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Variation in Penile and Clitoral Morphology in Four Species of Moles

By

Adriane Watkins Sinclair

A dissertation submitted in partial satisfaction of the

requirements for the degree of

Doctor of Philosophy

in

Integrative Biology

in the

Graduate Division

of the

University of California, Berkeley

Committee in charge:

Professor Stephen E. Glickman, Chair

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Professor Gerald R. Cunha

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Spring 2014

Abstract

Variation in Penile and Clitoral Morphology in Four Species of Moles

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Adriane Watkins Sinclair

Doctor of Philosophy in Integrative Biology

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Most eutherian mammals possess sexually dimorphic external genitalia. Males have a penis that is traversed to near the tip by a urethra, a scrotum that encloses the testes, and a long anogenital distance. In females anogenital distance is short, and the typical clitoris is usually markedly smaller than the penis, and is frequently "internally" situated with the urethra exiting independent of the clitoris. In addition, the clitoris is associated with an externally visible vaginal opening (at least during the breeding season). This sexual dimorphism is usually associated with the presence (males) or absence (females) of androgens during development of the external genitalia. Females with naturally "masculinized" external genitalia challenge the typical mammalian androgen-dependent masculinization theory and are the focus of this dissertation. Research as to how they "do," or "do-not" fit the current widely accepted theory of sexual differentiation may reveal novel mechanisms of sexual differentiation.

Since adult external genitalia are the endpoints of sexual differentiation, developmental processes can be inferred from examination of adult morphology, providing that anatomy of external genitalia has been (is) accurately described and interpreted. The first revelation incurred in my study was that all previous reports on mole external genitalia were in error in regard to the following terms: penis, clitoris, penile clitoris, phallus, prepuce and urethra. Accordingly, by way of correcting errors of previous literature, my first task was a detailed anatomic and morphometric analysis of external genitalia in four species of moles. For an even broader perspective I also reviewed morphology of external genitalia of mouse, human and spotted hyena. Accurate morphological descriptions of external genitalia are the essential pre-requisite to a full understanding of the comparative anatomy of mole external genitalia and the potential role of hormones in development of the external genitalia in these species.

This dissertation is focused on four species of moles. All are members of the family Talpidae, in the order Insectivora. Three of these species defy the conventionally obvious visual distinctions between males and females in so far as the perineal appendage described previously as "penis" and "penile clitoris" is of similar size in males and females. Unfortunately, the visible perineal appendage in these male and female moles is prepuce. A major question is why the prepuce is similar in size in males and females. One theory is that the ovarian interstitial gland is capable of producing androgen. Of the three mole species with "masculinized" female external genitalia, broad-footed moles (*Scapanus latimanus*) do not possess an ovarian interstitial gland, star-nosed moles (*Condylura cristata*) have an ovarian interstitial gland, and in hairy-tailed moles (*Parascalops breweri*) ovarian structure has never been investigated. For these three mole species, it is difficult for the casual observer to distinguish between males and females during the non-breeding season, when the vaginal opening of female moles is "closed." The fourth species examined in the present investigation is the Japanese shrew mole (*Urotrichus talpoides*), which does not possess an ovarian interstitial gland. In Japanese shrew moles, the distinction between males and females is obvious, since the male prepuce is much larger than the female prepuce, and the latter does not have a urethra exiting its tip. Thus, sexual dimorphism of the external genitalia in Japanese shrew moles appears to follow the typical mammalian pattern.

Intriguing work has been done on mole species concerning the ovarian interstitial gland, its resemblance to testicular tissue, and its ability to produce androgens in relation to the corresponding presence of a "penile clitoris" in some mole species. However, other mole species that do not possess this ovarian interstitial gland also display a "penile clitoris" that is similar in size and shape to male external genitalia. Of course, this erroneous discussion of "penile clitoris" from the literature actually deals with the female prepuce. Discussion of the role of the ovarian interstitial gland in masculinization of mole external genitalia, first and foremost requires a detailed accurate anatomic analysis of the structure of the so-called "penile clitoris". Unfortunately no comparative work has adequately addressed the role of androgens derived from the ovarian interstitial gland in development of external genitalia in the different mole species. For the first time, my accurate detailed description of mole genital morphology provides the opportunity to address this question.

The penis and clitoris of typical mammals are strikingly different, anatomically complex organs composed of epithelial tissue, connective tissue, vascular tissue, nerves, cartilage, and bone that are organized into specific and precise morphological patterns. The common developmental history, architecture, and composition of the penis and clitoris across most mammalian species allow for multiple features to be used to assess sexual dimorphism of the various components that constitute male and female external genitalia. The size and location of several key anatomic features were noted with the aid of three-dimensional reconstructions for a more detailed comparison between the different mole species. In addition to these internal anatomical measurements, anogenital distance, a trait that is modulated by androgen action *in utero*, was used as another measure of "genital masculinization". Measures of prepuce length, termed "phallus" length in previous publications, were used to investigate the degree of sexual dimorphism in the external genitalia in my study. Ovarian tissue was examined histologically for the presence or absence of an interstitial gland (a potential source of androgen). I discovered in the breeding season, Japanese shrew moles display a large glandular tissue structure attached to the ovary that has never before been reported. The Japanese shrew mole penis is much larger

than the clitoris, is vastly anatomically different from the clitoris, and males have a longer anogenital distance than females presenting a typical mammalian pattern of sexual dimorphism, presumably based upon the presence versus absence of adequate androgen levels. Broad-footed, star-nosed, and hairy-tailed moles have notable morphological variation in the penis and clitoris between these species as well as between the sexes. However, similar to star-nosed moles (that possess an ovarian interstitial gland), female broad-footed moles displayed several masculine morphological characteristics and an anogenital distance equal to the males' despite lacking an ovarian interstitial gland. This suggests that either development of the external genitalia is partially androgen-independent in these species or in females androgen production may be coming from another source. Lastly, I compared different patterns of external genitalia in human, mouse, 4 species of mole, and spotted hyena in relation to known endocrine profiles, and mechanisms of morphogenesis/differentiation noting gaps in the data.

In summary, my research provides the first accurate descriptions of the gross and histologic anatomy of male and female mole external genitalia. Also, comparative mouse-mole studies reported here have validated the remarkable similarity in external genitalia anatomy between these two species and have led to the conclusion that all previous literature on mole external genitalia suffers from consistent anatomical error. Being able to set the record straight allows for the first time an accurate definition of mole external genitalia anatomy and its relation to endocrine parameters.

This work is dedicated to:

Dr. Jim Sinclair,

Your passion for science inspired me, your love and support kept me going.

Candace Sinclair,

Your tireless belief in me has repeatedly lifted me up and taken me forward, thanks for never letting me fall.

Kirstin Sinclair-Kollasch,

My lifelong playmate, supporter, and sister with the biggest heart.

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I want to thank Dr. Gerald Cunha and Dr. Laurence Baskin for giving me a job in a great lab even though the degree wasn't yet achieved so I could continue my passion for research while writing the dissertation. And thank you Dr. Cunha for the endless hours of reading and editing this document, I've learned a lot from you.

My family, you cheered me on no matter what, thank you for always being there for me.

Chapter 1: "Masculinization" in Female Moles

A. Introduction:

In 1935, L.H. Matthews described the unusual "penile clitoris" of the European mole (*Talpa europaea*), and speculated that the morphology of this organ was dependent upon secretion of androgen from an ovarian interstitial gland during critical stages of development (Matthews, 1935). Female European moles displayed a traditional follicular ovary during the breeding season, but the ovarian interstitial gland, with histological characteristics reminiscent of testicular tissue, displayed a marked expansion in size in the adult female mole during the non-breeding season. Matthews did not have the opportunity to observe embryonic tissues in these moles. However, on the basis of the maintenance of rudimentary epididymes in female European moles, Matthews hypothesized that androgenic ovarian interstitial tissue was present during fetal life, as well as in adult females, and that presence of such tissues correlated with the unusual "masculinization" of the "clitoris" in European moles. Matthews' suggestion anticipated the Jost model, as described below, by more than a decade.

Whitworth *et al.* subsequently confirmed Matthews' speculation regarding the potential for androgenic secretion by the ovarian interstitial gland (Whitworth et al., 1999). They discovered that significantly higher levels of testosterone were produced by the ovarian interstitial gland during the non-breeding season than the ovarian portion through *in vitro* incubation of the separated ovarian and ovarian interstitial gland portions. Thus, both the presence of a "penile clitoris" and the simultaneous occurrence of this unusual ovarian structure, were extended to a variety of moles within the family Talpidae (Rubenstein et al., 2003; Zurita et al., 2003; Carmona et al., 2008). However, in 2003 Rubenstein *et al.* reported that although several North American mole species, including the star-nosed mole (*Condylura cristata*) and the shrew-mole (*Neurotichus gibbsii*), displayed both an ovarian interstitial gland and a "penile clitoris", there were several additional North American mole species that possessed a "penile clitoris" in the absence of an ovarian interstitial gland i.e., broad-footed moles (*Scapanus latimanus*) and Pacific coast moles (*Scapanus orarius*) (Rubenstein et al., 2003).Therefore, at this time, there is an apparent paradox in the literature: that broad-footed moles and Pacific coast moles possess a "penile clitoris", in the absence of a structure that could account for the secretion of androgen. In addition, although Wood (1914) published a limited array of cross-sections through the "clitoris" of the European mole (Wood-Jones, 1914), and Rubenstein *et al.* presented preliminary cross-sections through the "penis" and "clitoris" of the broad-footed mole (Rubenstein et al., 2003), there has been no systematic analysis of external genitalia morphology, and no systematic comparison of penile and clitoral morphology in any mole species. With the rare exceptions just noted, mole researchers have merely noted the presence of a large "clitoris," with an opening for the passage of urine at the tip. Unfortunately, all previous research on mole external genitalia has consistently suffered from a complete misconception of the anatomy of external genitalia. In a broader sense this emerged from generally inadequate and inaccurate descriptions of external genitalia morphology in several species, especially in "rodent-like" animals (mouse, rat, and moles). Thus, earlier literature on the mole external genitalia is incorrect with regard to the basic definitions of "penis", "clitoris", "penile clitoris", prepuce, preputial space and urethra. Accordingly, the present dissertation will accurately define external genitalia morphology in mice and in moles and discuss potential sources of androgen in three

species of North American moles: broad-footed moles, star-nosed moles, and hairy-tailed moles (*Parascalops breweri*), as well as the Japanese shrew mole (*Urotrichus talpoides*).

Note that in the discussion above the terms, "clitoris", "penis" and "penile-clitoris" are denoted with quotation marks. This convention will be used throughout this dissertation to denote the erroneous identification of these structures by virtually all previous investigations of external genitalia in moles. Accordingly, a major focus of this dissertation is to provide accurate definitions for the mole penis, phallus, clitoris, prepuce, preputial space and urethra so that these structures can the correctly assessed in moles and other species and related to relevant endocrine parameters.

B. Previous Misconceptions of External genitalia Anatomy:

Confusion regarding the anatomy of external genitalia of moles is a symptom of general imprecision more broadly in external genitalia of other species as well. For example, earlier descriptions of mouse external genitalia are inaccurate. Indeed, an illustration from "The Anatomy of the Laboratory Mouse" website (http://www.informatics.jax.org/cookbook/figures/figure9.shtml) shows a drawing of the adult mouse perineum in which the prominent perineal elevation is labeled penis (Fig. 1.1).
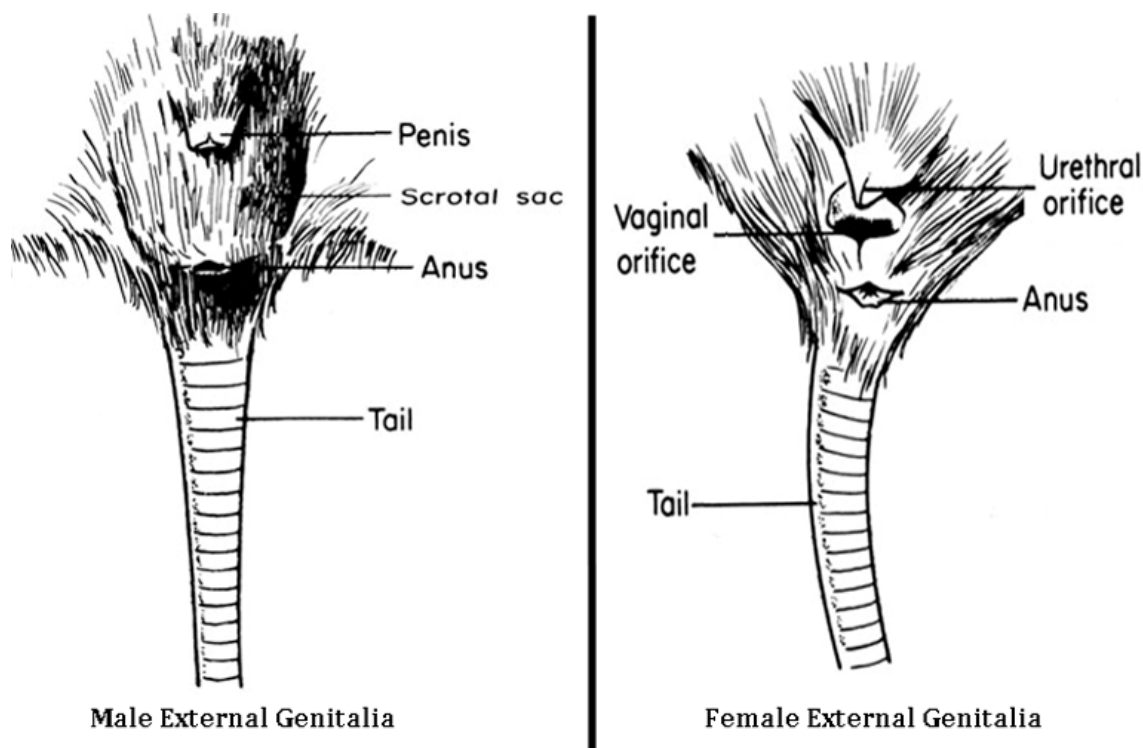
Figure 1.1 Illustrations of mouse external genitalia from the website "The Anatomy of the Laboratory Mouse". Note in the drawing of the male external genitalia (left) that the perineal elevation is incorrectly labeled penis. The drawing of the female external genitalia (right) correctly labels the urethral orifice, but fails to indicate that the perineal elevation in the female is the prepuce.

This is incorrect. The perineal elevation is the prepuce. The mouse and mole penis are for the most part an "internal organ" that only projects beyond the prepuce during urination and mating. Figure 1.2 is a reasonably accurate depiction of the anatomical relationship of the mouse penis to the prepuce. Another example of the imprecision of earlier literature of mouse external genitalia anatomy can be found in Figure 1.3A. Note in Murakami's figure 1.3A that the mouse penis is depicted as having a urethra opening near the tip of a blunt glans penis (Murakami, 1987). Figure 1.3B is a scanning electron micrograph of the adult mouse penis. Note that the mouse penis is not blunt distally, but instead has a prominent distal projection called the male urogenital mating protuberance (MUMP), and that the urethra does not open at the tip of the penis, but instead opens approximately 1mm from the distal tip of the adult mouse penis (Yang et al., 2010; Rodriguez et al., 2012; Weiss et al., 2012; Blaschko et al., 2013). Recent studies from the Baskin laboratory, with which I have been associated for over a year, have for the first time provided accurate comprehensive descriptions of mouse external genitalia (Yang et al., 2010; Rodriguez et al., 2012; Weiss et al., 2012; Blaschko et al., 2013). First and foremost, my investigation of mole external genitalia will begin with a description of external genitalia of a prototypic rodent/insectivore-like animal, namely the mouse, as external genitalia anatomy of the mouse serves as the most detailed and accurate model of rodent/insectivore external genitalia.
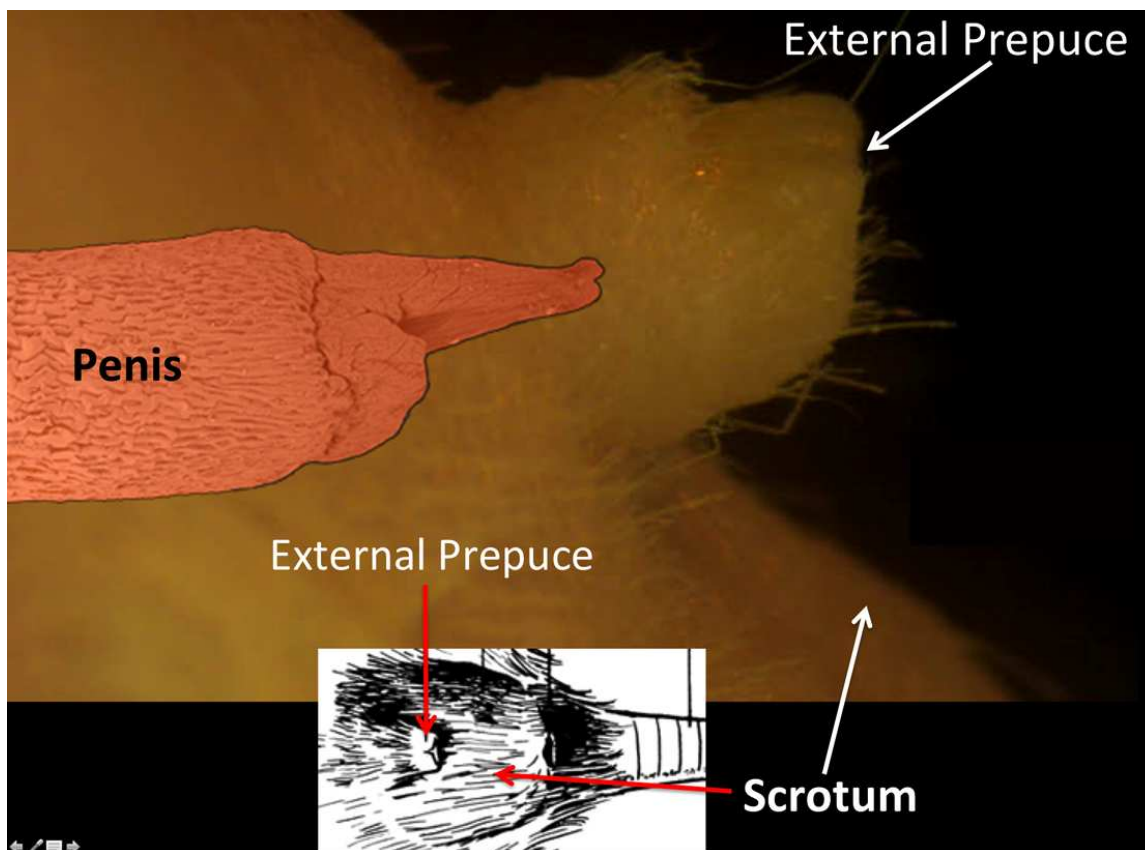
Figure 1.2. The overall photo is a side view of the adult male mouse prepuce (labeled External Prepuce) with a colorized scanning electron micrograph of the penis superimposed in roughly the correct position to illustrate that the penis is an "internal organ". The inset below shows a drawing of the mouse external genitalia correctly labeled.

Fig. 1. Schematic illustration of the development of the penis of normal and *Tfm* mice. Large dots, erectile tissue; small dots, condensed mesenchymal cells; cross-hatching, glans penis or clitoridis; *C*, corpus cavernosum penis; *CG*, corpus cavernosum glandis; *CU*, corpus cavernosum urethrae; *D*, distal segment of the os penis; *H*, hyaline cartilage; *M*, membrane bone; *P*, proximal segment of the os penis; *PG*, preputial gland; *PP*, prepuce; *U*, urethra.

Figure 1.3. (A) Murakami's diagram of the adult mouse penis (Murakami, 1987). Note in (A) that the mouse penis is depicted as blunt distally with the urethra opening near the distal tip. (B) SEM of adult mouse penis. Note the MUMP projecting distally. The urethral meatus (red arrow) forms by closure of the ventral cleft in the MUMP ridge ~1mm from the tip of the penis. Note spines on the surface of the penis.

C. Definition of External Genitalia Anatomy in Mice and Moles:

Through comparison of the anatomy of mouse and mole external genitalia, it is possible to precisely and accurately define the various components of that constitute the external genitalia of these species and thus revise the misconceptions of the mole external genitalia literature. Accordingly, in this section I propose a logical and verifiable anatomy of the penis, clitoris, phallus, "penile clitoris", prepuce, preputial space and urethra and in so doing describe the key features that distinguish each of these structures. The following terms have been used incorrectly in the mole external genitalia literature: penis, phallus, clitoris, prepuce, preputial space and urethra as indicated in Table 1.1.

Table 1.1. Summary of correct and mis-used/incorrect anatomical terms used by investigators of mole external genitalia.

Incorrect Mole Terminology	Correct Terminology
"Penis"	Prepuce
"Clitoris"	Prepuce
"Penile Clitoris"	Prepuce
"Phallus"	Prepuce
Female Urethra	Female Urethra and Preputial space
Male Urethra	Male Urethra and Preputial space
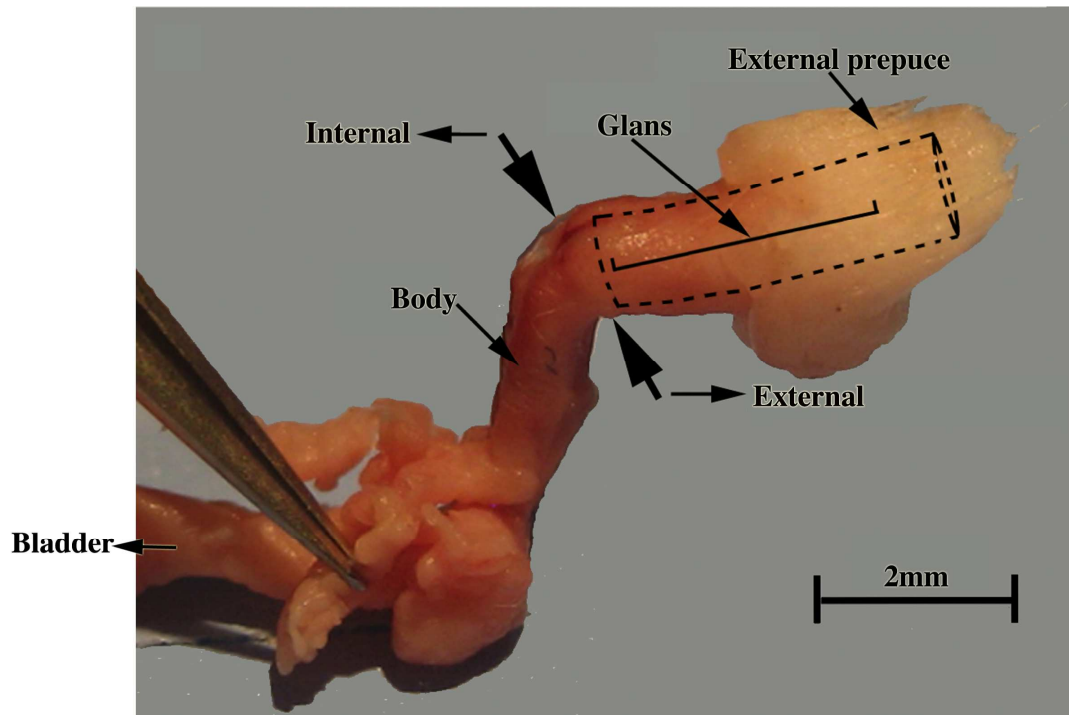
Figure 1.4. Photograph of the adult mouse penis. Large opposed arrows denote the boundary between the "internal" body of the mouse penis and the "external" glans, indicated by ⌐____⌐ and not seen because it lies within the preputial space (dotted lines). The structure held by the forceps is a corpus cavernosa. Its contralateral pair also can be seen. Both are attached to the pubic bones.
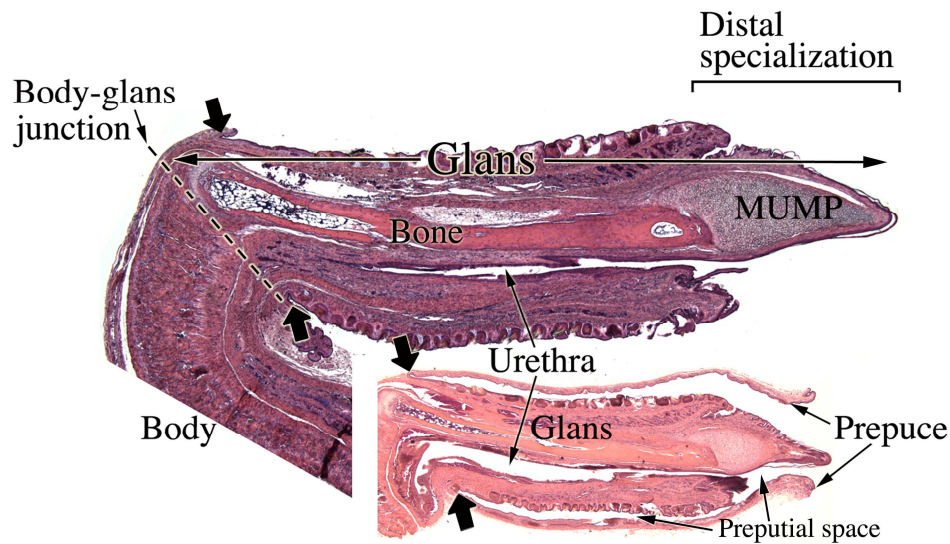
Figure 1.5. Mid-sagittal sections of the adult mouse penis. Lower section depicts the glans penis within part of the preputial space. Note the proximal attachment of the prepuce indicated by the opposed large arrows. In the upper section the prepuce has been removed but its proximal attachment is indicated by opposed large arrows. The junction between the "internal" body and the "external" glans is indicated by the dotted line.

Penis. The penis of mice and moles is an "internal organ" typically not seen in external views of the perineum. In the resting state the tip of the penis is situated within the preputial space a considerable distance from the tip of the prepuce (Figs. 1.2, 1.4 & 1.5) (See chapter 2 for mole data). One defining feature of the penis is that its external surface is a stratified squamous epithelium adorned by penile spines in mice (Fig. 1.3B) and without spines in the case of moles. The penile surface epithelium is devoid of hair follicles in both species. The penile urethra is completely surrounded by penile stroma and traverses through the penis to open at the urethral meatus into the preputial space (Figs. 1.5, 1.6, 2.2, 2.17). Another unique feature of the penis is the presence within its substance of well-defined erectile bodies in mice and moles (Fig. 1.6). The mouse and mole penises are appropriately divided into an external portion that projects from the body wall housed within the preputial space as well as an internal portion deep to the body surface, namely the attachments of the corpora cavernosa to the pubic bones (Fig. 1.4) and the attachment of the corpus spongiosum to the under surface of the urogenital diaphragm (human) (Fig. 1.10). Phallus is a generic term for the penis. During mating the penis projects outward beyond the prepuce, thus depositing semen into the vagina. While the specific details of urination are unknown in mice and moles, urine is expelled (a) either from the penile urethral meatus into the preputial space or (b) the penis is first extruded beyond the opening of the prepuce so that urine can exit cleanly from the penile urethra beyond the prepuce. None of the features of the penis are shared with the prepuce.
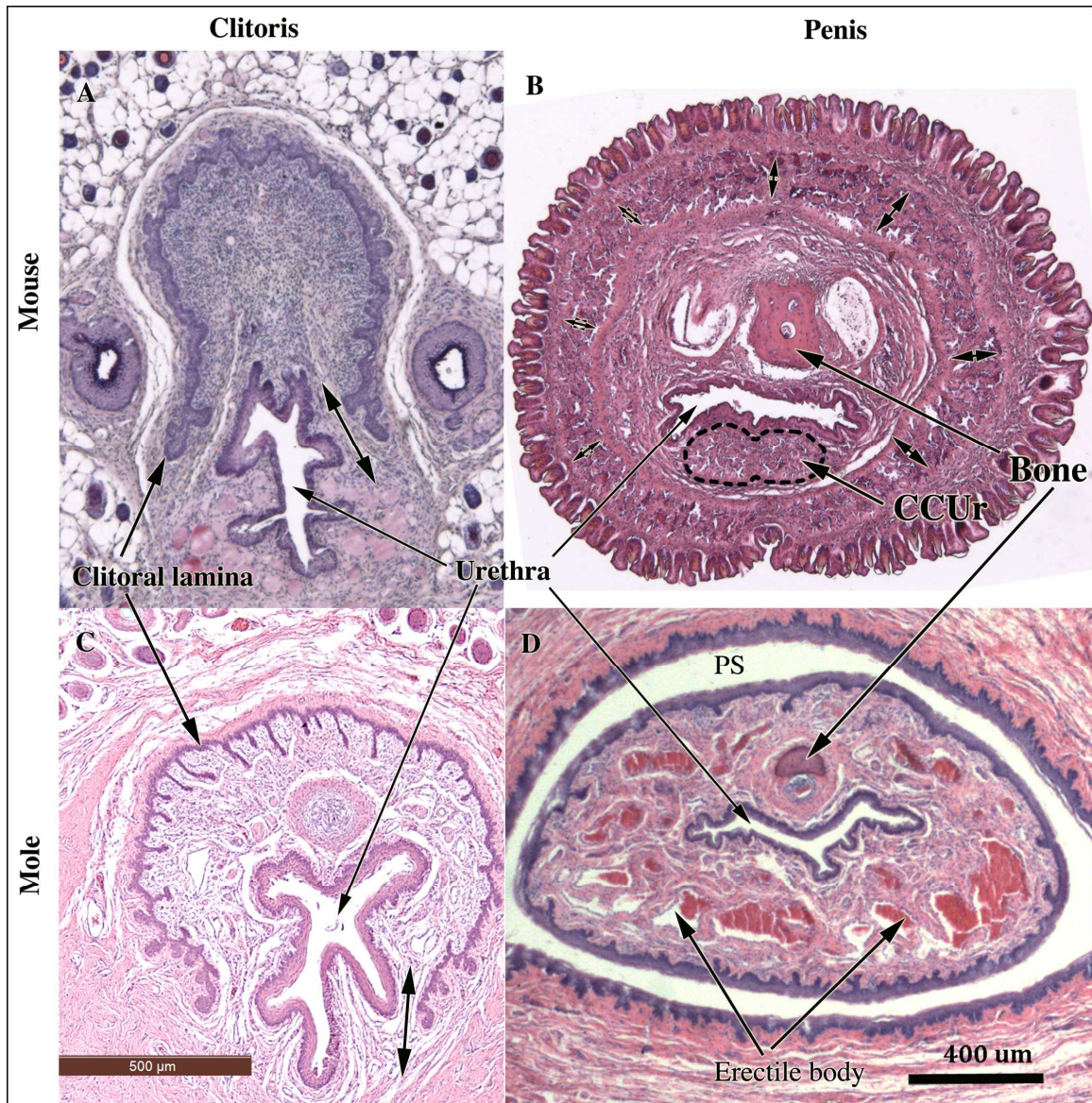
Figure 1.6. Sections of the adult mouse (A) and broad-footed mole (C) clitoris and adult mouse (B) and broad-footed mole (D) penis. The clitoris of both species is defined by an inverted U-shaped clitoral epithelial lamina with the urethra partly within the confines of the clitoral lamina and partly ventral to the clitoral lamina. In contrast, the penis of both species is oval to circular in contour, and the urethra resides completely within the substance of the penis. The penis of the mouse and certain mole species (broad-footed and hairy-tailed moles) contains an os penis (labeled bone) and well-defined erectile bodies (double-headed arrows =corpus cavernosum glandis, CCUr= corpus cavernosum urethrae). In the case of the mole penis (D) the erectile body (appropriately called corpus cavernosum glandis consists of blood filled spaces as indicated by the arrows. Note that clitoral stroma is confluent with ventral stromal (double-headed arrows).

Male prepuce. The male prepuce is a circumferential fold of skin with epithelium on both inner and outer surfaces that in mice and moles surrounds and houses the external portion of the penis (Fig. 1.7). In mice and moles, the prepuce is anatomically quite different in extent relative to the human male prepuce, which merely covers the distal aspect of the external (pendulous) portion of the penis (Figs. 1.10 & 1.11). The external prepuce of the male mouse and the prepuce of the male mole forms a prominent elevation in the perineum whose external surface is covered by a stratified squamous epidermis bearing hair follicles (Figs. 1.2, 1.4, 1.7, 2.4, 2.10, 2.11, 2.16, 2.17). The inner surface of the male mouse and mole prepuce, which defines the preputial space, is lined by a glabrous (non-hair-bearing) stratified squamous epithelium, which proximally reflects onto the penile surface and thus is continuous with penile surface epithelium at the internal-external penile junction (Figs. 1.5 & 1.7). A defining feature of the male mouse and mole prepuce is the complete absence of erectile bodies (Figs. 1.7, 2.4, 2.10, 2.11, 2.16, 2.17). The ducts of the right and left preputial glands open on the inner surface of the prepuce near the preputial meatus (not illustrated). It is unlikely (but unknown) that the mouse or mole prepuce enters the vagina during mating. As can be seen, the prepuce and the penis of mice and moles are distinctively different anatomical structures.

Male preputial space. The space defined by the inner epithelium of the prepuce. The penis lies within the preputial space (Figs. 1.5, 1.6, 1.7, 2,2, 2.7, 2.11, 2.13, 2.14, 2.17).

Female prepuce. The prominent elevation in the female perineum cranial to the vaginal meatus of both mice and moles is the female prepuce (Figs. 1.1, 1.8A, 2.3, 2.4, 2.10) and not the clitoris, which like the penis is an "internal organ" (Fig. 1.8). Examination of serial sections of the perineal elevation of female mice and moles reveals a histology virtually identical to that of the male prepuce (Fig. 1.7). The outer surface of the female prepuce (called the "penile clitoris" in the mole literature) is a stratified squamous epidermis bearing hair follicles (Fig. 1.7). The inner surface of the female prepuce, which defines the preputial space, is lined by a glabrous (non-hair-bearing) stratified squamous epithelium (Fig. 1.7). The ducts of the right and left preputial glands open on the inner surface of the female prepuce near the preputial meatus. The female urethra opens into the preputial space as will be described below. Both male and female prepuces are devoid of erectile bodies in mice and moles (Fig. 1.7).

