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Diurnal moths have larger hearing organs: Evidence from comparative 3D morphometric study on geometrid moths

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Tympanal organs, crucial for anti-bat defence in moths and key for taxonomy, are often overlooked due to their fragility during dissection. Using micro-CT, we analyzed the tympanal organs of 19 geometrid species, comparing diurnal and nocturnal species to understand how predators, like bats and diurnal birds or lizards, influence tympanal morphology and its allometric relationship with body size. We hypothesized that diurnal moths, with reduced anti-bat function, would have smaller tympanal organs, irrespective of body size.

Allometry was tested using phylogenetic linear regression and tympanal volume was compared across diurnal and nocturnal moths relative to the abdominal volume. We used 3D geometric morphometry, followed by comparative analysis of the shape and size of ansa, a unique “mechanical” for geometrids.

Contrary to our hypothesis, diurnal moths had significantly larger tympanal organs, with no allometric relationship with body size. Activity patterns had no significant effect on ansa shape and size, but nocturnal species exhibited convergence, suggesting potential auditory functions.

This study shows how daily activity patterns and predator-prey interactions shape sensory adaptations, with larger tympanal organs of diurnal species potentially reflecting adaptations to detect lower “non-bat” frequency. It also highlights non-invasive imaging techniques for studying delicate anatomical features in museum specimens.

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Abstract:

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Keywords: predation pressure, hearing organs, Geometridae moths, bats, micro-CT

Introduction:

The animal world is either diurnal, nocturnal, or crepuscular; as the adaptations to function in one temporal activity pattern reduce the efficiency of functioning and survival in another [1]. Nocturnality is presumed to be associated with adaptations to function in low-light and low-temperature conditions. However, in predominantly nocturnal taxa, like geckoes and moths, some clades have shifted to diurnality and have distinct adaptations to diurnal conditions like camouflage [2], photopic vision [3,4] aposematic wing colouration [5]and hearing sensitivity to lower 'non-bat' frequency [6]. The factors that cause a shift in the individual clades could largely be predators, climate, and competition [7], due to changes in information transmission in the sensory environment [8].

Predators are an important selective force in all terrestrial food webs. Prey species may invest proportionally more in defence, avoid interactions with their predators or follow alternative strategies for survival. For moths, the predation pressure from their most formidable predator, bats, vary in a predictable manner among nocturnal and diurnal species, influencing their daily activity pattern and hearing sensitivity[9]. This is reflected in the positive correlation between nocturnal flight activity and hearing sensitivity to ultrasound frequencies [10].

Diurnal moths show marked degeneration of the ultrasonic hearing, and some of them are reported to be 'bat-deaf' [6]. There are reports of predator released moths with no ears, e.g. the Polynesian Pyralid *Lathroteles obscura* [11] ; and [12] described extremely poor sensitivity in female wingless geometrids. Hearing sensitivity is often closely linked to body size, with larger animals generally having larger hearing organs and lower frequency hearing, as observed in lizards, frogs, mammals, and birds [13–15]. It is plausible that similar patterns might be observed in moths, where larger species may possess more developed hearing organs but exhibit lower sensitivity to ultrasound frequencies. In fact, studies suggest that larger moth species, such as those in the Noctuidae family, tend to have larger tympanal organs, but their sensitivity to higher frequency bat calls may be reduced compared to smaller moth species [16]. However, the evolution of hearing sensitivity in moths is not solely dictated by size. Other factors, including ecological niche, temporal and geographical isolation from bats, and the presence of other predators, could also influence hearing organ development and functionality.

Hearing organs in insects have diverse functions, such as intraspecific communication, parasitic host localization, and predator avoidance [17]. One specialized form of these organs, the tympanal organs, typically consists of a thin membrane or tympanum backed by an air-filled sac, and has evolved independently in at least seven different insect orders [18]. In most tympanate insects, the highest sensitivity is between 30-60KHz, i.e within the bat calling frequencies [19,20]. This evolutionary pressure is particularly evident in nocturnal moths, where the multiple origins of hearing organs are thought to be a defense mechanism against insectivorous bats[19–22]. Most moths including Noctuoidea, Geometroidea and Pyraloidea possess ultrasound sensitive ears [23]. As such, we would expect moths that are isolated from bats geographically or temporally to exhibit lower sensitivity to ultrasound.

