

Finding the clitoris: using comparative anatomical methods to describe and
characterize the clitorises of alpacas (*Vicugna pacos*)

by

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A Paper Presented to the
Faculty of Mount Holyoke College in
Partial Fulfillment of the Requirements for
the Degree of Bachelors of Arts with
Honors

Department of Biological Sciences

South Hadley, MA 01075

to be submitted in May 2024

This paper was prepared
under the direction of
Professor Patty Brennan
for eight credits.

ACKNOWLEDGEMENTS

Patty Brennan, thesis advisor

Rachel Keeffe, advisor and post-doctoral fellow in Brennan Lab

Heather Hamilton, microscopy facility director

Rebeccah Lijek, second thesis reader

Barbie Diewald, third thesis reader

Morning Beckons Farm for providing specimens for research.

Funding through a faculty grant for PLRB, the Brennan Lab is funded by NSF

CAREER Grant IOS: 2042260

Funding through Mount Holyoke College Lynk Funding granted to Grace

Thompson in summer 2023.

Live alpaca research allowed through animal teaching permit Camelid

Immunogenics Alpaca Teaching Proposal #002 granted to Dr. Steve Purdy of

University of Massachusetts Amherst. Approved 16 November 2022.

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Abstract

The clitoris, like many female reproductive organs, is a vastly understudied organ with a long history of misunderstanding. Despite poor representation in scientific literature, the clitoris plays an important role in pleasure, behavior, and reproduction among vertebrate animals. Clitoral stimulation has been linked to induced ovulation and increased rates of fertilization in domesticated agricultural animals. Alpaca (*Vicugna pacos*) clitoris anatomy has not been previously described despite the prevalence of alpaca farms across the world. Alpacas have unusually long copulation for an Artiodactyl (over 20 minutes on average), and a novel pattern of deep intromission with the penis causing abrasion and hematoma of the female mucosal layer along the upper reproductive tract and uterine horns. Through methods of dissection, histology, CT segmentation, and *in vivo* observation, we found that alpacas have a well-developed clitoris consisting of the primary components of corpus cavernosum, vestibular bulbs, extensive vascularization and innervation, and clitoral muscles. In addition to these expected structures, there is an unusual cartilage prong in the vulvar commissure and a clitoral lamina of unknown origin and composition that partially encircles the cartilage. These are possible subjects of future study.

Introduction

The clitoris, like many female reproductive organs, is a vastly understudied structure with a long history of misunderstanding and lack of representation in scientific literature (Ah King et al 2014). In humans, the clitoris has been called the source of hysteria in females, targeted as a barrier or distraction to successful intercourse, and is a site of desperate confusion for many (Di Marino & Lepidi 2014; Gross 2022). Just as the clitoris has been socially mistreated, it has similarly been disregarded in research. The clitoris is an organ included in the female reproductive tract but notably absent in numerous anatomical, histological, and medical texts. I have attempted to refer to texts such as *Atlas of Microscopic Anatomy: A Functional Approach* by Ronald A. Bergman, Adel K. Afifi, and Paul M. Heidger, *Atlas of Human Histology* by Mariano S.H. di Fiore, and *Reproduction in Farm Animals* by E.S.E. Hafez and B. Hafez – each of which has a section on female reproductive anatomy in humans or domesticated agricultural animals but has no mention of the clitoris. Similarly, popular and easily available sources, such as Wikipedia and Planned Parenthood, definitively state that a clitoris is present in “mammals, ostriches, and a limited number of other animals” and that it usually “lacks any reproductive function” or that “it’s pretty much just there to make you feel good” (Wikipedia; Planned Parenthood). It’s not necessarily wrong to say that it’s present in only a few species because the presence has not been confirmed in other species. However, it is incorrect to say that it lacks reproductive function. Typically, if mentioned in a text regarding humans, the clitoris is cited to be the only organ dedicated solely to pleasure

despite its role in copulation and reproduction through production of mucosa/lubrication, activation of immune response, and cultivation of an environment prepared to receive sperm.

Despite the small volume of publications dedicated to it, the clitoris does play an important role in pleasure, reproduction, copulation, and sometimes ovulation making it a valuable focus of study. The clitoris is homologous to the penis, an organ present and easily evident in the majority of mammals (Di Marino & Lepidi 2014; Gross 2022). Therefore, it can be presumed that most species where males have penises would also have females with clitorises (Brennan & Orbach 2020). However, there is insufficient explicit research as of yet to substantiate and validate this supposition. Despite this knowledge, as recently as 2020, research cited a lack of evidence for a clitoris in snakes and birds which has since been disproven by Folwell et al's (2022) discovery of the clitoris in snakes, and Brennan's personal observations of the clitoris in birds. Literature regarding humans has shown that the clitoris has a role in vaginal tenting (unconscious lifting of the cervix and uterus deeper into the body when the elastic vagina is stretched and aroused), lowered pH, increased temperature, and lubrication production – all of which facilitate copulation and a sperm environment that is advantageous to fertilization (Levin 2019). Yet more studies attempting to link the clitoris to reproductive function have shown that in some species, clitoral stimulation is essential for inducing ovulation (Pacličev et al 2016) and/or increasing rates of fertilization (Randel et al 1975).

Vicugna pacos (alpaca) is a domesticated animal about which there is no scientific literature expressly describing the clitoris. At the time of this writing, there is no published text describing the alpaca clitoris in detail. Alpacas are a primarily domesticated species often bred in the Americas for luxury textile fibers, meat, and labor. There are a number of factors that point toward possible presence and functionality of a clitoris in alpacas. First, male alpacas have a large well-developed penis which means it is likely that there is a similarly evolved clitoris in female alpacas given that the two structures are homologous and develop from the same embryonic tissue. Another point for a likely well-developed clitoris is because it could be a partial trigger for ovulation. Alpacas are not spontaneous ovulators like humans, rather, they are induced ovulators which signifies their need for an external stimulus to spur ovulation. According to a 2016 study by Pačičev et al, alpaca ovulation is male-induced either through mechanical stimulation of the vaginal wall, introduction of Ovulation Induction Factor (OIF) in the seminal plasma, or both. Brennan et al (2024) further supports this physiochemical trigger theory. Therefore, the clitoris could play a role in stimulating ovulation through tactile stimulation. A third argument for the likelihood of a functional clitoris in alpacas is that they demonstrate copulatory behavior between females (Miranda-de la Lama & Villarroel 2023; personal observation) which possibly points toward a presence of a clitoris that results in pleasurable interactions thereby reinforcing same sex sexual behavior, as is the case in dolphins and bonobos (Brennan et al 2024). Alpacas engage in unusually lengthy copulation (20-40 minutes) during which

females lie prone in the cush position or on their sides seemingly just patiently waiting for the male to finish. They often begin snacking on nearby hay or grass. Finally, copulation in alpacas involves intromission of the penis all the way to the end of the uterine horns, causing extensive abrasion of the entire female reproductive tract, particularly the upper tract including cervix, uterus and uterine horns (Brennan et al 2024) yet there is no evident female disturbance or irritation despite the prolonged copulation (Alpaca mating behaviour 2018). Perhaps a functional clitoris receiving pleasurable sensational input allows for tolerance of this intrusive and lengthy copulation.

While the alpaca clitoris is mentioned in only a few texts, there is no description of its location or functionality beyond a rare diagram with an arrow labeled “clitoris” or a couple of sentences describing it to be (perhaps) exclusively in the vulvar commissure. There is a mention in one photograph that the clitoris was enlarged with no other clarification in the text (Cebra et al 2014), and a photograph pointing at the supposed location of the clitoris that shows the entrance of the vestibule, with no visible clitoris and no explanatory text (Aba 2017). There are sources that vaguely refer to a cartilaginous core but don’t present theories for the form, function, or evolutionary significance of the structure (Smuts & Bezuidenhout 1987; Rodriguez et al 2014). As an organ included in the female reproductive tract with likely significance in ovulation and fertilization in this domesticated and bred species, the details of the alpaca clitoris are worth research and discovery in order to have more information pertinent to

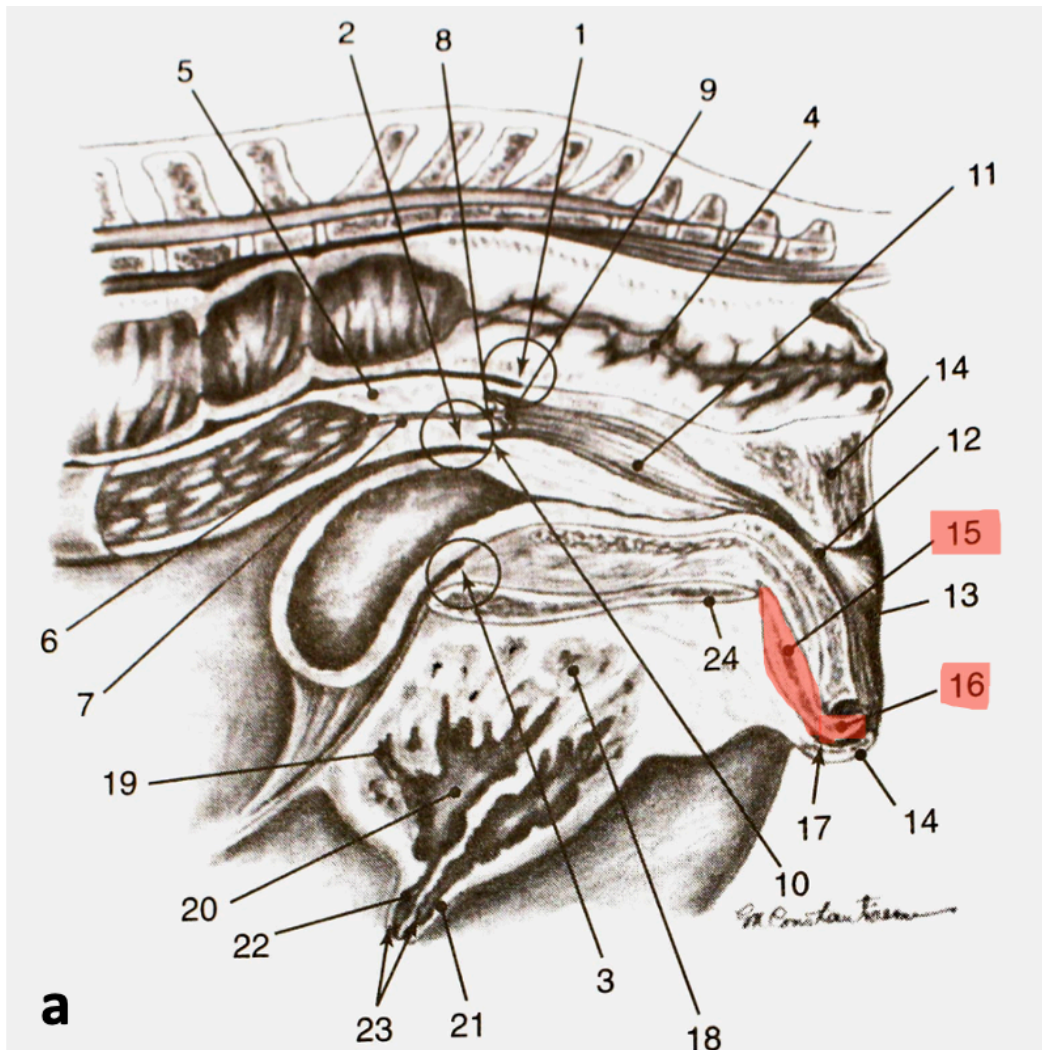
breeding, veterinary care, overall wellbeing, and additional literature to add to the canon of clitoral research across species.

The purpose of this research is to analyze and describe the alpaca clitoris by determining shape, size, attributes, and functionality through use of dissection, CT, histology, observation, and comparison to other mammals with described clitorises. Secondary aims of this research are to add to the small, but growing, body of literature on the clitoris and to reduce the imbalance of research pertaining to male reproductive organs more than female reproductive organs.

In this study, we predicted that there is a clitoris present in female alpacas that has extensive erectile tissue and significant innervation indicating functionality.

Clitoral anatomy

There are no currently existing comprehensive diagrams or analysis of the alpaca clitoris which renders it necessary to compare dissections to any diagrams of reproductive tracts of somewhat similar species. For reference, I have used a diagram of a mare from *Large Animal Theriogenology* (Youngquist & Threlfall 1997) to provide comparison to the dissection of alpacas because, similar to alpacas, mares have a vulvar commissure that partially contains the clitoris (Fig. 1). I also use literature describing the human clitoris (Di Marino & Lepidi 2014) and dolphin clitoris (Brennan et al 2022) to gain understanding of the structures that hallmark a clitoris and indicate functionality.



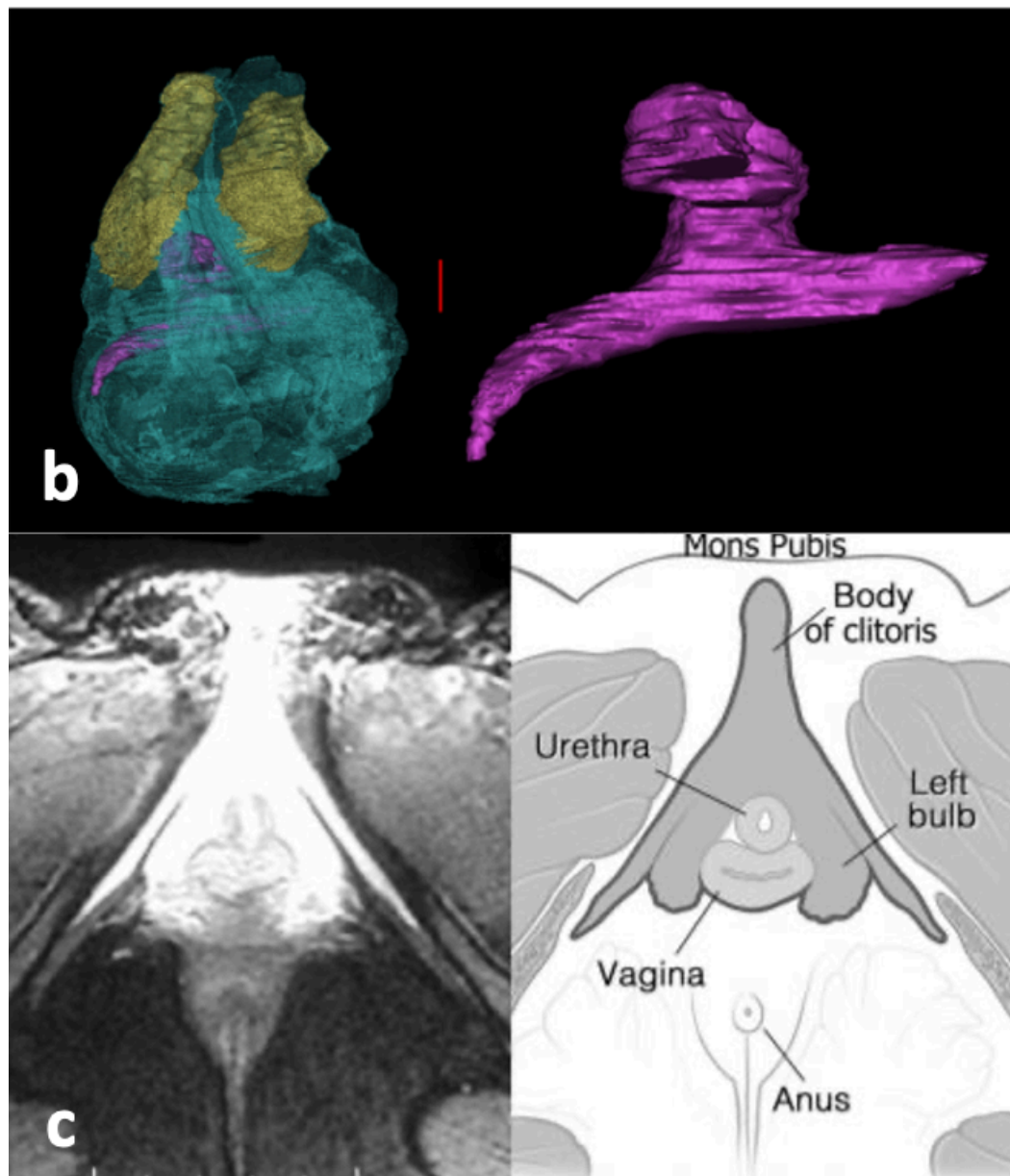


Figure 1. Clitoral diagrams from species with known and characterized clitorises. (a) diagram of a mare with the clitoris highlighted in red (Youngquist & Threlfall 1997), (b) segmentations of a CT scan of a dolphin clitoris (purple) within the reproductive tract (left) and isolated (right) (Brennan lab), (c) CT image of a human female genitalia where the clitoris is in bright white and compared to an illustration on the right (O'Connell et al 2005).

Clitoral anatomy in mammals is marked by the presence of a number of structures whose anatomical description and functional capability are enumerated below. Clitoral tissue is primarily erectile tissue of two types: corpus cavernosum and corpus spongiosum or vestibular bulb tissue, of which both have a unique

appearance as a spongy holey tissue that stretches with manipulation and inflation. The erectile tissue is studded with arteries, veins, nerve bundles, sinuses, lacunae, corpuscles, glands. Corpus spongiosum differs from spongy tissue by being more dense and sometimes having a tunica albuginea containing the tissue. Clitorises have two crura that anchor the erectile tissue of the corpus cavernosum and stretch from the body of the clitoris to the ischiocavernosus muscles or to some point along the pelvis (insertion location varies among species) (Di Marino & Lepidi 2014). Their purpose seems to be stabilizing and anchoring the erectile tissue and containing corpus cavernosum tissue when engorged. There is also an extensive nerve network that is responsible for smooth muscle activation and sensory information indicating presence of vestibular bulbs made of erectile tissue akin to corpus spongiosum. Whether a corpus spongiosum is completely homologous to the spongiosum of males is not certain but the clitoris has a described vestibular erectile tissue that is likely derived from spongiosum (Di Marino & Lepidi 2014). Henceforth, corpus spongiosum will be referred to as vestibular bulb tissue, spongy tissue, or vestibular bulbs for accuracy given the lack of defined homology. Two paired muscles (bulbospongiosus and ischiocavernosus) are present in mammal clitorises that control blood flow into or out of the clitoris to aid in tumescence.

In humans, the body of the clitoris divides into two segments: one short region that lies along the pubis and a longer arm that extends sagittally. The shorter segment of the clitoral body is formed by the joining of the crura and the segment bends upward along the pubic symphysis. Where the tunica albuginea of

the crura merge, a septum is formed that feeds into the cavernous commissure which consists of a double corpora cavernosa (Di Marino & Lepidi 2014). These corpora converge into a glans at the distal end of the clitoris. The longer segment of the clitoral body forms a bend that can be referred to as the elbow. Its degree of angle varies but the elbow of the segment is connected to the pubis by a suspensory ligament and retrocrual fascia (Di Marino & Lepidi 2014).

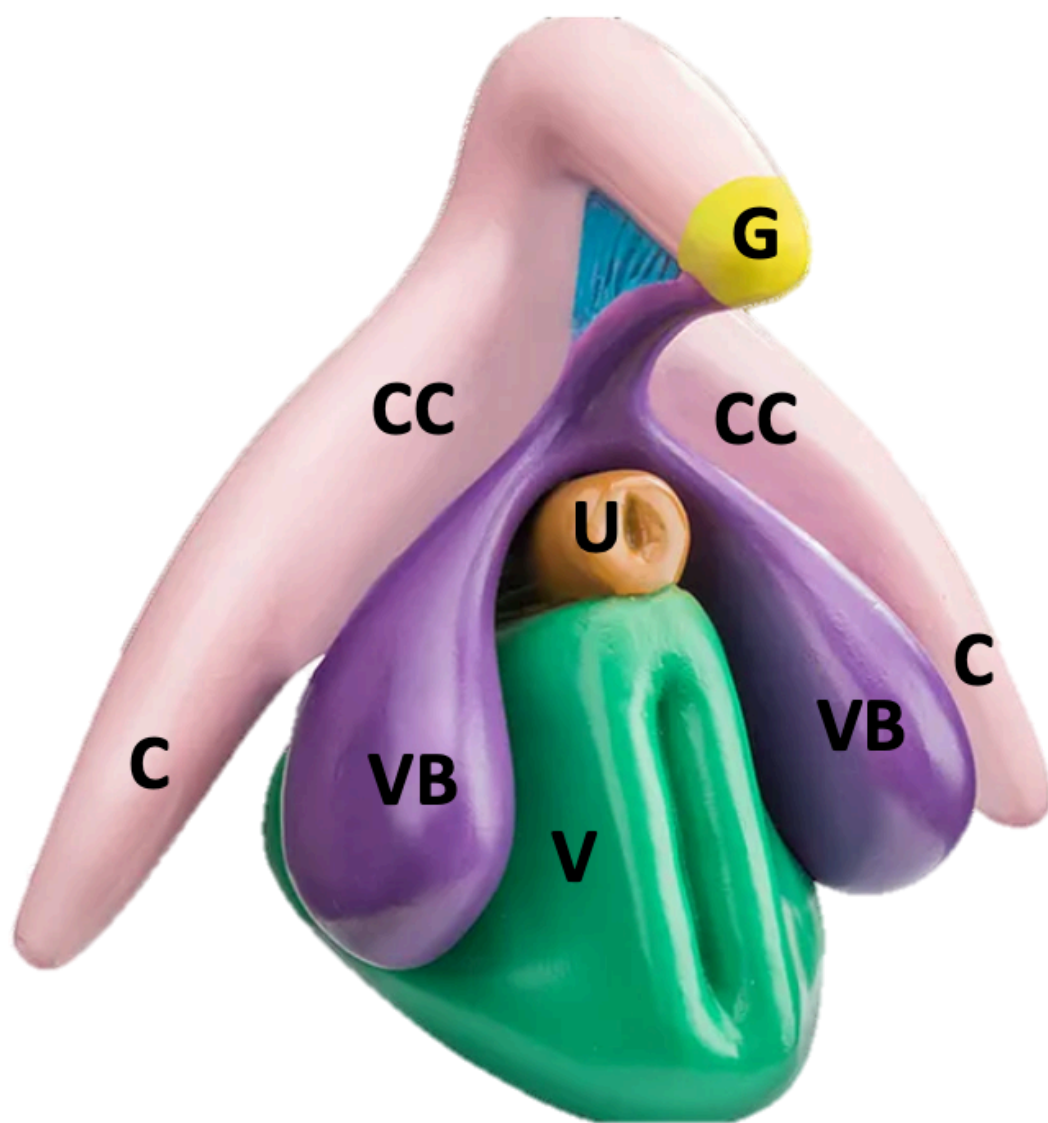


Figure 2. Diagram of human clitoris illustrating the major structures. G, glans clitoris; CC, corpus cavernosum; C, crura; VB, vestibular bulb; U, urethra; V, vagina. Model from Daniel Haag-Wackernagel.

Cavernous tissue or corpus cavernosum is a tissue with intricate venous sinus networks through which blood (or lymph fluid in some organisms) flows to pressurize and cause tumescence of the erectile tissue. The endothelial cells of this tissue can be tightly joined or have looser junctions but all rest on a basal lamina which can be useful to look for after tissue has been prepared with a hematoxylin and eosin Y (H&E) stain during histology (Di Marino & Lepidi 2014). Arterioles, venules, and nerve endings run through the sinus walls in this erectile tissue and indicate functionality of the clitoris. This tissue is composed primarily of collagen and smooth muscle fibers that can be oriented longitudinally or transversely in the sinuses (Di Marino & Lepidi 2014). One element of corpus cavernosum making it distinguishable from corpus spongiosum or vestibular bulb tissue is the thick fibrous connective tissue envelope, tunica albuginea, that surrounds the cavernosum. The purpose of the tunica is to resist the swelling of the tissue during tumescence to oppose over-engorgement and increase pressure within the tissue which results in a firmer structure. In the clitoris, the albuginea is typically a single-layer structure with fibers running vaguely circularly, though not fully enclosing, in an inconsistent pattern around the corpus cavernosum. Occasionally there is a region with an additional layer with fibers running longitudinally in the additional outer layer. The two-layer tunica albuginea is more consistently present in the penis where there is significantly more lengthening and pressurizing during tumescence. The clitoris has less elongation and therefore needs less of the outer layer's longitudinal restrictive fibers. Another element that can distinguish corpus cavernosum is the lower quantity of

lacunae, small pockets lined with a thin epithelium and surrounded by collagenous fibers and smooth muscle fibers. These fill with blood to tumesce the erectile tissue. These are significantly more concentrated in corpus spongiosum or vestibular bulb tissue than corpus cavernosum.

Spongy tissue/corpus spongiosum/vestibular bulb tissue is similar to the corpus cavernosum in its holey appearance and ability to be infiltrated with fluid (blood or lymph) during tumescence. However, it is less reliant on sinuses that become engorged and has more lacunae that are interspersed throughout the tissue in no particular organization (Di Marino & Lepidi 2014). It is theorized that the smooth muscle surrounding lacunae are responsible for filling and emptying the lacunae while the collagen fibers take the role of maintaining the filled or emptied state (Di Marino & Lepidi 2014). Vestibular bulb tissue is not enveloped by a tunica albuginea which leaves it more vulnerable to damage, most commonly occurring during labor. In mammals, the homology of the corpus spongiosum of the penis with the corpus spongiosum of the clitoris is unclear, but it is likely that the tissue of the vestibular bulbs in addition to the glans clitoridis, contain the corpus spongiosum of the clitoris.

In humans, each crura clitoridis has an extension of the superficial perineal artery that both vascularizes the erectile tissue and is responsible for the engorgement of the tissue when stimulated (Di Marino & Lepidi 2014). Each crus connects on one end to the ischial arch via ischiocavernosus muscles and increases in diameter until, at the other end, they merge and form a singular pillar that attaches to the pubic symphysis (Di Marino & Lepidi 2014). The crura fill

with blood (or lymph) and are highly pressurized and nearly immobile thanks to the tunica albuginea.

The clitoris is typically innervated by the cavernous nerve and pudendal nerve network originating in the pelvis and terminating in two branches called the perineal nerve and dorsal nerve of the clitoris (Di Marino & Lepidi 2014). The perineal nerve divides into a sensory superficial perineal nerve terminating in the perineum, labia majora, and distal end of the urethra, and a motor/sensory deep perineal nerve activating the ischiocavernosus and bulbospongiosus muscles as well as terminating in some parts of the corpus spongiosum or vestibular bulb tissue (Di Marino & Lepidi 2014). The dorsal nerve of the clitoris is less studied but, in humans, seems to follow a similar pathway to the perineal nerve though it incorporates autonomous fibers that hallmark the cavernous nerve. Both types of erectile tissue and the glans are innervated by nerve endings from these nerve networks resulting in an extremely sensitive clitoris.

On the ends of many nerve endings are corpuscles that are sensitive to tactile input and relay information to the nerves they attach to (Di Marino & Lepidi 2014). Genital corpuscles are highly complex and diverse with a range of specific characteristics ranging from mechanoreception, proprioception, hormonal influence, and location in the dermis, epithelium, or connective tissue.

Much of the clitoris also contains a variety of glands that are responsible for secreting lubricating fluid (Bartholin's glands) and other fluids (Di Marino & Lepidi 2014).

The clitoris contains two paired muscles called the ischiocavernosus muscles and bulbospongiosus muscles. The ischiocavernosus muscles incorporate and envelope the crura and insert on the ischial tuberosity (Di Marino & Lepidi 2014). Fiber bundles for this paired muscle are obliquely oriented around the crura which indicates that the primary purpose of the muscles are to squeeze blood out of the crura during detumescence (Di Marino & Lepidi 2014). The other paired muscle in the clitoris is the bulbospongiosus muscle which inserts at the central tendon of the perineum and terminates in clitoral fascia, suspensory ligament, around the superficial dorsal vein of the clitoris and around the urethra (Di Marino & Lepidi 2014). Given the termination around a vein, it is evident that, like the ischiocavernosus muscles, this part of the bulbospongiosus muscle serves to control blood flow to the erectile tissue though, the bulbospongiosus muscle encourages backflow of blood into the erectile tissue rather than the outflow and evacuation of the erectile tissue as is the function of the ischiocavernosus muscle.

