Original Paper

Brain, Behavior and Evolution

Brain Behav Evol
DOI: 10.1159/000348834

Received: August 2, 2012
Returned for revision: September 9, 2012
Accepted after second revision: February 10, 2013
Published online: April 24, 2013

Brain Size and Morphology of the Brood-Parasitic and Cerophagous Honeyguides (Aves: Piciformes)

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Key Words
Brain size · Brood parasitism · Hippocampus · Piciformes · Volumetrics

Abstract
Honeyguides (Indicatoridae, Piciformes) are unique among birds in several respects. All subsist primarily on wax, are obligatory brood parasites and one species engages in 'guiding' behavior in which it leads human honey hunters to bees' nests. This unique life history has likely shaped the evolution of their brain size and morphology. Here, we test that hypothesis using comparative data on relative brain and brain region size of honeyguides and their relatives: woodpeckers, barbets and toucans. Honeyguides have significantly smaller relative brain volumes than all other piciform taxa. Volumetric measurements of the brain indicate that honeyguides have a significantly larger cerebellum and hippocampal formation (HF) than woodpeckers, the sister clade of the honeyguides, although the HF enlargement was not significant across all of our analyses. Cluster analyses also revealed that the overall composition of the brain and telencephalon differs greatly between honeyguides and woodpeckers. The relatively smaller brains of the honeyguides may be a consequence of brood parasitism and cerophagy ('wax eating'), both of which place energetic constraints on brain development and maintenance. The inconclusive results of our analyses of relative HF volume highlight some of the problems associated with comparative studies of the HF that require further study.

Introduction

Honeyguides (Indicatoridae, Piciformes) have been of great interest to humans for centuries. As early as the 17th century [Chapin, 1924; Friedmann, 1955], writers documented the remarkable guiding behavior of honeyguides in which a series of calls and flight displays are used to 'guide' humans to a bees' nest. This unique behavior benefits not only the honeyguide, which would otherwise be unable to access the contents of the bees' nest, but also the people following the honeyguide. Indeed, following hon-

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eyguides reduces the search time of human honey hunters by 64% [Isack and Reyer, 1989]. Thus, this relationship between honeyguides and people appears to be mutually beneficial.

Guiding is not, however, the only unique aspect of the life history of honeyguides. For example, honeyguides feed on beeswax, a diet known as cerophagy. Some other avian taxa feed occasionally on beeswax and can digest it [Horne and Short 1990; Place and Stiles, 1992], but the honeyguides are the only truly cerophagous species. Wax is difficult to digest [Friedman and Kern, 1956], so honeyguides possess several gastrointestinal adaptations for cerophagy [Short and Horne, 2001; Downs et al., 2002]. Specializing on bees' nests not only affects the digestive system, but also has consequences for aspects of honeyguide behavior. For example, the bees' nests have to be located. African honey bees frequently abandon their nests [Schneider, 1990; McNally and Schneider, 1992; Short and Horne, 2001; Spiewok et al., 2006], which results in a constantly changing spatial distribution of nests. Thus, honeyguides must forage over relatively large areas and may have to remember the locations of both active and inactive nests in order to forage efficiently.

Apart from behaviors related to finding and feeding at bees' nests, honeyguides are also obligate brood parasites [Short and Horne, 2001]. In other words, to successfully reproduce, honeyguides must parasitize the nests of other species. The hatchling honeyguides attack host chicks as soon as they hatch, stabbing them to death with the aid of paired hooks at the tips of the beak [Spottiswoode and Koorevaar, 2012]. There is no evidence of a host offspring surviving in a nest parasitized by a honeyguide [Short and Horne, 2001].

Phylogeny, brood parasitism and foraging behavior all have effects on brain evolution in birds [Timmermans et al., 2000; Lefebvre et al., 2002; Iwaniuk, 2004; Iwaniuk and Hurd, 2005; Morrand-Ferron et al., 2007; Boerner and Krüger, 2008; Lefebvre and Sol, 2008], but there are no published data on honeyguide brains. Here, we present the first study of relative brain and brain region size of honeyguides in comparison to their relatives: barbets (Megalaimidae and Lybiidae), woodpeckers (Picidae) and toucans (Ramphastidae). If brain evolution in honeyguides has been mainly driven by selection for a flexible, innovative foraging technique like finding honey and guiding other species to it, we predict that honeyguides will have a relatively large brain and enlarged telencephalon [Lefebvre and Sol, 2008]. Conversely, brood-parasitic cuckoos have significantly smaller brains, relative to body size, than nonparasitic species [Iwaniuk, 2004; Boerner and Krüger, 2008], so if honeyguide brain evolution is mainly driven by brood parasitism, we predict that honeyguides will have relatively small brains. Lastly, the hippocampal formation (HF), a structure that plays a critical role in avian spatial cognition [Bingman, 1993; Colombo and Broadbent, 2000; Colombo et al., 2001; Smulders, 2006], is likely enlarged due to the spatial processing demands of the constantly changing temporal and spatial distribution of active bees' nests [Schneider, 1990; McNally and Schneider, 1992; Short and Horne, 2001; Spiewok et al., 2006] and brood parasitism [Sherry et al., 1993; Reboreda et al., 1996].

Materials and Methods

Brain Size Measurements

Brain volumes were measured from 327 specimens representing 63 species (n = 1–12 per species) with representatives of all five piciform families (table 1), by filling the endocranial cavity of skulls via the foramen magnum with a 50:50 mixture of sizes 8 and 9 lead shot. Brain size measures obtained with this method yield strong correlations (>0.95) with that of fresh brains [Iwaniuk and Nelson 2002] and are not influenced by potential variation related to freezing, desiccation or perfusion, which can affect fresh brains [Healy and Rowe, 2007].

We checked for the possibility that the combination of data from multiple sources might bias our conclusions [Healy and Rowe, 2007] by comparing our data with that of Mlikovsky [1989] for the 10 species present in both sources; no significant difference was detected (paired $t = 0.03$, d.f. = 9, $p = 0.98$). We therefore also included data for an additional 10 species from Mlikovsky [1989] to increase the total number of piciform species analyzed to 73 (table 1).

Body masses of all species were taken from the museum specimens themselves where available and the data for the remaining species were obtained from Dunning [2008].

Brain Specimens and Histology

To examine variation in the relative size of brain regions, we obtained specimens from several sources. Brains from 2 adult male lesser honeyguides *(Indicator minor)* from southern Zambia were loaned to us from the Alfred Denny Museum, University of Sheffield (ADM3256710, ADM3256711). The heads of these two honeyguide specimens were immersion fixed in 5% formaldehyde and remained in fixative until the brain was extracted several months later. We also obtained a yellow-bellied sapsucker *(Sphyrapicus varius)* and a downy woodpecker *(Picoides pubescens)* from wildlife rehabilitators. Both of the woodpeckers were immersion fixed in 4% buffered paraformaldehyde for at least 1 week. Lastly, we obtained a scaly-throated honeyguide (*Indicator variegatus*, USNM638140) of unknown sex, a female yellow-rumped tinkerbird (*Pogoniulus bilineatus*, USNM632982) and a male emerald toucanet (*Aulacorhynchus prasinus*, USNM540590) from the Division of Birds of the National Museum of Natural History (Washington, D.C., USA). All three of these specimens were adults. As with most museum collections, these specimens were immersion

Table 1. Sample sizes (n), body mass (BM) and brain volume (BV) for all five piciform families

