OXFORD

Original Article

Modification of vertebral regions explains heart position in arboreal colubrids (Serpentes: Colubridae)

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ABSTRACT

Recent research into the snake vertebral column has highlighted the importance of considering regionalization and its implications for the life history of snakes. Our research delves into the distinctions in vertebral column morphology and regionalization within the snake family Colubridae, comparing arboreal and terrestrial species. Our results provide significant support for dissociation between two pre-cloacal regions: the 'pre-atrial' (anterior to heart) and the 'post-atrial' (posterior to heart). Furthermore, the two ecological groups display distinct proportional compositions between the two pre-cloacal regions and the one post-cloacal region (caudal); arboreal species tend to possess a higher proportion of caudal vertebrae, whereas terrestrial species exhibit a higher proportion of post-atrial vertebrae. Our study reveals that the axial morphology of arboreal species is distinct from that of terrestrial species; however, this was not attributable to a convergent evolution process. By estimating the evolutionary history of vertebral number in each region between the ecological groups, we find each region has a unique pattern, further highlighting evolutionary disassociation between the regions. This study adds to the growing evidence on regionalization of the pre-cloacal snake vertebral column and underscores the importance in continuing to develop our understanding of snake evolution.

Keywords: adaptation; arboreality; axial elongation; axial skeleton; body plan; ecomorphology; regionalization; vertebrae

INTRODUCTION

The intriguing physical form of snakes has long fascinated researchers, with their highly elongate and limbless body plan. Being elongate poses unique challenges particularly regarding cardiovascular physiology. Heart position has been considered an adaptive trait with differing heart positions being attributed to two main factors: the snake's environmental habitat and its phylogenetic history. Arboreal snakes, which frequently climb vertically, are thought to have hearts that are adaptively positioned more anterior in the body cavity (closer to the head), to maintain hydrostatic blood pressure to the head (Lillywhite 1987, Seymour 1987, Badeer 1998, Lillywhite *et al.* 2012). Terrestrial snakes are considered generalized and have a heart more posterior than arboreal snakes (Seymour 1987, Faber *et al.* 2016), and more anterior than aquatic snakes; which typically have a centrally located heart that presumably minimises the cardiac workload required to circulate blood throughout the body,

which is facilitated by the release of gravitational pressure in the water (Lillywhite and Lillywhite 2017, Sherratt *et al.* 2022). Debate over whether the tail should be included (as opposed to using only snout-vent length) in the calculation of relative heart position has resulted in conflicting opinions among scientific publications (see Gartner *et al.* 2011, Lillywhite and Seymour 2011). The influence of phylogeny on heart position has also been demonstrated through analysis of a diverse range of arboreal, terrestrial, fossorial, and semi-aquatic snakes (Gartner *et al.* 2010). Such analysis suggests that heart position is heavily influenced by the phylogenetic history of a given species. These findings provide important insights into the role that ecology plays on cardiovascular location and function.

More recently, the position of the heart has been considered relative to the vertebral column. Simultaneously, Sherratt *et al.* (2019) and Hampton (2019a, b) recognized that the heart's position with respect to the number of vertebrae pre- and post-atria

was an important trait in understanding ecological differences in elapid sea snakes. This novel approach, considering organ position relative to vertebrae, contrasted with previous methods based on total body length or snout-vent length (e.g. Seymour and Lillywhite 1976, Gartner *et al.* 2010). These studies on sea snakes strongly supported the hypothesis that a snake's body plan is regionalized, an observation made by Head and Polly (2015), who showed that the vertebrae vary in shape along the vertebral column and this variation could be partitioned into three or four distinct (unnamed) regions. The heart was proposed to align with a regional boundary dividing the pre-cloacal vertebral column into two hypothetically disassociated regions (Hampton 2019a, Sherratt *et al.* 2019, Sherratt and Sanders 2020). Developmental mechanisms involving the presence and positioning of *Hox* boundaries and heterochronic changes in segmentation were proposed to underlie the independent evolution of pre- and post-atrial regions in sea snakes (Sherratt *et al.* 2019, Sherratt and Sanders 2020). Building on this, Hampton *et al.* (2022) solidified the significance of the heart as a marker of the transition between vertebral regions by identifying a clear transition in vertebrae form at the location of the heart's apex, labelling the pre-atrial vertebrae as the 'cervical' region. With these new considerations of regionalization, Sherratt *et al.* (2022) showed that the heart position of *Hydrophis* sea snakes is more centrally located due to a larger number of vertebrae in this pre-atrial region, which in the most extreme species is due to their specialized diet of burrowing eels requiring an elongated forebody region (Hampton 2019b, Sherratt *et al.* 2019). They demonstrated that the shifting heart position is due to different rates of evolution between the fore and hind body of aquatic snakes: faster evolutionary rates in the pre-atrial region, which elongate the 'neck', are responsible for the central heart position in aquatic snakes. The varied rates of evolution observed between the pre-atrial and post-atrial regions can only occur when there is a dissociation between the two modules (Polly *et al.* 2001). Hampton and Meik (2023) built on Head and Polly's (2015) study and demonstrated the presence of four pre-cloacal vertebral regions based on vertebral shape: the 'cervical', which are the anterior 5–10% of vertebrae with hypapophyses; the 'anterior thoracic', which is divided from the 'posterior thoracic' by the heart apex; and the lumbar, which extend caudally to the cloaca. They did not consider the tail. They also found some species did not possess 'cervical' regions and therefore only had three precloacal vertebral regions, of which the heart still defined the regional boundary between the 'anterior thoracic' and the 'posterior thoracic'. Although these studies have provided insight into the regionalization and importance of the heart as a regional boundary in snakes, it remains unclear how this translates to ecological differences observed in body shape among most snake clades.

Ecological factors have been found to influence the diverse body forms of snakes (e.g. Pough and Groves 1983, Houssaye *et al.* 2010, Sherratt *et al.* 2019, 2022) but studying this phenomenon can be challenging due to the generalist ecology of many snake species (Johnson 1955, Gartner *et al.* 2010, Houssaye *et al.* 2010). However, arboreal snakes, which require morphological specializations, present an excellent opportunity to investigate the effect of ecology on body form (Martins *et al.* 2001, Pizzatto *et al.* 2007, Hampton 2011). Many snake clades have

independently evolved arboreality, with evident convergent evolution towards a long, gracile body form with a prehensile tail (e.g. Gans 1974, Lillywhite *et al.* 2000, Pizzato *et al.* 2007, de Alencar *et al.* 2017, Harrington *et al.* 2018). The colubrids exhibit a higher incidence of arboreality compared to other snake families, encompassing more than half of all arboreal snake species (Shine 1991, Harrington *et al.* 2018). The diverse ecological groups present across the Colubridae clade provide a useful evolutionary experiment to study adaptation of the axial skeleton for an arboreal lifestyle while controlling for phylogenetic relationships.

In this paper, we aim to improve the understanding of the evolutionary diversity of snake body shape and the developmental mechanisms responsible for the heart position in arboreal snakes; this is based on the knowledge that differences in the number and size of vertebrae in body regions result from heterochronic changes in segmentation and rate of pre-somitic mesoderm growth (e.g. Gomez *et al.* 2008, Gomez and Pourquié 2009), leading to variation in vertebral column morphology. Our study examines differences in vertebral column regionalization between arboreal and terrestrial species within the colubrids. We hypothesize that the pre-atrial and post-atrial regions are dissociated, which has led to a high degree of variation in the number of vertebrae between species. Specifically, we hypothesize the two ecological groups will possess different proportional compositions among regions, with arboreal species comprising proportionally more caudal vertebrae and terrestrial species having proportionally more post-atrial vertebrae. We also examine the influence of arboreality on vertebral column morphology. We hypothesize that axial skeleton morphology of arboreal species will be distinct from that of terrestrial species, and convergent evolution with specialization for the arboreal niche has led to decreased variation in axial form among these species. Finally, we estimate the evolutionary history of vertebral number in each body region between arboreal and terrestrial species to provide comparison with that observed among terrestrial and aquatic elapid snakes (Sherratt *et al.* 2022). Results from this study are expected to further our understanding of regionalization of the snake body plan and how it plays a role in species diversification and niche evolution.