Morphology of the clitoris can provide clues to the functionality of the clitoris as is evident from the study on dolphin clitorises conducted by Brennan et al (2022). By analyzing the positioning of the clitoris and finding it towards the anterior aspect of the vaginal entrance, it is evident that physical contact and stimulation are possible and likely. Similar analysis of lacunae and extensible collagenous connective tissue indicate functionality of the clitoris because they signify an ability of the lacunae to fill with blood to aid tumescence and of the collagen to stretch or maintain structure during tumescence. Histology of the

tissue allowed Brennan et al to find free nerve endings that are hypothesized to be low threshold mechanoreceptors and are present in the clitorises of guinea pigs and mice (2022). Another structural finding that points to functionality of the clitoris in dolphins is the spatial orientation of the clitoral arteries close to nerves which, in humans, function in orgasm. Clearly, analyzing and describing the morphology of the clitoris provides evidence of functionality (or lack thereof). In addition, dolphin clitoris' presence of sensory genital corpuscles and a much thinner skin over the body of the clitoris compared to the skin of the adjacent genital opening may facilitate stimulation of the free nerve endings found right below the skin in the dermal papillae.

According to Tibary and Anouassi (1997), alpacas have a small clitoris which consists of a corpus cavernosum clitoridis surrounding a cartilaginous core. Tibary and Anouassi include an image and diagram of an alpaca reproductive tract that points to a clitoris but the shape is very unclear and the surrounding tissues are unidentified thereby making it difficult to adequately analyze the clitoris. The desire to clarify the lack of certainty regarding components of the clitoris and their location within the genitalia and reproductive tract are motivators for this research.

Clitoral function

Despite being commonly described as the only organ whose sole purpose is pleasure, the clitoris actually does have reproductive function in addition to its capacity for pleasure. When stimulated, the clitoris initiates a series of

physiological changes to the reproductive tract as well as biochemical changes adjusting the environment for sperm. Physiological changes may include increased heart rate and blood pressure, increase in lubrication and tissue oxygenation, and vaginal tenting (Levin 2019). Biochemically, humans' vaginal environment changes by slightly decreasing pH and increasing lubrication (Levin 2019). These adjustments allow for less painful copulation, facilitated motion for the penis, and an environment ready for sperm reception (Levin 2019). The clitoris also stimulates an immune response that can initiate uterine remodeling and facilitate maternal-embryo signaling (Katila 2012).

Study organism - alpacas (*Vicugna pacos*)

Alpacas are even-toed ungulates in the Artiodactyla order and Camelidae family. They are closely related to llamas, camels, guanacos, and vicunas, though they are more closely related to New World camelids like llamas, guanacos, and vicunas than Old World camelids including Bactrian camels, dromedary camels, and wild Bactrian camels. Alpacas are a species domesticated from vicunas in the Andean region of South America and a species that underwent extreme hybridization with llamas and population decrease during the Spanish conquest in the early 1500s (Miranda-de la Lama & Villarroel 2023). 90% of the global alpaca population exists in small communities across Peru where people benefit from alpaca meat, fiber, and labor (Miranda-de la Lama & Villarroel 2023). There has been a recent increase in breeding alpacas to satisfy the increasing demand for both the fine high quality alpaca fiber and nutritious gourmet alpaca meat. In

South America, alpacas live mostly in highlands and coastal regions of Peru where they subsist on difficult-to-digest fibrous grasses during the dry season and leafy vegetation in the wet season. Due to the seasonality of their diet and high altitudes of their environments, alpacas are evolved to easily store fat and water during the wet season and have high hemoglobin oxygen affinity to aid in low oxygen altitudes (Miranda-de la Lama & Villarroel 2023).

Alpacas are vocal animals who bleat to communicate danger and whose males emit a sound called “orgling” during copulation (Miranda-de la Lama & Villarroel 2023; Brennan et al 2024). The mating season for alpacas is tied to seasonality given that there is more food readily available for energy for gestation, however, in captive controlled environments where diet is not seasonally fluctuating, alpacas may mate and reproduce during any part of the year. Most male and female alpacas are sexually mature by 12 months old but male alpacas’ penises can take about 3 years to lose their penis-preputial attachment that inhibits full penile intromission and, therefore, inhibits successful fertilization (Miranda-de la Lama & Villarroel 2023). Female alpacas are induced ovulators which means that they do not spontaneously ovulate, rather, there must be an external stimulation to induce ovulation. For alpacas, this is likely due to a combination physiochemical input. Physical/tactile stimulation is delivered from the mounted male and penile intromission while chemical input is from beta nerve growth factor (beta-NGF), the ovulation-inducing factor (OIF) and most abundant protein in the male alpaca's seminal fluid (Kershaw-Young et al 2012).

Beta-NGF is the chemical key to inducing ovulation in alpacas because it starts a surge of luteinizing hormone (LH) within hours of administration (Kershaw-Young et al 2012). The mode of mechanism of NGF causing ovulation is still debated. Initially, the hypothesis was that beta-NGF is absorbed into the bloodstream from the uterus or reproductive tract, travels to the pituitary and/or hypothalamus, initiates release of LH which travels through the bloodstream down to the ovaries to induce ovulation (Kershaw-Young et al 2012; Maranesi et al 2021). However, in some species, introduction of NGF alone is insufficient to ensuring ovulation. It must be paired with some sort of clitoral or vaginal stimulation. This led to a differing theory on the mechanism of action of NGF, one in which NGF from seminal fluid initiates the production of yet more NGF from the uterine wall and both types of NGF are absorbed into the bloodstream. However, they would only need to travel to the ovary rather than reach the pituitary and/or hypothalamus to induce ovulation by stimulating GnRH production (Maranesi et al 2021). A spike in secretion of LH is the hormonal shift essential for initiating expulsion of the ovum from the ovarian follicle leaving behind a corpus luteum that slowly releases progesterone until stores are exhausted. Where beta-NGF is synthesized in the male differs between species, though in alpacas, it is produced in the prostate (Maranesi et al 2021). NGF is identifiable in the seminal plasmas of cow, sheep, goat, human, pig, horse, rabbit (Kershaw-Young et al 2012). The concentration of NGF in seminal plasma is much higher in camelids than in other species (Maranesi et al 2021).

During copulation, females lie still and prone in the cush position (also referred to as sternal recumbency) or on their side with their pelvis elevated to accommodate the male kneeling behind her. When females are in this position, it is not uncommon for them to be mounted by males or females, though it seems that ovulation is not triggered by female-female interactions (Miranda-de la Lama & Villarroel 2023). As mentioned earlier, alpaca copulation is unique because the penis intromits past the vagina and cervix, all the way into each of the uterine horns to the uterine papillae sequentially (Brennan et al 2024). This type of intrauterine insemination is found only in alpacas, though. A few other species, including the horse (*Equus equus*), pig (*Sus scrofa*), dog (*Canis domesticus*), mouse (*Mus musculus*), and rat (*Rattus rattus*) demonstrate some form of intromission into the cervix or insemination that pushes ejaculate through the cervix into the uterus (Brennan et al 2021), but not the intrahorn intromission found in alpacas.

Alpaca penises are long and small in diameter with a thick tunica albuginea that maintains a narrow structure even when the penis is fully erect (Brennan et al 2021). As the penis emerges, it circles, probing for the entrance to the vagina. Once entered, it circles in a clockwise direction until it is through the female's long cranial vagina and reaches the cervix where it intromits past the two or three cervical rings (Brennan et al 2021). There is a cartilaginous tip of hyaline cartilage whose theorized purpose is to aid in pushing past the female's constrictive cervix and allow for pressure against a pad in the uterine horns that helps direct the penis into each small uterine horn (Brennan et al 2021). The

cartilage-tipped penis pushes all the way to the distal tips of the uterine horn to the uterine papillae which signals the tubal ostium at the junction of the uterine horn and long oviducts (Brennan et al 2024; Brennan et al 2021). Alpacas are dribble ejaculators which means they continuously produce a small amount of ejaculate over the whole duration of copulation wherever the penis travels. The cartilage on the tip of the penis also causes significant abrasion to the uterine epithelium causing bloodiness and abrasion (Fig. 3) (Brennan et al 2024). The females seem unbothered by this intrusive copulation.

Theories that relate this uniquely abrasive copulation and the importance of beta-NGF hypothesize that the abrasions allow the OIF to enter the circulatory system more quickly thereby effectively inducing ovulation which secondarily results in fertilization (Brennan et al 2024). Thibary and Annouassi (1997) found that injection of seminal fluid alone was only 87% effective for achieving fertilization whereas natural mating that causes abrasion and introduction of seminal fluid achieved a 100% fertilization rate. Therefore, an explanatory theory for this cartilaginous tip's abrasion and the significantly more effective fertilization in natural insemination posits that the hard cartilaginous tip of the penis creates abrasion not made in artificial insemination enabling a quicker absorption of the beta-NGF OIF in seminal fluid. Therefore, abrasion in the uterine horns paired with the seminal beta-NGF OIF in seminal fluid is an essential partnership for successful reproduction in alpacas.

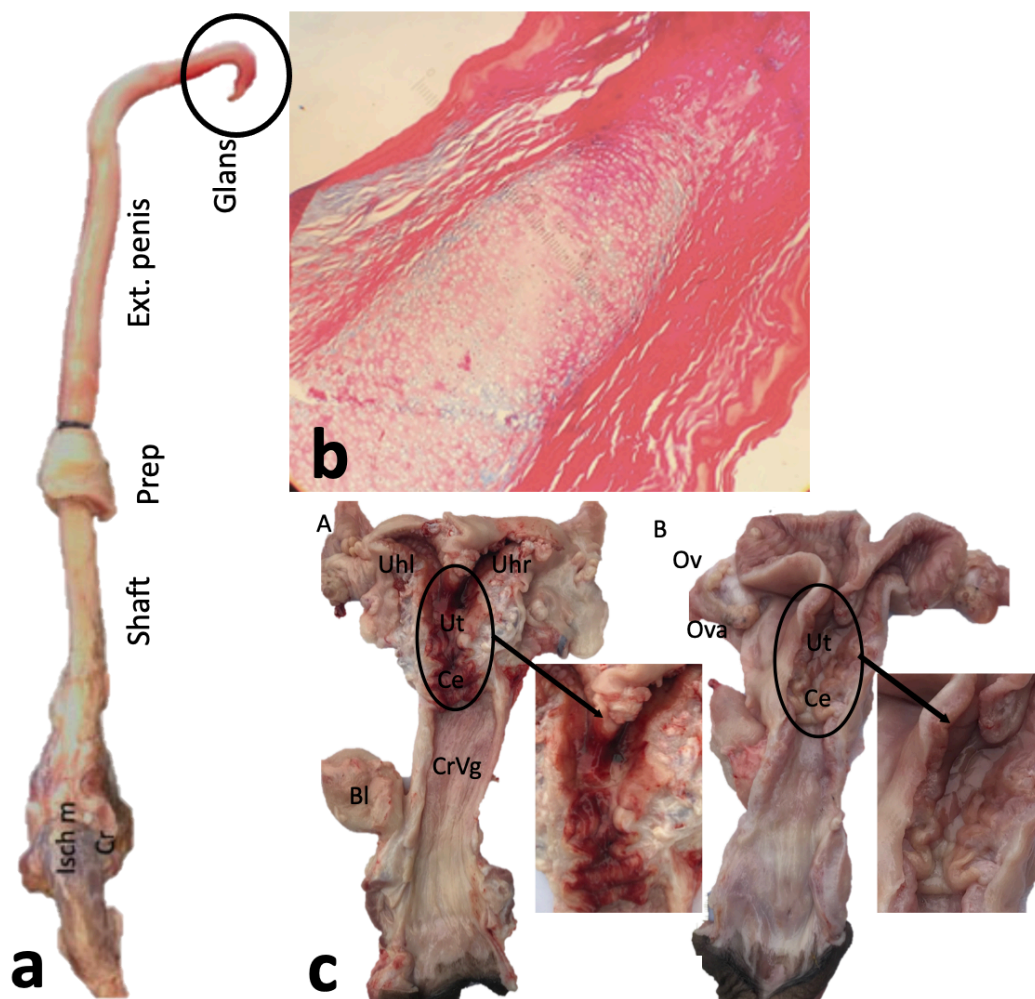


Figure 3. Alpaca penile anatomy and a comparison of abrasion in alpaca uterus post-copulation and after no copulation. (a) Alpaca penis morphology from Brennan et al, 2021. Isch m, ischiocavernosus muscle; Cr, crura; Shaft, internal segment of penis; Prep, prepuce; Ext. penis, external segment of penis; Glans, cartilaginous hooked glans and tip of penis (Brennan et al 2021) (b) histological section of glans showing hyaline cartilage from H&E stain at 40x magnification, (c) two female reproductive tracts where A is immediately post-copulation with obvious abrasion, B is not post-copulation and has no abrasion. Uhl, uterine horn left; Uhr, uterine horn right; Ut, uterus; Ce, cervix; CrVg, cranial vagina; Bl, bladder; Ov, ovary; Ova, ovarian follicles.

Another theory for the importance of the uterine abrasion during copulation is immune-based. As suggested by Brennan et al (2024), the abrasion incites a rapid immune response that could eliminate dead and dying sperm post-copulation, support tissue repair and angiogenesis (which makes a hospitable environment for an embryo and physically remodels the uterine tissue), and

induce immunological tolerance to the fetus. Copulatory wounding of the uterine epithelium has been shown to cause the production of pro-inflammatory cytokines that recruit leukocytes and macrophages to the site of abrasion (Katila 2012). Macrophages induce a remodeling of the uterine endometrium and prepare the maternal immune system for the incoming embryonic invasion (Katila 2012). Endometrial remodeling includes increased uterine receptivity to the blastocyst and uterine angiogenesis for improved vascularization in the uterus and (later) the fetus (Brennan et al 2024). The result is an environment prepared for embryo implantation. Leukocytes terminate any remaining sperm in the tract and harmful pathogens that were introduced to the uterine horns during copulation (Brennan et al 2024). The combination of the endometrial remodel and unnecessary sperm and pathogen removal supports the theory that copulatory wounding from penile intromission is actually beneficial to successful fertilization of female alpacas rather than harmful.

As previously mentioned, alpacas are part of the family *Camelidae* (Fig. 4) which diverges first into Old World camelids and New World camelids and then diverges into the three genera of *Camelus*, *Lama*, and *Vicugna*.

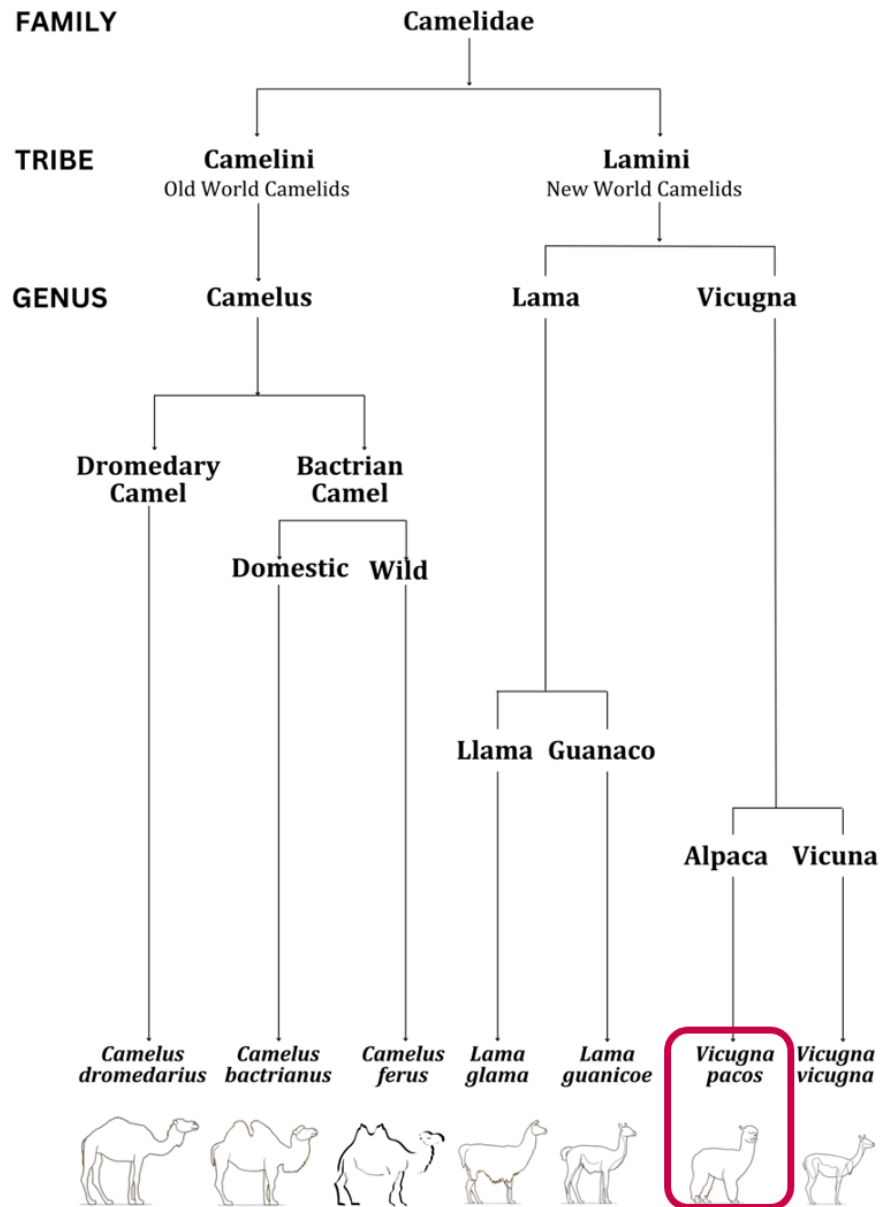


Figure 4. Phylogeny of *Camelidae* (Kishore et al 2024). Alpacas (*Vicugna pacos*) highlighted within a pink square.

New World camelids comprise the *Lama* and *Vicugna* genera within which are llamas (*Lama glama*) and guanacos (*Lama guanicoe*), alpacas (*Vicugna pacos*) and vicuñas (*Vicugna vicugna*), respectively. Vicuñas and guanacos are considered to be the wild species whereas llamas and alpacas are considered the domesticated species. They have a long history of close working relationships

with South American folks who use the animals for meat, textiles, labor, tools, and indigenous ritual practices (Vilá & Arzamendia 2020; Youngquist & Threlfall 2007). New World camelids live in grassy highlands of the Andes mountain range of South America, primarily in Peru. Domesticated species typically live on small farms where there may be herds of 30-300 individuals or they live on large farms that have flocks of 200-800 individuals (Youngquist & Threlfall 2007). Small farms often have multiple species living together (alpaca, llama, cow, pig, etc.) and employ less consistent management techniques such as shearing, breeding, or grassland conservation whereas large farms typically have more strict organization of species and tighter herd management techniques (Youngquist & Threlfall 2007). Breeding of alpacas and llamas is similar in that, in South America, they are both “seasonal breeders” (Youngquist & Threlfall 2007) from December to March which means that parturition occurs during the rainy season after a 345 day gestation period. In North America where food and water are typically more managed and consistent throughout the year regardless of the seasons, mating is not seasonal. For both llamas and alpacas across the Americas, parturition is timed very precisely to be during the day so the cria has an opportunity to be cleaned, dried off, and taught to stand before nightfall. Breeding for both species entails introducing a male to a flock of females who will either display behavior indicating receptivity to copulation or behavior that indicates a strong opposition to copulation. Receptivity to copulation is often due to presence of a mature ovarian follicle. Both llamas and alpacas have similar reproductive tracts and genitalia which explains the similar mating procedures. To date, there is

no explicit description of llama clitorises though it is mentioned in a few anatomical and agricultural texts.

Old World camelids comprise fully domesticated dromedary camels (*Camelus dromedarius*), domesticated Bactrian camels (*Camelus bactrianus*), and wild Bactrian camels (*Camelus ferus*). These species are significantly larger than the New World camelids, they have one to two humps, live in herds where there is a single dominant male among many females and offspring, and are home in Africa or central Asia (Kishore et al 2024). Dromedary camels have one hump and live mostly in Africa and the Middle East. Bactrian camels have two humps and primarily reside in central Asia (Kishore et al 2024). Like males of New World camelid species, Old World camelid males also have a prepuce with a cartilaginous tip that aids in entrance to the vagina, intromission past the cervix, and abrasion of the female's uterine horns. Mating entails the male mounted above a female in sternal recumbency (cush position) with his penis probing circularly for the vaginal entrance. Copulation typically lasts between 7-35 minutes and entails a series of small thrusts, grunting, and occasionally saliva dribbling from the male's mouth (Elwishy 1988). Like their closely related *Lama* and *Vicugna* individuals, dromedary and Bactrian camel males are dribble ejaculators depositing small amounts of sperm across the entire duration of mating. Old World and New World camelids continue to be similar in their status as induced ovulators whose highest rates of fertilization (90-100% and 93% respectively) occur when seminal plasma is introduced intramuscularly within the uterus (Kershaw-Young et al 2012).

Methods & Materials

All clitoral specimens are from female reproductive tracts sourced from Morning Beckons Farms where the alpacas were euthanized to be processed for food. After euthanization, reproductive tracts were removed and given to the Brennan lab for research carried out under IACUC permit #BR-59-0820. Live alpaca research was allowed through animal teaching permit Camelid Immunogenics Alpaca Teaching Proposal #002 granted to Dr. Steve Purdy of University of Massachusetts Amherst, approved 16 November 2022.

This research is founded on four central methods: gross dissection, paraffin histology, CT scanning, and *in vivo* observation and measurement. By taking a multi-faceted approach, details at multiple levels of analysis are possible and each method can support or further describe findings from the others.

Dissection

Gross dissection of the alpaca reproductive tract highlights macroscopic structures such as erectile tissue, crura, urethra, glans clitoris, muscle, and connective tissue. We can begin understanding the orientation and spatial relationship between each structure. This aids in describing larger aspects of the clitoris and how the macroscopic structures relate to other elements of the reproductive tract. We performed gross dissection of the clitoris of six females, collected tissues for histological processing from four females, and used two specimens for CT scanning and segmentation.

During each dissection, the goal was two-fold. First, we aimed to explore and understand the gross anatomy and context of the caudal vagina. We examined the deep clitoris that had been cut away from the body during removal to search for crura, erectile tissue, fat, and muscle. We measured and examined the vulvar commissure in search of the elusive cartilaginous core referred to in a couple of texts. We peeled back the vaginal lumen in search of erectile tissue and to determine how cranial the erectile tissue extended from the entrance of the vagina. The second goal of dissection was to collect samples for histological processing. Dissection was carefully documented by photography and diagrams in a lab notebook to retain knowledge of where each section derived (Fig. 5; Fig. 6). The later dissections were informed by need for greater detail or clarity in certain portions of the clitoris.

Histology

We performed paraffin histology on four specimens but only three provided sufficient data. One specimen was lost due to issues with dehydration. Sections for histology from the first specimen were obtained by making a midsagittal cut, sectioning one side in transverse sections and the other side in parasagittal sections, and collecting the tissue in cassettes. Tissue was photographed in the cassette to facilitate later reconstruction of the entire organ (Fig. 5). These samples were processed in a BioWave for dehydration, clearing, and infiltration before embedding, microtoming, and staining, but later processed

with standard paraffin protocols when the BioWave was found to be ineffective for our needs.

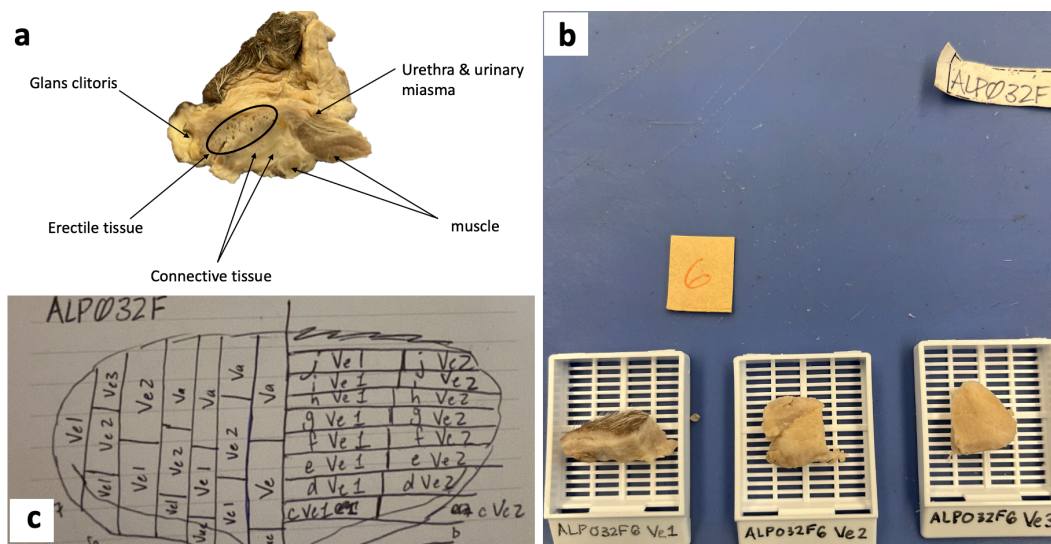


Figure 5. Images from dissection and transition to histological preparations. (a) dissection image of clitoris facing a mid-sagittal cut with labeling of visible reproductive tract and clitoral anatomy structures, (b) three specimens cut sagittally from clitoral dissection and placed into cassettes for histology, (c) drawn diagram of clitoral dissection for paraffin histology preparation.

In each specimen, the sectioning of the sample differed to allow for a more diverse array of perspectives. As mentioned above, the first sample was examined with a mid-sagittal cut while others were examined in transverse sectionings or with sagittal sectionings both at the midline and more laterally. For the fourth specimen, ALP003F, we used only a strip of the reproductive tract spanning the width of the vulvar commissure (~2 inches) and extending cranially (~5 inches) in order to more closely examine the cartilaginous structure (Fig. 6). These samples were processed using traditional dehydration, clearing, and infiltration techniques.

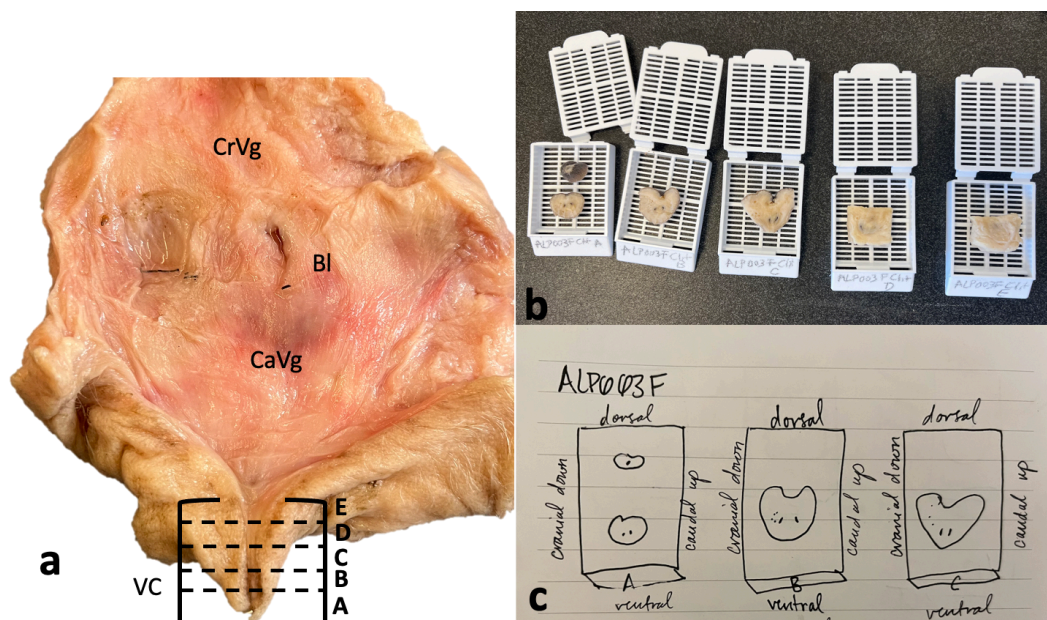


Figure 6. Dissected specimen and histological preparation with diagram. (a) specimen ALP003F, brackets indicate whole of dissection and sampling for histology, dashed lines indicate sectioning for histology, (b) cassettes with dissected specimens from ALP003F, (c) diagram of cassettes indicating directionality within anatomy. CrVg, cranial vagina; Bl, bladder; CaVg, caudal vagina; VC, vulvar commissure,

Paraffin histology of clitoral tissue and surrounding reproductive tract tissue allows for a more detailed analysis of the tissue composition of the clitoris. Initially, we used a BioWave Pro to accelerate the process but after discovering that it was insufficiently dehydrating the samples we transitioned to using traditional dehydration, clearing, and infiltration techniques. All samples underwent traditional paraffin protocols (detailed in Appendix 1) to embed, slice and mount sections of tissue from different regions of the clitoris. Stains consisted of hematoxylin and eosin Y (H&E), Masson's trichrome, and elastin/Verhoeff van Gieson elastin stain. The H&E stain allows for differentiation of nucleated cells and general tissues by highlighting cell orientation and types of cells. Masson's Trichrome is used to color collagen (blue), smooth muscle (red), vascularization, and innervation more clearly. It makes visible the hallmark functional features of

clitoral tissue which are erectile tissue containing lacunae, high amounts of collagen, and presence of nerves. Elastin stain or Verhoeff's stain allows visibility of elastin threads in the connective tissue. After these histological preparations, brightfield microscopy can illuminate how much blood flow the erectile tissue seems to receive, the ratio of smooth muscle to erectile tissue in the vaginal wall, amount of innervation, and the composition of connective and erectile tissue.