Family	Species		n	BM, g	BV, mm^3
Indicatoridae	Thick-billed honeyguide	*Indicator conirostris*	1	26	500
	Least honeyguide	*Indicator exilis*	2	21	400
	Greater honeyguide	*Indicator indicator*	1	52	850
	Spotted honeyguide	*Indicator maculatus*	3	49	800
	Lesser honeyguide	*Indicator minor*	2	27	665
	Scaly-throated honeyguide	*Indicator variegatus*	1	51	800
Lybiidae	Naked-fronted barbet	*Gymnobucco calvus*	3	55	1,250
	Black-collared barbet	*Lybius torquatus*	3	60	1,160
	Speckled tinkerbird	*Pogoniulus scolopaceus*	4	16	440
	Yellow-breasted barbet[1]	*Trachyphonus margaritatus*	2	64	1,200
	Crested barbet	*Trachyphonus vaillantii*	3	73	1,630
	Pied barbet	*Tricholaema leucomelas*	3	35	980
Megalaimidae	Brown barbet	*Calorhamphus fuliginosus*	3	42	1,100
	Blue-throated barbet	*Megalaima asiatica*	3	91	1,530
	Lineated barbet	*Megalaima lineata*	4	161	2,010
	Great barbet[1]	*Megalaima virens*	1	202	2,200
	Brown-headed barbet	*Megalaima zeylonica*	3	106	1,900
	Fire-tufted barbet	*Psilopogon pyrolophus*	5	129	2,210
Picidae	Maroon woodpecker	*Blythipicus rubiginosus*	1	84	3,104
	Pale-billed woodpecker	*Campephilus guatamalensis*	8	241	5,940
	Buff-spotted woodpecker	*Campethera nivosa*	4	38	1,580
	Chestnut-colored woodpecker	*Celeus castaneus*	4	109	2,110
	Greater flameback	*Chrysocolaptes lucidus*	1	142	4,960
	Northern flicker	*Colaptes auratus*	11	153	3,060
	Great spotted woodpecker[1]	*Dendrocopos major*	36	82	2,762
	Middle spotted woodpecker[1]	*Dendrocopos medius*	4	59	2,077
	Cardinal woodpecker	*Dendropicos fuscescens*	5	26	1,220
	Common flameback	*Dinopium javanense*	8	79	2,670
	Lineated woodpecker[1]	*Dryocopus lineatus*	1	180	4,300
	Black woodpecker[1]	*Dryocopus martius*	6	250	7,700
	Pileated woodpecker	*Dryocopus pileatus*	10	335	6,800
	Eurasian wryneck	*Jynx torquilla*	10	28	940
	Buff-necked woodpecker	*Meiglyptes tukki*	2	47	1,810
	Golden-fronted woodpecker[1]	*Melanerpes aurifrons*	1	55	2,200
	Red-bellied woodpecker	*Melanerpes carolinus*	11	77	2,160
	Red-headed woodpecker	*Melanerpes erythrocephalus*	7	72	1,780
	Acorn woodpecker	*Melanerpes formicivorus*	10	65	1,990
	Lewis' woodpecker	*Melanerpes lewis*	10	94	2,220
	Hispaniolan woodpecker	*Melanerpes striatus*	10	75	2,040
	West Indian woodpecker[1]	*Melanerpes superciliaris*	3	85	2,600
	Ashy woodpecker	*Mulleripicus fulvus*	2	200	4,430
	Black-backed woodpecker	*Picoides arcticus*	4	72	3,150
	Red-cockaded woodpecker	*Picoides borealis*	3	44	1,660
	Lesser spotted woodpecker[1]	*Picoides minor*	4	20	1,200
	Downy woodpecker	*Picoides pubescens*	8	25	1,210
	Ladder-backed woodpecker	*Picoides scalaris*	10	33	1,380
	Three-toed woodpecker	*Picoides tridactylus*	5	50	2,630
	Hairy woodpecker	*Picoides villosus*	10	83	2,950
	Yellow-throated woodpecker	*Piculus flavigula*	3	62	1,630
	Ochre-collared piculet	*Picumnus temminckii*	2	11	600
	Grey-headed woodpecker	*Picus canus*	8	150	3,680
	Green woodpecker[1]	*Picus viridis*	3	200	4,213

Table 1 (continued)

Family	Species		n	BM, g	BV, mm^3
	Rufous piculet	*Sasia abnormis*	2	8	580
	Red-naped sapsucker	*Sphyrapicus nuchalis*	5	46	1,270
	Yellow-bellied sapsucker	*Sphyrapicus varius*	10	50	1,300
	Checkered woodpecker	*Veneliornis mixtus*	1	34	1,414
	Little woodpecker	*Veneliornis passerinus*	2	31	1,450
Ramphastidae	Grey-breasted mountain toucan	*Andigena hypoglauca*	3	298	5,000
	Plate-billed mountain toucan	*Andigena lamnirostris*	2	335	4,350
	Groove-billed toucanet	*Aulacorhynchus sulcatus*	7	173	2,740
	Saffron toucanet	*Baillonius bailloni*	3	139	2,940
	Black-spotted barbet	*Capito niger*	3	55	1,350
	Red-headed barbet	*Eubucco bourcierii*	1	34	1,062
	Black-necked aracari	*Pteroglossus aracari*	7	232	3,480
	Chestnut-eared aracari	*Pteroglossus castanotis*	5	310	3,770
	Lettered aracari	*Pteroglossus inscriptus*	5	126	2,500
	Collared aracari	*Pteroglossus torquatus*	10	225	3,500
	Keel-billed toucan	*Ramphastos sulfuratus*	12	425	4,860
	Toco toucan	*Ramphastos toco*	6	540	6,170
	White-throated toucan	*Ramphastos tucanus*	7	530	5,980
	Channel-billed toucan	*Ramphastos vitellinus*	12	362	4,810
	Spot-billed toucanet	*Selenidera maculirostris*	8	139	2,910
	Toucan barbet	*Semnornis ramphastinus*	5	98	1,910

[1] Data from Mlikovsky [1989].

fixed as a whole in 10% formalin for several weeks to months and then transferred to 70% ethanol. Although ethanol storage does lead to significant brain shrinkage, the tissue is often in good condition and can be used for analyses of relative volume [Iwaniuk, 2010, 2011].

As described above, all of the specimens were immersion fixed. Although perfusions result in more rapid fixation of tissue and generally more consistent staining across a tissue block [Beach et al., 1987], immersion fixation can still result in good quality histology when perfusions are not possible. Beach et al. [1987] suggested that formaldehyde can only penetrate 1–2 mm in tissue blocks, but formaldehyde can, in fact, penetrate much deeper than that, provided that the specimens are left in the fixative for an extended period of time [Fox et al., 1985]. Indeed, immersion fixation can yield high-quality specimens for measuring brain region volume, cytoarchitecture and immunohistochemistry [Iwaniuk and Wylie, 2007; Iwaniuk et al., 2009; Gutiérrez-Ibáñez et al., 2011; Wylie et al., 2011; Corfield et al., 2012] even for large brains [Hof et al., 2000; Manger et al., 2003; Wang et al., 2008; Raghanti et al., 2009].

All specimens were processed using the same protocols: the brain was extracted and weighed, photographs taken and the brain stored in 30% sucrose in 0.1 M phosphate-buffered saline (pH = 7.4). After 24–48 h in sucrose solution, the brains were embedded in gelatin, serially sectioned at a thickness of 40 μm on a freezing stage microtome and the sections collected in 0.1 M phosphate-buffered saline. Every second section was mounted onto gelatinized slides, stained with thionin and coverslipped with Permount.

We also compiled data on woodpecker brain volumetrics from Portmann [1947] and Volman et al. [1997]. Unlike our own preparations, Portmann [1947] did not serially section brains to examine the relative size of individual brain regions. Instead, the brains were grossly dissected with a razor blade into four main components: telencephalon, optic lobes, cerebellum and brainstem. Each of the brain regions was then weighed to the nearest milligram. The data of Volman et al. [1997], in contrast, are based on histological sections from perfused woodpeckers, but the brains were processed in a similar fashion to ours. Although only information on the telencephalic and HF volumes are presented by Volman et al. [1997], this provided valuable information on the relative size of the HF, which could be compared with data from the piciform brains that we sectioned.

Volumetric Measurements

Volumes of brain regions of our histological specimens were measured using ImageJ [Rasband, 1997–2011] from photographs taken of every sixth section throughout the brain (i.e. every third section we collected). Across the entire brain, we measured the volumes of the telencephalon, cerebellum, optic lobes and brainstem, which included the pons, medulla, tegmentum and thalamus (see Appendix). These divisions correspond to the same regions measured by Portmann [1947]. The optic lobe measurement consisted of the entire optic tectum as well as the tegmental portions underlying the third ventricle. The brainstem was then measured as the pons, medulla, thalamus and the medial parts of the tegmentum that do not extend into the optic lobes.

