

Development of the Cloaca, Hemipenes, and Hemiclitores in the Green Anole, *Anolis carolinensis*

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Key Words

Anolis · Cloaca · Development · External genitalia · Genital tubercle · Hemipenes · Reptiles · Squamates

Abstract

In most amniotes, the intromittent organ is a single phallus; however, squamates (lizards, snakes, and amphisbaenians) have paired hemiphalluses. All amniotes studied to date initiate external genital development with the formation of paired genital swellings. In mammals, archosaurs, and turtles, these swellings merge to form a single genital tubercle, the precursor of the penis and clitoris; however, in squamates, the paired genital buds remain separate, giving rise to the hemiphalluses (hemipenes in males and hemiclitores in females). Although the molecular genetics and sexual differentiation of the genital tubercle have been investigated in mammals and birds, little is known about hemiphallus development. Here we describe development of the cloaca and hemiphallus in the green anole, *Anolis carolinensis*. Each hemiphallus originates as a protuberance that emerges at the ventral base of the hindlimb bud. Development of the hemipenes resembles penis development; however, differ-

ences exist in their tissue composition, morphogenesis, and gene expression patterns. These findings reveal aspects of phallus development that appear to be evolutionarily labile, both within squamates and more broadly among reptiles, and identify features that are conserved across amniotes. Our results, together with parallel studies in other reptilian taxa, suggest potential mechanisms for the diversification of external genital form.

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Evolution of the phallus, an intromittent organ capable of depositing sperm into the female reproductive tract, facilitated the transition from external to internal fertilization in amniotes. Males of most amniote clades, including mammals, crocodylians, birds, and chelonians, have a single phallus at the anterior margin of the cloaca (or terminus of the urogenital sinus in mammals) that delivers sperm to the female reproductive tract [Gadow, 1887; King, 1979a; Kelly, 2002; Herrera et al., 2013]. By contrast, male squamates (lizards, snakes, and amphisbaenians) develop paired phalluses, or hemipenes, on the lateral margins of the cloaca [Gadow, 1887; King, 1979a;

Kelly, 2002]. Within Squamata, male hemipenis morphology has been used in phylogenetic analyses based on its extensive variation and on the premise that the hemipenes are under only sexual but not environmental selective pressures [Arnold, 1986a, b; Klaver and Böhme, 1986; Keogh, 1999; Böhme and Ziegler, 2009; Jadin and Parkhill, 2011; Das and Purkayastha, 2012; Köhler et al., 2012].

Squamates have elaborate and diverse external genital anatomy. Although the morphological details of adult hemipenes have been well characterized, little is known about hemiphallus development or sexual differentiation [Raynaud and Pieau, 1985; Klaver and Böhme, 1986; Rosenberg et al., 1989, 1991; Mouden et al., 2000; Ruiz and Wade, 2002; Lovern et al., 2004; Roscito and Rodrigues, 2012]. Studies investigating the embryonic expression of steroid hormone receptors and the effects of hormone treatment on developing green anoles indicate that early stages of hemiphallus development are similar in both sexes (monomorphic), and at later stages both androgens and estrogens are necessary for development of sexually dimorphic anatomy [Lovern et al., 2004; Holmes and Wade, 2005; Beck and Wade, 2008]. This pattern is consistent with the biphasic nature of mammalian external genital development; in mice, an initial phase of morphogenesis patterns the indifferent genital tubercle, which then undergoes sexually dimorphic development under the influence of sex steroids [Glenister, 1954; Bellinger, 1981; Fraser and Sato, 1989; Ammini et al., 1997; Seifert et al., 2008].

Many of the genetic mechanisms that regulate formation of the phallus also have conserved functions in the development of other appendages such as the limbs, mammary glands, hair, feathers, and lungs [reviewed in Roelink, 1996; Pispá and Thesleff, 2003; Wu et al., 2004; Andrew and Ewald, 2010]. The genes of this ‘appendage toolkit’ that function in murine external genital development include Sonic hedgehog (*Shh*), the Hox paralogs *HoxA13/D13*, bone morphogenetic protein 4 (*Bmp4*), fibroblast growth factor 10 (*Fgf10*) and receptor 2 (*Fgfr2*), both canonical (via β -catenin) and non-canonical Wnt family members, and the distal-less transcription factors *Dlx5/6* [reviewed in Cohn, 2011]. *Shh* has been shown to be expressed in the cloacal endoderm of a number of vertebrates and plays an essential role in outgrowth and patterning of the mouse external genitalia [Haraguchi et al., 2001; Perriton et al., 2002; Lin et al., 2009; Miyagawa et al., 2009; Seifert et al., 2010]. *Bmp4* regulates patterning, differentiation, growth, and apoptosis in many embryonic tissues, including the external genitalia [Suzuki et al., 2003; Herrera et al., 2013]. Growth factor signaling via the

Fgf10-Fgfr2 pathway, which also functions in limb, lung, and gut development, has been shown to regulate urethral morphogenesis in the mouse [Xu et al., 1998; Sekine et al., 1999; De Moerloose et al., 2000; Ohuchi et al., 2000; Weaver et al., 2000; Revest et al., 2001; Petiot et al., 2005; Sala et al., 2006; Berg et al., 2007]. Although the genetic mechanisms of external genital development have been investigated in species with a single phallus, comparatively little is known about the molecular mechanisms that regulate morphogenesis of the paired hemipenes in males and the hemiclitores in females.

Here we investigate development of the hemipenes and hemiclitores in the green anole, *Anolis carolinensis*. Anoles are an emerging model system for comparative embryonic development and have been well studied as models for sexual dimorphism [Winkler and Wade, 1998; Ruiz and Wade, 2002; Holmes and Wade, 2005; Beck and Wade, 2008; Cohen et al., 2012; Wade, 2012; Sanger et al., 2013, 2014]. We describe the embryology of the external genitalia in green anoles, from the initiation of budding through sexual differentiation. Each hemiphallus develops from a ridge of somatopleure (lateral plate mesoderm and surface ectoderm) that protrudes from the ventral base of the hindlimb bud. Male and female external genitalia develop similarly until stage 10, approximately 8–11 days post-oviposition (dpo) [Sanger et al., 2008a], after which morphogenesis of sexually dimorphic hemipenes and hemiclitores occurs. We also analyze the expression patterns of *shh*, *bmp4*, and *fgfr2* in *A. carolinensis*; the orthologs of these genes have been shown to mediate development of the cloaca and external genitalia in mammals. Our results highlight some similarities between phallus and hemiphallus development, as well as differences in their tissue composition, morphogenesis, and gene expression patterns. These findings identify potential mechanisms involved in the evolution of external genitalia and also reveal aspects of external genital development that appear to be relatively labile, both within squamates and among amniotes.