CT scanning & segmentation

CT scanning is a third method of discovering the 3-dimensional structure of the clitoris. After staining two alpaca reproductive tracts spanning from vulva to cervix or from vulva to urinary meatus in baths of decreasing iodine concentration (detailed in Appendix 2) to soak the soft tissue for adequate contrasting (also known as diceCT or diffusible iodine-based contrast-enhanced computed tomography), the CT scan produces digital files that allow isolation and differentiation of tissue types thereby allowing analysis and determination of the shape of the organ. To begin this process, in August 2023, we prepared an iodine solution of 3.5% iodine and soaked two specimens, replacing the iodine two times on August 24th and September 19th (3.5 weeks each). Specimens were shipped to the University of Florida for CT scanning on November 8th, after 11 weeks in iodine. While dissection analyzes macromorphological structures and histology highlights micromorphological structures, CT scanning takes an even bigger perspective of the organ relative to the surrounding tissues and structures. It provides digital slices as images that can be processed and segmented in Slicer

5.6.1 allowing us to move incrementally through the specimen in three different planes distinguishing structures by comparing tissue density and appearance to identify and map complete *ex vivo* structures like corpus spongiosum, corpus cavernosum, crurae, muscle, adipose tissue, and various connective tissues. This specification provides information on where the edges of the clitoris are, how the clitoral tissue intercepts the vaginal wall or nearby muscles, and how extensive the organ is.

CT segmentation in Slicer 5.6.1 distinguishes grayscale and tissue consistency on three distinct planes of the specimen. We digitally paint (segment) each distinguishable region creating a singular three dimensional model with each painted region visible as a distinct structure within the specimen. To determine then what each structure is, we turn to the grayscale and tissue characteristics as clues. Grayscale is based on the density of the tissue with more white being more dense, more black being less dense.

One of the specimens was segmented by Rachel Keeffe, the postdoctoral fellow in the Brennan Lab. She was unfamiliar with any of the structures of the clitoris or reproductive tract but very familiar with the process of CT segmentation and Slicer 5.6.1. This essentially meant she was segmenting blind, just based on tissue appearance rather than what she was expecting to find. I segmented the other specimen. I knew what structures I wanted to find and expected to find but I was less familiar with the application. Upon finishing the segmentation, we compared the two specimens to corroborate findings and determine what each structure was based on our combined knowledge of Slicer

function and alpaca clitoris/reproductive anatomy from the other methods used in this study. For example, knowing from dissection that there seemed to be a hard cartilaginous structure in the vulvar commissure was helpful because when we saw on the CT that there was a small bright white prong within the vulvar commissure, we could paint it, visualize it within the context of the organ, and understand that it was the same structure found in dissection.

***In vivo* Observation**

A visit to Dr. Steve Purdy's alpaca farm in Western Massachusetts allowed for data collection of the dimensions of the glans clitoris or vulvar commissure before presence of males, in presence of males, and immediately after copulation. We also observed two male/female matings and one female/female mounting event.

Results

Dissection, histology, CT scans, and observation indicate presence of a well developed functional clitoris in alpacas.

Dissection

Dissection revealed erectile tissue, crura, and a glans clitoridis buried in the vulvar commissure, and a small (1-1.5cm) cartilaginous structure (Fig. 7; Fig. 21). Sagittal and transverse slices through the clitoris and caudal vagina reveal erectile tissue and dense connective tissue (Fig. 7). It was unclear how far

cranially the erectile tissue extended in the vagina. Initial dissections seemed to imply that it reached beyond the urinary meatus but later dissections, histology, and CT confirm that the erectile tissue does not extend so far, it does not reach the urinary meatus.

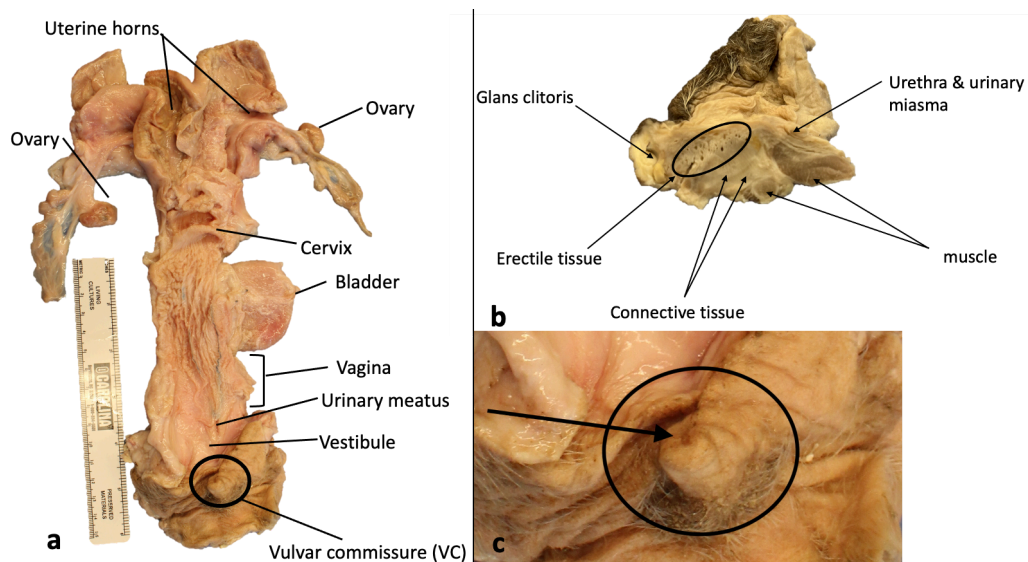


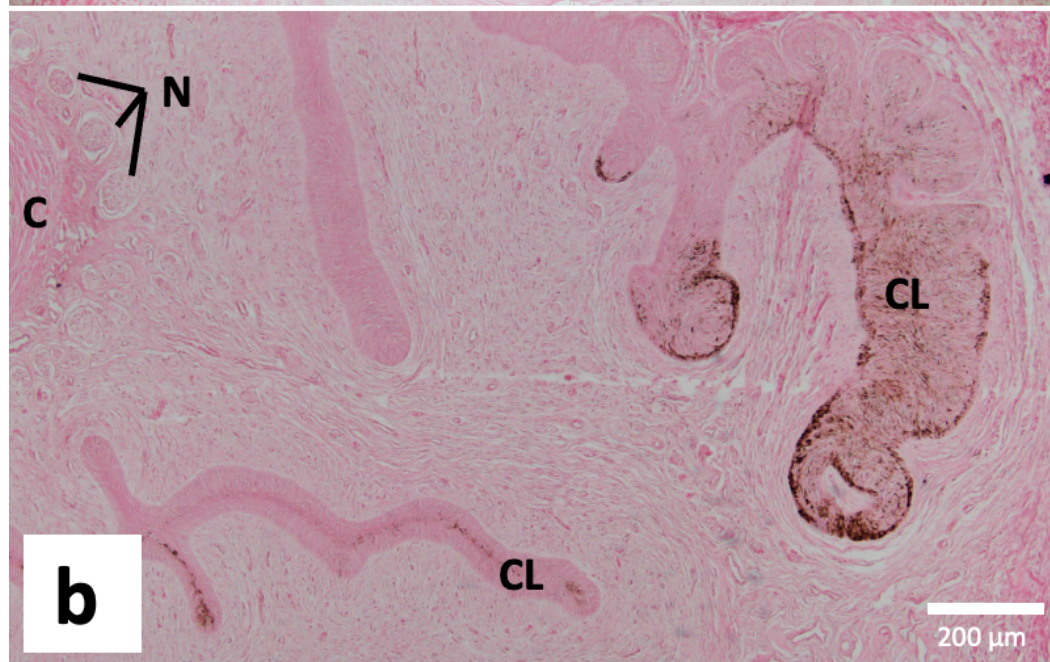
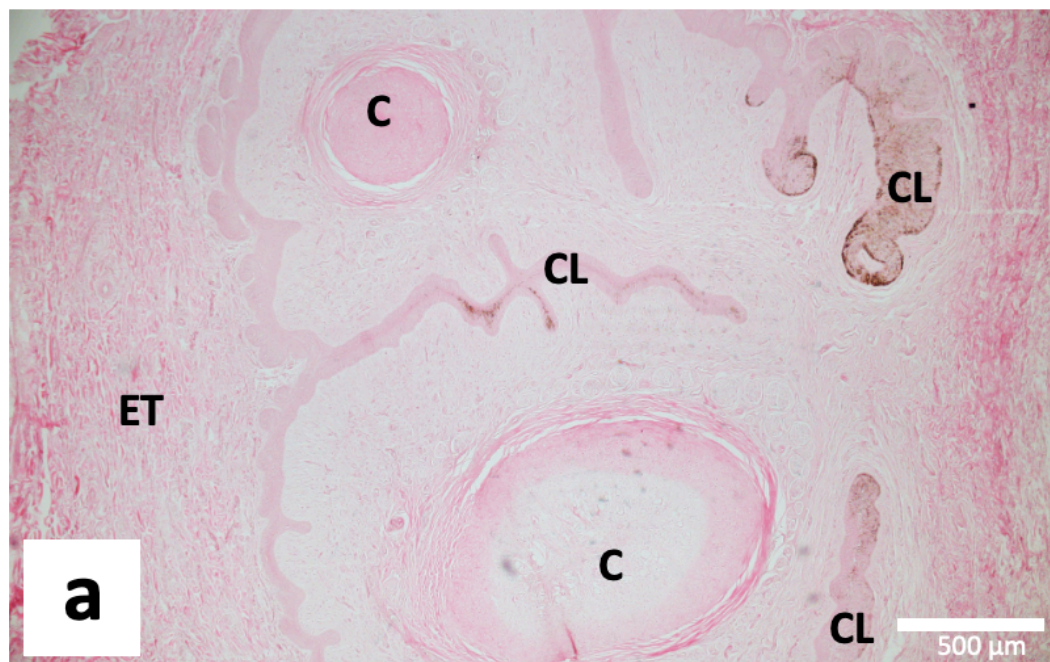
Figure 7. Female alpaca reproductive tract, midsagittal cross-section, cartilage in vulvar commissure. (a) entire female reproductive tract, (b) dissection image of clitoris facing a mid-sagittal cut with labeling of visible reproductive tract and clitoral anatomy structures, (c) encircled vulvar commissure with arrow pointing to dark spot characteristic of head of distal end of cartilaginous structure.

Variation between specimens was primarily at the vulvar commissure and in dimensionality of the reproductive tract.. The size and prominence of the vulvar commissure varied across specimens as did the length of the caudal vagina, cervix, and cranial vagina. This may be explained by differences in body size but we do not have data on the body mass of the alpacas from which these specimens were extracted.

Histology

Paraffin histology highlights vascularization and innervation throughout the erectile tissue, especially concentrated in distal parts of the erectile tissue in the vulvar commissure and in a fairly superficial ring around the vulva. In general, there is evidence of disorganized connective tissue and lacunae which are hallmarks of corpus spongiosum tissue immediately posterior to the vulvar commissure and extending laterally. Small smooth muscle filaments are interspersed throughout the corpus spongiosum and there are more substantial bundles adjacent to corpus spongiosum tissue as well as along the crura that indicate presence of bulbospongiosus muscle. This erectile tissue has extensive lacunae of different sizes adjacent to arteries and veins that supply the blood for tumescence.

The following few figures reference histological preparations demarcated in Figure 6.



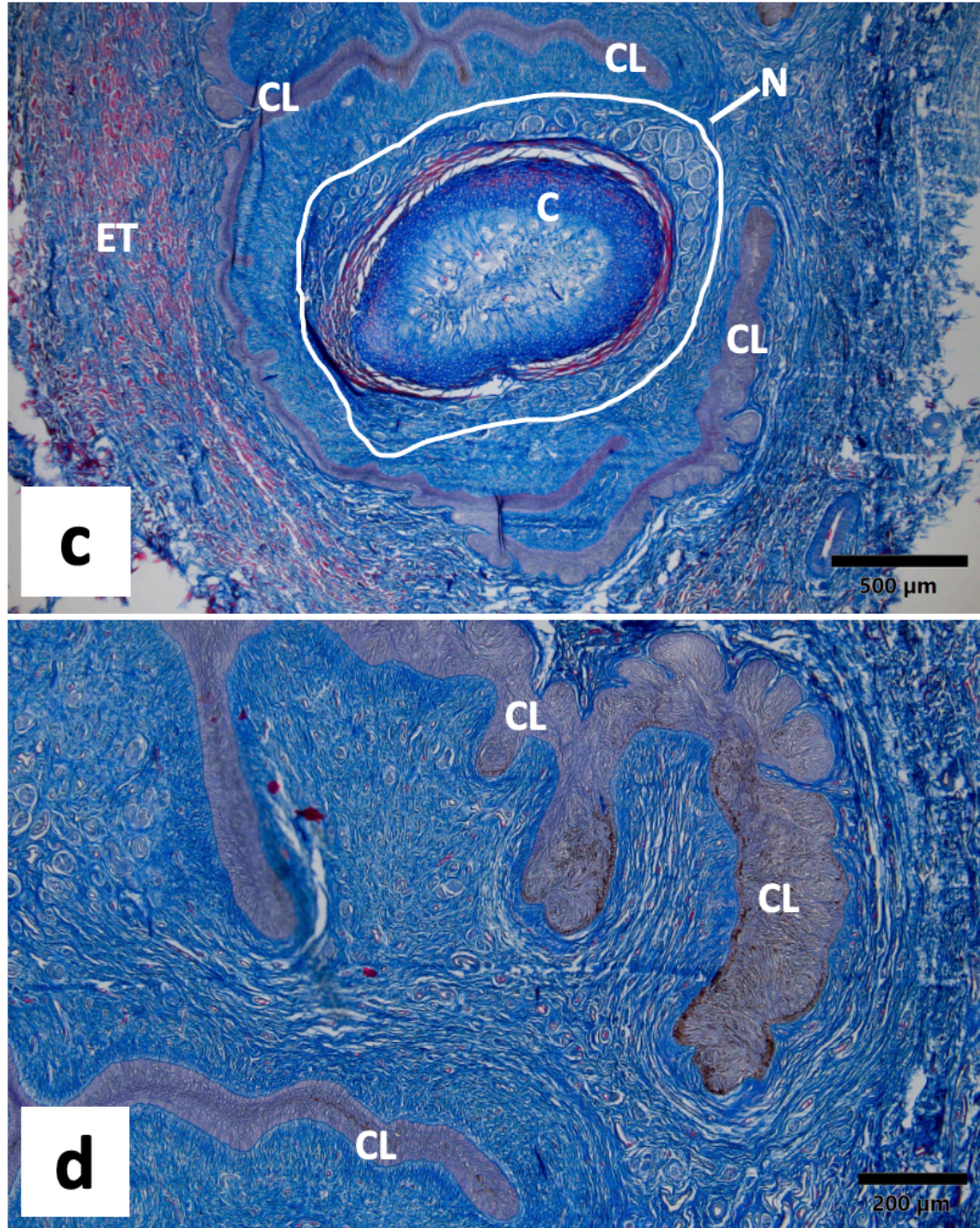
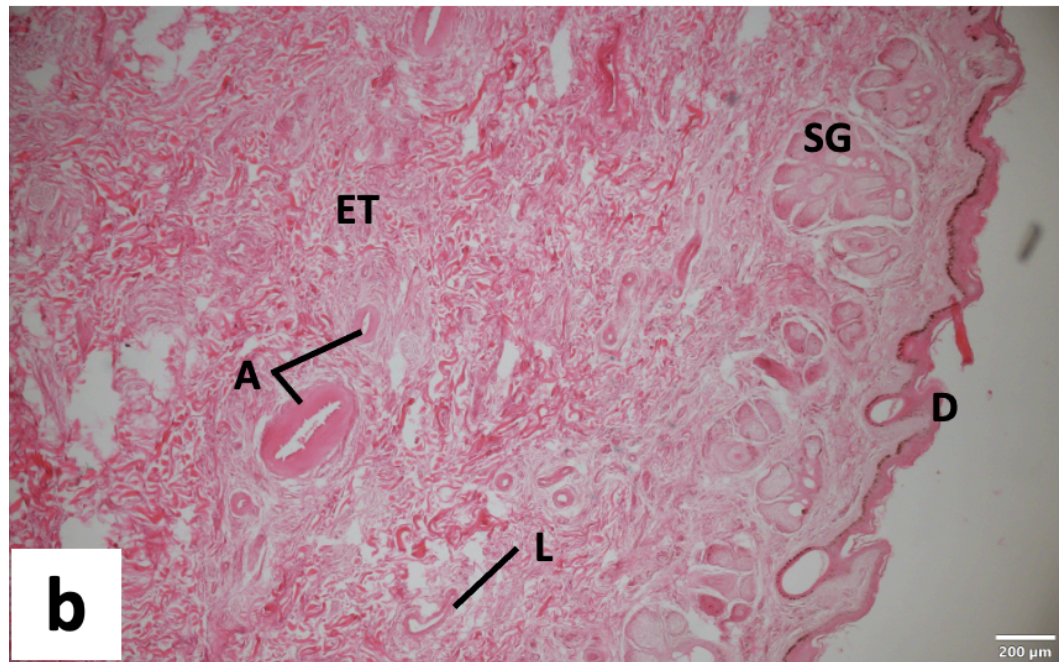
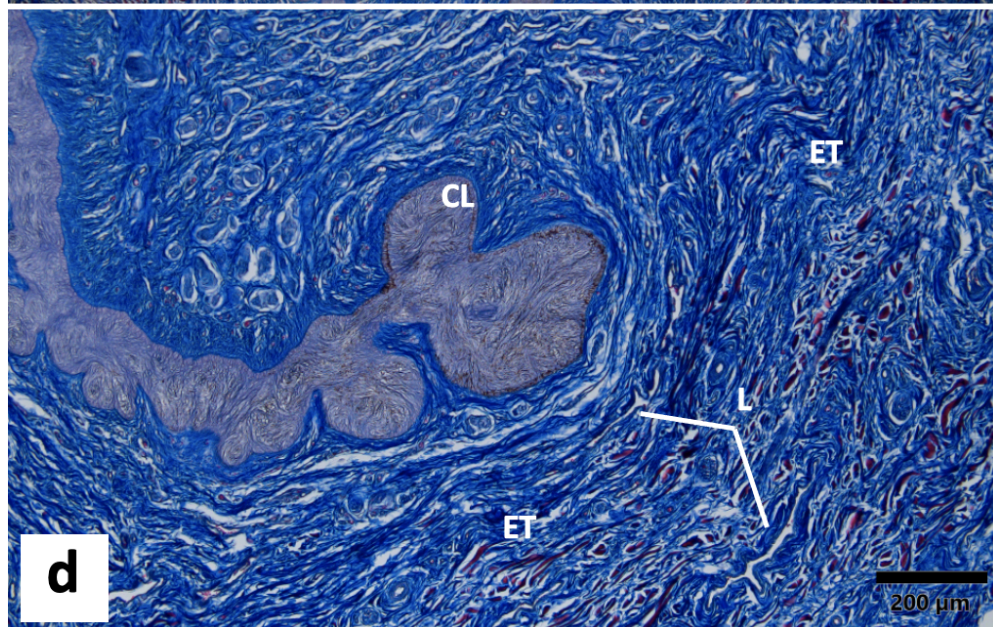
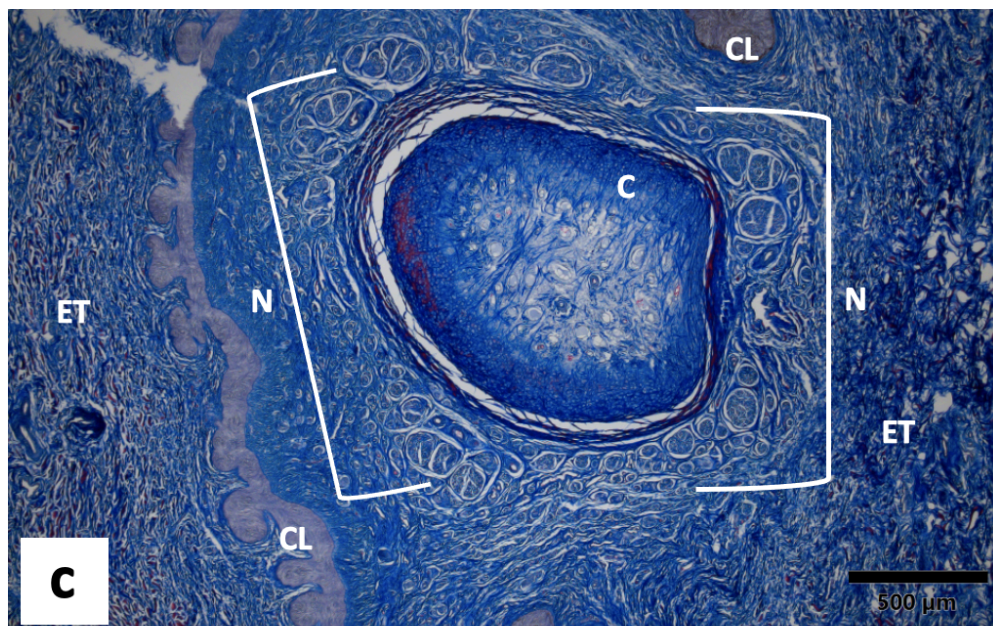


Figure 8. Histological preparation of distal tip of vulvar commissure (labeled A in Figure 6) with images taken by OlympusBX51 using brightfield microscopy. (a) H&E stain, 5x magnification, (b) H&E stain, 10x magnification, (c) Masson's Trichrome stain, 5x magnification, (d) Masson's Trichrome stain, 10x magnification. C, cartilage; CL, clitoral lamina; ET, erectile tissue; N, nerve bundles.

These histological preparations of the distal-most tip of the vulvar commissure indicate presence of a clitoral lamina surrounding two cartilage prongs. As evidenced in the H&E stains, the clitoral lamina has a higher density

of nuclei in the terminal ends indicating location of cell proliferation. Erectile tissue exists exclusively outside the clitoral lamina and is composed of collagen and smooth muscle fibers on the left side of 8c which is the anterior aspect of the vulvar commissure.





