Title

Histological features of the vomeronasal organ in the giraffe, *Giraffa camelopardalis*

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Running title

Vomeronasal organ in giraffes

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ABSTRACT

The vomeronasal organ (VNO) that preferentially detects species-specific substances is diverse among animal species, and its morphological properties seem to reflect the ecological features of animals. This histological study of two female reticulated giraffes (*Giraffa camelopardalis reticulata*) found that the VNO is developed in giraffes. The lateral and medial regions of the vomeronasal lumen were covered with sensory and non-sensory epithelia, respectively. The vomeronasal glands were positive for periodic acid-Schiff and alcian blue (pH 2.5) stains. The VNO comprises several large veins like others in the order Cetartiodactyla, suggesting that these veins function in a pumping mechanism in this order. In addition, numerous thin-walled vessels located immediately beneath the epithelia covering the lumen entirely surrounded the vomeronasal lumen. This sponge-like structure might function as a specific secondary pump in giraffes.

Research Highlights

- ✓ The vomeronasal organ in giraffes contained cartilage, sensory and non-sensory epithelia, nerve bundles, glands, veins and an artery.
- ✓ A specific ensemble of numerous vessels immediately beneath the epithelia covered the lumen.

INTRODUCTION

Most tetrapods use the olfactory system to detect chemical substances in the external environment. The olfactory system of mammals comprises the main olfactory and vomeronasal systems, and a vomeronasal organ (VNO) processes various species-specific chemicals (Halpern, 1987). Variations in morphology of the VNO are also species specific and seem to reflect the behavioral and ecological features of animals (Halpern, 1987).

Giraffes (*Giraffa camelopardalis*) are the tallest terrestrial mammals and belong to the order Cetartiodactyla. Male giraffes in search of estrous females lower the head to nuzzle the rumps of females, catch urine in their mouths and then raise their head to savor it (Dagg and Foster, 1976; Pratt and Anderson, 1985). The behavior in giraffes is generally considered as a flehmen response in which pheromones are transferred into the VNO. However, a VNO has not been established in giraffes, although the morphological and histological features of the VNO have been determined in other species of the same order such as cattle (Adams, 1986; Salazar et al., 1997, 2008), goats (Besoluk et al., 2001; Takigami et al., 2000), sheep (Kratzing, 1971; Salazar et al., 2007; Ibrahim et al., 2013), deer (Park et al., 2014), moose (Vedin et al., 2010), camels (Karimi et al., 2014; Ibrahim et al., 2015) and pigs (Salazar et al., 1997). As the vomeronasal system plays a critical role in the mating of animals that show flehmen behavior (Døving and Trotier, 1998), knowledge about the VNO in giraffes is important to comprehend the reproduction and social communication of giraffes. The present histological study reveals some properties of the VNO in adult giraffes.

MATERIALS AND METHODS

Animals

The heads of two captive reticulated female giraffes (*G. camelopardalis reticulata*) aged eight and nine years at the time of death in December, 2015 (Obihiro Zoo, Obihiro, Japan) and July, 2014 (Asahiyama Zoo, Asahikawa, Japan) were frozen within 24 hours of death and stored at Obihiro University of Agriculture and Veterinary Medicine. The heads were dissected without destroying the skull, and the VNO was sampled approximately 10 cm from the incisive papilla within the reach of a surgical knife.

Histological procedures

The VNO was trimmed into small blocks, fixed by Bouin's fluid and then embedded in paraffin using standard procedures. Specimens were cut frontally into 5-μm-thick sections, deparaffinized, stained with hematoxylin-eosin, periodic acid-Schiff or alcian blue (pH 2.5) and assessed using a Microphot-FX (Nikon, Tokyo, Japan) equipped with a Digital Sight DS-5M camera (Nikon). Sections were also stained with hematoxylin-eosin and photographed using a fluorescence Microphot-SA (Nikon) equipped with a G2E filter block and a Digital Sight DS-5Mc camera (Nikon) to determine autofluorescence.