We chose geometrid moths as our model group due to well-documented diel activity patterns [24] and their use in testing hearing sensitivity in diurnal moths [25]. While adult geometroid moths are primarily nocturnal, several unrelated lineages across families Uraniidae, Sematuridae, and Geometridae, exhibit diurnal activity[26–28]. This transition has occurred independently in Lepidoptera multiple times [3,27,29]. The strictly diurnal geometrid moth *Archiearis parthenias*, which is temporally isolated from bats, is sensitive to around 12KHz and practically deaf beyond 25 KHz [25], comparable to sympatric noctuid moths [12,30]. The tympanal organ is an important taxonomic character for geometrids, with ansa being a prominent unique character of the family [31,32] (Fig.1c, and Supplementary Video 1). The ansa, thought to protect the tympanum from mechanical damage caused by pressure from surrounding organs, particularly the oesophagus and flight muscles, has a wide base that strengthens the inner wall of the cavus, helping maintain tension across the tympanum [31]. The variation in ansa shape and size at the species and higher taxonomic level suggests it may serve functions beyond just a mechanical structure. However, research on diel activity in moths, function of hearing organ anatomy and predator pressures, remains limited.

In the above context, we wanted to ask, using a non-destructive 3D morphometry: 1) Does the size of the tympanal organ show an allometric relationship with body size? 2) Is there a difference in the size of the tympanal organs between diurnal and nocturnal moths? 3) How do the shape and size of the ansa vary between diurnal and nocturnal species? The overarching question guiding this study is whether these differences are linked to specialized hearing adaptations for detecting predators. We hypothesize that the structure of the tympanal organs is driven more by diel activity patterns than by body size, with tympanal organs being smaller in diurnal moths due to reduced anti-bat function. Also, we hypothesize that the ansa plays a crucial role in auditory function—not just for mechanical strength—meaning its size and shape should vary depending on the moth's activity pattern.

Material and Methods:

We selected 19 geometrid species (7 diurnal, 1 cathemeral and 11 nocturnal) from eight different subfamilies based on their relatedness in the phylogenetic tree [33,34] from different geographical locations. Listed in Table (S1).

Micro-CT Imaging and Image Processing:

All the specimens were imaged using a Nikon XT H 225 micro-CT scanner. Specimens were positioned in the sample holder following the method described by [35] to minimize noise caused by the insect pin.

All imaging was performed using a molybdenum target. The first four specimens were imaged with the following parameters: 74 kV beam energy, 94 µA beam current, 500 ms exposure time, 9,998 projections, and 8-frame averaging per projection. Each scan required 11 hours. The remaining 15 specimens were imaged using adjusted settings: 80 kV beam energy, 84 µA beam current, 1.4 s exposure time, 4,476 projections, and 4-frame averaging per projection, reducing the scan time to 7 hours. Further testing showed that lowering the frame averaging to 2 did not significantly affect image quality, allowing the scan time to be further reduced to 3.5 hours.

The voxel size of the reconstructed datasets ranged from 5 µm to 18 µm. To enhance image quality and minimize deformation caused by desiccation, staining and critical point drying should be considered for future studies, although these methods require fresh specimens. Reconstructions were generated from the projection images using Nikon CT Pro 3D Version XT 6.9.1.

Segmentation was performed using VGSTUDIO MAX 2024.3. A spherical region of interest (ROI) was used for segmentation, as this approach facilitates the extraction of the tympanal organs due to their roughly spherical shape. When possible, excess material was manually removed from the ROI. The voxel-based 3D datasets were then converted into high-quality mesh models by first generating an isosurface model, customized for each dataset to achieve optimal results. To facilitate further analysis and manage file sizes, the final mesh models were reduced to 300,000 vertices and were exported as WRL and PLY files

Morphometric measurement and analyses

The body length was used as a proxy for body size, as commonly used in morphometric studies[36,37]. We used ImageJ software [38] for this purpose. Further we measured abdomen width and abdomen length using Leica S9D stereo microscope, using Leica Flexacam C5 camera and Leica Microsystems 's Enersight software v. 2024. For volumetric measurement of the tympanal organ, we used landmark based method on the 3D reconstructed model in the software AGMT-3D [39]. We performed a phylogenetically informed linear regression to understand the relationship between body size and tympanal organ volume, while considering the relatedness of the species. To analyze the shape and size of the ansa, we used the *Geomorph* package in R [40], to do landmark-based 3D geometric morphometry on the 3D models of the tympanal organs. We digitized 10 homologous landmarks on the ansa of the tympanal organs of all the species (Fig. S2). Then we performed a General Procrustes Analysis, to remove the effects of scale on the landmarks, followed phylogenetic Principal Component analysis to compare and visualize the patterns the shape of ansa and compared the centroid size of the 3D model to compare the size of ansa, among the diurnal, cathemeral and nocturnal species. For the above analysis, we considered the relatedness of the species in our study based on the established phylogeny of geometrid moths [33,34]. We used Wilcoxon signed rank test, for pairwise comparisons in the analyses. We did not include the measurements for the cathemeral species *Dysphania percota* for any comparative analysis between diurnal and nocturnal taxa.

