

Relationship between flightlessness and brain morphology among Rallidae

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Japan Society for the Promotion of Science, Grant/Award Number: 18K06397 and 20H01409

Abstract

Studies have suggested that the brain morphology and flight ability of Aves are interrelated; however, such a relationship has not been thoroughly investigated. This study aimed to examine whether flight ability, volant or flightless, affects brain morphology (size and shape) in the Rallidae, which has independently evolved to adapt secondary flightlessness multiple times within a single taxonomic group. Brain endocasts were extracted from computed tomography images of the crania, measured by 3D geometric morphometrics, and were analyzed using principal component analysis. The results of phylogenetic ANCOVA showed that flightless rails have brain sizes and shapes that are significantly larger than and different from those of volant rails, even after considering the effects of body mass and brain size respectively. Flightless rails tended to have a wider telencephalon and more inferiorly positioned foramen magnum than volant rails. Although the brain is an organ that requires a large amount of metabolic energy, reduced selective pressure for a lower body weight may have allowed flightless rails to have larger brains. The evolution of flightlessness may have changed the position of the foramen magnum downward, which would have allowed the support of the heavier cranium. The larger brain may have facilitated the acquisition of cognitively advanced behavior, such as tool-using behavior, among rails.

KEYWORDS

3D geometric morphometrics, Aves, foramen magnum, phylogenetic ANCOVA, rails, tool-using behavior

1 | INTRODUCTION

Brain morphology is associated with behavior, sensory receptive ability, cognitive ability, and phylogeny of the species (Butler & Hodos, 2005). The importance of sensory and cognitive processing abilities is reflected in the volume of associated brain regions (Jerison, 1974). For example, the Wulst, located in the dorsal telencephalon of the avian brain, is the region that processes visual information such as binocular vision and tactile information from the bill; it is particularly well-developed among the predominantly nocturnal Strigiformes and Caprimulgiformes (Iwaniuk & Wylie, 2006; Wylie et al., 2015).

Many recent studies have claimed that brain morphology, that is, brain size and shape, and flight ability, that is, volant or flightless, in

Aves are interrelated (Gold & Watanabe, 2018; Kawabe et al., 2013; Kulemeyer et al., 2009; Marugán-Lobón & Buscalioni, 2009). For example, Kulemeyer et al. (2009) studied the relationship between the position of the foramen magnum, head posture, and differences in foraging ecology among species in Corvidae, and found that *Corvus* species had more sustained flight abilities than *Pica* or *Garrulus* species, which correlated with a more horizontal head posture and an upward positioned foramen magnum. The cerebellum is the brain area that coordinates muscle movement and balance during flight in Aves (Gill, 2006; Iwaniuk et al., 2004), and its size has been reported to be related to flight ability and posture, including underwater flight such as in Sphenisciformes (Boire & Baron, 1994; Knoll & Kawabe, 2020; Ksepka et al., 2012; Walsh et al., 2013).

However, whether the flight ability influences the morphology and the size of the brain remains debatable. The evolution of secondary flightlessness in Aves is known in several taxonomic groups, and secondarily flightless birds have been reported to have smaller brains than those of closely related volant species (Bennett & Harvey, 1985). On the contrary, Iwaniuk et al. (2004) examined the effect of flightlessness on relative brain size in nine taxonomic groups and found no significant difference between the brain sizes of flightless and volant species in six taxonomic groups including Rallidae. They suggested that the correlation between a relatively small brain size and flightlessness was not a general trend in Aves (Iwaniuk et al., 2004). These discrepancies in conclusions between previous studies may be partially due to the lack of appropriate use of phylogenetic comparative methods, which account for phylogenetic relationships when considering interspecific comparisons (Felsenstein, 1985).

Other factors may also contribute to brain size. Larger brain size in Aves is thought to be correlated with higher cognitive abilities that allow for the acquisition or innovation of complex foraging skills, such as tool-using behavior (Lefebvre et al., 1997; Overington et al., 2009; Shumaker et al., 2011; Wyles et al., 1983). Species with relatively high cognitive ability such as Psittaciformes and Passeriformes have a more developed pallium size than that of other species (Gill, 2006). New Caledonian Crow (*Corvus moneduloides*) is a species of Corvidae in Passeriformes that exhibits tool-using behavior in the wild (Hunt, 1996; Matsui et al., 2016), and has a larger brain compared to those of other species in Passeriformes (Cnotka et al., 2008). Overington et al. (2009) found a positive relationship between innovativeness in foraging technique and brain size in 76 avian families and supported the hypothesis that large brains allow for the production of novel behavior patterns (Ksepka et al., 2020; Lefebvre & Bolhuis, 2003).

The Rallidae (rails) is an ideal taxonomic group to use phylogenetic comparisons for assessing the effects of flight ability on brain size and shape, and the effects of brain size on cognitive ability. Rails are distributed worldwide, and adapted to a remarkably diverse range of environments, including forests, wetlands, grasslands, and oceanic and coral islands (Kirchman, 2012; Slikas et al., 2002; Taylor, 1998). Rallidae is a taxonomic group where the evolution of secondary flightlessness has occurred independently and repeatedly within the phylogeny (Garcia-R et al., 2014; Iwaniuk et al., 2004; Kirchman, 2012; McNab & Ellis, 2006; Roff, 1994). Particularly, rails living on the islands tend to evolve to become flightless (Slikas et al., 2002; Taylor, 1998). In addition, the Rallidae is also a taxonomic group in which tool-using behavior has been observed. Okinawa rail (*Gallirallus okinawae*) is the only species in Rallidae whose tool-using behavior has been confirmed by direct observation (Miyazawa & Shimada, 2017; but see also Woinarski et al., 1998). They break the shells of large snails by hitting them against anvil stones for consuming them (Miyazawa & Shimada, 2017). As has been found in other Aves, the cognitively advanced behavior, such as tool-using behavior by Okinawa rail may have been facilitated by the larger brain. Iwaniuk et al. (2004) reported that in Rallidae, several flightless rails whose

brain volume exceeded the predicted brain volume were observed, albeit not statistically significant (table 4 in Iwaniuk et al., 2004). Thus, in Rallidae, it is possible to hypothesize that species that have evolved secondary flightlessness have significantly larger brains than those of volant species. Even if this hypothesis is correct, however, if the number of independent evolutions within a taxonomic group is small, the phylogenetic comparison method will not lead to statistically significant results (Adams & Collyer, 2018). Therefore, the Rallidae, which has evolved flightlessness independently multiple times within a single taxonomic group, is the ideal study group to test this hypothesis (Garcia-R et al., 2014; Kirchman, 2012).

The development of computer technology has allowed for endocast extraction from computed tomography (CT) images (Ashwell & Scofield, 2008; Balanoff et al., 2016; Early, Iwaniuk, et al., 2020; Early, Ridgely, & Witmer, 2020; Iwaniuk et al., 2005; Kawabe et al., 2009, 2013; Knoll & Kawabe, 2020; Torres & Clarke, 2018; Zelenitsky et al., 2008). This is a non-invasive method that can be used to reconstruct brain volume, surface area, and shape from crania, facilitating the study of brain morphology even in rare collections of specimens. Although it has become possible to use endocasts to non-invasively estimate the volume of specific brain regions such as the olfactory bulb, optic lobes, hyperpallia, and optic tecta (Early, Iwaniuk, et al., 2020; Early, Ridgely, & Witmer, 2020; Knoll & Kawabe, 2020; Torres & Clarke, 2018), the manual reconstruction of digital endocasts has been time-consuming. Recently, however, a method to semi-automatically extract endocasts from CT data was developed (Michikawa et al., 2017; Ogiwara et al., 2018).

The purpose of this study is to test whether the hypothesis that flight ability, that is, volant/flightlessness, affects brain morphology (size and shape) is valid in the Rallidae where secondary flightlessness has evolved independently multiple times within a single taxonomic group. Since there is only one species identified as tool-using (Okinawa rail), statistical comparison to the non-tool-using species was not conducted, but this study possibly allows discussions of the evolutionary relationship among flightlessness and behaviors that require high cognitive abilities.

