Smith, T. D., Downing, S. E., Rosenberger, V. B., Loeffler, J. R., King, N. A., Curtis, A. A., Eiting, T. P., & Santana, S. E. (2024). Functional microanatomy of the vomeronasal complex of bats. The Anatomical Record, 1–28. https://doi.org/10.1002/ar.2555728

Functional microanatomy of the vomeronasal complex of bats

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Funding: National Science Foundation grants IOS-2202272; IOS-2202271, and IOS-2202273.

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ABSTRACT

Recently, Yohe and Krell (The Anatomical Record, vol. 306:2765–2780) lamented the incongruence between genetics and morphology in the vomeronasal system of bats. Here, we studied 105 bat species from 19 families using histology, iodine-enhanced CT, and/or microCT. We focused on structural elements that support a functional peripheral vomeronasal receptor organ (vomeronasal organ, VNO), together comprising the "vomeronasal complex." Our results support prior studies that describe a functional VNO in most phyllostomid bats, miniopterids, and some mormoopids (most known *Pteronotus* spp.). All of these species (or congeners, at least) have vomeronasal nerves connecting the VNO with the brain and some intact genes related to a functional VNO. However, some bats have VNOs that lack a neuroepithelium and yet still possess elements that aid VNO function, such as a "capsular" morphology of the vomeronasal cartilages (VNC), and even large venous sinuses, which together facilitate a vasomotor pump mechanism that can draw fluid into the VNO. We also show that ostensibly functionless VNOs of some bats are developmentally associated with ganglionic masses, perhaps involved in endocrine pathways. Finally, we demonstrate that the capsular VNC articulates with the premaxilla or maxilla, and that these bones bear visible grooves denoting the location of the VNC. Since these paraseptal grooves are absent in bats that have simpler (bar-shaped or curved) VNCs, this trait could be useful in fossil studies. Variable retention of some but not all "functional" elements of the vomeronasal complex suggests diverse mechanisms of VNO loss among some bat lineages.

1 Introduction

The vomeronasal system (or accessory olfactory system) is a novel special sense that evolved in terrestrial vertebrates (Eisthen, 1997), and has cellular and functional similarities to the olfactory system (or main olfactory system). Both the vomeronasal and olfactory systems detect odorants using bipolar sensory neurons positioned in the nasal cavity (Weiler and Benali, 2005). Although both systems detect odorant molecules associated with social behavior (Kelliher, 2007), the vomeronasal system is thought to be specialized for molecules of relatively lower volatility and heavier molecular weight, whereas the olfactory system excels in detecting volatile (airborne) odorants (Baxi et al., 2006). Olfactory nerves carry axonal projections to the olfactory bulb, the first connection in the central nervous system; vomeronasal nerves convey axons to the accessory olfactory bulb, which is closely congruent with the main olfactory bulb.

Among numerous functions, the vomeronasal system is thought to be more specialized for detecting sociosexual signals than the main olfactory system, though the latter likely has overlapping, or at least synergistic function (Achiraman et al., 2010). Loss of the vomeronasal system has been documented in numerous vertebrates, most notably in all extant birds (Halpern and Martinez-Marcos, 2003). Perhaps the most intriguing examples of vomeronasal reduction have been identified among the orders Chiroptera and Primates. Numerous species of each order have completely lost the accessory olfactory bulb in the brain and the peripheral receptor site, the vomeronasal organ (VNO). In both orders, species that do possess a VNO vary in its microanatomy, to the extent that some bats and primates possess only a rudimentary VNO (reviewed in Bhatnagar and Meisami, 1998). The lack of a functional vomeronasal system in catarrhine primates (Old World monkeys, apes and humans) is

hypothesized to be the result of a "sensory trade-off;" social signals detectable via trichromatic vision (e.g., sexual skin swellings) may have obviated the need for odorant-mediated signals (e.g., Liman and Innan, 2003; Zhang and Webb, 2003; Webb et al., 2004).

No similar overarching hypotheses exist to explain vomeronasal system loss in bats. Unlike primates, extant bats within both of the two major, early-diverging clades (suborders Yangochiroptera and Yinpterochiroptera) exhibit significant variation in presence and absence of anatomical structures related to vomeronasal system functionality. As a result, for many decades, it has been unclear whether a functional vomeronasal system has re-evolved more than once in bats or has been lost independently many times in different bat families (Wible and Bhatnagar, 1996; Yohe et al., 2017). Genetic evidence has provided some resolution to this question. One of two vomeronasal genes, the vomeronasal-type 1 receptor gene (V1R), has been identified in bats and is thought to be under relaxed selection pressure in at least some bat lineages (Yohe et al., 2017). V1R genes encode for a receptor that activates the Transient receptor potential cation channel, subfamily C, member 2 (Trpc2), which appears to be essential for detection of odorants by the vomeronasal system (Liman et al., 1999). Regarding genes encoding for Trpc2, Yohe et al. (2017) concluded pseudogenization has independently evolved at least 13 times in bats.

Multiple independent losses of a functional vomeronasal system in bats may indicate selection pressure on this system is low across the entire chiropteran order, or that it may vary in different groups. However, an ecological explanation for these hypotheses has been elusive (Bhatnagar, 1980; Yohe et al., 2023). To add confusion, a small number of bats retain an epithelial tube that is likely a vomeronasal organ homologue, but do not possess a neuroepithelial region in the tube and lack the large glands that empty in the VNO of other bats that have a morphologically functional VNO (Cooper and Bhatnagar, 1976; Bhatnagar, 1980). Yohe and Krell (2023) advocated for the investigation of numerous anatomical features at or surrounding the peripheral receptor organ of the vomeronasal system, such as glands and vasculature. These features were previously investigated using histology (e.g., Cooper and Bhatnagar, 1976; Bhatnagar, 1980; Bhatnagar et al., 2006), but have more recently been identified using diffusible-iodine contrast enhanced computed tomography (diceCT) (Yohe et al., 2017). The absence or presence of these structures, which collectively were referred to as the "vomeronasal complex" by Bhatnagar (1980), may shed more light on the nature of VNO loss in multiple lineages of bats as well as the "mismatch" between genomic and morphological data (Yohe and Krell, 2023).

1.1 Definitions of vomeronasal complex components

Vomeronasal organ (VNO)

Here, VNO refers to the epithelial tube that is adjacent to or surrounded by the vomeronasal cartilages (VNCs), whether it bears functional characteristics (neuroepithelium) or is a presumptive rudiment (Fig. 1; Bhatnagar, 1980). Since there are numerous ducts that run longitudinally in the nasal septum of mammals, including bilateral ducts of anterior medial septal glands (Bojsen-Møller, 1964) or other septal glands (Smith et al., 2001a), ontogenetic data may be required to recognize the VNO, especially in the cases in which the organ undergoes rudimentation (Bhatnagar et al., 1996; Smith et al., 2012). The VNCs are key structures to aid in identifying the VNO because they surround the VNO in many

species, or at least maintain a stable spatial relationship in species where the organ becomes rudimentary (e.g., Smith and Bhatnagar, 2000; Smith et al., 2012).

Vomeronasal cartilage (VNC)

VNCs, sometimes referred to as anterior paraseptal cartilages, are paired inferomedial cartilages of the nasal capsule that surround or are near the VNO (Fig.1). Rostrally they typically merge with the lamina transversalis anterior, a plate of cartilage that serves to support the rostral end of the nasal cavity (de Beer, 1937). VNCs are commonly present, whether or not they actually encapsulate the VNO. Although when a VNO is absent or rudimentary, the term "paraseptal cartilage" is more commonly used to refer to VNCs, they are nonetheless homologous.

When the VNO is surrounded by the VNC, or by a bony capsule instead, the organ is subject to pressure exerted by adjacent venous blood sinuses that can compress the luminal contents, causing fluid to leave the VNO (Eccles, 1982); conversely, sympathetic innervation can cause vasoconstriction of these vessels, and because the VNO is positioned between the blood sinuses and the encircling cartilage or bone, the VNO lumen then expands and a syringe-like suction draws fluid into the lumen (i.e., vomeronasal "pump" mechanism – Meredith and O'Connell, 1979). In at least some mammals, the VNCs rest within a groove in the maxillary bone, termed the "vomeronasal groove" (Garrett et al., 2013). Here we employ the term paraseptal groove to refer to this same surface feature, emphasizing that these depressions are closely adjacent to the septum when present.

Vomeronasal glands

Chemical stimuli perceived by the VNO are fluid-borne, as is the case for the main olfactory neuroepithelium, which detects odorants suspended within the secretions of Bowman's glands. In mammals with a functional VNO, glands superior, lateral and/or posterior to the VNO communicate with the organ's lumen and are thought to perform functions similar to Bowman's glands (Lee et al., 2008).

Vomeronasal neuroepithelium and vomeronasal nerves

In vertebrates with a functioning VNO, a presumably essential feature of the vomeronasal complex is a neuroepithelium that contains bipolar vomeronasal sensory neurons that connect to axons in the surrounding lamina propria, which travel in vomeronasal nerve bundles (Fig.1) to the accessory olfactory bulb (Bhatnagar and Meisami, 1998). The neuroepithelium is frequently paired with a lateral, less extensive nonsensory epithelium, also termed the receptor-free epithelium (RFE, Breipohl et al., 1979), but some bats and other mammals also lack the RFE. It is also noteworthy that the VNO of some bats and primates transiently possesses a neuroepithelium during prenatal development (Smith and Bhatnagar, 2000; Smith et al., 2012).

In light of great increase in the number of recognized bat species over the past several decades (Simmons and Cirranello, 2024) and the rapid pace of recent discovery on evolutionary genetics of their vomeronasal system (Yohe et al., 2017; Yohe and Krell, 2023), our knowledge of interspecific variation in

vomeronasal complex pertinent anatomy requires an update. Here, we use a large sample of bat species to search for key elements of the vomeronasal complex, described above, that signal functionality of this chemosensory organ. We review prior findings and make novel observations on a previously studied histological sample (Bhatnagar, 1980), newly histologically sectioned specimens, and specimens imaged using diffusible-iodine contrast enhanced computed tomography (diceCT; Gignac et al., 2016). In addition, we examine the osteology of the palate using microcomputed tomography (microCT) to see if structures of the vomeronasal complex leave visible impressions on the maxillary and/or premaxillary bone, as has been observed in other mammals (Garrett et al., 2013).

2 Materials and Methods

2.1 Sample and visualization methods

One hundred thirty-seven specimens, representing 105 species from 19 families of bats were studied using three different visualization methods, histology (light microscopy), diceCT, and microCT (Table 1; Table S1). Most are adults, but selected fetuses were also studied to establish ontogenetic characteristics. All specimens that were diceCT scanned were also microCT-scanned. Some individual specimens were studied using all three methods, some were studied by histology alone, and some were only available in microCT scans alone. A total of 74 specimens were histologically sectioned, most of which were previously prepared and studied (Cooper and Bhatnagar, 1976; Bhatnagar, 1980; Bhatnagar et al., 2006; Bhatnagar and Smith, 2007). Twenty-five specimens were sectioned recently for unrelated purposes (e.g., Eiting et al., 2014; Smith et al., 2021) or were sectioned in the lab of KP Bhatnagar but had never been described regarding VNO morphology; this grouping includes 14 species that have never been examined histologically. Newly prepared specimens were paraffin embedded, sectioned, and stained similarly to prior samples (Bhatnagar and Smith, 2007). Every 4^{th} to 5^{th} section was mounted (depending on specimen size) and slides were stained alternately with hematoxylin-eosin or Gomori trichrome procedures. Intervening sections were saved for other procedures. In this study, selected intervening sections (~ every 20th section) were mounted and stained with a combined alcian blue 2.5 (AB)-periodic acid-Schiff (PAS) protocol. Some unstained sections were available from specimens previously studied by Bhatnagar (1980) and these too were prepared here.

The AB-PAS method is used to demonstrate different types of mucous secretions that cannot be detected using stains such as hematoxylin-eosin. This protocol uses two different histochemical stains since some mucous substances cannot be detected by one stain alone (Smith and Pinkstaff, 1982). Under this method, mucous secreting glands either appear blue (alcian blue) or magenta (periodic acid-Schiff) when viewed by light microscopy; acidic mucins are AB+, and neutral mucins are PAS+ (Humason, 1979). These mucins likely have multiple functions, such as generalized protection of deeper (connective) tissues; this may be particularly true for acidic mucins, which are secreted during exposure to allergens or microbes (Casado et al., 2005). More relevant to the current study, mucous secretions are essential to odorant detection, and glandular hyposecretion is one possible cause of olfactory dysfunction (Zhao et al., 2023). Odorant uptake depends, in part, on solubility within mucous, and solubility is dictated by mucin macromolecules that give mucous its gel-like nature (Yang et al., 2007; Kennel et al., 2019). Although the functional implications are unknown, glands of VNO are known to produce acidic and/or neutral mucins (Roslinski et al., 2000; Kondoh et al., 2020). We thus sought to determine the nature of mucins in vomeronasal glands, and to contrast these to other nearby nasal glands. When combined, AB

can mask PAS positivity (Smith and Pinkstaff, 1982), so selected sections from all species were mounted on glass slides and stained with PAS only.

The histological specimens were viewed and photographed using a camera (Axiocam MRc 5 Firewire, Zeiss, Oberkochen, Germany) attached to a photomicroscope (Leica DMLB, Leica Microsystems, Wetzlar, Germany) for high-magnification views (25–630 x) and a second camera (Axiocam MRc 150 Firewire, Zeiss, Oberkochen, Germany) attached to a stereomicroscope (Zeiss Stemi, Zeiss, Oberkochen, Germany) for low-magnification views (0.64–1.6 x). In selected specimens, ImageJ (Fiji, version 6.1.1) was used to measure cross-section area (in mm^2) of the vomeronasal neuroepithelium of the right VNO from micrographs photographed at x200; scaled to an image of a stage micrometer photographed at the same magnification. Cross-sectional areas were multiplied by intersectional distances and summed to compute neuroepithelial volume (in mm^3), to provide estimates of metric variability among species (Table S1).

DiceCT scans were accomplished at the University of Washington, Seattle (Santana, 2008). We used a Skyscan 1172 µCT-scanner (Bruker MicroCT, Belgium) for all the imaging procedures. The scanning resolution (14–30 µm) depended on specimen size, and we ran all scans at 50 kV and 800 µA with a 0.25 mm aluminum filter. We scanned each specimen prior to iodine staining. We then submerged each bat head in a 1% w/v aqueous Lugol's iodine solution until all tissues of interest were clearly visible in µCT cross sections (8–35 days). We verified iodine uptake by scanning each specimen every 3–5 days during the staining process and refreshed the iodine solution as needed. The diceCT scans were originally optimized to reveal muscle tissue (Santana, 2008), but to a varying degree revealed soft tissues of interest in the present study.

Specimens studied by microCT alone were scanned at the American Museum of Natural History Microscopy and Imaging Facility (https://www.amnh.org/research/microscopy-and-imaging-facility) using a GE V|tome|x micro-CT scanner with a 240 mV high power directional X-ray tube. Scan resolution depended on the size of the specimen and resolution required to resolve nasal skeleton and ranged from 0.0112mm - 0.0495 mm.

2.2 Assessment of the vomeronasal complex structures and related osteological correlates

The sample was studied to assess micro- or macroscopic features that reflect the presence of vomeronasal cartilages (VNCs), by either direct observation (histology or diceCT) or indirect observation (microCT). Whenever possible, osteology as visualized by microCT was evaluated within the context of histology of diceCT of the same species (and the same specimen, when possible). Our specific osteological evaluation was for the presence or absence of paraseptal grooves. These are defined as restricted depressions in the premaxilla or maxillary bone that are bilateral to the midline nasal septum, or site of septal articulation in microCT slices. To be identified as a distinct groove, each depression had to have raised medial and lateral margins, as previously described in primates (Garrett et al., 2013). Selected specimens were three-dimensionally reconstructed using Amira version 2020.3.1 software to depict the grooves, when present, as longitudinal furrows.

