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Anatomical Specializations for Enhanced Olfactory Sensitivity in Kiwi, *Apteryx mantelli*

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Key Words
Allometry · Brain evolution · Comparative neuroanatomy · Olfaction · Olfactory bulb · Olfactory epithelium

Abstract
The ability to function in a nocturnal and ground-dwelling niche requires a unique set of sensory specializations. The New Zealand kiwi has shifted away from vision, instead relying on auditory and tactile stimuli to function in its environment and locate prey. Behavioral evidence suggests that kiwi also rely on their sense of smell, using olfactory cues in foraging and possibly also in communication and social interactions. Anatomical studies appear to support these observations: the olfactory bulbs and tubercles have been suggested to be large in the kiwi relative to other birds, although the extent of this enlargement is poorly understood. In this study, we examine the size of the olfactory bulbs in kiwi and compare them with 55 other bird species, including emus, ostriches, rheas, tinamous, and 2 extinct species of moa (Dinornithiformes). We also examine the cytoarchitecture of the olfactory bulbs and olfactory epithelium to determine if any neural specializations beyond size are present that would increase olfactory acuity. Kiwi were a clear outlier in our analysis, with olfactory bulbs that are proportionately larger than those of any other bird in this study. Emus, close relatives of the kiwi, also had a relative enlargement of the olfactory bulbs, possibly supporting a phylogenetic link to well-developed olfaction. The olfactory bulbs in kiwi are almost in direct contact with the olfactory epithelium, which is indeed well developed and complex, with olfactory receptor cells occupying a large percentage of the epithelium. The anatomy of the kiwi olfactory system supports an enhancement for olfactory sensitivities, which is undoubtedly associated with their unique nocturnal niche.

Introduction

Although olfaction was traditionally thought of as being poorly developed in birds based on the small size of their olfactory bulbs [Hill, 1905], more recent anatomical, physiological, and behavioral studies have established that olfaction is an important sensory modality in birds [Bang, 1960, 1971; Bang and Cobb, 1968; Caro and Balthazart, 2010; Michelsen, 1959; Tucker, 1965; Wenzel, 1987]. Indeed, in those birds in which olfactory thresholds have been determined, sensitivities to odors are similar to those of some mammals [Clark et al., 1993; Smith and Paselk, 1986; Snyder and Peterson, 1979; Stattleman et al., 1975; Waldvogel, 1989; Walker et al., 1986]. Behavioral studies have also demonstrated that olfaction is important in mediating many types of behaviors, including navigation [Holland et al., 2009; Papi, 1990; Wallraff, 2003, 2004], reproduction [Hagelin et al., 2003; Whittaker et al., 2013],

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individual recognition [Bonadonna and Nevitt, 2004; De Leon et al., 2003], nest recognition [Bonadonna and Bretagnolle, 2002; Bonadonna et al., 2003a, b, 2004], communication [Hagelin et al., 2003], and mate choice [Whittaker et al., 2011, 2013]. In addition, olfaction plays an important role in foraging in vultures *(Cathartes melambrotus)* [Graves, 1992], ravens *(Corvus corax)* [Harriman and Berger, 1986], parrots (*Strigops habroptilus* [Hagelin, 2004] and *Lorius garrulus flavopalliatus* [Roper, 2003]), procellariiforms, (shearwaters, petrels, and albatrosses) [Hutchison and Wenzel, 1980], and kiwi *(Apteryx mantelli)* [Benham, 1906; Cunningham et al., 2009; Wenzel, 1968, 1971].

In birds, odors are detected in the third nasal chamber, which is lined with olfactory sensory epithelium containing the olfactory receptor neurons [Bang, 1971]. The epithelium is supported on a sheet of cartilage that can be highly convoluted in some species to form spiral-shaped olfactory tubercles. The olfactory receptor neurons in this epithelium give rise to the olfactory nerves that make synapses with mitral and tufted cells in the olfactory bulb on the ventral aspect of the brain. Olfactory information is then transmitted to other areas of the brain where odorants are processed and perceived [Bang, 1971; Firestein, 2001]. The basic structure of the olfactory bulb is highly conserved across vertebrates, but the size and laminar cytoarchitecture vary considerably among birds [Bang and Cobb, 1968; Cobb, 1960a; Nieuwenhuys, 1967]. Some species have small, conjoined bulbs with thin layers that are barely visible, whereas in others the olfactory bulbs are large and complex with clear cellular laminae. Among birds, the relative size of the olfactory bulbs is correlated with a number of ecological factors: larger olfactory bulbs have been associated with water activity, nesting strategy, diet, and nocturnal or crepuscular activity [Bang, 1971; Cobb, 1960b; Healy and Guilford, 1990].

Kiwi (*Apteryx* sp.) are entirely ground dwelling and uniquely adapted for their nocturnal activities in native forests of New Zealand. Kiwi have a specialized auditory system, possibly tuned to locating prey using auditory cues [Corfield et al., 2011, 2012a], and have developed whisker-like facial bristles to help navigate a nocturnal environment [Cunningham et al., 2010], together with a highly regressed visual system [Martin et al., 2007]. In addition, kiwi have a long, slightly curved beak, with nostrils at the tip and a specialized bill tip organ, all used to locate and extract hidden invertebrate prey including earthworms and beetle larvae from soil, leaf litter, rotting wood, and damp sand [Colbourne, 1983; Cunningham et al., 2007, 2009, 2013; Martin et al., 2007; Potter, 1989; Reid et al., 1982; Taborsky and Taborsky, 1995; Watt, 1971]. Likely as a result of their many unique sensory adaptations, the brain of the kiwi has also undergone many changes, including an enlarged telencephalon [Corfield et al., 2008] resulting from enlargements to specific telencephalic regions [Corfield et al., 2012b]. In addition, the principal sensory trigeminal nucleus (PrV) and the nucleus basorostralis (Bas) [Cunningham et al., 2013], both of which process tactile information from the beak, are enlarged, whereas there is a reduction of all visual nuclei [Corfield, 2009; Martin et al., 2007].

In kiwi, olfaction is particularly important for finding food, and they are able to locate it over short distances using their sense of smell alone or in combination with remote touch [Benham, 1906; Cunningham et al., 2009; Wenzel, 1968, 1971]. Uniquely, kiwi appear to have a mechanism to transport odorants back to the olfactory concha at the base of their beak; air is rapidly inhaled through their nostrils, producing a loud 'sniffling' sound [Castro et al., 2010; Wenzel, 1971]. In addition, both the feces and the feathers of kiwi have a strong pungent odor, possibly originating from their large uropygial gland, which would provide ideal cues for social communication or even individual recognition [Bonadonna et al., 2007; Castro et al., 2010; Soini et al., 2013], although strong behavioral evidence for this function is lacking. Further supporting the idea that kiwi rely heavily on olfaction, their olfactory bulbs and nasal turbinates have been reported to be unusually large [Bang, 1971; Bang and Cobb, 1968; Cobb, 1960a; Corfield et al., 2012b; Craigie, 1930; Kraabe, 1959; Owen, 1839; Parker, 1891]. Nevertheless, this suspected enlargement has not been documented using appropriate quantitative and phylogenetic methods.

