Developmental Basis of Phallus Reduction during Bird Evolution

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Summary

Background: One of the most puzzling events in evolution is the reduction and loss of the phallus in birds. All birds reproduce by internal fertilization, but only ~3% of birds have retained a phallic capable of intromission. A number of hypotheses have been proposed for the evolutionary mechanisms that drove phallus reduction; however, the underlying developmental mechanisms are unknown.

Results: We investigated genitourinary development in two sister clades of birds, Galliformes (land fowl), most of which lack an intromittent phallus, and Anseriformes (waterfowl), which have well developed phallus in some species these elongated coiled organs can exceed the length of the body [23]. These morphological changes led to changes in copulation strategies in different lineages. For example, male birds that lack a prominent intromittent organ (IO) transfer sperm by apposing the cloaca with that of the female, in a maneuver known as the "cloacal kiss," which requires cooperation of the female [10, 11]. By contrast, males with an IO can manipulate females and even forcibly copulate with unwilling females, a behavior that is well documented in waterfowl [10, 13, 24].

While selective pressures for elaborate phallus morphologies are obvious, it is less clear how selection could favor reduction or loss of an IO. A number of hypotheses have been proposed for the adaptive significance of genital reduction, although the precise nature of the evolutionary mechanisms is unclear due to the paucity of ecological or experimental data [10, 11, 19, 21, 22]. One of the most influential hypotheses for genital diversity, the "lock and key" hypothesis, argues that rapid evolution of genitalia facilitated speciation through breeding incompatibility (i.e., only males and females of the same species can fit their genitalia together) [7]. Although anatomical barriers to hybridization can in theory lead to reproductive isolation, experimental studies have failed to support the idea that strict mechanical incompatibility has been a driving force in the evolution of genital form [2, 6, 7]. Alternatively, Mayr proposed that genital morphology undergoes frequent changes due to pleiotropic effects [9]. It is unclear why neutral changes resulting from pleiotropy would affect genitalia disproportionately [2], although studies of the genetic control of genital development have identified a multitude of developmental mechanisms (including cis-acting DNA regulatory elements) that are shared by other organ systems [25–28], which raises the possibility that modulating gene

Introduction

Few morphological characters in the animal kingdom rival the diversity, complexity, and evolvability of male external genitalia [1]. Among animals with internal fertilization, genital morphology evolves rapidly, and the size, shape, and anatomical details of the external genitalia can show dramatic variation, even between closely related species [2]. The basis of this diversity has been the subject of debate, and a number of evolutionary mechanisms have been proposed to account for the rapid divergence of genital form [1–8].

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expression in structures such as limbs could have collateral effects on the genitalia [29] A third hypothesis argues that diversification of external genitalia has been driven by sexual selection acting on variation that affects sperm competition, sensory features of genital morphology, female choice, and sexually antagonistic coevolution (i.e., males and females evolve adaptations and counteradaptations to gain control over copulation and fertilization) [2, 6, 7, 12, 30].

At a developmental level, little is known about the molecular genetic mechanisms of external genital evolution. Recent progress in the developmental biology of mammalian external genitalia has led to the identification of genetic pathways that control outgrowth and patterning of the penis [28, 31–42]. These discoveries, together with independent advances in avian phylogenetics, reproductive ecology, and comparative morphology [12, 20, 21, 43], create a new opportunity to investigate the developmental genetic basis of genital evolution in birds. Here we report that the same mechanisms that regulate mammalian penis development also operate in bird external genitalia, and that the proliferative cues that direct genital outgrowth are conserved in chick (a galliform) and duck (an anseriform) embryos despite striking differences in their adult genital morphologies. Although early development of the genital tubercle (the precursor of the phallus) is conserved in chicks and ducks, we find that chicks have a unique domain of cell death distally. This domain of cell death is associated with ectopic activation of the gene encoding bone morphogenetic protein 4 (Bmp4), which is known to induce apoptosis in other organs. Experimental inactivation of Bmp signaling in chick embryos blocks apoptosis and prevents regression of the genital tubercle. Furthermore, ectopic application of Bmp protein to the distal tip of duck genital tubercles leads to a chick-like pattern of apoptosis in ducks. We then extended our study to the most basal group of birds, the Paleognathae, and to the sister group to the birds, the Crocodylia, both of which have intromittent phalluses. The results show that ducks retained the plesiomorphic pattern of Bmp4 expression and cell death, whereas chickens evolved a novel domain of Bmp-mediated cell death that causes regression of the phallus before hatching. Taken together, our results suggest that reduction of the intromittent phallus in galliform birds evolved not by disruption of outgrowth signals but by de novo activation of cell death in the developing genital tubercle.

