Sexual Development

Sex Dev
DOI: 10.1159/000363758

Published online: June 20, 2014

Development of Hemipenes in the Ball Python Snake *Python regius*

Francisca Leal[a] Martin J. Cohn[a–c]

Departments of [a]Biology and [b]Molecular Genetics and Microbiology, Genetics Institute, and [c]Howard Hughes Medical Institute, University of Florida, Gainesville, Fla., USA

Key Words
Cloacal swellings · Genital development · Homology · Squamates · Spermatic sulcus

Abstract
Within amniotes, external copulatory organs have undergone extensive morphological diversification. One of the most extreme examples is squamate (lizards and snakes) hemipenes, which are paired copulatory organs that extend from the lateral margins of the cloaca. Here, we describe the development of hemipenes in a basal snake, the ball python *(Python regius)*. Snake hemipenes arise as a pair of lateral swellings on either side of the caudal part of the cloaca, and these paired outgrowths persist to form the left and right hemipenes. In non-squamate amniotes, external genitalia form from paired swellings that arise on the anterior side of the cloaca, which then fuse medially to form a single genital tubercle, the anlagen of the penis or clitoris. Whereas in non-squamate amniotes, Sonic hedgehog *(Shh)*-expressing cells of the cloacal endoderm form the urethral or sulcus epithelium and are required for phallus outgrowth, the hemipenes of squamates lack an endodermal contribution, and the sulcus does not express *Shh*. Thus, snake hemipenes differ from the genital tubercles of non-squamate amniotes both in their embryonic origins and in at least part of patterning mechanisms, which raises the possibility that hemipenes may not be direct homologs of the unpaired amniote penis. Nonetheless, we find that some developmental genes show similar expression patterns in snake hemipenes buds and non-squamate genital tubercles, suggesting that homologous developmental mechanisms are involved in aspects of external genital development across amniotes, even when these structures may have different developmental origins and may have arisen independently during evolution.

Lizards and snakes (order Squamata) belong to one of most basal reptile lineages, the Lepidosauria [Crawford et al., 2012; Fong et al., 2012; Wang et al., 2013]. Although reptile phylogenetic relationships were debated in the pregenomic era [deBraga and Rieppel, 1997; Zardoya and Meyer, 1998, 2001], recent phylogenomic analyses of amniotes consistently place turtles as the sister group of archosaurs (birds and crocodilians), and lepidosaurs (lizards, snakes and tuataras) as the sister group of turtles and archosaurs [Fong et al., 2012; Shaffer et al., 2013; Wang et al., 2013] (fig. 1A). Therefore, the lepidosaurs represent a key clade for understanding the evolution of amniote copulatory organs.

KARGER

E-Mail karger@karger.com
www.karger.com/sxd

1661–5425/14/0000–0000$39.50/0

Martin J. Cohn
Department of Molecular Genetics and Microbiology and Department of Biology
University of Florida, PO Box 103610
Gainesville, FL 32610 (USA)
E-Mail mjcohn@ufl.edu

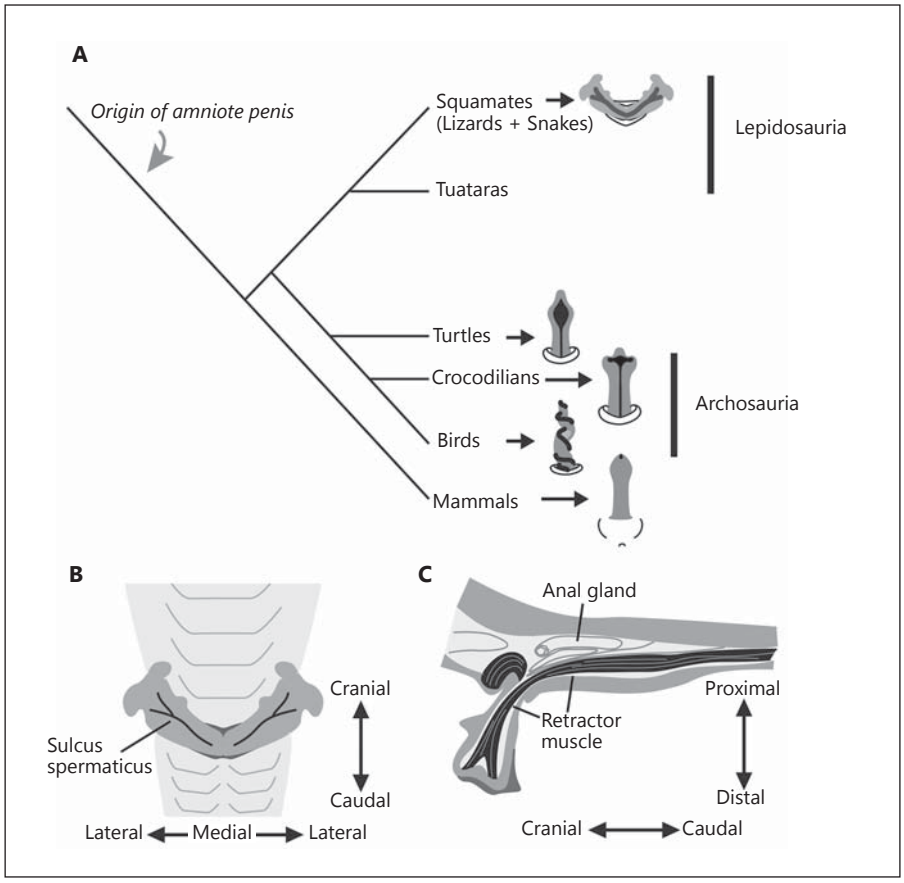

Fig. 1. A Genital morphology of amniote crown groups. Phylogenetic relationships based on the consensus of recent molecular phylogenomic analyses [Crawford et al., 2012; Wang et al., 2013]. Within the Lepidosauria, it is unclear whether hemipenes emerged before or after the divergence of tuataras and squamates, as tuataras have been reported to have rudimentary, non-intromittent cloacal swellings. Note that the penis depicted in birds represents only that of paleognaths and anseriforms; in other birds, the penis is reduced or absent. **B** Schematic diagram shows the external view of the everted snake adult hemipenes. The sulcus spermaticus can be seen on the postero-medial side of each hemipenis. **C** Schematic diagram of a sagittal section through a single hemipenis in an everted position. Note that the retractor muscle arises from the tail and attaches to the tip of the hemipenis. Illustration modified from Dowling and Savage [1960].

In contrast to the single median penises of turtles, crocodilians and birds [Gadow, 1887; Raynaud and Pieau, 1985; Lombardi, 1998], squamates have paired penises, known as hemipenes (fig. 1B), which are stored in individual cloacal pockets and can be everted for copulation [Gadow, 1887; Dowling and Savage, 1960; Raynaud and Pieau, 1985; Pough et al., 2001; Vitt and Caldwell, 2009]. Squamate hemipenes are used individually, in alternation, during copulation and are then pulled back into the caudal hemipenial pockets by means of a main retractor muscle that runs along the main axis of each hemipenis (fig. 1C). A unique feature found in snakes is a pair of anal glands positioned dorsal to each hemipenis and caudal to the cloaca [Dowling and Savage, 1960] (fig. 1C). Like other reptilian copulatory organs, each hemipenis has a spermatic furrow or sulcus along which sperm can travel from the male cloaca into the female cloaca. The primitive condition for lepidosaur genitalia remains equivocal, given that the tuatara, the most basal extant lepidosaur lineage and the sister group of the Squamata [Townsend et al., 2004; Crawford et al., 2012], has been described both as lacking intromittent organs altogether [Raynaud and Pieau, 1985; Pough et al., 2001] and as having rudimentary hemipenes [Vitt and Caldwell, 2009]. Nonetheless, the phylogenetic position of lepidosaurs raises the possibility that paired external genitalia are the primitive condition for amniotes; however this is debatable, as all other amniotes with external genitalia have a single (unpaired) penis [King and McLelland, 1981; Lombardi, 1998; Kardong, 2012].

The homology of squamate hemipenes relative to the penises of turtles, crocodilians, birds, and mammals is unresolved [Kelly, 2004]. One hypothesis is that squamate hemipenes originated by division of the ancestral unpaired reptilian penis along the midline of the urethral plate [Raynaud and Pieau, 1985]. However, if hemipenes are primitive, then the median penis of all other amniotes may have evolved by fusion of the hemipenes at the ventral midline. Another possibility, considered here, is that the transition from unpaired to paired (or vice versa) penile structures evolved not by modification of the ancestral organ, but rather by loss of the primitive penis and de novo development of a novel intromittent organ.

Developmentally, the penises of alligators, turtles, birds, and mammals begin as paired outgrowths on either side of the cranial part of the cloacal membrane that then merge to form a single genital tubercle shortly after outgrowth is initiated [Raynaud and Pieau, 1970, 1985; Perriton et al., 2002; Herrera et al., 2013]. If the paired genital swellings of amniote embryos are homologous, then one possibility is that each squamate hemipenis would be homologous to each of the bilateral halves of an otherwise unpaired penis. Furthermore, each half of the split urethral plate could give rise to each of the spermatic sulci on the left and right hemipenis. In this scenario, the spermatic sulcus in squamates would be homologous to the urethral groove or spermaticus sulcus of the unpaired reptilian penis. However, previous anatomical and embryological studies of urogenital development in lizards and snakes give little support to the hypothesis of homology between the ancestral unpaired reptilian penis and the squamate hemipenes [Raynaud and Pieau, 1985]. In addition to the obvious anatomical differences between squamate hemipenes and the unpaired penises of other reptiles, birds and mammals, it has been reported that hemipenes have a different origin during early cloacal and genital development [Raynaud and Pieau, 1970, 1985; Rosenberg et al., 1989]. A central argument is that hemipenes in lizards and snakes arise from latero-caudal swellings in relation to the cloacal membrane [Raynaud and Pieau, 1970, 1985; Rosenberg et al., 1989], whereas the unpaired reptilian penis in turtles and archosaurs was argued to arise from anterior paired (cranial) swellings [Raynaud and Pieau, 1985]. Further controversy concerned the origin of the spermatic sulcus and its relation to the urethral groove of other reptiles [Raynaud and Pieau, 1970, 1985].