Results:

The phylogenetic linear regression revealed no significant allometric relationship of tympanal organ volume with body size in geometrid moths (Adjusted R-square = 0.06, $p > 0.05$) (Fig. 2a). This suggests that the size of the tympanal organ does not scale proportionally with the overall body size in this group. Notably, the diurnal moths were found to be significantly smaller in body size compared to their nocturnal counterparts (Fig. 2b). Despite the absence of a significant allometric relationship, we observed that diurnal moths possess significantly larger tympanal organs than nocturnal species ($p < 0.05$) (Fig. 3).

In terms of the ansa, no significant differences were found in the overall shape or size across taxa (Fig. 4). However, we did observe some evidence of convergence in the size of the ansa among the nocturnal species. The inter-quartile range for the ansa size in diurnal moths was 0.197, while for nocturnal moths it was 0.09 (Fig. 5). The micro-CT scans also reveal that the ansa is a hollow with a narrow tube-like structure in the middle (Fig.6 and Supplementary video 2). This indicates that while the ansa size does not vary significantly across all species, there may be a trend towards similar size characteristics within nocturnal geometrid moths, possibly reflecting a shared evolutionary adaptation related to hearing sensitivity.

Discussion:

In this study, we investigated how auditory structures in moths change as a function of body size, and the extent to which certain morphological traits predict hearing sensitivity. Our findings revealed several interesting patterns in the tympanal organ and ansa characteristics of geometrid moths. Despite no significant allometric relationship between tympanal organ volume and body size, we found that diurnal moths tend to have larger tympanal organs—despite being smaller overall—pointing to potential evolutionary adaptations that help them navigate different abiotic and biotic factors, such as avoiding diurnal predators like birds and lizards. In essence, our result means that the tympanal organ, originally assumed to function as an anti-bat hearing organ in nocturnal species, has adapted a new function in diurnal environment. The structure could still have a hearing function, potentially being adapted to different predator pressure on lower “non-bat” frequency and the larger size could serve more effectively this purpose.

Interestingly, the shape and size of the ansa do not differ significantly across taxa, though there is some evidence of convergence in the size of the ansa among nocturnal moth species. This suggests that the ansa may be playing a previously unexplored role in auditory function.

These findings highlight the complex evolutionary dynamics between predator-prey interactions and morphological adaptations in moths. Moths seem to be fine-tuning their auditory systems in response to their specific ecological niches, whether to optimizing hearing for bat evasion or adapting to the demands of diurnal activity. This research deepens our understanding of the role sensory organs play in survival and reveals how predators drive the evolution of highly specialized traits.

Relationship between tympanal organ size and body size

Geometridae moths, being winged insects, rely more on their hearing based defence system to avoid bats [12]. The shift from nocturnal to diurnal behaviour has occurred independently multiple times across different subfamilies of geometrids, demonstrating parallel evolution. Diurnal species, though exposed to different environmental pressures, face similar predation threats, which may drive independent evolutionary trends such as the increase in the tympanal organ size, but decrease in body size. This phenomenon has been observed in Geometridae and other Lepidoptera families, where the transition from nocturnal to diurnal flight suggests convergent adaptation to similar selective pressures [27,29,41]. Preliminary data from the Uraniidae family show that the diurnal species have visually bigger tympanal organs also (our observation). Smaller body sizes in day-flying moths have been observed in temperate areas, and those could be an adaptation to evade bird predation [42]. This, however, needs to be tested in a wider geographical context. While the auditory systems of diurnal moths might be used for intraspecific competition or defense against birds, there is not yet sufficient evidence to confirm either of these suggestions.

Tympanal organ size and hearing sensitivity

In anurans and lizards, larger tympanic membranes generally produce peak vibration amplitudes at lower frequencies than do smaller tympanic membranes [13,14] like how the area of a drum head affects its pitch [43]. Our approach in scaling of auditory structures and how their dimensions relate to hearing performance, gives valuable insights into how body size influences both the morphology of hearing organs and auditory capabilities. Different taxonomic groups may have developed unique adaptations to address size-dependent constraints on hearing. For example, larger moths may also rely on other defense mechanisms like increased flight speed or improved camouflage to evade bat predation [44]. Additionally, examining deviations from proportional scaling between key auditory structures across insect species can reveal major evolutionary divergences and highlight instances of convergence in the evolution of the insect ear.