In this study, we examined the relative size of the olfactory bulb in kiwi using a large data set which includes emus, ostriches *(Struthio camelus),* rheas *(Rhea americana),* 3 tinamou species *(Nothura darwinii, Tinamus major*, and *Rhynchotus rufescens),* and 2 extinct moa species *(Anomalopteryx didiformis* and *Dinornis novaezealandiae).* Our statistical methods also account for phylogeny. In addition, we examined the anatomy of the olfactory system, including the cytoarchitecture of the olfactory bulbs and turbinates, to determine if any other modifications besides size are present in the olfactory system of kiwi that would improve olfactory function.

Methods

Ethical Statement

North Island brown kiwi *(A. mantelli)* were obtained from the Department of Conservation in Northland, New Zealand; all specimens used in the study were provided postmortem by conserva-

Table 1. Comparisons of brain, telencephalon, and olfactory bulb size in 8 bird species across different studies

Bird	Species	n	Brain, mm^3	Tel, mm^3	OB, mm^3	% Tel	% OB of Tel	Source
Pigeon	*Columba livia*	6	2,152.63	1,040.76	8.54	48.35	0.82	Ebinger and Lohmer, 1984
		1	2,306.95	1,245.72	7.78	54.00	0.62	Boire, 1989
		3	1,705.66	893.05	7.26	52.36	0.81	Corfield et al., 2012b
Common quail	*Coturnix coturnix*	10	877.90	419.09	1.83	47.74	0.44	Rehkamper et al., 1991
		1	810.81	369.40	0.75	45.56	0.20	Boire, 1989
Ring-necked pheasant	*Phasianus colchicus*	10	3,864.86	1,999.35	5.24	51.73	0.26	Rehkamper et al., 1991
		1	2,761.58	1,579.09	5.75	57.18	0.36	Boire, 1989
Wild turkey	*Meleagris gallopavo*	1	7,712.36	3,764.53	6.49	48.81	0.17	Boire, 1989
		3	5,274.02	2,914.66	7.37	55.26	0.25	Corfield et al., 2012b
Carrion crow	*Corvus corone*	5	9,573.53	7,167.48	2.01	74.87	0.03	Mehlhorn et al., 2010
		7	9,382.24	7,019.03	1.98	74.81	0.03	Rehkamper et al., 1991
Eurasian jay	*Garrulus glandarius*	2	3,735.33	2,545.26	1.04	68.14	0.04	Mehlhorn et al., 2010
		3	3,806.30	2,596.73	1.05	68.22	0.04	Rehkamper et al., 1991
House sparrow	*Passer domesticus*	4	954.88	637.75	0.48	66.79	0.08	Mehlhorn et al., 2010
		4	954.87	637.56	0.48	66.77	0.08	Rehkamper et al., 1991
Red-winged tinamou	*Rhynchotus rufescens*	1	3,013.88	1,704.73	6.83	56.56	0.40	Cunningham et al., 2013
		1	3,377.41	1,971.68	10.43	58.38	0.53	Boire, 1989

The percentage of the brain occupied by the telencephalon (% Tel) and the percentage of the telencephalon occupied by the OB (% OB of Tel) together with the source of the data are shown. Tel = Telencephalon; OB = olfactory bulb.

tion authorities and wildlife veterinarians. Because kiwi were not killed for this study, no university ethics approvals were required to undertake this research. North Island brown kiwi are protected in New Zealand, and permission to use these specimens for research was obtained under permits 37258-DOA, NO-16732-FAU, NO-18095-DOA, WC-17552-DOA, and WE-333-RES from the New Zealand Department of Conservation.

Specimens

The cytoarchitecture of olfactory regions was examined in the brains of 5 North Island brown kiwi (3 adults and 2 juveniles, 1 week and 2 months old). In addition, the olfactory turbinate from 1 adult North Island brown kiwi was examined. Data concerning the sizes of the brain, the telencephalon, the 'brainrest' (which includes the brainstem, midbrain, thalamus, and cerebellum), and the olfactory bulbs are shown in online supplementary table S1 (see www.karger.com/doi/10.1159/000365564) and represent all data that include separate measures of the volumes of different brain regions currently available for birds. Online supplementary table S1 also indicates the common name and order of each species. Data were compiled from the studies of Ebinger and Lohmer [1984], Corfield et al. [2012b], Boire [1989], Cunningham et al. [2013], Pistone et al. [2002], Rehkamper et al. [1991], Carezzano and Bee De Speroni [1995], Mehlhorn et al. [2010], Fernandez et al. [1997], and Alma and Bee De Speroni [1992]. Additional data on olfactory bulb size in California quail *(Callipepla californica),* black-winged stilt *(Himantopus himantopus),* South Island oystercatcher *(Haematopus finschi),* ostrich *(S. camelus),* and red-winged tinamou *(R. rufescens)* were obtained from the same specimens used in the studies of Cunningham et al. [2013] and Corfield et al. [2012c].

To minimize the effects of differential shrinkage among studies, our analysis used only the relative size of a brain structure; for example, we compared the size of the olfactory bulb relative to that of the telencephalon. It is likely that all regions of a brain will shrink by the same amount, and therefore comparison of relative brain sizes reduces the amount of error when combining data from multiple studies [Corfield et al., 2012c]. Indeed, when comparing, for example, the size of the brain and the olfactory bulb in pigeons between the studies of Ebinger and Lohmer [1984] and Corfield et al. [2012b], we found some differences in the sizes of both structures but the percentage of the telencephalon occupied by the olfactory bulb was nearly identical (table 1). Where data from multiple studies were available for the same species, comparisons of the percentage of the telencephalon and olfactory bulbs showed that for most species the overall the variation between studies was low (table 1).

Moa (Dinornithiformes) are extinct flightless birds endemic to New Zealand that, together with kiwi, are part of the ratite group [Davies, 2003b]. Having evolved alongside kiwi, it is of interest to determine whether kiwi and moa share similar olfactory abilities. Skulls of one *D. novaezealandiae* (LB7082) and one *A. didiformis* (LB5548) were obtained on loan from the Auckland Museum, Auckland, New Zealand, and digital endocasts were generated from CT scans (see below).

Kiwi Brain and Olfactory Turbinate Processing

All specimens were immersion-fixed in 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS). To section the olfactory turbinate, which is lined with olfactory epithelium, all bone encapsulating the olfactory turbinate was first removed. The calvarium was then removed to expose the brain and an incision was made to separate the olfactory bulb from the telencephalon. The brain was removed from the skull, leaving the olfactory bulb in place. The olfactory bulbs, which were still connected to the olfactory turbinate and free of any bone, were then processed.