2 | METHODS

2.1 | Specimens and CT scanning

Our sample consisted of crania from 49 specimens of Rallidae from 18 genera and 25 species (Table 1). The specimens were deposited at the Smithsonian National Museum of Natural History (Washington, DC, USA), the National Museum of Nature and Science, Japan (Tokyo, Japan), and Yamashina Institute for Ornithology (Abiko, Japan). Twenty-one volant and four flightless species were included in the sample: *Gallirallus okinawae*, *G. owstoni*, *Habroptila wallacii*, and *Porzana palmeri*. Only one tool-using species, *G. okinawae*, has been identified in the Rallidae (Miyazawa & Shimada, 2017). All extant flightless rails are island species (Slikas et al., 2002; Taylor, 1998). Therefore, it should be noted that even if a significant correlation

TABLE 1 List of species analyzed in the present study and associated information of the specimens

No.	Species	Flight ability (v/f)	Main habitat (c/i)	Collection	Specimen number	Voxel size	CS	Brain size (mm ³)	Body mass (g)
1	<i>Amaurolimnas concolor</i>	v	c	USNM	613,962	M	36.18	1590	95.0
2	<i>Amaurornis flavirostra</i>	v	c	USNM	642,379	S	32.93	1250	88.8
3	<i>Amaurornis phoenicurus</i>	v	c	YIO	71,174	M	39.30	2090	180.0
4	<i>Aramides cajanea</i>	v	c	USNM	612,266	M	46.52	3270	403.0
5	<i>Coturnicops noveboracensis</i>	v	c	USNM	556,930	S	25.87	589	52.5
6	<i>Crex crex</i>	v	c	USNM	490,297	M	33.81	1230	169.0
7	<i>Fulica americana</i>	v	c	USNM	610,793	M	43.77	2820	651.0
8	<i>Gallinula chloropus</i>	v	c	USNM	291,703	M	39.55	2420	503.0
9	<i>Gallinula chloropus</i>	v	c	USNM	490,331	M	44.51	2130	415.0
10	<i>Gallirallus okinawae</i>	f	i	YIO	71,240	M	41.67	3170	442.0
11	<i>Gallirallus owstoni</i>	f	i	USNM	501,064	M	38.19	1980	241.0
12	<i>Gallirallus philippensis</i>	v	c	NSMT	AS2557	M	36.64	1730	184.0
13	<i>Habroptila wallacii</i>	f	i	USNM	557,026	L	50.87	4480	1000.0
14	<i>Micropygia schomburgkii</i>	v	c	USNM	622,265	M	NA	NA	NA
15	<i>Laterallus albigularis</i>	v	c	USNM	612,271	S	30.17	906	41.9
16	<i>Pardirallus maculatus</i>	v	c	USNM	562,744	M	35.33	1510	130.0
17	<i>Porphyryla martinica</i>	v	c	USNM	610,789	M	29.87	2140	218.0
18	<i>Porzana carolina</i>	v	c	USNM	290,425	S	31.98	940	74.8
19	<i>Porzana cinerea</i>	v	c	NSMT	AS2546	S	39.34	NA	NA
20	<i>Porzana palmeri</i>	f	i	USNM	289,243	S	28.71	830	32.5
21	<i>Porzana pusilla</i>	v	c	YIO	60,918	S	27.62	707	35.4
22	<i>Rallina eurizonoides alvarezi</i>	v	c	USNM	561,972	M	36.08	1540	110.0
23	<i>Rallus elegans</i>	v	c	USNM	499,392	M	43.58	2790	415.0
24	<i>Rallus longirostris</i>	v	c	USNM	525,875	M	38.24	1900	266.0
25	<i>Ortygonax sanguinolentus sanguinolentus</i>	v	c	USNM	645,406	M	NA	NA	NA

Note. The voxel size: L = 257.73×257.73×200 μm, M = 103.09×103.09×200 μm, S = 66.89×66.89×200 μm.

Abbreviations: c, continent; CS, centroid size; f, flightless; i, island; NSMT, National Museum of nature and science (Tokyo, Japan); USNM, National Museum of Natural History (Washington, DC, USA); v, volant; YIO, Yamashina Institute for Ornithology (Abiko, Japan).

between flightlessness and larger brains is found in rails, this study cannot distinguish and discuss whether the larger brains are due to flightlessness or to island dwelling (Sayol et al., 2018).

Each cranium was scanned using a LaTheta LCT-100 CT scanner (Hitachi Aloka Medical). Three-dimensional (3D) images of each cranium were reconstructed with the voxel size of either 257.73×257.73×200, 103.09×103.09×200, and 66.89×66.89×200 μm, depending on the size of the cranium. The image processing software ImageJ (Schneider et al., 2012) was used to convert the files to RAW files. The endocranial surface was semi-automatically extracted from the cranial CT images (Michikawa et al., 2017). Briefly, a seed was placed in a cranial cavity and the cavity was extracted using a region-growing algorithm. Here, openings due to foramina and nerve canals were automatically closed, assuming that the cranial cavity is the largest cavity in the CT images (see Michikawa et al., 2017 for the details of the algorithm). The extracted surface of the endocranial cavity was then transferred to the image processing

software Geomagic XOS (3D Systems) to digitize the anatomical landmarks and to obtain their brain sizes measured in volume (mm³).

2.2 | 3D geometric morphometrics

First, a total of 12 cranial landmarks (a–l) were digitized on the external surface of each cranium using Geomagic XOS, and a coordinate transformation was performed. The coordinate transformation was unnecessary as 3D GM is a coordinate-free analysis. However, to facilitate the digitization of the bilateral landmarks, we firstly translated and rotated each cranium to the common anatomical coordinate system as shown in Figure 1. The median sagittal plane was calculated based on the midpoints of the bilateral pair of landmarks. X, Y, and Z axes were defined as the normal vector to the median sagittal plane, the vector in the sagittal plane perpendicular to the vector was defined by the landmarks *k* and *l*, and the cross product

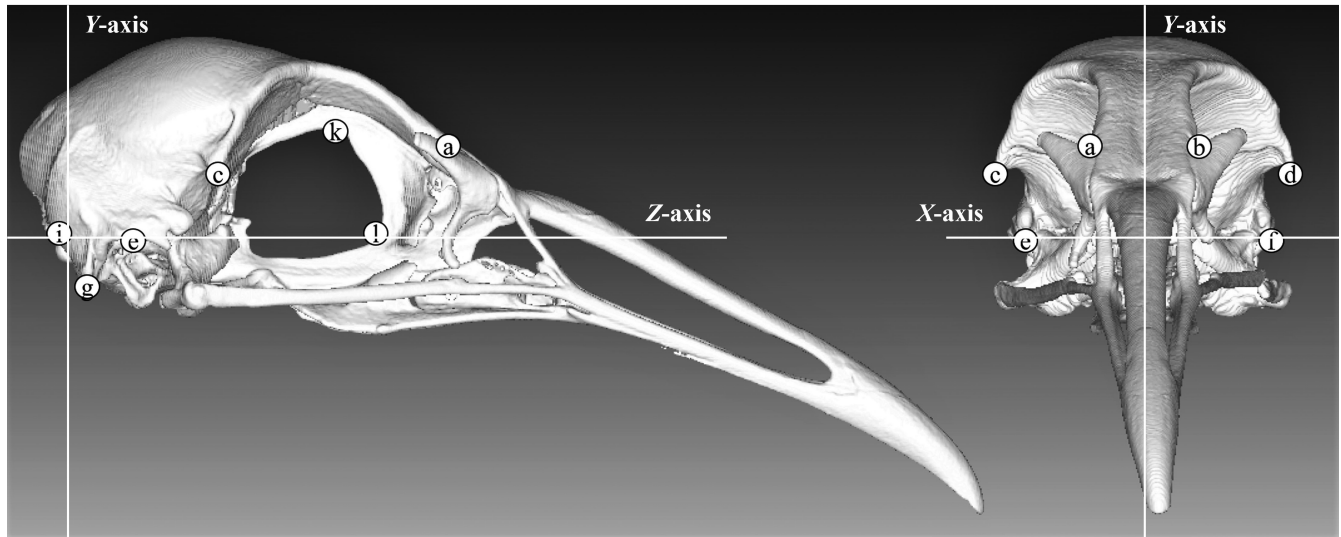


FIGURE 1 Cranial landmarks used for coordinate transformation. Left: Lateral view, right: Front view. Points a–l indicate the placement of the cranial landmarks used for coordinate transformation. The lines in the figure represent the X, Y, and Z axes with the foramen magnum as the origin.