MATERIAL AND METHODS

Sampling

We sampled terrestrial ($N = 16$) and arboreal species ($N = 16$) from across the Colubridae (Fig. 1), with species selected because of specimen availability (digital or physical), subfamily diversity, and known ecology (Supporting Information, Table S1). Where possible, ecology was assigned as identified using the following sources: Henderson and Hoevers (1977), Wilson and Mena (1980), Dowling (1990), Strussmann and Sazima (1993), Capula *et al.* (1997), Reams and Gehrmann (2002), da Costa Prudente *et al.* (2007), Fogell (2010), Campbell and Flores-Villela (2012), Steen *et al.* (2012), Achille and Achille (2015), Harrington *et al.* (2018), Bury *et al.* (2020), de Assis *et al.* (2020), Le Duc *et al.* (2021), and IUCN (2023) (details in Supporting Information, Table S1). Species were chosen so that each colubrid subfamily was represented by arboreal and terrestrial representatives (where possible). Although specimens of all
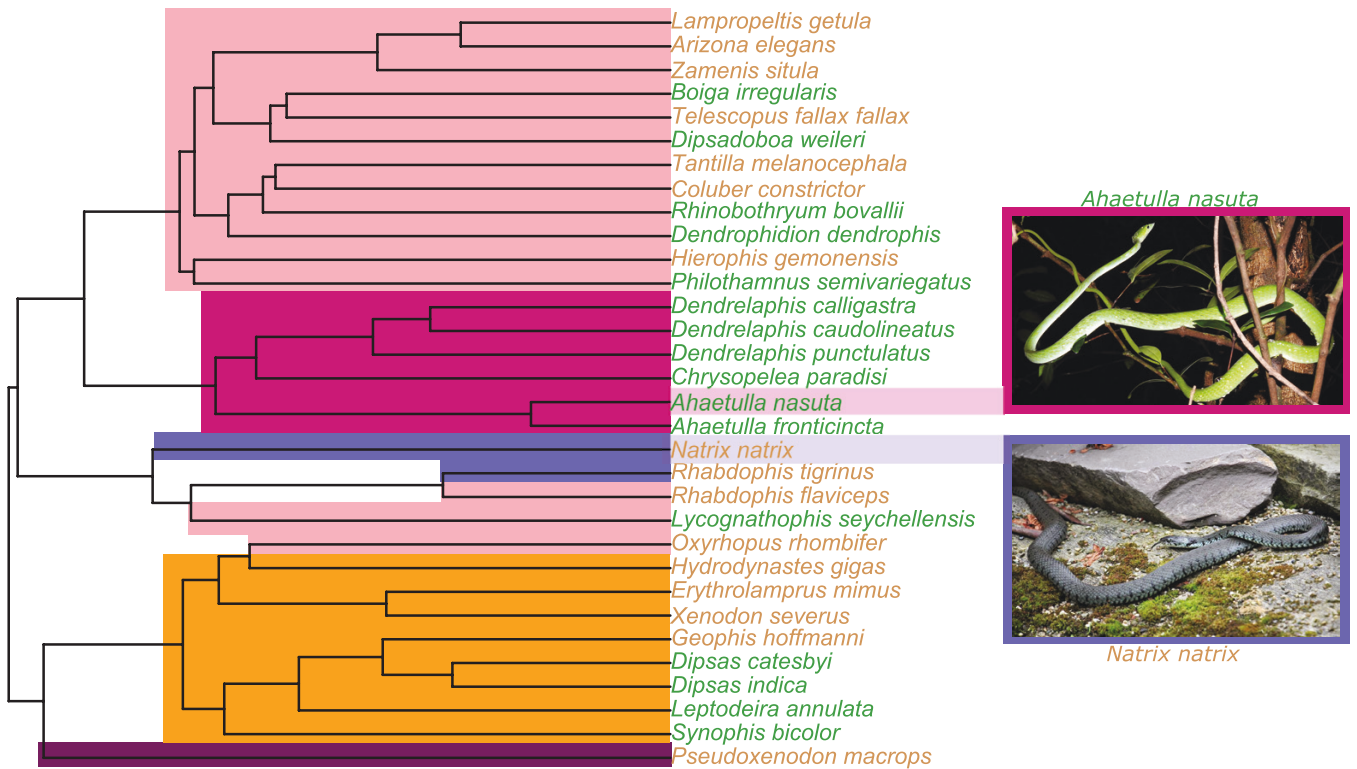
Figure 1. Phylogenetic tree adapted from Zaher *et al.* (2019) and pruned to the species included in this study. Tip labels are coloured by ecology (arboreal species denoted in green and terrestrial species in brown for all figures) and the branches are coloured by subfamily (dark pink = Ahaetuliinae; light pink = Colubrinae; orange = Dipsadinae; purple = Natricine; dark purple = Pseudoxenodon). Inset: images illustrate representative examples of both an arboreal (Kasambe 2017, green vine snake *Ahaetulla nasuta*, published on Wikimedia commons, licensed under CC BY-SA 4.0) and a terrestrial species (Pixabay 2016, grass snake *Natrix natrix*, published on Stockvault, licensed under CC0 1.0).

the same sex were not accessible, every effort was made to evenly spread mixes of sex across both ecological groups to minimise the influence of sex-specific variation. Specimens examined were alcohol preserved and housed in Natural History Museum collections (South Australian Museum, University of Michigan Museum of Zoology, American Museum of Natural History and Museum of Vertebrate Zoology). Those loaned from the South Australian Museum's herpetology collection were scanned using X-ray micro-computed tomography (CT) using a Bruker Skyscan 1276 micro-CT at Adelaide Microscopy. New scan data generated for this study were deposited on Morphosource (https://www.morphosource.org/projects/000445258). The remaining specimens were sampled as micro-CT scans available on Morphosource (details in Supporting Information, Table S1). Avizo 9.0.1 (Thermo Fisher Scientific) was used to digitally segment the isosurface representing bone and simplify the models down to 1 million faces.

Vertebra morphometrics

Three-dimensional (3D) landmarks were used to derive vertebra length from the micro-CT scans. Using Stratovan Checkpoint 2020.10.13.0859 (Stratovan Corporation 2020), one person (T.N-H.) landmarked the entire vertebral column, commencing at the first vertebra following the atlas-axis complex and terminating at the final caudal vertebra. The position of the cloaca was noted to identify the end of the pre-cloacal region. Landmarks were placed at the most posterior, dorsal tip of the spinal process (see Fig. 2). This location was chosen as it was least affected by different coiling positions and was able to be consistently identified in all specimens.

We prepared the landmark coordinate data for analysis by using the *geomorph* package 4.0.0 (Baken *et al.* 2021, Adams *et al.* 2022) in R Statistical Environment 4.0.1 (R Core Team 2021; RStudio Team 2021). To extract the length of individual vertebra, we used Pythagorean theorem implemented by the 'interlmkdist' function within the *geomorph* package. Relative vertebra lengths were calculated by dividing vertebra length by the sum of all vertebra lengths. The successive lengths of the pre-cloacal vertebra were plotted to produce a concave, fourth-degree polynomial 'intracolumnar profile' (see Fig. 3). To allow for variation in the number of vertebrae between species, while simultaneously preserving the total profile length, we fitted fourth-degree polynomials to each of the profiles implemented through the 'lm' and 'poly' functions within the *stats* package (Sherratt and Sanders 2020, Sherratt *et al.* 2022).

