

A Smaller Habenula is Associated with Increasing Intensity of Sexual Selection

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Abstract

The habenula is a small structure in the brain that acts as a relay station for neural information, helping to modulate behaviour in response to variable and unpredictable stimuli. Broadly, it is evolutionarily conserved in structure and connectivity across vertebrates and is the most prominent bilaterally asymmetric structure in the brain. Nonetheless, comparative evolutionary studies of the habenula are virtually non-existent. Here, we examine the volumes of the medial and lateral habenular subregions, in both hemispheres, across a group of Australian agamid lizards in the genus *Ctenophorus*. In males, we found bilaterally asymmetrical selection on the lateral habenula to become smaller with increasing intensity of sexual selection, possibly as a mechanism to increase aggressive responses. In females, we found bilaterally symmetrical selection on both the medial and lateral subregions to become smaller with increasing sexual selection. This is consistent with sexual selection increasing motivation to reproduce and the habenula's well-character-

ized role in controlling and modifying responses to rewarding stimuli. However, as there are currently no studies addressing habenular function in reptiles, it is difficult to draw more precise conclusions. As has happened recently in biomedical neuroscience, it is time for the habenula to receive greater attention in evolutionary neuroscience.

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Introduction

Located in the approximate centre of the brain is a small structure, the habenula, that acts as “coordinator” of sorts for signals being sent from the front of the brain to the back. It filters and sorts information and helps coordinate behavioural responses, especially to complex and unpredictable situations. The internal structure of the habenula and the connections it makes across the brain are, broadly, consistent across vertebrates [Bianco and Wilson, 2009; Aizawa et al., 2011; Freudenmacher et al., 2020].

The role of the habenula appears, broadly, to be one of behavioural flexibility: the ability to modulate and change behaviours in response to dynamic situations and stimu-

li [Mizumori and Baker, 2017]. It is heavily involved in behavioural responses to positive and negative reinforcement [Schultz, 1998], spatial memory acquisition and retrieval [Lecourtier et al., 2004], maintaining accurate circadian rhythms [Baño-Otálora et al., 2017], sexual and maternal behaviour [Modianos et al., 1974; Malacarne and Vellano, 1982; Matthews-Felton et al., 1995], and even play [van Kerkhof et al., 2013]. These roles are achieved largely through modulating monoamine signalling based on forebrain activity. This has perhaps best been studied in the context of dopamine, but the habenula also modulates the activity of serotonin, norepinephrine, and acetylcholine.

In all vertebrates, the habenula is divided into two subregions; in mammals, birds, and reptiles these are the medial and lateral subregions. The lateral habenula is the more extensively studied of the two regions, primarily (but not exclusively) in mammals. Behaviourally, it is thought to be involved in the ability to generate and modify behavioural strategies to respond to both rewarding and aversive stimuli [Ullsperger and von Cramon, 2003; Matsumoto and Hikosaka, 2007; Boulos et al., 2017; Sosa et al., 2021]. The medial habenula is less well studied but appears to be involved in regulating behaviours such as impulsivity, attention, and fear response [Viswanath et al., 2014]. There also remain many functions, such as those the habenula has in maternal and female sexual behaviour, that have not been identified as more dependent on one subregion.

A striking feature of the habenula is its pronounced bilateral asymmetry across vertebrates. Although not present in every species, there is frequently a volumetric difference between hemispheres in habenular volume [Bianco and Wilson, 2009]. Where this bilateral asymmetry is present, whether the larger habenula is in the left or the right hemisphere varies depending on species, as does whether it is the medial or lateral habenula that is asymmetric. There is currently no consistent explanation as to the functional implications of this asymmetry [Bisazza et al., 1998; Guglielmotti and Cristino, 2006; Stancher et al., 2018], despite it being the most prominent and well-documented volumetric asymmetry in the vertebrate brain.

Among the vertebrate lineages, the habenula is most poorly studied in reptiles. Connectivity studies indicate that the connections formed by the lateral and medial habenulae in reptiles are consistent with those observed in other vertebrates, suggesting that habenula function in reptiles ought to be broadly similar to that observed in other animals [Distel and Ebbesson, 1981; Hoogland, 1982; Díaz and Puelles, 1992]. Like other vertebrates, vol-

umetric asymmetry of the habenula is present seemingly haphazardly across reptiles. In some species, the left medial habenula is larger than the right, apparently due to projections from the parietal eye that project only to the left habenula [Engbretson et al., 1981; Korf and Wagner, 1981]; this is currently the only kind of bilateral asymmetry reported in reptile habenulae. Why the projections from the parietal eye result in this kind of asymmetry in some species and not others remains unclear, and whether other forms of bilateral asymmetry, present in other vertebrate groups, are present in reptiles remains unknown.