Materials and Methods

Anolis carolinensis Embryo Collection

Freshly laid eggs were generously provided from Dr. S. Tonia Hsieh or obtained from a breeding colony at Harvard University that was maintained according to guidelines approved by the Institutional Animal Care and Use Committee of Harvard University. Husbandry details are described in Sanger et al. [2008b]. Briefly, eggs were incubated in covered petri dishes with damp vermiculite at 27 °C until appropriate stages [Sanger et al., 2008a]. Embryos were harvested and dissected in cold PBS, staged accord-

ing to Sanger et al. [2008a], and fixed overnight in 4% paraformaldehyde at 4°C. The following day, embryos were dehydrated in a graded methanol series and stored in 100% methanol at -20°C.

Scanning Electron Microscopy and Fluorescent Imaging

Lower bodies of *A. carolinensis* embryos used for scanning electron microscopy (SEM) and whole mount fluorescent imaging were rehydrated through a graded methanol series to PBS. Tissue used for morphological analysis was stained with SYBR safe DNA stain (Invitrogen) for 2 h, washed briefly in PBS, and photographed under epifluorescence. Embryos used for SEM were post-fixed in 1% glutaraldehyde at 4°C for at least 12 h. Following fixation, samples were washed with PBS, osmicated in 2% osmium tetroxide for 1 h, and dehydrated to 100% ethanol. Samples were then critical point dried, mounted on stubs, and gold/palladium sputter-coated. Images were captured on a Hitachi S-4000 FE-SEM, and all figures were assembled using Adobe Creative Suite.

Histology

Embryos were transferred from methanol to 100% ethanol, permeabilized in Citrisolv (2 × 20 min) and infiltrated with wax (3 × 45 min) at 65°C before being embedded in fresh wax. A mixture of Paraplast Xtra and Paraplast Plus (1:1 by weight) was used for wax embedding. Sections were cut 10 μm thick on a Leica microtome. Slides were dewaxed and rehydrated, stained in Harris' Hematoxylin and 1% Eosin Y, dehydrated, mounted, and cover-slipped.

In situ Hybridization

For genetic nomenclature of *Anolis* species see Kusumi et al. [2011]. Coding sequences of *A. carolinensis shh*, *bmp4*, and *fgfr2* were isolated by RT-PCR amplification of cDNA generated from whole embryos. Primers were designed based on alignments of coding sequences in the human, mouse, chick, and *Anolis* genomes using ClustalW (*Anolis* sequences available from Ensemble: *shh* – ENSACAG00000011060; *bmp4* – ENSACAG00000017900; *fgfr2* – ENSACAG00000017206). PCR products were gel-purified and cloned into the pSC-A-amp/kan vector using the StrataClone PCR cloning system, and sequences were confirmed by NCBI BLAST analysis. The cDNA inserts were amplified by PCR using M13 forward and reverse primers, gel-purified, and used as templates for transcription of digoxigenin-labeled antisense riboprobes. Whole mount in situ hybridization was performed according to published methods [Nieto et al., 1996] with the following modifications: BM purple (Roche) was used as a color substrate in place of NBT/BCIP, Triton X-100 was replaced with Tween-20 in KBTB solution, and the concentration of Triton X-100 in NTMT solution was increased from 0.1 to 1%.

Results

Initiation of External Genital Development

In order to characterize morphological development of the hemiphallus and cloaca in *A. carolinensis*, we examined the external genitalia of embryos from stages 3–14 using SEM, fluorescent microscopy, and histology. The first post-ovipositional stage of development for

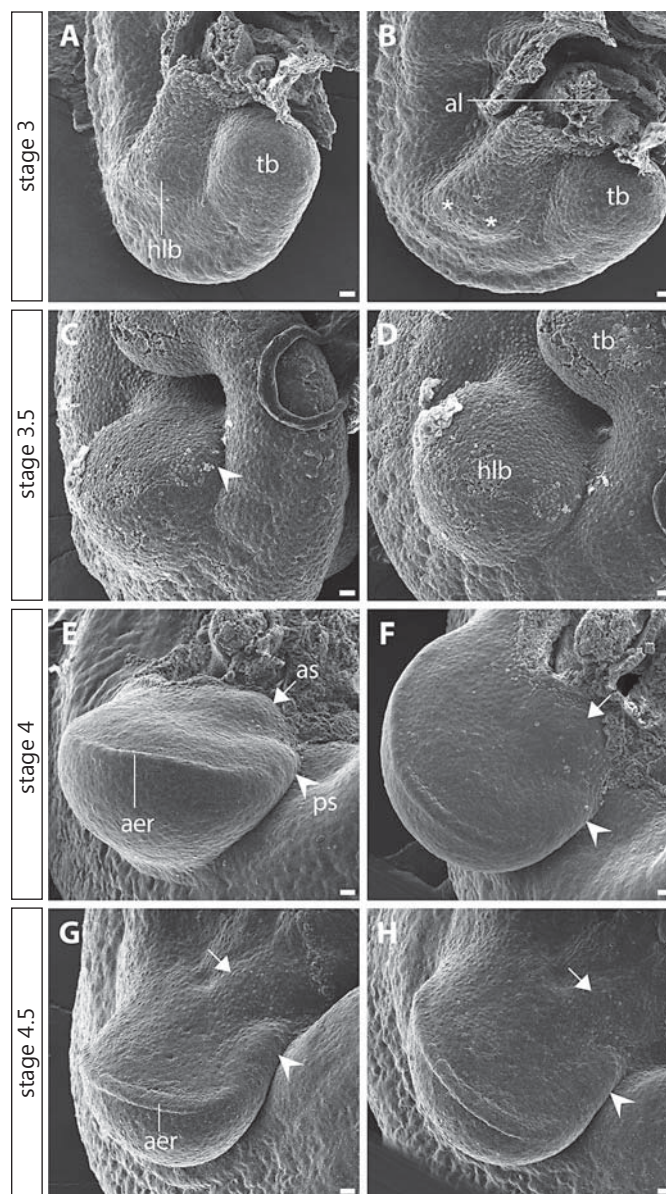


Fig. 1. Coordinated initiation of hindlimb, cloaca, and hemiphallus development. **A, B** Initiation of hindlimb development in a stage 3 embryo. The hindlimb bud (hlb) emerges between the allantois (al) and tailbud (tb). A subtle cleft divides cranial and caudal regions of the bud (asterisks in **B**). **C, D** The stage 3.5 hindlimb bud is rounded with a small protuberance (arrowhead) at its base. **E, F** Stage 4 hindlimb bud showing phallic swelling (ps, arrowhead), anterior swelling (as, arrow) and apical ectodermal ridge (aer). **G, H** The positions of the phallic and anterior swellings shift at stage 4.5. The hindlimb bud is dorsoventrally compressed, the apical ectodermal ridge is more pronounced, and the anterior and phallic swellings are situated at more medial positions than at the previous stage. **B, D, F, and H** are rotated views of **A, C, E, and G**, respectively. Scale bars = 20 μm.