In addition to these larger brain regions, we also measured the volumes of several telencephalic regions (see Appendix). More specifically, we measured the sizes of the: nidopallium, mesopallium, hyperpallium, striatum, arcopallium, entopallium and HF. We could not reliably identify the nucleus basalis across all specimens, so this region is subsumed by the nidopallium. All other telencephalic regions (e.g. septum, area corticoidea dorsolateralis) were lumped into a measurement we refer to as 'other'. To identify the borders of all telencephalic regions we referred to avian brain atlases [Karten and Hodos, 1967; Puelles et al., 2007]. The borders of the HF were similar to those of Volman et al. [1997] and Sherry et al. [1989]. More specifically, our measurement of the HF included both the hippocampus proper and the parahippocampal area. The HF was readily distinguished from the surrounding regions, for the most part, based on cell density. As with other species, a clear border could be seen from the dorsal tip on the lateral ventricle running to the dorsal surface of the telencephalon, separating the hippocampus and hyperpallium in more rostral regions and the hippocampus and the caudodorsolateral pallium more caudally. Ventrally, the HF was readily delineated from the septum by the presence of a clear border and marked change in cell density.

Statistical Analyses

Both body mass and brain volume were $\log_{10}$ transformed prior to analysis. To test for differences in relative brain size among the five recognized families of piciforms, we first ran a generalized linear model using species as independent data points and body mass, family membership and their interaction term as covariates of brain volume. The best-fit model was selected by the lowest Akaike Information Criterion value, as calculated in JMP v 9.0.2 (SAS Institute). We also calculated an ordinary least-square linear regression across all piciform species and ran an analysis of variance on the residuals derived from this regression using family membership as a grouping variable.

Because phylogeny can exert a significant effect on brain evolution [Harvey and Pagel, 1991], we also used a phylogenetic generalized least-square (PGLS) approach. Interfamilial relationships were based on Benz et al. [2006] and Livezey and Zusi [2007] (fig. 1) with additional resolution within the honeyguides obtained from Sibley and Ahlquist [1990] and the other piciform families from a range of other studies [Barker and Lanyon, 2000; Weibel and Moore, 2002; Moyle, 2004; Eberhard and Bermingham, 2005; Webb and Moore, 2005; Benz et al., 2006; Moore et al., 2006; Garcia-Trejo et al., 2009; Patané et al., 2009; Moore et al., 2011]. The placement of ten species was uncertain because they were not included in any comprehensive phylogenetic study that we could find. Topological inaccuracies can lead to incorrect conclusions in such analyses [Symonds, 2002], so we excluded these species from our PGLS models.

The phylogenetic tree and data matrices were constructed in the PDAP:PDTREE module [Midford et al., 2005] of the Mesquite software package [Maddison and Maddison, 2011] and the PDAP software package (available from T. Garland). As with the analyses described above, all brain volume and body mass data were $\log_{10}$ transformed prior to analysis. In addition, we also entered the residuals derived from the least-square regression of brain volume and body mass across all piciforms (see above). Because the phylogeny was constructed from multiple sources, all branch lengths were set at 1, which adequately standardized the data [Garland et al., 1992]. We then applied two models of evolutionary change in our PGLS analyses using the MATLAB program Regressionv2.m (available from T. Garland): Brownian motion and Ornstein-Uhlenbeck (OU) [Lavin et al., 2008; Gutiérrez-Ibanez et al., 2009; Swanson and Garland, 2009]. Akaike Information Criterion values were then used to determine which model best fit the data [Lavin et al., 2008; Gutiérrez-Ibanez et al., 2009].

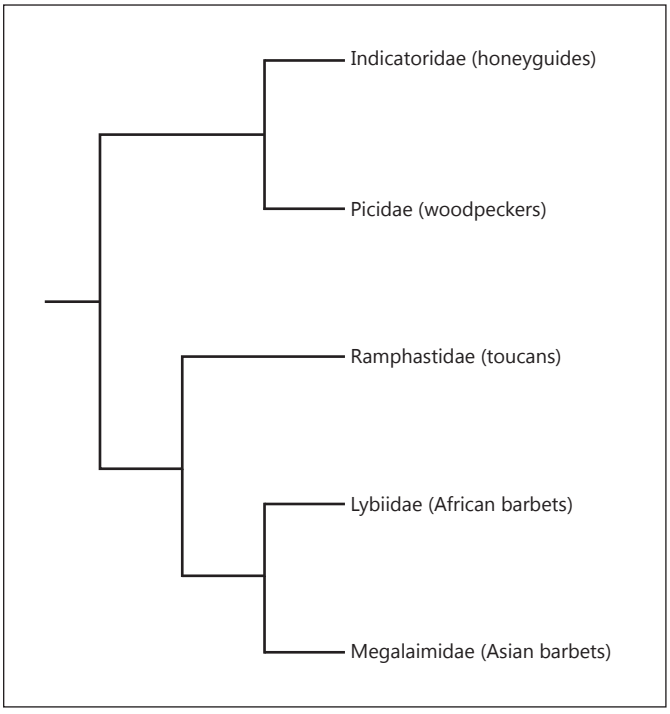

Fig. 1. A phylogenetic tree depicting the relationship among the five currently recognized piciform families [Livezey and Zusi, 2002; Benz et al., 2006].

The relative sizes of the different brain regions measured were expressed as proportions (brain region/total brain volume or telencephalic region/total telencephalon) because we obtained specimens from such a diverse array of sources and differential shrinkage among specimens was a concern. The small sample sizes of the barbets and toucans (both n = 1) precluded us from performing detailed statistical analyses across all groups, but unpaired comparisons were possible between honeyguides and woodpeckers for the larger brain regions [Portmann, 1947] and HF volumes. For the downy woodpecker (table 2), which we measured and was included in Volman et al. [1997], we took the average of the two HF measurements as representative of the species.

The proportional sizes of the brain regions were also examined through the use of hierarchical cluster analysis [Rehkamper et al., 2003; Iwaniuk and Hurd, 2005; Gutiérrez-Ibáñez et al., 2011]. The proportional sizes of the four main brain regions, telencephalon, optic lobes, cerebellum and brainstem, or the telencephalic regions (see above) were included. We present the results of an average linkage method, but the results were qualitatively similar using Ward's and other linkage methods as implemented in JMP v 9.0.2 (SAS Institute).

Table 2. Telencephalic and HF volumes for the specimens examined in this study and the study by Volman et al. [1997]

Family	Common name	Species	n	Telencephalon, mm^3	HF, mm^3	Source
Indicatoridae	Lesser honeyguide	*Indicator minor*	2	328.4 (326.01–330.86)	30.30 (27.63–32.98)	This study, ADM3256710, ADM3256711
	Scaly-throated honeyguide	*Indicator variegatus*	1	337.0	32.21	This study, USNM638140
Lybiidae	Yellow-rumped tinkerbird	*Pogoniulus bilineatus*	1	131.1	11.86	This study, USNM632982
Picidae	Red-bellied woodpecker	*Melanerpes carolinus*	5	1,425.3 (1112.8–1673.0)	71.8 (51.0–98.7)	Volman et al. [1997]
	Red-headed woodpecker	*Melanerpes erythrocephalus*	5	897.9 (712.1–1133.9)	31.4 (24.1–41.5)	Volman et al. [1997]
	Downy woodpecker	*Picoides pubescens*	7	661.8 (495.2–770.7)	33.9 (19.9–45.5)	Volman et al. [1997]
	Downy woodpecker	*Picoides pubescens*	1	698.8	43.08	This study
	Hairy woodpecker	*Picoides villosus*	5	1,596.8 (1438.7–1900.8)	74.8 (62.9–91.2)	Volman et al. [1997]
	Yellow-bellied sapsucker	*Sphyrapicus nuchalis*	1	696.9	54.67	This study

ADM = Alfred Denny Museum, Sheffield; USNM = National Museum of Natural History, Washington, D.C., USA.