Studies looking at variation in reptile brain anatomy have used the habenula as a “control” region, measuring it alongside regions that are hypothesized to vary according to a predictor of interest [Crews et al., 1996, 1997; Coomber et al., 1997; Kabelik et al., 2006, 2008; Hews et al., 2012]. This is likely due to the ease of measuring the habenula; its structure is easily discernible from most common nuclear stains. We initially used the left habenula as a control in a previous study examining patterns of sexual dimorphism in the preoptic area and ventromedial hypothalamus in response to the intensity of sexual selection [Hoops, 2016]. However, the study, which was conducted in dragon lizards (Agamidae, *Ctenophorus* spp.) [Hamilton et al., 2015], unexpectedly found that habenular volume varied with sexual selection. Only the left habenula was measured, however, and it was not subdivided into the medial and lateral subregions. To better understand habenular evolution, and to be able to draw conclusions regarding bilateral asymmetry, here we examine differences in habenular volume in response to the intensity of sexual selection, looking at both the left and right habenulae and both the medial and lateral subdivisions.

Methods

Animal Acquisition

Two hundred and twelve specimens were collected from the wild in Australia and transported to the Australian National University in Canberra, Australia. Sample sizes per species and sex (male/female) were: *Ctenophorus caudicinctus slateri* (8/10), *C. cristatus* (9/7), *C. fionni* (10/9), *C. fordi* (10/10), *C. gibba* (8/6), *C. isolepis gularis* (10/9), *C. modestus* (11/7), *C. nuchalis* (11/3), *C. ornatus* (8/8), *C. pictus* (13/10), *C. rufescens* (10/10), and *C. salinarium* (9/10). Lizards were maintained in outdoor enclosures with ad libitum access to food (wild insects and supplementary domestic crickets) and water. Specimens were euthanized and perfused according to standard protocol [Hoops, 2015]. Brains, excluding the olfactory bulbs, were extracted from the skulls and stored at 4°C until processing.

Table 1. Averaged phylogenetic linear models

Region	Hemisphere	Factor	Male			Female		
			Estimate	SE	RI	Estimate	SE	RI
Lateral habenula	Left	BV	0.641	0.256	0.72	0.937	0.285	
		SDC	-0.194	0.098	0.44			
		BSSD	-1.791	0.848	0.46	-2.566	0.778	
	Right	BV	0.578	0.268	0.63	0.905	0.250	1.00
		SDC	-0.152	0.917	0.20	-1.438	0.614	0.35
		BSSD	-1.487	0.118	0.16	-0.142	0.076	0.59
Medial habenula	Left	BV	0.858	0.213	1.00	1.050	0.109	1.00
		SDC	-0.094	0.091	0.13	-0.084	0.032	0.68
		BSSD	-0.842	0.791	0.14	-0.626	0.259	0.58
	Right	BV	0.844	0.214	1.00	1.064	0.106	1.00
		SDC	-0.109	0.090	0.16	-0.106	0.033	0.91
		BSSD	-0.817	0.800	0.13	-0.637	0.244	0.58

Factors in bold are directly correlated with the response variable. Factors in bold italics are inversely correlated with the response variable. RI values are absent when there is only one model in the set $\Delta\text{AICc} \leq 4$. BSSD, body size sexual dimorphism; BV, brain volume; RI, relative importance; SDC, sexual dichromatism; SE, standard error.

