Experimental evolution reveals divergence in female genital teeth morphology in response to sexual conflict intensity in a moth

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Abstract

The rapid evolutionary divergence of male genital structures under sexual selection is well documented. However, variation in female genital traits and the potential for sexual conflict to drive the coevolution between male and female traits has only recently received attention. In many lepidopterans females possess genital teeth (collectively, signa). Comparative studies suggest these teeth, involved in the deflation of spermatophores, may have coevolved with male spermatophore thickness via sexually antagonistic coevolution in a contest over the rate of deflation of spermatophores within the reproductive tract. We tested the hypothesis that sexual conflict should generate coevolution between genital teeth and spermatophore morphology by examining these traits under experimental manipulation of sexual conflict intensity. Using micro-CT scanning, we examined spermatophore and teeth morphology in populations of the Indian moth, *Plodia interpunctella*, which had been evolving for 110 generations under different adult sex-ratio biases. We found divergence in female signa morphology in response to sexual conflict: females from female-biased populations (reduced sexual conflict) developed wider signa. However, we found no evidence of coevolution between signa traits and spermatophore thickness as reported from comparative studies.

Keywords: experimental evolution; signa; coevolution; spermatophore.

Introduction

There is tremendous diversity in male genital morphology among internally fertilising taxa, even amongst highly related species (Eberhard, 1985, Hosken et al., 2004, Simmons, 2014). There is now widespread empirical support for sexual selection as a primary force in generating this variation (Hosken et al., 2004, Hosken et al., 2018). Genital evolution via sexual selection may operate through several mutually non-exclusive mechanisms. First, cryptic female choice, whereby females mate multiply and bias paternity toward males best able to stimulate them during copulation (Eberhard, 1985). Second, male-male competition (Simmons, 2001), in which male structures serve to avoid sperm competition by removing rival sperm (Waage, 1979) or aid in the transfer of manipulative seminal fluid (Hotzy et al., 2012). However, the reproductive interests of both males and females are unlikely to be shared. While males benefit from manipulating female reproduction to increase their reproductive success, this may be costly for females, generating sexually antagonistic coevolution (SAC)(Arnqvist et al., 2005).

Surprisingly, female genital morphology has long assumed to be relatively invariant and of lesser functional importance than male genitalia. As a result, it has been largely overlooked (Ah-King et al., 2014). However, recent interest in SAC has revealed comparable diversity in female genitalia in a variety of taxa (Brennan et al., 2007, Puniamoorthy et al., 2010, Orbach et al., 2018), and their coevolution with male genitalia. Evidence for SAC of genital morphology comes from phylogenetic and intraspecific comparisons (Brennan et al., 2007, Rönn et al., 2007, Yassin et al., 2013, Dougherty et al., 2017). Experimental evolution has rarely been used (but, see Simmons et al., 2011, Hopwood et al., 2016), despite being a powerful tool to dissect coevolutionary relationships.

The Lepidoptera are an ideal taxon to explore sexually antagonistic genital evolution. Female receptivity to mating is determined partly by stretch receptors in the reproductive tract (Sugawara, 1979), which identify the presence and volume of a spermatophore. Males can thus delay female remating by ensuring the female tract is full. Males do this by transferring primarily non-nucleate 'cheap filler' sperm (Cook et al., 1999), and by encasing them in a thick spermatophore that persists in the female's reproductive tract (bursa, Video S1), thereby reducing female receptivity to further mating. Furthermore, the spermatophores of many species bear a chitinous process (CSP), the function of which is unclear. The CSP does not degrade, potentially reducing female receptivity or preventing rival males from correctly aligning their spermatophores (Drummond, 1984).

In many lepidopterans the females possess a number of sclerotized teeth protruding from the bursal wall, 'signa'. Phylogenetic analyses among species suggest that signa may have evolved as a counter-adaptation to male manipulation, by deflating the spermatophore and reducing the stimulus that prevents females from remating (Sánchez et al., 2014). There may be costs to male-imposed monandry: females of many species benefit from mating multiply (Arnqvist et al., 2000) and failures of spermatophore alignment are common in the Lepidoptera, resulting in functionally infertile matings (Drummond, 1984, García-González, 2004). Histological studies provide direct evidence that in several lepidopteran species the signa pierce and rupture the spermatophore (Galicia et al., 2008). Phylogenetic analyses demonstrate that spermatophore walls are thicker in polyandrous species with signa, compared to monandrous species without signa. Conversely, monandrous species with signa have thicker spermatophore walls than polyandrous species with signa (Sánchez et al., 2014). However, to date no studies have used experimental evolution to examine the role of sexual conflict in female signa evolution within species.