Variation in the shape and size of the ansa

Auditory structures are shaped by a combination of phylogenetic, developmental, and physical constraints. Phylogenetic preconditions, such as the presence of ossicles in mammals and the columella in non-mammalian vertebrates, limit the available auditory structures. Developmentally, there are internal relationships between features, like how the dimensions of the bulla in rodents align with earlier tympanic membrane growth [45]. Physical constraints, such as the material properties of structures like the tympanic membrane and ossicles, also play a role. For evolutionary analysis, it is essential to consider both ontogenetic and phylogenetic factors, as structural changes result from a balance of adaptation and inherent constraints, with ontogeny ultimately reflecting phylogenetic history.

This framework can be extended to the convergent evolution of auditory mechanisms in insects and mammals [46]. The ansa in geometrid moths may function similarly to the middle ear ossicles in mammals, with variations in the ossicles influencing hearing sensitivity across mammalian species[47,48]. Similarly, the convergent shape and size of the ansa in nocturnal geometrid moths, and the fact that it is a hollow structure, could suggest that the ansa does not only have a mechanical function, but could also be an adaptation for enhanced sensitivity to higher frequencies in the tympanal organ. However, further investigation is needed to fully understand the relationship between these structures and their evolutionary implications.

To evaluate the adaptation versus constraints hypothesis, a comparative and functional analysis of various insect auditory systems is essential. Only by examining a broad range of species can we identify the shared acoustic properties as well as the functional differences between them, shedding light on the diversity of hearing systems in terms of size and frequency sensitivity.

Conclusion

In conclusion, as the first study to use 3D geometric morphometry on insect cuticular structures the results of this study highlight the complex interplay of ecological pressures, evolutionary constraints, and morphological adaptations in geometrid moths. The lack of a significant allometric relationship between tympanal organ size and body size, alongside the larger tympanal organs in diurnal moths, suggests that environmental factors, such as predator-prey interactions, may drive adaptations in auditory structures. The potential for convergent evolution, especially in the size and shape of the ansa across nocturnal species, further underscores the role of selective pressures in shaping auditory morphology. We gain valuable insights into how body size influences both hearing capabilities and evolutionary trends. Ultimately, our study emphasizes the importance of considering environmental and phylogenetic constraints when interpreting the evolution of auditory structures and opens avenues for further investigation into the convergent mechanisms of hearing across taxa.

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Figure 1

Anatomy of the tympanal organs in geometrid moths

The tympanal organ of the diurnal geometrid moth, *Milionia delicatul*a, a diurnal South-East Asian species in subfamily Ennominae where a) shows the lateral side of the specimen, which was scanned, showing the opening of the abdominal tympanal organ (red circle); b) shows the non-invasive micro-CT scan of the specimen clearly illustrating the tympanal organ (yellow) lodged in the abdominal cavity; c) is the 3D reconstruction of the tympanal organ, showing the tympanal cavity on the outer side and d) shows the ansa (yellow) on the inner wall of the tympanal cavity with the tympanic membrane or tympanum (red).