There have been no comparative developmental or molecular studies of reptile external genitalia development that address the question of homology among reptilian copulatory organs; most of the arguments published to date have been based on anatomy and histology of reptilian genital development. Here, we investigate the early embryology and molecular mechanisms of squamate hemipenes development and address the question of homology by comparing 2 distantly related squamates, pythons (this study) and the green anole lizard [Gredler et al., this issue], with turtles [Larkins and Cohn, this issue], alligators [Gredler et al., this issue], birds [Herrera et al., 2013, and this issue], and mammals [Perriton et al., 2002]. Our results show that, relative to other amniotes, the snake hemipenes arise from a different region of the embryonic cloaca. We also found that, by contrast to other amniotes, the formation of the spermatic sulcus does not involve formation of a urethral plate or any contribution of the cloacal or urodeal endoderm. However, our gene expression analysis during snake hemipenes formation shows that a subset of developmental genes is shared between squamate hemipenes and the mammalian genital gene regulatory network. The results presented here shed new light on the evolutionary origin of hemipenes and raise new questions about the mechanisms of genital outgrowth in squamate reptiles.

Materials and Methods

Embryo Collection

Freshly laid eggs from *Python regius* were purchased from Ben Cole Reptiles. The eggs were incubated at 31°C until the desired stages were reached. Seven different embryonic stages were collected, beginning immediately after oviposition and continuing until 15 days after oviposition. The embryos were staged by external morphological features, following the staging table for the embryonic development of *P. sebae* [Boughner et al., 2007] and *P. reticulatus* by [Raynaud, 1972].

Gene Cloning and in situ Hybridization

Embryos processed for in situ hybridization and histology were dissected in PBS, fixed in 4%, paraformaldehyde, dehydrated in a graded methanol series, and stored in methanol at –20°C. Embryos processed for RNA or DNA extraction were dissected in PBS and frozen in TRIzol (Life Technologies). RNA and genomic DNA isolation was performed using the TRIzol reagent following the manufacturer's instructions. The cDNA was generated using Amv retrotranscriptase (New England Biolabs) using 2 μg of the extracted RNA as a template.

PCR was performed using cDNA for cloning *Shh*, *Ptch1*, *Tbx4*, *Fgf8*, *Fgfr2*, and *Fgf10*, and genomic DNA for cloning *Hoxd13*, *Hoxa13*, *Msx2*, *Bmp4*, *Wnt7a*, and *Wnt5a*. PCR fragments were ligated into a pGEM-T easy vector (Promega). After Sanger sequencing, gene homology was determined initially by a BLAST search analysis, using vertebrate RNA Refseqs as the searchable database, and then confirmed by molecular phylogenetic analyses. Antisense mRNA probes were synthesized using either T7 or Sp6 RNA polymerases and were labeled with dioxigenin-UTP (Roche). Whole mount in situ hybridization was performed as previously described [Nieto et al., 1996] with the following modifications: the KTBT buffer contained 1% Tween-20 instead of Triton X-100, the NTMT buffer contained 1% Triton X-100, 10% dimethylformamide was added to staining solution, and proteinase K concentrations ranged from 10 up to 70 μg/ml [Laufer et al., 1997], with higher concentrations being used for older stages.

Histology and Scanning Electron Microscopy

Python embryos previously fixed in 4% PFA were refixed in Bouin solution and then dehydrated to 70% ethanol to capture bright-field microphotographs before processing for histology or scanning electron microscopy (SEM). Cross/transverse and sagittal serial histological sections were made of the pelvic region where

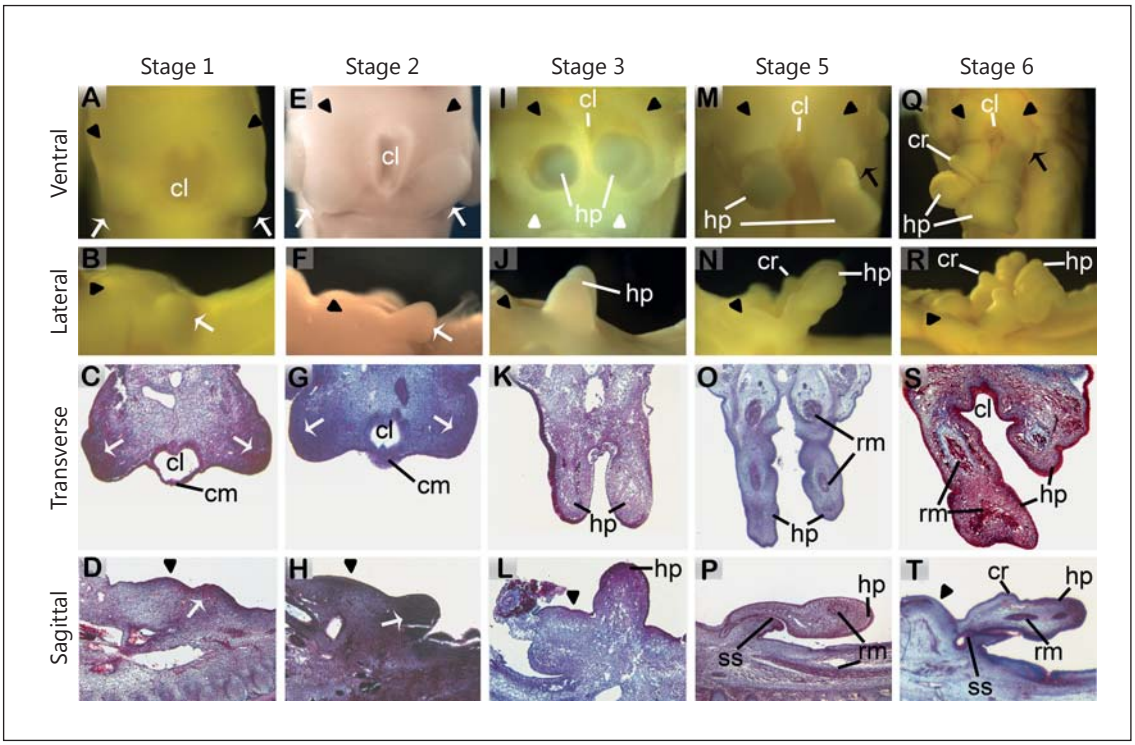

Fig. 2. Morphological development of hemipenes in ball pythons. Stages are shown at top. Bright-field microphotographs show the development of the external cloaca and hemipenes from stages 1–6 (top 2 rows) as well as histological sections (bottom 2 rows). Bright-field images were taken from ventral (**A**, **E**, **I**, **M**, **Q**), and lateral (**B**, **F**, **J**, **N**, **R**) views; histological sections were made in transverse (**C**, **G**, **K**, **O**, **S**) and sagittal (**D**, **H**, **L**, **P**, **T**) planes relative to the main body axis (both hemipenes are shown in transverse sections and one hemipenis is shown in sagittal sections). In transverse sections **A–F**, note that hemipenes anlagen (white arrows) arise at the sides of the cloacal membrane (cm) and there is no connectivity between the hemipenes and the cloacal endoderm. At stage 5 (**O**, **P**) the retractor muscle (rm) develops inside the hemipenis (hp), and the sulcus spermaticus (ss) forms a furrow that connects to the external cloacal opening. Black arrowheads: cranial lips; white arrowheads: caudal lip; black arrows: anal glands. cl = Cloaca; cr = cranial hemipenial ridge.

hemipenes development was taking place. These samples were embedded in paraffin wax and 10 μm sections were cut. Sections were stained with Masson Trichrome Kit (Thermo Scientific). For SEM imaging, the samples were dehydrated through a graded ethanol series to 100%, then critical-point dried and sputter coated. Lateral and ventral views were taken for all samples. SEM was performed at the University of Florida Interdisciplinary Center for Biotechnology Research electron microscopy core.

Results

Developmental Morphology of the Python Hemipenes Hemipenes Are Formed from Paired Swellings That Arise Latero-Caudal to the Cloacal Membrane

In order to characterize the embryonic origin and early development of external genital organs in pythons, we performed scanning electron microscopic and histological analyses of the cloacal region in a developmental series of python embryos. External genital outgrowth begins at stage 1, when a pair of swellings is initiated caudal to, and on either side of, the cloaca (fig. 2A–C, 3A, B). A pair of cranial swellings can also be observed at stage 1, and these swellings give rise to the cranial lip of the cloaca (fig. 2A, B, D, 3A, B). Beneath the cloacal membrane, the embryonic cloaca can be observed between the hemipenes swellings, but, in contrast to mammals, birds and turtles, there is no connectivity between the genital swellings and the cloacal epithelium (fig. 2C, D). At stages 2 and 3, the lateral swellings become larger and protrude distally to form 2 discrete genital tubercles, the buds that will form the hemipenes (fig. 2E–L, 3C–F). Analysis in whole-mount and in histological sections shows that the hemipenes buds do not emerge from the cloacal membrane, and we could not detect a contribution of the endodermal cloacal epithelium to the hemipenes buds (fig. 2A–G, 3A–D). Between stages 3 and 4, a pair of small caudal swellings can