Histology and diceCT scans were used to glean novel information about soft tissue elements of the vomeronasal complex. In a portion of the sample, many elements of the vomeronasal complex were

previously described (Cooper and Bhatnagar, 1976; Bhatnagar, 1980; Wible and Bhatnagar, 1996; Bhatnagar et al., 2006; Bhatnagar and Smith, 2007). Prior observations are tabulated here, and updated in cases where previous reports were incomplete, or new observations are made. Some previously studied structures are newly described regarding functional characteristics. Specifically, vomeronasal complex elements are categorized as follows:

1. *Vomeronasal organ*

   Previously, the VNO has been described in bats as either "well-developed," "rudimentary," or "absent" with some intermediate conditions occasionally used (e.g., "moderately" developed) (e.g., see Cooper and Bhatnagar, 1976; Bhatnagar, 1980). Here, we assume that a neuroepithelium is essential to VNO function as a chemosensory organ, and we use the term "neuroepithelial VNO" to refer to those that possess both a neuroepithelium and bundle axons departing the basal aspect of the neuroepithelium. We refer to bilateral epithelial tubes that lack a neuroepithelium, *and* are coextensive with vomeronasal cartilages as "putative rudimentary VNOs." If ontogenetic samples were available to show tubular VNOs were also present with vomeronasal nerves at an earlier developmental stage, we refer to the adult tubes as "rudimentary VNOs." This distinction is possible because all vertebrates that possess VNOs in adults transiently pass through a stage in which the primordial VNO exists as a tube that is incompletely supported by cartilage (Dieulafe, 1906); in some mammals the VNO loses its central nervous system connections but are known to retain epithelial remnants in the form of tubes lacking a neuroepithelium (Smith et al., 2001a, b). A third state, VNO absence, has evolved multiple times within the order Mammalia (e.g., Smith et al., 2001b; Bhatnagar et al., 2001).

2. *Vomeronasal cartilage*

   Wible and Bhatnagar (1996) described the VNC as absent, "bar-shaped" or a third state in which the VNC cross-sectionally had the shape of an "O," "J," "C," or "U." Here, we modify these descriptors with reference to the VNC's role in VNO stimulus acquisition (i.e., vomeronasal "pump" mechanism). Any VNC that surrounds the VNO both medially and inferiorly is referred to as a "capsular" VNC, with the assumption that the cartilage encircles and supports the VNO as well as the nearby tissues (venous sinuses, nerves). Such a cartilage might be O-, C-, U-, or J-shaped. Here, VNCs that are straight or slightly curved in cross-section are referred to as "simple" in morphology.

3. *Vomeronasal glands*

   Here, we re-evaluate glands and their communications, specifically whether glands in the VNC region empty into the lumen of the VNO, if present, or whether their ducts empty into the nasal cavity, and thus should properly be called "septal glands."

4. *Vomeronasal neuroepithelium and vomeronasal nerves*

The vomeronasal neuroepithelium is distinguished by the presence of basal cells, apical supporting cells, and an intermediate region comprising the cell bodies of bipolar sensory neurons (Stowers and Spehr, 2015). All bats previously described to have a VNO neuroepithelium have vomeronasal nerves that connect the VNO to the AOB. Prior studies have also described small populations of ganglionic masses that are found near the VNO of vomeronasal nerves (Bhatnagar, 1980).

3 Results

3. 1 Osteology of the palate and morphology of the vomeronasal cartilages across bat families

3.1.1 Pteropodidae

All pteropodids have a similarly smooth osseous palate in the region caudal to the incisive foramina (i.e., paraseptal grooves are absent). The vomeronasal cartilages are simple in morphology, cross-sectionally triangular or bar-shaped. In all species, toward the caudal half they are simple upright bars (Fig. 2a), and this is easily detectable in diceCT slices of some specimens (e.g., *Epomophorus wahlbergi*, Fig. 2b), but some scans did not permit detection of cartilage (*Rousettus aegyptiacus*). When cartilage is easily detected, it is far less radio-opaque than adjacent mucosa or connective tissues, including bone (Fig. 2b).

3.1.2 Rhinolophidae

The VNC of both *Rhinolophus lepidus* and *Rh. eloquens* articulates with the premaxilla, creating an indentation on the lateral sides of the palatine process (Fig. 2c). There is no discernable groove on the maxilla more caudally. In both species the VNC appears slightly curved; the lateral margin does not appear to curve upward based on diceCT slices (Fig. 2c). The VNC is capsular in *Rh. lepidus,* with a slight upward curve of the lateral margin (Fig. 2c), although the palate does not bear a distinct groove.

3.1.3 Hipposideridae

In all hipposiderids in our sample, the VNC articulates with caudally projecting palatine process of the premaxilla, as in *Rhinolophus* spp. Cross-sectionally, the premaxilla exhibits an indentation on the superior surface where the VNC articulates in *Hipposideros* spp. (Fig. 2c). There is no discernable groove on the maxilla more caudally in *Hipposideros* spp. (Figs. 3a, b). VNCs are vertically elongated and curve laterally toward the palate. In *Hipposideros lankadiva*, the lateral edge of the VNC curves upward slightly (Fig. 3d), and the morphology might be described as capsular; this is not the case for *Hi. caffer* (Fig. 3c, inset). MicroCT slices and 3-dimensional reconstructions of the palate of *Aselliscus tricuspidatus* also reveal deep bilateral paraseptal grooves along the palatine process of the premaxilla.

3.1.4 Rhinonycteridae

Cross-sectionally, the premaxilla exhibits an indentation on the superior surface where the VNC articulates in *Triaenops afer* (Fig. 2d). DiceCT slices did not allow resolution of the lateral edges of the VNC in *Tr. afer.*

3.1.5 Megadermatidae

In *Megaderma lyra*, the VNC is capsular in morphology, "cupping" the rudimentary VNO (Fig. 2e). The VNC of *Cardioderma cor* is similar (Fig. 2f-h). In *Lavia frons,* the VNC is a simple curved rod in cross-section (Fig. 2i). There is pronounced groove on the maxilla where the VNC articulates in *Me. lyra* (Fig. 2e), and a more subtle depression in the same location in *Ca. cor*. In *La. frons*, no impression is discernable on the maxilla where the VNC articulates.

The lumen of a likely tubular VNO rudiment in *Cardioderma cor* is clearly visible in diceCT slices, cupped by the cartilage (Figs. 2f-h). The VNC that surrounds it is strongly curved; but, while it curves beneath the putative VNO, it does not rise up laterally to "encapsulate" it. A specimen of *Megaderma spasma,* examined only by microCT, has large soft tissue masses residing dorsal to shallow paraseptal grooves. A second specimen of *Ca. cor,* examined only by microCT, has large soft tissue masses residing dorsal to prominent paraseptal grooves. In *Lavia frons,* no paraseptal grooves could be identified in two specimens using microCT. No paraseptal grooves are apparent in *Macroderma gigas* based on microCT-based osteological observations.

3.1.6 Rhinopomatidae

The VNC of both *Rhinopoma* spp. is capsular in morphology, and the newly described morphology of *Rh. hardwickii* is identical to prior descriptions of *Rh. microphyllum* (Cooper and Bhatnagar, 1976; Wible and Bhatnagar, 1996): Rostrally the VNC is J-shaped, laterally opened. Moving caudally, the VNC becomes C-shaped and closes laterally, becoming O-shaped. For most of its length, the VNO is surrounded by the O-shaped VNC. However, caudally the VNC becomes C-shaped again; this last transition is seen in Fig. 2j ("O"-shaped on left side of figure; "C"-shaped on right side of figure). The capsular VNCs articulate with the maxilla, where this bone exhibits pronounced paraseptal grooves. Three dimensional reconstructions from microCT scan volumes reveal these correspond to deep bilateral concavities at the rostral end of the palatal processes of the maxillary bone (not shown).

3.1.7 Craseonycteridae

No histology or diceCT of *Craseonycteris thonglongyai* is available to us to assess VNC morphology.

MicroCT slices of *Craseonycteris thonglongyai* reveal a subtle depression located adjacent to septal cartilage and mucosa. The septal cartilage is assumed to be central within the septum, since no bone is observed there (Fig. 2k). The soft tissue mass at the base of the septum is relatively wide, but is uniform in grayscale, permitting no discernment of cartilage morphology. However, the soft tissue region is proportionally wide enough to house a capsular VNC, and potentially a VNO.

3.1.8 Molossidae

In all species in which the VNC could be visualized in by histology (*Molossus* spp.; *Tadarida mexicana*) or diceCT (*Mops condylurus* and *Eumops glaucinus*) the VNCs are superoinferiorly elongated, and slightly curved (Figs. 3a, b; Fig. S1a). In *Tadarida mexicana,* histology reveals the VNCs make no contact with bone. Paraseptal grooves are not seen on the premaxillary of maxillary bones in any molossid in our sample except one adult *Platymops setiger*, in which there are paired oval depressions just caudal to the incisive foramen.

3.1.9 Vespertilionidae

Previously, VNCs were described as bar-shaped in cross-section in all vespertilionids (Bhatnagar, 1980; Bhatnagar et al., 2001). Based on new histological or diceCT observations, this is also the case for *Corynorhinus townsendii, Hypsugo crassulus* (Fig. S1b), *Lasiurus borealis, Myotis velifer* (Fig. 4c)*, Nycticeinops schlieffeni* (Fig. S1c), *Parastrellus hesperus* (Fig. 4d), and *Scotophilus dinganii* (Fig. 4e). In most species, the VNCs make no direct contact with the hard palate, and no paraseptal grooves are visible. In histologically sectioned *Perimyotis subflavus* and *Plecotus rafinesquii*, the caudal end of the bar-shaped VNCs make direct contact with the maxilla and leave slight rounded impressions in cross-section. Most caudally, the VNCs of *Pe. subflavus* are surrounded by bone of the maxilla. MicroCT scan slices series and reconstructions reveal no paraseptal grooves in the osseous palate of any vespertilionid bats except *Miniopterus* spp.

3.1.10 Miniopteridae

In *Miniopterus australis*, *Mi. magnater* and *Mi. schreibersii,* the VNC is capsular ("C"- or "J"-shaped), and a distinct groove is visible in the maxilla where the VNC articulates (Figs. 4f, 5). In all histologically sectioned *Miniopterus,* the VNO and VNC can also be tracked next to the palatine process of the premaxillae, rostral to the paraseptal groove. The duct of the vomeronasal organ opens just rostral to the premaxilla (Fig. 5b).

In both *Mi. magnater* specimens and in *Mi. australis*, the VNC ends before the caudal end of the VNO, and the VNC merges with the palate. The VNO and VNC end nearly at the same level in *Mi. schreibersii.* In one *Mi. magnater* specimen, the most caudal part of the VNC is a small nodule embedded in the palate, suggesting the caudal end either degenerates or is ossified with age, as has been described for some other mammals (e.g., Garrett et al., 2013).

In *Mi. inflatus,* the VNC is not easily visualized, but the VNO lumen is visible in diceCT scan slices. Like all *Miniopterus* spp. the VNOs reside above a groove in the maxilla and are separated from the maxilla by space sufficient for cartilage (Fig. 4f), so a capsular VNC is likely.

3.1.11 Emballonuridae

In *Rhynchonycteris naso,* histological sections reveal no paraseptal grooves in the rostral end of the palate, which is formed solely by the maxilla. The VNCs are simple in form, slightly curved anteriorly and straight upright bars in cross-section. The VNCs end rostral to the bony palate, making no contact with bone. Histology of *Balantiopteryx* spp. reveals simple VNCs, slightly curved in *B. io* and straight bars in *B. plicata.* The VNCs have no overlap with the maxilla in either *Balantiopteryx* spp., and no paraseptal grooves are detected on the bony palate. In *Saccolaimus saccolaimus*, VNCs are upright bars in cross-section, transitioning caudally to more rounded rod-shaped ending.

No paraseptal grooves are visible in the rostral palate of *Centronycteris centralis, Cormura brevirostris, Diclidurus scutatus, Emballonura alecto,* or *Saccolaimus flaviventris* based on microCT scans.

3.1.12 Mystacinidae

Although it was previously reported that the VNC is lacking in this species (Wible and Bhatnagar, 1996), we can provide a correction here. In histological sections of *Mystacina tuberculata*, the VNCs are vertical, simple bars in cross-section. They have a slight curvature rostrally in that the inferior margin deviates laterally. They are mostly positioned rostral to the maxillary part of the hard palate, in the region of the incisive foramen. The rostral end of the palatine processes of the maxilla end in a median spine. The very caudal ends of the VNCs create shallow grooves bilaterally on the sides of the median spine.

3.1.13 Furipteridae

No paraseptal grooves are visible in the rostral palate of *Furipterus horrens.* This implies the lack of a capsular VNC, but no histological material is available for confirmation.

3.1.14 Natalidae

In *Natalus stramineus*, the VNCs are vertical, simple bars in cross-section, with no curvature; caudally they end as rounded bars. The VNCs articulate extensively with the palate. The palate (maxillary part) has a raised shelf in the midline which articulates with the nasal septum, and the VNCs articulate on the lateral sides of this raised shelf, leaving slight concave impressions in cross-section. However, the VNCs do not articulate with the palate more laterally, and no paraseptal grooves are evident in cross-section.

3.1.15 Noctilionidae

Based on histological sections, the VNCs are vertical, simple bars in cross-section, with no curvature] in *Noctilio leporinus*. The VNCs articulate extensively with the palate but create no paraseptal grooves on the palate more laterally. The VNCs are bar-shaped (simple) in morphology.

3.1.16 Nycteridae

The VNCs were previously considered absent (Bhatnagar, 1980). He described "ventrolaterally-directed prongs" of the nasal septum which he considered potentially related to the VNCs. We can elaborate on these prior observations in that these same cartilaginous projections lead to a nearly separate bar on the animal's right side, with perichondrial bone matrix observed ventrally, whereas on the left side the prong disappears into a mass of bone (Fig. S2).

There are no distinct impressions of VNCs on microCT slice series.

3.1.17 Phyllostomidae

Prior work has established capsular VNCs in all known phyllostomids except *Brachyphylla cavernarum* (e.g., Bhatnagar, 1980; Bhatnagar et al., 2006; Bhatnagar and Smith, 2007). In *Brachyphylla cavernarum,* the VNCs are bar-shaped rostrally with a slight depression facing the rudimentary VNO (the latter described below). Caudally, the VNCs are slightly curved. In specimens studied histologically for the first time, the degree to which the VNCs overlapped the VNO rostrocaudally varied. In *Uroderma bilobatum* and *Micronycteris megalotis*, the VNC extended caudally beyond to caudal end of the VNO. In *Monophyllus redmani* and *Macrotus waterhousii*, the VNC ends rostral to the ending of the VNO. In *Ma. waterhousii*, caudal parts of the VNC are ossified and create extended lateral "lips" of the paraseptal grooves.

Most other phyllostomids in our sample exhibit paraseptal grooves in the osseous palate (Figs. 6a, b, f-h; Fig. 7), in particular on the palatal processes of the maxilla. The maxilla forms a raised "shelf" or platform in which deep paraseptal grooves are seen in *Chiroderma villosum* (Figs. 6d, e) and *Sturnira lilium*. A raised maxillary raised shelf is also seen in *Phyllostomus hastatus,* but it is proportionally shorter relative to palatal length, and with shallow grooves; diceCT of the same specimen did not allow clear visualization of VNC morphology. Although most of the length of the VNCs are located rostral to the horizontal plates of the maxilla in a vampire bat species, the caudal end of the VNCs create paraseptal grooves; these grooves are at least partially on raised shelves in *Diaemus youngi* and *Diphylla ecaudata,* but not in *Desmodus rotundus.*

Uroderma bilobatum bears no evidence of paraseptal grooves on the maxillary bone, in either histology or microCT slices (Fig. 6c). Histology of *Uroderma bilobatum* clearly reveals the capsular VNC is spatially elevated from the palate, but unlike other species such as *Chiroderma villosum* and *Sturnira lilium,* the slightly raised shelf in the midline of the palate is not directly in contact with the VNCs. A single sample of *Vampyressa bidens* bears no discernable groove, only slight bilateral depressions just anterior to the incisive foramina. However, diceCT of this specimen does permit visualization of a tall, oval VNO lumen bilaterally, with venous sinuses lateral to the lumina.

