Morphogenesis and Patterning of the Phallus and Cloaca in the American Alligator, *Alligator mississippiensis*

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Abstract

In most animals, reproduction by internal fertilization is facilitated by an intromittent organ, such as the penis in amniote vertebrates. Recent progress has begun to uncover the mechanisms of mammalian external genital development; however, comparatively little is known about the development of the reptilian penis and clitoris. Here, we describe the development of the phallus and cloaca in the American alligator, *Alligator mississippiensis*. The embryonic precursor of the penis and clitoris is the genital tubercle, which forms by the budding of genital mesenchyme beneath the ventral body wall ectoderm, adjacent to the cloacal membrane. The cloacal lips develop from another pair of outgrowths, the lateral swellings. Early development of the alligator phallus, cloaca, and urogenital ducts generally resembles that of other reptiles, suggesting that differences in adult reptilian phallus and cloacal anatomy arise at later stages. The phallic sulcus is derived from the cloacal endoderm, indicating that the crocodilian sulcus is functionally and developmentally homologous to the mammalian urethra. Initial external genital outgrowth and patterning occur prior to temperature-dependent sex determination. Our analysis of alligator phallus and cloaca development suggests that modifications of an ancestral program of urogenital development could have generated the morphological diversity found in the external genitalia of modern amniotes.

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The emergence of external genitalia facilitated the transition from external fertilization (e.g. spawning) to internal fertilization, a key innovation in the evolution of terrestriality. In most amniotes, an intromittent organ functions to transfer sperm from the male into the female reproductive tract. Although many of the morphogenetic and molecular mechanisms of phallus development are beginning to be understood in mammals, comparatively little is known about development of the external genitalia in other amniotes.

Reptilian phalluses have diverse morphologies. A single midline phallus, the male penis and female clitoris, occurs in most reptiles. Male squamates (lizards and snakes) have 2 hemipenes that function as intromittent organs [Arnold, 1986; Card and Kluge, 1995; Böhme and Ziegler, 2009; Gredler et al., this issue; Leal and Cohn, this issue]. Some birds have a single medial intromittent phallus, but the majority of avian species lack an intromittent

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phallus altogether [King, 1979]. Archosauria is comprised of birds and crocodilians (alligators, crocodiles, and gharials); recent molecular phylogenetic analyses support testudines (turtles) as the sister group to archosaurs [Chiari et al., 2012; Fong et al., 2012; Wang et al., 2013], although the turtle-archosaur clade remains controversial [reviewed in Hedges, 2012]. Male turtles and crocodilians have a single penis that is thought to develop by similar mechanisms [Gadow, 1887; Moens, 1912; King, 1979].

During amniote copulation, the male intromittent organ acquires and maintains rigidity in order to direct sperm into the female reproductive tract [Gadow, 1887; King, 1979; Powell, 2000; Kelly, 2002]. Erection and eversion by hydrostatic pressure and muscle contraction function to achieve turgor and to create a functional channel for sperm transfer [Hart and Melese-D'Hospital, 1983; Schmidt and Schmidt, 1993; Andersson and Wagner, 1995; Kelly, 2002, 2004, 2013; Hsu et al., 2005; Cabrera et al., 2007]. Hydrostatic pressure is produced by blood vasculature in penises of crocodilians, turtles and mammals [Zug, 1966; King, 1979; Kelly, 2004; Moore et al., 2012], by lymphatic vessels in bird penises, when present [King, 1979], and by both blood and lymph in squamates [Dowling and Savage, 1960]. Internally, crocodilian, turtle, and mammalian penises have large fibroelastic, vascular, and lacunar tissues that support vascular erection [Zug, 1966; King, 1979; Kelly, 2004]. Two regions of erectile tissue are present; the corpus spongiosum is flexible, fibrous tissue that surrounds the penile sulcus in non-mammalian amniotes and the penile urethra in mammals, and the corpora cavernosa are large, paired regions of highly vascularized tissue that expand upon increased blood flow [Reese, 1915; Zug, 1966; King, 1979; Powell, 2000; Kelly, 2004, 2013; Cabrera et al., 2007]. The base of the alligator penis is formed from paired penile bodies called crura [Gadow, 1887; Reese, 1924; Kelly, 2013]. Proximally, each crus is connected to the pelvic girdle and adjacent cloacal muscles; distally, the crura fuse to form the shaft of the corpus cavernosum [Powell, 2000; Kelly, 2013]. In crocodilians, eversion of the phallus is achieved by muscle contraction [Reese, 1915; Powell, 2000; Kelly, 2013], and vascular dilation inflates the distal portion of the penis (glans) and the tissue adjacent to the penile sulcus [Gadow, 1887; Reese, 1915; Cabrera et al., 2007; Ziegler and Olbort, 2007; Moore et al., 2012].

In birds, turtles and mammals, development of the phallus begins with the emergence of paired genital swellings between the hindlimb buds [Raynaud and Pieau, 1985; Perriton et al., 2002; Herrera et al., 2013; Larkins

and Cohn, this issue]. These swellings fuse to form the genital tubercle, the embryonic anlage of the penis in males and the clitoris in females [Raynaud and Pieau, 1985; Perriton et al., 2002; Herrera et al., 2013; Larkins and Cohn, this issue]. In non-mammalian amniotes, the adult phallus resides inside the proctodeum, which is formed by the cloacal lips and is the caudal-most of the 3 cloacal chambers [Gadow, 1887; King, 1979; Raynaud and Pieau, 1985]. Proximal or anterior to the proctodeum, the urodeum is the excretory cloacal chamber and generally receives the urogenital ducts and the bladder [Gadow, 1887; Raynaud and Pieau, 1985]. The coprodeum connects to the rectum to function with the digestive system, and is the most anterior cloacal chamber [Gadow, 1887; Raynaud and Pieau, 1985].

Morphogenesis of the phallus is physically, temporally, and molecularly linked to development of the cloaca [Mo et al., 2001; Dravis et al., 2004; Yucel et al., 2007; Seifert et al., 2009b; Suzuki et al., 2012; Xu et al., 2012]. A portion of the cloacal epithelium extends into the genital tubercle to form the penile sulcus (also known as the penile groove, phallic sulcus or sulcus spermaticus) in birds and turtles and the urethra in mammals [Seifert et al., 2008, 2009b; Herrera et al., 2013; Larkins and Cohn, this issue]. Genetic knockout experiments in mice have demonstrated that molecular perturbation of cloacal development affects morphogenesis of the genital tubercle, and subsequently formation of the penis/clitoris [Perriton et al., 2002; Miyagawa et al., 2009; Seifert et al., 2009a; Xu et al., 2012].

The developmental mechanisms that mediate formation of the phallus have been studied most thoroughly in the mouse. An evolutionarily conserved suite of gene networks regulates organogenesis throughout the embryo, and a number of these pathways have been implicated in external genital development. Members of the fibroblast growth factor (Fgf) family of signaling molecules mediate external genital development and formation of the urethral tube in mice [Haraguchi et al., 2000; Petiot et al., 2005]. Differential expression of Hox genes throughout the embryo creates a combinatorial code that confers identity on many organ systems, and the paralogous *Hoxd13* and *Hoxa13* genes are required for normal development of distal appendages, including the digits and external genitalia [Sordino et al., 1996; Kondo et al., 1997; Warot et al., 1997; Morgan, 2003; Scott et al., 2005]. Sonic hedgehog *(Shh)* encodes a secreted signaling molecule that is expressed in the embryonic endoderm and its derivatives, and is required for cloacal septation and for proper patterning and outgrowth of the murine genital tubercle [Bitgood and McMahon, 1995; Perriton et al.,

2002; Seifert et al., 2008, 2009a, 2010; Miyagawa et al., 2009; Runck et al., 2014]. Ligands and receptors of the bone morphogenetic protein (Bmp) family of patterning genes frequently interact with *Shh*, are common mediators of cell death during development, and have been demonstrated to regulate external genital morphogenesis in both mice and chickens [Bitgood and McMahon, 1995; Roberts et al., 1995; Sukegawa et al., 2000; Suzuki et al., 2003; Sasaki et al., 2004; Bandyopadhyay et al., 2006; Seifert et al., 2008; Herrera et al., 2013; Lu et al., 2013]. Other genes implicated in external genital development include members of the Wnt/β-catenin pathway [Schwabe et al., 2004; Lin et al., 2008; Draaken et al., 2012; Guo et al., 2014], Dlx genes [Suzuki et al., 2008] and Eph/ephrins [Dravis et al., 2004].