Results

Chicks and Ducks Initiate Early Outgrowth of Genital Tubercles, but Chick Tubercles Arrest and Regress

Reanalysis of avian external genital morphology in light of recent genomic studies of bird interrelationships suggests that reduction of the phallus likely occurred in stem galliforms after their divergence from anseriforms (Figure 1A). Comparison of male genital morphology in two anseriforms, domestic graylag goose (Anser anser) and Pekin duck (Anas platyrhynchos), and in two galliforms, domestic chicken (Gallus gallus domesticus) and Old World quail (Coturnix coturnix), reveals striking differences (Figure 1B). Geese and ducks have elongated, coiled phalluses with dermal spines, whereas chickens and quails have only rudimentary phallic swellings on the anterior margin of the cloacal wall (Figure 1B). Although highly reduced, chicken and quail phallic swellings retain proximodistal and dorsoventral polarity and a ventral sulcus, suggesting that early genital patterning may be conserved in galliform embryos. To determine when these morphological differences arise, we used scanning electron microscopy to investigate genital development in a staged series of chick and duck embryos. External genital development begins at the same embryonic stage (stage 26) in chicks and ducks (Figures 1C and 1D). Despite the internal position of the phallus within the cloaca in adult birds, embryonic development of the genital tubercle occurs externally and resembles the mouse genital tubercle at early stages (Figures 1C–1H) [32]. Development of the phallus begins with the initiation of paired genital swellings between the anterior and posterior cloacal swellings (Figures 1C and 1D). By stage 28, the paired swellings have merged to form a single genital tubercle in both chicks and ducks (Figures 1E–1H). Outgrowth of the tubercle continues until stage 35 in chicks, when development arrests, but in ducks the phallus continues to grow and extend from the cloaca (Figures 1I–1L). In chicks at stage 45, the rudimentary phallus is visible only as a bulge on the inferior margin of the cloacal collar (Figure 1M). Ducks at the same stage have a pronounced phallus that begins to form the characteristic coiled pattern (Figure 1N). Thus, initiation and early outgrowth of the genital tubercle occurs in both chicks and ducks, but in chickens, outgrowth arrests and the tubercle then regresses within the cloaca.

Mechanisms of Genital Tubercle Outgrowth Are Retained in Chicks

The precocious arrest of chick phallus development suggested that the molecular mechanisms that regulate genital tubercle outgrowth are disrupted. In mice, sustained outgrowth of the genital tubercle requires Sonic hedgehog (Shh) and Hoxa13/d13 [28, 32–34, 38, 41, 45]. Given that loss of Shh or Hoxa13 and Hoxd13 function in mouse embryos results in arrest of genital tubercle outgrowth, which mimics the situation in chicks, we tested whether these pathways are disrupted in chick genital tubercles. Unexpectedly, strong expression patterns of Shh, Hoxa13, and Hoxd13 are well conserved in chick and duck genitalia, despite the failure of chick genital development to progress beyond the early bud stage (Figure 2). Shh is expressed along the ventral sulcus in chick and duck at stage 29 and persists through stage 35 (Figures 2A–2D). To determine whether mesenchymal cells respond to Shh, we examined Ptch1 and Ptch2, transcriptional targets of Hedgehog [47]. Both genes show strong expression in mesenchyme on either side of the sulcus as late as stage 35 (Figures 2E–2L), indicating activation of the Hedgehog pathway. Hoxd13 and Hoxa13 are expressed throughout the genital tubercles of both chick and duck embryos at stage 29 (Figures 2M and 2N, 2O, and 2R), and expression weakens in both species by stage 35 (Figures 2O and 2P, 2S, and 2T). Thus, despite the extreme reduction of the galliform phallus, the mechanisms known to direct early outgrowth of the genital tubercle have been retained in chicks.