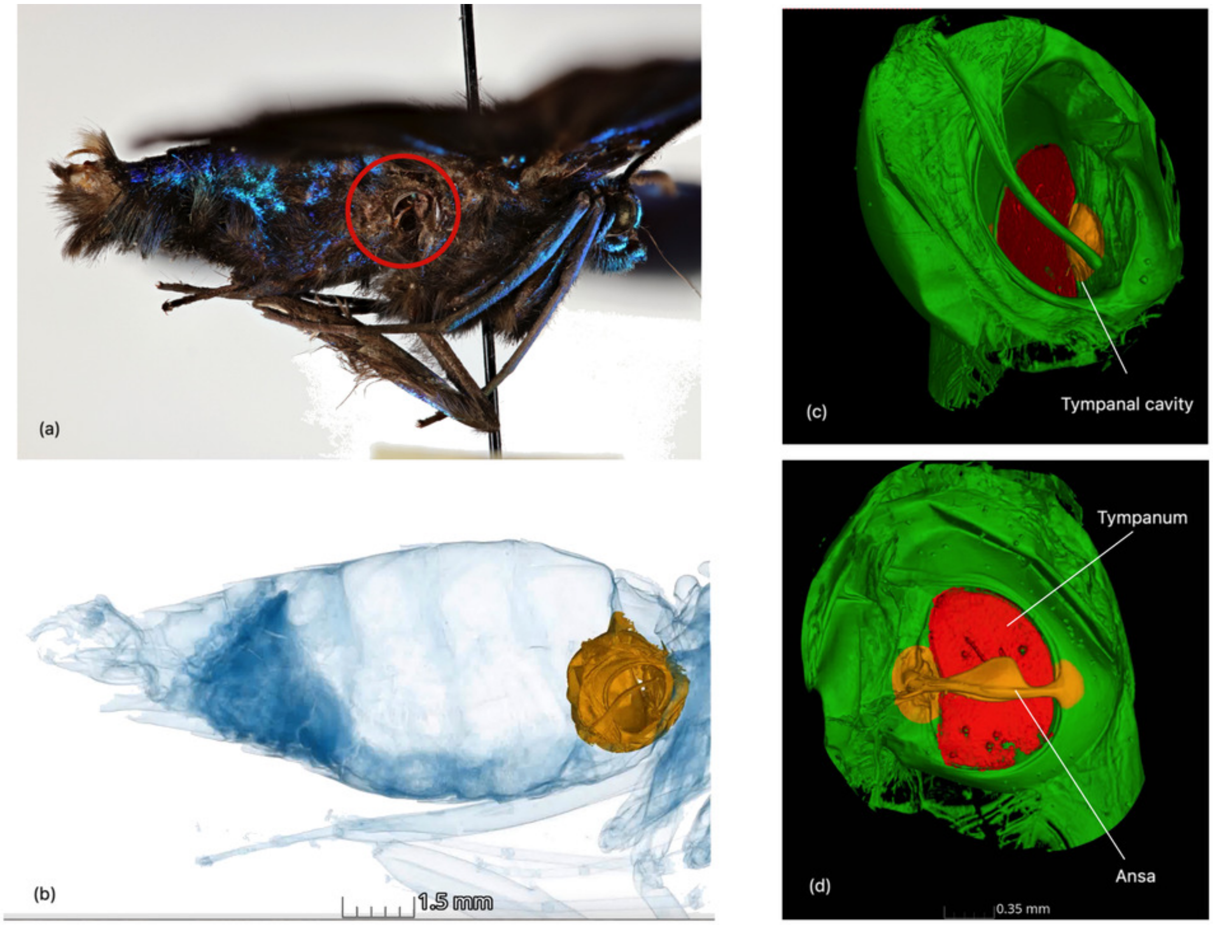

Figure 2

Allometry of body size with tympanal organ size and comparison of body size among diurnal and nocturnal taxa

a) The plot shows the results of a phylogenetic linear regression, where the relationship between Body size and Tympanal volume is assessed while accounting for phylogenetic relatedness. Each data point corresponds to a species, and the regression line shows the best fit for the relationship between the traits depicting the non-significant ($p>0.31$) allometric relationship; b) The graph shows comparison of the body size of diurnal and nocturnal moths, with diurnal moths being significantly smaller than the nocturnal species ($p<0.05$).