In two phyllostomids available for study only by microCT data, *Platalina genovensium* and *Chrotopterus auritus*, there are osteological features relating to vomeronasal cartilage articulation and, radio-opaque soft tissue masses that are consistent with VNOs. In *P. genovensium* there are prominent paraseptal grooves present. In *Ch. auritus*, the soft tissue masses reside against raised "shelves" that may constitute vomerine alae.

3.1.18 Mormoopidae

In histological sections of *Mormoops megalophylla*, the capsular VNC (described in more detail in Bhatnagar, 1980) articulates with a caudal projection of the premaxilla. In a microCT-scanned specimen, the caudal projection flattens near its articulation with the maxilla, and there the premaxilla exhibits bilateral paraseptal grooves. In *Mo. blainvillei*, paraseptal grooves are also visible, but it is difficult to visualize VNC morphology in diceCT slices (Fig. 6i).

In histologically sectioned *Pteronotus macleayii* and *Pt. quadridens*, the capsular VNCs extensively overlap the caudal end of the premaxilla, the incisive foramen, and the maxilla. These cartilages are associated with paraseptal grooves in the maxillary bone only (especially well-defined grooves in (*Pt. macleayii*).

3.1.19 Thyropteridae

In histologically sectioned *Thyroptera tricolor*, the VNC is capsular. Rostrally, it is positioned above the incisive foramen; passing caudally, the VNCs are positioned within distinct paraseptal grooves, which are readily seen in microCT frontal slices as well as 3-dimensional reconstructions (Fig 8).

TABLE 1 here

3. 2 Histology-based and diceCT-based microanatomical results across bat families

For Pteropodidae, Rhinonycteridae, Craseonyteridae, and Furipteridae, no new histologically sectioned were studied in this report. DiceCT of *Triaenops afer* (Rhinonycteridae) and newly examined pteropodids did not permit detection of VNOs, if they were present.

3.2.1 Rhinolophidae

Rhinolophus lepidus was examined previously in detail by Cooper and Bhatnagar (1976). In this study, we prepared selected unstained sections of the same specimen using the AB-PAS procedure. The glands near the posterior end of the rudimentary VNO, which communicate with the VNO lumen, are AB+/PAS-. The VNO does exhibit unicellular glands in its epithelium, previously reported to be PAS + (Cooper and Bhatnagar, 1976). Here we observed dark purple stained mucins in these cells, suggesting AB reactivity (denoting presence of acidic mucins) in addition to the PAS staining (Fig. 9a).

There are numerous venous sinuses in the vicinity of the VNO, but they appear to be part of vessels that ascend along the septum, from the palate to “roof” of the nasal cavity, a pattern noted previously for many bats (Smith et al., 2022).

DiceCT permitted soft tissue observations of *Rhinolophus eloquens.* In this species, adjacent to the VNC on each side, there are lumina visible; on one side there is a radio-opaque lining that surrounds the lumen, which may indicate an epithelial tube – a possible VNO rudiment (Fig. 2c).

3.2.2 Hipposideridae

In *Hipposideros lankadiva*, the rudimentary VNO (simple cuboidal, ciliated in structure) has AB+ mucous adherent to its apical cilia (Fig. 9b, inset). The source of the mucous is unclear, since nearby glands that connect to the VNO are AB-/PAS+ (Fig. 9b); however, these gland masses are not as densely packed with acini as nearby septal glands, which are also AB-/PAS+. Few unstained sections remained for study, and we cannot exclude the possibility that there are AB + glands somewhere along the rostrocaudal length of the VNO, or possibly unicellular AB+ glands in the epithelial tube itself. There are scattered venous sinuses seen in cross-section; most are not distributed lateral to the VNO.

Based on diceCT slices, no epithelial tubes adjacent to the VNC could be detected in *Hi. caffer*.

3.2.3 Megadermatidae

Fetal and adult *Megaderma lyra,* previously described in Smith et al. (2012) were reexamined here regarding neuronal cells in the VNO of all specimens. We confirm the observations of Cooper and Bhatnagar (1976) regarding small clusters of neuron-like cell bodies in the VNO wall; some of these have a ganglionic appearance, and these are easily seen in all fetal specimens, where they are associated closely with vomeronasal and perhaps other (e.g., cranial nerve 0) nerve bundles (Fig. S3). No discrete neuroepithelium, as contrasted with a lateral RFE, is seen at any age.

A putative VNO in *C. cor,* viewed in diceCT slices, was described above. Histological confirmation is required since the type of epithelium that lines the lumen cannot be resolved. However, the lumen opens rostrally in the nasal cavity (Fig. 2f), as does the vomeronasal organ of many bats. Based on diceCT scans, *Lavia frons* lacks any clear epithelial structures. Large paraseptal soft tissue masses are visible in microCT slices of *Macroderma gigas* and *Megaderma spasma;* these masses could contain VNOs or VNO rudiments.

3.2.4 Rhinopomatidae

Our observations on a newly sectioned specimen of *Rhinopoma microphyllum* match the findings of Bhatnagar et al. (1980) on this species. The VNO of *Rh. hardwickii* matches the same description. At high magnification the VNO may be described the same way in both species, the VNO lacks a distinct sensory epithelium, and is uniformly lined with stratified cuboidal columnar or simple columnar epithelium (changing throughout rostrocaudal length in both species). The walls of the VNO are lined with stratified columnar or cuboidal epithelium. Caudally, the epithelial lining thins, and branches into multiple ducts. Apically, both species bear short cilia (kinocilia), coated with AB+ mucins, facing the VNO lumen (Fig. 10a).

The tissues adjacent to the VNO, packed together within an enclosed VNC for nearly the entire length of the VNO, are similar in both species. The majority tissue type is adipose. Numerous venous sinuses surround the VNO, at some intervals neighboring it on all sides. In both species, some of these are cross-sectionally as large or larger than the VNO. No compound glands are found near the VNO. Although there are large lightly PAS + gland masses dorsal to the VNC on each side, these do not transmit ducts to the VNO. We concur with Bhatnagar (1980) that these are septal glands of the nasal cavity.

Like Bhatnagar (1980), we find that no nerve bundles near the VNO transmit back to the septum; we found small ganglion-like masses within the confines of the capsular VNC in *Rh. hardwickii*, as described previously for *Rh. microphyllum.*

3.2.5 Molossidae

In the newly sectioned *Molossus pretiosus,* there are tubular simple cuboidal/columnar epithelial structures lateral to the VNC; without developmental data, their identity remains uncertain. However, given that these are not symmetrical, and occur in greater numbers on the left than on the right sides, it seems doubtful that these are VNO homologies. Given that, it seems *Mo. pretiosus* resembles *Mo. molossus* in lacking a VNO.

In *Tadarida mexicana,* there are no nerves emanating from, or gland ducts communicating to the rudimentary VNO. Venous sinuses are present, but they are relatively small and appear to ascend as they course posteriorly.

No structures resembling tubular epithelial tubes are visible in diceCT slices of *Mops condylurus* or *Eumops glaucinus*.

3.2.6 Vespertilionidae

Newly sectioned vespertilionids examined here, including *Corynorhinus townsendii, Myotis velifer* and *Parastrellus hesperus,* support prior conclusions that no structures resembling vomeronasal organs are found in adults of this family. No epithelial structures resembling VNOs could be detected in diceCT scan slices of specimens studied using diceCT, but not histology (*Glauconycteris argentata, Hypsugo crassulus, Neoromicia capensis, Nycticeinops schlieffeni, Scotophilus dinganii*).

3.2.7 Miniopteridae

In both newly studied histologically sectioned *Miniopterus* spp.*, Mi. australis* and *Mi. magnater* (Fig. 11a, b), the VNO has a thick inferolateral neuroepithelium, and a thinner lateral receptor-free epithelium. The neuroepithelium has multiple rows of sensory neurons; the vomeronasal nerve bundles are prominent in the deeper lamina propria (Fig. 11b). The receptor-free epithelium bears short cilia. Neuroepithelial volume was measured in the two *Mi. magnater* specimens at 0.031 and 0.041 mm^3 (Table S1).

Large gland masses are seen dorsal to the VNO, and large venous sinuses are found next to the receptor-free-epithelium (Fig. 11a). Two species (*Mi. magnater* and *Mi. schreibersii*) that were stained with the AB/PAS method present similarly (Table 2). Most vomeronasal and septal gland acini are AB-/PAS-, but sparse AB+ and PAS+ parts of some gland acini are observed.

3.2.8 Emballonuridae

Previous descriptions of *Balantiopteryx io* established the presence of putative bilateral VNO rudiments (Bhatnagar, 1980); its structure (not previously described) is simple cuboidal epithelium. Whether *Ba. plicata* also possesses such epithelial tubes in the vicinity of the VNC is unclear; some epithelial tubes are present near the VNC, but tissue preservation is poor, preventing description of morphology.

Bhatnagar (1980) described large vomeronasal glands dorsal to the putative VNO in *Balantiopteryx io*. Although the VNOs end closer to the gland masses in this specimen, we do not detect direct unions between the VNO and glands (Fig. S4). Instead, these glands, which are lightly PAS + and AB-, instead appear to empty into the nasal cavity at intervals along the length of the bulging gland masses. Some internal ducts may transmit secretions rostrally, but do not approach the VNO. Because of lack of communication with the VNO, these are likely instead septal glands. In *Balantiopteryx plicata*, there is a similar glandular tissue mass dorsal to the VNCs, also emptying into the nasal fossa.

Rhynchonycteris naso lacks an identifiable VNO. There are gland masses dorsal to VNC, which are AB-/PAS-. These glands clearly open straight into the nasal cavity.

We have no additional findings on the emballonurid *Saccolaimus saccolaimus*, which was previously found to lack a VNO.

3.2.9 Mystacinidae

Our observations on *Mystacina tuberculata* support a prior assertion that they lack a VNO (Wible and Bhatnagar, 1996).

3.2.10 Natalidae

Our observations on *Natalus stramineus* match prior findings: the species lacks a VNO (Bhatnagar, 1980).

3.2.11 Noctilionidae

Our observations on *Noctilio leporinus* matched previous observations made by Bhatnagar (1980) on *Noctilio leporinus*: the species lacks a VNO.

3.2.12 Nycteridae

Previously, the VNO was reported to be absent *Nycteris thebaica* (Bhatnagar, 1980; Wible and Bhatnagar, 1996); this is reaffirmed upon additional inspection.

3.2.13 Phyllostomidae

In most phyllostomids, glands communicating with the organ on the dorsal side are intensely PAS+, and to a greater degree than septal glands (Figs. 9c-f; 10b-d; Table 2). Many species also exhibit sparse AB reactivity in vomeronasal glands (Table 2). *Sturnira lilium* shows AB reactivity of all nasal glands in the vicinity of the VNO, but is also in a poorer state of preservation than most other specimens prepared with the AB-PAS procedure. All species had AB+ secretions coating the epithelial apex. Some of the specimens have PAS+ sensory neurons in the neuroepithelium (Figs. 10b, c).

As reported by Bhatnagar (1980), all phyllostomids except *Brachyphylla cavernarum* have a VNO with a ventromedial neuroepithelium and a lateral receptor-free epithelium. The thickness of the neuroepithelium varied greatly, as did the number of rows of sensory neurons within it. Fig. 10b-d illustrates this well, showing the thinner neuroepithelium of *Carollia perspicillata* and *Macrotus waterhousii* compared to that of *Diaemus youngi*. Volume of the VNO neuroepithelium varies greatly, from 0.0133 mm^3 (*Micronycteris megalotis*) to 0.149 mm^3 (*Anoura geoffroyi*, data from Bhatnagar and Smith, 2007). *Anoura* spp. and *Diaemus youngi* had the largest neuroepithelial volumes (0.10 to 0.149 mm^3; Table S1). In specimens studied histologically for the first time, the morphology of the receptor-free epithelium varied. In *Ma. waterhousii* and *Mi. megalotis,* this epithelium was simply cuboidal/columnar and ciliated. In *Monophyllus redmani* and *Uroderma bilobatum*, the receptor-free epithelium varies across the length of the VNO between simple or stratified cuboidal and has no cilia, or sparse patches (in *Ur. biolatum*).

Here, a fetal *Brachyphylla cavernarum* is described, for the first time, for comparison to the adult specimen. The fetal *Brachyphylla* (Fig. 12a) has bilateral VNOs are bilateral epithelial tubes adjacent to the base of the nasal septum, and just above the vomeronasal cartilages, identical to descriptions of the adult (Bhatnagar, 1980). The fetal VNO wall is densely cellular, with nerve bundles departing the dorsal side (Fig. 12b). Intermingled among nerve fascicles are large cell bodies of a ganglionic appearance.

In the adult, additional observations are possible that expand on descriptions of Bhatnagar (1980; Fig. 12c). The epithelial morphology of the VNO wall varies from pseudostratified columnar epithelium to simple columnar/cuboidal. Caudally, the epithelial tubes divide into multiple lumina caudally; they end as blind-ended branched tubes. We do not detect communications with glands. The VNO is somewhat thicker on its dorsal side in many sections, and includes sparse cells that have a bipolar appearance (Fig. 12d, arrows). At the caudal end of the VNO in adult *Brachyphylla cavernarum*, large bundles of nerve fascicles can be seen ascending within the septal mucosa (Fig. 13a). Nested among nerve fascicles are ganglionic cell bodies that are well vascularized; a capillary is visible penetrating the center of one nerve-ganglia bundle (Fig. 13b). There are numerous blood-filled sinuses at both ends of the nerve (Fig. 13b-d).

3.2.14 Mormoopidae

Pteronotus quadridens has AB + glands communicating with it; septal glands are also AB +/ PAS -.

Two specimens of *Pt. macleayi*, previously described by Bhatnagar et al. (2006) were studied further to quantify neuroepithelial volume. Female VNO neuroepithelial volume is 0.017 mm^3; for the male neuroepithelial volume is 0.0162 mm^3 (Table S1).

3.2.15 Thyropteridae

No additional findings are reported on the VNO of *Thyroptera tricolor*, which was previously described as rudimentary (Bhatnagar, 1980). Laterally, the VNO is not encircled by cartilage, but it is here that large venous sinuses are adjacent to the VNO. One specimen of *Thyroptera discifera*, examined by microCT only, appears to reveal the lumina of paired VNOs positioned against paraseptal grooves (Fig. S5).

TABLE 2 here

4 DISCUSSION

The absence of the VNO in some bat species has been known for over a century (Broom, 1895, 1897). Loss of a functional vomeronasal system in many of the same species was subsequently confirmed when they were also shown to lack an accessory olfactory bulb (e.g., Frahm and Bhatnagar, 1980; and see review by Meisami and Bhatnagar, 1998). Subsequently, genetic data linked to functionality of an ion channel that facilitates pheromone detection largely matched the trends described based on morphological data (Yohe et al., 2017, 2018a). Here, we have made novel observations on bats regarding specific elements of the vomeronasal complex.

4.1 Histological observations: Newly described species and re-evaluation of key functional elements

4.1.1 Implications of phylogenetic patterns

Fourteen bat species were newly described here by histology. In addition, newly sectioned specimens of previously studied species, including *Carollia perspicillata, Glossophaga soricina*, and *Rhinopoma microphyllum*, confirm prior morphology-based conclusions about these species (functional vomeronasal system for both phyllostomids and a rudimentary VNO in *Rh. microphyllum*).

Our observations also confirmed several prior conclusions at the family level. First, it has been previously established that the family Phyllostomidae is unique in the nearly ubiquitous presence of a functional VNO as indicated by both morphological (e.g., Bhatnagar, 1980; Bhatnagar and Smith, 2007) and genetic (Yohe et al., 2018b) data. In this study, we verify presence of a vomeronasal complex complete with all elements indicating VNO function (neuroepithelium, vomeronasal nerves, communicating vomeronasal glands, capsular VNC, and venous sinus(es) in *Micronycteris megalotis,*

Monophyllus redmani, and *Uroderma bilobatum*. We also confirm that *Brachyphylla cavernarum* possesses a VNO rudiment (Bhatnagar 1980), but ontogenetic data suggest intriguing novel functions are possible (see below). Unfortunately, histology or diceCT are as yet unavailable for another phyllostomid genus, *Choeroniscus*, that bears evidence for relaxed selection pressure on Trpc2 genes (Yohe et al., 2017, 2018b).