The similar expression patterns of outgrowth-promoting genes in chick and duck genitalia led us to investigate whether the precocious arrest of genital development in chicks involves a failure of genital tubercle cells to respond to proliferative cues. Quantitative analysis of cell proliferation in chick and duck genital tubercle mesenchyme revealed no significant differences (p = 0.6) in their mitotic indices, despite the obvious differences in outgrowth at stage 35 (see Figure S1 available online). These results indicate that chick genital mesenchyme exhibits a normal response to the growth-promoting genes described above.
Increased Apoptosis Is a Synapomorphic Character of Galliform Genital Tubercles

Appendage outgrowth requires a balance between cell proliferation and cell death, and truncation can result from either decreased proliferation or increased cell death [33]. To determine whether increased apoptosis could underlie the arrest of chick genital development, we compared patterns of cell death in the genital tubercles of galliform, anseriform, paleognath, and crocodilian embryos. LysoTracker Red staining shows that chicks and ducks have similar patterns of apoptosis at stage 29, with labeled cells along the sulcus in both species (Figure S2), in a pattern similar to that reported for E11.5 mice [32]. Comparison at stage 36, however, revealed marked differences: chick genital tubercles showed widespread apoptosis throughout the distal mesenchyme and along the sulcus, whereas duck genitalia had few labeled cells in the sulcus or distal region of the tubercle (Figures 3A and 3C). To determine whether the differences in apoptosis between chick and duck genital tubercles are conserved features of Galliformes and Anseriformes, respectively, we examined additional members of each clade. Quail genital tubercles at stage 29 showed an apoptotic pattern similar to that seen in chick, whereas goose genitalia at this stage showed an apoptotic pattern resembling that of duck (Figure S2). By stage 36, quail genital tubercles showed extensive apoptosis throughout the distal mesenchyme and sulcus, whereas goose genital tubercles exhibited very low levels only along the sulcus (Figures 3B and 3D). These results indicate that the galliforms examined here (chicken and quail) underwent a late wave of apoptosis in the distal genital tubercle, which coincides temporally with the arrest of outgrowth and onset of regression.

If the evolutionary reduction of galliform external genitalia was driven by increased apoptosis, then the pattern of cell death observed in chick and quail should be a synapomorphic (shared derived) character, whereas the duck and goose pattern should be plesiomorphic (ancestral). We tested this hypothesis by extending our study to the most basal clade of birds, the Palaeognathae, and to the sister group to birds, the Crocodilia. Analysis of the emu, a paleognath, showed that there is low-level cell death along the sulcus at stage 36, as seen in ducks and geese (Figure 3E). In comparably staged alligator embryos, the genital tubercles show even fewer apoptotic cells than in emus, ducks, and geese (Figure 3F).
Thus, the wave of cell death in the distal genital tubercle appears to be a synapomorphy of galliforms.

**Chicken Genital Tubercles Have a Derived Pattern of Bmp4 Expression**

The finding that regression of galliform genital tubercles is associated with apoptosis, rather than loss of an outgrowth signal, raised new questions about the molecular mechanism responsible for the distal cell death. Bone morphogenetic proteins (Bmps) have been shown to induce apoptosis in a number of developmental contexts, including the external genitalia of mice [31, 48–50]. To determine whether endogenous differences in Bmp activity could underlie the differences in apoptosis between galliform and anseriform genitalia, we compared expression of Bmp2, Bmp4, and Bmp7 (ligands for BmpR1a, which controls apoptosis of the mouse genital tubercle [31]) in chick and duck embryos. Chicks and ducks showed different patterns of Bmp4 and Bmp7 expression at stage 29 (Figures 3G–3P), before differences in phallus development could be detected (Figures 1I and 1J). In chicks, strong expression of Bmp4 was detected along the entire proximodistal axis of the genital tubercle (Figure 3G), whereas in duck genitalia, Bmp4 was expressed in the cloaca and at the base of the genital tubercle, but not at the distal tip (Figure 3H). Bmp7 also showed strong expression along the chick genital tubercle at stage 29, particularly at the distal tip, but comparatively little expression was seen in duck genitalia (Figures 3I and 3J).

