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Immunohistological study of the unexplored vomeronasal organ of an endangered mammal, the dama gazelle (*Nanger dama*)

Mateo V. Torres¹  | Irene Ortiz-Leal¹  | Andrea Ferreiro² | José Luis Rois² | Pablo Sanchez-Quintero¹ 

¹Department of Anatomy, Animal Production and Clinical Veterinary Sciences, Faculty of Veterinary, University of Santiago de Compostela, Lugo, Spain

²Marcelle Nature Park, Outeiro de Rei, Lugo, Spain

Correspondence

Pablo Sanchez-Quintero, Department of Anatomy, Animal Production, and Clinical Veterinary Sciences, Faculty of Veterinary, University of Santiago de Compostela, Av Carballo Calero s/n, 27002 Lugo, Spain.
Email: pablo.sanchez@usc.es

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Abstract

Dama gazelle is a threatened and rarely studied species found primarily in northern Africa. Human pressure has depleted the dama gazelle population from tens of thousands to a few hundred individuals. Since 1970, a founder population consisting of the last 17 surviving individuals in Western Sahara has been maintained in captivity, reproducing naturally. In preparation for the future implementation of assisted reproductive technology, certain aspects of dama gazelle reproductive biology have been established. However, the role played by semiochemical-mediated communications in the sexual behavior of dama gazelle remains unknown due partially to a lack of a neuroanatomical or morphofunctional characterization of the dama gazelle vomeronasal organ (VNO), which is the sensory organ responsible for pheromone processing. The present study characterized the dama gazelle VNO, which appears fully equipped to perform neurosensory functions, contributing to current understanding of interspecies VNO variability among ruminants. By employing histological, lectin-histochemical, and immunohistochemical techniques, we conducted a detailed morphofunctional evaluation of the dama gazelle VNO along its entire longitudinal axis. Our findings of significant structural and neurochemical transformation along the entire VNO suggest that future studies of the VNO should take a similar approach. The present study contributes to current understanding of dama gazelle VNO, providing a basis for future studies of semiochemical-mediated communications and reproductive management in this species.

Abbreviations: Aa, arteries; AB, alcian blue; AOB, accessory olfactory bulb; BP, buccal papillae; BSA, bovine serum albumin; CB, calbindin; CR, calretinin; CYK8, cytokeratin 8; DAB, diaminobenzidine; DP, dental pad; EN, neuron-specific enolase; HE, hematoxylin-eosin; HRP, horseradish peroxidase; IB, incisive bone; ID, incisive duct; IP, incisive papilla; LEA, *Lycopersicon esculentum* agglutinin; Lp, lamina propria; MCV, mucomicrovillar complex; NCD, caudal nasal nerve; NVN, vomeronasal nerves; OMP, olfactory marker protein; PAS, periodic acid Schiff; PGP 9.5, protein gene product 9.5; PR, palatal rugae; PV, parvalbumin; RE, respiratory epithelium; RM, respiratory mucosa; SBA, soybean agglutinin; SC, septal cartilage; SE, sensory epithelium; SG, secretagogin; STA, *Solanum tuberosum* agglutinin; UEA, *Ulex europaeus* agglutinin; V, vomer bone; V1R, vomeronasal receptor 1; V2R, vomeronasal receptor 2; VC, vomeronasal cartilage; VD, vomeronasal duct; VG, vomeronasal gland; VNO, vomeronasal organ; VNS, vomeronasal system; VR, ventral recess; Vv, veins; VVA, *Vicia villosa* agglutinin.

Mateo V. Torres and Irene Ortiz-Leal are joint first authors.

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Research Highlights

- This exhaustive immunohistological study of the vomeronasal organ (VNO) of the dama gazelle provides the first evidence of notable differences in the expression of neuronal markers along the rostrocaudal axis of the VNO.
- This provides a morphological basis for the implementation of pheromones in captive populations of dama gazelle.

KEYWORDS

chemocommunication, G-proteins, immunohistochemistry, lectins, vomeronasal

1 | INTRODUCTION

Dama gazelle (*Nanger dama*), found primarily in northern Africa, is one of the most threatened and rarely studied gazelle species (Abáigar et al., 2019). Unique physiological, ecological, and behavioral adaptations allow dama gazelle, a member of the Sahelo-Saharan ungulate family, to thrive in the arid and semi-arid environments of north Africa (Durant et al., 2014). Three subspecies of dama gazelle can be distinguished according to their geographic distribution and coat color: addra gazelle (*Nanger dama ruficollis* Smith, 1827), mhorh gazelle (*Nanger dama mhorh* Bennett, 1833), and dama gazelle (*Nanger dama dama* Pallas, 1766).

Human pressures, including habitat degradation associated with increased grazing by domestic livestock (Devillers et al., 2005) and intense hunting activity (Cloudsley-Thompson, 1992; Grottenberger & Newby, 1986), have depleted the dama gazelle population. Currently, dama gazelle is considered a critically endangered species and is on the red list of the International Union for Conservation of Nature. Since 1970, a founder population of the last 17 surviving individuals from Western Sahara has been maintained in captivity (Alados et al., 1988), where this species has reproduced naturally (Holt et al., 1996). The remaining wild populations are very small and extremely fragmented (Lamarque et al., 2007), and the prospects of dama gazelle are not expected to improve in the future. The quality of its habitat is expected to continue deteriorating, and the hunting activity that has severely undermined its population remains uncurbed (Bronson, 2009). Additionally, frequent fights between males have been reported in this species when populations are reintroduced into the natural habitat (Cassinello & Pieters, 2000). In the specific case of the mhorh subspecies, which is the subject of this study, it has been considered extinct in the wild since 1968. In Morocco and Senegal, there are small populations in semi-freedom conditions, from reintroductions that were made from the captive population, which comes from four founder specimens, one male and four females. At present, the population of this subspecies exceeds 400 individuals, distributed in different zoos in Europe, the United States, and the Arabian Peninsula.

Maintaining sustainable populations of endangered wildlife species is challenging, and dama gazelle maintenance is even more difficult due to the issue described above. Because reproduction is

fundamental to species survival, understanding the reproductive mechanisms of endangered species is a high priority for conservation efforts. Enormous differences in the specific reproductive mechanisms exist across species, even among species that belong to the same phylogenetic clade (i.e., family; Wildt et al., 2001). Although certain aspects of dama gazelle reproductive biology have been established in preparation for the future implementation of assisted reproductive technology (Arregui et al., 2021; Pickard et al., 2001, 2003; Wojtusik et al., 2017), to our knowledge, no information exists regarding the role played by semiochemical-mediated communications in the sexual behavior and reproductive physiology of dama gazelle. Sexual attraction; mother–young interactions; estrus indication, induction and synchronization; puberty acceleration; postpartum anestrus; hormonal stimulation; and penile erections are all influenced by pheromones in ruminants (Archunan et al., 2014). Pheromones are also involved in stress regulation mechanisms in mammals. Therefore, pheromones may be able to reduce male aggressiveness when species are reintroduced to native habitats (Archer et al., 2022).

There is plenty of scope for the application of pheromones to the reproduction and management of dama gazelle populations; however, effective pheromone application first requires the neuroanatomical and morphofunctional characterization of the dama gazelle vomeronasal organ (VNO), the sensory organ responsible for processing semiochemicals as pheromones (Kunkhyen et al., 2017; Powers & Winans, 1975), kairomones (Fortes-Marco et al., 2013; Isogai et al., 2011), and major histocompatibility complex molecules (Leinders-Zufall et al., 2000, 2014). The VNO consists of two tubular, blind-ending, elongated structures located at the base of the nasal septum (Poran, 1998). The vomeronasal duct (VD) is located inside the VNO and establishes a direct communication with the nasopalatine duct and through it with the external environment (Takami, 2002). The VD is lined by vomeronasal epithelium, composed of pheromone-sensing receptor neurons (Eisthen, 1992; Zufall et al., 2002). Pheromones enter through the nasal or oral cavities, dissolve in mucus, and enter the VNO, where they activate the vomeronasal neuroepithelium (Sankarganesh et al., 2022). Transduction occurs in the neuroreceptors of the vomeronasal epithelium, and the pheromone signal is conveyed by vomeronasal nerves (NVN) to the accessory olfactory bulb (AOB), which serves as the initial central

nervous system center for integrating pheromonal information (Halpern, 1987; Mori, 1993; Ortiz-Leal et al., 2022). The morphological and immunohistochemical study of the dama gazelle VNO will answer questions regarding the existence of functional communications between the VNO and the environment and whether the VNO is fully equipped for neurosensory functions, which requires a complete external envelope, a differentiated neuroepithelium, and a parenchyma with the necessary glandular, vascular, and nervous elements (Salazar & Quinteiro, 1998; Salazar, Sánchez Quinteiro, et al., 1997).

Dama gazelle belong to the order Artiodactyla, suborder Ruminantia, and infraorder Pecora. Within Pecora, dama gazelle belong to Bovidae family, which has the largest number of species. Other families in this group are the Antilocapridae (pronghorns), Giraffidae, Moschidae (musk deer), and Cervidae. Among these families, anatomical and histological studies have been conducted in the giraffe VNO (Hart & Hart, 2023; Kondoh, Nakamura, et al., 2017), and a specific study of glycoconjugates was conducted in the musk deer VNO (Kondoh et al., 2020). The Cervidae family has been studied more extensively, and reindeer (*Rangifer tarandus*) (Bertmar, 1981), elk (*Alces alces*) (Vedin et al., 2010), sika deer (*Cervus nippon*) (Matsubara et al., 2019), and Korean roe deer (*Capreolus pygargus*) (Park et al., 2014; Shin et al., 2017) have all been found to feature functional VNOs.

Although more extensive studies have been conducted in the bovid VNO, these studies have focused on domestic species, such as cow (*Bos taurus coreanae*) (Adams, 1986; Jacobs et al., 1981; Jang et al., 2021; Salazar et al., 2008; Taniguchi & Mikami, 1985), goat (*Capra aegagrus hircus*) (Ladewig & Hart, 1980; Park et al., 2013; Yang et al., 2021), and sheep (*Ovis orientalis aries*) (Barrios et al., 2014; Ibrahim, 2018; Ibrahim et al., 2013; Kratzing, 1971; Salazar et al., 1998, 2000; Salazar et al., 2007; Salazar, Lombardero, Alemán, & Sánchez Quinteiro, 2003), with few studies examining the VNOs of wild bovids. However, substantial differences between wild and domestic bovids were discovered in the investigation of alcelaphine antelopes. Three of the antelope species studied, *Damaliscus iunatus*, *Alcelaphus buscelaphus*, and *Connochaetes taurinus*, either lacked the flehmen response or had no direct communication between the oral cavity and the VNO via the incisive duct (ID), despite a general histology study showing a developed VNO (Hart et al., 1988). These striking morphofunctional observations corresponded to a recent study of ancV1R gene expression (Zhang & Nikaido, 2020), which is considered to be a reliable diagnostic indicator of VNO function. AncV1R expression was inactivated in seals, sea otter, giant otter, fossa, owl monkey, and alcelaphine antelopes. Beyond antelopes, information regarding VNO function in wild ruminants is limited to the morphofunctional study of the water buffalo (*Bubalus bubalis*) VNO (Emam et al., 2016), the study of glandular secretions by *Gazella subgutturosa* (Abood & Hussein, 2018), and a computerized tomographic study of the saiga antelope (*Saiga tatarica*) nasal cavity, which revealed the presence of a VNO (Clifford & Witmer, 2004). These observations reveal wide morphological diversity in the family Bovidae, indicating that the extraction of information regarding the anatomy and histology of the

vomeroneasal system (VNS) across species can be challenging and risky (Salazar et al., 2016).

The present study was designed to define the morphological and immunohistochemical characteristics of the dama gazelle VNO. This study serves as the first necessary step for implementing reproductive species management using chemical communication and will contribute to filling existing gaps in knowledge regarding VNO function within the family Bovidae. We conducted a comprehensive morphofunctional study of the dama gazelle VNO using histological, histochemical, and immunohistochemical techniques. We performed an exhaustive study along the entire longitudinal axis of the VNO, evaluating differences in the microscopic structures and immunohistochemical labeling patterns along this axis. The wide range of differences that we found suggests that future studies of the VNO should take a similar approach and highlights the important structural and neurochemical transformations exhibited across the entire VNO.

2 | MATERIALS AND METHODS

2.1 | Samples

To carry out our study, we had a healthy 11-month-old male dama gazelle, subspecies mhorh (*Nanger dama mhorh*), which was donated by the Natural Park “Marcelle Natureza” (Outeiro de Rei, Spain) due to its death by accidental causes, specifically a neck fracture when the animal was engaged in social play. The animal was part of a breeding program to create reproductive groups so that the offspring can be transferred to progressive and controlled pre-release facilities in their native habitat. In order to be successful in the repopulation, the behavior and living conditions in the park are as similar as possible to those in their natural habitat.

Less than 12 h after the animal's death, the head was separated from the carcass and the skin, tongue, jaws, teeth, nose, and eyes were removed. A cross-section was made with the aid of a rotary saw to separate the snout, where the VNOs are located, from the rest of the skull. In the snout sample, the bony sides of the nasal cavity and the nasal turbinates were removed. This allowed to identify the location of the VNOs on both sides of the basal part of the nasal septum. Subsequently, a transverse cut was made to divide the nasal septum centrally to facilitate the entry of the fixative into the VDs. The resulting samples were immersed in freshly prepared Bouin's fluid and, after 24 h, transferred to 70% ethanol. The following day, the VNO was dissected, and multiple cross-sections were made equidistant along their entire length in order to analyze the characteristics of each area. This procedure was carried out under a Zeiss OPMI 1 surgical microscope. The bone tissue surrounding the VNO ventrally and medially was dissected from all samples, with the exception of those at the most rostral levels. These rostral pieces were decalcified for a week to microscopically study the topographic relationship of the VNO with the ID. The decalcifying agent used was Shandon TBD-1 Decalcifier (Thermo,

Pittsburgh, PA), and it was applied while stirring continuously at room temperature. All samples were marked and embedded in paraffin, thus obtaining the corresponding blocks. They were cut with a Leica Reichert Jung microtome with a thickness of 5–7 μm , depending on the tissue to be processed.

2.2 | Histological staining

In order to be able to visualize the different components of the analyzed tissue, we used hematoxylin–eosin (HE) as general staining. Additionally, periodic acid–Schiff (PAS) and alcian blue (AB) stains were used to identify neutral and acidic mucopolysaccharides, respectively.

2.3 | Lectin histochemical staining

Lectins, naturally occurring carbohydrate-binding molecules, can be used to detect glycoconjugates associated with certain cell populations in the tissues tested. For this reason, we have used the following five lectins in our research:

1. UEA (Vector-L1060), which comes from gorse, *Ulex europaeus*, and marks the L-fucose pathway (Kondoh et al., 2018).
2. LEA (Vector-B1175), which comes from tomato, *Lycopersicon esculentum*, and recognizes N-acetyl-glucosamine (Salazar, Lombardero, Cifuentes, et al., 2003).
3. VVA, which is isolated from fodder vetch, *Vicia villosa*, binds specifically to N-acetylgalactosamine structures (Tomiya et al., 2018).
4. SBA (Vector-B1235), which comes from fodder soybean, *Glycine max*, binds preferentially to oligosaccharide structures with N-acetylgalactosamine and to galactose residues (Park et al., 2012).
5. STA (Vector-B1165), which is isolated from potato, *Solanum tuberosum*, binds oligomers of N-acetylglucosamine and some oligosaccharides containing N-acetylglucosamine and N-acetylmuramic acid (Chun et al., 2023).