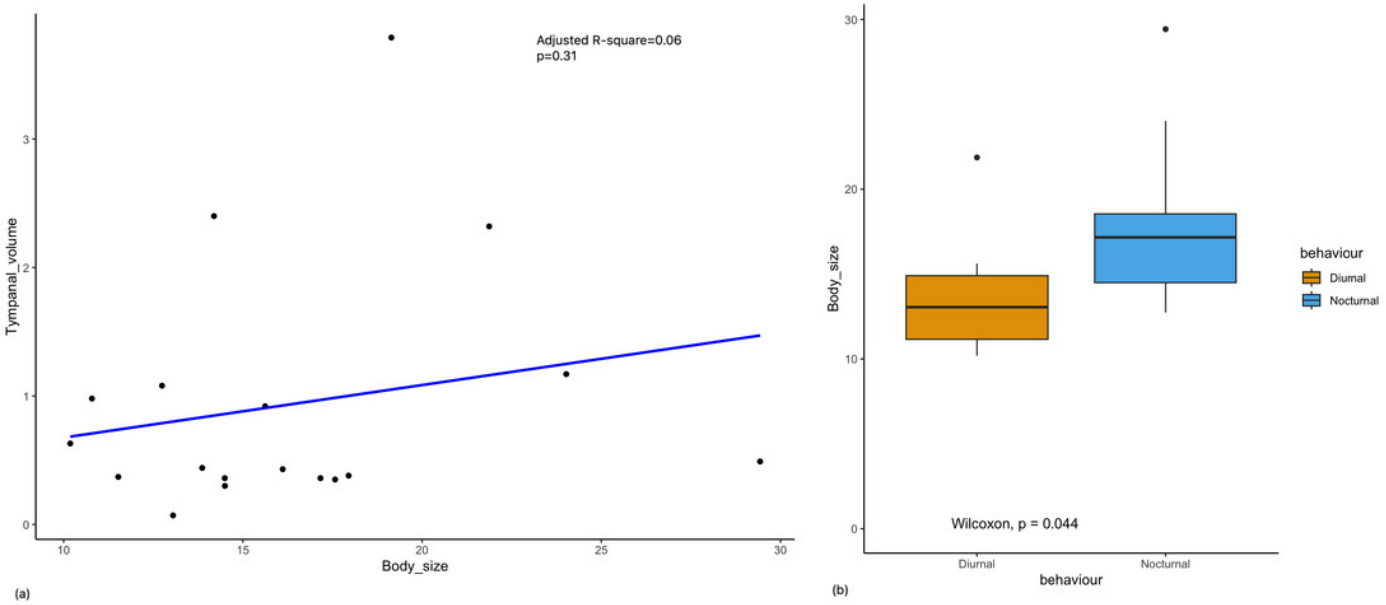

Figure 3

Comparison of the tympanal organ size between diurnal and nocturnal moths

a) The graph shows the comparison of volume ratio (tympanal volume: abdomen volume) of the diurnal and nocturnal species. The diurnal species have significantly larger tympanal volume ($p<0.012$) than the nocturnal species; b) and c) shows the micro-CT images of a diurnal (*Aletis concolor*) and a nocturnal (*Ectropis crepuscularia*) species, to visually represent the size of the tympanal organs compared to the abdomen size respectively.

