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Title: Patterns of intracolumnar size variation inform the heterochronic mechanisms underlying extreme body shape divergence in microcephalic sea snakes

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Abstract

Sea snakes (Hydrophiinae) that specialise on burrowing eel prey have repeatedly evolved tiny heads and reduced forebody relative to hindbody girths. Previous research has found that these ‘microcephalic’ forms have higher counts of precaudal vertebrae, and postnatal ontogenetic changes cause their hindbodies to reach greater girths relative to their forebodies. We examine variation in vertebral size along the precaudal axis of neonates and adults of two species. In the non-microcephalic *Hydrophis curtus*, intracolumnar patterns take the form of symmetrical curved profiles, with longer vertebrae in the midbody (50% of body length) relative to distal regions. In contrast, intracolumnar profiles in the microcephalic *H. macdowelli* are strongly asymmetrical curves (negative skewness) due to the presence of numerous, smaller-sized vertebrae in the forebody (anterior to the heart). Neonate and adult *H. macdowelli* specimens both exhibit this pattern, implying an onset of fore- versus hindbody decoupling in the embryo stage. Based on this, we suggest plausible developmental mechanisms involving the presence and positioning of *Hox* boundaries and heterochronic

changes in segmentation. Tests of our hypotheses would give new insights into the drivers of rapid convergent shifts in evolution, but will ultimately require studies of gene expression in the embryos of relevant taxa.

Introduction

Evolutionary shifts in vertebrate body form are often driven by distinct developmental genetic mechanisms that underpin the number, shape and relative size of vertebrae along the axial column (Richardson et al. 1998). Embryonic somites give rise to ossified vertebrae and are formed during somitogenesis according to the clock and wavefront model, in which a travelling wavefront determines the axial position of somites and a segmentation clock controls the periodicity of their formation (Pourquié 2003; Gomez et al. 2008; Pourquié 2011). The segmentation clock and wavefront progresses at different rates across lineages resulting in highly variable vertebral counts that are known to be tightly linked to functional ecology in some taxa (e.g., in fish: Lindsey 1978). In snakes, many more smaller-sized vertebrae are formed because an accelerated clock rate relative to the overall developmental rate means that fewer cells are delineated to form a somite during each oscillation (Gomez et al. 2008; Gomez and Pourquié 2009). In addition to meristic changes, vertebrae differentiate according to the expression boundaries of *Hox* transcription factors, which control the positions of cervical, thoracic and caudal regions. Finally, differential rates of postnatal somatic growth among axial regions can generate variation along the body axis even in the absence of segmental and homeotic (*Hox*) changes.

Morphological analyses of vertebral number and size provide a powerful approach for inferring the relative influence of segmentation, homeotic regionalisation, and differential postembryonic growth in the evolution of axial variation (e.g., Polly et al. 2001; Ward and Brainerd 2007; Muller et al. 2010; Ward and Mehta 2010; Head and Polly 2015; Sherratt et al. 2019). Axial elongation is generally associated with an increase in the number of vertebrae (Richardson et al. 1998), however the evolutionary correlation between vertebral number and body size is variable among snake species (e.g., Lindell 1994; Head and Polly 2007; Sherratt et al. 2019) and other elongate taxa including caecilians, bony fishes, squamates and salamanders (e.g., Renous and Gasc 1989; Ward and Mehta 2010; Bergmann and Irschick 2012; Bonett and Blair 2017). This discordance indicates that increased body length can be achieved in some elongate taxa through lengthening of vertebrae in one or more axial regions. Such variability in the size of individual vertebra along the axial column (so called

intracolumnar variation) has been characterised in taxa such as elongate amphibians (Wake 1980; Renous and Gasc 1989; Gillis 1997) and snakes (Johnson 1955; and often for paleontological inference, e.g., Christman 1975; Smith 1975; Polly, Head, and Cohn 2001; Sarris et al. 2012; McCartney 2015), but is an understudied aspect of the evolutionary development of body form.

This study aimed to understand the development of rapid and highly-replicated evolutionary changes in the body forms of fully marine sea snakes (Hydrophiinae). ‘Microcephalic’ sea snakes are characterised by tiny heads and reduced forebody girths that can be less than a third of their hindbody girths (where forebody and hindbody are delimited by the position of the heart, e.g. Sherratt et al. 2019). These extreme body shape changes are closely linked to a specialist diet of burrowing eel prey: narrow heads and forebodies allow microcephalic sea snakes to probe eel (particularly Ophichthidae) burrows on the sea floor (Guinea 1981; Takahashi 1981). Comparative phylogenetic analyses show that the microcephalic ecomorph has evolved at least seven times in the very rapidly speciating *Hydrophis* clade and its sister lineage *Microcephalophis* (Sanders et al. 2013; Sherratt et al. 2018). A morphometric study that explored the developmental mechanisms responsible for evolutionary shifts to microcephaly found evidence for changes in both somitogenesis and postnatal somatic growth (Sherratt et al. 2019). That is, microcephalic species have greater numbers of pre-cloacal vertebrae compared to most non-microcephalic species, and postnatal ontogenetic changes cause their hindbodies to reach greater sizes/girths relative to their forebodies in adulthood.

Here, we extend previous studies of the evolutionary development of body shape changes in sea snakes by examining variation in vertebral size along the body axis (intracolumnar variation) of neonate and adult representatives of microcephalic and non-microcephalic *Hydrophis* species. If the microcephalic species have smaller forebody relative to hindbody vertebrae in the neonate as well as in the adult, this would be suggestive of prenatal heterochronic changes that have not previously been described in snake embryos. If vertebrae have symmetrical profiles in the fore- and hindbody in the neonates of all species and in the non-microcephalic adult, and vary only in the microcephalic adults, variation in postnatal growth rates alone must account for the body shape changes. Most studies of developmental patterns in the evolution of the vertebral column have addressed either meristic variation in overall vertebral number driven by somitogenetic effects, or homeotic variation in the *Hox* gene expression boundaries that delimit clearly defined (e.g. cervical versus dorsal) axial regions. Our study examines both meristic changes and regional

differentiation to generate hypotheses for the evolutionary development of highly replicate shifts to extreme body shapes in sea snakes.