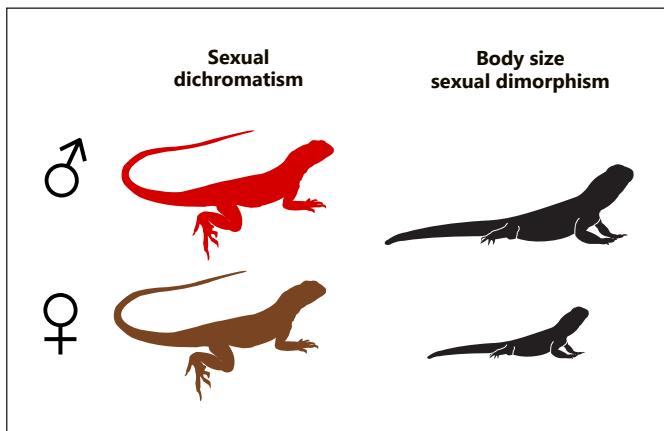


Fig. 1. The two forms of sexual dimorphism used in this study, which have been empirically linked to sexual selection, are differences between males and females in colour (sexual dichromatism) and body size (body size sexual dimorphism).

Habenula Measurement

Brains were sectioned coronally using a vibratome at 70 μm and then stained using the nuclear stain SYBR-green (Life Technologies Australia, Melbourne, VIC, Australia). Images of each section were captured using the image stitching function of the CellSens microscopy platform and a BX63 microscope (Olympus). The areas of both left and right lateral and medial habenulae were measured using the Count & Measure module in CellSens. Volumes were calculated by multiplying the areas by slice thickness and then summing across slices [Kabelik et al., 2006]. We

identified the medial and lateral habenulae by referring to the atlas of the swift dragon (*Ctenophorus modestus*) brain [Hoops et al., 2018], as well as atlases for other lizard species [Butler and Northcutt, 1973; Cruce, 1974; Medina et al., 1992; ten Donkelaar, 1998]. Measurements were conducted, blind to sex, by a single researcher (D.H.).

Sexual Dimorphism

The degree of sexual dimorphism in a species, i.e., a phenotypic difference between sexes, is widely associated with the strength of sexual selection [Andersson, 1994; Stuart-Fox and Ord, 2004; Fairbairn et al., 2008; Chen et al., 2012]. In lizards, sexual dimorphism in body size and in colour (sexual dichromatism; Fig. 1) have been empirically linked to the strength of pre-copulatory sexual selection [Cox et al., 2003; Sullivan and Kwiatkowski, 2007; Fairbairn et al., 2008; Cox and Calsbeek, 2009]. *Ctenophorus* dragons are among the most variable between species in both these measures [Chen et al., 2012] and are, therefore, an ideal group in which to test the impact of sexual selection on brain evolution. Values for sexual dichromatism and body size sexual dimorphism for the species included here are available from our earlier study [Hoops et al., 2017a]. For each measure, the relative difference between sexes is calculated using the formula $d = 2(M - F)/(M + F)$ [Hoops et al., 2017a]. If $d > 0$, males are more colourful/larger and if $d < 0$, females are more colourful/larger.

Analysis

The phylogeny used in this study is pruned from a published phylogeny of agamids [Chen et al., 2012]. Values for whole brain volume, included in our models as a size correction factor, are available from our earlier study [Hoops et al., 2017b]. These values were determined using MRI prior to sectioning and staining each