Table 3. The results of mixed model analyses of variance with body mass (g) and family as covariates of brain volume (mm^3) and one-way ANOVAs of family and residual brain volumes derived from a least-square linear regression of body mass against brain volume across all piciforms measured

Model	F	d.f.	p	r^2	AIC
Mixed model					
No phylogeny	51.33	4, 67	<0.01	0.95	–158.99
Brownian	0.36	4, 56	0.84	0.75	–99.77
OU	26.77	4, 56	<0.01	0.93	–144.57
Residuals					
No phylogeny	52.08	4, 68	<0.01	0.74	–163.26
Brownian	0.56	4, 57	0.69	0.04	–134.50
OU	13.69	4, 57	<0.01	0.37	–155.70

'Model' refers to whether no phylogeny or a phylogenetic generalized least-square approach was used with two different models of evolutionary change: Brownian motion or OU [Lavin et al., 2008]. AIC = Akaike Information Criterion, with lower values indicating a better fit of the model to the data.

Results

Relative Brain Size

No significant interaction effects between body mass and family membership were detected in any of our mixed models, so we included only the main effects of family and body mass for our three sets of analyses. In table 3, we present the results for the effect of family membership for species as independent data points ('no phylogeny') and our two models that incorporate phylogenetic relatedness. The best-fit model in our PGLS analysis is the OU model (table 3), which yielded a significant effect of family membership on brain volume, while controlling for body mass. Post hoc tests (Tukey-Kramer HSD) indicated that this was due to two differences in relative brain size: woodpeckers have significantly larger brains than all other families and the honeyguides have significantly smaller brains than all other families, except for the Asian barbets (fig. 2a).

We then calculated residuals derived from a common least-square linear regression across all piciforms using species as independent data points ($y = 2.038 + 0.661x$, $F = 272.75$, d.f. = 1, 71, $p < 0.01$, $r^2 = 0.79$) and performed analyses of variance grouped according to family. Again, the OU model was the best fit of the two PGLS models (table 3) and yielded a significant effect of family membership on relative brain volume. In a similar fashion to our mixed model, post hoc comparisons indicated that woodpeckers have significantly larger and the honeyguides have significantly smaller relative brain volumes than all other piciform families (fig. 2b).

Overall Brain Morphology

Examination of the external morphology of the brains (fig. 3) revealed substantial differences among piciform families. The woodpecker brain (fig. 3a) is dominated by the cerebral hemispheres, which surround and obscure much of the lateral aspect of the cerebellum. The olfactory bulbs, although prominent, are rounded at their rostral pole. The optic lobes are oriented in an almost parallel plane with the ventral surface of the brainstem.

Compared to the woodpecker, the honeyguide brain has a very different shape (fig. 3b). The honeyguide's cerebral hemispheres are not as rounded and do not extend

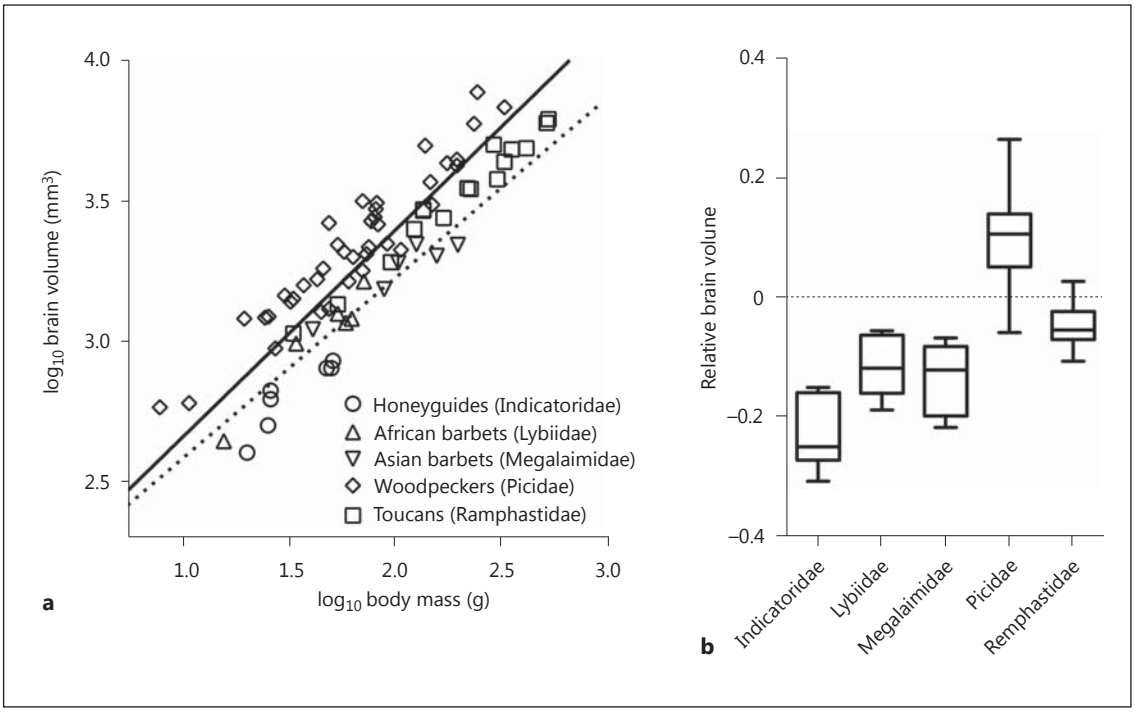

Fig. 2. a A scatterplot of $\log_{10}$ brain volume (mm^3) plotted against $\log_{10}$ body mass (g) of 65 species of piciforms. Each family is depicted by a different symbol. The solid line depicts the linear regression line using species as independent data points and the dashed line depicts a phylogeny-corrected linear regression line plotted back into the original data space following Garland and Ives [2000]. **b** A box and whisker plot (minimum and maximum) of the brain volume residuals derived from a least-square linear regression line of $\log_{10}$ brain volume and $\log_{10}$ body mass of all 65 piciform species measured. The residuals are grouped according to family.

as far caudally as those of the woodpecker and the lateral aspect of the cerebellum is clearly visible. The olfactory bulbs also appear much larger than in the woodpecker and are pointed at the rostral pole. The cerebellum appears to be larger, although this could be a reflection of the lack of a caudolateral expansion of the cerebral hemispheres. Lastly, the optic lobes are tilted at approximately a 45° angle relative to the brainstem.

The brain of African barbets, represented by the yellow-bellied tinkerbird, shares some features with that of the honeyguides (fig. 3c). Like the honeyguides, the tinkerbird has cerebral hemispheres that are not rounded, but are more pointed at the rostral end and do not envelop the lateral aspect of the cerebellum. The optic lobes are also tilted to a similar angle. The two main differences between the tinkerbird and the honeyguides appear to be the lack of enlarged olfactory bulbs or cerebellum in the tinkerbird.

Finally, the toucanet brain (fig. 3d) is different yet again from the previous three species. The cerebral hemispheres are somewhat expanded, not as much as the woodpecker, but certainly more than that of the tinkerbird and honeyguide. The optic lobes are tilted at an angle somewhere between 0 and 45°. Unfortunately, the olfactory bulbs were missing from this specimen, so we cannot comment on the size or shape of the olfactory bulbs in the toucanet.

Variation in Brain Region Size

Figure 4 depicts the proportional sizes of four main regions of the avian brain: telencephalon, optic lobes, cerebellum and brainstem. As discussed above, we selected these four regions so that we could compare our measurements with that of Portmann [1947]. The woodpeckers stand out as having proportionally larger telencephala (65–74% of total brain volume) and correspondingly smaller optic lobe and brainstem volumes compared to all other piciforms. No significant differences were detected between Portmann's [1947] woodpecker measurements and ours (unpaired t tests, all $p > 0.10$). In