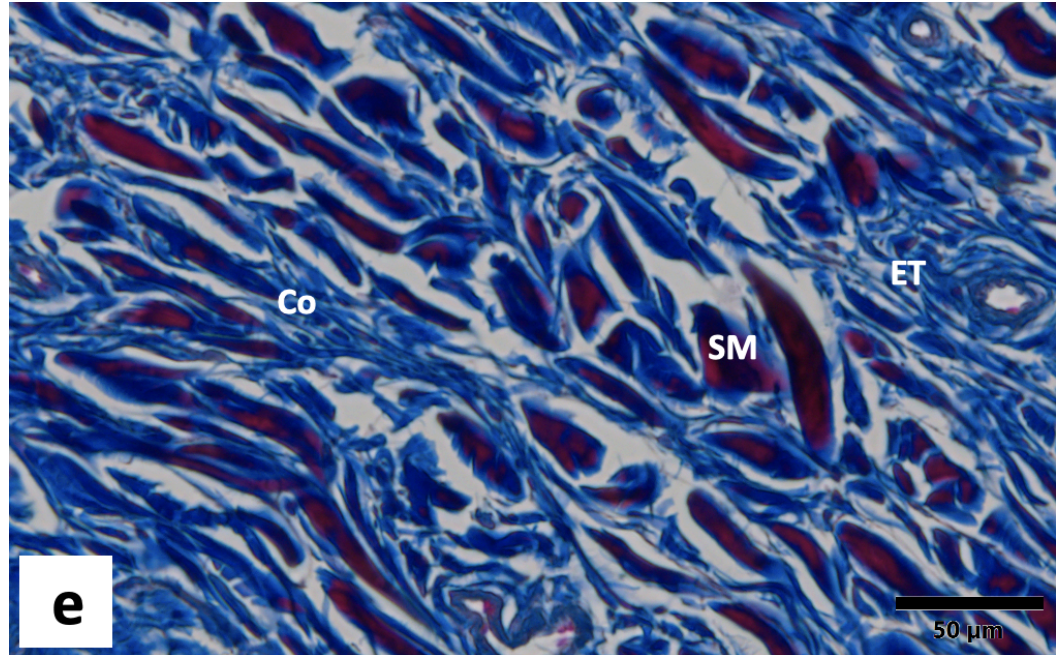
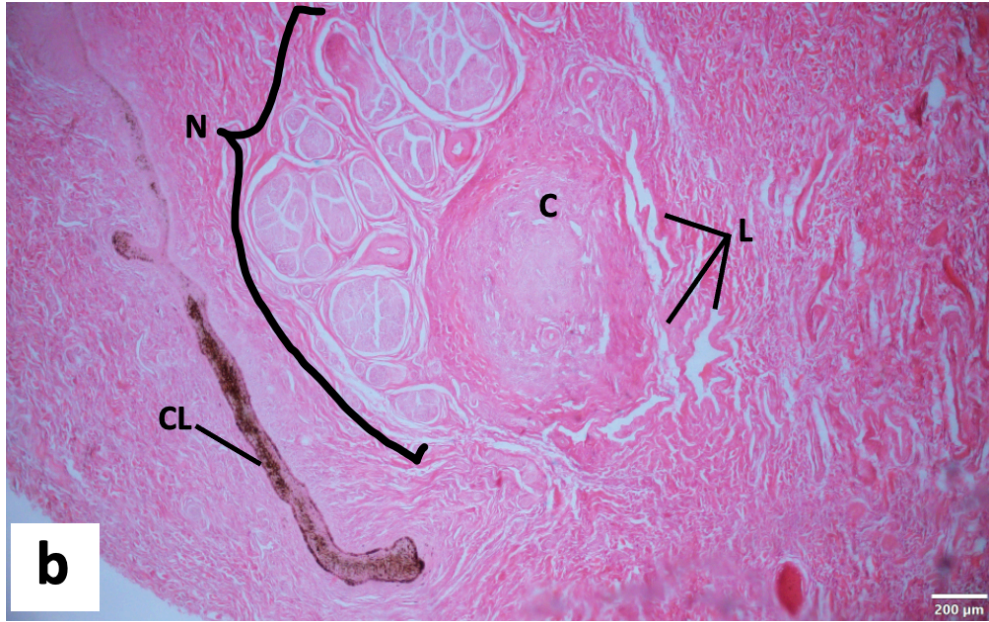
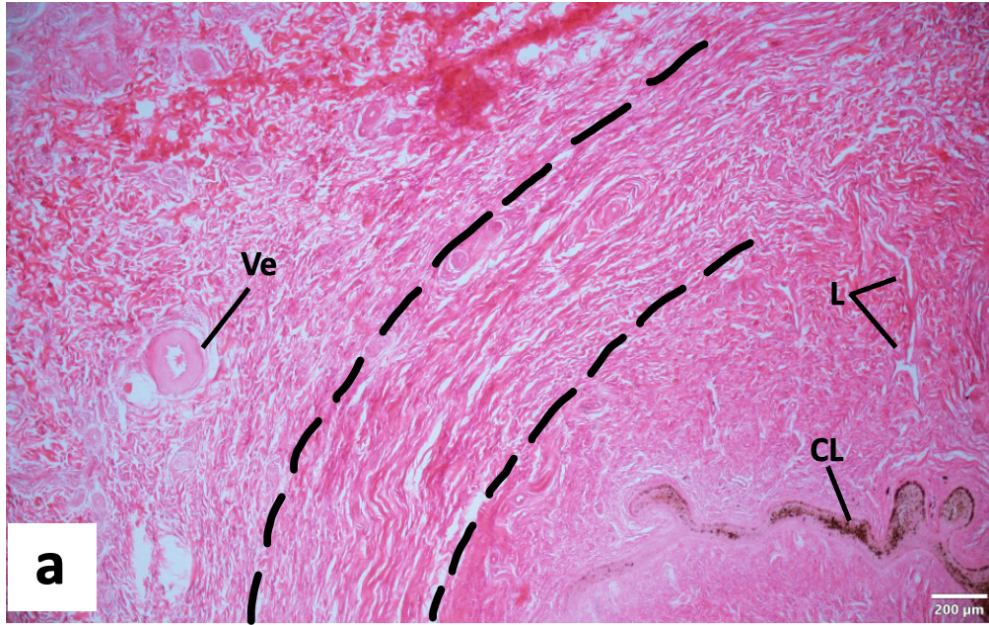
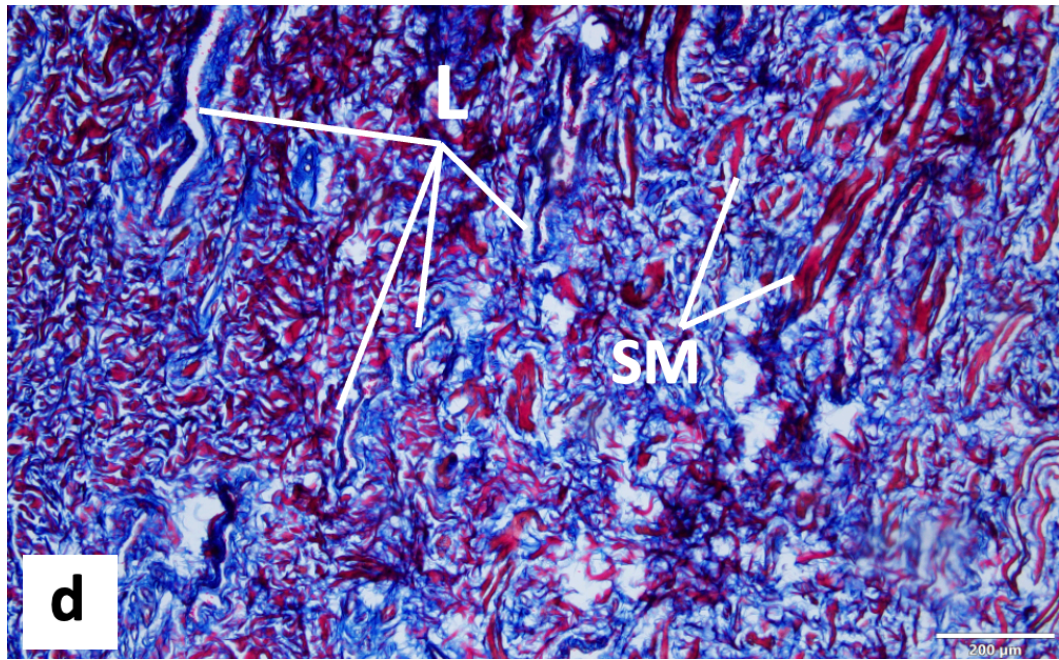
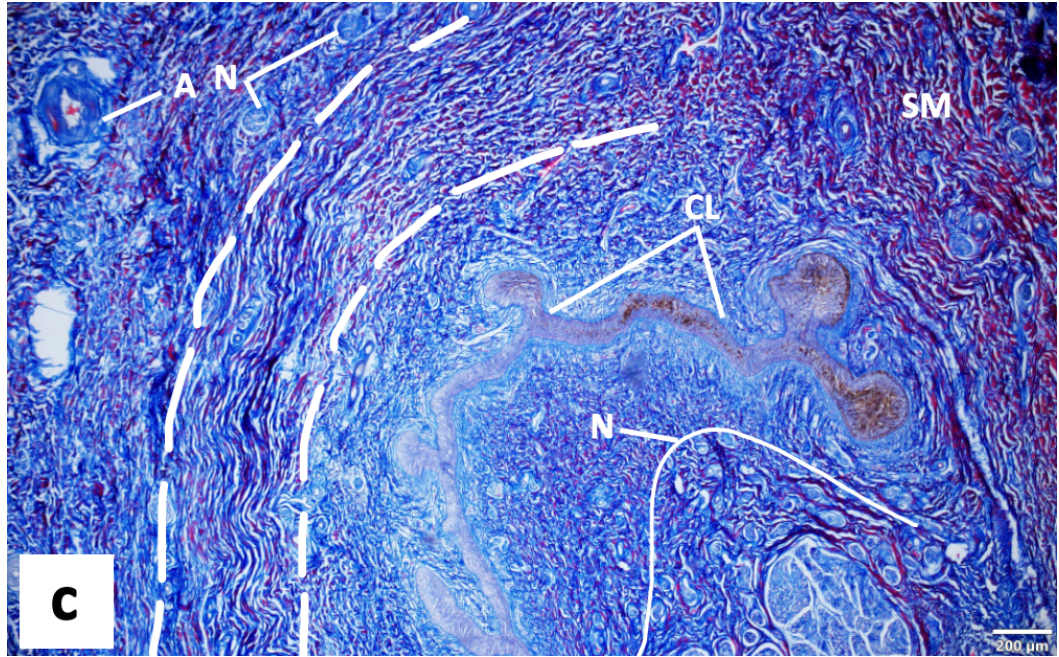


Figure 9. Histological preparations of the vulvar commissure, second-most distal sampling (labeled B in Figure 6). Images taken with OlympusBX51 using brightfield microscopy. (a) H&E stain, 5x magnification, (b) H&E stain, 5x magnification, (c) Masson's Trichrome stain, 5x magnification, (d) Masson's Trichrome stain, 10x magnification, (e) Masson's Trichrome stain, 50x magnification. Co, collagen; SM, smooth muscle bundles, ET, erectile tissue; CL, clitoral lamina; N, nerves; C, cartilage; A, artery; L, lacuna; SG, sebaceous gland; D, dermis; Co, collagen; SM, smooth muscle bundles.

These images from the distal portion of the vulvar commissure show that what was a double-headed cartilage at the very distal tip has now merged into a single cartilaginous prong. It is still surrounded by many nerve bundles and is partially encircled by the clitoral lamina. Outside of the clitoral lamina, there is dense erectile tissue studded with vascularization and lacunae that indicate functionality of tumesceable erectile tissue. Nerve bundles varying from ~ 0.1 mm to ~ 0.9 mm in diameter completely encircle the cartilage. Erectile tissue is primarily composed of collagen with some short smooth muscle bundles studded in the erectile tissue on the dorsal side of the vulvar commissure.





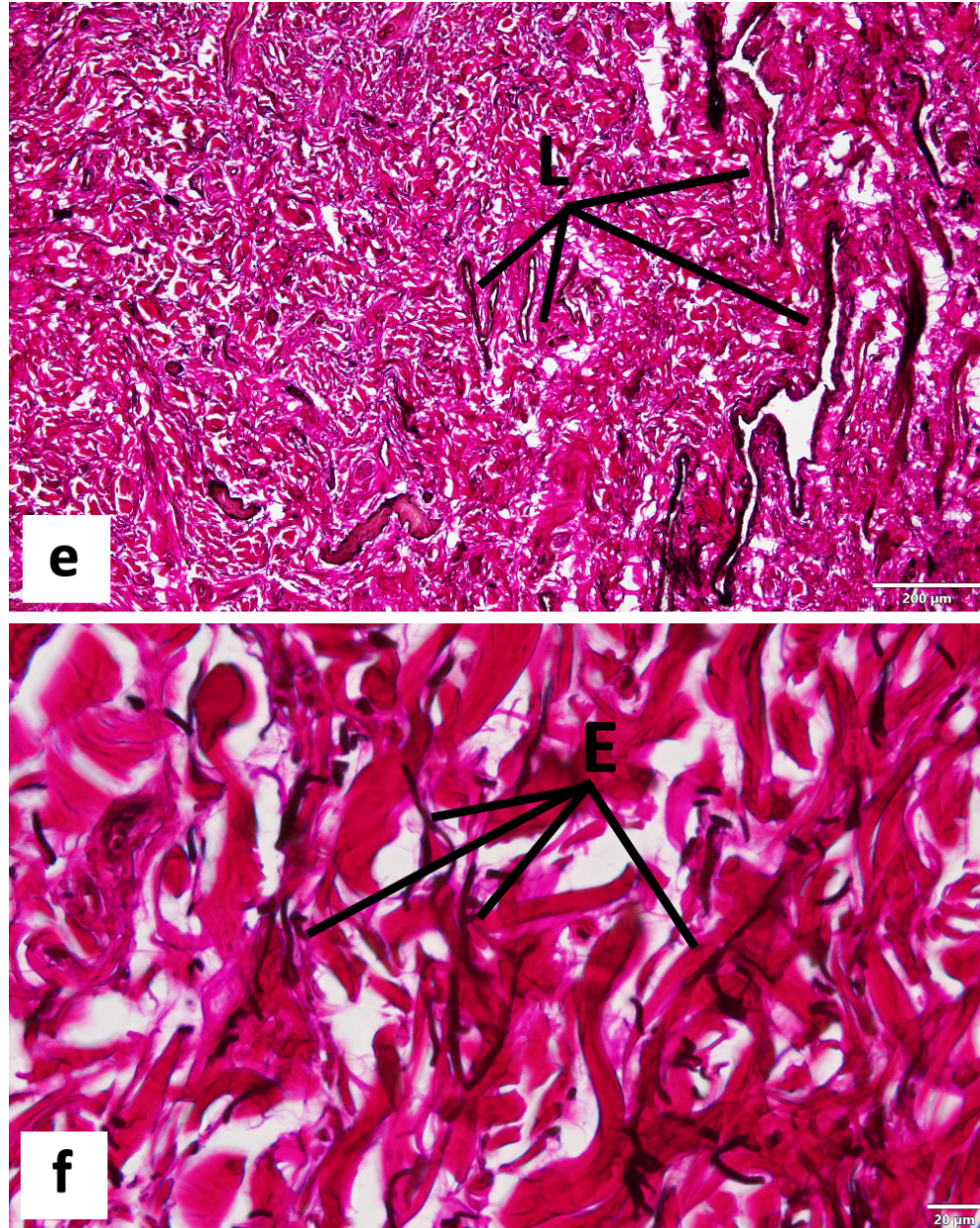
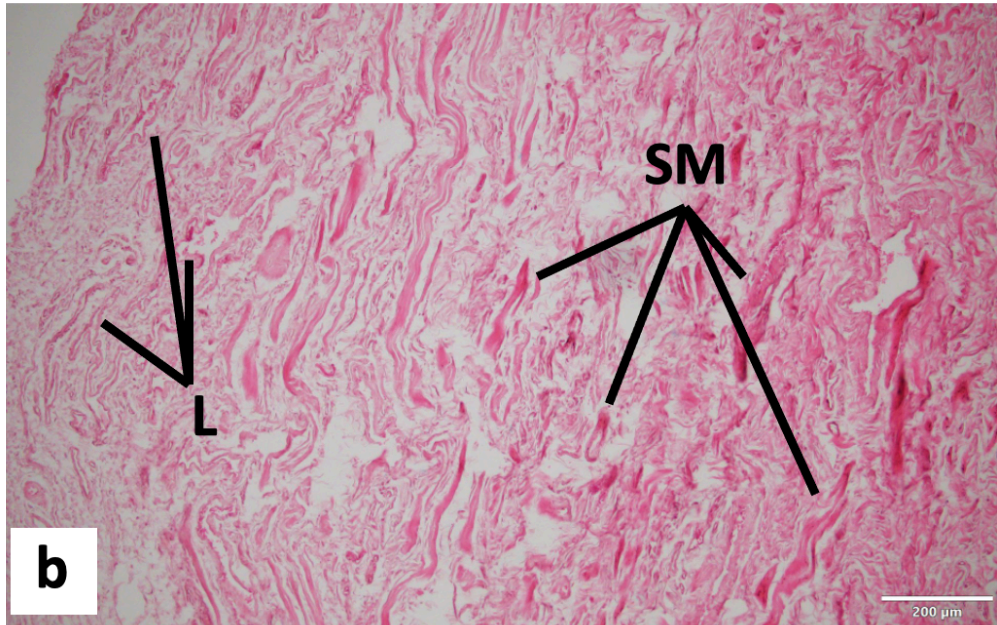
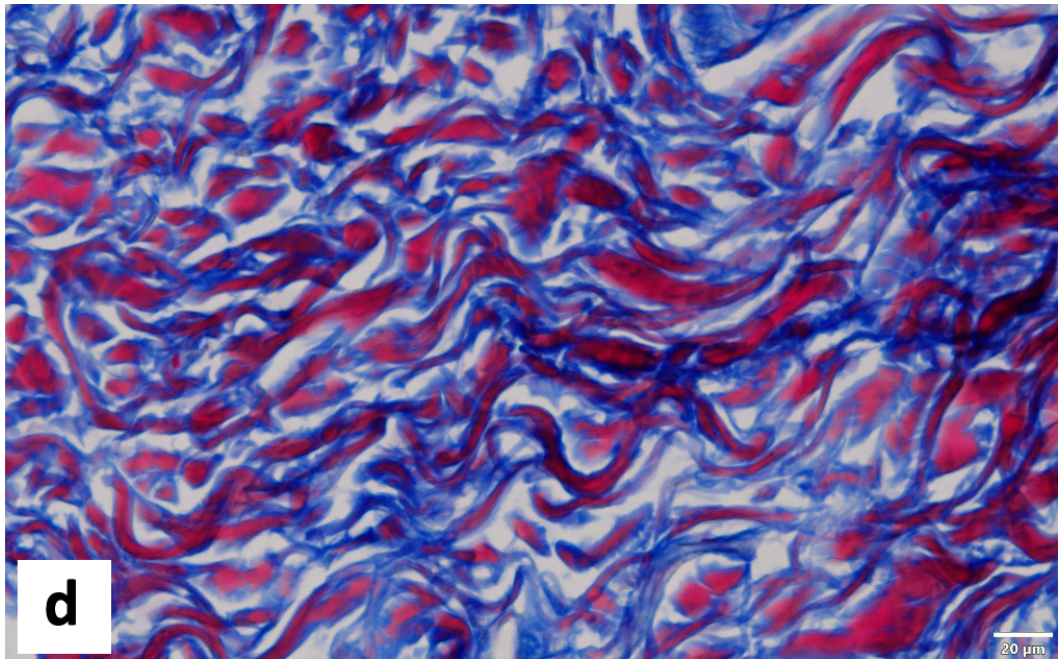
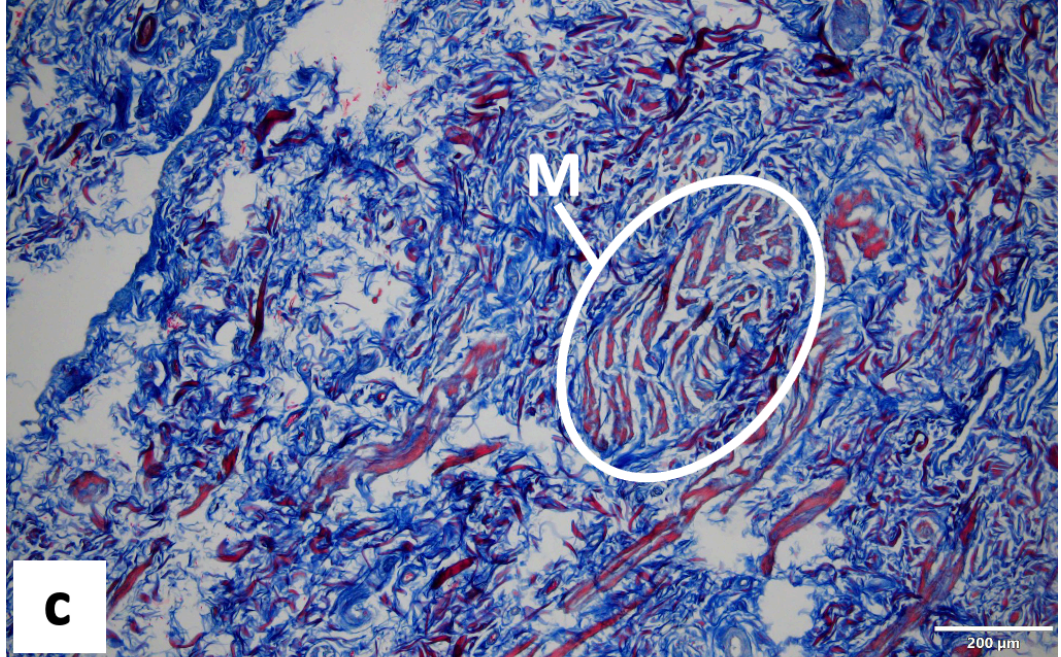


Figure 10. Histological preparations of the mid-vulvar commissure (labeled C in Figure 6). Images taken with OlympusBX51 using brightfield microscopy. (a) H&E stain, 5x magnification, dashed lines delimit shift in organization and density of erectile tissue, (b) H&E stain, 5x magnification, (c) Masson's Trichrome stain, 5x magnification, dashed lines delimit shift in organization and density of erectile tissue, (d) Masson's Trichrome stain, 10x magnification, (e) elastin stain, 10x magnification, (f) elastin stain, 50x magnification. A, artery; L, lacunae; CL, clitoral lamina; C, cartilage; N, nerve; SM, smooth muscle; E, elastin fiber.

Images of the mid-ventral commissure illustrate a changing structure of the clitoris. The diameter of the cartilage prong shrinks and becomes less

definitively cartilaginous and more collagenous. There are still well-developed nerves but only on the anterior of the cartilage. The clitoral lamina is also thinner and encircles only the anterior of the cartilage rather than nearly the entire circumference as seen in more distal sectionings. Erectile tissue outside the clitoral lamina is arranged in three bands. Deepest in the ventral commissure and closest to the clitoral lamina there is a loosely organized matrix of collagen that then shifts into a middle band with clearly organized longer strands of collagen fibers closely interspersed with smooth muscle fibers. The most superficial band is just deep to the dermis and is primarily disorganized collagen with intermittent vascularization and sebaceous glands. Short elastin fibers are evident in the collagenous matrix of erectile tissue. The fibers are not organized but their kinked shape indicates potential for extensibility thereby conveying tensile strength and functionality of the erectile tissue.





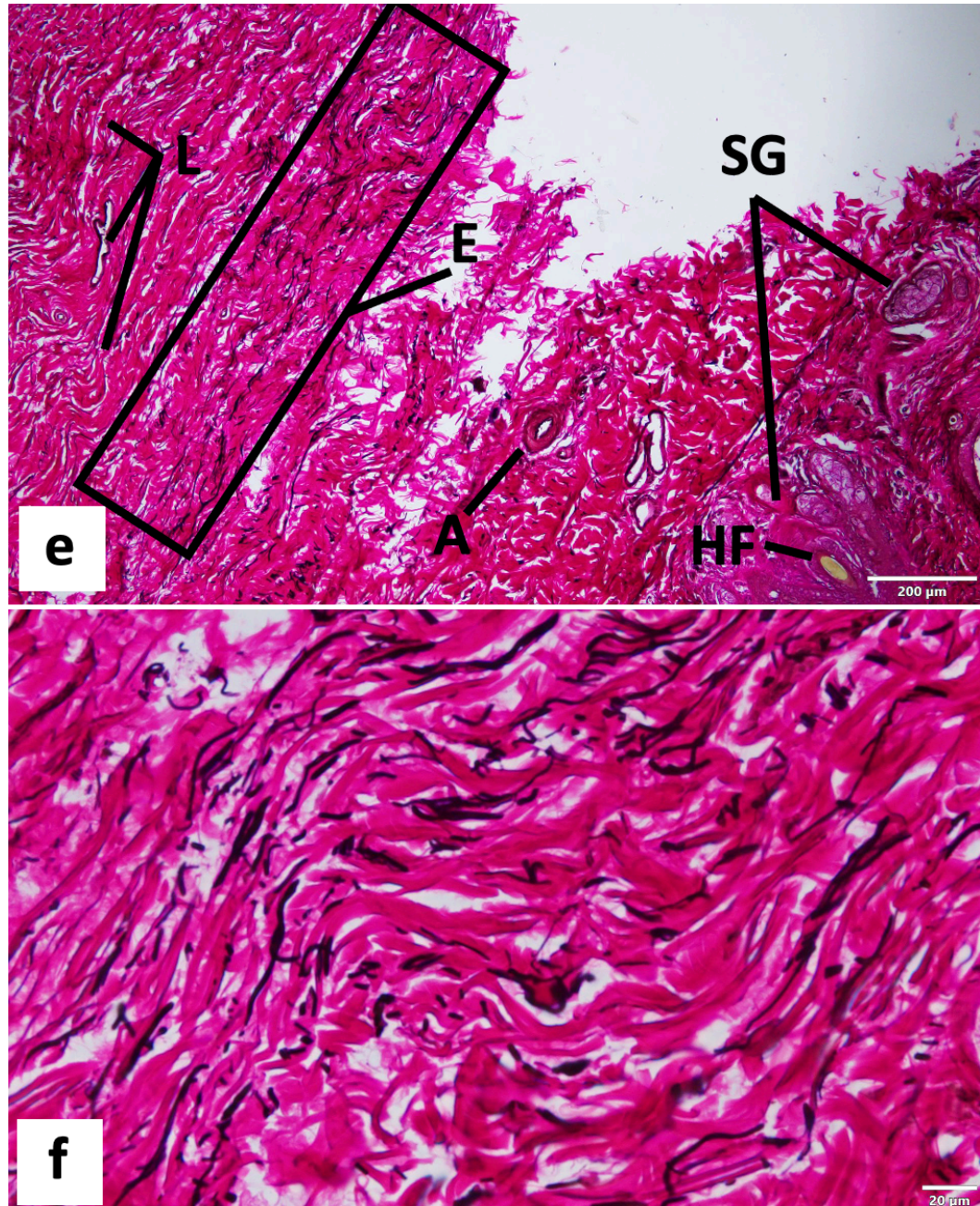
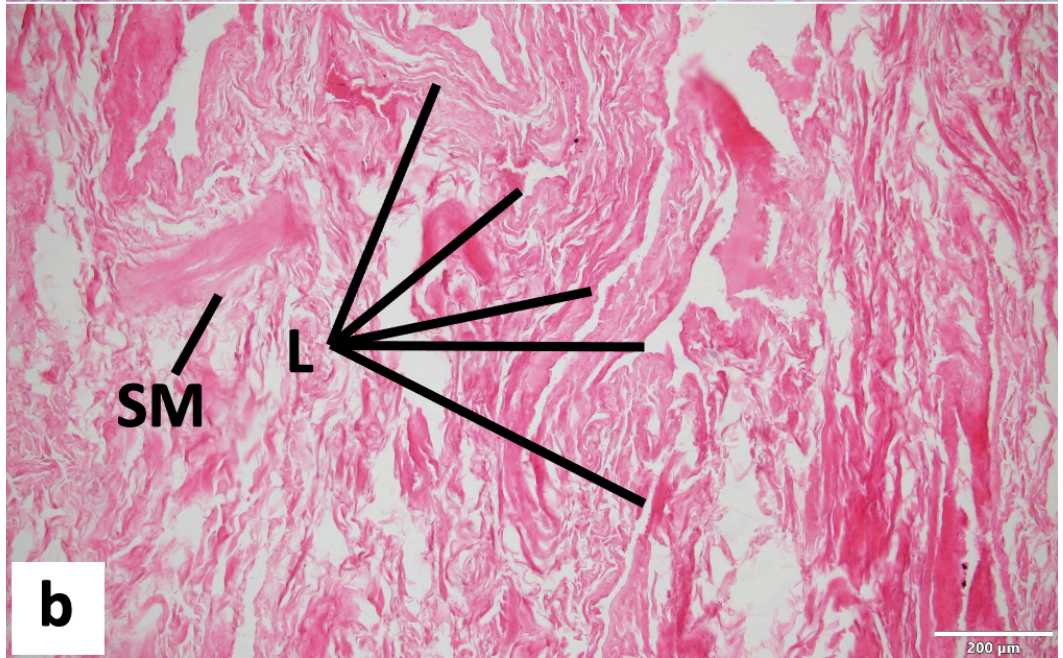
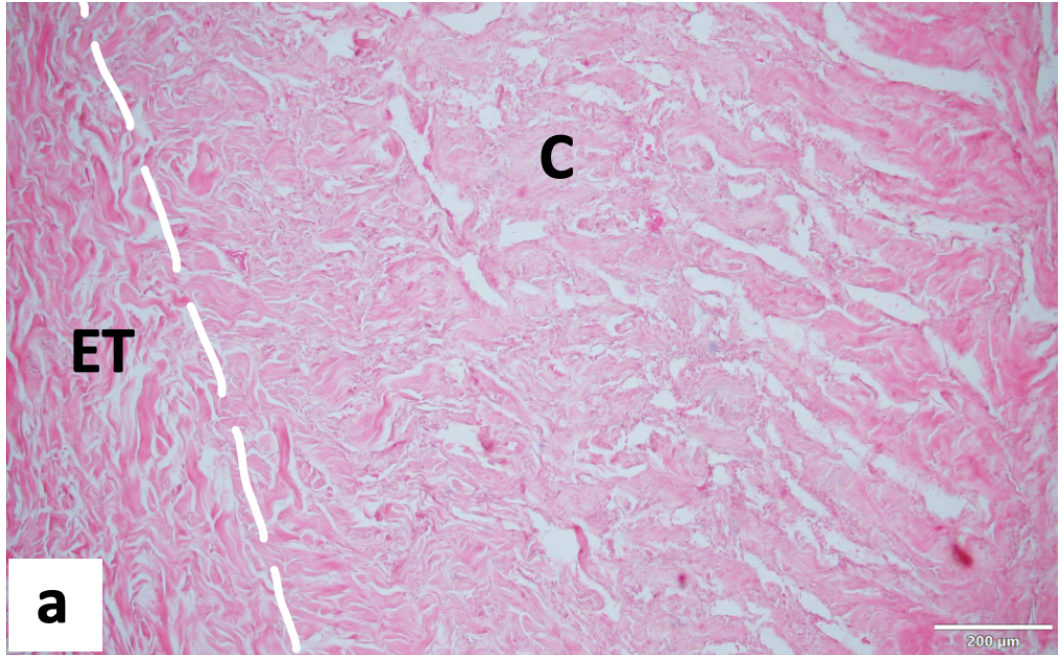


Figure 11. Histological preparations of the proximal vulvar commissure (labeled D in Figure 6). Images taken with Olympus BX51 with brightfield microscopy. (a) H&E stain, 5x magnification, large structure or gland within clitoral lamina of unknown composition and origin, (b) H&E stain, 10x magnification, erectile tissue matrix studded with smooth muscle fibers and lacunae, (c) Masson's Trichrome stain, 10x magnification, muscle fibers, (d) Masson's Trichrome stain, 50x magnification, smooth muscle fibers closely interspersed with collagen fibers in erectile tissue, (e) elastin stain, 10x magnification, elastin fibers surround lacunae and are interspersed in erectile tissue just deep to the dermis, (f) elastin stain, 50x magnification, elastin fibers clearly defined in erectile tissue. C, cartilage; N, nerve; CL, clitoral lamina; L, lacunae; SM, smooth muscle; M, muscle; E, elastin fibers; A, artery; SG, sebaceous glands; HF, hair follicle.

Images from the proximal section of the vulvar commissure indicate continued shrinkage of the cartilage diameter and morphing from hyaline cartilage into a collagenous matrix. Sizeable nerves remain bordering the cartilage and the clitoral lamina is smaller. There is a large structure within the clitoral lamina of unknown composition and origin. Frequent lacunae confirm continued presence of erectile tissue in the vulvar commissure and elastin fibers within erectile tissue indicate extensibility of the tissue. 11c contains a small muscle bundle indicative of a deep insertion of a muscle, likely the bulbospongiosus muscle. The bulbospongiosus muscle inserts along the vestibular bulbs which begin to merge with the glans clitoridis as the vulvar commissure meets the vagina at the vulva.