Female Prepuce   **Male Prepuce**

Mouse

Mole

PPGD

Preputial space

Tip of penis

Urethra

A   B   C   D

500 µm
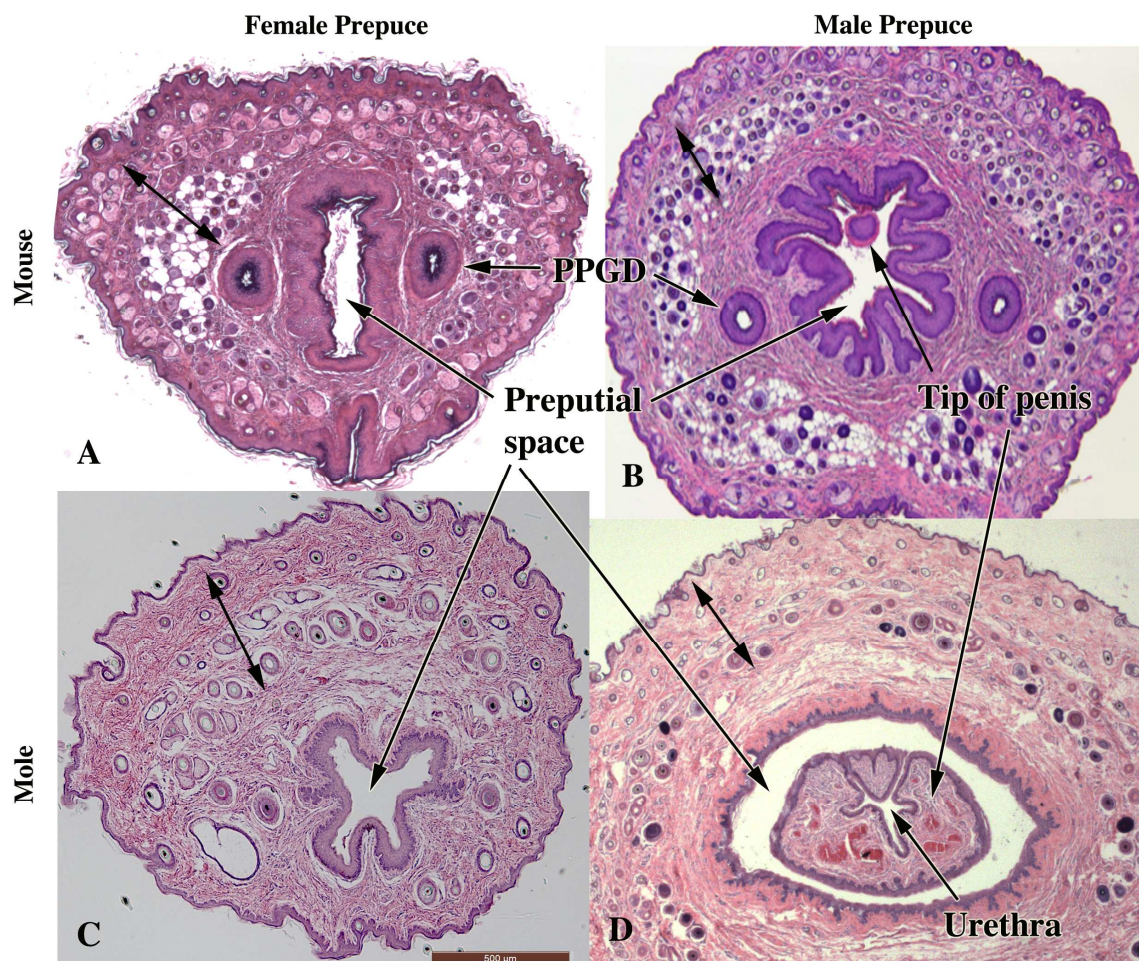
Figure 1.7. Sections of prepuces of adult female (A) and male (B) mice and adult female (C) and male (D) broad-footed moles. In both sexes and both species the skin covering the surface of the prepuce is stratified squamous and hair-bearing (double-headed arrows denote a stromal layer containing hair follicles). The preputial space is lined by a stratified squamous glabrous epithelium and contains the penis. The wall of the prepuce lacks erectile bodies. Note in (D) the urethra opening into the preputial space.

Clitoris. The clitoris of mice and moles is an "internal organ" (Fig. 1.8A) defined along most of its extent by an inverted U-shaped epithelial lamina (Figs. 1.6, 1.8C & D, 1.9D, 2.6, 2.7, 2.12, 2.15) even though the distal aspect of the clitoris is more complicated anatomically (Figs. 1.8B & E, 1.9A-C). For the most part, the mouse and mole clitoris is a stromal organ whose shape is defined by an inverted U-shaped clitoral epithelial lamina which is stratified, but non-cornified (Figs. 1.6, 1.8, 1.9, 2.6, 2.7, 2.12, 2.15). Although the clitoral stroma is highly vascular, distinct erectile bodies are not present in mice, while in moles a distinct corpus cavernosum is present though it is less well developed than in male moles (Figs. 1.6, 1.8, 1.9, 2.6, 2.7, 2.12, 2.15). The female urethra opens into the preputial space (Figs. 2.6, 2.12, 2.15). Along its entire proximal-distal extent the female urethra is associated with the clitoral stroma (Figs. 1.6, 1.8B-D, 2.6, 2.7, 2.12, 2.15). Distally the mouse and mole clitoral lamina and the urethra epithelium are fused into a common structure (Fig. 1.8E). Proximally, the clitoral lamina and the urethral epithelium are separate entities with the urethra partially within the confines of the U-shaped clitoral epithelial lamina (Fig. 1.8C-D). Thus, the female urethra is associated with clitoral stroma dorsally, but not ventrally (Figs. 1.6, 1.8, 2.6, 2.7, 2.12, 2.15). More proximally (that is deeper into the body) the female urethra departs ventrally from the clitoral lamina and thus is no longer associated with clitoral stroma (Fig. 1.8B-E).

Female urethra. Urine passing down the urethra from the bladder after traversing the urethra enters the preputial space and hence to the exterior onto the surface of the female prepuce (Figs. 1.6, 1.7, 1.8, 1.9, 2.6, 2.7, 2.12, 2.15). Hence, the female urethra and female preputial space are continuous with each other (Fig. 1.8B). The external meatus of the female prepuce, from which urine emerges, is definitely preputial space defined by the hair-bearing prepuce externally and the glabrous stratified squamous epithelium internally (Fig. 1.7A & C, 1.9A-C). The point where the urethra merges into the preputial space is difficult to discern, as there is no defining landmark. Taking a logical approach, we propose that the mouse and mole urethra is appropriately a stand-alone tubular structure lined by a non-cornified urethral epithelium (Figs 1.6A, 1.6C, 1.8D. 1.9D). As the stand-alone urethra extends distally the urethral epithelium fuses with the clitoral lamina (Figs. 1.8E, 1.9A-C), which distally merges with the well-defined stratified squamous, cornified, inner preputial epithelium (Fig. 1.7). Based upon this line of reasoning, we suggest that the dividing line between the female urethra and preputial space occurs at the point where the urethral epithelium is completely separate from the clitoral lamina (Figs. 1.8D & 1.9D).
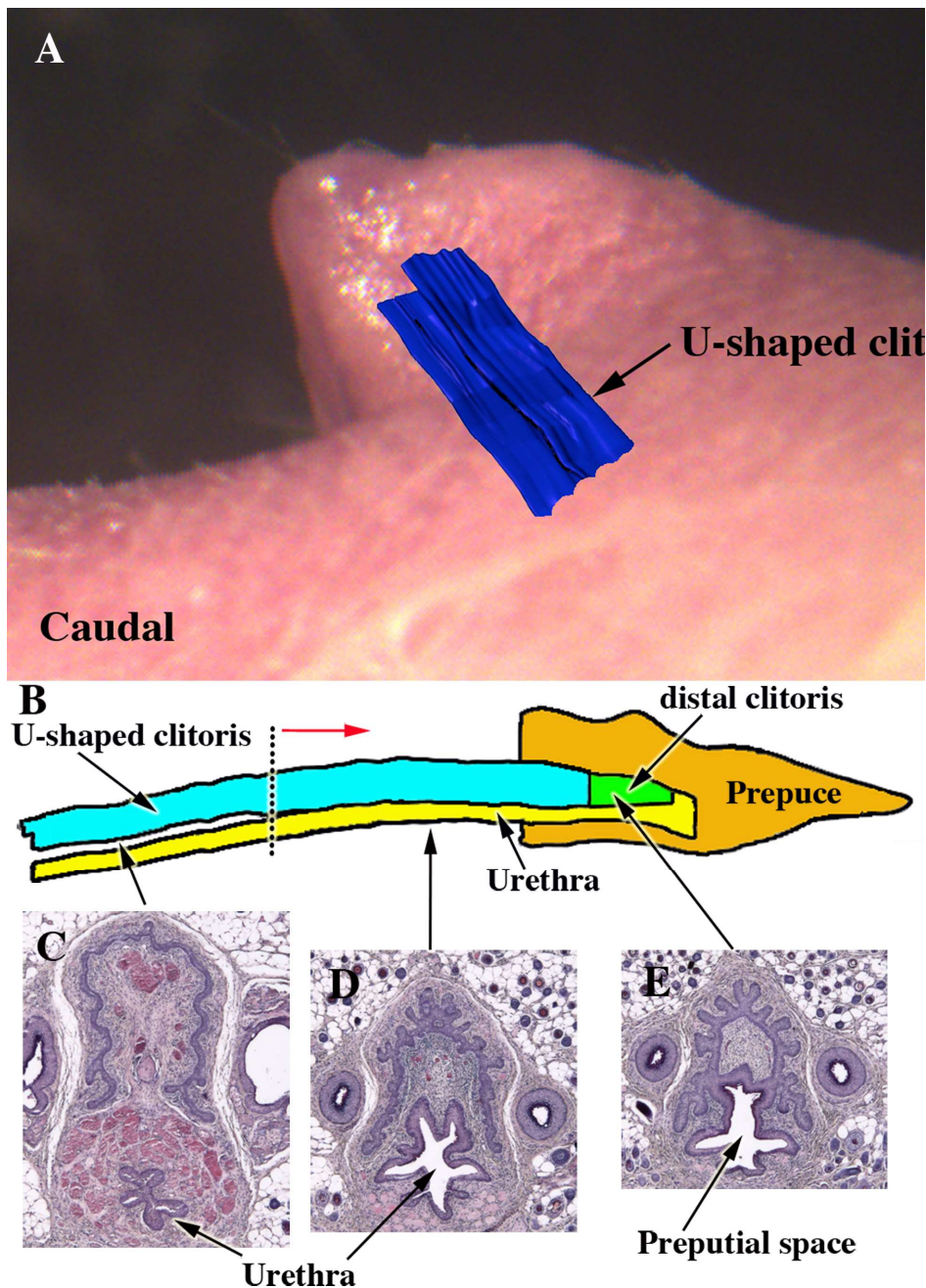
Figure 1.8 (A) Side view of the female mouse prepuce with a three dimensional reconstruction of the U-shaped clitoral epithelial lamina superimposed. The distal tip of the U-shaped clitoral epithelial lamina lies ~800μm from the distal tip of the female prepuce. (B) Drawing of the female mouse external genitalia. The urethra in yellow is ventral to the U-shaped clitoris proximally, but from the dotted line distally (red arrow) the urethra lies partly within the concavity of the U-shaped clitoral lamina and partly ventral to the U-shaped clitoral lamina. The U-shaped clitoris extends distally into the prepuce as a narrow epithelial defined structure (green). (C-E) Associated histological sections illustrate the morphology at the positions indicated by the arrows.
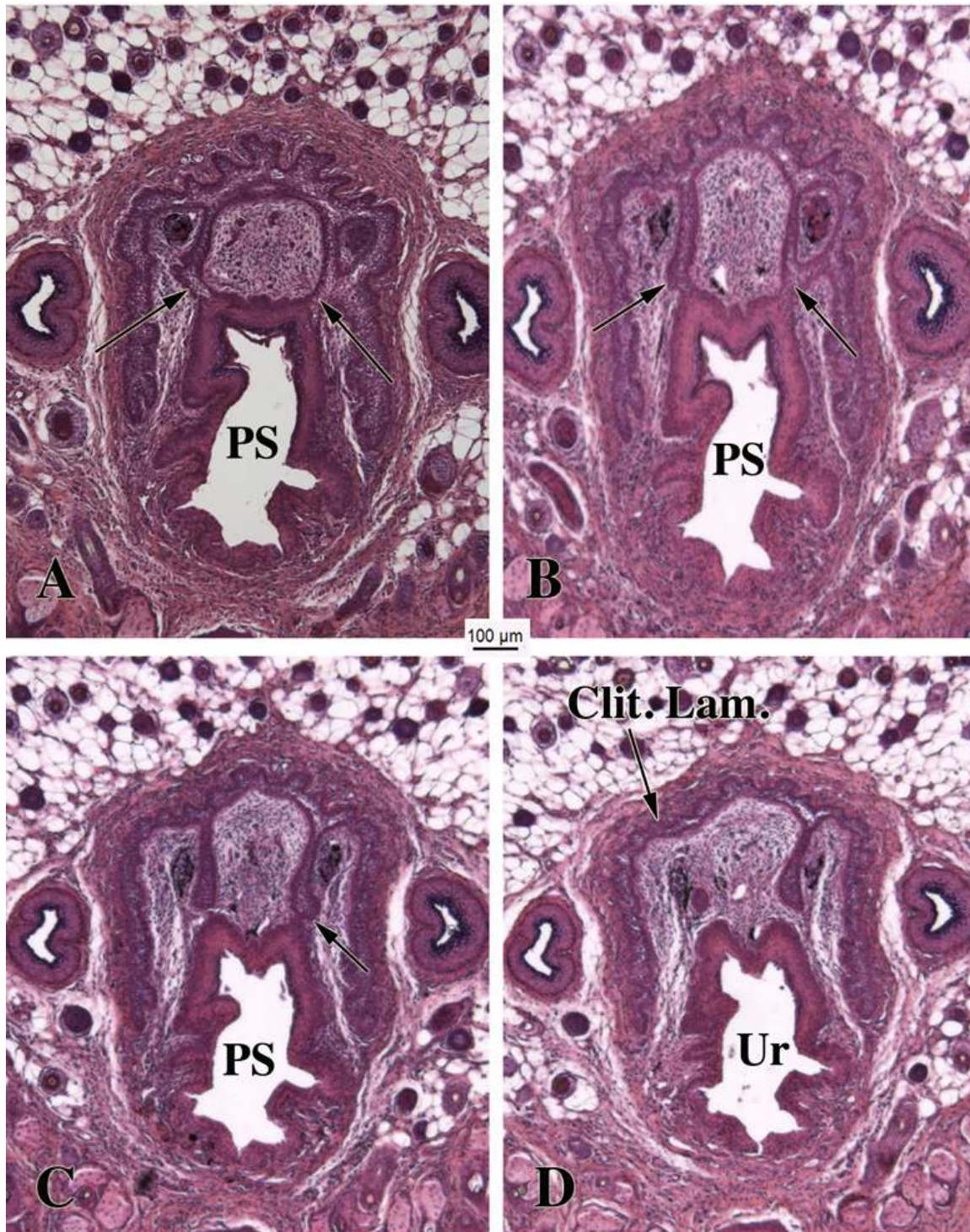
Figure 1.9. Serial sections through the junction of the preputial space (PS) (A-C) and the urethra (Ur) of an adult female mouse (D). The sections read from proximal to distal (A to D). Note that the urethra is a "stand alone" structure, not attached to other epithelia only in (D), while in more proximal sections (A-C), the epithelium defining the urethra is attached (arrows) to the emerging U-shaped clitoral lamina, which is also a "stand alone" structure in (D) only.

Concluding comments.   The anatomical concepts enunciated above have been made possible through knowledge of the detailed mouse external genitalia anatomy that has emerged over the last few years from studies in the Baskin lab, to which I am significant contributor and co-author (Blaschko et al., 2013) as well as in considerable data included in this dissertation. Comparative mouse-mole studies have validated the remarkable similarity in external genitalia anatomy between these two species and have led to the conclusion that all previous literature on mole external genitalia suffers from consistent anatomical error. Being able to set the record straight allows for the first time an accurate definition of mole external genitalia anatomy and its relation to endocrine parameters.

D. Comparison of anatomy of male external genitalia in mouse and human:

The terminology describing the mouse and human penis is quite different and must be thoroughly understood to avoid confusion and misconceptions. In both mouse and human, part of the penis lies below the body surface (internal) and part projects from the body wall (external). In humans the internal portion of the penis is comprised of the proximal attachment of the corpora cavernosa to the pubic bones and the proximal attachment of the corpus spongiosum to the under surface of the urogenital diaphragm. The external or pendulous portion of the human penis is called the shaft or body of the penis, which contains the corporal body and corpus spongiosum (Fig. 1.10A-B). The distal portion of the human penile shaft is called the glans and is the distal expanded portion of the corpus spongiosum (Fig. 1.10A), which is small relative to size of the shaft (Clemente, 1985). In the mouse the internal portion of the penis is unfortunately called the body of the penis and contains the corporal body and its attachments to the pubic bones (Fig. 1.10C). The external portion of the mouse penis lies within the preputial space, is called the glans (Rodriguez et al., 2011), and contains several erectile bodies as well as the os penis (Figs. 1.4, 1.5, 1.6B). The penile projection from the murine body wall, namely the glans, is situated within the preputial space, is relatively long with a shaft proximally, and a specialized distal region comparable to the human glans (Figs. 1.4 & 1.5) (Rodriguez et al., 2011; Weiss et al., 2012; Blaschko et al., 2013). Thus, the mouse glans is homologous to the pendulous human penile shaft as both are external projections of the body wall.
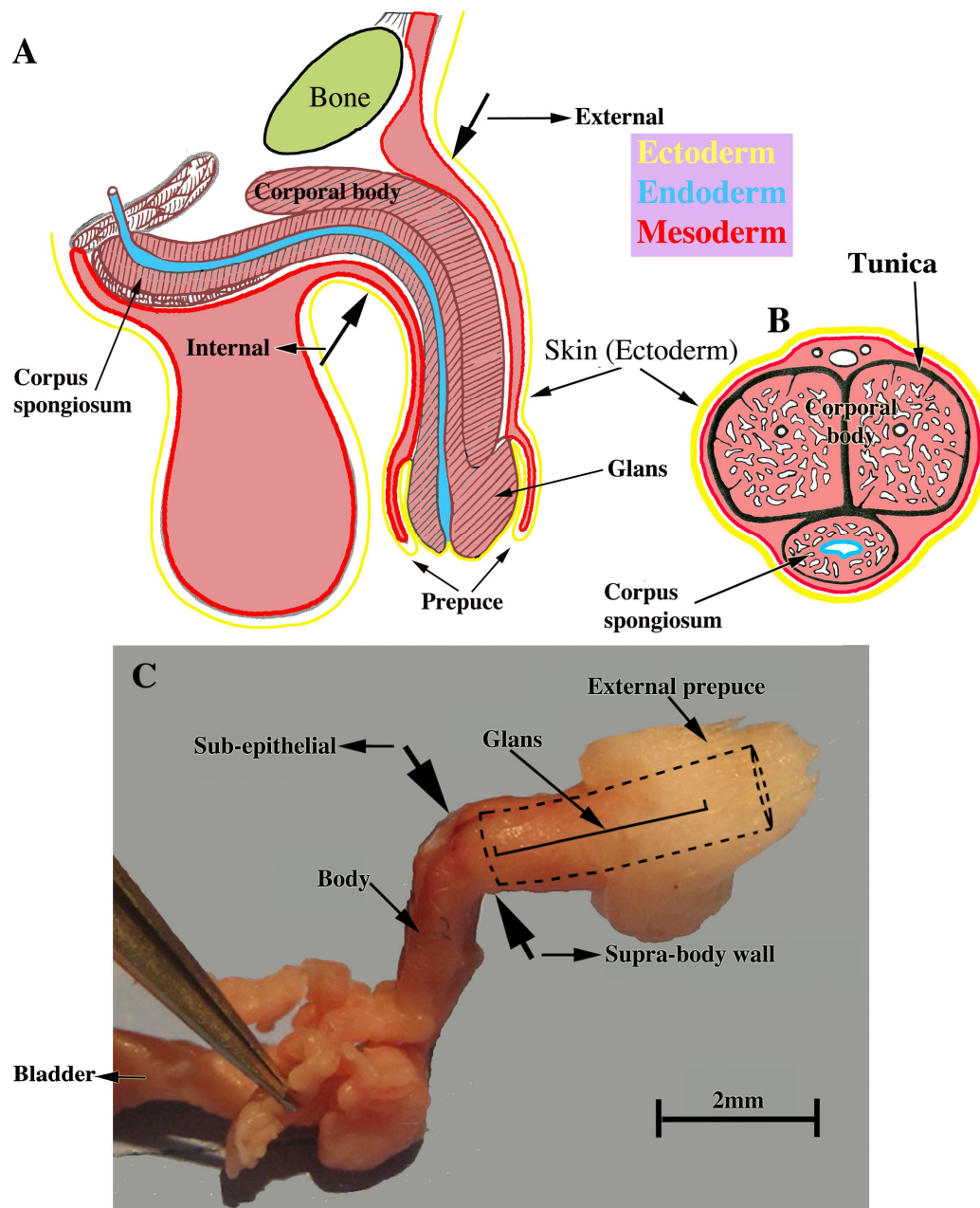
Figure 1.10. Drawings of human penis (A) in mid-sagittal view and (B) in transverse section to illustrate germ layer derivation of external genitalia components. The urethra (blue) is derived from endoderm. The skin (yellow) is derived from ectoderm. Note the ectoderm-endoderm junction in the urethral meatus. All structures circumscribed in red and shaded flesh color are derived from mesoderm and include erectile bodies, connective tissue, blood vessels, and smooth muscle. (C) Photograph of the adult mouse penis. Large opposed arrows denote the boundary between the "internal" body of the mouse penis and the "external" glans, indicated by ▭ and not seen because it lies within the preputial space (dotted lines).

        Preputial anatomy also differs markedly in mice versus humans. The external portion of the mouse penis (called the glans) resides within an extensive preputial space (Figs. 1.11B) defined by the attachment of the prepuce to the glans, which occurs proximally near the internal/external or glans/body junction in the mouse (Fig. 1.5). Distally the mouse prepuce is represented as the prominent hair-bearing elevation in the perineum (Figs. 1.2, 1.4, 1.11B) (Rodriguez et al., 2011). This terminology makes sense as the space created by this "external prepuce" houses the penis. In contrast, the human prepuce is attached to and covers only the distal aspect of the penis, namely the glans (Fig. 1.11C). Thus, the hair-bearing "traditional mouse prepuce" is clearly not homologous to the human prepuce. Recent study of the adult mouse penis revealed a series of ridges encircling the distal aspect of the glans penis. The distal projection of the mouse glans, the MUMP, is fused to the circumferential MUMP ridge to define the urethral orifice (Figs. 1.2, 1.3B, 1.11A) (Rodriguez et al., 2011). Proximal to the MUMP and MUMP ridge is another circumferential ridge, the internal prepuce (originally called the glanular ridge) (Rodriguez et al., 2011), which is integral to and encircles the glans penis (Figs. 1.3B, 1.11A) (Blaschko et al., 2013). The internal prepuce of the mouse has remarkable morphological homology with the human prepuce in so far as it is integral to the distal aspect of the glans and encircles and covers the glans penis (Fig. 1.11). Thus, the mouse actually has 2 prepuces: (a) the "traditional mouse prepuce" now called external prepuce and the internal prepuce (Fig. 1.11). The male mole prepuce, which forms an elevation in the perineum (Figs. 2.4, 2.10, 2.11, 2.16, 2.17) is homologous to the external prepuce of the mouse. The function of the internal mouse prepuce (like that of the human prepuce) is presumed to be male and/or female sexual stimulation. Animals built low to the ground, such as the mouse and mole, appear to need an extensive protective (external) prepuce to maintain penile cleanliness and protect the sensitive penis from foreign matter on the ground. Thus, the penis of mice and moles is housed deep within an extensive hair-bearing skin flap, the (external) prepuce. Clearly, the mole prepuce and external mouse prepuce are very different anatomically from the human prepuce.
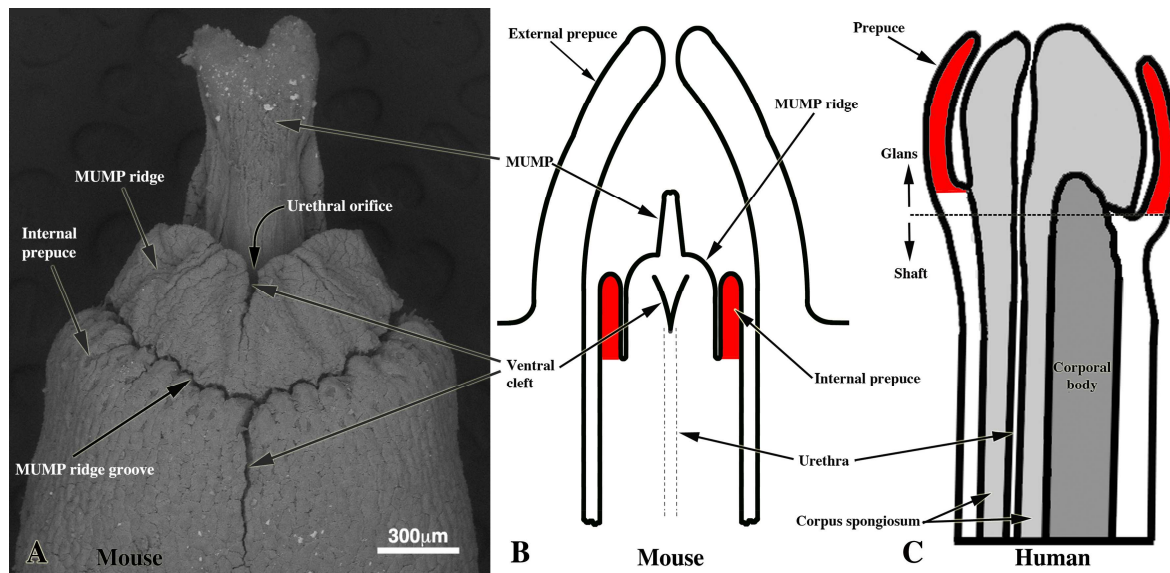
Figure 1.11. Re-evaluation of penile terminology to establish mouse-human homology. (A) SEM of the adult mouse penis. The penile glans lies within an extensive preputial space beginning at the opening of the preputial space distally in the hair-bearing prepuce (a prominent elevation in the perineum labeled external prepuce in (B) and ending proximally near the glans-body junction (See also Figs. 4 & 5). Drawings of mouse (B) and human (C) morphology demonstrating the homology of the human prepuce and the mouse "internal prepuce" (both red) in so far as both are integral to the distal penis and encircle the glans. (From Blaschko et al., 2014).

E. Comparison of anatomy of female external genitalia in mouse, mole and human:

The clitorises of mice, moles and humans are organs hidden from view, although to vastly different degrees. The human clitoris lies within the vaginal vestibule, which is demarcated by the labia minora and is in turn surrounded by the labia majora. Mice and moles do not have either labia minora or labia majora. The cranial aspects of the human labia minora are attached to the glans clitoris, which is surrounded dorsal-laterally by the prepuce of the clitoris (the homologue of the human penile prepuce) (Clemente, 1985) (Fig. 1.12). These details of human clitoral anatomy have little counterpart in the mouse (or mole). Indeed the mouse and mole clitoris is properly called an "internal organ" lying a considerable distance deep to the hair-bearing perineal elevation, which is the female mouse (or mole) prepuce (see Figs. 1.8A, 2.6, 2.12, 2.15).
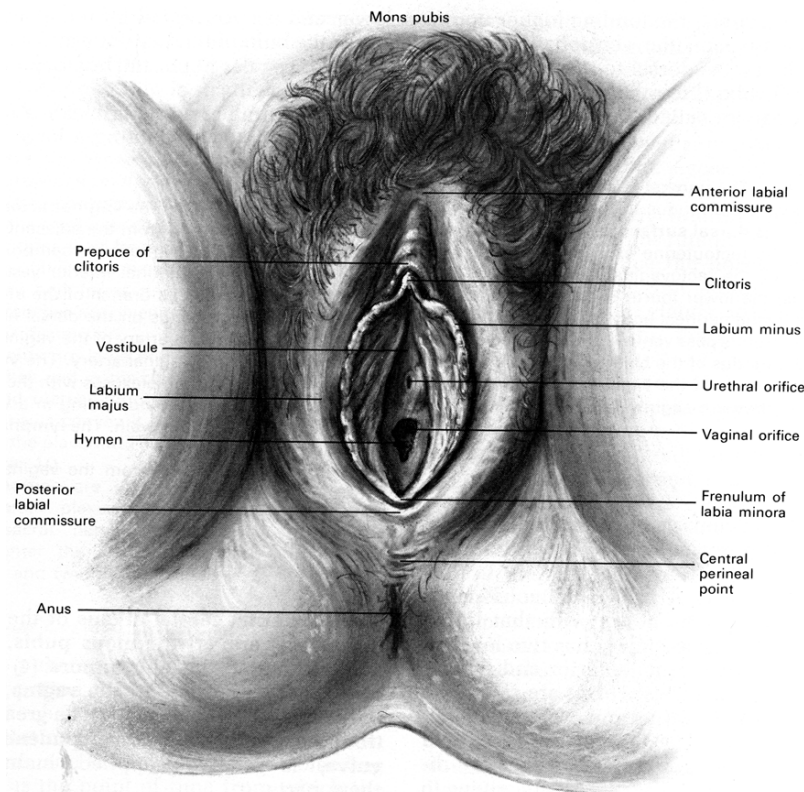
Figure 1.12. Drawing of human external genitalia from Grays Anatomy (Clemente, 1985). Note the prepuce of the clitoris.

The clitoris of mice and moles is defined by an inverted U-shaped epithelial lamina (Figs. 1.6A & C, 1.9D, 2.6, 2.7, 2.12, 2.15), even though the distal tip of the mouse clitoris is more complicated anatomically (Fig. 1.8B) (Weiss et al., 2012). While the urethra is completely separate from the human clitoris, in the mouse and mole the urethra resides partially within the confines of the U-shaped clitoral lamina (Figs. 1.6A & C, 1.8, 1.9D, 2.6, 2.7, 2.12, 2.15).

F. Dissertation Prospectus:

Throughout this dissertation the previous erroneous use of the terms mole "penis", "penile clitoris", "clitoris" and "phallus" will be designated in "quotation marks". Three species of mole (star-nosed, hairy-tailed, and broad-footed moles) have been described as having a large "penile clitoris," which is not present in Japanese shrew moles (Carmona et al., 2008). Literature on sex and species differences in clitoral and penile morphology of moles is limited and mostly inaccurate. Accordingly a detailed description of mole genital morphology is an essential pre-requisite to a full understanding of the role of hormones in development of the external genitalia. The Baskin lab has recently presented a morphometric comparison of male and female external

genitalia in the laboratory mouse based on serial sections and 3-dimensional reconstructions (Weiss et al., 2012; Schlomer et al., 2013). Based on the procedures outlined by Weiss *et al.*, a morphometric analysis of sex differences in the external genitalia morphology of moles is presented for the first time in this dissertation.

The literature review, that constitutes the next section of this chapter, begins with an account of the current theory of sexual differentiation of the urogenital system and the essential evidence that underlies the Jost theory. Research is then introduced on four mammalian species that do not appear to conform to this generally accepted theory. Finally I will discuss the research of this dissertation, as well as the contemporary literature concerning this subject for each of the four species of mole under investigation.

The second chapter is the investigation of penile and clitoral morphology in the four species of moles cited above. This was accomplished through detailed morphometric comparisons of clitoral and penile morphology. The third and final chapter is a discussion of the implications of these results for moles, mice and other mammals. The discussion summarizes the similarities and differences between male and female moles of the same species, as well as variation among other species (mice, spotted hyenas and humans), and correlates the level of penile or clitoral "masculinization" with existing evidence of androgen production.

G. Contemporary Understanding of Sexual Differentiation and Development of the External genitalia: The Jost Model

The developing fetus of eutherian mammals passes through a common indifferent stage before anatomical and physiological differentiation into the male or female phenotypes. Work by French embryologist Alfred Jost in the 1940's and 1950's established the primary theory of mammalian sexual differentiation (Jost, 1947, 1953). While the Jost model has subsequently been confirmed, expanded, and refined, the major points presented by Jost have remained central to our contemporary understanding of sexual development (Wilson et al., 1981b). The Jost model states that chromosomal sex elicits sexual differentiation of the gonadal primordia. Contemporary molecular data indicate that the Sry (sex-determining region Y) gene on the Y chromosome initiates a cascade of gene interactions that determine whether the indifferent gonad develops into a testis (Wilhelm et al., 2007; Sekido and Lovell-Badge, 2009)

In males, anti-Müllerian hormone (AMH) secreted by the fetal testes is responsible for the regression of the Müllerian ducts. Secretion of testosterone by the testes at specific times during fetal development is responsible for development of the Wolffian ducts into the epididymes, vas deferens, and seminal vesicles, and development of the prostate from the urogenital sinus. Testosterone and it's conversion to 5α-dihydrotestosterone (DHT) in target tissues causes the development of the genital tubercle into a penis and causes the genital swellings to fuse and develop into the scrotum in those animals that possess a scrotum (Yamada et al., 2003).

In females, the absence of testosterone secretion results in regression of the Wolffian ducts, while the absence of AMH allows the Müllerian ducts to persist and develop into the oviducts, uterus, cervix and upper vagina. In the absence of androgens, the genital tubercle develops into the clitoris, while the urogenital folds and swellings develop into the minor and major labia that border the vaginal opening (human) (Moore and Persaud, 2003). Based on this theory the female external genitalia develop as the result of the absence of gonadal cues during sexual differentiation leading to the view that female development is a passive process. However, passive development is impossible, and referring to feminine development as "passive" is simply a statement of ignorance.