Materials and Methods

Samples and X-rays

We examined intracolumnar size variance of vertebrae in three species of sea snake. Of these two are microcephalic species (*Hydrophis macdowellii* and *H. obscurus*), and one is a non-microcephalic species (*H. curtus*) (Table 1). These species are phylogenetically distant and have characteristically different body shapes and body sizes, as demonstrated in two recently published studies (Sherratt, Rasmussen, and Sanders 2018; Sherratt et al. 2019). Regarding the maximum total lengths recorded for these species (details in Sherratt, Rasmussen, and Sanders 2018), *H. macdowellii* (1160mm) and *H. obscurus* (1200mm) are shorter than *H. curtus* (1760mm). By contrast, the species-maximum number of pre-cloacal vertebrae is higher in *H. obscurus* (217) and *H. macdowellii* (193) than in *H. curtus* (141) (details in Sherratt et al. 2019). With respect to body shape, the relative girth, which is measured as a ratio of the girth at a point three-quarters down the body from the neck, over the girth at the neck (in line with first ventral scale, for details see Sherratt, Rasmussen, and Sanders 2018) is high in *H. macdowellii* (3.1) and *H. obscurus* (3.05) compared to *H. curtus* (1.2). This ratio is the determinant of *H. macdowellii* and *H. obscurus* being microcephalic species.

Specimens were obtained from the herpetology collections at South Australian Museum (SAM), Adelaide Australia, Queensland Museum (QM), Brisbane Australia, the Field Museum of Natural History (FMNH), Chicago USA, and University of Copenhagen Zoological Museum (ZMUC), Copenhagen Denmark. Sampling was limited by museum availability. We sampled adults and neonates for each species (Table 1), with neonates defined according to the size range given in Fry et al. (2001). Digital X-rays were made using a Faxitron LX-60 digital X-ray machine at University of Adelaide Health and Medical Sciences facility. Following a previous study (Sherratt et al. 2019), metal pins were placed into the preserved specimens at the posterior-most tip of the heart, to mark the boundary between fore- and hindbody, and cloaca, to mark the end of the body (Figure 1).

Measurements and analysis

Intracolumnar size variance in vertebrae was examined by measuring all vertebrae from the first pre-cloacal vertebra after the atlas to the last vertebra anterior to the cloaca. Tail vertebrae were not examined in this study because the vertebrae behind the cloaca represents a separate body region not under examination here. We measured the length of each vertebra from the X-rays using the multipoint tool in ImageJ v.1.52i (Schneider et al. 2012). Landmarks were placed medially along the vertebral column at the anterior limit of the centrum of each vertebra to capture vertebra length. Vertebral width could not be measured due to the changing orientation of the vertebral column (axial torsion) resulting from preservation. Coordinates (x,y) of the landmarks in millimetres were exported into the R statistical environment v.3.5.2 (R Development Core Team 2018) and inter-landmark distances were calculated to get the length of each vertebrae. Each X-ray was measured three times to assess and ensure repeatability, and the (Table S1), and the three replicates were averaged for each specimen and used in the following analyses.

To visually compare patterns of intracolumnar size variation among specimens (intracolumnar profiles), we standardised intracolumnar size variance of vertebrae by body size. This was done by dividing vertebra length by the sum of all vertebra lengths, resulting in relative vertebra length. This procedure adjusts for body size differences without changing the proportional differences in vertebrae size along the axial column (see Figure S1 for intracolumnar profiles prior to standardisation) and allows comparison of adult and neonate specimens for differences in their intracolumnar profiles. Then relative vertebra length was plotted against vertebra number (index). Due to differential growth of vertebrae along the axial column, this results in a curve approximated to a polynomial of the fourth degree with a positive inflection point. To quantitatively compare these intracolumnar profiles between species and ages, we calculated intracolumnar variability of vertebra length, expressed as a coefficient of variability, CV which is $100 \times (\text{standard deviation}/\text{mean})$ (sensu Johnson 1955).

We test whether microcephalic species have overall smaller forebody vertebrae relative to hindbody vertebrae in the neonate, which would indicate prenatal heterochronic changes. Forebody and hindbody are delimited by the posterior-most tip of the heart. To examine differences in the vertebral size between body regions, we tested whether vertebrae in the forebody (anterior to the posterior-most tip of the heart, herein “anterior to the heart” for short) are shorter on average compared to the hindbody (posterior to the heart) in each neonate. One-sided Welch’s t-tests (which takes into account uneven variances and numbers of vertebrae in each sample) were used to evaluate against the null hypothesis of no difference in the group means. We then compared these results to a one-sided Welch’s t-test

that tested whether vertebrae in the anterior 50% body region are shorter on average compared to the posterior 50% body region in each neonate.

Results

The three sampled *Hydrophis* species show distinct patterns of intracolumnar variation in vertebra size, which are strongly conserved within-species, through life stage (Figure 2). With respect to robusticity, the non-microcephalic species *H. curtus* has proportionally larger vertebrae along the whole axial column compared with *H. macdowellii* and *H. obscurus*. Furthermore, the coefficient of variability (CV) of vertebrae size is low in *H. curtus* (14.9-19.16/14.93.5-19.16 and 16.4), and high in *H. macdowellii* (19.01-27.32/18.9 and 26.2) and *H. obscurus* (22.10-28.45).

In *H. curtus*, the anterior-most and posterior-most vertebrae are similarly sized, creating a symmetrical profile centred roughly around the midbody (50% of body length) when graphed (Figure 2). The adults and neonate *H. curtus* show the same pattern (Figure 2). In neonate *H. curtus* we cannot reject the null hypothesis that the average vertebra length of the anterior half of the body is shorter than in the posterior half ($t = 0.39$, $df = 121.96$, $p\text{-value} = 0.65$), since they are slightly larger in the anterior (Figure 3A). There is a small and significant difference in average vertebra length between the forebody (anterior to heart) and hindbody (posterior to heart) in neonate *H. curtus* ($t = -2.84$, $df = 59.81$, $p\text{-value} = 0.0031$), where forebody vertebrae are smaller on average (Figure 3B), but this could be due to the low proportion of vertebrae in the forebody (43/137).

By contrast, both *H. macdowellii* and *H. obscurus* present a striking asymmetrical intracolumnar profile (Figure 2), and *H. obscurus* is most exaggerated owing to the longer vertebral column. The adult specimens also exaggerate the curve profile seen in the neonates (Figure 2). In both species the vertebrae in the hindbody region (below the heart) appears to show a similar profile to that found in *H. curtus*. However, the vertebrae in the forebody show a marked size reduction and coupled with the greater number of vertebrae in this region results in a long, shallow slope (Figure 2). The resulting overall profile is strongly asymmetrical (negative skewness- in mathematical terms), resulting in a maximum vertebra size two thirds of the way along the body. Vertebrae are significantly smaller in the anterior 50% than the posterior 50% of the body in neonate *H. macdowellii* ($t = -5.46$, $df = 185.71$, $p\text{-value} < 0.001$, Figure 3C; and other specimens, Figure S2), and also significantly smaller in

the forebody (anterior to heart) than the hindbody (posterior to heart) in neonate *H. macdowellii* ($t = -7.55$, $df = 150.31$, $p\text{-value} < 0.001$, Figure 3D; and other specimens, Figure S3). Similarly for neonate *H. obscurus* vertebrae in the anterior 50% are smaller than the posterior 50% (-10.62 , $df = 212.74$, $p\text{-value} < 0.001$, Figure 3E; and other specimens, Figure S2) and smaller in the forebody than the hindbody ($t = -9.24$, $df = 211.68$, $p\text{-value} < 0.001$, Figure 3F; and other specimens, Figure S3).