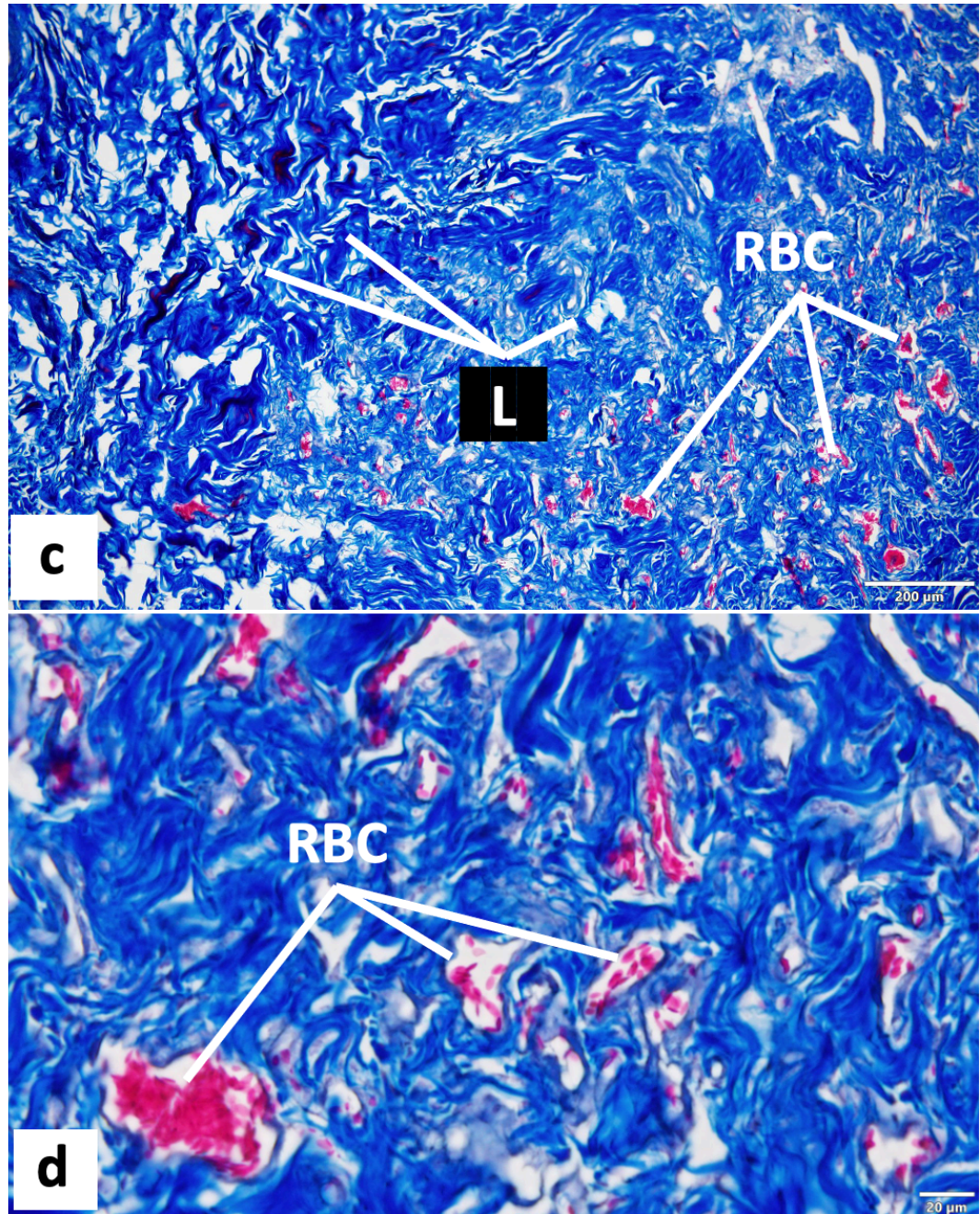


Figure 12. Histological preparations of the most proximal segment of vulvar commissure (labeled E in Figure 6). Images taken using Olympus BX51 under brightfield microscopy. (a) H&E stain, 50x magnification, dashed line delimits border of erectile tissue and morphing cartilage, (b) H&E stain, 10x magnification, erectile tissue with frequent lacunae and a smooth muscle bundle, (c) Masson's Trichrome stain, 10x magnification, erectile tissue with lacunae, (d) Masson's Trichrome stain, 50x magnification, small lacunae filled with red blood cells. ET, erectile tissue; C, cartilage; SM, smooth muscle; L, lacunae; RBC, red blood cells.

The most proximal section of the vulvar commissure indicates no presence of hyaline cartilage but there is a clear region of collagenous matrix forming in the wake of the cartilage. Erectile tissue remains collagenous and studded with lacunae and smooth muscle fibers. The sinus networks contain red blood cells indicating that the tissue was filled with blood thereby confirming erection capability.

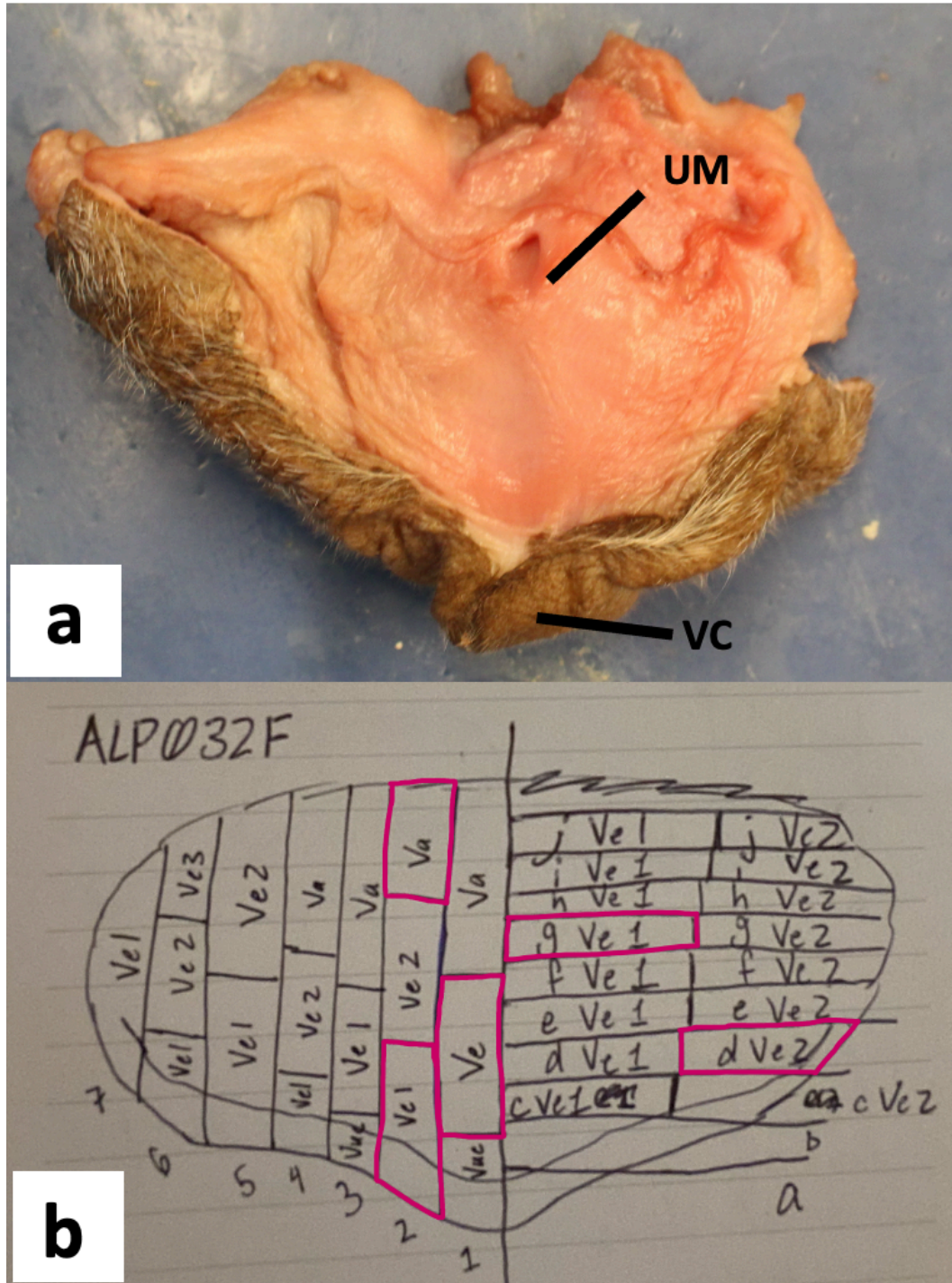
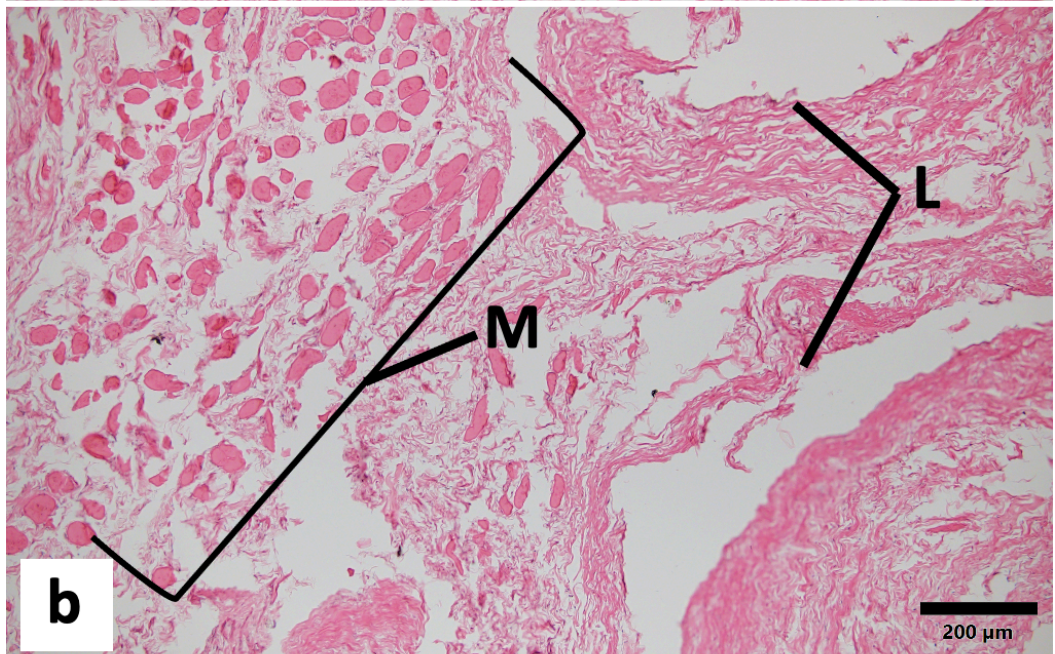
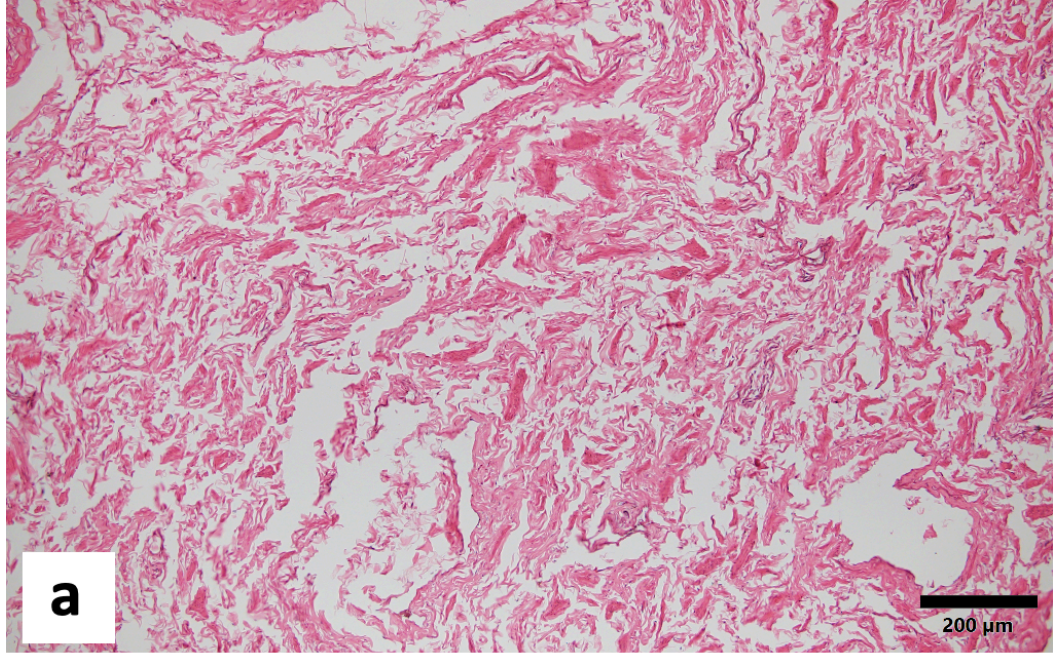


Figure 13. ALP032F specimen and diagram for histology. (a) specimen before dissection for histology, (b) diagram of dissected specimen, highlighted sections comprise the images in figures below. UM, urinary meatus; VC, vulvar commissure.



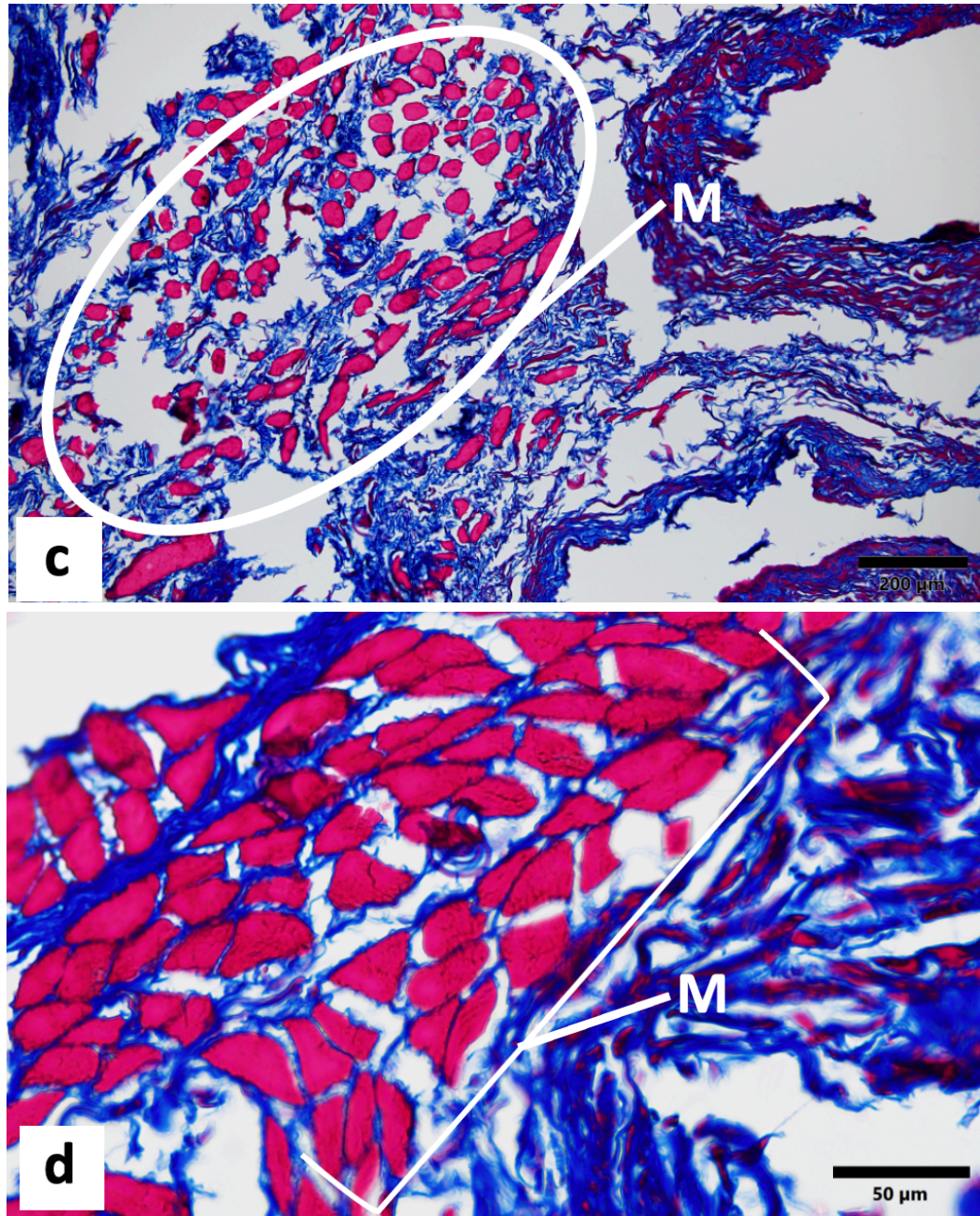
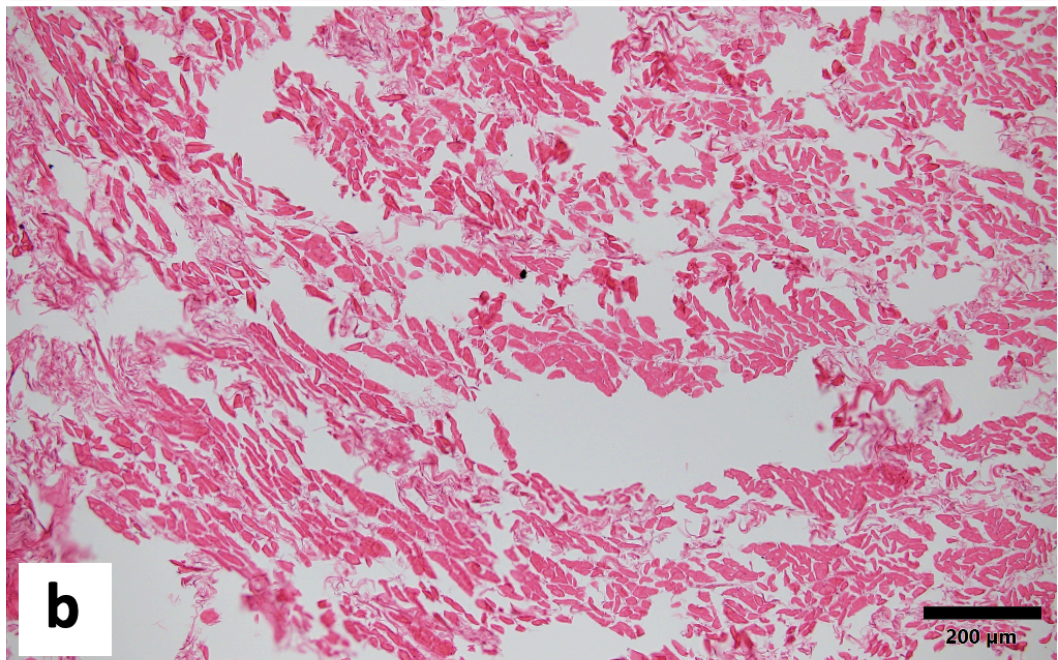
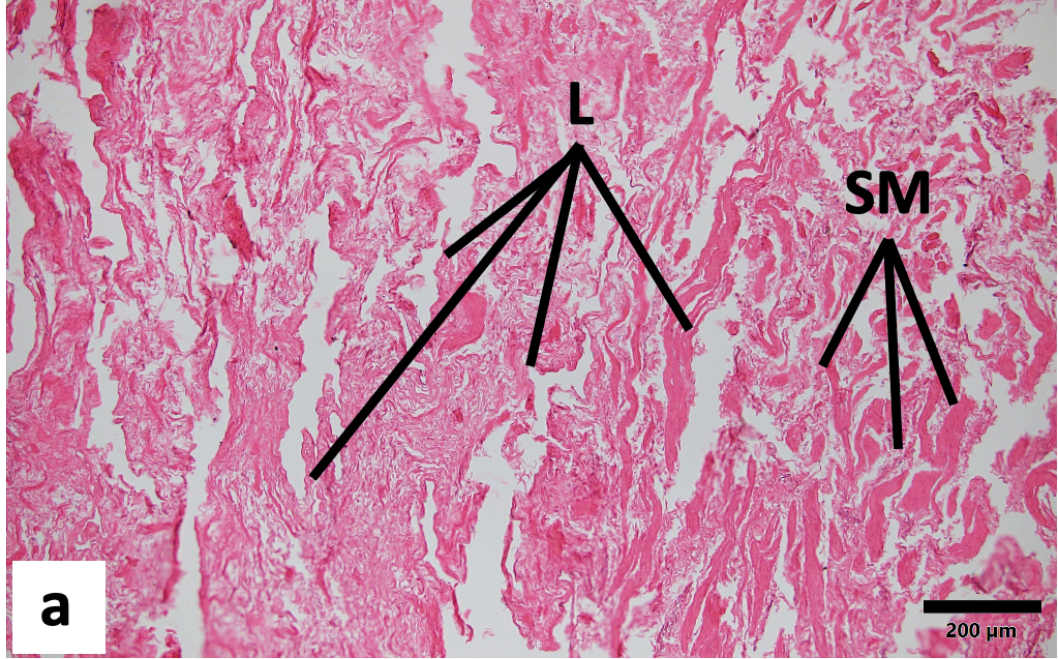


Figure 14. Histological preparations of the mid-sagittal caudal vagina and clitoris (labeled 1Ve in Figure 13). Images taken with Olympus BX51 using brightfield microscopy. (a) H&E stain, 10x magnification, erectile tissue, (b) H&E stain, 10x magnification, transverse muscle and erectile tissue with large lacunae, (c) Masson's Trichrome stain, 10x magnification, transverse muscle bundle embedded within erectile tissue, (d) Masson's Trichrome stain, 50x magnification, transverse muscle bundles adjacent to collagenous erectile tissue. M, muscle; L, lacunae.

In the midline of the caudal vagina, large lacunae and loose erectile tissue matrix are evidence of erectile tissue. Transverse muscles likely indicate

bulbospongiosus muscle adjacent to the spongy tissue of vestibular bulbs. Large gaps in tissue in 14c are not lacunae, rather, they are shredded tissue from preparation issues. If they were lacunae, there would be a continuous epithelium lining the gap.



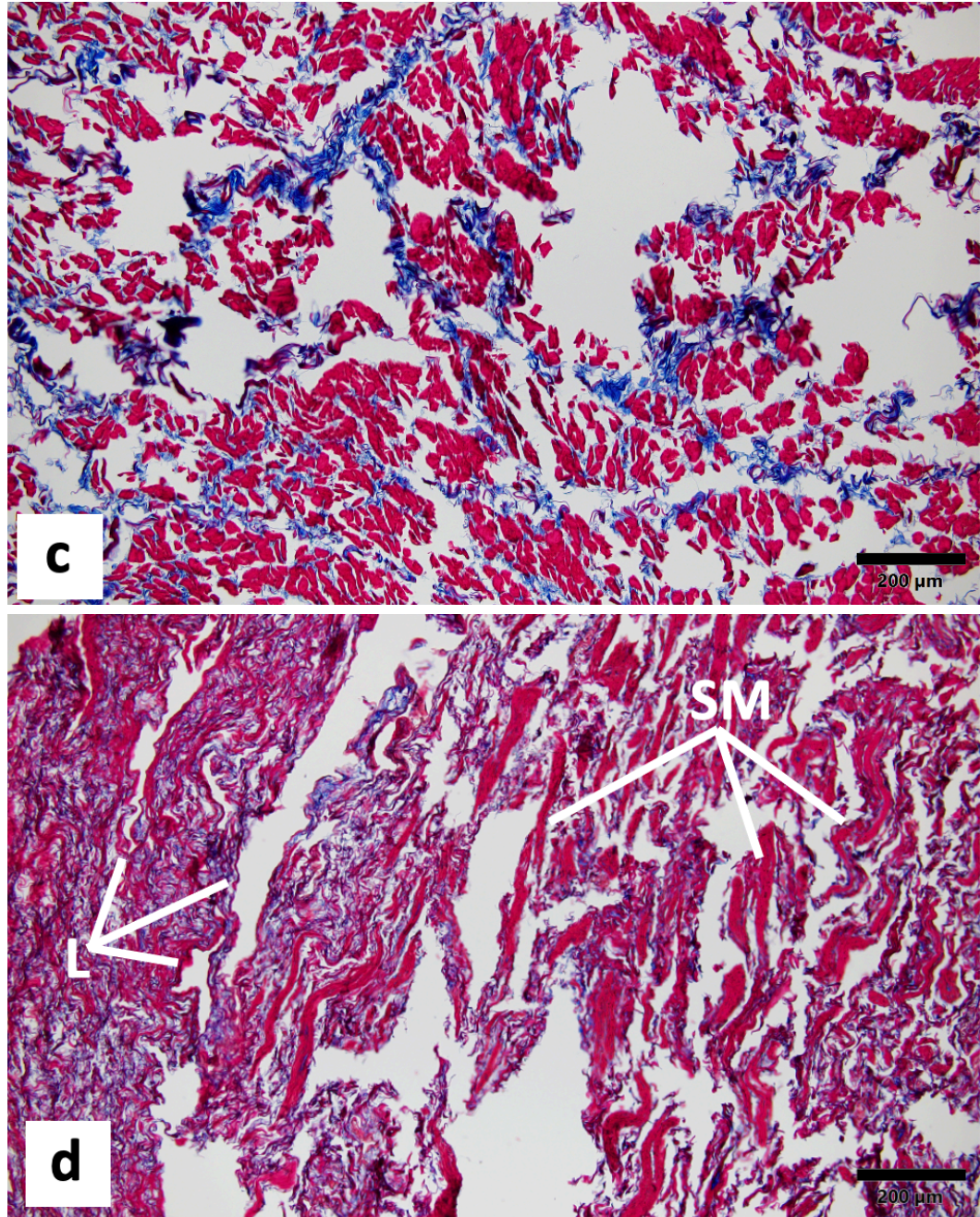
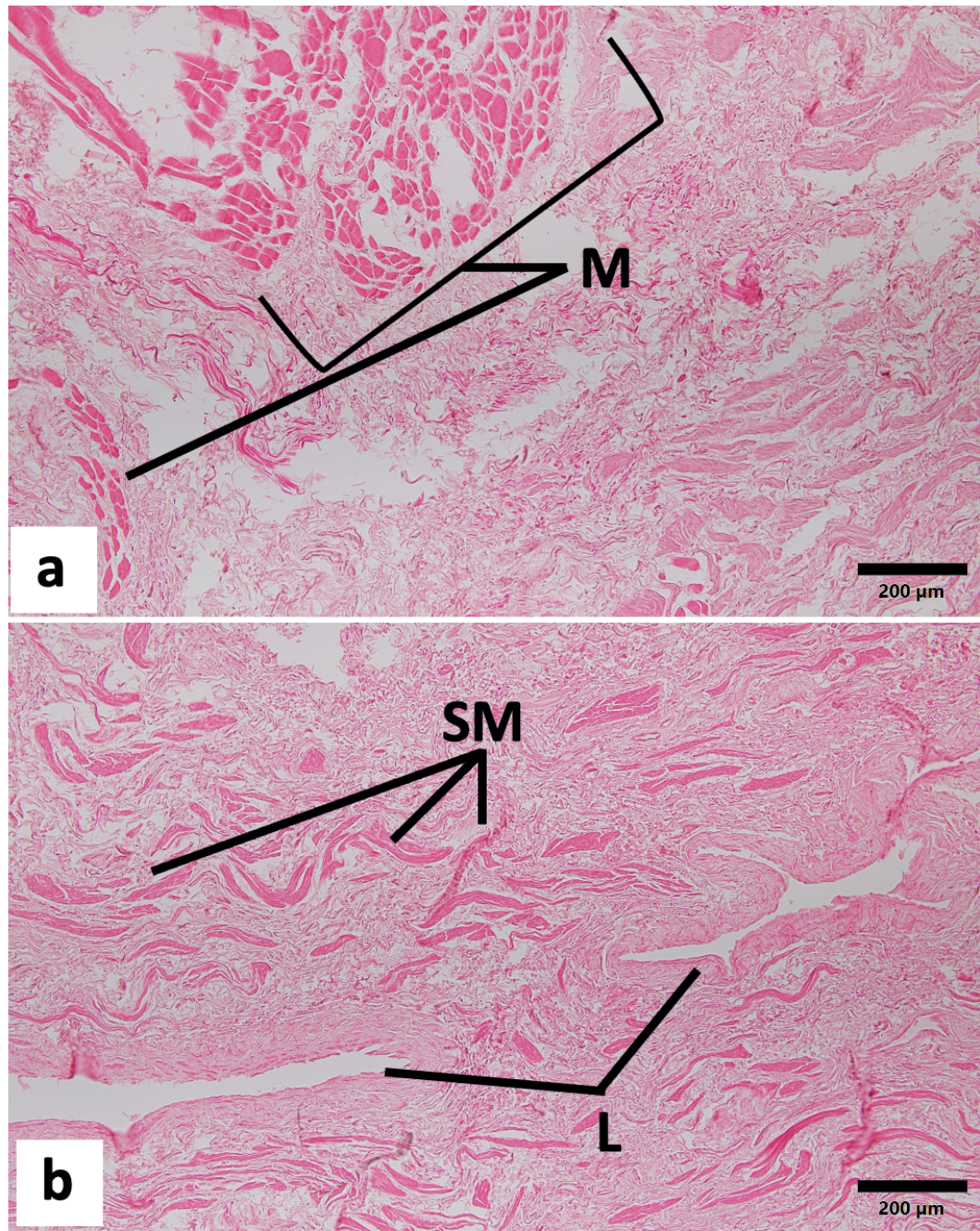


Figure 15. Histological preparations of the caudal vagina adjacent to the urinary meatus (labeled 2Va in Figure 13). Images taken with Olympus BX51 using brightfield microscopy. (a) H&E stain, 10x magnification, erectile tissue with large lacunae and longitudinal smooth muscle bundles, (b) H&E stain, 10x magnification, transverse muscle, (c) Masson's trichrome stain, 10x magnification, transverse muscle, (d) Masson's trichrome stain, 10x magnification, erectile tissue with large lacunae and longitudinal smooth muscle bundles. L, lacunae; SM, smooth muscle.