H. Evidence for the Jost Model:

Fetal or Neonatal Gonadectomy. In his work, Alfred Jost employed fetal gonadectomy to eliminate the presence of androgens in the developing fetus and demonstrated that if XX and XY rabbit fetuses were castrated *in utero* before sexual differentiation, they proceeded to develop ducts and external genitalia with the female phenotype (Jost, 1947, 1953). Subsequent neonatal mouse castration studies have led to the concept that fetal testicular androgens specify penile identity and then subsequently elicit penile specific morphogenesis (Rodriguez et al., 2012).

Exposure to Androgens During Sensitive Periods of Development. Female mammals that were exposed to androgens, such as testosterone propionate (TP), during critical stages of gestation exhibited "masculinized" external genitalia. The magnitude of this shift toward the male phenotype varies by species, timing, and amount of androgen administration. Prenatally androgenized females may exhibit any or all of the following features: an enlarged clitoris, an increased anogenital distance, an absence of a vaginal opening, and occasionally a pseudoscrotum. Such "masculinization" has been observed in a variety of species, including the rat (Rhees et al., 1997; Wolf et al., 2002; Welsh et al., 2008), mouse (Gandelman et al., 1979; Yucel et al., 2003), sheep (Clarke et al., 1976; Wood and Foster, 1998; Jackson et al., 2008; Roberts et al., 2008), primate (Wells and van Wagenen, 1956; Goy et al., 1988; Herman et al., 2000), and guinea pig (Phoenix et al., 1959). Women with congenital adrenal hyperplasia (CAH) are exposed to endogenous androgens *in utero* and exhibit some degree of "masculinization" of the external genitalia (Merke and Bornstein, 2005).

Whereas treatment with an androgen such as TP during gestation results in masculinization of the external genitalia of female mammals, it is 5α-dihydrotestosterone (DHT) that is required for the virilization of the external genitalia and urogenital sinus (UGS). In the developing genital tubercle and UGS testosterone is converted locally to DHT by the enzyme 5α-reductase. Exposure of male rats and mice *in utero* to inhibitors of 5α-reductase causes hypospadias, a cleft prepuce, and a reduced anogenital distance (Imperato-McGinley et al., 1985; Anderson and Clark, 1990; Iguchi et al., 1991; Clark et al., 1993). Human males (XY) with 5α-reductase deficiency have testes that produce T but are unable to convert T to DHT in the target tissues. These males have epididymes, vas deferens, and seminal vesicles due to the actions of T in stabilizing and virilizing the Wolffian ducts, but are born with ambiguous external genitalia

having more of a female than a male phenotype. Accordingly, most 5α-reductase deficient infants have been raised as females (Moore et al., 1975; Imperato-McGinley, 1984).

Administration of Anti-Androgens During Sensitive Periods of Development. In other studies, anti-androgens have been administered to pregnant females to block androgenic activity during fetal sexual differentiation resulting in demasculinization of the external genitalia of the male offspring with little to no change to the female offspring. The demasculinizing effects of the anti-androgen varies by species, duration, and amount of treatment, and produces effects ranging from males with a fully female appearing external genitalia to males with hypospadias, decreased penis size, underdeveloped scrotum, failure of preputial separation from the glans penis, and reduced anogenital distance. This has been shown in a variety of species such as: rats treated with finasteride (Imperato-McGinley et al., 1992; Bowman et al., 2003), mice treated with flutamide (Silversides et al., 1995; Kojima et al., 2002), sheep treated with flutamide (Jackson et al., 2008) primates treated with flutamide (Herman et al., 2000), primates treated with finasteride (Prahalada et al., 1997), guinea pigs treated with cyproterone acetate (Goldfoot et al., 1971; Thornton et al., 1991) and rabbits treated with finasteride (Kurzrock et al., 2000).

Androgen-receptor mutant animals. Tfm mice are insensitive to androgens due to a mutation in the gene encoding the androgen receptor (He et al., 1990) and exhibit completely feminized external genitalia (Weiss et al., 2012). Male Tfm mice have testes, normal or elevated serum testosterone and produce AMH. Consequently, they lack both the Wolffian duct and Müllerian duct derivatives, and develop female external genitalia with a blind-ending vagina (Lyons and Hawkes, 1970; Cunha, 1975a). These findings in the Tfm male mouse have been confirmed in the male AR null mouse (Rodriguez et al., 2012). Androgen receptor mutations and their effects in mice are similar to the human Androgen Insensitivity Syndrome (Quigley et al., 1995; Ahmed et al., 2000).

I. Naturally "Masculinized" Females: Challenging Jost's Model:

Some female mammals exhibit varying degrees of natural genital "masculinization." If the Jost Model is true, androgens must have been circulating during critical stages of sexual differentiation/development to produce "masculinization" of females. Such female "masculinization" has been observed in the ring-tailed lemur, the spotted hyena, and various moles, which are the focus of this dissertation.

Prosimians. In several species of Prosimian primates, females have an elongated erectile clitoris, fully or partially traversed by the urethra, and resemble the male penis in size and appearance. Also, an imperforate vagina may be present during non-breeding seasons or until puberty (Hill, 1953; Dixson, 1998). At the present time, only the external genitalia morphology of the ring-tailed lemur (*Lemur catta*) has been studied in detail. These lemurs do not have a pseudo-scrotum, and the vaginal opening is imperforate until puberty and thereafter remains open. Males have greater phallic length and width and a greater anogenital distance than females. Males are generally heavier than females, but there is size monomorphism in other bodily

measurements. The clitoris is traversed by fused corpora cavernosa, like the male, and is encapsulated by a *tunica albuginea*. The urethra opens on the ventral surface of the glans clitoris, and there is no corpus spongiosum. An os clitoris is present that is smaller than the male os penis (Drea and Weil, 2008).

Female lemurs (*L. catta)* play as vigorously as males and engage in scent-marking and territorial defense like males. Females are more aggressive than males in intergroup encounters, and there is strict female social dominance over males (Jolly, 1966; Drea, 2007). It has been found that seasonal increases in female aggression are associated with related increases in androstenedione and estrogen, indicating that sex steroids may play an activating role in female aggression (Drea, 2007). Additionally, concentrations of androstenedione, testosterone, and estrogen are higher during pregnancy than during the time before conception or after parturition, which may indicate steroids play an organizational role as well (Drea, 2009). No studies have been performed to investigate whether anti-androgen treatment during gestation would produce a female with typical female mammalian external genitalia, or a male with genitalia either demasculinized to resemble the *L. catta* female phenotype or the typical mammalian female phenotype.

The Spotted Hyena. Female spotted hyenas (*Crocuta crocuta*) exhibit the most profoundly "masculinized" external genitalia of any female mammal. Female spotted hyenas lack a vaginal opening and have a pseudo-scrotum. The peniform clitoris is similar in size to the male penis even though subtle differences in external shape are apparent (Cunha et al., 2014). The penile clitoris is traversed to the tip by a central canal called the urogenital sinus (UGS) and is used for urination, copulation, and parturition (Matthews, 1939; Frank and Glickman, 1994; Cunha et al., 2003; Cunha et al., 2014). Females are generally larger than the males, are more aggressive, and are socially dominant (Kruuk, 1972). The female spotted hyenas can also display erections similar to the males, and such erections are used in meeting ceremonies (Kruuk, 1972; East et al., 1993; Holekamp and Smale, 1998). The internal urogenital system of female spotted hyenas is morphologically similar to the typical female mammal, and the peniform clitoris is slightly shorter, thicker, less angular, and has a larger urogenital meatus than the male penis (Frank et al., 1990; Glickman et al., 1992; Drea et al., 1998; Cunha et al., 2003). Concerning the internal morphology of the phalli, both sexes possess a corporal body (fused corpus cavernosa) surrounded by a thick *tunica albuginea*. The male urethra is surrounded by corpus spongiosum and the *tunica albuginea*, while these elements do not surround the female UGS. Retractor muscles are located ventral to the urethra in males, and dorsal-lateral to the UGS in females. The larger, highly folded, female UGS resides in the most ventral position in the female peniform clitoris (Matthews, 1939; Neaves et al., 1980; Cunha et al., 2003; Cunha et al., 2005; Cunha et al., 2014). Thus, while the female penile clitoris is similar in size to the male penis, there is a distinct female phenotype for this species encompassing internal and external morphology (Cunha et al., 2014).

Non-pregnant female spotted hyenas have testosterone concentrations within the range of other female mammals, but this value is markedly increased during gestation when fetuses are exposed to significant quantities of androgen *in utero*. The latter is a result of the conversion of maternal androstenedione to testosterone by the placenta (Glickman et al., 2006). However, the development of a scrotum and the formation of a phallus are androgen-independent in both males

and females (Cunha et al., 2014). *In utero* exposure to flutamide, an anti-androgen, and finasteride, a 5-reductase inhibitor that blocks the conversion of T to DHT, failed to convert the external genitalia of female spotted hyenas to the typical female phenotype. However, in male fetuses this cocktail of "anti-androgens" elicited a shift in internal and external morphology of the penis to the female phenotype, indicating that androgens contribute to sex differences in external morphology (Drea et al., 1998) and internal phallic structure (Cunha et al., 2005; Cunha et al., 2014). Thus, non-androgenic mechanisms may be responsible for formation and growth of the external genitalia of the spotted hyena, which is inconsistent with the Jost model of sexual differentiation.

European, North American, and Asian Moles. As noted previously, in 1935 Matthews published a monograph on the reproductive physiology the European mole (Matthews, 1935). He noted the presence of a "penile clitoris" and the unusual structure of the ovary. The ovary was characterized as polar, with typical follicular ovarian tissue at one end and an ovarian interstitial gland at the other. The follicular portion is typical of female mammals, while the ovarian interstitial gland is concentrated in the medulla and hilus region of the ovary. Blood vessels enter the ovarian interstitial gland at the opposite end from the ovary. The ovarian bursa does not touch the follicular region, instead forming a pouch around the ovary, but does lie on the surface of the ovarian interstitial gland. Matthews reported that there were seasonal changes in the morphology of the ovary. Females captured during the non-breeding season had an ovarian interstitial gland noticeably larger than the follicular portion, whereas females captured during the breeding season had a follicular region and an ovarian interstitial gland of roughly equal size.

These observations encouraged further study of this unusual structure. A similar ovarian interstitial gland, with similar seasonal changes, was found in several other species of mole (Mossman and Duke, 1973; Burgos et al., 1988; Sanchez et al., 1996; Beolchini et al., 2000; Carmona et al., 2008). When the cellular structure of the European mole ovarian interstitial gland was investigated, the interstitial cells of the ovarian interstitial gland were almost indistinguishable from the Leydig cells of testes. The medullary cords within the ovarian interstitial gland are spherical and much shorter than typical male testicular cords. The cells defining the outer surface of the medullary cords generally resemble immature Sertoli cells but lack the specialized Sertoli cell intracellular junctions. The medullary cords also lack germinal cells and overall resemble fetal male testicular cords (Jimenez et al., 1993; Beolchini et al., 2000).

Other features of the ovary also have a masculine appearance. The ovarian bursa covering the follicular ovary portion is typical ovarian surface epithelium consisting of a monolayer of cuboidal cells. The ovarian surface epithelium changes where it overlies the ovarian interstitial gland and is similar to the *tunica albuginea* of males consisting of a multilayer of flattened connective tissue cells with abundant collagen fibers (Jimenez et al., 1993).

European moles, Spanish moles (*Talpa occidentalis*) and Roman moles (*Talpa romana*) all possess an ovarian interstitial gland along with rudimentary epididymes attached to the ovarian bursa by connective tissue. This epididymal tissue is located at the ovarian interstitial gland pole and not the follicular ovarian pole (Jimenez et al., 1993; Jimenez et al., 1996; Sanchez et al., 1996). In female Spanish moles the Wolffian ducts degenerate just before birth but the

cranial portion of the Wolffian ducts persists and begins to grow several days after birth. At 15-20 days postpartum, the time when the Leydig cells differentiate in the ovarian interstitial gland and testosterone is detected in the blood, the Wolffian duct remnants begins to take on epididymal features (Zurita et al., 2003). From puberty onward the rudimentary epididymes continue to grow slowly throughout life, but do not undergo any changes from breeding to non-breeding seasons (Jimenez et al., 1996).

The resemblance of the ovarian interstitial gland to male testicular tissue led researchers to investigate whether the ovarian interstitial gland produced androgens. When plasma concentrations of testosterone were measured by radioimmunoassay in Spanish moles, Jimenez *et al.* (1993) found that there were indeed significant concentrations of testosterone circulating in females, with higher concentrations in adult and juvenile females from the non-breeding season, which correlated with ovarian interstitial gland weight (Jimenez et al., 1993). Further research on juveniles found little/no detectable testosterone in the blood plasma of females 0-10 days postpartum (dpp). However, testosterone began to increase after 10 dpp such that by 30+ dpp the testosterone levels were higher than in non-breeding-season males of the same age (Zurita et al., 2003). Non-breeding-season female European moles had testosterone levels higher than pregnant breeding-season females and were in the same range as non-breeding-season males. There was no significant difference in the level of androstenedione between breeding- and non-breeding-season females. They did, however, have measurable levels of androstenedione indicating it may be a substrate for the metabolic production of testosterone. When the follicular ovary and ovarian interstitial gland portions were separated and incubated with progesterone or androstenedione, the ovarian interstitial gland metabolized these steroid hormone substrates primarily to testosterone, while the follicular ovary made some estradiol but little to no testosterone (Whitworth et al., 1999).

Detailed observations of fetal gonadal development in European moles, Roman moles, and Spanish moles have been made (Beolchini et al., 2000; Barrionuevo et al., 2004; Zurita et al., 2007). Zurita *et al.* studied the Spanish mole and found that in female fetuses germ cells reside primarily in the cortex, not the medulla/ovarian interstitial gland region, with meiosis starting postnatally (Zurita et al., 2003). The asymmetrical distribution of primordial germ cells is not due to selective colonization, and it is believed that the delay in meiosis in females may permit testis-like development in the absence of oocyte-derived inhibitory factors. In the European mole, Barrionuevo *et al.* discovered that mesonephric cell migration, which is required for correct testis differentiation, occurs at the same time in males and females (Barrionuevo et al., 2004). This causes the formation of medullar cords, suggesting testis-like development within the ovarian interstitial gland. Peri-tubular myoid cells, unlike Sertoli or Leydig cells, are testis-specific and have no homologous counterpart in the ovary. In the developing female fetus, myoid cells are found in the medullary/ovarian interstitial gland region, and not the cortex/follicular ovarian region. The formation of medullar cords, vascular system, and the *tunica albuginea,* also initiate at the same time in male and female moles. Cytodifferentiation of pre-Sertoli cells, Leydig cells and myoid cells is delayed in the female but not in the male according to typical mammalian testicular differentiation.

Female moles possessing this ovarian interstitial gland lack the SRY gene, and there is no AMH gene expression during female gonadal development, indicating that neither is responsible

for the presence of the ovarian interstitial gland (Jimenez et al., 1993; Sanchez et al., 1996; Zurita et al., 2003). Carmona *et al.* investigated the expression patterns of genes that are known to be involved in mammalian sex determination and differentiation in the European mole (Carmona et al., 2009). They discovered that the Sertoli-like cells in the female mole ovarian interstitial gland do not express SOX9, which is typically up-regulated by SRY during testicular development and is believed to be essential for testis development. This may be a reason female mole Sertoli-like cells never fully differentiate into Sertoli cells.

The external genitalia of female moles are characterized by a vaginal opening that remains open for the period of mating and parturition before closing for the non-breeding season (Matthews, 1935). All species of mole studied to date that possess an ovarian interstitial gland also have a "hypertrophied clitoris" that is traversed by the "urethra" (Wood-Jones, 1914; Rubenstein et al., 2003; Zurita et al., 2003). Development of the "penis" and "penile clitoris" has been investigated in the European mole. Wood-Jones found that fetal developmental stages during the elongation of the genital tubercle, urethral groove closure, and the formation of the prepuce, take place in exactly the same manner in both sexes (Wood-Jones, 1914).

J. Research Proposed in This Dissertation:

Females with naturally "masculinized" external genitalia are of special interest for this dissertation. Research as to how they "do," or "do-not" fit the current widely accepted theory of sexual differentiation, may reveal other mechanisms involved in sexual differentiation. An essential aspect of this dissertation is an accurate description and definition of the terms penis, clitoris, phallus, urethra and prepuce as previous investigators have incorrectly described these terms, especially in moles. Intriguing work has been done on mole species concerning the ovarian interstitial gland, its resemblance to testicular tissue, its ability to produce androgens in relation to the corresponding presence of a "penile clitoris" in some mole species. However, very little work has been done in this area on mole species of North America. No detailed analysis of the structure of any "penile clitoris" has been performed on any species of mole, and no comparative work has been done to try to understand the presence or absence of the "penile clitoris" with or without an ovarian interstitial gland in the different mole species. There has been no investigation into whether female moles lacking an ovarian interstitial gland produce androgens.

The following goals have been the focus of this dissertation.

1. Accurate descriptions of the gross and histologic anatomy of male and female mole external genitalia, and thus correction of unacceptable and misleading anatomy pervasive throughout the mole literature.
2. Review of gross and histologic anatomy of male and female mouse external genitalia with new data added.
3. Review of anatomy of male and female human external genitalia.
4. Morphometric analysis with three-dimensional reconstruction of male and female mole external genitalia.

5.   Correlation of male and female mole external genitalia with the presence or absence of an ovarian interstitial gland (a potential source of androgen).

6.   Discussion of different patterns of external genitalia in human, mouse, four species of moles and spotted hyena in relation to known endocrine profiles and mechanisms of morphogenesis/differentiation with discussion of gaps in the data.

7.   Discussion of future perspectives for advancing the field.

Given that the presence or absence of androgens during development play a central role in most species in determining masculine versus feminine differentiation of the external genitalia, a major focus within the Discussion will be devoted to the status of knowledge on androgen sources and androgen action in the species under consideration. In this regard, data indicate that androgenic treatment of pregnant rhesus monkeys can produce a clitoris in female offspring that mimics the structure of the penis (Wells and van Wagenen, 1956). Studies in rats, mice and humans further substantiate the idea that exogenous and endogenous androgens can masculinize female external genitalia to variable degrees (Merke and Bornstein, 2005; Al-Maghribi, 2007; Rodriguez et al., 2012). In stark contrast, the female spotted hyena has a profoundly "masculinized" clitoris whose formation and growth are androgen-independent (Cunha et al., 2003; Cunha et al., 2014). Whether information from these species is relevant to mole external genitalia is a subject of this dissertation.

For the purpose of this dissertation, female and male representatives of four different species of mole were obtained: (1) The broad-footed mole, which does not possess an ovarian interstitial gland but does have a "penile clitoris"; (2) The star-nosed mole, which does have a ovarian interstitial gland and a "penile clitoris"; (3) The Japanese shrew mole, which does not possess either an ovarian interstitial gland, or "penile clitoris"; and (4) The hairy-tailed mole. There is no published work on the reproductive anatomy of the latter species. The magnitude of sexual dimorphism was assessed in each species by performing detailed morphometric comparisons of clitoral, penile and preputial morphology. A concise review of the literature and my research perspectives for each species is given below.

Broad-footed mole (*Scapanus latimanus*). The ovaries of the broad-footed mole lack an ovarian interstitial gland even though females of this species possess a prominent "phallus" traversed by the "urethra". Anatomy of external genitalia of this species reported previously is riddled with errors, and thus the first order of this dissertation will be an accurate description of the relevant anatomy. There is also no significant difference in "phallus" length or anogenital distance in males and females (Mossman and Duke, 1973; Rubenstein et al., 2003). There are no published data on blood hormone levels, IHC of steroidogenic enzymes in relation to male and female "phallic" morphology such as clitoral shaft length compared to penile length. While not published, and not conclusive data, Nicki Rubenstein did test a couple blood serum samples of non-breeding season adult females for androstenedione and testosterone via radioimmunoassay and found high levels of these hormones.

Based on the previous work completed on broad-footed moles and the Jost model, I expected to find that the clitoral anatomy may have some masculine features such as a corpus cavernosum surrounded by a tunica, but not complete masculinization, which would include a

clitoris with a free tip projecting into the preputial space, an os clitoris, and no ventral tethering of the clitoral shaft. Additionally, I anticipated finding a vaginal opening during the breeding season in adult females, which remains imperforate the remainder of the year. Male morphological characteristics in the clitoris would likely be less well developed than in their penile counterparts. A reasonable prediction is that the male penis would contain a well-developed corpus cavernosum, thick tunica, corpus spongiosum, os penis, and that the penis projects freely into the preputial space without ventral tethering, and a urethra contained within the organ. The anogenital distance of males and females would not be significantly different from each other, and they would have a similar phallus length as was found in a previous study (Rubenstein et al., 2003). Similarly, based on previous studies, I expected to confirm that female broad-footed moles lack an ovarian interstitial gland and will present a typical mammalian follicular ovary for a seasonally breeding species. The male would also present a typical mammalian testis with associated changes in size, spermatogenic function, and Leydig cell androgen production for a seasonally breeding species.

Star-nosed mole (*Condylura cristata*). Star-nosed moles have been reported to have an ovarian interstitial gland and a prominent "phallus" traversed by the "urethra" (Mossman and Duke, 1973; Rubenstein et al., 2003). As above, the anatomy of external genitalia of this species reported previously is full of errors, and thus the first order of this dissertation will be an accurate description of the relevant anatomy. To date, there are no published data in this species on anogenital distance, "phallus" length, internal phallic morphology, IHC analysis for steroidogenic enzymes, blood hormone levels, or the presence/absence of an epididymes near the ovarian interstitial gland.

Star-nosed moles from the non-breeding season, but not the breeding season, were supplied by Drs. Kenneth Catania and Diana Bautista of Vanderbilt University. Due to this, studies of seasonal changes in certain traits were not performed. Based on the previous research done on star-nosed moles and the Jost model, I hypothesize that the star-nosed mole clitoris may possess more masculine characteristics than the broad-footed mole clitoris, with traits such as a better-developed corpus cavernosum and tunica, and perhaps an os clitoris, but not complete transformation of the clitoris into a penis. The male penis is expected to be fully masculinized and anatomically similar to the penis of the broad-footed mole. The difficulty in external identification of the sex of several mole species, such as the star-nosed and shrew-mole (*Neurotrichus gibbsi*), has resulted in the designation of "sex unknown" for many of the specimens in the Museum of Vertebrate Zoology at the University of California, Berkeley. Based on this and data from other mole species, I anticipated that there would be no statistically significant difference in anogenital distance or phallic length between males and females. In accord with previous studies, I postulate that the ovary of the non-breeding season female would have an ovarian interstitial gland of comparable size to the follicular ovarian portion probably containing medullary cords. I may find evidence of rudimentary epididymes near the ovarian interstitial gland portion as has been found in other mole species possessing an ovarian interstitial gland (Matthews, 1935; Jimenez et al., 1993; Sanchez et al., 1996).

Japanese shrew mole (*Urotrichus talpoides*). Carmona *et al.* (2008) reported that female Japanese shrew moles possess normal mammalian ovaries with typical ovarian epithelium and that this species lacks a "penile clitoris". As above, the anatomy of external

genitalia of this species will be accurately described for the first time so that the features of external genitalia of this species can be intelligently discussed. No other information concerning external genitalia, gonadal tissue, or blood hormones has been published. Male and female Japanese shrew moles from the breeding season were obtained from Dr. Shinohara of the University of Miyazaki, Japan. Based on previous work and on the Jost model, I hypothesize that the clitoris of the Japanese shrew mole would be markedly smaller than the male penis and that it would lack masculine characteristics in internal morphology. The penis would be typical of mammalian males and would be anatomically similar to the penises of the other mole species being studied. The breeding season female would possess a perforate vaginal opening and a typical mammalian ovary with normal ovarian epithelium.

Hairy-tailed mole (*Parascalops breweri*). There are currently no publications on the external genitalia, gonadal tissues, adrenal tissues, or blood hormone concentrations in this species. Two male and two female non-breeding season hairy-tailed mole specimens were provided by Dr. Ken Catania and Diana Bautista of Vanderbilt University. Though the sample size was small, the presence or absence of masculine traits in the morphology of the external genitalia is highly conserved across individuals in a species so that I believe my observations on this species will be representative and valid. I anticipate the external genitalia of male and female hairy-tailed moles will be similar to those of the broad-footed mole in that the male should possess the typical traits of a mammalian penis. I predict that the female hairy-tailed mole will possess a clitoris similar to that of the female broad-footed mole in that its anatomy should have some masculine characteristics.

Chapter 2: Morphology of the External Genitalia of Four Species of Moles

Introduction:

Most eutherian mammals possess external genitalia that are sexually dimorphic. Males have a penis that is traversed to near the tip by a urethra, and a scrotum that encloses the testes. The typical clitoris is usually markedly smaller than the penis (and frequently is "internally" situated with the urethra exiting near the clitoris and not at its tip). In addition, the clitoris is associated with an externally visible vaginal opening (at least during the breeding season).

This dissertation is focused on four species of moles. All are members of the family Talpidae, in the order Insectivora. Three of these species defy the conventionally obvious visual distinctions between males and females, i.e., both the "clitoris" and the "penis" are of substantial size, and females appear to urinate through the tip of the "clitoris". These three species include broad-footed moles (*Scapanus latimanus*), star-nosed moles (*Condylura cristata*) and hairy-tailed moles (*Parascalops breweri*). For these species, it is difficult for the casual observer to distinguish between males and females during the non-breeding season, when the vaginal opening of female moles is "closed." The fourth species examined in the present investigation is the Japanese shrew mole (*Urotrichus talpoides*). In Japanese shrew moles, the distinction between males and females is obvious, since the "penis" is much larger than the "clitoris", and the latter does not appear to have a urethra exiting its tip (Carmona et al., 2008). Thus, sexual dimorphism of the external genitalia in Japanese shrew moles appears to follow the typical mammalian pattern.

In the paragraph above, I have designated penis and clitoris as "penis" and "clitoris". As stated above, the reason for this convention is that over the years there has been considerable confusion in the terminology of male and female rodent/insectivore external genitalia (especially in moles). A critical goal of this dissertation is to provide explicit definitions for the terms, penis, clitoris, penile clitoris, urethra and prepuce.

Development of the Mammalian External genitalia. Initially, the embryonic ambisexual external genitalia are the same in both sexes and are composed of three perineal structures, the embryonic genital tubercle, genital swellings, and urogenital folds (Fig. 2.1). The genital tubercle lies cranial to the urogenital ostium, which is the opening of the urogenital sinus into the amniotic cavity. The urogenital folds are lateral to this opening followed by the more laterally situated genital swellings (human). In males, the genital swellings migrate caudally and fuse to form the scrotum. The genital tubercle lengthens, forming the penis. A urethral groove forms on the ventral aspect of the genital tubercle and is bounded laterally by the urogenital folds, now called the urethral folds. Distal to the urethral grove is a solid epithelial plate, the urethral plate, which extends distally into the glans penis. Canalization of the urethral plate extends the urethral groove distally. The urethral folds grow and fuse in the midline as the phallus elongates creating the tubular urethra with the urethra opening at the tip of the glans penis. In female development the genital tubercle enlarges to a much lesser extent to form the clitoris, the urogenital folds do not fuse but instead form the labia minora (human) on either side

of the urethral and vaginal openings. The genital swellings, which also do not fuse in human females, form the labia majora (Figs. 1.12 &. 2.1) (Moore and Persaud, 2003; Yamada et al., 2003). This description of external genitalia development, taken from the literature on development in humans, applies in large part to other mammals even though certain features (labia minora and labia majora) are characteristic of humans and not seen in other species.
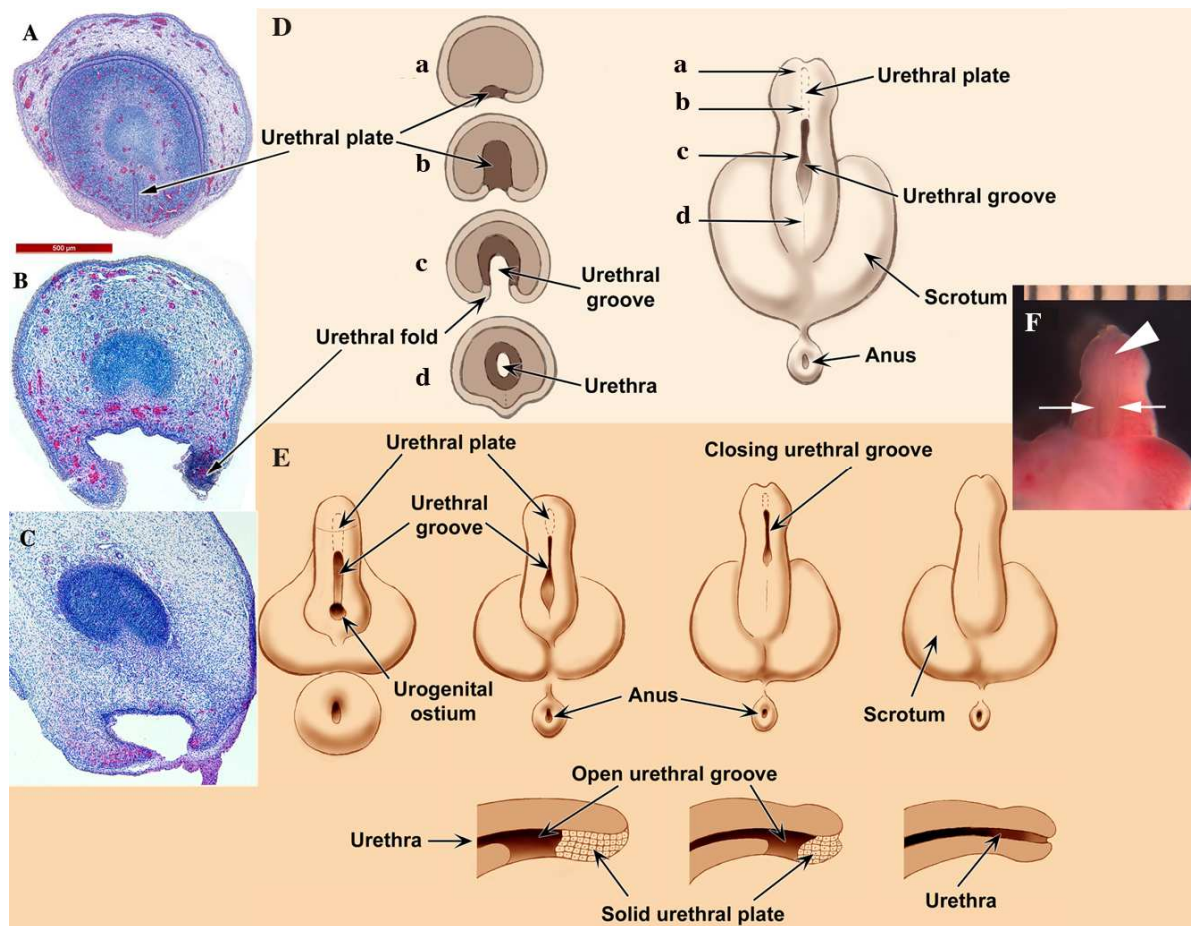
Figure 2.1. Human penile urethral development depicted in diagrams (D & E), transverse sections (A, B, C) and a photo (F). Note the solid urethral plate (A & a-b), open urethral groove (B & c), which terminates distally at the urethral plate. (C) shows initiation of epithelial fusion. Transverse sections at positions (a-d) illustrate the solid epithelial urethral plate (a & b) and its canalization (c). Fusion of the urethral folds and mesenchyme confluence across the midline is depicted in (d). Figure (E) depicts proximal to distal fusion of the urethral groove and distal "retraction" of the urethral plate. Transverse sections (A, B, C) and photo (D) are from a 12-week human fetal penis. (F) is a photo of a penis of a 12 week human fetus showing the open urethral groove (opposed small arrows) and the urethral plate (arrowhead).

The prepuce of both male and female mice emerge from preputial swellings that are initially located lateral to the embryonic genital tubercle (Cunha, 1975b). The preputial swellings grow to the midline and fuse with each other in both sexes and then grow distally to eventually completely cover embryonic genital tubercle, thus rendering the penis or clitoris as "internal organs" (Perriton et al., 2002; Petiot et al., 2005; Seifert et al., 2008). As the prepuce grows over the penis or clitoris, a layer of epithelial cells, the preputial lamina, demarcates the prepuce from the glans. In males, typically around the time of puberty, the preputial lamina canalizes to form a preputial space between the glans penis and the prepuce (Hunter, 1935; Kanagasuntheram and Anandaraja, 1960). This separation of the prepuce from the glans appears to be affected by androgens and has been used as an indicator of pubertal development (Korenbrot et al., 1977).

Internal Morphology of the Adult Mammalian Penis. The external and internal anatomy of the penis has been studied in only a small percentage of the more than 4,000 species of extant eutherian mammals. However, there appears to be a common basic architecture of the adult eutherian penis. A large portion of the interior of the penis is composed of the corpora cavernosa (corporal body), the corpus spongiosum or other erectile bodies. The bilateral corpora cavernosa are attached proximally to the pubic bones (Fig. 1.4) and are located dorsally within the penile shaft eventually fusing in the midline as they extend distally to form the corporal body (Fig. 1.10B). The corporal body is surrounded by a fibrous sheath, the *tunica albuginea* (Fig. 1.10B), and depending on the species, terminates at the os penis (Figs. 1.6B) or continues to near the distal end of the penile shaft but not into the glans (human). The corpus spongiosum is located ventral to the corpora cavernosa (Fig. 1.10), is often traversed by the penile urethra, and is usually surrounded by a fibrous sheath. The mouse homologue of the human corpus spongiosum is called the corpus cavernosum urethrae (Fig. 1.6B) (Rodriguez et al., 2011). Increased blood flow to these corporal bodies, constrained by the surrounding tunica, is responsible for penile erection. The glans of the human penis is a distal expansion of the corpus spongiosum. The penile urethra terminates, typically as a simple slit, at or near the tip of the glans in most species. In species such as rats, mice, guinea pigs, and cats the surface of the glans or the penile shaft is adorned with keratinized epithelial spines (Williams-Ashman, 1990).