Second, prior work has established that absence of a vomeronasal system is characteristic of many vespertilionid bats, as evidenced by the lack of a VNO in all species examined to date. Here, detailed examination of five additional vespertilionids confirms prior observations that a functional vomeronasal system is absent in this family; *Corynorhinus townsendii, Myotis velifer,* and *Parastrellus hesperus* all lack a VNO (even as rudiment), a capsular VNC, as well as vomeronasal glands or venous sinuses in the immediate lateral vicinity to the VNC.

Third, our findings also confirm prior observations in miniopterids in that both *Miniopterus australis* and *Mi. magnater* possess all elements of the vomeronasal complex in a fully developed state. This confirms the inference of a functional vomeronasal system in these species based on accessory olfactory bulb presence (Bhatnagar and Meisami, 1998) and supports genetic data on other miniopterids (Yohe et al., 2017). In addition, miniopterids possess a capsular VNC, venous sinuses lateral to the VNO, vomeronasal glands, and vomeronasal nerves.

Fewer specimens of other families are newly studied here. Our findings support most prior conclusions that in all known emballonurids and molossids, the vomeronasal system is nonfunctional (Bhatnagar and Meisami, 1998; Yohe et al., 2017). One conflicting interpretation may be found in Orr et al. (2016), who categorized the VNO as present (and not rudimentary) in one molossid (*Eumops auripendulus*) but rudimentary or absent in all other molossids. The latter has been asserted previously regarding this bat family, based on the presence of rudimentary VNOs at most, and the absence of an accessory olfactory bulb in all studied species. Of the sources listed to support their categorization of VNO functional state (see Tables S1-2 in Orr et al., 2016), only Hayden et al. (2014) mention a VNO as "present" in *Eumops auripendulus.* However, the sources that Hayden et al. provide (Wible and Bhatnagar, 1996; Bhatnagar and Meisami, 1998) on VNO presence/absence do not state a VNO (functional or rudimentary) is present in this species. Accordingly, we consider it remains the case that no molossids that have been investigated to date possess a functional VNO. The putative VNO rudiment in *Tadarida mexicana* (Bhatnagar, 1980) requires further ontogenetic investigation.

4.1.2 Neural elements

The presence of a neuroepithelium in a newly studied phyllostomid and two *Miniopterus* spp. was not surprising. A more specific observation on several specimens with vomeronasal neuroepithelia, that some vomeronasal sensory neurons are PAS+, has not been reported previously in bats to our knowledge. In mice, this is thought to typify aging neuroepithelia (Mechin et al., 2021). As relatively long-lived mammals, it may be the case that many bats bear this age-related change, but since the neuroepithelia are nonetheless well-populated with sensory neurons, there is no indication of age-related decline.

We also report the first evidence that VNOs of species lacking accessory olfactory bulbs or intact genes relating to VNO function nonetheless have ganglionic and perhaps other neuronal bodies associated with nerves that connect to the VNO in fetuses, and then persist postnatally. It should be added that ganglionic masses, termed vomeronasal ganglia, were observed in many bat species by Bhatnagar (1980). These occur in bats with and without a neuroepithelial VNO. This hints that there are additional functions for neural structures that develop near (and perhaps include prenatal cells of) the VNO. The highly vascularized state of the ganglionic-nerve complexes in adult *Brachyphylla* may indicate an endocrine function, as already hypothesized for terminal nerve ganglia (Ma et al., 2015).

4.1.3 Glandular structures

The exact function of vomeronasal gland secretions is unknown. It is hypothesized that the secretions of nasal or even orbital glands may produce or at least contain odorant-binding proteins that facilitate perception of semiochemicals through mechanisms that remain poorly understood (Stopková et al., 2016; Pelosi and Knoll, 2022; and see Hillenius and Rehorek, 2005). Patterns of vomeronasal gland mucins have been characterized by many investigators. There are reported phylogenetic patterns of mucin histochemistry in vomeronasal glands (Kondoh et al., 2020), but in general most mammals have been reported to exhibit reactivity of vomeronasal glands to the neutral mucin stain, PAS (e.g., Salazar et al., 1997; 2003; Roslinski et al., 2000; Kondoh et al., 2020).

In our new observations on gland masses of bats, we emphasize a major distinction between gland masses that are in proximity to rudimentary VNOs, and those that communicate with the VNOs bearing a neuroepithelium. In the case of the former, we note that some rudimentary VNOs are notably lacking gland duct communications. In VNOs such as those of *Brachyphylla* and *Rhinopoma,* a secretion transport function for these rudimentary tubes seems doubtful based on the lack of gland communications. In contrast, in *Hipposideros lankadiva, Megaderma lyra* and *Rhinolophus lepidus*, gland ducts that could convey secretions to the VNO lumen are clearly present. However, in all species with a neuroepithelial region of the VNO, at least portions of the vomeronasal gland are strongly reactive to mucin stains. Most bat species with a neuroepithelial VNO have intense PAS reactivity in vomeronasal glands. In addition, most of these same species have sparse acini with intense AB reactivity, or appeared to stain with both AB and PAS. Exceptions include *Sturnira lilium* and *Pteronotus quadridens,* both of which have strong AB reactivity in the vomeronasal glands (and each has a neuroepithelial VNO).

The precise function of mucins in the nasal airway remains uncertain, aside from some specific roles for clearance of the airways (Knowles & Boucher, 2002). Our results are consistent with other studies that contrast the nature of mucins in olfactory epithelium compared to respiratory epithelium (e.g., Kennel et al., 2019). In this sense, it is unsurprising that bats with functional VNOs have strong reactivity to neutral or acidic mucin stains, whereas nearby septal glands stain differently. Given that glands in bats with rudimentary VNOs lack strong reactivity to these same stains, or lack glandular communications, this is consistent with the hypothesis that mucin secretions are important for odorant transport, perhaps by dictating degree of solubility (Yang et al., 2007; Kennel et al., 2019). However, other functions remain possible, such as protection of delicate sensory tissue (Kennel et al., 2019).

A final implication of our findings is that glands near rudimentary VNOs are of uncertain homology. In many cases, they may actually be septal glands (e.g., in *Brachyphylla*), and vomeronasal

glands may have been evolutionarily lost. Bats with VNO rudiments bear certain similarities to the rudimentary VNO of humans and chimpanzees (*Pan troglodytes*). In humans, the VNO transiently bears sensory epithelium and is innervated during embryonic development, but becomes denervated and loses bipolar sensory neurons during later development (Smith and Bhatnagar, 2000). Postnatally, the epithelial wall of the human and chimpanzee VNO is simpler than that of a functional VNO, and bears kinocilia (Smith et al., 2001a). Roslinski et al. (2000) and Smith et al. (2001b) argued that this simplified VNO has been repurposed for a glandular function (recently, Kondoh et al., 2024, made a similar suggestion about the VNO in harbor seals). This might be true in at least some bats as well (*Hipposideros, Megaderma, Rhinolophus*). If so, the VNO might be important to the microenvironment of the nasal airway, perhaps contributing to mucosa protection or defense against microbes (but with a loss of ancestral function for chemoreception).

4.1.4 Venous sinuses

All bats with a neuroepithelial component of the VNO also possess prominent venous sinuses lateral to the VNO, which may be hypothesized to operationalize the "vomeronasal pump" to draw fluid into the VNO lumen and possibly also to expunge the lumen. In some bats, such as *Anoura* spp., the walls of these sinuses are very thick compared to communicating venous channels (Smith, unpublished obs.), perhaps indicating exceptional vasomotor control. It has previously been observed that in some phyllostomids the VNC may end and allow the VNO to stretch farther caudally with less medial support (Bhatnagar, 1980; Bhatnagar and Smith, 2007); here we find this is the case for additional phyllostomids, and some *Miniopterus* spp. This may indicate vasomotor influences do not completely depend on full cartilaginous support.

Whereas venous sinuses are consistently associated VNOs bearing neuroepithelia, they are also present in species with putative VNO rudiments, such as *Thyroptera tricolor*. This and other elements of a "functional" vomeronasal complex (see below) incongruously exist in more than a single bat species that seem to lack VNO function.

4.2 Key features of the vomeronasal complex identifiable by diceCT

The utility of diceCT for identifying venous sinuses (Smith et al., 2022) and nasal epithelia (Yohe et al., 2017; Smith et al., 2021) has been well-documented. Less studied is the effectiveness of diceCT for detecting cartilage. Our results here indicate that the VNC may be recognized when it exhibits a contrast with other adjacent connective tissue, supporting the findings by Smith et al. (2021) who looked at other nasal cartilages. However, it sometimes evades description, indicating refined techniques in staining and/or scanning are needed.

Given adequate resolution, the VNO itself may be visible, though perhaps mainly its lumen (Yohe et al., 2017). Findings of the present study suggest that diceCT may reveal important clues about VNO morphology based on lumen shape. For example, the putative VNO lumen of *Cardioderma cor* seems relatively dilated. In some bats and New World primates, this coexists with a relatively thin epithelium, often without medial-lateral differentiation of receptor-free versus neuroepithelium (Bhatnagar, 1980; Smith et al., 2011a). In some species of New World monkeys (e.g., *Saguinus* spp.), the vomeronasal

epithelium is exceedingly thin, with sparse relatively immature sensory neuron populations, and an extremely large VNO lumen; these VNOs have only small populations of mature sensory receptor neurons (Smith et al. 2011b). Thus enlarged VNO lumina may be an indication of VNO functional regression. Conversely, the VNOs with very thick neuroepithelia have narrow crescent-shaped lumina; this too may be detected using diceCT, given adequate resolution.

4.3 Osteological correlates of the vomeronasal complex

In living mammals and their extinct relatives, subtle features of the palate have been used as indicators of vomeronasal organ presence (Garrett et al., 2013; Crompton et al., 2017). In primates, trough-like impressions in the bony palate correspond to the articulation of VNCs with the dorsal surface of the bony palate, as demonstrated using histology (Garrett et al., 2013). The rostrocaudal lengths of these impressions can serve as proxies for dimensions of the vomeronasal organ itself in primates, allowing for robust analyses of mammals that are not readily available in very large cadaveric samples (e.g., Garrett and Steiper, 2014). In addition, the longitudinal furrows created by the VNC-bony palate articulation may prove instructive in detecting the presence or absence of a VNO in primate fossils. This potential utility of an osseous proxy for the VNO is of great interest here because bats resemble primates in great variability regarding presence or absence of the VNO.

The use of histology in the present study definitively shows the intimate relationship between a VNC with a capsular morphology and the presence of a cross-sectional groove in the maxillary or premaxillary bones. These cross-sectional grooves are three-dimensionally manifested as elongated furrows. However, with reference to histology, two caveats are clear in bats. First, some bats have a capsular VNC, but lack any osteological impressions (*Uroderma bilobatum*) or bear only slight impressions (e.g., *Vampyressa bidens*), thus requiring supporting histological or diceCT data. Second, histological data clearly reveal bat species lacking a neuroepithelial VNO (e.g., *Rhinopoma* spp.) may nonetheless possess a capsular VNC. Nonetheless, our findings show the traces of the vomeronasal complex such as the location of the VNC, and even ossified parts of it, can be detected in fossil bats.

4.4 Summing evidence on the vomeronasal complex

Numerous bats in which the VNO may be nonfunctional, at least as a major chemoreceptor organ, nonetheless possess two or three elements of the vomeronasal complex. This is the case in at least several yinpterochiropterans (*Rhinopoma hardwickii* and *Rh. microphyllum, Rhinolophus lepidus, Hipposideros lankadiva, Megaderma lyra* and *Cardioderma cor*). Among yangochiropterans, *Mormoops blainvillei,* possibly *Mo. megalophylla* and *Thyroptera tricolor* also possess two to three vomeronasal complex structures. However, all of these species lack a neuroepithelial VNO, and in most of these species, the accessory olfactory bulb (AOB), the first central connection of the vomeronasal system, is documented as absent (Frahm and Bhatnagar, 1980). On the other hand, morphology of the VNC is a particularly intriguing trait when cross-referenced with genetic data. All of the species mentioned above have a capsular VNC, but only a few have been studied genetically regarding retention of active genes that relate to VNO functionality. Our observations on *Mormoops blainvillei* are tentative, but based on osteological and diceCT data, it appears to resemble *Mo. megalophylla* in possessing a capsular VNC and a rudimentary VNO. Several of the *Hipposideros* and *Rhinolophus* species studied here have not been

studied genetically, but congeners have. Osteology of *Craseonycteris* suggests a capsular VNC may be present. In all of these bat genera, some active Trpc2 genes and multiple pseudogenes have been identified (Yohe et al., 2017).

The mosaic assemblage of vomeronasal complex structures in some extant bats suggests that relaxed selection pressure on V1R or Trpc2 genes leaves some functional morphological traits intact. This is particularly curious in *Thryoptera tricolor,* which possesses a capsular VNC with a large venous sinus lateral to it, but between these structures is a rudimentary VNO. To date, species of *Rhinopoma* and *Thyroptera* remain uninvestigated regarding V1R or Trpc2 genes. Further work should consider how skull modification relating to echolocation may have impacted the VNO, perhaps even influencing its presence or absence. Since parts of the vomeronasal complex of extant bats are without adjacent bony support, osteological clues are subtle. Furthermore, genetic studies (e.g., Yohe et al., 2017) and our present morphological work indicate that the VNO may vary in degree of functionality. Thus, further detailed osteological investigations using high resolution methods are required. Some evolutionary answers may hinge on extensive phylogenetic comparative analyses and/or fossil material that may or may not be presently available.

Why do some bats retain a rudimentary epithelial tube, while the vast majority of certain families (Pteropodidae, Vespertilionidae) lack any trace of it? Interesting parallels are found among primates, in which “typical” neuroepithelial VNOs are found in all known strepsirrhines (lemurs and lorises), whereas haplorhines (tarsiers, monkeys, apes, and humans) exhibit a range of VNO morphologies including rudiments, fully neuroepithelial (i.e., lacking the RFE) or absence. Smith et al. (2014) referred to the human VNO, a simple epithelial tube similar to that of some bats described here, as a “chronological vestige,” a structure which fulfills part of its function during development and persists as a rudiment. In the human VNO, and other mammals, migrating neurons use vomeronasal and terminal nerves as a latticework for their travel back to the forebrain through the cribriform plate (Schwarting et al., 2007). It seems likely that a similar transient function could exist for the rudimentary VNO of some bats. The ganglionic masses observed here have also been described in ontogenetic specimens of bats that lack VNOs, and they nest along terminal nerve bundles (Jastrow and Oelschläger, 2006); the nerve bundles observed here in *Brachyphylla* and *Megaderma* specimens could be cranial nerve 0 as well. Ganglionic masses are established during neuronal migration that follows the terminal nerve. The source of these neurons is thought to be the olfactory placode (but see Bhattacharyya and Bronner-Fraser, 2008, regarding some controversy). However, migration of neurons that become ganglionic masses or migrate further to reside in the hypothalamus (Schwanzel Fukuda et al., 1996; Schwanzel-Fukuda, 1999), may also continue from olfactory placode derivatives such as the VNO (Kjær and Fischer Hansen, 1996; Schwarting et al., 2007).

The persisting presence of ganglionic masses associated with axonal bundles, long known in adult bats (Bhatnagar, 1980), suggests postnatal functions, particularly in light of the degree of vascularity shown here in nerves near the VNO of *Brachyphylla cavernarum*. The postnatal function of terminal nerve ganglia remains a poorly studied topic, and bats present an interesting model for further exploration.