Discussion

The intracolumnar pattern of size variation in the non-microcephalic sea snake, *H. curtus*, is very similar to the intracolumnar patterns previously described in other snakes (Johnson 1955; Smith 1975; Polly, Head, and Cohn 2001; Sarris et al. 2012). In all of these species, vertebral size variation along the pre-caudal axis forms a symmetrical profile, with more robust vertebrae in the midbody (50% of body length) versus the anterior and posterior regions. In contrast, the microcephalic sea snakes, *H. macdowellii* and *H. obscurus*, show an asymmetrical profile (negative skewness) of intracolumnar variation that has not previously been reported in other studied snakes (or any other elongate vertebrate). This asymmetry is due to the presence of numerous, smaller-sized vertebrae in the forebody, and manifests as a long, shallow slope towards the heart and a profile similar to non-microcephalic snakes behind the heart. There is a greater disparity between fore- and hindbody segments in the adults versus neonates, which is consistent with external measurements of the postnatal ontogeny of fore- versus hindbody regions of these other species of microcephalic sea snakes.

Our results allow several inferences about the evolutionary development of extreme body shape changes in sea snakes. Head and Polly (2015) have proposed that the subtle morphological transition between fore- and hindbody regions in snake vertebral columns is suggestive of a *Hox* transition, and we have interpreted our data in light of this. However, although homeotic changes are implied by the decoupling of fore- versus hindbody growth in sea snakes (Sherratt et al., 2019, and this study Figure 2), additional heterochronic changes during somitogenesis (e.g., Gomez et al. 2008; Pourquié 2011) must account for the increased vertebral counts of microcephalic species compared to almost all non-microcephalic sea snakes. Hence, we believe that our data imply coordinated homeotic and somitogenetic changes (see Muller et al. 2010), and could be explained by one or more of the following hypotheses. Under the first hypothesis, an overall increase in somitogenesis rate (and/or increased axis length) in microcephalic relative to non-microcephalic sea snakes results in

higher vertebral counts across axial regions, and the presence of a *Hox* boundary approximate to the heart leads to the disparity in the size of vertebrae in fore- versus hindbody regions. The asymmetric intracolumnar profile in the neonate microcephalic *H. macdowellii* indicates that the heterochrony responsible for decoupling fore- and hindbody development is involved in the embryonic stage. Therefore, under this scenario, homeotic effects would be expected to lead to differential rates of prenatal somite maturation and/or vertebral growth. Alternatively, more smaller-sized segments can be formed in an axis of the same length (without coincident homeotic shifts) if clock rate is accelerated relative to overall axis growth. Hence, a heterochronic change in somitogenesis rate along the body axis provides a plausible and simpler explanation for the concomitance of reduced vertebral size and increased vertebral number in forebody versus hindbody patterning in the neonate *H. macdowellii*. However, under this scenario, homeotic effects in the postnatal stage are still needed to explain the ontogenetic increase in fore- versus hindbody size disparity (Fig 2, and Sherratt et al., 2019).

Studies of gene expression in the embryos of relevant taxa will ultimately be needed to test our hypotheses. A priority should be to examine whether the transition in relative fore- versus hindbody girth corresponds to the expression boundaries of the *HoxC8* and *HoxA7* genes implicated in the anterior morphological boundary in the snake body axis (Head and Polly 2015). If the relevant *Hox* boundaries are posteriorly shifted in microcephalic versus non-microcephalic species, this could account for the relative increase in forebody compared to hindbody vertebral counts in some species. Similarly, analyses of spatio-temporal expression patterns of clock and wavefront genes are needed to identify the heterochronic changes involved in somite formation. Several previous studies have considered the role of changes in the timing of onset or the overall rate of somitogenesis in the evolution of axial variation (Richardson et al. 1998; Ward and Brainerd 2007; Gomez et al. 2008). However, the rate of somitogenesis has been shown to decline along the vertebral column in model taxa (e.g. zebra fish: Schmidt and Starck 2004), and the evolution of an extreme gradient in somitogenesis rate along the axis has been reported in marsupials (Keyte and Smith 2012). Marsupial newborns show a dramatic rostral to caudal gradient in developmental maturation, resulting in hindlimbs that are underdeveloped compared to the forelimbs that are needed to crawl to the teat. A study of opossum development found that the rate of somitogenesis along the anterior-posterior axis slows to a greater extent in opossum compared to other studied amniote embryos (Keyte and Smith 2012). However, although experimental studies of model species have identified the genetic pathways involved in segmentation, the molecular changes

responsible for shifts in clock rates, either along the body axis or among species, remain to be known.

Studies of the developmental-genetic basis of body shape changes in viviparous sea snakes are not an easy prospect given the difficulties in obtaining embryos of known stages. Yet these efforts would give new insights into the drivers of rapid convergent shifts in evolution, and may provide the key to explaining the anomalously high speciation rates in the *Hydrophis* clade of sea snakes. At the same time, the recent and highly replicate origin of the microcephalic ecomorph offers a powerful opportunity to understand the roles and coordination of homeotic and somitogenetic changes in the evolution of new body shapes.

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Author Contributions

ES and KLS conceived the study; ES collected the data and performed the statistical analyses; ES and KLS wrote the manuscript.

Competing Interests

The authors declare that they have no competing interests.

Ethical Statement

Not applicable.

Data Accessibility

Data and R scripts available on Figshare (private link for review: <https://figshare.com/s/1e9827d36ce9ab090df5>).

Figure Captions

Figure 1 X-ray images of neonates of three species demonstrating the variation in size of vertebrae in the forebody (anterior to the posterior-most tip of the heart). A: a non-microcephalic species, *Hydrophis curtus* (QM J11175); B: a microcephalic species, *H. macdowelli* (QM J93156), the tail unfortunately missing on the specimen; C: a microcephalic species, *H. obscurus* (ZMUC R661253). Pins were used to mark the posterior tip of the heart and the cloaca (yellow circles). Heart is labelled. Pin length is 24mm. Details of specimens are given in Table 1.

Figure 2 Intracolumnar variation of the relative length of pre-cloacal vertebrae for adult and neonate specimens of *Hydrophis curtus*, *H. macdowelli*, and *H. obscurus*. Vertebra index is the sequential identifier for the measured vertebrae from head (left) to cloaca (right). Arrows denote heart position. See Figure S1 for these data prior to body-size standardisation of vertebra length.