We analysed genital evolution in replicate populations of the Indian meal moth, *Plodia interpunctella*, which have evolved for over 110 generations under differing adult sex-ratio biases (and therefore levels of sexual conflict). These populations have previously been assayed for 6 traits (Ingleby et al., 2010, McNamara et al., 2013). In male-biased populations, females mate more frequently, and subsequently males transfer more sperm in each ejaculate (Ingleby et al., 2010), which reduces female post-mating receptivity (Lewis et al., 2013).

In *P. interpunctella*, males clearly benefit from preventing female remating, especially as the proportion of offspring sired by the second male to mate is high (Brower, 1975). While there are no clear benefits to polyandry for females in terms of increased fecundity or fertility in this species (as is typical for many lepidopteran females in species with low remating rates (Torres-Vila et al., 2004)), females may be selected to remate to promote sperm competition (Keller et al., 1995). If sperm competitiveness is heritable, then females may gain indirect benefits from multiple mating via good- or sexy-sperm processes (Curtsinger, 1991, Keller et al., 1995, Yasui, 1997, McNamara et al., 2014). Indeed, male *P. interpunctella* exhibit adaptions for sperm competition (Cook et al., 1995, Ingleby et al., 2010). Moreover, the risk of infertile mating can be high in this species (Ryazanova, 2014), so a proportion of females will benefit from remating to acquire sufficient sperm for fertilisation.

Concordant with theories of signa-spermatophore co-evolution (Sánchez et al., 2014), we predicted that female genital teeth morphology should respond to experimental increases in the intensity of sexual conflict under male-biased sex ratios. In response, we also predicted that males from these male-biased populations should evolve spermatophores with thicker walls.

Methods

Moths were reared at the University of Exeter, Cornwall UK, on a diet of bran, yeast, honey and glycerol, and maintained at 28°C with a 16:8 h light:dark cycle (Ingleby et al., 2010). Two adult sex-ratio treatments, each with two replicates, were established from a stock population. The female-biased (1:3 male:female) and male-biased (3:1 males:female) treatments were maintained at 120 adults at each generation for approximately 110 generations (see Ingleby et al., 2010). Sex-ratio bias was only enforced at the adult mating stage. Larvae were reared under identical densities on ad libitum food.

Seven pairs of newly-emerged virgin males and females from each replicate were mated. After copulation, individuals were frozen, wings removed, and fixed in Opresol. The specimens were then shipped to the University of Western Australia.

We used X-Ray micro-CT scanning to examine male and female reproductive morphology (Mattei et al., 2015, Dougherty et al., 2017, Dougherty et al., 2017). Moths were imaged using a Zeiss VersaXRM ^{TM} Micro-CT scanner (Zeiss Corp., Oberkochen, Germany). Moth abdomens were rinsed three times in PBS for 10 minutes, stained for 24h in a 1% iodine solution (0.5g iodine powder in 50mL absolute ethanol), and dehydrated for 45 mins in increasing concentrations of ethanol (25, 50, 75 and 100% (twice)). Abdomens were scanned in 100% ethanol at 4× optical magnification, operating at 40 kV and 3 W. For each specimen, 1601 X-Ray projections were acquired through 360 degrees with a 15 s exposure per projection, resulting in a voxel size of 1.64 µm. Images were analysed in Avizo (Visualization Sciences Group, FEI Corp., OR).

For each specimen the signa and CSP were manually selected slice-by-slice, and then visualised in three dimensions (Video S1 & S2). We took three linear measurements for each female, using the 3D measure tool: tooth number, average tooth height, and teeth array width (Fig. 1). We took two spermatophore measurements: ampulla thickness and CSP volume. CSP volume was calculated by multiplying the number of voxels selected across all slices by the voxel size (Fig. 2). Spermatophore thickness was measured in two dimensions on a single slice approximately halfway down the ampulla (Video S2). The mean of four measurements taken at 90 degree intervals around the circumference of the ampulla was calculated. A camera binning of 2x was used to achieve a suitable signal to noise ratio, resulting in 1010 x 1010 pixels per image. No filter was used when collecting images. Secondary references were collected using the LE2 filter. Scan data was reconstructed using the Zeiss Reconstructor package (v10.6.2005, Zeiss). Prior to reconstruction, a standard centre shift and beam hardening correction was made, default recon filter was set to smooth (kernel size = 0.7) and no ring removal applied. All measurements were conducted by one person who was blind to the specimen's identity. Wing length was also measured (see McNamara et al., 2008).