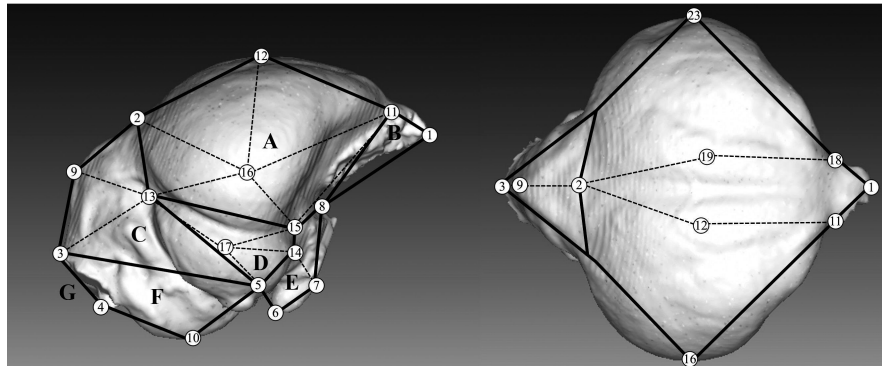


FIGURE 2 Brain landmarks used in 3D geometric morphometrics. Left: Lateral view, right: Dorsal view. The numbers represent the points on Table 2. The areas circled by solid lines (A–F) represent each of the following brain areas: A = telencephalon, B = olfactory bulb, C = cerebellum, D = optic tectum, E = diencephalon, F = myelencephalon. G represents the position of foramen magnum.

of X and Y axes respectively. Thus, the axes correspond to the mediolateral, superoinferior, and anteroposterior directions respectively (Figure 1).

Then, we digitized a total of 24 brain landmarks (1–24) on endocasts (Figure 2; Table 2) for 3D geometric morphometrics. Landmarks were defined according to Kawabe et al. (2013), so that the boundaries of the telencephalon, cerebellum, optic lobe, olfactory bulb, foramen magnum, pituitary gland, optic nerve, and medulla can be quantitatively compared among species. The brain landmarks were obtained using Viewbox 4 (dHAL Software, Kifissia, Greece).

2.3 | Principal component analysis (PCA) and phylogenetic ANCOVA (phylANCOVA)

Landmark coordinates of each specimen were normalized by centroid size for size-independent shape analysis and were registered using the Generalized Procrustes method (Rohlf & Slice, 1990). We

performed PCA based on variance–covariance matrix on Procrustes shape coordinates using Morphologika 2.5 (O'Higgins & Jones, 1998) to establish shape variation trends between endocasts. The same software was used to visualize the variation in shape explained by PC scores. For each species, the brain shape was defined as a set of 3D coordinates after size normalization and alignment using the Procrustes method.

Then, we depicted the transformation of the mean shape along each of the PC axes using the LandmarkSurfaceWarp module in Amira 5.2.1 software (FEI Visualization Sciences Group) based on Yamasaki et al. (2018). Additionally, the averaged wireframes of the endocasts of all the studied species were depicted. The extreme brain shapes were calculated by varying each PC to the maximum and minimum values that can be output by Morphologika 2.5, whereas the remaining PCs were fixed.

Considering the difference of phylogenies in the Rallidae, we used phylANCOVA to analyze whether the flight ability (volant/flightless) affects brain morphology (size and shape) (Early, Ridgely,

TABLE 2 Definitions of brain landmarks

No.	Anatomical description
1	Median anterior tip of the olfactory bulb
2	Median junction between the telencephalon and cerebellum
3	Median dorsal point of the foramen magnum
4	Median ventral point of the foramen magnum
5	Median junction between the hypophysis and mesencephalon
6	Median ventral tip of the hypophysis
7	Median junction between the optic nerve and hypophysis
8	Median junction between the telencephalon and optic nerve
9	Perpendicular at the midpoint between landmarks 2 and 3 to the dorsal margin of the cerebellum in lateral view
10	Perpendicular at the midpoint between landmarks 4 and 5 to the ventral margin of the mesencephalon in lateral view
11, 18	Most anterior tip of the telencephalon, right and left
12, 19	Perpendicular at the midpoint between landmarks 11 (18) and 2 to the dorsal margin of the telencephalon in lateral view, right and left
13, 20	Intersection of the telencephalon, cerebellum, and optic lobe, right and left
14, 21	Most anterior point of the optic lobe, right and left
15, 22	Intersection of the telencephalon, optic lobe, and diencephalon, right and left
16, 23	Most lateral point of the widest part of the telencephalon, right and left
17, 24	Most lateral point of the widest part of the optic lobe, right and left

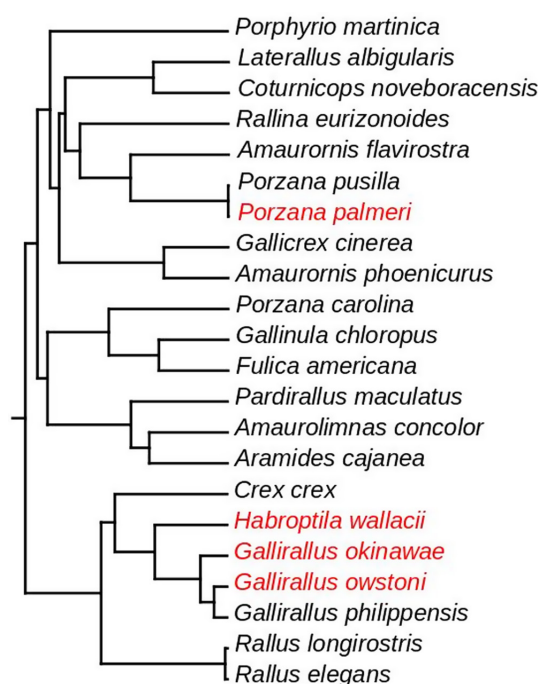


FIGURE 3 Phylogenetic tree of the 22 studied species for phylANCOVA. The species with black letters represent volant rails, and the species with red letters represent flightless rails. Flightless Okinawa rail (*G. okinawae*) is the only confirmed tool-using species.

& Witmer, 2020; Juarez et al., 2019). As discussed above, body mass may affect brain size, and brain size may affect brain shape (Kawabe et al., 2013; Marugán-Lobón & Buscalioni, 2009). Therefore, we used

phylANCOVA with brain size as the objective variable and flight ability and body mass as the explanatory variables to analyze the relationship between brain size and flight ability, and with brain shape as the objective variable and flight ability and brain size as the explanatory variables to analyze the relationship between brain shape and flight ability. To facilitate the interpretation of the morphological characteristics of the brain shape of volant/flightless rails, we used phylANCOVA with each of the PC with high contribution rates (PCs with a proportion of variance exceeding 10%) as the objective variable, and flight ability and brain size as explanatory variables to examine the effect of flight ability on the variation of each PC.

Phylogenetic data for the analysis were acquired by digitizing the time-calibrated tree in Figure 3 in the study by Garcia-R et al. (2014) using TreeSnatcher Plus (Laubach & Von Haeseler, 2007). The tree is essentially the most comprehensive phylogenetic tree for Rallidae. However, this phylogenetic tree lacks three species out of the 23 species treated in this study: *Porzana cinerea*, *P. palmeri*, and *Rallina eurizonoides alvarezii*. Thus, the phylogenetic position of *R. eurizonoides sepiaria* was used as an alternative of *R. eurizonoides alvarezii*. In addition, we hypothesized that *P. palmeri* diverged from *P. pusilla* 125,000 years ago (Slikas et al., 2002), and *P. palmeri* was added to the sister position of *P. pusilla* in the time-calibrated tree of Garcia-R et al. (2014) with a diverging age adjusted to 125,000 years ago. Hence, phylANCOVA were conducted for 22 species (Table 1; Figure 3), except for the *P. cinerea*, for which phylogenetic relationships could not be determined. We used the `procD.pgls` function (Adams, 2014; Blomberg et al., 2012) in the R package “geomorph ver 4.0.0” for the analysis (Adams & Otárola-Castillo, 2013; R

Development Core Team, 2013), using 1000 permutations. Data on body mass for each species were obtained from Iwaniuk and Nelson (2003), Ozaki (2010), and Dunning Jr (2007), and the average of the multiple data was used as a representative value for body mass of the species.

To visually confirm how the flight ability affects brain shape while considering the phylogeny, phylomorphospaces were plotted by mapping the obtained phylogenetic information onto two-dimensional scatter plots of PCs with a high proportion of variance, using `gm.prcomp` function in `geomorph` ver 4.0.0 (Adams & Otárola-Castillo, 2013; R Development Core Team, 2013).