Figure 3 Relative lengths of vertebrae by body region for exemplar neonates of *Hydrophis curtus* (QM J11175), *H. macdowelli* (QM J22453) and *H. obscurus* (ZMUC R661255). All specimens are shown in Figures S2 and S3. A, C, E: in the anterior and posterior halves of the body, divided at 50% of neck-vent-length. B, D, F: in the forebody (anterior to heart) and hindbody (posterior to heart). Asterisk denotes significant (* $p < 0.05$, ** $p < 0.001$) one-sided Welch's t-test indicating greater vertebrae size in the posterior/hindbody regions.

Table Captions

Table 1 Details of the specimens studied of the species *Hydrophis curtus*, *H. macdowelli*, and *H. obscurus*, including museum ID, body size (TL=total length and SVL=snout-to-vent length, in cm), the number of vertebrae in different body regions (forebody is anterior to heart, hindbody is posterior to heart) and total number of pre-cloacal vertebrae (excluding the atlas). QM = Queensland Museum, Brisbane Australia. SAM = South Australian Museum, Adelaide Australia. FMNH = Field Museum of Natural History, Chicago USA. ZMUC = University of Copenhagen Zoological Museum, Copenhagen Denmark.

Species	Museum ID	TL	SVL	forebody	hindbody	Total precloacal
<i>macdowelli</i>	SAM_R24236	54.6	48.4	73	114	187
<i>macdowelli</i>	QM_J22453	53.0	47.0	84	106	190

<i>macdowellii</i>	QM_J47243	86.7	77.9	85	106	191
<i>macdowellii</i>	QM_J93156	51.5	50.2	84	114	198
<i>curtus</i>	QM_J11175	31.8	27.8	43	94	137
<i>curtus</i>	FMNH_202011	80.4	72.5	42	95	137
<i>curtus</i>	FMNH_202032	66.4	59.6	41	92	133
<i>curtus</i>	FMNH_202086	73.1	64.3	40	92	132
<i>obscurus</i>	ZMUC_R661244	90.3	79.3	129	82	211
<i>obscurus</i>	ZMUC_R661253	49.3	43.8	125	89	214
<i>obscurus</i>	ZMUC_R661255	46.8	41.5	128	87	215

Supporting Information Legends

Figure S1 A graph showing the intracolumnar variation among adult and neonate specimens of *Hydrophis curtus*, *H. macdowellii*, and *H. obscurus*.

Figure S2 A graph showing the relative lengths of vertebrae in the anterior and posterior halves of the body (50% of neck-vent-length) among neonate specimens.

Figure S3 A graph showing the relative lengths of vertebrae in the forebody (anterior to heart) and hindbody (posterior to heart) regions of the body.

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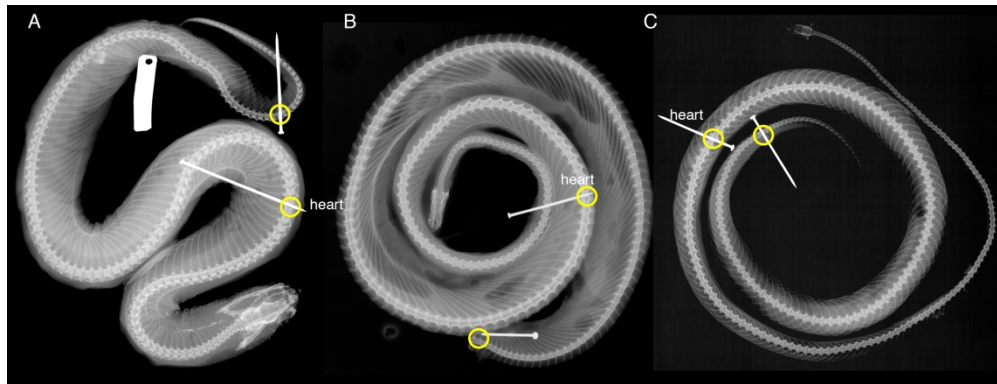


Figure 1 X-ray images of neonates of three species demonstrating the variation in size of vertebrae in the forebody (anterior to the posterior-most tip of the heart). A: a non-microcephalic species, *Hydrophis curtus* (QM J11175); B: a microcephalic species, *H. maddowelli* (QM J93156), the tail unfortunately missing on the specimen; C: a microcephalic species, *H. obscurus* (ZMUC R661253). Pins were used to mark the posterior tip of the heart and the cloaca (yellow circles). Heart is labelled. Pin length is 24mm. Details of specimens are given in Table 1.

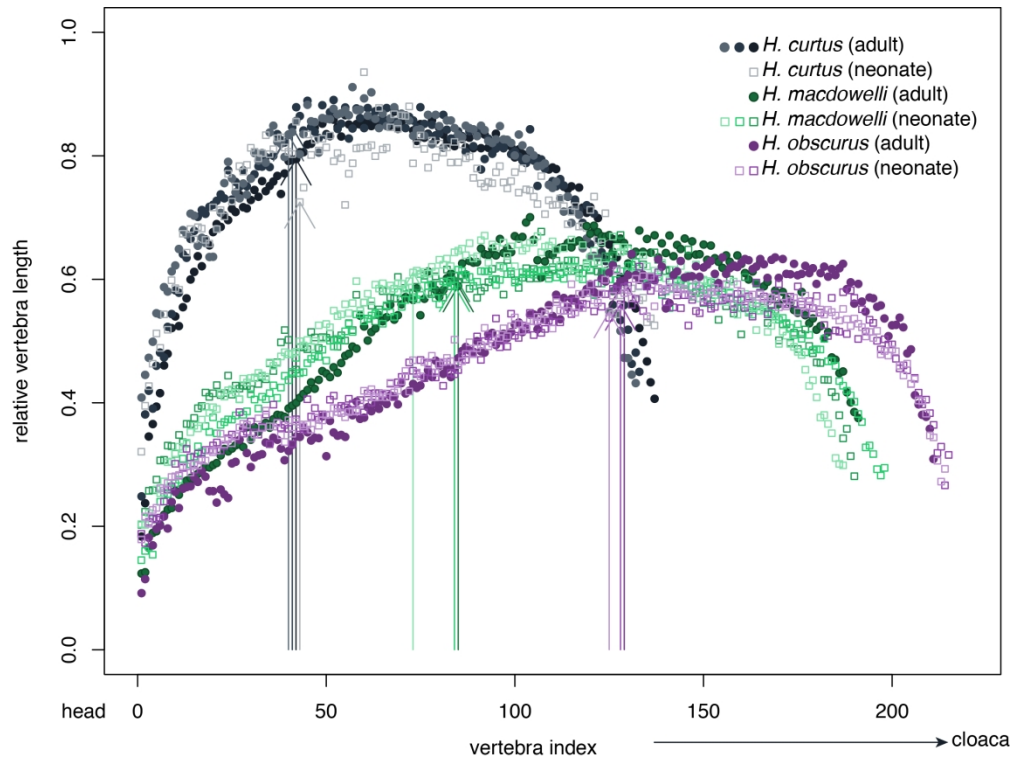


Figure 2 Intracolumnar variation of the relative length of pre-cloacal vertebrae for adult and neonate specimens of *Hydrophis curtus*, *H. maddowelli*, and *H. obscurus*. Vertebra index is the sequential identifier for the measured vertebrae from head (left) to cloaca (right). Arrows denote heart position. See Figure S1 for these data prior to body-size standardisation of vertebra length.