Heart position

We determined heart position in alcohol-preserved specimens by manually feeling the external body for a hard lump, making a small ventral incision at the estimated location of the heart, and then counting the ventral scales between the apex of the heart (posterior tip) and the back of the cranium, as number of ventral scales is proportional to number of vertebrae in these species (Alexander and Gans 1966). Heart position was also recorded as
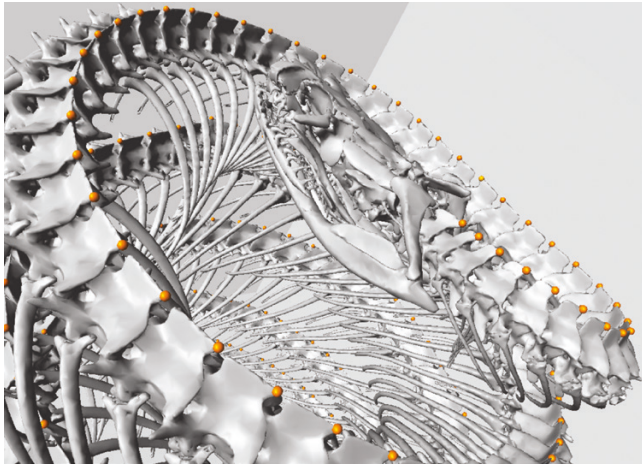
Figure 2. Landmarks (depicted in orange) were placed on the posterior, dorsal tip of the spinal process.
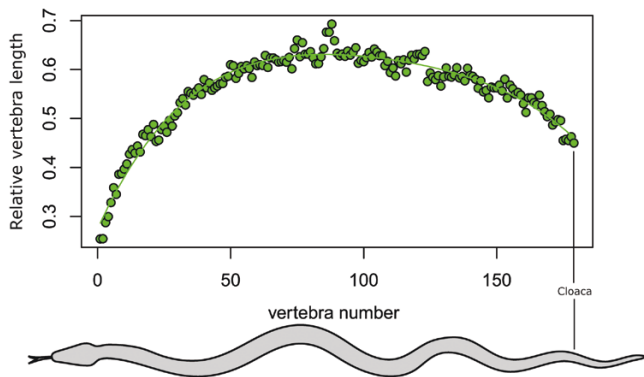
Figure 3. Example intracolumnar profile for a single specimen with relative lengths of individual pre-cloacal vertebra shown by the green points. The green line is the fitted fourth-degree polynomial used to estimate the profile shape. Note, the concave shape of the curve where the largest vertebrae are located at the middle of the body. The y-axis is the relative length of the vertebra compared to total pre-cloacal length, and the x-axis vertebra position. The snake shadow shows the direction of the head and tail on the profile.

a percentage along the axial skeleton, both in relation to the pre-cloacal region (Gartner *et al.* 2010) and total length (Seymour 1987). Expressing this data as a percentage along the axial skeleton is the conventional method (e.g. Seymour 1987, Gartner *et al.* 2010, Anderson and Secor 2015, Lillywhite and Lillywhite 2017) and facilitates straightforward conversion of any linear measurements into this format.

The snake body plan is herein referred to by regions: pre-atrial, post-atrial, and caudal. Pre-atrial refers to vertebrae between the head and the apex of the heart; post-atrial is the region between the apex of the heart and the cloaca; and caudal are the vertebrae posterior to the cloaca.

To include heart position within the intracolumnar profiles, vertebra length datasets were divided into pre-atrial and post-atrial regions. A fourth-degree polynomial was fitted to each region to create a regionalized intracolumnar profile following Sherratt *et al.* (2022), using the stats R package functions 'lm' and 'poly' (arguments: degree = 4, raw = TRUE). These stand-ardized profiles were then converted into a fixed number of

'landmarks' (15 in the pre-atrial and 20 in the post-atrial regions) that together described the shape of the profile (akin to a geometric morphometric approach). The x,y coordinates of these landmarks (x being the vertebra position and y being the relative vertebrae length) were used as variables in a principal component analysis (PCA) described later.

Vertebrae counts

Number of vertebrae in each body region—pre-atrial, post-atrial, and caudal—were tallied for the two ecological groups. The position of the heart and cloaca were noted such that the average position of the heart relative to the total vertebral column and relative to the pre-cloacal vertebral column could be calculated for both ecological groups. This allows comparison of the heart position with and without the inclusion of the tail. Boxplots implemented with the 'boxplot' function were used to visualize variation in vertebrae counts within each of the regions and overall between the two ecological groups.

Statistical analyses

A phylogenetic tree was obtained from Zaher *et al.* (2019) and pruned to include only the 32 species in this study using the 'keep.tip' function in *ape* R package v.5.7-1 (Paradis and Schliep 2018). The phylogeny is a penalized likelihood multi-gene tree of more than 1000 Caenophidian snakes with branch lengths scaled to unit time using an autocorrelated relaxed clock method (Zaher *et al.* 2019).

To determine if there was a significant difference between the relative heart positions of the ecological groups, a phylogenetic analysis of variance (ANOVA) was implemented by using the 'procD.pgls' function within *geomorph*, which performs a phylogenetic generalized least squares analysis for multidimensional data (Adams 2014). This analysis was conducted on the relative heart position for both tail included, and tail excluded. ANOVAs were also implemented to determine if the ecological groups differed significantly in the number of vertebrae overall and separately in each vertebral region. The statistical significance was assessed using a residual randomization permutation procedure (RRPP) using 1000 permutations.

In order to test for dissociation between the different vertebral regions, ratios of the vertebral counts in the regions were created (i.e. pre-atrial: post-atrial, pre-atrial: caudal, post-atrial: caudal, and pre-cloacal: caudal). These ratios were each implemented as the response variable against the total number of vertebrae in each species through the 'procD.pgls' function in *geomorph*. Statistical significance again was assessed through RRPP.

To compare the shape of regionalized intracolumnar profiles between species, we ordinated the profiles using a PCA implemented using 'gm.prcomp' within *geomorph*. We tested for convergence in intracolumnar profile shape (heart position included) using the Stayton (2015) approach. We calculated the C_1 statistic, which assesses whether the species of an ecological group have converged towards a more compact morphospace region compared to what would be anticipated under a Brownian motion model of evolution. C_1 explicitly measures the morphological distance among extant species compared to the distance among estimated ancestors. C_1 and associated statistical significance (permutation approach) was obtained through

implementation of the 'convSig' function in *convevol* v.2.0.1 (Stayton 2015, Zelditch *et al.* 2017).

An analysis of covariance (ANCOVA) was conducted to determine if there was a statistically significant difference between ecological groups in their intracolumnar profile shape when heart position was a covariate. Intracolumnar profile was represented by x,y, coordinates of the vertebral position (x) and cumulative relative size (y), which are used in the plot of Figure 3. This was implemented in the 'procD.pgls' function within *geomorph*. To test whether the ecological groups differed in the amount of intracolumnar profile shape diversity, we used a Procrustes variance (PV) disparity test implemented through 'morphol.disparity' function within *geomorph*, again with heart position as a covariate. The statistical significance of both the ANCOVA and disparity test were assessed using a RRPP using 1000 permutations.

Traitgrams were used to show estimations of the evolutionary history of each region and support dis/association results found in the region ratio ANOVAs. The proportion of vertebrae in a region was plotted against time, with internal nodes estimated through maximum likelihood, implemented in the 'phenogram' function within *phytools* v.1.2-0 (Revell 2012). A traitgram was made for each of the three regions (pre-atrial, post-atrial, caudal), with the branches coloured by subfamily and tip labels coloured by ecology.

RESULTS

Vertebral counts

In our study, including or excluding the tail does not change the observed pattern between arboreal and terrestrial species (Fig. 4); arboreal species have, on average, a heart position more posterior than terrestrial species, although this result is only significant when the tail is excluded (tail excluded: $R^2 = 0.1587$, $F_{(1,32)} = 5.6572$, $P = 0.032$; tail included: $R^2 = 0.0131$, $F_{(1,32)} = 0.3977$, $P = 0.516$). When the tail is excluded (Fig. 4B), there is a greater range of relative heart positions across both ecological groups and a greater difference in the mean relative heart position.