Some eutherian species possess a bony structure in the penis called the os penis (Figs. 1.6, 2.2, 2.7, 2.14) (Burt, 1960; Best and Schnell, 1974; Patterson, 1983; Dixson, 1987; Verrell, 1992; Hosken et al., 2001; Miller and Burton, 2001; Whidden, 2001; Baryshnikov et al., 2003; Ferguson and Larivière, 2004; Lüpold et al., 2004). While the shape and size of the os penis varies greatly between species, it is usually located dorsal to the urethra (Figs. 1.6, 2.2, 2.7, 2.14) and distal to the corpora cavernosa. In mice the os penis is located within the glans. In mice and moles the flaccid penis is hidden inside the prepuce (Figs. 1.4, 1.5, 2.2, 2.7, 2.11, 2.13, 2.14, 2.17).

The Clitoris. In some species (human), only the tip of the glans clitoris is visible externally (Fig. 1.12), while in rodents/insectivores the clitoris is an "internal organ" not visible externally (Figs. 1.8A, 2.6, 2.12, 2.15). Within the clitoris, erectile bodies may be absent, poorly defined, or if present are much smaller than their penile counterparts (human) (Clemente, 1985). In the mouse clitoris well-defined erectile bodies are absent even though the clitoris is highly vascular (Fig. 1.6) (Weiss et al., 2012). While the clitoris and urethra are closely associated

anatomically, in the mouse and mole the urethra lies partly within the U-shaped clitoral lamina and partly ventral to the clitoris (Figs. 1.6, 1.8B-E, 1.9, 2.6, 2.7, 2.12, 2.15) (Weiss et al., 2012).

Androgens and Sexual Differentiation of the External genitalia. Since the embryonic ambisexual genital tubercle of eutherian fetuses initially is morphologically identical in males and females, fetal sex can be determined via morphology of the gonads, which differentiate several days before sex differentiation of the external genitalia. Development of male external genitalia is not dependent on genotypic sex but is determined through the action of androgenic hormones signaling through the androgen receptor. Thus, genetically male XY fetuses lacking androgens or lacking functional androgen receptors will develop female external genitalia (Ohno, 1979; Wilson et al., 1995). Examples, as described in Chapter 1, include the testicular feminized (Tfm) mouse with a mutation in the gene encoding the androgen receptor (Lyons and Hawkes, 1970). Another mutation affecting development of the external genitalia in males (XY) involves the gene encoding 5α-reductase. Such 5α-reductase-deficient individuals are unable to convert testosterone to 5α-dihydrotestosterone (DHT) in the target tissues, which leads to feminization of the internal and external genitalia (Moore et al., 1975; Imperato-McGinley et al., 1979). Accordingly, treatment of experimental animals *in utero* with 5α-reductase inhibitors (finasteride) or various anti-androgens (cyproterone acetate or flutamide), results in a reduced anogenital distance in males, hypospadias, a cleft prepuce, and reduction in penile size (Imperato-McGinley et al., 1985; Anderson and Clark, 1990; Clark et al., 1993; Silversides et al., 1995; Kojima et al., 2002). Similarly, mutations in genes encoding proteins critical for synthesis of androgens or androgen action will lead to variable degrees of feminization (Wilson et al., 1983b; Lee et al., 2007). Unlike males, where the presence of DHT is necessary for virilization of external genitalia, there is no corresponding hormone required for the development of the genitalia into a female phenotype. If a XX female fetus or neonate is ovariectomized, it will continue to develop typical female external genitalia (Jost, 1953; Rodriguez et al., 2012). However, the external genitalia of females can be virilized by endogenous or exogenous androgens since clitoral tissues express androgen receptors (Bentvelsen et al., 1995; Hughes, 1998; Al-Maghribi, 2007; Rodriguez et al., 2012).

Anogenital Distance: An index of "androgenization." Anogenital distance is the distance between the genital papillae and the center of the anus, and is typically assessed in neonates. In rodents and humans, anogenital distance has been found to be approximately twice the length in males than in females (Graham and Gandelman, 1986; Vandenbergh and Huggett, 1995; Gray et al., 1999; Wolf et al., 2002; Salazar-Martinez et al., 2004; Thankamony et al., 2009). Moreover, administration of androgens during critical stages of development has been shown to increase female anogenital distance to that of males of the species (Gandelman et al., 1979; vom Saal, 1979; Rhees et al., 1997; Wolf et al., 2002; Wolf et al., 2004). Conversely, the administration of anti-androgens to males of several species has resulted in reduction of anogenital distance similar to that of females (Clark et al., 1993; McIntyre et al., 2001; Wolf et al., 2004; Welsh et al., 2008). Specific data concerning anogenital distance and "phallus" length have been reported for the European mole and indicate that, wereas there is some slight overlap, anogenital distance and "phallus" length are greater in males than in females (Wood-Jones, 1914; Matthews, 1935). Some researchers concerned that males are often substantially larger than females of the same species have introduced various correction factors for anogenital distance in

order to account for differences in body size between males and females, or size differences between control and treatment groups. Such correction factors typically employ anogenital distance as a function of body weight, or body length (Graham and Gandelman, 1986; Vandenbergh and Huggett, 1995; Gallavan et al., 1999). However, in general, sex differences in anogenital distance are much greater than sex differences in body weight, or body length, and even when appropriate corrections are applied, researchers have consistently supported the utility of anogenital distance as an indicator of androgen action or "androgen blockade" *in utero* in laboratory animals and humans.

Experimental and Clinical Evidence for Androgenic Influence on Anogenital Distance. Female rodents exposed to an androgen, such as testosterone propionate (TP) *in utero* have a significantly increased anogenital distance, which can approach that of normal males (Gandelman et al., 1979; vom Saal, 1979; Rhees et al., 1997; Wolf et al., 2002; Wolf et al., 2004). This effect has been shown to be permanent and dose dependent (Wolf et al., 2002; Hotchkiss et al., 2007). In addition, male rodents treated with anti-androgens *in utero* have a reduced anogenital distance (Clark et al., 1993; McIntyre et al., 2001; Wolf et al., 2004; Welsh et al., 2008). All of the above manipulations support the claim that androgens are the primary cause of sexual dimorphism of anogenital distance. Therefore, the degree of anogenital distance sexual dimorphism can be an indicator of androgen action *in utero*. The human clinical literature is also in accord with such effects in that females with congenital adrenal hyperplasia are virilized by endogenous androgens and have an increased anogenital distance (Callegari et al., 1987). European moles exposed *in utero* to testosterone propionate had an identical anogenital distance and "phallus" length in male and female offspring similar to that of untreated males (Godet, 1946), which suggests that the exogenous androgen increased anogenital distance in females to the male length.

Anogenital Distance and the Uterine Position Effect. While anogenital distance can be influenced by hormone manipulations or mutations affecting steroidogenesis, naturally occurring low levels of androgens in female fetuses arising from neighboring male fetuses have been shown to have measurable masculinizing effects on anogenital distance. Hormones can travel between adjacent fetuses (Meisel and Ward, 1981; Even et al., 1992; Vom Saal and Dhar, 1992). Accordingly, female fetuses developing between 1 male and 1 female (1M), between 2 males (2M), or between 2 females (0M) are exposed to different levels of sex steroids. Since sexual differentiation is mediated primarily by androgens early in development, elevated levels of androgens in 2M females causes development of masculine traits relative to 1M or 0M female fetuses. Intrauterine position affects behavior, physiology, as well as morphology (Ryan and Vandenbergh, 2002). In rodents, 2M females have a longer, more masculine, anogenital distance than 0M females (McDermott et al., 1978; vom Saal and Bronson, 1978; Richmond and Sachs, 1984; Vandenbergh and Huggett, 1995). Likewise, prenatal treatment with the anti-androgen flutamide abolishes this anogenital distance intrauterine position effect supporting the theory that increase in anogenital distance in 2M females is due to increased testosterone levels *in utero* (Clemens et al., 1978). This intrauterine position effect on anogenital distance is not an artifact of the genetic homogeneity inherent in laboratory animals, which may reduce natural variation in this trait allowing for low levels of androgens to have a noticeable effect. The wild female house mouse (*Mus musculus*), California mouse (*Peromyscus californicus*), and female domestic rabbit (*Oryctolagus cuniculus*) also show a greater anogenital distance in 2M females or in females

from male-biased litters (Zielinski et al., 1991; Palanza et al., 1995; Banszegi et al., 2009). Thus, low levels of androgens received by female fetuses via proximity to male fetuses have a measurable effect on the external genitalia morphology, specifically anogenital distance, in multiple species. This supports the use of anogenital distance as an indicator of masculinization caused by androgen action *in utero*.

Sexual Dimorphism In the External Genitalia of Moles. Previous publications on moles have measured the length of the external genitalia and termed it the "phallic", "clitoral", "penile clitoral" or "penile" length. As mentioned in Chapter 1, the perineal appendage projecting outward from the body is not the "phallus", "penis", or "penile clitoris", but instead is the prepuce in both male and female moles as discussed above. In both sexes the actual external genitalia (penis and clitoris) are "internal" organs residing deep within the preputial space (Fig. 2.2, 2.6, 2.11, 2.12, 2.14, 2.15, 2.17). Henceforth, "phallus," "penis," and "clitoris" in quotation marks will refer to incorrect designations from the literature, while the absence of quotation marks will represent our revised designation of these terms.
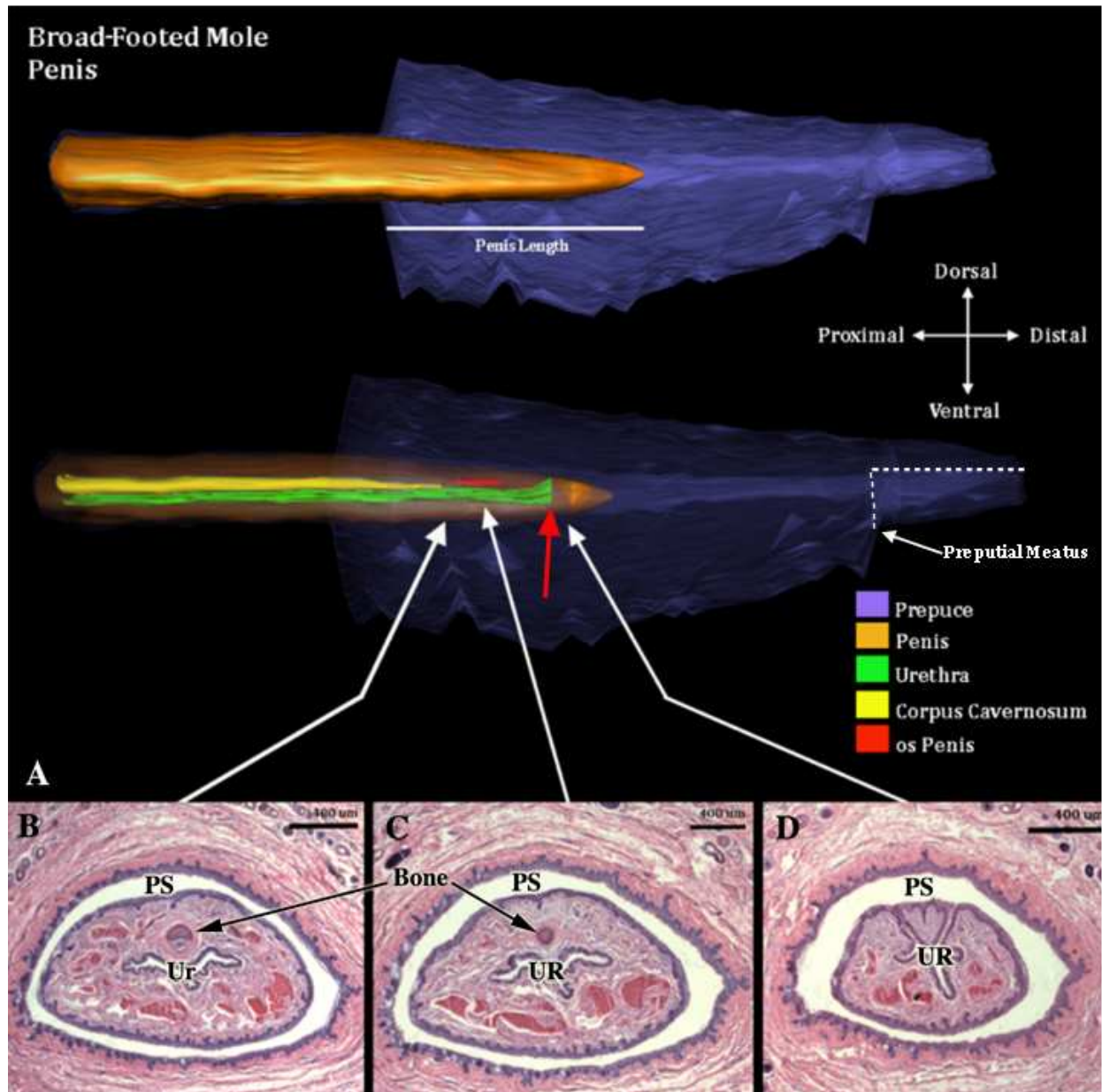
Figure 2.2. Three-dimensional reconstructions (A) of the adult broad-footed mole penis with transverse H&E stained sections (B-D). The reconstructions have been artificially elongated to better show the shapes and locations of the structures. The top figure is a side view of the reconstruction with the prepuce partially transparent showing the placement of the penis. The white line in the top figure labeled penis/prepuce overlap represents the amount of the penis external to the body wall that resides within the prepuce. Note that the distal tip of the penis is situated a considerable from the distal tip of the prepuce. The lower three-dimensional reconstruction is also a side view with the prepuce and penile surface epithelium partially transparent showing the structures inside the penis. The white dotted lines represent the length of the open ventral preputial groove with an arrow indicating the preputial meatus. White arrows indicate the corresponding locations of the H&E transverse sections. Histological sections (B-D) are in order from proximal to distal. Note in (D) that the urethra (UR) opens into the preputial space (PS). Red arrow in (A) denotes the opening of the urethra into the preputial space.

Multiple species of female moles have been described incorrectly as possessing a "penile clitoris", even though the Japanese shrew mole (*Urotrichus talpoides*), lacks a "penile clitoris" (Carmona et al., 2008). Female moles generally demonstrate seasonal vaginal opening that exists for only the period of mating and parturition before closing for the duration of the non-breeding season. In the literature, the "penile clitoris" of the European mole (*Talpa europea*) has been reported incorrectly to be traversed by a urethra, which exits near the tip of the "phallus" similar to the male (also incorrect). In agreement with the typical mammalian phenotype, anogenital distance and "phallus" length is greater in male versus female European moles (Wood-Jones, 1914; Matthews, 1935). Broad-footed moles (*Scapanus latimanus*) have also been shown to possess a prominent "phallus," which is traversed by the "urethra". However, in this species there was no significant difference in "phallic" length (actually preputial length) or anogenital distance in males and females (Rubenstein et al., 2003).

Development of the penis and penile clitoris has been investigated in the European mole. Wood-Jones found that elongation of the genital tubercle, urethral groove closure, and the formation of the prepuce take place in exactly the same manner in both sexes during embryonic development (Wood-Jones, 1914). Godet discovered that male and female offspring of pregnant European moles treated with testosterone propionate had an indistinguishable anogenital distance and "phallus" length (Godet, 1946).

Several, but not all, mole species possessing a "penile clitoris" also have an ovarian interstitial gland (Mossman and Duke, 1973; Carmona et al., 2008). In Spanish and European adult moles circulating levels of testosterone correlated with ovarian interstitial gland weight, with higher concentrations of testosterone during the non-breeding season when the ovarian interstitial gland was largest (Jimenez et al., 1993; Whitworth et al., 1999; Zurita et al., 2003). The presence of the ovarian interstitial gland and its production of androgens are in accord with the Jost theory of sexual differentiation, and the possibility that the "penile clitoris" in these species is formed as the result of androgens produced by the ovarian interstitial gland of the mother or developing fetus. However, the broad-footed mole and the eastern mole (*Scalopus aquaticus*) possess a "penile clitoris" but lack an ovarian interstitial gland (Rubenstein et al., 2003). So either the "penile clitoris" (prepuce) in these species may be caused by androgen production from some other source, or the development of the "penile clitoris" in these species is androgen-independent.

Review of Penile and Clitoral Morphology: Indices of Sexual Dimorphism. The penis and clitoris of typical mammals are strikingly different, anatomically complex organs composed of epithelial tissue, connective tissue, vascular tissue, nerves, cartilage, and bone that are organized into specific and precise morphological arrangements. The common developmental history, architecture, and composition of the penis and clitoris across most mammalian species allow for multiple features to be used to assess sexual dimorphism between the clitoris and penis. A list of key homologous features that are present in the penis but lacking in the clitoris are presented in recent papers from the Baskin lab (Yang et al., 2010; Rodriguez et al., 2011; Weiss et al., 2012), who created such lists for use in studies of wild-type and mutant strains of mice. In this dissertation, a table of similar homologous traits (See Table 2.1) has been prepared. However, two traits (that were used in mouse studies) were omitted as epithelial spines and cartilage are not observed in either males or females of any species of mole under study in my project.

Employing this scale, a penis with all nine features would represent a typical fully masculinized mammalian penis, and a clitoris lacking all of these features would represent a typical mammalian clitoris as is the case for the Japanese shrew mole. The size and location of several key features were noted with the aid of 3D reconstructions for a more detailed comparison between the different mole species as described below. As can be seen from this analysis of male/female sexually dimorphic features (Table 2.1), whereas the Japanese shrew mole exhibits a profound male/female dichotomy (score of 8 [male] versus 0 [female]), females of the other mole species exhibit variable degrees of masculine traits.

Table 2.1 Sexually dimorphic features in mole external genitalia.

Typical Male Traits (1)	Typical Female Traits (0)	BFM Male	BFM Female	SNM Male	SNM Female	HTM Male	HTM Female	JSM Male	JSM Female
Circular transverse profile	U shaped lamina	1	0	1	0	1	0	1	0
No tethering, freely mobile organ	Ventral tethering/ immobile	1	0	1	0	1	0	1	0
Urethra completely within penis	Urethra never entirely within clitoris	1	0	1	0	1	0	1	0
Os penis	No os clitoris	1	0	0	0	1	1	0	0
Located in preputial space	Not located in epithelium-lined space	1	0	1	0	1	0	1	0
Distinct corpus cavernosa	Diffuse erectile tissue	1	1	1	1	1	1	1	0
Thick tunica	No tunica	1	1	1	1	1	1	1	0
Large organ size	Small organ size	1	1	1	1	1	1	1	0
Tip free	Tip tethered	1	0	1	1	1	1	1	0
Totals:		9	3	8	4	9	5	8	0

In addition to these internal anatomical measurements, anogenital distance, a trait that is modulated by androgen action *in utero*, was used as another measure of "genital masculinization". Measures of prepuce length, termed "phallus" length in previous publications, were used to investigate the degree of sexual dimorphism in the external genitalia of European and broad-footed moles and are incorporated into my study.

General Reproductive Anatomy of the Four Species Under Study:

Broad-footed mole (*Scapanus latimanus*). The ovaries of adult broad-footed moles lack an ovarian interstitial gland (Mossman and Duke, 1973). Rubenstein *et al.* confirmed the lack of an interstitial gland or medullary cords during both the breeding and non-breeding seasons (Rubenstein et al., 2003). However, their investigation of the clitoral anatomy showed that females possess a prominent "phallus" which is traversed by the "urethra". Male mean body length was greater than that of females, but there was no significant difference in "phallic" length or anogenital distance in males and females. However, no distinction was made between adult and juvenile animals in these measures (Rubenstein et al., 2003).

Star-nosed mole (*Condylura cristata*). Star-nosed moles have an ovary that is polar, with follicles at one end and interstitial tissue containing medullary cords at the other, termed the ovarian interstitial gland, or "ovotestis." Females of this species were also reported to display a prominent "phallus" traversed by the urethra (Mossman and Duke, 1973; Rubenstein et al., 2003).

Hairy-tailed mole (*Parascalops breweri*). There are currently no publications on the external genitalia or gonadal tissues of this species. However, female hairy-tailed moles possess a prepuce of similar size to that of the male.

Japanese shrew mole (*Urotrichus talpoides*). Carmona *et al.* reported that female Japanese shrew moles possess normal mammalian ovaries with typical ovarian architecture. This species lacks a "penile clitoris" and an interstitial ovarian gland (Carmona et al., 2008). Thus, the Japanese shrew mole is currently the only known species of mole to lack a "penile clitoris" and also lack an ovarian interstitial gland.

Final Comments

Ideally, this dissertation would include studies of the development of the external genitalia and the differentiation of the ovarian interstitial gland through the study of embryos at different stages of development. Unfortunately, the inaccessibility of trapping sites, due to ice and snow for the star-nosed and hairy-tailed moles during their breeding season prevented this option and prevented access to adult tissues from the breeding season. Finding pregnant broad-footed moles during the breeding season was also unsuccessful. This is in part be due to the lack of any effective live capture traps that can be placed underground inside a mole tunnel. Multiple individuals were recruited from four different golf course locations around the San Francisco Bay Area to assist in the capture of live broad-footed moles. Over the course of three breeding seasons, only three females were captured and none was pregnant. Little is known about mole breeding habits. Perhaps there is a reduction in digging new tunnels during this time, and currently the appearance of new mole hills is the only effective cue for locating the presence of a mole for successful capture. Early attempts at maintaining broad-footed moles in captivity were not possible, a problem that is not unique to this species (as revealed by correspondence with other researchers studying moles). To my knowledge, broad-footed moles have not bred in captivity. I was fortunate to be able to obtain Japanese shrew moles from the breeding season, but they were not pregnant. Our collaborator, Dr. Akio Shinohara, did not return to the field for trapping in the non-breeding season. Nonetheless, the research on the broad-footed mole, star-nosed mole, Japanese shrew mole, and hairy-tailed mole, as presented in this dissertation, offers a more comprehensive account of the reproductive morphology and endocrinology of these species than has been available to date.

Methods:

Subjects:

Mice. Adult wild-type CD-1 mice (Charles River Breeding Laboratories, Wilmington, MA, USA) were housed in polycarbonate cages (20 X 25 X 47 cm^3) with laboratory grade pellet bedding. Mice were given Purina lab diet and tap water ad libitum and sacrificed at 60 days of age. The Institutional Animal Care and Use Committee at the University of California, San Francisco approved all animal use protocols. The external genitalia were photographed to identify typical surface characteristics with a digital camera and a dissecting microscope. External genitalia were dissected and fixed in formalin, paraffin embedded and serially sectioned (transversely and longitudinally) at 7µm for histologic staining.

Penile surface details were elucidated via scanning electron microscopy (SEM). External genitalia were dissected and fixed in 2% glutaraldehyde 0.1M sodium cacodylate buffer at pH of 7.2 for 6 hours. The specimens were then fixed in 2% osmium tetraoxide for 2 hours, subsequently dehydrated in serial alcohol solutions and critical point dried in a Tousimis AutoSamdri 815 Critical Point Dryer (Tousimis, Rockville, MD). The specimens were then mounted on a stub with carbon tape, and images were obtained using a Hitachi TM-1000 Scanning Electron Microscope (Hitachi High Technologies America, Inc. Pleasanton, CA).

Broad-footed mole (*Scapanus latimanus*). Live broad-footed moles were obtained from golf courses in San Francisco and Moraga, California (Moraga Country Club, San Francisco Golf Club, Harding Park Golf Club, and the Presidio Golf Course) between 2008 and 2010. Moles were euthanized using 0.05 mL of Euthasol diluted with saline (from Virbac Animal Health Inc., 390mg Pentobarbital sodium and 50mg phenytoin sodium per mL), weighed and measured for head-body length and tail length. Animals were either perfused with 4% paraformaldehyde before dissection, or the lower torso was cut open to expose the reproductive organs and immersed in fixative. After a minimum of 48 hours of fixation, tissues were transferred sequentially through 30%, 50%, and hence to 70% ethanol before being stored at room temperature in 70% ethanol.

Males were divided into three categories: adult breeding season males (bsM), adult non-breeding season males (nbsM), and juvenile non-breeding season males (juvM). Seasonal classification was based on testicular weight and sperm production. The difference in testicular by weight from the breeding season to the non-breeding season is significant (mean ±stdev, bsM=0.386 ±0.42g, nbsM=0.014 ±0.017g, bsM n=2, nbsM n=12, F=581.288, p<0.0001). Histological examination of testes revealed breeding season males to have larger more developed interstitial cells and large seminiferous tubules containing developing sperm than did non-breeding individuals. Classification of males as either adult non-breeding season or juvenile was also determined by histological examination of preputial separation, the separation of the prepuce from the penis. Preputial separation has been found to be androgen-dependent and to occur near the onset of puberty (Korenbrot et al., 1977).

Females were also divided into three categories: adult breeding season females (bsF), adult non-breeding season females (nbsF), and juvenile non-breeding season females (juvF). As has been

previously noted by other researchers, the vagina was imperforate in all females except for those trapped in December, January, and February (Fig. 2.3) when an opening could clearly be observed visually (Rubenstein et al., 2003). This is consistent with the breeding season for this species, and accordingly these animals were categorized as breeding season adults (Gorman and Stone, 1990). Additionally, changes in uterine and oviductal size were used to classify females as breeding season, non-breeding season, or non-breeding season juveniles. Juvenile females were those just born in the last breeding season. Accordingly their uteri and oviducts are thin, semi translucent, and underdeveloped (not illustrated). Uteri and oviducts of adult breeding season females are significantly larger, more vascularized, and better developed, which coincided with vaginal patency. Non-breeding season adult females were classified as animals that are at least 1 year old, meaning that they had already gone through at least one breeding season. Thus, their uteri and oviducts have under gone the large increase in size during the breeding season. These structures do not regress in the non-breeding state, and thus can be distinguished from the uteri and oviducts of juvenile females. Females lacking a vaginal opening and whose uterus and oviducts were in an intermediate state between juvenile and breeding adult females were classified as non-breeding adult females. The difference in the diameter of the uterus and oviducts between the three age classifications is significant (Uterus: mean ±stdev, bsF=5.133 ±0.987μm, nbsF=2.675 ±0.497μm, juvF=1.254 ±0.559μm; bsF n=3, nbsF n=4, juvF n=13; F=50.441, p<0.0001) (Oviduct: mean ±stdev, bsF=3.450 ±0.304μm, nbsF=2.150 ±0.173μm, juvF=1.046 ±0.357μm; F=72.664, p<0.0001).
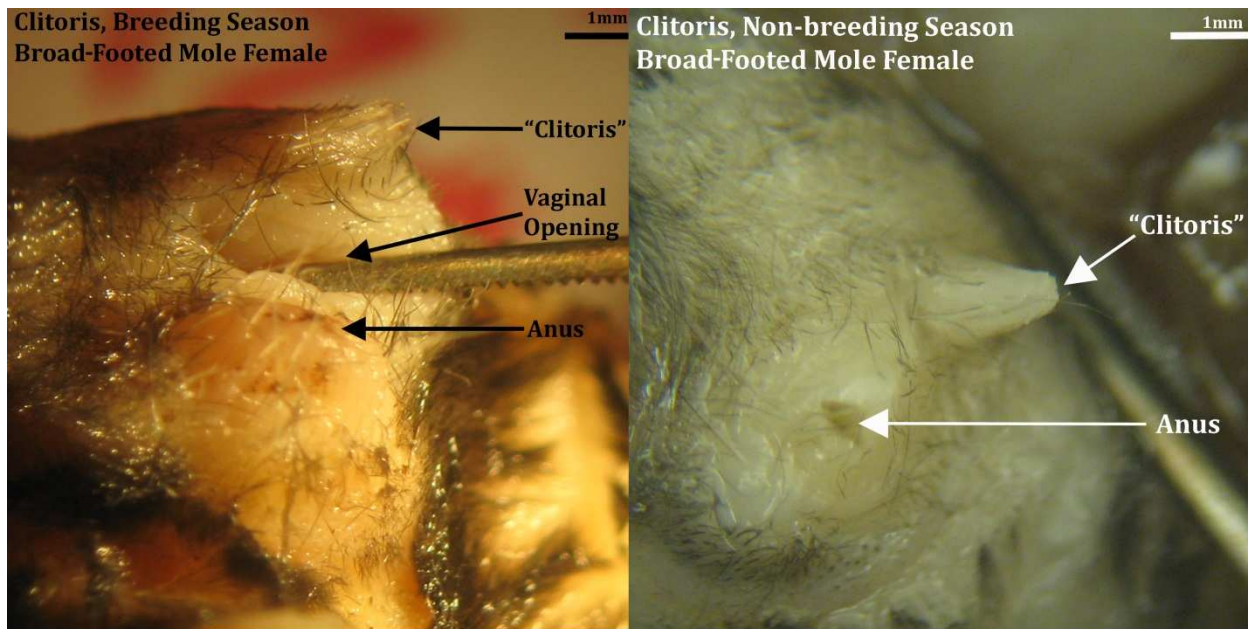

Figure 2.3. Broad-footed mole female external genitalia from the breeding and non-breeding seasons showing the presence and absence of the vaginal opening. Note that the term clitoris is placed in quotation marks as what is actually seen in these images is the prepuce that covers the clitoris.

Total number of animals in each category:

	Breeding Males	Breeding Females	Non-Breeding Adult Males	Non-Breeding Juvenile Males	Non-Breeding Adult Females	Non-Breeding Juvenile Females
Sample Size (n)	2	3	3	10	4	13

Star-nosed mole (*Condylura cristata*). Star-nosed moles were trapped in Emporium, PA by Dr. Kenneth Catania of Vanderbilt University and Dr. Diana Bautista of University of California, Berkeley as part of studies on the somatosensory physiology of this species. All animals were caught in June or July, during the non-breeding season, and females did not display a perforate vagina (Eadie and Hamilton, 1956). Moles were either euthanized and fixed immediately or were housed at Vanderbilt University for 1-3 months before euthanasia and fixed in 4% paraformaldehyde. After a minimum of 48 hours for fixation, tissues were transferred sequentially through 30%, 50%, and hence to 70% ethanol before being stored at room temperature in 70% ethanol. Total sample size: males n=7, females n=9.

Hairy-tailed mole (*Parascalops breweri*). Hairy-tailed moles were also trapped in Emporium, PA by Dr. Kenneth Catania of Vanderbilt University and Dr. Diana Bautista of University of California, Berkeley. Trapping of this species was incidental to their work on star-nosed moles, and no additional animals were obtained in future trapping attempts. All animals were caught in June or July. Females did not display a perforate vagina indicating they were in a non-breeding state consistent with the previous report of the late winter to early spring breeding season of this species (Eadie 1939). Moles were euthanized and fixed immediately in 4% paraformaldehyde with the lower torso cut open to expose the reproductive organs to fixative. After a minimum of 48 hours for fixation, tissues were transferred sequentially through 30%, 50%, and hence to 70% ethanol before being stored at room temperature in 70% ethanol. Total sample size: males n=2, females n=2.

Japanese shrew mole (*Urotrichus talpoides*). Japanese shrew moles were collected in Kagamisu, Miyazaki, Japan in March 2008 by Dr. Shinohara from the University of Miyazaki, Japan. Upon capture animals were euthanized, the abdominal cavity was opened to facilitate penetration of the 4% paraformaldehyde before being transferred to 70% ethanol and shipped to our laboratory. The animals were determined to be in a reproductively active state based on the perforate vagina in the females, the presence of developing sperm in the male testis and fully developed sperm in the epididymes. Based on these observations as well as the advice by Dr.

Shinohara, these animals were classified as breeding season individuals. Total sample size: males n=2, females n=2.