The UEA lectin was employed unmodified and identified with an anti-UEA antibody, whereas the other four lectins, VVA, STL, LEA, and SBA, were biotinylated. The following procedures were followed.

2.3.1 | Biotinylated lectins protocol

(i) The tissular endogenous peroxidase activity was blocked to avoid interference with the final developing step by incubating in a 3% H_2O_2 solution for 10 min. (ii) Sections were incubated for 30 min at room temperature in 2% bovine serum albumin (BSA), which prevents nonspecific binding. (iii) The slides were incubated in a humidified chamber overnight at 4°C in the biotinylated lectin diluted in 0.5% BSA at 1:200. The following day, samples were incubated (iv) for 90 min at room temperature in Vectastain ABC complex (Vector

Laboratories, Burlingame, CA, USA). Finally, (v) the sections were developed by incubating the sections in a solution of 0.05% diaminobenzidine (DAB) and 0.003% H_2O_2 for 5–10 min. The reaction was monitored with the microscope.

2.3.2 | UEA lectin protocol

The protocol for UEA began with the same first two steps. Subsequently, (iii) incubation with UEA lectin (1:60 in 0.5% BSA) was carried out for 1 h at room temperature; (iv) 3 washes of 5 min in 0.1 M phosphate buffer (PB, pH 7.2). (v) Afterwards the sections were incubated in a humidified chamber (overnight 4°C) in a peroxidase conjugated immunoglobulin against UEA (DAKO, P289; 1:50). Finally, the samples were developed (vi) by incubation in the same DAB solution as that employed in the biotinylated-lectins protocol. Controls were performed for both protocols, both without the addition of lectins and with the preabsorption of lectins, by using an excess amount of the corresponding sugar.

2.4 | Immunohistochemical staining

Using immunohistochemistry, the dama gazelle VNO was examined in-depth. The anti-G α i2 and anti-G α o antibodies are particularly useful because they label the transduction cascade for V1R and V2R vomeronasal receptors, respectively (Halpern et al., 1995). Antibodies were also used to detect olfactory marker protein (OMP), which is expressed in mature neurons in both olfactory subsystems (Rodewald et al., 2016). The calcium-binding proteins calbindin (CB), calretinin (CR), parvalbumin (PV), and secretagogin (SG) participate in the regulation of cytosolic free calcium ion concentrations in neurons. The distribution of proteins has previously been recognized as a useful neuronal marker for identifying specific brain regions and discrete neuronal subpopulations (Coppola & Disney, 2018; de Góis Morais et al., 2021; Kishimoto et al., 1993; Pérez-Revuelta et al., 2020). Cytokeratin 8 forms part of the intermediate filament cytoskeleton of epithelial cells. Accordingly, it is an effective marker for olfactory and vomeronasal sustentacular cells (Khan et al., 2021). Enolases are glycolytic enzymes that may be involved in cellular differentiation. γ enolase, specific of the nervous tissue, is a good marker for mature olfactory and vomeronasal neuroreceptor cells (Takahashi et al., 1984). PGP 9.5, also known as ubiquitin C-terminal hydrolase, is highly specific to neurons and to cells of the diffuse neuroendocrine system. In the VNO it identifies neuroreceptor cells but not supporting or basal cells (Taniguchi et al., 1993).

The protocol of the immunohistochemical labeling is explained in detail in Torres et al., 2021. Briefly, the initial step consisted of inhibiting endogenous peroxidase activity with 3% H_2O_2 . Then (ii) nonspecific binding was blocked for 30 min using 2.5% normal horse serum from the ImmPRESS Anti-Mouse IgG/Anti-rabbit IgG Reagent Kit (Vector Laboratories, CA, USA). (iii) Subsequently, the primary antibody was added to the corresponding dilution and incubated

overnight (4°C). The following day, (iv) the samples were incubated at room temperature for 20 minutes with the corresponding ImmPRESS VR Polymer HRP anti-rabbit IgG reagent. After (v) rinsing in Tris buffer (0.2 M pH 7.61) for 10 min, (vi) the samples were developed using DAB as a chromogen in the same manner as for lectins.

All immunohistochemical protocols were run with the appropriate controls. In the absence of a positive control from dama gazelle, we reproduced the whole histochemical procedure with mouse tissues known to express the respective antigens. Samples for which the primary antibody was replaced by antibody diluent were used as negative controls.

2.5 | Antibody characterization and specificity

Table 1 gives information on all antibodies, including their suppliers, dilutions, immunogen targets, and Research Resource Identifiers

(RRID). In each instance, the immunostaining patterns produced by these antibodies in dama gazelles were comparable to those previously observed in a variety of mammalian species. References pertinent to each antibody are also listed in Table 1.

2.6 | Acquisition of images

The images that make up the different illustrations were taken with a Olympus SC180 digital camera coupled to a Olympus BX50 microscope. Adobe Photoshop CS4 (Adobe Systems, San Jose, CA, USA) was used to fine-tune brightness and contrast, balance levels, and crop or resize images for presentation. Photomicrographs of the entire VNO were taken as a mosaic of several photographs made with the 10× objective and merged with image-stitching software (PTGui Pro).

TABLE 1 Detailed information on the antibodies used in this study: Species of elaboration, dilution, catalog number, manufacturer, target immunogens, relevant reference for each antibody, Research Resource Identifiers (RRID) codes, and secondary antibody employed.

Antibody	1st Ab species/dilution	1st Ab catalog number /manufacturer	Immunogen	Reference	RRID	2nd Ab species/dilution, catalog number
Anti-Gαo	Rabbit 1:200	MBL-551	Bovine GTP Binding Protein Gαo subunit	Prince et al. (2009)	AB_591430	ImmPRESS VR HRP Anti-rabbit IgG Reagent MP-6401-15
Anti-Gαi2	Rabbit 1:200	Santa Cruz Biotechnology sc-7276	Peptide mapping within a highly divergent domain of Gαi2 of rat origin	de la Rosa-Prieto et al. (2010)	AB_2111472	ImmPRESS VR HRP Anti-rabbit IgG Reagent MP-6401-15
Anti-CB	Rabbit 1:6000	Swant CB38	Rat recombinant calbindin D-28k	Hermanowicz-Sobieraj et al. (2018)	AB_10000340	ImmPRESS VR HRP Anti-rabbit IgG Reagent MP-6401-15
Anti-CR	Rabbit 1:6000	Swant 7697	Recombinant human calretinin containing a 6-his tag at the N-terminus	Adrio et al. (2011)	AB_2619710	ImmPRESS VR HRP Anti-rabbit IgG Reagent MP-6401-15
Anti-SG	Rabbit 1:1000	Gift from Ludwig Wagner (University of Vienna, Austria)	Recombinant human secretagogin	Alpár et al. (2012)	AB_1079874	ImmPRESS VR HRP Anti-rabbit IgG Reagent MP-6401-15
Anti-PV	Rabbit 1:400	Proteintech 26521-1-AP	Parvalbumin peptide	Hsu et al. (2018)	AB_2880541	ImmPRESS VR HRP Anti-rabbit IgG Reagent MP-6401-15
Anti-OMP	Mouse 1:200	Santa Cruz Biotechnology sc-365818	Amino acids 1–163 human OMP (B-6)	Goncalves and Goldstein (2020)	AB_10842164	ImmPRESS VR HRP Anti-mouse IgG Reagent MP-6402-15
Anti-CYK88	Rabbit 1:200	Proteintech 17514-1-AP	CYK8 peptide	Hou et al. (2015)	AB_2134726	ImmPRESS VR HRP Anti-rabbit IgG Reagent MP-6401-15
Anti-PGP 9.5	Rabbit 1:200	Proteintech 14730-1-AP	UCHL1/PGP9.5 fusion protein Ag6490	Ren et al. (2021)	AB_2210497	ImmPRESS VR HRP Anti-rabbit IgG Reagent MP-6401-15
Anti-EN	Mouse 1:100	Santa Cruz Biotechnology sc-21738	Amino acids 416–433 human γ-enolase	Matayoshi and Otaki (2011)	AB_627513	ImmPRESS VR HRP Anti-mouse IgG Reagent MP-6402-15

Abbreviations: CB, calbindin; CR, calretinin; CYK8, cytokeratin; NE, neuron-specific enolase; HRP, horseradish peroxidase; OMP, olfactory marker protein; PGP 9.5, protein gene product 9.5; PV, parvalbumin; SG, secretagogin.

3 | RESULTS

3.1 | Macroscopic study

The VNO of dama gazelle consists of two tubular, elongated structures located at the base of the nasal septum, extending from the level of the incisive papilla to a level corresponding approximately to the rostral border of the lacrimal bone. To account for any structural or neurochemical alterations that might occur along the longitudinal axis of such a lengthy structure, we conducted a serial histological study, resulting in the definition of 15 transverse levels. These levels are depicted in Figure 1a, which shows a lateral view of the head of a dama gazelle.

In artiodactyls, communication between the VD and the external environment is indirect, requiring the opening of the VD into the ID (also known as the nasopalatine duct). The ID typically opens into the oral cavity via the incisive papilla and extends into the nasal cavity through an aperture in the ventral recess. However, the incisive papilla has not been found in some species of the family Bovidae. Therefore, we assessed whether an incisive papilla was present in dama gazelle to determine whether functional communication could occur between the VD and the ID. Our examination of the dama gazelle hard palate (Figure 1b) revealed the opening of the ID in a well-defined incisive papilla.

The decalcification of the two rostral blocks (L1–L2) allowed for the examination of topographic relationships between the VD and ID,

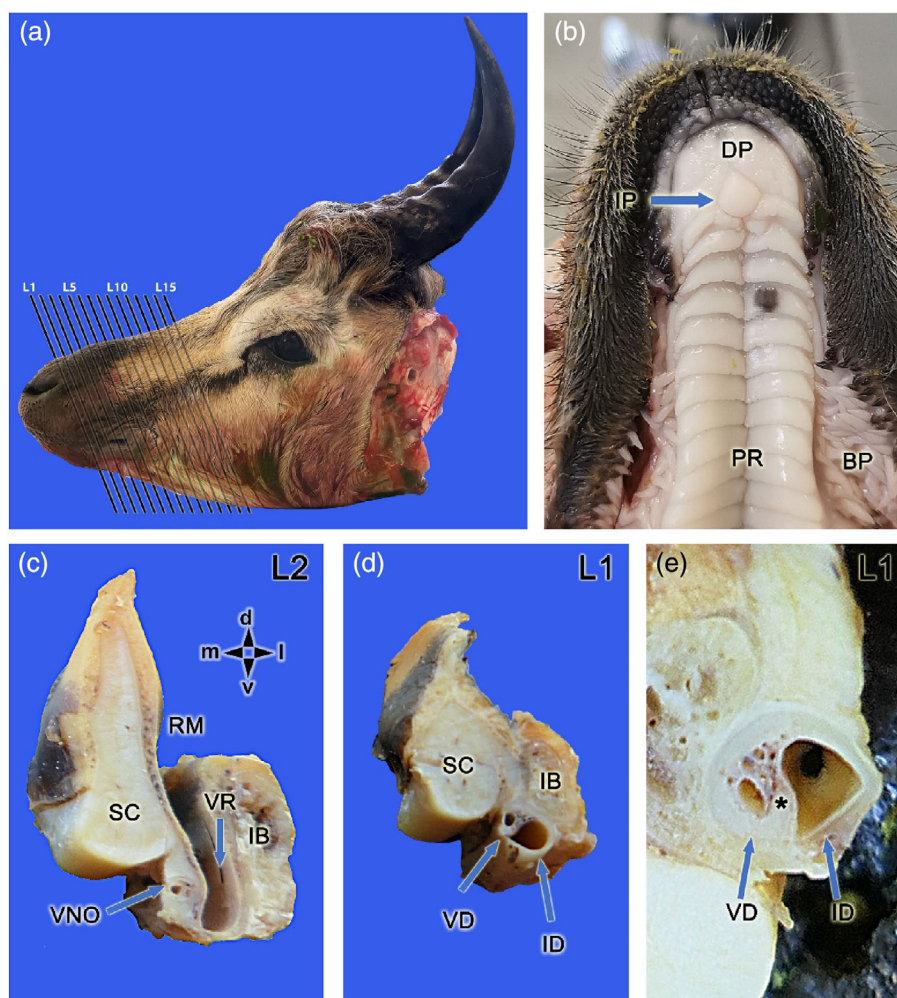


FIGURE 1 Dissection of the dama gazelle vomeronasal organ (VNO). (a) The lateral view of the head displaying the 15 transverse levels that correspond to the histological series depicted in Figure 2. (b) Ventral view of the palate, showing the opening of the incisive duct (ID) in the incisive papilla. (IP). (c–d) Sections of the decalcified nasal cavity at the first two levels, as shown in (a), to study the communication between the VNO and the external environment. The most rostral level (L1) is shown in (d). The wide and lateral ID runs parallel to the smaller vomeronasal duct (VD). Both passages are encased in a common cartilaginous envelope. However, the communication between the VD and the ID has not been established. The topographical relationship with the midline septal cartilage (SC) and the lateral incisive bone (IB) can also be seen. The next caudal level (L2) is shown in (c). The cartilaginous envelope of the VNO is thick, and its dorsal process contacts the SC. At this stage, the ID has opened to the ventral recess of the nasal cavity (VR). (e) Higher magnification of the vomeronasal L1 reveals the unique cartilaginous envelope of both the VNO and the ID. The asterisk indicates the formation of erectile tissue in the VNO, which is made up of numerous blood vessels positioned between the VD and the ID. BP, buccal papillae; DP, dental pad; PR, palatal rugae; RM, respiratory mucosa. Orientation: d, dorsal; l, lateral; m, medial; v, ventral.

in addition to the septal cartilage of the nasal cavity and the incisive bone (Figure 1c,d). The two ducts run parallel to each other, occupying a region that corresponds to the palatal fissure. The ID was considerably larger in caliber than the VD. Despite being separated by a broad band of erectile tissue, both passages are encased in a common cartilaginous envelope (Figure 1e). At the L2 level, the ID opens to the ventral recess of the nasal cavity, whereas the VD remains encased in a large cartilaginous envelope (Figure 1c). The obtained sections did not permit the macroscopic identification of communication between ducts; hence, this issue was not able to be clarified until the serial histologic examination of the VNO was performed.

3.2 | Microscopic study

3.2.1 | Histological study

The serial histological study of the VNO comprised 15 levels (Figure 2), allowing for the discrimination of changes in the shape and dimensions of its three primary components: the VD, the vomeronasal cartilage encapsulating the VD, and the parenchyma between the VD

and the cartilage. The use of the term “parenchyma” does not correspond to the classic sense of a parenchymal viscera. The VNO, originating from the ectoderm, is not a viscera. Rather, it corresponds to the definition of the predominant functional component of an organ.

The VNO cartilage consists of a single piece that varies in shape across levels. The cartilage generally consists of a circular section complemented by a prominent dorsal projection that directly contacts the septal cartilage along the entire length of the VNO. The rostral-most part of the VNO cartilage has the clearest circular section, but from L4 to L9, the capsule cavity is slightly flattened dorso-ventrally. In the caudal part of the VNO, the cartilage undergoes striking modifications. In particular, at levels L10–L12, a visible increase in the cavity size is observed, coinciding with the end of the VD. After L12, the cartilage acquires a triangular section and undergoes a rapid reduction in its lumen, such that the extent of the parenchyma progressively decreases. At the most caudal level considered (L15), the lumen contains only the main branches of the VNO innervation and irrigation. The remarkable development of the vascular component of the VNO, which occupies the lateral regions of the majority of sections, can be observed in the parenchyma, composed primarily of massive venous sinuses.