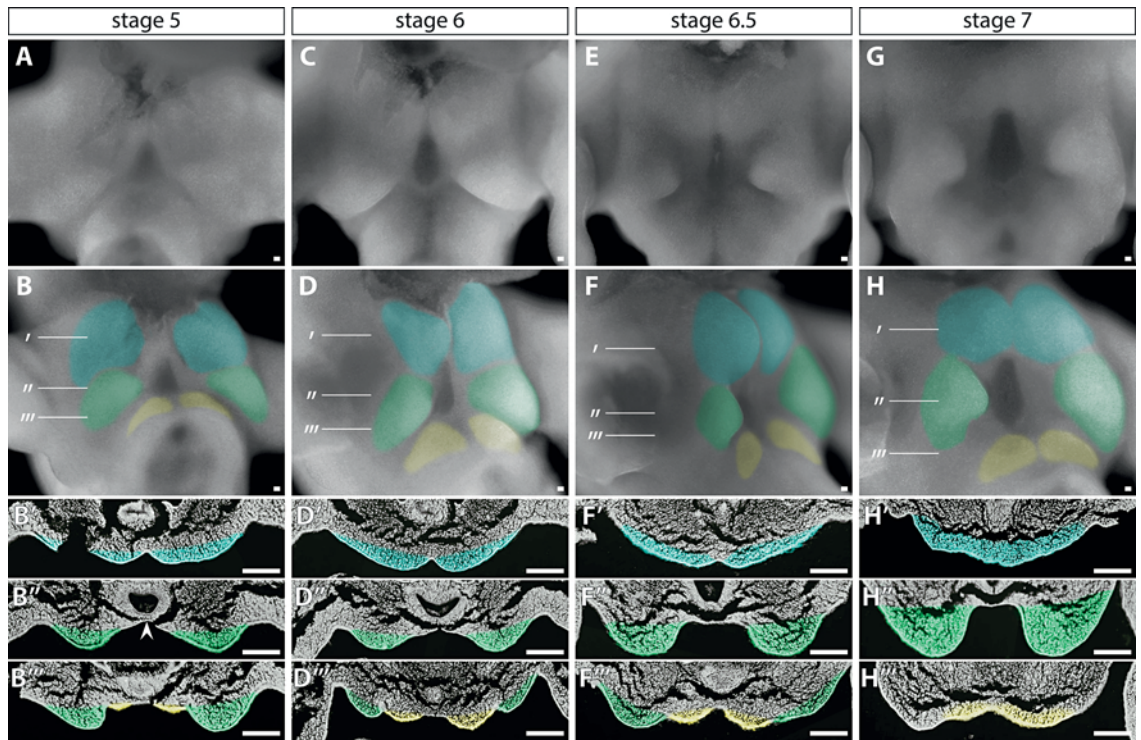


Fig. 2. Maturation of 3 pairs of external genital swellings. **A–H** Ventral views of the developing hemiphallus and cloaca at stage 5 (**A–B'''**), stage 6 (**C–D'''**), stage 6.5 (**E–F'''**), and stage 7 (**G–H'''**). **B, D, F, and H** are oblique views of the embryos in **A, C, E, and G**, respectively, that have been pseudocolored to annotate the anterior (blue), phallic (green), and posterior (yellow) swellings. White lines in pseudocolored panels indicate the transverse plane of section for histology panels below (**B'–B'''**, **D'–D'''**, **E'–E'''**, **H'–H'''**).

The cloacal membrane (arrowhead) is indicated in **B''**. The anterior shift of the phallic swellings from stages 5–7 is illustrated in the bottom 2 rows; the size of the phallic (green) area increases at the level of the cloacal membrane, moving from **B''** to **H''**, and concomitantly decreases at a more posterior level shown in the bottom panel from **B'''** to **H'''**. The anterior cloacal lip is formed by fusion of the anterior swellings at stage 7 (**G–H'''**). Scale bars = 50 μ m.

Anolis species is characterized by presence of a hindlimb bud [Sanger et al., 2008a]. In *A. carolinensis*, the early hindlimb buds emerge as swellings along the lateral body wall, between the allantois and tail bud, at stage 3 (fig. 1A). The hindlimb buds at this stage show a subtle asymmetry along the anteroposterior axis (fig. 1B, asterisks). A number of morphogenetic changes occur caudally in the embryo during the first dpo. The hindlimb bud becomes more spherical (fig. 1C, D), and a small mound of cells emerges on the posterior-ventral side of the hindlimb bud near its junction with the body wall (fig. 1C, arrowhead). By stage 4 (0–1 dpo, 'early limb bud' stage), an apical ectodermal ridge has formed at the dorsoventral boundary of the hindlimb bud (fig. 1E). There are 2 convexities on the ventral side of the stage 4 hindlimb bud; a phallic swelling forms proximally on the posterior-ventral side of the hindlimb bud (fig. 1E, F), and a separate swelling arises slightly anterior and proximal to the phallic swelling, near

the junction between the hindlimb bud and body wall (fig. 1E, F). By stage 4.5, the hindlimb bud undergoes slight dorsoventral flattening, and the apical ectodermal ridge becomes more pronounced (fig. 1G, H). The phallic swelling is located at the junction of the hindlimb bud and the ventral body wall, and the anterior swelling remains positioned medial and cranial to the phallic swelling (fig. 1G).

The positions of the anterior and phallic swellings shift gradually from lateral to medial between stages 5 and 9. At stage 5 (0–3 dpo, 'late limb bud' stage), the phallic swellings are located on the ventral body wall, adjacent to, but distinct from, the proximal hindlimb buds (fig. 2A). A third pair of external genital protuberances, the posterior swellings, emerges caudal and medial to the phallic swellings (fig. 2B). These 3 sets of paired structures are the anlagen of, from cranial to caudal, the anterior cloacal lip, the hemipenes in males and hemicloitores in females, and the posterior cloacal lip (fig. 2B–B'''). Morphogenesis of

these external genital structures occurs in concert with development of the cloaca. The position of the future cloacal orifice is marked by the cloacal membrane, a transient embryonic structure defined by the direct contact of endoderm and ectoderm (fig. 2B'', arrowhead).

At stage 6 (3–5 dpo, 'paddle-shaped bud' stage), the left and right anterior swellings meet at the ventral midline, cranial to the cloacal membrane (fig. 2C). At this stage, the phallic swellings have enlarged, especially on the medial side, appear pointed at the apex and, relative to previous stages, are positioned further cranially with respect to the cloacal membrane (fig. 2C–D''', compare fig. 2A, C). The anterior halves of the posterior swellings lie medial to the phallic swellings and caudal to the cloacal membrane (fig. 2D, D'''). One day later, the phallic swellings are larger than at stage 6 and are situated at a higher axial position relative to the cloacal membrane (fig. 2E–F'''). By stage 7 (4–7 dpo, 'digital plate' stage) the anterior swellings have fused to form the anterior cloacal lip, while the posterior swellings have increased in size but are still unfused (fig. 2G–H'''). The phallic swellings are larger and rounder than at previous stages (fig. 2H''').