Anti-olfactory marker protein immunohistochemical procedures

Specimens were histochemically processed as described (Kondoh et al., 2016). Briefly, 5-μm-thick deparaffinized sections were incubated with 0.3% H_2O_2 in methanol and then with 3% normal goat serum. The sections were then incubated at 4°C overnight with 2 μg/mL of anti-olfactory marker protein (OMP) antibody (sc-67219; Santa Cruz Biotechnologies, Santa Cruz, CA, USA), followed by 7.5 μg/mL of biotinylated anti-rabbit IgG antibody (BA-1000; Vector Laboratories, Burlingame, CA,

USA) for 60 min. Thereafter, the sections were immersed in avidin-biotin-peroxidase complex (PK-6100; Vector) for 30 min and then colored with Tris-HCl buffer containing 0.006% H_2O_2 and 0.02% 3,3’-diaminobenzidine tetrahydrochloride.

RESULTS

The soft tissue of the VNO was surrounded entirely by the vomeronasal cartilage at the long axis level (about 10 cm from the incisive papilla) (Fig. 1). Although the epithelia covering the wall of lumen were damaged because of postmortem and/or freezing changes, the histological details were sufficiently preserved to distinguish the sensory vomeronasal epithelium (VNE) and non-sensory epithelium (NSE) covering the medial and lateral regions of the lumen, respectively (Fig. 2A). The VNE possessed bipolar cells with a dendrite and an axon extending to the lumen and basal lamina, respectively (Fig. 2C), although the nuclear morphology in these cells seemed damaged due to freezing changes. Several small axon bundles were also located in the lamina propria near the VNE (Fig. 2C). No cells resembled sensory cells in the NSE (Fig. 2D). Furthermore, the VNE was positive for OMP, which is used broadly as a marker of mature olfactory neurons, and the NSE was negative for OMP (Fig. 2B). The medial lamina propria possessed some nerve bundles (Fig. 2E).

The lamina propria deep in the VNE and NSE possessed several and many acini and ducts of vomeronasal glands, respectively (Fig. 1). The vomeronasal glands were positive for both periodic acid-Schiff and alcian blue (pH 2.5) staining (Fig. 3).

Several vomeronasal veins (Fig. 4A) found in the lamina propria deep in the NSE, and a single vomeronasal artery (Fig. 4B) was located in the lamina propria around the ventral border region between the VNE and NSE. The artery was distinguished from the veins by an internal elastic membrane that emitted autofluorescence (Fig. 4C, D). Numerous venules and/or lymphatics possessing thin wall and endothelial cells were arranged around the circumference of all connective tissues immediately beneath the epithelia to form a spongy-like structure (Fig. 1, 4E).

DISCUSSION

The present study identified a developed VNO in giraffes, and found that the lateral and medial regions of the lumen are covered by a sensory VNE and NSE, respectively. These features are typical of the VNO of most mammals (Halpern, 1987). We found that the VNO of giraffes possesses the vomeronasal cartilage that surrounded the soft tissue throughout the examined length. This morphological feature of the cartilage seems similar to that of goats (Besoluk et al., 2001), sheep (Salazar et al., 2007), deer (Park et al., 2014) and moose (Vedin et al., 2010). However, whether or not the more posterior part is also completely surrounded by cartilage could not be estimated, because the shape of the cartilage varies depending on the location along the entire length (Salazar et al., 1997).

The VNO of other cetartiodactyl species comprises several veins within the lamina propria deep in the NSE (Kratzing, 1971; Adams, 1986; Salazar et al., 1997, 2007, 2008; Takigami et al., 2000; Vedin et al., 2010; Ibrahim et al., 2013, 2015; Park et al., 2014) like that of the giraffes shown herein. These veins are assumed to play a role in a pumping mechanism (Døving and Trotier, 1998) that uptakes odorants into the vomeronasal lumen of cetartiodactyla species.

Numerous thin-walled vessels that were located immediately beneath the VNE as well as the NSE, surrounded the vomeronasal lumen entirely in the giraffes. Although capillaries and small veins are irregularly-scattered in the lamina propria around the vomeronasal lumen in cattle (Adams, 1986; Salazar et al., 2008), sheep (Salazar et al., 2007), goats (Besoluk et al., 2001), moose (Vedin et al., 2010) and camels (Karimi et al., 2013), such a spongy structure contacting the vomeronasal lumen has not been detected in any other species. This ensemble of vessels might be a secondary pump that is specific to giraffes.