3 | RESULTS

We successfully extracted endocasts of 23 species in 16 genera, but the endocasts of *Micropygia schomburgkii* (Table 1, No. 14) and *Ortygonax sanguinolentus sanguinolentus* (Table 1, No. 25) were not generated due to damage. The PC scores and 24 landmark coordinates of the 23 species are presented in Tables S1 and S2 respectively. Data on 3D rendering of the crania and endocasts of the 23 species studied can be accessed using the following URL: <https://www.morphosource.org/projects/000433635>.

Eigenvalues, percentages of total variance explained, and cumulative proportion of variance explained of each PC score are shown in Table S3. The variances explained by PC1, PC2, and PC3 were 25.7%, 18.3%, and 13.8% respectively. The cumulative proportion of variance explained by PC1, PC2, and PC3 was 57.8%, providing a reasonable approximation of the total shape variation (Table S3).

The results of PCA of endocranial shape variation in Rallidae were presented in Figure 4. The points representing species in the same genus were distributed in close proximity on the scatter plot of phylomorphospaces of PC1 and PC2 (Figure 4a), but not in that of PC1 and PC3 (Figure 4b).

Variation in the brain shape along the PC1 axis is presented in Figure 5a. Decreasing PC1 scores involved the expansion of the maximal widths of the telencephalon, which was accompanied by caudal elongation of the cerebellum and reduction of the optic lobe (Figure 5a).

Variation in the brain shape along the PC2 axis was characterized by elongation or shortening of the telencephalon along the rostro-caudal axis (Figure 5b). Decreasing PC2 scores involved expanding the maximal width of the telencephalon, which was accompanied by flattening of the myelencephalon due to a more inferiorly positioned foramen magnum, which also result in the medulla and the region around the foramen magnum oriented downward (Figure 5b).

Variations in the brain shape along the PC3 axis were characterized by a lateral expansion or contraction of the telencephalon, and expansion or contraction of the rostrum and the olfactory bulb (Figure 5c). The lateral expansion of the telencephalon followed a decrease in the PC score, which was accompanied by an anterior and posterior shortening of the dorsal part of the telencephalon and contraction of the olfactory bulb. In addition, the anteroposterior

elongation of the dorsal portion of the telencephalon resulted in ventral rotation of the brain (Figure 5c).

As a result of `phylANCOVA`, the brain sizes of flightless and volant species differed even after considering the effects of body mass (Table 3a). The brain size increased as body mass increased ($p = 0.001$, Figure 6a), and the brain size of flightless species was significantly larger than that of volant species ($p = 0.004$, Figure 6a). Brain shapes of flightless and volant species also differed even after considering the effect of brain size (Table 3b). Brain shape varied as brain size increased ($p = 0.006$); however, the brain shape was significantly different depending on flight ability ($p = 0.001$). Flightless species had significantly larger PC1 scores ($p = 0.003$, Figure 6b), and smaller PC2 and PC3 scores ($p = 0.001$, Figure 6c; $p = 0.002$, Figure 6d, respectively) compared to phylogenetically closely related volant species, whereas no significant effect of the brain size was observed (Table 3c-e).

4 | DISCUSSION

The present study showed that the variation in brain shape across the Rallidae species was generally consistent with that reported for the entire class of Aves (Kawabe et al., 2013). Kawabe et al. (2013) suggested that the main brain shape variation trends in Aves are expansion or reduction of the telencephalon and elongation or shortening of the brain base and brain stem, and the same was observed in the present study in Rallidae. On the other hand, some variation in brain shape across the Rallidae species was found to differ from that reported for the entire class of Aves. No significant variation of the telencephalon was observed along with the anteroposterior direction. In addition, there was no significant variation trend in the shape of the brain base or brain stem in rails. In Kawabe et al. (2013), PC1 and PC2 corresponded to trends of the brain to rotate dorsally and ventrally, but in this study, these trends were associated with PC3. The difference between the results of this study and those of Kawabe et al. (2013) may be attributable to the fact that this study analyzed brain variation restricted to a single family of the Rallidae, whereas Kawabe et al. (2013) examined variation in all Aves phylogenies.

The results of `phylANCOVA` showed that flightless species tended to have significantly larger brain size than volant species in Rallidae, even after considering the effect of body mass (Table 3a). Iwaniuk et al. (2004) found that the observed brain volumes of several flightless rails exceeded the predicted brain volumes, although the difference was not statistically significant (table 4 in Iwaniuk et al., 2004). Contrary with the study claiming that secondarily flightless birds have relatively smaller brains than closely related volant species (Bennett & Harvey, 1985), our findings statistically supported the results of Iwaniuk et al. (2004) and the hypothesis that the rails that have evolved secondary flightlessness have significantly larger brain sizes than volant rails.

This study demonstrated that there was a significant variation in brain shape between flightless and volant species, even after

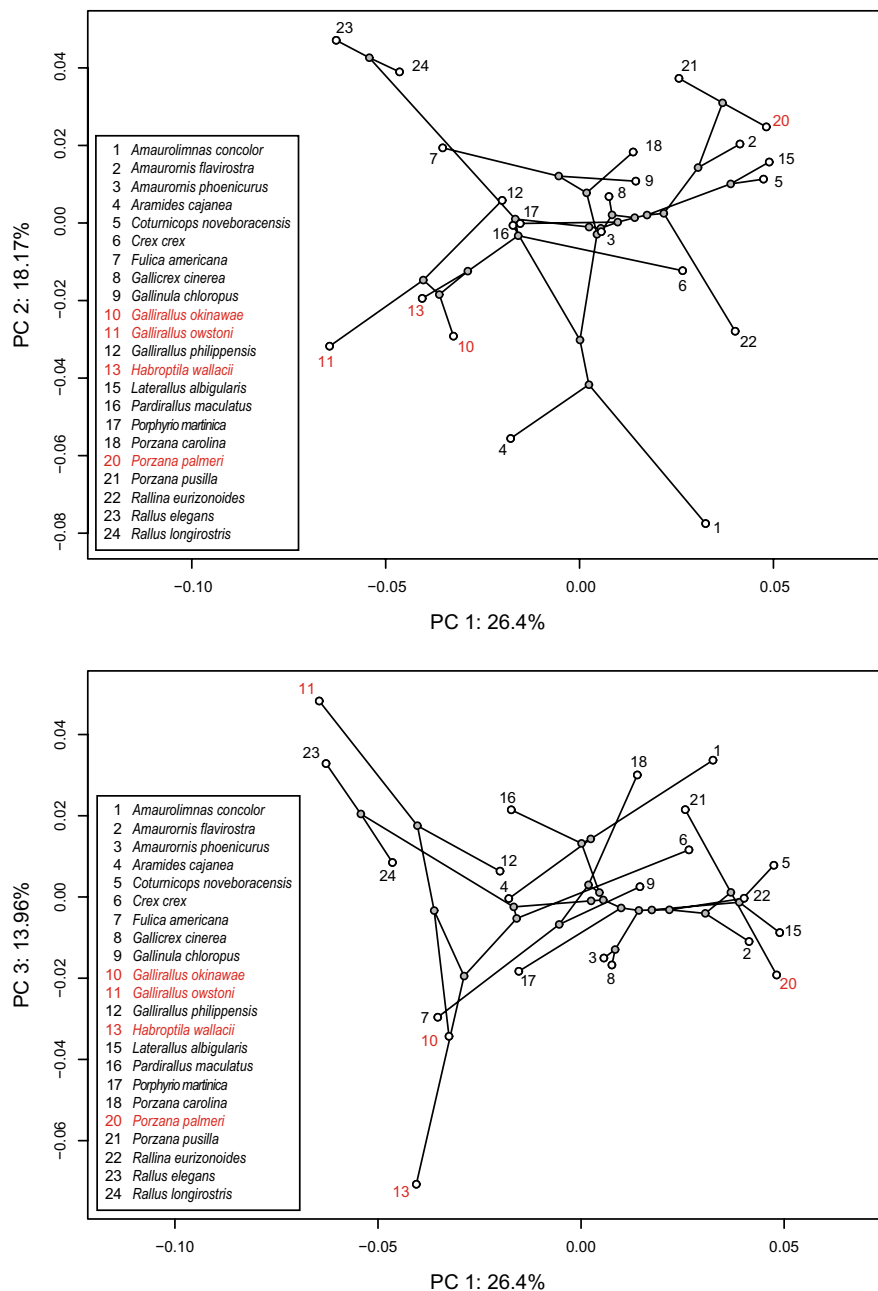


FIGURE 4 Phylomorphospace of the two principal components. (a) PC1 versus PC2 (top), and (b) PC1 versus PC3 (bottom). Each plot represents 23 different Rallidae species. Black numbers represent volant rails and red numbers represent flightless rails. The lines connecting the plots indicate the phylogenetic relationship. The gray plots represent the assumed ancestral species of the species connected by the lines. (%) Represents percentage of total variance explained by PCs. Flightless Okinawa rail (*Gallirallus okinawae*) is the only confirmed tool-using species.

considering the effect of brain size (Table 3b). The results of phylANCOVA showed that flightless rails have a wider telencephalon and more inferiorly positioned foramen magnum than closely related volant rails. These results suggest that there is a significant relationship between flight ability and brain morphology in rails, and thus, that our hypothesis that flight ability affects brain morphology is valid in the Rallidae was supported.

