**Differential growth of body segments explains ontogenetic shifts in organ position for the Diamondback Water Snake** *(Nerodia rhombifer)*

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Differential growth of body segments explains ontogenetic shifts in organ position for the
Diamondback Water Snake (*Nerodia rhombifer*)

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Differential growth of body segments explains ontogenetic shifts in organ position for the Diamondback Water Snake (*Nerodia rhombifer*)

Gretchen E. Anderson and Stephen M. Secor

**Abstract**

As snakes grow, their organs move anteriorly relative to body size. We explored a developmental explanation for the ontogenetic shift in the relative position of internal organs for snakes using the Diamondback Water Snake (*Nerodia rhombifer* [Hallowell, 1852]). With age, this water snake’s heart, liver, small intestine, and right kidney move anteriorly by 2.5-5.0 percentage points of snout-vent length. The number of precaudal vertebrae did not vary due to size or sex. The anterior edge of the heart, liver, small intestine, and right kidney were typically aligned within a span of 4-8 vertebrae that likewise did not differ as a function of size or sex. Snakes exhibited a positive relationship between the number of precaudal vertebrae and the vertebra number aligned with each organ. Total length, centrum length, centrum width, ball width, height, and mass eight vertebrae sampled at consistent vertebral number revealed that vertebrae in the middle region of the body grow at a greater rate than vertebrae at the anterior or distal ends of the body. For *N. rhombifer* the observed forward shift in relative organ positions is the product of regional differences in the growth of body segments. Predictably, these differences arise from a developmental program generated by the differential expression of *Hox* genes.
**Keywords:** Development, Diamondback Water Snake. *Nerodia rhombifer*, Ontogeny, Organ position
Introduction

Organ placement within the body cavity of snakes has been shown to vary interspecifically as a function of habitat and to be conserved phylogenetically (Lillywhite 1987; Seymour 1987; Gartner et al. 2010; Lillywhite et al. 2012). In addition, intraspecific variation in organ position has been identified as correlates of both sex and body size (Bergman 1956, 1961, 1962, 1963; Anderson and Secor 2015). Females possess more anteriorly-positioned hearts, livers, pancreas, and spleens, and more posteriorly-placed kidneys as compared to conspecific males, independent of body size, in species of viperids, elapid, and colubrid snakes (Bergman 1956, 1961, 1962, 1963; Collins and Carpenter 1969; Nasoori et al. 2014). It is suggested that for female snakes the forward shift of the heart, liver, pancreas, and spleen, and the distal placement of the kidneys provides more space between these sets of organs for developing embryos and eggs (Collins and Carpenter 1969).

Understandably, as a snake lengthens with age the absolute distance between its snout and each organ increases. However, the relative distance between the snout and organs (expressed as a fraction or percentage of snout-vent length; [SVL]) decreases with size. Across a diverse lineage of snakes that include two boids, a pythonid, two viperids, an elapid, and seven colubrids, the anterior edge of the heart and liver shifts forward in the body cavity by an average of 4.0±0.6% and 5.0±0.6% of SVL, respectively, from their position in neonates and juveniles to their position in adults (Bergman 1956; 1962; 1963; Anderson and Secor 2015). For example, the leading edge of the heart and liver for juvenile kingsnakes, Lampropeltis getula (L., 1766) of 25 cm SVL are positioned at 19.4% and 31.8% of SVL, respectively; whereas for an adult of 100 cm SVL, these
organs start at 14.9% and 24.3% of SVL, respectively (Anderson and Secor 2015). Similar forward shifts in relative position have likewise been observed for other organs (e.g., pancreas, gall bladder, spleen, and kidneys) (Bergman 1962, 1963; Anderson and Secor 2015). A study of ten species (two boids, a pythonid, two viperids, and five colubrids) revealed that the anterior edges of the small intestine and right kidney shift forward with age by an average of 6.1±1.1 and 2.0±0.8 percentage points, respectively, relative to SVL. In short, as a snake lengthens with age, many of its internal organs appear to move forward in the body cavity relative to body length (Bergman 1962, 1963; Anderson and Secor 2015).