Prior to sectioning, specimens were cryoprotected in 30% sucrose in PBS until they sank (usually 3–7 days) and embedded in a solution of 15% gelatin with 30% sucrose. The block was then placed in 4% paraformaldehyde overnight and then in 30% sucrose-PBS until it sank. Blocks were sectioned sagittally at a 50-μm thickness. Every second section was mounted serially onto subbed slides, stained with cresyl violet, dehydrated, and coverslipped with DePeX (Serva Electrophoresis) from xylene.

To obtain a 3-dimensional (3-D) model of the kiwi olfactory bulbs, fiducial points were required to align the sections. To create sections with fiducial points, the brain of one kiwi was placed into a custom-made mold which consisted of a plastic base with small holes drilled in a grid pattern. The brain was placed in the mold on a preset gelatin base, with the midline facing down. Small pins were inserted into the base of the mold so that they surrounded the brain and ran in a rostral-caudal direction. A 15% gelatin, 30% sucrose and PBS solution containing black fabric dye (to darken the gelatin solution) was then poured over the brain. Once set, the gelatin block, including the brain, was removed, trimmed, and placed, along with the pins, into 4% paraformaldehyde overnight. The following day the pins were removed and further processed as above. The end result was sections that contained holes in the gelatin that could be used for alignment [Corfield et al., 2012b].

Immunocytochemistry

Kiwi olfactory turbinate sections and alternating olfactory bulb sections were labeled with an anti-neural cell adhesion molecule (NCAM) antibody (clone 4d; Developmental Studies Hybridoma Bank, University of Iowa Department of Biology) [Frelinger and Rutishauser, 1986; Watanabe et al., 1986]. The NCAM antibody labeled the glomerular layer, the olfactory fila, the olfactory nerve, and olfactory receptor cells and was therefore used to identify and describe these structures in kiwi. Floating sections were first incubated for 10 min in 10% H_2O_2 in PBS:methanol (1:1) to block endogenous peroxidase activity and then washed in PBS (0.1 M, pH = 7.4) for 30 min. Sections were incubated overnight at room temperature with constant agitation in the primary antibody diluted 1:250 in a buffer consisting of 0.1 M PBS containing 2.5% normal horse serum and 0.4% Triton X-100. Incubation in the primary antibody was followed by 3 washes of 10 min each in PBS and then incubation in an anti-mouse biotinylated secondary antibody (1:300; Jackson ImmunoResearch Laboratories, Inc.) for 1 h at room temperature. Following 3 washes in PBS, the sections were incubated in horseradish peroxidase-streptavidin complex (Invitrogen) diluted 1:1,000. The sections were washed for a further 30 min in the incubation buffer and developed using diaminobenzidine tetrahydrochloride (DAB; 0.25 mg/ml) with 0.005% H_2O_2 and $CoCl_2$ to render the reaction product black. The sections were mounted onto subbed slides, dehydrated, cleared, and coverslipped with DePeX from xylene.

CT Scans of Moa Skulls

Moa skulls were scanned at the Green Lane Clinical Centre, Auckland, New Zealand, on a Philips Brilliance 16 CT scanner at 260 mAs at an X-ray energy of 120 kVp with a slice thickness of 1.0 mm [Corfield et al., 2008]. The resulting serial sections were saved as 16-bit TIFF files. Endocranial outlines for the olfactory bulbs, telencephalon, and brainrest were obtained for each section using AMIRA (v 5.2; Visage Imaging, San Diego, Calif., USA). The borders between the telencephalon and the rest of the brain, as well as between the telencephalon and the olfactory bulbs, were defined with the aid of Nissl-stained sagittal sections of emu and ostrich brains. Figure 1 shows examples of 3 different levels through a moa skull and illustrates the areas defined as the olfactory bulb, the telencephalon, and the brainrest.