There are two possible, but not exclusive, explanations for the result that the brain size of flightless rails was larger than that of

volant rails in proportion to their body mass. One is that (1) flightlessness reduced body mass in rails. According to previous studies, flightlessness has the potential to both increase and decrease body mass. Although volant species in Aves are subjected to strong selective pressure to reduce their body or head weight in general, such selective pressure is not observed in flightless birds (Gussekloo & Cubo, 2013). On the contrary, one of the morphological features associated with the evolution of flightlessness is the reduction in basal metabolic rate owing to the reduction in the size of the keel and the

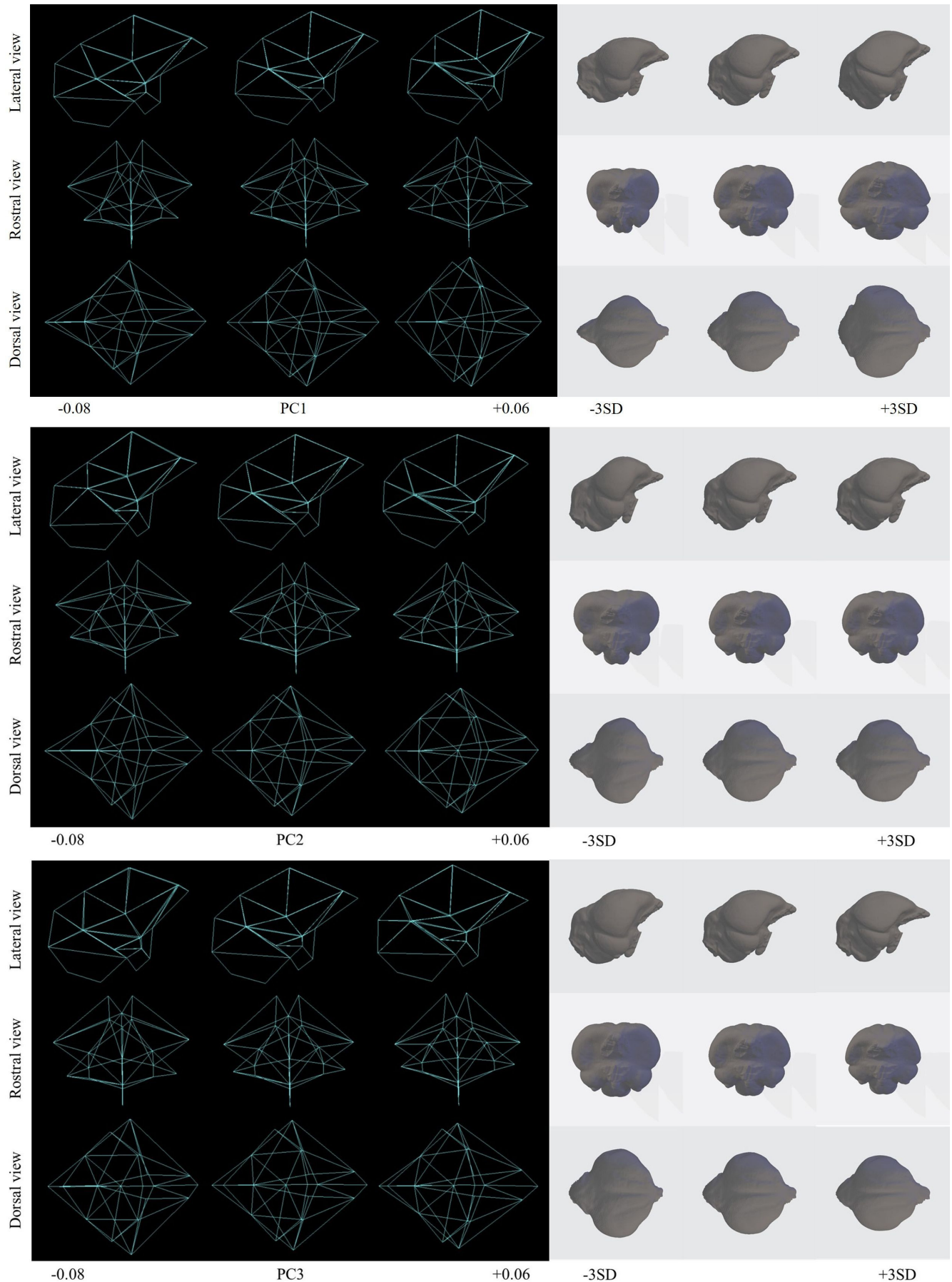


FIGURE 5 Variation trend of brain shape with changes in each PC. Left: The wireframes represent the extreme diagrams where a PC is varied by the maximum value (+0.06, -0.08). Right: The endocasts represent the extreme figures where a PC is varied by $\pm 3SD$. (a: Top), (b: Middle), (c: Bottom) variations of brain shape along PC1, 2, and 3 respectively

TABLE 3 Results of phylANCOVA. (a) The relationship between brain size and flight ability, (b) the relationship between brain shape and flight ability, (c–e) the relationship between PC1, 2, 3 and flight ability respectively

Variables	df	SS	MS	Rsqr	F	β	Z	p
(a) Model: <Brain size> ~ <Body mass> + <v/f>								
Body mass	1	84,530	84,530	0.814	124.306	4.317	5.372	0.001
v/f	1	6345	6345	0.061	9.330	-130.481	2.501	0.004
Residuals	19	12,920	680	0.124		898.307		
Total	21	103,795						
(b) Model: <Brain shape> ~ <Brain size> + <v/f>								
Brain size	1	0.000	0.000	0.127	9.689	-	2.471	0.006
v/f	1	0.002	0.002	0.624	47.625	-	2.453	0.001
Residuals	19	0.001	0.000	0.249				
Total	21	0.002						
(c) Model: <PC1> ~ <Brain size> + <v/f>								
Brain size	1	0.000	0.000	0.017	1.119	0.000	0.556	0.322
v/f	1	0.000	0.000	0.700	46.914	-0.023	2.998	0.003
Residuals	19	0.000	0.000	0.283		0.058		
Total	21	0.000						
(d) Model: <PC2> ~ <Brain size> + <v/f>								
Brain size	1	0.000	0.000	0.025	1.335	0.000	0.696	0.248
v/f	1	0.000	0.000	0.624	33.742	0.014	3.438	0.001
Residuals	19	0.000	0.000	0.351		-0.018		
Total	21	0.000						
(e) Model: <PC3> ~ <Brain size> + <v/f>								
Brain size	1	0.000	0.000	0.037	3.421	0.000	1.353	0.079
v/f	1	0.001	0.001	0.755	68.919	0.039	3.112	0.002
Residuals	19	0.000	0.000	0.208		-0.045		
Total	21	0.001						

Note. β values in the residuals row indicate intercepts for each model.

Abbreviations: β , regression coefficient; df, degrees of freedom; f, flightless; F, F-statistic; MS, mean sum of squares; p, p-value; Rsqr, coefficient of determination; SS, sum of squares; V, volant; Z, Z-statistics.

concomitant decrease in pectoral muscle mass (McNab, 1994), which accounts for the greatest weight in the avian body (Burton, 1985). In our study, the simple main effect of flight ability on body mass was not significant (Table S4a, $p = 0.818$). The other possibility is that (2) regardless of body mass, flightlessness enlarged brain size in rails. However, when body mass was excluded from the explanatory variables in the model in Table 3a, no significant main effect of flight ability on brain size was found (Table S4b, $p = 0.194$).