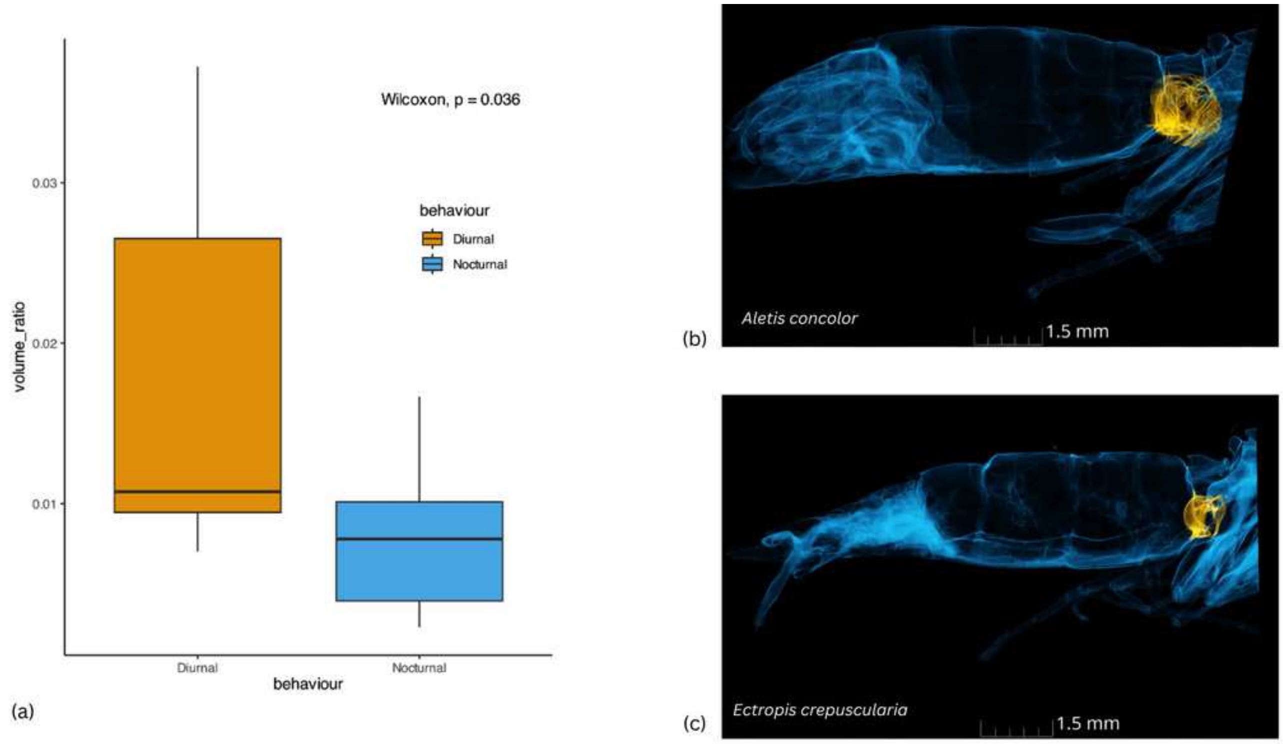

Figure 4

landmark based clustering of shape of ansa

The plot shows the projection of the landmark-based shape parameters of each species onto the first two principal components (PC1 and PC2), which account for 39.95% and 33.52% of the total variance, respectively. Each point represents a species, and the colors indicate different categories or groups within the dataset. There is no significant clustering observed among the diurnal or nocturnal species

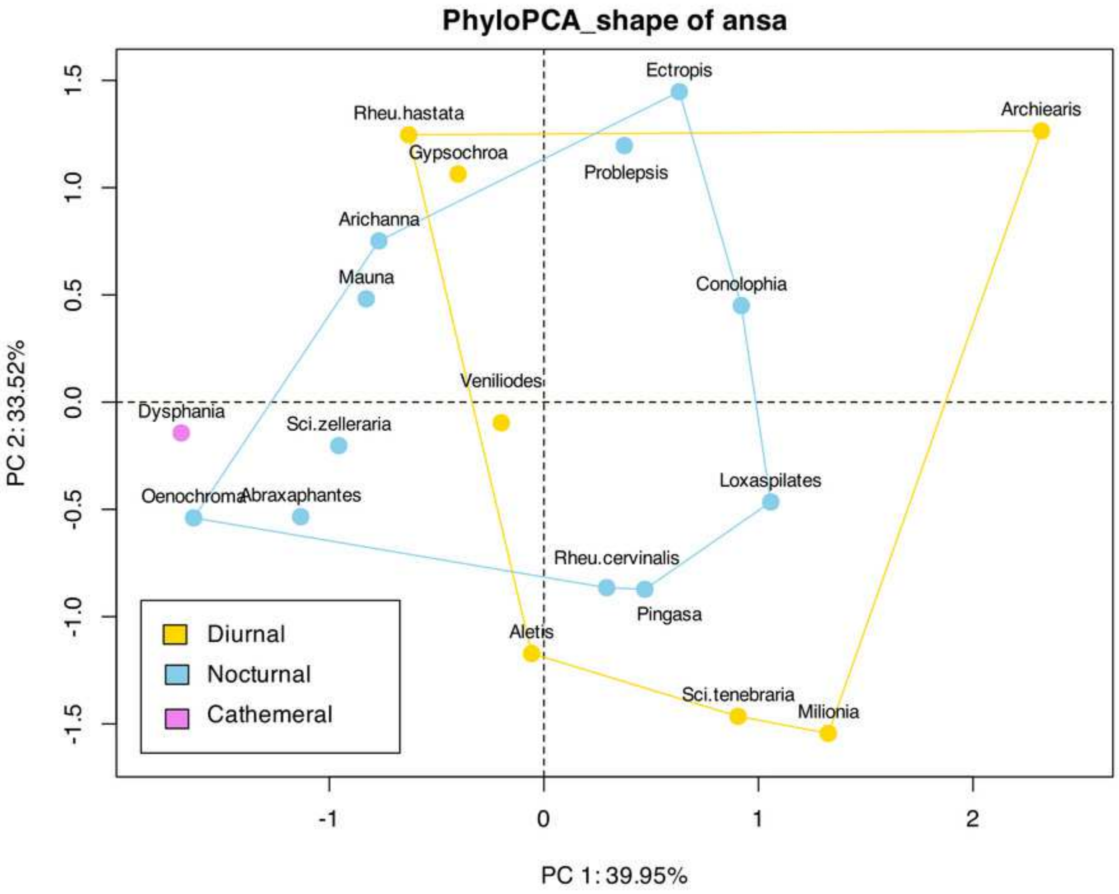

Figure 5

Comparison of the size of ansa in diurnal and nocturnal moths

The graph shows the comparison of Centroid size (Csize) of the 3D model of the ansa, which is an estimate of size of 3D model. There is no significant difference between the size of the ansa in diurnal and nocturnal taxa ($p>0.93$), but the nocturnal species point towards a convergence in size (Inter-quartile range=0.09).