Statistics

All analyses were conducted in R 3.0.1. Mixed-effects models (package "lme4" version_0.999999911–5) were used to account for the identities of population replicates. Population replicate was nested within sex ratio treatment and analysed as a random effect. We used standardised body size calculated separately for males and females (individual wing length – mean population wing length/standard deviation of population wing length). Some wings were damaged, thus there was a reduced sample size in some analyses (for both males and females, one individual from female- and three from male-biased populations). Dependent variables were optimally power transformed to maximize normality of residuals, and the exponents noted. Models were not reduced, except for non-significant interactions which were removed from final models (Engqvist, 2005). All fixed effects included in final models are indicated in Table 1.

Results

There was divergence in the width of the signa: females from male-biased populations developed narrow signa compared with females from the female-biased populations (Table 1 & 2). Although tooth number and height were not significantly affected by sex-ratio bias (Table 1 & 2), we note that tooth height was 20% greater in the male biased population. However, with only 2 replicate populations per treatment, the power to detect such a difference was compromised.

Spermatophore thickness and CSP size were not affected by sex-ratio bias. However, males transferred thicker spermatophores to larger females (estimate ± standard error = 1.25 ± 0.58; Table 1 and 2).

Discussion

We demonstrate clear evolutionary divergence in the shape of a female genital trait, the signa, via manipulation of sexual conflict intensity. Interestingly, there was no evidence of male coevolution of thicker spermatophores in response to changes in the signa, as has been implicated in phylogenetic comparisons among species of Lepidoptera (Sánchez et al., 2011, Sánchez et al., 2014).

Previous correlative studies have found that signa evolution is related to variation in female mating frequency, and the potential for sexual conflict (Galicia et al., 2008, Sánchez et al., 2011, Sánchez et al., 2014). Consistent with these findings, we were able to select for changes in signa morphology as a consequence of increased sexual conflict in *P. interpunctella*. Females from male-biased populations, in which sexual conflict intensity was increased, developed narrower signa. We also note that this structural shape change was associated with an increase in mean tooth height (although not significant, this may reflect low statistical power). This raises the possibility that in response to increased sexual conflict intensity, female signa are beginning to evolve longer teeth, with a narrower profile. We were, unfortunately, unable to quantify differences in the structural shape of the teeth, such as 'pointiness'. The teeth are extremely small, internalized structures that required the use of microCT imaging. While this technique was sufficient to obtain the gross measures of signa dimensions presented here, the images were not of sufficient quality for three-dimensional shape analysis. Refinement of microCT methodology for such analysis would be the next step in elucidating the functional value of these morphological changes. Analogues of genital teeth are found in other taxa, including the beetle, *Callosobruchus maculatus* (Cayetano et al., 2011, Dougherty et al., 2017). Unlike our study, however, manipulation of sexual selection intensity did not alter teeth morphology in seed beetles. The authors suggested that, in this case, it may be due to male (offensive) genital traits responding faster to selection than female (defensive) genital traits (Cayetano et al., 2011).

Interestingly, males transferred thicker spermatophores to heavier females, suggesting it is a plastic trait that males can adjust in response to a female's potential reproductive value, and/or sperm competition risk, given that heavier *P. interpunctella* females are more fecund and have a greater mating frequency (Gage, 1998). While male lepidopterans have been demonstrated to transfer more sperm to heavier females (Gage, 1998), it has never been shown that males plastically increase investment into spermatophore thickness and durability, per se.

Why is there no evidence of signa/spermatophore thickness coevolution in *P. interpunctella*? First, under SAC, the sex currently 'winning' the evolutionary contest may change through time (Kokko et al., 2014). Thus, it remains possible that a detectable male response is yet to evolve, or that females are currently enjoying an advantage. Furthermore, the potential costs for males of increasing their spermatophore thickness, compared to the relatively smaller investment of females into genital teeth, may also explain the patterns observed.