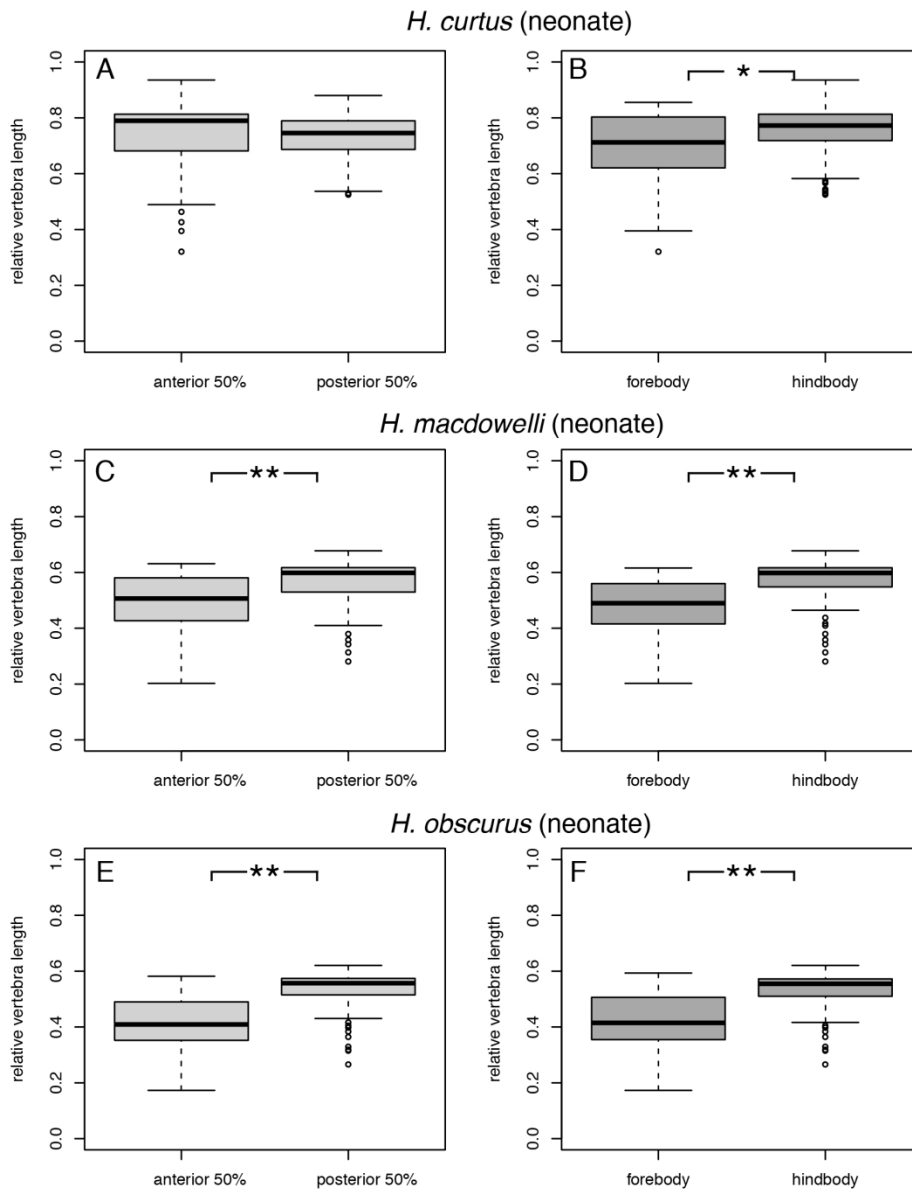


Figure 3 Relative lengths of vertebrae by body region for exemplar neonates of *Hydrophis curtus* (QM J11175), *H. maddowelli* (QM J22453) and *H. obscurus* (ZMUC R661255). All specimens are shown in Figures S2 and S3. A, C, E: in the anterior and posterior halves of the body, divided at 50% of neck-vent-length. B, D, F: in the forebody (anterior to heart) and hindbody (posterior to heart). Asterisk denotes significant (* $p < 0.05$, ** $p < 0.001$) one-sided Welch's t-test indicating greater vertebrae size in the posterior/hindbody regions.

Title: Patterns of intracolumnar size variation inform the heterochronic mechanisms underlying extreme body shape divergence in microcephalic sea snakes

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Abstract

Sea snakes (Hydrophiinae) that specialise on burrowing eel prey have repeatedly evolved tiny heads and reduced forebody relative to hindbody girths. Previous research has found that these ‘microcephalic’ forms have higher counts of precaudal vertebrae, and postnatal ontogenetic changes cause their hindbodies to reach greater girths relative to their forebodies. We examine variation in vertebral size along the precaudal axis of neonates and adults of two species. In the non-microcephalic *Hydrophis curtus*, intracolumnar patterns take the form of symmetrical curved profiles, with longer vertebrae in the midbody (50% of body length) relative to distal regions. In contrast, intracolumnar profiles in the microcephalic *H. macdowelli* are strongly asymmetrical curves (negative skewness) due to the presence of numerous, smaller-sized vertebrae in the forebody (anterior to the heart). Neonate and adult *H. macdowelli* specimens both exhibit this pattern, implying an onset of fore- versus hindbody decoupling in the embryo stage. Based on this, we suggest plausible developmental mechanisms involving the presence and positioning of *Hox* boundaries and heterochronic

changes in segmentation. Tests of our hypotheses would give new insights into the drivers of rapid convergent shifts in evolution, but will ultimately require studies of gene expression in the embryos of relevant taxa.

Introduction

Evolutionary shifts in vertebrate body form are often driven by distinct developmental genetic mechanisms that underpin the number, shape and relative size of vertebrae along the axial column (Richardson et al. 1998). Embryonic somites give rise to ossified vertebrae and are formed during somitogenesis according to the clock and wavefront model, in which a travelling wavefront determines the axial position of somites and a segmentation clock controls the periodicity of their formation (Pourquié 2003; Gomez et al. 2008; Pourquié 2011). The segmentation clock and wavefront progresses at different rates across lineages resulting in highly variable vertebral counts that are known to be tightly linked to functional ecology in some taxa (e.g., in fish: Lindsey 1978). In snakes, many more smaller-sized vertebrae are formed because an accelerated clock rate relative to the overall developmental rate means that fewer cells are delineated to form a somite during each oscillation (Gomez et al. 2008; Gomez and Pourquié 2009). In addition to meristic changes, vertebrae differentiate according to the expression boundaries of *Hox* transcription factors, which control the positions of cervical, thoracic and caudal regions. Finally, differential rates of postnatal somatic growth among axial regions can generate variation along the body axis even in the absence of segmental and homeotic (*Hox*) changes.

