

# Snakes: hatching of a model system for Evo-Devo?

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**ABSTRACT** Evo-Devo studies rely on a collection of animal model systems belonging to different phylogenetic branches to try and understand how organisms carrying a similar set of genes and pathways can develop into such a variety of shapes and sizes. The squamate clade, however, has only recently started to receive the attention it deserves in particular due to extreme morphological and metabolic aspects and, consequently, the important insights that it could bring in different fields. The recent sequencing of several squamate genomes as well as the generation of high quality transcriptomes for different snake tissues now provide the necessary tools to complement biological studies. Here, we briefly report on recent work involving developing snake embryos to illustrate their interest to assess vertebrate developmental mechanisms. We also discuss the relevance to use snake species as Evo-Devo model systems and potential ways to cross the important limitations intrinsically associated with developmental and genetic studies of these fascinating animals.

**KEY WORDS:** *Hox gene, snake, model organism, axial extension, vertebrae*

*Look like th' innocent flower, but be the serpent under 't*

*Lady Macbeth*

Snakes have always had a special position within the animal kingdom, as judged by the enormous number and diversity of associated symbols, in religious, mythological or historical contexts. The fact that these animals carry dual positive and negative representations - the good and the evil - echoes their morphological ambiguity as vertebrates crawling like worms. This particular phenotype, however, is only one of the many extreme forms found within vertebrates, a taxon that displays a large variety of organisms, while sharing almost the same set of developmental genes and pathways.

Ever since the mid 1980's, when molecular genetics started to revive the links between the fields of evolution and developmental biology, a shift in paradigm occurred whereby species-specific genes were no longer considered as the source of diversity, leading to the necessity to explain how comparable genetic materials can generate distinct organisms (discussed e.g. in (Carroll, 2005, De Robertis, 2008)). With the advent of large-scale comparative genomic analyses, some solutions to this conundrum start to emerge. For example, a high conservation of coding genes seems to be counterbalanced by important variations in their regulatory modalities, as exemplified by recent global comparisons between the mouse

and the human regulomes (Cheng *et al.*, 2014, Stergachis *et al.*, 2014, Yue *et al.*, 2014). However, a more comprehensive view of this question requires the experimental integration of as many species as possible, in particular from phylogenetically distant groups such that predictions formulated when studying particular cases can be readily challenged, corrected and enriched by considering potential variations thereof.

## 'Snakes' as model organisms?

In this context, various views have been proposed regarding the choice of strategies to best use the variety of animal species to understand their intimate molecular and mechanistic commonalities (see e.g. (Duboule, 2010, Milinkovitch and Tzika, 2007)). One extreme (reductionist) view is that the full understanding of a single organism, within a given taxon, will unravel all basic mechanisms at work and thus naturally explain all of their variants. Accordingly, efforts should all concentrate on the same model system. The opposite (holistic) view states that only the study of all species on earth will provide us with a satisfactory level of understanding since each of them will bring an essential contribution due to its

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*Abbreviations used in this paper:* AER, apical ectodermal ridge; dpo, days post oviposition; PSM, presomitic mesoderm; SMT, sperm-mediated gene transfer.

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evolutionary success. The evo-devo community currently implements a more reasonable and pragmatic strategy, whereby selected species are studied, if possible representative of all major groups such that results can be shared, compared and integrated into a general conceptual framework. The criteria to select such epistemic objects are however not trivial to define (see (Tzika and Milinkovitch, 2008) and often reflect a utilitarian view of the problem, as illustrated by qualifying species-specific traits as displaying experimental ‘advantages’ or ‘disadvantages’, which usually qualifies the capacity of an animal to deliver the expected results.