4.5 Conclusions

Both morphological and genetic evidence indicate a highly variable pattern of VNO loss among some bat lineages. This study brings together several tools for morphological observations on the VNO and related structures in a broader array of bats than previously examined. We identify functional elements of the vomeronasal complex and establish some traits exist even in bats that seem to lack functionality of the VNO as a chemoreceptor organ. We also document osteological features relating to structures of the vomeronasal complex, including visible paraseptal grooves on the hard palate. Extant bats do reveal limitations of this feature. For instance, at least one extant bat has a functional VNO but lacks these grooves (*Uroderma bilobatum*), and some bats have a capsular VNC but a rudimentary VNO. Nonetheless, these osteological proxies could detect important patterns in extant and fossil bats relating to VNO loss.

ACKNOWLEDGMENTS

We gratefully acknowledge our late friend and colleague Dr. Kunwar P. Bhatnagar, as well as Indu Bhatnagar and his former graduate student Dr. Greg Cooper, who provided so much of the histological material used in this study. We are also grateful to Dr. Eva Garrett for helpful discussions on osteological proxies for soft tissues and cartilage of the vomeronasal complex. We thank Jeff Bradley (Burke Museum Mammalogy Collection Manager) and Vaibhav Chhaya for assistance with specimen access and cataloguing.

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Figure legends

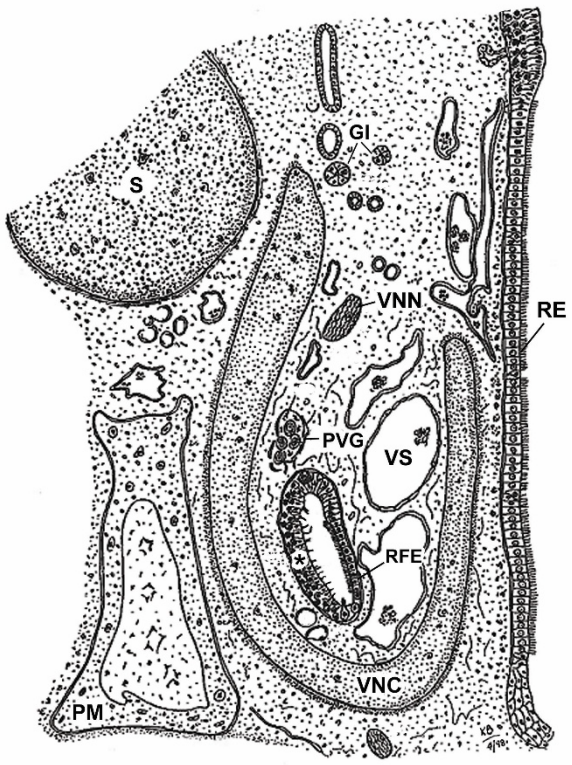

Figure 1: Elements of the vomeronasal complex in *Artibeus jamaicensis.* The vomeronasal complex at its greatest complexity includes a vomeronasal cartilage (VNC) with a "capsular" morphology that surrounds the following structures: a vomeronasal neuroepithelium (VNE), receptor-free epithelium (RFE), paravomeronasal ganglionic masses (PVG), venous sinuses (VS) and vomeronasal nerves bundles (VNN). Gl, glands of the vomeronasal organ; L, lumen of the vomeronasal organ; PM, premaxilla; RE, respiratory epithelia, S, septal cartilage. Modified after Bhatnagar and Meisami, 1998.

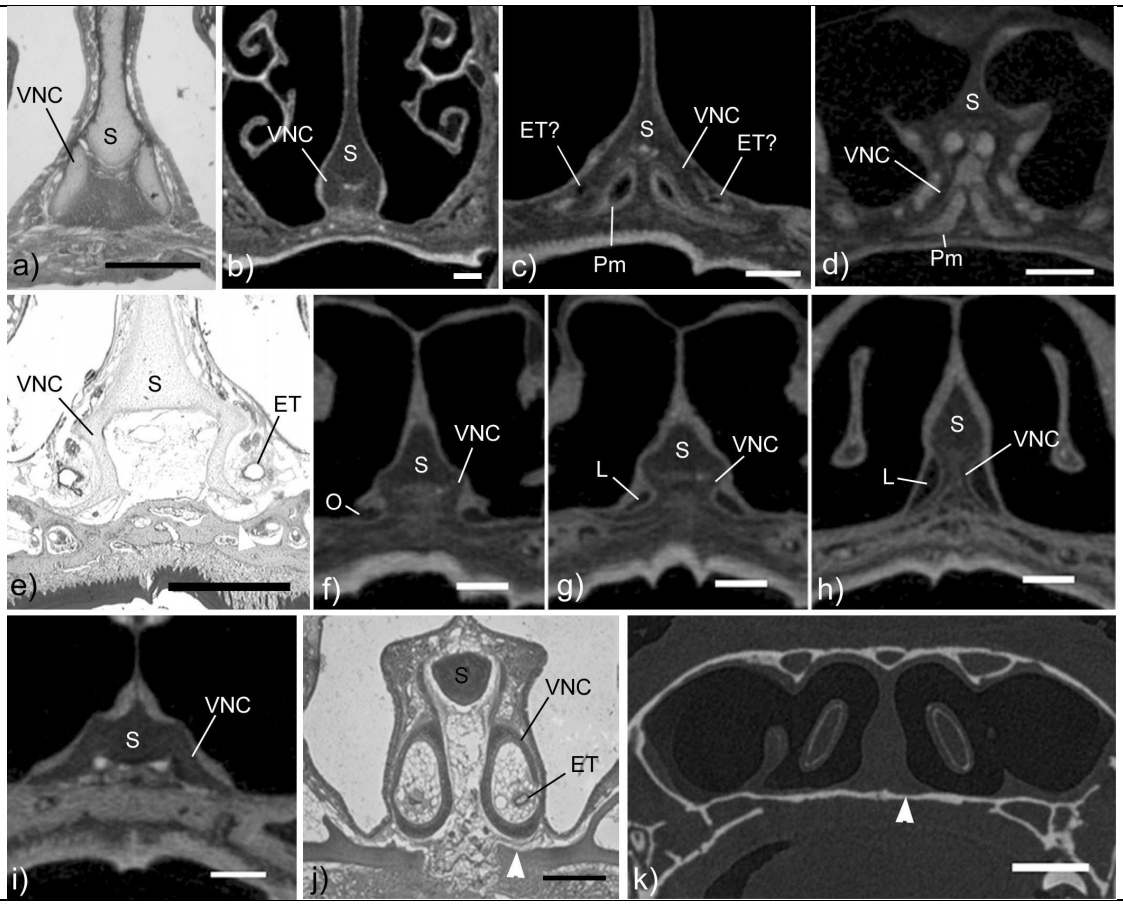

Figure 2: The vomeronasal cartilages (VNCs) in yinpterochiropteran bats varies in morphology. In pteropodids, such as *Macroglossus sobrinus* (a) and *Epomophorus wahlbergi* (b), it takes the form of a simple upright bar. In most other species, it has some degree of curvature: c) *Rhinolophus eloquens*, d) *Triaenops afer*, e) *Megaderma lyra*, f-h) *Cardioderma cor*, i) *Lavia frons*, j) *Rhinopoma microphyllum*, k) *Craseonycteris thonglongyai*. An example of simple, bar-shaped VNCs are clearly shown in *Ma. sobrinus* (a); this morphology is often easily detected using diceCT slices. In bats possessing a rudimentary vomeronasal organ (or epithelial tube, ET), the VNCs curve up laterally to assume a capsular morphology (see e, f, and j). L, greatly enlarged lumen of a presumed VNO rudiment in *Cardioderma cor*; S, septal cartilage. Scale bars, 0.5 mm.

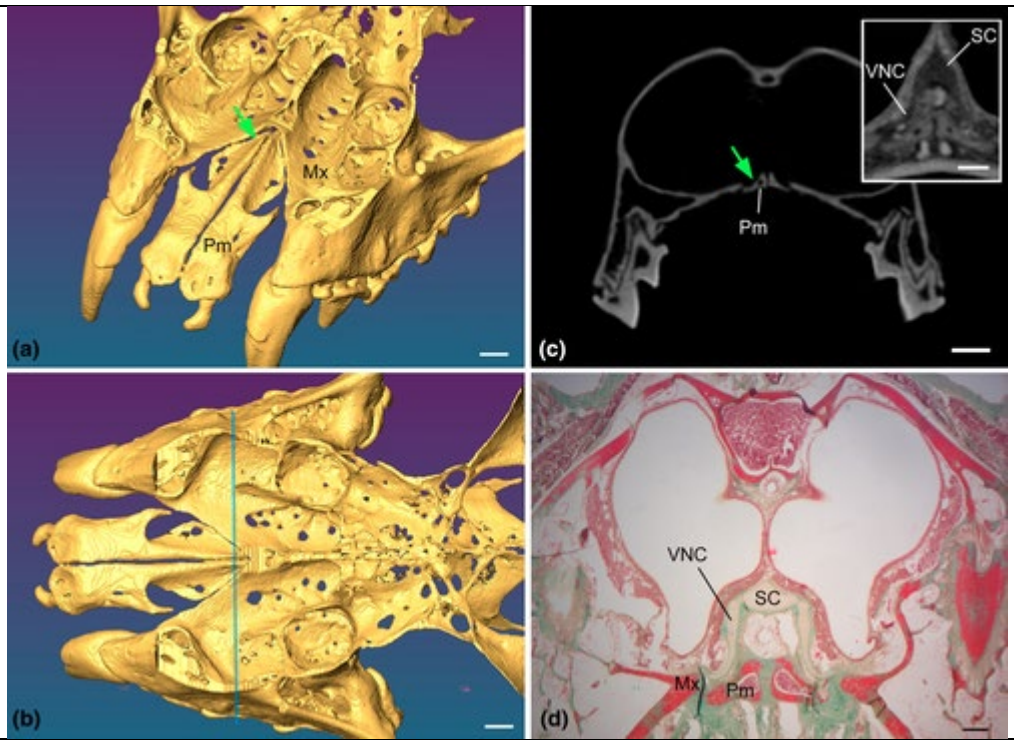

Figure 3: a, b) The osseous palate of *Hipposideros caffer*, showing an indentation (green arrow) into the caudal palatine process of the premaxilla (Pm). c) A microCT slice corresponding to the blue line shown in 2b reveals the same indentation in cross-section, and a diceCT slice of the same specimen at a matching level reveals the dark (more radiolucent) shape of the vomeronasal cartilage (VNC) rests against the premaxilla. d) A similar cross-sectional level of a histologically sectioned *Hipposideros lankadiva*, showing the same spatial relationship of the VNC and Pm. Mx, maxilla; S, septal cartilage. Scale bars: a-c, 0.5 mm, d, 300 µm; inset, 250 µm.

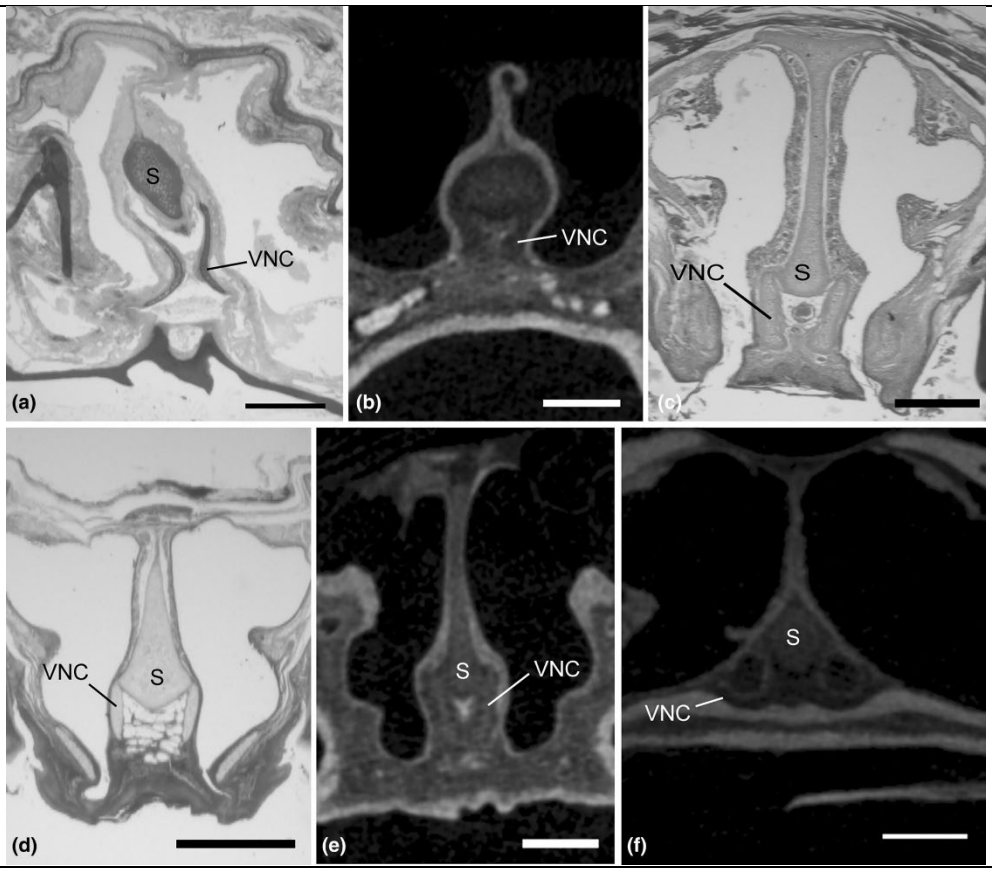

Figure 4: The vomeronasal cartilages (VNCs) in yangochiropteran bats. In many bats of this suborder, the VNC is "simple" in morphology. For example, VNCs are slightly curved in cross-section, as in *Molossus pretiosus* (a) or *Mops condylurus* (b). In many others it resembles an upright bar in cross-section, as in *Myotis velifer* (c), *Parastrellus hesperus* (d), or *Scotophilus dinganii* (e). In contrast, all known miniopterids have a VNC with a capsular appearance in cross-section, such as in the newly studied *Miniopterus inflatus* (f). Note the VNC can be discerned in diceCT based on a distinct radio-opacity of cartilage relative to adjacent soft tissues (b, e, f). S, septal cartilage. Scale bars, 0.5 mm.

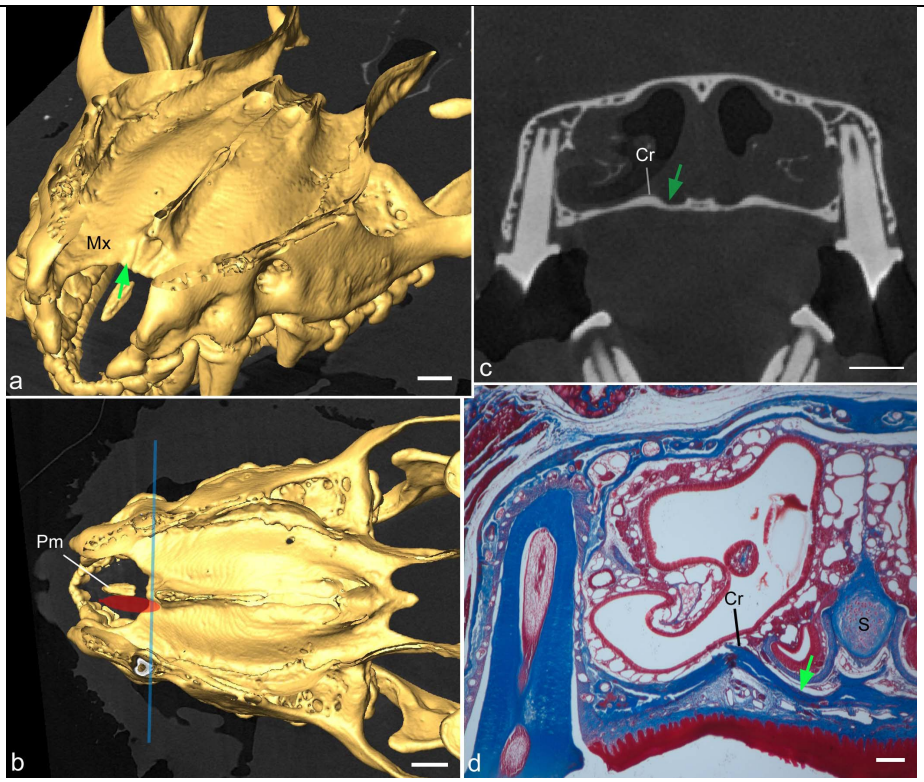

Figure 5: The osseous palate of *Miniopterus australis,* showing an indentation, or groove (green arrow) in the maxilla (Mx). c) A microCT slice corresponding to the blue line shown in b reveals the same groove in cross-section, and emphasizes that this groove is delimited laterally by a slightly raised crest (Cr). d) A similar cross-sectional level in *Mi. australis*, showing the same spatial relationship of the VNC and Mx using histology. After comparisons of histological sections to CT slices, it was possible to estimate the rostral caudal extent of the vomeronasal organ (see reddish overlay, shown in b), because the vomeronasal organ is coextensive with the middle remnant of the palatal process of the premaxilla (Pm). S, septal cartilage. Scale bars, a, 0.5 mm; c, 0.75 mm, b, 0.5 mm, d, 150 µm.