Morphological analyses of vertebral number and size provide a powerful approach for inferring the relative influence of segmentation, homeotic regionalisation, and differential postembryonic growth in the evolution of axial variation (e.g., Polly et al. 2001; Ward and Brainerd 2007; Muller et al. 2010; Ward and Mehta 2010; Head and Polly 2015; Sherratt et al. 2019). Axial elongation is generally associated with an increase in the number of vertebrae (Richardson et al. 1998), however the evolutionary correlation between vertebral number and body size is variable among snake species (e.g., Lindell 1994; Head and Polly 2007; Sherratt et al. 2019) and other elongate taxa including caecilians, bony fishes, squamates and salamanders (e.g., Renous and Gasc 1989; Ward and Mehta 2010; Bergmann and Irschick 2012; Bonett and Blair 2017). This discordance indicates that increased body length can be achieved in some elongate taxa through lengthening of vertebrae in one or more axial regions. Such variability in the size of individual vertebra along the axial column (so called

intracolumnar variation) has been characterised in taxa such as elongate amphibians (Wake 1980; Renous and Gasc 1989; Gillis 1997) and snakes (Johnson 1955; and often for paleontological inference, e.g., Christman 1975; Smith 1975; Polly, Head, and Cohn 2001; Sarris et al. 2012; McCartney 2015), but is an understudied aspect of the evolutionary development of body form.

This study aimed to understand the development of rapid and highly-replicated evolutionary changes in the body forms of fully marine sea snakes (Hydrophiinae). ‘Microcephalic’ sea snakes are characterised by tiny heads and reduced forebody girths that can be less than a third of their hindbody girths (where forebody and hindbody are delimited by the position of the heart, e.g. Sherratt et al. 2019). These extreme body shape changes are closely linked to a specialist diet of burrowing eel prey: narrow heads and forebodies allow microcephalic sea snakes to probe eel (particularly Ophichthidae) burrows on the sea floor (Guinea 1981; Takahashi 1981). Comparative phylogenetic analyses show that the microcephalic ecomorph has evolved at least seven times in the very rapidly speciating *Hydrophis* clade and its sister lineage *Microcephalophis* (Sanders et al. 2013; Sherratt et al. 2018). A morphometric study that explored the developmental mechanisms responsible for evolutionary shifts to microcephaly found evidence for changes in both somitogenesis and postnatal somatic growth (Sherratt et al. 2019). That is, microcephalic species have greater numbers of pre-cloacal vertebrae compared to most non-microcephalic species, and postnatal ontogenetic changes cause their hindbodies to reach greater sizes/girths relative to their forebodies in adulthood.

Here, we extend previous studies of the evolutionary development of body shape changes in sea snakes by examining variation in vertebral size along the body axis (intracolumnar variation) of neonate and adult representatives of microcephalic and non-microcephalic *Hydrophis* species. If the microcephalic species have smaller forebody relative to hindbody vertebrae in the neonate as well as in the adult, this would be suggestive of prenatal heterochronic changes that have not previously been described in snake embryos. If vertebrae have symmetrical profiles in the fore- and hindbody in the neonates of all species and in the non-microcephalic adult, and vary only in the microcephalic adults, variation in postnatal growth rates alone must account for the body shape changes. Most studies of developmental patterns in the evolution of the vertebral column have addressed either meristic variation in overall vertebral number driven by somitogenetic effects, or homeotic variation in the *Hox* gene expression boundaries that delimit clearly defined (e.g. cervical versus dorsal) axial regions. Our study examines both meristic changes and regional

differentiation to generate hypotheses for the evolutionary development of highly replicate shifts to extreme body shapes in sea snakes.

Materials and Methods

Samples and X-rays

We examined intracolumnar size variance of vertebrae in three species of sea snake. Of these two are microcephalic species (*Hydrophis macdowellii* and *H. obscurus*), and one is a non-microcephalic species (*H. curtus*) (Table 1). These species are phylogenetically distant and have characteristically different body shapes and body sizes, as demonstrated in two recently published studies (Sherratt, Rasmussen, and Sanders 2018; Sherratt et al. 2019). Regarding the maximum total lengths recorded for these species (details in Sherratt, Rasmussen, and Sanders 2018), *H. macdowellii* (1160mm) and *H. obscurus* (1200mm) are shorter than *H. curtus* (1760mm). By contrast, the species-maximum number of pre-cloacal vertebrae is higher in *H. obscurus* (217) and *H. macdowellii* (193) than in *H. curtus* (141) (details in Sherratt et al. 2019). With respect to body shape, the relative girth, which is measured as a ratio of the girth at a point three-quarters down the body from the neck, over the girth at the neck (in line with first ventral scale, for details see Sherratt, Rasmussen, and Sanders 2018) is high in *H. macdowellii* (3.1) and *H. obscurus* (3.05) compared to *H. curtus* (1.2). This ratio is the determinant of *H. macdowellii* and *H. obscurus* being microcephalic species.

Specimens were obtained from the herpetology collections at South Australian Museum (SAM), Adelaide Australia, Queensland Museum (QM), Brisbane Australia, the Field Museum of Natural History (FMNH), Chicago USA, and University of Copenhagen Zoological Museum (ZMUC), Copenhagen Denmark. Sampling was limited by museum availability. We sampled adults and neonates for each species (Table 1), with neonates defined according to the size range given in Fry et al. (2001). Digital X-rays were made using a Faxitron LX-60 digital X-ray machine at University of Adelaide Health and Medical Sciences facility. Following a previous study (Sherratt et al. 2019), metal pins were placed into the preserved specimens at the posterior-most tip of the heart, to mark the boundary between fore- and hindbody, and cloaca, to mark the end of the body.

Measurements and analysis

Intracolumnar size variance in vertebrae was examined by measuring all vertebrae from the first pre-cloacal vertebra after the atlas to the last vertebra anterior to the cloaca. Tail vertebrae were not examined in this study because the vertebrae behind the cloaca represents a separate body region not under examination here. We measured the length of each vertebra from the X-rays using the multipoint tool in ImageJ v.1.52i (Schneider et al. 2012). Landmarks were placed medially along the vertebral column at the anterior limit of the centrum of each vertebra to capture vertebra length. Vertebral width could not be measured due to the changing orientation of the vertebral column (axial torsion) resulting from preservation. Coordinates (x,y) of the landmarks in millimetres were exported into the R statistical environment v.3.5.2 (R Development Core Team 2018) and inter-landmark distances were calculated to get the length of each vertebrae. Each X-ray was measured three times to assess and ensure repeatability, and the (Table S1), and the three replicates were averaged for each specimen and used in the following analyses.

