Snake evolution and prospecting of snake venom

Vonk, F.J.

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Author: Vonk, Freek Jacobus
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Chapter 4: Axial Patterning in Snakes and Caecilians: Evidence for an Alternative Interpretation of the *Hox* Code

Woltering JM, Vonk FJ, Müller H, Bardine N, Tuduce IL, de Bakker MA, Knöchel W, Sirbu IO, Durston AJ and MK Richardson.

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It is generally assumed that the characteristic deregionalized body plan of species with a snake-like morphology evolved through a corresponding homogenization of *Hox* gene expression domains along the primary axis. Here, we examine the expression of *Hox* genes in snake embryos and show that a collinear pattern of *Hox* expression is retained within the paraxial mesoderm of the trunk. Genes expressed at the anterior and most posterior, regionalized, parts of the skeleton correspond to the expected anatomical boundaries. Unexpectedly however, also the dorsal (thoracic), homogenous rib-bearing region of trunk, is regionalized by unconventional gradual anterior limits of *Hox* expression that are not obviously reflected in the skeletal anatomy. In the lateral plate mesoderm we also detect regionalized *Hox* expression yet the forelimb marker *Tbx5* is not restricted to a rudimentary forelimb domain but is expressed throughout the entire flank region. Analysis of several *Hox* genes in a caecilian amphibian, which convergently evolved a deregionalized body plan, reveals a similar global collinear pattern of *Hox* expression. The differential expression of posterior, vertebra-modifying or even rib-suppressing *Hox* genes within the dorsal region is inconsistent with the homogeneity in vertebral identity. Our results suggest that the evolution of a deregionalized, snake-like body involved not only alterations in *Hox* gene *cis*-regulation but also a different downstream interpretation of the *Hox* code.

The patterning and morphogenesis of the vertebrate skeleton during embryonic development is regulated by *Hox* genes. In tetrapods, the *Hox* gene family consists of 39 closely related homeobox genes consisting of 13 paralogous gene groups organized in 4 clusters (named A, B, C and D) (reviewed in [135]. These genes are expressed in collinear spatial domains along the antero-posterior body axis and act as effectors of vertebral identity. The sequential expression pattern of different *Hox* genes is often referred to as "the *Hox* code" [136] which, combined with different inductive properties of the genes, provides positional information for the antero-posterior regionalization of the axial skeleton. Typically, *Hox* genes form nested expression series and the domains of anterior genes overlap with those of more posterior genes. Where several genes are co-expressed, posterior genes act dominantly over anterior genes, a form of phenotypic suppression called "posterior prevalence" [137]. The evolutionary conservation of the *Hox* patterning system is reflected in the fact that expression boundaries of *Hox* genes correlate with the same anatomical transitions in different vertebrate species [138,139] and changes in *Hox* expression are often suggested to underlie macro-evolutionary modifications of the bodyplan[140].

Evolution has produced an astonishing diversity of body shapes adapted to different lifestyles. An important contributor to the shape of animals with backbones is the number of bones (vertebrae) that make up this structure. Some animals have gone to extremes (Fig. 10), none more so than snakes, which have more vertebrae than any other living animal — often more than 300, with some species having more than 500. One of the most striking modifications of the vertebrate axial skeleton is the evolution of a 'snake-like', elongated body plan as adopted by at least 7 extant taxa of reptiles, fish and amphibians independently[141] (Fig. 10). The evolutionary transition to a snake-like body plan is accompanied by a profound deregionalization of the axial skeleton which includes loss or reduction of the limbs and limb girdles, as well as sternal elements [141,142]. These species have a greatly increased number of vertebrae[141,143,144] and, with the exception of the atlas, all of the pre-cloacal vertebrae are usually rib bearing. In contrast to non-elongated species, the dorsal (in squamates the rib-bearing region of the trunk skeleton is termed 'dorsal' instead of 'thoracic') region of the skeleton is extremely homogeneous with few or no differences between ribs and vertebrae along the antero-posterior axis. It has been hypothesized that this peculiar “deregionalized” limbless anatomy results from a corresponding homogenization of *Hox* expression domains along the snake’s primary axis[145]. In this study we set out to analyse *Hox* expression in detail, including previously unexamined posterior paralog members in the corn snake (*Pantherophis guttatus*) and to a lesser extent, because of material scarcity, in a caecilian amphibian (*Ichthyophis* cf. *kohtaoensis*), which evolved a comparable body plan independently.

Figure 10 | Body elongation, including increased numbers of vertebrae and a longer backbone, has evolved independently many times among vertebrates. a, Eastern glass lizard (*Ophisaurus ventralis*). b, European eel (*Anguilla anguilla*). c, Sao Tome caecilian ((*Schistometopum thomense*). d, Malayan pit viper (*Calloselasma rhodostoma*). Photos not to scale. (a,b,d, F.J.V. & M.K.R.; c, S. Blair Hedges.)

Results

Spatially collinear *Hox* expression in the snake somitic mesoderm

The axial skeleton of the corn snake consists of a total of 308 vertebrae (Fig. 11) and the anatomical subdivisions are listed in Table 1. Characteristic features include the complete absence of limb girdles, sternal elements, forelimbs and hindlimbs and the presence of homogeneous ribs on all but the 3 most anterior pre-cloacal vertebrae.