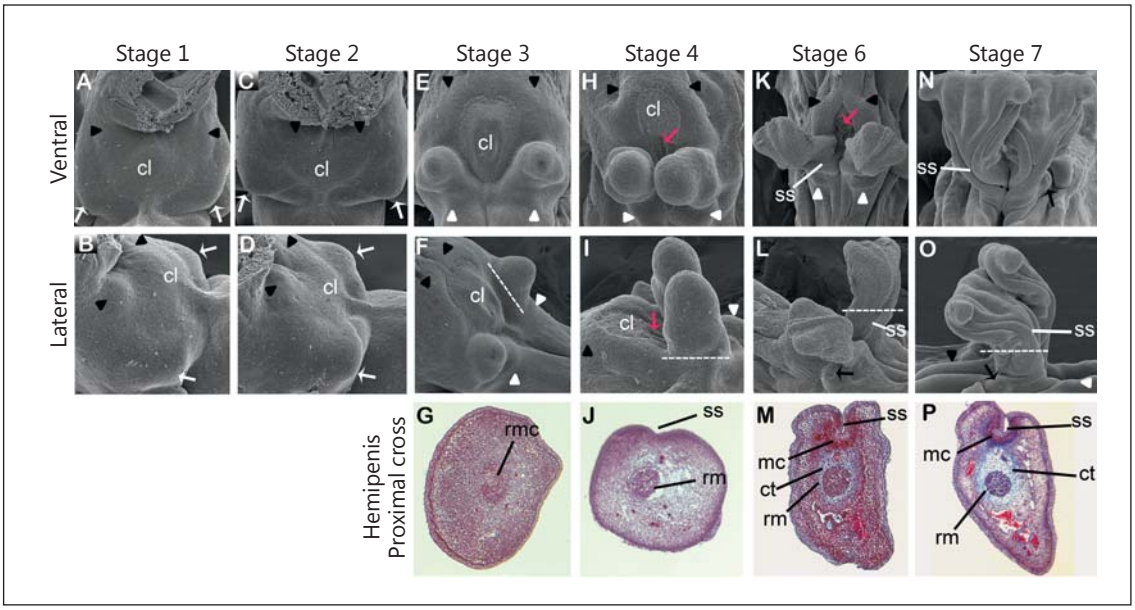

Fig. 3. Development of the external cloaca and hemipenes in ball pythons. Stages are shown at top. SEM (top 2 rows) and histological cross-sections of python hemipenes (bottom row). Dashed lines indicate planes of section. SEM analysis shows that the hemipenes buds develop lateral and caudal to the cloacal membrane (**A–F**). Histological cross-sections of the hemipenes (bottom row) show that the sulcus spermaticus starts to form at stage 4 as an invagination of the surface ectoderm on the medial side of the hemipenis (**J**). After stage 4, the sulcus spermaticus matures and invaginates further into the hemipenis and mesenchymal cell condensations differentiate into a U-shaped connective tissue subjacent to the sulcus epithelium (**M**, **P**). In **K**, **L**, the sulcus spermaticus can be seen invaginating into the medial side of the hemipenis at the base of the hemipenis stalk. At stage 7 (**N**, **O**), the sulcus spermaticus is well developed and bifurcates at the midlevel of each hemipenis. In the histological sections, the development of the retractor muscle can be observed as a mesenchymal condensation at the base of the hemipenis at stage 3, and then differentiating into muscle tissue (**G**, **J**). The retractor muscle is surrounded by connective tissue forming a connective tissue sheath at stages 6 and 7 (**M**, **P**). Black arrowheads: cranial lips; white arrowheads: caudal lips; white arrows: hemipenes buds; red arrows: cloacal opening; black arrows: anal glands. rmc = Retractor muscle condensation; mc = mesenchymal condensations underneath of the sulcus epithelium; ct = connective tissue surrounding the retractor muscle. For further abbreviations, see figure 2.

be observed posterior to the base of the hemipenes, and these swellings will form the caudal lip of the cloaca (fig. 2I, 3E, F). At stage 4, the cloacal membrane starts to rupture in a caudal to cranial direction (fig. 3H, I). Between stages 4 and 5, the cylindrically shaped hemipenes form a large median transverse fold over the cranial side of each tubercle (fig. 2M–P, 3H, I). This fold forms a small ridge, slightly lateral to the cranial side of the hemipenis (fig. 2Q, R). At stage 5, the anal glands appear at the base of each hemipenis on their lateral sides (fig. 2M, Q, 3L–O). Between stages 5 and 6, the cranial lip of the cloaca becomes better differentiated, forming a V-shaped ridge, and the cloacal membrane degenerates, leaving a large cloacal opening (fig. 2M–S, 3K). The distal ends of the hemipenes are smooth before stage 6; however, they then form 2 small buds on their medial and lateral aspects of the distal tip, which will form 2 distal outgrowths that result in each hemipenis developing a 'T'-shape (fig. 3N, O).

The Spermatic Sulcus Forms by Invagination of Surface Ectoderm on the Medial Side of Each Hemipenis

During early development of the python hemipenes, each hemipenis bud forms with no contribution from the urodeal (cloacal) epithelium (fig. 2E, I–T, 3H–O). The exact point at which the surface ectoderm of the genital tubercle abuts the cloacal endoderm was not clear from our embryological analysis.

The spermatic sulcus in pythons develops late, compared to the urethral plate development in mouse, bird, and turtle penises, and forms on the surface of, rather than internally in, python hemipenes. The spermatic sulcus first becomes detectable at stage 4, when the medial surface ectoderm at the base of each tubercle begins to invaginate slightly (fig. 3H–J). This coincides with the formation of a focal mesenchymal condensation beneath the epithelial invagination. The formation of the sper-

matic sulcus then follows a proximal to distal direction. At stages 5 and 6, the sulcus runs along the medial surface of the proximal tubercle and turns over the caudal surface at the midpoint of the tubercle (fig. 2P, T, 3K, L). At this stage, the sulcus has not yet reached the distal tip of the tubercle. In cross-sections of the hemipenis, the sulcus is evident as a U-shaped epithelial invagination overlying a dense condensation of mesenchymal cells (fig. 3M). By stage 7, the sulcus spermaticus has bifurcated approximately halfway along each hemipenis. Differentiation occurs first in the proximal part of the spermatic sulcus and then progresses distally towards the bifurcated tips, showing a mature connective tissue with abundant collagenous fibers underneath the epithelium (fig. 3N–P).

A unique characteristic of squamate hemipenes is the presence of the retractor muscle [Gadow, 1887; Dowling and Savage, 1960; Raynaud and Pieau, 1985; Kardong, 2012]. Anatomically, the retractor muscle in squamates originates from transverse processes of the first caudal vertebrae and inserts at the distal tip of the hemipenis [Gadow, 1887]. In python embryos, the retractor muscle is first observed at stage 3, as a condensation in the middle of each hemipenis closer to its base (fig. 3G). At stages 4 and 5, the condensation differentiates into muscle tissue along the hemipenis and into the proximal part of the tail (fig. 2O, P, 3J). The most distal part of the hemipenis, however, shows no signs of the retractor muscle at this stage. At stage 6, the retractor muscle starts to develop a circumferential layer of connective tissue around it and, by stage 7, this connective tissue is well developed, showing abundant collagenous fibers (fig. 3M, P). Although the hemipenis is not bifurcated in ball pythons, as it is in many other snakes [Dowling and Savage, 1960; Branch, 1986; Zaher, 1999], internally the retractor muscle begins to bifurcate at stage 6 (fig. 2S), and both ends reach the distal tip of the hemipenes by stage 7.

Molecular Development of Python Hemipenes

The Snake Hemipenis Develops in the Absence of *Shh* or *Fgf8*

The mouse genital tubercle develops from paired swellings on either side of the cranial margin of the cloacal membrane and, as the swellings merge early in development, they incorporate *Shh*-expressing cloacal endoderm into a urethral epithelial plate. In mouse, SHH produced by the endodermally derived urethral plate plays an essential role in genital tubercle outgrowth and functions as a tissue organizer [Perriton et al., 2002; Seifert et al., 2010]. The functions of SHH in this tissue include pattern specification at early stages and regulation of growth by controlling cell cycle kinetics [Seifert et al., 2010].

In pythons, however, we did not identify a tissue equivalent to the urethral plate during hemipenes development, nor did we detect *Shh* in the paired genital tubercles (fig. 4A). Absence of *Shh* in the early hemipenes buds, while unexpected, is consistent with our observation that the cloacal endoderm makes no contribution to the hemipenes. Within the cloacal endoderm situated between the hemipenes buds, we detected clear and restricted expression of *Shh*, but this domain never reaches the base or any part of the hemipenes during development.

In older hemipenes (stage 6), *Shh* is activated in 2 distal domains near the tip of each hemipenis (fig. 4C, E). Given that the sulcus spermaticus forms on the medial (sulcate) side of the hemipenis and the domains of *Shh* expression are located on the opposite (asulcate) side, these spots of *Shh* expression are not part of the developing sulcus spermaticus (fig. 4D, F). To determine whether signaling by other hedgehog family members could compensate for the absence of *Shh* in the sulcus, we examined the expression of the Hh target gene *Ptch1*, which acts as a readout of Hh pathway activation. We found that at early stages (stages 1–3) of hemipenes development, *Ptch1* is not detectable in the hemipenis bud (fig. 4B). *Ptch1* is strongly expressed, however, in the pericloacal mesenchyme, but this does not extend to the hemipenes (fig. 4B). At later stages, when the 2 domains of *Shh* appear in the distal epithelium of stage 6 hemipenes, we observed complementary domains of *Ptch1* beneath the patches of *Shh* expression (fig. 4C, G, E, I). At no stage did we detect *Ptch1* in or around the sulcus of the hemipenis (fig. 4B, H, J). Thus, there appears to be no hedgehog activity in the developing sulcus spermaticus or anywhere else in the early hemipenes buds prior to the appearance of the paired distal *Shh* domains at stage 6.

A second marker of the distal urethral epithelium in mouse is *Fgf8* [Haraguchi et al., 2000; Perriton et al., 2002]. Although *Fgf8* is expressed in the distal urethral epithelium, previous work showed that FGF8 protein is not translated, target genes are not activated, and deletion of FGF8 has no effect on genital development, suggesting that *Fgf8* mRNA is a marker of the distal urethral epithelium, but is not functionally relevant [Haraguchi et al., 2000; Seifert et al., 2009; Lin et al., 2013]. They also suggested that transcription of *Fgf8* in the distal urethral epithelium may be specific to mammals, as *Fgf8* expression was found in the distal urethral epithelium of mouse, pig and opossum genital tubercles, but was not detectable at the distal tip of the genital tubercle in turtles, alligators