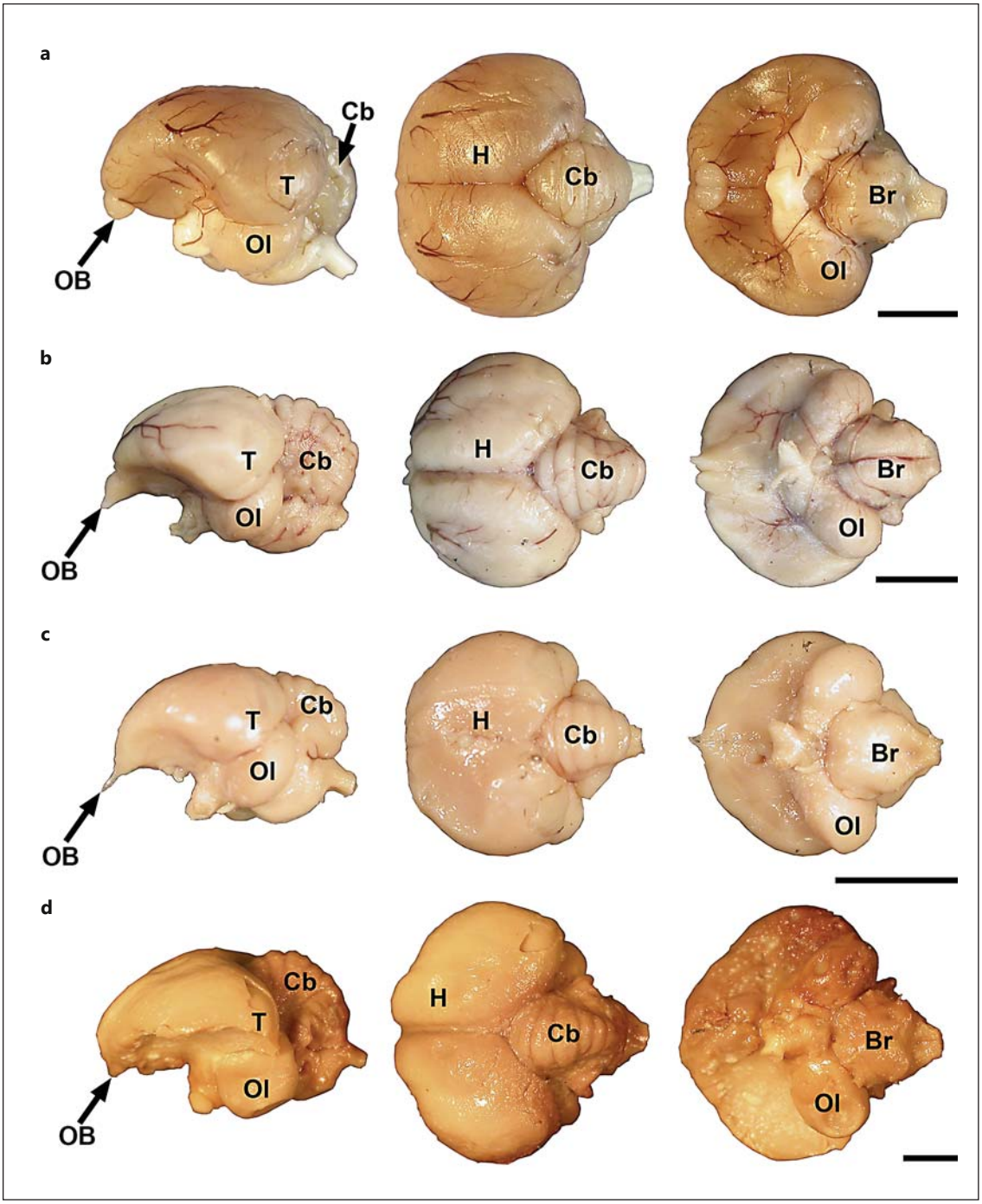

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Fig. 3. Photographs of four piciform brains in lateral (left), dorsal (middle) and ventral (right) aspects. Each row of photos is a representative of a different family. **a** A woodpecker (Picidae), the yellow-bellied sapsucker *(Sphyrapicus varius)*. **b** A honeyguide (Indicatoridae), the scaly-throated honeyguide *(Indicator variegatus)*, USNM638140. **c** An African barbet (Lybiidae), the yellow-rumped tinkerbird *(Pogoniulus bilineatus)*, USNM632982. **d** A toucan (Ramphastidae), the emerald toucanet *(Aulacorhynchus prasinus)*, USNM540590. Br = Brainstem; Cb = cerebellum; H = hyperpallium; OB = olfactory bulbs; Ol = optic lobes; T = telencephalon. All scale bars = 5 mm.

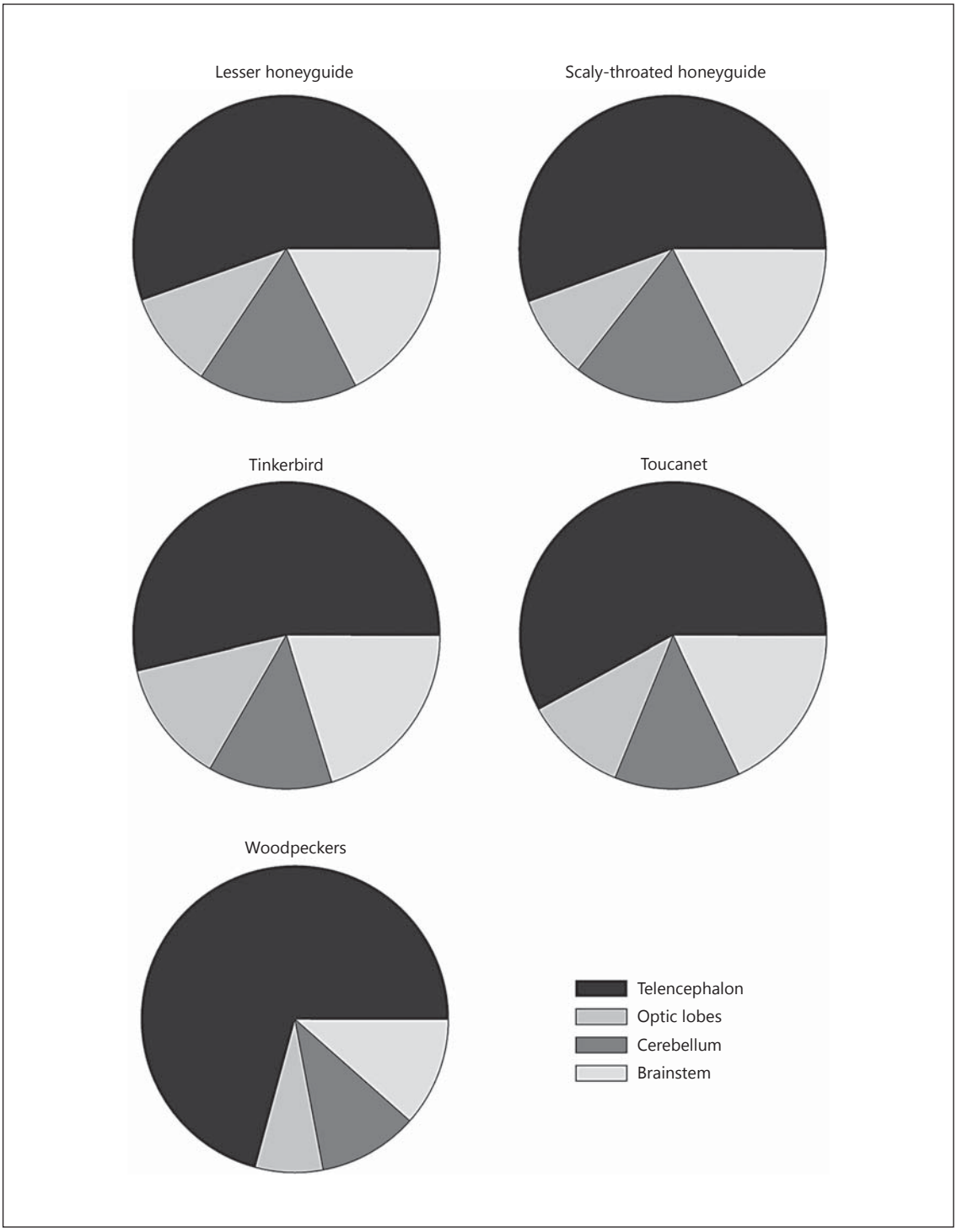

Fig. 4. Pie charts depicting the proportional sizes of the four main regions of the avian brain as defined by Portmann [1947]: telencephalon, optic lobes, cerebellum and brainstem.

