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SHORT NOTE



Spawning location is linked to the relative size of olfactory nerves in anurans

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The brain is the most important organ associated with demands on cognitive ability, and selection pressures have been implicated to explain variation in brain size and brain architecture in a wide range of taxa. Using phylogenetic comparative methods, we studied the effect of spawning location (terrestrial, lentic and lotic) on variation in the size of different brain parts among 43 Chinese anuran species. Spawning location was not significantly associated with relative brain size, and there were no links between spawning location and independent contrasts in size of the olfactory bulbs, telencephalon, optic tecta and cerebellum. However, the independent contrasts in size of the olfactory nerves differed significantly among the three types of spawning location. Our findings provide evidence that the brain structures underlying olfaction are linked to lifehistory attributes required for spawning in specific habitat types.

Key words: anurans, comparative method, olfactory nerves, spawning location

he brain is the main organ for the processing of information, and cognitive ability can be linked to brain architecture (Smeets et al., 1997; Striedter, 2005). Comparative studies have revealed various factors affecting the relative sizes of different brain structures across a wide range of taxa (Huber et al., 1997; Sol et al., 2002; Garamszegi et al., 2005; Dunbar & Shultz, 2007; Fitzpatrick et al. 2012; West, 2014; Kruska, 2014; Liao et al., 2015). For example, habitat complexity is positively correlated with forebrain and telencephalon size in fish (Huber et al., 1997). Linked with the fact that large brains are energetically costly (Allen & Kay, 2012), diet quality shows a significant effect on brain size variation in primates and carnivores (Dunbar & Shultz, 2007) and olfactory bulbs and optic tecta in cichlid fishes (Huber et al., 1997). Sexual selection (García-Peňa et al., 2013), domestication (Kruska, 2014) and social behaviour (i.e. invasion success, Sol et al., 2002; song complexity, Garamszegi et al., 2005) are further shown to impose selection pressures on the size of different brain structures.

In amphibians, brain size has previously been shown to be associated with habitat traits (Taylor et al., 1995; Gonda et al., 2010; Amiel et al., 2011; Jiang et al., 2015; Liao et al., 2015). The ability to find suitable spawning locations likely improves reproductive success and the survival of offspring, and different types of spawning sites are linked to differential life-history demands (such as migratory distances from spawning sites to summer/ winter grounds, physiological tolerances) which could be linked to cognitive demands. However, a study designed to test whether brain size and brain architecture is related to spawning location is as yet lacking. Here, we analysed whether spawning location explains observed variation in relative brain size and five brain structures (olfactory nerves, olfactory bulbs, telencephalon, optic tecta and cerebellum) across 43 species of anurans.

We obtained volumetric measures of brain parts of 200 males from 43 anuran species with known phylogenies during the breeding seasons 2007-2013 from Hengduan Mountains, China. Females remained unconsidered due to difficulties in capturing them for some species. Sample size per species ranged between 3 and 16 individuals (average 4.6 individuals), except for four species for which we had only two samples, and for two species which were represented only by single individual (Online Appendix 1). Following Liao et al. (2013), spawning location for each species was classified on a three-point scale: 1 – arboreal and terrestrial: spawning occurs mostly occur on trees or ground, eggs in foam nests; 2 - lentic aquatic: eggs in ponds; 3 - lotic aquatic: eggs in running water. We took individuals to the laboratory, and kept them in a rectangular tank (0.5 m × 0.4 m × 0.4 m). We deeply anesthetised all the individuals with benzocaine before killing them using double-pithing and preservation in 4% buffered formalin. We measured body size (snout-vent length: SVL) to the nearest 0.01 mm with a caliper, and body mass to the nearest 0.1 mg with an electronic balance within 2-8 weeks after preservation. We removed the brains, and weighed them with an electronic balance. The number of days spent in buffered formalin does not affect brain weight after correcting for body mass (Liao et al., 2015). The same

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Source	Sums of squares	df	Mean square	F	р	
Brain						
Breeding habitat	0.008	2	0.004	0.489	0.617	
Log body size	2.565	1	2.565	52.947	<0.001	
Olfactory nerves						
Breeding habitat	0.484	2	0.242	4.513	0.017	
Log body size	2.565	1	2.565	47.782	<0.001	
Olfactory bulbs						
Breeding habitat	0.049	2	0.024	0.986	0.383	
Log body size	0.862	1	0.862	34.806	<0.001	
Telencephalon						
Breeding habitat	0.007	2	0.003	0.317	0.730	
Log body size	0.493	1	0.493	40.915	<0.001	
Optic tecta						
Breeding habitat	0.038	2	0.019	1.371	0.266	
Log body size	0.313	1	0.313	22.797	<0.001	
Cerebellum						
Breeding habitat	0.018	2	0.009	0.253	0.778	
Log body size	0.447	1	0.447	13.312	<0.001	

Table 1. The influence of spawning location on variation on independent contrasts of (log) brain architectures across 43 anuran species when correcting log $_{10}$ (body size) using MANCOVA^a. ^a For testing evolutionary associations, the regression was forced through the origin.

people (SLL and WBL) performed all dissections, digital images and measurements.

