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Morphological Analysis of the Olfactory System of the Pig-Nosed Turtle, *Carettochelys insculpta*

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The turtle olfactory organ consists of the upper (UCE) and lower (LCE) chamber epithelium, projecting to the ventral and dorsal parts of the olfactory bulbs, respectively. The UCE is associated with glands, contains ciliated olfactory receptor neurons, and is assumed to detect odorants primarily in air, while the LCE is devoid of glands, contains microvillous olfactory receptor neurons, and is assumed to detect odorants primarily in water. Examining the olfactory system of the pig-nosed turtle, *Carettochelys insculpta*, this study found that both the upper and lower chambers of the nasal cavity were lined with sensory epithelium devoid of associated glands and contained ciliated olfactory receptor neurons. Moreover, the olfactory bulbs were not divided into dorsal and ventral parts. These results suggest that the olfactory system of the pig-nosed turtle is a single system specialized for detecting odorants in water.

Key words: olfactory bulb, olfactory organ, olfactory receptor neuron, olfactory system, turtle

INTRODUCTION

The olfactory system of vertebrates consists of the olfactory organ, the olfactory bulb, and the olfactory nerve connecting the two. In most terrestrial vertebrates, at least two types of olfactory organs can be distinguished, which are called the olfactory epithelium and the vomeronasal organ (Eisthen, 1997; Eisthen and Polese, 2007; Taniguchi and Taniguchi, 2014). The olfactory epithelium is composed of three types of cells: olfactory cells, supporting cells, and basal cells. The olfactory cells are bipolar neurons equipped with an axon and a dendrite. From the cell body, which has a round nucleus and is located between the nuclei of the supporting and basal cells, the axon and dendrite of an olfactory cell extend to the surface and basal side of the epithelium. Within dendrites, microtubules run parallel to the long axis of the cell and cilia are present at the tips of dendrites. The axons of olfactory cells converge, penetrate the basal lamina, and are enveloped by Schwann cells to form the olfactory nerve. Once they reach the olfactory bulb, the axons reassemble and enter the olfactory glomerulus, where they synapse with secondary neurons. The supporting cells of the olfactory epithelium are columnar cells extending from the basal lamina of the epithelium to the apical surface, with an oval nucleus located in the superficial layer of the epithelium. The supranuclear cytoplasm of supporting cells contains secretory granules that exit apically onto the surface of the epithelium which terminates in microvilli. Basal cells of the olfactory epithelium are irregularly shaped cells located just above the basal lamina and function as stem cells of olfactory cells that continue to turn over throughout life (Graziadei, 1973). The cellular composition of the vomeronasal organ is identical to that of the olfactory epithelium, but unlike olfactory cells, which have cilia, the sensory cells of the vomeronasal organ have microvilli at the tips of their dendrites. Axons of vomeronasal sensory cells project to the accessory olfactory bulbs by making vomeronasal nerves (Døving and Trotier, 1998).

The typical turtle olfactory organ consists of upper (UCE) and lower (LCE) chamber epithelium lining the upper and lower chambers of the nasal cavity, respectively. They are separated from each other by ridges of non-sensory epithelium (Parsons, 1959). Both the UCE and LCE contain olfactory receptor neurons (ORNs), supporting cells, and basal cells. In many turtles, the ORNs in the UCE have cilia at the tips of their dendrites, whereas those in the LCE lack cilia but have microvilli (Graziadei and Tucker, 1970; Hatanaka et al., 1982). Based on the topographic arrangement of the upper and lower chambers of the nasal cavity and the presence of associated glands in the UCE and their absence in the LCE, the UCE is considered to be the olfactory organ for detecting odorants in air, while the LCE detects them in water (Schwenk, 2008).

Like axons in other vertebrates, axons of turtle ORNs fasciculate to form olfactory nerve bundles when they leave olfactory organs. Axons originating from the UCE (LCE) constitute the lateral (medial) part of the olfactory nerves (Nakamuta et al., 2018). Both parts of the olfactory nerve connect to the olfactory bulb situated at the rostral pole of the telencephalon. The olfactory bulb is subdivided into dorsal and ventral parts. Furthermore, both parts of the olfactory bulb are separated into six layers histologically: the olfactory nerve, glomerular, external plexiform, mitral cell,

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internal plexiform, and granule cell layers (Nakamuta et al., 2016b). Axons originating from the LCE project to the olfactory glomeruli in the dorsal part of the olfactory bulb, whereas those from the UCE project to the olfactory glomeruli in the ventral part.