These images are from the caudal vagina just distal to the urinary meatus.

The erectile tissue has large lacunae signifying bulbospongiosum erectile

tissue/vestibular bulb tissue. Extensive transverse muscle bundles indicate bulbospongiosus muscle. As in the previous imaging of this specimen, large gaps between tissue lacking a continuous epithelium are not lacunae, they are evidence of issues in preparation.



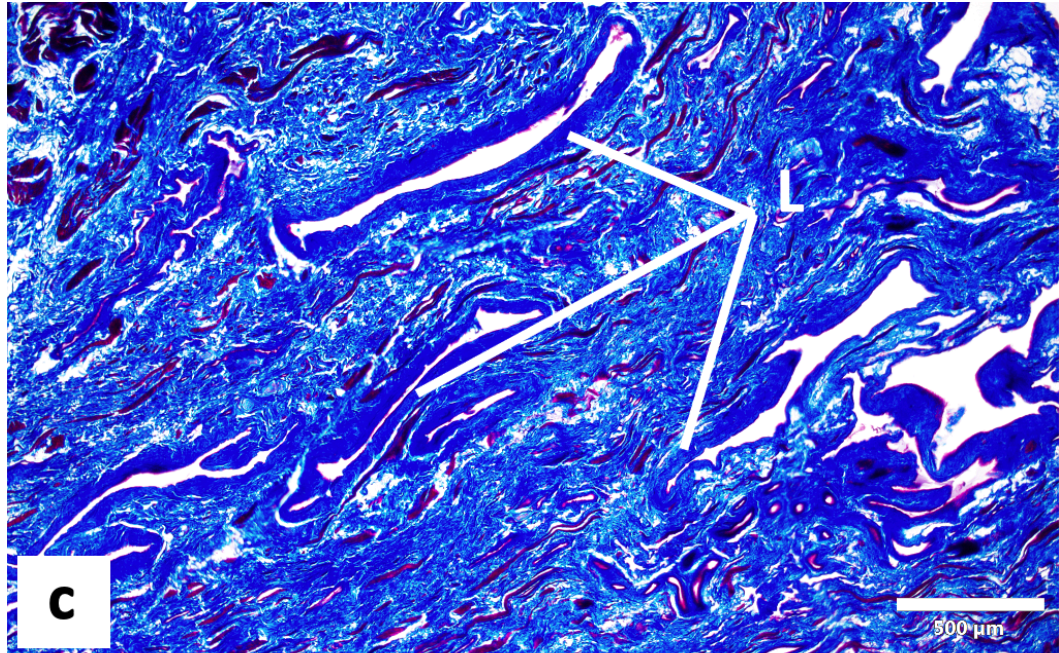


Figure 16. Histological preparations of the caudal vagina just lateral of the midsagittal line (labeled 2Ve1 in Figure 13). Images taken using Olympus BX51 with brightfield microscopy. (a) H&E stain, 10x magnification, transverse muscle bundle, (b) H&E stain, 10x magnification, (c) Masson's Trichrome stain, 5x magnification. M, muscle; SM, smooth muscle; L, lacunae.

Sectionings from this region of the superficial caudal vagina show transverse muscle indicative of bulbospongiosus muscle. There are also large lacunae amidst disorganized collagen and smooth muscle fibers typical of spongy erectile tissue.



Figure 17. Histological preparation of lateral caudal vagina (labeled dVe2 in Figure 13). Images taken with OlympusBX51 with brightfield microscopy. (a) H&E stain, 5x magnification. L, lacunae.

Erectile tissue in this lateral area of the vagina contains large lacunae and disordered collagenous matrix.

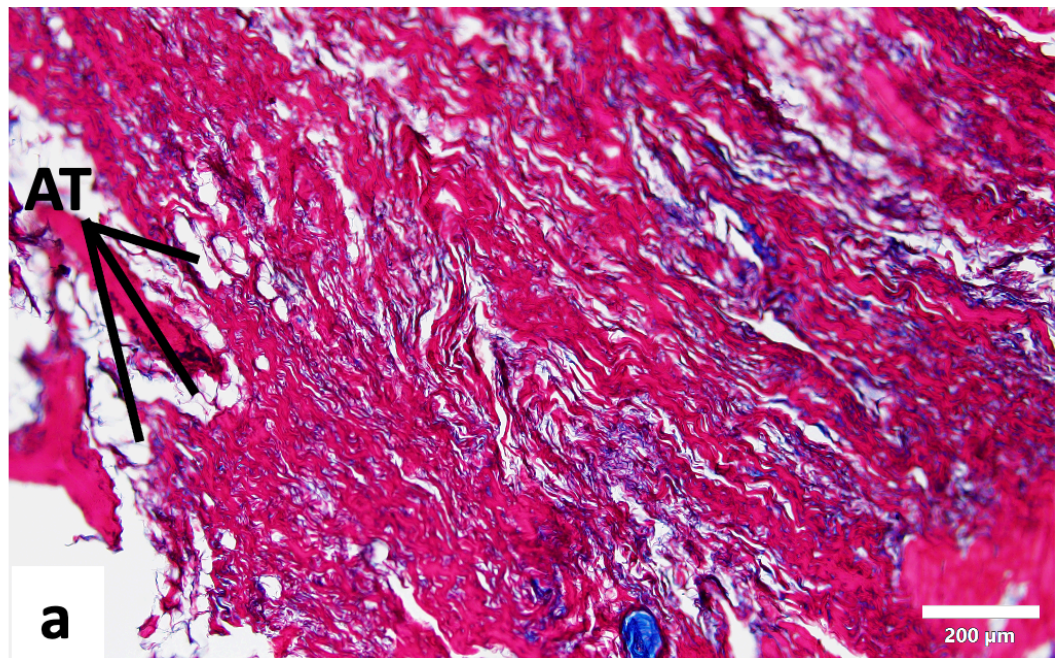
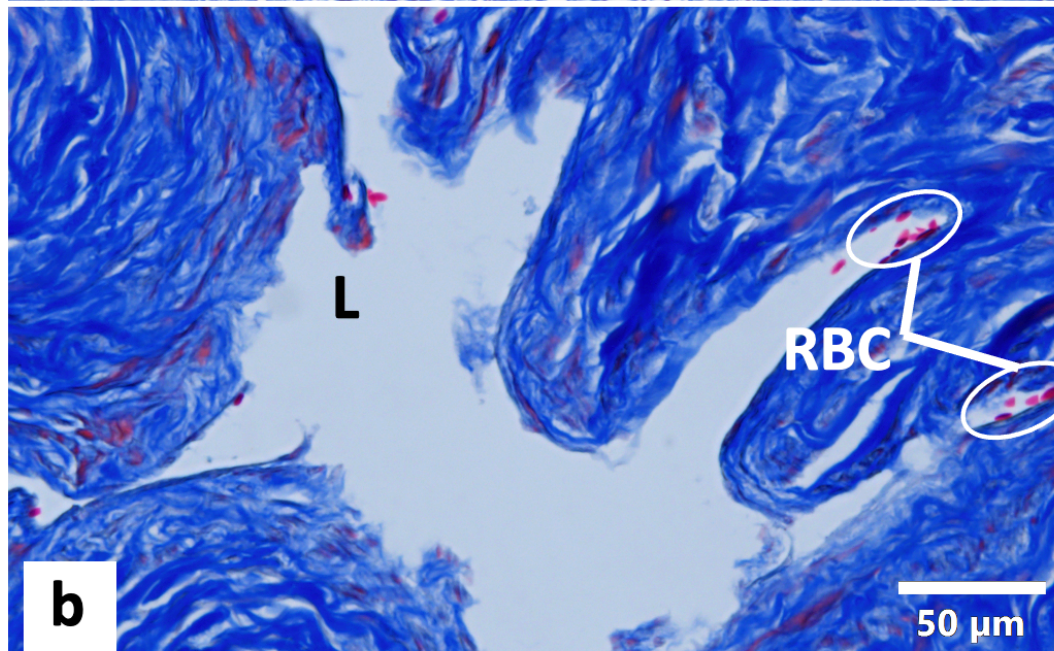
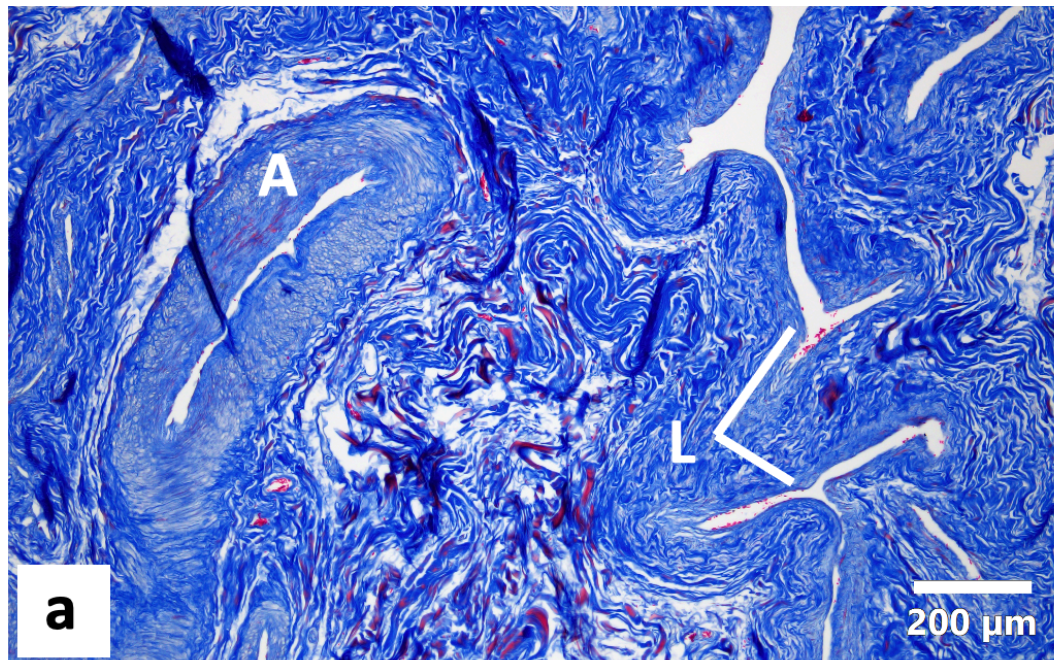


Figure 18. Histological preparation of mid-sagittal vagina just caudal to the urinary meatus (labeled gVe1 in Figure 13). (a) Masson's Trichrome stain, 10x magnification. AT, adipose tissue.

This image illustrates the dense connective tissue interspersed with adipose tissue. Though there were preparation issues with this region of the specimen, this image is relevant for its proof of erectile tissue extending midway to the urinary meatus.



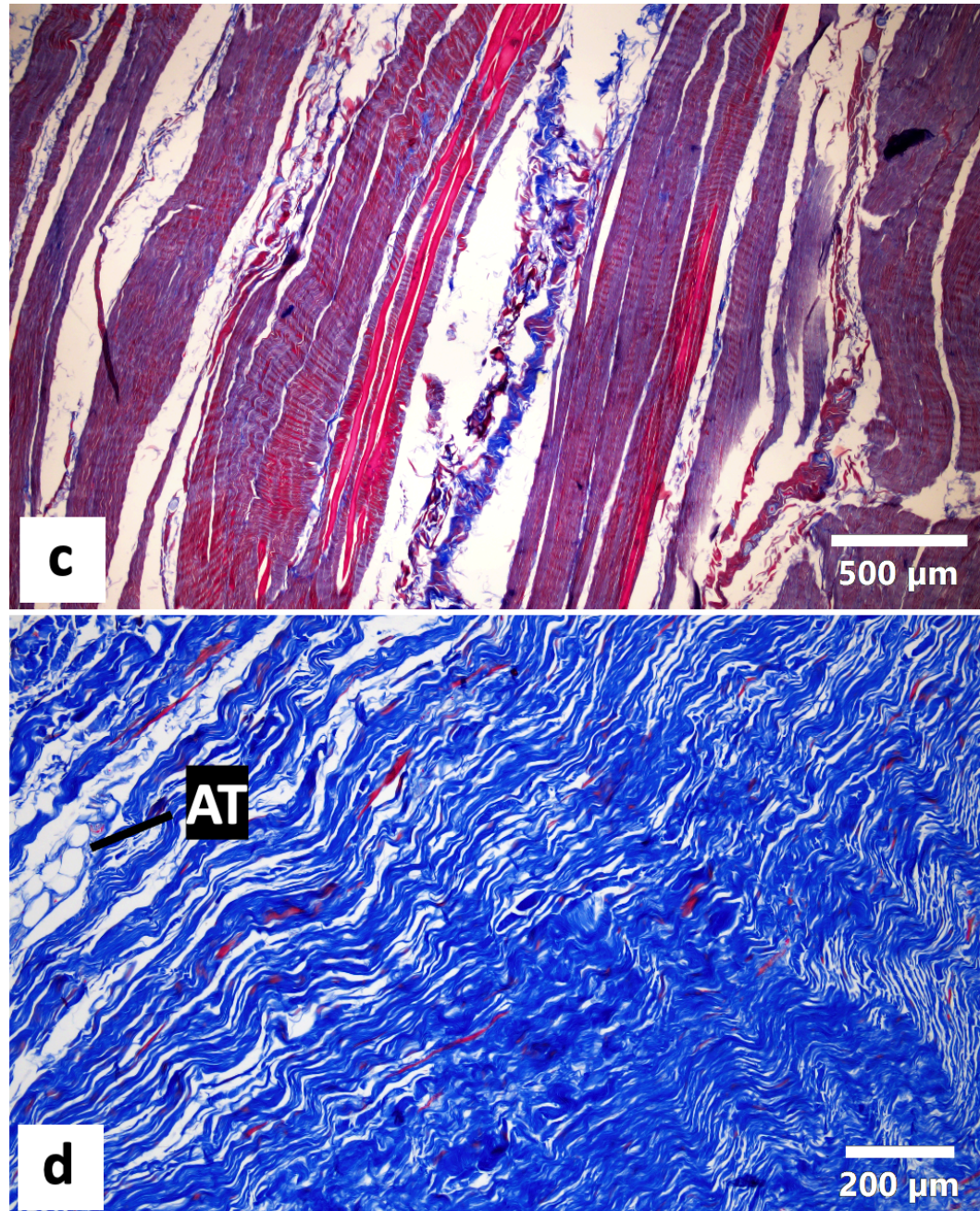
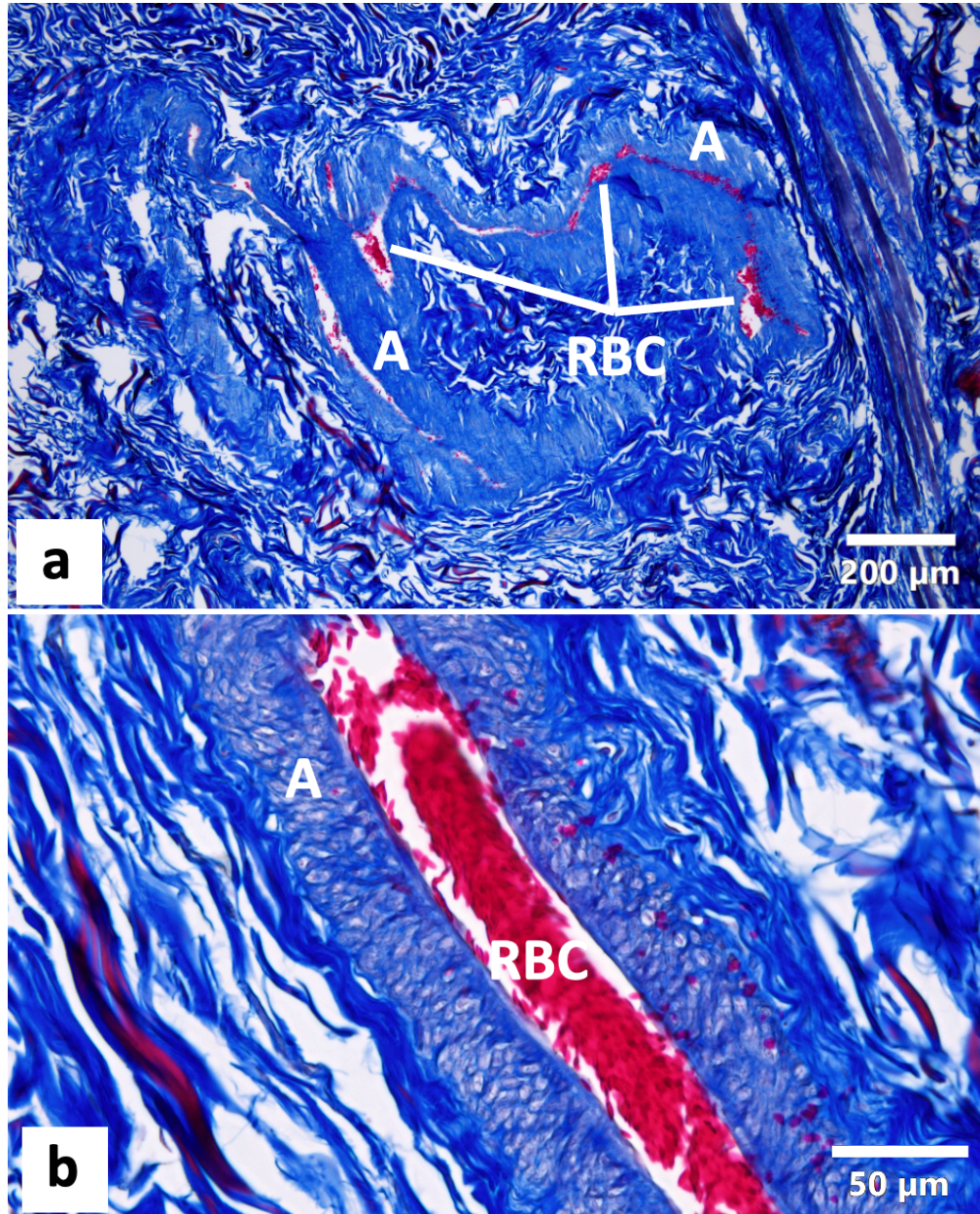


Figure 19. Histological preparations of regions associated with the vestibular bulb in the caudal vagina. Images taken with OlympusBX51 with brightfield microscopy. (a) Masson's Trichrome stain, 10x magnification, (b) Masson's Trichrome stain, 50x magnification, (c) Masson's Trichrome stain, 5x magnification, (d) Masson's Trichrome stain, 10x magnification. A, artery; L, lacunae; AT, adipose tissue.

These vestibular bulb sections show large arteries, presence of blood within sinus network and artery, collagen fibers (organized in parallel), lacunae, and some striated muscle fibers likely from the ischiocavernosus muscle given

that these sections are from the deeper more cranial vagina close to the urinary meatus.



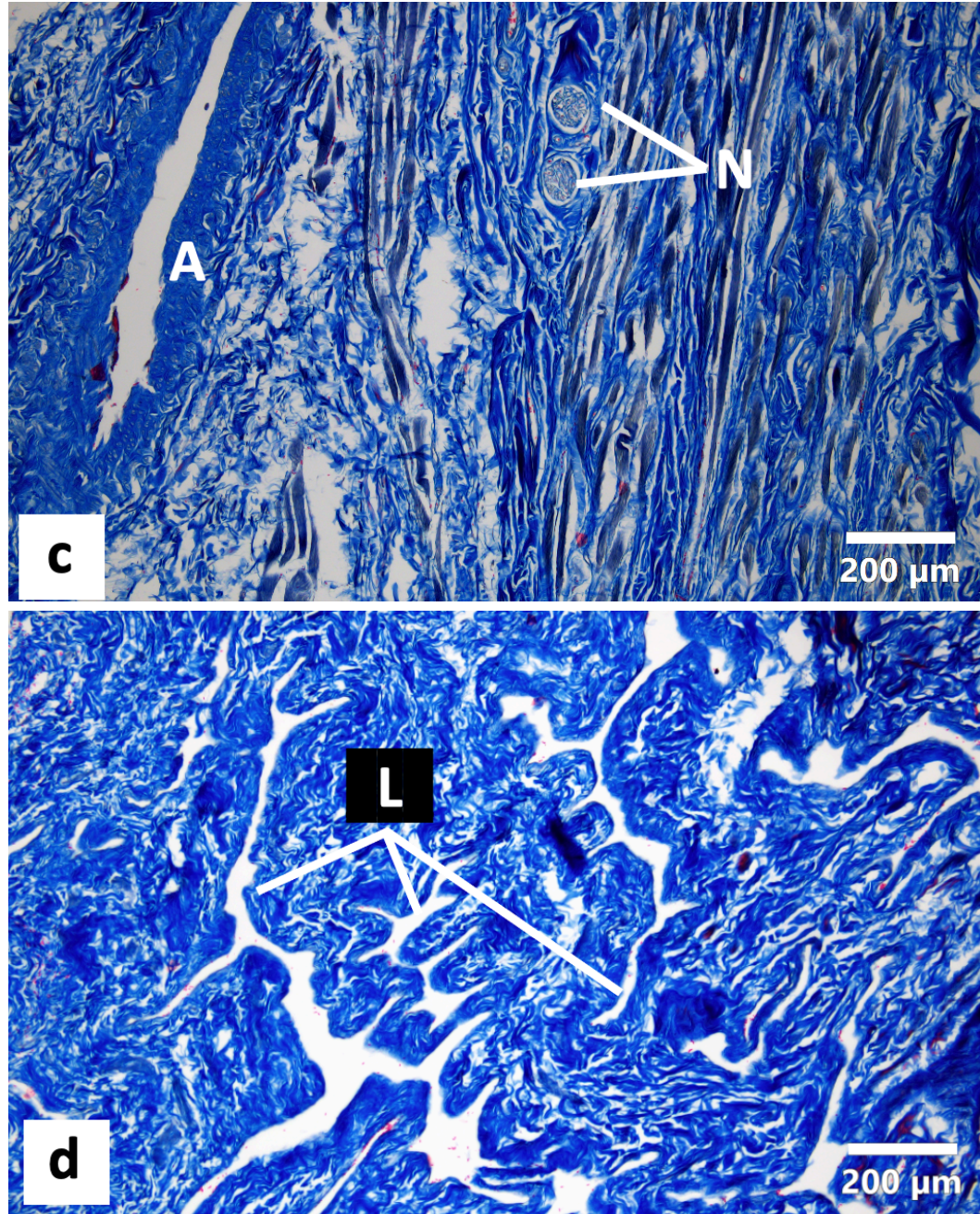


Figure 20. Histological preparations of regions associated with the vestibular bulb in the caudal vagina. Images taken with OlympusBX51 with brightfield microscopy. (a) Masson's Trichrome stain, 10x magnification, (b) Masson's Trichrome stain, 50x magnification, (c) Masson's Trichrome stain, 10x magnification, (d) Masson's Trichrome stain, 10x magnification. A, artery; RBC, red blood cells; N, nerve.

These regions of vestibular tissue have large lacunae and large arteries with evidence of recent blood influx. The proximity of arteries and lacunae paired

with the size of them explain the quick swelling and deswelling of the clitoris evident during observation.

As shown in the above images, all of the expected structures are present in the clitoris with the unexpected addition of a cartilaginous structure in the vulvar commissure and a clitoral lamina surrounding the cartilage and nerves. The tiny cartilaginous structure is no more than 1-1.7cm in length with a hard round pin head made of two tiny prongs just deep to the dermis of the vulvar commissure (Fig. 8; Fig. 21). Described in a singular sentence to be a “central core of cartilage” (Smuts & Bezuidenhout 1987) in the clitoris, our histology shows that it is actually a forked structure made of hyaline cartilage. The left cartilage prong is smaller in diameter than the right by a matter of roughly 2mm yet both are densely surrounded by nerves. Remarkably, this cartilage is entirely surrounded by more than 60 nerve bundles 0.1-0.9mm in diameter.