<u>Histological Analysis</u>. After fixation and transfer to 70% ethanol the internal reproductive organs were photographed in situ before being removed. Ovaries were removed with all immediate surrounding tissue left intact so as to prevent damage to the ovarian interstitial gland or other structures of interest. External genitalia were photographed after the hair surrounding the external genitalia had been plucked or trimmed under a dissecting microscope to allow a clear view. After photography, the external genitalia were dissected from the rest of the reproductive tract. All dissected tissues were subsequently stored separately in 70% ethanol at room temperature. Prepuce length was measured using electronic calipers to the nearest 0.01mm, from the tip of the phallus to where the mid portion of the structure connected with the body (Fig. 2.4), and the mean value for each individual was used for all further analysis. Anogenital distance was also measured using electronic calipers from the center of the anal sphincter to the middle of the genital shaft at the point where it connected to the body, and the mean value for each individual was used for all further analysis (Fig. 2.4). In female Japanese shrew moles anogenital distance was measured from the center anus to the skin on the opposite side of the vaginal opening since this species does not possess a penile clitoris. For histological analysis dissected genital tissues were dehydrated in a graded ethanol series, embedded in paraffin, and serially sectioned at 10µm for morphological analysis. Every 5th or 10th section was stained with hematoxylin and eosin (Harris Hematoxylin and Eosin Y Stain from American MasterTech Scientific Inc. Lodi, CA) according to standard procedures and was photographed through a microscope at 10x using a Cannon powershot A590 IS digital camera. Relevant structures from the photographed slides of one representative male and one female of each species were traced using Photoshop CS3. Traced lines were then used to create 3-dimensional reconstructions using Winsurf 3D software. Measurements of internal structures were achieved by tracking the structural changes in the serial sections of all specimens. The distal tip of the prepuce was used as the starting point and serial sections were counted as they proceeded proximally in the tissue. Measurements were taken when new structures, like bone, appeared or ended providing measurements as to placement and length of the structures within the phallus. The external length of the penis or clitoris, that portion of these organs projecting beyond the body wall and thus over-lapped by the prepuce (also called penis/prepuce overlap), was measured from distal penile or clitoral tip to the point where the external prepuce became contiguous with the body wall (Figs. 2.2, 2.6, 2.11, 2.12, 2.14, 2.15, 2.17).
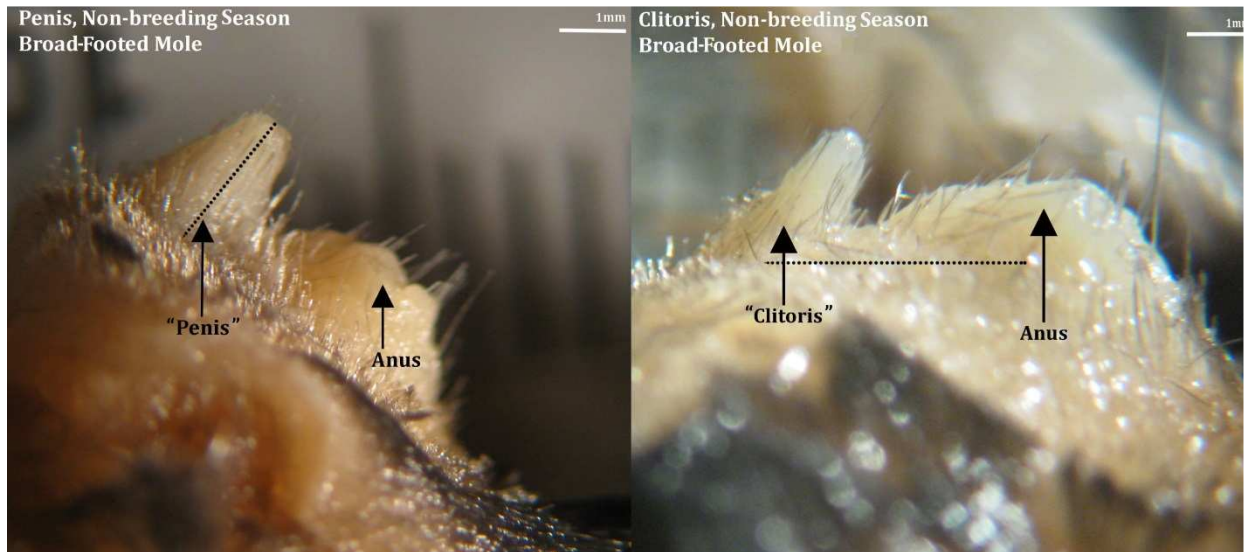
Figure 2.4 Broad-footed mole male and female external genitalia from the non-breeding season. Note that the structures labeled "penis" and "clitoris" are actually the prepuces of male and female moles, the penis and clitoris residing inside. The dotted line on the male image indicates the measurement with calipers for preputial length. The dotted line on the female image indicates the measurement with calipers for anogenital distance.

Results:

Mice:

Prepuce Length. Preputial length was determined from photographs of 8 male and female CD-1 mice (Fig. 2.5). Average male preputial length was 3.42mm (±0.17), while female preputial length was 2.14mm (±0.25), and this difference was statistically significant (Male n=4, Female n=4, $t(5)=8.697$, $p=0.0003$). However, male Cd-1 mice are larger than females. When prepuce length was normalized to body weight the difference in male versus female preputial length was no longer significant (Male n=4, Female n=4, $t(6)=1.801$, $p=0.1218$).
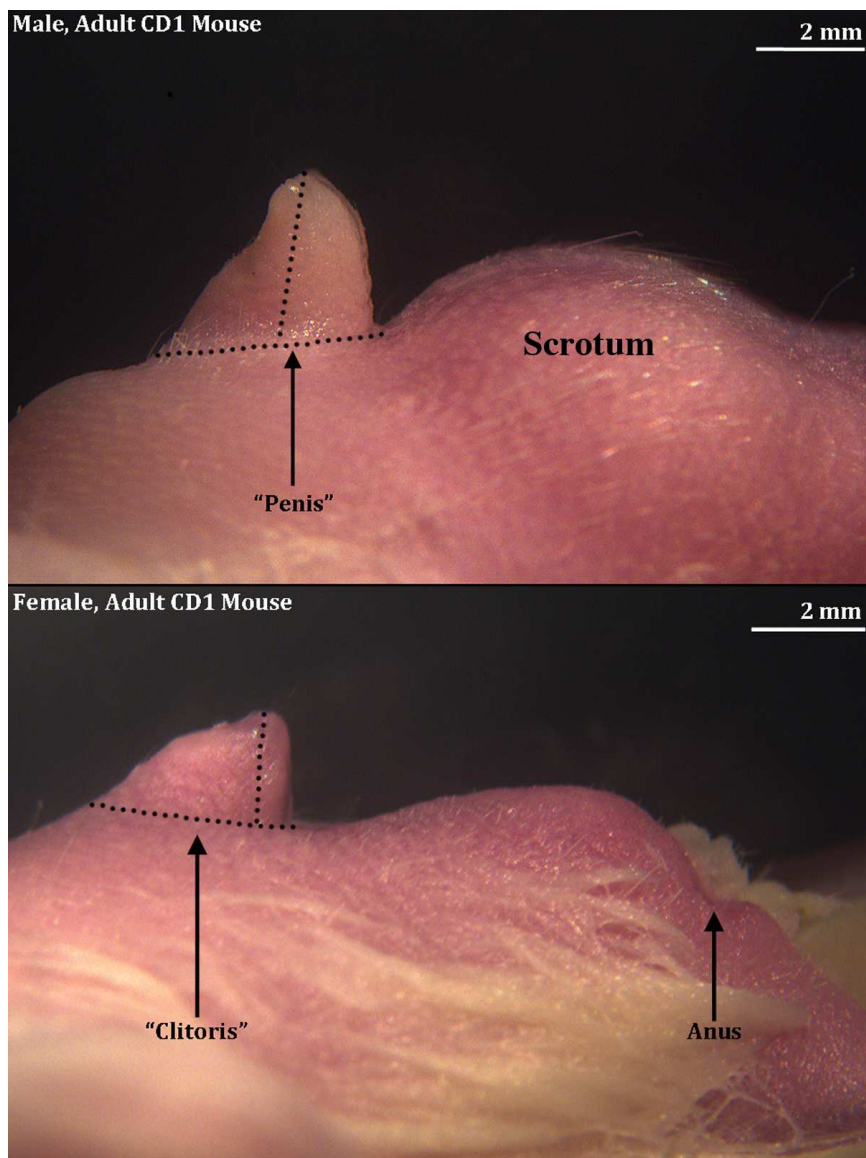
Figure 2.5. Side views of the male and female mouse prepuce. Horizontal lines demarcate the junction of the prepuce with the body surface. Vertical lines indicate the length of the prepuce. Preputial length is slightly longer in male versus female mice.

Broad-footed moles:

Body Weight and Body Length. Broad-footed mole body weight shows sexual dimorphism between males (mean 65.39 ±10.26g) and females (58.85 ±8.37g) (M n=20, F n=22, F=5.162, p=0.028). However, female body weight does not change by age (adult versus juvenile) or season (breeding versus non-breeding) (mean bsF=57.77 ±6.28g, nbsF=59.85 ±8.74g, juvF=54.83 ±6.62g, bsF n=3, nbsF n=4, juvF n=13, F=0.866, p=0.438). In males there is no difference in body weight between adult breeding and adult non-breeding season males, nor between adult breeding and juvenile non-breeding season males (mean bsM=77.15 ±6.86g, nbsM=74.73 ±9.12g, juvM=63.99 ±8.06g, bsM n=2, nbsM n=4, juvM n=10, F=3.743, p=0.052). However, when comparing only non-breeding adult and non-breeding juvenile males, there was a measurable difference in body weight (nbsM mean=74.73 ±9.12g, juvM mean=63.99 ±8.06g, p=0.0462). Body length measurements are monomorphic in broad-footed mole males (mean=140.69 ±14.30mm) and females (mean=141.77 ±10.57mm) (M n=18, F n=20, F=0.070, p=0.793). This monomorphism does not change based on female age or reproductive season (mean bsF=142.33 ±14.31mm, nbsF=136.78 ±4.82mm, juvF=143.40 ±6.87mm, bsF n=3, nbsF n=4, juvF n=11, F=1.025, p=0.383) nor male age or reproductive season (mean bsM=133.55 ±2.76mm, nbsM=160.00 ±9.54mm, juvM=143.08 ±15.38mm, bsM n=2, nbsM n=3, juvM n=9, F=2.559, p=0.122). This differs from the measures of body length found by Rubenstein *et al.* where female broad-footed moles were found to be significantly smaller than males (p<0.05). However, the body length measurements used by Rubenstein *et al.* included the tail length, while my measurements were from the snout to the base of the tail and not the tip of the tail (Rubenstein et al., 2003).

Prepuce Length. Female broad-footed moles possess a prominent prepuce that to the eye appears similar in size and shape to the male prepuce (Figs. 2.4). Prepuce length was measured twice for each animal and the mean value was used. There was no significant difference in preputial length between broad-footed males and females (mean, male=2.635 ±0.389mm, female=2.747 ±0.331mm) (M n=12, F n=18, F=0.713, p=0.406). No difference was observed between adult females and juvenile females (mean bsF=2.837 ±0.206mm, nbsF=2.942 ±0.379mm, juvF=2.665 ±0.360mm, bsF n=3, nbsF n=4, juvF n=11, F=1.032, p=0.380) nor between adult males and juvenile males (mean bsM=2.705 ±0.389mm, nbsM=2.560 ±0.071mm, juvM=2.701 ±0.463mm, bsM n=2, nbsM n=2, juvM n=7, F=0.092, p=0.913). In an attempt to control for body size as a confounding factor, prepuce length was normalized to either body weight (PLI 1) or body length (PLI 2). In the case of analysis by body weight (PLI 1), prepuce length showed a difference between broad-footed males and females with females having a slightly longer prepuce than males (M n=12, F n=18, F=4.774, p=0.038). There was no difference in PLI 1 between adult females and juvenile females (bsF n=3, nbsF n=4 juvF n=11, F=0.847, p=0.453) nor adult and juvenile males (bsM n=2, nbsM n=2, juvM n=7, F=0.149, p=0.865). When analyzed by body length (PLI 2) there was no difference between males and females (M n=11, F n=17, F=0.040, p=0.844) nor between adult females and juvenile females (bsF n=3, nbsF n=4, juvF n=10, F=0.542, p=0.596) or adult and juvenile males (bsM n=2, nbsM n=2, juvM n=6, F=0.730, p=0.537).

Penile/Preputial & Clitoral/Preputial Overlap. Penile/preputial or clitoral/preputial overlap denotes that portion of the penis or clitoris that projects beyond the body surface in the

resting state and thus is situated within the male or female prepuce (Figs. 2.2 & 2.6). This was obtained by measuring in serial sections the distance from the distal tip of the penis or clitoris to the point where the external prepuce became contiguous with the body wall. Clitoral/preputial overlap of the broad-footed mole is on average 1200μm (stdev=147) (Fig. 2.2), while penile/preputial overlap is 1475μm (stdev=177) (Fig. 2.6). Thus, the portion of the penis or clitoris that projects beyond the body surface is not significantly different between the sexes (M n=4, F n=5, F=4.190, p=0.110).

Additionally a percentage was calculated of the amount of penile/preputial or clitoral/preputial overlap relative to total preputial length. By this convention penile/preputial overlap represents less than half of total preputial length (41.07%, stdev=0.12%) in broad-footed male moles (Fig. 2.2). In contrast in female broad-footed moles clitoral/preputial overlap relative to total preputial length was 43.15% (stdev=8.48%) (Fig. 2.6). The difference between males and females is not significant (M n=4, F n=5, F=0.107, p=0.761).
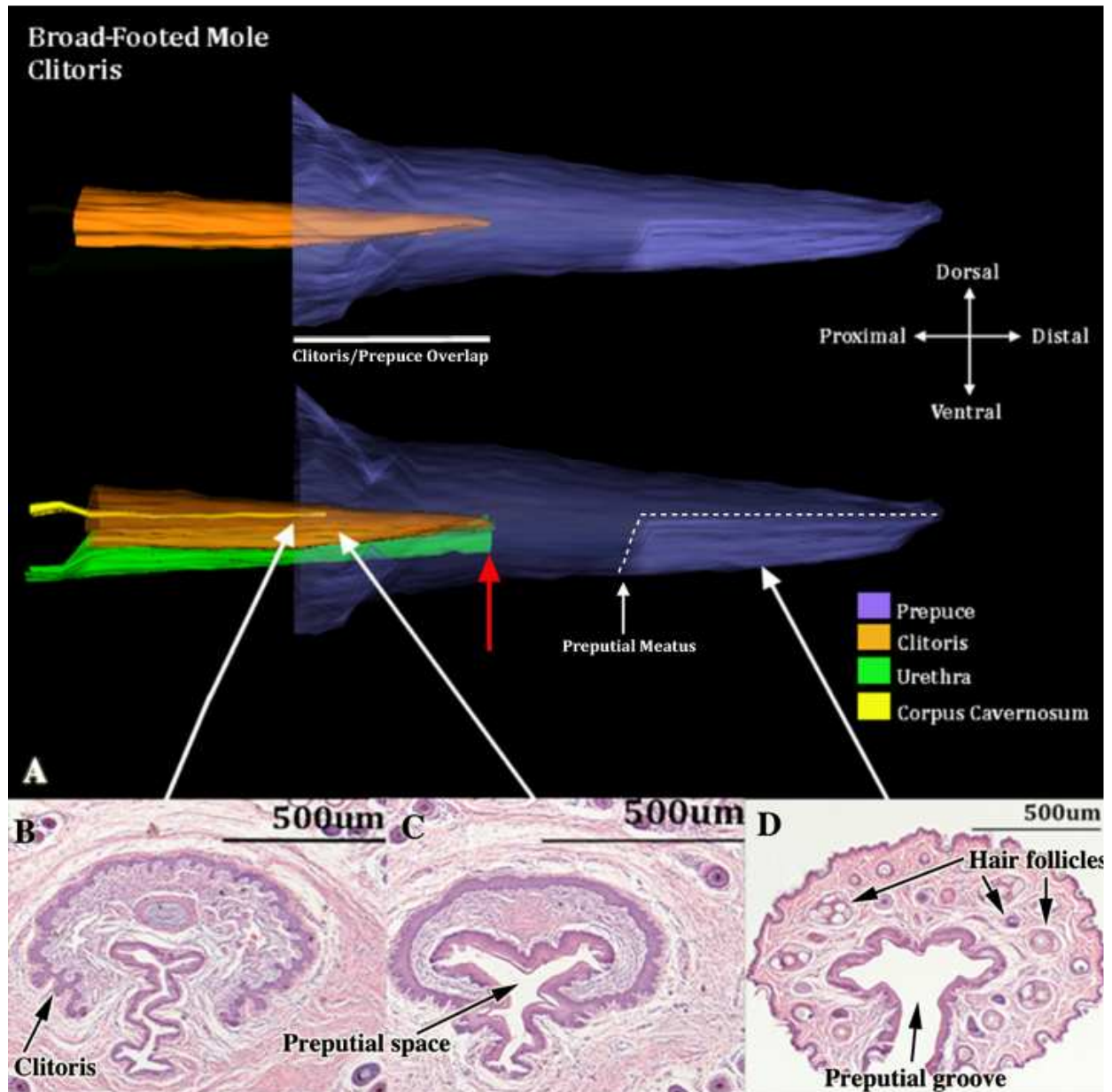
Figure 2.6 Three-dimensional reconstructions (A) of the adult broad-footed mole clitoris with transverse H&E stained sections (B-D) in order from proximal to distal. The components of the reconstructions have been artificially elongated to better show the shapes and locations of the structures. The top figure in (A) is a side view of the reconstruction with the prepuce partially transparent showing the placement of the clitoris. The lower three-dimensional reconstruction in (A) is also a side view with the prepuce and clitoris semi-transparent showing the urethra and corpus cavernosum. The white dotted lines represent the length of the open ventral preputial groove with an arrow indicating the preputial meatus. White arrows indicate the corresponding locations of the H&E transverse sections (B-D). (D) is a distal section showing prepuce only, which has a ventral preputial groove. Note hair follicles. In (B) note that the clitoris and urethra are separate structures. The urethra is partially circumscribed by the clitoral lamina. In (C), a transitional region, the internal space is appropriately labeled preputial space since the "clitoral lamina" and "urethra" are still fused. Red arrow in (A) denotes the opening of the urethra into the preputial space.

Preputial Attachment. As discussed previously the penis resides within the preputial space, is not attached or tethered to the prepuce in adults except where the inner preputial epithelium reflects onto the penile surface deep within the preputial space. Thus, the penis is mobile or capable of extending external to the prepuce during mating and urination. However, preputial separation (delamination of the preputial lamina) has not occurred in juvenile males allowing this to be an indicator of maturity. In contrast, the clitoris of the broad-footed mole is ventrally tethered to surrounding tissues in proximal regions where the clitoris is represented as an inverted U-shaped lamina in adults and juveniles (Fig. 2.7). Distally the epithelium of the clitoris is in contact with the epithelium of the prepuce, thus at no point does the clitoris lie in the preputial space unattached to the prepuce (Fig. 2.6).
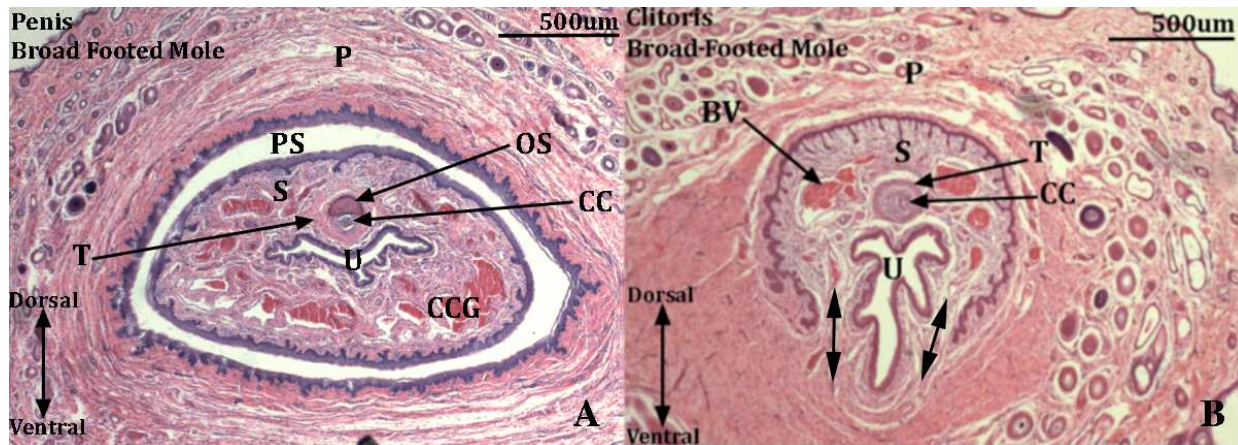
Figure 2.7. Transverse H&E stained sections of an adult broad-footed mole penis and clitoris. Letters for identification of pertinent structures are: CC (corpus cavernosum), CCG (corpus cavernosum glandis), OS (os penis), P (prepuce), PS (preputial space), S (shaft of clitoris or penis), T (tunica), U (urethra). Note that the penis is untethered and thus has freedom of movement within the preputial space. In proximal regions the clitoral stroma is broadly confluent ventrally with surrounding stroma (double-headed arrows) and thus is tethered and immobile in this region while distally the epithelium of the clitoris remains in contact with the epithelium of the prepuce.

Internal Penis/Clitoris Morphology. Broad-footed male moles possess an os penis, and females lack an os clitoris (os penis mean length=450µm (stdev=141 n=4) (Fig. 2.6). The penis and clitoris both possess a corpus cavernosum surrounded by a tunica (Figs. 2.2, 2.6, 2.7). In males the distal tip of the corpus cavernosum ends approximately 700µm from the distal tip of the penis (stdev=141µm, n=4), while in females it ends 363µm from the distal tip of the clitoris (stdev=95µm, n=5). In males the greater distance of the corpus cavernosum from the tip of the penis is accounted for by the os penis that lies immediately distal to the corpus cavernosum. The urethra is completely enclosed within the penis and is about 2/3 within the clitoral lamina throughout its length but never fully resides within the clitoral lamina (Figs. 2.6 & 2.7). The corpus spongiosum in humans and some other species surrounds the urethra in males and is absent in females. In other species such as mice a structure homologous to the corpus spongiosum, the corpus cavernosum urethrae, lies ventral to the urethra in males and is absent in females. I did not observe a corpus spongiosum or a corpus cavernosum urethrae in either male or female broad-footed moles. However, a complex network of blood vessels was observed throughout the length of the penis and clitoris with males having a somewhat greater or larger amount of blood vessels. We call this diffuse network of blood vessels the corpus cavernosum glandis of the male and female moles (Figs. 1.6 & 2.7). Close examination of the skin of the penis revealed that the dermis is exceptionally rich in collagenous fibers surrounding the corpus cavernosum glandis (Fig. 2.8). This dense band of stroma, lying just deep to the penile epithelium, may function in a similar manner to the tunica allowing for blood to fill the penis and not expand outward laterally but primarily longitudinally and may aid in rigidity of erection.
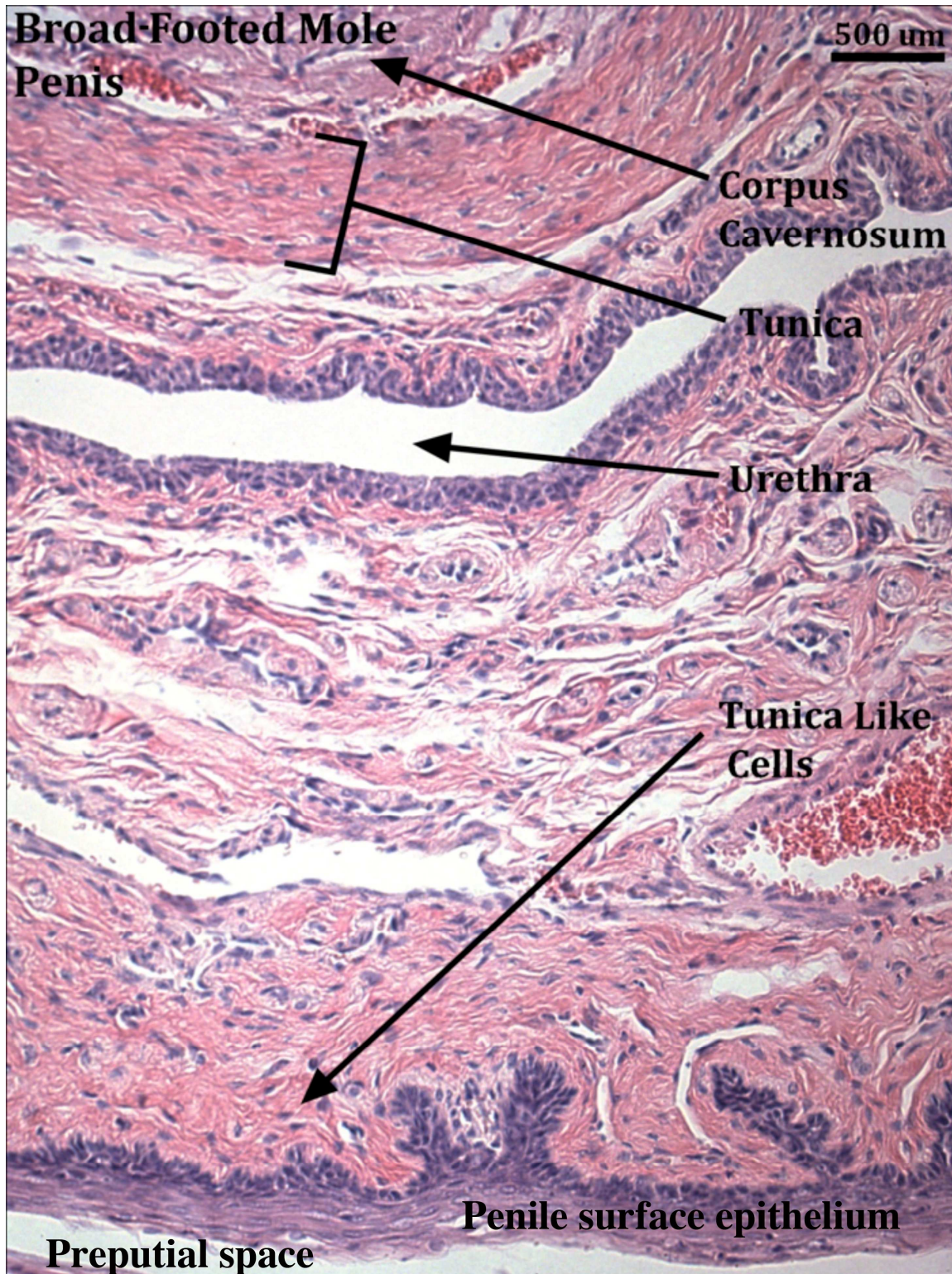
Figure 2.8. Transverse H&E stained section of the adult broad-footed mole penis displaying the band of tunica-like cells lying just deep to the penile epithelium.

Masculine Traits Score. As presented in the introduction of the Chapter, I adopted a list of 9 key homologous mole penile/clitoral features from a similar list created in the Baskin lab, UCSF that are generally present in the mammalian penis but lacking in the clitoris. The penis of the broad-footed mole possesses all 9 of the typical male features. The clitoris of the species is clearly different internally from the penis but does possess 3 out of the 9 typical male features: (a) large organ size of the clitoris, (b) the distinct corpus cavernosa, and (c) the thick tunica that surrounds the corpus cavernosa (Table 2.1 and Fig. 2.9).

Figure 2.9. Graph depicting the level of genital "masculinization" according to the 9 defined morphological traits. BFM=broad-footed mole, SNM=Star-nosed mole, HTM=Hairy-tailed mole, JSM=Japanese shrew mole.

Anogenital Distance. Anogenital distance was measured twice for each animal and the mean value used. No significant difference in anogenital distance was observed between broad-footed mole males and females (mean, male=2.454 ±0.474mm, female=2.259 ±0.655mm) (M n=12, F n=18, F=0.784, p=0.384). There was also no difference between adult broad-footed females a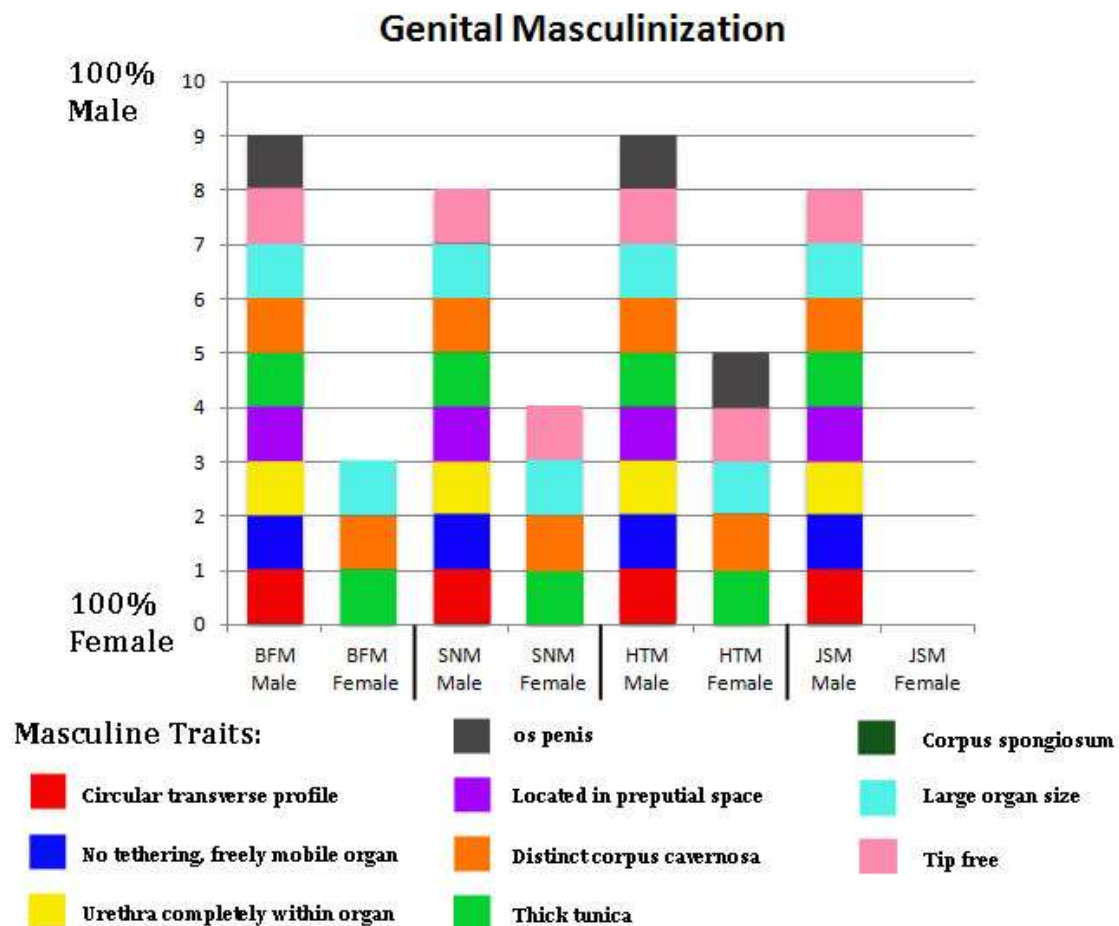nd juvenile females (mean bsF =2.800 ±0.654mm, nbsF=2.302 ±0.800mm, juvF=2.154 ±0.647mm, bsF n=3, nbsF n=4, juvF n=11, F=1.060, p=0.371) nor adult and juvenile males (mean bsM=2.535 ±0.191mm, nbsM=2.445 ±0.530mm, juvM=2.296 ±0.490mm, bsM n=2, nbsM n=2, juvM n=7, F=0.237, p=0.795). As discussed earlier in this chapter, other studies have used anogenital distance index for analysis in order to control for variations in body size. I performed an analysis of anogenital distance normalized to body weight (anogenital distance index 1) or body length (anogenital distance index 2). When analyzed by body weight (anogenital distance index 1) there was no difference between broad-footed males and females (M n=12, F n=18, F=0.730, p=0.401) nor between adult females and juvenile females (bsF n=3, nbsF n=4, juvF n=11, F=0.176, p=0.841) or adult and juvenile males (bsM n=2, nbsM n=2, juvM n=7, F=0.326, p=0.736). Similarly, when anogenital distance was analyzed by body length (anogenital distance index 2) there was no difference between broad-footed males and females (M n=11, F n=17, F=0.711, p=0.407) nor between adult females and juvenile females (bsF n=3, nbsF n=4, juvF n=10, F=2.341, p=0.142) or between adult and juvenile males (bsM n=2, nbsM n=2, juvM n=6, F=0.240, p=0.797).

Star-nosed moles:

Body Weight and Body Length. Star-nosed moles used in my study had already been decapitated for use in Dr. Catania or Dr. Bautista's research and thus prevented measurements of body weight or body length and therefore also prevented any measures of anogenital distance index or phallus length index. However, previous publications have noted that this species fails to show significant differences in body weight or body length between the sexes. Thirty adult males and eighteen females from Ithaca, New York, USA, were weighed throughout the year by Hamilton with males averaging 53.4 grams (39 to 70 grams) and females averaging 50.3 grams (35.2 to 77 grams) (Hamilton, 1931). Similarly, body length did not show a significant difference between the sexes with adults of either sex averaging 194.8mm (177.0 to 206.0) n=7 taken from Nova Scotia, Canada, and 180.0mm (161.0 to 191.0) n=22 from Pennsylvania, USA (Yates and Moore, 1990).

Prepuce Length. Female star-nosed moles possess a prominent prepuce similar to that of the male (Fig. 2.10). However, actual prepuce length was significantly different between the sexes with males having a greater prepuce length than females (mean ±stdev, male=5.350 ±0.626mm, female=3.622 ±0.514mm, M n=4, F n=5, F=20.819, p=0.0026).
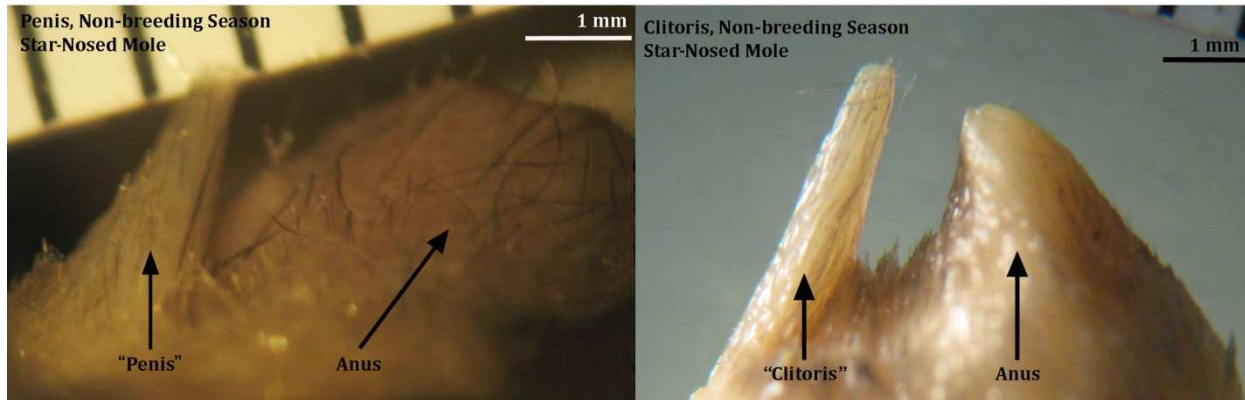
Figure 2.10. Star-nosed mole male and female external genitalia from the non-breeding season. Note that the structures labeled "penis" and "clitoris" are actually the prepuce of male and female moles. The penis and clitoris lie within the prepuce.

