Comparison of Vagina Morphologies between Little skates (*Leucoraja ocellata*) and Winter Skates (*Leucoraja erinacea*)

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ABSTRACT

The reproductive tract of skates has been studied for several decades, but despite the fact that skates have internal fertilization, there has not been any mention of a vaginal structure in this group that could be involved in copulation. For my thesis study, I examined two sister species of skates, Little and Winter skates, to answer several questions: 1): Is there a distinct contrasting structure in the cloaca of the female vs. the male skates to determine that they have a vagina? 2): If so, is there a significant difference in the shape and size of Winter and Little skate vaginas? 3): How does vaginal shape vary according to body size within each species, and Finally, because male skates have intromittent reproductive organs called claspers, we wanted to determine if female Little skates had adapted their vaginal tissue for copulation with the hooked claspers of the male Little skate, and compare these to the Winter skates, where the males have claspers that lack a hook. In order to do this, we examined the vaginal tissue using histology (stains: Masson's Trichrome and H & E). This work highlights the importance of examining female morphology in detail to understand the evolution of genitalia.

INTRODUCTION

Reproduction in vertebrates is a highly studied area, becoming particularly relevant in the modern era due to the decline of many species as a consequence of human impact and the rise of invasive vertebrate species. Studying reproduction is therefore crucial to understanding population growth and ecological compatibility; to figure out why some species thrive and others are wiped out.

However, despite the vast array of literature surrounding reproduction, copulatory organs, especially in females, tend to be ignored. Studies tend to focus on the "lock and key" hypothesis (Dufour 1844), which proposed that genitalia variation coevolved to keep reproduction between species. This hypothesis has led to vast amounts of literature being written about male genitalia, with a study finding that publications focusing on the "lock and key" hypothesis show a high amount of bias to only studying male genitalia (Ah-King 2014). Ah-King found that only a small percentage of the literature focuses solely on female genitalia, and researched the possible explanations behind this gap. Some scientists in the field claim that since vaginas are soft, amorphous structures, the internal female reproductive organs are more difficult to study than the external intromittent male organs. However, Ah-King found that there is a high amount of male bias in researchers, a bias that was found to be evenly distributed across genders of

publication authors. It seems that the centuries of unchallenged assumptions that female genitalia have a lack of variation or unimportance in sexual selection have led to a vast gap in the research; a gap that is fortunately being filled more and more with each passing year (Ah-King 2014).

By studying female genitalia, as well as genital variation, we are able to get a far deeper understanding of what drives sexual selection and the broader evolutionary adaptations that develop. With the large gap in research, and the large amount of assumptions that have gone mostly unchallenged, there have been discoveries in recent years that have revealed that the traditional understandings of vaginas are severely underinformed. Many avian species do not have external male genitalia, and so birds have not been subject to much morphological study of their genitalia. Ducks have long, corkscrew-like phalluses, and although the male genitalia had been studied previously, before Brennan et al 2007, no one had examined the duck vagina to determine how it interacted with the penis. When examined, the duck vagina was found to have a counterclockwise corkscrew formation, with pouches and turns that would make it incredibly difficult for the male penis to fit into. The hypothesis was posited that this was to protect female ducks from being inseminated by a male that they did not wish to mate with, as there tends to be more males than females, leading to violent mating rituals. This work was revolutionary and was a massive piece of counter-evidence to the “lock and key” hypothesis. Most recently, Keeffe et al 2025 examined female Burmese pythons, which had been originally described as not having a vaginal pouch.

However, upon examination of the reproductive aged females dissected in this study, there was a well-defined vaginal pouch that also had large intraspecific variation. Such large errors must be corrected and should not be allowed to stand unchallenged. Orr et al. 2022 researched bat genitalia, and discovered that in species with penile spines, the vaginal wall was thicker. This was theorized to have been an evolutionary advantage to protect the female reproductive system from physical damage during copulation. All of this to show that there is massive amounts of variation in female genitalia, from the presence of a vagina, to the shape, and all the way to the variation in thicknesses of the vaginal walls.

Most vertebrates have a cloaca that serves as the combined opening of the digestive and urogenital tract, with some species, i.e. squamates, having a described vagina or vaginal pouch connecting the cloaca and the oviducts (Sánchez-Martínez et al 2007). The vagina is defined as the terminal end of the reproductive tract that interacts with the male intromittent organ for internal fertilization (Keeffe and Brennan 2023). It is theorized to have evolved from oviduct tissues, and assists in the physical mechanisms of copulation, transporting or rejecting sperm, and can act as a defense organ from infection. The vaginal organ has a vast array of morphological variety across vertebrates, but certain structures are consistent across the groups. There is a mucosal lumen in the innermost layer, a dense layer of vascularized connective tissue, and a thick, longitudinal layer of muscles in the outermost layer of the vaginal wall (Keeffe and Brennan 2023).