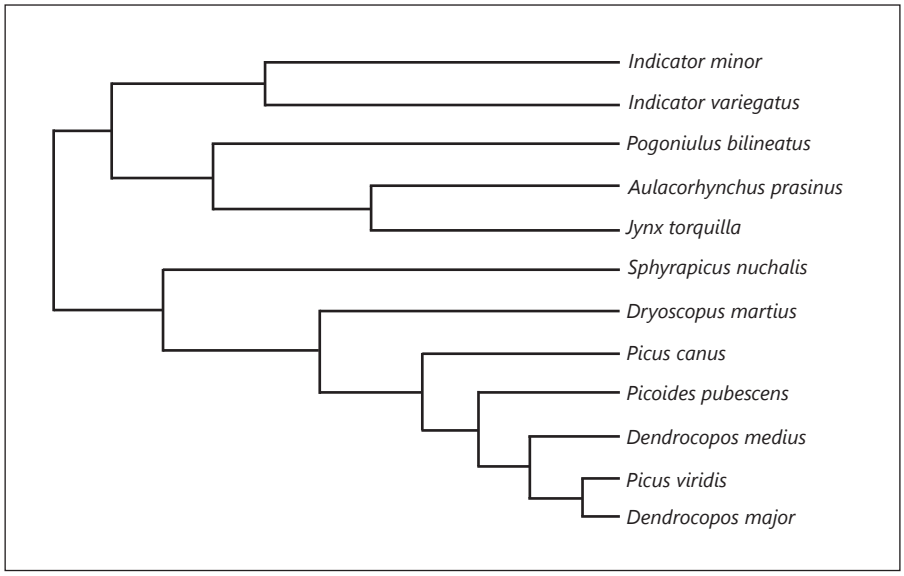

Fig. 5. A dendrogram resulting from a hierarchical cluster analysis of the proportional volumes of four brain regions measured in Portmann [1947] and our specimens (telencephalon, optic lobes, cerebellum and brainstem) using an average linkage method.

fact, our measurements fell within the distribution of Portmann's [1947] measurements for all four brain regions, thereby providing some confidence that this lack of significant difference was not solely due to small sample sizes. We therefore combined data sets and tested for significant differences in the proportional sizes of each of the four brain regions between woodpeckers and honeyguides.

Relative to total brain volume, woodpeckers have significantly larger telencephala ($t = 4.02$, d.f. = 8, $p = 0.004$) and smaller cerebella ($t = -6.17$, d.f. = 8, $p < 0.001$) and brainstems ($t = -3.46$, d.f. = 8, $p = 0.009$) than honeyguides. The relative size of the optic lobes, however, did not differ significantly between the two families ($t = -1.76$, d.f. = 8, $p = 0.12$). The PGLS analyses only corroborated two of these differences; woodpeckers still had smaller cerebella than honeyguides ($F = 11.84$, d.f. = 1, 8, $p = 0.001$) and there was no difference in the relative size of the optic lobes ($F = 0.01$, d.f. = 1, 8, $p = 0.83$). The other two comparisons, telencephalon ($F = 2.63$, d.f. = 1, 8, $p = 0.14$) and brainstem ($F = 1.05$, d.f. = 1, 8, $p = 0.34$), yielded no significant differences between woodpeckers and honeyguides. Thus, the only consistent, significant difference that we detected was the presence of a relatively large cerebellum in the honeyguides.

A hierarchical cluster analysis of the proportional sizes of all of the four brain regions corroborated these differences; honeyguides do not share a similar cerebrotype to that of their sister taxon, the woodpeckers (fig. 5). In fact, all of the woodpeckers, with the exception of the Eurasian wryneck *(Jynx torquilla)*, are grouped separately from all other piciform lineages. Within this mixed group of piciforms, the linkages among species did not reflect the phylogenetic relationships derived from molecular and morphological traits (fig. 1).

Variation in Telencephalic Region Size

Because the toucanet brain lacked olfactory bulbs (see above), we examined the relative size of all other telencephalic regions relative to the telencephalon minus the volume of the olfactory bulbs to facilitate comparisons across all groups. Overall, the composition of the telencephalon differed slightly across piciforms (fig. 6). The nidopallium comprised the largest portion of the telencephalon in all groups, but there were minor variations in the proportional sizes of the other brain regions. In the woodpeckers, for example, the entopallium was relatively small and the hyperpallium was relatively large compared to the other piciforms. Similarly, the striatum occupied a much larger amount of the tinkerbird telencephalon than in other piciforms.

A hierarchical cluster analysis of the proportional volumes of the eight telencephalic structures shown in figure 6 revealed a similar pattern to that of the entire brain (fig. 7). That is, the honeyguides were grouped separately from the woodpeckers. A reexamination of the data based on the two main clusters shown in figure 7 (cluster 1 = woodpeckers + toucanet, cluster 2 = honeyguides + tinkerbird) indicated that this was due to the honeyguides and tinkerbird having relatively larger arcopallial, entopallial,

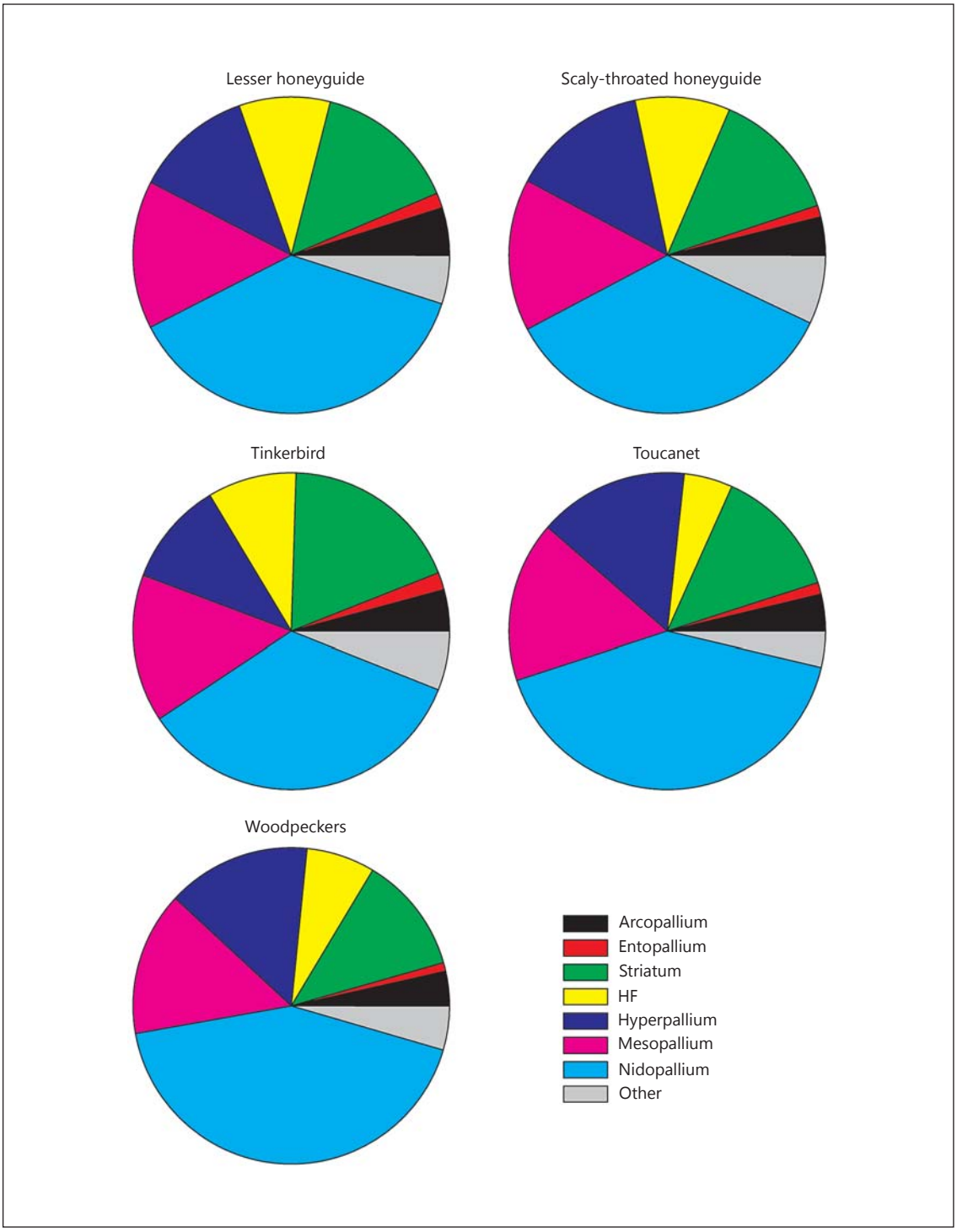

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Fig. 6. Pie charts depicting the proportional sizes of eight different telencephalic brain regions (see legend) relative to the total volume of the telencephalon without the olfactory bulbs.