In order to test the hypothesis that phallus development in crocodilians is controlled by the same mechanisms that pattern mammalian and avian external genitalia [Kondo et al., 1997; Warot et al., 1997; Haraguchi et al., 2000, 2001; Perriton et al., 2002; Morgan, 2003; Sasaki et al., 2004; Petiot et al., 2005; Scott et al., 2005; Seifert et al., 2008, 2009a, b; Miyagawa et al., 2009; Herrera et al., 2013], we performed a detailed analysis of American alligator *(Alligator mississippiensis)* phallus development from the time that outgrowth is initiated through the onset of sexual differentiation. We show that initiation and early development of the alligator genital tubercle is strikingly similar to that of turtles, birds, and mammals. Moreover, gene expression patterns in the developing alligator phallus are similar to those that have been observed for orthologous genes in turtles, birds, and mice, suggesting that the molecular mechanisms of phallus formation are conserved across amniotes. Morphogenesis of the cloaca occurs by interaction between the posterior limits of the digestive and excretory tracts, and a portion of the cloacal endoderm extends into the genital tubercle to form the phallic sulcus. Our results show that the crocodilian phallus and cloaca develop by morphogenetic and molecular mechanisms that closely resemble those of other amniotes. Viewed in a phylogenetic context, these similarities are consistent with the hypothesis that the genital tubercle of crocodilians is homologous to that of birds, turtles, and mammals.

Methods

Egg Collection and Embryo Dissection

A. mississippiensis eggs were provided by the Rockefeller Wildlife Refuge (RWR) in Grand Chenier, La., and the St. Augustine Alligator Farm Zoological Park (SAAFZP) in St. Augustine, Fla.,

USA. Crocodilians undergo temperature-dependent sex determination, and the thermosensitive period in *A. mississippiensis* occurs between Ferguson stages 21 [31–35 days post-oviposition (dpo)] and 24 (46–50 dpo) [Ferguson, 1985; Lang and Andrews, 1994]. *A. mississippiensis* embryos hatch at stage 28 (64–70 dpo). Eggs from RWR were incubated in outdoor tanks at the wildlife refuge for 2 weeks (stage 12) before being transferred to the University of Florida for incubation. To investigate development of the phallus and cloaca prior to sex determination, we incubated eggs at 33°C (which produces 100% male hatchlings, and maintains consistency in developmental timing) until they reached stages 12.5–21, then harvested and dissected them in cold PBS [Ferguson, 1985; Lang and Andrews, 1994]. Eggs from SAAFZP were incubated at 32°C for 1–2 months prior to transfer to the University of Florida where they were processed immediately for dissection. After removal from the egg, embryos at stage 23 and older were anesthetized and euthanized by intracoelomic injection of tricaine methylsulfonate (MS222) (0.7% MS222, pH 7.0 at 250 mg/kg for anesthesia, 0.4 ml of 50% MS222 for euthanasia) based on published methods [Conroy et al., 2009].

Tissue used for RNA extraction was stored in RNAlater at –20°C. Embryos used for histology were fixed overnight in 4% paraformaldehyde at 4°C, rinsed in PBS and dehydrated in a graded ethanol series, then stored in 70% ethanol at 4°C. For whole mount in situ hybridization, embryos were fixed overnight in 4% paraformaldehyde at 4°C, rinsed in PBS and dehydrated in a graded methanol series, then stored in 100% methanol at –20°C. Tissue used for scanning electron microscopy (SEM) was fixed and stored in 1% glutaraldehyde at 4°C.

Cell Death Analysis

Stage 15 embryos were quickly dissected in PBS and incubated in LysoTracker red (Molecular Probes) staining solution (25 μl LysoTracker red in 15 ml PBS) at 37°C for 30 min, then rinsed in PBS and fixed overnight in 4% paraformaldehyde at 4°C. Epifluorescent and brightfield images were acquired after fixation.

Scanning Electron Microscopy

After glutaraldehyde fixation, embryos were washed with PBS, osmicated in 2% osmium tetroxide for 1 h, and dehydrated to absolute ethanol. The embryos were then critical-point dried and mounted on stubs, sputter coated with gold/palladium, and imaged on a Hitachi S-4000 FE-SEM.

Histology

Paraffin histology was performed according to standard methods. Lower bodies were dehydrated to absolute ethanol, permeabilized in xylene, then infiltrated with and embedded in paraffin. Sections were cut 5 μm thick and slides were stained with Masson's trichrome (Richard Allen Scientific).

In situ Hybridization

RNA extracted (Qiagen RNeasy Plus Micro kit) from stage 15 *A. mississippiensis* tail and forelimb tissue was used to synthesize cDNA (BioRad iScript cDNA synthesis kit) that served as template for PCR cloning. Primers were designed from published sequences (Bmp4, EF527278; Shh, EF527277). PCR products were ligated into the pGEM-T Easy Vector (Promega), PCR-amplified using M13 primers, and used as templates for transcription of digoxigenin-labeled antisense riboprobes for *Bmp4* and *Shh*. The remaining

probes were generated by transcription from linearized plasmids for *Hoxd13 (Trachemys scripta,* kindly provided by C. Larkins) and *Fgfr2* and *Fgf10* (kindly provided by T. Iguchi). 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 KTBT solution, and the concentration of Triton X-100 in NTMT solution was increased from 0.1 to 1%.

Results

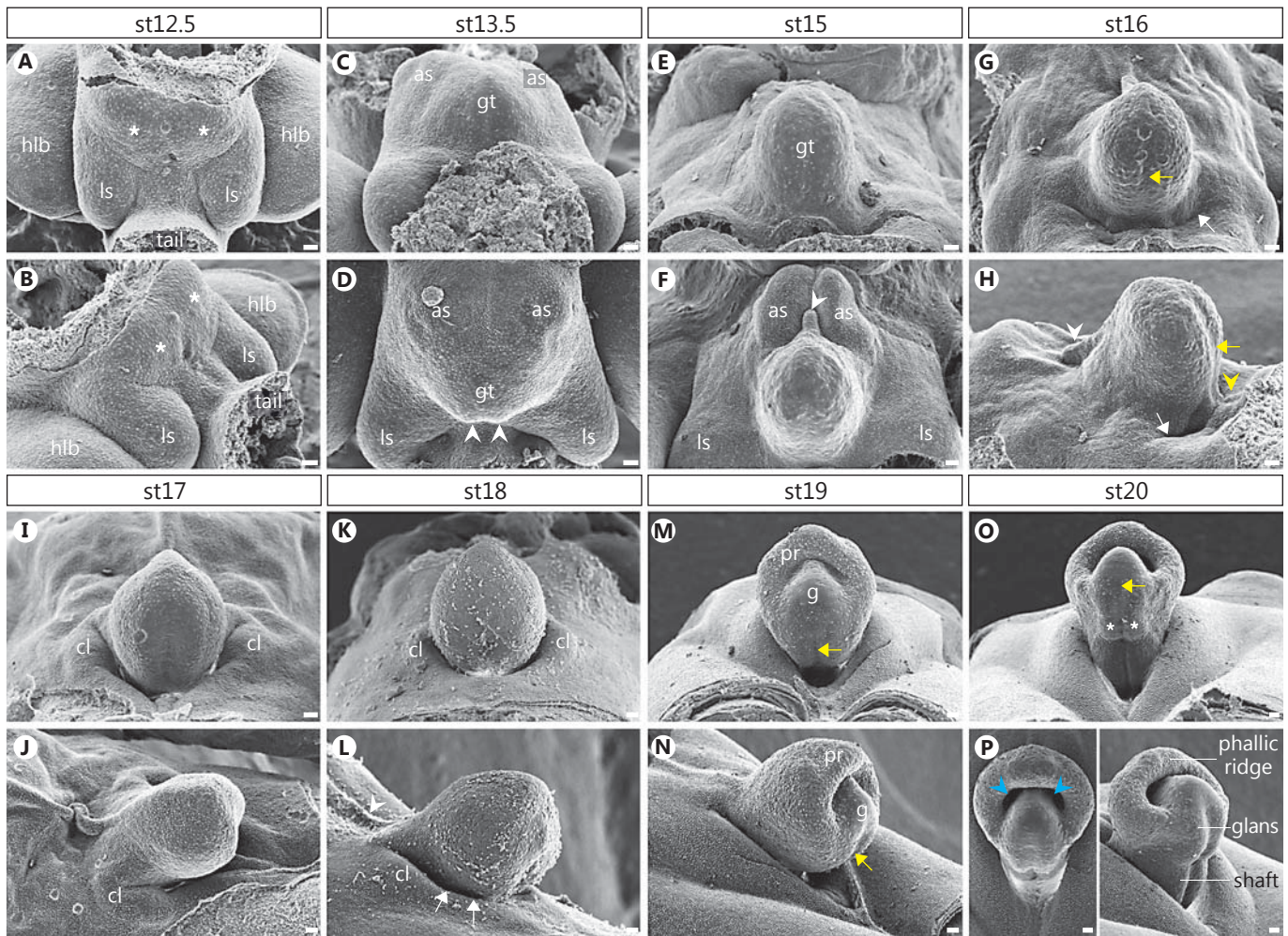*Early Development of the Alligator Phallus*