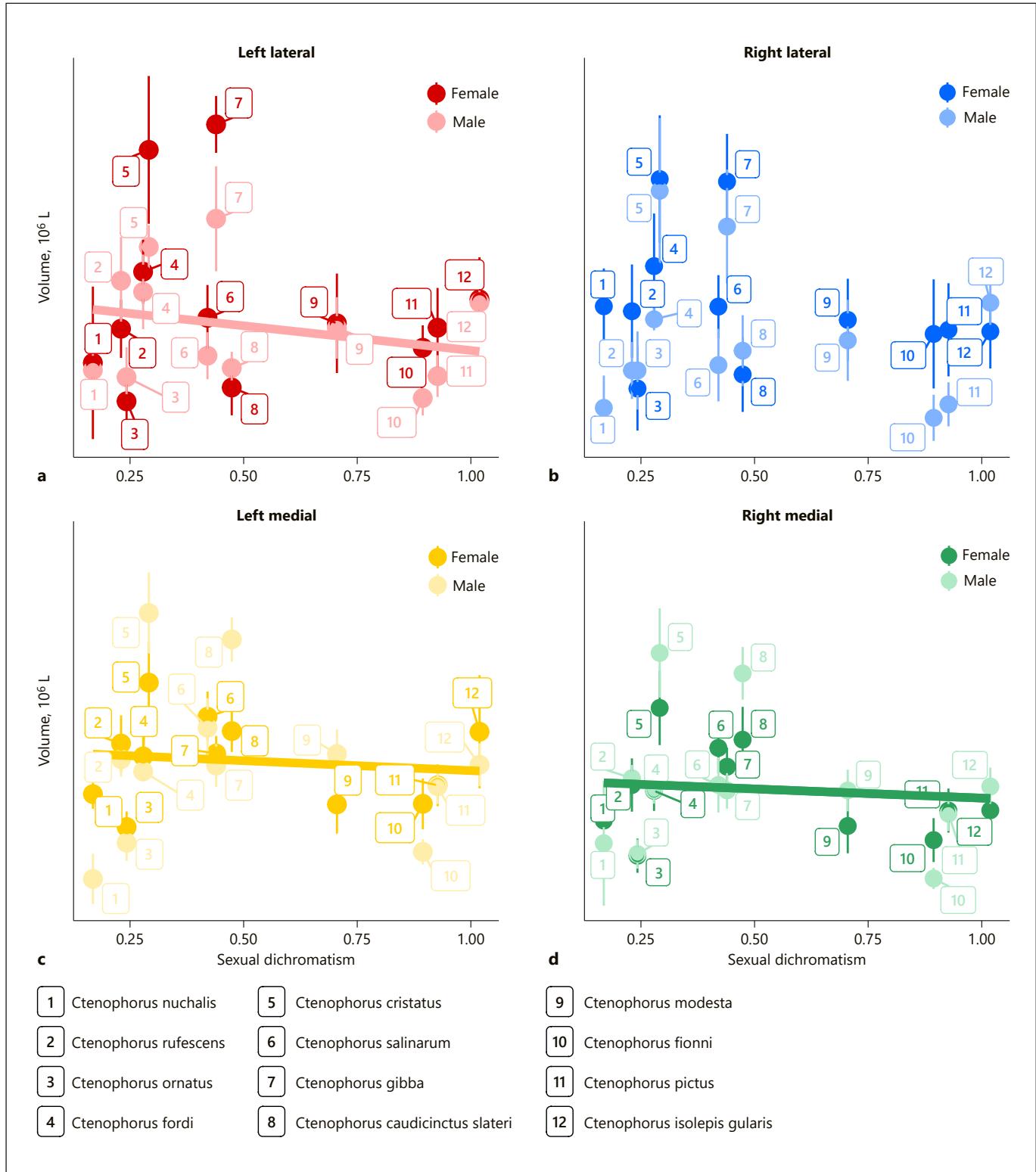


Fig. 2. The mean volumes of the medial and lateral habenulae with respect to increasing levels of sexual dichromatism, an index of sexual selection, in *Ctenophorus* dragon lizards. The left lateral habenula is negatively correlated with sexual dichromatism in males

(a), and both medial habenulae are negatively correlated with sexual dichromatism in females (c, d). Points show size-independent species means \pm standard error. Regression lines are shown for relationships that have a nonzero slope.

brain. Volumes were calculated automatically by aligning each brain to an average model of a lizard brain and summing the number of voxels (3D pixels) in the aligned brain-space [Janke and Ullmann, 2015; Hoops et al., 2017b].

For each brain region of interest, left lateral habenula, right lateral habenula, left medial habenula, and right medial habenula, we generated a phylogenetically corrected linear model using the package *phylolm* [Ho and Ane, 2014] in the computing environment R [R Development Core Team, 2014]. We used Brownian Motion as the evolutionary model as we previously found that it is the most likely model to represent brain evolution in *Ctenophorus* [Hoops et al., 2017b]. Each model included as independent factors sexual dichromatism, body size sexual dimorphism, and brain volume.

From each model we used the R package *MuMIn* [Barton, 2014] to generate models of all possible combinations of our independent factors and calculate the average of all models in which the corrected Akaike information criterion [Hurvich and Tsai, 1989] was within 4 units of the model with the lowest AICc value [Symonds and Moussalli, 2010; Grueber et al., 2011]. In this model average, predictors were considered significant if their 95% confidence intervals (CI) did not include 0. We also report relative importance, a percentage ranking of each factor in a model average that takes into account both the number of models in the average that include the factor, and the quality of those models based on their AICc [Symonds and Moussalli, 2010].