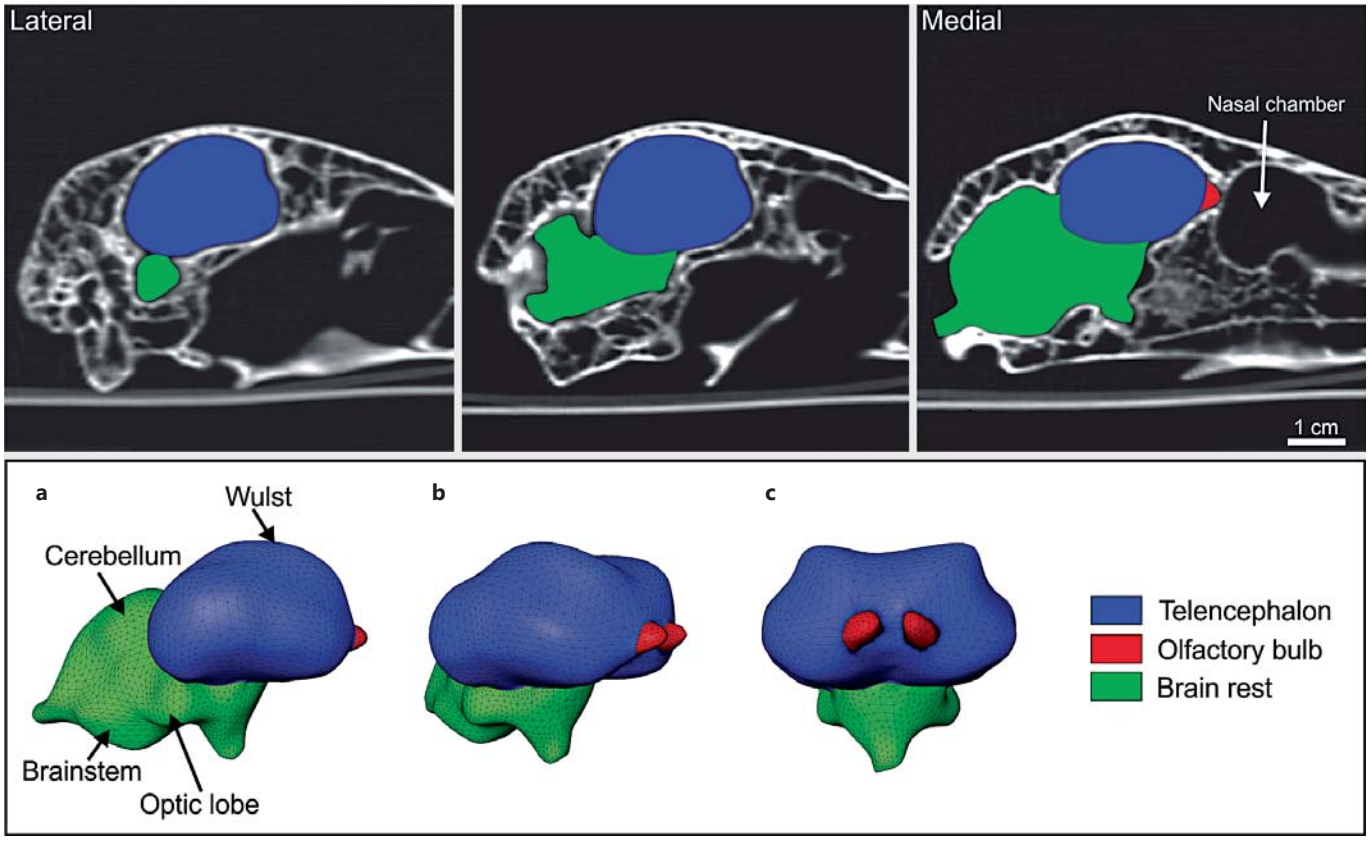
Volumetric Measurements and Analysis

Once each brain region was outlined in AMIRA, the stack of images was exported as a series of TIFF files, with the area of interest filled in black against a white background. These TIFF stacks were imported into ImageJ (v 1.34s; National Institutes of Health, USA) to obtain the cross-sectional area of the brain region. The cross-sectional areas were added for each brain region and then multiplied by the slice thickness and the number of sections between stack slices to obtain the volume. To examine scaling relationships, we plotted the $\log_{10}$-transformed volume of the olfactory bulbs against the $\log_{10}$-transformed brain volume minus the volume of the olfactory bulbs, the telencephalon volume minus the volume of the olfactory bulbs, and the brainrest volume.

Because phylogeny can significantly affect brain evolution [Harvey and Pagel, 1991], we first tested for phylogenetic signals using the PHYTOOLS package in R [Revell, 2009; Team, 2013]. The effect of phylogeny on all variables was tested using Blomberg's K [Blomberg et al., 2003]. We found a significant phylogenetic signal for all variables (randomization test, $p > 0.001$ for all variables); therefore, we used analyses that accounted for phylogenetic effects. Phylogenetic trees were constructed based on the relationships in the study of Hackett [2008] using Mesquite [Maddison and Maddison, 2011]. Additional resolution within orders was obtained for Passeriformes [Ericson et al., 2005], Charadriiformes [Mayr, 2011], Psittaciformes [Wright et al., 2008], Anseriformes [Donne-Gousse et al., 2002], Galliformes [Wang et al., 2013], and Tinamiformes [Bertelli and Porzecanski, 2004]. Because we reconstructed the phylogeny of all species from multiple sources, we used an arbitrary branch length model, which we then used to construct 'phylogeny-corrected' confidence intervals [Garland and Ives, 2000; Lavin et al., 2008] using the PDAP:PDTREE module of Mesquite [Midford et al., 2010].

3-D Modeling in AMIRA

Images of kiwi brain sections were first aligned according to the fiducial points using the Alignslice module in AMIRA. CT scans of the moa skulls did not require any image alignment. The LabelField module was used to segment out each brain region from each image using the brush tool and then each brain region was assigned to a material. A SurfaceGen module was attached to the LabelField module and the 3-D model was visualized by attaching a SurfaceView module.



Color version available online

Fig. 1. CT scans of the skull and reconstructions of the brain of a little bush moa *(A. didiformis)*. The top illustrates CT scans at 3 different levels through the skull, with colored (only in the online version) regions indicating the area designated to the olfactory bulb, telencephalon, and brainrest (brainstem, cerebrum, midbrain, and thalamus). **a–c** 3-D reconstructions of the moa brain from the CT scans. **a** Lateral view. **b** A more rostral view. **c** Rostral view.

Results

The structure of kiwi olfactory bulbs differs from that of other birds (fig. 2a). Instead of having an olfactory bulb that extends from the rostral pole of the telencephalon in a stalk-like or 'pedunculated' structure typical of other birds, kiwi olfactory bulbs resembled an extensive cortex-like sheet. 3-D reconstructions demonstrate that these cortical sheets form a cup-like structure that surrounds the frontal pole of the brain (fig. 2b). In contrast, the olfactory bulbs of the moa more closely resemble those of the emu, ostrich and tinamou, with an obvious pedunculated structure (fig. 1).

Of the 55 species from 17 orders of birds examined in this study, only 4 had olfactory bulb volumes that were above the phylogeny-corrected 95% CI (fig. 3). Kiwi olfactory bulbs were above the 95% CI when regressed against all 3 of the scaling variables: brain, telencephalon, and brainrest (fig. 3). This result demonstrates that the size of the olfactory bulbs in kiwi is larger than would be expected based on its brain size. Although kiwi olfactory bulbs were significantly outside the 95% CI in all 3 regression plots, they were closest to the line in the regression against the telencephalon, likely resulting from the enlarged telencephalon in the kiwi [Corfield et al., 2008]. Interestingly, the emu, 1 of 6 other paleognathous birds examined in this study, also had hypertrophied olfactory bulbs, which fell above the 95% CI when regressed against all 3 scaling variables (fig. 3). In addition, emu olfactory bulbs appeared in histological brain sections to be well developed (fig. 2). Nevertheless, not all paleognathines have large olfactory bulbs: the relative size of the olfactory bulbs in the 2 moa species was close to the regression line in all 3 plots, as were those of ostriches, rheas, and 2 of the 3 tinamou species examined (fig. 3). The relative olfactory bulb size of the third tinamou species, Darwin's