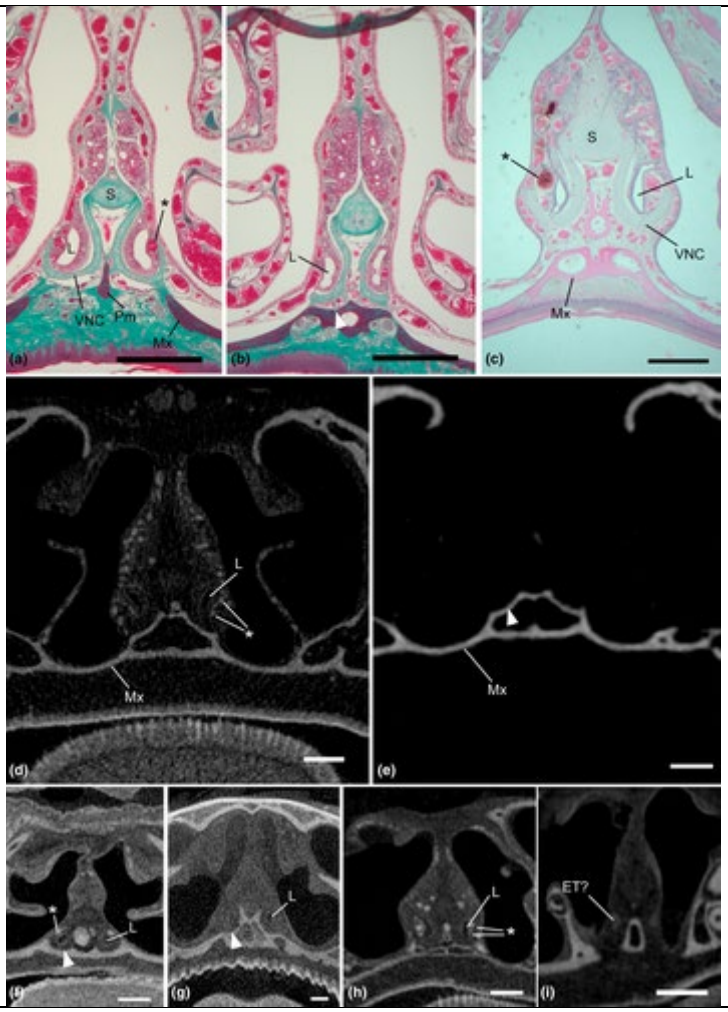

Figure 6: The vomeronasal cartilages (VNC) in yangochiropteran bats, including phyllostomids (a-h) and one mormoopid (i). Most phyllostomid bats possess a VNC with a capsular morphology, which surrounds the vomeronasal organ (L, lumen of organ) on its medial ventral and part of its lateral aspects. *Micronycteris megalotis* (a, b) has a typical VNC that articulates rostrally with the palatal process of the premaxillary (a, Pm), and caudally with a groove (b, white arrowhead) in the palatal processes of the maxillary bone (Mx). *Uroderma bilobatum* (c) is an exception, possessing a capsular VNC that is elevated above the maxilla, which therefore lacks a groove. DiceCT or even microCT permits detection of the paraseptal groove in *Chiroderma villosum* (d, e). *Lonchophylla handleyi* (f), *Phyllostomus hastatus* (g), and *Vampyressa bidens* (h) all exhibit a paraseptal groove on each side of the septum associated with a visible lumen of the VNO. Most phyllostomids have large venous sinuses (*) lateral to the vomeronasal organ (a, c). These are visible in some diceCT scans as radio-opaque masses (d, f, h). i) The mormoopid bat *Mormoops blainvillei* lacks a paraseptal groove but may possess a small (likely rudimentary) vomeronasal organ in the form of an epithelial tube (ET). Scale bars, 0.5 mm.

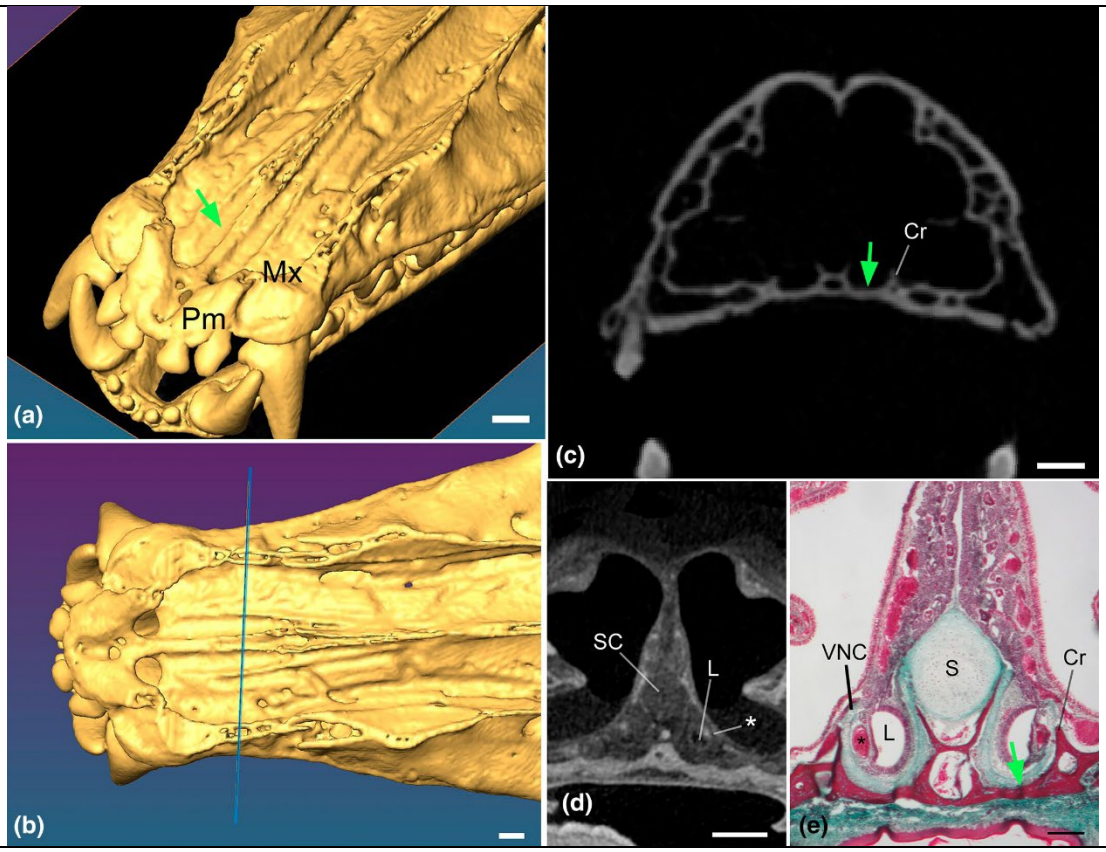

Figure 7: a, b) The osseous palate of *Leptonycteris yerbabuenae,* showing an indentation, or groove (green arrow) in the maxilla (Mx). c) A microCT slice corresponding to the blue line shown in b reveals the same groove in cross-section, and emphasizes that this groove is delimited laterally by a crest (Cr). d) A similar cross-sectional level in a histologically sectioned *Leptonycteris nivalis* specimen, showing the same spatial relationship of the VNC and Mx. Scale bars, a, 0.75 mm; b-d, 0.5 mm; c, 150 µm.

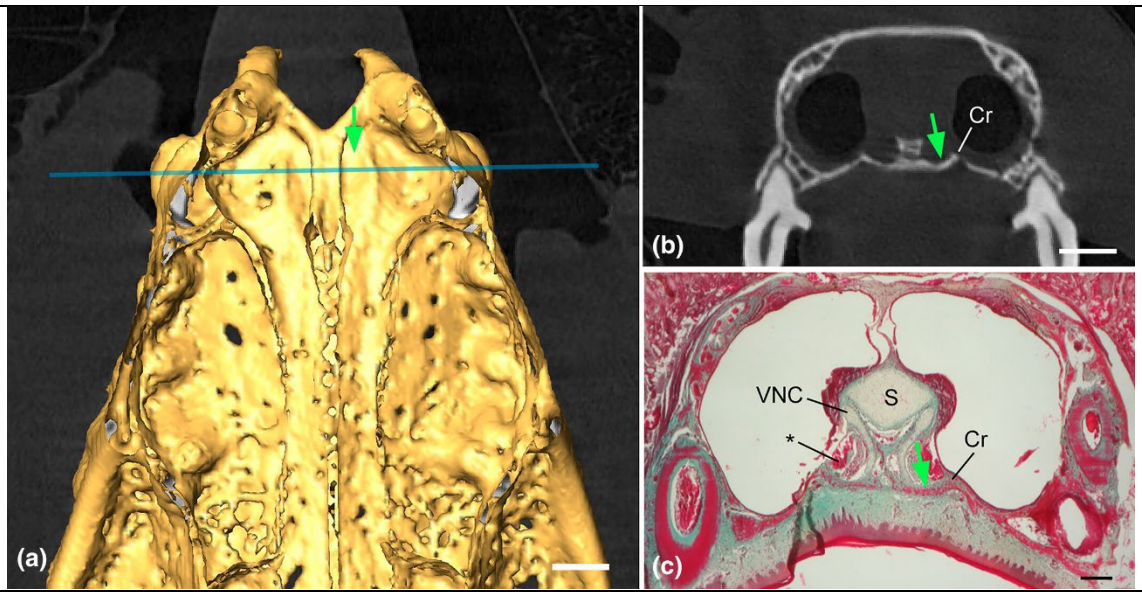

Figure 8: a) The osseous palate of *Thyroptera tricolor,* showing an indentation, or groove (green arrow) into the caudal palatine process of the premaxilla (Pm). b) A microCT slice corresponding to the blue line shown in b reveals the same groove in cross-section, and emphasizes that this groove is delimited laterally by a slightly raised crest (Cr). c) A similar cross-sectional level in a different, histologically sectioned *Th. tricolor* specimen, showing the spatial relationship of the groove with the vomeronasal cartilage (VNC). S, septal cartilage. Scale bars, a, b, 0.5 mm; c, 150 µm.

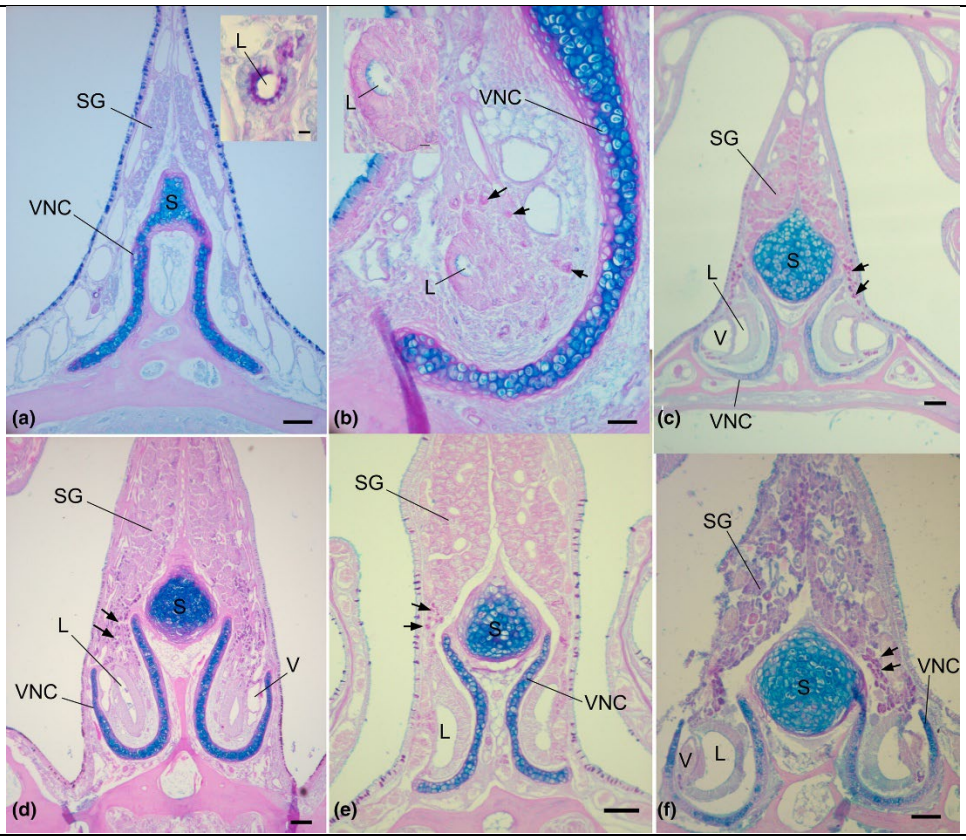

Figure 9: Alcian blue-periodic acid histology to reveal mucin histochemistry in the bat vomeronasal complex, including a) *Rhinolophus lepidus*, b) *Hipposideros lankadiva*, c) *Glossophaga soricina,* d, *Artibeus jamaicensis,* e) *Micronycteris megalotis,* f) *Leptonycteris nivalis*. *Rhinolophus lepidus* and *Hipposideros lankadiva* have vestigial vomeronasal organs, in the form of simple epithelial tubes, with few glands nearby. Insets reveal AB+ (blue) and PAS+ (magenta) vacuoles in epithelial tube of *Rh. lepidus,* and AB+ secretions coating cilia in *Hi. lankadiva*. In most phyllostomids, glands communicating with the organ on the dorsal side (arrows) are intensely PAS+ (arrows), and to a greater degree than septal glands (SG). L, lumen of vomeronasal organ; S, septal cartilage; VNC, vomeronasal cartilage. Scale bars, a, 100 µm; b, 50 µm; c, 100 µm; d, 150 µm; e, f, 100 µm; inset a, 20 µm; inset b, 10 µm.