The forward movement of the relative position of organs would serve to bring organs relatively closer to the head and further from the tail. The only adaptive rationale so far advanced for variation in organ position is the proposed hypothesis that arboreal snakes possess hearts more anteriorly placed within the body cavity to facilitate sufficient vascular pressure and perfusion to the head when vertically oriented (Lillywhite 1987; Seymour 1987). If, for the species studied by Bergman (1956, 1962, 1963) and Anderson and Secor (2015), individuals become more arboreal with age and size we could consider this proposal as an adaptive explanation for the apparent shift in heart position. However, there is no evidence that any of these species become more arboreal with age. Additionally, there is currently no adaptive explanation for the anterior shift of other organs, either to bring them relatively closer to the head or further from the tail. Since we are unable to develop an adaptive or functional explanation for the collective forward shifting in the relative position of organs, we shall consider a developmental explanation.
The ophidian axial musculoskeletal system is fairly simple, consisting of long series of largely undifferentiated vertebra and ribs, with their associated muscles. Within the body cavity, organs are serially arranged: anteriorly are the heart, lung, esophagus, and liver; followed in the middle region by the stomach, pancreas, spleen, gallbladder, and the start of the small intestine; and distally are the gonads, large intestine, and kidneys (Fig. 1). The location within the body cavity where each organ develops is embryonically pre-determined by a developmental program (Grapin-Botton and Melton 2000). This program of organ regionalization is initiated by the cross-talk involving transcription factors (e.g., homeobox \([\text{Hox}]\) genes) between the gut mesoderm and endoderm (Grapin-Botton and Melton 2000). For snakes, a result of this regionalization program is the positioning of organs within a particular span of body segments. Interspecific variation in organ position would therefore stem from differences in the particular body segment(s) within which organs develop, or to species-specific differences in the length of individual body segments. If each organ is developmentally fixed a particular set of body segments, then ontogenetic shifts in relative organ position must result from the differential growth of body segments.

Regional variation in body growth has been documented for three species of caecilians and the rat, *Rattus norvegicus* (Berkenhout 1769). For the caecilians, vertebrae in the middle region of the body grow at a greater rate than vertebrae in the most anterior and distal portions of the body, whereas for the rat, it is the sacral and caudal vertebrate that exhibits the highest rate of growth. (Wake 1980; Bergmann et al. 2006). Caudal vertebrae likewise experience ontogenetic variation in growth. Noted for several species of lizards, the proximal caudal vertebrate experience positive allometry, whereas the
distal vertebrae experiences negative allometry with size (Bergmann and Russell 2001; Bergmann et al. 2003, 2004)

The foregoing leads to two questions: first, do organs physically shift within the body cavity as a snake increases in size, or do they remain stationary, fixed to a body segment(s); and second, if the latter is true, are the shifts in position therefore a product of differential growth of body segments. To address these questions, we used the Diamondback Water Snake, *Nerodia rhombifer* (Hallowell 1852), to examine the position of organs with respect to vertebrae, and to determine if there are regional variations in vertebral growth rates. In this study we focused our attention on four organs; the heart, liver, small intestine, and right kidney. These organs span the length of the body cavity and have been documented to move anteriorly (relative to SVL) with an increase in body size for *N. rhombifer* (Anderson and Secor 2015). We shall demonstrate that, over a four-fold range in body length, organs did not exhibit any size-dependent changes in their alignment to body segments. Our measurements of eight precaudal vertebrae samples at constant vertebral numbers along the length of the body for each snake revealed regional differences in growth rates, thus providing an explanation for the anterior shift in relative organ position for *N. rhombifer* and other snakes.

**Methods**

**Specimen source and handling**

The diamondback water snake, one of the largest species of water snakes, inhabits a variety of aquatic habits in south-central United States and eastern Mexico (Gibbons and Dorcas 2004). It is an active foraging snake that feeds predominately on fish
(Mushinsky and Hebrard 1977; Gibbons and Dorcas 2004). The snakes used in this study were captured by hand from commercial catfish ponds in LeFlore County, Mississippi over a 15-year span. We housed water snakes in large tanks (3000 L) under a 14h:10h light:dark cycle at 25-28°C. Snakes were fed catfish weekly and had continuous access to water. The data used in this study originated from 209 water snakes that were originally used in other studies (Secor and Nagy 2003; Cox and Secor 2010; Secor and Bessler 2012; Secor et al. 2012). Snakes were dissected either fresh (after severing the spinal cord immediately posterior to the head) or after frozen storage. Snout-vent length, total length (TL) and body mass of the 209 snakes averaged (± 1 standard error) 69.8 ± 1.2 cm, 91.3 ± 1.6 cm, and 273.4 ± 12.8 g, respectively. Following a mid-ventral incision, we measured for all 209 snakes the distance from the tip of the snout to the anterior edge of the heart, liver, small intestine, and right kidney (Fig. 1). For 162 of these snakes we counted the number of ventral scales (starting from the gular scale and ending at the scale immediately anterior to the cloaca), and noted which scale was aligned closest to the anterior edge of each of the four organs. Following the removal of organs and skin, the carcass was dried and skeletonized in a dermestid colony. From 130 cleaned skeletons we counted the number of vertebrae and/or measured vertebral morphology. All animal care protocols and experimental procedures were conducted with the approval of the University of Alabama Institutional Animal Care and Use Committee.