All data and code used here have been deposited at the Open Science Framework (OSF, doi: 10.17605/OSF.IO/8UZNK).

Results

In the lateral habenula, both sexual dichromatism and body size sexual dimorphism predicted volume to different degrees in both sexes (Table 1). In males, the left lateral habenula decreased in volume with increases in both types of sexual dimorphism (Fig. 2a, 3a). However, the strength of sexual dimorphism and the volume of the right lateral habenula were not significantly related, indicating a bilaterally asymmetric effect (Fig. 2b, 3b). In females, only body size sexual dimorphism was an important predictor of lateral habenula volume (Fig. 2a, b, 3a, b). As body size sexual dimorphism increased, lateral habenula volume decreased, and this effect was consistent across both the left and right lateral habenulae.

The medial habenula showed a relationship with indices of sexual selection only in females (Table 1). Neither form of sexual dimorphism was important for predicting medial habenula volume in males. In females, both body size sexual dimorphism and sexual dichromatism were negatively correlated with medial habenula volume, and this was consistent for both the left and right medial habenulae (Fig. 2c, d, 3c, d).

Discussion

We originally conceived of measuring the habenula because we hypothesized it would not be affected by the intensity of sexual selection and would, therefore, be an appropriate “control” region [Hoops, 2016]. However, we found that there was a relationship between left habenula volume and sexual dichromatism, an index of sexual selection. Based on that finding, we designed this study, separating the habenula into its medial and lateral subregions and measuring them bilaterally. We found that the volumes of both subregions correlated with sexual selection, but differently between sexes. In females, both lateral habenulae and both medial habenulae were smaller in species that were more sexually dimorphic. The lateral habenula correlated with body size sexual dimorphism, while the medial habenula correlated with both indices of sexual selection. In contrast, in males only the left lateral habenula showed a significant correlation with sexual dimorphism; this region was smaller in species that were more dimorphic.

These results are in striking contrast to both the predictions we made previously [Hoops, 2016] and those of other studies, which hypothesized that the habenula is not involved in reproductive (and related) behaviour and is, therefore, an appropriate control region in studies of the neural control of these behaviours [Sakata et al., 2002; Kabelik et al., 2006]. Where a rationale is provided for the use of the habenula as a control, it is that the habenula is not known to express steroid hormone receptors [Coomber et al., 1997; Kabelik et al., 2008]. Indeed, studies of steroid receptor expression and steroid hormone concentration published up to recently validate this rationale: in lizards the habenula is depicted as being devoid of such expression [Morrell et al., 1979; Halpern et al., 1982; Young et al., 1994; Rhen and Crews, 2001; Tang et al., 2001; Rosen et al., 2002]. Furthermore, although functional studies in other vertebrate groups have linked the habenula with reproductive behaviours [Ogawa and Parhar, 2021], no such studies exist for reptiles.

Two more recent studies have demonstrated that there is in fact abundant expression of androgen receptors in the habenula in at least one genus of lizard. The first study found androgen receptor expression exclusively in fibres passing through the habenulae of the Eastern fence lizard (*Sceloporus undulatus*) [Moga et al., 2000]. Another study found equal or greater numbers of androgen receptor-expressing cells in the habenula compared to the medial preoptic nucleus and ventromedial hypothalamus, two regions traditionally associated with reproductive behav-