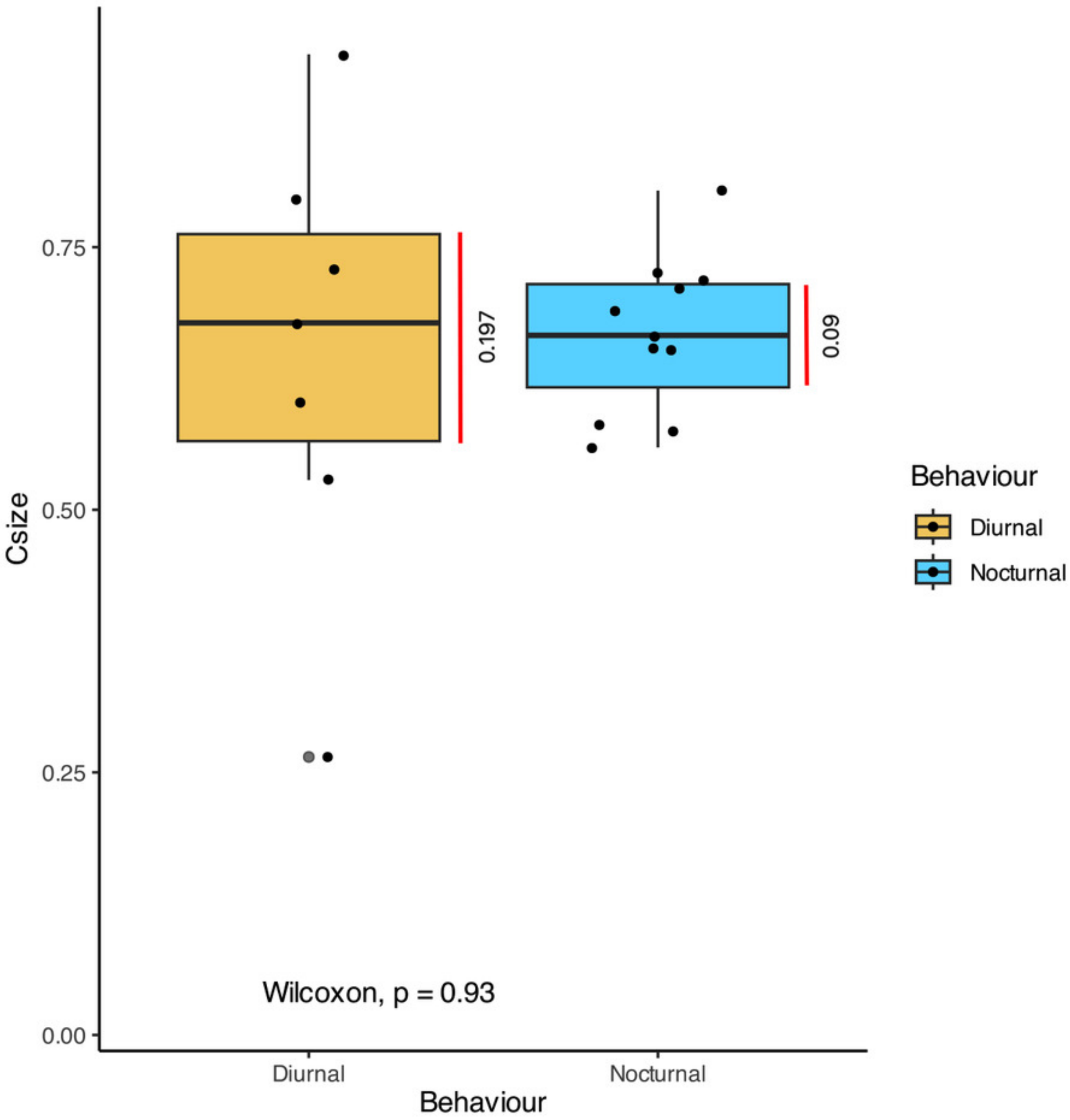
Csize
0.00
0.25
0.50
0.75
0.197
0.09
Wilcoxon, p = 0.93
Diurnal
Nocturnal
Behaviour
Behaviour
Diurnal
Nocturnal

Figure 6

Structure of ansa

Micro-CT scan of ansa of the tympanal organ of *Milionia delicatula*. The scan shows clearly that the structure is hollow (dark orange), being wider at the base, narrow in the middle, and slightly expanded on the apex. 3D video of the structure is available in Supplementary video