**Organ position in relation to vertebra position**
There is a correlation between the number of precloacal ventral scales and the number of precaudal vertebrae among alethinophidian snakes (excluding *Acrochordus* and many members of Hydrophinae) (Ruthven and Thompson 1913; Alexander and Gans 1966; Voris 1975). However, if each ventral scale does develop aligned to each somite, they are therefore not directly aligned with each vertebra given that each vertebra originates from adjoining portions of two consecutive somites (Aoyama and Asamoto, 2000). We still wanted to ensure that ventral scale number closely identifies the vertebra number aligned to each organ in our study. Therefore, we counted the number of ventral scales (from gular to vent) and the number of precaudal vertebrae from prepared skeletons of 80 snakes (mean SVL = 70.6±1.8 cm, range 28 – 107 cm). As illustrated in Results, we found a strong match between the number of precloacal ventral scales and the number of precaudal vertebrae. Therefore, we are confident of our use of ventral scale number to identify the vertebra closely aligned to the cranial edge of the heart, liver, small intestine, and right kidney.

**Differential growth of body segments**

To determine whether regions of the body grow at different rates, we measured the size and mass of eight precaudal vertebrae selected at roughly regular intervals of the vertebral column for 75 snakes (19 females and 56 males). The 75 snakes spanned a four-fold range in SVL (23.8 – 99.3 cm, mean = 61.6±2.6 cm) and were selected because they provide an ontogenetic series of snake sizes that are evenly distributed across the range of SVL. For each prepared skeleton, we counted the total number of precaudal vertebrae and measured and weighed the 10th, 25th, 45th, 65th, 85th, 105th, 125th, and 140th
vertebra (counting from the head). For each vertebra, we used digital calipers to measure (to 0.01 mm) total length (TL), centrum length (CL), centrum width (CW), ball width (BW), and height (H), and an analytical balance to measure mass (M; to 0.1 mg) (Fig. 2).

**Data analysis**

To demonstrate the relationships between organ position and SVL, we undertook linear regression (OLS) analyses of absolute (distance from snout to anterior edge) and relative (distance/SVL) organ position. The latter set of analyses served to illustrate the anterior shifting of organs relative to SVL with an increase in body size. We tested separately for the effects of SVL and sex on the number of precaudal vertebrae and on the vertebra number aligned to the leading edge of each organ applying general linear models (GLM). Since there were no significant interactions between SVL and sex, our models incorporate only the main effects of SVL and sex. To assess whether the number of ventral scales are equally matched to the number of vertebrae, we employed a pair t-test comparing the number of ventral scales with the number of vertebrae for 80 individuals. Given the observed variation among snakes in the number of vertebrae and the range of vertebrae aligned to each organ, we tested for a correlation (Pearson correlation) between the total number of vertebrae and the vertebrae number aligned to each organ. We followed those analyses with similar correlation tests to identify any relationships between organs in vertebrae alignment.

Prior to examining differences in growth among the selected vertebrae, we checked for data normality and linearity (Kolmogorov-Smirnov) for each vertebral position and measurement. Each measured dimension (total length, centrum length,
centrum width, ball width, and height) demonstrated normality and linearity, however
mass did not. We therefore log transformed vertebral mass and tested with log
transformed body mass (rather than SVL). Since sex was found not to be a significant
factor in any case, GLM analyses of vertebral dimensions and mass included the main
and interactive effects of SVL and vertebral position. Finding significant interactions
between SVL and vertebral position (to be presented) for each measurement, we
conducted t-tests to identify if there are significant differences in the scaling relationships
(i.e., slopes) between pairs of vertebral positions. For this set of analyses and others were
we are simultaneously undertaking multiple comparisons, we applied a Bonferroni
correction to our level of significance. In other cases, statistical significance was
established at α = 0.05. Results are reported as mean ± 1 SE and all statistical analyses
were performed using SAS (SAS, Cary, NC).