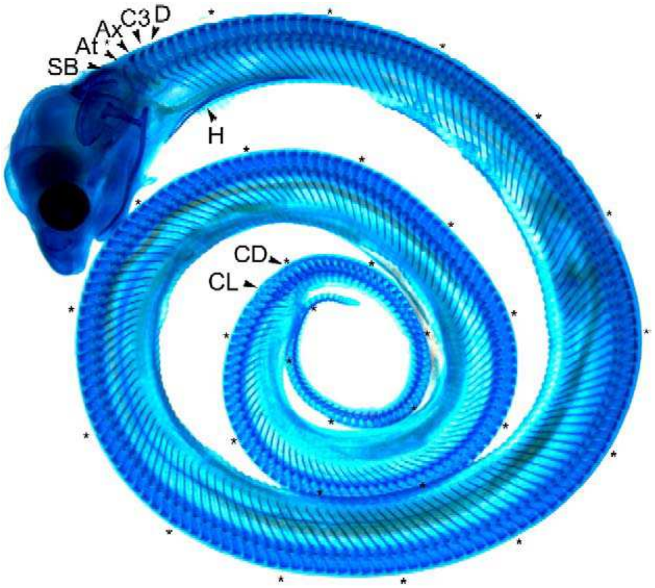
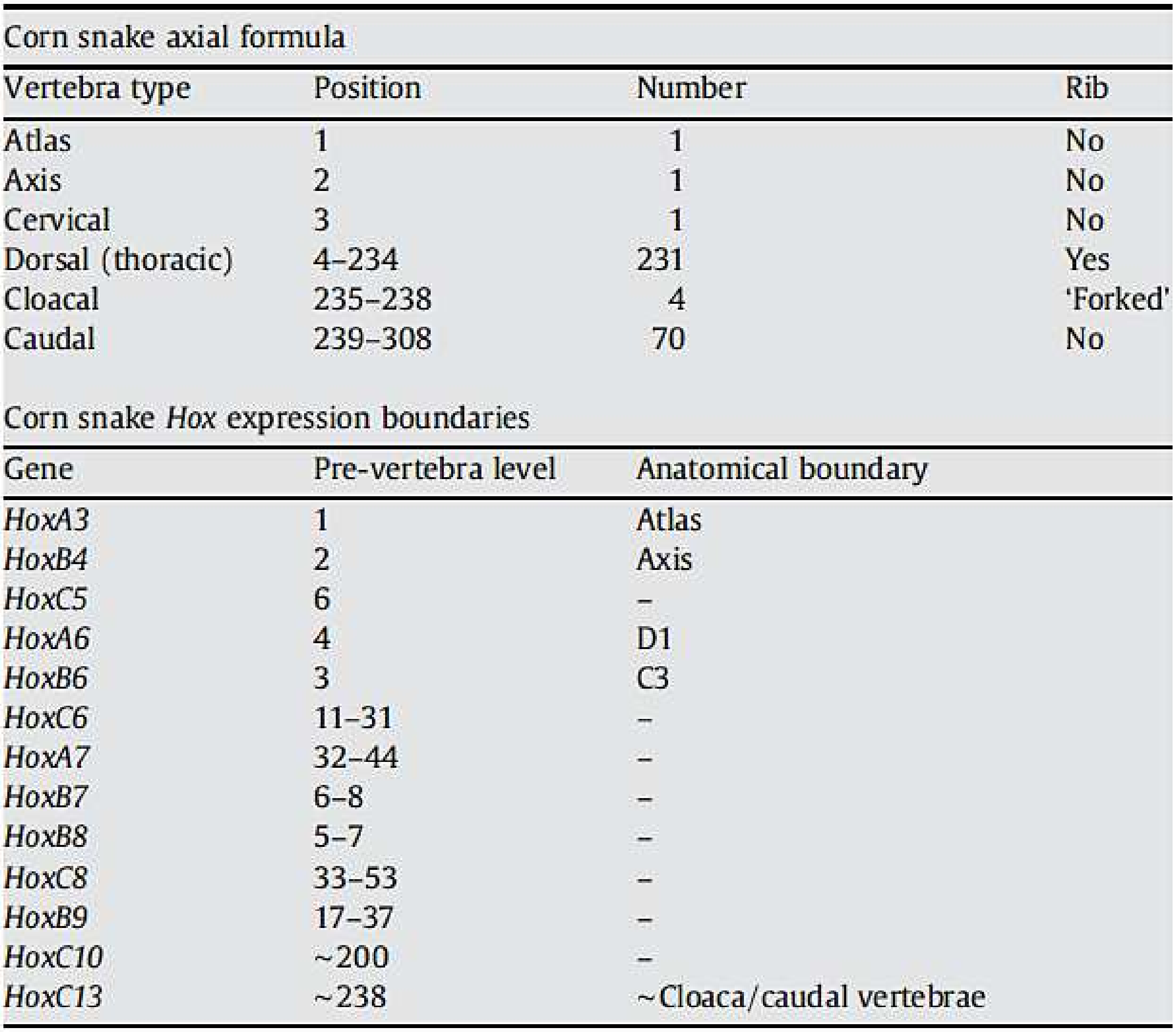


Figure 11 | Alcian blue staining of an advanced corn snake embryo to show the skeletal anatomy. The axiak skeleton consists of 308 vertebrae; the atlas (At), the axis (Ax), 1 additional cervical vertebrae (C3) and 231 dorsal vertebrae (D), 4 cloacal vertebrae (CL) and 70 caudal vertebrae (CD). The first doral, cloacal and caudal vertebrae are indicated. SB; skull bone, H; hyoid.

We examined the expression of a panel of *Hox* genes in tailbud stage embryos (see Supplementary Material, Fig. S1). For all *Hox* genes examined, we observe, as reported previously[145], a strong homogenous expression in the posterior part of the trunk, but also a clear spatially collinear expression in the anterior part of their expression domains (Fig. 12 A-T and Table 1).

Table 1 | Corn snake axial formula and anterior somitic *Hox* expression limits



Corn snake axial formula

Vertebra type	Position	Number	Rib
Atlas	1	1	No
Axis	2	1	No
Cervical	3	1	No
Dorsal (thoracic)	4–234	231	Yes
Cloacal	235–238	4	'Forked'
Caudal	239–308	70	No

Corn snake *Hox* expression boundaries

Gene	Pre-vertebra level	Anatomical boundary
HoxA3	1	Atlas
HoxB4	2	Axis
HoxC5	6	–
HoxA6	4	D1
HoxB6	3	C3
HoxC6	11–31	–
HoxA7	32–44	–
HoxB7	6–8	–
HoxB8	5–7	–
HoxC8	33–53	–
HoxB9	17–37	–
HoxC10	~200	–
HoxC13	~238	~Cloaca/caudal vertebrae

Indicated here are the vertebral types, their position and number, and whether they are rib-bearing, the lymphapophyses of the snake cloacal vertebrae are indicated as forked ribs. Anterior somatic Hox expression boundaries are given below. Where *Hox* expression boundaries coincide with an anatomical transition this is indicated. A pre-vertebrae range is given in case of a diffuse expression limit.

The anterior expression boundaries of the most anterior and most posterior *Hox* genes examined, *HoxA3*, *HoxB4* and *HoxC13*, coincide with homologous morphological boundaries in the mouse and chicken [4, 12] (Fig. 12 A, B, S, T and Table 1). They are located at the atlas, axis and cloaca respectively and are thus expressed at expected positions within regionalized parts of the snake trunk.