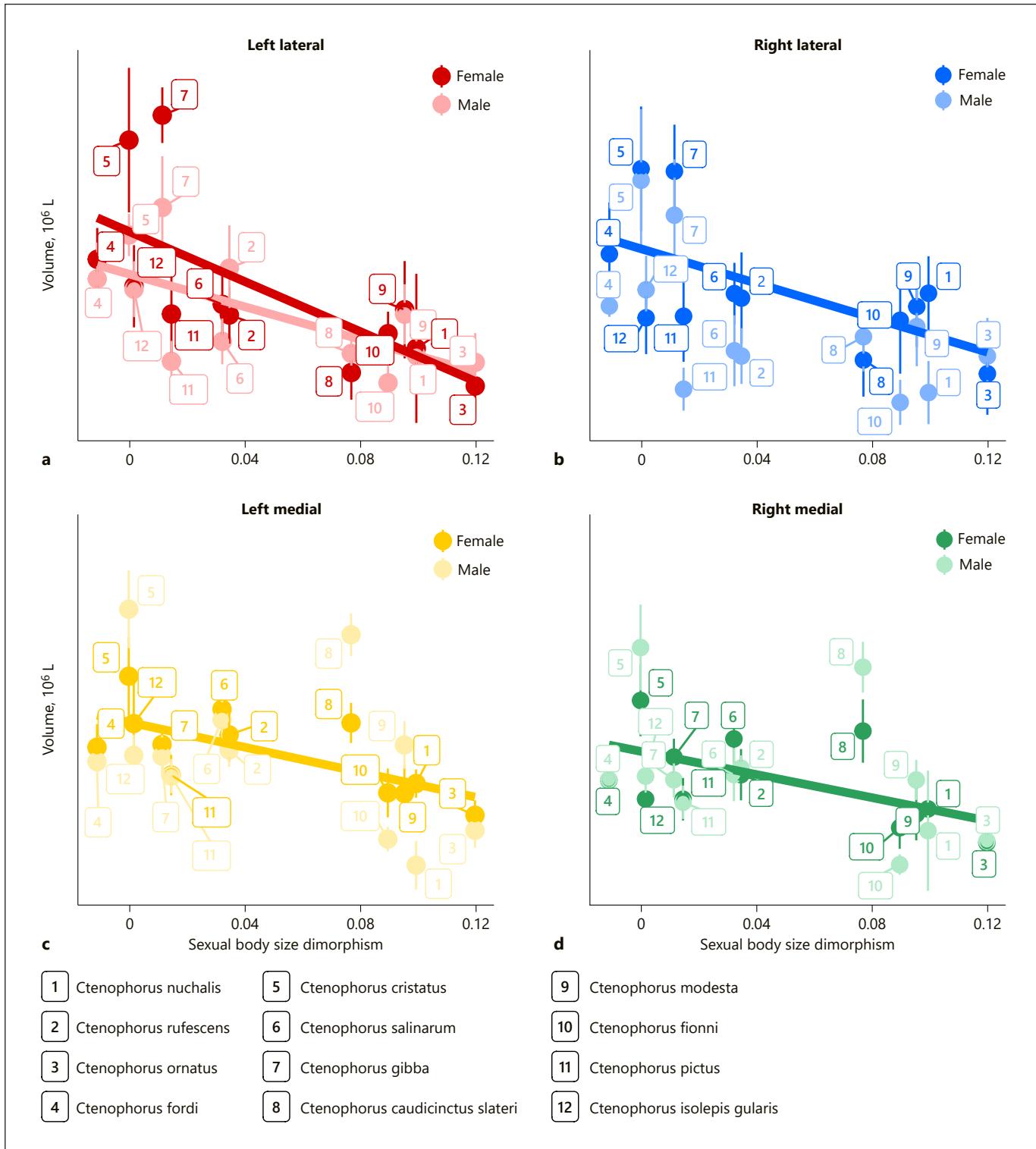


Fig. 3. The mean volumes of the medial and lateral habenulae with respect to increasing levels of body size sexual dimorphism, an index of sexual selection, in *Ctenophorus* dragon lizards. The left lateral habenula is negatively correlated with body size sexual dimorphism in both sexes (**a**), and the right lateral habenula and

both medial habenulae are negatively correlated with body size sexual dimorphism in females (**b-d**). Points show size-independent species means \pm standard error. Regression lines are shown for relationships that have a nonzero slope.

iour [Hews et al., 2012]. These findings directly contradict not only the earlier work but also each other, as the former detected expression only in fibres, while the latter found expression in cell bodies. This probably reflects the dramatic improvements in antibody binding efficiency and in immunohistochemistry methods that have been developed in the interim. It would be worth recharacterizing steroid hormone receptor expression in the habenula in a broad range of lizard species considering these findings.

The second study mentioned above [Hews et al., 2012] also compared the number of androgen receptor-expressing cells in the habenula of the Eastern fence lizard, which is sexually dichromatic, to that of the sexually monochromatic striped plateau lizard (*Sceloporus virgatus*). They found that androgen receptor-expressing cells were more abundant in the monochromatic species, a result which parallels our findings here that habenula volume tends to decrease with increasing sexual dimorphism. The habenula responds to both positive and negative reward, helping control and modify appropriate behavioural responses to diverse stimuli. Increased selection on sexual motivation may effectively require a dampening of motivation and drive to respond to other, diverse stimuli. Under this hypothesis, smaller habenulae and fewer habenular neurons that detect reproductive hormones are outcomes that might be predicted from selection that acts to specifically increase motivation to reproduce.