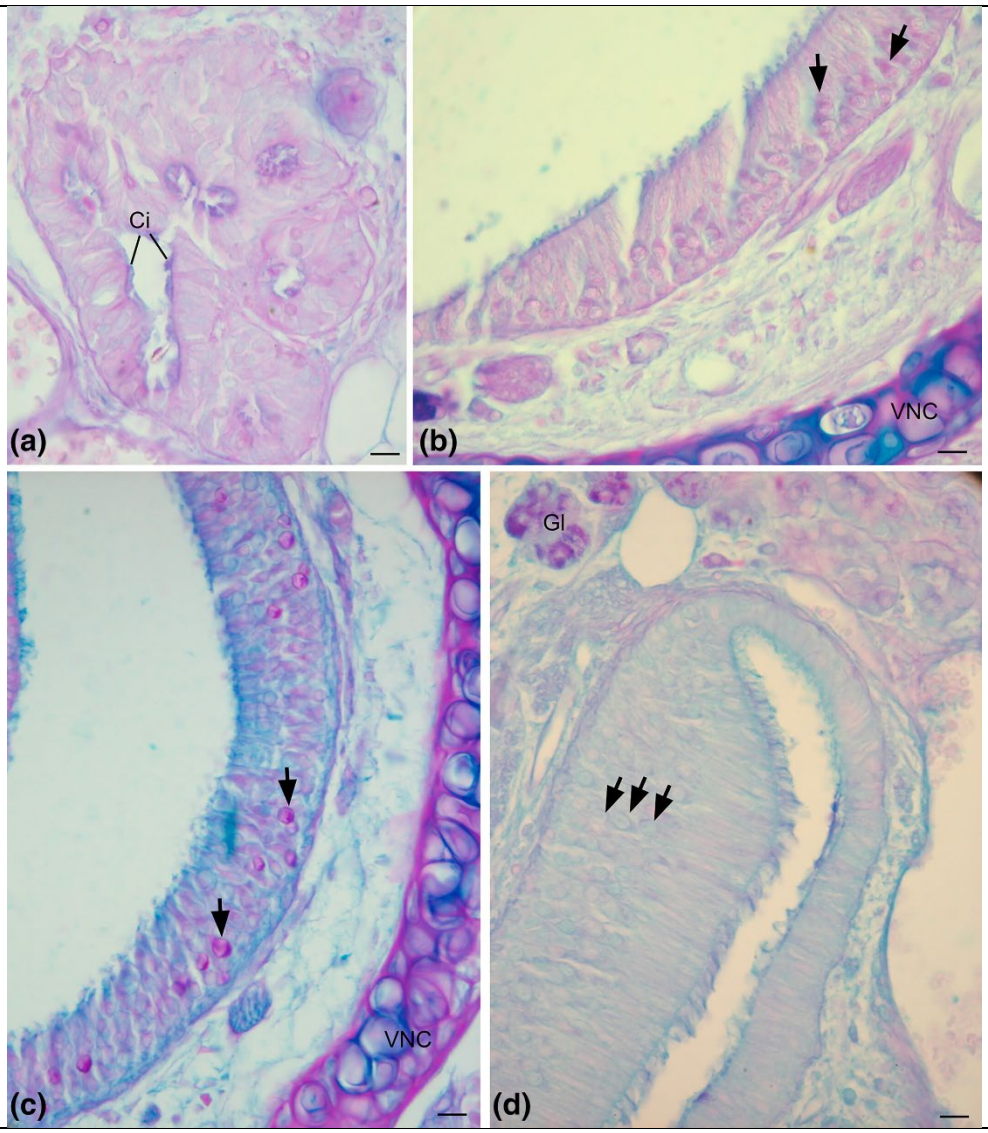

Figure 10: Alcian blue-periodic acid histology to reveal mucin histochemistry in the bat vomeronasal complex, including a) *Rhinopoma hardwickii*, b) *Carollia perspicillata*, c) *Macrotus waterhousii,* d, *Diaemus youngi*. In *Rh. hardwickii*, the vomeronasal organ is a simple columnar epithelial tube, with AB + cilia at the epithelial apex. *Ca. perspicillata* and *Ma. waterhousii* have PAS+ sensory neurons (arrows) in the neuroepithelium. In *Di. youngi*, the neuroepithelium is notably thick (note row of arrows indicating sensory neurons). All species had AB+ secretions coating the epithelial apex. Ci, cilia. Scale bars, a-d, 10 µm.

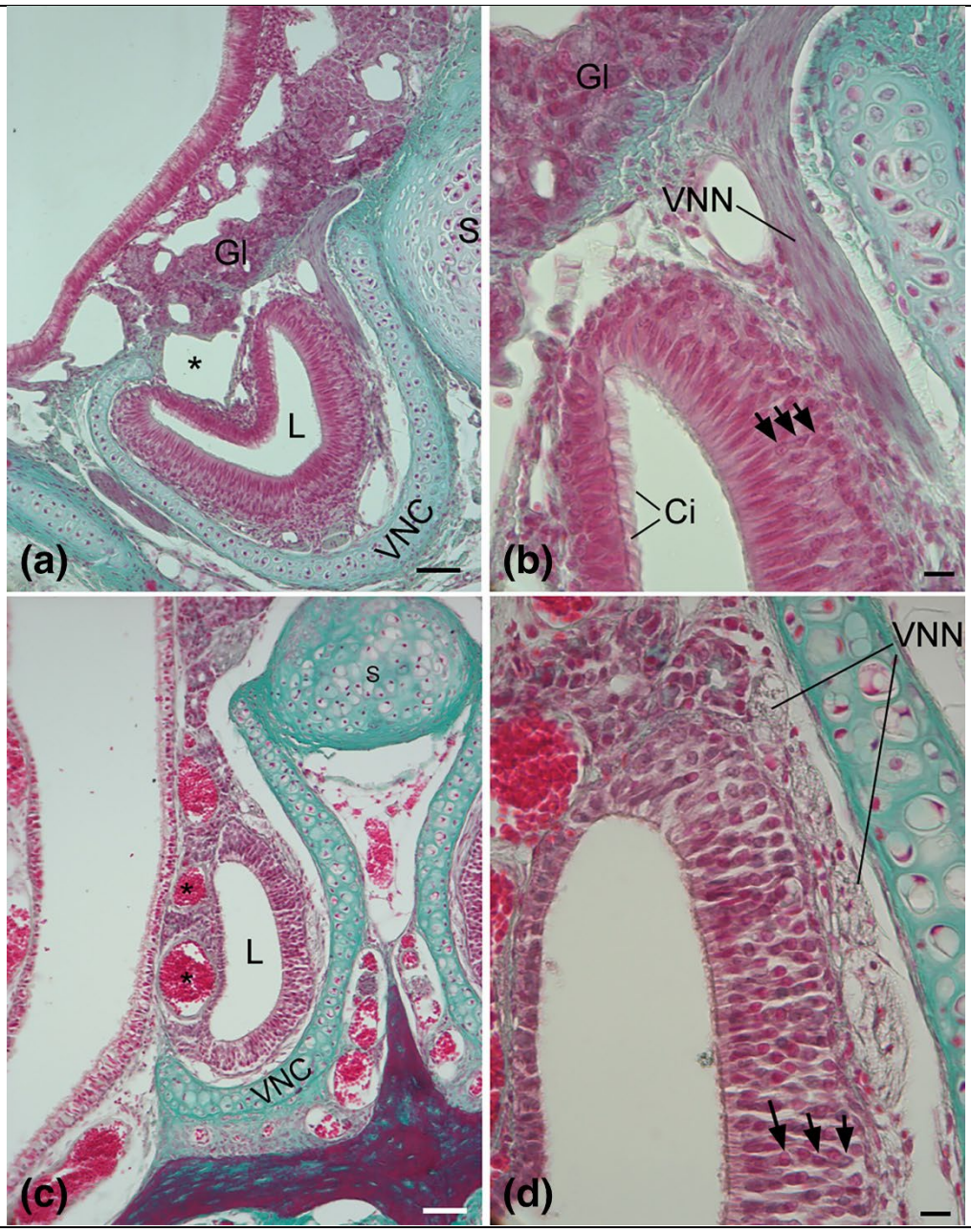

Figure 11: The neuroepithelial vomeronasal organ of *Miniopterus magnater* (a,b) and *Micronycteris megalotis* (c, d). In both species, functionality of the organ is strongly indicated by rows of round nuclei of bipolar sensory neurons (see arrows in b, d) within the medial neuroepithlium. The lateral epithelium is thinner and ciliated (Ci) and pseudostratified or simple columnar in structure (cilia are present in *M. megalotis,* but harder to visualize here. Other functional indicators are the vomeronasal nerves (VNN) approaching the neuroepithelial side, glands, and laterally positioned venous sinuses (*). VNC, vomeronasal cartilage. Scale bars, a, 50 µm; b, 10 µm; b, 50 µm; d, 10 µm.

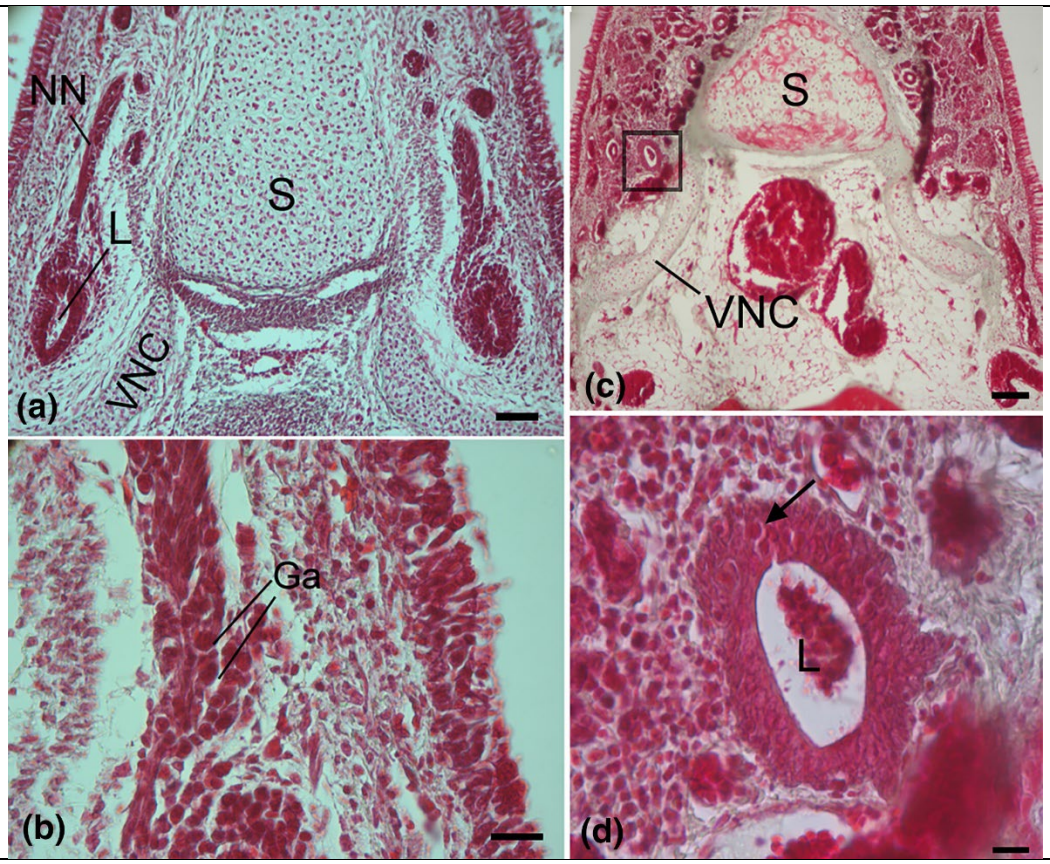

Figure 12: The VNO (L, lumen of VNO) in *Brachyphylla cavernarum*. a, b) In the fetus, the VNOs are bilateral epithelial tubes adjacent to the vomeronasal cartilages (VNC) near the nasal septum (S). Dorsal to the VNO, the vomeronasal nerve (NN) bundles have numerous cell bodies of a ganglionic appearance (Ga) embedded within them. c) In an adult, the VNO remains in a similar position. The VNO remains densely cellular on its dorsal wall, including sparse cells that have a bipolar appearance (d, arrows). Scale bars, a, 50 µm; b, 20 µm; c, 40 µm d, 10 µm.

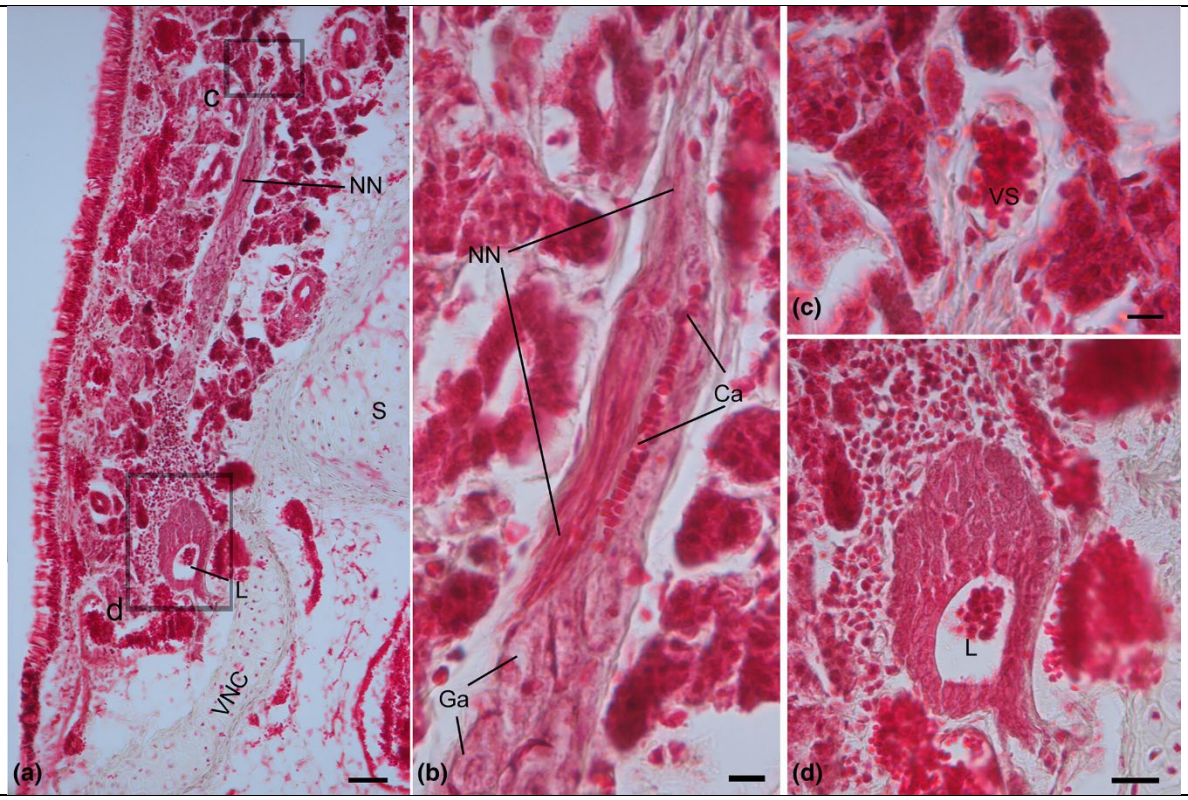

Figure 13: a) The caudal end of the rudimentary VNO (L, lumen of VNO) in adult *Brachyphylla cavernarum*. On each side, a larger bundle of nerve fascicles (NN) can be seen ascending within the septal mucosa. b) Nested among nerve fascicles are ganglionic cell bodies (Ga), and a capillary (Ca) is seen penetrating the center of the nerve-ganglia bundle. There are numerous blood-filled sinuses at both ends of the nerve, including adjacent to the VNO (c, d). S, septal cartilage. Scale bars, a, 50 µm; b, c, 10 µm; d, 20 µm.

Supplemental figure legends.

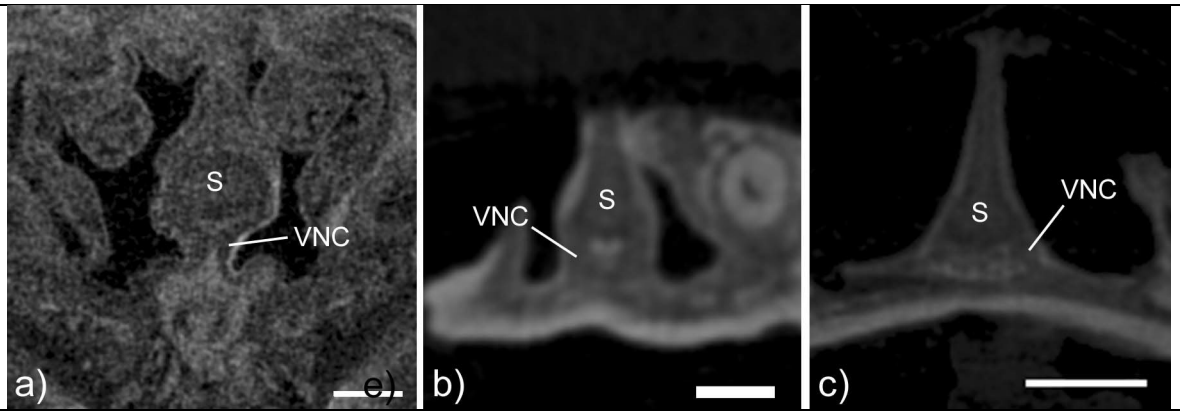

Figure S1: The vomeronasal cartilages (VNCs) in yangochiropteran bats, as viewed by diceCT. *a*) *Eumops glaucinus*, b) *Hypsugo crassulus*, c) *Nycticeinops schlieffeni*. Scale bars, 0.5 mm.