Arboreal species have significantly more vertebrae than terrestrial species (Fig. 5A), with ecology accounting for 50.8% of the variance observed in the number of vertebrae ($R^2 = 0.5080$, $F_{(1,32)} = 30.977$, $P = 0.001$). The two ecological groups differ in their distribution density of vertebrae across the three regions (Fig. 5), with arboreal colubrids possessing significantly more vertebrae in both their caudal ($R^2 = 0.5550$, $F_{(1,32)} = 37.419$, $P = 0.001$) and pre-atrial regions ($R^2 = 0.3131$, $F_{(1,32)} = 3.677$, $P = 0.001$). The difference between the ecological groups is not significant in the post-atrial region ($R^2 = 0.0553$, $F_{(1,32)} = 1.755$, $P = 0.193$). There are two terrestrial outliers with many more vertebrae in the post-atrial region, these are *Zamenis situla* (Linnaeus, 1758) and *Lampropeltis getula* (Linnaeus, 1766).
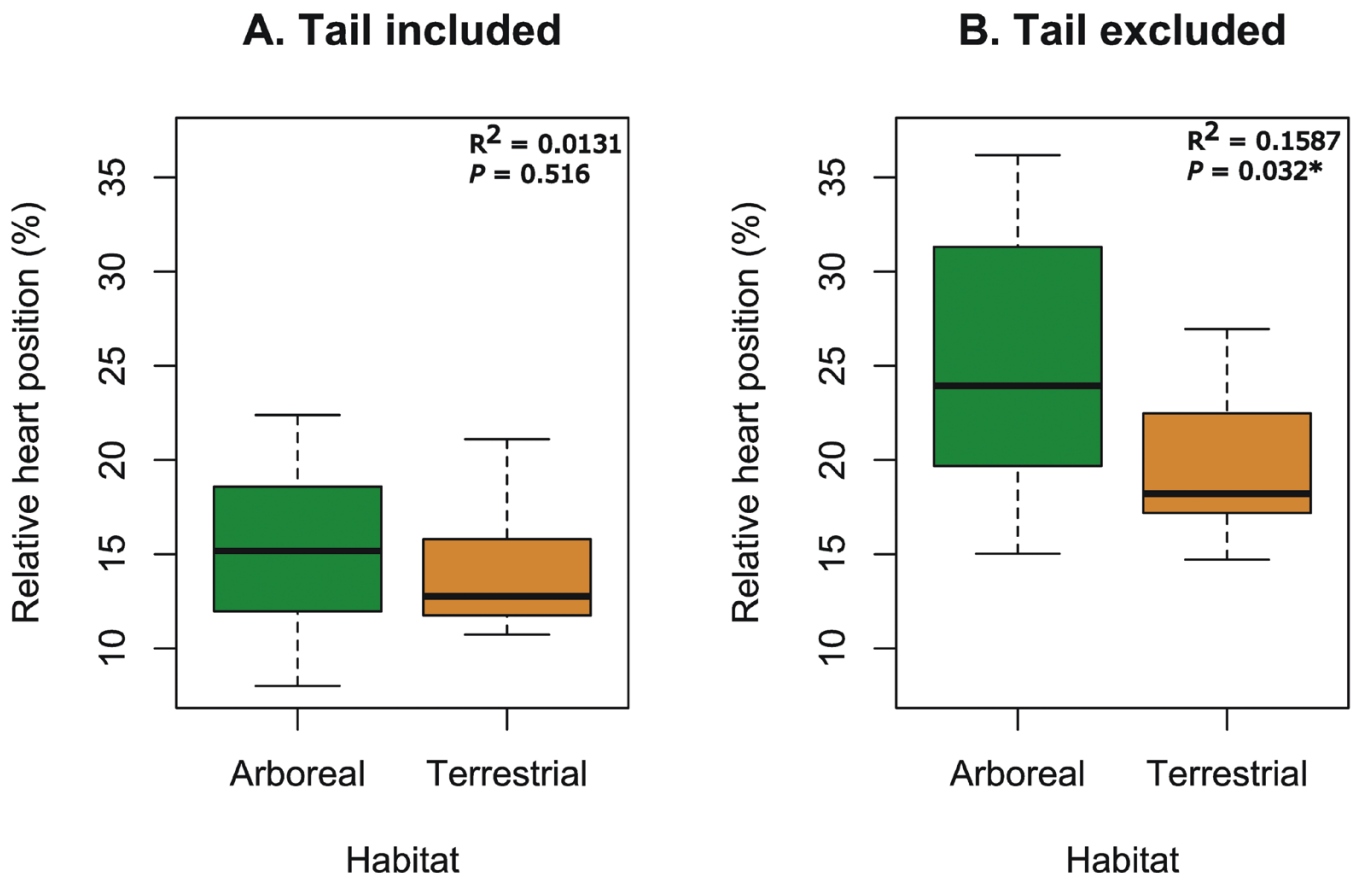
Figure 4. Relative heart position (% of body length) between arboreal and terrestrial species, demonstrating the effect of the inclusion (A) or exclusion (B) of tail vertebrae in the total count.
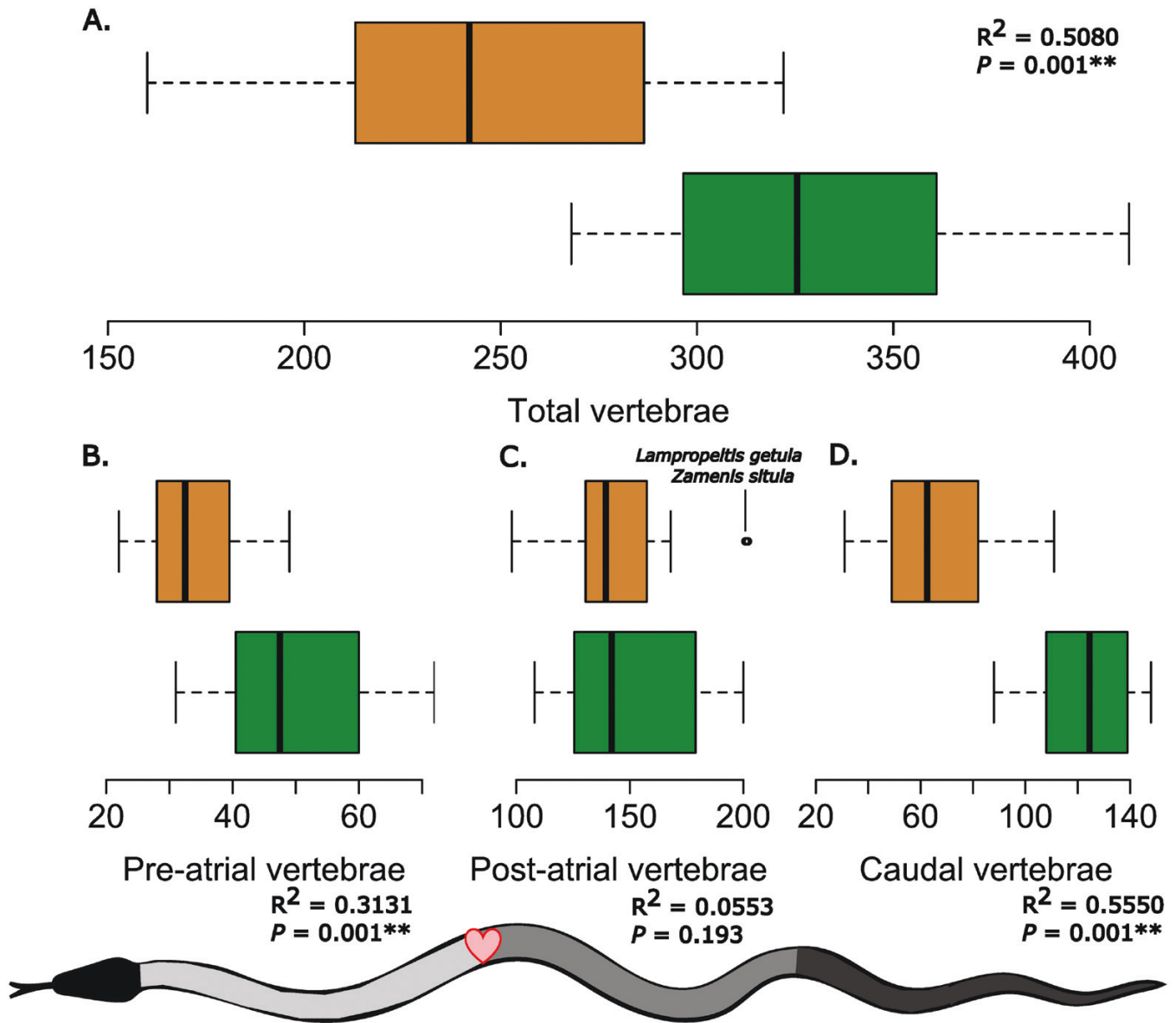
Figure 5. The total number of vertebrae between terrestrial and arboreal species (A). The number of vertebrae in the pre-atrial (B), post-atrial (C), and caudal (D) regions. ANOVA coefficient of determination (R^2) and significance (P) for each region is given. The snake shadow shows the direction of the head relative to the regions.

Our results support dissociation between the pre-atrial and post-atrial regions indicating that the number of vertebrae in the pre-atrial region can change without the number of vertebrae in the post-atrial region also changing (Fig. 6A). We found all other regions to be integrated with one another (Fig. 6B–D).

Intracolumnar profile

Ecology is a significant predictor of intracolumnar profile shape, whereas heart position alone is not (Table 1). The significance of the interaction term indicates that heart position influences the intracolumnar profile differently between ecological groups.