The relative sizes of the UCE and LCE vary among turtle species. Some turtles have a UCE almost as large as the LCE, including the snapping turtle *Chelydra serpentina* (Nakamuta et al., 2016a), whereas the UCE is far smaller than the LCE in turtles such as the Chinese soft-shelled turtle, *Pelodiscus sinensis* (Nakamuta et al., 2016b). The relative size of the dorsal and ventral parts of the olfactory bulb also varies among turtle species. In the snapping turtle olfactory bulb, the dorsal and ventral parts are almost equal in size, while the dorsal part is far larger in the Chinese soft-shelled turtle olfactory bulb; the dorsal part occupies the dorsal and lateral regions, and the ventral part occupies the ventromedial region of the olfactory bulb (Nakamuta et al., 2016a, b). These differences are thought to mirror the adaptation of chelonian olfactory systems to their diverse habitats, as turtles live in a wide variety of environments: some turtles are fully terrestrial and others are semiaquatic.

The pig-nosed turtle, *Carettochelys insculpta*, (Fig. 1) is the only known extant member of the genus *Carettochelys*, family Carrettochelyidae, and it inhabits the rainforests of New Guinea and northern Australia (Franklin, 2007). It swims in a manner like sea turtles: stroking both forelimbs simultaneously upward and downward, as if flying in water. Although the anatomical characteristics of the pig-nosed turtle have been reported (Walther, 1922), little attention has been paid to its olfactory system. Therefore, in this study, we analyzed its olfactory system using light and electron microscopy and obtained data suggesting that its olfactory organs and olfactory bulbs may constitute a single olfactory system specialized for detecting odorants in water.

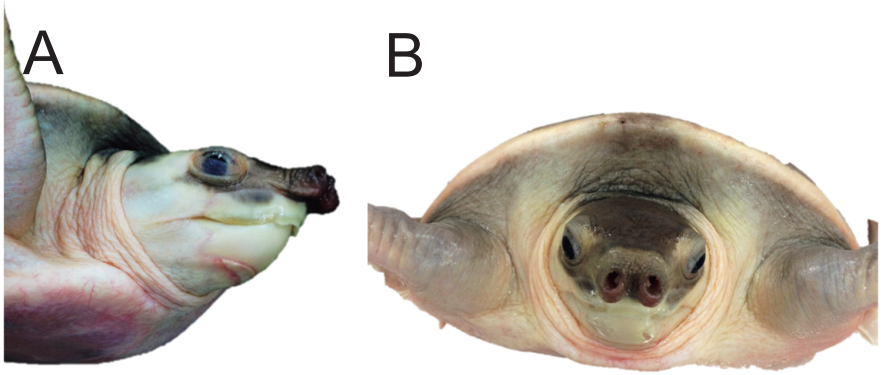

Fig. 1. Lateral **(A)** and frontal **(B)** views of the pig-nosed turtle. See large nostrils situated at the tip of the snorkel-like snout.

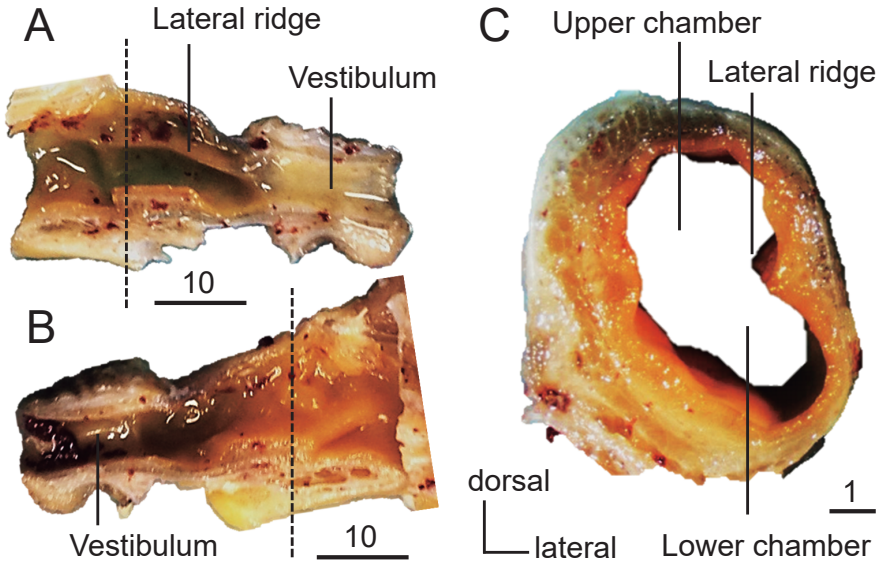

Fig. 2. Pig-nosed turtle nasal cavity. **(A)** Medial view of the lateral wall. **(B)** Lateral view of the medial wall. **(C)** Transverse section at the level indicated by dotted lines in **(A)** and **(B)**. The upper and lower chambers are incompletely separated by the ridges on the wall of the nasal cavity. Bars: 10 mm in **(A, B)**; 1 mm in **(C)**.