SEM was used to characterize development of the external genitalia in alligator *(A. mississippiensis)* embryos between stage 12.5, prior to the appearance of the genital tubercle, and stage 20, before the onset of sexual differentiation [staging was performed according to Ferguson, 1985; Lang and Andrews, 1994]. At stage 12.5 (14 dpo), genital mesenchyme and the overlying ectoderm protrude from the ventral body wall, anterior and lateral to the cloacal membrane (fig. 1A, B). A small crease in the midline of this early genital tissue delineates the position of the cloacal membrane, a transient structure formed where the cloacal endoderm meets the overlying surface ectoderm. An additional pair of swellings forms lateral to the cloacal membrane and caudal to the genital swellings, at the level of the posterior hindlimb bud (fig. 1A, B). By stage 13.5 (15 dpo), a single genital tubercle is apparent (fig. 1C, D). The apex of the genital tubercle is slightly bifid at this stage, which may represent its formation by fusion of the 2 genital swellings (fig. 1D). A third pair of swellings emerges anterior to the genital tubercle (fig. 1C, D). Outgrowth of the genital tubercle continues and, by stage 15 (18 dpo), it has a rounded, cylindrical appearance (fig. 1E). By stage 15, the genital tubercle has extended beyond the lateral swellings (fig. 1E, F). The anterior swellings, which are positioned immediately cranial to the base of the genital tubercle, are elongated anteroposteriorly and compressed laterally (fig. 1F). A small remnant of tissue, the cranial raphe, is evident between the 2 anterior swellings, at the base of the dorsal side of the genital tubercle (fig. 1F). At stage 16 (21 dpo), a fold has developed at the junction between the ventral side of the genital tubercle and the body wall (fig. 1G, H). The cranial raphe persists anterior to the genital tubercle (fig. 1H). An indentation of the surface epithelium along the ventral midline of the genital tubercle indicates the position of the phallic sulcus (fig. 1G, H). This furrow extends posteriorly into the

ventral body wall ectoderm between the genital tubercle and the tail (fig. 1H).

At stage 17 (22 dpo), the genital tubercle bends caudally, coincident with deepening of the depression between the genital tubercle and the ventral body wall (fig. 1I). Two crests of tissue are present at the lateral margins of this fold, forming the anlagen of the cloacal lips (fig. 1I, J). At stage 18, the cloacal lips are thinner mediolaterally and are closer to the genital tubercle than at stage 17 (fig. 1K, L). The posterior-most cloacal chamber, the proctodeum, appears as the cloacal lips emerge and the genital tubercle bends caudally (fig. 1L). The cranial raphe persists anterior to the genital tubercle (fig. 1L). At stage 19 (27 dpo), a crescent-shaped outgrowth, which we have termed the phallic ridge, develops along the dorsal margin of the distal genital tubercle (fig. 1M). The phallic sulcus is visible on the ventral side of the genital tubercle, extending into the proctodeal chamber (fig. 1M, N). The cloacal lips extend farther laterally than at previous stages (fig. 1N). At stage 20, the phallic sulcus extends distally into the glans, which appears to consist of 2 lobes and is distinct from the shaft of the phallus (fig. 1O, P). Maturation of the distal genital tubercle has resulted in formation of an arch-shaped pocket between the phallic ridge and the apex of the glans (fig. 1P).

Maturation of the Genital Tubercle

We used histology to examine the internal anatomy of the developing phallus. At stage 17, the phallic sulcus is a bilaminar epithelial plate that extends from the ventral surface into the center of the genital tubercle (fig. 2A–C). A ring of dense connective tissue, the anlage of the corpora cavernosa (alternatively known as fibrovascular bodies or corpora fibrosa), is visible in the mesenchyme dorsal to the phallic sulcus (fig. 2B, C). A second region of lacunar connective tissue, the corpus spongiosum, is positioned ventral to the corpus cavernosum (fig. 2C). At stage 18, the distal phallic sulcus appears as a bilaminar plate dorsally and the ventral margin is an open groove (fig. 2D). In the proximal genital tubercle, the dorsal and lateral sides of the phallic sulcus are bordered by the dense mesenchyme of the corpora cavernosa, and the center of the phallic sulcus begins to delaminate to form an internal lumen (fig. 2E). Blood vessels can be observed in mesenchyme lateral to the phallic sulcus (fig. 2E). The cranial raphe is present anterior to the genital tubercle (fig. 2F). Ventral and posterior to the genital tubercle, an epithelial invagination indicates formation of the proctodeum (fig. 2F). At stage 19, the corpora cavernosa are present in both the proximal and distal genital tubercle and are continuous with the paired penile

Fig. 1. Morphogenesis of the alligator phallus. Scanning electron micrographs of developing external genitalia in *A. mississippiensis* from stages 12.5 through 20. Panels in the first and third rows show ventral views of the external genitalia with anterior to the top. Panels in the second and fourth rows show alternate views of the embryos in the first and third rows, respectively. Alternate views are oriented laterally with anterior to the left, except for panels **D**, **F** and **P**, which show top views with anterior to the top. Outgrowth of the genital ectoderm and mesenchyme (white asterisks in **A** and **B**) forms the genital tubercle (gt), which is initially forked distally (white arrowheads in **D**). The lateral swellings (ls) develop into the cloacal lips (cl). The cranial raphe (white arrowheads in **F**, **H** and **L**) is located between the anterior swellings (as). The phallic sulcus (yellow arrows) is visible along the ventral midline of the genital tubercle, the proctodeum (white arrows) develops as a crease on the caudal side of the genital tubercle, and a caudal extension of the cloacal epithelium (yellow arrowhead) is visible between the genital tubercle and the tail. The phallic ridge (pr) develops at the dorsal side of the glans (g). At stage 20, the proximal glans is bifid (asterisks in **O**), and maturation of the phallic ridge has resulted in formation of a pocket in the distal glans (blue arrowheads). hlb = Hindlimb bud. Scale bars = 50 μm.

crura internally (fig. 2G–I). Each crus is positioned caudal and slightly dorsal to the ischium, and the sphincter cloacae muscles are visible between the crus and the ventral body wall (fig. 2I). A pair of large blood vessels is visible adjacent to the phallic sulcus on the ventral side of the genital tubercle (fig. 2G).