We also found that larger males produce larger non-gametic components of the spermatophore – the CSP. The CSP does not degrade in the female's bursa, potentially acting as a durable, mechanical means of reducing female receptivity or preventing rival males from aligning their spermatophores correctly for sperm transfer. The exact function of the CSP, however, remains unclear, as it did not respond to sexual conflict intensity, consistent with a previous phenotypic study in a moth from the same sub-family (McNamara et al., 2010).

As we found no clear evidence of SAC between signa and spermatophore, there exists the possibility that the observed divergence in signa morphology could be due to factors other than sexual conflict intensity. Because females in the FB populations have lower fecundity and fertility (S. Willis, unpublished data), in order to facilitate sperm transfer, there may have been reduced selection on females in these populations to rupture the spermatophore. We find this unlikely, however, because females do not increase their reproductive output by receiving more sperm in this species (Cook, 1999). Thus, there is no evidence that females experience sperm limitation. Further, comparative studies suggest that the role of sexual conflict in signa evolution is widespread (Sánchez et al., 2011, Sánchez et al., 2014).

In conclusion, female genital teeth are present in multiple invertebrate taxa. We demonstrate for the first time that these female genital traits can evolve in response to sexual conflict. However, in contrast to comparative studies in the same taxon, we were unable to find evidence of coevolutionary relationships between female tooth morphology and male spermatophore traits. We suggest, however, that a larger study with a great number of population replicates and samples should be conducted to more closely examine the divergence in signa morphology, and to further examine the potential for coevolution between signa and spermatophore morphology in this species.

Supporting Information

Video S1: Female bursa and spermatophore (in situ) and *Video S2: Male spermatophore* are located at https://figshare.com/s/f698b264c1972276d04e.

Data Accessibility

Data will be archived in Dryad, upon manuscript acceptance.

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Figure legend

Figure 1. Female signa, showing wide and short teeth (left) and narrow and tall teeth (right). The tooth height and teeth array width measurements are shown.

Figure 2. The spermatophore, with labels identifiying (a) the chitinous spermatophore process (b) the aperture through which sperm exit the spermatophore (c) the sperm-containing ampulla, which has been virtually sectioned at the approximate position at which spermatophore thickness was measured.

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Table 1. Model summary and untransformed means ± standard errors for genital and spermatophore traits in response to sex-ratio treatment. The exponent to which the dependent variable was transformed is noted. Effect sizes and their 95% confidence intervals for every variable in each model are also noted. Confidence interval ranges that do not overlap 0, and significant P values are highlighted in bold.

	Sex-ratio treatment				
	Female-biased	Male-biased	Effect size & 95% CI	X^2	P
Teeth number[a]					
Sex ratio bias	6.5 ± 0.34	5.5 ± 0.34	-20.28 (-45.08, 4.52)	2.44	0.11
Female size			5.32 (-7.58, 18.23)	0.62	0.43
Teeth array width[b] *(µm)*					
Sex ratio bias	257.39 ± 4.14	203.21 ± 8.01	**-3.42 (-6.16, -0.67)**	4.79	**0.029**
Female size			-0.28 (-0.98, 0.23)	0.98	0.32
Mean tooth height[c] *(µm)*					
Sex ratio bias	44.84 ± 2.73	55.43 ± 2.15	23.84 (-0.52, 48.16)	2.99	0.08
Female size			2.19 (-3.86, 8.95)	0.44	0.51

Spermatophore thickness[d] *(µm)*					
Sex ratio bias	6.28 ± 0.26	7.10 ± 0.49	0.39 (-1.59, 2.30)	0.14	0.70
Female size			**1.25 (0.18, 2.33)**	4.70	**0.03**
Male size			-0.29 (-1.33, 0.75)	0.26	0.61
Process size[e] *(µm^3)*					
Sex ratio bias	142.13 ± 3.67	153.10 ± 2.66	0.975 (-1.31, 3.26) x 10^{14}	0.58	0.45
Female size			2.06 (-3.21, 6.76) x 10^{13}	0.64	0.42
Male size			**1.17 (0.726, 1.62) x 10^{14}**	25.82	**<0.001**