At stage 35, Bmp4 continued to be expressed distally in chicks only (Figures 3G’–3H’). By this stage, Bmp7 and Bmp2 expression had diminished in both chick and ducks (Figures 3I’–3L’). To quantify levels of Bmp4 expression in chick and duck genital tubercles, we used quantitative real-time PCR (qRT-PCR) and found that chick genitalia have significantly higher levels of Bmp4 mRNA at stage 29 (>3-fold higher) and stage 35 (>2-fold higher; Figure 3Q). The sustained expression of Bmp4 in chick genitalia led us to ask whether Msx1 and Msx2, downstream effectors of Bmp signaling [48], show differential activity in chick and duck genital tubercles. At stages 29 and 35, Msx1 and Msx2 were expressed distally in chick genitalia (Figures 3M, 3M’, 3O, and 3O’), whereas the region of apoptosis occurs (Figure 3A), but neither gene could be detected in duck genitalia (Figures 3N, 3N, 3P, and 3P’). These findings show that distal activation of the Bmp4 pathway coincides spatially and temporally with the wave of cell death that occurs in galliform external genitalia.

The results described above raised the possibility that galliforms evolved a new domain of Bmp4 (and apoptosis) in their distal genital tubercles, but comparison of duck and chick genital development could not exclude the possibility that distal Bmp4 expression is a primitive character that was lost in anseriforms. Therefore, we examined Bmp4 expression in the emu, a basal bird with a well-developed phallus that does not undergo widespread distal apoptosis (Figure 3E). In emu embryos at stage 29, Bmp4 showed a duck-like pattern of expression: Bmp4 expression was detected in the cloacal collar and interdigitally in the hindlimbs, but not at the distal tip of the genital tubercle (Figures 3R and 3S). Taken together with our phylogenetic survey of apoptosis across archosaur (avian + crocodylian) external genitalia (Figures 3A–3F), these results indicate that Bmp4 expression and apoptosis in the distal genital tubercle evolved in the galliform lineage after its divergence from the anseriforms.

**Inhibition of Bmp Signaling Rescues Apoptosis and Prevents Regression of Chick Genital Tubercles**

Having established the relative timing of the evolutionary acquisition of distal Bmp4 expression, we went on to perform functional experiments to test the hypothesis that cell death in galliform external genitalia is caused by Bmp signaling. If this hypothesis is correct, then antagonism of Bmp activity should rescue cells from apoptosis. We applied beads soaked in Noggin protein (0.275 μg/μl), which binds Bmps and blocks their interaction with Bmp receptors [51], to one side of chick genital tubercles at stage 26. Noggin beads resulted in local inhibition of apoptosis within 24 hr, whereas the contralateral side showed no change in apoptosis (Figure 4A). Chick tubercles receiving control beads (soaked in PBS) showed no change in apoptosis on either side (Figure 4B). To confirm the specificity of Noggin treatments, we monitored expression of the Bmp target gene Msx1. On the Noggin-treated side of the genital tubercle, Msx1 was downregulated within 24 hr (the contralateral side, by contrast, continued to show strong expression of Msx1), indicating that Bmp signaling had been inactivated (Figure 4C). PBS control beads did not affect Msx1 expression (Figure 4D).

Interestingly, the distal tip of the genital tubercle appeared to extend further on the Noggin-treated side than on the contralateral side (Figure 4C), suggesting that increased cell survival resulted in sustained outgrowth. We therefore quantified proximodistal outgrowth of the bead side versus the contralateral side 24 hr after implantation of either a Noggin bead or PBS control bead. The difference between the bead side and contralateral side was ~6.5 times greater in [Figure 5].
Noggin-treated tubercles (100.8 μm) than in control tubercles (15.6 μm; p = 0.01; Figure 4E). Thus, antagonism of Bmp signaling in the chick genital tubercle resulted in significantly increased outgrowth. These results indicate that Bmp function is required for apoptosis and the arrest of outgrowth in chick genital tubercles.