In the Rallidae, it is more likely that not only one of these two possibilities occurred, but that flightlessness facilitated a decrease in body mass and an increase in brain size at the same time, so that no significant effects of flightlessness on body mass or brain size, respectively, were detected. A significant negative correlation between pectoral muscle mass and brain size (Isler & van Schaik, 2006) suggests that the saved basal metabolism, in turn, facilitated the development of a large brain relative to body mass in Aves. In the Rallidae, the fact that flightless species have approximately half the basal metabolic rate of volant species (McNab & Ellis, 2006) suggests

that flightless species are more likely to have a larger brain relative to their body mass than volant species. Thus, although the brain is also a major energy-consuming organ in Aves (Isler & van Schaik, 2006; Shiomi, 2022), the results of our study support the hypothesis that in the Rallidae, flightlessness reduced the necessity to expend energy on muscles required for flight, such as the pectoral muscles, thereby conserving basal metabolism, which in turn facilitated the development of a large brain relative to body mass (Isler & van Schaik, 2006).

Kulemeyer et al. (2009) pointed out that the position of the foramen magnum and their sustained flight abilities in Corvidae were correlated. They found that increased flight ability in *Corvus* species compared to *Pica* or *Garrulus* species was accompanied by more horizontal head posture, and more upward positioned foramen magnum than those of latter species (Kulemeyer et al., 2009; Kawabe et al., 2013). The flightless non-New Zealand ratites, such as *Struthio camelus*, have inferiorly positioned foramen magnum, and their cervical vertebrae support the skull vertically from below (Ashwell & Scofield, 2008). Our results show that flightless rails have more inferiorly positioned foramen

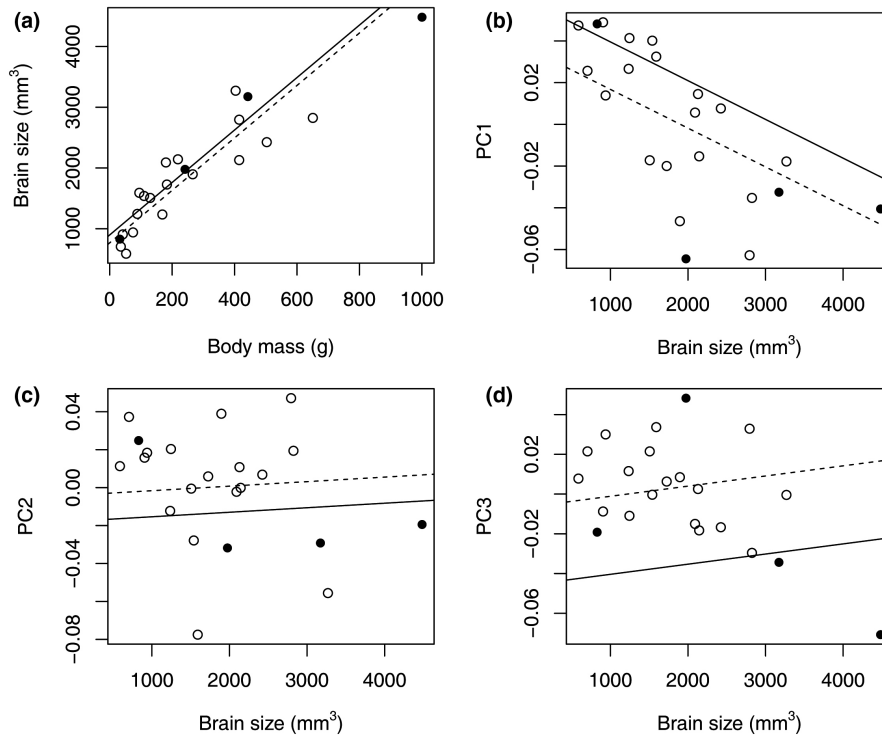


FIGURE 6 The relationship between body mass and brain size (a: Top left), and between brain size and PC1 (b: Top right), PC2 (c: Bottom left), PC3 (d: Bottom right) respectively. Black circles represent flightless species, and white circles represent volant species. Solid (flightless species) and dotted (volant species) lines represent PGLS predicted lines for each category. See Table 3 for the significance of the contribution of each explanatory variable

magnum compared to phylogenetically related volant rails support these previous findings. In Rallidae, the inferiorly positioned foramen magnum would have allowed firm support of heavier cranium acquired through flightlessness. However, it should be noted that this tendency does not necessary apply to all birds. For example, volant Woodcocks (*Scolopax rusticola*) have extremely inferiorly positioned foramen magnum (Marugán-Lobón & Buscalioni, 2009), and the extinct flightless New Zealand ratites, moa, had more superiorly positioned foramen magnum (Ashwell & Scofield, 2008).

Our study suggests that flightless rails have larger brain or enlarged telencephalon than volant rails, and that may have facilitated cognitively advanced behavior, such as tool-using behavior (Shumaker et al., 2011). The birds in Corvidae have a larger brain compared to those of other species in Passeriformes (Cnotka et al., 2008). The New Caledonian Crow (*Corvus moneduloides*), a species of Corvidae in Passeriformes, endemic to New Caledonian Island, exhibits tool-using behavior in the wild (Hunt, 1996). In addition, Hawaiian crow (*Corvus hawaiiensis*), an extinct species in the wild and originally endemic to Hawaii Island, also engaged in tool-using behavior for foraging in the captivity (Rutz et al., 2016). However, a larger brain alone does not necessarily translate to cognitively advanced behaviors (Jönsson et al., 2012). It is suggested that tool-using behavior in Corvidae was facilitated by island-specific ecological conditions, such as rich but embedded food resources and low predation risk (McNab, 1994; Rutz et al., 2016). In fact, Rook (*Corvus frugilegus*) does not exhibit tool-using behavior in the wild (Cnotka et al., 2008).

However, in an experimental environment where it is allowed to learn to make and use tools, Rook exhibits tool-using behavior that is similar to that of New Caledonian Crow (Bird & Emery, 2009). These studies suggest that large brain size and living in the islands facilitated cognitively advanced behavior, such as tool-using behavior (Lefebvre & Bolhuis, 2003; Overington et al., 2009).

Within the Rallidae, tool-using behavior has only been observed in the Okinawa rail (*Gallirallus okinawae*: Miyazawa & Shimada, 2017). In Okinawa Island, eutherian predators have never existed, and the island is inhabited by large-sized terrestrial snails, such as *Satsuma mercatoria* with shell diameters exceeding 40 mm (Nishi, 2015). For Okinawa rails, land snails are the most dependent food resource in any season (Kobayashi et al., 2018). It is impossible for Okinawa rails to swallow such large-sized snails directly, but they can break open the shell and eat the contents, making the snails, which are extremely abundant on the ground, a potentially nutritious food resource (Kobayashi et al., 2018; Miyazawa & Shimada, 2017). There is no other potentially high-nutrient and super-abundant food resource on Okinawa Island other than these large-sized terrestrial snails. Therefore, in the case of Okinawa rail, the larger brain size, flightlessness, and the environmental condition such as the absence of predatory mammals and the abundance of terrestrial food resources embedded in the hard shell may have enabled cognitively advanced tool-using behavior with respect to hitting the shells of snails on anvil stones to crack them, and feeding only on the soft contents of

the snail (Kobayashi et al., 2018; Miyazawa & Shimada, 2017; Rutz et al., 2016). The lack of such food resources may have been the reason why some island birds, such as moa or dodo, did not result in cognitive evolution, even there were no predatory mammals (Angst et al., 2017; Worthy & Holdaway, 2002). Further research on tool-using behavior in the field and under experimental conditions of flightless rails on the islands, such as *Gallirallus owstoni*, *Habroptila wallacii*, and *Porzana palmeri* for which tool-using behavior has not been confirmed, is required.

AUTHOR CONTRIBUTIONS

Tatsuro Nakao: concept, design, acquisition of data, data analysis, interpretation, and drafting of the original manuscript. Takeshi Yamasaki: acquisition of data, data analysis, and interpretation. Naomichi Ogihara: acquisition of data, data analysis, interpretation. Masaki Shimada: concept, design, interpretation. All the authors contributed to critical revision of the manuscript.

