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COMMENTS

THE DEVELOPMENTAL BASES OF LIMB REDUCTION AND BODY ELONGATION IN SQUAMATES

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Abstract.—Employing an integrative approach to investigate the evolution of morphology can yield novel perspectives not attainable from a single field of study. Studies of limb loss and body elongation in squamates (snakes and lizards) present a good example in which integrating studies of systematics and ecology with genetics and development can provide considerable new insight. In this comment we address several misunderstandings of the developmental genetic literature presented in a paper by Wiens and Slingluff (2001) to counter their criticism of previous work in these disciplines and to clarify the apparently contradictory data from different fields of study. Specifically, we comment on (1) the developmental mechanisms underlying axial regionalization, body elongation, and limb loss; (2) the utility of presacral vertebral counts versus more specific partitioning of the primary body axis; (3) the independent, modular nature of limbs and limb girdles and their utility in diagnosing genetic changes in development; and (4) the causal bases of hind limb reduction in ophidian and nonophidian squamates.

Key words.—Development, evolution, *Hox* genes, limb, limblessness, lizard, snake.

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As in many areas of evolutionary biology, there are a variety of approaches that can be used to investigate the origins of morphological diversity: one can make inferences based on morphological and/or ecological comparisons alone, but it is also desirable to investigate how the genetic modification of developmental programs has led to the generation of divergent phenotypes. Integrating these approaches has the potential to yield a more complete understanding of morphological evolution than is attainable through the use of individual methods alone.

One group in which this integrative approach may shed new light on evolutionary processes is the squamates (lizards and snakes). Many squamate groups exhibit a trend toward body elongation and limb reduction (Greer 1991), but few researchers have employed a robust phylogenetic framework and statistical analysis when testing hypotheses about this trend. Wiens and Slingluff (2001) addressed this problem with the family Anguidae, a diverse group of elongate lizards that show various stages of limb reduction and loss. Among many other conclusions they report a strong correlation between body elongation and limb reduction, and conclude that these features have repeatedly evolved concurrently, not serially, as has previously been suggested (e.g., Gans 1975; Lande 1978). Although we take no issue with their conclusions regarding the coincident evolution of an elongate body plan with limb reduction and loss, we believe they have misinterpreted recent molecular studies regarding the developmental bases of limb loss in squamates. Our goal here is to complement the findings of Wiens and Slingluff (2001) with a clarification of the developmental bases of limblessness in squamates. We discuss: (1) the developmental mechanisms underlying axial regionalization, body elongation, and limb loss; (2) the utility of presacral vertebral counts versus more specific partitioning of the primary body axis; (3) the independent, modular nature of limbs and limb girdles, and their utility in diagnosing changes in development; and (4) the causal bases of hind limb reduction in ophidians (snakes) and nonophidian squamates.

The goal of Wiens and Slingluff (2001) was to investigate the sequence of morphological and ecological changes associated with the transition from a ''lizardlike'' to a ''snakelike'' form, using molecular phylogenetic, morphometric, and comparative phylogenetic methods in the family Anguidae. They also used their data from anguid lizards to criticize a developmental model for the evolution of limblessness in snakes (Cohn and Tickle 1999). Although we agree that the data presented show that different developmental mechanisms may have been responsible for limb loss in anguids and snakes, their discussion of the developmental processes involved is misleading. We address each point mentioned above to clarify and expand on the results and conclusions of Wiens and Slingluff (2001). We note at the outset, however, that we only discuss Cohn and Tickle's empirical results, and do not necessarily endorse their conclusions regarding the phylogenetic origins of snakes, or the evolutionary rate at which they ascribe morphological changes to shifts in *Hox* gene expression boundaries.

(1) A common misinterpretation of Cohn and Tickle's (1999) study lies in the perception that a common developmental mechanism must link both body elongation and limb loss (e.g., Graham and McGonnell 1999; Greene and Cundall 2000; Wiens and Slingluff 2001). In fact, Cohn and Tickle's study deals solely with forelimb loss, hind limb reduction, and changes in the specification of regional identity within the vertebral column, and does not begin to explore the causal bases of body elongation. This confusion appears to have arisen from the fact that, as background in their Abstract, Cohn and Tickle (1999, p. 474) stated, ''Combined limb loss and trunk elongation is found in many vertebrate taxa, suggesting that these changes may be linked by a common developmental mechanism.'' This idea is addressed by neither their experiments nor their discussion. Instead, they investigated the expression patterns of three *Hox* genes, HOXC6, HOXC8, and HOXB5, in a python. In birds and mice the anterior expression boundaries of HOXC6 and HOXC8 are associated with specifying thoracic versus cervical vertebral