The flehmen response of giraffes is a behavioral sequence that starts with lowering the head to nuzzle the rumps of females, and ends with raising the head to savor the urine (Dagg and Foster, 1976; Pratt and Anderson, 1985). Blood circulation in the head and neck of giraffes is directly affected by

raising and lowering the head (Brøndum et al., 2009). Blood flow ceases and blood pressure in the cranial jugular veins progressively increases in the lowered heads of anesthetized giraffes. Raising the head immediately causes a large transient venous return (peak flow ~12 L/min) of blood that accumulates in the jugular vein in the lowered head and a consequent reduction in blood pressure in the jugular vein (Brøndum et al., 2009). These facts suggest that the blood pressure and volume in the veins of the head change during the flehmen response, and larger veins might be affected more and sooner. Therefore, the ensemble of numerous small vessels, a specific secondary pump in the VNO of giraffes, might play a role in the subsequent compression during the final phase of flehmen response.

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Conflict of Interest: The authors have no conflicts of interest to declare.

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FIGURE LEGENDS

Fig. 1. Topographical features of vomeronasal organ in eight-year-old female reticulated giraffe. Boxed area corresponds to Fig. 2A at higher magnification. A, artery; C, vomeronasal cartilage; G, vomeronasal gland; L, vomeronasal lumen; NSE, non-sensory epithelium; V, vein; VNE, sensory vomeronasal epithelium. *Ensemble of vessels surrounding vomeronasal lumen. Arrows and arrowheads, nerve bundles and ducts of vomeronasal glands, respectively. Left side of figure is lateral, and upper, dorsal. Bar = 500 μm.

Fig. 2. Histological properties of vomeronasal sensory (VNE) and non-sensory (NSE) epithelia. Hematoxylin-eosin staining (**A**) and anti-OMP immunostaining (**B**) of border area between VNE and NSE (box in Fig. 1). Dashed line indicates border. Arrow (**B**) indicates positive reaction. Hematoxylin-eosin staining of VNE (**C**) and NSE (**D**). Arrows and arrowhead (**C**) indicate axons and dendrites of sensory cells, respectively. Asterisk (**C**) indicates axon bundle the lamina propria. Nerve bundle within medial lamina propria (**E**). Bars = 50 (**A, B, E**) and 20 (**C, D**) μm.

Fig. 3. Histological properties of vomeronasal glands (arrows). Periodic acid-Schiff (**A**) and alcian blue (pH 2.5) (**B**) staining. Bars = 200 μm.

Fig. 4. Histological properties of vascular structures in vomeronasal organ. Vomeronasal vein (**A, C**) and artery (**B, D**). Panels (**C, D**) show autofluorescence images corresponding to panels (**A, B**), respectively. Autofluorescence shows internal elastic membrane (arrows) in artery (**D**) but not vein (**C**). Ensemble of vessels (**E,** asterisks) containing endothelial cells (arrows) in connective tissue immediately beneath epithelium (e). Bars = 50 (**A–D**) and 20 (**E**) μm.