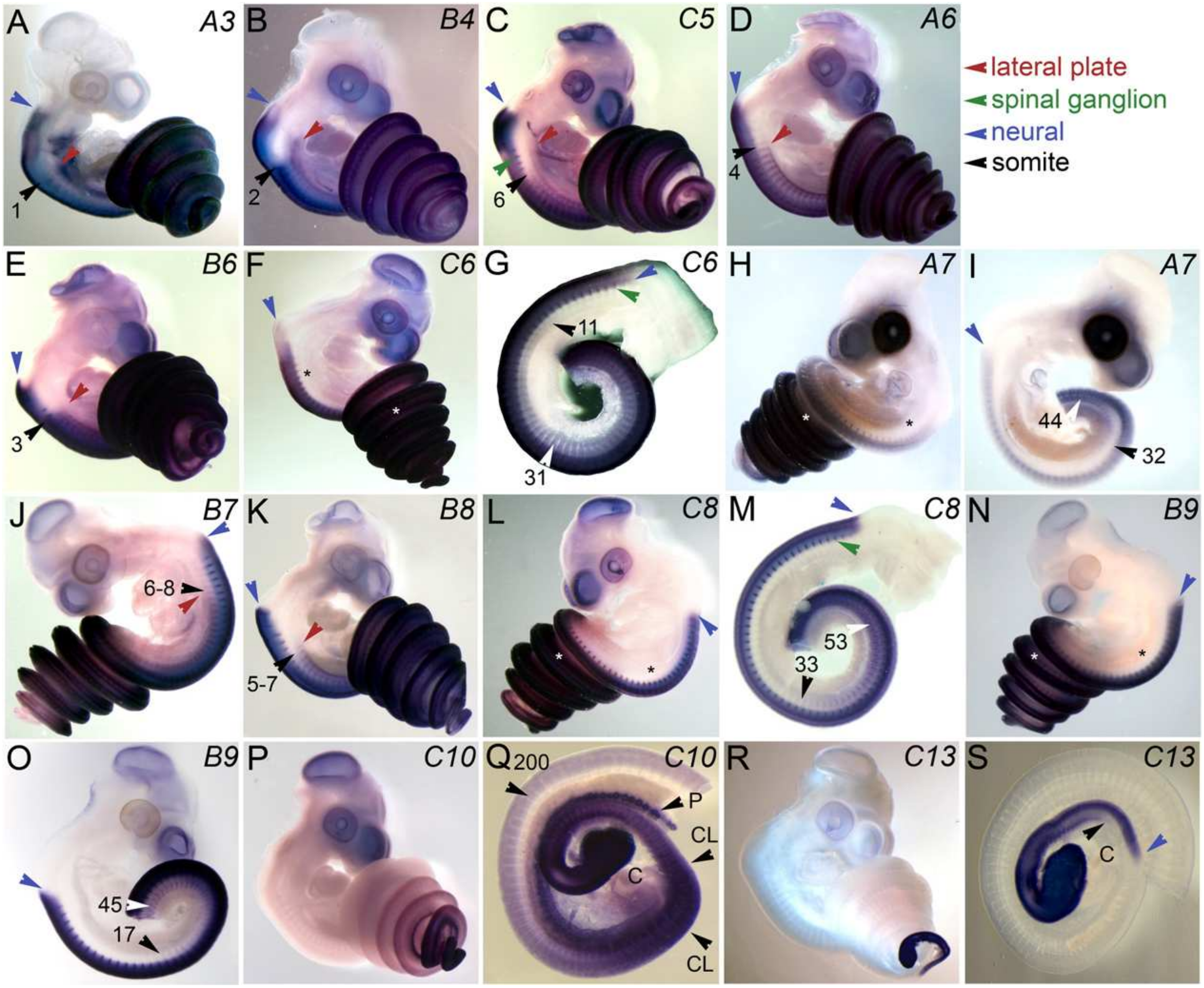


Figure 12 | *Hox* gene expression in corn snake embryos. All embryos are at the second day after oviposition. *Hox* gene names are indicated in the upper right corner of each picture. (A-E,J,K). Expression of *HoxA3*, *HoxB4*, *HoxC5*, *HoxA6*, *HoxB6*, *HoxB7* and *HoxB8*. These anterior genes have sharp anterior expression boundaries in the somitic mesoderm indicated with a black arrowhead and a number referring to the pre-vertebra level. Red arrowheads indicate the anterior expression boundaries in the lateral plate mesoderm and blue arrowheads indicate the anterior limit of expression in the neural tissue. In some panels an example of spinal ganglion expression is indicated with a green arrowhead. For these *Hox* genes additional expression data is provided in the supplementary data (Fig.S2). (F-I, L-O) Expression of *HoxC6*, *HoxA7*, *HoxC8* and *HoxB9*. These anterior genes

have gradual anterior expression limits in the somitic mesoderm which are difficult to visualize exactly in the whole mount views (F,H,L,N,O). In these whole mounts a black asterisk is placed in the anterior somitic domain where no expression is detected and a white asterisk is placed in the posterior domain where the genes are expressed robustly. The expression limits for these genes are indicated more precisely in flat mounts of the anterior trunk region (G,H,M,O). In these images anterior expression limits in the somitic mesoderm are indicated with a black arrowhead and a number indicating the pre-vertebra level. This somitic domain is indicated again more posteriorly in a region of more robust expression with a white arrowhead and the indication of pre-vertebra level. In some panels an example of spinal ganglion expression is indicated with a green arrowhead and the anterior start of the neural expression domain is indicated with a blue arrowhead. For these *Hox* genes additional expression data is provided in the supplementary material (Fig.S3-6). (P-Q) *HoxC10* expression in whole mount (p) and flatmount of trunk/tail transition (Q). HoxC10 is expressed in the somotic mesoderm anterior to the cloaca (indicated "C") and cloacal vertebrae (indicated with black arrowheads and "CL") anterior to approximately pre-vertebra 200 (indicated with a black arrowhead and number) in a region of the trunk that will give rise to rib bearing vertebrae. Expression in pronephric vesicles is indicated with a black arrowhead and "P". Additional *HoxC10* expression data is provided in the supplementary material (Fig.S7). (R,S) Expression of *HoxC13* in whole mount (R) and flat mount of trunk/tail transition (S). *HoxC13* is expressed in the somitic mesoderm up till approximately the cloaca (indicated "C"), expression indicated with a black arrowhead. The anterior limit of neural expression is indicated with a blue arrowhead.

The dorsal region in snakes extends for most of the body length and has remarkable similar vertebrae. Despite this very poor regionalization, we do detect collinear expression in the dorsal region of the *Hox* genes belonging to paralogous groups 5-9. These genes are differentially expressed in the trunk and have been experimentally linked to the patterning of the thoracic and lumbar region in mouse and chicken [138,146-148] (Fig 12 C-O, Supplementary Material Fig S2, 3 and Table 1). The anterior boundaries of the most anteriorly expressed of these paralogous genes are sharply demarcated (*HoxC5*, *HoxA6* and *HoxB6*) or decrease in intensity over several somites (*HoxB7* and *HoxB8*). Genes expressed more posteriorly (*HoxC6*, *HoxA7*, *HoxC8* and *HoxB9*) show unconventional gradual anterior limits of expression, slowly fading out over the course of approximately 10 somites, but being completely absent from the anterior part of the trunk paraxial mesoderm. We analyzed expression of several genes at earlier stages (~150 somites: *HoxA6*, *HoxB6*, *HoxC6* and *HoxB9*; data not shown) or later stages (~ 5 days:

HoxC8 and *HoxB9*; data not shown) and detected expression in the same pattern as observed in the stages shown in Fig. 12.