MATERIALS AND METHODS

The animal experiments were approved by the Animal Care and Use Committee of Iwate University (Authorization number A201720) and conducted in accordance with the Guide for the Care and Use of Experimental Animals. The olfactory organs and olfactory bulbs of a male pig-nosed turtle (body weight 1320 g, carapace length 20 cm) were used in this study. After anesthesia by an intraperitoneal injection of sodium pentobarbital (64.8 mg/kg), the turtle was euthanized by cardiac perfusion of Ringer's solution, followed by a fixative solution containing 2.5% glutaraldehyde in 0.1 M phosphate buffer.

The olfactory organs were dissected and processed for transmission and scanning electron microscopy, as described previously (Nakamuta et al., 2016b). Semi-thin sections were stained with toluidine blue and examined with a light microscope. The olfactory bulbs were embedded in OCT compound (Sakura Finetek, Tokyo, Japan) after cryoprotection in a sucrose gradient (10%, 20%, and 30%). Frozen sections were made at 20 μm thicknesses and stained with either hematoxylin–eosin or the Kluver–Barrera method.

RESULTS

As in other turtles, the nasal cavity of the pig-nosed turtle is roughly divided into upper and lower chambers by the ridges on the walls of the nasal cavity (Fig. 2). In this study, the epithelium lining the upper chamber will be referred to as the UCE and the epithelium lining the lower chamber as the LCE. Both UCE and LCE were composed of ORNs, supporting cells, and basal cells (Fig. 3A, B). Nuclei of the supporting cells, ORNs, and basal cells were situated in the apical, middle, and basal regions of these epithelia. It was difficult