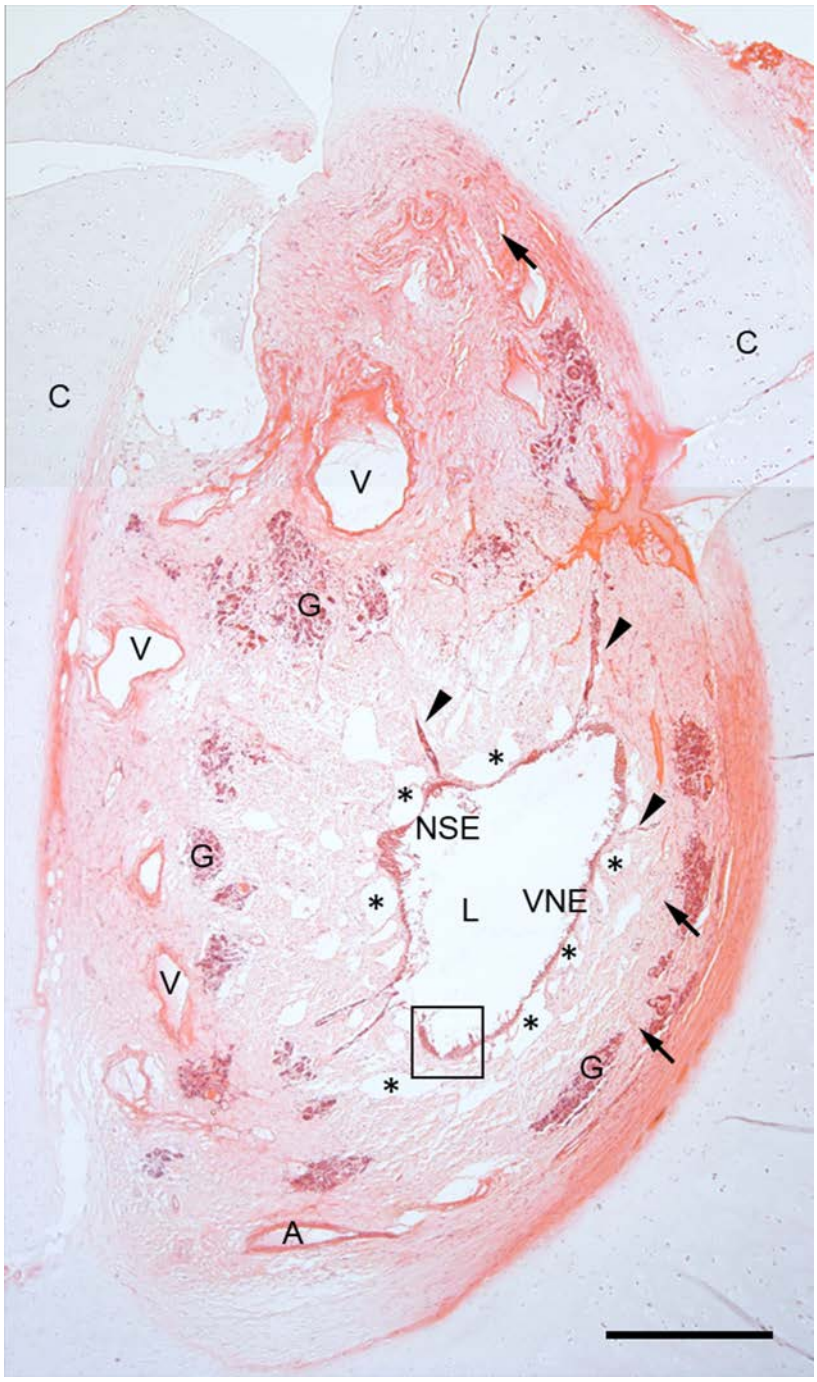

Fig. 1