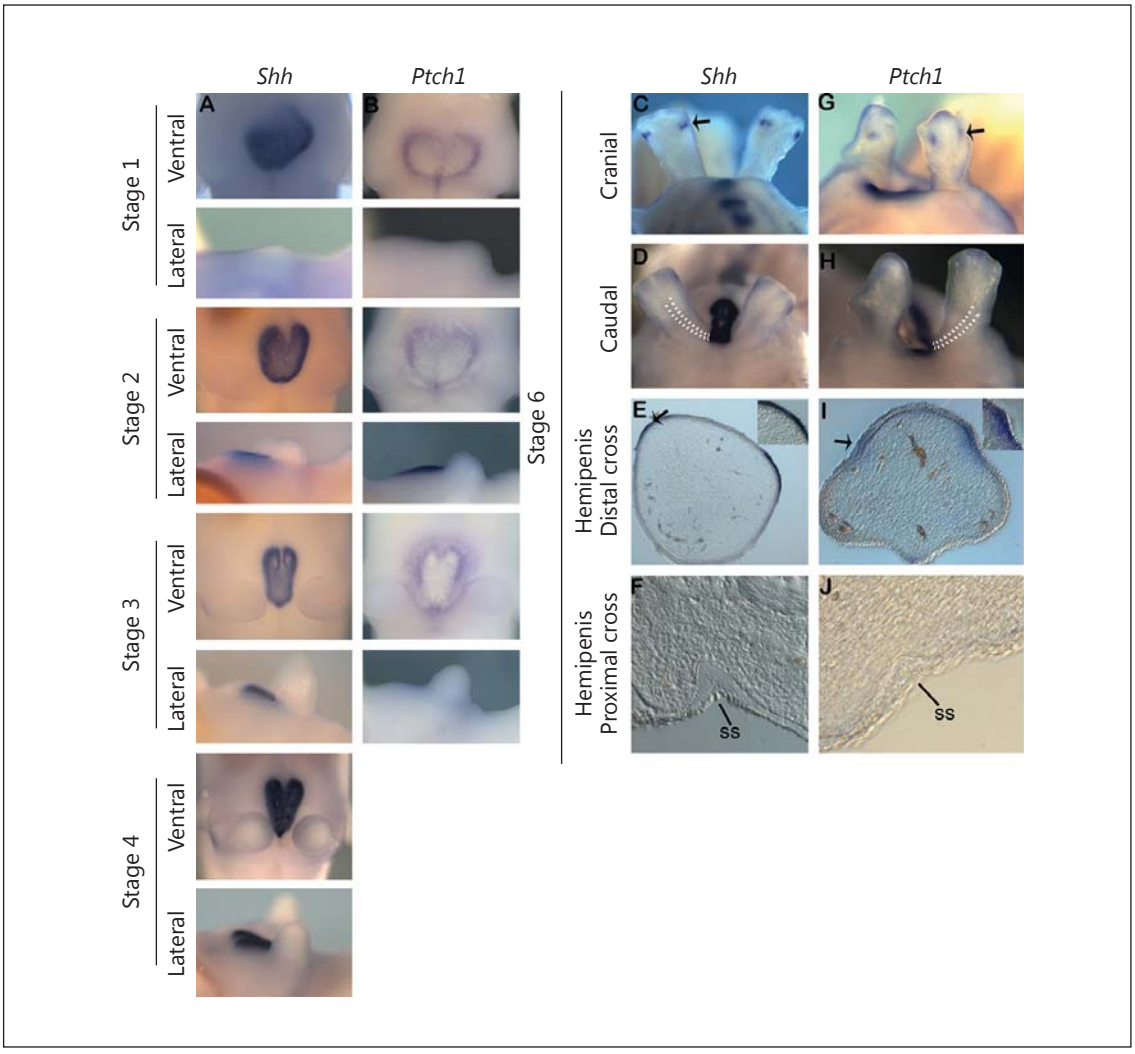

Fig. 4. Gene expression analysis of *Shh* and *Ptch1* during ball python hemipenes development. **A**, **B** Cloacal and genital regions shown in ventral and lateral views. In ventral views, anterior is to the top, and in lateral views, anterior is to the left. **A** *Shh* is expressed strongly in the cloacal endoderm, but no domain of *Shh* expression can be detected in the early hemipenes anlagen. **B** *Ptch1* is expressed in the pericloacal mesenchyme, and its expression is not detected in the early hemipenes buds. **C–J** At stage 6, expression of *Shh* (**C–F**) and *Ptch1* (**G–J**) during sulcus morphogenesis shows no contribution of *Shh*-expressing cells to the sulcus spermaticus epithelium. Top 2 rows show cranial and caudal views of whole mount in situ hybridizations. Bottom 2 rows show hybridizations on cryosections cut transverse (cross) to the long axis of the hemipenes at proximal and distal levels. **E**, **I** Distal sections pass through the 2 *Shh* and *Ptch1* expression domains. **F**, **J** Proximal sections pass through the developing sulcus spermaticus. **C**, **G** In cranial view of the asulcate surface, 2 *Shh* (**C**) and *Ptch1* (**G**) expression domains can be observed at the distal hemipenis (black arrows). Cryosections at the level of these domains show that *Shh* is expressed in the epithelium (**E**), and *Ptch1* expression (**I**) is located in the epithelium and the underlying mesenchyme (black arrows). **D**, **H** In caudal view, the dotted white lines mark the developing sulcus spermaticus, and the posterior and medial sides (sulcate face) of the hemipenes are visible. Note that neither *Shh* nor *Ptch1* (**D**, **F** and **H**, **J**, respectively) are detectable in the developing sulcus spermaticus.

or birds [Seifert et al., 2009; Herrera et al., 2013]. Consistent with these findings, we did not find *Fgf8* expression in the developing hemipenes of pythons, although *Fgf8* was consistently expressed in the cloacal endoderm in all stages analyzed (fig. 5A).

Fgf10 and *Fgfr2* Are Expressed during Hemipenes Development in Pythons

Fgf10, which regulates urethral tubulogenesis in mammals, is expressed in the python cloaca, but not in the hemipenes buds at stages 1 and 2 (fig. 5B). *Fgf10* expres-

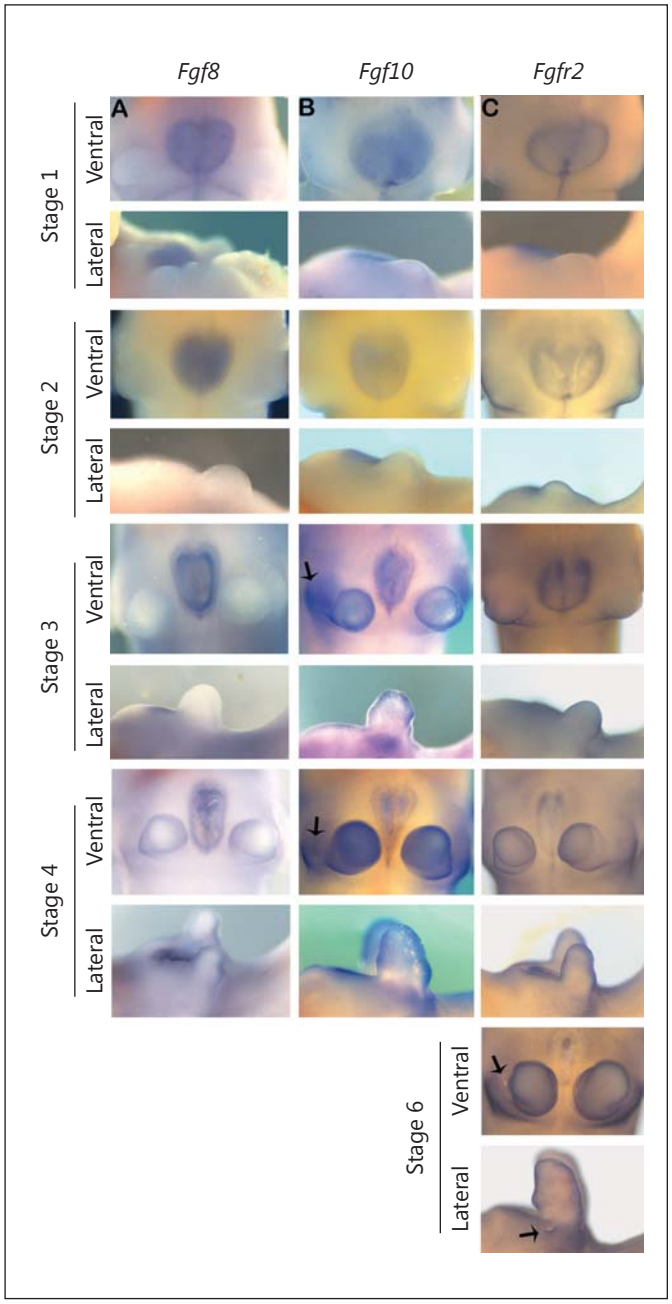

Fig. 5. Gene expression analysis of *Fgf8, Fgf10 and Fgfr2.* Two rows show lateral and ventral views of cloacal and genital region for each stage (stages 1–6). In ventral views, anterior is to the top and in lateral views, anterior is to the left. **A** *Fgf8* is not detectable in the hemipenes at any developmental stage (stages 1–4), but strong expression was detected in the cloacal endoderm at all stages. **B** *Fgf10* expression can be detected only in the cloacal membrane at stages 1 and 2. After stage 3, the expression in the cloaca is maintained and the hemipenes and anal gland primordia show strong expression (black arrows). **C** *Fgfr2* is expressed in the cloacal membrane from stage 1. Specific hemipenial expression of *Fgfr2* is first seen at stage 3, and strong expression persists through stage 6 in the hemipenes and the anal gland anlagen.

sion in the hemipenes first becomes detectable at stage 3 in the mesenchyme of the distal half of the hemipenes (fig. 5B). We also detected the expression of *Fgf10* in the developing anal glands on the lateral side of each hemipenis, near the point where each hemipenis merges with the body wall (fig. 5B).

We then examined the expression of *Fgfr2*, which encodes the primary receptor for FGF10 and is required for urethral development in mice. We used an *Fgfr2* probe that targets the transmembrane tyrosine kinase domain, which is present in both the iiib and iiic isoforms. We detected expression of *Fgfr2* in the cloacal endoderm, but not in the hemipenes buds at stage 1 (fig. 5C). *Fgfr2* expression becomes detectable in the surface ectoderm of the hemipenes buds at stages 2 and 3 (fig. 5C). *Fgfr2* weakens in the cloaca after stage 4 but remains active in the surface ectoderm of the hemipenes over the next several stages, showing strong expression through stage 6 (fig. 5C).