Digital images of the dorsal, ventral, left and right sides of the brain were taken through a Motic Images 3.1 digital camera mounted on a Moticam 2006 light microscope at 400× magnification. For paired structures, we only measured the width of the right hemisphere and doubled the volume estimate. We measured the width, height and length of the entire brain and five brain structures (olfactory nerves, olfactory bulbs, telencephalon, optic tectum and cerebellum) from digital photographs using tpsDig 1.37 software (Fig. 1). The volumetric estimates of different brains were obtained using the ellipsoid model volume=(L*W*H) π / 6*1.43 (see Liao et al., 2015). For each brain, this procedure was repeated five times and the average of five measurements was used as the final estimate. Repeatability of all measures and inter-measurer repeatability for all brain traits were high (Liao et al., 2015). To further assess the accuracy of our estimates, we estimated intra-measurer repeatabilities by three measurements for one randomly picked specimen from each of the 43 species. Intraspecific variability across the five brain structures showed that heterogeneity in variability across them would not bias the results (detailed data not shown). All metric variables were log₁₀-transformed to meet distributional assumptions. We multiplied all data by 1000 prior to log transformation because some of the measurements were smaller than 1 (Sokal & Rohlf, 1995). Because body size differs significantly among species and large bodied species have bigger brains (Liao et al., 2015), we used log₁₀ (body size) as a covariate to control for brain-body size allometry in all analyses.

To control for phylogenetic effects we employed comparative analyses by independent contrasts (Purvis & Rambaut, 1995). We used the phylogeny of Pyron & Wiens (2011) to reconstruct phylogenetic tree for the 43 species (Fig. S1). Following the suggestions of Pagel (1992), branch lengths were first arbitrarily set to one. Felsenstein (1985) provides the details of the general procedure for estimating the character values in the ancestors. Contrasts in SVL, brain size and the size of all brain parts were independent (Liao et al., 2015). With 43 species at the tips of the reconstructed trees, 42=(43-



Fig. 1. Dorsal, ventral and lateral views of anuran brain. Shown are the measures (length, width and height) that were taken from each of the five brain parts (*viz*. olfactory nerves, olfactory bulbs, telencephalon, optic tectum and cerebellum).



Fig. 2. The mean (±SE) relative olfactory nerves size among three types of spawning locations in 43 anurans species when correcting phylogenetic effects. Residuals were generated from regression of \log_{10} (olfactory nerves) on \log_{10} (body size).

1) pairs of contrasts for size measures (brain, brain parts and SVL) could be computed for the node pairs sharing an immediate common ancestor, and then re-scaled and analysed as suggested by Garland et al. (1992). We rescaled spawning location and then mapped its state onto the phylogenetic tree. We used maximum parsimony OSX (Zeng & Liu, 2011) to estimate the variable states in the ancestral nodes of the phylogeny. We ran a multivariate general linear model (MANCOVA) using independent contrasts in log₁₀ (mean brain size) and log₁₀ (size of brain structures) as dependent variables, and spawning location as fixed factors, and independent contrasts in log₁₀ (SVL) as covariate to test the effect of spawning location on variation in brain size. All analyses were performed by using Type III sums of squares tests with the SPSS (21.0) statistical package.

We found no significant effect of spawning location on independent contrasts in relative sizes of brain structures (MANCOVA; spawning location: Wilks' $\lambda_{12,}_{66}$ =0.641, p=0.204; body size: Wilks' $\lambda_{6,33}$ =0.293, p<0.001). Subsequent univariate tests showed that spawning location did not explain significant amounts of variation in relative brain size, and relative size of olfactory bulbs, telencephalon, optic tecta and cerebellum. However, spawning location showed a significant effect on the relative size of olfactory nerves, with lotic aquatic spawning species having significantly larger olfactory nerves than species from the other categories (Tukey's post hoc test: p<0.05, Fig. 1; Table 2).

Our results uncover the fact that spawning location explains a significant amount of variation in the relative size of olfactory nerves, but that this relation is also linked to differential body sizes. Species with lotic aquatic spawning location have larger olfactory nerves than species with arboreal, terrestrial and lentic spawning locations. Hence, the association between spawning location and relative sizes of olfactory nerves give support for the assertion that natural selection stemming from difference in spawning location might contribute to the evolution of brain architecture. Differences in migratory distances between terrestrial sites and spawning sites, physiological tolerances and breeding modes could affect the size of specific brain structure. In the study, we observed the size of olfactory nerves varying by two orders of magnitude across 43 species (Online Appendix 2). The main function of olfactory nerves is to receive olfactory signals (Striedter, 2005). However, it is as yet unknown whether the increased size of olfactory nerves in species with lotic spawning might be linked to olfactorial abilities in turbulent flow.

Comparative studies suggest that the mechanisms behind brain evolution can be explained by ecological factors characterising the species (Huber et al., 1997; Striedter, 2005; Garamszegi et al., 2005; Dunbar & Shultz, 2007; Liao et al., 2015). In amphibians, habitat types affect variation in size of different brain structures. For example, Taylor et al. (1995) found that fossorial species have relatively larger olfactorial bulbs than hylids and ranids. Also, arboreal species have a larger telencephalon than species from other habitat classes, and predation risk affects the size of optic tecta while diet affects telencephalon size (Liao et al., 2015). Interestingly, spawning location only affects the olfactory nerves, supporting the mosaic hypothesis of brain size evolution (Barton & Harvey, 2000).

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