The results described above led us to ask whether, during the evolution of Galloanserae, activation of Bmp signaling could have been sufficient to induce apoptosis in a genital tubercle fated to form an intromittent phallus. We tested this hypothesis by applying beads loaded with Bmp2 protein (a paralog of Bmp4 that also signals through BmpR1a [52]) to the genital tubercles of duck embryos at stage 26, the stage when Bmp is required for apoptosis in chicks. Eight hours after application of a Bmp2 bead, duck genital tubercles showed ectopic apoptosis on the treated side of the tubercle, whereas application of control beads had no effect on cell death (Figure S3). Taken together, these results show that distal Bmp activity is both necessary and sufficient for apoptosis and regression of the phallus (Figure 4F).
Discussion

During the evolution of birds, an intromittent phallus was reduced or lost in multiple lineages, including at least once in galliforms and again in the common ancestor of neoaves [19, 21, 22]. Consequently, ∼97% of extant bird species lack an intromittent phallus [10, 11, 22]. Our comparative study of external genital development in birds shows conservation of the signals that promote initiation and early outgrowth of the genital tubercle across galliform, anseriform, and paleognath lineages, but our data from chick embryos suggest that galliforms are unique in their expression of Bmp4 in the distal genital tubercle. Experimental manipulations of Bmp signaling in chick and duck embryos show that this derived domain of Bmp activity is necessary and sufficient for the wave of distal apoptosis that causes regression of the genital tubercle in galliforms. The results presented above suggest that a new region of Bmp4 expression arose in the galliform lineage after its divergence from anseriforms, and that this resulted in a domain of apoptosis that arrests genital outgrowth and causes regression of the phallus.

Evolution of Phallus Reduction

The marked divergence of genital morphology between anseriforms and galliforms raises questions about the selective pressures that led to phallic reduction. Loss of intromittent organs is an evolutionary paradox: How can reduction of a structure that facilitates internal fertilization have a positive effect on reproductive fitness? It is unlikely that reduction of the phallus resulted in improved delivery of sperm. Darwin first proposed that variable characters with no apparent adaptive value were not subject to natural selection, but that these traits...
could be stabilized in a population if they confer an advantage “over other individuals of the same sex and species, in exclusive relation to reproduction” [53]. Copulation with males lacking an intromittent phallus requires female cooperation, such as presentation of the cloaca and eversion of the vagina [10, 11]. Accordingly, by selecting males with reduced or nonintromittent phalluses, females can control paternity, which is consistent with the idea that female choice played a role in reduction and loss of the phallus [10, 11, 14, 19, 22, 43].

An alternative to the sexual selection hypothesis is that reduction of phallus size may have been a secondary or pleiotropic effect of developmental changes to other characters [9]. The molecular mechanisms of appendage development have been deeply conserved during animal evolution, and the gene networks that regulate development of external genitalia also control formation of a wide range of other structures, including limbs, gut, nervous system, muscle, and integumentary appendages such as feathers and scales. Moreover, tissue-specific expression of a number of genes expressed in the genital tubercle, including Bmp4, Mx2, and Hoxd13, has been shown to be controlled by enhancers that also direct transcription in other regions of the embryo. In mouse, an evolutionarily conserved enhancer located more than 45 kb 5’ to the Bmp4 promoter can drive expression in the genital tubercle, digit tips, dorsal root ganglia, and whisker hairs [25]. Similarly, Mx2 expression in the genital tubercle ectoderm and in the apical ectodermal ridge of the limb buds is regulated by a shared cis-regulatory element [27], as is Hoxd13 expression in the digits and the genital tubercle [54].