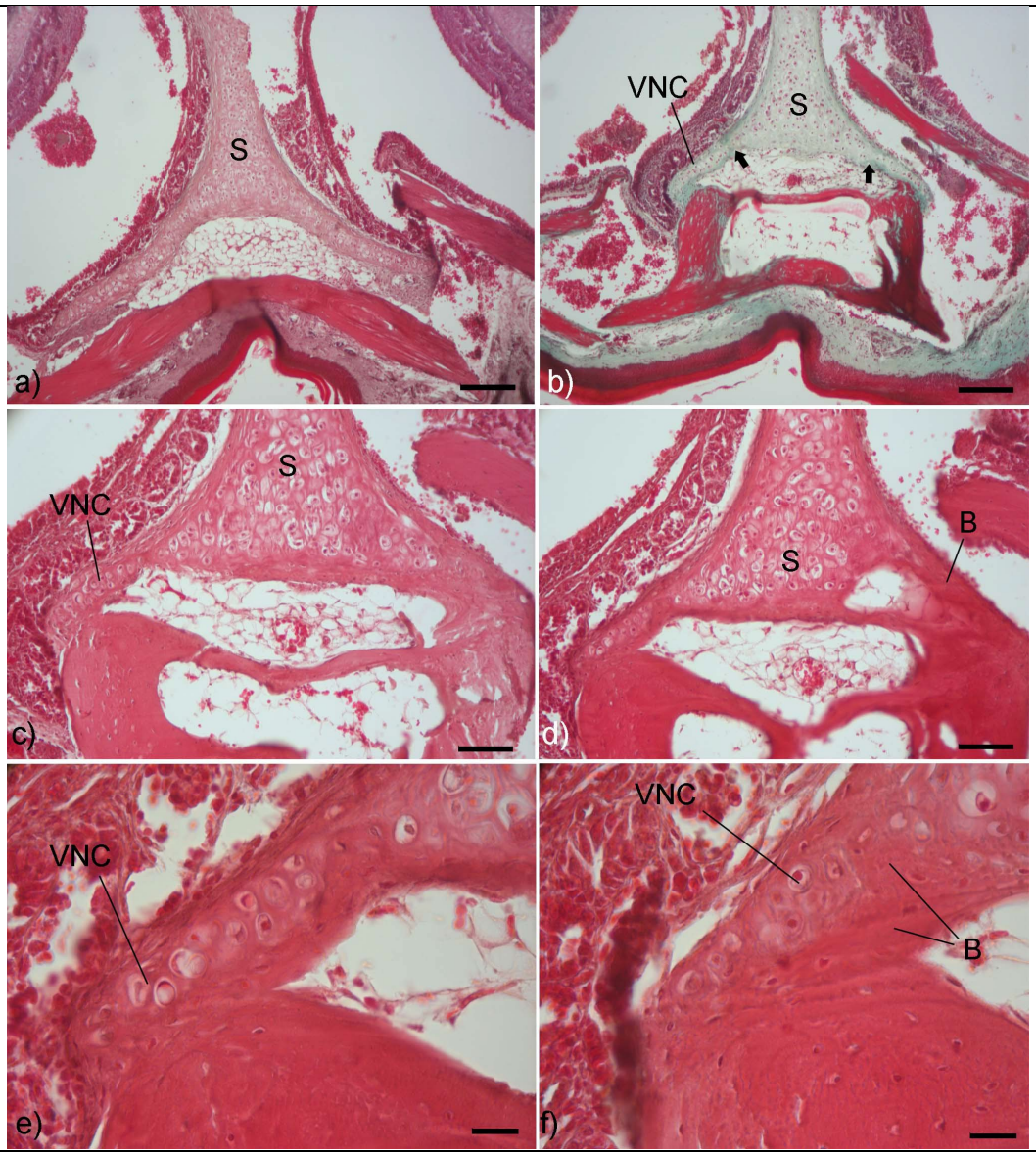

Figure S2: The vomeronasal cartilages (VNCs) of a histologically sections adult *Nycteris thebaica.* In the rostrocaudal series, the VNCs connect with bilateral projections that are connected to the base of the septal cartilage (a). b) More caudally, these projections begin to have partial separation (arrows) from the septum, and then c) become completely separated on the right (left side of figure). d) On the opposite side, the VNC seems to "disappear" into a mass of bone (B). e and f) Most caudally, the right VNC becomes smaller, and embedded within bone. Scale bars, a, b, 150 µm; c, d, 75 µm ; e, f, 20 µm.

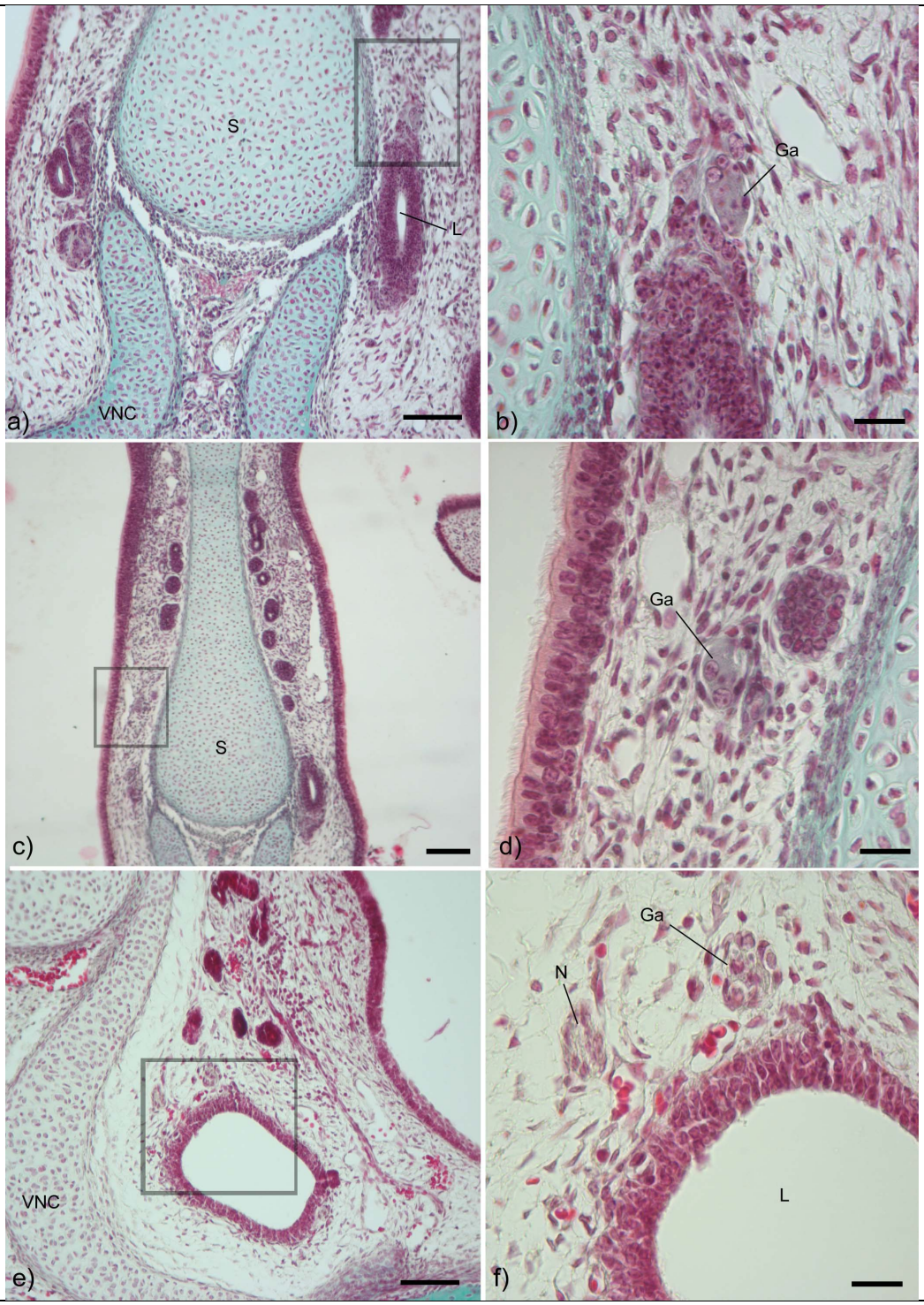

Figure S3: The vomeronasal organ (L, lumen of vomeronasal organ) and associated structures in two fetal *Megaderma lyra,* at 21.5 mm (a-d) and 37 mm (e, f) crown-rump length. At both ages, large ganglionic masses (Ga) are seen dorsal to the vomeronasal organ (a, b), and also ascending to even more dorsal levels within septal mucosa (c, d). In the larger fetus, the lumen is proportionally larger while the epithelium of the vomeronasal organ is thinner with fewer rows of cell nuclei, but nerves (N) and ganglia are still visible nearby (f). Scale bars, a, 75 µm; b, d, f, 20 µm; c, 100 µm; e, 75 µm.

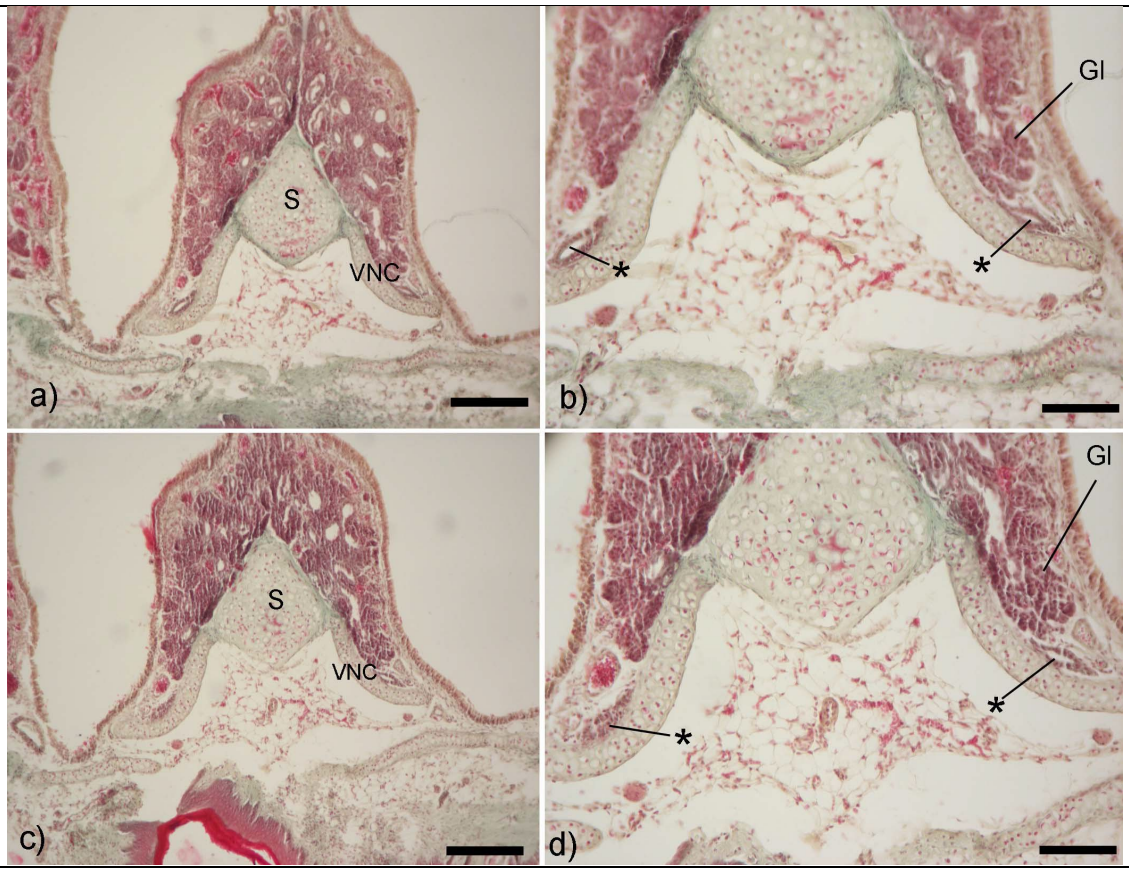

Figure S4: The vomeronasal complex of *Balantiopteryx io.* a) Vomeronasal cartilages (VNCs) are slightly curved. b) A rudimentary vomeronasal organ (*) rests just dorsal to the VNC; gland masses (Gl) are dorsal to the VNO. c and d) show the same structures are a more caudal level. Throughout the length of the VNO, no communication of glands ducts to its lumen could be detected. Scale bars, a, c, 200 µm; b, d, 100 µm.

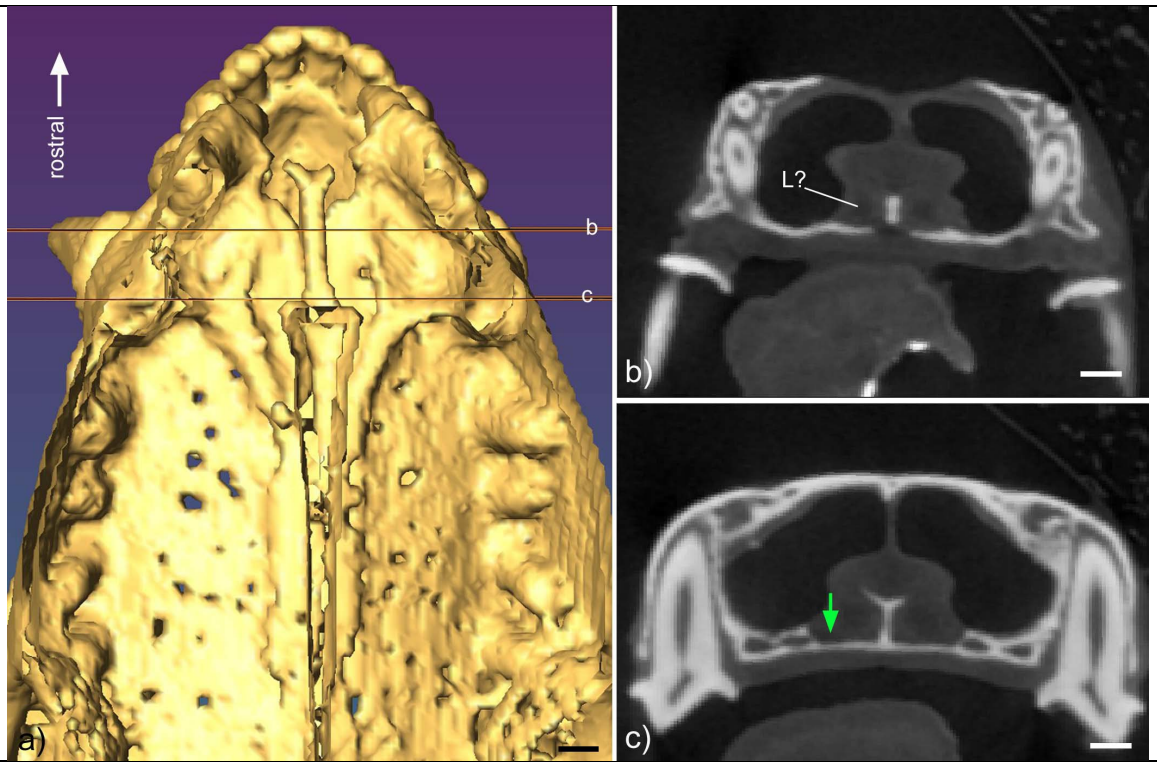

Figure S5: A dorsal view of the palate of *Thyroptera discifera,* showing bilateral depressions where vomeronasal cartilages likely articulate. Cross-sectional levels for b & c are indicated. b) A rudimentary vomeronasal organ (*) rests just dorsal to the grooves. c) At a more caudal level, the grooves (green arrow) are deep. Scale bars, a-c, 1 mm.