Early Development of the Cloaca

Morphogenesis of the cloaca in *A. carolinensis* is linked to the development of the external genital swellings. At stage 5, the phallic swellings are positioned on the ventral body wall adjacent to the posterior limit of the coelomic cavity (fig. 3A). Cranial to the phallic swellings, the anterior swellings extend from the body with a similar proximodistal length but are slightly longer along the ventral midline than are the phallic swellings (fig. 3B). At this stage, the posterior swellings are just beginning to emerge caudal to the phallic swellings (fig. 3A, B). The Wolffian or mesonephric duct traverses the dorsal wall of the coelom (fig. 3B). At its posterior limit, the Wolffian duct dilates and contacts the cloacal horn, a lateral projection of the dorsal cloaca, forming a y-shaped structure in the sagittal plane (fig. 3B). The posterior margin of the hindgut approaches the ventral body wall to form the urodeal chamber of the embryonic cloaca (fig. 3C). The allantois connects to the urodeum on the ventral side (fig. 3C).

At stage 6, the phallic swellings extend further from the ventral body wall than the anterior swellings; however, the presence of the anterior but not phallic swellings in the lateral-most sections indicates that the anterior swellings are wider mediolaterally than the phallic swellings (fig. 3D, E). The mesenchymal cells underlying the surface ectoderm of each of the 3 sets of external genital swellings appear to be more tightly packed than the sur-

rounding areas, resulting in comparatively dense areas of mesenchyme (e.g. phallic swellings in fig. 3E). The Wolffian duct connects the mesonephric tubules to the cloaca, and its dilated terminus remains in contact with the cloacal horn (fig. 3E). A small projection, the ureteric bud, extends cranially from the dorsal wall of this bulbous portion of the Wolffian duct (fig. 3E). Metanephric tubules develop adjacent to the ureteric bud (fig. 3D). The allantois dilates anterior to the urodeum, and the hindgut constricts immediately adjacent to the urodeum, forming the coprourodeal fold and delineating the coprodeum chamber of the cloaca (fig. 3F, white asterisk). The urodeum has expanded anteroposteriorly along the ventral body wall, and the entire ventral face of the urodeum is now parallel to the surface ectoderm (fig. 3F).

By stage 7 the posterior swellings are visibly distinct from the body wall, and the anterior swellings have fused to form the anterior cloacal lip (fig. 3G, H). The mesenchyme of the external genital swellings remains dense, and the ureteric bud enlarges cranially to form the embryonic ureter (fig. 3H). The allantois and hindgut remain connected to the urodeum, and the urodeal chamber has expanded in the craniocaudal direction (fig. 3H, I).

At stage 8 (6–9 dpo, 'digital condensation' stage), an elaboration of the Wolffian duct forms the posterior ureter, which extends caudally from the cloacal horn under the posterior cloacal swellings (fig. 3K). The endoderm of the urodeum abuts the surface ectoderm to form the cloacal membrane (fig. 3L). As in the mouse, the cloacal membrane of *A. carolinensis* is defined by epithelial-epithelial contact; no mesenchyme exists between the endoderm and ectoderm. The urodeum dilates to form a more rounded chamber (fig. 3L).

By stage 9 (7–9 dpo, 'early digital web reduction' stage), the posterior swellings have fused to form a single posterior cloacal lip (fig. 3M). The distal portion of each phallic swelling tilts slightly caudally at stage 9 (fig. 3N). The posterior ureter extends towards the tail in the mesenchyme beneath the posterior cloacal swellings (fig. 3N). The allantois constricts at its junction with the urodeum; this constriction will later develop into the blind-ending bladder (fig. 3O). The posterior side of the ventral urodeal wall, which forms the cloacal membrane, extends caudally, and the entire ventral side of the urodeum is larger anteroposteriorly than at stage 8 (fig. 3O).

Sexual Differentiation of Male Hemipenes

Three important structures develop in the stages before the external genitalia undergo sexual differentiation. First, a morphological ridge develops along the proxi-

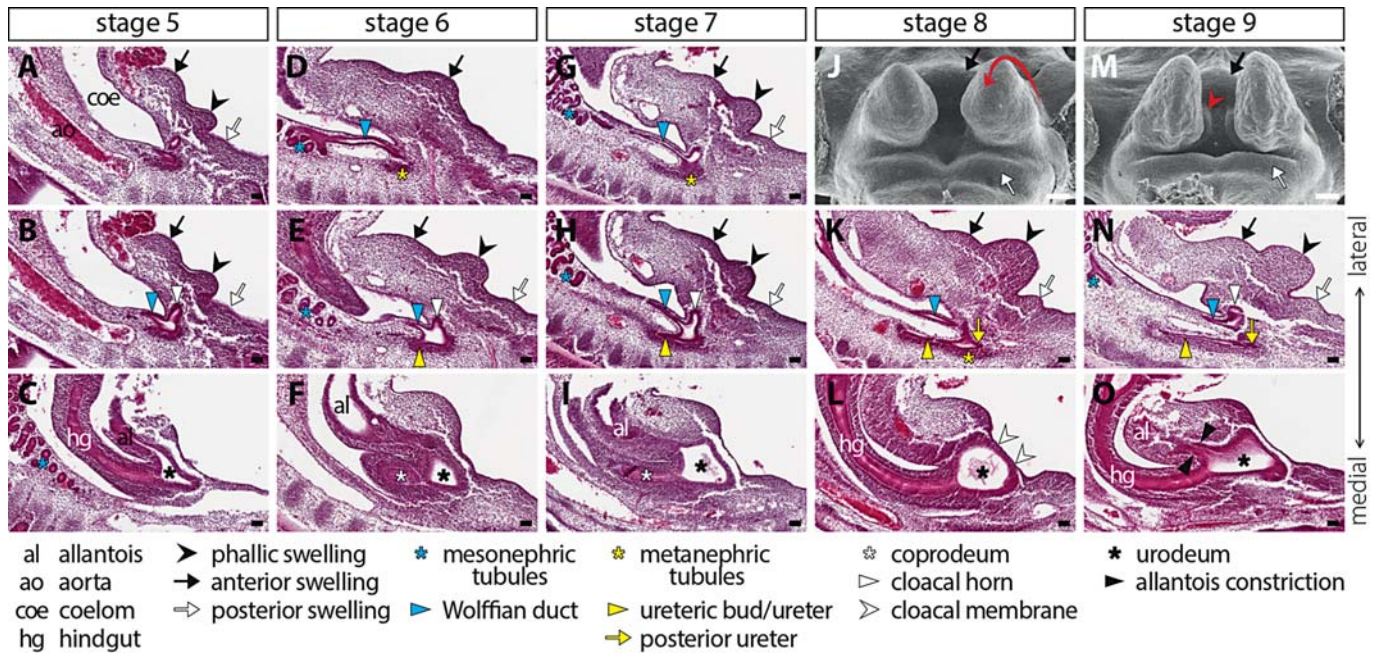


Fig. 3. Development of the cloaca and urogenital ducts. **A–I** Sagittal histological sections through the developing external genital swellings and cloaca at stage 5 (**A–C**), stage 6 (**D–F**), and stage 7 (**G–I**). Phallic swellings (black arrowheads), anterior swellings (black arrows), and posterior swellings (white arrows) are initially positioned on the body wall adjacent to the caudal limit of the coelom (coe). Note the dense mesenchyme underneath the surface ectoderm of the external genital swellings in stages 5–7 (e.g. under the arrow and arrowhead in **A**, **B**, and **E**). The urodeum (black asterisk) is formed at the posterior limit of the hindgut (hg) and allantois (al) (**C**). Constriction of the caudal hindgut delineates the coprodeum (white asterisk). The Wolffian duct (blue triangle) connects the mesonephric tubules (blue asterisk) to the cloacal horn (white triangle).