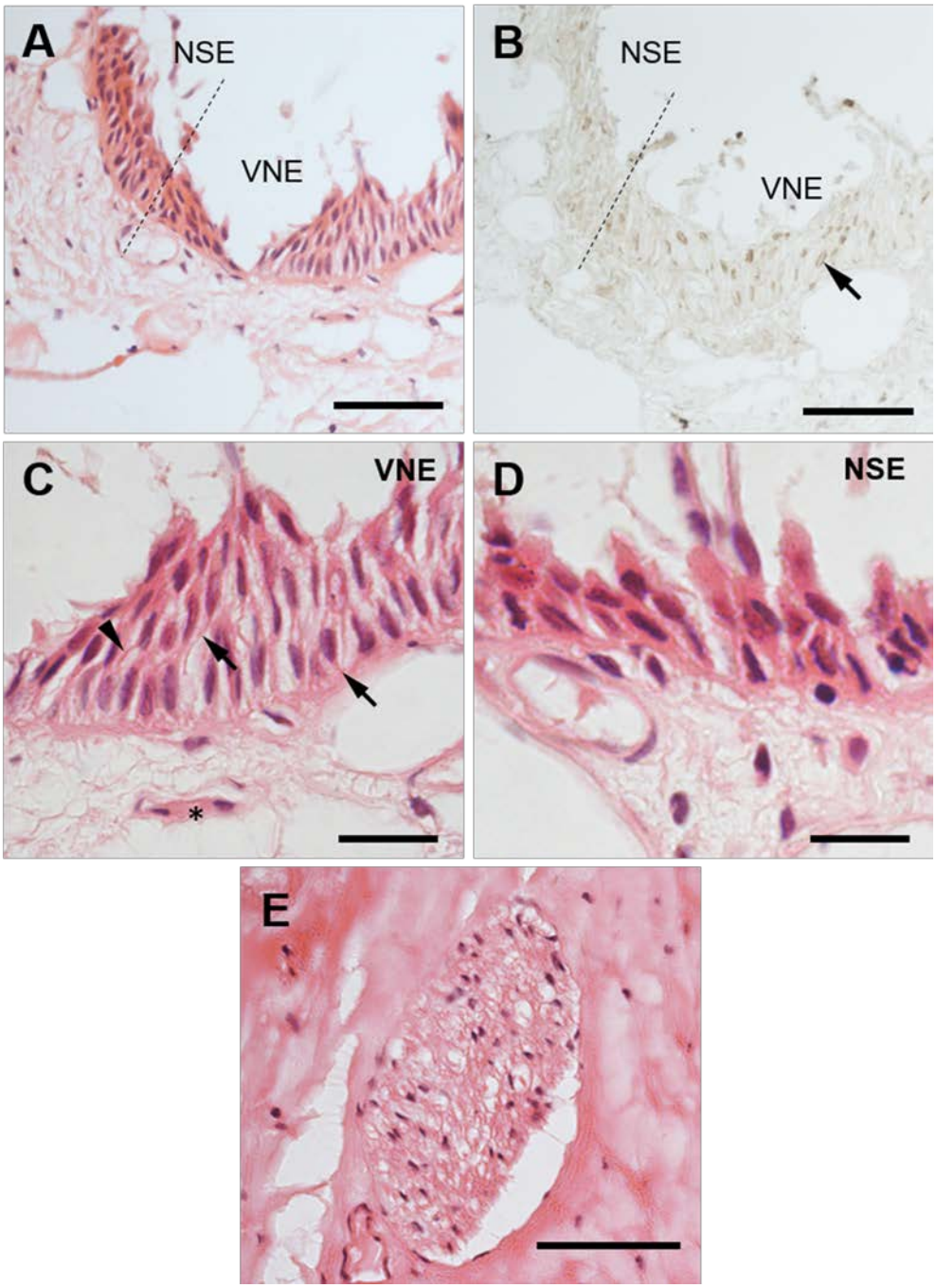

Fig. 2

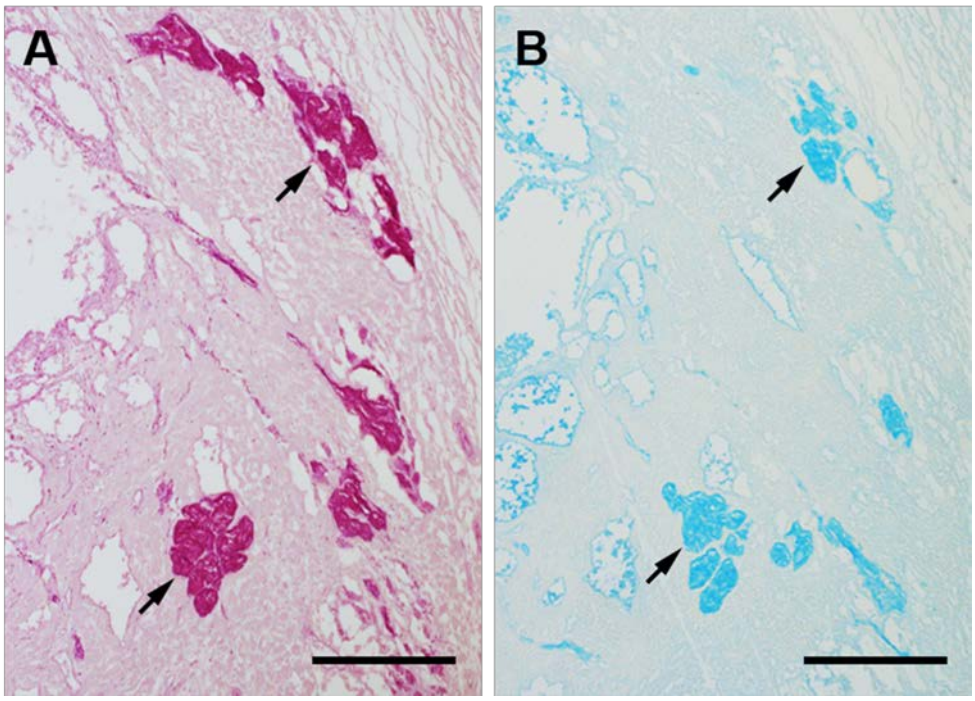