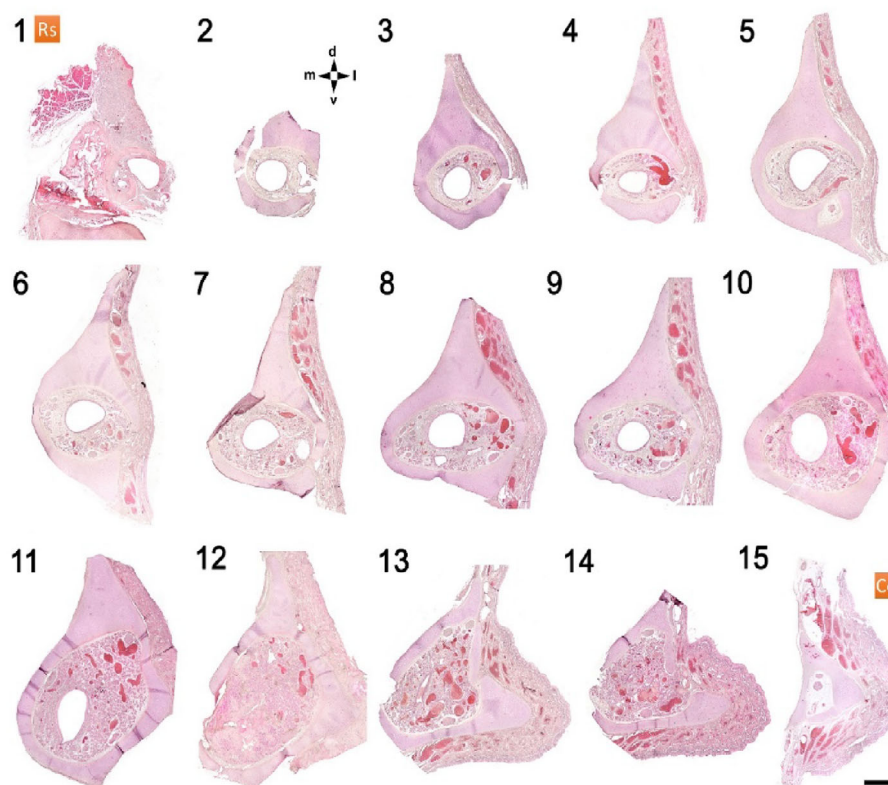


FIGURE 2 Serial histological study, stained with hematoxylin–eosin, enabled the definition of 15 transverse levels of the VNO from rostral (1) to caudal (15). Changes in the shape and dimension of the cartilage and vomeronasal duct are observed throughout the histological series. Variations in the main structural features of the VNO's parenchyma, particularly its vascular component, are noticeable. Level 1 represents the more rostral (Rs) section, depicting the confluence of both vomeronasal and incisive ducts within the palatal fissure. Levels 2 and 3 depict the rostral part of the VNO, while levels 5 and 6 correspond to the central part of the VNO where the two main epithelia lining the vomeronasal duct are already differentiated. Level 10 indicates the caudal part of the VNO where the vomeronasal duct acquires a larger caliber. Level 13 marks the caudal termination of the vomeronasal duct, which is no longer present in the next and last two levels. Directionality is indicated by the following: cd, caudal; d, dorsal; l, lateral; m, medial; rs, rostral; v, ventral. Scale bar: 500 μ m.

The serial histological study of the L1 block (Figure 3) demonstrated the existence of functional communication between the VNO and the ID (Figure 3a). At this level, the VD has a much narrower diameter than the ID, and both ducts increase their calibers caudally. No glands are observed in the surrounding parenchyma. Instead, vast venous sinuses were observed. Throughout the entire L1 level, both ducts are enclosed by a single cartilaginous capsule, the medial branch of which contacts the vomer bone (Figure 3c,e). Only in the most rostral sections is the cartilage surrounding the VD separated into two distinct parts (Figure 3a). The epithelial lining of both ducts changes remarkably throughout this rostral level. Rostrally, the VD is covered by a squamous simple epithelium (Figure 3a,c), but more caudally, the VD is covered by a pseudostratified epithelium comprising two cell layers (Figure 3e). Two cell types could be differentiated: large cuboidal cells with more superficial nuclei and elongated cells morphologically similar to neuroreceptors (Figure 3f). Rostrally, the lateral ID consists of a well-developed, stratified squamous epithelium (Figure 3b); however, at the level of communication between the VD and ID, squamous simple epithelium is observed (Figure 3d). At the most caudal sections of the L1 level, the epithelium transitions into a pseudostratified lining that is rich in superficial caliciform cells and resembles the respiratory mucosa (Figure 3g).

At both the L2 and L3 levels, the VD is circular in shape (Figure 4a,d). At the L2 level, the lateral VNO parenchyma contains large venous sinuses (Figure 4a), whereas the remaining parenchyma contains almost no glands or vessels, consisting primarily of connective tissue and a few small branches of the NVN (Figure 4a). The lateral epithelium of the VD shows basal cells for the first time, covered by a pseudostratified epithelium formed by two layers of rounded or oval cells with nuclei darkly stained by hematoxylin staining and individual superficial cells with translucent nuclei (Figure 4b). The medial epithelium shows similar characteristics to those observed at the L1 level, with polyhedral cells containing rounded nuclei in the apical position and a simple layer of basal cells (Figure 4c). At the L3 level the vomeronasal cartilage presents a sharp dorsal projection and encircles the entire VNO. Compared with the previous level, the parenchyma presents the great development of the glandular component, concentrated in the dorsolateral quadrant, together with the venous sinuses (Figure 4d). The lateral epithelium is similar to that observed in the previous level (Figure 4e), whereas changes in the organization of the medial vomeronasal epithelium are observed. The geometrically shaped cells from the previous level are replaced with cells containing elongated nuclei and cytoplasm and arranged in an incipient columnar manner, representing a transition to the typical vomeronasal receptor epithelium (Figure 4f).

Level L5 (Figure 5a–e) corresponds to the central VNO, where the vomeronasal cartilage presents a lateral opening and a ventral recess for the caudal nasal nerve (NCd) is observed (Figure 5a). At this level, the VD adopts a characteristic crescent shape, and this is the first level at which the medial epithelium shows the typical morphology of a full developed sensory epithelium (SE). The basal cells are not very conspicuous, and the neuroreceptor cell somas are large and rounded, containing distinct nuclei, eosinophilic cytoplasm, and

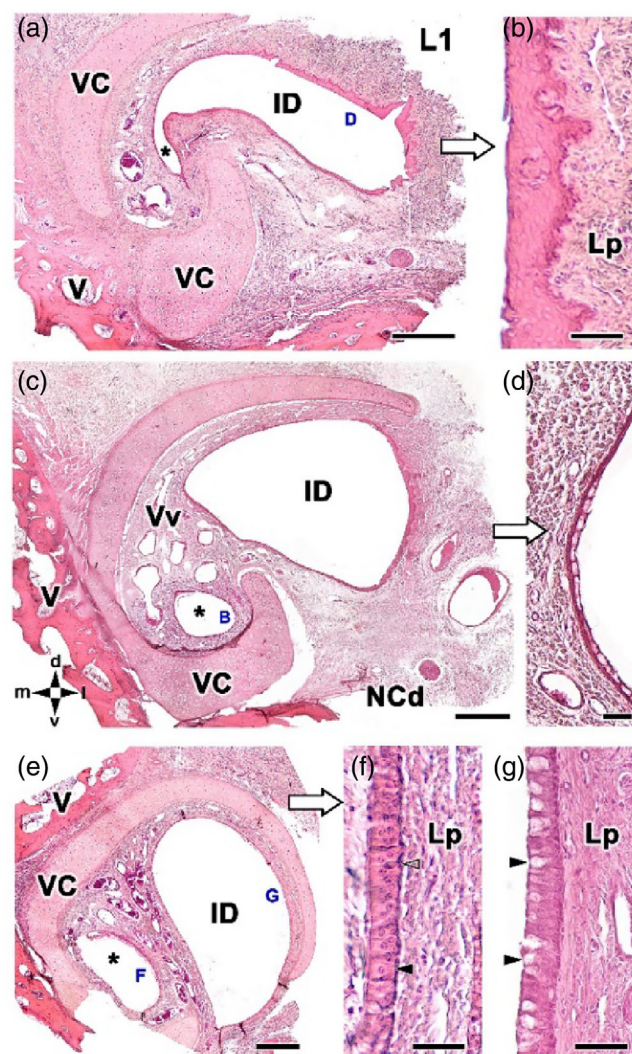


FIGURE 3 Histological study of the communication between the vomeronasal organ (VNO) and the incisive duct (ID). (a) A rostral section in block L1 demonstrates the existence of functional communication between the VNO and the ID (*). In this section, the vomeronasal cartilage (VC) is divided into two distinct parts. Both ducts are enclosed by a single cartilaginous capsule, with its medial branch contacting the vomer bone (V). There are no observed glands in the surrounding parenchyma, but large venous sinuses (Vv) can be seen. The caudal nasal nerve (NCd) runs ventrally to the ID. (b) At higher magnification, the ID is covered by a well-developed stratified squamous epithelium, mainly in the lateral part. (c) In a more caudal section than above, the VD is now complete, although it is noticeably smaller than the ID. (d) Upon closer examination of the VD, it can be observed that it is lined by a layer of simple squamous epithelium. (e) A more caudal section at the L1 level shows the enlargement of the VD. Additionally, the lining of both the vomeronasal and ID has undergone a change, as can be seen in higher magnification images (f) and (g). (f) The vomeronasal epithelium is pseudostratified and consists of two cell layers. Two cell types are differentiated: large cuboidal cells (black arrowhead) and elongated cells (open arrowhead) that appear to be neuroreceptors. (g) In contrast, the ID epithelium is a denser pseudostratified lining with superficial caliciform cells (arrowheads). Stain: hematoxylin–eosin. Lp, lamina propria. Orientation: d, dorsal; l, lateral; m, medial; v, ventral. Scale bars: a, c, e = 500 μ m, b, d, f, g = 50 μ m.

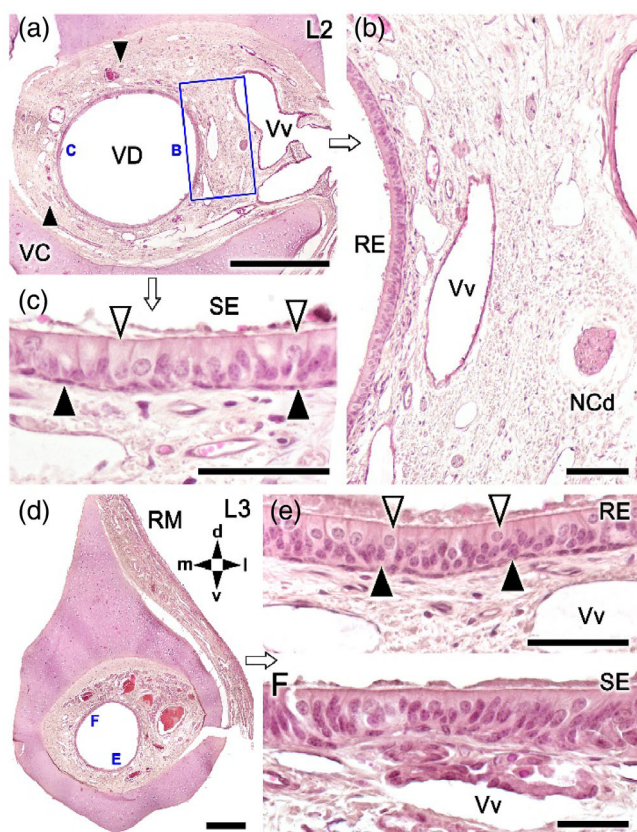


FIGURE 4 Histological study of the vomeronasal organ (VNO) at the rostral levels L2 (a–c) and L3 (d–f). (a) At the L2 level, the VD is circular. The lateral VNO parenchyma contains large venous sinuses that extend into the lateral opening of the vomeronasal cartilage. The rest of the parenchyma has minimal glands or vessels, primarily consisting of connective tissue and a few small branches of the NVN. (b) An enlarged view of the boxed area in (a), showing the RE at higher magnification. The lateral epithelium of the VD shows basal cells for the first time, covered by a pseudostratified epithelium formed by two layers of rounded or oval cells with dense nuclei, stained by hematoxylin staining. Additionally, individual superficial cells with translucent nuclei are present. (c) The medial epithelium shows characteristics similar to those observed at the L1 level. It presents polyhedral cells with rounded nuclei located in the apical position (open arrowhead) and a simple layer of basal cells (black arrowhead). (d) At level 3, the vomeronasal cartilage presents a distinct dorsal projection and encloses the entire VNO. Compared with the previous level, the parenchyma at this level shows significant development of the glandular component, which is concentrated in the dorsolateral quadrant along with the venous sinuses. (e) The lateral epithelium at this level is similar to the one observed in the previous level with superficial cuboidal cells (open arrowheads) and dark nucleated cells (black arrowheads) located in a basal position. (f) The geometrically shaped cells from the previous level are replaced with cells containing elongated nuclei and cytoplasm. These cells are arranged in an incipient columnar manner, representing a transition to the typical vomeronasal receptor epithelium. RE, respiratory epithelium; RM, respiratory mucosa; SE, sensory epithelium; VD, vomeronasal duct; Vv, veins; directionality is indicated by the following: d, dorsal; l, lateral; m, medial; v, ventral. Scale bars: a, d = 500 μ m, b = 100 μ m, c, e, f = 50 μ m.

processes that extend toward the luminal surface (Figure 5b). The sustentacular cells are strongly stained and densely packed close to the luminal surface (Figure 5b). In the respiratory epithelium (RE), a few polyhedral cells containing translucent cytoplasm can be observed in the apical zone. The medial parenchyma is devoid of glands but contains numerous NVN branches (Figure 5d), whereas the lateral region of the VD is connected to tubular glands that open in the dorsal commissure (Figure 5e).

At a more caudal level, L9 (Figure 5f–j), the lateral commissure in the VC is almost completely closed, and the VD has a reduced diameter with a circular shape. The parenchyma shows the increased development of vascular erectile tissue (Figure 5f). The NCd is located within the parenchyma, and numerous thick NVNs run medially. Although the VD lumen no longer displays a crescent shape, three-quarters of the epithelial lining display neuroreceptors (Figure 5g). The SE shows a clear distinction between neuroreceptor cells located in the basal region and the apical sustentacular cells, which contain dense nuclei (Figure 5i,j). The RE (Figure 5h) resembles that at the central level (L5; Figure 5c), although fewer superficial polyhedral cells can be observed at L9.

Figure 6 shows the main histological features of the caudal VNO. At level L10 (Figure 6a–d), the cartilaginous envelope is complete. Compared with level L9, the parenchyma in level 10 is remarkably enlarged by the presence of copious glandular tissue and large blood vessels. The SE and RE are clearly differentiated (Figure 6b,c), and similar to level L9, the SE in level 10 contains clearly defined neuroreceptor cells (Figure 6d). However, the SE in level 10 also contains a higher density of sustentacular cells than the SE in L9. Level L13 (Figure 6e–h) corresponds with the caudal extremity of the VD, and the medial SE and the lateral RE remain clearly differentiated (Figure 6f). In the SE, in addition to neuroreceptor and sustentacular cells, superficial globose cells can be observed (Figure 6g). The basal cells are less numerous at level L13 than in previous levels. The RE maintains its structure and does not contain basal cells (Figure 6h). At level L14 (Figure 6i,j), the parenchyma is composed of venous sinuses, abundant glandular tissue, and nerve bundles, and the VD is absent, but several tubular epithelial structures can be detected, which correspond to the earliest epithelial invaginations that, like gloved fingers, give rise to the VD (Figure 6j). These ducts are lined exclusively with RE. At the most caudal level studied, L15 (Figure 6k), the cartilaginous sheath is triangular in shape and contains branches of the NCd, veins, and arteries.