Bmp4, Wnt5a and Wnt7a Signaling Molecules Are Expressed during Hemipenes Development

We next examined the expression of genes encoding BMP and WNT signaling proteins, which have been shown to play roles in the regulation of patterning and outgrowth of the mouse genital tubercle [Perriton et al., 2002; Suzuki et al., 2003; Seifert et al., 2009]. At stage 1, the hemipenis bud shows restricted expression of *Bmp4* at the most distal part of the hemipenial swellings (fig. 6A). As the hemipenes buds continue to grow, the expression of *Bmp4* remains strong and restricted to the distal part of the hemipenis (fig. 6A). We also detected the expression of *Bmp4* in the cloacal endoderm in all the stages analyzed. *Wnt5a* is expressed strongly in the emerging hemipenes buds at stages 1 and 2 (fig. 6B). After stage 3, *Wnt5a* expression becomes expressed in the lateral anal glands and in the cranial and caudal cloacal swellings (fig. 6B). *Wnt7a* is expressed weakly in the genital tubercle ectoderm at stages 1 and 2 (fig. 6C). After stage 3, *Wnt7a* expression fades in the cloaca and becomes stronger in the hemipenes, showing the highest expression over their lateral surfaces (fig. 6C).

The Developing Python Hemipenes and the Developing Mouse Penis Share a Common Set of Transcription Factors

The transcription factors HOXD13 and HOXA13 have been implicated in mouse and human genital tubercle outgrowth [Kondo et al., 1997; Mortlock and Innis, 1997; Warot et al., 1997]. We detected strong expression of

Hoxd13 and *Hoxa13* in the hemipenes buds of python embryos. *Hoxd13* shows broad expression in the pericloacal field, including the cranial and the lateral swellings of the hemipenes buds. From stage 1, *Hoxd13* expression is concentrated at the cranial swellings and in the hemipenes tubercles, but the domain does not extend proximally to the base of the tubercles (fig. 7A). At stage 6, there is strong expression of *Hoxd13* in the primordia of the cranial lip of the cloaca (derived from anterior cloacal swellings), but most of the hemipenial expression is gone by this stage, except for a domain of strong expression at the hemipenial cranial ridge (fig. 7A). In contrast to *Hoxd13*, expression of *Hoxa13* is restricted only to the distal part of the hemipenes from stage 1 through stage 4, and no expression can be detected on the cranial cloacal swellings (fig. 7B).

Msx2 is expressed strongly in the mouse genital tubercle, marking the distal mesenchyme [Seifert et al., 2010]. Similarly, the python genital tubercles show restricted expression of *Msx2* in the distal domains of the hemipenes from stages 1 to 4 (fig. 7C). *Msx2* expression was not detected in the cranial or caudal swelling, but slight pericloacal expression was observed at all stages (fig. 7C).

Finally, we examined the expression of *Tbx4* during python hemipenes development. Similar to the patterns described in mouse [Chapman et al., 1996], we observed a broad expression of *Tbx4* in the lateral hemipenial and cranial cloacal swellings (fig. 7D). As the hemipenes and primordium of the cranial cloacal lip differentiate, the expression of *Tbx4* becomes restricted to those structures, with the strongest expression detected in the hemipenes (fig. 7D).

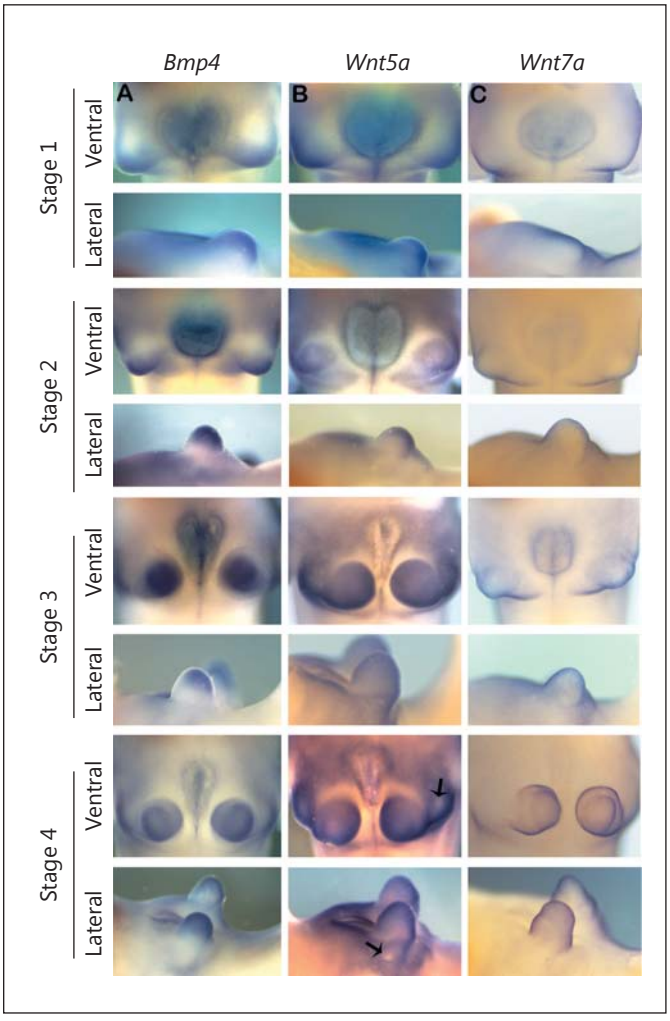

Fig. 6. Gene expression analysis of *Bmp4, Wnt5a* and *Wnt7a*. Stages 1–4 are shown in 2 rows in ventral and lateral views. In ventral views, anterior is to the top and in lateral views, anterior is to the left. **A** *Bmp4* shows a very restricted expression in the mesenchyme of the distal tip of the hemipenes buds; it is later expressed in the anal gland primordia. **B** *Wnt5a* is expressed in a broad domain, although it is restricted to the hemipenes buds at early stages (stages 1 and 2). Later, *Wnt5a* is expressed strongly in the anal gland primordia (black arrows) and the cranial and caudal lip primordia. **C** *Wnt7a* is expressed weakly at stages 1 and 2, but after stage 3, it is detectable in the hemipenes ectoderm, with strongest expression laterally. At stages 1 and 2, *Wnt7a* is expressed in the cloacal membrane.

Discussion

Developmental Origin of Snake Hemipenes and the Sulcus Spermaticus

The results reported here show that development of the snake hemipenes begins with the emergence of a pair of lateral swellings that lie on each side of the caudal region of the cloacal membrane. The hemipenial swellings are separate from the cranial cloacal swellings, which do not participate in the formation of the hemipenial anlagen, but instead form the cranial lip of the cloaca. In ball pythons, the genital swellings grow distally, initially forming smooth cylinder-like genital tubercles, but these do not incorporate the cloacal/urodeal endoderm. Our histological and SEM analysis indicates that the sulcus spermaticus in ball pythons forms in by invagination of the medial side of the hemipenis surface ectoderm, which differs from the endodermal sulcus (and urethra) of other amniotes. The ectodermal invagination that gives rise to the sulcus spermaticus proceeds in a proximal-distal di-

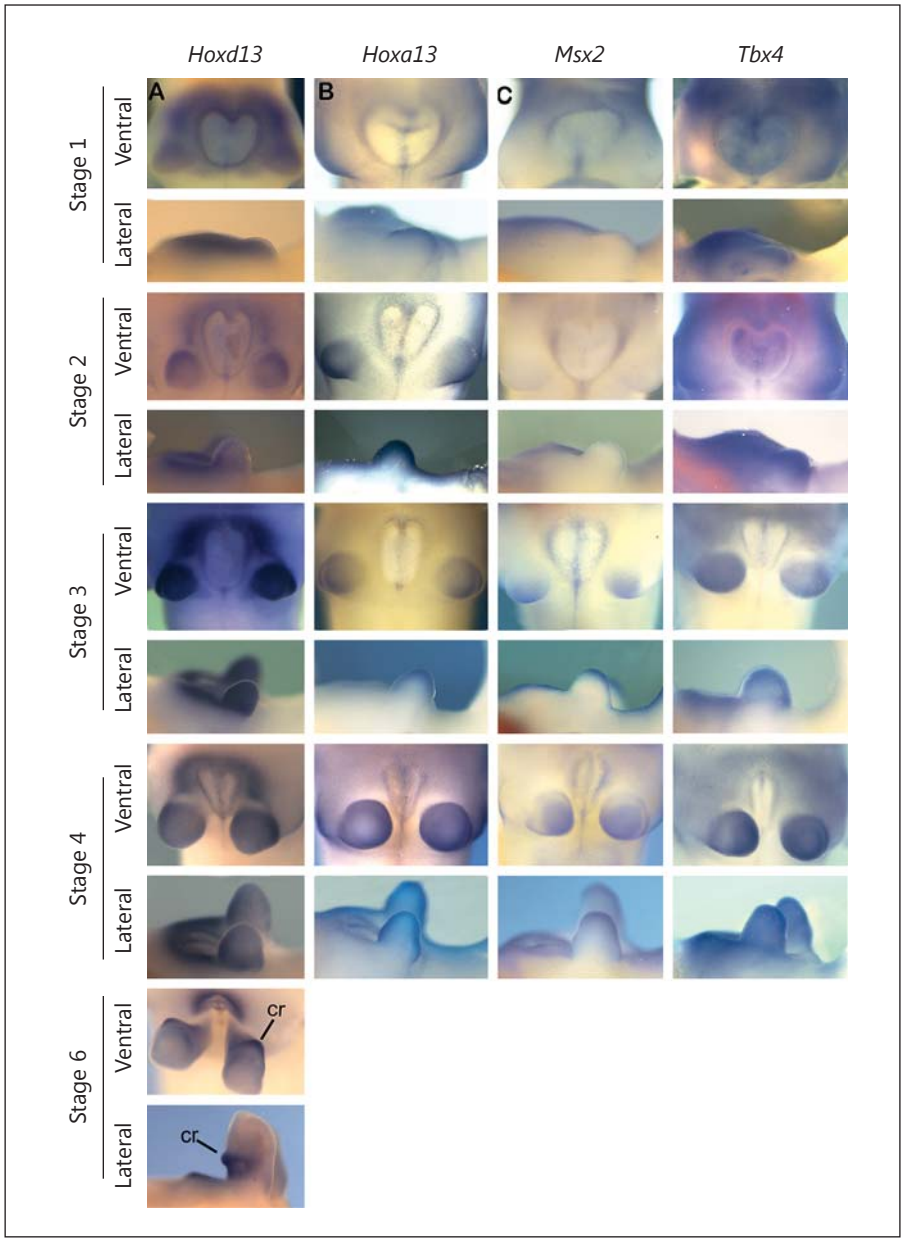

Fig. 7. Gene expression analysis of *Hoxd13, Hoxa13*, *Msx2* and *Tbx4* during ball python hemipenes development from stages 1 to 6. Ventral and lateral views are shown for each gene. In ventral views, anterior is to the top and in lateral views, anterior is to the left. **A** *Hoxd13* is expressed from early stages of external cloacal development and marks the anterior swellings, pericloacal mesenchyme and the hemipenes buds. A horseshoe pattern of expression is seen in the cranial cloacal lip and hemipenes. At stage 6, hemipenial expression of *Hoxd13* is weak, but a strong domain of expression is observed in the hemipenial cranial ridge. **B** *Hoxa13* expression is detected early in hemipenes development and is restricted to the hemipenes buds at all stages. **C** *Msx2* expression is first detectable at stage 2 and is restricted to the distal hemipenis mesenchyme. Pericloacal expression is detected at all stages. **D** *Tbx4* has a broad expression domain in the external cloaca, but shows no expression caudal to the hemipenis bud. Later in development, *Tbx4* expression is strongest in the hemipenes and in the developing cranial lip of the cloaca.