Metanephric tubules (yellow asterisk) surround the ureter (yellow triangle) which develops from the posterior Wolffian duct. **J–L** Stage 8 hemiphalluses and cloaca investigated by SEM (**J**) and sagittal histology (**K**, **L**). The phallic ridge (red arrow) develops on the asulcal side of the hemiphallus. The cloacal membrane (white arrowheads) forms by direct contact between the endoderm and surface ectoderm. The posterior ureter (yellow arrow) develops from an extension of the caudal ureter. **M–O** Stage 9 hemiphalluses and cloaca investigated by SEM (**M**) and sagittal histology (**N**, **O**). The posterior cloacal lip (white arrow) forms at stage 9. The secondary phallic buds are present (red arrowhead). The allantois constricts in diameter immediately adjacent to the urodeum (black triangles). ao = Aorta. Scale bars = 20 μ m.

modistal axis of the dorsal side of each phallic bud at stage 8 (fig. 3J). Second, at stage 9, a small medial bud (the secondary phallic bud) emerges on the cranial margin of each phallic bud (fig. 3M). Lastly, surface ectoderm along the ventral midline of each phallic bud invaginates to form the initial sulcus spermaticus.

A previous study demonstrated that the gonads of *A. carolinensis* begin to differentiate at incubation days 9 and 10 [Holmes and Wade, 2005]. At stage 9.5, the male and female gonads can be distinguished histologically, although there is no obvious dimorphism between male and female hemiphalluses [Holmes and Wade, 2005]. Sexually dimorphic development of *A. carolinensis* external genitalia begins at stage 10 (8–11 dpo, ‘digital webbing partially reduced’ stage). Proximodistal outgrowth of the hemipenes is sustained in males, and both

the secondary phallic buds and anterior cloacal lip continue to develop (fig. 4A). The sulcus spermaticus deepens as the surface epithelium continues to invaginate (fig. 4A).

By stage 12 (12–14 dpo, ‘digital pad’ stage), the phallic ridge of male embryos has grown considerably and is visible as a large swelling that extends proximodistally along the asulcal side and over the apex of each hemipenis (fig. 4B). Surface ectoderm invaginates further into the sulcus spermaticus which now forms a contiguous groove with the inferior margin of the posterior cloacal lip (fig. 4B). The secondary phallic buds have protruded further by this stage, decreasing the width of the cloacal furrow between the hemipenes (fig. 4B).

In male embryos at stage 14 (15–18 dpo, ‘scale anlagen’ stage), the phallic ridge remains detectable on the asulcal

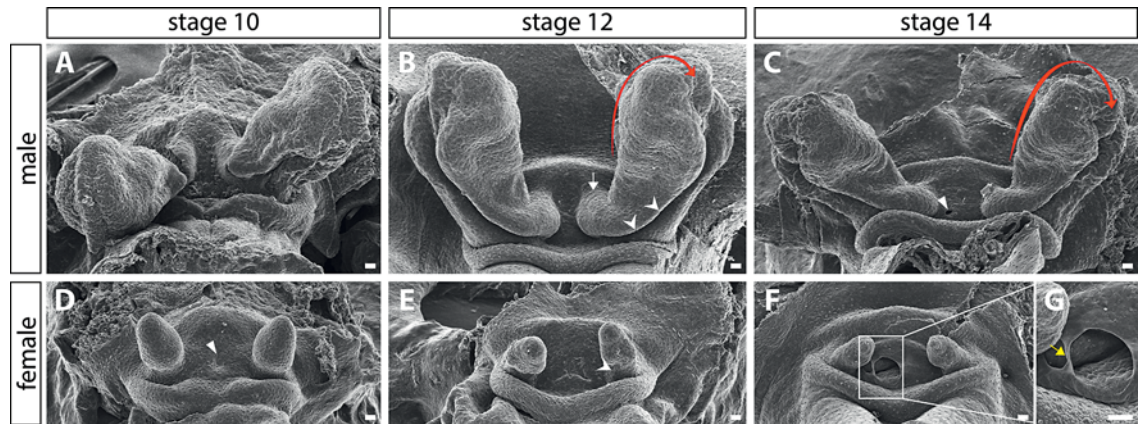


Fig. 4. Sexually dimorphic development of the hemipenes and hemiclitores. **A–C** SEM of developing male hemipenes at stage 10 (**A**), stage 12 (**B**), and stage 14 (**C**). The sulcus spermaticus (arrowheads) matures by continued invagination of the surface ectoderm into the truncus of each hemipenis between stages 10 and 12. The phallic ridge (red arrow) terminates at the distal tip of the hemipenis at stage 12 and on the sulcal side of the hemipenis, between the 2 sides of a bifurcated sulcus spermaticus, at stage 14. The secondary phallic buds (arrow in **B**) are present at the base of the hemipenes. The cloacal membrane (triangle in **C**) begins to rupture at

stage 14. **D–G** SEM of female hemiclitores at stage 10 (**D**), stage 12 (**E**), and stage 14 (**F**). An indentation (triangle in **D**) in the epithelium between the hemiclitores indicates the position of the cloacal membrane. Note the vestigial sulcus spermaticus (arrowhead in **E**). The female cloacal membrane ruptures at stage 14, connecting the urodeum to the newly formed proctodeum via the formation of the uroproctodeal fold. **G** Epithelial cells appear to be stretched across the rupturing cloacal membrane (yellow arrow), suggesting the possibility of mechanical stress. Scale bars = 20 μ m.

side of each hemipenis (fig. 4C, red curved arrow). Its growth over the apex of the hemipenis results in a bifurcation of the distal portion of the sulcus spermaticus (fig. 4C). A small cloacal opening is present (fig. 4C).

Sexual Differentiation of Female Hemiclitores

The stage 10 hemiclitores of females are smaller than stage 10 hemipenes, and the secondary phallic buds have regressed (fig. 4D). Although the anterior cloacal lip has grown slightly (but less than in males at this stage), the lateral aspects of the posterior cloacal lip are generally larger (compare fig. 4A, D). An indentation of the surface epithelium appears at the site of the cloacal membrane, where the urodeal endoderm meets the ventral ectoderm (fig. 4D).