Fig. 3

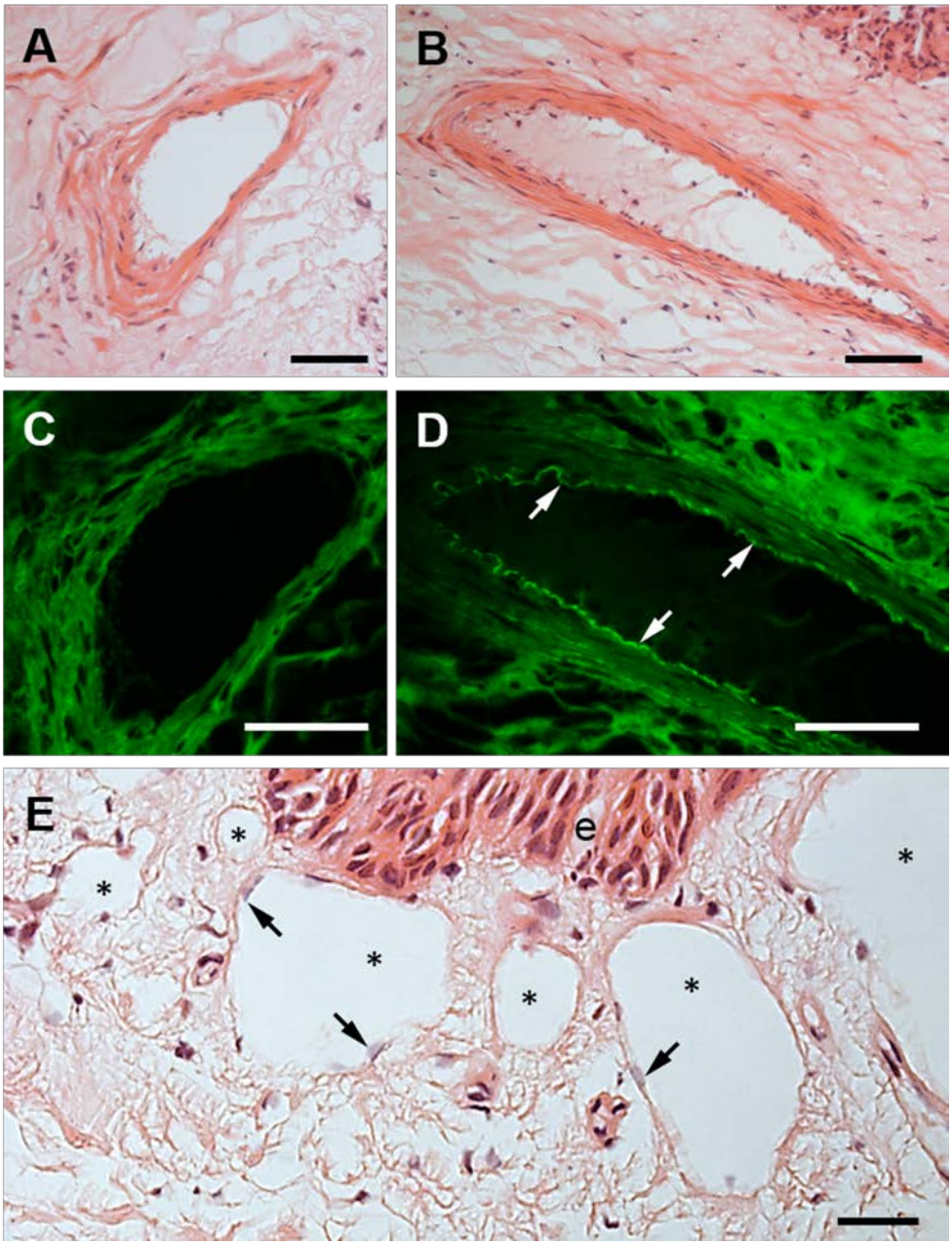