identity (Oliver et al. 1988; Burke et al. 1995; Shashikant et al. 1995) and determining the axial position of the forelimb bud (Cohn et al. 1997), whereas HOXB5 is expressed throughout the trunk up to the atlas, the first cervical vertebra. In the python examined, all three of these genes are expressed throughout the trunk up to the first postcranial somite that will later form the atlas. Forelimbs develop at the cervical/thoracic junction, which correlates with the anterior boundary of HOXC6 expression (Burke et al. 1995). Cohn and Tickle (1999) attributed the loss of forelimbs in snakes to an anterior shift of thoracic identity, which disrupts the cervical/thoracic junction.

It is important to recognize that this reasoning applies only to forelimb loss, not to body elongation. *Hox* genes are transcription factors that specify the identity of different axial regions. They are not growth factors, do not control rates of somitogenesis, and are therefore unlikely to be causally involved in elongation of the body. In vertebrates, elongation of the body is accomplished either by adding vertebrae to specific axial regions or lengthening individual vertebral elements (Burke et al. 1995). Such changes presumably occur through heterochronic changes in the rate or duration of somite segmentation from presomitic mesoderm that either increase the total number of somites or lengthen individual somites, since somites are the precursors of vertebrae (Gilbert 2000; Jouve et al. 2000). Although the underlying mechanism for this is still undetermined, it is presumably related to heterochronic changes in the segmentation clock, the molecular mechanism responsible for controlling the timing of somitogenesis in vertebrates (Pourquié 2001, 2003).

(2) The use of presacral vertebral counts is a common practice in herpetology for species diagnosis and often serves as a measure of body elongation (e.g., Stokeley 1947; Greer 1987; Caputo et al. 1995; Greer et al. 2000; Wiens and Slingluff 2001). However, since the model proposed by Cohn and Tickle (1999) deals with *homeotic* changes in regional identity within the vertebral column, not *meristic* changes in the number of vertebrae, presacral vertebral counts would be more informative if the number of vertebrae of each type present within each region of the vertebral column (cervical, thoracic, lumbar) was specified. Snakes often possess more than 300 vertebrae but only one clearly identifiable presacral region, the thoracic region, although some (apparently chimeric) anterior vertebrae also possess the ventral hypophyses typical of cervical vertebrae (Cohn and Tickle 1999; Zaher and Rieppel 1999). Other elongate squamates, including anguids, possess either two or three identifiable regions in their presacral vertebral column (Romer 1956; T. Sanger, pers. obs.). Cohn and Tickle propose that the loss of axial regionalization in snakes is due to *homeotic* shifts in the expression patterns of HOXC6 and HOXC8, genes whose expression is normally associated with the thoracic region (Oliver et al. 1988; Burke et al. 1995; Shashikant et al. 1995), and do not address mechanisms dealing with *meristic* changes in the number of vertebrae. Acknowledging the distinction between homeotic and meristic changes in this context allows one to recognize that forelimb loss due to changes in axial *Hox* gene expression is not likely to be common among nonophidian squamates because most species retain cervical vertebrae. However, partial anteriorization of *Hox* gene expression boundaries could well be found during the development of other limbless groups with reduced numbers of cervical vertebrae, such as amphisbaenians and other limb-reduced lizard species, none of which have yet been examined.

As explained above, the developmental basis of meristic changes in vertebral number likely involves independent, unrelated changes in the segmentation clock (Pourquié 2001, 2003). Also, since the positioning of pelvic girdles is determined by *Hox* gene expression boundaries, not the dissociated process of somitogenesis, their position alone may not accurately reflect changes in the developmental processes leading to elongation of the body. Thus, although Wiens and Slingluff (2001) suggest that trunk elongation and tail elongation represent two different ways in which body length is increased, changes in both these parameters are the consequence of changes in a single underlying developmental process, the rate and duration of somitogenesis (Pourquié 2001, 2003).