Snakes lack a lumbar region and rib-bearing vertebrae are present up to the cloaca. Genes from the *Hox-10* group suppress thoracic and induce lumbar fate in the mouse and expression of these genes is normally absent in the thoracic mesoderm[138,149-151]. In the corn snake by contrast, we detect expression of *HoxC10* within the rib-bearing part of the trunk (Fig. 12 Q, R, Fig. S4) and this expression thus conflicts with its presumed function as a rib suppressor (at least in snakes). As it has been suggested that *Hox-10* genes exert their rib suppressing activity in the pre-somitic mesoderm [150], we analyzed embryos at earlier stages and we also detected *HoxC10* expression in the pre-somitic mesoderm of rib-bearing somites (Supplementary Material Fig. S4).

Contrary to expectations, therefore, we find that the somitic mesoderm, which will give rise to the dorsal (thoracic) homogeneous rib-bearing part of the skeleton, is regionalized by *Hox* genes that are known to possess vertebra-modifying or even rib-suppressing activity in the mouse. Despite this regionalization, the dorsal skeleton shows no or very little regionalisation of vertebral anatomy.

Comparison of *Hox* expression at the cervico-dorsal transition in snake and lizard embryos

Snakes have no well-defined transition from trunk to neck (cervico-dorsal) which in other squamates is marked by the position of the forelimb girdle (squamates often have rib-bearing vertebrae in the neck region hence presence of ribs is not a useful anatomical marker)[140,142]. The expression boundaries of *HoxC5*, *HoxA6*, *HoxB6*, *HoxC6* and *HoxB8* are located around the cervico-dorsal (thoracic) transition in mouse and chicken[138,148,152,153] and expression of the three *Hox-6* paralogs coincides at the level of 1st thoracic vertebra[138,153]. In the corn snake *HoxA6* is expressed as far anterior as pre-vertebra 4, *HoxB6* as far as pre-vertebra 3 while *HoxC6* is expressed gradually up to approximately pre-vertebra 11 (Fig. 13 and Supplementary Material Fig. S2). The expression of these genes thus seems shifted relative to each other, possibly in relation to the deregionalization of the snake body plan. However, due to lack of *Hox* expression data from non-snake squamates these differences in expression could also represent a synapomorphic squamate character. Therefore, we investigated the expression boundaries of *hoxC5*, *HoxC6*, *HoxB6* and *HoxB*8 in the bearded dragon lizard (*Pogona vitticeps*) and of *HoxA6* in the green anole lizard (*Anolis carolinensis*) (Fig. 14). These species have 4 rib-less neck vertebrae and the forelimbs are positioned between vertebra 6 and 7 (data not shown). *HoxC5* and *HoxB8* have anterior expression boundaries at the level of the 6th pre-vertebra, *HoxA6* is expressed at the level of 5th pre-vertebra (the first rib-bearing vertebra), *HoxC6* at the 7th pre-vertebra and *HoxB6* at the 3rd pre-vertebra (Fig. 14 A-E). This shows that a relatively normal cervico-dorsal transition, although slightly elongated, is patterned in snakes (Fig. 14 F). Interestingly, the expression of *HoxA6* corresponds to the transition from rib-bearing to rib-less vertebrae at pre-vertebra level 4 in the snake and pre-vertebra 5 in the anole lizard. However, all the other skeletal anatomical features associated with this transition are absent in snakes.