In addition to the number of vertebrae, the ecological groups differ in the relative size of the vertebrae along the vertebral column (Fig. 7). Principal component (PC) analysis of the intracolumnar profile shapes of each species shows variation in two main axes. PC1 (74.5%, Fig. 7) represents the number and relative size of vertebrae where bodies with many vertebrae that are homogenously sized are at the minimum (PC1 min) and short-bodied snakes with a large change in relative vertebrae size along the profile are at the maximum (PC1 max). PC2 (19%) represents the position of the heart, with more posteriorly placed hearts at the minimum (PC2 min) and more anteriorly located hearts at the maximum (PC2 max). The terrestrial species exhibit more diversity along PC1, whereas most of the diversity for the arboreal species occurs along PC2. However, the ecological groups do not differ significantly in overall morphological disparity ($PV_{arb} = 33\ 331.17$, $PV_{terr} = 41\ 462.5$, $P = 0.553$), meaning they occupy the same amount of morphospace. Furthermore, we do not find any evidence of convergence in intracolumnar profile form among arboreal species; however, there is statistically significant convergence among the terrestrial species (arboreal: $C_1 = 0.1817$, $P = 0.270$; terrestrial: $C_1 = 0.228$, $p = 0.023$).
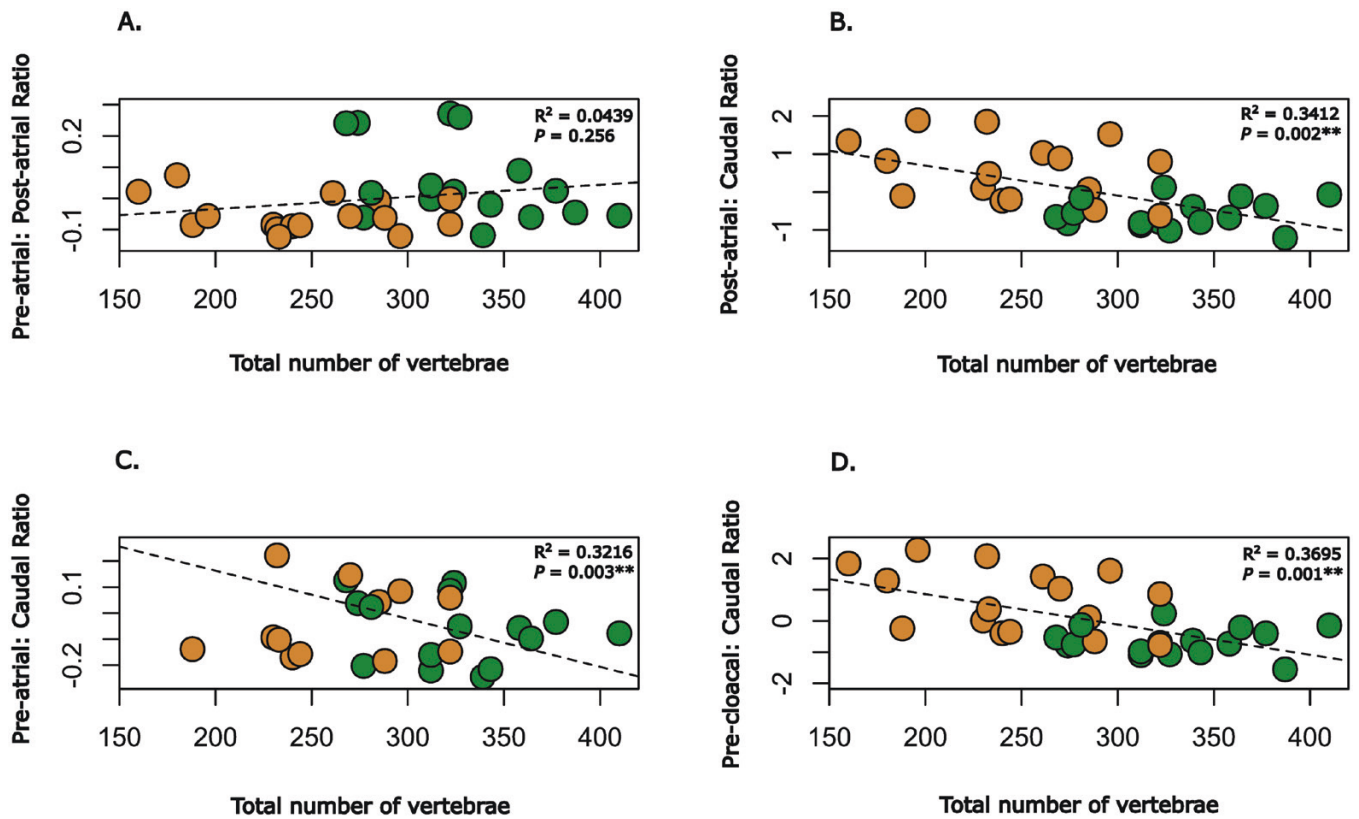
Figure 6. Scatterplots of the phylogenetic generalized least squares (PGLS) for the total number of vertebrae against pre-atrial:post-atrial ratio (A); post-atrial:caudal ratio (B); pre-atrial:caudal ratio (C); pre-cloacal:caudal ratio (D). PGLS coefficient of determination (R^2) and significance (P) of each test is given.

Table 1. ANCOVA of intracolumnar profile shape predicted by ecological groups (ecology) with heart position as a covariate (f1 = intracolumnar profile ~ heart position * ecology). Where intracolumnar profile is the x,y coordinates of vertebra position and cumulative relative size as seen in Figure 3. Degrees of freedom (d.f.), sum of squares (SS), mean square (MS), coefficient of determination (R^2), test statistic (F), effect size (Z), and P-value based upon the F statistic (Pr) for each factor are provided

	d.f.	SS	MS	R^2	F	Z	Pr
Heart position	1	1825.858	1825.858	0.016972	0.670 818	0.264 306	0.412
Ecology	1	14 155.92	14 155.92	0.131 583	5.20 087	1.80 986	0.024
Heart position: ecology	1	15 388.28	15 388.28	0.143 038	5.653 638	1.779 306	0.032
Residuals	28	76 211.41	2721.836	0.708 407			
Total	31	107 581.5					

Evolutionary history of vertebral regions

The three regions differ substantially in terms of their estimated evolutionary history of the proportion of vertebrae in each region (Fig. 8). There does not seem to be a strong influence of phylogeny on the proportion of vertebrae in any of the regions, as shown by the branches coloured by subfamily being pulled in different directions along the y-axis. In the pre-atrial region (Fig. 8A), the two ecological groups show a mixed dispersal along the y-axis with no clear pattern. Whereas in the post-atrial region (Fig. 8B), the terrestrial snakes typically possess a larger proportion of vertebrae compared to arboreal species. The arboreal snakes, however, possess a greater number of caudal vertebrae both in terms of proportion and overall, when compared to terrestrial species (Fig. 8C).