Fig. 4

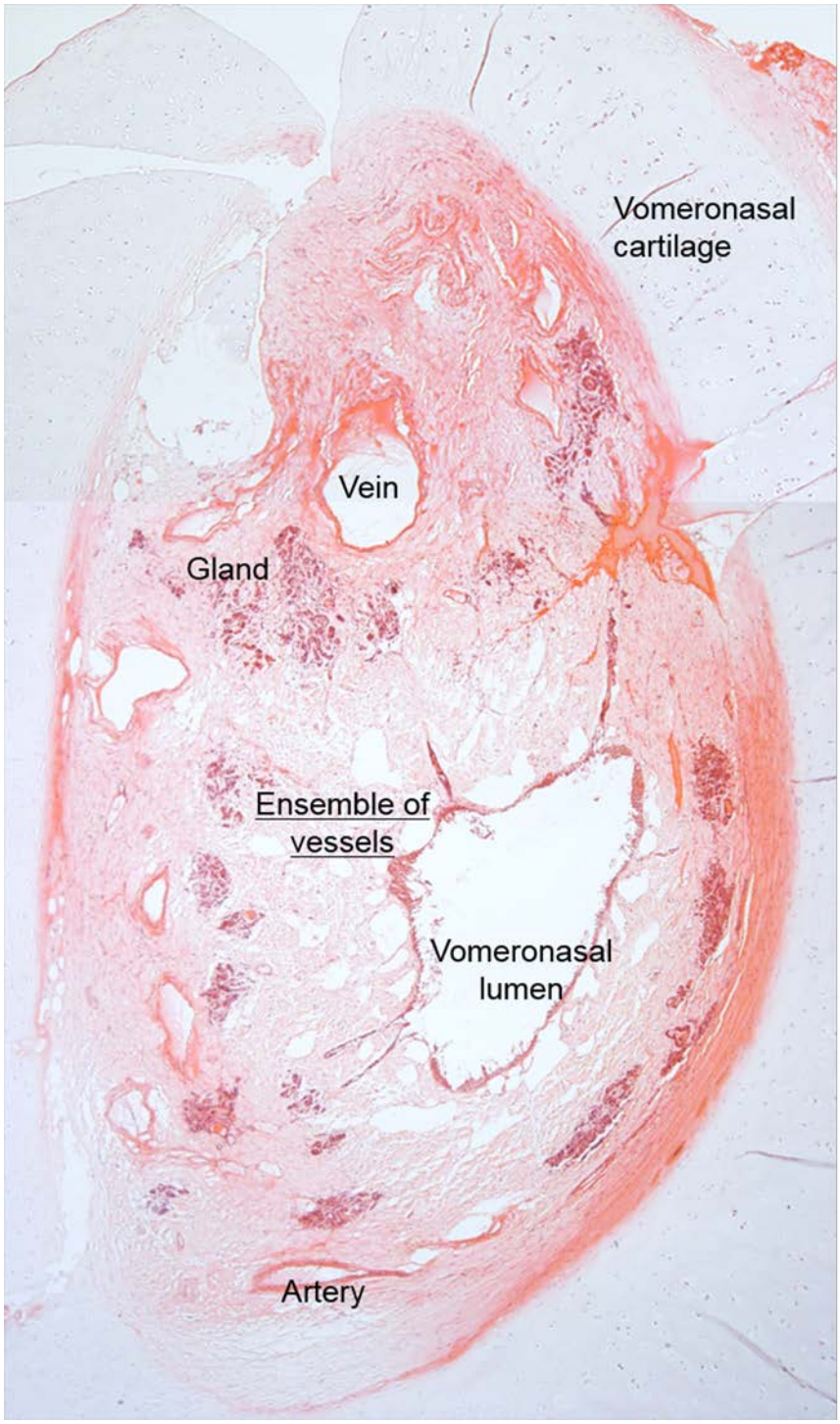

Fig. Abstract