rection and then bifurcates distally, giving rise to a Y-shaped sulcus spermaticus. Raynaud and Pieau [1985] reached the same conclusions about the origin of the hemipenial anlagen and the spermatic sulcus in their study of the European green lizard *(Lacerta viridis)*, slow-worm serpentiform lizard *(Anguis fragilis)* and the dice snake *(Natrix tessellata)*. Our gene expression analysis further supports the hypothesis that the origin of the sulcus spermaticus is independent of the urodeal endoderm. In pythons, as in other amniotes, *Shh* is expressed in the cloacal endodermal cells (including the urodeum), but transcripts could not be detected in the medial hemipenial epithelium during the sulcus formation. Similarly, *Ptch1*, which is a target and transcriptional readout of hedgehog signaling, was not detectable in the mesenchyme adjacent to the sulcus epithelium. Thus, our histological data show no continuity between the cloacal epithelium and the hemipenes and, with the exception of the small distal domains that appear at late stages (stage 6), we see no evidence of *Shh*-expressing or Shh-responding

cells in the hemipenes. Direct cell-lineage tracing will be required to determine definitively whether the cloacal endoderm contributes to the sulcus; however, our data are suggestive that hemipenis outgrowth and patterning occurs in the absence of endoderm or *Shh* expression.

Hemipenis Developmental Gene Regulatory Network

Our study reveals that patterns of gene expression during python hemipenes development share some similarities with the gene regulatory network that controls mouse genital development; however, striking differences are associated with the unique anatomy of hemipenes. Of particular interest is the finding that python hemipenes develop in the absence of cloacal endoderm and lack of expression of *Shh* and *Fgf8*, 2 genes expressed in the endodermally derived urethral epithelium in the mouse [Perriton et al., 2002; Seifert et al., 2010; Cohn, 2011]. *Shh* is essential for genital tubercle patterning and outgrowth in mice. Deletion of *Shh* at early stages of mouse development results in the absence of external genitalia (although paired genital swellings begin to develop but then arrest) and inactivation at later stages results in hypospadias [Perriton et al., 2002; Lin et al., 2009; Miyagawa et al., 2009; Seifert et al., 2010]. Given the essential role of *Shh* in mouse, the absence of *Shh* during early hemipenes development is remarkable and raises new questions about the molecular mechanisms that regulate outgrowth and patterning of the hemipenes. The absence of mRNA for the hedgehog target gene *Ptch1* during hemipenes development indicates that the role of *Shh* has not been taken over by another hedgehog gene, as there appears to be no hedgehog signal transduction within the hemipenes. Although *Shh* signaling does not occur for most of hemipenes outgrowth, *Shh* is activated at late stages in the distal epithelium of the hemipenes, where 2 lateral buds form at the tip of each hemipenis. These outgrowths are not associated with the sulcus, which remains negative for *Shh*. The expression of *Ptch1* in the mesenchyme beneath the *Shh*-expressing epithelium suggests a late role of *Shh* pathway in the outgrowth of the distal buds at the terminus of the hemipenes.

Our finding that *Fgf8* is not detectable in python hemipenes is consistent with the previous report that *Fgf8* is a marker of the distal urethral epithelium only in mammals, where it is transcribed, but not translated [Haraguchi et al., 2000; Seifert et al., 2009; Lin et al., 2013]. Although *Fgf8* is not expressed during ball python hemipenes development, *Fgf10* and *Fgfr2* are expressed widely in the hemipenis bud. These genes regulate urethral tube formation in mice [Petiot et al., 2005]. In python hemipenes, the expression of both genes is weak at early stages, but *Fgf10* increases later. The low-level, ubiquitous expression of both genes in hemipenes contrasts sharply with their expression pattern in other amniotes, where *Fgf10* is expressed adjacent to the *Fgfr2iiib* domain on either side of the sulcus/urethra. We saw no such regionalization of *Fgf10* or *Fgfr2* expression in the sulcus-forming region of hemipenes, consistent with the divergent origin and mode of sulcus formation in squamates. Our *Fgfr2* RNA probe recognizes the tyrosine kinase domain of both *Fgfr2* isoforms and, therefore, the expression pattern in the surface epithelium and underlying mesenchyme should reflect both the *FgfR2iiib* and *FgfR2iiic* isoforms.

In the mouse genital tubercle, *Wnt5a* and *Bmp4* expression are sustained by the action of *Shh* from the urethral plate [Perriton et al., 2002]. *Wnt5a* positively regulates proximodistal outgrowth of the tubercle, whereas *Bmp4* acts as a negative regulator [Yamaguchi et al., 1999; Suzuki et al., 2003; Seifert et al., 2009]. In ball python hemipenes, *Wnt5a* and *Bmp4* show the highest expression in the distal tip mesenchyme, resembling the patterns described for mouse. The absence of *Shh* expression during hemipenes outgrowth suggest that, in contrast to the mouse genital tubercle, sustained expression of *Bmp4* and *Wnt5a* is not controlled by *Shh* during hemipenes development. In addition to the hemipenes buds, the primordia of the cranial and caudal lips of the cloaca (cranial/anterior cloacal and caudal/posterior cloacal swellings) are marked by the expression of *Wnt5a*, which suggests that *Wnt5a* may play a role in outgrowth and differentiation of the external cloaca. We also studied the expression of *Wnt7a*, which has been proposed to have a role in differentiation of mouse genital skin [Chiu et al., 2010]. We detected *Wnt7a* expression over the lateral side of the hemipenes, but little expression was observed on the medial side of the hemipenis bud. This polarized pattern of *Wnt7a* expression is reminiscent of the ectodermal *Wnt7a* expression in the dorsal limb bud and in the mouse genital tubercle, where *Wnt7a* is expressed more strongly on the dorsal side than the ventral [Chiu et al., 2010].

A further similarity between python and other amniote (mice, turtles and archosaurs) penis development is the expression of posterior *Hox* genes. Python hemipenes express abdB-related *Hox* genes from the *HoxD* and *HoxA* cluster. The transcription factors *Hoxd13* and *Hoxa13* show strong expression in the early hemipenes buds, and later *Hoxd13* becomes restricted to a subdistal domain of the hemipenis, where a hemipenial ridge arises. These expression patterns suggest that, as in the mouse penis, posterior Hox transcription factors are involved in

the outgrowth and patterning of python hemipenes. The expression of *Hoxd13* also is very strong in the primordium of the cranial lip of the cloaca, which indicates a potential role in the development of the python external cloaca. By contrast, *Hoxa13* shows strong expression in the hemipenes, but is not detectable in either the cranial or caudal lips of the cloaca. Thus, whereas *Hoxd13* may play a role in external cloacal and genital development, any role of *Hoxa13* would be restricted to the hemipenes.

Two additional homeobox-containing genes, *Msx2* and *Tbx4,* show expression patterns similar to those reported for the mouse genital tubercle [Seifert et al., 2010]. *Msx2* is expressed in the mesenchyme at the distal tip of the hemipenis bud. Sustained expression of *Msx2* in hemipenes is likely to be independent of SHH signaling, as neither *Shh* nor *Ptch1* is detected in the vicinity of the *Msx2* domain. A broad domain of *Tbx4* expression was observed in the mesenchyme of the entire cloacal field, and later, *Tbx4* shows strong expression in the entire hemipenes. *Tbx4* also marks the cranial lip, but not the caudal lip, of the external cloaca. The finding that 3 pairs of swellings (cranial/anterior cloacal, genital and caudal/posterior cloacal swellings) express different combinations of transcription factors suggests that these swellings have distinct molecular identities that are specified early in their development. In this respect, anteroposterior regionalization of the cloacal and genital swellings may be controlled by a mechanism that is similar to that which regionalizes the branchial arches, the serially repeated outgrowths at the anterior end of the gut. The conserved expression of a subset of appendage development genes in the genital tubercles and limbs of amniotes raises the possibility that conserved regulatory elements direct their expression in these outgrowths; however, the notable absence of *Shh*, a key outgrowth factor in the genital tubercles of non-squamate amniotes, suggests that squamates may have evolved novel mechanisms to sustain outgrowth of their external genitalia.

Evolutionary Origin of the Hemipenes

Our analysis of the developmental origin of the hemipenial buds, particularly the finding that python hemipenes lack a contribution of the cloacal endoderm, points to fundamental developmental differences between snake hemipenes and the unpaired penis of non-squamate amniotes. The findings that python hemipenes develop on the posterior side of the embryonic cloaca, whereas the paired genital swellings of non-squamate amniotes develop on the anterior side of the cloaca suggest that the squamate hemipenes could have different embryonic origins, which raises questions about the homology of hemipenes and unpaired amniote penises.

Analysis of external genital anatomy in a phylogenetic context suggests that a single, unpaired penis is the primitive condition for amniotes, and this was retained in each of the major clades of reptiles (including birds) and mammals (fig. 8). However, the phylogenetic origin of the hemipenes is not entirely clear, due, in large part, to the equivocal status of copulatory organs in the sister group of Squamata, the tuataras [King and McLelland, 1981; Gauthier et al., 1988; Lombardi, 1998; Vitt and Caldwell, 2009]. Tuataras are the most basal extant lepidosaurs, although there is a great diversity of non-squamate Lepidosauromorpha reptiles (i.e. Lepidosauria + extinct relatives) in the fossil record, and little is known about their external copulatory organs [Gauthier et al., 1988; Evans, 2003]. A number of questions about the origin of the hemipenes remain to be answered. Did hemipenes evolve within Squamata or earlier in the Lepidosauromorpha lineage? Did tuataras lose their external copulatory organs secondarily? To our knowledge, there is no detailed analysis of the anatomy of the tuatara cloaca or its development, but anecdotal reports in the literature describe tuataras as either completely lacking copulatory organs or still conserving 'rudimentary' hemipenes [King and McLelland, 1981; Raynaud and Pieau, 1985; Lombardi, 1998; Pough et al., 2001; Vitt and Caldwell, 2009]. Therefore, interpretation of cloacal and genital morphology in tuataras is an important consideration in any hypothesis about hemipenes origins.