Results

Relative organ position and vertebrae number

Distances from the snout to the anterior edge of each organ increased as a linear
function of SVL (F values = 3779-19338, \( r^2 = 0.949-0.990, P \) values < 0.0001).
However, the relative position of each of the four organs decreases as a function of SVL
(F values = 21.6-80.3, \( r^2 = 0.096-0.283, P \) values < 0.0001) (Fig 3). Taken from these
relationships; for a young water snake of 25 cm SVL, the leading edges of the heart,
liver, small intestine, and right kidney are positioned at points approximately equivalent
to 21.3%, 32.2%, 60.5%, and 79.2% of SVL, respectively. For an adult snake of 100 cm
SVL, the same organs are positioned at points equivalent to 18.9%, 30.0%, 55.5%, and 76.0% of SVL, respectively.

For 130 cleaned skeletons, the number of precaudal vertebrae ranged from 142-150, averaging 146.1 ± 0.2 (Fig. 4). We found that neither SVL nor sex (101 males, 29 females) had a significant effect (SVL $F=0.12$, $P>0.732$; sex $F=0.04$, $P>0.850$) on the number of precaudal vertebrae.

Alignment of organs and vertebrae

For 80 water snakes, our counts of precaudal ventral scales were significantly matched (paired t-test; $t=-1.49$, $P>0.139$) with the subsequent counts of precaudal vertebrae from prepared skeletons (Fig. 5). For each individual, the difference between the number of precloacal ventral scales (mean 145.7 ± 0.3) and the number of precaudal vertebrae (mean 145.5 ± 0.3) averaged 1.24±0.11 (range = 0 – 3). Therefore, we believe that our ventral scale counts accurately identify the specific vertebra aligned with the cranial edge of each organ.

Across the four-fold range in SVL, we found that the vertebra number aligned with each organ did not vary as a function of SVL or sex (SVL $F$ values = 0.00-0.18, $P$ values = 0.676-0.979; sex $F$ values = 0.00-2.51; $P =$ values 0.115-0.997) (Fig. 5). For these snakes, the anterior edge of the heart was generally aligned with vertebra 26, 27, 28, or 29 (77% of individuals), though it ranged between vertebra 23 and 33 (Fig. 6A). The leading edge of the liver ranged in its alignment between vertebra 36 and 50, with 85% of observations between vertebra 42 and 47 (Fig. 6B). The anterior edge of the small intestine ranged in its alignment between vertebra 73 and 90, with a majority (74%)
aligned to vertebra 77 through 84 (Fig. 6C). For the majority (77%) of right kidneys, the anterior edge was aligned to vertebra 105 through 112, though ranging from vertebra 101 to 122 (Fig. 6D).

This variation in the vertebra aligned to the anterior edge of each of the four organs is due in part to the variation among snakes in the number of precaudal vertebrae. This was demonstrated by plotted the aligned vertebra number for each organ against the number of precaudal vertebrae (Fig. 7). For each organ, there was a significant correlation ($r = 0.404-0.477$, $P$ values $< 0.0001$) between the aligned vertebra and the number of precaudal vertebrae. The trend is that for snakes with fewer precaudal vertebrae (e.g., 140 – 144) to have their organs aligned with a vertebra at the lower end of the range, and vice versa.

Driven by these relationships there was also significant correlations ($r = 0.238-0.498$, $P$ values $< 0.0012$) between organs in their alignment, with the exception of the liver and right kidney ($r = 0.191$, $P = 0.015$). In general, if a snake’s heart was more anteriorly placed compared to other snakes, so was its liver, small intestine, and right kidney (Fig. 8).

**Differential Growth of Body Segments**

Total length, centrum length, centrum width, ball width, and height of the eight selected vertebrae increased as a linear function of SVL (Table 1). Vertebral mass (logged) likewise increased ($P < 0.0001$) linearly with body mass (logged) at each position (Fig. 7F). In addition to SVL, there was a significant effect of vertebrae position ($F$ values $= 5.07-18.47$, $P$ values $< 0.0001$), and significant interactions between SVL and position ($F$
= 17.07-152.8, \( P \) values < 0.0001) for each measurement. The former results illustrates that the selected vertebrae differ in size along the vertebral column of these snakes, and the latter findings demonstrate that the scaling relationships (i.e., scopes) between vertebrae dimension and SVL vary among the eight vertebrae.

