAOB, as well as the volume of the pAOB GL appears to be similar in these species, suggesting no differences between them in the usage of non-volatile pheromones. Thus, the anatomical differences in the conformation of the AOB reported here are unlikely to be due to environmental factors. Instead, they point to the existence of idiosyncratic species-specific differences in VNS-dependent aspects of behaviour.

### AOB differences and phomonal sensing

It is widely accepted that the size and degree of specialization of a sensory structure correlate with the relevance that the corresponding sensory modality has for the realization of the 'umwelt' (organism/environment relationship) of a given organism (Jerison, 1973). In the case of olfaction, the main olfactory bulb (MOB) of nocturnal, olfaction-dependent species tends to be larger than the OB of more visually dependent diurnal species, a trait observed in mammals (Barton et al. 1995). In a similar way, we might expect that the volume of the GL of a given AOB subdomain, which reflects the number and size of the glomeruli it contains, very likely correlates with the frequency and diversity of the phomone-dependent behaviours mediated by such subdomains.

As we stated above, the most striking differences between *O. degus* and *O. lunatus* concern the volume of the GL in the aAOB, which is markedly reduced in the latter species. Previous studies have shown that in *O. degus* the aAOB GL features more and larger glomeruli than the pAOB GL, indicating an enhanced sensory activity of the aAOB over the pAOB in this species (Suárez & Mpodozis, 2009a). We were unable to perform equivalent measurements in *O. lunatus*, due to difficulties in the stereological quantification of glomeruli. However, our qualitative observations indicate that unlike *O. degus*, in *O. lunatus* the aAOB GL exhibit glomerular profiles of similar size to those located at the pAOB. Altogether, these observations indicate that the aAOB subdomain is less active in *O. lunatus* than in *O. degus*, while the pAOB subdomain seems to be equally active in both species. Given that the aAOB activity is mainly driven by volatile pheromones (Inamura et al. 1999; Inamura & Kashiwayanagi, 2000; Leinders-Zufall et al. 2000; Sugai et al. 2006), we conclude that the sensing of volatile pheromones is less frequent, less intense and/or less relevant in *O. lunatus* than in *O. degus*.

In addition, a remarkable aspect of our results is that while in *O. lunatus* the GL show no significant bias between AOB subdomains, the M/T cells, whose dendrites also contribute to the glomeruli, show a definite bias in number towards the aAOB. This bias is similar in magnitude and direction to the one found in *O. degus*. We think that this apparent incongruence might be explained by the pattern of connections of M/T cells inside the AOB. AOB M/T cells differ from MOB M/T cells in that the former are not restricted to innervate only one glomerulus, but the latter are (Del Punta et al. 2002). Instead, AOB M/T cells perform synaptic contacts with 6–10 glomeruli sharing innervation from vomeronasal nerve (VNN) neurons expressing a common vomeronasal receptor (Del Punta et al. 2002; Hovis et al. 2012). This property might allow AOB M/T cells to stay post- and pre-synaptically active, hence alive, even if some of the glomeruli they innervate become less active or even obliterated. A more detailed study focused on how the bias

in the M/T cells number arises during ontogeny may help to clarify this latter point.

### AOB bias and social environment

Social interactions in mammals rely to a great extent upon semiochemical interchanges (for review, see Liberles, 2014). This observation led us to hypothesize that species exhibiting different degrees of sociality would have differences in the specialization of the VNS. Interestingly, even though *O. degus* and *O. lunatus* are closely related species, they exhibit marked differences in their social behaviours. On the one hand, *O. degus* exhibits a very rich social system. Individuals of this species live in groups composed of a dominant male and two–five females, plus their offspring. Members of a group share the same burrow system, including a communal nest site, and exhibit a variety of cooperative behaviours, such as communal play, construction and maintenance of burrows, anti-predatory surveillance and alarm calls emission (Ebensperger & Bozinovic, 2000; Ebensperger et al. 2004, 2006; Jesseau et al. 2009). In contrast, *O. lunatus* individuals do not share burrows nor do they form communal groups or engage in cooperative behaviours. However, males and females exhibit a partial overlap in their household domains, reflecting that social bonds, up to a certain degree, also take place in this species (Sobrero et al. 2014). Such contrasting social behaviours very likely imply differences in the frequency and diversity of the phomonal interactions that take place during the lifespan of individuals of each species. In *O. degus*, the conformation of the social ensemble likely involves a high frequency and a variety of interactions in which phomonal cues, along with olfactory cues, are rehearsed. In *O. lunatus*, in which social interactions seem to be less relevant, a comparatively low frequency of phomonal interactions can be expected.

### Can AOB plasticity be driven by social behaviour?

In this context, it is relevant to note that the neural components of the vertebrate olfactory system exhibit a remarkable activity-dependent neuronal plasticity. More specifically, it has been repeatedly shown that glomerular maturation, size and number, and even the overall size of the MOB, are dependent on olfactory activity at early and advanced stages of ontogeny, in animals as distant as fishes, rodents and humans. (Sullivan et al. 1989; Zou et al. 2004; Li et al. 2005; Kerr & Belluscio, 2006; Valle-Leija et al. 2012; Cheetham et al. 2016; Negoias et al. 2017; Scheib et al. 2019) In the case of the VNS, recent studies in mice reported that the pool (in terms of different chemosensitive types) of VN neurons is subject to considerable changes in composition after prolonged exposition to different combinations of semiochemicals (Xu et al. 2016). In mice, the AOB glomeruli underwent an increment in the size of the VNN axonal arborization and a delay in its coalescence after prolonged

exposition to pheromones during early postnatal development (Hovis et al. 2012).

There is also evidence suggesting that social activity could be a driving factor of the plasticity of the AOB components. In mice, male–male social interactions led to changes in the excitatory/inhibitory synaptic balance between mitral and granular AOB cells (Cansler et al. 2017). In the solitary tree shrews, *Tupaia glis*, the total AOB volume becomes significantly smaller in individuals raised in captivity than in wild animals, likely as a consequence of the disruption of normal social interactions during captivity (Frahm et al. 1984). In hamsters, the size of synaptic contact zones and the somal area of M/T cells in the AOB become larger in animals raised in social conditions compared with those raised in isolation (Matsuoka et al. 1994, 1998). Our own preliminary observations indicated that degus raised in captivity features at adulthood a less marked bias between AOB subdomains, especially at the GL (data not shown).

In summary, our results, together with the previous evidence, lead us to conjecture that: (i) in octodontid rodents, the orchestration of social behaviours involves active volatile pheromonal signalling; and (ii) the frequency and relevance of the social interactions enacted in the life mode of these species may lead the AOB to reach different structural outcomes in different species, both in ontogeny and phylogeny, in what appears to be a ‘Baldwinian’ case of canalization of phenotypic plasticity (Baldwin, 1896; Pigliucci et al. 2006). It is worth noting that this latter process seems to act specifically over certain AOB components, namely the GL, leaving others, the M/T cells, unaffected.

Altogether, the evidence suggests that the structure of the VNS system can be shaped, in a very specific and direct manner, by the behavioural demands entailed in the lifestyle of different species. The mechanisms underlying this process deserve to be studied more. In particular, the comparative study of the ontogeny of the VNS in different species may serve to validate or discard these conjectures.

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## Author contributions

PFA conceived and performed the experiments, analysed the results and co-wrote the manuscript. SED performed the experiments and analysed the results. RS provided the materials and analysed the results. JM conceived the experiments, supervised the project, and wrote the manuscript.

## Data availability statement

The data and images that support the findings of this study are available from the corresponding authors upon reasonable request.

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