DISCUSSION

Our study examined differences in vertebral column morphology and regionalization between arboreal and terrestrial species within the Colubridae. The results support the hypothesis that the pre-atrial and post-atrial regions are dissociated, and the high degree of variation in the number of vertebrae between species is different among these regions. Specifically, our data support the hypothesis that the two ecological groups possess different proportional compositions among regions, with arboreal species comprising proportionally more caudal vertebrae and terrestrial species having proportionally more post-atrial vertebrae. We showed that axial skeleton morphology of arboreal species is distinct from that of terrestrial species, but not due to convergent evolution. Through estimating the
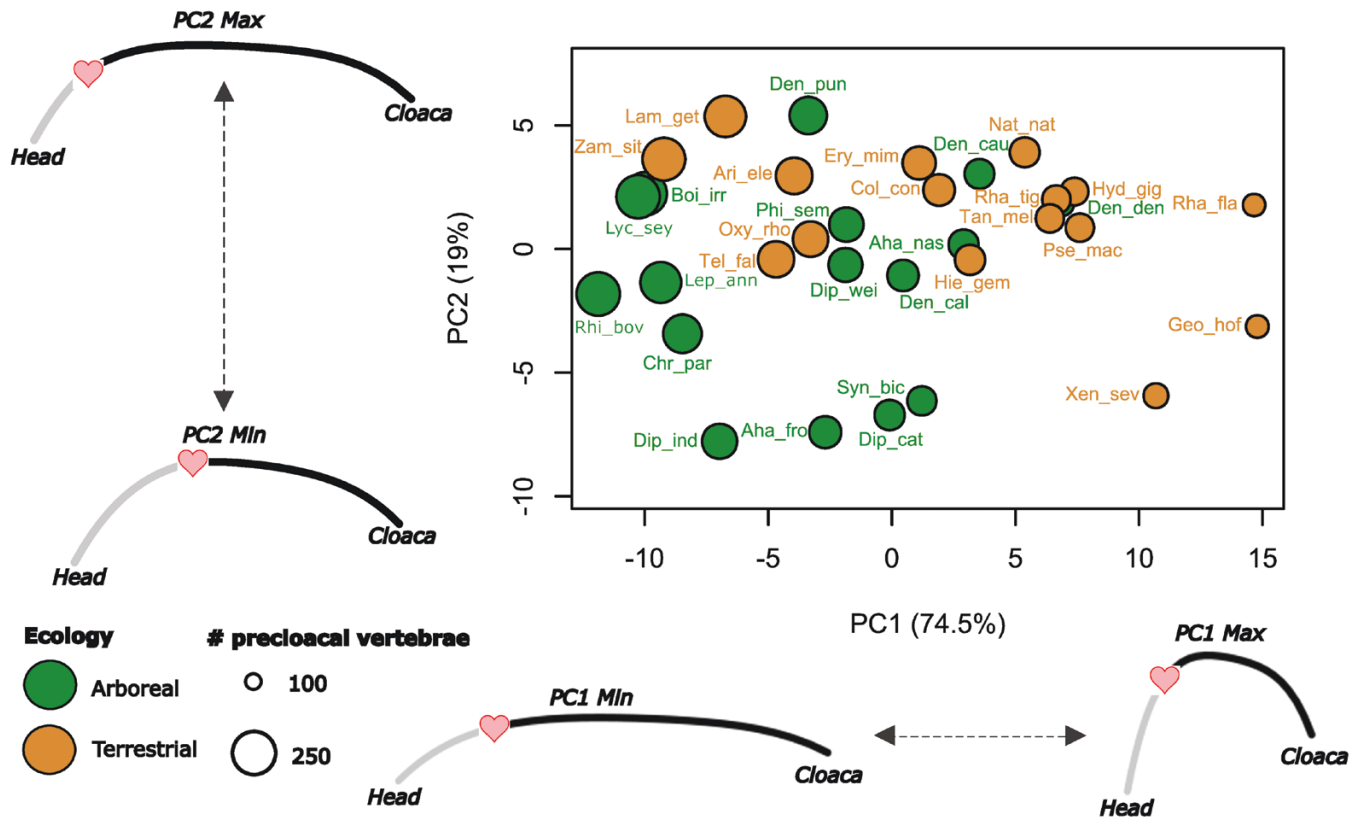
Figure 7. Principal components (PC) 1 and 2 scatterplot of the standardized precloacal intracolumnar profile shapes for 32 species (i.e. Fig. 3). Point size is scaled to number of precloacal vertebrae. Intracolumnar profile shapes representing minimum and maximum PC scores for each axis are given beside and below the axis (labelled PC1 min, PC1 max, etc.). Pre-atrial region shown in grey and post-atrial shown in black.

evolutionary history of vertebral number in each body region between arboreal and terrestrial species, we find each region has a unique pattern, which further supports the evolutionary disassociation of these regions. Our study builds upon the findings of recent studies (Head and Polly 2015, Hampton 2022, Sherratt *et al.* 2022, Hampton and Meik 2023) that suggest regionalization of the snake body plan has played an important role in species diversification and niche evolution of this radiation.

Heart position debate

Our results show that arboreal colubrids possess a significantly more posteriorly positioned heart, but only when the tail is excluded (i.e. heart position relative to snout-vent length) (Fig. 4). The position of the heart in arboreal snakes has been a topic of debate among researchers (see Gartner *et al.* 2010, 2011, Lillywhite and Seymour 2011). Most previous studies have examined heart position across a broad phylogenetic sample to make inferences on ecological differences, with some claiming arboreal species have a slightly more posterior heart position and others claiming the opposite pattern (Seymour 1987, Gartner *et al.* 2010). There are two main factors which explain the conflicting results found in these two studies: (1) inclusion/exclusion of the tail and (2) species sampling. The effect of including/excluding the tail has been examined thoroughly in Gartner *et al.* (2011). As for the effect of species sampling, Anderson and Secor (2015) found that colubrids possess a more anterior heart position than species from other families. Hence in Gartner *et al.* (2010), which used a predominantly

colubrid sample, arboreal species were found to have a slightly more posterior heart position. Whereas Seymour (1987) used colubrid species for the arboreal ecological group and primarily non-colubrid species for the terrestrial representation, and found arboreal snakes to have a more anterior heart position. The results from Seymour (1987) were likely driven by colubrids broadly having more anterior heart positions. Hence our study within a single family (Colubridae) provides the opportunity to control phylogenetic variation in heart position and use the same sample to also examine the influence of including the tail.

Our detailed analysis indicates that heart position is influenced by both phylogenetic history and ecology, and the heart position varies by differential addition of vertebrae to the three vertebral regions. This is also shown in Hampton and Meik (2023), Sherratt *et al.* (2020), and Sherratt *et al.* (2022), albeit focussing on other clades. Previous studies of heart position claim heart position is an adaptive trait of physiology; a more anterior heart can better supply blood to the head when the snake is vertically climbing (Seymour 1987, Badeer 1998). Although we do not debate this functionality, we argue that terrestrial snakes are generalists, and many can climb vertically like arboreal snakes. Similarly, arboreal snakes do not spend their entire day vertical, but rather a very small portion of their day (Faber *et al.* 2016). It is for these reasons that we propose that in colubrids it is not the heart position that is the target for selection, but rather the vertebral column and modification to different axial regions to suit an arboreal lifestyle.
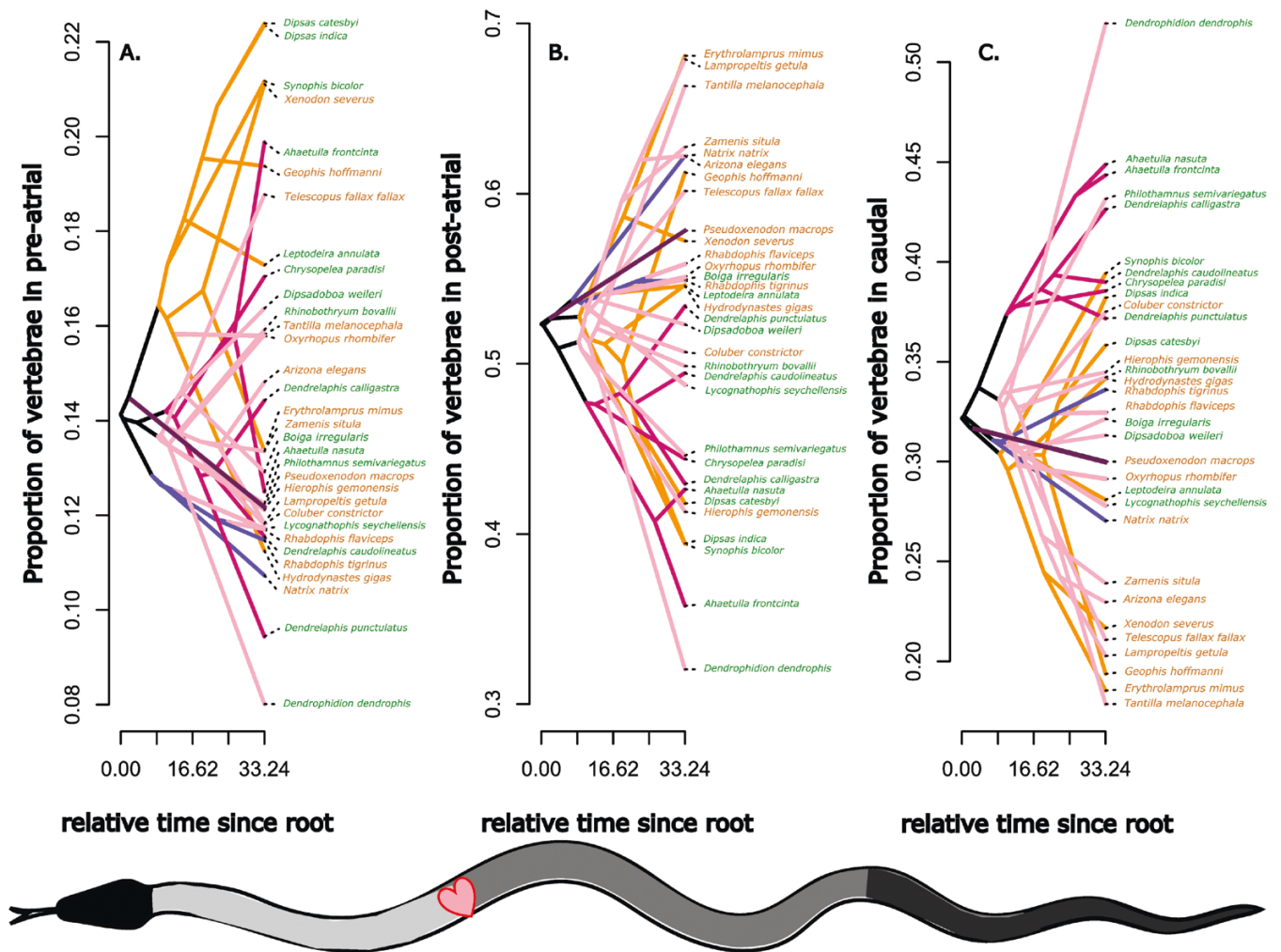
Figure 8. Traitgram showing the proportion of vertebrae in the pre-atrial (A), post-atrial (B), and caudal (C) regions mapped onto the consensus tree of 32 species. Branches are coloured by subfamily (dark pink = Ahaetuliinae; light pink = Colubrinae; orange = Dipsadinae; purple = Natricine; dark purple = Pseudoxenodon) and species name tip labels are coloured by ecology (terrestrial = brown, arboreal = green).