In the latter example, the length of the digits and the penis scale together in response to changes in the quantity of Hoxd13 expression [28]. Therefore, modulation of gene regulation in other organ systems has the potential to have pleiotropic effects on gene expression in the genital tubercle (and vice versa). However, even if reduction of the intromittent phallus arose due to pleiotropic effects of Bmp4 regulatory evolution in another cell population and therefore was not the primary target of selection, persistence of a reduced phallus in galliforms would have required stabilizing selection. Thus, we propose that regression of the intromittent phallus in galliforms arose due to acquisition of a novel domain of Bmp4 expression in the distal region of the genital tubercle, and that stabilization of this domain may have been due to selection for males with small or nonintromittent phalluses.

A Developmental Mechanism for Reduction of the Phallus in Galliform Birds

Outgrowth of appendages generally requires induction and maintenance of cell proliferation as well as attenuation of apoptosis in order to keep cell death below the rate of cell proliferation. Regionalized regulation of cell death plays an important role in morphological sculpting of appendages, influencing processes and traits such as digit differentiation, joint cavitation, removal of interdigital webbing, urethra formation, and limb and genital length. Activation of apoptosis by Bmp signaling has been implicated in a number of evolutionary reductions, such as loss of teeth in birds [55] and reduction of forewings in soldier ants [56]. Reciprocally, inhibition of the apoptotic activity of Bmps through upregulation of Bmp antagonists (e.g., Gremlin) and/or survival cues (e.g., Fgf8) has been implicated in other evolutionary innovations, such as retention of interdigital webbing in bat wings. Our analysis of signals known to promote outgrowth of the mouse genital tubercle, including Shh and Hoxd13a/a13, revealed surprising conservation between galliforms and anseriforms, suggesting that galliforms have not lost the mechanisms for external genital development. Indeed, quantification of cell division in chick and duck genital tubercles showed that there is no significant difference in cell proliferation. This is consistent with our previous report that, in the mouse, Shh promotes outgrowth of the genital tubercle by regulating cell-cycle kinetics [33]. By contrast, galliforms have a derived pattern of apoptosis at the distal tip of the genital tubercle, and this coincides with a domain of Bmp4 expression not detected in anseriforms or paleognaths. These results indicate that the distal expression of Bmp4 and the resulting increase in apoptosis in the distal region of the genital tubercle are a synapomorphic characteristic of galliforms.

The ability of the Bmp antagonist Noggin to prevent cell death and to sustain outgrowth of galliform genital tubercles provides a causal link between the distal domain of Bmp signaling and apoptotic regression of the phallus. Furthermore, our finding that a distal wave of apoptosis can be induced by experimental activation of Bmp signaling at the distal tip of duck genital tubercles demonstrates that Bmp activity alone is sufficient to induce cell death and regression of a genital tubercle that otherwise is fated to form an elongated phallus. Thus, Bmp signaling in the distal genital tubercle of Gallus can both induce and control apoptosis in the phallus during embryonic development. These findings are consistent with studies of Bmp function in mouse external genital development. Deletion of Bmpr1A results in reduced cell death in the mouse genital tubercle, which leads to hyperplasia of the penis [31]. Moreover, Noggin knockout mice have excessive Bmp signaling, which causes reduction of the penis [31]. Taken together, our findings support the hypothesis that evolutionary reduction of an intromittent phallus in galliform birds resulted from the acquisition of a new distal domain of Bmp activity in the developing genital tubercle.

Interestingly, cracids are an exception to the galliform rule in that they have a fully intromittent phallus (although not as pronounced as in anseriforms [21]). In light of current phylogeny [20], cracids may have re-evolved an intromittent phallus (Figure 1A), which would suggest that galliforms have retained the competence to redevelop a phallus from a rudimentary genital organ. Our findings that (1) the molecular machinery for genital tubercle outgrowth has not been disrupted in chicks, (2) regression of the phallus is caused by Bmp-mediated apoptosis, and (3) antagonism of Bmp signaling is sufficient to block apoptosis and rescue outgrowth raise the possibility that modulating Bmp activity may have been sufficient for reemergence of an intromittent phallus.

Whether the same developmental mechanism that we identified in galliforms also played a role in the independent reductions of the phallus in neoaves remains to be tested. Comparative studies of birds have the potential to identify the developmental mechanisms responsible for a number of other major morphological transitions, such as how genital outgrowth is sustained for such an extensive period in waterfowl, how coiling of the penis and vagina is regulated, what determines the direction of the coiling, and how the microanatomy of penile spines and other dermal ornamentation is specified in birds.