We also examined horizontal sections of the caudal VNO (Figure 7), revealing how the caudal extremity of the VD gives way to a large parenchymal region rich in glandular tissue and venous sinuses and flanked on both sides by cartilage (Figure 7a). Some vomeronasal glands (VGs) are observed in the ventral parenchyma, but venous sinuses predominate (Figure 7b). At a dorsal level, the NVN has a remarkable number of longitudinal branches (Figure 7c). The glandular tissue is serous in nature (Figure 7d).

A serial histological study was conducted using specific stains for glandular tissue. AB (Figure 8a–c) was used to identify acidic mucins,

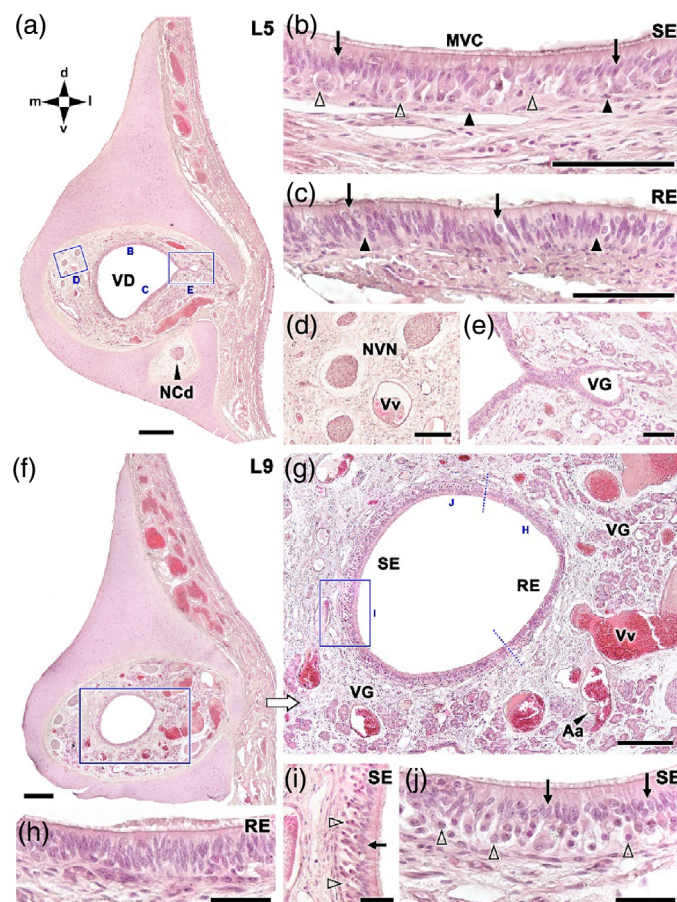


FIGURE 5 Histological features of the central (L5) and caudal (L9) parts of the dama gazelle vomeronasal organ (VNO). (a) Centrally, the most prominent features of the vomeronasal cartilage are its lateral opening and the presence of a ventral recess for the caudal nasal nerve (NCd). At this level, the VD adopts a distinct crescent shape. (b) The medial epithelium displays the typical morphology of a fully developed sensory epithelium. The presence of basal cells is not prominent (black arrowheads), while the soma of neuroreceptor cells (open arrowheads) are rounded with clear nuclei, eosinophilic cytoplasm, and processes extending toward the surface. They are large cells, in contrast to sustentacular cells, which are strongly stained (black arrows), and densely packed near the luminal surface. A mucomicrovillar complex (MVC) covers the luminal surface of the SE. (c) Polyhedral cells with translucent cytoplasm can be observed in the apical zone of the respiratory epithelium (RE) (arrows). Conversely, cells with dark-stained ellipsoidal nuclei can be found throughout the epithelium (arrowheads). (d) The medial parenchyma contains numerous NVN branches. (e) The glandular tissue is located laterally in the parenchyma, and it is less abundant than at the anterior level. Tubular glands (VG) release their secretions into channels that lead to the vomeronasal duct (VD). (f) At a more caudal level, L9, the VC is nearly closed. The VD features a smaller diameter and circular shape, indicating increased development of vascular erectile tissue. The NCd is situated within the parenchyma, and numerous thick NVNs run medially. (g) At higher magnification (inset in (f)), the transitions between the sensory and respiratory epithelia are marked with two dotted dashed lines. Approximately three-quarters of the duct's epithelial lining consists of neuroreceptor cells. (h) The RE resembles that of the central level (L5), although there are fewer polyhedral cells visible. The epithelium's surface has numerous cilia. (i, j) The SE shows a marked differentiation between neuroreceptor cells in the basal part (open arrowheads) and sustentacular cells with densely packed nuclei (black arrows). On the luminal surface of the SE, the presence of microvilli is striking. Aa, arteries; Vv, veins; directionality is indicated by the following: d, dorsal; l, lateral; m, medial; v, ventral. Scale bars: a, f = 250 μ m, b = 250 μ m, b–e = 100 μ m, h–j = 50 μ m.

whereas PAS stain (Figure 8d–l) was used to identify neutral mucins in the tissue. Both stains revealed epithelial glands on the surface of the respiratory mucosa of the nasal cavity, the vomeronasal cartilage (Figure 8a,d,l), and the mucomicrovillar complex that covers the luminal surface of the VD epithelium (Figure 8b,j). However, a clear distinction was observed between the types of secretions produced from glands within the VNO parenchyma. Beginning at level L3 (Figure 8f) and extending through the caudal VNO, generalized PAS staining of the glandular component was observed, whereas AB only

stained the central VNO (level L6) and was restricted to a small group of glands in the dorsal parenchyma (Figure 8b,c,l). At level L3, only a small group of ventral glands were stained with PAS, whereas by level L4, these VGs extended and occupied most of the dorsal parenchyma. Starting at level L6 and continuing through the caudal VNO (Figure 8d,e), VGs could be found throughout the parenchyma surrounding the VD, filling the spaces between venous sinuses. At the most caudal levels of the VD (Figure 8h,k), PAS-positive glands were highly expressed.

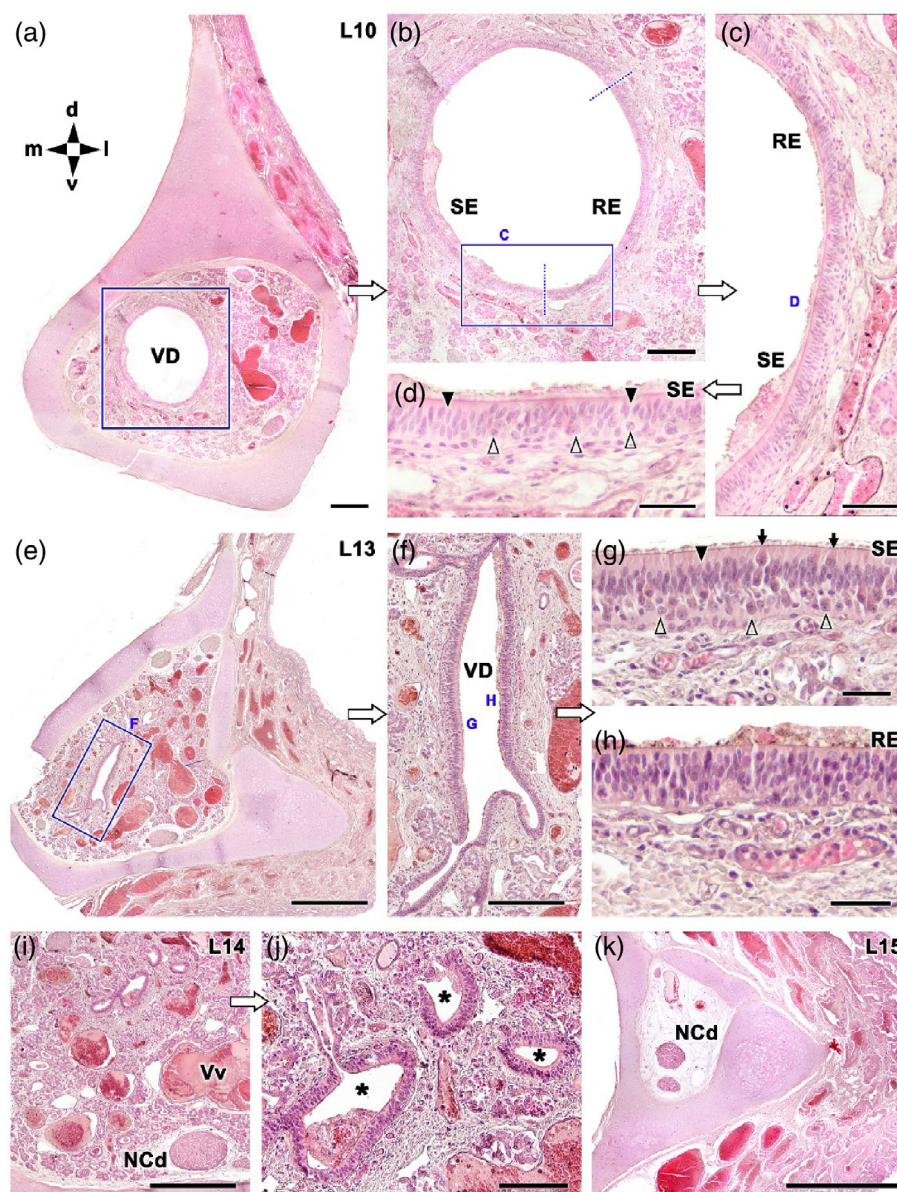


FIGURE 6 Histological features of the caudal levels of the dama gazelle vomeronasal organ (VNO). (a) At level L10, the cartilaginous envelope is complete. In comparison to level L9, the parenchyma in L10 is significantly enlarged owing to the abundant presence of glandular tissue and large blood vessels. (b, c) The SE and RE are clearly differentiated. (d) At higher magnification, the SE is similar to level L9, displaying clearly defined neuroreceptor cells (open arrowheads). However, the SE in level L10 also reveals a higher density of sustentacular cells (black arrowheads). (e) Level L13 corresponds with the caudal extremity of the VD. (f) Even at this more caudal level, the medial sensory and the lateral respiratory epithelia remain clearly differentiated. (g) In the sensory epithelium, aside from the receptor (open arrowhead) and sustentacular cells (black arrowhead), superficial globose cells (arrows) can be observed. Basal cells are not as abundant as in the previous levels. (h) The respiratory epithelium remains structurally intact and does not contain basal cells. (i) At level L14, the parenchyma consists of venous sinuses, abundant glandular tissue, and nerve bundles, with the VD being absent. (j) At higher magnification, tubular epithelial structures (asterisk) can be detected. These structures correspond to the earliest epithelial invaginations, which give rise to the VD, resembling gloved fingers. The ducts are lined exclusively by respiratory epithelium. (k) At the most caudal level studied (L15), the cartilaginous sheath is triangular and contains branches of the caudal nasal nerve (NCd), as well as veins and arteries. RE, respiratory epithelium; SE, sensory epithelium; Vv, veins; directionality is indicated by the following: d, dorsal; l, lateral; m, medial; v, ventral. Scale bars: e, k = 500 μ m; a, i = 250 μ m; f = 250 μ m; b, c, j = 100 μ m; d, g, h = 50 μ m.

3.2.2 | Immunohistochemical study

The immunolabeling pattern against G protein subunits (Figure 9) is especially revealing because expression of the $\alpha 2$ and αo subunits in the VNO is thought to correspond to the effective expression of the

two major families of vomeronasal receptors, V1R and V2R, respectively.

G α i2 protein immunolabeling (Figure 9a–k) varies markedly throughout the VNO (level L2). At the most rostral level, L2, G α i2 can be faintly detected throughout the entire VD epithelium (Figure 9a),

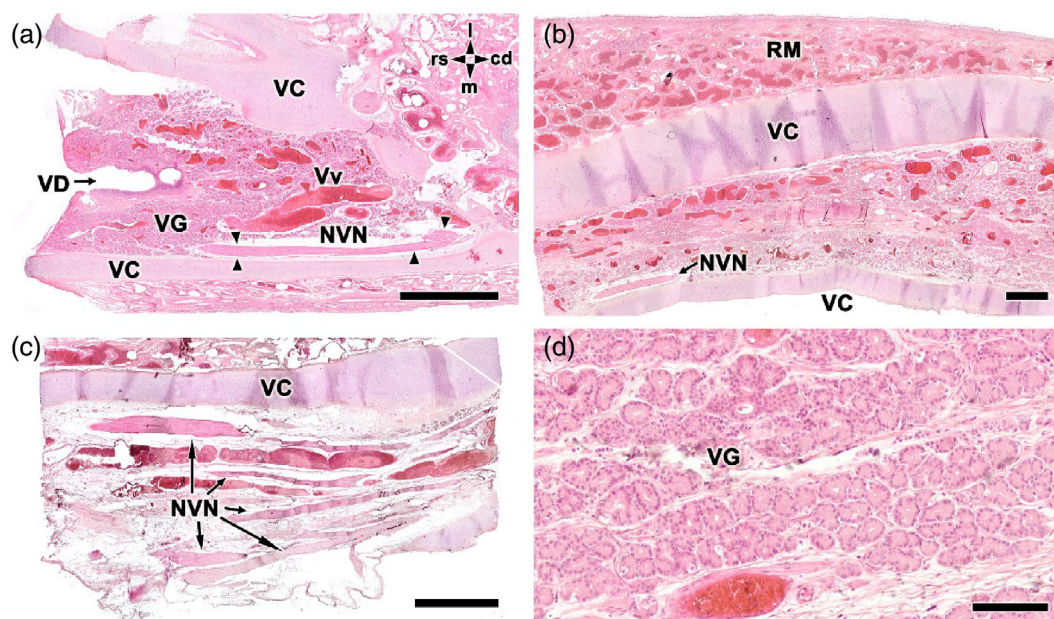


FIGURE 7 Horizontal sections of the caudal vomeronasal organ (VNO). (a) The caudal extremity of the VD transitions into a large parenchymal region rich in glandular tissue and venous sinuses (Vv) flanked by cartilage on both sides. The vomeronasal nerves (NVN) are visible in the medial parenchyma of the organ. (b) Ventrally, the VC remains, and some VG can be seen in the parenchyma; however, venous sinuses predominate. (c) Dorsally, venous sinuses are accompanied by numerous longitudinal branches of the NVNs. (d) Higher magnification view of the serous glandular tissue (VG) in the caudal part of the VNO. RM, respiratory mucosa; directionality is indicated by the following: cd, caudal; l, lateral; m, medial; rs, rostral. Scale bars: a, c = 1 mm, d = 100 μ m.

likely due to the presence of scattered cells with neuroepithelial morphology and strongly labeled processes (Figure 9d). At the next level studied (L3), *Gai2* results in strong immunolabeling of the NVN (Figure 9b), which continues through the entire VNO. At the L3 level, neuroreceptor cells remain scattered, but they possess large and strongly stained processes (Figure 9e). At central levels of the VNO (L5 and L6), the difference between *Gai2*-immunopositive SE and immunonegative lateral epithelium becomes evident (Figure 9c,g). The SE presents with typical features, including a continuous layer of neuroreceptor-positive somata with neatly stained cytoplasm and dendritic boutons (Figure 9f,h,i). Level L10 shows an important modification, with almost no *Gai2*-positive immunolabeling along the entire perimeter of the VD epithelium (Figure 9j). However, at higher magnification, scattered *Gai2*-immunopositive cells can be observed among *Gai2*-immunonegative cells (Figure 9k).