Hemipenes Origin and Homology

Our developmental analysis of ball python hemipenes identifies several embryological and molecular differences that raise the possibility that squamate hemipenes may be an evolutionary novelty that arose in the Squamata lineage and, as such, may not be direct homologs to the unpaired amniote penis. Alternatively, if these structures are homologous to the unpaired penises of other amniotes, then several changes occurred after the divergence of the lepidosaur lineage, including a posterior shift in the topographic position of the paired genital swellings relative to the cloaca (or an anterior shift of the cloaca relative to the hemipenes), loss of the endodermal signaling region and associated Shh expression, and the loss of the endodermally derived sulcus and evolution of an ectodermal sulcus.

Despite the aforementioned differences in the developmental genetic mechanisms of hemipenes formation compared to mouse genital tubercle development, there are numerous similarities in the expression patterns of genes encoding signaling molecules, such as *Bmp4*, *Wnt5a*

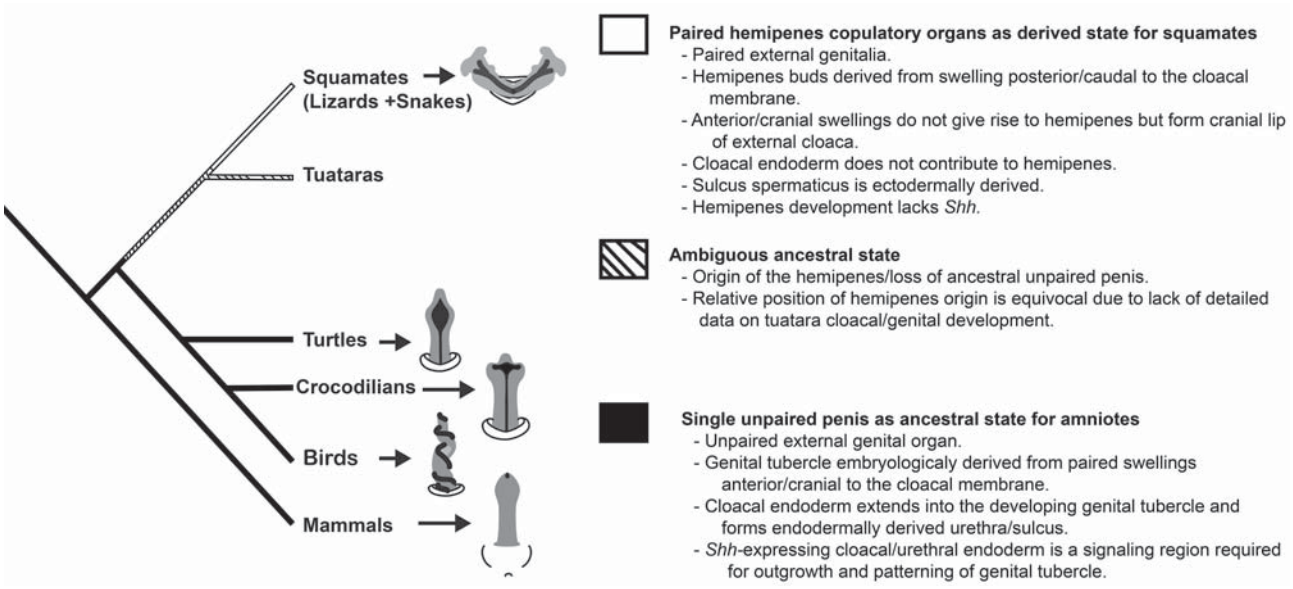

Fig. 8. Model for the evolution of amniote external genitalia. Shared anatomical and developmental characters are listed for each lineage. In this scenario, the unpaired medial penis is supported as the ancestral state. The exact location of the hemipenes origin is equivocal within lepidosaurs (squamates + tuataras), and therefore, the ancestral state for the Lepidosauria lineage is depicted as ambiguous. Data for amniote genitalia embryology, morphology and gene expression is derived from published morphological descriptions in mammals [Perriton et al., 2002], birds [Herrera et al., 2013], crocodilians [Gredler et al., this issue], turtles [Raynaud and Pieau, 1985; Larkins and Cohn, this issue], tuataras [Raynaud and Pieau, 1985; Pough et al., 2001] and squamates [Raynaud and Pieau, 1985].

and *Wnt7a* as well as transcription factors, such as *Hoxd13*, *Hoxa13*, *Tbx4*, and *Msx2*. However, those similarities are not necessarily indicative of direct structural homology of the lizard and snake hemipenes with the amniote unpaired penis. The promiscuity of the gene network associated with appendage formation, which is activated at different times and in different places in vertebrate development, is equally consistent with these similarities arising by co-option and subsequent regulatory divergence of the evolutionarily ancient appendage-forming cassette. Indeed, the battery of genes implicated in external genital development not only pattern the mouse genital tubercle, but also other vertebrate appendages, such as limbs, integumentary appendages (e.g. feathers and scales), teeth, glands, gut appendages, and tails [Minelli, 2000, 2002, 2003; Chuong and Homberger, 2003; Gilbert, 2010; Cohn, 2011].

We considered the hypothesis that the ancestral amniote penis is conserved but modified in Squamata, where the unpaired penis split to form each hemipenis. We looked for embryological or molecular evidence that each hemipenis consists of one half of a penis, perhaps resulting from failed fusion of the paired genital swellings. We found no developmental signatures that would support this hypothesis, and some of our data could be interpreted as contradicting the idea that a primitive median phallus divided to form the hemipenes. The results presented here raise an alternative hypothesis, in which the unpaired penis was lost at some point in lepidosauromorph evolution and that hemipenes are an evolutionary innovation for the Squamata lineage. Fusion of paired genital swellings at the midline and over the cloacal membrane is a crucial morphogenetic event for penis development in other amniotes (mice, turtles, and archosaurs), and this fusion is accompanied by the inclusion of the cloacal endoderm into the amniote genital tubercle [Raynaud and Pieau, 1985; Perriton et al., 2002; Herrera et al., 2013; Gredler et al., this issue; Larkins and Cohn, this issue]. The inclusion of endodermal tissue into the amniote genital tubercle is essential for the formation of the urethral plate that drives outgrowth and later differentiates into the tubular urethra of mammals and the spermatic furrow in birds, alligators and

turtles. In squamates, however, none of those morphogenetic events take place, and the hemipenes lack a urethral plate or any endodermal contribution.

Additionally, we found that the spermatic sulcus in squamates is ectodermally derived, and other mesenchymal and endodermal genes that are expressed in association with the amniote urethral plate, such as *Fgfr2* in the epithelium and *Bmp4* and *Fgf10* in the mesenchyme [Haraguchi et al., 2000; Perriton et al., 2002; Petiot et al., 2005; Gredler et al., this issue; Larkins and Cohn, this issue], show no association with the sulcus spermaticus in python hemipenes development. Therefore, the sulcus spermaticus not only differs at the level of its tissue morphogenesis compared to the amniote seminal furrow/tubular urethra, but also at the level of the expression of developmental genes.

If hemipenes are an evolutionary innovation that followed the loss of an unpaired median penis, then this could have involved a change in the fate of the unpaired medial penis precursors in squamates. Our embryological and gene expression data might shed light onto this problem. During early stages of python cloacal development, a pair of cranial/anterior swellings develop at a pericloacal position, near the site at which paired genital swellings of the unpaired medial penis form in other amniotes. Similar to the genital swellings in mammals, birds, alligators, and turtles, but in contrast to the hemipenial swellings, the cranial/anterior swellings in all squamates fuse at the midline and form the cranial lip of the cloaca [Raynaud and Pieau, 1985]. Furthermore, the cranial swellings express genes that are expressed in the genital swellings in other amniotes, such as *Hoxd13* and *Wnt5a*. Therefore, it is plausible that squamates conserve the precursors of the unpaired medial penis of other amniotes; however, they might have no contribution to the formation of the hemipenes. Instead, they could contribute to the formation of the cranial lip of the external cloaca.

The evolution of novel intromittent organs in vertebrates is not uncommon [Lombardi, 1998]. In other vertebrates (e.g. the anuran amphibian *Ascaphus truei*, the tailed frog; all limbless caecilian amphibians), novel intromittent organs evolved adjacent to the cloaca, and studies of these organs have suggested that they have no direct homologs in other lineages that have evolved genital organs [Lombardi, 1998; Gower and Wilkinson, 2002]. These neomorphic genital structures are distinct from the modifications of preexisting (nongenital) organs for use in intromission, such as the fin specializations that have evolved in chondrichthyans and in some teleost fishes [Lombardi, 1998].

If hemipenes are an innovation, then this would imply that the evolution of this novel copulatory organ involved the co-option of ancient appendage development pathways, similar to the ones that control the development of median phalluses, in other amniotes as well as the acquisition of novel mechanisms for hemipenes development. Therefore, our findings that *Shh* does not appear to be involved in hemipenes development and that there is no equivalent to the urethral plate tissue organizer in squamates suggest that the hemipenes form under the control of different outgrowth signals than those operating in the ancestral amniote penis (fig. 8). Comparisons of gene expression networks and their associated *cis*-regulatory mechanisms in hemipenes and unpaired penis development should illuminate how some of the same genes that would otherwise be controlled by hedgehog signaling (as in the genital tubercles of other amniotes) are regulated in the squamate hemipenes. Although only a handful of organ-/tissue-specific DNA regulatory elements active in the mouse genitalia have been discovered to date [Spitz et al., 2003; Menke et al., 2008; Sagai et al., 2009], further studies of the degree of conservation versus novelty in the functions of those regulatory sequences will be required to uncover the gene regulatory landscape that orchestrates hemipenes development.