For each measurement there were significant differences (\( t = 3.11-27.95, \ P \) values < 0.001) between slopes among the eight vertebral positions (Fig. 9). The trend was fairly consistent among measurements. Slopes were the highest for vertebrae in the middle region of the body (vertebrae 65 and 85), followed by the vertebrae that proceed and follow these two (45 and 105), followed by vertebrae 25 and 125, and then by vertebrae 140 and 10 (Fig. 9). Vertebral mass likewise followed this trend, as vertebrae in the middle region of the body exhibited a greater increased in mass with body mass, than vertebrae closer to the head and tail (Fig. 9F). In short, as \textit{N. rhombifer} increases in length, its vertebrae in the middle region of the body increase in all dimensions and in mass at a faster rate than vertebrae at the anterior and distal ends of the body.

**Discussion**

Snakes experience with growth an anterior shifting in the position of their internal organs relative to body size (Bergman 1956, 1962, 1963; Anderson and Secor 2015). This phenomenon is apparently conserved across broad taxonomic lineages, habitat preferences, feeding habits, and sex (Anderson and Secor 2015). Hypothesizing a developmental mechanism underlying the ontogenetic shift in relative organ position, we examined variation in organ-vertebra alignment and vertebrate size as a function of body
size. For the diamondback water snake, organ alignment to vertebrae did not change with size. However, there were distinct differences in how vertebrae grow among different regions of the body. Vertebrae in the center of the body grew at a greater rate, while those vertebrae of the anterior region grew at a slower rate. This ontogenetic pattern in development produces the basic mechanism for the observed movement in relative organ position, a trait potentially characteristic of snakes.

**Variation in vertebrae number**

Snakes exhibit distinct variation in the number of precaudal (=trunk) vertebrae evident at the family (2-fold), genus (1.8-fold), and species (1.5-fold) level (Alexander and Gans 1966; Voris 1975). Across diverse families of snakes (e.g., Typhlopidae, Boidae, Hydrophiidae, and Colubridae), the two-fold range in precaudal vertebra number typically spans 130-150 vertebrae at the low end of the range and 250-300 vertebrae at the high end (Alexander and Gans 1966; Voris 1975). The range and variation in the number of precaudal vertebrae we observed for *N. rhombifer* is similar to that noted for species both within families phylogenetically distant from *Nerodia* (e.g., Typhlopidae, Uropeltidae, Boidae, and Hydrophiidae) and those more closely related (e.g., other species of *Nerodia* and species of *Regina* and *Thamnophis*) (Ruthven and Thompson 1913; Wright and Wright 1957; Alexander and Gans 1966; Voris 1975). Wright and Wright (1957) documented that *N. rhombifer* possesses 133-150 ventral scales, a range wider than we observed (142-150). The fact that all snakes used in this study were collected from the same locality and that the number of vertebrae has a strong genetic
basis (Arnold 1988) would explain why we observed a smaller range in the number of ventral scales.

Intraspecific variation in vertebral number has been identified as a function of size and sex. *Nerodia rhombifer* displays great sexual size dimorphism, with adult females 2-3 times greater in mass than adult males (Gibbons and Dorcas 2004). It is proposed that larger female size in snakes is a product of selection for larger clutches and thus greater reproductive success (Shine 1994). For vertebrate groups that lack distinct regionalization of the vertebral column (e.g., snakes), body size is strongly correlated to the number of vertebrae (Head and Polly 2007). This phenomenon of pleomerism has been documented for colubrid, viperids, and elapid snakes across multiple continents (Lindell, 1994). For sexually dimorphic snakes with females being the larger sex, the trend is for females to possess more precaudal vertebrae than males (Shine 2000). However, for New World natricines (which includes *Nerodia*), this is not the case, as males and females do not differ in the number of vertebrae regardless of size (Shine 2000). Our findings agree with this assessment; we found no effect of either sex or size on the number of precaudal vertebrae. A caveat of this outcome is that we included individuals of all age classes in our analysis and it may only be for full-grown adults that any match between body size and vertebral number would be evident. In this study, we limited body size to a maximum of 100 cm SVL, as this is the maximum size that includes both male and female snakes. When we selected the data for snakes > 80 cm SVL (thereby including the largest males) and included data (not used in the present study) from even larger females (101-118 SVL), the outcome is the same; there is no relationship between body size and the number of precaudal vertebrae.
Organ and vertebrae association