Dissociation of the pre- and post-atrial regions

Our results support the dissociation of the pre- and post-atrial regions, whereas all other tested regions (post-atrial and caudal, pre-atrial and caudal, and pre-cloacal and caudal) show weak patterns of association (evolutionary integration; Fig. 6A vs. Fig. 6B–D). This is perhaps better visually interpreted from the traitgrams (Fig. 8), where the distribution of species across the y-axis is not consistent between the two regions. Disassociation of the pre- and post-atrial regions has also been found in elapid snakes (Sherratt *et al.* 2022) and more broadly across 13 families of snakes (Hampton and Meik 2023). Together these studies strongly indicate the pre- and post- atrial regions are evolutionarily decoupled in Alethinophidian snakes. We encourage research into this phenomenon among species of Scolecophidia.

The finding of dissociation between the pre- and post-atrial regions raises the question of whether calling the pre-cloacal vertebrae collectively a 'trunk' is still appropriate. Typically, it has been thought that the cervical vertebrae of snakes are only those without ribs (Hoffstetter and Gasc 1969). However, the plesiomorphic condition is a cervical region that extends to the

forelimb girdle, as in limbed squamates. Cohn and Tickle (1999) said in pythons 'anterior vertebrae have both ribs (a thoracic feature) and ventral hypophyses (generally a cervical feature)', such that 'the entire truck resembles an elongated thorax'. Yet others have argued for 'cervicalisation' of the trunk (Caldwell 2001, Scanlon 2004), since the anterior vertebrae are typically distinguishable from the other pre-cloacal vertebrae by possessing distinct narrow, elongate hypapophyses (Smith 1975, Hampton *et al.* 2022). Hampton and Meik (2023) show that heart position is correlated with a regional transition between the 'anterior thoracic' and the 'posterior thoracic'. Our study builds on the growing evidence that the region anterior to the heart, previously called the forebody (Sherratt *et al.* 2019, Sherratt and Sanders 2020) and herein termed the pre-atrial (Sherratt *et al.* 2022) is, regardless of semantics, a distinct evolutionary region which is subject to adaptive responses to environmental shifts.

Regionalization in the body musculature of snakes has been investigated to identify the neck-trunk boundary (Tsuihiji *et al.* 2012). The authors concluded that the anatomical structures usually associated with the neck-trunk boundary are disassociated

in snakes, but there was evidence of regionalization in axial musculature, which was not reflected in the 'apparently homogenised series of vertebrae posterior to the well-differentiated atlas-axis complex' (Tsuihiji *et al.* 2012). However, they did not quantitatively examine the axial skeleton, and we propose that axial musculature should be reconsidered with respect to the shape variation of the axial skeleton, particularly in the vicinity of the heart, to better understand this boundary. Given the above discussion and findings of recent studies showing the pre-atrial region as distinct, we propose the re-definition of cervical vertebrae in snakes to consider this region, and in doing so use of 'cervical' for the vertebral region anterior of the heart; however, we acknowledge that the recent study by Hampton and Meik (2023) considered the cervical vertebrae to be separate from the 'anterior thoracic' region, which is their term for the pre-atrial vertebrae.

Ecological differences in composition of axial regions

Pre-atrial
The appearance of a more posteriorly positioned heart in the arboreal species, when only considering pre-cloacal vertebrae (Fig. 4B), is due to them having proportionally more vertebrae in the pre-atrial region (Fig. 5B). Increasing the number of vertebrae in the pre-atrial region to gain a longer body with a more posteriorly positioned heart is how marine-adapted elapids of the *Hydrophis* clade have evolved (Sherratt *et al.* 2022). However, not all arboreal colubrids have added vertebrae to the pre-atrial region compared to their terrestrial relatives (Fig. 8A), indicating this is not the sole developmental strategy for body elongation in arboreal species. There is a high degree of variation among the pre-atrial region of arboreal colubrids, possessing as low as 8% and as high as 22% of their total vertebrae in this region. This observed variation suggests that there may be additional factors which are influencing the pre-atrial region, perhaps such as hunting method (e.g. active, ambush), the complexity of the environment (i.e. how much the arboreal species must stretch to reach branches), or a trade-off in vertebrae counts with the caudal region.

Post-atrial
Although arboreal and terrestrial colubrids have similar numbers of post-atrial vertebrae (Fig. 5C), proportionally the terrestrial species have a greater allocation of their total axial skeleton within this region (Fig. 8B). This region thus appears to be evolutionarily conserved across the colubrid species sampled. These results contrast with the study of terrestrial and aquatic elapids, which showed great differences in the total and proportional number of vertebrae in the post-atrial region (Sherratt *et al.* 2022). There is a lack of comparative studies considering the axial skeleton of snakes with respect to post-atrial diversity to offer further comparisons, but in squamates this region exhibits substantial variation (Bergmann and Irschick 2012, Head and Polly 2015). Further research into the post-atrial region of snakes is needed to understand the mechanisms driving vertebral variation and if there has been adaptation for different functional needs. It is predicted that species that ingest long prey items, such as snakes and eels, would need a proportionally longer post-atrial region and internal organs as a structural adaptation (Jackson *et al.* 2004). Anderson and Secor (2015) have shown

there is variation in the position of internal organs in this region; however, a more targeted study of diet and habitat is needed to test this. Furthermore, the study by Head and Polly (2015) indicates a possible additional region occurring at approximately 85% pre-cloacal body length, this was also supported in the comprehensive study by Hampton and Meik (2023). We suggest further studies examine other organ positions, namely the posterior tip of the right lung, and their possible association with an additional regional boundary.

Caudal
The caudal region is where the arboreal and terrestrial species show the most variation. Arboreal species have both a greater number of vertebrae in the caudal region (Fig. 5D) and a greater relative proportion (Fig. 8C) compared to the terrestrial species. Although previous studies have not necessarily considered the axial skeleton, this pattern of long-tailed arboreal snakes is well reported across most snake clades (Vitt and Vanglider 1983, Martins *et al.* 2001, Pizzato *et al.* 2007, Hampton 2011, Lawing *et al.* 2012, Sheehy *et al.* 2016, de Alencar *et al.* 2017, Tingle and Garland 2021). Long tails are more effective as a counterweight by grasping branches during cantilevering and striking, as they have a greater overall mass and provide a longer moment arm to prevent downwards body pitch (Tingle and Garland 2021).

Since tail length is known to differ substantially between the sexes (Shine 2000), it is important to consider this as a limitation of the present study. Although our study is limited with all species only represented by a single specimen, every effort was made to balance the spread of males and females across the two ecological groups to reduce bias. Males are known to have longer tails, which enables them to adequately store their hemipenes and may assist in copulation, whereas the females typically have shorter tails since their comparative sexual organs, hemiclitori, are smaller (Folwell *et al.* 2022). Shine (2000) found some evidence of males having shorter trunks and longer tails, whereas females possess longer trunks and shorter tails. He suggested there may be a developmental mechanism which limits the species to an overall number of vertebrae and therefore the two sexes balance their allocation of vertebrae in different ways. If this is so, sex-specific selective pressures may influence this variation with the female requiring a longer trunk to carry young and the male needing a longer tail to store the hemipenes. Since we found integration between the post-atrial and caudal region, this supports the need for further investigation into this relationship. Unfortunately, the present study could not control sex-specific differences and further studies of intraspecific sex-specific vertebral columns is necessary to test this hypothesis. Regardless of sex, arboreal colubrids have a significantly greater allocation of vertebrae to their caudal regions than seen in terrestrial species, and this is the primary reason for their overall longer bodies.