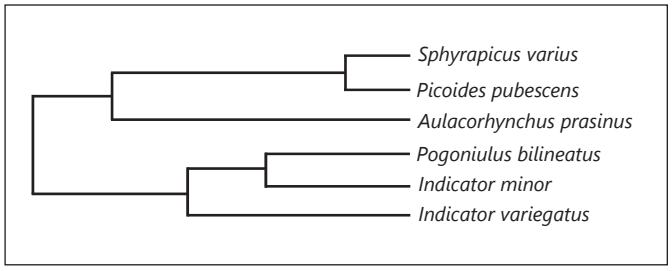

Fig. 7. A dendrogram resulting from a hierarchical cluster analysis of the proportional volumes of four brain regions measured in Portmann [1947] and our specimens (telencephalon, optic lobes, cerebellum and brainstem) using an average linkage method.

striatal and HF volumes and smaller hyperpallial and nidopallial volumes than the woodpeckers and toucanet.

With the inclusion of the data of Volman et al. [1997], we had a total of five woodpecker species to compare with the two honeyguides (fig. 8a). The honeyguides had a HF that was significantly larger than that of the woodpeckers (no phylogeny: $t = 3.41$, d.f. = 5, $p = 0.02$; PGLS: $F = 7.65$, d.f. = 1, 5, $p < 0.05$) relative to telencephalon size. The relative HF volume of the tinkerbird was similar to that of the two honeyguides (fig. 8a), but because we only had a single specimen, we could not determine whether the tinkerbird differed significantly from the honeyguides or woodpeckers. Note that the toucanet was not included in this comparison because their data included the olfactory bulbs in the telencephalic volume, but the toucanet lacked olfactory bulbs (see above).

To further examine the relative size of HF across piciforms, we constructed a scatterplot of HF volume and telencephalon volume (fig. 8b). Although both of the honeyguides sit above the regression line, so do at least two of the woodpeckers and none of the species fall outside of the 95% confidence interval. Thus, based on the scatterplot, we cannot conclude that the honeyguides have a relatively enlarged HF.

Discussion

Overall, our results indicate that honeyguides have relatively smaller brains than other piciforms, as well as a brain and telencephalic composition that is markedly different to that of their sister clade the woodpeckers. Our analysis of relative HF, however, provided mixed results, depending on how we examined relative HF. Honeyguides therefore supported at least one of our predictions, a reduction in relative brain size, and they clearly have a divergent brain size and morphology from that of their sister clade the woodpeckers [Sibley and Ahlquist, 1990; Short and Horne, 2001; Benz et al., 2006; Livezey and Zusi, 2007]. It should, however, be noted that in our comparisons of brain composition we only examined two species within a single genus. There are 17 recognized species of honeyguides that belong to 4 genera [Short and Horne, 2001]. Although all species are cerophagous and brood parasitic, they likely vary behaviorally and ecologically from one another. Our examination of two species might not reflect the brains of all honeyguide species. Nevertheless, this is the first study on honeyguides and provides some insight into how the unique life history of these species is correlated with brain size and composition.

Brain Size and Composition

As discussed above, one of the possible reasons for these differences is the honeyguides' reliance on beeswax. To digest beeswax, honeyguides have a prolonged gut transit time and increased levels of lipases and other enzymes [Downs et al., 2002]. Interestingly, other vertebrates with prolonged gut passage times and/or diets that include foods that are difficult to digest generally have relatively small brains [Aiello and Wheeler, 1995; Isler and van Schaik, 2009]. Comparative studies of gut and brain size in primates even led to the development of the 'expensive brain hypothesis' [Aiello and Wheeler, 1995], which proposes that because the development of both the gastrointestinal tract and the brain are expensive, species evolve either a large gut and a relatively small brain, or vice versa. Isler and van Schaik [2006] explicitly tested the expensive tissue hypothesis in birds and found that although gut mass did not yield a significant relationship with relative brain size across all birds, crude fiber content was negatively correlated with relative brain size within some orders, including the Piciformes. Thus, the energetic demands of cerophagy may have played a role in the evolution of relatively small brains in honeyguides.

Brood parasitism may also have driven the evolution of honeyguide brains. Just as large guts and relatively small brains are associated with one another, so too are brood parasitism and relatively small brains [Iwaniuk 2004; Boerner and Krüger, 2008]. In cuckoos, brood-parasitic species tend to have relatively smaller brains than non-parasitic species [Iwaniuk, 2004; Boerner and Krüger, 2008]. Similarly, the brood-parasitic paradise whydah *(Vidua paradisaea)* has a slightly smaller brain size than that

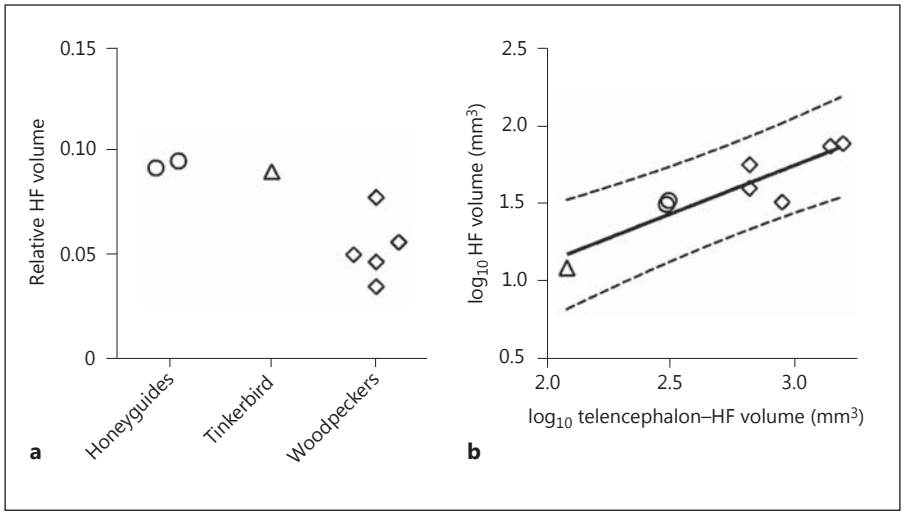

Fig. 8. a A univariate scatterplot of relative HF volume expressed as a proportion of total telencephalic volume grouped according to taxon. **b** A scatterplot of HF volume plotted against telencephalic (minus HF) volume. The solid line indicates the least-square linear regression line and the dotted lines are the upper and lower bounds of the 95% prediction interval. Note that the symbols match those of **a**.

predicted by its body mass [Iwaniuk, 2004], and among 33 species of Icteridae, the brood-parasitic cowbirds (*Molothrus* spp.) had relatively small brains [Overington, 2011]. Brood parasitism has evolved seven times independently in birds [Johnsgard, 1999; Davies, 2000; Sorenson and Payne, 2001] and, including the present study, this trend has been found in at least four of these parasitic clades. Why brood parasites have relatively smaller brains than other species, however, remains unclear. Boerner and Krüger's [2008] evolutionary path analysis suggests that in cuckoos, relative brain size decreased prior to the evolution of brood parasitism and other life history traits (e.g. migration, habitat preference). Similar changes would be difficult to discern in piciforms because the divergence between the woodpeckers and honeyguides coincides with the evolution of brood parasitism as well as many other life history traits (e.g. cerophagy).

One aspect of brood parasites that might be relevant to relative brain size is that they tend to develop much more rapidly than their hosts, a feature that allows many of them to kill or remove host eggs and/or nestlings [Davies, 2000]. A recent study of developmental staging in several brood parasites, including honeyguides, showed that this is at least partially due to internal incubation such that at laying the embryos are advanced by 31 h relative to their host species [Birkhead et al., 2011]. The shorter incubation period of honeyguides and other brood parasites has significant implications for how the brain develops. Across birds, the duration of incubation is positively correlated with relative brain size; longer incubation periods are found in species with relatively larger brains [Iwaniuk and Nelson, 2003]. Honeyguides, and other brood parasites, might therefore have relatively smaller brains because of this shortened incubation period and rapid development.