The matching between the number of pre-cloaca ventral scales and precaudal vertebrae for *N. rhombifer* and other snakes stems from a pattern of repeated series of trunk units that include a vertebra, attached ribs, adjoining muscles, spinal nerves, a band of lateral scales, and a single ventral scale. Body segments are also associated with individual organs, as illustrated by the alignment of organs to a specific span of vertebrae independent of body size (Fig. 6). Thus, the relative forward movement of organs is not due to the shifting forward from one body segment to the next. The association between organ position (i.e., the anterior edge of the organ) and vertebral number is not precise, and varies within a span of 10 - 20 vertebrae. This variation is due in part to the variation in the number of precaudal vertebrae (Fig. 7) and the spatial association of organs (Fig. 8; Anderson and Secor 2015).

The ventral scale number aligned with the leading edge of organs in *N. rhombifer* is similar to those previously noted by Garrigues (1962) for *N. erythrogaster* (Forster 1771), *N. sipedon* (L. 1758), and several species of *Thamnophis*. That study illustrated the variation in the ventral scales aligned to internal organs among North American colubrids. For example, the anterior edge of the heart was aligned with ventral 21 for *Heterodon nasicus* (Baird and Girard 1852) and with ventral 47 for *Pituophis melanoleucus* (Daudin 1803), while the anterior border of the right kidney ranged from ventral 87 for *Heterdon platyrhinos* (Sonnini and Latreille 1801) to 186 for *Lampropeltis getula* (Garrigues 1962). When combining the variation in relative organ position as a function of habitat, phylogeny, and sex, with the three-fold range in the number of
precaudal vertebrae, it is evident that the developmental placement of snake organs during embryonic segmentation is a product of multiple selective and genetic forces.

**Segmentation and differential growth**

The ophidan postcranial (and precaudal) axial skeleton is characterized by reduced regionalization (“deregionalization”) and is constructed as a homogenous series of vertebrae and connecting pair of ribs (Woltering et al. 2009). The distinct regionalization of the axial skeleton for the majority of vertebrates is the product of differential expression of homeobox (*Hox*) genes that are arranged serially in domains along the antero-posterior axis of the body (Woltering et al. 2009). Boundaries of *Hox* gene expression denote the transition between distinct regions (cervical, thoracic, etc.) of the axial skeleton (Burke et al. 1995). The lack of such regionalization and transitions in snakes was earlier explained by a homologous anterior expansion of *Hox* genes that code for thoracic rib-bearing segments, and the suppression of *Hox* coding for the development of cervical vertebrae, forelimb girdle, and forelimbs (Cohn and Tickle 1999). Later studies identified a more vertebrate-conserved collinear pattern of *Hox* gene expression that includes boundaries that would normally code for distinct regional differentiation of the axial skeleton (Woltering et al. 2009; Di-Poi et al. 2010). Those studies proposed that the disassociation between *Hox* gene expression and axial phenotype in snakes stems from downstream alterations in regulatory pathways (Woltering et al. 2009; Di-Poi et al. 2010).

Both of these hypotheses and the described “deregionalization” of the snake’s axial skeleton have been debated recently by Head and Polly (2015). Their geometric morphometric analysis of vertebral morphology for 40 species of snakes of diverse taxa
revealed three to four regions of the ophidian axial skeleton that are distinctly different in vertebral shape. Although the vertebral morphology of snakes exhibits less serial variation and is more gradual in transition between regions than limbed squamates (i.e., lizards), they both possess the same degree of regionalization. When Head and Polly (2015) mapped *Hox* gene expression boundaries for *Pantherophis guttatus* (from Woltering et al. 2009) to their regional models of precaudal vertebrae for this snake, they found fairly close matching between expression boundaries and identified sites of regional transition (within 5-10 vertebrae). They hypothesized that *Hox* gene expression and function, along with regional phenotype of the axial skeleton, are conserved traits among amniotes and that the evolution of the snake body plan stemmed from developmental modifications that resulted in the loss of limbs and the addition of serial repeats of body segments (Head and Polly 2015).