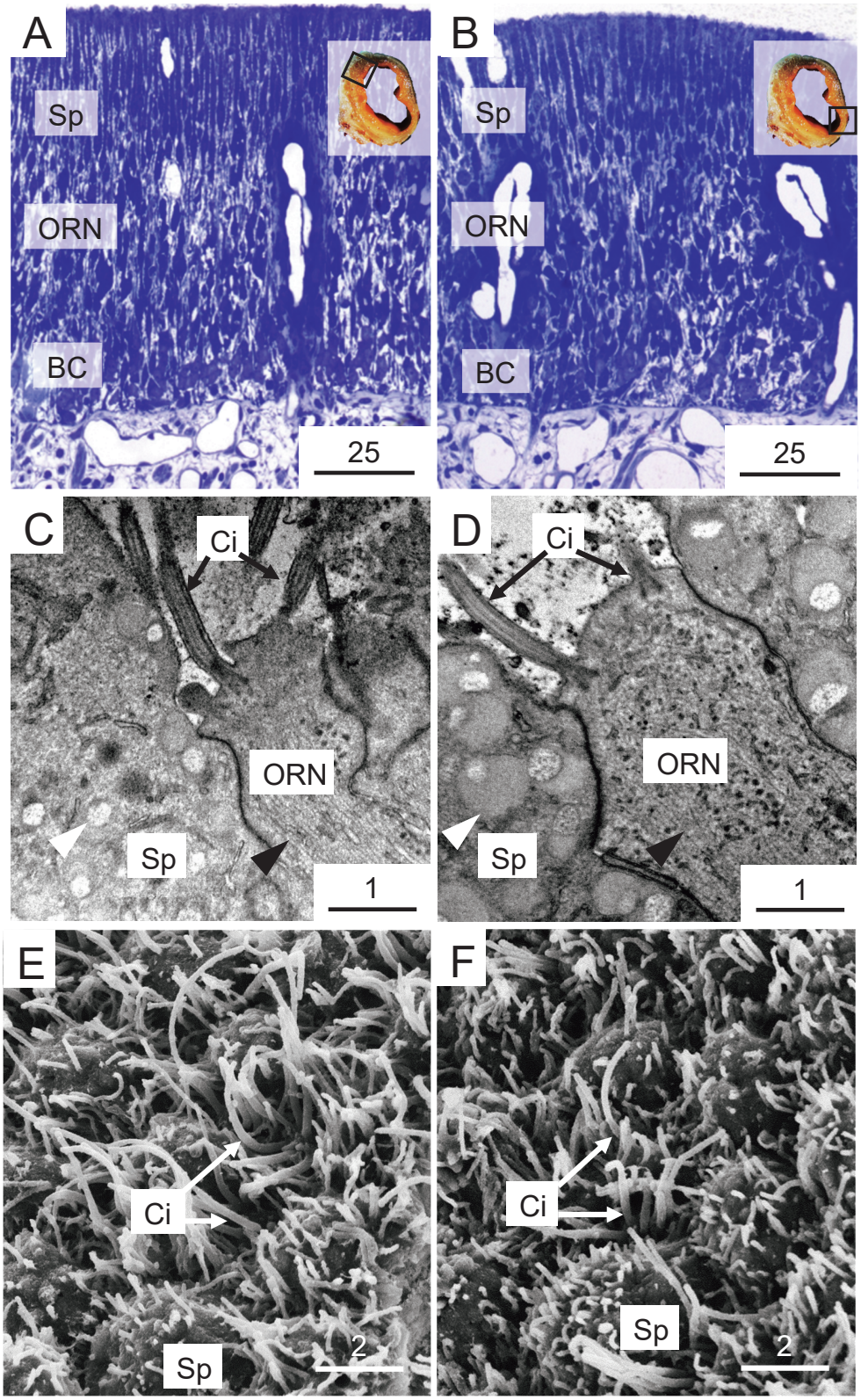

Fig. 3. Epithelium lining the pig-nosed turtle olfactory organ. Toluidine blue-stained semithin sections through the upper **(A)** and lower **(B)** chamber epithelia, showing the nuclei of supporting cells (Sp), olfactory receptor neurons (ORN), and basal cells (BC). See absence of associated glands in the lamina propria. Insets show where tissues were collected. Transmission electron micrographs of the apical part of the upper **(C)** and lower **(D)** chamber epithelia. Scanning electron micrographs of the luminal surface of the upper **(E)** and lower **(F)** chamber epithelia. The ORN of both upper and lower chamber epithelia contain microtubules (black arrowheads) in their cytoplasm and have cilia (Ci) at the tip of their dendrites. Supporting cells (Sp) contain secretory granules (white arrowheads) in their cytoplasm and bear microvilli at their apical ends. Bars: 25 μm in **(A, B)**; 1 μm in **(C, D)**; 2 μm in **(E, F)**.