(3) Limbs and limb girdles represent distinct developmental modules that develop semiautonomously and can evolve independently (McGonnell 2001). However, Wiens and Slingluff (2001, p. 2313) use the presence of pectoral girdle elements in anguids to reject the model of Cohn and Tickle (1999) by stating, ''Expansion of *Hox* gene domains would explain the complete absence of all limb girdle elements, but not the absence of limbs alone.'' Though we agree that anterior shifts in *Hox* gene expression have probably not been involved in limb reduction or loss in anguids, this statement is misleading since it makes several assumptions not recognized by Wiens and Slingluff (2001). For example, *Hox* gene expression is independently regulated in lateral plate and paraxial mesoderm (Cohn et al. 1997). The clavicle, humerus, ulna, radius, carpals, and metacarpals all develop from lateral plate mesoderm (Chevallier 1977; Gilbert 2000; but see McGonnell et al. 2001) and would all therefore be affected by shifts in *Hox* expression patterns in this tissue. The scapula, however, forms from both paraxial and lateral plate mesoderm (Huang et al. 2000), so *Hox* domain shifts in only one tissue would not necessarily lead to the elimination of girdle elements derived from the other. Shifts in *Hox* expression domains can account for the development of limb-possessing, girdleless forms (e.g., Ahlberg 1992), and to limbless, girdle-possessing forms (e.g., Belting et al. 1998). Cohn and Tickle (1999) describe a mechanism that could be responsible for the loss of both pectoral girdle and forelimb bones by noting that *Hox* gene expression boundaries have expanded anteriorly in ''*both* paraxial and lateral plate mesoderm'' (our italics) in pythons. It should be noted that snakes are the only squamates, with the exception of some amphisbaenians, known to lack both pectoral and pelvic girdles. Other limbless squamates retain both sets of limb girdles (Greer 1997).

(4) Cohn and Tickle (1999) postulated that different developmental mechanisms were responsible for forelimb loss and hind limb reduction in snakes. Although they remark that shifted *Hox* gene expression domains may have been involved in hind limb reduction by disrupting the polarizing ability of the early limb bud, they primarily attribute the reduction of hind limbs to a deficit in apical ectodermal ridge (AER) formation early in limb development. The AER is an organizing

structure responsible for maintaining distal outgrowth of the growing limb bud through the secretion of fibroblast growth factors (FGFs; Gilbert 2000; Sun et al. 2002). Though Wiens and Slingluff (2001) did not discuss the developmental differences between forelimb loss and hind limb reduction, we feel that differences between these processes are important in understanding the Cohn and Tickle model of snake evolution and the larger trend across squamates. Lack, or early degeneration, of the AER has been suggested to play a role in limb reduction or truncation in other squamates such as *Anguis fragilis* (Raynaud 1962, 1974; Raynaud et al. 1995), *Ophisaurus apodus* (Rahmani 1974), and three species of *Scelotes* (Raynaud and Van den Elzen 1976). In *Anguis fragilis*, cell proliferation has been shown to diminish significantly after AER degeneration (Raynaud and Kan 1992), and cell death and limb bud degeneration begins shortly after loss of the AER (Raynaud 1974; Raynaud and Van de Elzen 1976; Lande 1978). Treatment of the lateral plate mesoderm or early limb buds of pythons (Cohn and Tickle 1999) and *Anguis fragilis* (Raynaud et al. 1995, 1998) with FGF2 recovers limb bud outgrowth, indicating that an FGF may be one of the factors lacking in these limbless forms. In summary, it is important to recognize that shifts in axial *Hox* gene expression domains are likely not alone responsible for the reduction of hind limbs in squamates, and that programmed degeneration of the AER is a more plausible mechanism for the arrested development and degeneration of early limb buds.

Wiens and Slingluff (2001) have laudably completed one of the first statistical analyses of the relationships between body elongation, limb reduction, and limb loss within squamates, and convincingly shown that these changes occurred concurrently, not serially, as previously suggested. We do not wish to challenge any statistical correlation between body elongation and limb reduction, for this association seems well established, but hope to supplement these observations with a more accurate discussion of the developmental mechanisms underlying these changes. In light of these clarifications it seems that at least two different developmental mechanisms for limb loss may be responsible for the convergent trend observed in different groups of squamates: (1) shifting *Hox* gene expression domains in paraxial and lateral plate mesoderm, which has so far only been observed in a snake, and (2) loss and/or degeneration of the AER during limb bud development, leading to the early degeneration of limb primordia. In making this distinction we note that, during early snake evolution, limbs may well have initially been truncated due to premature degeneration of the AER, as in anguids, after which shifts in *Hox* gene expression secondarily resulted in disruption of the cervical/thoracic junction, and the final loss of forelimbs. It also seems that one need only invoke one developmental mechanism, involving heterochronic changes in the segmentation clock, to explain changes in body length, although the morphological outcomes of these changes (i.e., the number of vertebrae of each type) vary greatly in different lineages of squamates. Thus the repeated, concurrent association between body elongation and limb reduction in anguids, as demonstrated by Wiens and Slingluff (2001), may well be due to constraints imposed by the mechanics of locomotion and/or common patterns of environmental selection, yet involve changes in quite separate, dissociated developmental mechanisms in each lineage.

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