Gao immunolabeling (Figure 9l–q) was observed throughout the VD epithelium, starting at level L2 (Figure 9l). At central levels, L5 and L7, *Gao*-immunolabeled NVN are observed, with no discrimination between lateral and medial epithelia (Figure 9m,o). Not all neuroreceptor cells of the epithelium are *Gao*-positive, as immunopositive cells are interspersed among identifiable *Gao*-negative regions (Figure 9n). At a more caudal level, L10, no *Gao* immunostaining is observed in the VD epithelium, similar to the lack of *Gai2* immunolabeling at this level (Figure 9p). However, at higher magnification, a few *Gao*-positive neuroreceptor cells can be observed in the lateral RE (Figure 9q).

Neuron-specific enolase (NE) is a neuron-specific maturity marker that produces a distinctive labeling pattern in both the NVN and SE, although NE distribution varies across VNO levels (Figure 10a–g). In the rostral VNO (level L3), mature neuroreceptor cells can be observed, interspersed throughout the dorsomedial SE (Figure 10a,d). At central levels L5 (Figure 10b) and L6 (Figure 10e), clear differentiation can be observed between the NE-immunopositive medial SE and the NE-immunonegative lateral RE. NE immunolabeling is present on the NVN and less intensely on the NCd. In the SE, neuronal somas of varying sizes and degrees of NE immunolabeling are spread over the entire basal layer (Figure 10c), with occasional neuronal somas appearing atypically proximal to the lumen (Figure 10h). At a more caudal level, L10, the density of NE immunolabeling in the SE decreases (Figure 10f), but at higher magnification (Figure 10g), a high density of NE-immunopositive neuroreceptor cells can be observed in the SE. An antibody against the OMP (Figure 10i–n) produces diffuse immunolabeling in the VD epithelium throughout the rostral (L2, L3) and central levels (L6) of the VNO (Figure 10k). At higher magnification, OMP immunolabeling appears more intense in individual neuroreceptor cells (Figure 10l–n). Anti-OMP does not immunolabel any cells in the caudal VD epithelium beyond level L10.

Protein gene product 9.5 (PGP 9.5) is a calcium-binding protein that can be used to characterize the neuroreceptor elements of the VNO (Figure 11a–e). At a central level (L7), anti-PGP 9.5 immunolabels the NVN and the SE (Figure 11a), including all neuroreceptor cells in the basal layer and their dendritic knobs (Figure 11b). At a more

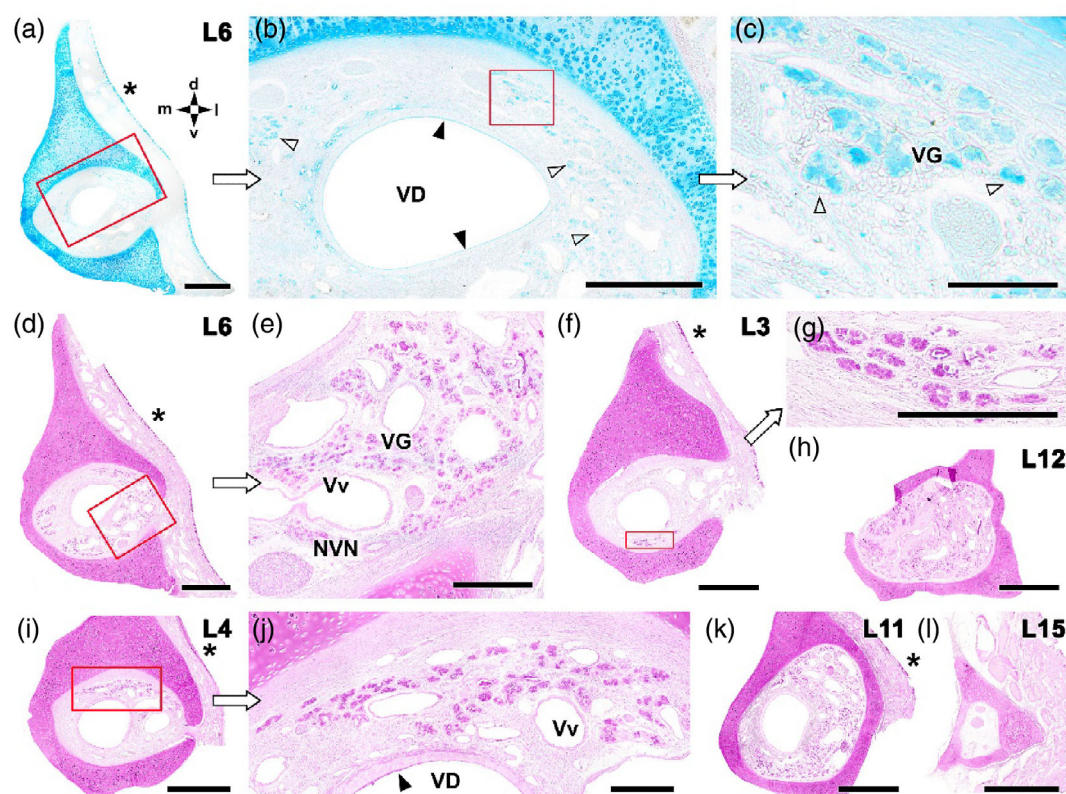


FIGURE 8 Glandular component of the gazelle dama vomeronasal organ (VNO). (a) Alcian Blue staining strongly marks the VC and the respiratory mucosal surface of the nasal cavity (asterisk). (b) At higher magnification of a section corresponding to the central L6 level, faint staining can be observed in a small group of glands located in the dorsal part of the parenchyma (open arrowheads) (c) At higher magnification of the box in (b), the serous nature of the VG can be observed. The luminal surface of the RE exhibits similar staining (arrowhead). (d) At the same level L6, PAS staining reveals significant glandular tissue development, encircling the entire VD. The VC and nasal cavity mucosa (asterisk) exhibit intense staining. (e) The enlarged inset in (d) displays the serous morphology of the VG. The veins basal lamina (Vv) and the connective tissue in the NVNs are slightly marked. (f) The more rostral levels exhibit a lower degree of VG development. At L3, the glands are located ventrally and possess a serous composition (g). (i) At level L4, VG are located dorsally, occupying most of the dorsal parenchyma (j). (h, k) At the most caudal levels of the VD (L12, L11, respectively), PAS-positive glandular development reaches its maximum expression. (l) The most caudal level of the VNO studied has no VGs. Directionality is indicated by the following: d, dorsal; l, lateral; m, medial; v, ventral. Scale bars: a, d, f, h, i, k, l = 500 μ m; e, j = 150 μ m; b, c, g = 100 μ m.

caudal level, L11, the general immunolabeling pattern is similar to the pattern observed in the rostral VNO (Figure 11c); however, compared with the immunolabeling pattern observed in the central level L7, PGP 9.5-immunopositive cells in the caudal SE are not as uniformly arranged and sometimes aggregate in clusters (Figure 11d). In the RE, anti-PGP 9.5 only labels a few isolated cells (Figure 11e). Cytokeratin 8 (CYK8) is a general epithelial cell marker (Figure 11f–j). Anti-CYK8 produces a strong, almost homogeneous staining pattern in the SE and RE throughout the VNO, starting at the rostral levels L2 (Figure 11f) and L3 (Figure 11g). However, a few isolated cells are not CYK8-positive (Figure 11h,j).

Anti-calbindin (anti-CB, Figure 12a,b) stains the NVN and the SE. Most of the neuroreceptor cells in the basal SE are CB-immunopositive (Figure 12a), but many neuroreceptor cells in the apical layers remain unstained (Figure 12b). Anti-calretinin (anti-CR, Figure 12c–e) produces a similar immunolabeling pattern as anti-CB, with strong labeling of the NVN and the SE (Figure 12c,d), with only a

few neuroreceptor cells remaining unstained (Figure 12e). Anti-secretagogin (anti-SG, Figure 12f,g,h,i,l) produces labeling in both the SE and RE, primarily in basal layers. In contrast to other marker antibodies, anti-SG does not label projections or dendritic boutons and produces a more diffuse pattern (Figure 12h,i). In the caudal level L10, anti-SG does not stain the VD epithelia (Figure 12j). Anti-parvalbumin (Figure 13i,k) diffusely labels the NVNs and vomeronasal epithelia throughout the VNO.

The results of the immunohistochemical study are schematically summarized in Figure 13.

3.2.3 | Lectin histochemical study

All of the lectins employed in the present study labeled the VGs and the muco-microvillar complex, which forms the superficial borders of both the SE and RE (Figure 14a–m). At levels L3 and L8, the lectin

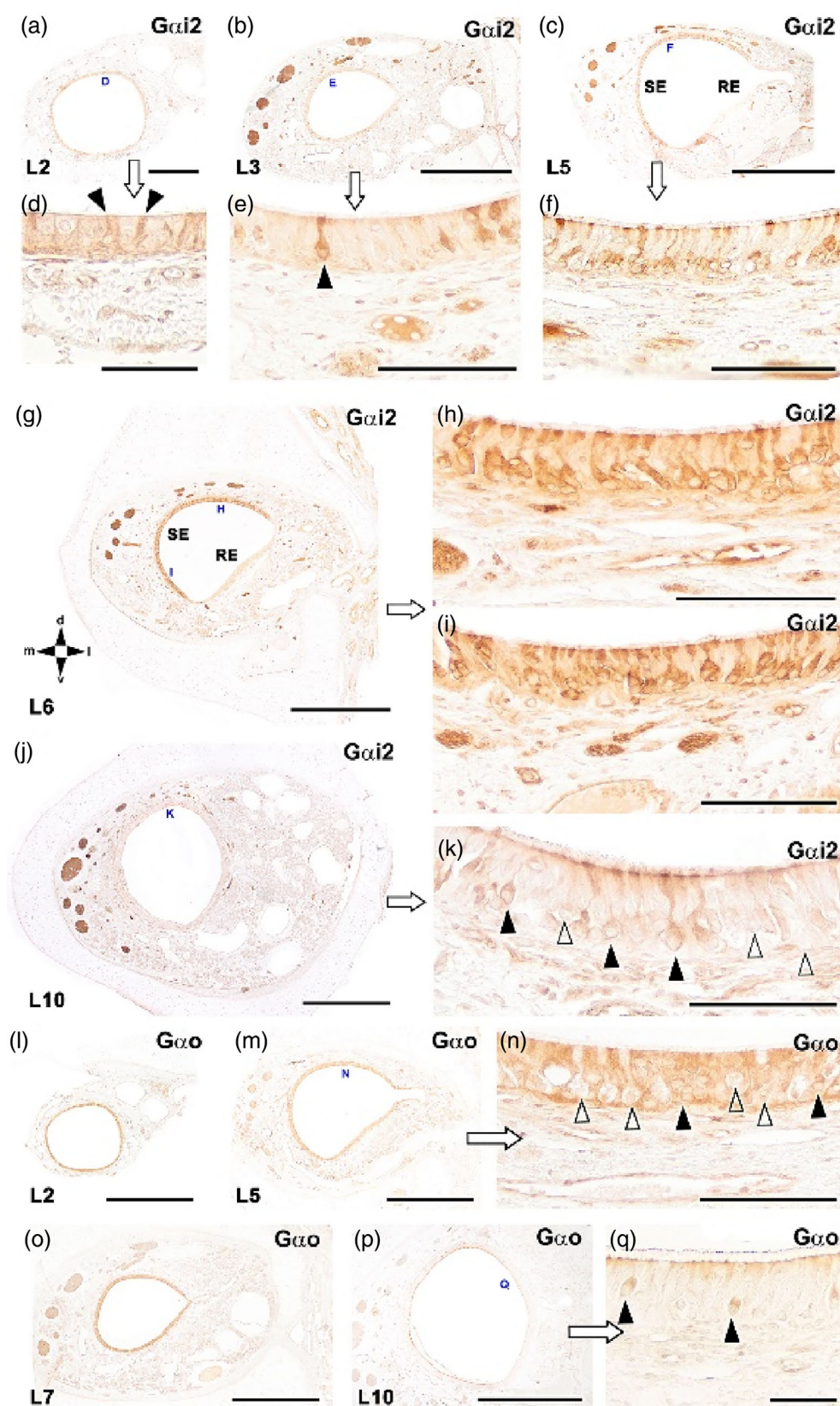


FIGURE 9 Legend on next page.

soybean agglutinin (SBA, *Glycine max*) strongly labeled the VG (Figure 14a,e). At a central level (L6), *Vicia villosa* agglutinin (VVA) faintly labeled the VG (Figure 14b). *Lycopersicon esculentum* agglutinin (LEA) labeled the vomeronasal epithelia, NVN, and VG but did not label the NCd (Figure 14c,d). At central levels (L4 and L5), *Solanum tuberosum* agglutinin (STA, Figure 14f,i) stained the vomeronasal epithelia, NVN, and VG (Figure 14k,n). *Ulex europaeus* agglutinin (UEA) stained both the vomeronasal epithelia and VG (Figure 14g,h,j,l,m) but did not stain NVN at any level (Figure 14g,j).

4 | DISCUSSION

Maintaining healthy and sustainable populations of endangered species, such as dama gazelle, contributes to the preservation of overall biodiversity and ecological balance within an ecosystem. Reintroductions of dama gazelles carried out in semi-freedom conditions, initially result in population growth, but their reproductive success begins to decline over time. The cause is unknown, but concern points to inbreeding depression or reproductive management issues in the reserves. The population is growing progressively in captivity, which will allow for the reinforcement of populations in their natural habitat in the future.

Chemical communications among individuals of the same species can play significant roles in reproductive behaviors. This communication can involve pheromones, which are chemical substances released by one individual that affect the behavior or physiology of another individual of the same species (Liberles, 2014). In many mammals, pheromones play crucial roles in mating behaviors, including mate attraction and selection. For example, female mice release pheromones that attract male mice and increase their sexual behaviors (Fu et al., 2015), and male hamsters release pheromones that stimulate female ovulation and receptivity (Richardson et al., 2004). Nowadays there is plenty of scope for the application of bovine pheromones (Landaeta-Hernández et al., 2023; Rekwot et al., 2001). However,

before pheromones can be used to manage dama gazelle reproduction, the neuroanatomical and morphofunctional characterization of the dama gazelle VNO is necessary. The present study offers the first attempt, to our knowledge, to characterize the dama gazelle VNO. Across mammals, the VNS has been found to display wide morphofunctional diversity (Meisami & Bhatnagar, 1998; Smith et al., 2001; Smith, Garrett, et al., 2011; Wysocki, 1979), requiring the results of this study to be contextualized with regard to the interspecies variability observed in ruminants and in mammals, in general, which will provide further insights into the ecology, evolution, and conservation biology of dama gazelle.

Across all mammals, prior studies of the VNO have often been limited to the central part of the organ, ignoring changes along the rostrocaudal axis. However, numerous studies have evaluated spatial modifications in the VNO by performing serial macroscopic and histological sections, allowing for these anatomical and histological changes to be characterized (Bertmar, 1981; Kondoh et al., 2021; Ortiz-Leal et al., 2020; Salazar et al., 1995; Salazar, Lombardero, Alemañ, & Sánchez Quinteiro, 2003; Tomiyasu et al., 2022; Torres et al., 2020; Vedin et al., 2010). In the current study of the dama gazelle VNO, we extended this approach to include a spatial study of the immunohistochemical and lectin-histochemical features of the VD and NVN, allowing us to integrate morphological and functional observations along the entire length of the VNO.