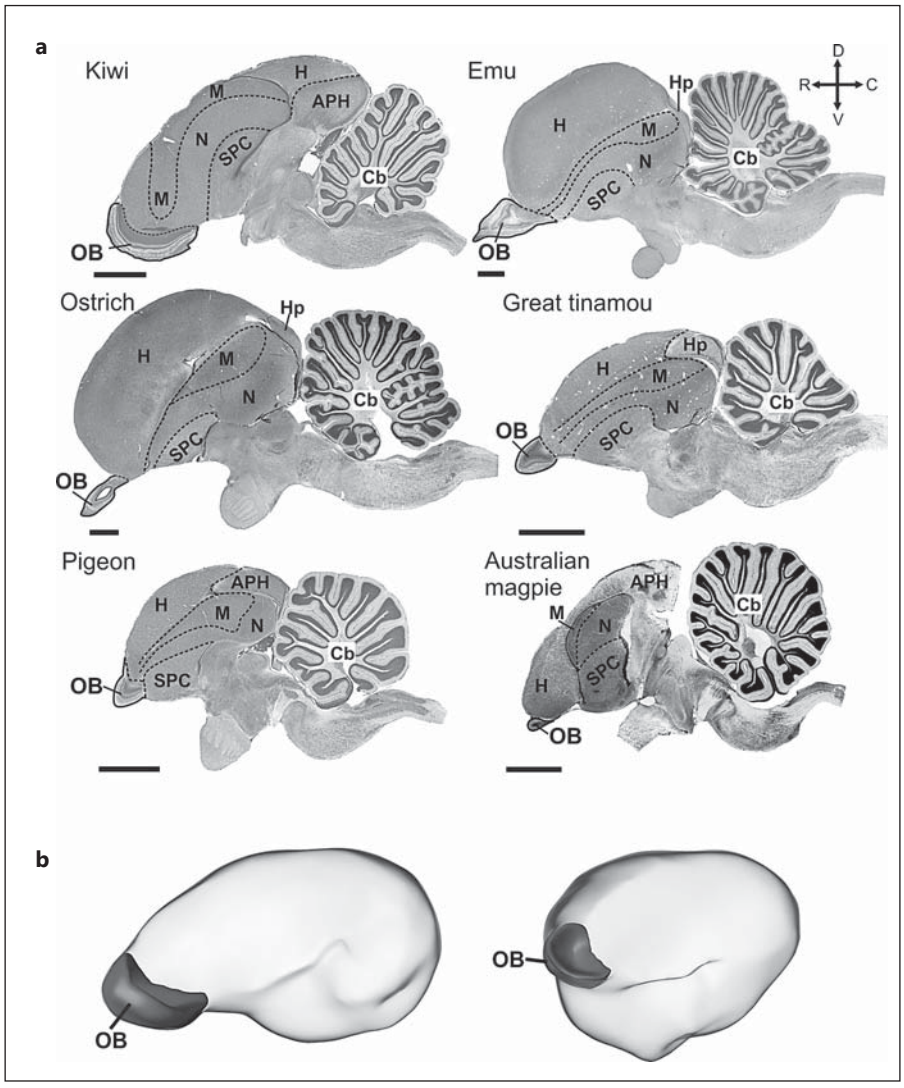


Fig. 2. Structure and size of the olfactory bulb in the kiwi *(A. mantelli),* emu *(Dromaius novaehollandiae),* ostrich *(S. camelus),* great tinamous *(T. major),* pigeon *(Columba livia),* and Australian magpie *(Gymnorhina tibicen).* **a** Sagittal sections of the 6 species stained with cresyl violet showing the olfactory bulb (OB). **b** Lateral view (left) and more caudal view (right) of a 3-D reconstruction of the telencephalon and olfactory bulb in the kiwi. The telencephalon is displayed as a transparent object, while the olfactory bulb is colored grey. Cb = Cerebellum; M = mesopallium; H = hyperpallium; Hp = hippocampus; N = nidopallium; SPC = striatopallidal complex; V = ventricle; APH = area parahippocampalis; D = dorsal; V = ventral; R = rostral; C = caudal. Scale bars = 4 mm.

nothura *(N. darwinii),* was somewhat enlarged but still within the 95% CI.

In addition to the kiwi and emu, the 2 gruiform species examined in this study *(Fulica armillata* and *Porphyrio porphyrio melanotus)* also had hypertrophied olfactory bulbs in all 3 regression plots (fig. 3). All Passeriformes and some Psittaciformes and Galliformes had hypotrophied olfactory bulbs. An example of a histological section of the relatively small olfactory bulbs of the Australian magpie *(Cracticus tibicen),* which is typical of most Passeriformes, can be seen in figure 2.

In kiwi brain sections stained with cresyl violet, the external plexiform, mitral cell, internal plexiform, granule cell, and periventricular layers were clearly visible (fig. 4a). The layers appeared better defined in the kiwi than in the pigeon. In particular, the mitral cell layer, which was organized as a relatively thin, compact layer with clear external and internal plexiform layers flanking it, is much more prominent in kiwi than in pigeons. In the mitral cell layer of the pigeon, cells were more loosely packed and the external and internal plexiform layers were less well defined. The glomerular layer, olfactory fila, and olfactory nerve were labeled by the NCAM antibody and could easily be identified in kiwi (fig. 4b).

The structure of the turbinates in kiwi, which are lined with olfactory epithelium, is large and complex (fig. 5) [Bang, 1971]. The olfactory bulbs are in very close contact with the olfactory epithelium and, instead of having a well-defined single olfactory nerve, as in many other