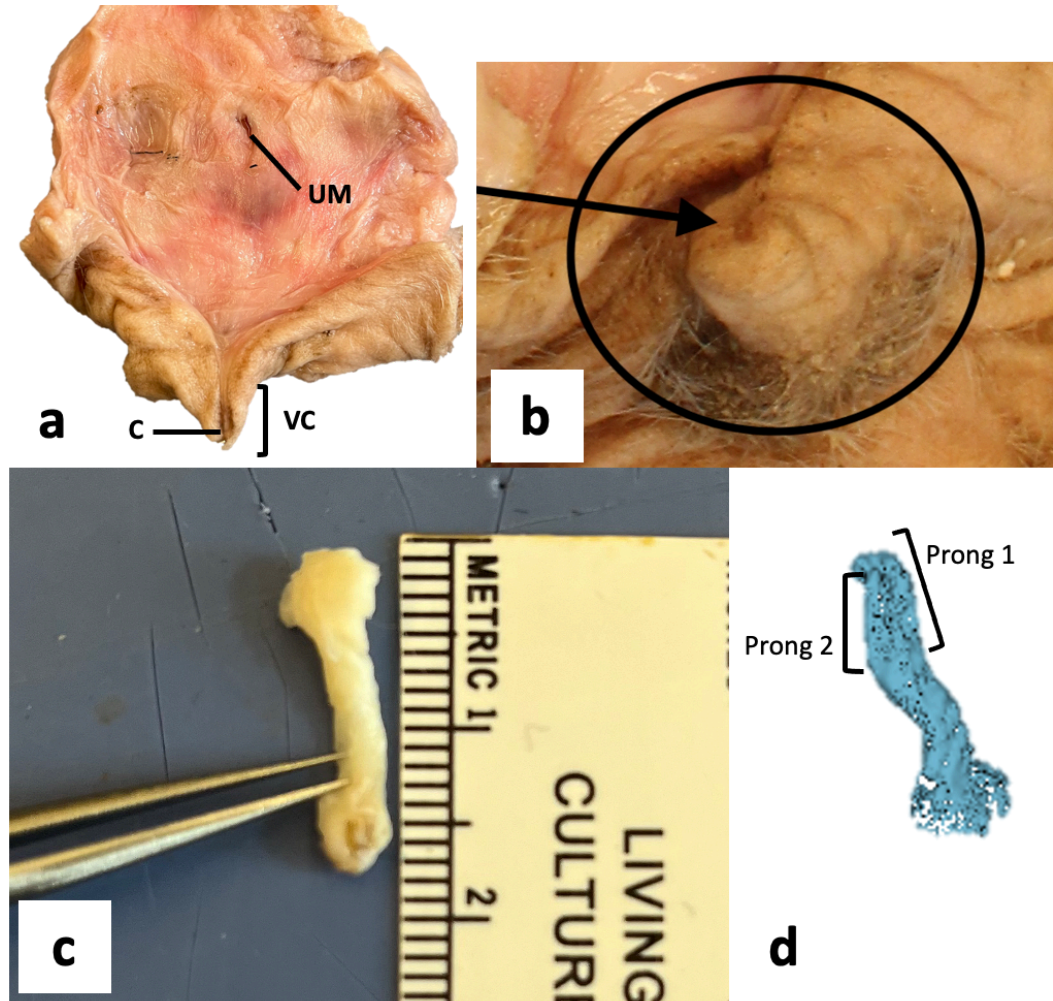
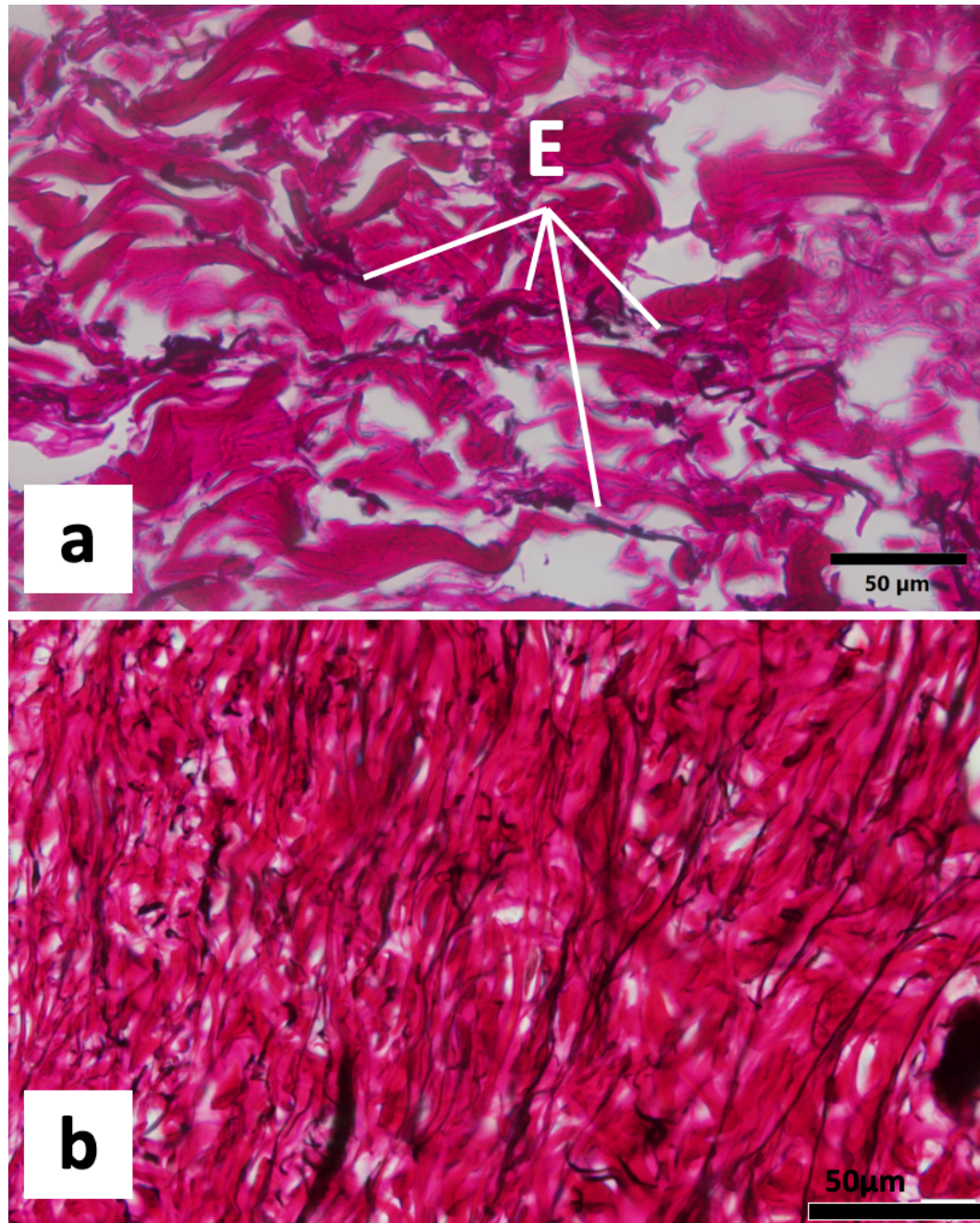


Figure 21. Cartilaginous structure in vulvar commissure. (a) caudal vagina and vulva, (b) arrow indicating head of cartilage on vulvar commissure, (c) excised cartilage (1.7cm), (d) CT segmentation of cartilage illustrating two distinct prongs at head of cartilage. UM, urinary meatus; C, cartilage; VC, vulvar commissure.

Elastin is often present in tissues that undergo significant stress from stretch and tension. Tissues that perform this sort of function and have well-researched elastin are articular cartilage, and lungs. Despite not typically being in that list of tissues, presence of elastin in the erectile tissue should be expected given that the tissue is extensible which requires tensile strength and flexibility. The Brennan Lab found extensive elastin fibers in the dolphin clitoris.

In comparison of alpaca and dolphin clitorises, alpacas have shorter, less dense elastin fibers in the clitoral erectile tissue (Fig. 22).



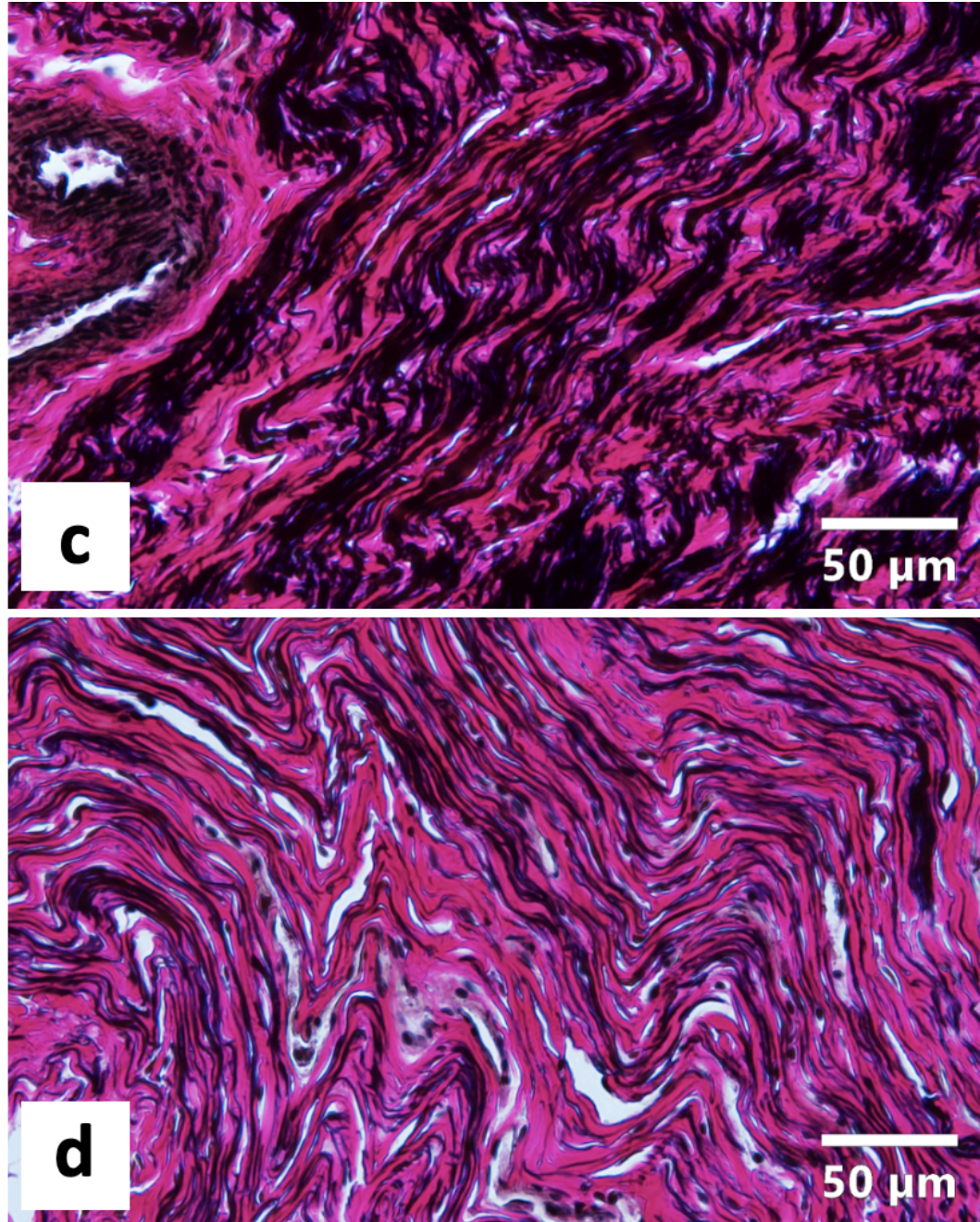


Figure 22. Comparison of elastin in alpaca clitorises and dolphin clitorises. Images taken with OlympusBX51 with brightfield microscopy at 50x magnification of erectile tissue stained with elastin stain. (a) short wavy elastin strands in distal-most tip of alpaca vulvar commissure (b) elastin fibers in erectile tissue of distal vulvar commissure of alpaca, (c) dense elastin in dolphin clitoris, (d) long organized elastin stains in dolphin clitoris (Brennan Lab).

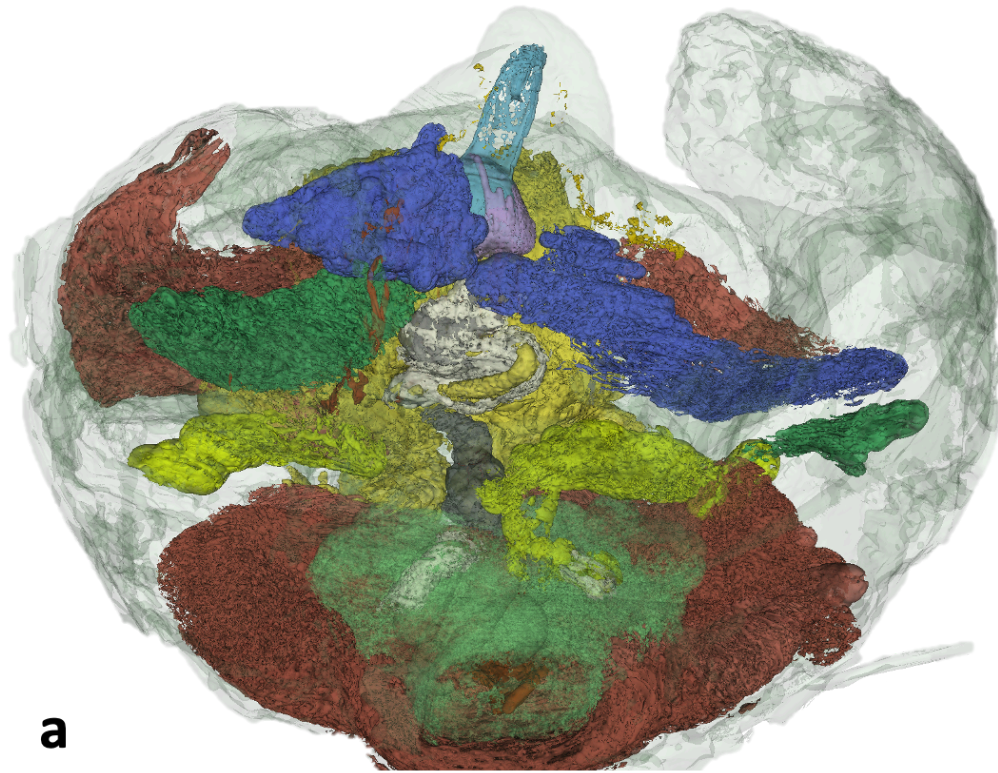
CT segmentations

CT scans of two alpaca specimens show extensive corpus cavernosum extending cranially from the base of the cartilage until diverging into two arms that form the crura of the clitoris. Our specimens were collected from alpacas in such a way that it is unclear to us where exactly the crurae connect within the pelvis. The crura seems to be wrapped in a tunica albuginea forming a patchwork pattern that is of inconsistent thickness and coverage on the crura. Corpus spongiosum extends laterally from the midline to form a ring around the caudal vagina or vestibule. There is evidence of vascularization and innervation in both types of erectile tissue through visible blood vessels and nerves. Two muscles connect to the erectile tissue. Adjacent to the crura are two branches of small ischiocavernosus muscles responsible for contraction that sustains high blood pressure during erection. The bulbospongiosus muscle lies along the vestibular bulbs. CT confirms that the cartilage within the vulvar commissure is a two-pronged structure that merges into one at the cranial juncture with the rest of the vulva and vagina.

Comparison of the two specimens after segmentation by Rachel Keeffe (ALP041F) and myself (ALP022F) revealed that we both found the same structures despite differences in our knowledge of the anatomy. The corroboration of structures between the two specimens is indicative that the same primary structures of the clitoris exist across individuals. However, there was also variation between the two clitorises that can be explained by differences in the extraction of the specimens, problems with the staining (perhaps some tissues in

one specimen were more or less stained than in the other), or simply by minor variation between individuals.

Of the two specimens we collected CT scans of, one (ALP022F) has a large pathology at the base of the cartilage that could have impacted the sizing and growth of the cartilage but there remains two distinct prongs of cartilage.



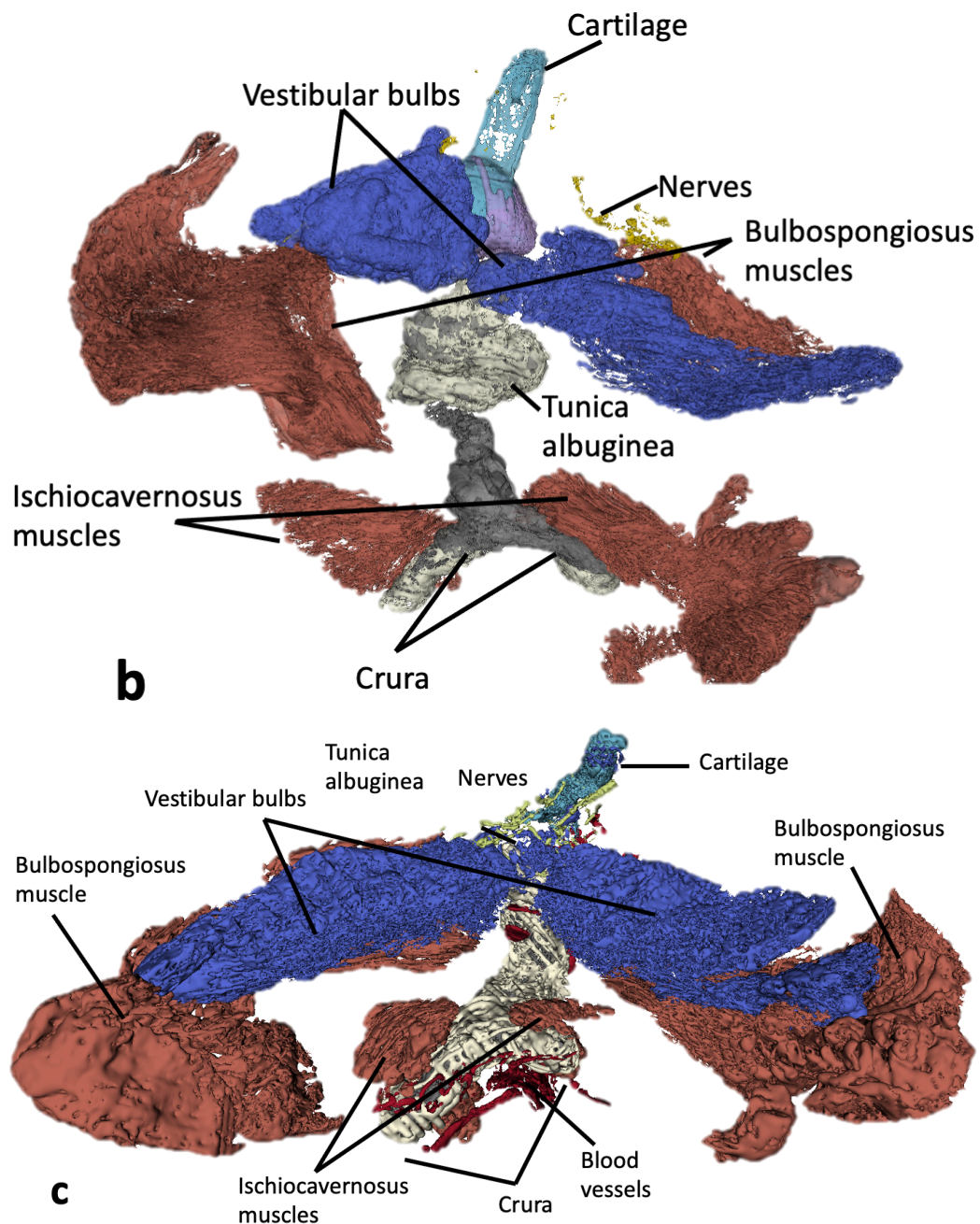


Figure 23. CT segmentations of clitoris. (a) entire segmentation of excised clitoris, vagina, vulva, (b) segmentation of clitoris from specimen ALP022F with labeled primary components, (c) segmentation of clitoris from specimen ALP041F with labeled primary components.

In vivo Observation

Initially, females and males were in separate flocks in distinct parts of the farm where there was no contact. The female flock included four females: Kahlua,

Rianiti, Irene, and Amy. We took advantage of no male presence in the barn to individually measure each female's vulvar commissure by length, width, and thickness (Table 1; Fig. 24). For each alpaca, we photographed the vulva and took an ultrasound reading of the vulvar commissure. The ultrasound of the vulvar commissure was intended to determine size and superficiality of cartilage (Fig. 25). After an incident of gates being left open, an ultrasound on Amy found that she was 25-30 days pregnant. At the time of clitoral and mating data collection, an ultrasound of Amy indicates that she remained 33-38 days pregnant.

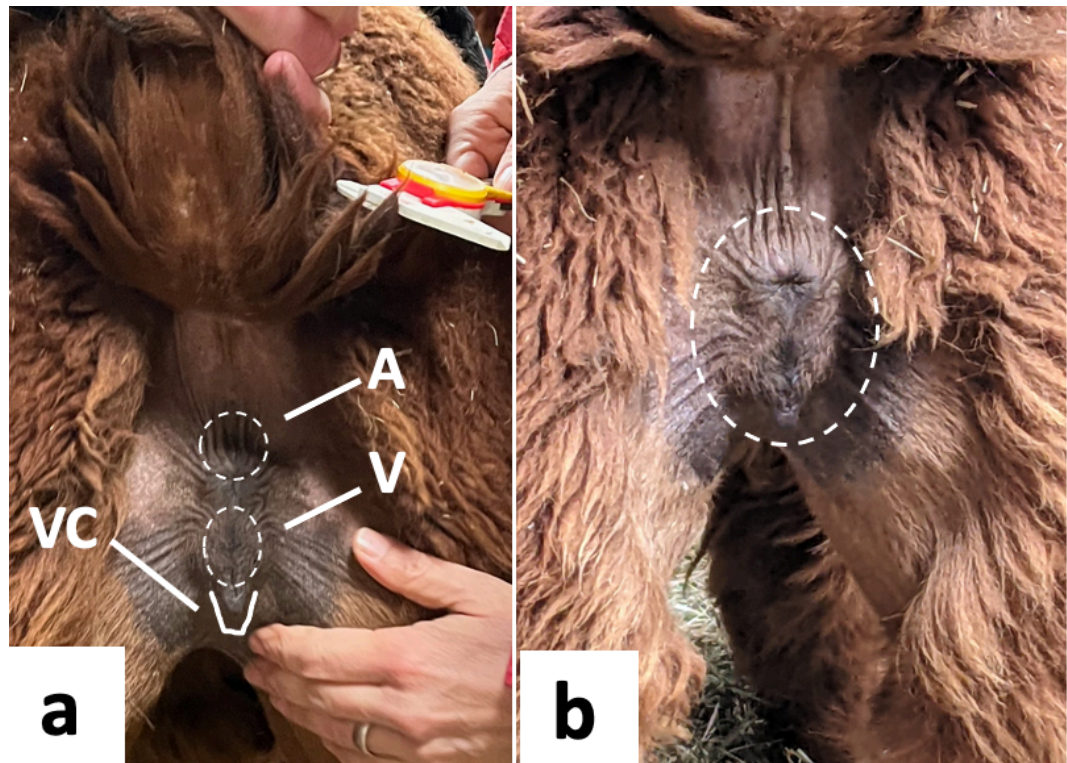


Figure 24. Anatomy of alpaca external genitalia with non-erect and erect clitoral ring (Kahlua). (a) non-erect clitoris, (b) erect clitoral ring around vulva. R, rectum; V, vulva; VC, vulvar commissure.

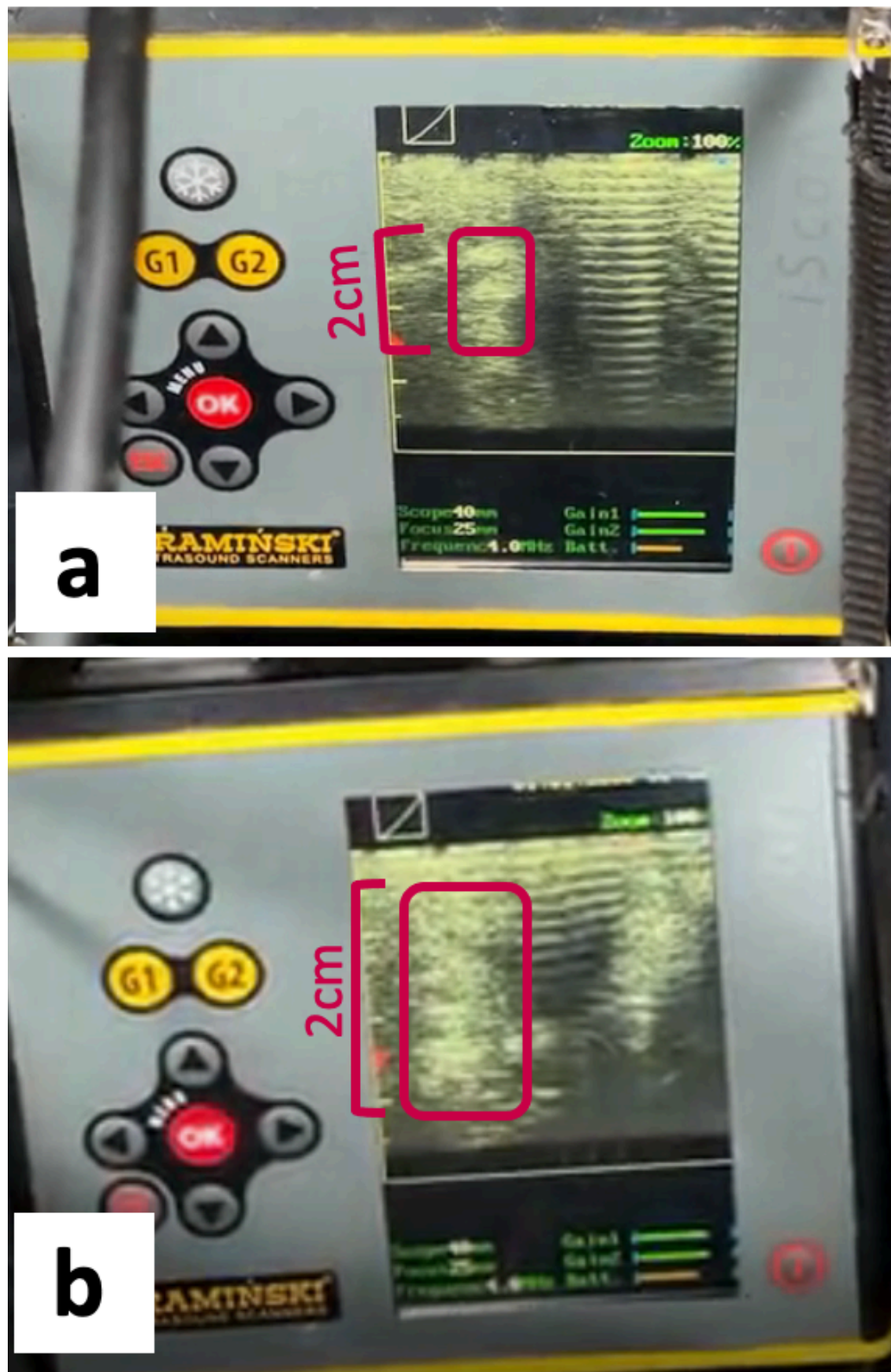


Figure 25. Ultrasound readings of cartilage as ~2cm long in vulvar commissure (a) in Kahlua (a) and Rianiti (b).

Measuring the vulvar commissure before males were introduced to the flock, was intended to gather data that reflected an non-erect clitoris. However, upon touch by hands or the calipers, the clitorises began to tumesce and visibly swell which impacted the accuracy of our data collection (Fig. 24). We anticipated simply seeing the vulvar commissure that points directly downward from the base of the vaginal opening. It is clear that there is a firm pad about 2.5cm anterior to the vulvar commissure below the vaginal opening. Additionally, when aroused, a ring around the vaginal opening visibly tumesced (Fig. 24) indicating that it is the vestibular bulb tissue evident in histology and CT segmentation.

When not erect, the vulvar commissure containing the glans clitoris points downward from the vagina and varies in shape and dimension across alpacas (Table 1). Within seconds of tactile contact from measuring the length, width, and thickness of the vulvar commissure with calipers, the glans clitoris began to swell and the size of the firm pad just behind the glans also began to tumesce. This was consistent across all four alpacas in our sample group. As quickly as the area became enlarged, it also reduced in size when tactile stimulation was removed. In the presence of a male, a receptive female's clitoris also began to quickly swell which caused the vulvar commissure to no longer point downward, rather, it lifted to a nearly 90 degree angle such that it protruded directly outward from the vaginal opening (Fig. 28). The firm point also grew in size and a swelling appeared in a ring around the vagina. This remained until after copulation ceased.

After each female alpaca was measured, we brought the flock of males into the barn but kept them in a paddock about 30 feet away from the female

flock. We then repeated the measurements of the vulvar commissure as before to note whether the physical proximity of the males or the males' vocalizations had any effect on female arousal. No ultrasound was taken because the cartilage itself cannot change during erection.

Following measurements of all the females (Table 3 in Appendix 3), we commenced behavior testing. One male, Fireball, was introduced to the flock of females to determine which females would be receptive to copulation or display signs of disinterest. Receptivity to copulation indicates a mature follicle. Amy, the pregnant alpaca, was exceptionally opposed to copulation and demonstrated this by humming and running away from Fireball. Kahlua also displayed disinterest by walking away when approached. Rianiti was approached but was not as receptive as Irene, demonstrated by her walking away and lack of immediate assumption of the cush position. During this period of interacting with the females, Fireball's penis began to protrude making small probing circular motions.

Fireball mounted Irene who had quickly dropped into the cush position indicating receptiveness to copulation. Once mounted, Fireball orgled consistently and began inching forward closer to Irene's rump. It took him about six minutes to reach the necessary angle for intromission. During these moments, his penis was probing and running into the hay as well as Irene's rectum, rump, and vagina. Once the penis entered the vagina, there was a period of 24 minutes of mating during which Fireball continuously orgled, made small thrusting movements, and frequently had dilated nostrils. Irene seemed unperturbed and shifted to lay on her side during copulation.

Directly after copulation, we measured Irene's vulvar commissure (Table

1).

Alpaca	CR diameter (mm) before males	CR diameter (mm) after copulation with male	CR diameter (mm) after copulation with female	% increase after copulation with male	% increase after copulation with female
Rianiti	18.8-18.4	24.6-28.0	20.0	41.4	7.53
Irene	16.6	38.4	34.0	131.3	104.82

Table 1. Measurements of vulvar commissure and clitoral ring diameter before copulation and post-copulation. Clitoral ring diameter references the ring of tumescence around the vulva when the clitoris is erect.

We took a sample of the sperm from Irene's vagina to analyze seminal fluid viscosity and vaginal abrasion.