Acknowledgements

We thank B. Cole for supplying freshly laid ball python eggs. We are grateful to O. Tarazona and M. Gredler for discussions about external genitalia evolution in reptiles and for comments on an earlier draft of the manuscript, and K. Kelley and K. Backer-Kelley at the Electron Microscopy ICBR core at UF for their assistance in preparing and imaging of SEM samples. Francisca Leal is supported by an HHMI international student research fellowship. This project was supported by the National Science Foundation (NSF IOS-0843590), the National Institute of Environmental Health Sciences (R01-ES017099), and the Howard Hughes Medical Institute (to M.J.C.).

References

Boughner JC, Buchtová M, Fu K, Diewert V, Hallgrímsson B, Richman JM: Embryonic development of Python sebae – I: Staging criteria and macroscopic skeletal morphogenesis of the head and limbs. Zoology (Jena) 110:212–230 (2007).

Branch WR: Hemipenial morphology of African snakes: a taxonomic review. Part 1. Scolecophidia and Boidae. J Herpatol 20:285–299 (1986).

Chapman DL, Garvey N, Hancock S, Alexiou M, Agulnik SI, et al: Expression of the T-box family genes, *Tbx1–Tbx5*, during early mouse development. Dev Dyn 206:379–390 (1996).

Chiu HS, Szucsik JC, Georgas KM, Jones JL, Rumballe BA, et al: Comparative gene expression analysis of genital tubercle development reveals a putative appendicular Wnt7 network for the epidermal differentiation. Dev Biol 344:1071–1087 (2010).
Chuong CM, Homberger DG: Development and evolution of the amniote integument: current landscape and future horizon. J Exp Zool B Mol Dev Evol 298:1–11 (2003).
Cohn MJ: Development of the external genitalia: conserved and divergent mechanisms of appendage patterning. Dev Dyn 240:1108–1115 (2011).
Crawford NG, Faircloth BC, McCormack JE, Brumfield RT, Winker K, Glenn TC: More than 1000 ultraconserved elements provide evidence that turtles are the sister group of archosaurs. Biol Lett 8:783–786 (2012).
deBraga M, Rieppel O: Reptile phylogeny and the interrelationships of turtles. Zool J Linn Soc Lond 120:281–354 (1997).
Dowling HG, Savage JM: A guide to the snake hemipenes: a survey of basic structure and systematic characteristics. Zoologica 14:17–28 (1960).
Evans SE: At the feet of the dinosaurs: the early history and radiation of lizards. Biol Rev Camb Philos Soc 78:513–551 (2003).
Fong JJ, Brown JM, Fujita MK, Boussau B: A phylogenomic approach to vertebrate phylogeny supports a turtle-archosaur affinity and a possible paraphyletic lissamphibia. PLoS One 7:e48990 (2012).
Gadow H: Remarks on the cloaca and on the copulatory organs of the amniota. Philos T R Soc B 178:5–37 (1887).
Gauthier J, Estes R, De Queiroz K: A phylogenetic analysis of Lepidosauromorpha, in Estes R, Pregill G (eds): The Phylogenetic Relationships of the Lizard Families, pp 15–98 (Stanford University Press, Palo Alto 1988).
Gilbert SF: Developmental Biology, ed 9 (Sinauer Associates, Sunderland 2010).
Gower DJ, Wilkinson M: Phallus morphology in caecilians (Amphibia, Gymnophiona) and its systematic utility. Bulletin of The Natural History Museum. Zoology Series 68:143–154 (2002).
Haraguchi R, Suzuki K, Murakami R, Sakai M, Kamikawa M, et al: Molecular analysis of external genitalia formation: the role of fibroblast growth factor *(Fgf)* genes during genital tubercle formation. Development 127:2471–2479 (2000).
Herrera AM, Shuster SG, Perriton CL, Cohn MJ: Developmental basis of phallus reduction during bird evolution. Curr Biol 23:1065–1074 (2013).
Kardong KV: Vertebrates: Comparative Anatomy, Function, Evolution (McGraw-Hill, New York 2012).
Kelly DA: Turtle and mammal penis designs are anatomically convergent. Proc Biol Sci 271 Suppl 5:S293–S295 (2004).
King AS, McLelland J: Form and Function in Birds, vol 2 (Academic Press, London 1981).
Kondo T, Zákány J, Innis JW, Duboule D: Of fingers, toes and penises. Nature 390:29 (1997).
Laufer E, Pizette S, Zou H, Orozco OE, Niswander L: BMP expression in duck interdigital webbing: a reanalysis. Science 278:305 (1997).
Lin C, Yin Y, Veith GM, Fisher AV, Long F, Ma L: Temporal and spatial dissection of Shh signaling in genital tubercle development. Development 136:3959–3967 (2009).
Lin C, Yin Y, Bell SM, Veith GM, Chen H, et al: Delineating a conserved genetic cassette promoting outgrowth of body appendages. PLoS Genet 9:e1003231 (2013).
Lombardi J: Comparative Vertebrate Reproduction (Kluwer Academic, London 1998).
Menke DB, Guenther C, Kingsley DM: Dual hindlimb control elements in the *Tbx4* gene and region-specific control of bone size in vertebrate limbs. Development 135:2543–2553 (2008).
Minelli A: Limbs and tail as evolutionarily diverging duplicates of the main body axis. Evol Dev 2:157–165 (2000).
Minelli A: Homology, limbs, and genitalia. Evol Dev 4:127–132 (2002).
Minelli A: The Development of Animal Form: Ontogeny, Morphology, and Evolution (Cambridge University Press, Cambridge 2003).
Miyagawa S, Moon A, Haraguchi R, Inoue C, Harada M, et al: Dosage-dependent hedgehog signals integrated with Wnt/β-catenin signaling regulate external genitalia formation as an appendicular program. Development 136:3969–3978 (2009).
Mortlock DP, Innis JW: Mutation of *HOXA13* in hand-foot-genital syndrome. Nat Genet 15:179–180 (1997).
Nieto MA, Patel K, Wilkinson DG: In situ hybridization analysis of chick embryos in whole mount and tissue sections. Methods Cell Biol 51:219–235 (1996).
Perriton CL, Powles N, Chiang C, Maconochie MK, Cohn MJ: Sonic hedgehog signaling from the urethral epithelium controls external genital development. Dev Biol 247:26–46 (2002).
Petiot A, Perriton CL, Dickson C, Cohn MJ: Development of the mammalian urethra is controlled by Fgfr2-IIIb. Development 132:2441–2450 (2005).
Pough FH, Andrews RM, Cadle JE, Crump ML, Savitzky AH, Wells KD: Herpetology, ed 2 (Prentice Hall Inc., Upper Saddle River 2001).
Raynaud A: Étude embryologique de la formation des appendices postérieurs et de la ceinture pelvienne chez le Python réticulé Python reticulatus (Éd. du Muséum, 1972).
Raynaud A, Pieau C: Contribution à l'étude des premiers stades de la formation des organes copulateurs chez les reptiles. Mémoires du Muséum national d'histoire naturelle. Nouvelle série. Série A. Zoologie. 58. Fasc. 3 (1970).
Raynaud A, Pieau C: Embryonic Development of the Genital System (John Wiley and Sons, New York 1985).
Rosenberg HI, Bauer AM, Russell AP: External morphology of the developing hemipenes of the dwarf chameleon, *Bradypodion pumilum* (Reptilia: Chamaeleonidae). Can J Zoolog 67:884–890 (1989).
Sagai T, Amano T, Tamura M, Mizushina Y, Sumiyama K, Shiroishi T: A cluster of three long-range enhancers directs regional Shh expression in the epithelial linings. Development 136:1665–1674 (2009).
Seifert AW, Yamaguchi T. Cohn MJ: Functional and phylogenetic analysis shows that *Fgf8* is a marker of genital induction in mammals but is not required for external genital development. Development 136:2643–2651 (2009).
Seifert AW, Zheng Z, Ormerod BK, Cohn MJ: Sonic hedgehog controls growth of external genitalia by regulating cell cycle kinetics. Nat Commun 1:23 (2010).
Shaffer HB, Minx P, Warren DE, Shedlock AM, Thomson RC, et al: The western painted turtle genome, a model for the evolution of extreme physiological adaptations in a slowly evolving lineage. Genome Biol 14:R28 (2013).
Spitz F, Gonzalez F, Duboule D: A global control region defines a chromosomal regulatory landscape containing the *HoxD* cluster. Cell 113:405–417 (2003).
Suzuki K, Bachiller D, Chen YP, Kamikawa M, Ogi H, et al: Regulation of outgrowth and apoptosis for the terminal appendage: external genitalia development by concerted actions of BMP signaling [corrected]. Development 130:6209–6220 (2003).
Townsend T, Larson A, Louis E, Macey JR: Molecular phylogenetics of squamata: the position of snakes, amphisbaenians, and dibamids, and the root of the squamate tree. Syst Biol 53:735–757 (2004).
Vitt LJ, Caldwell JP: Herpetology: An Introductory Biology of Amphibians and Reptiles, ed 3 (Acedemic Press, Burlington 2009).
Wang Z, Pascual-Anaya J, Zadissa A, Li W, Niimura Y, et al: The draft genomes of soft-shell turtle and green sea turtle yield insights into the development and evolution of the turtle-specific body plan. Nat Genet 45:701–706 (2013).
Warot X, Fromental-Ramain C, Fraulob V, Chambon P, Dollé P: Gene dosage-dependent effects of the *Hoxa-13* and *Hoxd-13* mutations on morphogenesis of the terminal parts of the digestive and urogenital tracts. Development 124:4781–4791 (1997).
Yamaguchi TP, Bradley A, McMahon AP, Jones S: A *Wnt5a* pathway underlies outgrowth of multiple structures in the vertebrate embryo. Development 126:1211–1223 (1999).
Zaher H: Hemipenial morphology of the South American xenodontine snakes: with a proposal for a monophyletic Xenodontinae and a reappraisal of colubroid hemipenes. Bull Am Mus Nat Hist 240:1–168 (1999).
Zardoya R, Meyer A: Complete mitochondrial genome suggests diapsid affinities of turtles. Proc Natl Acad Sci USA 95:14226–14231 (1998).
Zardoya R, Meyer A: The evolutionary position of turtles revised. Naturwissenschaften 88:193–200 (2001).