Interestingly, we found a bilaterally asymmetrical pattern of selection in male dragons. The left lateral habenula was smaller in more dimorphic *Ctenophorus* species, while the right lateral habenula showed no relationship to sexual dimorphism. The lateral habenula has recently been identified as important for the positive reinforcement of aggressive behaviour in inter-male aggression, at least in mammals [Golden et al., 2016; Flanigan et al., 2017]. Vertebrates, including lizards, are more likely to respond to a conspecific aggressively when viewed through the left eye, a phenomenon known as left-eye preference [Bisazza et al., 1998; Rogers and Andrew, 2002]. In lizards, due to the complete contralateral decussation of optic fibres in the optic chiasm [Butler and Northcutt, 1971], this indicates a preference for aggressive behaviour from the right hemisphere, including the right lateral habenula [Hews and Worthington, 2001]. Aggressive behaviour towards conspecifics is frequently associated with reproduction and mate competition [Cooper et al., 1987; Andersson, 1994; Ariyomo and Watt, 2012; McEvoy et al., 2013], which is a mechanism

of sexual selection [Baird et al., 1997; Sinervo et al., 2000; Irschick et al., 2007]. Our results suggest, therefore, that sexual selection has acted to increase the relative volume of the right lateral habenula over the left, which is consistent with the role of sexual selection in promoting male-male aggression.

We found more extensive correlations between habenula volume and indices of sexual selection in females. Both habenula subregions, in both hemispheres, were negatively correlated with at least one form of sexual dimorphism. Across vertebrates, sexual selection, and sexual conflict in particular, have well-documented influences on female brain structure in a range of species [Cummings, 2012, 2015, 2018]. However, to our knowledge, this is the first evidence that sexual selection may alter brain structure in female lizards [Hoops et al., 2017a]. In some mammals, the habenula is important for initiating female soliciting behaviours [Modianos et al., 1974, 1975; Matthews-Felton et al., 1995], and in this context it may be important in species with a relatively low intensity of sexual selection or sexual conflict, where we see reductions in brain regions involved in male sexual behaviour [Hoops et al., 2017a]. We note that the slope of the regression lines in the female medial habenulae is shallow, and, therefore, this does not appear to be a strong effect. Nonetheless, this effect, and the potential for sexual selection to alter brain structure in female lizards, deserves additional attention.

In both males and females, it is difficult to draw any strong conclusions regarding the specific behaviours under selection based on our results. Unfortunately, the data we rely on regarding the function of the habenula are necessarily drawn from other vertebrates, principally mammalian model systems. The habenula is measured frequently as a “control” region in lizard neuroscience [Crews et al., 1996, 1997; Coomber et al., 1997; Kabelik et al., 2006, 2008; Hews et al., 2012; Hoops, 2016]; however, studies designed to probe the function of this complex and phylogenetically conserved structure are lacking. As the habenula has received renewed interest in basic and biomedical neuroscience research in the last 20 years [Geisler and Trimble, 2008; Viswanath et al., 2014], we suggest that this region should be a prime target for future studies in evolutionary neuroscience. Its phylogenetically conserved connectivity and structure, its prominent bilateral asymmetry, and the correlation it shows with the intensity of sexual selection indicate that significant advances in understanding this region have the potential to come from further studies in an evolutionary neuroscience context.

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Statement of Ethics

All data used in this study were gathered from experiments that were approved by the Animal Experimental Ethics Committee of the Australian National University (protocol No. A201149).

Conflict of Interest Statement

Like all academics, the authors of this study are conflicted inasmuch as researcher quality, including for funding and employment opportunities, is gauged based on publication output. Other than that, all authors declare no conflicts of interest.

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Author Contributions

This study was conceived and designed by D.H., M.J.W., and S.J.K. D.H. collected and analysed the data and drafted the manuscript. All authors contributed to interpreting the results and editing the manuscript.

Data Availability Statement

The data and code that support the findings of this study are available at the Open Science Framework (OSF, doi: 10.17605/OSF.IO/8UZNK).

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