Within vertebrate amniotes, while mammals are well represented as model systems, the huge and diverse group of sauropsids is clearly underrepresented in laboratories, besides some bird species. Several rather limited taxons such as chelonians and crocodylians have received some attention recently, for very specific purposes (Green *et al.*, 2014, Milinkovitch *et al.*, 2013, Nagashima *et al.*, 2009, Shaffer *et al.*, 2013, Wang *et al.*, 2013), yet the importance of Squamata is certainly not reflected in the number of species used efficiently at the bench, despite the recent releases of genome sequences for the green anole lizard (Alfoldi *et al.*, 2011), the Burmese python (Castoe *et al.*, 2013) the king cobra (Vonk *et al.*, 2013) or other snakes (Gilbert *et al.*, 2014), as well as several high quality transcriptomes (see e.g. (Schwartz *et al.*, 2010, Tzika *et al.*, 2011). Since a long time, however, lizards and snakes have been used to address successfully a number of specific questions, for example related to ecological research (Shine and Bonnet, 2000), to the emergence of viviparity (Murphy and Thompson, 2011) or to understand parthenogenesis (Lutes *et al.*, 2010).

More recently, next generation sequencing has allowed to re-examine two research fields where snakes are starring for many years, i.e. the evolution of the venom system (Fry *et al.*, 2006, Menez *et al.*, 2006) and its potential use in drug discovery (Harvey, 2014), as well as the extraordinary metabolic adaptations of some species in response to adverse environmental conditions. Many species of snakes produce venom that is released upon biting a prey. While this liquid is best known for its dangerous properties, it also contains peptides of great biomedical potential such as for example painkillers, anesthetics or regulators of blood pressure. Recently, the genome of the king cobra was released and the transcriptome from the venom glands established, showing that toxin genes were co-opted by various mechanisms and that these genes massively expanded through gene duplications, leading to venom protein neo-functionalization (Vonk *et al.*, 2013). This work not only provided insights into venom toxin evolution but also represents a powerful resource for identifying peptides of potential pharmacological interest.

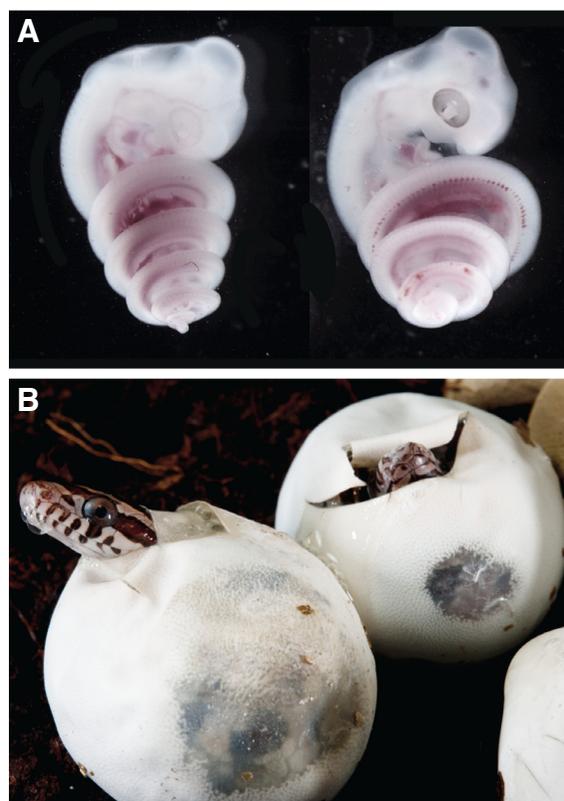
Along with the king cobra genome, the Burmese python genome was also released and brought some important information regarding the remarkable adaptations that this snake species has experienced in its metabolism (Castoe *et al.*, 2013). Indeed, the python has evolved a mechanism to cope with extreme feeding conditions, intercalating long periods of fasting with the ingestion of very large meals. One to three days after a large food intake, the python’s small intestine, stomach, liver, pancreas, heart, lungs and kidneys undergo a 0.5 to two-fold increase in their size. However, once digestion is completed, these organs shrink back to their original dimensions. Genome sequencing together with transcriptome analysis of four organs before and after feeding, showed that rapid and massive transcriptional responses occur in multiple organ systems, which can coordinate changes in organ size and function. Interestingly, the homologs of

these genes in humans are associated with metabolism, development, pathology and chromatin remodeling (Castoe *et al.*, 2013).