ACKNOWLEDGMENTS

We thank Dr. Isao Nishiumi from National Museum of Nature and Science, Japan, and Dr. Helen James and Dr. Christopher Milensky from National Museum of Natural History, USA for providing cranium samples. We thank Dr. Takashi Nagamine and Dr. Yumiko Nakaya from Conservation and Animal Welfare Trust for facilitating our research on the relationship between tool-using behavior and flightlessness in the Okinawa rail. We also thank Dr. Soichiro Kawabe from Fukui Prefectural University, Japan and Dr. Dean C. Adams from Iowa State University, USA, for their instruction and comments on 3D geometric morphometrics. This research was partially supported by a Grant-in-Aid for Scientific Research (C) (no. 18K06397 to T.Y.) and Grant-in-Aid for Scientific Research (B) (no. 20H01409 to M.S.).

CONFLICT OF INTEREST

The authors have no conflicts of interest to declare.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are openly available in (Skulls and brain endcasts of rails [Rallidae]) at [<https://www.morphosource.org/projects/000433635>], reference number (000433635).

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REFERENCES

- Adams, D.C. (2014) A generalized K statistic for estimating phylogenetic signal from shape and other high-dimensional multivariate data. *Systematic Biology*, 63(5), 685–697.
- Adams, D.C. & Collyer, M.L. (2018) Phylogenetic ANOVA: group-clade aggregation, biological challenges, and a refined permutation procedure. *Evolution*, 72(6), 1204–1215.
- Adams, D.C. & Otárola-Castillo, E. (2013) Geomorph: an R package for the collection and analysis of geometric morphometric shape data. *Methods in Ecology and Evolution*, 4, 393–399.
- Angst, D., Chinsamy, A., Steel, L. & Hume, J.P. (2017) Bone histology sheds new light on the ecology of the dodo (*Raphus cucullatus*, Aves, Columbiformes). *Scientific Reports*, 7(1), 1–10.
- Ashwell, K.W.S. & Scofield, R.P. (2008) Big birds and their brains: paleoneurology of the New Zealand moa. *Brain, Behavior and Evolution*, 71(2), 151–166.
- Balanoff, A.M., Bever, G.S., Colbert, M.W., Clarke, J.A., Field, D.J., Gignac, P.M. et al. (2016) Best practices for digitally constructing endocranial casts: examples from birds and their dinosaurian relatives. *Journal of Anatomy*, 229(2), 173–190.
- Bennett, P.M. & Harvey, P.H. (1985) Brain size, development and metabolism in birds and mammals. *Journal of Zoology*, 207(4), 491–509.
- Bird, C.D. & Emery, N.J. (2009) Insightful problem solving and creative tool modification by captive nontool-using rooks. *Proceedings of the National Academy of Sciences*, 106(25), 10370–10375. Available from: <https://doi.org/10.1073/pnas.0901008106>
- Blomberg, S.P., Lefevre, J.G., Wells, J.A. & Waterhouse, M. (2012) Independent contrasts and PGLS regression estimators are equivalent. *Systematic Biology*, 61(3), 382–391.
- Boire, D. & Baron, G. (1994) Allometric comparison of brain and main brain subdivisions in birds. *Journal of Brain Research*, 35(1), 49–66.
- Burton, P.J.K. (1985) Musculature. In: Campbell, B. & Lack, E. (Eds.) *A dictionary of birds*. Vermillion: Buteo Books, pp. 365–368.
- Butler, A.B. & Hodos, W. (2005) *Comparative vertebrate neuroanatomy: evolution and adaptation*. New Jersey: John Wiley & Sons.
- Cnotka, J., Güntürkün, O., Rehkämper, G., Gray, R.D., & Hunt, G.R. (2008). Extraordinary large brains in tool-using new Caledonian crows (*Corvus moneduloides*). *Neuroscience Letters*, 433(3), 241–245. Available from: <https://doi.org/10.1016/j.neulet.2008.01.026>
- Dunning, J.B., Jr. (2007) *CRC handbook of avian body masses*. Florida: CRC Press.
- Early, C.M., Iwaniuk, A.N., Ridgely, R.C. & Witmer, L.M. (2020) Endocast structures are reliable proxies for the sizes of corresponding regions of the brain in extant birds. *Journal of Anatomy*, 237(6), 1162–1176.
- Early, C.M., Ridgely, R.C. & Witmer, L.M. (2020) Beyond endocasts: using predicted brain-structure volumes of extinct birds to assess neuro-anatomical and behavioral inferences. *Diversity*, 12(1), 34.
- Felsenstein, J. (1985) Phylogenies and the comparative method. *The American Naturalist*, 125(1), 1–15.
- García-R, J.C., Gibb, G.C. & Trewick, S.A. (2014) Deep global evolutionary radiation in birds: diversification and trait evolution in the cosmopolitan bird family Rallidae. *Molecular Phylogenetics and Evolution*, 81, 96–108.
- Gill, F.B. (2006) *Ornithology*, 3rd edition. New York: W.H. Freeman.
- Gold, M.E.L. & Watanabe, A. (2018) Flightless birds are not neuroanatomical analogs of non-avian dinosaurs. *BMC Evolutionary Biology*, 18(1), 1–11.
- Gussekloo, S.W. & Cubo, J. (2013) Flightlessness affects cranial morphology in birds. *Zoology*, 116(2), 75–84.
- Hunt, G.R. (1996). Manufacture and use of hook-tools by new Caledonian crows. *Nature*, 379(6562), 249–251. Available from: <https://doi.org/10.1038/379249a0>
- Isler, K. & van Schaik, C.P. (2006) Costs of encephalization: the energy trade-off hypothesis tested on birds. *Journal of Human Evolution*, 51, 228–243.
- Iwaniuk, A.N., Dean, K.M., & Nelson, J.E. (2005). Interspecific allometry of the brain and brain regions in parrots (Psittaciformes): comparisons with other birds and primates. *Brain, Behavior and Evolution*, 65(1), 40–59. Available from: <https://doi.org/10.1159/000081110>
- Iwaniuk, A.N. & Nelson, J.E. (2003) Developmental differences are correlated with relative brain size in birds: a comparative analysis. *Canadian Journal of Zoology*, 81(12), 1913–1928.