By stage 19, the proctodeal chamber has grown larger ventrolaterally, extending farther along the base of the genital tubercle than at stage 18 (fig. 2I). The surface epithelium between the newly formed phallic ridge and glans appears thicker and more stratified than neighboring cell populations (fig. 2H, I). At stage 20, the phallic sulcus remains a bilaminar epithelial plate, except at the dorsal tip, where a small lumen has formed along the proximodistal axis of the genital tubercle (fig. 2J, K). An epithelial invagination and thickening is apparent in the proctodeal
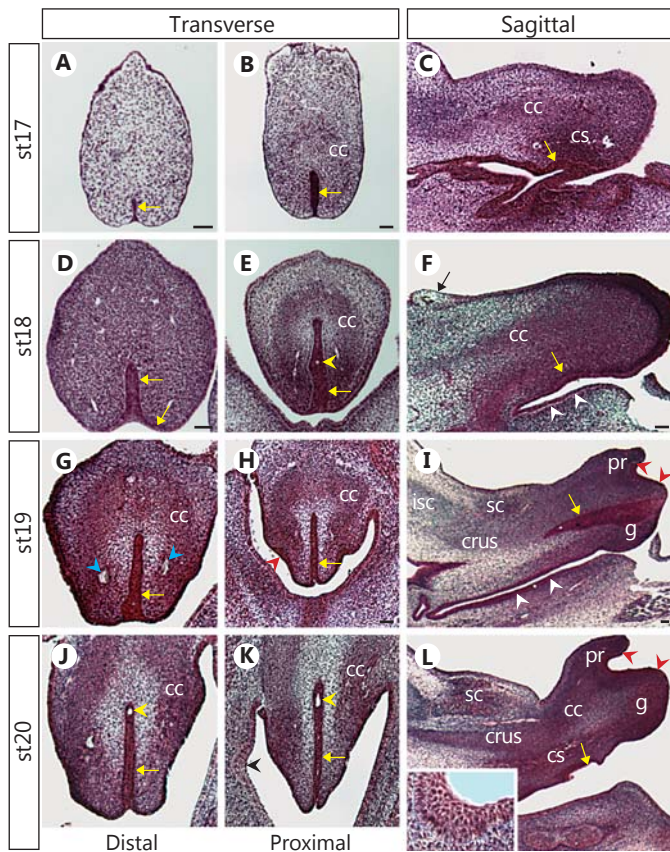
Fig. 2. Internal development of the alligator genital tubercle. Histological sections of the developing genital tubercle at stages 17–20. Transverse (cross) sections are perpendicular to the long (proximodistal) axis of the genital tubercle. **A**, **D**, **G**, and **J** are through the distal tip of the tubercle and **B**, **E**, **H**, and **K** are proximal sections. Sections in **C**, **F**, **I**, and **L** are through the sagittal plane of the main body axis and the genital tubercle. The phallic sulcus (yellow arrows) is a bilaminar epithelial plate, which delaminates (yellow arrowheads) at its dorsal margin at stage 20. The proximal end of the corpus cavernosum (cc) abuts the crus, and the corpus spongiosum (cs) is visible immediately adjacent to the phallic sulcus. Blood vessels (blue arrowheads) can be seen in the genital tubercle mesenchyme. The cloacal gland (black arrowhead in **K**) develops inside of the proctodeal epithelium (white arrowheads). The sphincter cloacae (sc) muscles develop ventral to the crus. Thickened epithelium (red arrowheads, **inset** in **L**) develops between the phallic ridge (pr) and glans (g). The black arrow denotes the cranial raphe. isc = Ischium. Scale bars = 50 μm.

epithelium, adjacent to the proximal genital tubercle; this marks development of the cloacal musk glands (fig. 2K). The sphincter cloacae muscles have matured and the crura are larger, extend farther anteriorly towards to the ischium, and are better defined than at stage 19 (fig. 2L). The corpus cavernosum abuts the distal margin of the

crus, and the corpus spongiosum is positioned immediately interior to the phallic sulcus (fig. 2L). The depression between the phallic ridge and the glans is lined by thickened epithelium (fig. 2L, inset). The anteroposterior extent of the proctodeum has increased and further separates the phallus from the proctodeal epithelium (fig. 2L).
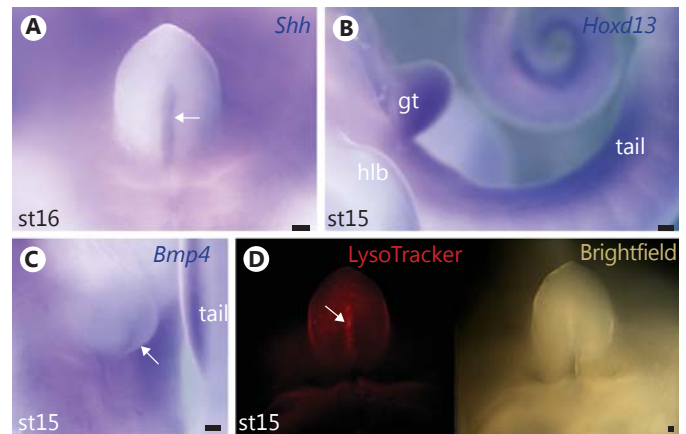
Gene Expression and Cell Death

Fgf signaling regulates development of a closed urethral tube in mice; *Fgf10* is expressed in the genital tubercle mesenchyme adjacent to the urethral plate, and its receptor, *Fgfr2*, is expressed in the mouse urethral plate epithelium [Petiot et al., 2005]. Loss of function mutations in either of these genes result in the formation of an open urethral sulcus instead of a closed urethral tube; in mammals, this type of birth defect is defined as hypospadias [Haraguchi et al., 2000; Petiot et al., 2005]. Since the crocodilian sulcus has the same function and embryonic origin as the mammalian urethra, we investigated the expression of *Fgf10* and *Fgfr2* in the developing alligator phallus. At stage 12.5, whole mount in situ hybridization for *Fgf10* showed staining in the mesenchyme on either side of the cloacal membrane, in the region of the genital tubercle primordia (fig. 3A). *Fgfr2* is expressed in a complementary pattern along the cloacal epithelium, between the 2 *Fgf10* domains (fig. 3B). By stage 13, *Fgf10* is expressed in the mesenchyme of the genital tubercle on either side of the cloacal/sulcus epithelium (fig. 3C). *Fgfr2* expression occurs along the sulcus epithelium and cloacal membrane of the genital tubercle, and extends caudally along the ventral surface ectoderm (fig. 3D). *Fgfr2* transcripts can also be detected in the ectoderm along the lateral margins of the genital tubercle (fig. 3D). An additional broad domain of *Fgfr2* expression is present anterior to the genital tubercle, in the region where the anterior swellings will form (fig. 3D). At stage 14, the *Fgf10* expression domain has expanded laterally within the genital tubercle mesenchyme, but no expression is detectable in the lateral swellings (fig. 3E). Each of the *Fgfr2* expression domains present at stage 13 persists through stage 14, and the expression domain along the lateral ectoderm of the phallus appears broader and stronger than at previous stages (fig. 3F). At stage 15, transcription of *Fgf10* is visible in the genital tubercle on either side of the phallic sulcus, and this expression extends posteriorly along the margin of the proctodeum (fig. 3G). *Fgfr2* continues to be expressed in the phallic sulcus/cloacal membrane, in the ectoderm of the lateral swellings, in a broad domain anterior to the genital tubercle, and on the surface ectoderm of the ventrolateral sides of the genital tubercle (fig. 3H).
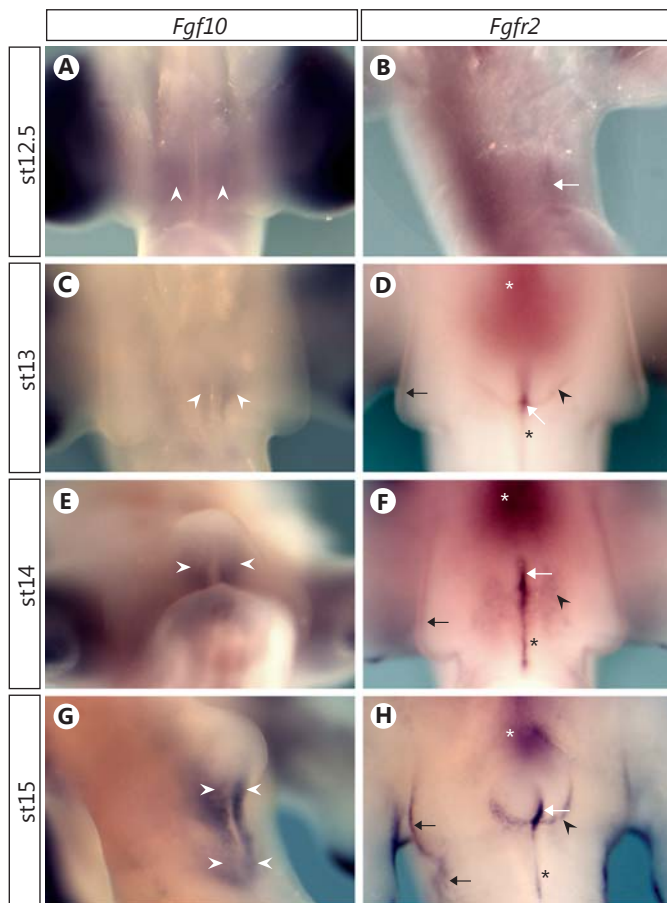
Fig. 3. *Fgf10* and *Fgfr2* expression in the developing alligator external genitalia. Whole mount in situ hybridization for *Fgf10* (**A**, **C**, **E**, **G**) and *Fgfr2* (**B**, **D**, **F**, **H**) on *A. mississippiensis* genital tubercles at stages 12.5 (**A**, **B**), 13 (**C**, **D**), 14 (**E**, **F**), and 15 (**G**, **H**). *Fgf10* mRNA was detected in the mesenchyme of the lateral swellings (white arrowheads in **A**), paired mesenchymal domains in the genital tubercle at stages 13–15 (white arrowheads in **C**, **E**, **G**), and in an additional caudal pair of regions between the genital tubercle and the tail at stage 15 (bottom white arrowheads in **G**). *Fgfr2* mRNA was detected in the developing cloacal membrane at stage 12.5 (white arrow in **B**) and in the phallic sulcus at stages 13–15 (white arrows in **D**, **F**, **H**). At stages 13–15 (**D**, **F**, **H**), *Fgfr2* was also detected in a thin domain extending from the base of the phallic sulcus towards the tail (black asterisks), in lateral regions of the surface epithelium of the genital tubercle (black arrowheads), in a diffuse area anterior to the genital tubercle (white asterisks), and in the epithelium of the lateral swellings (black arrows) including a caudally extended domain at stage 15 (bottom black arrow in **H**).