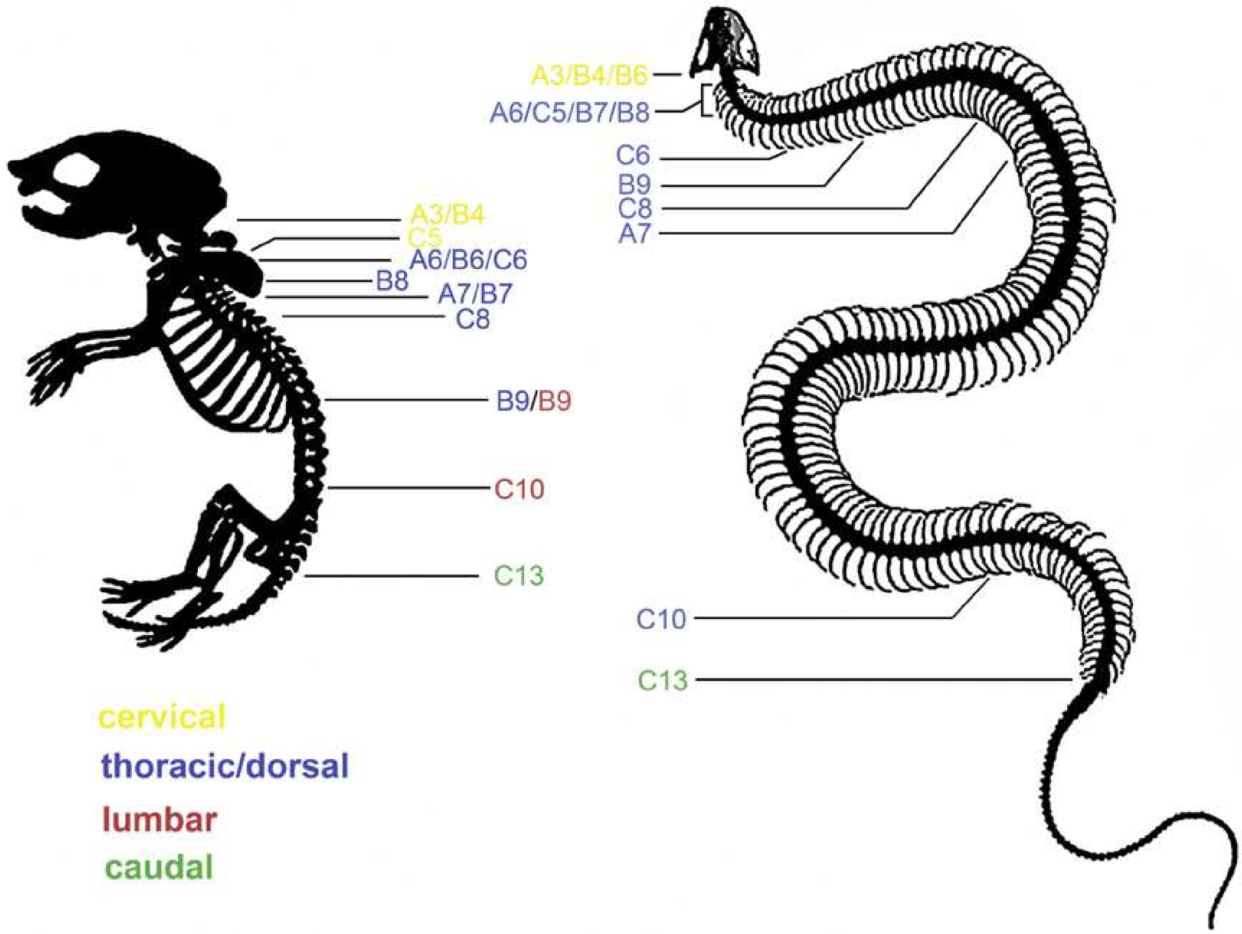


Figure 13 | Schematic visualization of the approximate anterior mesodermal limits of *Hox* gene expression in the mouse and snake. Schematic drawing of a mouse and a snake skeleton, with indications of the approximate anterior boundaries of *Hox* gene expression, demonstrating a conservation of global collinear *Hox* expression zones in the snake despite absence of a correspondingly regionalized skeleton. Colours indicate the predominant anatomic region the genes are associated with. For precise levels of *Hox* expression zones in the snake, see Table 1, for the exact mouse expression data, refer to the cited literature. For the mouse, expression data from [138] were used for *HoxB4*, *HoxC5*, *HoxC6* and *HoxB7*. *HoxA7* is according to [154], *HoxA3* according to[139], *HoxC13* according to[155], *HoxA6* according to[153], *HoxB6* according to[152], *HoxB8* and *HoxC8* according to[148], and *HoxC10* according to[151]. The relative position of *HoxB9* is mapped according to its expression at the lumbar/thoracic transition in chicken[138]. In the mouse *HoxB9* expression appears however to be dynamically shifting along this axis, with at earlier stages more anterior and at later stages more posterior expression than indicated here [146,156]. Genetically the *Hox*-9 genes have been demonstrated to be involved in the patterning of the thorax as well as the suppression of anterior thoracic fate in the lumbar region [146,156], therefore the gene is indicated in the mouse as being both thoracic and lumbar.

Conservation of global collinear *Hox* expression domains in a caecilian amphibian

Caecilian amphibians have independently evolved a snake like postcranial skeleton. We analysed *Hox* gene expression in embryos from a caecilian (*Ichthyophis* cf. *kohtaoensis*) which has a skeleton consisting of 126 vertebrae with the atlas being the only pre-cloacal rib-less vertebra (Fig. 15, axial formula given in Table 2). We examined expression of *HoxC5*, *HoxC6*, *HoxB8*, *HoxC8* and *HoxC13* and analyzed their expression in Brauer stage 26 embryos (Supplementary Material Fig. S1). The global expression patterns of these genes are comparable to snakes and they exhibit spatially collinear expression within the trunk (Fig. 16, Supplementary Material Fig. S5 & Table 2). Similarly to snakes, the expression domains of *HoxC5*, *HoxC6*, *HoxB8* and *HoxC8* are not linked to morphology in the axial skeleton in the dorsal region and *HoxC13* expression corresponds to the transition into the caudal region.

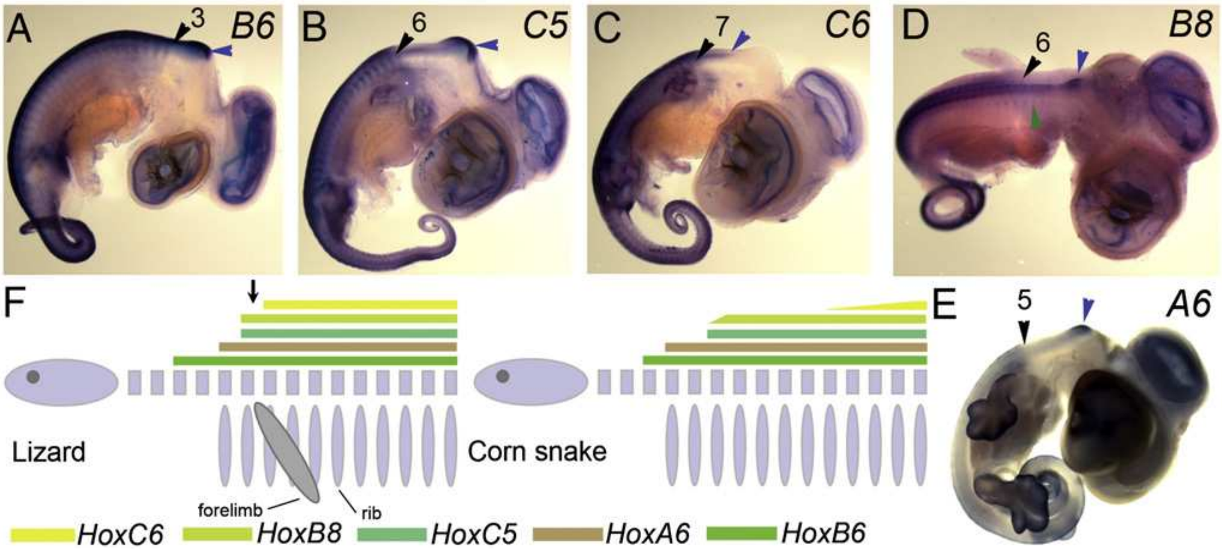


Figure 14 | *Hox* gene expression in lizard embryos and comparison of snake and lizard cervico-dorsal transition *Hox* patterning. (A-E) Expression of *HoxC6*, *HoxB6* and *HoxB8* in embryos of bearded dragon lizard and *HoxA6* in the green anole lizard. Expression in the somitic mesoderm is indicated with black arrowheads and numbers referring to the pre-vertebra levels. Anterior boundaries of neural expression are indicated with a blue arrowhead. *Hox* genes are indicated in the upper right corner of each image. In the panel for *HoxB8* (D) an example of spinal ganglion expression is indicated with a green arrowhead. (f) Schematic visualization of *Hox* expression patterns in the cervico-dorsal transition in lizard and corn snake. The arrow indicates the position of the transition between cervical and dorsal region in the lizard. Despite the absence of most of the morphological skeletal features corresponding to the cervico-dorsal transition, a regionalized pattern of *Hox* expression is preserved. Note that *HoxA6* expression corresponds to the start of the rib-bearing region in both snakes and lizards.