By stage 12, the hemiclitores have undergone further regression (fig. 4E). The female remnant of the sulcus spermaticus persists as a small indentation along the sulcal base (fig. 4E). The posterior cloacal lip is now larger in females than in males, and a pronounced indentation of the surface epithelium can be seen at the site of the cloacal membrane. Both the anterior and posterior cloacal lips extend outward from the body wall and are much more pronounced than in males (fig. 4E).

By stage 14, the female hemiclitores have continued to regress and begin to bend medially towards the cloacal

membrane and caudally towards the posterior cloacal lip, which also folds slightly inward at the lateral margins (fig. 4F). The third chamber of the cloaca, the proctodeum, accommodates the hemipenes and hemiclitores internally between the anterior and posterior cloacal lips. As the cloacal membrane begins to rupture, forming the cloacal orifice, it delineates the boundary between urodeum and proctodeum (fig. 4F). Interestingly, rupturing of the cloacal membrane appears to occur asymmetricaly or randomly, and cells often appear to be stretched across the opening (fig. 4F, G).

*Gene Expression in Developing *A. carolinensis* External Genitalia*

In order to compare the molecular mechanisms of hemiphallus and cloacal development in *A. carolinensis* to those described for the mouse, we examined expression of genes implicated in mouse external genital and cloacal development in the developing genitalia of *A. carolinensis* at stages 8 and 9. We focused on the expression patterns of 3 genes, *sonic hedgehog* (*shh*), *bone morphogenetic protein 4* (*bmp4*), and *fibroblast growth factor receptor 2* (*fgfr2*), the orthologs of which are known to regulate outgrowth (*Shh*, *Bmp4*), patterning (*Shh*), cell death (*Bmp4*), cell proliferation (*Shh*, *Fgfr2*), urethral tubulo-

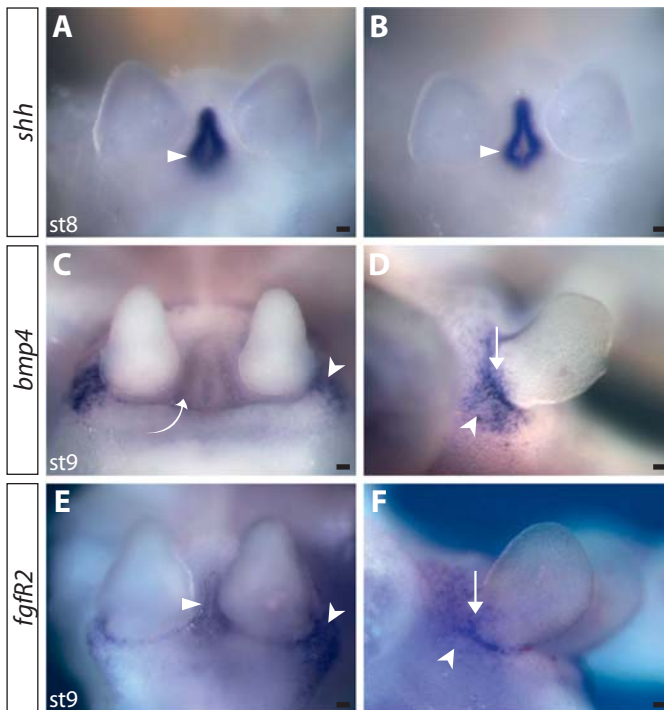


Fig. 5. Gene expression in the developing cloaca and hemiphallus. Whole mount in situ hybridization showing expression of *shh* at stage 8 (A, B), *bmp4* at stage 9 (C, D), and *fgfr2* at stage 9 (E, F). A, C, and E show ventral views of cloacal and genital buds; B shows a ventral view of the cloacal membrane and apical view of the genital buds; D and E show lateral views of the right genital bud. Transcription of these genes was detected in 4 distinct expression domains: the cloacal membrane (triangle, *shh* and *fgfr2*); the surface epithelium adjacent to the cloacal membrane (curved arrow, *bmp4*); the ectoderm between the anterior and posterior cloacal lips (arrowhead, *bmp4* and *fgfr2*), and the lateral side of the proximal hemiphallus (arrow, *bmp4* and *fgfr2*). Scale bars = 20 μ m.

genesis (*Shh*, *Fgfr2*), and preputial development (*Fgfr2*) during mouse external genital morphogenesis.

Strong expression of *shh* was observed in the cloaca of *A. carolinensis* at stage 8 (fig. 5A, B). Although the sulcus spermaticus is present at this stage, it was negative for *shh*, consistent with our finding that the sulcus is derived from an invagination of surface ectoderm and that cloacal endoderm does not contribute to the hemipenes or hemiclitores (fig. 4). In *A. carolinensis* embryos at stage 9, we detected *bmp4* transcripts lateral to each hemiphallus, between the margins of the anterior and posterior cloacal lips (fig. 5C, D, arrowheads). The pericloacal *bmp4* domain extends to the proximal, lateral side of each hemiphallus (fig. 5D, arrow), and weak expression was observed in the surface ectoderm between each hemiphallus and the margin of the cloaca (fig. 5C, curved arrow). *Fgfr2* is ex-

pressed on the surface ectoderm between the anterior and posterior cloacal lips (arrowhead) as well as in a small domain on the proximolateral margin of each hemiphallus (fig. 5D, arrow) at stage 9, similar to the pattern observed for *bmp4* (fig. 5E, F). Unlike *bmp4*, however, *fgfr2* is expressed in the cloacal membrane, in a pattern similar to that of *shh* (fig. 5E, triangle; compare with fig. 5A, B).

Discussion

Ontogeny of the Squamate Hemiphallus

External genital development in the green anole involves coordinated morphogenesis of 3 sets of paired outgrowths: the anterior cloacal, the phallic, and the posterior cloacal swellings. Development of these buds is associated with outgrowth of the hindlimb buds. In the first stage of hindlimb development, a protuberance forms on the posterior-ventral side of the base of each hindlimb bud. It is possible that this single swelling gives rise to both the phallic and anterior swellings, which are distinguishable by stage 4, but its location at the posterior margin of the hindlimb bud suggests that it is the early phallic swelling alone and that the anterior swelling is developing independently at this stage. Following initiation, the anterior swellings on the left and right sides of the cloaca fuse medially to form the anterior cloacal lip, whereas the phallic swellings remain unfused and develop into the male hemipenes or female hemiclitores. A third pair of outgrowths, the left and right posterior swellings, appears on the ventral body wall immediately caudal to the developing cloacal membrane; these buds merge to form the posterior cloacal lip. The similarities between the pattern of external genital and cloacal morphogenesis in the green anole and that described for other squamates, such as the slow worm (*Anguis fragilis*), the European green lizard (*Lacerta viridis*), the dice snake (*Natrix tessellata*), and the ball python suggest that gross development of the external genitalia generally is conserved across squamates [Gadow, 1887; King, 1979a; Leal and Cohn, this issue; Raynaud and Pieau, 1985].