Transformation exponents: [a]*2.28;* [b]*0.6;* [c]*1.12;* [d]*1.2;* [e] *6.68*

Table 2. Means ± standard errors for individual sex-ratio population replicates

	Sex-ratio population replicate			
	FB1	FB2	MB1	MB2
Teeth number	6.43 ± 0.48	6.50 ± 0.62	5.40 ± 0.51	5.67 ± 0.71
Signa width (µm)	256.08 ± 7.02	256.75 ± 5.40	231.74 ± 7.61	182.96 ± 6.15
Mean tooth height (µm)	37.35 ± 1.67	52.67 ± 3.88	60.01 ± 2.67	56.42 ± 3.14
Spermatophore thickness (µm)	6.03 ± 0.48	6.76 ± 0.24	5.62 ± 0.72	7.55 ± 0.76

Process size (μm^3)	150.69 ± 3.64	133.95 ± 5.80	144.91 ± 2.70	155.24 ± 5.50

Figure 1.

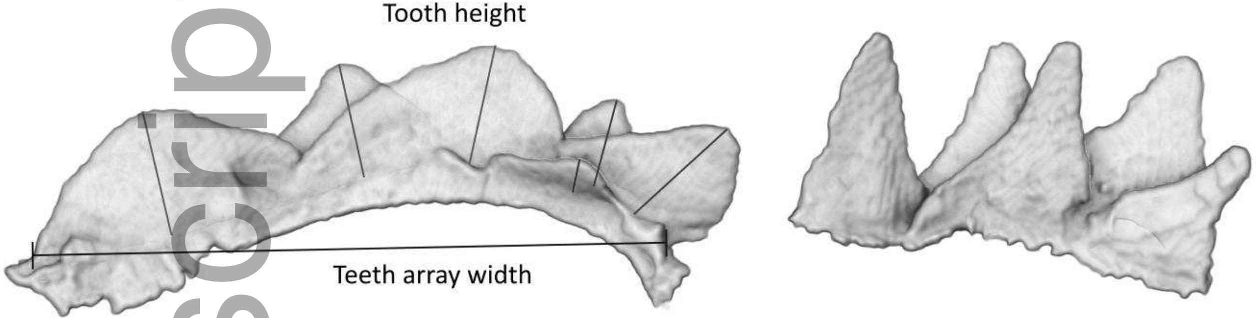
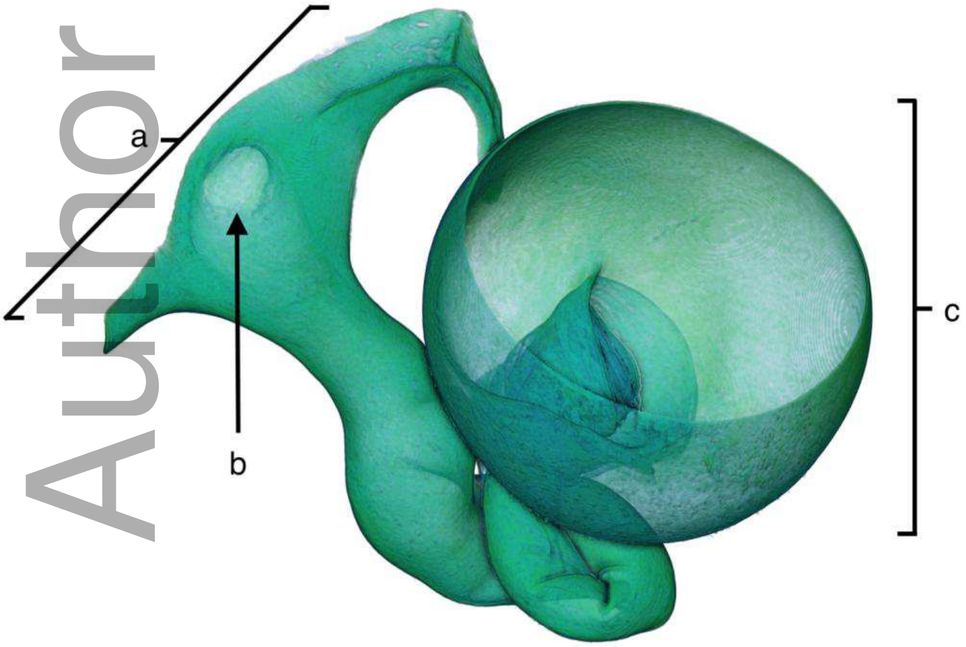
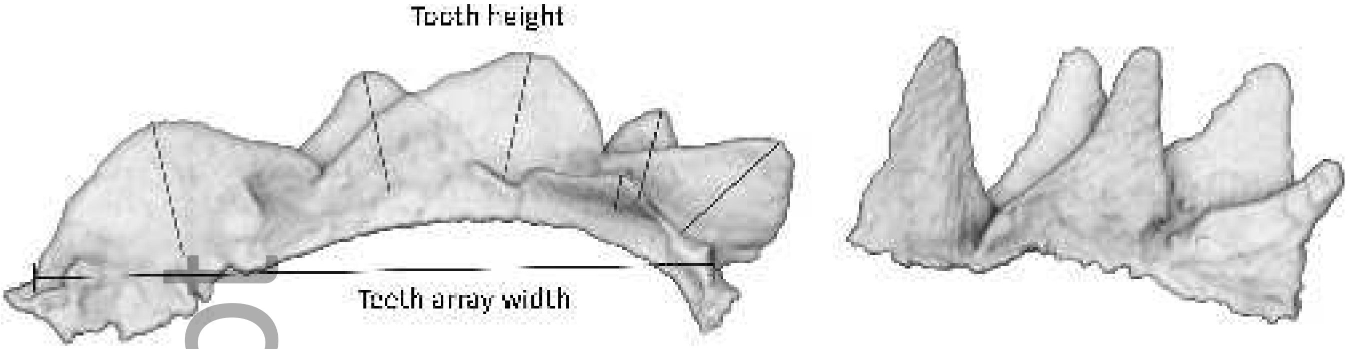