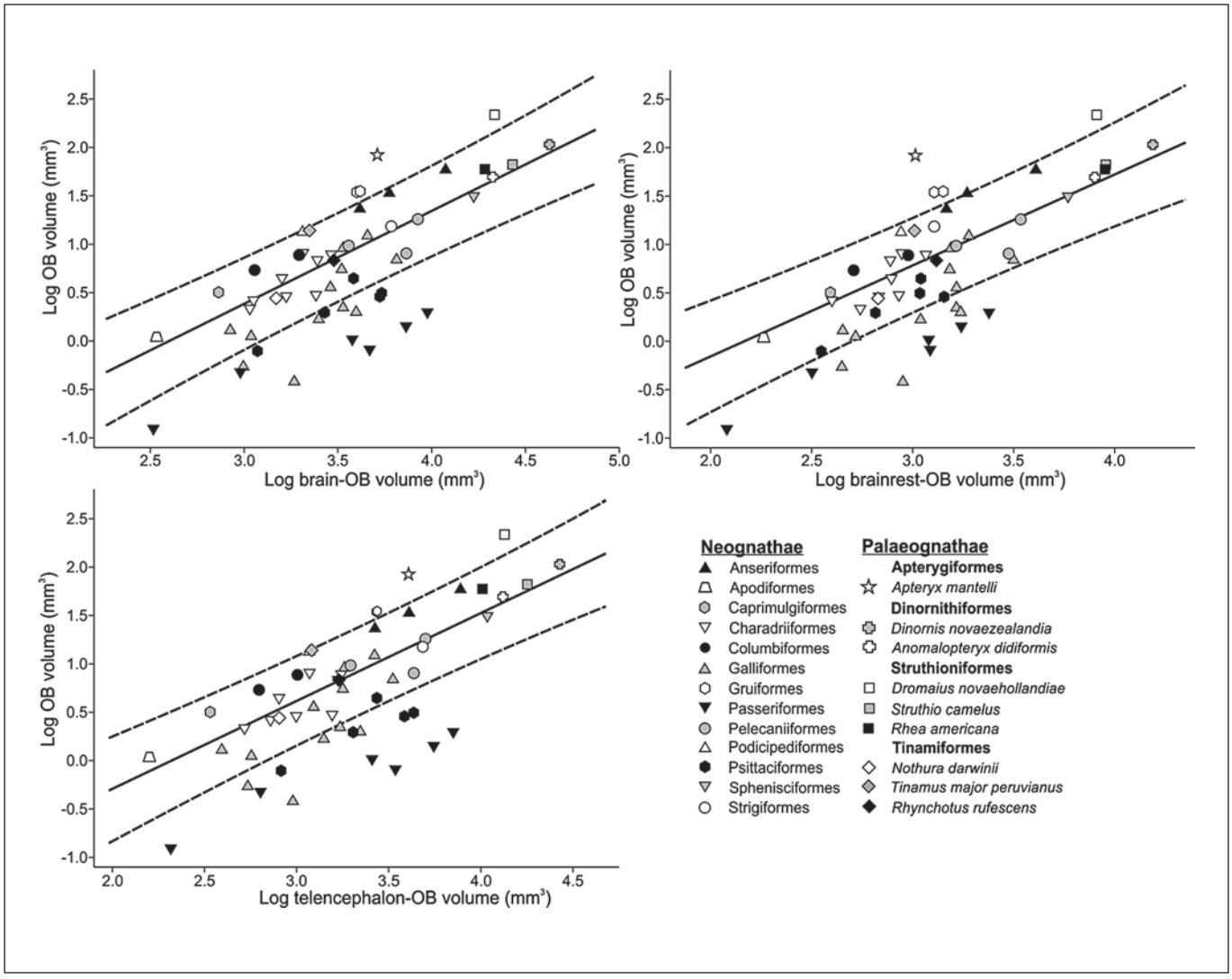


Fig. 3. Scatterplots of the log olfactory bulb (OB) volumes relative to the volume of the brain, telencephalon, and brainrest (brainstem, cerebrum, midbrain, and thalamus), all measured in cubic millimeters. OB volume is not included in brain and telencephalon volumes. Solid lines indicate least-squares linear regression lines and broken lines indicate the phylogeny-corrected 95% CI using the PDAP:PDTREE module of Mesquite [Midford et al., 2010]. The values for the 2 moa species were obtained from CT scans of the skull.

birds, kiwi have 2 or 3 fiber bundles that leave the epithelium and enter the olfactory bulb at different points. In the sensory epithelium, only the olfactory receptor cells and nerve fibers were labeled with the anti-NCAM antibody (fig. 6). Much of the surface of the nasal cavity was lined with sensory epithelium, with nonsensory epithelium restricted to the most anterior regions of the nasal cavity (fig. 6).

Discussion

In this study, we built on previous work examining the anatomy of the kiwi olfactory system [Bang, 1971; Bang and Cobb, 1968; Craigie, 1930; Kraabe, 1959; Owen, 1839; Parker, 1891] to advance our understanding of the neuroanatomical basis of improved olfactory sensitivities in birds. In particular, we aimed to further examine variations in the relative size of the olfactory bulbs in birds to determine if the presumed enhanced olfactory abilities in

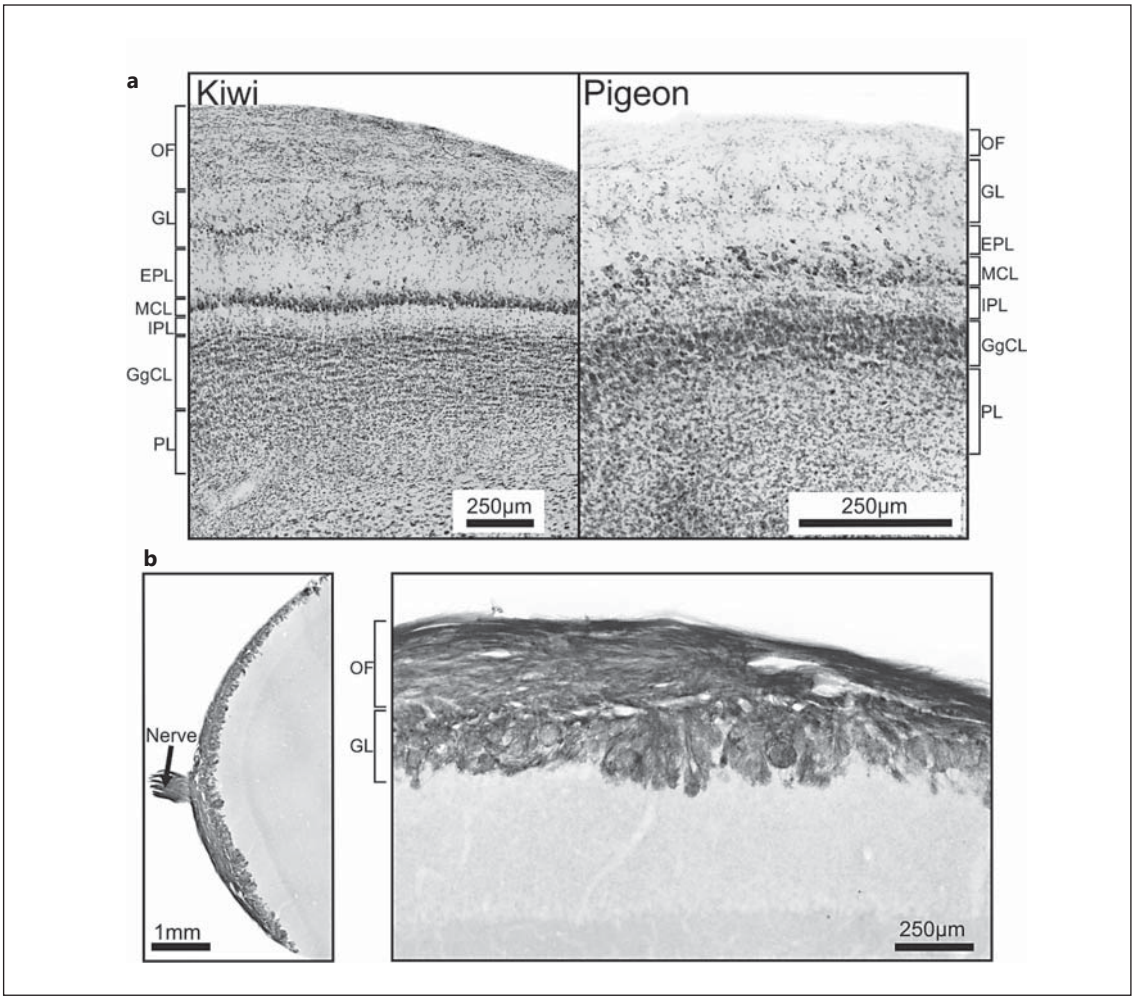


Fig. 4. Laminar structure of the olfactory bulb in kiwi *(A. mantelli)*. **a** Sagittal sections stained with cresyl violet of the olfactory bulb in kiwi and pigeons *(C. livia)*. The layers are indicated on the left and right, respectively. **b** Immunocytochemical labeling of the olfactory bulb of kiwi using an anti-NCAM antibody. Labeling appears to be present in the glomerular cells, olfactory fila, and olfactory nerve. On the left, the extent of the glomerular layer can be seen, and on the right is a magnified view. OF = Olfactory fila; GL = glomerular layer; EPL = external plexiform layer; MCL = mitral cell layer; IPL = internal plexiform layer; GgCL = granule cell layer; PL = periventricular layer.