- Iwaniuk, A.N., Nelson, J.E., James, H.F., & Olson, S.L. (2004). A comparative test of the correlated evolution of flightlessness and relative brain size in birds. *Journal of Zoology*, 263(3), 317–327. Available from: <https://doi.org/10.1017/s0952836904005308>
- Iwaniuk, A.N. & Wylie, D.R. (2006). The evolution of stereopsis and the Wulst in caprimulgid birds: a comparative analysis. *Journal of Comparative Physiology A*, 192(12), 1313–1326. Available from: <https://doi.org/10.1007/s00359-006-0161-2>
- Jerison, H.J. (1974) On the meaning of brain size. *Science*, 184, 677–679.
- Jønsson, K.A., Fabre, P.H. & Irestedt, M. (2012) Brains, tools, innovation and biogeography in crows and ravens. *BMC Evolutionary Biology*, 12(1), 1–12.
- Juarez, B.H., Speiser, D.I. & Oakley, T.H. (2019) Context-dependent evolution of ostracod morphology along the ecogeographical gradient of ocean depth. *Evolution*, 73(6), 1213–1225.
- Kawabe, S., Shimokawa, T., Miki, H., Matsuda, S. & Endo, H. (2013). Variation in avian brain shape: relationship with size and orbital shape. *Journal of Anatomy*, 223(5), 495–508. Available from: <https://doi.org/10.1111/joa.12109>
- Kawabe, S., Shimokawa, T., Miki, H., Okamoto, T. & Matsuda, S. (2009). A simple and accurate method for estimating the brain volume of birds: possible application in paleoneurology. *Brain, Behavior and Evolution*, 74(4), 295–301. Available from: <https://doi.org/10.1159/000270906>
- Kirchman, J.J. (2012) Speciation of flightless rails on islands: a DNA-based phylogeny of the typical rails of the Pacific. *The Auk*, 129(1), 56–69.
- Knoll, F. & Kawabe, S. (2020) Avian palaeoneurology: reflections on the eve of its 200th anniversary. *Journal of Anatomy*, 236(6), 965–979.
- Kobayashi, S., Morita, Y., Nakaya, Y., Nagamine, T., Onuma, M., Okano, T., et. al. (2018). Dietary habits of the endangered Okinawa rail. *Ornithological Science*, 17(1), 19–35. Available from: <https://doi.org/10.2326/osj.17.19>
- Ksepka, D.T., Balanoff, A.M., Smith, N.A., Bever, G.S., Bhullar, B.A.S., Bourdon, E. et al. (2020) Tempo and pattern of avian brain size evolution. *Current Biology*, 30(11), 2026–2036.
- Ksepka, D.T., Balanoff, A.M., Walsh, S., Revan, A. & Ho, A.M.Y. (2012) Evolution of the brain and sensory organs in Sphenisciformes: new data from the stem penguin *Paraptenodytes antarcticus*. *Zoological Journal of the Linnean Society*, 166(1), 202–219.
- Kulemeyer, C., Asbahr, K., Gunz, P., Frahnert, S. & Bairlein, F. (2009) Functional morphology and integration of corvid skulls—a 3D geometric morphometric approach. *Frontiers in Zoology*, 6(1), 1–14.
- Laubach, T. & Von Haeseler, A. (2007) TreeSnatcher: coding trees from images. *Bioinformatics*, 23(24), 3384–3385.
- Lefebvre, L. & Bolhuis, J.J. (2003) Positive and negative correlates of feeding innovations in birds: evidence for limited modularity. In: Reader, S.M. & Laland, K.N. (Eds.) *Animal Innovation*. New York: Oxford University Press, pp. 39–61.
- Lefebvre, L., Whittle, P., Lascaris, E. & Finkelstein, A. (1997) Feeding innovations and forebrain size in birds. *Animal Behaviour*, 53(3), 549–560.
- Marugán-Lobón, J. & Buscalioni, Á.D. (2009). New insight on the anatomy and architecture of the avian neurocranium. *The Anatomical Record*, 292(3), 364–370. Available from: <https://doi.org/10.1002/ar.20865>
- Matsui, H., Hunt, G.R., Ogihara, N., McGowan, K.J. & Mithraratne, K. (2016) Adaptive bill morphology for enhanced tool manipulation in new Caledonian crows. *Scientific Reports*, 6(1), 1–11.
- McNab, B.K. (1994). Energy conservation and the evolution of flightlessness in birds. *The American Naturalist*, 144(4), 628–642. Available from: <https://doi.org/10.1086/285697>
- McNab, B.K. & Ellis, H.I. (2006) Flightless rails endemic to islands have lower energy expenditures and clutch sizes than flighted rails on islands and continents. *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology*, 145(3), 295–311.
- Michikawa, T., Suzuki, H., Moriguchi, M., Ogihara, N., Kondo, O. & Kobayashi, Y. Automatic extraction of endocranial surfaces from CT images of crania. (2017). *PloS one*, 12(4), e0168516: Available from: <https://doi.org/10.1371/journal.pone.0168516>
- Miyazawa, K. & Shimada, M. (2017). Anvil use by Okinawa rail *Gallirallus okinawae*. *Japanese Journal of Ornithology*, 66(2), 153–162. Available from: <https://doi.org/10.3838/jjo.66.153>
- Nishi, H. (2015) *Handbook of snails*. Tokyo, Japan: Bun-ichi Co., Ltd.
- Ogihara N., Amano H., Kikuchi T., Morita Y., Suzuki H., Kondo O. (2018) Digital reconstruction of Neanderthal and Early *Homo sapiens* endocasts. In: Bruner E., Ogihara N., Tanabe H. (eds) *Digital endocasts. Replacement of Neanderthals by Modern Humans Series* pp. 9–32. Tokyo: Springer. Available from: https://doi.org/10.1007/978-4-431-56582-6_2
- O'Higgins, P. & Jones, N. (1998). Facial growth in *Cercocebus torquatus*: an application of three-dimensional geometric morphometric techniques to the study of morphological variation. *The Journal of Anatomy*, 193(2), 251–272. Available from: <https://doi.org/10.1046/j.1469-7580.1998.19320251.x>.
- Overington, S.E., Morand-Ferron, J., Boogert, N.J. & Lefebvre, L. (2009) Technical innovations drive the relationship between innovativeness and residual brain size in birds. *Animal Behaviour*, 78, 1001–1010.
- Ozaki, K. (2010) *Conservation biology of Okinawa rail Gallirallus okinawae*, PhD Doctoral dissertation, Toho University (in Japanese).
- R Development Core Team. (2013). *R: a language and environment for statistical computing*. Version 3.0.1. R Foundation for Statistical Computing, Vienna. Available at: <http://cran.Rproject.org>
- Roff, D.A. (1994) The evolution of flightlessness: is history important? *Evolutionary Ecology*, 8(6), 639–657.
- Rohlf, F.J. & Slice, D. (1990) Extensions of the Procrustes method for the optimal superimposition of landmarks. *Systematic Biology*, 39(1), 40–59.
- Rutz, C., Klump, B.C., Komarczyk, L., Leighton, R., Kramer, J., Wischnewski, S. et al. (2016) Discovery of species-wide tool use in the Hawaiian crow. *Nature*, 537(7620), 403–407.
- Sayol, F., Downing, P.A., Iwaniuk, A.N., Maspons, J. & Sol, D. (2018) Predictable evolution towards larger brains in birds colonizing oceanic islands. *Nature Communications*, 9(1), 1–7.
- Schneider, C.A., Rasband, W.S., & Eliceiri, K.W. (2012). NIH image to ImageJ: 25 years of image analysis. *Nature Methods*, 9(7), 671–675. Available from: <https://doi.org/10.1038/nmeth.2089>
- Shiomi, K. (2022). Possible link between brain size and flight mode in birds: does soaring ease the energetic cost of the brain? *Evolution*, 76(3), 649–657. Available from: <https://doi.org/10.1111/evo.14425>
- Shumaker, R.W., Walkup, K.R., & Beck, B.B. (2011). *Animal tool behavior: the use and manufacture of tools by animals*. Maryland: JHU Press. Available from: <https://doi.org/10.1179/0197726113Z.0000000007>
- Slikas, B., Olson, S.L., & Fleischer, R.C. (2002). Rapid, independent evolution of flightlessness in four species of Pacific Island rails (Rallidae): an analysis based on mitochondrial sequence data. *Journal of Avian Biology*, 33(1), 5–14. Available from: <https://doi.org/10.1034/j.1600-048x.2002.330103.x>
- Taylor, B. (1998) *Rails: a guide to the rails, crakes, gallinules and coots of the world*. New Haven: Yale University Press. Available from: <https://doi.org/10.2307/4089617>
- Torres, C.R. & Clarke, J.A. (2018) Nocturnal giants: evolution of the sensory ecology in elephant birds and other palaeognaths inferred from digital brain reconstructions. *Proceedings of the Royal Society B*, 285(1890), 20181540.
- Walsh, S.A., Iwaniuk, A.N., Knoll, M.A., Bourdon, E., Barrett, P.M., Milner, A.C. et al. (2013) Avian cerebellar floccular fossa size is not a proxy for flying ability in birds. *PLoS One*, 8(6), e67176.
- Woinarski, J.C.Z., Fisher, A., Brennan, K., Morris, I., Willan, R.C. & Chatto, R. (1998) The chestnut rail *Eulabeornis castaneoventris* on the Wessel and English Company Islands: notes on unusual habitat and use of anvils. *Emu-Austral Ornithology*, 98(1), 74–78.

- Worthy, T.H. & Holdaway, R.N. (2002) *The lost world of the moa: prehistoric life of New Zealand*. Christchurch: Indiana University Press.
- Wyles, J.S., Kunkel, J.G. & Wilson, A.C. (1983) Birds, behavior, and anatomical evolution. *Proceedings of the National Academy of Sciences*, 80(14), 4394–4397.
- Wylie, D.R., Gutiérrez-Ibáñez, C., & Iwaniuk, A. (2015). Integrating brain, behavior, and phylogeny to understand the evolution of sensory systems in birds. *Frontiers in Neuroscience*, 9, 281. Available from: <https://doi.org/10.3389/fnins.2015.00281>
- Yamasaki, T., Aoki, S. & Tokita, M. (2018) Allometry and integration do not strongly constrain beak shape evolution in large-billed (*Corvus macrorhynchos*) and carrion crows (*Corvus corone*). *Ecology and Evolution*, 8(20), 10057–10066.
- Zelenitsky, D.K., Therrien, F., & Kobayashi, Y. (2008). Olfactory acuity in theropods: palaeobiological and evolutionary implications.

Proceedings of the Royal Society B: Biological Sciences, 276(1657), 667–673. Available from: <https://doi.org/10.1098/rspb.2008.1075>

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How to cite this article: Nakao, T., Yamasaki, T., Ogihara, N. & Shimada, M. (2022) Relationship between flightlessness and brain morphology among Rallidae. *Journal of Anatomy*, 00, 1–13. Available from: <https://doi.org/10.1111/joa.13690>