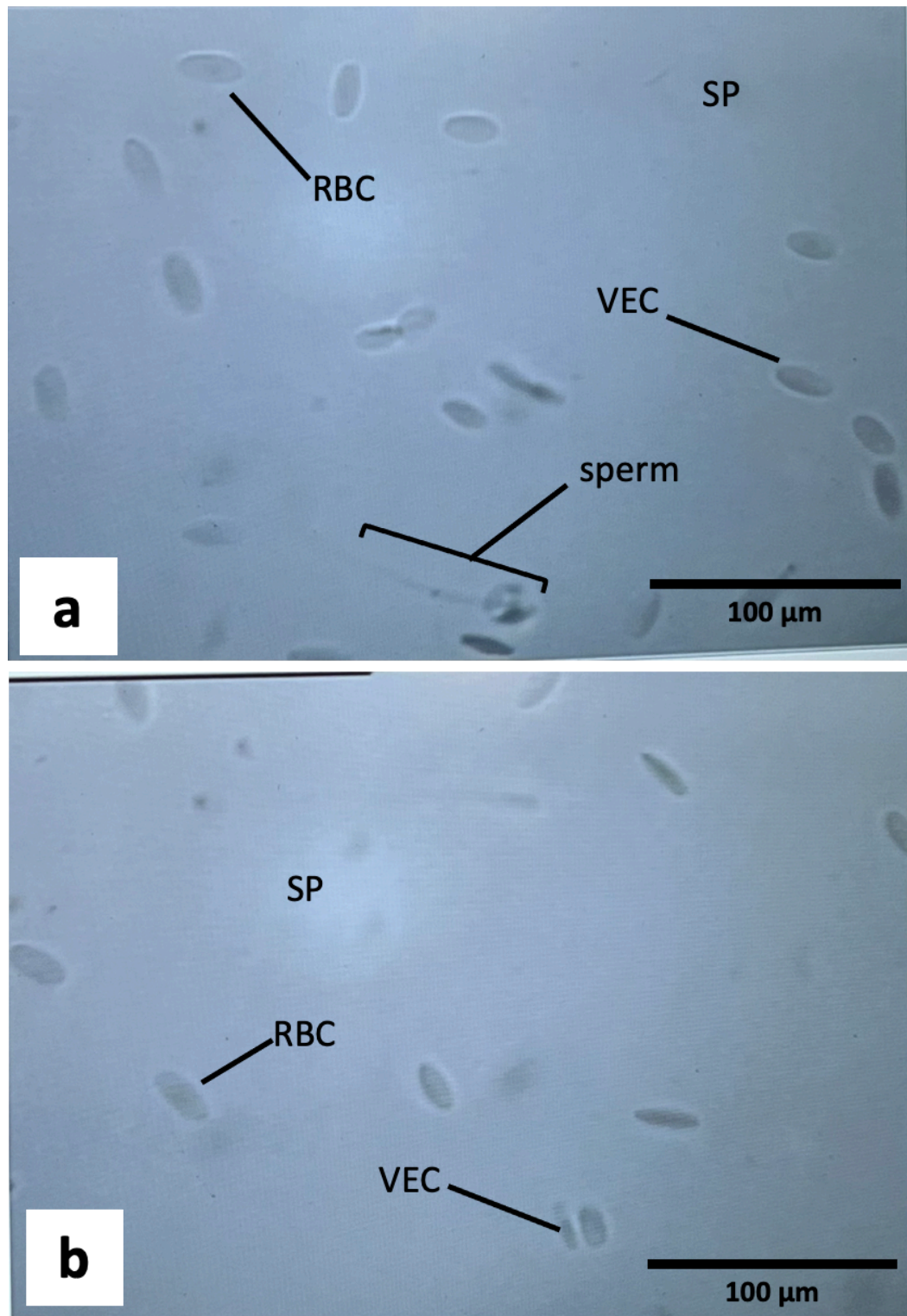


Figure 26. Seminal fluid extracted from female's vagina post-copulation. Viewed under microscope at 40x magnification. (a) distinct spermatozoa in lower center of image, multiple RBCs indicate abrasion, (b) no evident sperm, moderate RBCs and VECs indicate abrasion. RBC, red blood cell; SP, seminal plasma; VEC, vaginal epithelial cell.

After a short period of rest and inactivity for the alpacas while we evaluated the seminal fluid, we returned to the female flock to introduce J.D., another male. Amy and Kahlua repeated their aversion or disinterest and J.D. immediately rushed toward Irene who quickly returned to the cush position despite copulating with Fireball recently. However, when J.D. approached Irene, Rianiti began spitting at the male and vocalizing. The spitting and aggressive maneuvers are indications of displeasure and opposition to copulation. Upon removal of the male from Irene, Rianiti mounted Irene and began thrusting and intermittently nuzzling Irene's neck (a gesture unobserved in the male/female behavior). The clitorises of both females were tumesced and erect as if in the presence of a male (Table 1).

After Rianiti removed herself from Irene, J.D. approached her and mounted her. They walked and stood in this mounted position for a few minutes before Rianiti dropped into the cush position. J.D. took about 1.5 minutes to enter the vagina, orgled nearly consistently, and thrust intermittently throughout the copulation. Notably, during J.D. and Rianiti's mating, Irene moved to lie closer to Rianiti's head and occasionally Rianiti would nuzzle Irene's neck.

During copulation, neither J.D. nor Rianiti were disturbed by exploratory palpation by observers. We found that Rianiti's vulvar commissure was erect and had lifted 90 degrees which placed it firmly against the penis as it thrust and entered the vagina. This revealed that the position of the erect vulvar commissure was in full direct contact with the penis (Fig. 27). The degree of contact paired

with the positional shift from erection is clear evidence that the clitoris in alpacas is a functional organ.

After 31 minutes, we halted copulation and harvested seminal fluid from Rianiti's vaginal canal.

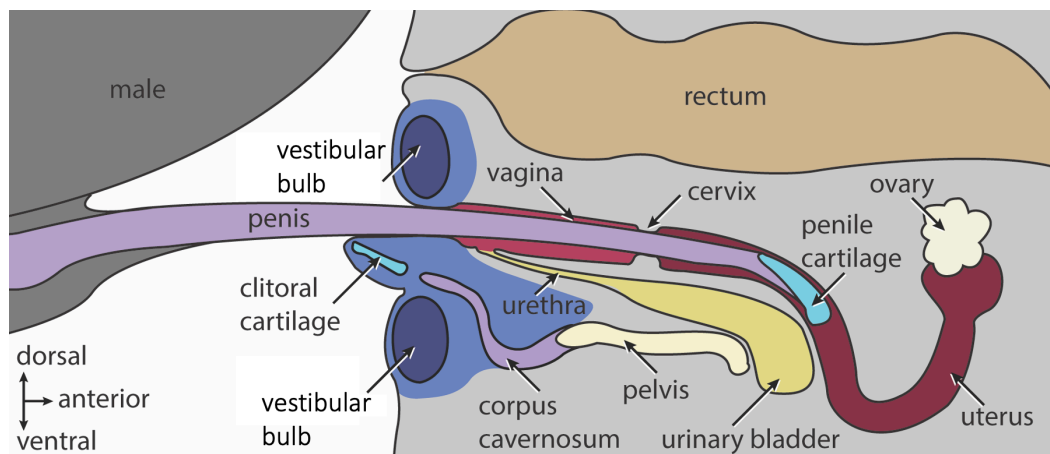


Figure 27. Conjugation of penis relative to clitoral cartilage. This diagram was created by Rachel Keeffe. It is for visualization purposes, it is not to scale.

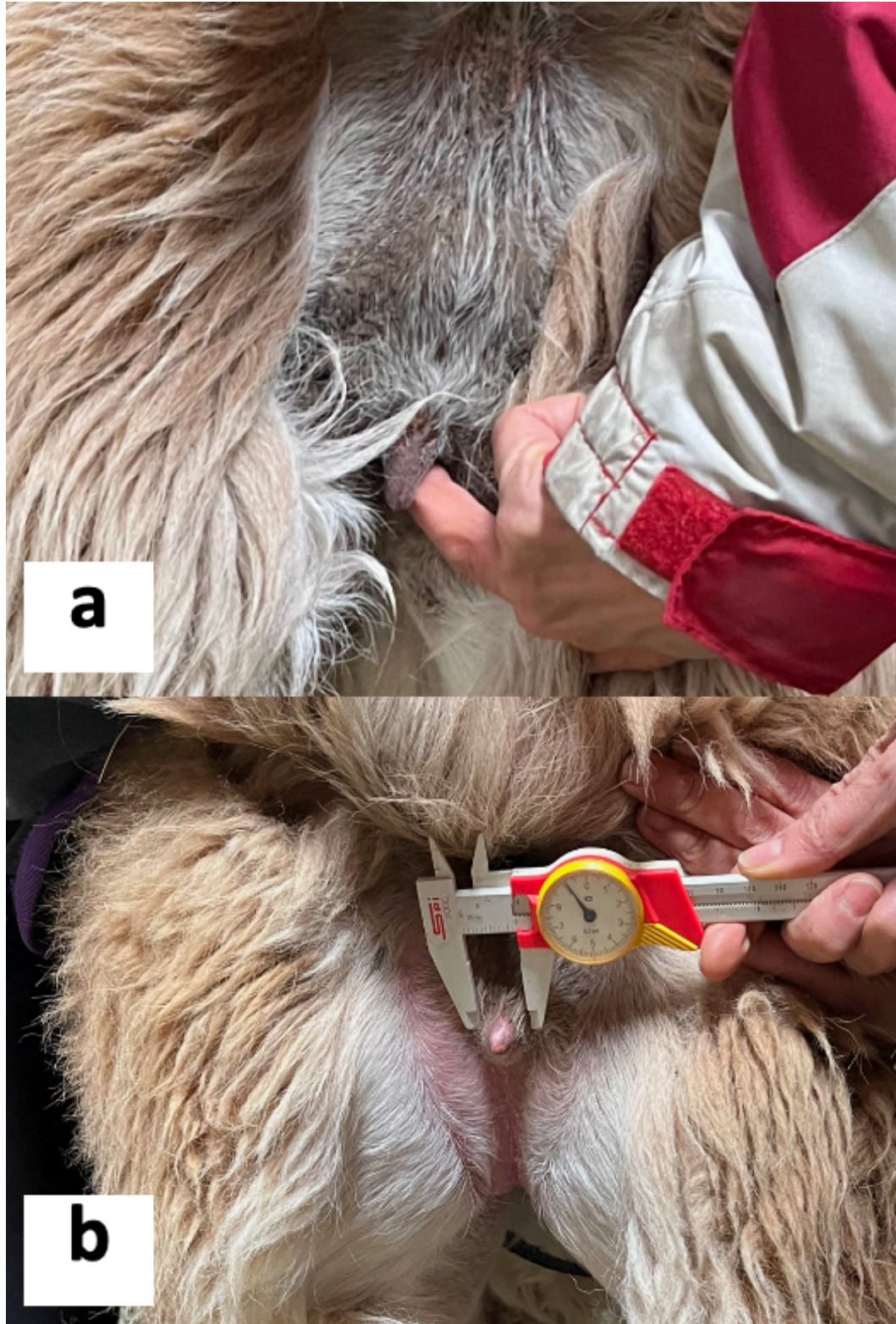


Figure 28. Non-erect and erect vulvar commissure (Rianiti) (a) Rianiti with non-erect clitoris during measurement before introduction to males, (b) Rianiti with erect clitoris – swollen, lifted 90 degrees – after contact with calipers.

Given the observation that an erect clitoris protrudes outward, during female/female copulatory imitation, there must be contact between the clitoris of the mounted female and the abdomen of the mounting female. This supplements the theory that the clitoris is functional, receives tactile stimulation and sensation. Depending on the amount of physical stimulation received by the mounting female, it may also indicate that there is more to clitoral arousal than just tactile stimulation.

Collection of seminal fluid post-copulation showed evidence of motile sperm, vaginal epithelial cells, and red blood cells (Fig. 26). Vaginal epithelial cells and red blood cells indicate evidence of the abrasion of the reproductive tract from the males' penis' cartilaginous tip.

During the initial measurement of the females' vulvar commissures, we also took ultrasounds that revealed the small cartilage structure. These images helped corroborate the size of the cartilage and the superficiality of the structure.

Discussion

Our results show that alpacas have a well-developed clitoris with extensive erectile tissue, multiple nerve endings, and novel cartilage and laminal structures. The clitoris is likely functional as supported by *in vivo* observations.

Our multifaceted approach of dissection, histology, CT segmentation, and observation in order to examine and characterize the clitoris allowed for analysis at multiple scales of magnification and served to corroborate findings across methods. Dissection provided an overview of the gross anatomy and allowed for a

rough draft or blueprint of the organ. Histology allowed for a much more detailed illustration of the smaller structures, confirmation of the gross structures identified during dissection, and description of the entire clitoris in incremental slices. Smaller structures revealed by histology included nerves, cartilage, clitoral lamina, lacunae, vascularization, collagen, elastin, and smooth muscle. CT segmentation alongside dissection and histology created an opportunity for 3D analysis of individual structures within the greater context of the organ and surrounding tissues. By isolating the vestibular bulbs in CT segmentation, it is evident that they do not extend to the urethra but do form a ring around the vaginal opening. Finally, *in vivo* observation was an opportunity to see the organ in action and let the knowledge gained from previous methods coalesce into the functional active organ involved in copulation.

Terminology - vestibular bulbs vs corpus spongiosum

As mentioned, the clitoris and penis are homologous structures in males and females. However, the homology of specific tissue types, such as corpus spongiosum, within these organs has not been determined. Therefore, until embryonic and developmental research can confirm or deny the homology of corpus spongiosum tissue in the clitoris and penis, bulbs of tissue appearing as corpus spongiosum are typically referred to as vestibular bulbs (Di Marino & Lepidi 2014; Turnhout et al 1995).

Immune Response

Physical and chemical stimulation of the female reproductive tract triggers cascades of responses to facilitate reproduction. These cascades can include uterine remodeling to accommodate pregnancy, amplification of potential maternal-embryonic signaling pathways, induction of ovulation, and sperm preservation or termination. In alpacas, the uterine abrasion caused by penile intromission during copulation may be a method of quickening the immune response by physically harming the uterine lining and recruiting immune cells. Additionally, the penis' messy exploratory phase before entering the vagina can help explain the importance of the female's intercourse-instigated immune response perhaps aided in part by an aroused clitoris.

Cartilage

The presence of cartilage in the alpaca clitoris is unusual. Within the entire taxonomic group of Artiodactyls (encompassing species from alpacas to buffalos to whales), there are no species other than alpacas with a baubellum or clitoral os. In fact, cartilage and bone in the clitoris and penis are only known to exist in primates, rodents, insects, carnivores, and bats (Lough-Stevens et al 2017). Alpacas are part of none of those groups. Therefore, it's a bit of a mystery as to why there is a pronged cartilaginous structure within the clitoris.

Given that the clitoris and penis are homologous structures developing from the same embryonic tissue a reasonable first assumption is that this hyaline cartilage in the clitoris is explainable by homology. However, sexual dimorphism is not uncommon and there is evidence of species whose males have a baculum

but the females' baubellum differs significantly in structure or is simply not present (Lough-Stevens et al 2017). This indicates that the baubellum may evolve somewhat independently of the baculum (Lough-Stevens et al 2017). Despite this variability, it seems to be unique to camelids because, at the time of this writing, a cartilaginous core or forked cartilage structure has not been described in clitorises of any artiodactyl species other than alpacas (Brennan et al 2021). In addition, histological examination of the cartilaginous penis tip does not show the extensive innervation we find in the cartilage of the clitoris (Brennan et al 2021).

A secondary method to rationalizing the cartilage is to propose a function. The cartilaginous structure and extensive nerves within the vulvar commissure revealed during histology and CT scanning in conjunction with the observed angle change during erection indicate related function. The cartilage likely supplements blood pressure-driven tumescence by providing further support to the vulvar commissure. The nerves surrounding the cartilage are quite superficial given the thinness of the vulvar commissure which indicates sensation is registered during contact from the penis during copulation. This could provide a theory as to why the intrauterine abrasion from mating does not result in evolution of aversion to copulation.

Elastin

Erectile tissue around the vulvar commissure stained with Verhoeff's stain showed short wavy strands of elastin amongst collagen fibers in the connective tissue. Presence of elastin provides tissues with increased elasticity, reversible

high extensibility, and reduced stiffness (Trębacz & Barzycka 2023). Elastin forms from tropoelastin synthesized from “fibroblasts, smooth muscle cells, chondrocytes, or endothelial cells” (Karsdal et al). This synthesis occurs pre-natally through adolescence and the elastin produced during that period is all that is made during a lifetime. It can wear out in old age, becoming less flexible and less resistant to extreme stretch or stress (Trębacz & Barzycka 2023). Elastin is more present in tissues that experience significant stress and stretch such as the lungs, arteries, and articular cartilage (Trębacz & Barzycka 2023). Though most reference texts cite these three structures as most abundant in elastin, genitalia is notably absent despite its extraordinary extensibility. The penis is a highly elastic structure as are the vagina and clitoris which makes them prime candidates for further elastin research. The Brennan Lab had slides on hand from previous research on dolphin clitorises that show copious amounts of elastin (Fig. 22). Compared to the alpaca clitoris, the dolphin has much longer strands and significantly denser bundles of elastin. In the alpaca, elastin strands are somewhat infrequent and are roughly contained within a band just superficial to the clitoral lamina and cartilage but deep to muscle, vestibular bulb tissue, and the epithelium. Despite their short length, the small elastin fibers in the alpaca clitoris could still maintain a significant role in supporting the flexibility of the tissue. Articular cartilage has a “fine and dense network of elastin fibers located around the chondrocytes” (Trębacz & Barzycka 2023) which works to retain tissue shape despite stretch forces in the cartilage. Where the elastin forms a looser network more superficially, it serves as reinforcement of the tissue to multidirectional

strain (Trębacz & Barzycka 2023). Elastin in alpacas is present where the space surrounding the cartilage chondrocytes and matrix superficial to the epithelium are one and the same due to the small thin nature of the vulvar commissure.

Elastin here is certainly a loose web rather than a dense network which would indicate resistance against multidirectional strain. Given that tumescence stretches the vulvar commissure in every direction outward, perhaps the elastin aids in tissue resilience during erection. More research is warranted for the role of elastin in genitalia.

Clitoral lamina

The clitoral lamina in alpacas and other species remains somewhat of a mysterious structure of unknown function and content. Studies examining moles (*Scapanus latimanus*), mice (*Mus musculus*), and rats (*Rattus rattus*) (Cunha et al 2020; Liu et al 2018; Sinclair et al 2016) reveal images of a similar lamina surrounding the bulbs of erectile tissue in the clitoris and penis. The clitoral lamina in these species is a product of fusion of preputial swellings fuse with the genital tubercle during development (Cunha et al 2020). The lamina initially connects to the groove that precedes a formed urethra but, as the groove morphs into a mature urethra, the ends of the lamina detach from the urethra creating a small gap that fills with mesenchymal cells (Cunha et al 2020; Liu et al 2018). These studies examined the structure as it developed prenatally which illustrated that the clitoral lamina derives from ectodermal tissue. Cunha et al (2020) and Liu et al (2018) also did a material analysis and came to preliminary results showing

the lamina to contain high amounts of keratin. It seems that this clitoral lamina is developed as the female matures and the erectile tissue is formed (Cunha et al 2020) though there is no theory of a possible function.

Despite the similarities of the clitoral lamina in moles, mice, and rats to that of the alpaca, there are notable differences. In rodents, the lamina surrounds the urethra in addition to erectile tissue. In alpacas, the lamina is not associated with the urethra at all. Another key difference is that the clitoral lamina in rodents surrounds mesenchymal tissue while the os clitoridis lies outside of the clitoral lamina (Cunha et al 2020; Liu et al 2018). Conversely, alpacas' clitoral lamina surrounds cartilage and minimal mesenchymal tissue. Most of the erectile mesenchymal tissue exists outside of the lamina. Given that there is no proposed theory for function of the clitoral lamina and that the research of this thesis does not encompass developmental characteristics of the alpaca clitoris, it is unclear what the significance of these differences is. Further research could benefit from a developmental emphasis and/or an analysis of the material components of the lamina to help propose function or confirm that it is simply a remnant of fusing tissues.

Homosexuality

Homosexual behavior is common among many species. Various species of marine mammals (Brennan et al 2022), primates, hoofed mammals, rodents, birds, and marsupials exhibit homosexual behavior through a myriad of methods (Bagemihl 1999; Rufo & Ottoni 2021). This behavior has been criticized as

abnormal because it cannot lead to reproduction which must render it evolutionarily disadvantageous. This is an incomplete understanding. To some degree, homosexual behavior is indicative of some form of sensation and function in a pleasurable context. While the type of sensation and the organism's associated feeling is indeterminable simply from observation of homosexuality, there is undeniably a reason for this behavior. Elective clitoral stimulation and other homosexual behaviors that are pleasant can be theorized to prevent evolution of aversion to copulation, factor into dynamics of dominance or bond between members of a group, and contribute to tension relief within the group (Bagemihl 1999; Rufo & Ottoni 2021). Homosexuality in alpacas is consistent with other research of species with functional clitorises and serves as further evidence of functionality.

Conclusion

As evidenced by presence of all primary components of a clitoris and observation of *in vivo* tumescence and detumescence, we determine that alpacas have a clitoris spanning the vulvar commissure and caudal vagina. Extensible erectile tissue of corpus cavernosum and spongy tissue studded with smooth muscle fibers, nerves, arteries, veins, and lacunae comprise the crura and vestibular bulbs. There are adjacent ischiocavernosus and bulbospongiosus muscles that likely aid in erection. Extensive nerve bundles surrounding the cartilage within the vulvar commissure indicate potential for sensation and relaying information. Further research is warranted for the developmental origin

and composition of the clitoral lamina, evolutionary relevance of cartilage in alpacas, muscle function of the clitoral muscles, and specific mechanisms of relevance of the clitoris in reproductive function.

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Appendix 1 - paraffin histology protocol**Dehydration, clearing, infiltration with BioWave**

Paraffin Tissue Processing (2mm or less)								
Dehydration & Clearing								
Description	User Prompt (on/off)	Time (min)	Power (Watts)	Temp (°C)	Load Cooler (off/auto/on)	Vacuum Bubble Pump	Steady Temp Pump (on/off)	Steady Temp temp (°C)
70% EtOH Dehydration	On	7	100	40	Off	Off	On	40
95% EtOH Dehydration	On	5	100	40	Off	Off	On	40
100% EtOH Dehydration	On	5	100	40	Off	Off	On	40
100% EtOH Dehydration	On	5	100	40	Off	Off	On	40
100% HistoClear	On	10	250	40	Off	Off	On	40
Increase SteadyTemp temperature to 60°C								
100% HistoClear	On	10	650	60	Off	Off	On	60

Remove cassettes from BioWave Pro, begin infiltration in the incubator set to 60°C (or 5-10°C above the melting temperature of the paraplast).

Infiltration	
Description	Time (min)
50% EtOH/50% paraplast	15
100% paraplast	30
100% paraplast	30
100% paraplast	60

Dehydration, clearing, infiltration without BioWave*Dehydration*

Description	Time (hr)
70% EtOH	1

90% EtOH	1
100% EtOH	1
100% EtOH	1
100% EtOH	4/overnight

Clearing

Description	Time (min)
1:1 HistoClear/EtOH	30
100% HistoClear	30
100% HistoClear	30

Infiltration

Description	Time (min)
1:1 HistoClear/Paraffin	15
100% Paraplast	30
100% Paraplast	30
100% Paraplast	60

Embedding

Embed each specimen in paraplast using a Shandon-Thermo HistoCentre 2 embedding center. We use paraplast to embed the specimens. Take a photo and draw a diagram to document the orientation of each section in the embedded block.

Sectioning

Section each paraplast block with Leica RM2135 microtome into slices 6µm or less. Place sections in a water bath at 42°C (or 5-10°C below the melting temperature of the paraplast) to mount on pre-cleaned microscope slides.

Staining*Clearing & hydration*

Description	Time (min)
100% HistoClear	5

100% histoclear	2
100% EtOH	2
95% EtOH	2
70% EtOH	2
50% EtOH	2
35% EtOH	2
H2O	2

Hematoxylin & eosin Y

Description	Time (min)	Recipe
Hematoxylin	20 sec	1:1 Hematoxylin A/Hematoxylin B Hematoxylin A: 95% alcohol, 5% Hematoxylin Hematoxylin B: 4% 29% ferric chloride, 95% H2O, 1% hydrochloric acid
H2O	rinse	
35% EtOH	2	
50% EtOH	2	
70% EtOH	2	
95% EtOH	2	
Eosin Y	2.5	Eosin Y, C.I. 45380 0.5g 95% ethanol 100mL
95% EtOH	2	
100% EtOH	2	
100% EtOH	2	
100% HistoClear	2	
100% HistoClear	5	

Masson's Trichrome

Description	Time (min)	Recipe
Masson A	1.5	0.3g acid fuchsin, 0.7g ponceau de xyloidine, 1.0mL glacial acetic acid, 100mL dH ₂ O
H ₂ O	rinse	
Masson B	1.5	2.50g aqueous phosphomolybdic acid (APA), 250mL dH ₂ O
Masson C	15s	2.5g aniline blue, 100mL, 1.0mL glacial acetic acid
H ₂ O	x2 rinses	
1% Acetic Acid	2.5	1% glacial acetic acid, 99% dH ₂ O
1:1 Acetic Acid/EtOH	30sec	50% 1% acetic acid, 50% 100% EtOH
100% EtOH	2	
100% EtOH	2	
100% HistoClear	5	
100% HistoClear	5	

Verhoeff Van Giesen Elastin Stain

Description	Time	Recipe
Verhoeff's solution	1 hr	20mL 5% alcoholic hematoxylin, 8mL 10% aqueous ferric chloride, 8mL iodine solution
dH ₂ O rinse	x2-3	
2% ferric chloride	1-2 min	
dH ₂ O rinse	x3	
solution D	1 min (discard solution after use)	5% sodium thiosulphate
running tap water	5 min	

Counterstain I	3-5 min	
95% EtOH	2 min	
100% EtOH	2 min	
100% EtOH	2 min	
100% HistoClear I	3 min	
100% HistoClear II	3 min	

Microscopy

Using an Olympus BX51 light microscope, set up Kohler illumination and use brightfield microscopy to image slides under 5x, 10x, and 50x magnification. Captured images using CellSens.

Appendix 2 - diceCT preparation, adapted from Dr. Jaimi A. Gray

For up to two weeks each, dehydrate specimens by placing in solutions of 70% EtOH, 50% EtOH, 30% EtOH.

To stain in iodine, prepare 15% Lugol's iodine and Sorensen's buffer.

- Lugol's iodine: 5g I₂, 10g KI, 100mL dH₂O
- Sorensen's buffer: 37.76g Na₂HPO₄, 1L dH₂O AND 36.20g KH₂PO₄, 1L dH₂O

Mix the two solutions (Lugol's iodine, Sorensen's buffer) to meet target percentage based on specimen size (smaller specimens in 1.25 target %, larger specimens in 3.75 target %).

Target %	Volume of 15% Lugol's	Volume of DI H ₂ O	Volume of buffer	Total volume
1.25	8.33	41.67	50	100
2.50	16.67	33.33	50	100
3.75	25	25	50	100

Table and recipes from Dr. Jaimi Gray.

Soak specimen in solution until appropriately stained. The duration varies for specimen size. If the solution around the specimen becomes pale, refresh. This indicates that the iodine is not fully saturated into the tissue and needs more time to stain.

Place specimen in 30% EtOH for up to 24 hours before CT scan.

Appendix 3 - *in vivo* alpaca observation measurements & demographic data

Alpaca	Age (yrs)	Weight (lbs)
Kahlua	17	162
Amy	8	135
Rianiti		201
Irene	13	156

Table 2. Age and weight of female alpacas.

	Alpaca	VC length (mm)	VC width (mm)	VC thickness (mm)	Clitoral ring diameter (mm)
<i>Before introduction of male</i>	Kahlua	10.6-11.6	15.1-12.1	7.1-4.6	na
	Amy	14.4-17.5	13.1-13.2	9.5-9.2	na
	Rianiti	14.9-16.1	17.6-14.6	19.5-13.2	18.8-18.4
	Irene	20.5-19.6	17.4-15.6	14.0-12.5	16.6
<i>After copulation with male</i>	Kahlua	na	na	na	na
	Amy	na	na	na	na
	Rianiti				24.6-28.0
	Irene				38.4
<i>After copulation with female</i>	Kahlua	na	na	na	na
	Amy	na	na	na	na
	Rianiti				20.0
	Irene				34.0

Table 3. Measurements of the vulvar commissure from four alpacas.