kiwi [Cunningham et al., 2009; Wenzel, 1968, 1971] are reflected in the size of their olfactory bulbs. Although data on the size of olfactory bulbs for kiwi and a number of other birds is available in Bang and Cobb [1968], the methods available at the time may have resulted in inaccurate measures of olfactory bulb sizes. Indeed, Bang and Cobb [1968] used a millimeter ruler to determine the greatest diameter of one olfactory bulb and the corresponding cerebral hemisphere to produce a ratio. Given the cortex-like external appearance of kiwi olfactory bulbs, the method used by Bang and Cobb [1968] would have included part of the nidopallium and therefore dramatically overestimated the bulb diameter. In addition, telencephalon size is highly variable among avian species [Corfield et al., 2008; Iwaniuk et al., 2004; Portmann, 1947] and is indeed enlarged and elongated in kiwi [Corfield et al., 2008, 2012b], further adding to the inaccuracy of this method. In the current study, we obtained data from histological sections and examined these data using phylogenetically corrected statistics, allowing accurate examination of the size of olfactory bulbs in birds. We also normalized olfactory bulb sizes to 3 different scaling

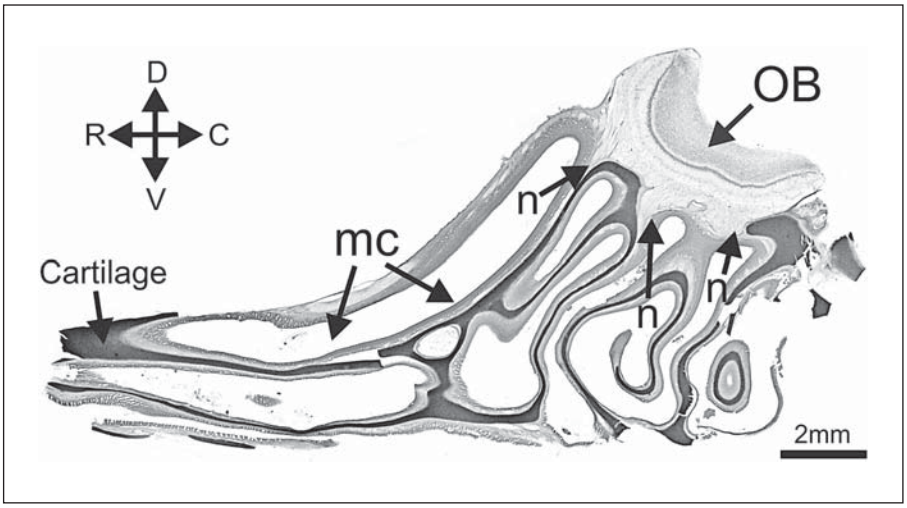


Fig. 5. Olfactory nerve and structure of the turbinate and olfactory epithelium in the kiwi *(A. mantelli),* stained with cresyl violet. The section is on the sagittal plane and is from a medial location. OB = Olfactory bulb; mc = maxillary concha; n = nerve bundles; D = dorsal; V = ventral; R = rostral; C = caudal.

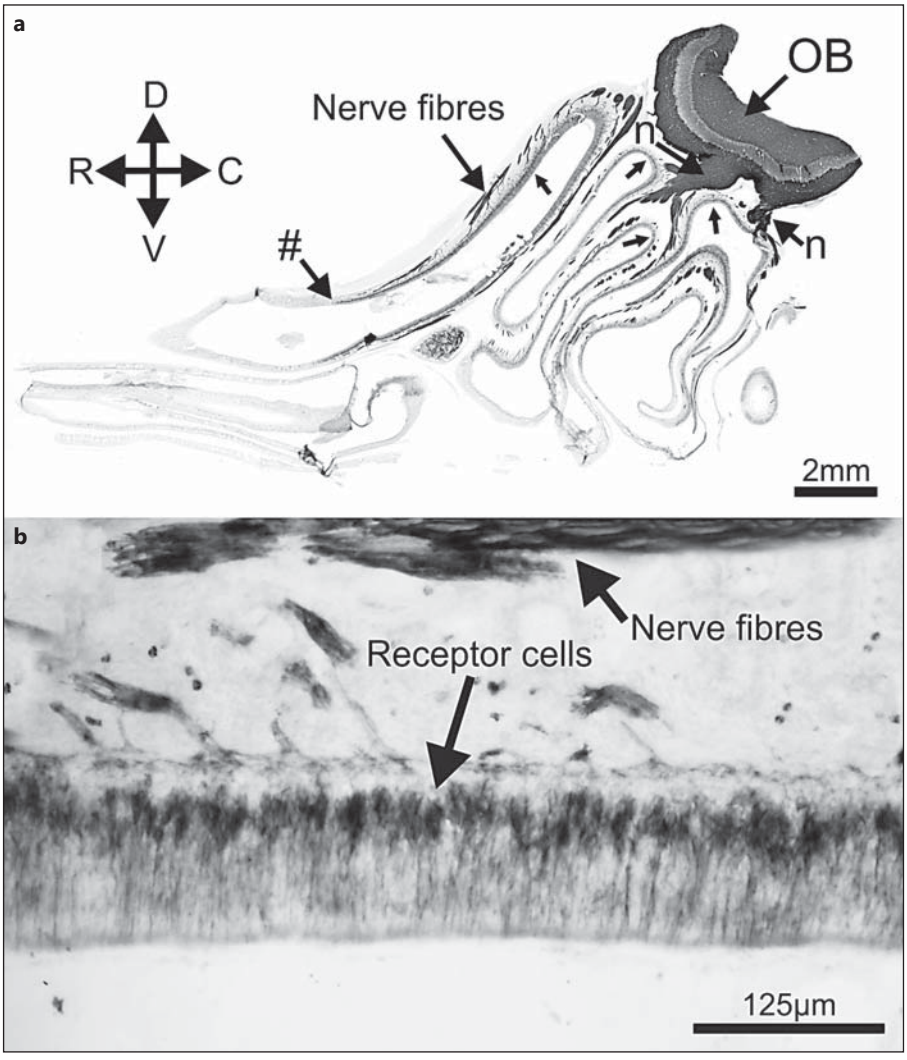


Fig. 6. NCAM-like immunocytochemical labeling of the olfactory nerve, turbinates, and olfactory epithelium in the kiwi *(A. mantelli).* **a** The anti-NCAM antibody labels the olfactory nerve and olfactory receptor cells. The section is on the sagittal plane and is from a medial location. Small arrows indicate examples of areas with olfactory receptor cells. **b** Higher-magnification image of the olfactory epithelium showing the labeled receptor cells. # Indicates a boundary between the olfactory sensory epithelium and nonolfactory 'respiratory' epithelium. n = Nerve bundles; OB = olfactory bulb; D = dorsal; V = ventral; R = rostral; C = caudal.

variables including the brainrest, allowing olfactory bulb sizes across species to be thoroughly explored. In addition to examining the size of the olfactory bulbs, we employed histological and immunocytochemical techniques to examine the cytoarchitecture of the olfactory bulb and epithelium of the kiwi to investigate the possible presence of other anatomical features in addition to size that may improve olfactory acuity. Our approach, therefore, strengthens our understanding of the kiwi olfactory system by using improved quantification techniques and statistical analysis, providing the data necessary to better understand the link between neuroanatomy and olfactory capabilities.