Fig. 4. Gene expression and cell death in the embryonic alligator phallus. **A–C** Whole mount in situ hybridization for *Shh* at stage 16 (**A**), *Hoxd13* at stage 15 (**B**) and *Bmp4* at stage 15 (**C**). **A** Ventral view with anterior to the top. **B** Lateral view with anterior to the left. **C** Ventrolateral view with anterior to the top. Expression in the phallic sulcus (white arrows) was detected for *Shh* at stage 16 and for *Bmp4* at stage 15. At stage 15, *Hoxd13* showed expression in 3 domains of the posterior embryo: the genital tubercle (gt), the hindlimb bud (hlb) and the ventral side of the tail. A region of *Bmp4* expression was detected in the tail at stage 15. **D** Whole mount fluorescent (left) and light (right) micrographs of a stage 15 American alligator genital tubercle stained with LysoTracker red to reveal apoptotic cells. LysoTracker red staining was detected in the distal region of the phallic sulcus (white arrow), which co-localizes with the expression of *Bmp4*. Scale bars = 20 μm.

We next assayed alligator genital tubercles at stage 15/16 for expression of 3 other genes, *Shh*, *Hoxd13*, and *Bmp4*, which are known to be involved in development of mouse and bird external genitalia. *Shh* is expressed in the phallic sulcus (fig. 4A), and *Hoxd13* is expressed throughout the genital tubercle, as well as in the tail (fig. 4B). *Bmp4* is expressed weakly in the region of the phallic sulcus, and a stronger domain exists in the distal tip of the tail (fig. 4C). In addition to playing a role in the patterning of multiple developmental systems, *Bmp4* has been shown to promote apoptosis in the genital tubercle of mouse, chick, and duck embryos [Suzuki et al., 2003; Herrera et al., 2013]. To determine whether *Bmp4* expression is associated with cell death in the phallic sulcus of alligator embryos, we performed LysoTracker red staining at stage 15. We detected a region of LysoTracker-labeled cells in the phallic sulcus, which co-localizes with the region of *Bmp4* expression in the developing phallus (fig. 4D).

Cloacal Development

In mammals, development of the phallus occurs together with morphogenesis of the cloaca [Seifert et al., 2008, 2009a]. Histological analysis was used to investigate alligator cloacal development and to determine its relationship to morphogenesis of the phallus. At stage 17, the

cloaca is a single chamber (fig. 5A). The allantois connects to the ventral cloaca and has a thinner epithelium than the heavily stratified cloacal (urodeal) epithelium. Anterior to the cloaca, the urorectal septum separates the allantois and hindgut (fig. 5A). The caudal side of the urodeum is separated from the surface ectoderm by a layer of dense mesenchyme (fig. 5A). The ventral portion of the posterior cloacal epithelium extends into the proximal genital tubercle, forming the anlage of the phallic sulcus (fig. 5B). The cloacal gland (or cloacal musk gland) is visible posterior to the urodeum, adjacent to the developing proctodeum (fig. 5B). On the dorsal side of the cloaca, a region of the urodeum evaginates to form a channel that will connect the cloacal sinus to the Wolffian (mesonephric) duct (fig. 5B). Ventral to the developing cloaca, a condensation of mesenchymal cells indicates formation of the crura (fig. 5A).

By stage 19, the coprodeum has begun to form cranial and dorsal to the urodeum (fig. 5C, D). The uroproctodeal fold marks the junction of the urodeum and proctodeum (fig. 5C). The Wolffian duct joins the proctodeum at the base of the phallus, caudal to the uroproctodeal fold (fig. 5C). The allantois remains in contact with the urodeum, and caudal growth of the urorectal septum has resulted in reduction of the anteroposterior length of the urodeum (compare fig. 5A and C). The urethra connects the urodeum to the phallic sulcus, which extends to the distal tip of the glans (fig. 5C). The corpus cavernosum is visible along the length of the phallus; proximally it connects to the crus, and distally it abuts the thickened ectoderm between the phallic ridge and glans (fig. 5C, D). The corpus spongiosum is visible as a population of dense connective tissue along the shaft of the phallus, between the phallic sulcus and the crus (proximally) or corpus cavernosum (distally) (fig. 5D). The sphincter cloacae muscles are visible just ventral to the crura (fig. 5C, D). The genital tubercle projects from the anlage of the ventral proctodeal wall, and the cloacal gland extends internally on the dorsal side of the proctodeum (fig. 5D).

The proctodeal chamber has enlarged by stage 20 (fig. 2H, 5E). The coprodeal and rectal epithelia are heavily stratified and convoluted (fig. 5E). The ligamentum ramus connects the dorsal side of the caudal ischium to the crus (fig. 5F). The sphincter cloacae muscles have subdivided into the pars superficialis, pars medialis and pars intermedius (fig. 5F). The corpus cavernosum is visible in the distal shaft, and the corpus spongiosum is positioned between the crus and the phallic sulcus (fig. 5E, F). By this stage, the phallic sulcus has formed an open groove (fig. 5E).
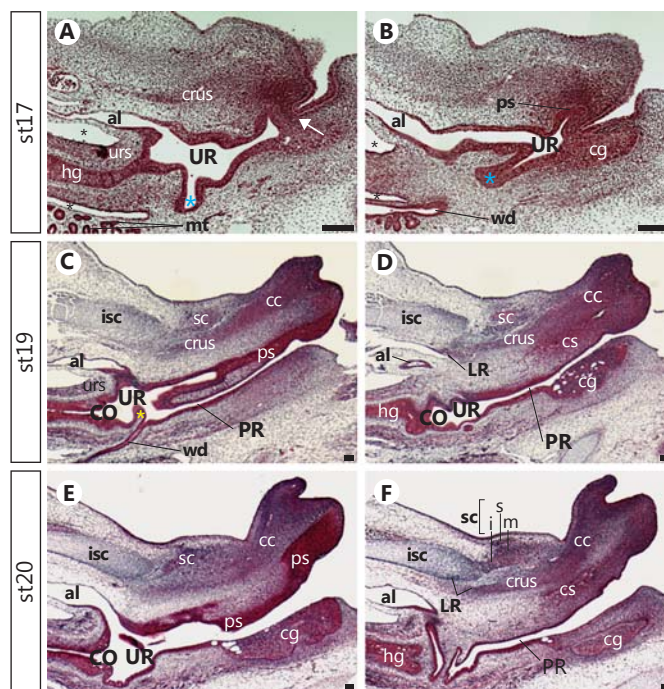