Penile/Preputial & Clitoral/Preputial Overlap. Penile/preputial or clitoral/preputial overlap denotes the portion of the penis or clitoris that projects beyond the body surface in the resting state and thus is situated within the male or female prepuce (Figs. 2.11 & 2.12). Penile/preputial or clitoral/preputial overlap is measured by serial sections from the distal tip of the penis or clitoris to the point where the external prepuce became contiguous with the body wall. Clitoral/preputial overlap of the star-nosed mole is on average 2232μm (stdev=416) (Fig. 2.12), while penile/preputial overlap is 2005μm (stdev=219) (Fig. 2.11). Thus, the portion of the penis or clitoris that projects beyond the body surface is not significantly different between the sexes (M n=3, F n=5, F=0.497, p=0.512).

Additionally a percentage was calculated of the amount of penile/preputial or clitoral/preputial overlap relative to total preputial length. By this convention penile/preputial overlap represents less than half of total preputial length (46.57%, stdev=11.89%) in star-nosed male moles (Fig. 2.11). In contrast in female star-nosed moles clitoral/preputial overlap relative to total preputial length was 66.95% (stdev=9.49%) (Fig. 2.12). This difference was statistically significant (M n=3, F n=5, F=7.267, p=0.036).

Figure 2.11. Three-dimensional reconstructions (A) of the adult star-nosed mole penis with transverse H&E stained sections (B-D). The components of the reconstructions are not to scale as they have been artificially elongated to better show the shapes and locations of the structures. The top three-dimensional reconstruction is a side view with the prepuce partially transparent showing the placement of the penis. The three-dimensional reconstruction is also a side view with the prepuce and penis semi-transparent showing the structures inside the penis. The white dotted lines represent the length of the open ventral preputial groove with an arrow indicating the preputial meatus. White arrows indicate the corresponding locations of the H&E transverse sections. Histological sections (B-D) are in order from proximal to distal. Note that the urethra is absent in sections (A & B), but present in section (C) indicating that the urethral meatus is proximally located (red arrow).
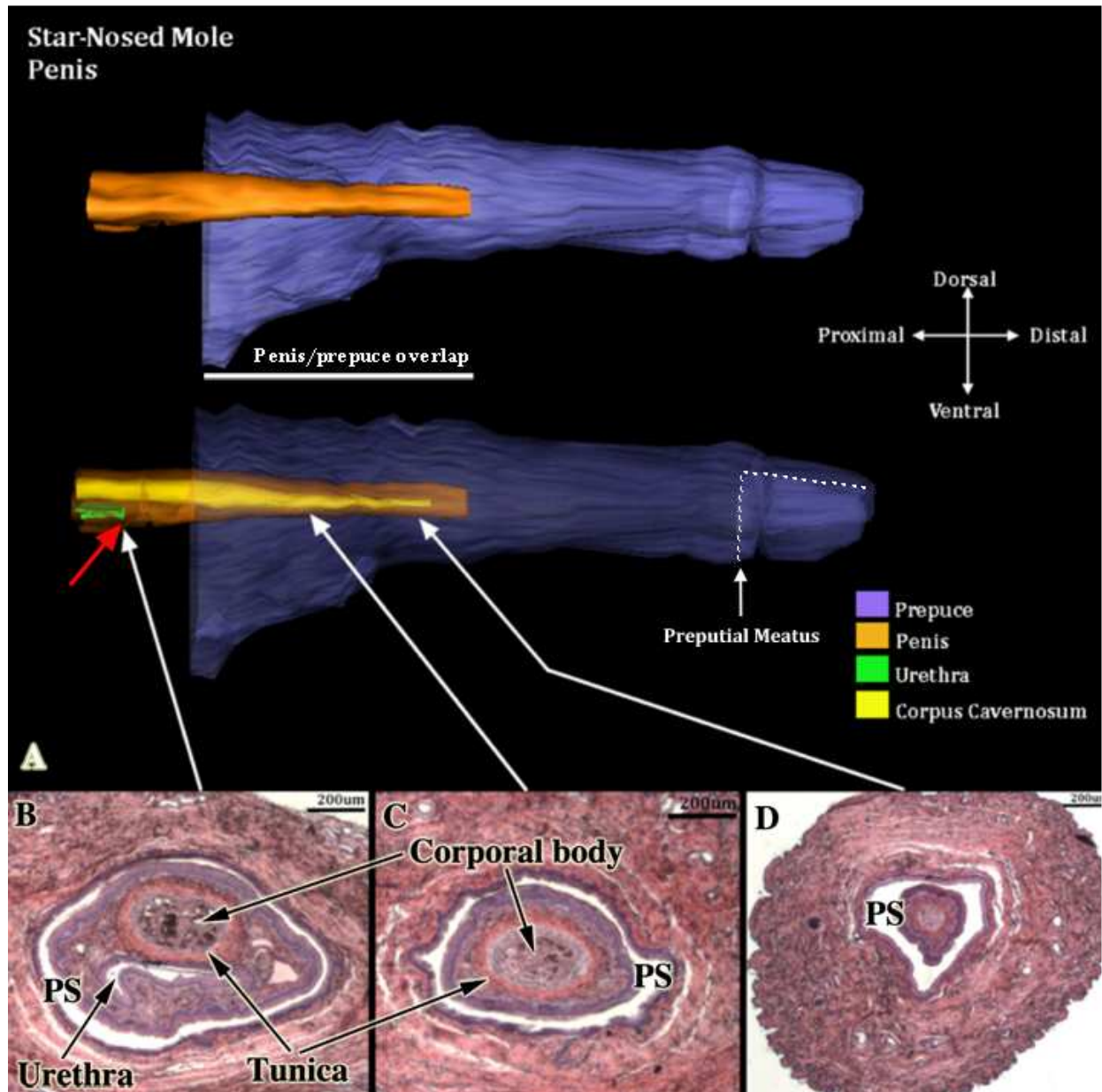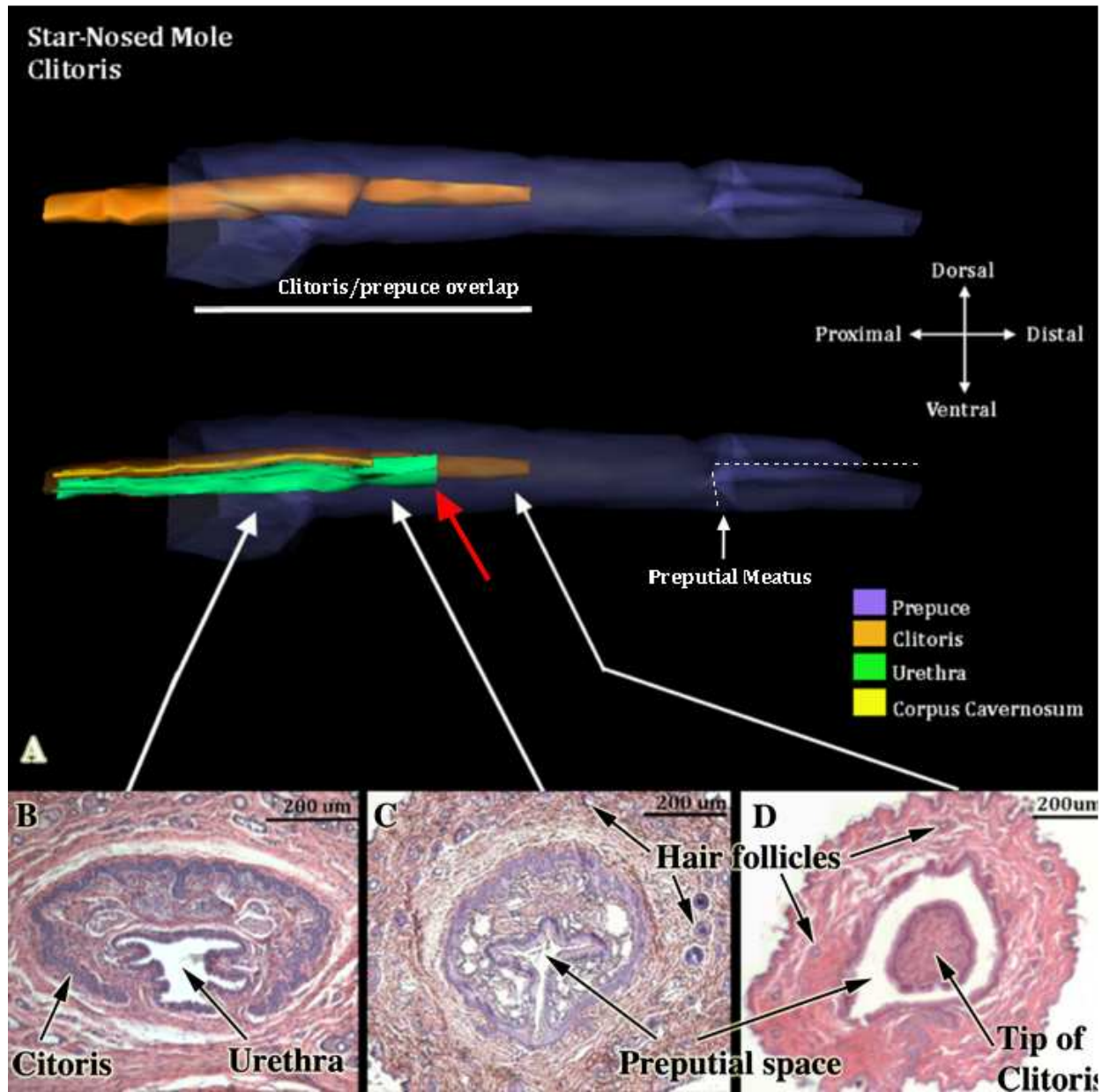
Figure 2.12. Three-dimensional reconstructions of the adult star-nosed mole clitoris with transverse H&E stained sections. The components of the reconstructions are not to scale as they have been artificially elongated to better show the shapes and locations of the structures. The top figure is a side view of the reconstruction with the prepuce partially transparent showing the placement of the clitoris. The lower figure is also a side view of the reconstruction with the prepuce and clitoris partially transparent showing the urethra and corpus cavernosum. The white dotted lines represent the length of the open ventral preputial groove with an arrow indicating the preputial meatus. White arrows indicate the corresponding locations of the H&E transverse sections. Red arrow in (A) denotes the opening of the urethra into the preputial space. Note in (D) that the distal aspect of the clitoris projects freely into the preputial space and thus is presumably mobile.

Preputial Attachment. The star-nosed mole penis resides within a preputial space and is not attached to the prepuce in adults (Fig. 2.13). The proximal portion of the U-shaped clitoris is tethered ventrally as a result of confluence of clitoral stroma with ventral stroma. However, the distal portion of the clitoris is completely surrounded by epithelium and projects freely into the preputial space (Fig. 2.12A & D). Thus, the tip of the clitoris is un-tethered for approximately 417.5μm (SD=159, n=4). However, one female not included in this measurement did have the tip of the clitoris attached to the prepuce along its entire length. Proximally the clitoral attachment to the prepuce forms an inverted U-shaped lamina as in the other mole species and the ventral portion of the clitoral stroma is continuous with the stroma of the surrounding tissue (Figs. 2.12 & 2.13).

Internal Penis/Clitoris Composition. The star-nosed mole penis and clitoris are similar in so far as neither possesses an os penis or os clitoris. Both also possess a corpus cavernosum surrounded by a tunica. However, similar to the broad-footed mole, the tunica and corpus cavernosum are larger in males than in females (Figs. 2.12 & 2.13). In males the distal tip of the corpus cavernosum ends at approximately 180μm from the distal tip of the penis (stdev=72, n=4), while in females it ends 228μm from the distal tip of the clitoris (stdev=55, n=5). The urethra resides completely within the penis and is on average fully 90% within the clitoris throughout its length (Fig. 2.11 & 2.13). Corpus spongiosum or corpus cavernosum urethrae were absent in both male and female star-nosed moles. However, similar to the broad-footed mole, there is a diffuse network of blood vessels throughout the length of the penis and clitoris, which I have termed the corpus cavernosum glandis of the mole. Males have a noticeably greater number of this network of blood vessels than females. Close examination of the skin of the penis revealed a dermis exceptionally rich in collagenous fibers surrounding the corpus cavernosum glandis (Fig. 2.13) As with the broad-footed mole this dense band of stroma, lying just deep to the penile surface epithelium in star-nosed moles may function in a similar manner to the tunica allowing for blood to fill the penis and not expand outward laterally but primarily longitudinally and may aid in rigidity of erection.
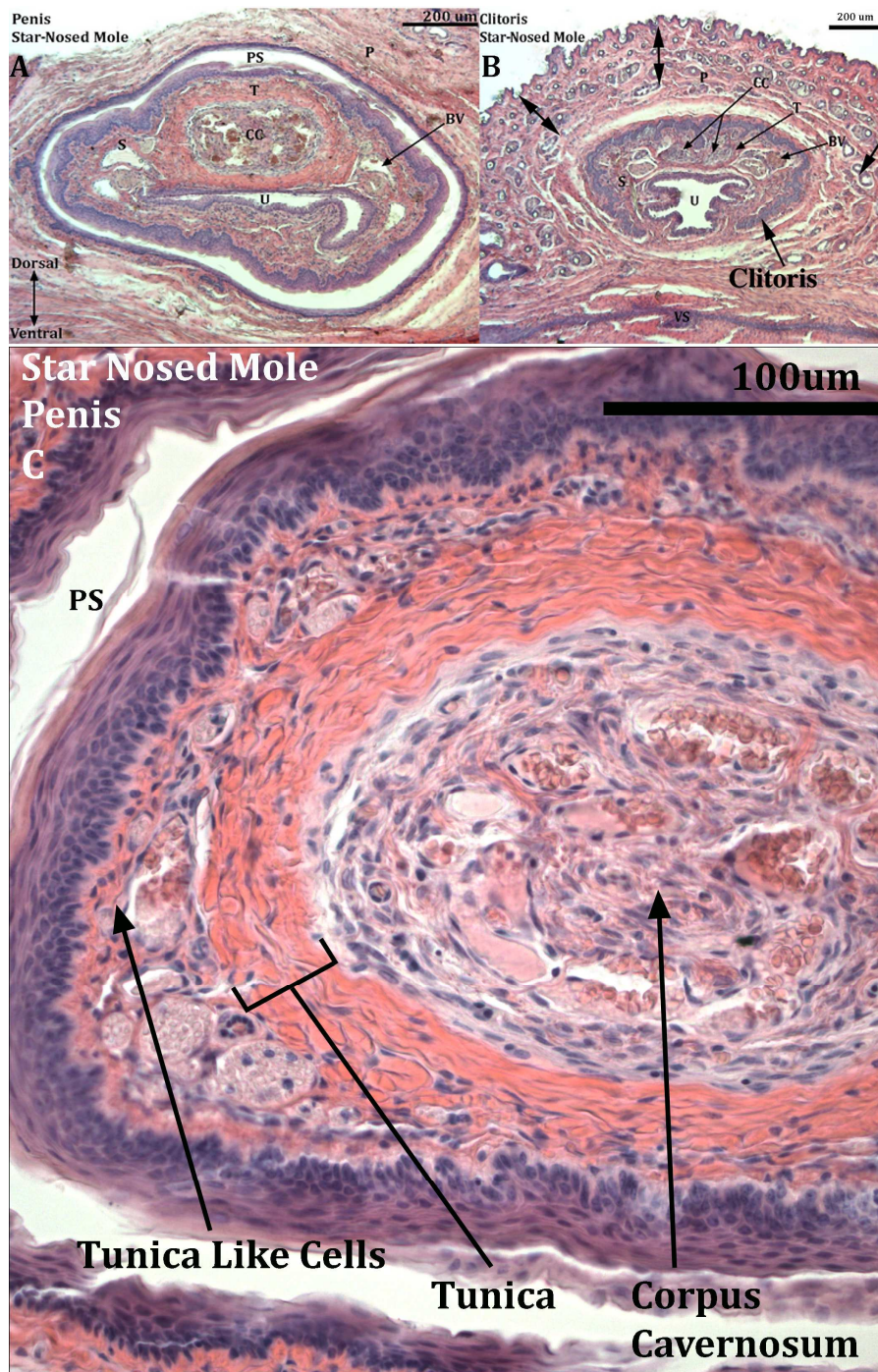
Figure 2.13. Transverse H&E stained sections of an adult star-nosed mole penis (A & C) and clitoris (B). Letters for identification of pertinent structures are: BV (blood vessel), CC (corpus cavernosum), P (prepuce), PS (preputial space), S (shaft of clitoris or penis), T (tunica), U (urethra), VS (closed vaginal seam). Dorsal double-headed arrows in (B) = hair follicles of the female prepuce. Note that the penis is untethered and free to move within the preputial space, whereas the clitoris is tethered ventrally via confluence of clitoral stroma with ventral stroma. (C) is a high magnification photo of the penis distal to the urethra.

Masculine Traits Score. The star-nosed mole penis possesses 8 out of the 9 common masculine characteristics of the mammalian penis. It lacks only the os penis. In contrast the clitoris of the star-nosed mole possesses 4 out of the 9 masculine characteristics, displaying a large organ size, a freely mobile distal tip of the clitoris, a distinct corpus cavernosa, and the thick tunica that surrounds the corpus cavernosa tissue (Figs. 2.9 & 2.13 and Table 2.1)

Anogenital Distance. There is no significant difference in anogenital distance between male and female star-nosed moles (mean ±stdev, male=2.410 ±0.079mm, female=2.297 ±0.119mm, M n=3, F n=4, F=1.969, p=0.219).

Hairy-tailed moles:

Body Weight and Body Length. The hairy-tailed moles received from Dr. Catania or Dr. Bautista had already been decapitated, which prevented measurements of body weight or body length and therefore also prevented any measures of anogenital distance index or phallus length index normalized to body weight or length. However, previous publications have stated that this species does exhibit some sexual dimorphism in body weight and length. Eadie measured 41 males and 19 non-reproductive females collected from New Hampshire, USA with males averaging 54.5 grams of body weight to the female 47.5g. Male body length averaged 155mm to the female 147mm (Eadie 1939). Conner reported the means and extremes for 22 males and 12 non-reproductive females from New York, USA with males weighing 51.5g (45.5 to 62.8g) and females 45.4g (41.0 to 49.9g). Male body length was 164.8mm (155.0 to 173.0mm) and females 158.6mm (151.0 to 166.0mm) (Conner, 1960).

Prepuce Length. Female hairy-tailed moles possess a prominent phallus similar in size and shape to the male (Figs. 2.14 & 2.15). Prepuce length was similar in males (2.55mm & 2.60mm) and females (2.65mm & 2.57mm).

Penile/Preputial & Clitoral/Preputial Overlap. Penile/preputial or clitoral/preputial overlap denotes the portion of the penis or clitoris that projects beyond the body surface in the resting state and thus is situated within the male or female prepuce (Figs. 2.14 & 2.15). It is measured by serial sections from the distal tip of the penis or clitoris to the point where the external prepuce became contiguous with the body wall. Clitoral/preputial overlap of the hairy-tailed mole is noticeably longer at 2200µm & 2150µm (Fig. 2.15), than the penile/preputial overlap at 1100µm & 1180µm in length (Fig. 2.14).

Additionally in hairy-tailed moles a percentage was calculated of the amount of penile/preputial or clitoral/preputial overlap relative to total preputial length. By this convention penile/preputial overlap represents less than half of total preputial length 43.14% & 45.38% (Fig. 2.14) in hairy-tailed male moles. In contrast in female hairy-tailed moles clitoral/preputial overlap relative to total preputial length was much greater at 83.02% & 83.66% (Fig. 2.15).

Figure 2.14. Three-dimensional reconstructions (A) of the adult hairy-tailed mole penis with transverse H&E stained sections (B-D). The components of the reconstructions are not to scale as they have been artificially elongated to better show the shapes and locations of the structures. The top three-dimensional reconstruction is a side view with the prepuce partially transparent showing the placement of the penis. The lower three-dimensional reconstruction is also a side view with the prepuce and penis semi-transparent showing the urethra, corpus cavernosum, and os penis. The white dotted lines represent the length of the open ventral preputial groove with an arrow indicating the preputial meatus. White arrows indicate the corresponding locations of the H&E transverse sections. Note that the urethra does not extend to the distal tip of the penis (A & D). Red arrow in (A) denotes the opening of the urethra into the preputial space.
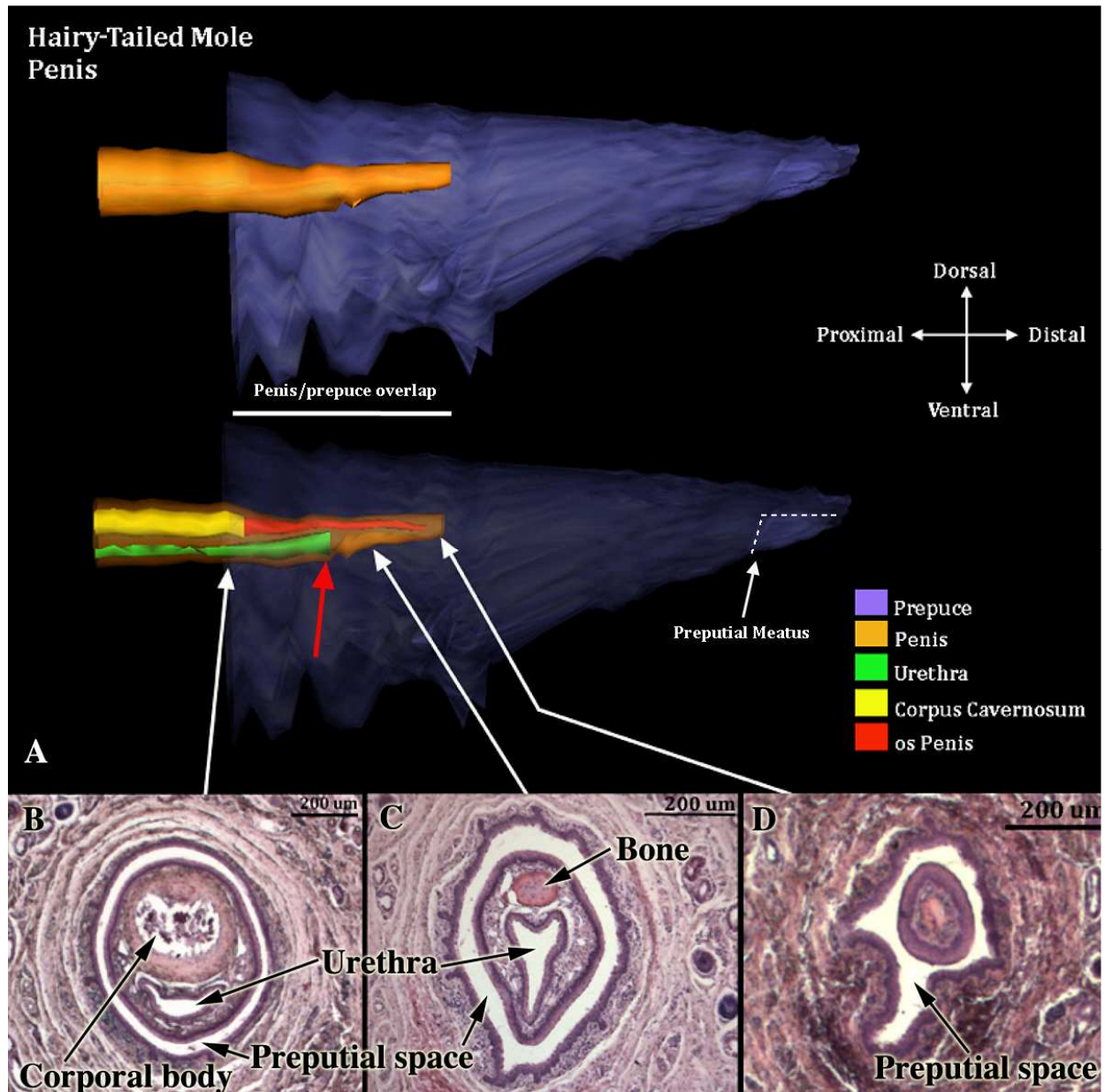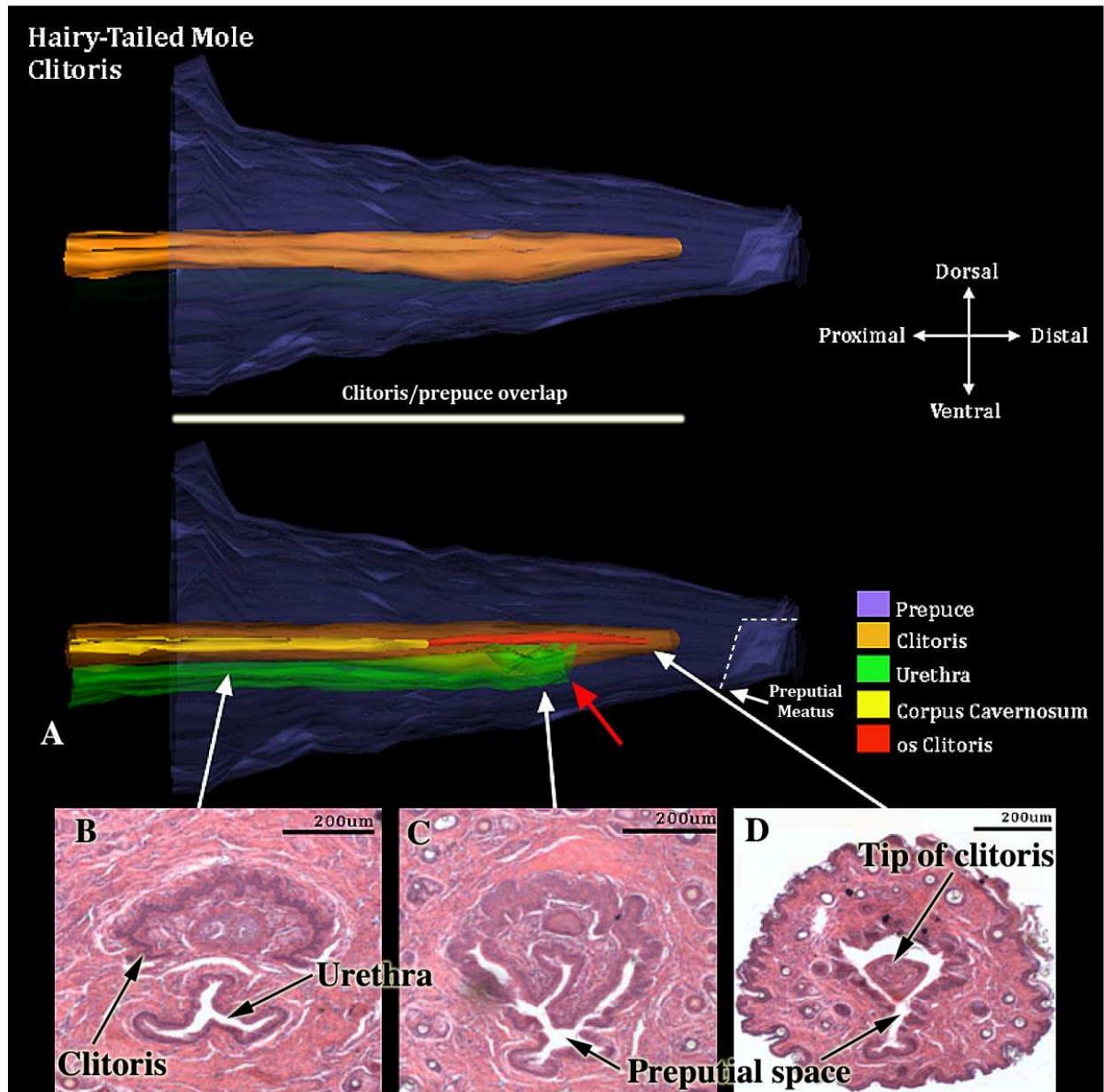
Figure 2.15. Three-dimensional reconstructions (A) of the adult hairy-tailed mole clitoris with transverse H&E stained sections (B-D). The components of the reconstructions are not to scale as they have been artificially elongated to better show the shapes and locations of the structures. The top three-dimensional reconstruction is a side view with the prepuce partially transparent and the urethra absent showing the placement of the clitoris. The lower three-dimensional reconstruction is also a side with the prepuce and clitoris partially semi-transparent showing the urethra, corpus cavernosum, and os clitoris. The white dotted lines represent the length of the open ventral preputial groove with an arrow indicating the preputial meatus. White arrows indicate the corresponding locations of the H&E transverse sections. Histological sections (B-D) are in order from proximal to distal. Labeling of the urethra and prepuce are as described. Red arrow in (A) denotes the opening of the urethra into the preputial space. Note that the distal tip of the clitoris project freely into the preputial space (D).

      Preputial Attachment. The penis resides within a preputial space and is not attached to the prepuce in adults except proximally where the inner preputial epithelium reflects onto the penile surface. In females proximally the stroma within the U-shaped clitoris is confluent with ventral stroma and thus the U-shaped clitoris is tethered (Fig. 2.15), even though the distal tip of the clitoris is "free," (not attached to the prepuce) within the preputial space (Fig. 2.15D). Indeed, the clitoral tip is un-tethered for 400μm and 260μm (n=2).

      Internal Penis/Clitoris Composition. Both male and female hairy-tailed moles possess bone within the penis and clitoris, respectively, which lies distal to the corpus cavernosum. The os penis is 650μm & 720μm long and the os clitoris is 750μm & 600μm (Figs. 2.14 & 2.15). Both sexes also possess a corpus cavernosum surrounded by a tunica, which is larger in males than in females. In males the distal tip of the corpus cavernosum ends at approximately 700μm & 790μm from the distal tip of the penis, while in females it ends 900μm & 750μm from the distal tip of the clitoris. The urethra resides completely within the penis in males and is only about 1/4 within the U-shaped clitoral lamina (Figs. 2.14 & 2.15). Corpus spongiosum and corpus cavernosum urethrae were not observed in both male and female hairy-tailed moles. However, similar to the broad-footed and star-nosed moles, there is a network of blood vessels throughout the length of the penis and clitoris, termed the corpus cavernosum glandis of the mole. Males have a noticeably greater number of these blood vessels than females but do appear to have fewer overall than the penis of the broad-footed mole and star-nosed mole.

      Masculine Traits Score. The hairy-tailed mole penis possesses all 9 of the typical masculine characteristics. The clitoris of the species is clearly different internally from the penis but does possess 5 out of the 9 masculine characteristics displaying (a) a large organ size, (b) a freely mobile distal tip of the clitoris, (c) an os clitoris, (d) a distinct corpus cavernosa, and (e) a thick tunica that surrounds the corpus cavernosa (Figs. 2.9, 2.14, 2.15 and Table 2.1).

Japanese shrew moles:

      Body Weight and Body Length. Japanese shrew mole body weight appears to be monomorphic with males weighing 17.2g & 17.7g and females weighing 18.5g & 17.2 g. Body length is also monomorphic between males (93.5mm & 94.0mm) and females (90.0mm & 94.0mm).

      Prepuce Length. The male Japanese shrew mole possesses a prominent prepuce similar to what has been observed in other mole species, but the female does not possess any visible external genitalia other than the vaginal opening (Fig. 2.16). Therefore, no preputial length measurement was able to be taken for females but male preputial length measured 2.10mm & 2.45mm.
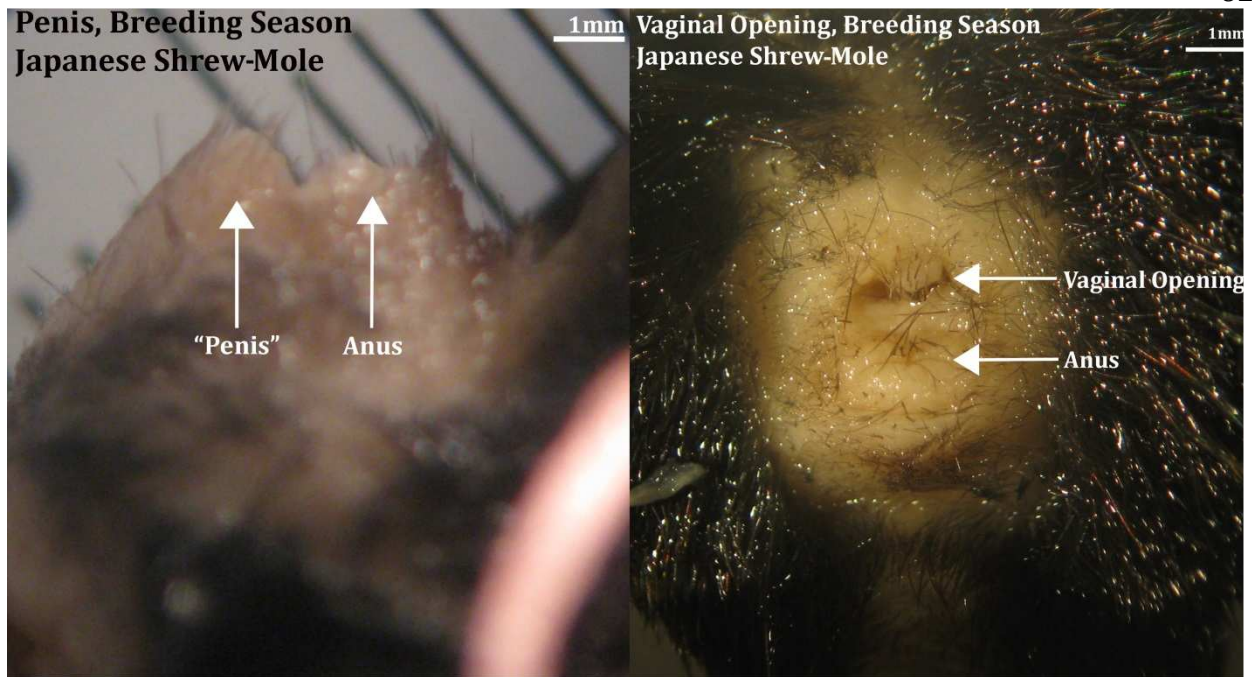
Figure 2.16. Japanese shrew mole male and female external genitalia from the breeding season. Note that the structure labeled "penis" is actually the prepuce of the male mole with the penis residing inside.