Bmp Regulation and the Evolution of Bird Morphology

Modulation of Bmp expression has been implicated in evolution of at least four morphological innovations in birds: feathers [57], toothlessness [55], beak shape [58, 59], and,
now, phallic reduction. It is interesting that the phallus is one of several avian structures, including teeth and the most anterior and posterior digits, that undergo partial development during embryogenesis and then regress. It is possible that this also reflects gene regulatory elements that are shared among multiple organ systems. If transcription of a gene in a developing organ is regulated by an enhancer that also regulates gene expression in the progenitors of an organ that has been lost during evolution, then that early phase of expression (and perhaps an early phase of development) could be maintained in both cell populations. Such conservation of gene regulatory mechanisms may underlie the occasional reemergence of structures that have been lost during evolution.

Experimental Procedures

Embryo Collection
Fertilized chicken, quail, duck, goose, and emu eggs were obtained from commercial suppliers, and alligator eggs were obtained from the Rockefeller National Wildlife Refuge. Chicken, quail, goose, and duck eggs were incubated in a humidified incubator at 38 °C, and emu eggs were incubated at 36.4 °C. Stages of development were determined using the Hamburger-Hamilton staging series for comparison of multiple anatomical landmarks [44]. Alligator eggs were incubated at 33 °C to induce male development.

Scanning Electron Microscopy
Genital tubercles were dissected in PBS, fixed in 1% glutaraldehyde in PBS, and stored at 4 °C. Specimens were then critical-point dried, mounted on metal studs, sputter coated with gold particles, and imaged on a Hitachi S4000 scanning electron microscope.

RNA Isolation, cDNA Synthesis, and RT-PCR
Genital tubercles were dissected from staged embryos and stored in RNAlater. Tissue samples were collected from each embryo for genomic DNA extraction and genotyping using sex-specific primers [60]. Male tubercles were pooled and RNA was extracted using a RNeasy Mini Kit (Qiagen). RNA quantity and purity were determined using a NanoDrop ND-1000. For each pooled sample, cDNA was synthesized from 2 μg of total RNA according to manufacturer’s specifications (Bio-Rad). Total RT-PCR reactions were performed using SYBR Green qPCR Master Mix (Bio-Rad). The primers used for RT-PCR were as follows: Bmp4 (F 5'-AATCCACACCAACACGCTCATC-3', R 5'-AGGACACCCCTTGACTATCAT-3'), chick β-actin (F 5'-TGATGACTCTGGTATGTGTTAC-3', R 5'-CTCTCGGTGTTGTGGTAGAG-3'), duck β-actin (F 5'-GCTCCCTGTCACCTCCTC-3', R 5'-GCTGCTGATACCTTCACCATTCC-3'). Relative level of expression was calculated using the ΔΔ Ct method.

In Situ Hybridization
Embryos harvested for in situ hybridization were fixed in 4% paraformaldehyde (PFA) at 4 °C overnight and dehydrated in a graded methanol series. Whole-mount RNA in situ hybridization was performed as described by Nieto et al. [61] with the following modifications: Proteinase K concentration was increased to 70 μg/ml, following the protocol of Laufert et al. [62]; KBTB buffer contained 1% Triton X-100; alkaline phosphatase buffer (NBT/BCIP Kit Elite; Vector Labs) according to the manufacturer’s instructions. Cell counts were made by positioning a counting box (6,670 mm2) in each embryo. Significance was determined by ANOVA.

Detection of Apoptotic Cells
Apoptotic cells were localized using LysoTracker Red (Molecular Probes/Invitrogen). Embryos were rinsed briefly in PBS and incubated in LysoTracker Red (1:200) in PBS at 37°C for 30 min and then washed briefly in PBS before fixation in 4% PFA. Samples were rinsed in methanol to remove background, viewed and photographed under epifluorescence, and then photographed again in the same plane of focus with bright-field illumination.

References
Evolutionary Reduction of the Penis in Birds