Fig. 5. Ontogeny of the alligator cloaca. Histology of the developing alligator cloaca and genital tubercle. All panels are sagittal sections stained with Masson's trichrome. **A, B** At stage 17, the allantois (al) and hindgut (hg) are separated by the urorectal septum (urs). An evagination (blue asterisks) of the dorsal urodeum (UR) lies in close proximity to the developing Wolffian duct (wd), into which the mesonephric tubules (mt) empty. The phallic sulcus (ps) extends from the urodeum into the genital tubercle, and a population of condensed cells is visible between the urodeum and the surface ectoderm (white arrow in **A**). The cloacal gland (cg) develops on the dorsal wall of the proctodeum. **C, D** By stage 19, the urodeum connects with the coprodeum (CO) on its anterior side and the proctodeum (PR) on its posterior side, and the uroproctodeal fold (yellow asterisk in **C**) projects between the urodeum and proctodeum. The phallic sulcus is adjacent to the corpus spongiosum (cs). The corpus cavernosum (cc) is visible in the mesenchyme of the distal genital tubercle, the sphincter cloacae (sc) muscles are positioned ventral to the crus, and the ligamentum ramus (LR) connects the crus to the ischium (isc). **E, F** At stage 20, the sphincter cloacae muscles have begun to differentiate into the pars superficialis (s), pars medialis (m) and pars intermedius (i). Black asterisks = peritoneum. **A, B** Scale bars = 20 μm. **C–F** Scale bars = 50 μm.

Differentiation of the Alligator Penis

To investigate early sexual differentiation of the penis, we used SEM to examine embryos at 2 stages: during (st23) and after (st25) the thermosensitive period [Smith and Joss, 1993]. Initial development of the phallus occurs externally, but at the time of sexual differentiation, it is partially enclosed within the proctodeum. At stages 19
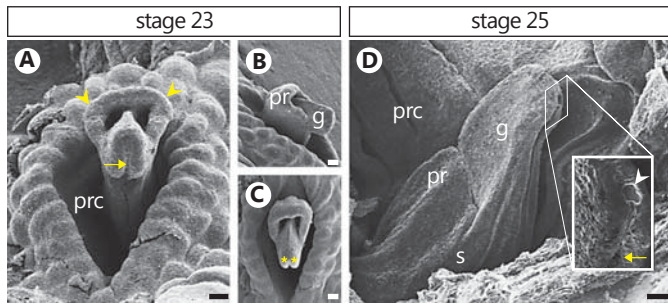
Fig. 6. Sexual differentiation of the alligator penis. Scanning electron micrographs of American alligator genital tubercles incubated at a male-producing temperature during (stage 23) and after (stage 25) the thermosensitive period of sexual differentiation. **A** Ventral view with anterior to the top. **B** Lateral view with anterior to the top left. **C** Top view with anterior to the top. **D** Lateral view with anterior to the left. **A–C** During the thermosensitive period, the shaft of the genital tubercle is enclosed within the proctodeum (prc), while the glans (g) and phallic ridge (pr, yellow arrowheads in **A**) remain outside of the cloaca. The phallic sulcus (yellow arrow) extends distally from the base of the phallus into the glans, which is bifid distally (yellow asterisks in **C**). **D** After the thermosensitive period, the penis is composed of the phallic ridge, glans, and shaft (s), and is enclosed within the proctodeum. The penile sulcus (**inset** in **D**, yellow arrow) is visible along the ventral midline of the penis. In the distal glans, the lateral margins of the surface epithelium are in contact at the midline over the penile sulcus, and a circular opening (**inset** in **D**, white arrowhead) is visible at the terminus of the penile sulcus. Scale bars = 100 μm.

and 20, the genital tubercle has begun to withdraw into the proctodeum; the ventral side of the shaft lies within the proctodeum, but the dorsal side is visible externally and is contiguous with the ventral body wall ectoderm (fig. 1N, P). By stage 23, the proctodeal chamber encloses the shaft on all sides, while the phallic ridge and glans are still located outside of the proctodeum (fig. 6A–C). The glans elongates and is now longer than the phallic ridge, whereas the stage 20 glans and phallic ridge are similarly sized (compare fig. 6B to 1P). Bifurcation of the distal glans occurs as the phallic sulcus extends distally into the glans (fig. 6A, C). The lateral margins of the phallic ridge extend cranially and the medial portion remains tethered to the underlying glans (fig. 6A). By stage 25, the penis is enclosed within the proctodeum and is regionalized into the shaft, phallic ridge and glans (fig. 6D). The glans constitutes approximately half of the length of the male phallus (fig. 6D). The phallic sulcus remains a continuous structure along the ventral side of the shaft and glans (fig. 6D). The lateral margins of the penile sulcus make contact along the ventral midline of the distal glans, re-

sulting in the appearance of a morphological tube instead of a groove, while the terminus of the sulcus is an open groove (fig. 6D, inset).

Discussion

Ontogeny of the Alligator Phallus

Morphogenesis and anatomy of crocodilian genitalia are generally thought to resemble that of turtles; however, since the late 19th and early 20th centuries, few studies have investigated penis, clitoris, and cloaca development in crocodiles and alligators [Clarke, 1891; Reese, 1908, 1915, 1924; Moens, 1912; Ferguson, 1985]. Our analysis of external genital development in the American alligator provides new insight into morphogenesis of the embryonic genital tubercle and ontogeny of the cloaca. Early development occurs via the formation of 3 sets of outgrowths: the genital tubercle, lateral swellings, and anterior swellings. Budding of the external genitalia from the pericloacal region of the ventral body wall begins at stage 12.5, and the genital tubercle, the anlage of the penis and clitoris, is apparent by stage 13.5. The lateral swellings develop at the level of the posterior hindlimb bud, caudal and lateral to the genital swellings, and develop into the cloacal lips. Development of the cloacal lips from the lateral swellings has been described in caimans, lending support to our hypothesis that the cloacal lips in *A. mississippiensis* also are derived from the lateral swellings [Reese, 1924]. Maturation of the cloacal lips forms the proctodeum, the cloacal chamber that encloses the adult phallus. The anterior swellings are evident at stage 13.5, cranial to the genital tubercle.

The unpaired, medial phallus in non-squamate amniotes develops from the cloacal epithelium and the somatopleure (lateral plate mesoderm and surface ectoderm) on the left and right sides of the cloaca [Raynaud and Pieau, 1985; Perriton et al., 2002; Herrera et al., 2013 and this issue; Larkins and Cohn, this issue]. In birds, turtles and mice, the left and right somatopleural portions of the genital tubercle emerge as morphologically distinct paired outgrowths, the genital swellings, that fuse to form the genital tubercle [Raynaud and Pieau, 1985; Perriton et al., 2002; Herrera et al., 2013; Larkins and Cohn, this issue]. Although we found similar projection of external genital tissue adjacent to the cloacal membrane in the alligator, we could not resolve whether the tubercle arises from 2 morphologically distinct buds or a single region of outgrowth. Other reports of crocodilian phallus development describe a single genital eminence [Reese, 1910,

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Alligator Phallus and Cloaca
Development

1924; Ferguson, 1985 and citations therein], although it is possible that embryos in those studies were examined after initiation stages, when paired buds are apparent in other taxa. On the homology of amniote intromittent organs, Gadow [1887] argues that the 'original duplicity' of the unpaired (avian, turtle, crocodilian, and mammalian) phallus is manifested in paired anatomical structures such as the copulatory nerve supply and vasculature, corpora cavernosa, and crura. We posit that in all amniotes with a single phallus, the genital tubercle develops by coordinated outgrowth of homologous regions of lateral plate mesoderm and surface ectoderm adjacent to the cloacal membrane.