To visually compare patterns of intracolumnar size variation among specimens (intracolumnar profiles), we standardised intracolumnar size variance of vertebrae by body size. This was done by dividing vertebra length by the sum of all vertebra lengths, resulting in relative vertebra length. This procedure adjusts for body size differences without changing the proportional differences in vertebrae size along the axial column (see Figure S1 for intracolumnar profiles prior to standardisation) and allows comparison of adult and neonate specimens for differences in their intracolumnar profiles. Then relative vertebra length was plotted against vertebra number (index). Due to differential growth of vertebrae along the axial column, this results in a curve approximated to a polynomial of the fourth degree with a positive inflection point. To quantitatively compare these intracolumnar profiles between species and ages, we calculated intracolumnar variability of vertebra length, expressed as a coefficient of variability, CV which is $100 \times (\text{standard deviation}/\text{mean})$ (sensu Johnson 1955).

We test whether microcephalic species have overall smaller forebody vertebrae relative to hindbody vertebrae in the neonate, which would indicate prenatal heterochronic changes. Forebody and hindbody are delimited by the posterior-most tip of the heart. To examine differences in the vertebral size between body regions, we tested whether vertebrae in the forebody (anterior to the posterior-most tip of the heart, herein “anterior to the heart” for short) are shorter on average compared to the hindbody (posterior to the heart) in each neonate. One-sided Welch’s t-tests (which takes into account uneven variances and numbers of vertebrae in each sample) were used to evaluate against the null hypothesis of no difference in the group means. We then compared these results to a one-sided Welch’s t-test

that tested whether vertebrae in the anterior 50% body region are shorter on average compared to the posterior 50% body region in each neonate.

Results

The three sampled *Hydrophis* species show distinct patterns of intracolumnar variation in vertebra size, which are strongly conserved within-species, through life stage (Figure 2). With respect to robusticity, the non-microcephalic species *H. curtus* has proportionally larger vertebrae along the whole axial column compared with *H. macdowellii* and *H. obscurus*. Furthermore, the coefficient of variability (CV) of vertebrae size is low in *H. curtus* (14.9-19.16/14.93.5-19.16 and 16.4), and high in *H. macdowellii* (19.01-27.32/18.9 and 26.2) and *H. obscurus* (22.10-28.45).

In *H. curtus*, the anterior-most and posterior-most vertebrae are similarly sized, creating a symmetrical profile centred roughly around the midbody (50% of body length) when graphed (Figure 2). The adults and neonate *H. curtus* show the same pattern (Figure 2). In neonate *H. curtus* we cannot reject the null hypothesis that the average vertebra length of the anterior half of the body is shorter than in the posterior half ($t = 0.39$, $df = 121.96$, $p\text{-value} = 0.65$), since they are slightly larger in the anterior (Figure 3A). There is a small and significant difference in average vertebra length between the forebody (anterior to heart) and hindbody (posterior to heart) in neonate *H. curtus* ($t = -2.84$, $df = 59.81$, $p\text{-value} = 0.0031$), where forebody vertebrae are smaller on average (Figure 3B), but this could be due to the low proportion of vertebrae in the forebody (43/137).

By contrast, both *H. macdowellii* and *H. obscurus* present a striking asymmetrical intracolumnar profile (Figure 2), and *H. obscurus* is most exaggerated owing to the longer vertebral column. The adult specimens also exaggerate the curve profile seen in the neonates (Figure 2). In both species the vertebrae in the hindbody region (below the heart) appears to show a similar profile to that found in *H. curtus*. However, the vertebrae in the forebody show a marked size reduction and coupled with the greater number of vertebrae in this region results in a long, shallow slope (Figure 2). The resulting overall profile is strongly asymmetrical (negative skewness- in mathematical terms), resulting in a maximum vertebra size two thirds of the way along the body. Vertebrae are significantly smaller in the anterior 50% than the posterior 50% of the body in neonate *H. macdowellii* ($t = -5.46$, $df = 185.71$, $p\text{-value} < 0.001$, Figure 3C; and other specimens, Figure S2), and also significantly smaller in

the forebody (anterior to heart) than the hindbody (posterior to heart) in neonate *H. macdowellii* ($t = -7.55$, $df = 150.31$, $p\text{-value} < 0.001$, Figure 3D; and other specimens, Figure S3). Similarly for neonate *H. obscurus* vertebrae in the anterior 50% are smaller than the posterior 50% (-10.62 , $df = 212.74$, $p\text{-value} < 0.001$, Figure 3E; and other specimens, Figure S2) and smaller in the forebody than the hindbody ($t = -9.24$, $df = 211.68$, $p\text{-value} < 0.001$, Figure 3F; and other specimens, Figure S3).

Discussion

The intracolumnar pattern of size variation in the non-microcephalic sea snake, *H. curtus*, is very similar to the intracolumnar patterns previously described in other snakes (Johnson 1955; Smith 1975; Polly, Head, and Cohn 2001; Sarris et al. 2012). In all of these species, vertebral size variation along the pre-caudal axis forms a symmetrical profile, with more robust vertebrae in the midbody (50% of body length) versus the anterior and posterior regions. In contrast, the microcephalic sea snakes, *H. macdowellii* and *H. obscurus*, show an asymmetrical profile (negative skewness) of intracolumnar variation that has not previously been reported in other studied snakes (or any other elongate vertebrate). This asymmetry is due to the presence of numerous, smaller-sized vertebrae in the forebody, and manifests as a long, shallow slope towards the heart and a profile similar to non-microcephalic snakes behind the heart. There is a greater disparity between fore- and hindbody segments in the adults versus neonates, which is consistent with external measurements of the postnatal ontogeny of fore- versus hindbody regions of these other species of microcephalic sea snakes.