The arboreal vertebral column
Arboreal colubrids are known for their physical appearance of being elongate and gracile (Harrington *et al.* 2018). While previous research on arboreal vipers has demonstrated that arboreal environments necessitate a more specialized and constrained morphology compared to terrestrial dwellers (de Alencar *et al.* 2017), our results do not support convergent evolution towards a specific arboreal colubrid body form. This could also be

explained by most vipers sharing comparable ecological roles, such as being ambush predators, whereas arboreal colubrids exhibit diversity in their ecological niches. We found that arboreal colubrid species showed as much diversity in axial profile shape as seen in terrestrial species. This suggests that arboreal colubrids might have attained arboreal adaptations through diverse evolutionary routes, leading to a broader diversity in body form compared to vipers. Harrington *et al.* (2018) provided support of this concept claiming arboreality evolved many times within the colubrids. Their results demonstrated colubrids exhibit a minimum of 13 distinct instances of arboreal origins, surpassing the estimated seven occurrences predicted in vipers (Harrington *et al.* 2018). Unlike the vipers, the arboreal colubrids of our study do not demonstrate convergent evolution, despite their conspicuous physical similarities.

One of the most common traits of arboreal snakes across all families is elongation, which has been previously examined using external linear measurements (Lindell 1994, Tingle and Garland 2021, Banci *et al.* 2022). Our findings provide evidence for the elongation of vertebral columns in arboreal colubrids occurring through an overall increase in the number of vertebrae, with arboreal colubrids on average possessing slightly over 300 vertebrae in total (Fig. 5A). However, whether this characteristic is consistent across families remains uncertain as conflicting results have been reported in different studies on arboreal vipers. Although some studies have supported the elongation of arboreal vertebral column through increased number of vertebrae in vipers (Hampton 2011), others have contradicted this concept (Tingle and Garland 2021). Further supporting our results, Banci *et al.* (2022) also identified that increase in body and tail vertebrae counts corresponded to the degree of arboreality in most species. Arboreal colubrids seem to possess the most vertebrae in their post-atrial region (~150), then their caudal region (~120), and the fewest vertebrae in their pre-atrial region (~45). The pre-cloacal vertebrae in arboreal colubrids form a consistently small and homogeneous size distribution across the vertebral column, resulting in a distinct long, flat intracolumnar profile compared to the terrestrial colubrids with more heterogeneous vertebrae sizes, indicating an ecological influence on profile shape despite the absence of convergent evolution.

Increasing the number of vertebrae provides the opportunity for varied muscular attachments, as seen in Jayne (1982) where arboreal species possess a more slender, elongated musculus semispinalis-spinalis, which may enhance arboreality-specific behaviours, such as dorsiflexion of the vertebral column for cantilevering (Jayne 1988). This is further enabled since many small vertebrae allow greater flexibility and tighter coiling (Jayne 1982, Jones and Pierce 2016). This adaptation also enhances locomotion across delicate arboreal structures and more efficient cantilevering since the longer axial muscles produce an elongated lever arm for the muscle to act through (Lillywhite *et al.* 2000). This is further supported though the slender appearance of arboreal snakes (Vitt and Vanglider 1983, Guyer and Donnelly 1990, Martins *et al.* 2001, Pizzatto *et al.* 2007, Feldman and Meiri 2013, de Alencar *et al.* 2017). This slender appearance is facilitated through smaller vertebrae and is conserved irrespective of total body size (Tingle and Garland 2021). The slender morphology of arboreal snakes offers numerous selective advantages, including enhanced gap-bridging capacity,

enhanced locomotion on narrow and fragile canopy twigs, and effective camouflage. The unique vertebral column of arboreal colubrids, composed of numerous small and similarly-sized vertebrae, underlies their physical appearance and enables their successful survival in arboreal environments.

From these intracolumnar profiles, it is possible to make inferences on the developmental mechanism underlying the arboreal body shape. Firstly, invoking the 'clock and wavefront' model (Pourquié 2003, 2011), the many small vertebrae occur under the control of a molecular oscillator by increasing the speed of somitogenesis (Gomez *et al.* 2008, Gomez and Pourquié 2009). Therefore, through heterochronic changes in somitogenesis the long slender intracolumnar profile of arboreal colubrids, or indeed any other body shape of snakes, can be modelled. Secondly, the ecological variation in heart position relating to physiology can be achieved by differentially laying down somites in each axial region. Regionalization of the snake body plan is determined by *Hox* expression boundaries as in other vertebrates (Woltering *et al.* 2009), and morphological changes in the vertebral shape along the axial column appear to coincide with some anterior *Hox* boundaries (Head and Polly 2015). In mammalian models, the position of the heart along the anterior-posterior axis is clearly understood to result from *Hox* function (reviewed in Lescroart and Zaffran 2018) and thus the snake body plan is expected to have similar mechanisms. The finding that there are distinct morphological changes in vertebrae adjacent to the snake's heart (Hampton 2022, Hampton and Meik 2023) provides strong evidence that the heart can be used to infer regional boundaries. However, it is not yet understood how homeotic control of heart position explicitly relates to the pre- and post-atrial regions identified here and elsewhere (e.g. Head and Polly 2015, Hampton 2022, Sherratt *et al.* 2022, Hampton and Meik 2023). Our study joins those aforementioned to provide the substantial evidence that these regions are evolutionarily distinct and disassociated warrants further investigation into both *Hox* gene boundaries and organ position along the snake vertebral column.

Recommendations for future research

The axial skeleton of snakes has largely been dismissed for its perceived uniform morphology, with little attention given to potential vertebral regions. Furthermore, the great number of vertebrae often precludes studies of intracolumnar variation. Yet research that has attempted to quantify the diversity have shown apparent and appreciable variation not just within the axial skeleton, but among species (Johnson 1955, Christman 1975, Smith 1975, Sarris *et al.* 2012, McCartney 2014, Head and Polly 2015, Sherratt and Sanders 2020, Sherratt *et al.* 2022, Hampton and Meik 2023). We encourage future studies to consider other ecological transitions in snake evolution. As an example, the intracolumnar profile of the short-bodied colubrid species *Xenodon severus* (Linnaeus, 1758) in this study is very short with extremely heterogeneously-sized vertebrae along the column. This specimen exhibited a very similar intracolumnar profile to *Acanthophis antarcticus* (Shaw & Nodder, 1802), a similarly shaped ambush elapid examined in Sherratt *et al.* (2022). From this we predict these species demonstrate adaptive morphology similar to the typically short and stout bodied snakes, such as vipers. Additionally, their unusually posterior

heart positions warrant further investigation. Another area are the fossorial species, which are typically small and may present the first instance of vertebral subtraction. Fossorial elapids in Sherratt *et al.* (2022) typically have the fewest vertebrae. Yet it has been shown that among typhlopids, the characteristically fossorial snakes of Scolecophidia, there is a dissociation between the number of vertebrae and the total length of the snake (Head and Polly 2007), but to which region those extra vertebrae have been added is unknown. Therefore, although it is known that snake body size and number of vertebrae varies substantially among species, and these traits are generally correlated (Lindell 1994), the deviations from this correlation (e.g. Head and Polly 2007, Sherratt *et al.* 2019), and where precisely those differences occur—the region of the body—is the next step in understanding snake evolution.

SUPPORTING INFORMATION

Supplementary data is available at *Zoological Journal of the Linnean Society* online.

ACKNOWLEDGEMENTS

AUTHOR CONTRIBUTIONS

Tamika Nash-Hahn (Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Validation, Visualisation, Writing – original draft, Writing – review and editing), Natasha Stepanova (Data curation, Writing – review and editing), Alison R. Davis Rabosky (Data curation, Writing – review and editing) and Emma Sherratt (Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualisation, Writing – original draft, Writing – review and editing). All authors read and approved the final manuscript.

FUNDING

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

DATA ACCESSIBILITY

Morphological data and R code are available from the Figshare Repository (DOI: 10.6084/m9.figshare.23206838). The novel micro-CT slice data are stored in the digital archives of the South Australian Museum and archived on MorphoSource (www.morphosource.org) project ID: 000445258.

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