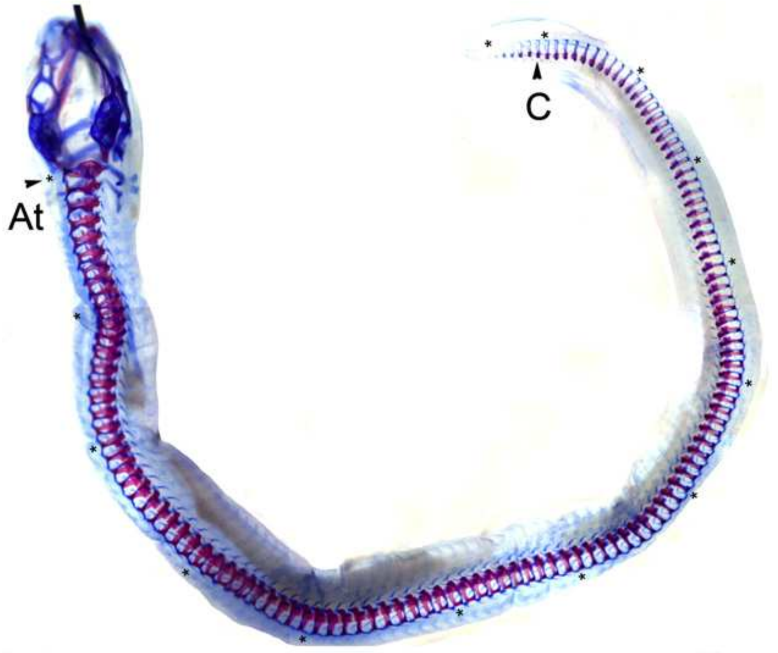
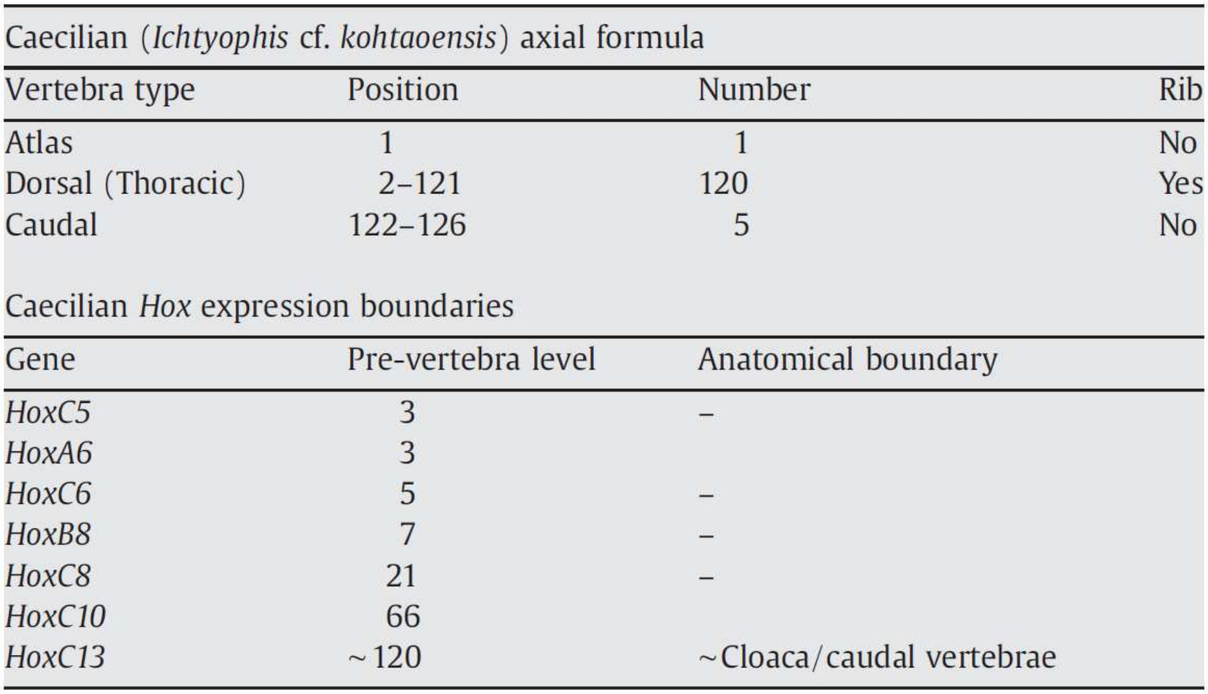


Figure 15 | Alcian blue - Alazarin red staining of an advanced caecilian embryo to show the skeletal anatomy. The axial skeleton consists of 126 vertebrae, the atlas (At), 121 cervical / dorsal vertebrae and 5 caudal vertebrae. Each tenth vertebra is marked with an asterisk. C; cloaca.

Table 2 | Caecilian axial formula and anterior somitic *Hox* expression limits.



Caecilian (*Ichtyophis* cf. *kohtaoensis*) axial formula

Vertebra type	Position	Number	Rib
Atlas	1	1	No
Dorsal (Thoracic)	2–121	120	Yes
Caudal	122–126	5	No

Caecilian *Hox* expression boundaries

Gene	Pre-vertebra level	Anatomical boundary
HoxC5	3	–
HoxA6	3	
HoxC6	5	–
HoxB8	7	–
HoxC8	21	–
HoxC10	66	
HoxC13	~120	~Cloaca/caudal vertebrae

Indicating here are the vertebral types, their position and number, and whether they are rib-bearing. Anterior somitic *Hox* expression boundaries are given below. Where *Hox* expression boundaries coincide with an anatomical transition this is indicated.