The anterior swellings also appear for only a short period of development, forming in association with the genital tubercle and cranial raphe. The cranial raphe that we observed in association with the anterior swellings resembles an epithelial tag that has been described in development of the chicken external genitalia [Bakst, 1986]. Our analysis suggests that the anterior swellings are either incorporated into the cranial side of the growing genital tubercle, regress, or contribute to the cloacal lips. While it is possible that cells of the anterior swellings give rise to the phallic ridge later in development, the fate and function of these swellings remains unclear. Fate-mapping experiments will be necessary to determine the definitive fate of the anterior swellings in *A. mississippiensis.*

Our data demonstrate that, at stage 19, a crescent-shaped ridge forms on the dorsal side of the distal glans. It has been suggested that formation of this tissue may reflect homology with the mammalian prepuce [Reese, 1924]. A comparable feature has been described in turtles; Raynaud [1985] characterizes the 'ridge on the phallic primordium' of *Testudo graeca* as a 'horseshoe-shaped fold that envelops the urodeal furrow *(sulcus)* and lobes *(bifurcated distal tubercle)*', and a similar structure has been recently described in the developing red-eared slider turtle, *T. scripta* [Larkins and Cohn, this issue]. Based on its morphological resemblance to the ridge on the turtle genital tubercle, we favor the use of the term 'phallic ridge' to describe this structure in the crocodilian phallus. Among adult crocodilians, this structure has been referred to as the cuff in the American alligator [Moore et al., 2012], the head in the broad-snouted caiman (*Caiman latirostris,* together with the adjacent portion of the glans [Nuñez Otaño et al., 2010]), the base in the American alligator (with all other phallic tissue except the distal-most projection of the glans [Allsteadt and Lang, 1995]), and the glans penis in the spectacled caiman (*Caiman crocodilus*, with the central portion of the glans [Reese, 1924; Cabrera et al., 2007]).

The position, morphology, and relative size of the phallic ridge appear to vary among crocodilian species. In the Nile crocodile, Australian freshwater *(Crocodylus porosus)*, and Australian saltwater *(Crocodylus johnsoni)* crocodiles, the proximodistal length of the phallic ridge is more than half of the length of the penis, and the morphological transition between the ridge and the neighboring glans is gradual [Webb et al., 1984; Ziegler and Olbort, 2007]. In contrast, the spectacled, smooth-fronted *(Paleosuchus trigonatus)*, and Cuvieri dwarf *(Paleosuchus palpebrosus)* caimans have a shorter phallic ridge that is positioned at the distal tip of the penis, resulting in a comparatively thin phallus with a bulbous extremity [Ziegler and Olbort, 2007; Cabrera and García, 2010]. The phallic ridge of the smooth-fronted caiman extends completely over the glans, whereas the glans is longer than the ridge in the Cuvieri dwarf caiman [Ziegler and Olbort, 2007]. Finally, the phallic ridge of Chinese *(Alligator sinensis)* and American alligators is short relative to the length of the penis, similar to the pattern in caimans, although it is positioned more proximally, approximately halfway along the length of the phallus [Allsteadt and Lang, 1995; Ziegler and Olbort, 2007; Moore et al., 2012]. In order to facilitate future comparisons between the sexes and among species of crocodilians, we propose that the term phallic ridge be used to describe this structure. Future analysis of phallic ridge development among different species will help identify homologous structures within the crocodilian phallus.

Morphogenesis of the Alligator Cloaca and Phallic Sulcus

Formation of the phallus is associated with cloacal development. The vertebrate cloaca comprises 3 chambers; the coprodeum is the anterior-most cloacal chamber and communicates with the digestive system, the urodeum performs urinary and excretory functions, and the proctodeum is the caudal-most chamber and houses reproductive structures [Gadow, 1887; King, 1979]. Our data show that the early embryonic cloaca in *A. mississippiensis* is composed of the urodeum; its anterior wall is connected to the allantois on the ventral side and the hindgut on the dorsal side. Cranial to the urodeum, the urorectal septum separates the allantois and hindgut. In mammals, caudal elongation of the urorectal septum (or, alternatively, medial migration of lateral folds) results in formation of distinct urogenital and anorectal tracts [Qi et al., 2000; Seifert et al., 2009a; Kluth, 2010; Xu et al., 2012]. Our findings support previous reports that the urorectal septum grows caudally during development of the croco-

dilian cloaca but never reaches the proctodeal epithelium. A consequence of the early arrest of urorectal septum descent in crocodilians is persistence of a shared urinary and anorectal chamber at the terminal end of the cloaca, in contrast to the distinct urogenital and anorectal orifices that develop as a result of complete cloacal septation in mammals [Gadow, 1887; Reese, 1908, 1910, 1924; Seifert et al., 2009a].

In squamates, turtles, and mammals, the allantois has been reported to contribute to the bladder [Gadow, 1887; King, 1979; Raynaud and Pieau, 1985; Beuchat, 1986]. Similar to birds, the crocodilian allantois regresses before hatching; storage of urine occurs in a large chamber formed by the post-hatch fusion of the coprodeum and urodeum [Gadow, 1887; Kuchel and Franklin, 2000]. Our data demonstrate that the allantois is still present at stage 20, but its epithelium is less stratified than that of the adjacent urodeal epithelium. This finding suggests that the transition between cloacal and allantoic epithelia becomes increasingly pronounced due to concomitant thinning of the allantois epithelium and stratification of the urodeal epithelium, which is consistent with previous descriptions of caiman and alligator cloacal development [Reese, 1908, 1910, 1915, 1924].

Early communication between the cloaca and mesonephros is established in *A. mississippiensis* by mechanisms similar to those that have been described for other reptiles. At stage 17, the craniolateral wall of the urodeum evaginates dorsally and anteriorly to form a small vestibule, and the Wolffian ducts have not yet contacted the developing cloaca. This structure is similar to the embryonic 'urogenital pocket' of the turtle cloaca and the 'cloacal horn' that forms in squamate embryos [Raynaud and Pieau, 1985]. We posit that this region contacts the developing Wolffian duct in crocodilians, as in turtles [Raynaud and Pieau, 1985]. By stage 19, the Wolffian duct opens into the proctodeum adjacent to the uroproctodeal fold. These findings are consistent with previous reports that the genital ducts of crocodilians empty caudally into the proctodeum and not the urodeum, a trait that is unique among reptiles [Gadow, 1887; Forbes, 1940; King, 1979; Ferguson, 1985; Kuchel and Franklin, 2000; Oliveira et al., 2004; Cabrera et al., 2007; Cabrera and García, 2010].

The phallic sulcus forms as an extension of the endodermally-derived cloaca into the developing phallus as a bilaminar epithelial plate that first extends proximodistally and subsequently ventrodorsally through the genital tubercle. A similar developmental progression occurs in development of the mouse urethra; a population of cells from the embryonic cloaca develops inside the genital tubercle as a bilaminar epithelial plate which later opens to form the tubular urethra [Perriton et al., 2002; Seifert et al., 2008]. The data presented here and in our studies of external genital development in turtles and birds [Herrera et al., 2013 and this issue; Larkins and Cohn, this issue] are consistent with the hypothesis that the phallic sulcus of non-squamate reptiles is homologous to the urethral tube of mammals, with the primary difference being the formation of an open groove in the former and a closed tube in the latter.

Molecular Genetic Mechanisms of Alligator External Genital Development

A number of similarities exist between limb and external genital development, including shared patterns of gene expression [reviewed in Cohn, 2011]. The expression patterns of Hox genes confer positional identity on undifferentiated embryonic tissue, particularly with respect to axial position [Burke et al., 1995; Roberts et al., 1995; Kondo et al., 1997; Warot et al., 1997; Mansfield and Abzhanov, 2010]. *Hoxd13* is an AbdB-related Hox gene that is required for the development of distal appendages in vertebrates, including the digits, fins, tail, terminal hindgut, and genital tubercle [Sordino et al., 1996; Kondo et al., 1997; Warot et al., 1997; Morgan, 2003; Scott et al., 2005]. In mice, expression of *Hoxd13* in the digits and genital tubercle is controlled by a conserved *cis* regulatory sequence located within the global control region, an enhancer-containing region upstream of *Hoxd13* [Gonzalez et al., 2007]. In *A. mississippiensis*, we found expression of *Hoxd13* in the genital tubercle and distal limb buds (as well as the tail). These results are consistent with the hypothesis that *Hoxd13* defines the terminus or distal domain of appendages [Warot et al., 1997], and suggest that the gene regulatory controls identified in mice are conserved in alligators.