Penile/Preputial Overlap. Penile/preputial overlap denotes the portion of the penis that projects beyond the body surface in the resting state and thus is situated within the prepuce (Fig. 2.17). It is measured by serial sections from the distal tip of the penis to the point where the external prepuce became contiguous with the body wall. Penile/preputial overlap of the Japanese shrew mole is 1480μm & 1690μm. The female does not possess any visible external genitalia other than the vaginal opening (Fig. 2.16).

Additionally in Japanese shrew moles a percentage was calculated of the amount of penile/preputial overlap relative to total preputial length. By this convention penile/preputial overlap represents was 70.48% & 68.98% total preputial length (Fig. 2.17).
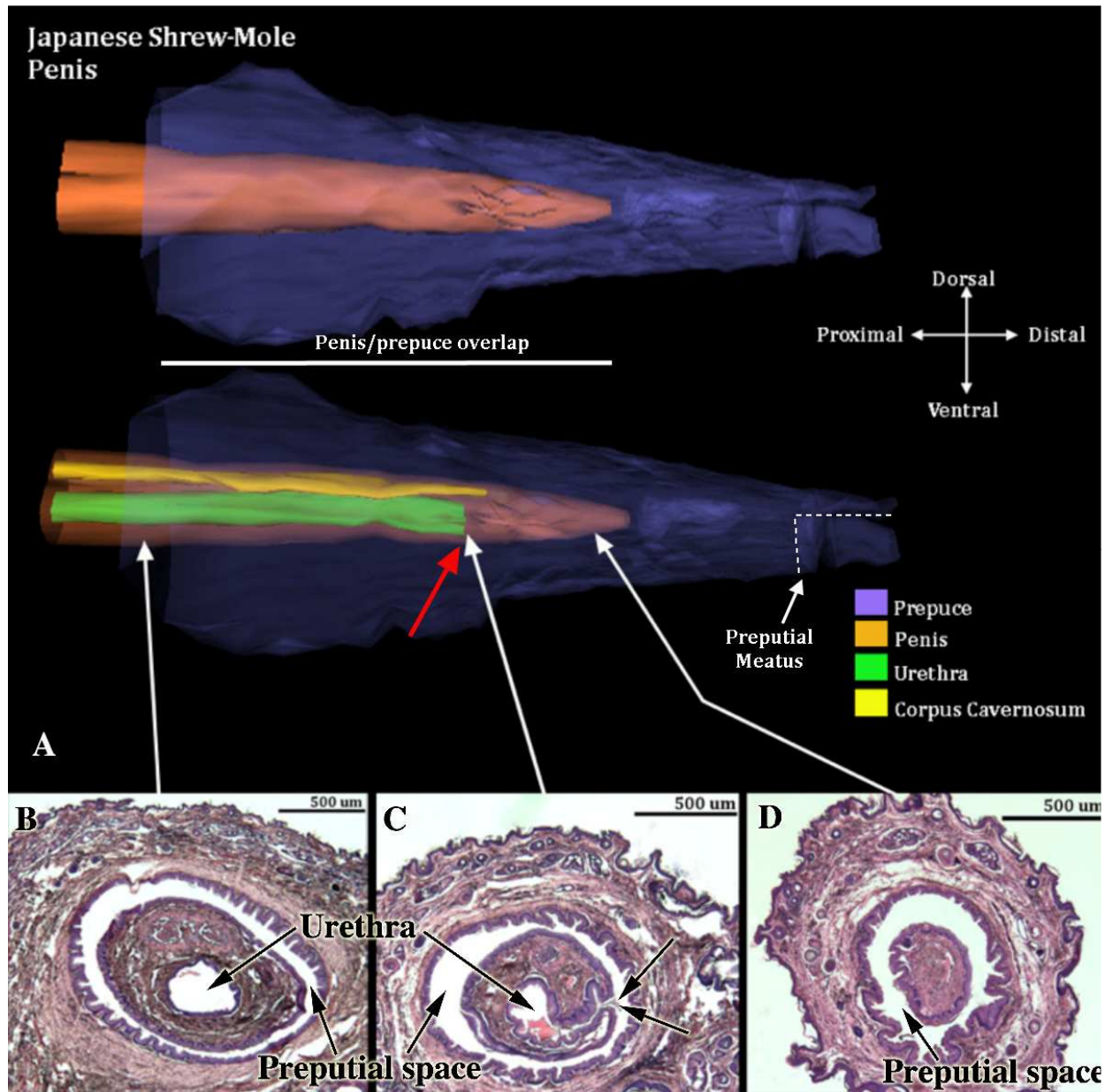
Figure 2.17.   Three-dimensional reconstructions (A) of the adult Japanese shrew mole penis with transverse H&E stained sections (B-D). The components of the reconstructions are not to scale as they have been artificially elongated to better show the shapes and locations of the structures. The top three-dimensional reconstruction is a side view with the prepuce partially transparent showing the placement of the penis. The lower three-dimensional reconstruction is also a side view with the prepuce and penis partially semi-transparent showing the urethra and corpus cavernosum. The white dotted lines represent the length of the open ventral preputial groove with an arrow indicating the preputial meatus. White arrows indicate the corresponding locations of the H&E transverse sections. Histological sections (B-D) are in order from proximal to distal and show the penis within the preputial space. Note in (C) that the urethra is opening into the preputial space (arrows). Note in (A & D) that the urethra does not extend to the tip of the penis.

Preputial Attachment. The penis resides within a preputial space and is not attached to the prepuce in the adult male. The clitoris is not external therefore it is not attached to a prepuce and is an immobile organ due to ventral confluence of clitoral stroma with surrounding tissue.

Internal Penis/Clitoris Composition. Japanese shrew moles do not have an os penis. There is a distinct corpus cavernosum surrounded by a tunica, which ends distally 260μm & 300μm from the distal tip of the penis. The urethra does not exit at the very distal tip of the penis, instead it exits ventrally into the preputial space approximately 350μm & 475μm from the distal tip of the penis (Fig. 2.17A & D). Subsequently as the urethra proceeds proximally it remains completely enclosed within the penis (Fig. 2.17). The vascular network throughout the penis is not as extensive as that seen in male or female broad-footed moles and therefore is not deemed a corpus cavernosum glandis in this species. The female Japanese shrew mole has no external phallus; however, internally there appear to be some rudimentary erectile structures dorsal to the urethra (Fig. 2.18 C&D). The urethra joins the vaginal opening very near its distal end but proximally separates from the vagina and is partially enclosed by the parentheses-shaped lamina (Fig. 2.18). No other structures are clearly identifiable in regards to the phallus characteristics in this investigation.
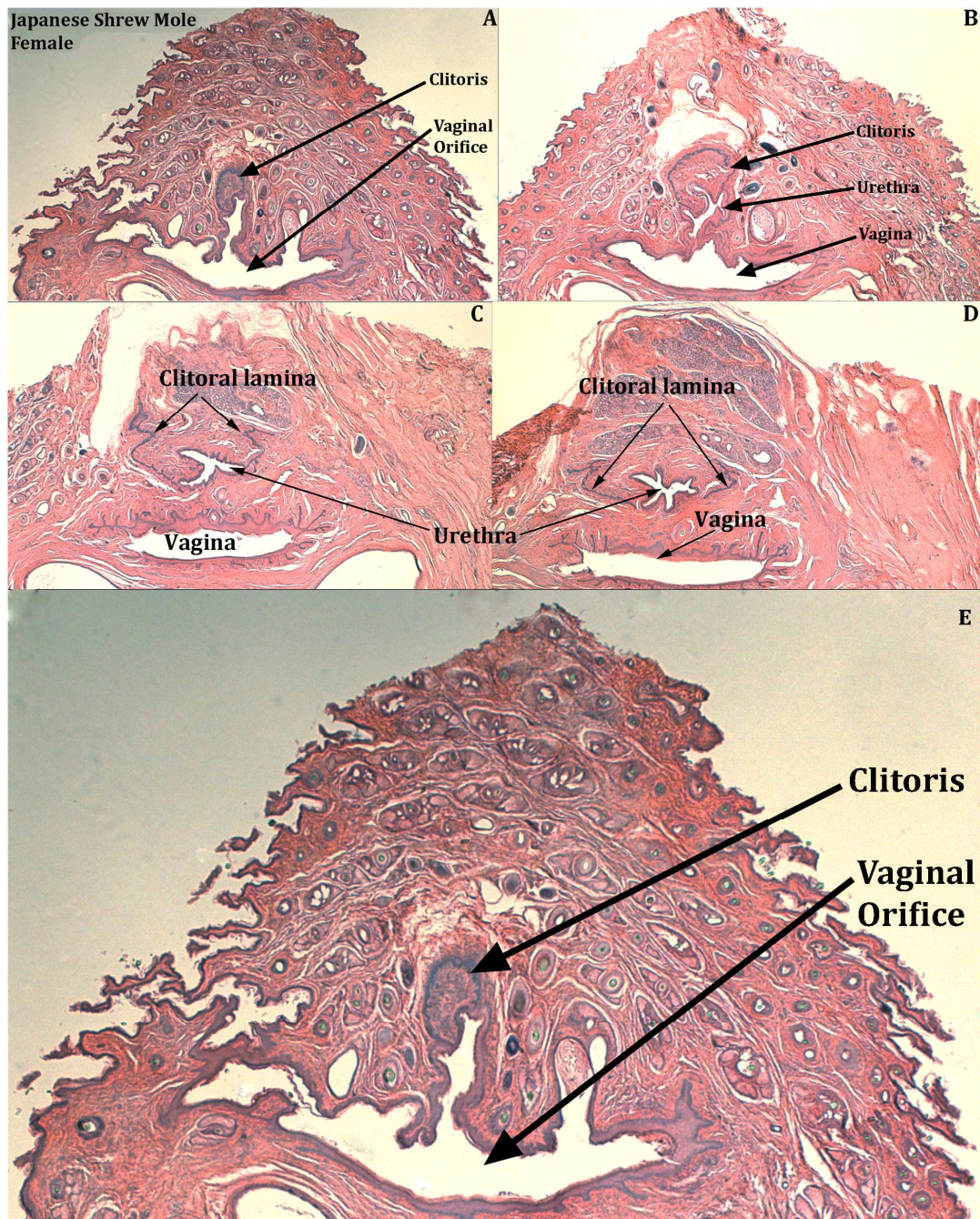
Figure 2.18. Transverse H&E stained sections of adult Japanese shrew mole female genitalia identifying the distal beginning of the clitoris and the connection of the urethra to the vaginal orifice (E). Sections (A-D) progress distal to proximal from left to right. In (A & E) note that the U-spaded clitoral lamina is fused to the urethra, which opens into the distal aspect of the vagina (E). In (B) the U-shaped clitoral lamina is attached to the urethra only on its left side, and the connection of the urethra with the vagina can be seen. In C & D) the U-shaped clitoral lamina is represented merely as its right and left pieces that flank the urethra.

Masculine Traits Score. The Japanese shrew mole penis possesses 8 out of the 9 masculine characteristics lacking only the os penis. The clitoris of this species possesses none of the 9 masculine characteristics making it, by these measures, fully feminized external genitalia (Figs. 2.9, 2.17 and Table 2.1).

Anogenital Distance. The distance from the anus to the vaginal opening in Japanese shrew mole females is 1.25mm & 1.45mm. When measured from the anus to the opposite side of the vaginal opening where the rudimentary clitoris would be located, as seen by sectioning, the anogenital distance is approximately 1.85mm & 2.0mm. In males the average distance is 2.45mm & 2.75mm and is longer than either measurement for the females.

Ovarian Histology:

The typical mammalian ovary consists of a surface epithelium, sometimes a *tunica albuginea*, followed by the ovarian cortex. The ovarian cortex is generally a thick zone of cellular stroma that contains oocytes, follicles, corpora lutea, and interstitial gland tissue. The inner core of the ovary is the ovarian medulla, which contains the large blood and lymph vessels, all or part of the rete ovarii, and some interstitial tissue. While the *tunica albuginea* may be visible, the border between the medulla and cortex is almost never distinct. The ovarian hilus is the portion of the ovarian medulla to which the ovarian ligament is attached, and through which the blood and lymph vessels enter and does not contain any ovarian follicles. The rete ovarii is generally groups of tubules located in the hilus of the ovary, but may extend through the medulla or be isolated in the mesovarium adjacent to the hilus. The rete is often continuous with the transverse ductules through which it contacts the longitudinal duct of the epoophoron, a remnant of the mesonephric duct. The ovary may lie within an ovarian bursa, a sac of connective tissue and ligaments surrounding the ovary and connected with the oviducts (Mossman and Duke, 1973).

Broad-footed mole ovary. The ovaries of the broad-footed mole in the breeding and non-breeding seasons appear typical of most mammals. The ovary lies within an ovarian bursa and displays typical ovarian epithelium without a thick or distinctive *tunica albuginea*. The cortex shows numerous follicles at various stages of development with interstitial and stromal cells scattered throughout. The medulla of the ovary is more centrally located, contains blood and lymph vessels as well as part of the rete ovarii and is connected to the ovarian hilus (Fig. 2.19). This is consistent with previous reports of the broad-footed mole ovarian anatomy (Mossman and Duke, 1973; Rubenstein et al., 2003).
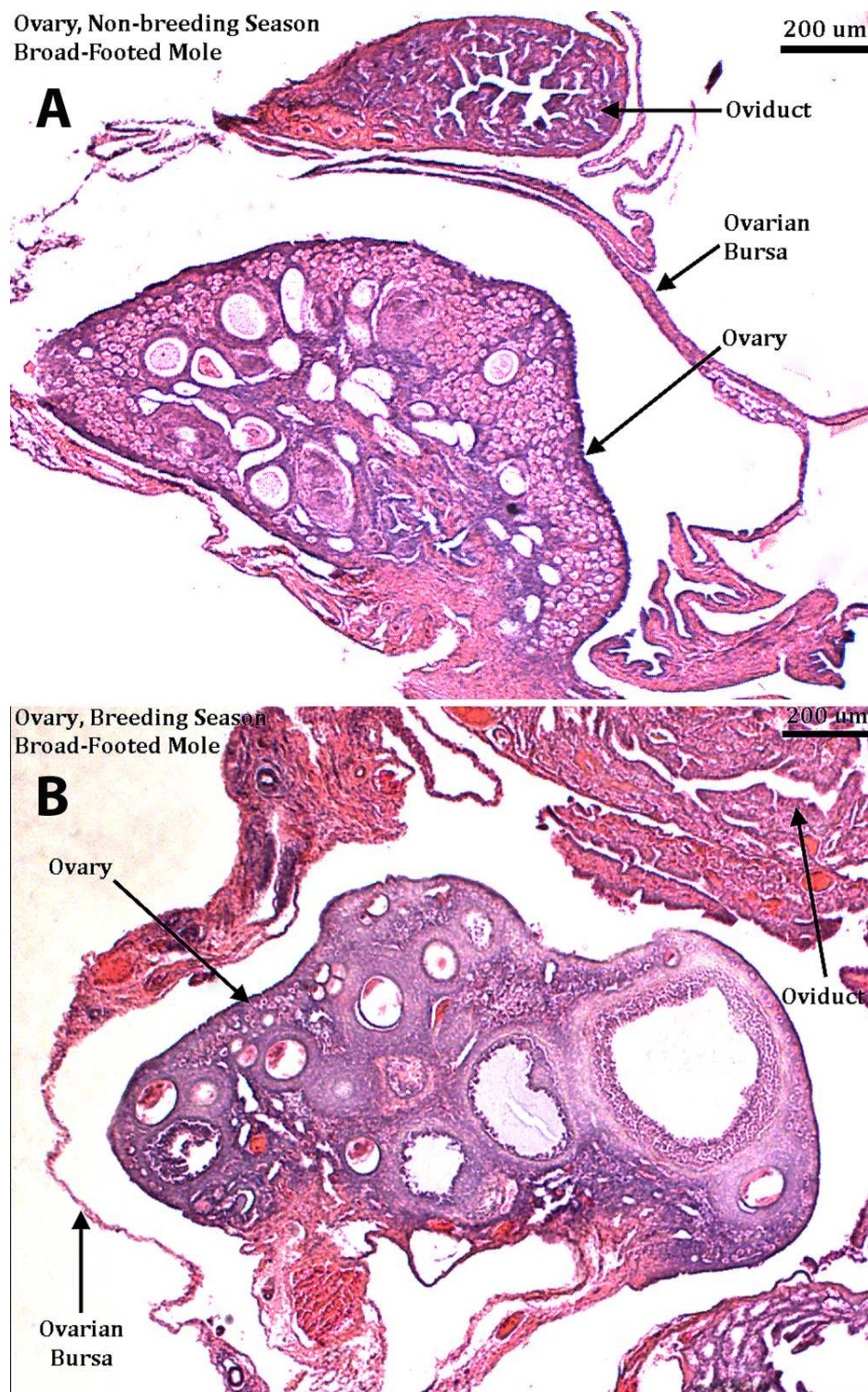
Figure 2.19. Transverse H&E stained sections of adult broad-footed mole ovary from (A) the non-breeding season and (B) the breeding season. Note the follicles present at various stages of development, the ovarian bursa connected to the oviducts and surrounding the ovary but not lying over the ovarian epithelium.

Star-nosed mole ovary. The ovary of the star-nosed mole in the non-breeding state is significantly different than the typical mammalian ovary. The ovary is polar with one side (the cortex) presenting as a typical follicular ovary with follicles at various stages of development. The opposite side of the ovary (interstitial gland) is equal in size or larger than the follicular side but lacks follicles and instead is composed of small spherical compact structures, called medullary cords, that are embedded in a dense matrix of interstitial tissue with Leydig-like cells (Fig. 2.20). These medullary cords bear some resemblance to fetal seminiferous tubules, do not contain germ cells, and are surrounded by a monolayer of flattened myoid cells (Fig. 2.21). This interstitial gland comprises the majority of the ovarian medulla so that most of the lymph and blood vessels and rete ovarii do not reside within the medulla. While the cortex (follicular ovary) and the medulla (interstitial gland) portions of the ovary are anatomically distinct, they are not physically separated from each other but intermingle slightly (Fig. 2.20- 2.21). Around the outer edge of the interstitial gland can be seen a dense multilayer of flattened cells forming a tunica in a similar manner to the male testis. These observations are consistent with what has previously been reported for the ovary of the star-nosed mole in the non-breeding season (Mossman and Duke, 1973; Rubenstein et al., 2003).
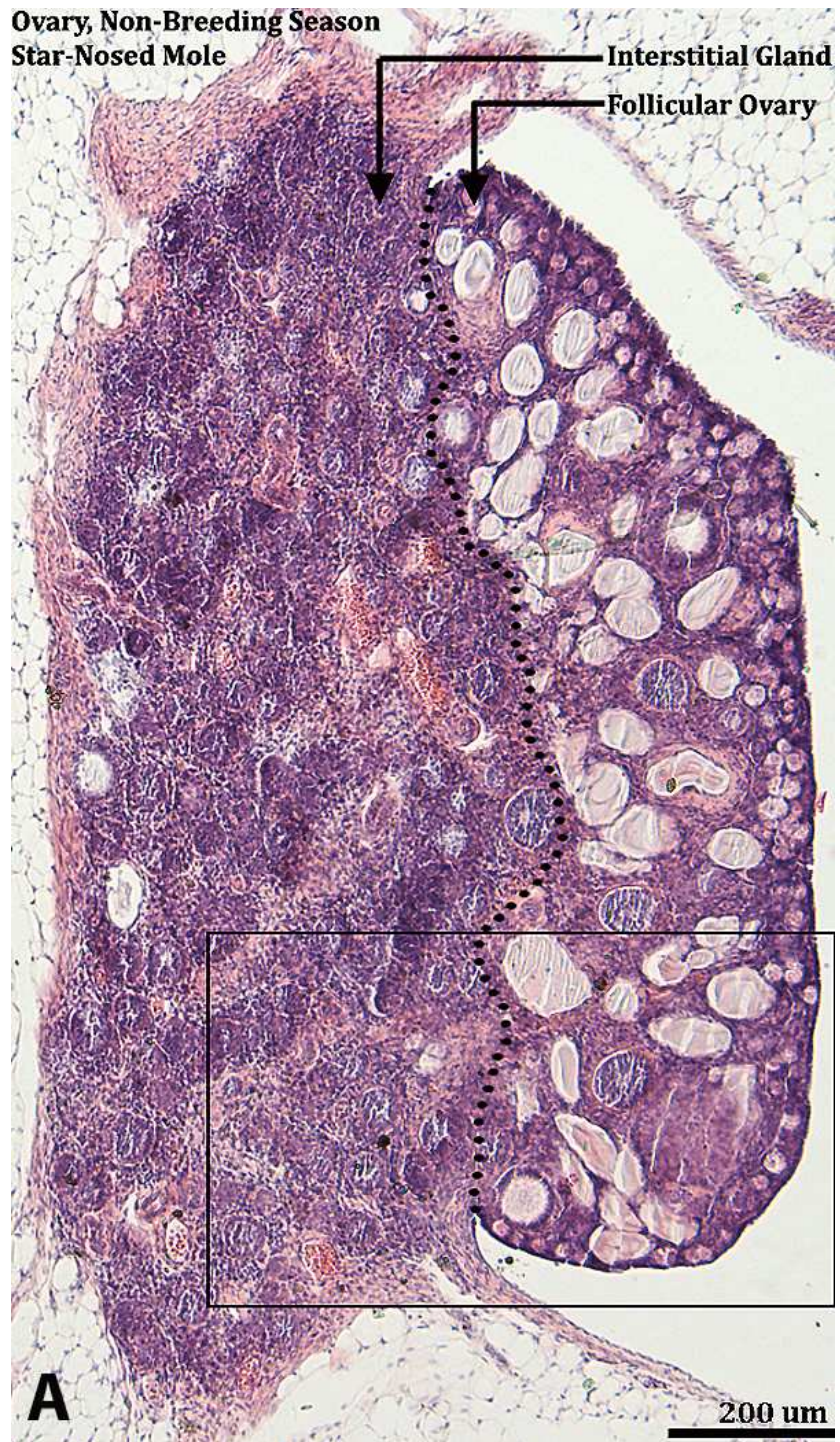
Figure 2.20. Transverse H&E stained section of adult star-nosed mole ovary from the non-breeding season. Note the ovary is polar with the right half, demarcated by the dotted line, presenting as a normal ovary with follicles while the left half, the interstitial gland, is composed of interstitial and Leydig type cells and possessing small spherical structures known as medullary cords.

Figure 2.21. Enlargement of the black box region from figure 2.20 of the transverse H&E stained section of adult star-nosed mole ovary from the non-breeding season. Note the difference between normal ovarian primordial follicles and the spherule structures, the medullary cords, which are similar to immature testicular cords.

Hairy-tailed mole ovary. Unfortunately hairy-tailed mole gonadal tissue was not available for investigation as it was damaged during shipment or storage. No other publications to date have investigated the ovarian anatomy of the hairy-tailed mole so it remains unknown whether they possess an ovarian interstitial gland.

Japanese shrew mole ovary. I was only able to obtain the ovary of the Japanese shrew mole from the breeding season. A previous report of the ovarian morphology of this species was from the non-breeding season and reported a typical mammalian ovary with follicles and an inconspicuous medulla forming a typical ovarian hilus (Carmona et al., 2008). These observations are partially true for the ovaries of the two breeding season females I examined. The breeding season Japanese shrew mole has a relatively normal appearing ovary with follicles in the cortex and a central medulla of blood and lymph vessels that connect to the ovarian hilus. An ovarian bursa surrounds the ovary and is connected to the ovarian hilus. One major difference was the presence of a large glandular structure attached to the ovary that was equal or slightly larger in size than the ovarian portion. The gland was very uniform in cellular type, and close examination of the cellular structure in this glandular region showed a resemblance to adrenal cortex cells, notably their large cytoplasm packed with small vacuoles of uniform size. A few species have an everted corpus luteum where the granulosa layer becomes enlarged to form a bulb-like projection attached to the rest of the ovary by a narrow isthmus with its surface covered by the cells of the granulosa layer. This has been seen in the pangolin (*Manis*) and four species of bats (Mossman and Duke, 1973; Gopalakrishna and Badwaik, 1988). However, no covering of granulosa cells is seen in the ovarian glandular structure of the Japanese shrew mole, which was not pregnant and did not have remnants of an ovulated follicle. Other species have been observed to form masses of gonadal adrenal-like tissue comparable in size to corpora lutea, and have been recognized as accessory adrenals because they have all the characteristics of an adrenal gland, except that they always lack medullary tissue. Preliminary studies support the production of hormones from these tissues. While small clumps of this tissue type are common in the ovary, a large mass of gonadal adrenal tissue has been found only in the nine-banded armadillo (*Dasypus novemcinctus*), the Asiatic elephant (*Elephas maximus*), all sciurids except the red squirrel, and in the patas monkey (*Erythrocebus patas*). While the gland attached to the Japanese shrew mole ovary may be a similar structure, gonadal adrenal type tissue usually occurs just outside the ovary in close association with the epoophoron or rete ovarii. In the Japanese shrew mole this glandular structure is clearly part of the ovary (Mossman and Duke, 1973). Also, I was unable to find any information on the seasonality of gonadal adrenal type tissue in other species, but it would appear that this gland of the Japanese shrew mole is present in the breeding season but absent in the non-breeding season. This large glandular tissue of the Japanese shrew mole cannot at this time be defined as either everted corpus luteum, adrenal type tissue, or some other yet unidentified glandular tissue. This is the first time it has been observed or reported on in this species.
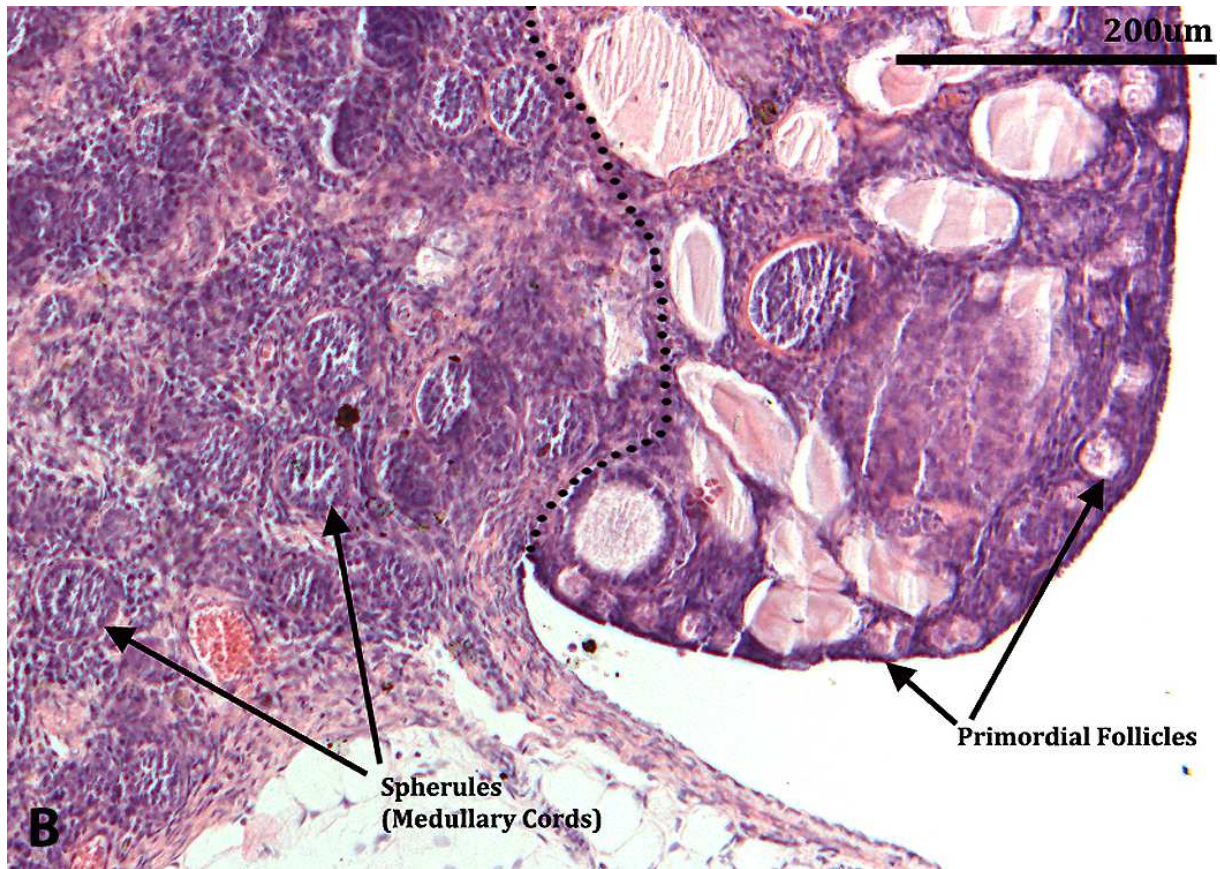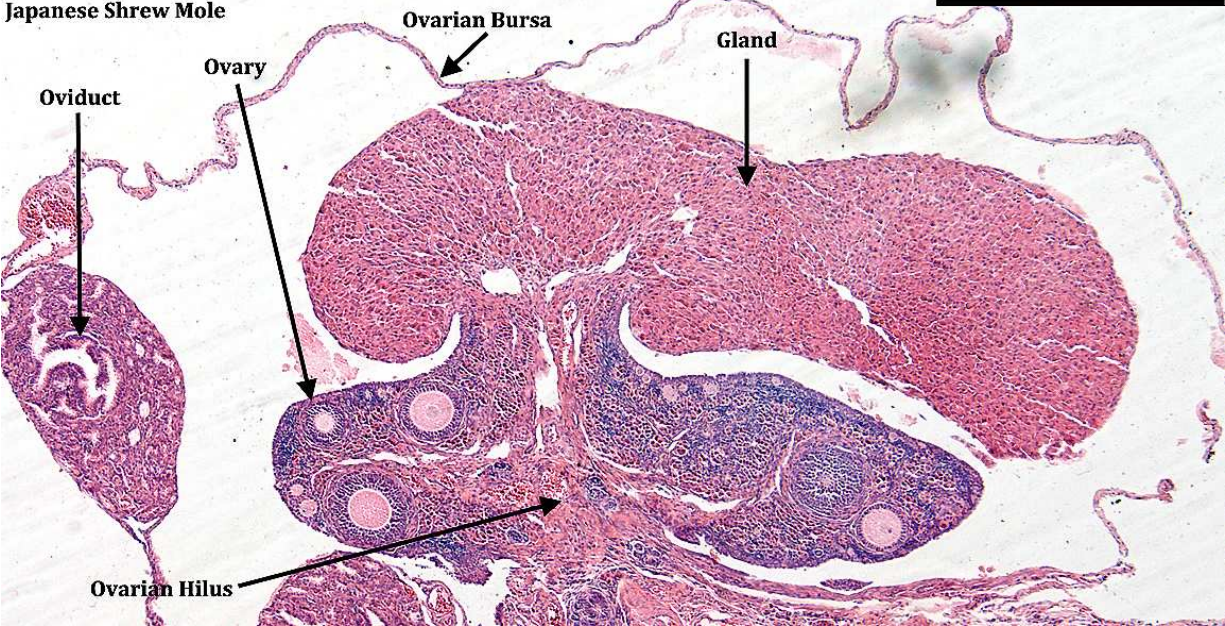
Figure 2.22. Transverse H&E stained section of adult Japanese shrew mole ovary from the breeding season. Note the ovary with follicles and its typical ovarian hilus. However, note that the ovary is attached to a glandular structure that is equal or slightly larger in size to the ovary.

Chapter 3: Discussion, Concluding Remarks and Future Directions

The prerequisite of correct anatomy:

The most fundamental issue in discussing the comparative anatomy of external genitalia in moles and other species is accurate description of the relevant anatomy so that correct comparisons can be made across species. At the onset of my study of mole external genitalia, it was apparent that the terminology used by previous investigators was in error. Detailed accurate descriptions of mouse external genitalia has recently emerged from the Baskin laboratory (Yang et al., 2010; Rodriguez et al., 2011; Rodriguez et al., 2012; Weiss et al., 2012; Blaschko et al., 2013) and have served as a model for understanding the anatomy of mole external genitalia. Critical analysis and comparison of the anatomy of mouse and mole external genitalia has demonstrated that virtually the entire previous mole literature on external genitalia is incorrect with regard to the following terms: penis, clitoris, penile clitoris, phallus and urethra. This problem in mis-identification of the anatomy of rodent/insectivore external genitalia is not unique to the mole literature as indicated in Figure 1, a modern drawing of the mouse perineum in which the male prepuce is labeled as penis in the "Anatomy of the Laboratory Mouse" website. Needless to say, any discussion of the comparative anatomy and endocrinology of external genitalia requires correct anatomy. Thus, for the first time I have provided correct descriptions of the mole penis and prepuce, which provides an opportunity for an appropriate discussion of the comparative anatomy and endocrinology of the species under consideration.

The most grievous error in the mole literature on external genitalia is the mis-identification of the male prepuce as the "penis" and the female prepuce as "penile clitoris". Likewise, previous mole literature refers to the prepuce as "phallus", which is synonymous with penis and thus is also incorrect. For both mice and moles the elevation in the perineum of males and females is the prepuce, which is covered with hair externally and is devoid of erectile bodies. In both male and female moles the prepuce is a tubular structure, and in males the preputial space houses the penis. In female moles, the U-shaped portion of the clitoris is generally deep (interior) to the preputial space and in close association with the urethra (Fig. 1.8B-E). In contrast, the distal portion of the clitoris of star-nosed and hairy-tailed moles projects freely into the preputial space.

In males (both in mice and moles) the inner preputial epithelium is continuous with the surface epithelium of the penis in the depth of the preputial space where the inner preputial epithelium reflects onto the surface of the penis. The penis of the mouse and mole is for the most part an internal organ not visible externally except presumably during urination and mating. In mice the surface epithelium of the penis is covered with spines (and not hair) (Yang et al., 2010; Blaschko et al., 2013), whereas the surface of the mole penis is devoid of spines (and hair). The distinctive feature of the penis of all species is that it is traversed by the penile urethra and contains several well-defined erectile bodies whose names vary from species to species (corporal body, corpus spongiosum [human], corpus cavernosum glandis, MUMP corpora cavernosa, cavernosum urethrae [mouse]). In mice and in broad-footed and hairy-tailed moles an os penis is

also present (although absent in star-nosed and Japanese shrew moles), while in humans bone or cartilage is absent.