The olfactory bulbs in kiwi are the largest of any bird examined in this study and are undoubtedly associated with enhanced olfactory sensitivity to enable kiwi to function in their nocturnal ground-dwelling niche. Olfactory bulb sizes have been correlated with olfactory capabilities in a number of vertebrate species, and results suggest that olfactory bulb size is a functional rather than phylogenetic adaptation. Among birds, Procellariiforms have some of the largest relative olfactory bulb sizes [Bang and Cobb, 1968], and their well-developed olfactory abilities have been well characterized, particularly with respect to olfactory-based foraging strategies. For example, several species of storm petrels (*Oceanodroma* sp.), prions (*Pachyptila* sp.), and gadfly petrels (*Procellaria* sp.) are able to detect dimethyl sulfide, caused by the breakdown of phytoplankton, at great distances [Nevitt, 1999; Nevitt et al., 1995, 2004]. In 3 mammalian species examined in one study, the percentage of the brain occupied by the olfactory bulb was greatest in dogs, followed by goats and humans (0.31, 0.18, and 0.01%, respectively), with the enlargement in dogs being associated with their reliance on olfaction to find prey and in reproductive activities [Kavoi and Jameela, 2011]. Also among mammals, the size of olfactory bulbs is correlated with habitat type and home range size in Carnivora [Gittleman, 1991]; nocturnal primates and insectivores tend to have larger olfactory structures than diurnal species [Barton et al., 1995]. In fishes, too, olfactory bulb size varies greatly among species, and size is correlated with ecological niche [Yopak et al., 2014]. Indeed, the largest olfactory bulbs were found in pelagic sharks and deep-sea fish and the smallest were found in reef-associated teleost species. Overall then, the size of the olfactory bulbs is related to olfactory capabilities across vertebrates. Although the physiological mechanism linking relative olfactory bulb size with the ability to detect or discriminate odors is unclear [Roper, 1999], larger olfactory bulbs contain more mitral cells and possess thicker functional layers [Wenzel and Meisami, 1987], suggesting some correlation between the size and structure of the olfactory bulb and olfactory acuity [Clark et al., 1993].

In kiwi, the structure of the olfactory bulbs appears to be unique in that it is not pedunculated but instead forms a cortex-like sheet surrounding the rostral pole of the brain, adjacent to and in direct contact with the nidopallium. In addition, the olfactory bulbs are also in close proximity to the nasal turbinates. Instead of a single nerve bundle leaving the epithelium and entering the olfactory bulbs, as in most other avian species, in kiwi, 2 or 3 nerve fiber bundles leave the epithelium and are distributed over the surface of the olfactory bulbs. Whether these unique anatomical features are related to olfactory acuity is yet to be determined; however, they are likely associated with the substantial enlargements of olfactory structures seen in kiwi. Indeed, in addition to large olfactory bulbs, procellariiform seabirds, particularly snow petrels *(Pagodroma nivea),* have massive turbinates that are nearly in direct contact with the olfactory bulbs, and the olfactory nerve shows a branching pattern similar to that of kiwi [Wenzel, 1971, 1987]. Wenzel [1971] suggested that the massive size of the turbinates and olfactory bulbs in snow petrels imposes space constraints, leading to their unique nerve branching pattern, and this may also be the case in kiwi.

Modern birds (neornithines), with their heavy reliance on visual and auditory input, are thought to be descended from ancestors that were more reliant on olfaction [Wenzel, 1971; Zelenitsky et al., 2011]. This hypothesis is supported by data suggesting that earlier diverging groups, such as Procellariiformes (petrels and albatrosses) and Apterygiformes (kiwi), have proportionally larger olfactory bulbs than do groups such as Passeriformes [Wenzel, 1971]. In the current study, however, we found that most birds that are considered to have diverged earlier than other avian groups, i.e. ostriches, tinamous, turkeys, and peacocks [e.g. Hackett et al., 2008], did not have hypertrophied olfactory bulbs. One species that does have hypertrophied olfactory bulbs and is considered a basal bird is the emu. It is possible, therefore, that a well-developed olfactory system was retained in kiwi and emus and lost in rheas, ostriches, and cassowaries. We are unaware of any behavioral evidence concerning the use of olfaction in emus and know of no explanation for why this sense would have remained developed in emus but been reduced in, for example, ostriches, which appear to have comparable behaviors and occupy a reasonably similar niche [Davies, 2003a]. Although it is hard to rule out the

possibility that phylogeny has played a part in the enlarged olfactory bulbs in kiwi, it is more likely that the large olfactory bulbs in kiwi have evolved in association with specific ecological factors, particularly their nocturnal behavior and feeding modality [Healy and Guilford, 1990]. In a nocturnal environment, and in the absence of reliable vision [Martin et al., 2007], a well-developed olfactory system may facilitate the detection and localization of prey, as shown by Wenzel [1968, 1971] and Cunningham et al. [2009]. It is interesting to note, however, that moa evolved in the same environment as kiwi, yet they appear to have been less reliant on olfaction [Ashwell and Scofield, 2008]. Again, this is probably a result of differences in feeding behaviors and diet, with moa feeding on fibrous twigs and leaves taken from low trees and shrubs [Burrows, 1989].

Overall, our results strongly suggest that kiwi olfactory capabilities are extremely good. Indeed, all olfactory structures in kiwi are large and well developed: they have a high number of functionally intact olfactory receptor genes in their genome [Steiger et al., 2008, 2009] and they have adaptations such as a long beak with nostrils at its tip. Therefore, together with their specialized auditory and tactile systems [Corfield et al., 2011, 2012a; Cunningham et al., 2007, 2009, 2013], kiwi appear to have evolved an olfactory system well tuned to utilizing olfaction to function in their nocturnal and ground-dwelling niche. This reliance on olfactory rather than visual information is reminiscent of the situation of many nocturnal mammals. As such, it represents a clear example of convergent evolution in which a common set of perceptual challenges presented by the nocturnal forest floor environment have resulted in similar sensory specializations. In a niche such as this, perhaps olfaction is the most reliable and important sensory modality.

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