These sequencing efforts have recently triggered some coordination amongst various laboratories investigating such aspects of snake biology, to try and federate forces in snake research (Castoe *et al.*, 2012). In addition, the past few years have also seen a handful of papers starting to use snakes as a model system in a modern evo-devo context, i.e. using molecular and/or genomic tools to look at snake embryos with the aim of integrating the data within vertebrates at large. By ‘snake embryos’, we mean embryos derived from various species of snakes, sometimes displaying high evolutionary distances. Indeed it isn’t yet clear whether one particular species will be favored as a paradigm in the future, or if the different scientific interests at stake will lead to the study of as many distinct species. Below, we briefly mention these studies and propose some considerations regarding the actual and future status of snakes as model systems in developmental biology.

### Manipulating snake embryos

Like their body shape, the development of snake embryos is fascinating in many respects (Fig. 1). Snakes likely arose from ancestral lizards that adopted a burrowing lifestyle and became



**Fig. 1. Snakes as model system for developmental studies.** (A) Corn snake embryos dissected at two different developmental stages: 4.5 days post oviposition (dpo) and 6.5 dpo, showing their elongated body plan. (B) Two corn snakes eclosing from their eggs (copyright Michel M. Milinkovitch, University of Geneva). Due to their oviparous or ovoviviparous strategies, snake embryos up to rather late developmental stages are difficult to obtain and a fortiori, to manipulate.

adapted accordingly by changing their morphological and physiological characteristics, leading to elongated and limbless vertebrates (Vidal and Hedges, 2004). Such species must have undergone remarkable selective pressure to modify critical developmental mechanisms in this respect, and hence these animals should present excellent opportunities to better understand how developmental processes and pathways can be changed to generate a large variety of shapes.

The burrowing lifestyle of snake ancestors was accompanied by a considerable reduction of limbs, up to their complete disappearance. The understanding of the process underlying the loss of appendages would certainly bring critical insights into the early steps of limb field determination, budding and development. Also, it may give us a hint as to why this phenomenon arose repeatedly throughout evolution. Some snakes, however, display limb remnants, traces of their tetrapod ancestors. This is the case of pythons, a large and almost tailless snake that preys by constriction, which most likely maintained vestigial hindlimbs as pelvic spurs to assist during copulation (Held I, 2014). Such intermediate forms are key to understand the transition towards a limbless animal. Interestingly, snake limb buds were shown to differ from tetrapod buds by lacking an apical ectodermal ridge (AER) (Cohn and Tickle, 1999), the signaling center that normally controls the proximal to distal growth of limb buds (see (Tabin and Wolpert, 2007)). However, the reason why an AER fails to further develop in snakes is still unknown.

Snakes have a very elongated body plan with multiple segments, displaying similar morphologies. The study of this extreme vertebrate formula may thus shed light on the nature of those mechanisms regulating the extension, segmentation and patterning of the major body axis. By looking at components of the segmentation clock (Pourquie, 2003) in developing corn snake embryos, a step was taken towards understanding the mechanism behind the large number of snake vertebrae. Gomez *et al.*, (Gomez *et al.*, 2008) indeed reported that, while somites were generated in snakes by using the same mechanism than in any other vertebrates, a large number of relatively small somitic condensations were produced, due to both a slower shrinkage of the mass of presomitic mesoderm (PSM) and a segmentation clock that progresses four times faster than in the mouse (Gomez and Pourquie, 2009).

### **Hox genes in the evolution of the serpentiform body plan**

Changes in the expression specificity and/or function of *Hox* genes, a family of transcription factors involved in the patterning of the body plan in all animals, have been proposed as potential causes in the evolution of a serpentiform morphology (see refs in (Woltering, 2012)). It was reported that snakes have the same set of *Hox* genes as any other amniote, with the notable exception of *Hoxd12*, which is absent from all snakes sequenced thus far. In contrast, a *Hoxc3* gene is found in all Squamata, which in turn is not present in mammals (Di-Poï *et al.*, 2010, Vonk *et al.*, 2013). However, it is unlikely that this small change in *Hox* gene content would have contributed to the generation of the snake-like body plan. The absence of *Hoxd12*, a gene mostly involved in tetrapod limb development, may rather reflect a consequence of limblessness in snakes (e.g. (Kmita *et al.*, 2002)). Instead, either modifications in the ability of *Hox* proteins to properly regulate downstream target genes, or changes in the transcriptional regulation of *Hox* genes

themselves may have accompanied the evolutionary transition towards these extreme morphologies.