Our results allow several inferences about the evolutionary development of extreme body shape changes in sea snakes. Head and Polly (2015) have proposed that the subtle morphological transition between fore- and hindbody regions in snake vertebral columns is suggestive of a *Hox* transition, and we have interpreted our data in light of this. However, although homeotic changes are implied by the decoupling of fore- versus hindbody growth in sea snakes (Sherratt et al., 2019, and this study Figure 2), additional heterochronic changes during somitogenesis (e.g., Gomez et al. 2008; Pourquié 2011) must account for the increased vertebral counts of microcephalic species compared to almost all non-microcephalic sea snakes. Hence, we believe that our data imply coordinated homeotic and somitogenetic changes (see Muller et al. 2010), and could be explained by one or more of the following hypotheses. Under the first hypothesis, an overall increase in somitogenesis rate (and/or increased axis length) in microcephalic relative to non-microcephalic sea snakes results in

higher vertebral counts across axial regions, and the presence of a *Hox* boundary approximate to the heart leads to the disparity in the size of vertebrae in fore- versus hindbody regions. The asymmetric intracolumnar profile in the neonate microcephalic *H. macdowellii* indicates that the heterochrony responsible for decoupling fore- and hindbody development is involved in the embryonic stage. Therefore, under this scenario, homeotic effects would be expected to lead to differential rates of prenatal somite maturation and/or vertebral growth. Alternatively, more smaller-sized segments can be formed in an axis of the same length (without coincident homeotic shifts) if clock rate is accelerated relative to overall axis growth. Hence, a heterochronic change in somitogenesis rate along the body axis provides a plausible and simpler explanation for the concomitance of reduced vertebral size and increased vertebral number in forebody versus hindbody patterning in the neonate *H. macdowellii*. However, under this scenario, homeotic effects in the postnatal stage are still needed to explain the ontogenetic increase in fore- versus hindbody size disparity (Fig 2, and Sherratt et al., 2019).

Studies of gene expression in the embryos of relevant taxa will ultimately be needed to test our hypotheses. A priority should be to examine whether the transition in relative fore- versus hindbody girth corresponds to the expression boundaries of the *HoxC8* and *HoxA7* genes implicated in the anterior morphological boundary in the snake body axis (Head and Polly 2015). If the relevant *Hox* boundaries are posteriorly shifted in microcephalic versus non-microcephalic species, this could account for the relative increase in forebody compared to hindbody vertebral counts in some species. Similarly, analyses of spatio-temporal expression patterns of clock and wavefront genes are needed to identify the heterochronic changes involved in somite formation. Several previous studies have considered the role of changes in the timing of onset or the overall rate of somitogenesis in the evolution of axial variation (Richardson et al. 1998; Ward and Brainerd 2007; Gomez et al. 2008). However, the rate of somitogenesis has been shown to decline along the vertebral column in model taxa (e.g. zebra fish: Schmidt and Starck 2004), and the evolution of an extreme gradient in somitogenesis rate along the axis has been reported in marsupials (Keyte and Smith 2012). Marsupial newborns show a dramatic rostral to caudal gradient in developmental maturation, resulting in hindlimbs that are underdeveloped compared to the forelimbs that are needed to crawl to the teat. A study of opossum development found that the rate of somitogenesis along the anterior-posterior axis slows to a greater extent in opossum compared to other studied amniote embryos (Keyte and Smith 2012). However, although experimental studies of model species have identified the genetic pathways involved in segmentation, the molecular changes

responsible for shifts in clock rates, either along the body axis or among species, remain to be known.

Studies of the developmental-genetic basis of body shape changes in viviparous sea snakes are not an easy prospect given the difficulties in obtaining embryos of known stages. Yet these efforts would give new insights into the drivers of rapid convergent shifts in evolution, and may provide the key to explaining the anomalously high speciation rates in the *Hydrophis* clade of sea snakes. At the same time, the recent and highly replicate origin of the microcephalic ecomorph offers a powerful opportunity to understand the roles and coordination of homeotic and somitogenetic changes in the evolution of new body shapes.

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Author Contributions

ES and KLS conceived the study; ES collected the data and performed the statistical analyses; ES and KLS wrote the manuscript.

Competing Interests

The authors declare that they have no competing interests.

Ethical Statement

Not applicable.

Data Accessibility

Data and R scripts available on Figshare (private link for review: <https://figshare.com/s/1e9827d36ce9ab090df5>).

Figure Captions

Figure 1 X-ray images of neonates of three species demonstrating the variation in size of vertebrae in the forebody (anterior to the posterior-most tip of the heart). A: a non-microcephalic species, *Hydrophis curtus* (QM J11175); B: a microcephalic species, *H. macdowelli* (QM J93156), the tail unfortunately missing on the specimen; C: a microcephalic species, *H. obscurus* (ZMUC R661253). Pins were used to mark the posterior tip of the heart and the cloaca (yellow circles). Heart is labelled. Pin length is 24mm. Details of specimens are given in Table 1.

Figure 2 Intracolumnar variation of the relative length of pre-cloacal vertebrae for adult and neonate specimens of *Hydrophis curtus*, *H. macdowelli*, and *H. obscurus*. Vertebra index is the sequential identifier for the measured vertebrae from head (left) to cloaca (right). Arrows denote heart position. See Figure S1 for these data prior to body-size standardisation of vertebra length.

Figure 3 Relative lengths of vertebrae by body region for exemplar neonates of *Hydrophis curtus* (QM J11175), *H. macdowelli* (QM J22453) and *H. obscurus* (ZMUC R661255). All specimens are shown in Figures S2 and S3. A, C, E: in the anterior and posterior halves of the body, divided at 50% of neck-vent-length. B, D, F: in the forebody (anterior to heart) and hindbody (posterior to heart). Asterisk denotes significant (* $p < 0.05$, ** $p < 0.001$) one-sided Welch's t-test indicating greater vertebrae size in the posterior/hindbody regions.

Table Captions

Table 1 Details of the specimens studied of the species *Hydrophis curtus*, *H. macdowelli*, and *H. obscurus*, including museum ID, body size (TL=total length and SVL=snout-to-vent length, in cm), the number of vertebrae in different body regions (forebody is anterior to heart, hindbody is posterior to heart) and total number of pre-cloacal vertebrae (excluding the atlas). QM = Queensland Museum, Brisbane Australia. SAM = South Australian Museum, Adelaide Australia. FMNH = Field Museum of Natural History, Chicago USA. ZMUC = University of Copenhagen Zoological Museum, Copenhagen Denmark.

Species	Museum ID	TL	SVL	forebody	hindbody	Total precloacal
<i>macdowelli</i>	SAM_R24236	54.6	48.4	73	114	187
<i>macdowelli</i>	QM_J22453	53.0	47.0	84	106	190

<i>macdowellii</i>	QM_J47243	86.7	77.9	85	106	191
<i>macdowellii</i>	QM_J93156	51.5	50.2	84	114	198
<i>curtus</i>	QM_J11175	31.8	27.8	43	94	137
<i>curtus</i>	FMNH_202011	80.4	72.5	42	95	137
<i>curtus</i>	FMNH_202032	66.4	59.6	41	92	133
<i>curtus</i>	FMNH_202086	73.1	64.3	40	92	132
<i>obscurus</i>	ZMUC_R661244	90.3	79.3	129	82	211
<i>obscurus</i>	ZMUC_R661253	49.3	43.8	125	89	214
<i>obscurus</i>	ZMUC_R661255	46.8	41.5	128	87	215

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