4.1 | Macroscopic anatomy

The external morphology of the VNO is defined by the shape of its external capsule. In dama gazelle, the external capsule is cartilaginous, encompasses the entire VNO, and is topographically oriented such that the medial wall faces the vomer bone. These features are common to all ruminants in which the VNO has been studied. The shape of the dama gazelle VNO is relatively peculiar, as the VNO in most ruminants appears ovoid, with the major axis in the dorsoventral

FIGURE 9 Immunohistochemical study of G-proteins in the vomeronasal organ (VNO) of dama gazelle. (a) Immunolabeling of Gαi2 protein in the rostral VNO (level L2) faintly marks the entire VD epithelium. At higher magnification (d) it can be seen that this labeling is mainly due to the presence of scattered cells with neuroepithelial morphology and highly stained processes (arrowhead). (b) At the next level studied (L3), the vomeronasal duct, except for its lateral part, continues to show diffuse immunolabeling. This is in contrast to the strong immunolabeling present in the vomeronasal nerves, which remains consistent throughout the entire VNO. At higher magnification (e), the epithelium still shows scattered neuroreceptor cells. However, in comparison to the previous level, these cells possess larger and more intensely stained processes (arrowhead). (c) At the central level of the VNO (L5), the difference between the immunopositive sensory epithelium and the immunonegative lateral epithelium becomes evident for the first time. At higher magnification (f), the sensory epithelium exhibits its characteristic features: a continuous layer of neuroreceptor-positive somata with neatly stained cytoplasm and dendritic boutons. (g) At the L6 level, the vomeronasal duct acquires its crescent shape, and the difference between the SE and RE becomes more noticeable. (h, i) The entire length of the SE presents a similar microscopic structure. (j) The L10 level reflects a major modification, with minimal Gαi2-positive immunolabeling along the entire perimeter of the VD epithelium. (k) However, at higher magnification, scattered Gαi2-immunopositive cells can be observed among Gαi2-immunonegative cells (open arrowheads). (l) Immunolabeling with the anti-Gαo antibody was present throughout the VD epithelium, from the most rostral L2 level. (m, o) At levels L5 and L7, NVNs are immunolabeled, and there is no distinction between the lateral and medial epithelia. (n) At higher SE magnification at L5, it becomes apparent that not all neuroreceptor cells in the epithelium are Gαo-positive. There are distinct Gαo-negative regions (open arrowheads) interspersed between the immunopositive Gαo cells (black arrowheads). (p) At the L10 level, there is no immunostaining of the VD epithelium, as was the case with Gαi2. (q) However, higher magnification reveals the presence of Gαo-positive neuroreceptor cells, specifically in the lateral RE (arrowheads). Directionality is indicated by the following: d, dorsal; l, lateral; m, medial; v, ventral. Scale bars: c, g, j, l, m, o, p = 500 μm; a, b = 250 μm; e, f, h, i, k, n = 100 μm; q = 50 μm; d = 25 μm.

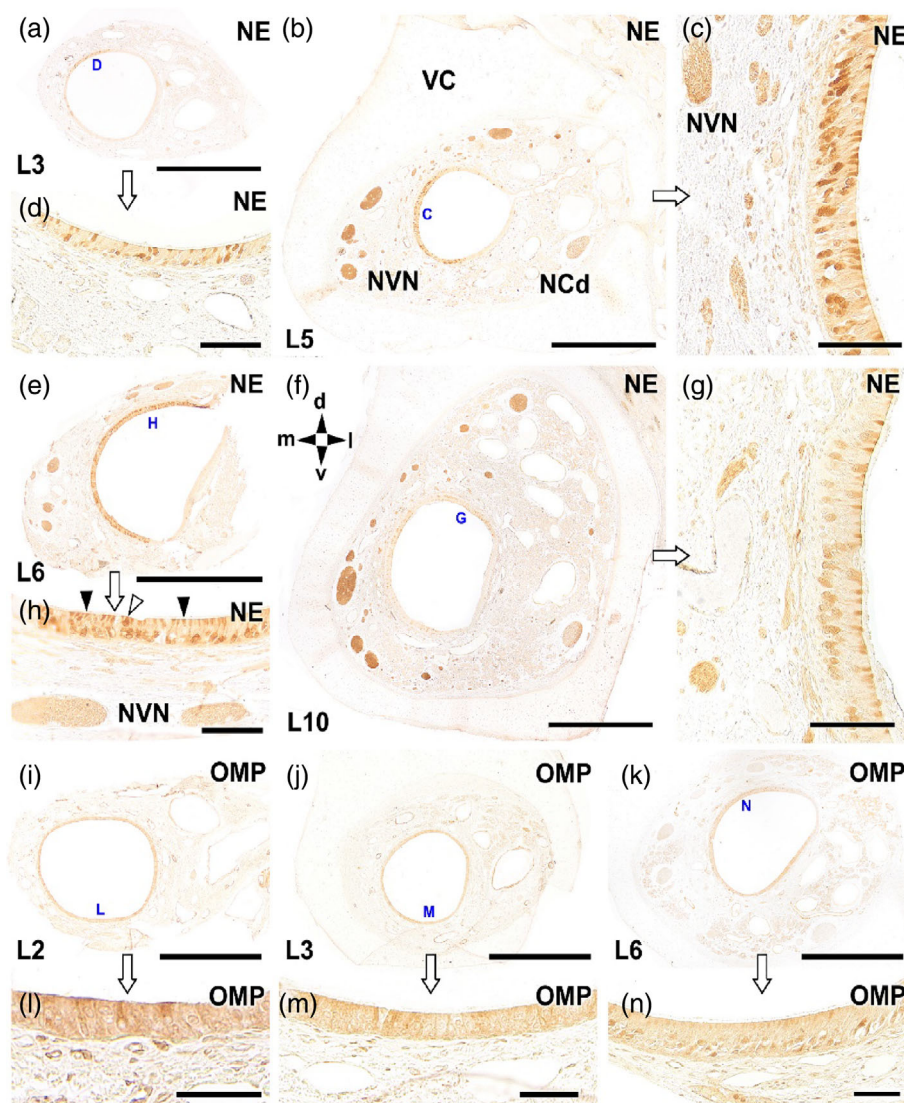


FIGURE 10 Analysis of neuronal maturity in the vomeronasal organ (VNO) of the dama gazelle. Neuron-specific enolase (NE) is a neuron-specific maturity marker, and it distinctively labels both the vomeronasal nerves (NVN) and the sensory epithelium (SE). However, its distribution varies depending on the level of study. (a) In the rostral part of the VNO (L3), mature neuroreceptor cells are interspersed throughout the dorsomedial SE. These neuroreceptor cells can be observed at higher magnification (d). (b) At central level L5, the immunopositive medial SE and the immunonegative lateral RE are clearly differentiated. Immunolabeling is present on both the NVNs and the NCd nerve, but the labeling is less intense in the latter. Higher magnification of the SE (c) reveals neuronal somas of varying sizes and degrees of immunolabeling that extend throughout the entire basal layer. (e) The next caudal level, L6, displays a comparable arrangement of the SE. At higher magnification (h), the apical zone of the SE contains the dendritic knobs of the receptor cells (black arrowheads) and individual neurons whose somas are atypically positioned next to the lumen (open arrowhead). (f) At a more caudal level, L10, the density of immunolabeling decreases in the SE. However, higher magnification of the SE (g) reveals a high density of NE-immunopositive neuroreceptor cells. (i–k) The OMP antibody produces diffuse immunolabeling in the VD epithelium at the rostral levels (L2, L3) (i, j) and central level (L6) (k). (l, m, n) At higher magnification, it is noticeable that immunolabeling is more intense in individual neuroreceptor cells. Directionality is indicated by the following: d, dorsal; l, lateral; m, medial; v, ventral. Scale bars: a, b, e, f, i–k = 500 μ m; c, d, g, h, l–n = 100 μ m.

orientation (Hart et al., 1988; Ibrahim et al., 2013; Jacobs et al., 1981; Park et al., 2014; Vedin et al., 2010), whereas the major axis of the ovoid dama gazelle VNO is lateromedial. However, notable variations in shape have been observed along the rostrocaudal axis of the organ in ruminant species, which have been examined using complete histological series, mainly cow (Salazar et al., 2008) and sheep, the latter more homogeneous in all its length (Salazar et al., 2007).

Compared to these species, dama gazelle presents the largest degree of morphological variation. Another remarkable feature of the dama gazelle VNO is the presence of indentations in the lateral wall of the cartilage, which allows for the passage of vessels and nerves. Ruminant species present a wide lateral communication at rostral levels of the VNO. However, the central region of the cow VNO displays a wide lateral gap, which is lacking in the central region of the sheep

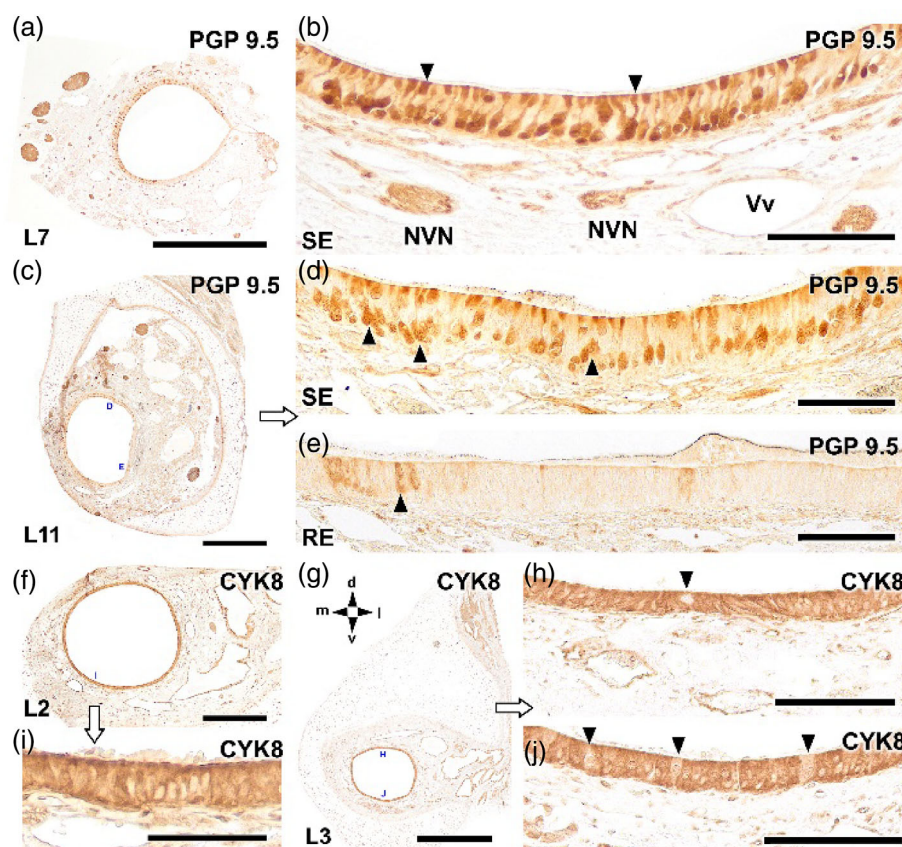


FIGURE 11 Characterization of the vomeronasal epithelium of the dama gazelle with anti-PGP 9.5 and CYK8. (a) Calcium-binding protein PGP 9.5 enables the characterization of the neuroreceptor elements in the sensory epithelium (SE). At the central level L7, anti-PGP 9.5 immunolabels the vomeronasal nerves (NVN) and SE. (b) Immunolabeling involves all neuroreceptor cells in the basal layer and their dendritic knobs (arrowheads). (c) At a more caudal level L11, the general immunolabeling pattern is similar to that observed in the rostral VNO. (d) At higher magnification, a significant number of PGP 9.5 immunopositive cells can be observed in the SE. However, compared to the previous level, these cells are not uniformly arranged. In fact, they are sometimes found in clusters (arrowheads). (e) In the RE, anti-PGP 9.5 only labels a few isolated cells (arrowhead). (f) Anti-cytokeratin 8 (CYK8) is a widely used marker for identifying epithelial cells. It provides a robust and almost homogenous staining pattern in the SE throughout the VNO, including its more rostral levels L2 (f) and L3 (g). (h–j) In the VD epithelium, there are a few isolated cells that are not CYK8-positive (arrowheads). Directionality is indicated by the following: d, dorsal; l, lateral; m, medial; v, ventral. Scale bars: a, c, g = 500 μ m; f = 250 μ m; b, d, e, h, j = 150 μ m; i = 50 μ m.

VNO (Salazar et al., 2007, 2008). The central region of the dama gazelle VNO displays a moderate lateral gap, which coincides with the formation of an internal recess that exclusively accommodates the main trunk of the NCd.

Data regarding how the VD communicates with the external environment is limited because the accurate determination of this communication requires the evaluation of a complete histological series using decalcified samples. Our observations in the dama gazelle VNO are consistent with available evidence in other ruminants, including both bovine (e.g., cow and sheep; Salazar et al., 2007, 2008), and non-bovine (e.g., moose) species (Vedin et al., 2010). In all cases, the VD opens into the ID and becomes incorporated on its medial side such that both ducts share the same cartilaginous capsule. In the dama gazelle VNO, we identified functional incisive papilla, which is not a feature common to all Antilopinae, as the three species of alcelaphine antelopes studied by Hart et al. (1988).

4.2 | Microscopic anatomy

4.2.1 | Vomeronasal epithelia

Our extensive serial histological study has allowed us to determine differences in the epithelial lining of the VD along the rostrocaudal VNO axis. The medial epithelium of the VD is home to the neuroreceptor cells responsible for semiochemical detection (Dennis et al., 2004; Mombaerts, 2004), whereas the lateral RE is home to the cells that produce the mucus required for chemoreception (Breipohl et al., 1979; Takami et al., 1995) and the growth factors that modulate neuroreceptors (Getchell et al., 2002).