In the mouse, the role of *Hox* genes in rib cage development has been extensively studied and *Hox* paralogous groups 5 to 9 were shown to organize the thoracic skeleton in a collinear manner (McIntyre *et al.*, 2007). Mutations within each of these genes generated somewhat overlapping mutant phenotypes, with abnormal phenotypes affecting progressively more posterior parts of the thoracic vertebrae. Therefore, different combinations of *Hox* genes are necessary to generate morphologically distinct vertebral identities (McIntyre *et al.*, 2007). The first detailed analysis of *Hox* gene expression specificities in the corn snake revealed that the observed segmental uniformity did not reflect a homogenization of *Hox* gene expression domains. Somehow unexpectedly, these expression domains were also found to be organized in a collinear manner along the anterior to posterior axis, as in the mouse (Fig. 2) (Woltering *et al.*, 2009). However, recent analyses of snake vertebrae morphology (Head and Polly, 2015) have shown that snakes have four different types of pre-cloacal vertebrae (like other vertebrates) and hence the de-regionalization of the primaxial skeleton (including dorsal ribs) may not be as pronounced as previously thought. In contrast, an important transformation comes from the loss of the abaxial rib cage (the ventral part, including the sternum).

Both *Hoxc10* and *Hoxa10*, which in mammals encode proteins with a strong rib-repressing activity (Wellik, 2003), were nevertheless unexpectedly found transcribed in somites of early corn snake embryos, at body levels corresponding to rib-bearing vertebrae (Di-Poï *et al.*, 2010, Woltering *et al.*, 2009), suggesting that such proteins were no longer able to repress rib formation. Further work showed that the snake *Hoxa10* protein was nevertheless able to repress rib formation when overexpressed in the mouse presomitic mesoderm, suggesting that the snake *Hoxa10* protein can still exert this particular rib-suppressing property. One potential explanation to this conundrum was provided by the presence of a single polymorphism in the *Hox* binding site of a target gene enhancer element, making the latter unresponsive to the presence of *Hox10* transcription factors. In this case, therefore, a mutation in a binding site rather than a protein loss of function caused the persistence of ribs at places where they had disappeared in other amniotes (Guerreiro *et al.*, 2013).

Ectopic activation of *Hox* genes produces homeotic transformations, where a part of the body takes the identity of another one (ref. in (Duboule and Morata, 1994)). Likewise, changing *Hox* gene expression in time and space in the course of evolution, *via* regulatory modifications may have induced significant morphological changes. As other developmental loci implementing complex regulations (Simons *et al.*, 2006), amniote *Hox* clusters tend to be poor in repeated elements, a situation perhaps selected to prevent interferences with long-range regulations by global enhancers (see (Duboule, 2007)). In this context, the fact that all squamates display rather high amounts of transposable elements within *Hox* clusters, which are also substantially larger than their amniote counterparts (Di-Poï *et al.*, 2009, Di-Poï *et al.*, 2010), suggests that such elements may have had some consequence upon *Hox* gene regulation. For example, the corn snake *Hoxd13* and *Hoxa13* genes have significantly low levels of expression during tail bud development (Di-Poï *et al.*, 2010), the place where somites are added during body extension. Because these genes are involved in regulating axial extension (Young *et al.*, 2009), see (Young and

Deschamps, 2009), their altered regulation, in conjunction with a rapid segmentation clock (Gomez *et al.*, 2008), could account for (part of) the elongated snake body plan (Di-Poi *et al.*, 2010, Woltering, 2012).