At a finer scale, subtle variation exists in the spatiotemporal origins of squamate phallic and cloacal swellings (e.g. their positions relative to the hindlimb buds) and in the late microanatomical patterning of the hemipenes. Derivation of the phallic anlagen from the anlagen of the anterior cloacal lip, partitioning of a single initial extra-cloacal swelling, and independent development of the anterior and phallic swellings are examples of different mechanisms that have been proposed to explain the on-

togeny of hemipenes and anterior cloacal lip [Raynaud and Pieau, 1985]. We have found that from the onset of external genital development, the anterior and phallic swellings protrude as distinct structures in the green anole. In the slow worm, the anlagen of the hemipenes and anterior cloacal lip initially appear to develop as a single protuberance; however, Raynaud and Pieau [1985] argue that the presence of a small furrow separating the 2 paired swellings from the onset of their development is evidence for separate developmental origins. Differences among these studies may reflect real differences between species and, therefore, it remains possible that these structures arise independently in some species and originate from a common bud in others. Cell fate mapping will be necessary in order to definitively distinguish the cellular origins of these tissues.

Posterior Appendage Development: Limbs and Genitalia

Morphogenesis of the cloaca and hemiphalluses is associated with hindlimb development. When the phallic anlagen of the green anole first appear, the hindlimb buds have just formed and are approximately the same size as the forelimb buds. One of the most striking examples of homoplasia (convergence, parallelism, and reversal) is limb reduction/limb loss and elongation of the body which has evolved independently at least 26 times within Squamata [Brandley et al., 2008]. In the slow worm, a legless lizard, the phallic anlagen arise as part of paired lateral swellings immediately adjacent to the cloacal membrane, of which the medial portion will develop into the hemipenes and the lateral portion will form the hindlimb buds (which eventually regress) [Raynaud and Pieau, 1985]. Therefore, initiation of genital and hindlimb bud development occur together in the slow worm, whereas in both the green anole and the European green lizard, hemiphallus morphogenesis occurs after hindlimb buds develop, prior to formation of a footplate [Gadow, 1887; Raynaud and Pieau, 1985]. Thus, in the green anole and the European green lizard, the external genitalia form at a relatively late stage of hindlimb development as compared to the slow worm. In the dice snake, which lacks limb buds altogether, lateral swellings develop on each side of the cloacal membrane, and the posterior portion forms the phallic anlagen [Raynaud and Pieau, 1985]. Thus, although genital swellings and hindlimb buds develop in close proximity to one another, their patterning is independent to the extent that one structure can be modified or even lost without affecting the other.

We found that in the green anole, the genital buds that form the hemipenes and hemiclitores first arise caudal to

the cloacal membrane, but these phallic swellings either migrate or are displaced anterior to the level of the midpoint of the cloacal membrane for the remainder of development. The same pattern of posterior genital initiation that we observed in the green anole has been reported in limb-reduced squamates, such as the dice snake, the lesser microteiid lizard *Calyptommatus sinebrachiatus*, and the ball python [Raynaud and Pieau, 1985; Roscito and Rodrigues, 2012]. By contrast, genital swellings of birds and mammals emerge lateral to the cloacal membrane [King, 1979a; Perriton et al., 2002; Seifert et al., 2009; Herrera et al., 2013]. This pattern of genital initiation (immediately adjacent to the cloacal membrane) also occurs in limbed and limb-reduced squamates, such as the European green lizard, the slow worm, and the gymnophthalmid lizard *Nothobachia ablephara* [Raynaud and Pieau, 1985; Roscito and Rodrigues, 2012]. Thus, the development of genital swellings caudal to the cloacal membrane in the green anole is not a general feature of squamates; rather, the position at which genital swellings emerge relative to the cloaca is a variable character that does not correlate strictly with limbs or length of the body.

Together, these studies suggest that there may be modularity both in the timing of limb and genital bud initiation and in the relative positioning of these buds along the anteroposterior and dorsoventral axes. Nonetheless, we suggest that this variation occurs within a limb-genital field of somatopleure adjacent to the developing cloaca.

Development of the Cloaca

The vertebrate cloacal membrane contains both endodermal and ectodermal cells, although the boundary between these derivatives is often difficult to identify. The cloaca is divided into 3 chambers; the coprodeum connects to the digestive system, the urodeum functions in urinary homeostasis, and the proctodeum performs roles involved in copulation/reproduction [Gadow, 1887; King, 1979b]. The boundaries between chambers are known as cloacal folds. Along the craniocaudal axis, the coprourodeal fold separates the coprodeum from the urodeum which is then separated from the proctodeum by the uroproctodeal fold. In early green anole embryos, the coprodeum is formed from the caudal hindgut and is separated from the urodeum by a coprourodeal fold, similar to other reptiles [King, 1979b]. The allantois connects to the urodeum ventral to the coprourodeal fold, while the Wolffian ducts and ureter connect on the dorsal side. Squamates generally have a reduced proctodeal chamber, a faint uroproctodeal fold, and a prominent coprouro-

deal fold [King, 1979b]. Our finding that *A. carolinensis* has a well-developed coprourethral fold that appears at stage 6 is consistent with the pattern found in other squamates.

Both the embryologic origin and the adult morphology of the bladder vary greatly within vertebrates [Gadow, 1887; King, 1979b; Raynaud and Pieau, 1985; Beuchat, 1986]. Many squamates lack a bladder or have a vestigial bladder stalk, while other species have an allantois-derived bladder that lies on the ventral side of the cloaca and does not connect to the ureters [Raynaud and Pieau, 1985; Beuchat, 1986]. We have found that the allantois connects to the developing cloaca on the ventral side of the hindgut, and other researchers have indicated that green anoles have a functional bladder [Beuchat, 1986]. Our results suggest that the bladder of *A. carolinensis* also is derived from the allantois.

Sexual Differentiation of the Hemipenes and Hemiclitores

We have identified 2 developmental structures, the secondary phallic buds and the phallic ridge, that appear to be necessary for development of functional male hemipenes. Each of these structures is present in early embryos but later regresses in females and persists in males. The secondary buds on the base of the hemipenes grow towards the midline in males, restricting the size of the cloacal opening, and this smaller outlet may function in directing the flow of sperm towards the sulcus spermaticus during copulation. Similarly, the phallic ridge is present early in development but is lost in females at the earliest stage of sexual dimorphism. In males, this ridge persists and its growth appears to cause bifurcation of the sulcus spermaticus. Previous studies demonstrated that the 2 openings of the sulcus spermaticus align with each of the 2 female oviducts during copulation [Conner and Crews, 1980; Tokarz and Slowinski, 1990]. Some snake species lack a bifurcate sulcus spermaticus; instead, each hemipenis contains a single groove that traverses the proximodistal length of each hemipenis [Dowling and Savage, 1960]. If a bifurcate sulcus spermaticus forms as a consequence of phallic ridge invasion, as we propose, then we would predict that a simple sulcus spermaticus develops due to loss of the phallic ridge or a premature arrest in its maturation.