The SE in the rostral junction between the VD and the ID is characterized as a squamous simple epithelium. Caudal to this junction, the SE transitions into a pseudostratified epithelium, comprising two cell layers consisting primarily of polyhedral cells with scattered receptor cells. At level L3, geometrical cells are replaced by elongated cells,

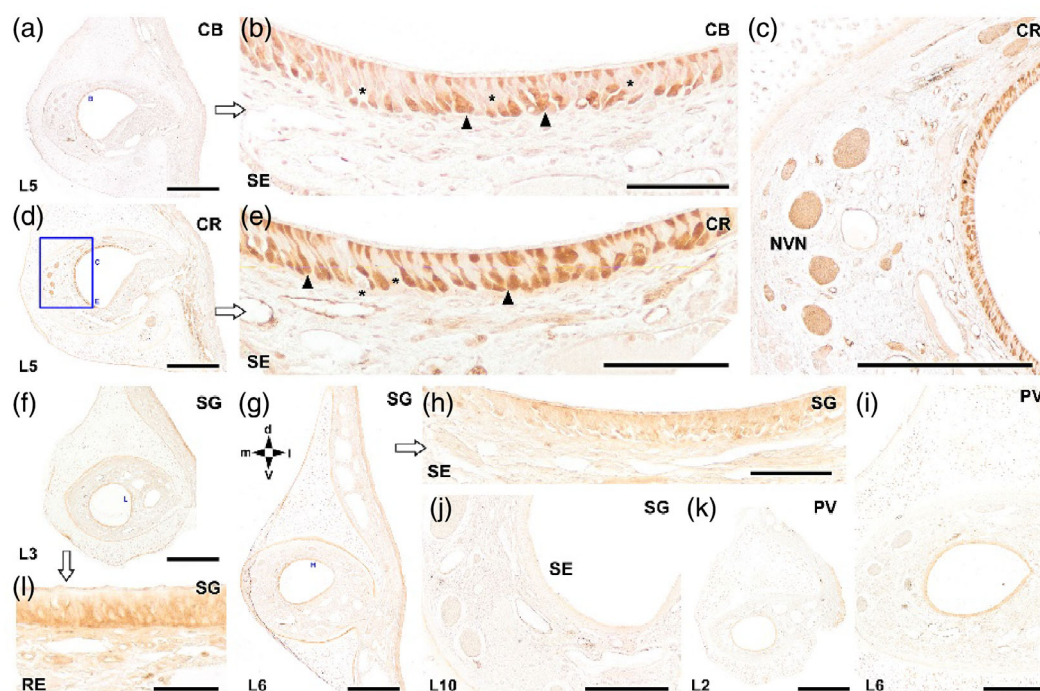


FIGURE 12 Characterization of the dama gazelle vomeronasal organ (VNO) by calcium-binding proteins. (a) Anti-calbindin (CB) stains the NVNs and the SE. (b) Most of the neuroreceptor cells in the basal SE are CB-immunopositive (arrowheads). However, many neuroreceptor cells in the apical layers remain unstained (asterisks). (c, d) Anti-calretinin (CR) produces a labeling pattern similar to that of CB, showing strong labeling of the NVNs and SE. (e) Only a few neuroreceptor cells remain unstained (asterisk). (f, g) Anti-secretagogin (SG) produces labeling in both vomeronasal epithelia, primarily among basal cells (h, i). In contrast to other markers, SG does not label projections or dendritic boutons, resulting in a more diffuse pattern. At the caudal level L10, anti-SG does not stain the VD epithelia. (i, k) Anti-parvalbumin (PV) diffusely marks the NVNs and vomeronasal epithelia throughout the VNO. Directionality is indicated by the following: d, dorsal; l, lateral; m, medial; v, ventral. Scale bars: a, d, e, f, g, k = 500 μ m; c, i, j = 250 μ m; b, e, h = 100 μ m; l = 50 μ m.

representing the transition to the typical vomeronasal receptor epithelium. At level L5, the medial epithelium begins to display the typical VNO morphology, characterized by a fully developed SE in a crescent-shaped lumen. At levels L7–L9, the VD displays a reduced diameter with a circular shape, and three-quarters of the epithelial lining contain neuroreceptors with a clear distinction observed between the basal neuroreceptor cells and the apical sustentacular cells, which have dense nuclei. This cellular organization is preserved throughout the caudal portion of the VD, although increases in the development of the parenchyma and the size of the VD are observed at L10 until the abrupt end of the VD at L12–L13. The squamous epithelium of the RE observed in the rostral levels is replaced by two layers of rounded or oval cells with scattered superficial cells featuring translucent nuclei until L4. At L5, a few polyhedral cells with translucent cytoplasm can be observed in the apical zone, but the features at this level are mostly typical for RE. This pattern can be observed throughout the caudal extremity of the VD, and even the earliest epithelial invaginations that give origin to the VD are lined exclusively with RE.

Remarkable changes in the lumen shape along the entire VNO length have not previously been described for other ruminants. In the cow, Adams (1986) describes the crescent shape as the predominant one, except for the anterior part of the organ. He describes a rostral VNO featuring a cuboidal stratified epithelium containing cells with

lightly stained cytoplasm, which coincides with our observations for the rostral VNO of dama gazelle. Likewise, descriptions of the cow neuroepithelium, (Jang et al., 2021; Salazar et al., 2008; Taniguchi & Mikami, 1985) which contains basal, neuroreceptor, and sustentacular cells, and the organization of the cow RE coincide with our observations of these structures in dama gazelle. Similar observations have been made for other Bovinae, such as goat (Park et al., 2013), sheep (Ibrahim, 2018; Ibrahim et al., 2013; Kratzing, 1971; Salazar et al., 1998, 2000; Salazar et al., 2007; Salazar, Lombardero, Alemañ, & Sánchez Quinteiro, 2003), and water buffalo (Emam et al., 2016), in addition to other ruminants, such as giraffe (Kondoh, Nakamura, et al., 2017), reindeer (Bertmar, 1981), elk (Vedin et al., 2010), and Korean roe deer (Park et al., 2014). These data indicate that this organization represents a widely conserved evolutionary pattern in ruminants.

4.2.2 | The vascular pump

The vomeronasal pump is unique to mammals and is responsible for moving pheromone molecules from the external environment to sensory cells within the VNO, enhancing its chemosensory capabilities. The pumping mechanism is powered by vasomotor movements, which



FIGURE 13 Schematic representation of neuromarker expression in the vomeronasal sensory epithelium along the rostrocaudal axis of the vomeronasal organ (VNO). The occurrence of sensory epithelium and respiratory epithelium along the VD is shown in blue. A fully differentiated epithelium is shown in dark blue, while light blue indicates an epithelium that has not yet adopted the typical characteristics of both sensory and respiratory epithelium. Brown indicates the expression of the two subunits of the G-proteins, $G\alpha o$ and $G\alpha i2$ in the SE, with dark brown corresponding to full expression in the epithelium and light brown representing the diffuse presence of labeled neuroreceptor cells. Dark and light gray represent the OMP expression pattern. Calcium-binding proteins are represented in dark green in levels where the epithelium shows strong immunolabeling, and in light green where their expression is lower.

can suck stimuli into the VNO and actively expel the contents of the VD. These mechanisms are activated by fibers in the nasopalatine nerve, which cause the constriction of blood vessels within the VNO capsule (Meredith & O'Connell, 1979), reducing the blood volume in the cavernous tissue and creating a pressure differential that expands the VNO lumen and draws in fluid from the region around the duct opening (Meredith et al., 1980). Stimuli enter the VNO via the mucus stream flowing past the VD from the ID. Effective vomeronasal stimuli are mucus-soluble chemicals or substances that become mucus-soluble when bound to carrier molecules, such as those secreted by VGs (Krishna et al., 1995).

The development of the vascular component in the dama gazelle VNO is observable starting at the rostral levels, where the lateral parenchyma is completely occupied by blood vessels. Moving caudally, the vessels extend toward the dorsal and ventral areas and, finally, in the caudal sections, occupy the entire parenchyma, which we observed in both transverse and horizontal sections. This pattern of vascular development is common among those ruminant species in which the vascular component has been studied (Salazar et al., 1998, 2008; Vedin et al., 2010). In dama gazelle, we observed that the vomeronasal neuroepithelium extends nearly to the caudal interdigitations of the VD, which arise directly from VGs in that region, a feature that we are not aware of being described in other species. Therefore, the presence of a more developed vasculature in the caudal extremity of the VNO is hypothesized to contribute to the generation of strong negative pressure, which would be necessary for external stimuli to reach the receptors located in the caudal VNO. The ability of stimuli

to reach caudal receptors would depend both on the vascular pup activity and on the physicochemical properties of stimulus molecules. A gradient of dispersion is likely established along the length of the duct, such that only those substances that are highly soluble in glandular secretions reach the caudal extremity, whereas less soluble substances remain in the rostral regions. Consistent with this concept, we hypothesize that the specificity of vomeronasal receptors along the VD is adapted to accommodate the differential migration of substances along the duct, similar to how compounds are eluted along a chromatography column.

4.2.3 | Vomeronasal glands

The vomeronasal epithelia in the VNO are coated with a mucous fluid produced by VGs, which is necessary to carry external stimuli to the neuroepithelium (Sankarganesh et al., 2022). This mucous fluid contains various enzymes able to transform the structures of molecules, which can determine the ability of these molecules to activate neuroreceptors, influencing olfactory (Nagashima & Touhara, 2010). The mucous fluid also contains enzymes with antibacterial and antioxidative properties (Ding & Xie, 2015).

Mucous fluid properties differ across mammal species (Kondoh et al., 2020) and are often characterized using AB, which stains the acidic polysaccharides synthesized and secreted by VGs, and PAS, which stains neutral polysaccharides. Initial studies examining VGs concluded that they only secreted neutral compounds in all species

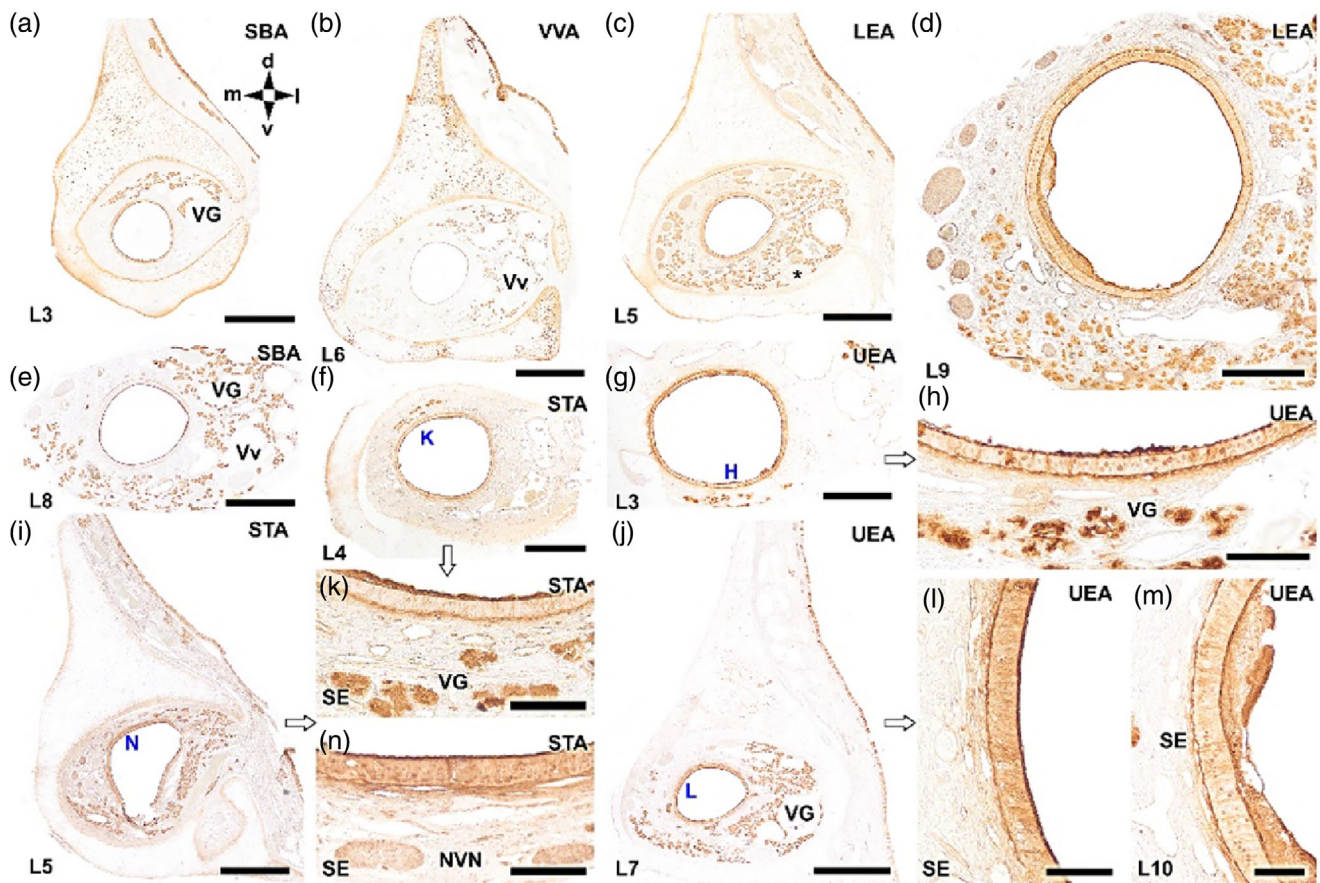


FIGURE 14 Lectin-histochemical study of the dama gazelle vomeronasal organ (VNO). All of the lectins employed labeled the vomeronasal glands (VGs) and the muco-microvillar complex, that is, the superficial border of both vomeronasal epithelia. (a, e) Labeling of the VNO at levels L3 and L8 using the lectin SBA (*Glycine max*). In both cases, the VG are labeled strongly. (b) VVA (*Vicia villosa*) labeling at the central level L6 of the VNO reveals a faint labeling of the VG. (c, d) LEA (*Lycopersicon esculentum*) labels the vomeronasal epithelia, the NVNs, and the VG. However, LEA does not stain the NCd (asterisk in c). (f, i, k, n) STA (*Solanum tuberosum*) lectin labeling at the central levels (L4, L5) of the VNO stains the vomeronasal epithelia, vomeronasal nerves (NVN), and VG. (g, h, j, l, m) UEA (*Ulex europaeus*) lectin stains vomeronasal epithelia, VG, but hardly stains NVNs. Vv, veins; directionality is indicated by the following: d, dorsal; l, lateral; m, medial; v, ventral. Scale bars: a–c, e, i, j = 500 µm; d, f, g = 250 µm; h = 100 µm; k–n = 50 µm.

examined due to the ability of PAS but not AB to stain glandular tissue (Cuschieri & Bannister, 1974; Loo & Kanagasunteram, 1972; Oikawa et al., 1993, 1994; Salazar et al., 1996). Their results reflected the positivity of the glandular tissue to the PAS marker, while AB produced a negative result. However, in 1997, Salazar and collaborators showed AB-positive VGs for the first time in the VNO of cow and horse, which also featured PAS-positive glands (Salazar, Quinteiro, & Cifuentes, 1997). Subsequently, several authors obtained similar results, detecting both types of VGs, in all ruminants studied, including cow (Jang et al., 2021; Salazar et al., 2008), sheep (Salazar et al., 2000, 2007), goat (Yang et al., 2021), sika deer (Kondoh et al., 2020), giraffe (Kondoh, Nakamura, et al., 2017), and goitered gazelle (Aboud & Hussein, 2018). The degree of glandular development and the intensity of the response to both PAS and AB were similar for all studied species.

Our observations in dama gazelle reveal a clear distinction between these two types of VGs. PAS produced a generalized staining

of the glandular component, from the rostral level L3 to the caudal end of the VNO, whereas AB only stained the central part of the VNO (level L6), restricted to a small group of VGs in the dorsal parenchyma. This result highlights the importance of examining the entire VNO when making comparisons across animals, as extrapolations may not be possible using information obtained from limited areas. For example, AB-positive VGs were recently identified in musk shrews (Kondoh et al., 2020), despite no evidence of AB-positive VGs in this species during initial studies (Oikawa et al., 1993).

4.2.4 | Innervation

The distribution of the two main VNO innervation components, unmyelinated NVN fibers and myelinated NCd fibers, have not been well studied in ruminants. However, our observations in dama gazelle are comparable to those described in other bovine species (Salazar

et al., 2007; Salazar, Quinteiro, & Cifuentes, 1997; Vedin et al., 2010). Our serial histological study revealed that starting in the most rostral levels (L3), sensory innervation can be observed in the medial parenchyma, indicating significant sensory innervation for the rostral neuroepithelium, even though the rostral levels lack the layered structure or receptor density characteristic of the central levels.

4.3 | Immunohistochemical study

Given the remarkable variations observed in the structure of the VD and its epithelial lining, we conducted an immunohistochemical assessment of VNO markers along the entire length of the VNO rather than limiting this analysis to the central VNO.