### Still a long way to go

While these different results suggest some avenues to investigate the drastic modification that occurred in the snake body plan, they nevertheless remain at a rather descriptive or theoretical level. As in many such cases of emerging evo-devo model organisms, hypotheses can be proposed but their experimental verifications can hardly be carried out. For instance, slowing down the segmentation clock while keeping the same developmental pace should in principle give less somites yet of a larger size. Likewise, strongly activating *Hox* group 13 genes in the early tail bud should lead to a snake of a shorter body length. The same holds true for the genomic tools and their application based on snake genome sequences, such as ChIP-seq, DNA methylation analyses or chromatin structure experiments. While they may reveal essential variations between snakes and other amniotes, the functional significance of such variations will be difficult to ascertain.

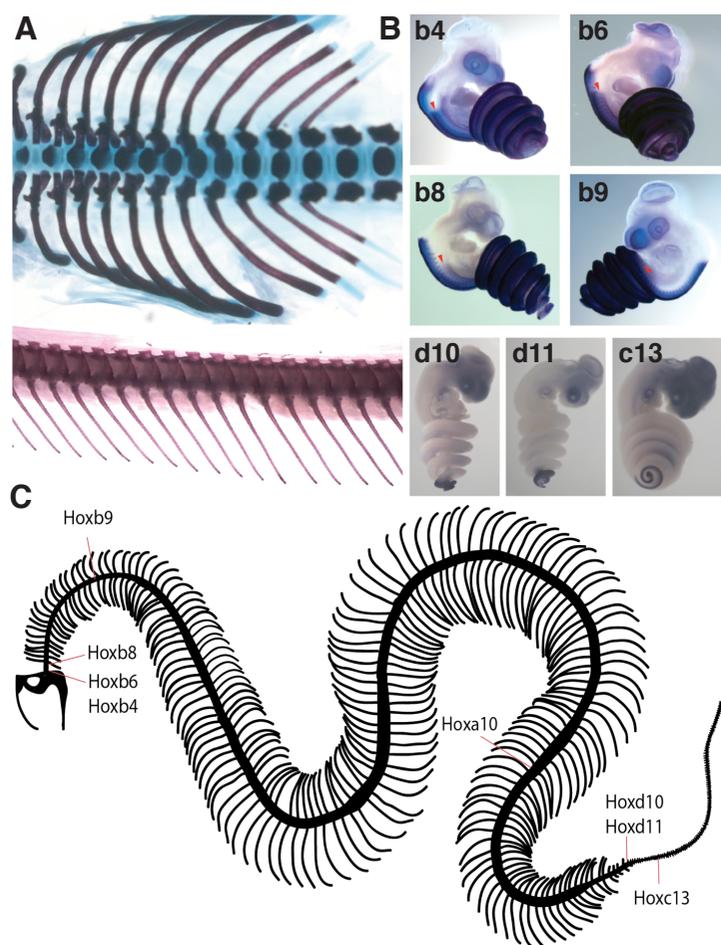
Unfortunately, none of these experiments can be carried out in snakes thus far, mostly due to the biology of these animals, their oviparous or ovoviviparous reproductive strategies and the time needed to reach sexual maturation. An alternative approach

is to use a system amenable to functional studies such as mice, where snake elements (genes, enhancers, binding sites) can be introduced and analyzed. For example, assuming that a mutation rendered a snake *Hox* protein unable to properly interact with target genes, this protein as well as its 'corrected' version could be assayed in a transgenic mouse context to see if a similar effect is scored. Likewise, a mutation within a snake enhancer sequence, which would make a target gene unresponsive to a *Hox* protein could be introduced within the cognate mouse sequence. Also, to address cases where differential *Hox* gene regulation is observed between snakes and mice, large pieces of the snake genome could be introduced into mice such as to see if the variability is encoded into the transposed piece or by upstream regulators. In all these cases, however, the results will mostly tell us about how the mouse can accommodate snake material, rather than truly demonstrating a causative effect of the transgenic material upon the extreme snake morphologies. To answer the latter question, hypotheses must be functionally checked in the appropriate context: the snakes themselves.