Determining the extent to which cerophagy and/or brood parasitism is responsible for evolutionary changes in honeyguide brain size and composition is not possible at present, but it is clear that honeyguides have a markedly different brain from that of their sister taxon the woodpeckers. These differences were clear in comparisons of individual brain regions as well as hierarchical cluster analyses. Overall, woodpeckers have significantly larger brains, relative to body size, and significantly smaller relative cerebellar volumes than honeyguides. In our cluster analyses (fig. 5), woodpeckers were consistently grouped together and widely separated from the honeyguides. The only exception to this pattern was the Eurasian wryneck. Although the relative brain size of the wryneck is similar to that of other woodpeckers, this species lacks the same expansion of the telencephalon found in other species and the wryneck grouped with the other piciforms in our cluster analyses (fig. 5). Wrynecks are basal to all other woodpeckers [Sibley and Ahlquist, 1990; Benz et al., 2006; Livezey and Zusi, 2007], so this suggests that the differences between honeyguides and woodpeckers in the relative size of the telencephalon and cerebellum may have coincided with the early evolution of woodpeckers.

Relative HF Size

HF size was significantly larger than that of woodpeckers when we expressed it as a proportion of the telencephalic volume (fig. 8a), but a scatterplot of the HF and

telencephalon data suggested the HF size is only slightly larger than that of some woodpeckers (fig. 8b). Due to our small sample sizes, we could not perform detailed statistical analyses on the HF data, so it is unclear whether the HF of honeyguides is, in fact, enlarged. This is perhaps surprising because honeyguides appear to rely heavily on spatial memory for both their diet and reproductive cycle. For example, bees' nests are patchily distributed in the environment and African bee colonies are highly mobile and prone to both disturbance-induced and seasonal absconding of nests [Spiewok et al., 2006]. African bees' nests therefore represent a spatially and temporally variable food source and this could pose significant demands on the spatial memory of honeyguides. Similarly, other brood-parasitic species have relatively larger HF volumes than nonparasitic species, irrespective of sex [Reboreda et al., 1996]. The underlying reason for this HF enlargement is thought to be that brood parasites have a more 'spatially complex' life history in that they must keep track of the location and status of multiple host nests [Sherry et al., 1993; Reboreda et al., 1996; Sherry, 2006]. Given that our hypothesis was that these two key features of honeyguide life history would drive an increase in relative HF size, why do our results not necessarily support this hypothesis?

One possibility is that by combining our data with that of Volman et al. [1997], we have contributed a potentially confounding variable to our dataset [Roth et al., 2010]. Unlike our immersion-fixed specimens, the woodpeckers used in Volman et al. [1997] were perfused. This could have led to differences in the amount of shrinkage in HF or the telencephalon as a whole, but our measurements of a downy woodpecker that was immersion fixed fell within the range of volumes of specimens reported by Volman et al. [1997] (table 2). Similarly, if the borders that we used to define the HF were different from those of Volman et al. [1997], we would also have expected to see a difference between the two downy woodpecker measurements. Thus, fixation and the borders of HF could cause extra 'noise' in our dataset, but this seems to be an inadequate explanation for why the HF is larger in honeyguides in one analysis and not another.

A second possibility is that the spatial ecology of honeyguides is not different to that other piciform birds. The assumption that interspecific differences in relative HF size reflect spatial abilities has been an area of controversy over the years [Bolhuis and Macphail, 2001; Brodin and Bolhuis, 2008; Roth et al., 2010]. One of the several issues raised was the notion of describing some species as more specialized food cachers than others and the mixed results of comparative studies of caching behavior and relative HF size [Brodin and Lundborg, 2003; Lucas et al., 2004; Brodin and Bolhuis, 2008; Roth et al., 2010]. In fact, one of the studies that failed to detect a difference in relative HF volume in relation to food caching behavior was the study of woodpeckers by Volman et al. [1997]. Specifically, the scatter hoarding red-bellied woodpecker *(Melanerpes carolinus)* did not have a significantly larger HF than the larder-hoarding red-headed woodpecker *(Melanerpes erythrocephalus)* or the noncaching downy and hairy *(Picoides villosus)* woodpeckers. Thus, despite marked differences in presumed spatial abilities, no significant difference in relative HF volume was found. Because the spatial memories of piciforms have yet to be tested, we do not know the extent to which spatial abilities vary among piciform species, if at all. Although honeyguides do have a unique life history, the patchiness of their food sources or finding host nests might not pose a significantly greater challenge to their spatial abilities than woodpeckers' finding spatially scattered prey or suitable areas for nesting.

Finally, it is worth noting that even if we do accept that the HF is expanded in honeyguides, this could reflect a decrease in the size of other telencephalic regions and not necessarily an expansion of the HF [Ward et al., 2012]. For example, if HF size is constant and the sizes of the nidopallial and mesopallial areas change, any calculation of relative HF volume would necessarily change. Our examination of proportional sizes of several telencephalic regions suggests that nidopallial, hyperpallial and striatal regions are smaller in the honeyguides than the woodpeckers (fig. 6), but an adequate test of this hypothesis requires much larger sample sizes than what was available for this study. Addressing this question of whether it is HF that enlarges or other regions that shrink might shed further insight into the complex relationship between HF and spatial ability, which has been the source of great controversy over the years [Bolhuis and Macphail, 2001; Brodin and Bolhuis, 2008; Roth et al., 2010].

Acknowledgments

All of the methods reported in this paper adhere to the Canadian Council of Animal Care regulations for animal research. We would like to thank the curatorial staff of the National Museum of Natural History and the Zambia Wildlife Authority (for research permits) without whom this study would not have been possible, Ted Garland for providing PDAP and Regressionv2.m for our statistical analyses and two anonymous reviewers for improving our manuscript. Support for this study was provided by NSERC Discovery Grants to D.R.W., L.L. and A.N.I. and a Royal Society Dorothy Hodgkin Fellowship to C.N.S.

Appendix

The data used in the volumetric analyses of brain regions among piciform birds.

Species	Brain	Cb	BrSt	OL	TeO	T	OB	A	E	St	H	HF	M	N	Source
Aulacorhynchus prasinus	1,075.77	142.07	301.83	116.67	80.80	623.83		23.57	7.45	83.28	95.59	31.06	102.22	257.76	This study
Dendrocopos major	2,703	279.84	294.15	165.36		1,963.65									Portmann [1947]
Dendrocopos medius	2,061	220.05	210.60	140.40		1,490.40									Portmann [1947]
Dryocopus martius	7,979	636.00	788.64	400.68		6,153.30									Portmann [1947]
Indicator minor	571.11	87.44	155.26	61.27	44.17	323.85	2.16	15.74	5.32	47.00	39.81	27.63	46.78	126.55	This study
Indicator minor	603.55	109.76	151.34	58.81	43.62	327.18	3.68	15.98	4.77	48.14	38.49	32.98	51.63	117.52	This study
Indicator variegatus	597.53	107.87	147.93	52.88	41.26	332.20	4.84	13.05	3.92	44.71	46.22	32.21	51.49	116.89	This study
Jynx torquilla	804	92.02	129.47	87.74		494.88									Portmann [1947]
Picoides pubescens	997.53	110.11	180.26	68.10	47.95	698.83	2.55	21.90	5.73	88.27	101.93	43.08	99.20	307.60	This study
Picus canus	3,465	350.24	304.47	240.79		2,569.09									Portmann [1947]
Picus viridis	4,384	418.91	480.59	264.71		3,220.21									Portmann [1947]
Pogoniulus bilineatus	244.28	31.94	78.17	31.75	23.26	130.67	0.47	5.56	2.31	24.18	13.91	11.86	19.63	45.32	This study
Sphyrapicus variegatus	1,074.85	140.28	232.53	90.27	67.28	696.91	4.66	27.65	6.33	79.32	102.95	54.67	105.67	288.10	This study

All of the data provided in this study are given in mm^3. Data derived from Portmann [1947] are given in mg because the original dataset only included masses. Cb = Cerebellum; BrSt = brainstem; OL = optic lobe; TeO = optic tectum; T = telencephalon; OB = olfactory bulbs; A = arcopallium; E = entopallium; St = striatum; H = hyperpallium; M = mesopallium; N = nidopallium. For details on the borders used to define these regions, see the accompanying text.

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