*Hox* gene associated regionalization of the vertebral column of *N. rhombifer* is suggested by the regional variation in growth allometries and modest differences in morphology. Although the precaudal vertebrae of this snake are all similar in shape, there are subtle differences. Notable is that the anterior most vertebrae (first 25-30) possess a relatively longer ventral spine (i.e., hypapophysis), equal in length to the dorsal neural spine. Beyond this point the hypapophysis is reduced and the postzygapophyses extend more laterally, broadening the vertebrae. Closer to the tail (last 20-25 precaudal vertebrae), the vertebrae gradually decrease in size before transitioning to caudal vertebrae. The differential growth rates of vertebrae with ontogeny for *N. rhombifer* could stem from: (1) differences in the *Hox* gene group or groups link to different regions of the precaudal vertebral column; (2) differential expression (temporal and/or spatial).
within a *Hox* group; and/or, (3) regional differences in downstream regulation in phenotype expression. Regardless of the developmental mechanism(s), the fact that vertebrae in the middle region of the body grow at a faster rate than those of the anterior region provides the simple explanation for the anterior shifting of organs relative to size. In short, as the distance between the organ and cloaca increases at a relatively greater rate than the distance between the snout and organ, the organ moves forward relatively with an increase in length.

The differential growth of mid-body vertebrae compared to anterior and distal precaudal vertebrae begs for a functional explanation. What potential selective purpose would be served by more rapid expansion of the mid body? The mid body, spanning vertebra 65-105, houses the stomach, distal liver, pancreas, spleen, gall bladder, small intestine, gonads, and anterior portions of the large intestine and right and left kidneys. A positive allometric growth of stomach length would enable larger water snakes to consume relatively larger fish. When plotted against SVL, stomach length of *N. rhombifer* does increase allometrically as function of SVL (S.M. Secor, unpublished data). Selective benefits for the gall bladder, spleen, and pancreas would be minor considering their small size and that they are attached to the start of the small intestine. However, the small intestine might benefit with a greater length, thereby ensuring the complete digestion and absorption of the meal. An added or alternative advantage is a larger space with maturity for the development of eggs and embryos. Although this would not convey an advantage to males for housing their testes due to their relatively smaller size, it would provide a selected benefit for increasing female fecundity (Collins
and Carpenter 1969). *Nerodia rhombifer* are viviparous and young develop within the body cavity that spans vertebrae 85-130 (S.M. Secor, personal observations).

Taking a different approach, the regionalization in vertebral growth rates may stem in part, or entirely, from constraints in growth for vertebrae in the anterior and distal regions of the body. For the distal most subcaudal vertebrae, the thinning of the body and the transition to caudal vertebrae may impose a relaxation in growth rate compared to vertebrae anterior to these. As mentioned, the anterior most vertebrae possess a longer ventral spine. This spine and attached musculature and the need for greater lateral flexibility of the neck region for prey capture might drive the selection for the biomechanical advantage of a comparatively lower rate of vertebral growth.

Given the conserved nature of their body plan, the intra and interspecific variation in the number of vertebrae, and the noted differences in organ position, snakes are an excellent model to explore evolutionary, developmental, and biomechanical based questions of vertebral structure and organ positioning. Further examination of vertebral growth patterns and organ positions in snakes would serve to ascertain whether differential growth of vertebrae is conserved among snake lineages and test the before mentioned hypotheses of differential growth. Adding detailed mapping of *Hox* gene domains would illuminate the developmental mechanisms of regionalization of the snake's body. Gained from these studies would be insight into the nature of the interspecific and ontogenetic variation in the developmental interplay between segmental growth and organ position.

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Literature cited


Figure legends

Figure 1. Exposed internal organs of *Nerodia rhombifer*. Illustrated are the measurements taken from the snout to the anterior edge of the heart (H), liver (L), small intestine (SI), and right kidney (RK).

Figure 2. *Nerodia rhombifer* vertebra depicting the measured dimensions for total length (TL), centrum length (CL), centrum width (TW), ball width (BW), and height (H).

Figure 3. Relative position (distance from snout/snout-vent length (SVL)) of the anterior edge of the heart (◆), liver (▲), small intestine (■), and right kidney (●) as a function of snout-vent length for *Nerodia rhombifer*. For each organ, there was a significant negative relationship between relative organ position and SVL. With an increase in body length, relative positions of organs shifted anteriorly.