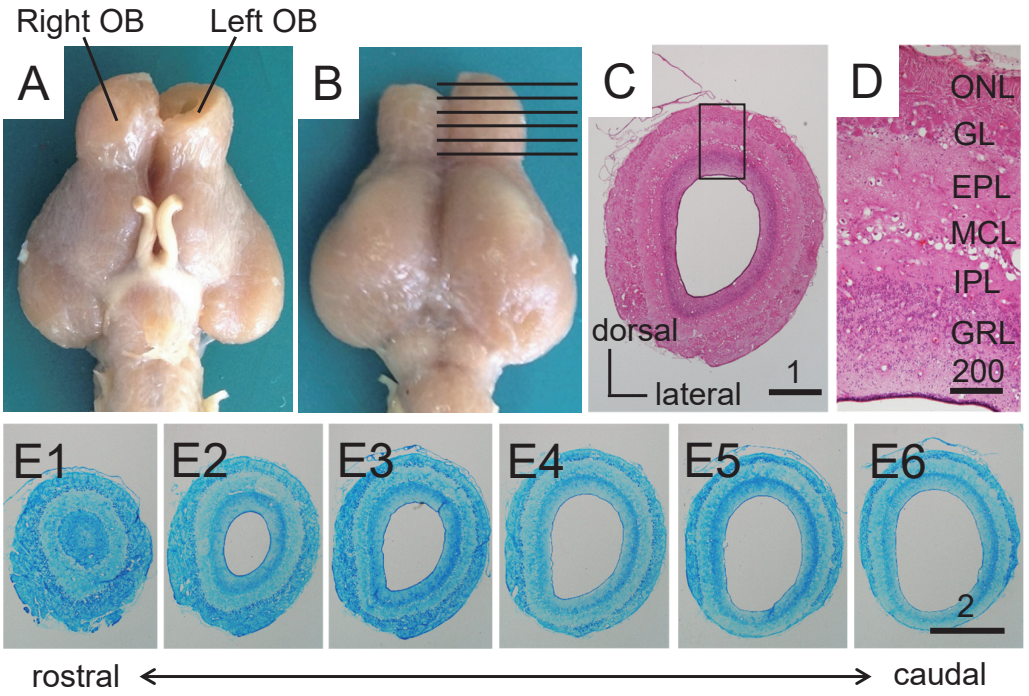

Fig. 4. Pig-nosed turtle olfactory bulb. Ventral **(A)** and dorsal **(B)** views. Hematoxylin-eosin-stained transverse section through the olfactory bulb **(C)** and higher magnification view of the boxed area **(D)**. Kluver Barrera-stained transverse sections arranged from rostral to caudal at 1 mm intervals **(E1–E6)**. Olfactory nerve layer (ONL), glomerular layer (GL), external plexiform layer (EPL), mitral cell layer (MCL), internal plexiform layer (IPL), granule cell layer (GRL). Bars: 1 mm in **(C)**; 200 μm in **(D)**; 2 mm in **(E1–E6)**.

to distinguish the UCE from the LCE because no associated glands were observed in the lamina propria. In addition, non-sensory epithelium separating the UCE and LCE was not discernible. In the lamina propria of both the UCE and LCE, blood vessels and nerve bundles were noted. Furthermore, electron microscopic observations of the UCE and LCE revealed the presence of ORNs bearing cilia at the tips of their dendrites in each epithelium (Fig. 3C–F). The ORNs contained longitudinally arranged microtubules and were distinguished from the supporting cells containing secretory granules in their cytoplasm. Apical ends of the supporting cells were equipped with microvilli in each epithelium.

The right and left olfactory bulbs were situated at the rostral pole of the brain (Fig. 4A, B). By the histological examination of olfactory bulbs, the olfactory nerve, glomerular, external plexiform, mitral cell, internal plexiform, and granule cell layers were distinguished around the olfactory ventricle (Fig. 4C, D). However, no obvious division into dorsal and ventral parts was found in thorough examinations of the olfactory bulbs from the rostral to caudal ends (Fig. 4E1–E6).

DISCUSSION

No associated glands were found in the sensory epithelia lining the upper and lower chambers of the nasal cavity of the pig-nosed turtle. This implies that both the UCE and LCE of this species correspond to the LCE of other turtles and detect odorants primarily in water. The tetrapod vertebrate olfactory organ is generally associated with glands (Taniguchi and Taniguchi, 2014) that play important roles in olfaction by dissolving odorants in air with their secretory products, which helps them to bind to receptors (Getchell and Getchell, 1992). However, the functional separation (detecting odorants in air or in water) in the turtle olfactory organ is thought to be incomplete, as suggested by the incomplete separation of the upper and lower chambers of the nasal cavity. Indeed, the LCE of some turtles can detect volatile odorants in addition to water-soluble odorants (Hatanaka and Matsuzaki, 1993). Thus, the absence of the associated glands in the olfactory organ of the pig-nosed turtle does not necessarily indicate the inability to detect odorants in air. Further analyses, including analyses of olfactory receptor genes, are needed to elucidate the function of olfactory organs in this species.

In the olfactory organs of many turtle species, the ORNs bear cilia in the UCE but not in the LCE (Graziadei and Tucker, 1970; Hatanaka et al., 1982; Saito et al., 2000; Nakamuta et al., 2016a). However, the ORNs of both the UCE and LCE bear cilia in the pig-nosed turtle olfactory organ, as shown here. Ciliated ORNs are also present in both the UCE and LCE in the olfactory organ of the Chinese soft-shelled turtle (Nakamuta et al., 2016b). However, in that turtle, the presence of associated glands in the UCE clearly distinguishes it from the LCE, although the UCE cannot be distinguished from the LCE by the fine structure of ORNs (Nakamuta et al., 2016c). Furthermore, soft-shelled turtle olfactory bulbs, which receive projections from the UCE and LCE, are clearly divided into dorsal and ventral parts (Nakamuta et al., 2016c). Whether the fine structural feature of ORNs shared by the two turtle species in the superfamily Trionychoidea is a trait of their common ancestors or the result of convergent evolution in highly aquatic turtles is currently unknown and warrants further investigation.

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COMPETING INTERESTS

The authors declare no competing interests.

AUTHOR CONTRIBUTIONS

NN, SN, and YY collected data, analyzed data, made interpretations, and wrote the manuscript. HK made interpretations and wrote the manuscript.

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