We used specific antibodies against the α subunits of the Gi2 and Go proteins to characterize the expression of the vomeronasal receptors, V1R and V2R, respectively (Dulac & Axel, 1995; Ryba & Tirindelli, 1997). An investigation of G protein expression and distribution in the goat VNS revealed, for the first time, that the Go pathway was absent in some species (Takigami, 2000), a finding that was subsequently confirmed in numerous mammals, including Laurasiatheria and Primates (Suárez et al., 2011). However, discrepancies between gene sequencing and immunohistochemical studies have been documented, particularly in the order Carnivora. Dennis et al. (2003), studying the dog, and Ortiz-Leal et al. (2020), studying the fox, both observed immunopositive labeling in the vomeronasal SE using both anti-Gai2 and anti-Gao antibodies, despite the apparent absence of functional V2R genes in the genomes of both species (Kukekova et al., 2018; Young & Trask, 2007). However, new sequencing studies focused on olfactory and vomeronasal genes are likely to result in the addition of new genes to the vomeronasal repertoire, which was initially defined using a whole-genome approach. Recently Kondoh et al. (2022) detected using genome assembly the presence of functional V2R genes in the genomes of cattle, goats, sheep, and pigs, which previously were thought to be absent from these species.

The study by Kondoh et al. (2022) was limited to the expression of Gai2 and Gao in the AOB, the first integrative center for the VNS in the brain, without performing immunohistochemistry in the VNO itself. Our study in dama gazelle examined both Gai2 and Gao immunopositivity in the VNO and throughout the vomeronasal epithelium. By performing a serial immunohistochemical study along the entire VD, we were able to confirm a lack of consistent expression for both markers in the whole SE, as a significant portion of the VD did not express either protein. In particular, the large segment extending from L9 to L13, despite having a well-characterized neuroepithelium, lacked any immunolabeling for either protein, indicating that the receptors expressed in this caudal third of the VD are likely not either V1R or V2R and suggesting that the molecules detected by V1R and V2R do not reach the caudal end of the VD. We hypothesize that only low-molecular weight compounds that are highly soluble in the vomeronasal mucus can be detected in the caudal VD, likely by receptors that have not yet been described. The absence of vomeronasal receptor expression in such a large stretch of the VNO could explain why

previous studies of G-protein expression in the ruminant VNO, including goat (Takigami, 2000), sheep (Salazar et al., 2007), and sika deer (Matsubara et al., 2019), have failed to detect Gao immunopositivity, as these studies are typically performed in a single, either central or caudal level, where these proteins were not detected in dama gazelle.

Kondoh et al. (2022), in their study of the cow AOB, did not detect Gao immunopositivity, and Gao was also not detected in the dog (Salazar et al., 2013) or fox AOB (Ortiz-Leal et al., 2022); however, the lack of Gao expression in the AOB does not preclude V2R expression in the VNO, as information from these receptors may project to areas of the olfactory bulb other than the AOB. In the fox olfactory bulb, Gao expression in the NVN was found to project not to the AOB but to the olfactory limbus, a transition zone between the main olfactory bulb and the AOB (Ortiz-Leal et al., 2023).

While the presence of functional V2R genes in artiodactyls aligns with our detection of Gao-immunoreactivity in the dama gazelle VNO, we must acknowledge the possibility of Gao protein serving a different role in the VNO. For instance, it could potentially be involved in cell-to-cell communication in other parts of the nervous system, as described by Jiang and Bajpayee (2009), which may not be directly related to transduction. Nonetheless, this observation would mark the first piece of evidence for this particular function in the vomeronasal neuroepithelium of any mammal, and it does not align with the immunolabeling pattern we observed in the dama gazelle VNO. The immunolabeling pattern spans dendritic processes, the cell body (soma), and the axons that make up the NVN, consistent with typical patterns seen in species where both G-proteins are involved in the transduction cascade (Halpern & Martinez-Marcos, 2003).

The neuronal maturity of the VNO neuroepithelium in dama gazelle was analyzed using anti-OMP, a widely used molecular marker for olfactory neurons in both the olfactory mucosa and the VNO in various species (Rodewald et al., 2016; Sasuga et al., 2013; Smith, Dennis, et al., 2011). The functional properties of OMP remain under debate, but it has been shown to play roles in neuronal maturation and stimulus-response selectivity (Buiakova et al., 1996). In dama gazelle, OMP-positive immunolabeling could be observed in the rostral vomeronasal SE, but no OMP staining was observed in the caudal levels L10–L13, similar to the pattern observed for G protein labeling. Anti-OMP stained cell bodies more intensely than dendrites, a pattern that was also observed in sheep (Ibrahim, 2018), cow (Jang et al., 2021), and roe deer (Shin et al., 2017). However, OMP staining in the goat showed the reverse pattern, with dendrites staining more intensely than cell bodies (Park et al., 2013; Yang et al., 2021). Reported anti-OMP immunolabeling patterns in the RE vary across ruminants. No OMP-positive immunolabeling was detected in the sheep RE (Salazar et al., 2007), whereas the roe deer features OMP-positive ciliated and basal cells (Shin et al., 2017). By contrast, dama gazelle show an intermediate pattern, with diffuse labeling and only a few ciliated OMP-positive cells in the RE, and a similar intermediate pattern has been reported in both cow and Korean black goat (Park et al., 2013; Yang et al., 2021).

To better understand the neurochemical properties of the vomeronasal neuroepithelium, we used several neuronal markers. PGP 9.5 is

a neuronal biomarker expressed in sensory paraneurons that plays a vital role in ubiquitin regulation. PGP 9.5 is expressed in olfactory receptor cells, taste bud cells, and vestibular hair cells (Iwanaga et al., 1992; Johnson et al., 1994). Our PGP 9.5 labeling in dama gazelle is consistent with labeling observed in sheep (Ibrahim, 2018), cows (Jang et al., 2021), Asian roe deer (Park et al., 2014) and Korean black goats (Park et al., 2013). PGP 9.5-positive cells show strong immunoreactivity in their soma, dendrites, and axons, which extend through the lamina. Additionally, some PGP 9.5-positive ciliated cells were observed in the RE, as reported in all studied ruminants except sheep.

Our investigation along the rostrocaudal axis of the VNO revealed PGP 9.5 expression in the caudal SE (L10–L13), a region lacking G proteins and OMP expression. The presence of PGP 9.5 immunolabeling confirms the neuroepithelial nature of this epithelial tissue, as indicated by histological results, and suggests that this region expresses receptors other than those in the V1R and V2R families.

NE, a brain-specific isozyme of the glycolytic enolase enzyme, is consistently found in the cytoplasm of mature neurons, including sensory neurons (Iwanaga et al., 1989). To our knowledge, no prior immunohistochemical studies examining NE have been performed in ruminants. Our observations in dama gazelle are consistent with the prior immunolabeling pattern reported for a wide variety of mammals, including rodents and humans (Takami et al., 1993; Wang et al., 1994), and extend our understanding of NE positivity throughout the entire SE, confirming the neurosensory nature of the caudal third of the VD indicated by PGP 9.5 staining.

Calcium-binding proteins modulate calcium signaling and the transmission of sensory information and serve as specific markers of vomeronasal receptor cells. Although their presence has been identified in the VNO of a wide range of mammals groups, including Rodentia (Jia & Halpern, 2003; Kishimoto et al., 1993; Torres et al., 2020), Marsupialia (Jia & Halpern, 2004; Torres et al., 2022), Canidae (Ortiz-Leal et al., 2020), Soricidae (Malz et al., 2000), and Hominidae (Johnson et al., 1994), no available information exists regarding calcium-binding proteins in the VNO of ruminants. By studying four calcium-binding proteins, CB, CR, SG, and parvalbumin, we were able to fill an important gap in the study of VNO neurochemistry for a large group of mammals. In addition, our study represents the first description of SG in the mammalian VNO (Wagner et al., 2000). Of the four markers we studied, CB and CR provided the best characterization of the structural elements that form the neuroreceptor epithelium of the VNO, as SG and parvalbumin were detected in both the SE and RE. In dama gazelle, anti-parvalbumin staining was similar to the pattern observed in the mouse VNO, which was moderate but less intense than CB or CR staining (Kishimoto et al., 1993).

Antibodies against both CB and CR stained a subpopulation of vomeronasal receptor neurons in the dama gazelle VD. The somata of the immunoreactive neurons were located in the basal and middle regions of the VNO SE, in contrast with the apical position of CB-positive somata in the rat VNO (Jia & Halpern, 2003). Thick apical dendrites, piriform somata, and axons were clearly stained by both markers in the dama gazelle VNO. In addition, CB- and CR-

immunoreactive axons were observed in the nerve bundles in the lamina propria and in the NVN, a pattern that gradually appeared in the SE at rostral levels L3–L4 before appearing throughout the SE from levels L5 to L9. Similar to G-protein and OMP expression, calcium-binding protein expression was not detected in the caudal SE.

The immunohistochemical study also included the specific characterization of epithelial tissue by examining the expression of CYK8, a type II intermediate filament protein (Franke et al., 1981). In dama gazelle, anti-CYK8 broadly identified neuroreceptor cells and sustentacular cells across the SE and RE. However, anti-CYK8 did not produce homogeneous labeling in the anterior VNO, with no staining observed in the apical polygonal cells, whose functional significance remains unknown. We did not find any references to this cell type in the VNO literature. Most cytokeratin investigations in the VNO have used cytokeratin cocktails. In dogs, Dennis et al. (2003) found that cytokeratin immunolabeling was restricted to basal cells, whereas in opossum, Poran (1998) found a more diffuse pattern, similar to the pattern observed in dama gazelle but with greater intensity detected in the RE than in the SE.

4.4 | Lectin histochemical study

Lectins are a type of agglutinin capable of binding to certain glycoconjugates found on the cell surface (Brooks, 2023). The luminal surface of the VD is covered by a layer of glycocalyx, comprised of carbohydrates and proteins, allowing lectins to be particularly useful for investigations of the VNS (Plendl & Sinowatz, 1998). Lectins are often used to identify sensory cells, the RE, and glandular components of the VNO (Halpern et al., 1998). If we limit our focus to the nervous tissue, it is remarkable how certain lectins are unique to the vomeronasal pathway. Certain lectins are unique to the neuronal tissues found in the vomeronasal pathway, able to recognize the dendrites, somas, axons, and terminal projections of neuronal cells (Chun et al., 2023; Kondoh et al., 2018; Kondoh, Kamikawa, et al., 2017; Salazar et al., 1994). However, studies of the VNS using lectins must recognize that labeling patterns are highly specific, with notable differences even between species as phylogenetically close as the rat (Salazar & Sánchez Quinteiro, 1998) and the mouse (Salazar et al., 2001). Therefore, cross-species extrapolations should be avoided.

In our investigation of the dama gazelle VNO, we examined five lectins to reveal the glycoconjugate-binding patterns in the SE, RE, NVN, and VGs. All of the lectins used stained VGs and the mucro-microvillar complex, which covers the luminal surface of both the SE and RE. In addition, each lectin stained specific structures of the VNO and exhibited individual localization patterns and labeling intensities. SBA produced intense labeling of the glandular tissue throughout the VNO, starting in the rostral extremity. Similarly, VVA stained the VGs in the central VNO but with a much lower intensity than SBA. Neither SBA nor VVA stained the VD epithelia or NVN axons. LEA and STA strongly labeled the vomeronasal epithelium, VGs, and NVN but did not label the NCd. UEA labeling was concentrated in the vomeronasal epithelium and VGs, with no staining observed in the parenchymal

NVN. In contrast to the proteins detected in the immunohistochemical study, no noticeable differences were observed in the lectin-histochemical labeling patterns along the rostrocaudal axis of the VNO.

These labeling patterns obtained using these lectins in dama gazelle have both similarities and differences compared with the labeling patterns obtained using these lectins in the VNO of other ruminant species, including sheep (*Ovis orientalis aries*) (Ibrahim et al., 2013; Salazar et al., 2000, 2007), cow (*Bos taurus coreanae*) (Jang et al., 2021), goat (*Capra aegagrus hircus*) (Park et al., 2013; Yang et al., 2021), and Asian roe deer (*Capreolus pygargus*) (Park et al., 2014; Shin et al., 2017).

In dama gazelle, SBA produced labeling similar to that observed in sheep (Ibrahim, 2018) with staining observed only in VGs and the luminal rim of the vomeronasal epithelium. SBA produced moderate staining in the vomeronasal SE of other ruminant species, such as cow (Jang et al., 2021), goat (Park et al., 2013; Yang et al., 2021), and roe deer (Park et al., 2014). In cow, SBA also moderately stains the NVN (Jang et al., 2021).

In dama gazelle, VVA only labeled the superficial border of the epithelium and VGs, although less intensely than SBA. By contrast, in cow (Jang et al., 2021), and to a lesser extent, in the sheep (Ibrahim et al., 2013) VVA moderately stains the vomeronasal epithelium and NVN.

LEA stained numerous vomeronasal structures in dama gazelle, including the vomeronasal epithelial rim, vomeronasal epithelium, VGs, and NVN. In cow, sheep, and roe deer, LEA stains the same structures in a similar fashion (Ibrahim et al., 2013; Jang et al., 2021; Salazar et al., 2000; Shin et al., 2017). Interestingly, STA staining produces the same pattern as LEA staining, labeling the luminal rim, vomeronasal epithelium, VGs, and NVN in dama gazelle, roe deer, and cow (Jang et al., 2021; Shin et al., 2017). Finally, the UEA lectin marked three vomeronasal structures in the VNO of the dama gazelle: the luminal rim, the vomeronasal epithelium, and the VGs. UEA labeled three vomeronasal structures in the dama gazelle VNO: the luminal rim, the vomeronasal epithelium, and VGs. This pattern was also observed in sheep, goat, roe deer, and cow, although faintly (Ibrahim et al., 2013; Jang et al., 2021; Park et al., 2013; Salazar et al., 2000; Shin et al., 2017). In cow, UEA also stains the NVN (Jang et al., 2021).

In summary, our study has contributed to filling an existing gap in knowledge regarding the structure of the VNO in wild ruminant species, as little information on the histological and neurochemical organization of the ruminant VNS is currently available. By investigating the dama gazelle VNO, we have gained valuable insights into the structure and function of this critical sensory structure in this endangered species. Because dama gazelle is a threatened species, the information obtained from our study can be used to design and implement programs utilizing pheromones to enhance breeding success and increase genetic diversity in captive populations, which may be critical for the survival of the species. Our work provides a solid morphological foundation for future research on this topic.

Finally, our study also significantly contributes to the general study of the VNO. By conducting a comprehensive histological and immunohistochemical study of the organ, we identified notable differences in the organization of the VD and the expression of various neuronal markers along the rostrocaudal VNO axis. These findings highlight the importance of considering such differences when conducting future studies of the VNO in other species. Overall, our study underscores the critical roles played by the VNO in mediating social and reproductive behaviors in dama gazelle and highlights the need for further research to better understand this unique sensory structure in other wild ruminant species.

AUTHOR CONTRIBUTIONS

Mateo V. Torres: Conceptualization; investigation; writing – original draft; methodology; writing – review and editing; formal analysis. **Irene Ortiz-Leal:** Conceptualization; investigation; writing – original draft; methodology; writing – review and editing; formal analysis. **Andrea Ferreira:** Investigation; methodology; data curation. **José Luis Rois:** Data curation; methodology; investigation. **Pablo Sanchez-Quintero:** Conceptualization; investigation; funding acquisition; writing – original draft; methodology; writing – review and editing; formal analysis; project administration; data curation; supervision; resources.

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CONFLICT OF INTEREST STATEMENT

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

DATA AVAILABILITY STATEMENT

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

ETHICS STATEMENT

All the animals employed in this study dead by natural causes.

ORCID

Mateo V. Torres  <https://orcid.org/0000-0002-7671-3379>

Irene Ortiz-Leal  <https://orcid.org/0000-0002-8468-3659>

Pablo Sanchez-Quintero  <https://orcid.org/0000-0002-9891-4817>

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