This point raises again the general nature of model systems in biological studies and the necessity to develop the appropriate tools in a large variety of representative animal species. In this context, some important obstacles must still be removed before snakes become a fully useful model system in developmental biology. A species would be required that can reproduce all year long, lay many eggs at a not-too-late developmental stage with, if possible, some

level of inbreeding. Yet the most difficult obstacle to overcome will be to make snakes genetically modifiable. For the moment, it is difficult to imagine how this could be achieved efficiently and sperm-mediated gene transfer (SMGT; see (Smith and Spadafora, 2005) may be the most promising approach to develop. This approach, however, has not proven particularly successful in other animal species thus far.

The relationship between evo-devo research and the realm of animal model systems is complex and reflects the variety of mindsets making this large community of scientists. Often, a species is selected due to one particular trait, which may help understand a question of wide interest due to the accumulation of knowledge derived from other well-established systems such as e.g. digit reduction in tetrapods (Cooper *et al.*,



**Fig. 2. Hox gene expression and its relationship to the snake axial skeleton.** (A) Dorsal view of a mouse rib cage (top), compared to a snake rib cage (lateral view, bottom). Unlike the snake (but see (Head and Polly, 2015), the mouse rib cage displays a variety of rib morphologies, as established by the succession of *Hox* gene expression domains (e.g. (Kessel and Gruss, 1991)). (B) *Hox* gene expression territories during the development of the extended body axis in the corn snake. A collinear distribution is observed in the snake, despite the lack of a clear segmental regionalization. For example, *in situ* hybridization of 2.5 to 3.5dpo corn snake embryos with *Hoxb4*, *Hoxb6*, *Hoxb8*, *Hoxb9*, *Hoxd10*, *Hoxd11* and *Hoxc13*-specific probes show that the anterior boundary of gene expression shifts successively towards more posterior regions of the embryo, in a collinear manner (data from (Di-Poi *et al.*, 2010, Woltering *et al.*, 2009)). Anterior limits of gene expression for *Hoxb4* to *Hoxb9* are indicated with a red arrowhead. (C) Schematic representation of an adult snake skeleton, with the approximate positions of anterior limits of mesodermal *Hox* gene expression, as transposed from expression studies in embryos. Selected *Hox* gene expression anterior boundaries are indicated (Woltering *et al.*, 2009).

2014). Likewise, a deep mechanistic understanding, at the cellular level, of one precise developmental situation can be successfully complemented by the analysis of as many variants as possible within closely related species (Arnoult *et al.*, 2013, Sommer, 2009). Alternatively, new species can be developed as genuine model systems, by complementing a fair knowledge of the biology of the animal by modern genomic tools and a potential for genetic analyses (Sato and Levine, 2005). It is however necessary that reliable functional approaches be developed and adapted to each model and to critically assess their efficiency and relevance (see (Kok *et al.*, 2014). At the other end of the spectrum, PCR products can be amplified from any animal species and gene expression recorded and compared, leading to more or less significant inferences (Duboule, 2005).

After all, the heuristic value of a given animal model is hardly predictable and can only be judged retrospectively, by both the number and resilience of general concepts extracted from them and readily extended to a larger group of species, ideally to all. For example, the chick and the importance of crest cells (Le Douarin, 2004), apoptosis in the nematode *C. elegans* (Hengartner and Horvitz, 1994), homeotic transformations (Lewis, 1978) or innate immunity (Lemaitre *et al.*, 1996) in *Drosophila* and the existence of parental imprinting in mouse (Barton *et al.*, 1984, McGrath and Solter, 1984), to name only a few, are trademarks of 'classical' models used in developmental biology over the past 40 years. It may take some more years for the serpent to emerge from under the flower and join the pantheon of model systems for developmental biology and genetics. Nothing does strictly prevent it to reach that stage, as long as enough scientists have time and interest to invest into this endeavor and that they can find the appropriate funding, which may be soon the most difficult obstacle to cross.

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