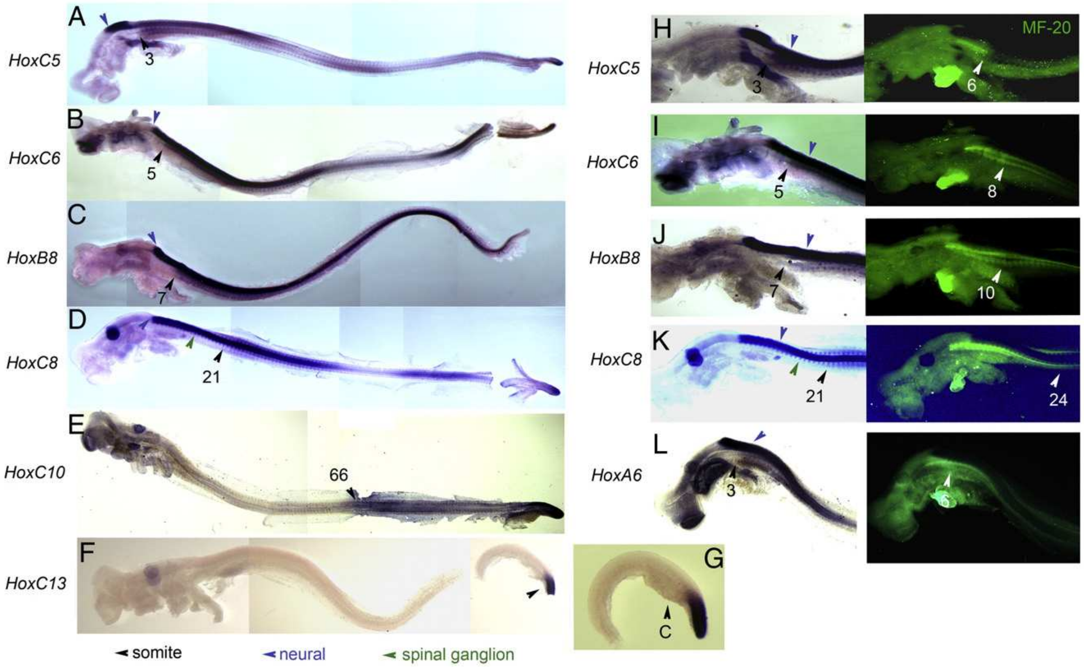


Figure 16 | (page 52) *Hox* gene expression in Brauer stage 26 caecilian embryos. (A-E) Shown are flat mounted embryos with the yolk removed (for whole mount unprocessed example see Supplementary Material Fig. S1). *Hox* genes are indicated on the left. Anterior boundaries *Hox* gene expression in the somitic mesoderm is indicated with black arrowheads and pre-vertebrae levels are indicated with numbers. The anterior boundary of neural expression is indicated with a blue arrowhead and in the panel for *HoxC8* (d) an example of spinal ganglia expression is indicated with a green arrowhead. (G) Close up of the tail plus posterior trunk region of *HoxC13* expression, "C" indicates the position of the cloaca. (H-L) Fluorescent immune staining with the somite marker MF-20 antibody together with *Hox* gene in situ hybridization. In the brightfield close ups of head plus anterior trunk, the anterior *Hox* gene expression boundaries in the somites are indicated with black arrowheads and numbers indicating the pre-vertebra level. In the fluorescent microscopy views of the same samples showing the somite specific staining of the MF-20 antibody, the anterior level of *Hox* expression is marked with a white arrowhead and a white number referring to the somite number.

Hox and *Tbx5* expression in the snake lateral plate mesoderm

In addition to the homogenous, extended rib cage, another typical feature of snake-like species is the lack of limbs. *Hox* genes are involved in limb bud induction and it has been shown that the forelimb buds develop at the anterior boundary of *Hox-9* gene expression in the chick lateral plate mesoderm[157]. In the corn snake, we do observe collinear expression of *HoxA*, *HoxB* and *HoxC* genes within the lateral plate somatopleure (Fig. 12, Supplementary Material Fig. S2, 3 Fig. 16 A, D; lateral plate expression of all "thoracic genes" investigated, except *HoxB4, HoxB6* and *HoxA3*, was verified by cryosectioning: Supplementary Material Fig. S2, 3 and data not shown). In general the axial level of *Hox* gene expression coincides or is located very near the axial level of the somitic boundary. For *HoxC6* and *HoxC8,* which have very gradual anterior limits in the somites, we observe similar gradual anterior limits in the lateral plate (Supplementary Material Fig. S3 and data not shown).

HoxB9 is expressed in the lateral plate mesoderm up to the same axial level as in the somitic mesoderm (Fig. 17 A, D). This position could thus correspond to a vestigial forelimb domain. We investigated the expression of the forelimb specific marker *Tbx5* [158,159] in the corn snake to see if any such domain could be detected at the molecular level. Unexpectedly however, *Tbx5* is expressed throughout the entire antero-posterior extent of the somatopleure (Fig. 17 B, E and Supplementary Material Fig. S6).

Thus, despite regionalized collinear *Hox* gene expression in the lateral plate mesoderm, no forelimb buds are induced in the snake and the forelimb gene *Tbx5* shows a very strong deregionalized expression that seems independent from the *Hox* expression pattern in the lateral plate (Fig. 17 F).