The stage of sexual differentiation of the external genitalia varies among squamates and is thought to be controlled by the relative timing of gonadal development [Raynaud and Pieau, 1985]. Our findings indicate that sexually dimorphic development occurs early in the green

anole, which supports previous findings [Winkler and Wade, 1998; Ruiz and Wade, 2002; Lovern and Wade, 2003; Lovern et al., 2004; Holmes and Wade, 2005; Beck and Wade, 2008; Cohen et al., 2012; Wade, 2012]. Development of sexually dimorphic structures, including external genitalia, in squamates has been linked to differences in sex steroid activity [Crews, 1980], and experimental studies have demonstrated the role of testosterone in sexual differentiation of anole genitalia [Winkler and Wade, 1998; Lovern et al., 2004; Beck and Wade, 2008]. Although hemipenes demonstrate some plasticity in response to hormones during breeding season, organizational hormone activity is essentially complete by hatching [Ruiz and Wade, 2002; Lovern et al., 2004]. In *A. carolinensis*, embryonic male hemipenes express high levels of androgen receptor mRNA and low levels of estrogen receptor α , while female hemiclitores have low androgen receptor and high estrogen receptor α expression, similar to the pattern that occurs in mammalian external genital development. This suggests that sexually dimorphic development of the hemipenes and hemiclitores is mediated by both androgen and estrogen [Beck and Wade, 2008; Cohen et al., 2012].

A bifurcate sulcus and constricted cloacal opening are important for the efficient transfer of sperm during copulation. We found these regions to be the most dimorphic structures in male and female genitalia, and we predict that these regions of the developing male hemipenes may show enriched androgen receptor expression. A recent study [Köhler et al., 2012] described divergent external genital morphologies in 2 closely related species of *Anolis*; one species has a unilobate hemipenis and the other has a bilobed hemipenis, similar to the green anole. It was suggested that the bilobed hemipenis functions more efficiently than a single-lobed phallus in simultaneous delivery of sperm to both oviducts [Köhler et al., 2012]. Based on the importance of the phallic ridge in formation of a bilobed hemipenis, we hypothesize that modifications to development of the phallic ridge may underlie some of the variation in hemipenis morphology, especially the number of lobes at the apex of the hemipenis.

Molecular Mechanisms of Hemiphallus Development

In mice, *Shh* is expressed in the cloacal endoderm, and lineage-tracing experiments have demonstrated that the urethral and rectal epithelia develop from cloacal endoderm with no contribution of mesodermal or ectodermal cells [Seifert et al., 2008]. The cloacal epithelium of *A. carolinensis* embryos strongly expresses *shh*, but the hemiphallus does not express this endodermal marker,

consistent with our histological evidence that the sulcus spermaticus is derived only from ectodermal cells. In mice, deletion of *Shh* results in persistent cloaca and absence of external genitalia (the paired genital swellings are initiated, but they fail to grow out or fuse to form a phallus) [Haraguchi et al., 2001; Perriton et al., 2002]. Thus, both green anoles and *Shh* null mice have an endodermal cloacal opening flanked by paired genital swellings comprised of only ectoderm and mesoderm; however, mice cannot form a phallus in the absence of *Shh*. These findings indicate that *Shh*-independent initiation of external genital development and formation of paired genital swellings are developmental mechanisms shared across Amniota. However, the ability of the hemiphallus to continue developing beyond the initiation stage without *Shh*-expressing cells is a difference in the gene regulatory network that controls anole phallus development.

In chick and mouse embryos, expression of *Shh* in hindgut epithelium induces *Bmp4* in the surrounding mesenchyme [Bitgood and McMahon, 1995; Roberts et al., 1995; Sukegawa et al., 2000]. Epithelial-mesenchymal interactions mediated by these factors are required for development of the gut, urogenital system, and external genitalia [Bitgood and McMahon, 1995; Roberts et al., 1995; Sukegawa et al., 2000; Suzuki et al., 2003; Sasaki et al., 2004]. In the developing external genitalia of *A. carolinensis*, there are 3 domains of *bmp4* expression: the surface ectoderm lateral to each developing hemiphallus (between the margins of the anterior and posterior cloacal lips), the proximal, lateral side of each hemiphallus, and around the cloacal endoderm. The *bmp4* domain around the cloacal membrane lies immediately adjacent to the *shh*-expressing region, indicating that *shh* in the cloacal endoderm could potentially regulate expression of *bmp4* in the pericloacal mesenchyme. Bmps also are important regulators of appendage outgrowth. Given that *Bmp4* is expressed in the mesenchyme of the developing limbs and genital tubercle of mouse embryos, where they act as negative regulators of bud outgrowth [Haraguchi et al., 2001; Perriton et al., 2002; Bandyopadhyay et al., 2005], the lack of *bmp4* expression in the mesenchyme of the hemiphallus is another divergent feature of anole genital development.

In green anole embryos, *fgfr2* is expressed in the surface ectoderm on the lateral margins of the cloacal lips and phallic buds and in the cloacal endoderm. It has been hypothesized that the ectodermal domains of *Fgfr2* may contribute to urethral tube closure in mice [Petiot et al., 2005], and therefore it is surprising to find *fgfr2* active in similar paired ectodermal domains in an amniote with-

out a urethra. The lateral ectoderm of the cloacal lips in *A. carolinensis* has overlapping *bmp4* and *fgfr2* expression, whereas in mice, *Bmp4* expression is excluded from the regions of ectodermal *Fgfr2*. One potential explanation for the co-localization of *fgfr2* and *bmp4* at the lateral ectoderm of the anole external genitalia may be that *bmp4* acts to repress *fgfr2*-mediated outgrowth around the developing cloaca. If these paired ectodermal tissues develop from the same cells in anoles and mice, it is tempting to speculate that decoupling the expression of *Fgfr2* and *Bmp4* may have played a role in the evolution of a tubularized urethra. If this were the case, then we would predict that other non-mammalian amniotes, all of which lack a tubularized urethra, might have overlapping regions of *Fgfr2* and *Bmp4* expression in the lateral ectoderm of the developing external genitalia. Based on this mechanism, evolution of a tubular urethra may have resulted from loss of *Bmp4* expression from the ectoderm adjacent to the embryonic phallus.

It is noteworthy that in all vertebrates studied to date, development of a midline phallus is associated with formation of an endodermal sulcus/urethra, whereas the paired lateral phalluses of squamates lack this endodermal contribution and the sulcus forms from surface ectoderm. This phenomenon may reflect an evolutionary developmental constraint in which cloacal endoderm becomes incorporated into the phallus only when the paired genital swellings fuse, as in non-squamate amniotes. Further research into the developmental interactions between external genital and cloacal morphogenesis, particularly involving comparisons of vertebrates with different genital morphologies, should shed light on the role of the cloacal endoderm in external genital development.

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