Figure 2.





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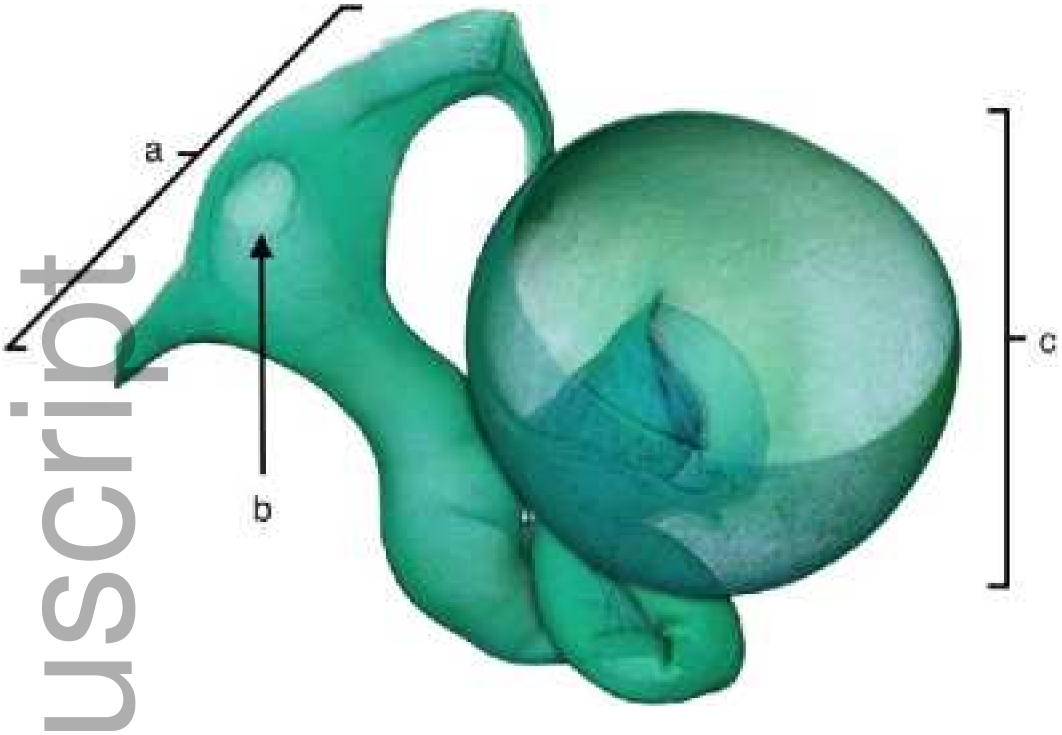

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