The clitoris of mice and moles is characterized by an inverted U-shaped epithelial lamina that partially circumscribes clitoral stroma. The clitoris of mice and Japanese shrew moles is a deeply located internal organ defined by a clitoral lamina. In contrast, in broad-footed, hairy-tailed and star-nosed moles the clitoris projects a considerable distance into the preputial space (1200-2232µm). Additionally, the clitoris of the hairy-tailed and star-nosed moles has a freely mobile tip within the preputial space. The female urethra is partially circumscribed by the clitoral lamina and partly ventral to the clitoral lamina (Fig. 1.8B-E). Again, the clitoris of mice and moles defined in part by the U-shaped clitoral lamina is vastly different from the hair-bearing female prepuce, which is a hair-bearing elevation in the perineum.

In the mole literature, investigators have described the "urethra" opening to the exterior on the surface of the "penis" or "penile clitoris", which we now know to be the mole prepuce. Thus, previous use of the term "urethra" is also incorrect in the mole literature. In males the urethra lies within the penis and accordingly opens into the preputial space proximal to the distal aspect of the penis. Thus, the male urethral meatus is continuous with the preputial space. While nothing is known about how male moles urinate, it is essential to recognize that urine emerges to the exterior through the preputial meatus. Thus, the pathway of urine in male moles is as follows: bladder, penile urethra, penile urethral meatus, preputial space, and preputial meatus to the exterior. The caveat is that if during urination the penis is extended beyond the preputial meatus, then urine would be expelled directly to the exterior via the penile urethral meatus. This is an important distinction not appreciated in previous mole literature.

My re-evaluation of the anatomy of mole external genitalia is congruent with that of the mouse and allows for the first time an informed discussion of the comparative anatomy and endocrinology of external genitalia across multiple species.

Endocrine assumptions:

Perhaps the most basic concept regarding sex differentiation of the external genitalia is that androgens play a central role. The Jost theory states that in the presence of androgens, the male pattern emerges, while in the absence of androgens the female pattern develops. While this idea is correct and applicable to many species, other mechanisms are possible in so far as certain aspects of external genitalia development may be androgen-independent. In the case of the spotted hyena, mouse and some mole species, the apparent "masculinization" of female external genitalia raises two possibilities: (a) that "masculinization" of female external genitalia has been elicited by endogenous androgens or (b) that the "masculinized" female external genitalia emerged via androgen-independent mechanisms. Given the incomplete nature of the endocrine literature in moles, it is difficult to distinguish between these alternatives. However, if endogenous androgens are responsible for "masculinization" of female external genitalia, my assumption is that there should be a source of androgens and more important there should be some manifestation of androgen actions in other organs such as the presence of female prostate

or epididymis or an elongated anogenital distance. In the absence of such corroborating information of androgen action, we must entertain the idea that certain masculinizing events are androgen-independent.

Anogenital Distance:

Human. Anogenital distance is longer in males than in females in all phase of life (Salazar-Martinez et al., 2004; Thankamony et al., 2009) and is presumed to be androgen-dependent.

Hyena. Anogenital distance has never been measured in spotted hyenas.

Mouse. Anogenital distance is longer in males than in females (Graham and Gandelman, 1986; Vandenbergh and Huggett, 1995) and is androgen-dependent as discussed above.

Mole. For broad-footed and star-nosed moles there is no significant difference in anogenital distance in males and females when normalized to body weight or body length. No information on anogenital distance is available for hairy-tailed moles. Anogenital distance is longer in male than female Japanese shrew moles (Table 3.1). Thus, sexual dimorphism in anogenital distance in Japanese shrew moles, mice and humans implies the presence of adequate androgen levels in males and inadequate androgen levels in females. The importance of this conclusion and the similarity of mole versus mouse and human data strongly suggest that there are biologically relevant differences in androgen levels during development of the external genitalia in male and female Japanese shrew moles. The important corollary to this idea has profound implications regarding the androgen-dependency (or the lack thereof) in structures such as the male and female prepuce, in so far as similarities in size/length of the male and female prepuce are likely to be androgen-independent.

Table 3.1. Summary of masculine features observed in female moles.

	Ovarian Intersitial Gland	Anogenital Distance	Prepuce Length	Clitoral-Preputial Overlap	Free Clitoral Tip	Os clitoris	Urethral Location*
Star-nosed mole	Yes	M=F	M>F	Shorter but substantial	yes	no	90%
Hairy-tailed mole	ND	ND	M=F	Longest	yes	yes	25%
Broad-footed mole	No	M=F	M=F	Shorter but substantial	no	no	66%
Japanese shrew mole	No	M>F	M>F	none	no	no	never

* Urethral Location: The urethra becomes partially enclosed within clitoral stroma as defined by the U-shaped clitoral lamina for much of the clitoral length. The amount of the urethra that resides within the clitoral stroma is defined as the urethral location.

Male Prepuce:

  How does it develop? The description of preputial development below is taken from the mouse literature and, given many similarities, is likely to apply to development of the mole prepuce. While mouse/mole versus human prepuce are vastly different anatomically (Fig. 1.11), development of the prepuce is similar in all 3 species. The prepuce is a circumferential fold of skin with epithelium on both its inner and outer surfaces. In mice and moles the prepuce (external prepuce in the case of mouse) surrounds and houses the penis. The outer surface of the prepuce is covered in hair similar to that of the surrounding abdominal skin. In humans the prepuce is quite different in that it merely covers the distal aspect of the glans penis and is devoid of hair. During development, the mouse prepuce originates from small swellings lateral to the genital tubercle. These bilateral preputial swellings grow ventrally around the genital tubercle to fuse in the ventral midline and then grow distally along the embryonic genital tubercle toward its distal tip (Perriton et al., 2002; Petiot et al., 2005). In rodents/insectivores the ventral fused preputial folds eventually overgrow the genital tubercle to completely cover the penis. As the prepuce grows over the penis, a layer of epithelial cells, the preputial lamina, demarcates the prepuce from the glans. In male mice, typically around the time of puberty, the preputial lamina canalizes to form a preputial space between the glans penis and the prepuce (Hunter, 1935; Kanagasuntheram and Anandaraja, 1960). This preputial separation occurs around the time of puberty and appears to be an androgen-dependent event (Korenbrot et al., 1977). In similar fashion, formation of the human prepuce involves the ventral growth and midline fusion of preputial folds (Favorito et al., 2012).

Female Prepuce:

  How does it develop? While there is no specific literature on the development of the female prepuce in mice or any other species to my knowledge, in female mice the prepuce develops in a similar (if not identical) fashion to that of the male prepuce. This conclusion is based upon the following facts: (a) Development of the ambisexual pattern of external genitalia is virtually identical in male and female mice during the period 11 to 16 days of gestation, resulting in the formation of the genital tubercle and preputial swellings. (b) Ventral midline fusion of the preputial swellings and subsequent "over-growth" of the female genital tubercle by the preputial swellings is virtually identical in male and female mice. By birth, male and female external genitalia seen grossly and by scanning electron microscopy are remarkably similar with the exception that anogenital distance is greater in males and newborn male external genitalia are slightly larger than their female counterparts. This information has been verified by Dr. Gerald R. Cunha, who has dissected tens of thousands of male and female mouse embryos and neonates.

Size of the Prepuce

Human Prepuce. In human males the prepuce is attached to the distal aspect of the penis, covers the glans only, and does not have hair. In human females the small glans clitoris is surrounded dorsal-laterally by the prepuce of the clitoris also lacking hair. Thus, in humans the penile prepuce is much larger than clitoral prepuce. The profound sexual dimorphism in the size and morphology of the prepuce in humans is believed to be androgen-dependent based upon several lines of evidence, the most compelling being X/Y males having mutations in the androgen receptor (Wilson et al., 1981a; Wilson et al., 1983b). Form of the external genitalia in such androgen-resistant males is distinctly feminine, with a clitoris and presumably a clitoral prepuce (clitoral hood) (Wilson et al., 2011).

Hyena Prepuce. The prepuce of the male spotted hyena is similar to that of the human male in that it is attached to the distal aspect of the penis covering the glans only. In contrast to humans, the external portion of the hyena prepuce is hair-bearing, but to a much lesser extent than that of the ventral body. Size and shape of the prepuce of male and female spotted hyenas is similar. Thus, it is likely that development and growth of the prepuce of male and female spotted hyenas are androgen-independent (Cunha et al., 2014).

Mouse Prepuce. Mice are built low to the ground and presumably for that reason male mice have an extensive external prepuce to protect the penis from foreign matter on the ground. Therefore, the mouse penis is housed deep within a hair-bearing elevation of the perineum, called the external prepuce. Male mice also have an internal prepuce, which is integral to and completely surrounds the distal aspect of the glans penis (Blaschko et al., 2013). The external prepuce of the male mouse is clearly homologous to the mole prepuce. The external prepuce of mice is "slightly" longer in males than in females, but this slight difference is ameliorated when accounting for the sex difference in body weight. We conclude that development and growth of external prepuce in male and female mice is androgen-independent for the following reasons: (a) Size and morphology of male and female mouse prepuce is remarkably similar even though there is no evidence in female mice of androgen-dependent masculinization of other urogenital organs. Thus, the similar size of the female and male mouse prepuce is attributed to an androgen-independent mechanism. Accordingly, growth of the male prepuce may be androgen-independent as well. This conclusion is supported by the fact that androgen production during fetal and neonatal periods is substantial in males and nearly undetectable in female mice. Thus, in the apparent absence of sufficient androgens the female mouse prepuce grew to a size equivalent to that of the male, signifying that development of the prepuce is androgen-independent in female mice and probably also in male mice.

Mole Prepuce. Male and female prepuces are similar in size and shape in star-nosed, broad-footed and hairy-tailed moles. In contrast, female Japanese shrew moles lack an external perineal (preputial) projection (Table 3.1). This does not mean that the female Japanese shrew mole lacks a prepuce, but instead that the prepuce of the female Japanese shrew mole does not project distally from the perineal body wall. In all the other moles investigated male and female prepuces are prominent elevations in the perineum. A stratified squamous epidermis bearing hair follicles covers the external surface of male and female mole prepuces. In males and females the inner surface of the prepuce is non-hair bearing stratified squamous epithelium. In males the

preputial space extends proximally until the inner preputial epithelium reflects onto the penile surface. Likewise, in female star-nosed and hairy-tailed moles the clitoris projects into the preputial space and thus the inner epithelium of the prepuce reflects onto the surface of the clitoris, with the inverted U-shaped clitoral lamina being more deeply placed (Figs. 2.12D & 2.15D). In contrast, in Japanese shrew moles the clitoris is completely deep to the preputial space (Fig. 2.18). In broad-footed moles the clitoris is connected to the prepuce along its entire length, never residing in a preputial space even at its distal tip (Fig. 2.6).

Given the incomplete information regarding androgen action in mole development, what can be inferred regarding endocrine parameters of preputial development from existing data? It is important to note that in star-nosed and hairy-tailed females the distal end of the clitoris projects into the preputial space similar to the penis of these species. Thus, just like the male the inner preputial epithelium of star-nosed and hairy-tailed mole females reflects onto and thus is continuous with clitoral surface epithelium. The inference here is that the preputial lamina separated into inner and outer layers to such an extent as to define a freely mobile, untethered glans clitoris and an untethered penis. Preputial separation in males has been shown to be androgen-dependent (Korenbrot et al., 1977), and thus the projection of the clitoral glans into the preputial space of star-nosed and hairy-tailed mole females may be a manifestation of androgen action. In female star-nosed moles androgens could be derived from the ovarian interstitial gland. The presence or absence of an ovarian interstitial gland is not known for hairy-tailed moles. In broad-footed moles, that lack an ovarian interstitial gland, the distal end of the clitoris is tethered as is that of the mouse and thus indicative of an absence of androgen action. However, male and female anogenital distance was equal in both broad-footed and star-nosed moles, often an indicator of androgens acting on female mammals during development. This lack of sexual dimorphism in anogenital distance may indicate androgens are present in broad-footed mole females during development and possibly affect the size of the prepuce but not the distal tip of the clitoris residing within a preputial space as the clitoris tip does in star-nosed and hairy-tailed moles. The complete lack of a protuberant prepuce (perineal elevation) in female Japanese shrew moles is the most extreme sexual dimorphism in the moles I have studied, and thus is the most similar to the vast difference in prepuce size seen in humans. The absence of a clitoral projection into the preputial space in Japanese shrew moles correlates with the lack of an ovarian interstitial gland and differences in anogenital distance implying the absence of androgen action in females. Thus, in Japanese shrew moles the obvious interpretation for the absence of a protuberant female prepuce (perineal elevation) is that androgen levels are insufficient to "masculinize" the prepuce and that preputial sexual dimorphism seen in Japanese shrew moles is (like in the human) androgen-dependent. Unfortunately, for females of all four mole species there is no additional corroborating information regarding other biological effects of androgens such as retained epididymis or female prostate. Thus, it appears that preputial characteristics (size and morphology), while androgen-independent in mice and spotted hyenas, are not able to be determined to be androgen-dependent or independent for female star-nosed, broad-footed and hairy-tailed moles. In contrast, sexual dimorphism of prepuce of humans and Japanese shrew moles appears to be androgen-dependent.

In all four mole species studied there was a range in the length of the prepuce between males and females (Table 3.1). In star-nosed moles preputial length was significantly longer in males versus females (p=0.0026). Star-nosed moles could not be measured for body weight or

length as previously stated. However, as previous publications have reported that body weight and body length do not vary significantly between the sexes, it is unlikely such measures would change the results (Hamilton, 1931; Yates and Moore, 1990). Since star-nosed moles possess an ovarian interstitial gland, and the anogenital distance is equal between the males and females, sexual dimorphism in prepuce length may be androgen-dependent. Preputial length in male and female hairy-tailed moles did not differ significantly. Thus, my findings on preputial length are in accord with those of previous investigators who took into account body weight and body length (Eadie, 1939; Conner, 1960). In broad-footed moles preputial length was the same between males or females. Also no difference was observed when comparing adults or juveniles of either sex, and this trait was monomorphic when adjusted for body length. Slight variation in preputial length was seen (p=0.038) between male and female broad-footed moles only when compared against differences in body weight, with preputial length being slightly longer in females versus males. As broad-footed female moles have a typical mammalian ovary, this suggests that growth of the prepuce is also androgen-independent in this species. Yet if we consider that broad-footed mole male and female anogenital distance is similar, the possibility that androgens are being produced from some other source than an ovarian interstitial gland to affect preputial length cannot be ruled out. Male Japanese shrew moles have a prominent prepuce, while females have none. As this species has been reported to lack an ovarian interstitial gland in the non-breeding season, these data suggest that preputial length may reflect androgen action in the case of males and the lack thereof in females as appears to be the case for male and female prepuce in humans (Wilson et al., 1983a; Wilson et al., 1983b; Carmona et al., 2008). This sexual dimorphism is also present in the anogenital distance of Japanese shrew moles supporting the theory of androgen action in male but not female Japanese shrew moles causing the greater preputial length.

Thus, the prepuce length and morphology of moles displayed quite a bit of variation between the species, some more closely resembling the extreme sexual dimorphism of humans and others more monomorphic like that of the mice and spotted hyena.

Penile Anatomy:

While there is diversity in the anatomy of the eutherian penis (Simmons and Jones, 2007), the common basic architecture of the penis is the presence of well-defined erectile bodies and a penile urethra. The corpora cavernosa and corpus spongiosum comprise a large portion of the interior of the human penis. In other species these two erectile bodies may be remarkably similar to those of humans (as in the case of the spotted hyena) (Cunha et al., 2003; Cunha et al., 2014). Alternatively, other species may have different erectile bodies with different names. For example, the mouse has four erectile bodies: corpora cavernosa forming the corporal body within the "body of the penis", and the corpora cavernosa glandis, MUMP corpora cavernosa and the corpus cavernosum urethrae within the so-called "glans" (see Fig. 1.4). The bilateral corpora cavernosa of attach proximally to the pubic bones and fuse as they extend distally into the penile shaft to form a common midline corporal body, which is dorsally situated within the penis. The corporal body is surrounded by a fibrous sheath called the *tunica albuginea*. The corporal body either terminates distally at the os penis in certain mole species or continues to the shaft-glans

junction (human and hyena). The corpus spongiosum lies ventral to the corpora body, is also usually surrounded by a fibrous sheath, and completely surrounds the penile urethra in humans (Clemente, 1985). The corpus spongiosum in humans is expanded proximally as the bulb of the penis and expanded distally to form the glans (Clemente, 1985). The bulb or the penis in humans is attached to the under surface of the urogenital diaphragm. In mice the homologue to the corpus spongiosum is called the corpus cavernosum urethrae (Rodriguez et al., 2011). In contrast to the human corpus spongiosum, the mouse corpus cavernosum urethrae does not surround the urethra, but instead lies immediately ventral to the urethra. The mammalian penis is traversed by the urethra, which terminates at or near the distal tip of the glans in most species. In some species (mouse, rat, cat and others) the surface of the glans or shaft is adorned with keratinized epithelial spines (Williams-Ashman, 1990; Simmons and Jones, 2007). Some species possess a bony structure within the penis called the os penis, which lies dorsal to the urethra. An os penis is present in broad-footed and hairy-tailed moles, and an os clitoris is present in hairy-tailed moles.

Clitoral Anatomy:

The clitoris is the homologue of the penis and accordingly shares a common developmental history and anatomy. The most detailed description of the clitoris is found in the human medical literature. However, unlike the penis, the clitoris is not traversed by the urethra. The female urethral meatus opens to the exterior ventral to the clitoris. Accordingly the urethra is associated with the ventral vagina wall (Clemente, 1985). Those clitorises containing well defined erectile bodies can be divided into three general regions: (a) the distal glans, (b) the middle corpus or shaft, and (c) proximally the bifurcated segment comprising the attachment of the clitoral corpora cavernosa to the pubic bones (Clemente, 1985). Like the penis, the human clitoris is basically composed of two erectile bodies: (a) the corpora cavernosa, which are attached proximally to the pubic bones and fuse distally to form the body of the clitoris, and (b) the bulbs of the vestibule, commissure of the bulbs, which fuse distally to form the freely mobile glans clitoris, all of which are homologous to the corpus spongiosum of the penis (Moore, 1985; Clemente, 2001). In some species, like humans, only the tip of the glans clitoris is visible externally, while in rodents/insectivores the clitoris is a wholly "internal organ" deeply placed within or deep to the preputial space as discussed above. The clitoris may contain erectile tissue homologous to that of the penis, as is the case for humans, or may be devoid of anatomically defined erectile bodies as is the case for the mouse. A distinctive feature common to the clitoris of the mouse and the 4 species of moles studied herein is the presence of an inverted U-shaped clitoral epithelial lamina, which is associated with the urethra. The presence of other structures such as bone is rare in mammalian females, but an os clitoris is present in female mice and in hairy-tailed moles (Weiss et al., 2012) but not in the other species of moles studied herein, humans or in spotted hyenas (Cunha et al., 2003; Cunha et al., 2014).

Phallus Size and Urethral Location:

Human. The human external genitalia exhibit a large degree of sexual dimorphism. Human males possess a pendulous penis, while in females only the very tip of the glans of the clitoris is seen externally. The urethra in males traverses the penis ending distally as a simple slit at the tip of the glans. In females the urethral opening lies ventral to the clitoris and is never associated with the clitoris (Clemente, 1985). This difference in the size of human external genitalia has been shown to be androgen-dependent (Wilson et al., 1981a; Wilson et al., 1983a; Wilson et al., 1983b).

Hyena. Male and female spotted hyenas have a large pendulous phallus. The penis is slightly longer than the clitoris. Studies of the spotted hyena utilizing prenatal treatment with anti-androgens or prepubertal castration have shown that formation of the male and female phallus and subsequent growth of the penis and clitoris is for the most part androgen-dependent (Cunha et al., 2014). In both male and female spotted hyenas the urethra traverses to the tip of the phallus. In females a better name for the "urethra" is urogenital sinus as the canal through the clitoris transmits urine as well as the pup at parturition (Cunha et al., 2014). In males the urethra opens on the dorsal surface of the glans penis, while in females it opens on the ventral surface of the glans clitoris (Cunha et al., 2014). Also, the penile urethra is surrounded by a thick *tunica albuginea* and the corpus spongiosum in male spotted hyenas, but is not constrained by either of these structures in females (Cunha et al., 2003; Cunha et al., 2014). While initial formation of the embryonic phallus and subsequent growth of the phallus in male and female hyenas is androgen-independent, considerable sexual dimorphism of external and internal phallic morphology has been reported and shown to be dependent upon androgen action (Cunha et al., 2003; Cunha et al., 2005; Cunha et al., 2014) based upon prenatal treatment with anti-androgens or mibolerone, a synthetic androgen. Homologous male and female phallic structures such as retractor muscles, *tunica albuginea*, shape of the urethra/UGS, presence or absence of the corpus spongiosum, and position of the urethral meatus (dorsal versus ventral) have been shown to be androgen-dependent in spotted hyenas (Cunha et al., 2005; Cunha et al., 2014).

Mouse. Overall size of the mouse penis and clitoris are vastly different with the penis being a much larger organ (Rodriguez et al., 2011; Weiss et al., 2012). The mouse penis is an "internal organ" in the resting state with the tip of the penis being situated a considerable distance from the distal tip of the external prepuce (Fig. 1.2). The mouse clitoris is also an "internal organ" lying deep to the female prepuce. The mouse clitoris is defined by an inverted U-shaped epithelial lamina and is mostly a stromal organ devoid of anatomically defined erectile bodies (Weiss et al., 2012). The penis is traversed by the urethra, which opens on the ventral side of the penis at the base of the MUMP (Rodriguez et al., 2011). In female mice the urethra only partially resides within the clitoral region defined by the inverted U-shaped lamina (Fig. 1.8B-E), and the female urethra opens into the preputial space as described above (Fig. 1.9). Based upon experiments involving neonatal castration/ovariectomy plus treatment with oil vehicle or DHT, many anatomic features (organ size, erectile bodies, cartilage, bone) were shown to be androgen-dependent in male and female mouse phalluses (Rodriguez et al., 2012). However, an important point illustrated in Rodriguez *et al.* is that the presence (males) or absence (females) of prenatal androgens specifies penile or clitoral organ identity on the undifferentiated embryonic genital tubercle.

Mole. The mole penis is an "internal organ" (like the mouse) with the tip of the penis in resting state being situated a considerable distance from the distal tip of the prepuce. The clitoris of mice and the Japanese shrew mole is also an "internal organ" lying deep to the prepuce, while in star-nosed, and hairy-tailed moles the distal aspect of the clitoris projects into the preputial space (Figs. 1.8A, 2.12, 2.15, 2.18), with the inverted U-shaped epithelial lamina being situated deep to the preputial space. In broad-footed moles the clitoris is similar in size to that of star-nosed and hairy tailed moles, also displaying the inverted U-shaped epithelial lamina, but the distal aspect of the clitoris does not project into the preputial space (Fig. 2.6). The actual size of the penis and clitoris in moles has never been measured before, as all previous mole research incorrectly designated prepuce as "penis" (males) or "penile clitoris" (females). In broad-footed moles and star-nosed moles there is no significant difference in the length of the penis or clitoris projecting into the preputial space (penile or clitoral/preputial overlap) (Table 3.1). Unfortunately, I did not have the opportunity of measuring total penile and clitoral length. In hairy-tailed moles clitoral/preputial overlap was nearly double that of penile/preputial overlap (Figs. 2.14 & 2.15). For male Japanese shrew moles the penile/preputial overlap was similar in length to broad-footed, star-nosed and hairy-tailed mole males, whereas female Japanese shrew moles had no measurable clitoral length. Considering that the penile/preputial or clitoral/preputial overlap was monomorphic in broad-footed moles, that lack an ovarian interstitial gland, as well as in star-nosed moles, that have an ovarian interstitial gland, this feature apparently is not dependent on androgens in these species. However, because anogenital distance is equal between male and female star-nosed and broad-footed moles, the presence of androgens during development and hence the effect of androgens on this trait cannot be wholly excluded. In Japanese shrew moles clitoral/preputial overlap is not applicable, whereas in male Japanese shrew moles this measure is comparable to that of the other mole species and indicative of androgen action. This is supported by the sexual dimorphism in anogenital distance in Japanese shrew moles.

The penis of all four mole species contained a urethra (completely enclosed within the penis) that exited proximal to the distal penile tip. In contrast, the spatial relationship of the female urethra to the U-shaped clitoral lamina of all species of mole studied (as well as the mouse) varies on a proximal to distal basis. Proximally, the urethra of mice and moles is completely ventral to the U-shaped clitoral lamina as described for the mouse (Weiss et al., 2012) and verified for the mole (Fig. 1.8). However, progressing distally the female urethra takes a dorsal trajectory in both mice and moles to become partially enclosed within clitoral stroma as defined by the U-shaped clitoral lamina. In the most distal location the spatial relationship of the urethra to the U-shaped clitoral lamina varies in mole species (Table 3.1). Distally the urethra in the female star-nosed mole resided approximately 90% within the clitoral stroma defined by the inverted U-shaped lamina, and the urethra opened into the preputial space as described above (Fig. 2.12). In the broad-footed mole approximately 2/3 of the urethra was situated (Fig. 2.6), while in hairy-tailed female moles only about 1/4 of the urethra was enclosed within the clitoral stroma (Fig. 2.15). It is interesting that the star-nosed mole female, which has an ovarian interstitial gland, is the mole species with the greatest amount of the urethra contained within clitoral lamina, thus making it more similar to the placement of the male urethra. Perhaps androgens determine the extent to which the urethra is "embraced" by the clitoral lamina. This suggestion is supported by the studies in which neonatal female mice were treated with DHT (Rodriguez et al., 2012).

Bone:

      Human. Human male and female external genitalia lack bone.

      Hyena. Hyena male and female external genitalia lack bone.

      Mouse. Both males and females possess a bony structure in the phallus (os penis and os clitoris, respectively). The os penis is vastly longer and thicker than the os clitoris. Testicular feminized mice (Tfm) are unable to respond to androgens, and yet develop an os clitoris, which suggests that formation of the bone is an androgen-independent event (Murakami, 1987; Rodriguez et al., 2012), even though growth of the os penis and os clitoris is dependent on androgens (Rodriguez et al., 2012). This conclusion is based upon neonatal castration studies or treatment of developing male mice with anti-androgens, which reduced os penis length. Similarly, treatment of neonatal female mice or embryonic rats with androgen induced an os clitoris or increased length of the os clitoris (Glucksmann and Cherry, 1972; Glucksmann et al., 1976; Rodriguez et al., 2012).

      Mole. Moles displayed great variation between species and sex concerning the presence of an os penis or os clitoris (Table 3.1). In broad-footed moles, males possess an os penis but females (which have a normal ovary) lack an os clitoris, suggesting that formation of the os penis is androgen-dependent. In star-nosed moles, which do possess an ovarian interstitial gland, neither males nor females possess bone in their external genitalia. In hairy-tailed moles both males and females possess bone (os penis and os clitoris) and thus resemble the mouse, while in Japanese shrew moles neither sex possess a bone. Given the paucity of data on the role of androgens in bone formation and growth in moles, no unifying concept can be formulated. However, given the diversity of bone presence or absence in moles, it is likely that several different mechanistic scenarios are at play.

Erectile Bodies:

      Human. The human penis contains two erectile bodies: the corpora cavernosa and the corpus spongiosum. The clitoris also contains two erectile bodies homologous to the elements within the penis. The considerable sexual dimorphism in anatomy is clearly dependent upon the presence or absence of androgens in humans (Wilson et al., 1981a; Wilson et al., 1983b).

      Hyena. Male spotted hyenas have three erectile bodies within their external genitalia: corpora cavernosa, the corpus spongiosum and the distal glanular erectile bodies (Cunha et al., 2003; Cunha et al., 2014). The corpora cavernosa and corpus spongiosum of the male spotted hyena are similar to their human counterparts. As in humans, the hyena corpora cavernosa fuse in the midline to form the corporal body, which is surrounded by a thick *tunica albuginea*. The *tunica albuginea* also surrounds the urethra and associated corpus spongiosum in males. The distal glanular erectile bodies are located in the glans penis and in males surround the urethra and have a distinct chisel shape. Female spotted hyenas have only two erectile bodies: the corpora

cavernosa and the distal glanular erectile bodies (the corpus spongiosum is absent in female spotted hyenas). In females the *tunica albuginea* only surrounds the corporal body. The female distal glanular erectile bodies are located in the glans, are located dorsal to the urethra and have a blunt shape. The sexual dimorphism described above is androgen-dependent based upon studies of prenatal treatment of pregnant spotted hyenas with anti-androgens (Cunha et al., 2005; Cunha et al., 2014). As an aside, the difference in the shape/position of the distal glanular erectile bodies appear to define the sex differences in the shape of the male and female glans upon erection (Cunha et al., 2014)

Mouse. The mouse penis contains 4 erectile bodies: corpora cavernosa forming the corporal body within the "body of the penis", and within the so-called "glans" the corpora cavernosa glandis, MUMP corpora cavernosa and the corpus cavernosum urethrae. The mouse clitoris does not contain anatomically defined erectile bodies, even though the clitoris of the mouse neonate has undifferentiated mesenchymal precursors of at least some of the erectile bodies (Rodriguez et al., 2011; Weiss et al., 2012). Based upon neonatal gonadectomy plus oil or DHT treatment, the development of erectile bodies is androgen-dependent in male and female mice. The absence of erectile bodies in male testicular feminized mice (Tfm) further supports this conclusion (Murakami, 1987; Rodriguez et al., 2012).

Mole. Classical corpus spongiosum or corpus cavernosum urethrae were not observed in any of the mole species studied. Broad-footed mole males and females possess a corporal body surrounded by the *tunica albuginea*. The corpus cavernosum is larger in males than in females. The corpus cavernosum glandis of the mole (named for the first time in this dissertation), is a complex network of blood vessels observed throughout the length of the glans penis and clitoris with males having a somewhat greater or larger amount of these blood vessels. A dense band of stroma rich in collagenous fibers was observed lying just deep to the penile surface epithelium surrounding this network of blood vessels, and may function in a similar manner to the *tunica albuginea,* allowing blood to fill the penis and not expand outward laterally but primarily longitudinally; this may contribute to the rigidity of the erection (Figs. 2.8 & 2.13). Both male and female star-nosed moles possess a corpus cavernosum surrounded by a tunica, which is also larger in males than in females. Similar to the broad-footed mole, a corpus cavernosum glandis was also observed as a diffuse network of blood vessels throughout the length of the glans penis and clitoris. The corpus cavernosum glandis was more prominent in male than female star-nosed moles. Male and female hairy-tailed moles possess a corpus cavernosum surrounded by a *tunica albuginea*, which was larger in males than in females. Similar to the previous two species, both male and female hairy-tailed moles displayed a corpus cavernosum glandis, which is larger in males than females. The corpus cavernosum glandis of male hairy-tailed moles appeared to be less developed than that of broad-footed or star-nosed moles. In male Japanese shrew moles there is a distinct corpus cavernosum surrounded by a tunica. Whereas there are several blood vessels throughout the glans penis suggestive of the corpus cavernosum glandis, they are less developed than that of broad-footed or star-nosed moles. Defined erectile bodies in female Japanese shrew moles are extremely rudimentary structures bounded by parentheses-shaped epithelial lamina that may or may not be erectile tissue. In both male and female broad-footed, hairy-tailed and star-nosed moles the corpus cavernosa is surrounded by a tunica (in the same location as in mice and spotted hyenas suggesting that the development of this erectile body is

androgen-independent). However, as in mice, this erectile tissue is smaller in females than males, suggesting its size is dependent on androgens.

Concluding comments:

This study represents the most in depth investigation and characterization of male and female mole external genitalia and the first to accurately describe the anatomy of mole external genitalia in broad-footed, star-nosed, hairy-tailed and Japanese shrew moles. Moreover, through examination/review of mouse, spotted hyena and human external genitalia, I have discussed the anatomy and endocrinology of external genitalia development in a broad perspective. An important advance embodied in this dissertation is the use of three-dimensional reconstruction and morphometric analysis to understand three-dimensional patterning of the external genitalia and verify differences or similarities of size/pattern in four species of male and female moles that exhibit interesting diversity/similarity of external genitalia size/pattern. I have noted in the four mole species the presence/absence of the ovarian interstitial gland, a potential source of androgens, and whether the presence/absence of this structure correlates with external genitalia size/pattern. Finally, using corroborating data regarding the possible presence or absence of androgens during development (anogenital distance, bone, female epididymis or female prostate), I discussed inferences regarding the role of androgens in development of the mole external genitalia.

Future Directions:

Given the obvious gaps in information on actual androgen levels during the development of mole external genitalia, my discussion of whether the development of external genitalia are androgen-dependent or androgen-independent is based upon inference from the facts at hand. Thus, further investigation is required to determine whether the fetus or the newborn mole is exposed to androgens in each of these species. In this regard, to advance this area of research, future studies should be directed to answering the following questions.

1. What is the status of androgen synthesis by the testes, ovaries, adrenals and placenta during fetal and neonatal development in moles? This should include an examination of the presence or absence of the appropriate steroidogenic enzymes, and should also relate to the presence/absence of the ovarian interstitial gland and whether it is present developmentally.

2. What is the status of 5α-reductase in developing mole external genitalia?

3. How does the ontogeny of androgen receptors within developing external genitalia correlate with the presence or absence of androgens in fetal and neonatal male and female moles and presumed/inferred androgen action?

4. What is the effect of perinatal "androgen blockade" either through use of anti-androgens or neonatal gonadectomy on development of mole external genitalia?

5. What is the effect of perinatal administration of DHT on the development of male and female external genitalia?

6. How do any/all of the experimental interventions suggested above relate to differences in the development of individual elements that constitute male and female external genitalia in the various mole species?

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