In mice, lineage-tracing experiments have demonstrated that the urogenital and anorectal epithelia are derived from *Shh*-expressing endodermal cells [Seifert et al., 2008]. Our finding that *Shh* is expressed in the developing phallic sulcus in *A. mississippiensis*, together with histological data demonstrating that the sulcal epithelium is contiguous with the cloacal epithelium, is consistent with the alligator sulcus having an endodermal origin. *Shh* is expressed in the embryonic phallic sulcus of birds and turtles [Herrera et al., 2013 and this issue; Larkins and Cohn, this issue], suggesting that mechanisms of genital tubercle patterning are conserved among amniotes. Although our study examined a small subset of genes im-

plicated in mammalian genital development, the results suggest that the Shh pathway is active in the crocodilian genital tubercle. Epithelial-mesenchymal interactions between the (endodermal) epithelially-expressed *Shh* and mesenchymally-expressed *Bmp4* are required for development of the gut, urogenital system, multiple epithelial appendages, and external genitalia [Bitgood and McMahon, 1995; Roberts et al., 1995; Sukegawa et al., 2000; Suzuki et al., 2003; Sasaki et al., 2004; Bandyopadhyay et al., 2006; Seifert et al., 2008; Herrera et al., 2013; Lu et al., 2013]. Therefore, our detection of *Bmp4* expression in the phallic sulcus of the alligator genital tubercle at stage 15 was somewhat unexpected; however, *Bmp4* has been shown to promote apoptosis in the genital tubercle of mouse, chick, and duck embryos [Suzuki et al., 2003; Herrera et al., 2013]. Cell death analysis in alligator genital tubercles revealed apoptosis in the phallic sulcus at stage 15, in a region that overlaps with *Bmp4* expression. Taken together with the studies cited above, this finding raises the possibility that *Bmp4* expression could act to promote apoptosis during development of the open phallic sulcus.

In mice, *Fgf10* is expressed in the genital tubercle mesenchyme, and its primary receptor, *Fgfr2,* is expressed in both the endodermally-derived urethral epithelium and along the genital (surface) ectoderm [Haraguchi et al., 2000; Perriton et al., 2002; Petiot et al., 2005]. Genetic knockout studies have demonstrated that these regions of *Fgf10* and *Fgfr2* expression mediate maturation and proliferation of the urethral epithelium, development of a closed urethral tube, and formation of the ventral aspect of the prepuce [Haraguchi et al., 2000; Petiot et al., 2005]. In the American alligator, *Fgf10* is expressed in the mesenchyme neighboring the cloacal membrane during initial outgrowth of the external genitalia. *Fgfr2* is expressed in the adjacent cloacal (and later, sulcus) epithelium, and an additional region of *Fgfr2* expression occurs in the ventral and lateral surface ectoderm of the genital tubercle. The similarity of these expression patterns in alligator and mouse genital tubercles suggests that *Fgf10-Fgfr2* signaling may play conserved roles in maturation of the (phallic) sulcal epithelium, outgrowth of the genital tubercle, and development of the ventral phallus in *A. mississippiensis*.

We identified 3 additional domains of *Fgfr2* expression in the alligator genital tubercle: a caudal extension of the cloacal membrane, the surface ectoderm of the lateral swellings, and in a broad domain anterior to the genital tubercle. Over a century ago, it was reported that a portion of the cloacal endoderm extends into the tail of crocodilian embryos to form the transient post-anal gut [Reese,

1910]. Thus, the caudal domain of *Fgfr2* expression that we observed in alligators may reflect formation of a post-anal gut. Anterior to the genital tubercle, a broad region of *Fgfr2* expression was observed in the region of the anterior swellings. However, *Fgfr2* expression in this region appears to be mesenchymal, in contrast to the epithelial domains of *Fgfr2* activity in the phallic sulcus and genital ectoderm. Since *Fgf10* is expressed only in mesenchymal tissue and Fgf signaling generally proceeds by epithelial-mesenchymal crosstalk, a different Fgf ligand would be required to activate *Fgfr2* anterior to the genital tubercle [Peters et al., 1992; De Moerlooze et al., 2000; reviewed in Itoh, 2007]. Future work investigating the expression patterns and functions of different Fgf family members in external genital development may shed light on the roles of these unique *Fgfr2* expression domains in alligators.

Environmental Influence on Development of the Crocodilian Phallus

Alligators undergo temperature-dependent sex determination, and the development of sexually dimorphic structures, including the external genitalia, is sensitive to environmental cues [Ferguson, 1985; Hutton, 1987; Smith and Joss, 1993; Lang and Andrews, 1994; Allsteadt and Lang, 1995; Western et al., 1999, 2000]. *Fgfr2* and *Fgf10* are required for normal development of the phallus in mice [Haraguchi et al., 2000; Petiot et al., 2005], and our findings suggest that these genes might have conserved functions in crocodilian external genital development. We showed previously that *Fgfr2* expression in mouse external genitalia is responsive to treatment with anti-androgens [Petiot et al., 2005], and other studies have demonstrated its ability to mediate androgen signaling in the prostate, which has led to it being called an 'andromedin' [Lu et al., 1999]. Nile crocodile embryos treated with testosterone in ovo develop larger penises than controls [Ramaswami and Jacob, 1963], and American alligators collected from Florida lakes with high levels of endocrine disrupting chemicals (EDC) have smaller penises than animals living in uncontaminated environments [Guillette et al., 1996, 1999; Gunderson et al., 2004]. In light of earlier demonstrations that deletion of *Fgfr2* or *Fgf10* in mice causes feminization of male external genitalia, and that *Fgfr2* is a target of androgen signaling, the results presented here raise the possibility that EDC-induced downregulation of *Fgfr2* may be a mechanism by which environmental contaminants induce feminization of the phallus in crocodilian embryos.

External genital morphology is sexually dimorphic in hatchlings of some crocodilian species, such as the Aus-

tralian freshwater and saltwater crocodiles [Webb et al., 1984; Hutton, 1987; Lang and Andrews, 1994; Allsteadt and Lang, 1995; Ziegler and Olbort, 2007]. The penises and clitorises of other species, including the Indian gharial *(Gavialis gangeticus)* and the American alligator, historically have been considered relatively monomorphic [Webb et al., 1984; Hutton, 1987; Lang and Andrews, 1994; Allsteadt and Lang, 1995; Ziegler and Olbort, 2007]. However, more recent studies have identified subtle differences in size and shape of the hatchling penis and clitoris in species that were thought to have monomorphic external genitalia [Webb et al., 1984; Lang and Andrews, 1994; Allsteadt and Lang, 1995; Nuñez Otaño et al., 2010]. Morphometric analyses have demonstrated that the penis is significantly longer and more rounded (measured at the phallic ridge) than the clitoris in American alligator [Allsteadt and Lang, 1995] and broad-snouted caiman [Nuñez Otaño et al., 2010] hatchlings. In addition to sexual dimorphism, temperature-dependent intrasex differences in phallus shape have also been described; among male hatchlings, those incubated at higher temperatures have more rounded penises than lower-temperature males, and the same trend applies for hatchling clitorises [Allsteadt and Lang, 1995]. Thus, despite the bimodal nature of temperature-dependent sex determination, incubation temperature influences external genital morphology on a finer scale within an individual sex than between sexes. We found that the alligator phallic ridge undergoes morphological changes during the thermosensitive period; these changes could be mediated by incubation temperature independent of the gonads. Sex determination in flies is determined by chromosomes and is temperature-independent [Baker and Ridge, 1980]; however, development of abdominal pigmentation is sexually dimorphic and is thermosensitive [Gibert et al., 2007]. Transgenic experiments have identified a gene network that modulates both melanin production in females and development of sex combs in males [Gibert et al., 2007]. This raises the intriguing possibilities that sex determination could involve both genetic and thermosensitive mechanisms, and that thermosensitive genes could act on both local patterning and sex determination in reptiles. Testing of these hypotheses has the potential to elucidate some of the more subtle mechanisms by which morphogenesis of crocodilian external genitalia is influenced by the environment.

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