Figure 4. The number of precaudal vertebrae plotted against snout-vent length (SVL) for male (●) and female (◯) *Nerodia rhombifer*. Note the lack of any effect of body size or sex on the number of precaudal vertebrae.

Figure 5. Number of precloacal ventral scales (anterior to the cloacal vent) plotted against the number of precaudal vertebrae for *Nerodia rhombifer*. Demonstrated is the close match between the number of precloacal ventral scales and precaudal vertebra.

Figure 6. The ventral scale number aligned with the anterior edge of the heart (A), liver (B), small intestine (C), and right kidney (D) plotted against snout-vent length for male (●) and female (◯) *Nerodia rhombifer*. Note the lack of an effect of body size or sex on the ventral scale number (= vertebral number) that is aligned with the anterior edge of each organ.

Figure 7. Ventral scale number aligned with the anterior edge of the heart (A), liver (B), small intestine (C), and right kidney (D) plotted against the number of precaudal vertebrae for *Nerodia rhombifer*. Note the effect of the total number of precaudal
vertebrae on the ventral scale number aligned to the anterior edged of the four organs. For snakes with fewer precaudal vertebrae, each organ is positioned at the lower end of the range of aligned ventral scales.

Figure 8. The position (ventral scale alignment) of each organ plotted against the position of the other three organs for *Nerodia rhombifer*. Significant correlations existed between heart and liver (A), heart and small intestine (B), heart and right kidney (C), liver and small intestine (D), and small intestine and right kidney (F).

In most cases, if an individual possess an organ relatively more distal in the body cavity (compared to other individuals), its other organs were likewise more distally positioned.

Figure 9. Regression slopes (± 1SE) plotted for each vertebral position for vertebral total length (A), centrum length (B), central width (C), ball width (D), height (E), and mass (F) for *Nerodia rhombifer*. Slopes are generated from non-transformed meristic measurements against SVL for total length, centrum length, centrum width, ball width, and height. Regression slopes for mass was generated from the plotting of log transformed mass against logged body mass. For each measurement, matching letters above means indicate slopes that are not significantly different. Error bars are not noted if smaller than symbol for mean values. Note that vertebrae in the middle region of the body (e.g., 45, 65, 85, and 105) possessed greater slopes (i.e., greater growth rates) than vertebrae at the cranial and caudal ends of the body (e.g., 10, 25, 125, and 140).
Table 1. Linear regression equations for total length (TL), centrum length (CL), centrum width (CW), ball width (BW), height (H), and log-transformed mass (M) for the 10th, 25th, 45th, 65th, 85th, 105th, 125th, and 140th vertebra as a function snout-vent length (x) for HL, CL, CW, BW, and H, and a function of log-transformed body mass (x) for M for *Nerodia rhombifer*.

<table>
<thead>
<tr>
<th></th>
<th>10th</th>
<th>25th</th>
<th>45th</th>
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<th>105th</th>
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<tbody>
<tr>
<td>TL</td>
<td>0.059x + 0.366</td>
<td>0.071x + 0.393</td>
<td>0.088x + 0.242</td>
<td>0.096x + 0.133</td>
<td>0.100x – 0.097</td>
<td>0.094x – 0.091</td>
<td>0.083x – 0.033</td>
<td>0.073x – 0.055</td>
</tr>
<tr>
<td>CL</td>
<td>0.048x + 0.390</td>
<td>0.062x + 0.219</td>
<td>0.073x + 0.092</td>
<td>0.078x + 0.012</td>
<td>0.080 – 0.136</td>
<td>0.077x – 0.159</td>
<td>0.069x – 0.059</td>
<td>0.064x – 0.214</td>
</tr>
<tr>
<td>CW</td>
<td>0.023x + 0.971</td>
<td>0.037x + 0.739</td>
<td>0.051x + 0.394</td>
<td>0.054x + 0.293</td>
<td>0.051x + 0.316</td>
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<td>0.040x + 0.417</td>
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<tr>
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<td>0.024x + 0.537</td>
<td>0.024x + 0.493</td>
<td>0.021x + 0.493</td>
<td>0.019x + 0.402</td>
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<tr>
<td>H</td>
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</tr>
</tbody>
</table>

TL, CL, CW, BW, and H are expressed in mm. M is expressed in mg.