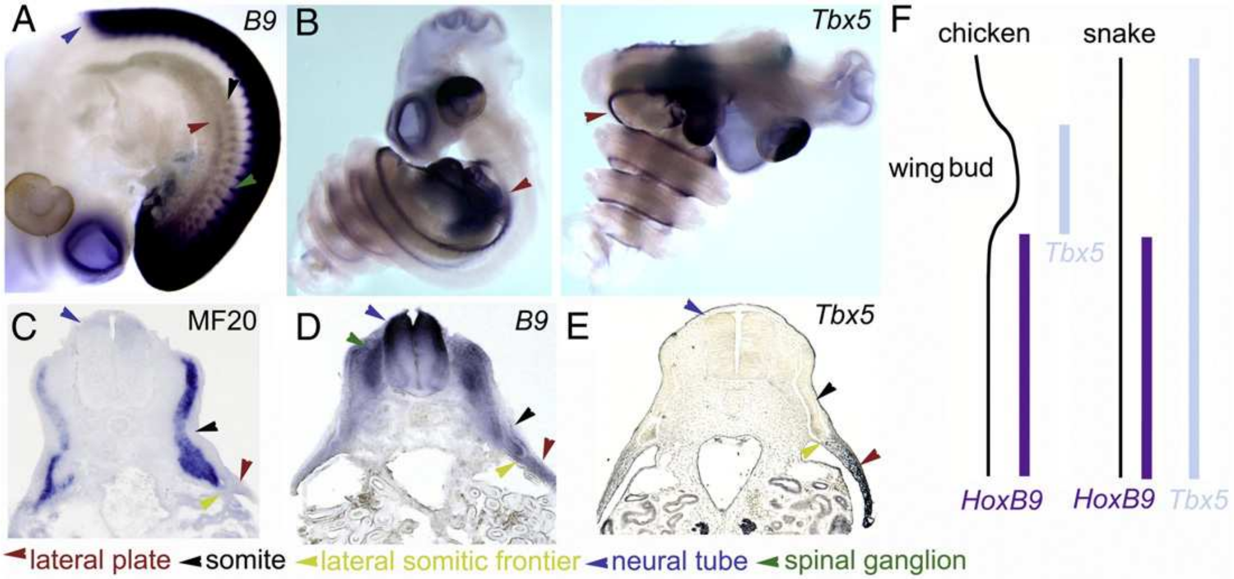


Figure 17 | Expression of *HoxB9* and *Tbx5* in the snake lateral plate mesoderm. All tissue shown is from embryos at the second day after oviposition. (A) *HoxB9* is expressed in the snake with an anterior limit in the lateral plate (red arrowhead) and somitic mesoderm (black arrowhead) around pre-vertebra 17. The anterior limit of neural tube expression is marked with a blue arrowhead and an example of spinal ganglion expression is marked with a green arrowhead. (B) The forelimb marker *Tbx5* is expressed throughout the antero-posterior extent of the pre-cloacal lateral plate somatopleure in the corn snake. Expression starts more anterior than *HoxB9* at the head-trunk transition (marked with a red asterisk). The general lateral plate expression domain is indicated with red arrowheads. (c) To determine the position of the lateral somitic frontier in the transversal sections immunostaining with MF20 antibody specific for somitic mesoderm was performed. The cryosection shown was taken in the anterior trunk. The lateral somitic frontier is indicated with a yellow arrowhead. (D) *HoxB9* expression in transversal cryosection taken around pre-vertebra 40 shows expression in lateral plate and somitic mesoderm. (E) *Tbx5* expression in transversal wax section taken at the mid-axial level shows *Tbx5* expression in the lateral plate mesoderm. (F) Schematic comparison of *HoxB9* and *Tbx5* expression in the lateral plate. In the chicken *HoxB9* and *Tbx5* are expressed mutually exclusive at these stages of development with *Tbx5* being anterior and *HoxB9* posterior. In the snake, *Tbx5* expression starts more anterior than *HoxB9* but overlaps posteriorly throughout the pre-cloacal region of the trunk with *HoxB9* expression. Thus, despite the "normal" collinear pattern of *HoxB9* expression, *Tbx5* is not regionalized to a forelimb domain but expressed more posterior than expected. Lateral plate, somitic, spinal ganglion and neural tube staining as well as the position of the lateral somitic frontier are indicated with different colour arrowheads. Additional snake *HoxB9* expression data is provided in the Supplementary Material (Fig. S5) and additional chicken and snake *Tbx5* data in Fig.S8.

Discussion

Snakes put their long bodies to good use when moving through dense vegetation, along tree branches or in water, which they do with incredible grace. Constricting snakes use their coils to suffocate prey, whereas venomous snakes are like a coiled spring that can strike prey at a distance and with terrifying speed. Snakes are among the most successful of vertebrate groups, both in terms of number of species and geographic distribution[160]. Elongated bodies have certainly contributed to this success. Model species (such as mice) will always be the mainstay of research because scientists can engineer changes in their genomes and look for effects on the body plan under controlled conditions[161]. Biologists are nevertheless increasingly considering non-model organisms. In snakes, natural selection has done the genetic engineering for us, and so we can study them as experiments performed by evolution. Investigating snakes hand-in-hand with model species will provide a holistic view of evolution and development. Snake embryos are not easy to work with in the lab because it is difficult to open their eggs without damaging the embryo, and obtaining eggs from snakes is not always easy. But if these technical challenges are overcome, and snake genome sequences become widely available, a new era of 'evo-devo' research may open up.

We have described a collinear pattern of *Hox* expression along the primary axis in snake and caecilian embryos. At the anterior and posterior regionalized parts of the axis we find that *Hox* gene expression coincides with expressed anatomical transition. Genes, such as *HoxA3*, *HoxB4*, *HoxA6*, and *HoxC13* have anterior expression boundaries corresponding to clear morphological transitions in the axial skeleton (atlas, axis, rib-bearing region and dorsal-caudal transition).

We however also detect collinear *Hox* expression boundaries within the dorsal homogenous rib-bearing part of the skeleton that are not correlated with transitions in the morphology of the axial skeleton. Genes such as *HoxC6*, *HoxA7*, *HoxC8*, *HoxB9* and *HoxC10*, that normally act to modify the axial skeleton, display regionalized expression in “silent” domains (i.e. not reflected in anatomy), suggesting that they have lost their ancestral effects on vertebral regionalisation. The diffuse anterior expression boundaries of these genes in the snake also may reflect loss of involvement in the regionalization of the axial skeleton.

Although snakes lack a neck region, as defined in squamates by the positioning of the forelimb girdle, the comparison *Hox* gene expression in lizards indicates that a relative normal neck region is patterned. Interestingly, *HoxA6* expression coincides with the beginning of the rib-bearing region in both lizards and snake, and our observations do not rule out the possibility that a single *Hox* gene is responsible for rib formation. Apparently, though, the mechanism of posterior prevalence, according to which more posterior *Hox* genes modify these ribs or suppress rib formation completely, is not operational.

The snake lateral plate mesoderm shows a collinear pattern of *Hox* expression as has been implicated in the positioning of the limbs in chicken. No limb buds are present in corn snakes however, and neither is any molecular trace of a discrete forelimb. In contrast to the potential upstream regionalizing signal provided by differential *Hox* expression (especially *HoxB9*), the expression of *Tbx5* is highly deregionalized.

In summary, our data suggest that the axial expression patterns of *Hox* genes in the somitic and lateral plate mesoderm of the snake and caecilian do not induce the same downstream responses as they do in the mouse. And so, while some ancestral features of the ‘*Hox* code’ are retained in the two long-bodied lineages studied here, they result in a remarkably different phenotype from that seen in the mouse.

We provide evidence that, besides changes in expression domains through alterations in *cis*-regulation, alterations in downstream gene interpretation may also play an important role in the evolution of a snake-like body plan. These changes in downstream response to the *Hox* pattern could, in principle, occur at two levels. First, the response of downstream genes could be altered through changes in *cis*-

regulatory sequences leading to a different response to *Hox* genes. Alternatively, changes in *Hox* coding sequences could lead to the differential activation of target genes. The contribution of coding sequence evolution to evolutionary modifications has received ample attention recently [162,163] and it has been shown that for instance changes in *Ubx* coding sequence were essential for changes in invertebrate bodyplans[164,165]. The possibility exists that in snakes and caecilians a similar mechanism is involved in changes in their body plan. Transgenic gain-of-function assays in mouse should reveal whether snake and caecilian *Hox* genes do indeed have different inducing properties from those of their mouse orthologs.

Material & Methods

Corn snake and bearded dragon embryos were obtained from private breeders in the Netherlands and caecilian embryos were collected from the wild in Thailand, in accordance with local and international regulations. Alizarin red and alcian blue staining was carried out according to standard protocols, and embryos were cleared in methyl salicylate.

Genes were cloned by PCR from *Patherophis spiloides* cDNA or *Pantherophis guttatus* genomic DNA and caecilian genomic DNA and are deposited in genebank under accession numbers GQ176238-GQ176263.

Immunostaining and histology was carried out according to standard procedures. The MF-20 antibody was obtained from the Developmental Studies Hybridoma Bank, University of Iowa. *In situ* hybridization was carried out according to standard protocols. Pre-vertebral axial formulae were determined by somite counting and, if visible in the *in situ* hybridization wholemounts, the position of the first spinal ganglion. In corn snakes and bearded dragons, we used the amniote standard formula where the 1st pre-vertebra forms from somite 5-6. In caecilian we determined (by Alcian blue staining and the position of the first spinal ganglion) that the 1st vertebra forms from somites 3-4, which is the same as in *Xenopus laevis.*

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