While most teleost fishes have external fertilization and therefore lack a vagina, cartilaginous fish have internal fertilization through the use of intromittent organs known as claspers. Claspers are believed to be the oldest intromittent organs, and although they are believed to have evolved from the pelvic fins, there is some controversy around this hypothesis. Fossil evidence was presented in 2014 that dermal bony claspers develop separately from the pelvic fins in gnathostomes, a trait which appeared to be lost, then gained as cartilaginous claspers in stem chondrichthyans (Long et al 2014). However, a study of the molecular development of Little skate males showed that hormonal stimulation of the Hand2 and Shh gene causes the males to undergo a posterior pelvic fin expansion during in vivo development, something that is not present in female skates, giving evidence to the interpretation that claspers do indeed arise from the pelvic fins in Elasmobranchs (O'Shaughnessy et al. 2015). A vagina has recently been described and is known to be present in the spiny dogfish shark. Hedrick et al (2019) found that there was a high statistical amount of vaginal variance within the spiny dogfish shark, again disproving the misconception that there is little to no morphological vaginal variance. Hedrick et al (2019) found that the new 3D automated landmarking technology provided far superior statistical analysis of vaginal structures, and that 2D imaging and analysis of these three-dimensional structures deters significant amounts of data. Using the 3D automated landmarking system, they were able to statistically determine that the vaginas of

spiny dogfish shark change in shape during pregnancy to become more asymmetric.

Little skates (*Leucoraja erinacea*) and Winter skates (*Leucoraja ocellata*) belong to a class of cartilaginous fish called Chondrichthyans, which include sharks, rays, and Chimera (ratfish). Little and Winter skates are sister taxa (Martinez et al. 2016), with a similar ecological niche (the mid to north Atlantic Ocean), a similar body plan, and are phylogenetically very close (Frisk and Miller 2009). Winter skates are much larger than their Little skate counterparts; the former can reach up to 110 cm in body length, and the latter reaches about 57 cm in length (Frisk and Miller 2006).

These two species have reproductive traits that are substantially different (Frisk and Miller 2009). Little skates have much longer claspers (male intromittent organs located in the pelvic fins) than Winter skates relative to body size, as well as much larger testes, despite Little skates being much smaller than Winter skates. In addition, Little skates have two hooks on the end of their claspers that are not present in male Winter skates, suggesting that male Little skates hook themselves to the vagina during copulation, while Winter skates do not appear to have that capability (Figure 2). These male traits suggest that Little skates may mate with multiple males more often than Winter skates, so more intense competition may be driving copulatory traits in this species (Frisk and Miller 2009).

Little and Winter skates, like all other chondrichthyans, have a cloaca: a single opening where both the end of the digestive tract opens into the coprodeum compartment, and the connection to the reproductive tract and urinary ducts are located in the urodeum. These skates are oviparous, meaning they lay egg cases and do not give birth to live young. Females have paired oviducts with oocytes that produce egg cases, which are later laid through the cloaca; they can have up to two egg cases, one in each oviduct, at a time (Figure 1) (Musick and Ellis 2011). During copulation, males insert their claspers in the female cloacal opening and deposit sperm internally (Musick and Ellis 2011). An anatomical novel on Elasmobranchs (Third Edition 1934) by J. F. Daniel describes an observed thickening in the "uterus" of elasmobranch females when the males have a hook on their clasper (pp. 309). Both can reproduce all year round, although it seems that Little skates have several-month periods in the Fall and Spring where they are more frequently pregnant, and Winter skates have a period of higher egg laying during the summer (Kittle 2025). Little skates become reproductive around age three, or when they reach 32 cm in length, and live to around 12 years, while Winter skates become reproductive at around 11 years and live to about 20 years. Little skates lay approximately 10-35 egg cases in a year, and Winter skates lay around 40 egg cases a year. Little skate egg cases gestate for approximately 5-6 months, whereas Winter skate egg cases gestate for up to 18 months (Bester 2025).

While there is a large quantity of literature surrounding the study of the reproductive habits of skates, including Little and Winter skates, there is an absence of any mention of a vaginal structure. Martinez et al. 2016 in a study of sexual dimorphism in these two taxa refers to measurements of this structure as “cloacal depth” or “cloaca” for their examinations of the female skate, with no description of how this “cloacal depth” differs from the male cloaca, only that it exists. The Kardong Comparative Vertebrate Anatomy textbook and laboratory guide, a staple in many anatomy classrooms- including my own- also fails to mention this vaginal structure in Chondrichthyans. There is mention of the oviduct, where sperm and fertilized eggs are transferred through to the shell gland, where the egg case is secreted and formed around the embryo, and then the uterus, a larger structure towards the end of the oviduct where either embryos or egg cases are held until oviposition, and the cloaca. We find no mention of this structure in any of the text or numerous diagrams in the Urogenital chapters (Kardong 2015, pp. 175-176). Musick and Ellis 2011, another in-depth piece of literature on the reproduction of Chondrichthyans, also discusses the morphology of the uterus and oviducts, with no discussion of the area connecting the uterus and the cloacal entrance. There is no consistent agreement for the terminology of this structure amongst publications, with terms like cloaca, urogenital sinus, and even cervix being used interchangeably (Somsap et al 2021 & citations therein). The term “vagina” has been used for other chondrichthyans in published papers

(Siqueira-Souza et. al 2024) (Dodd 1983); but no reference to a vagina in skate literature was found.

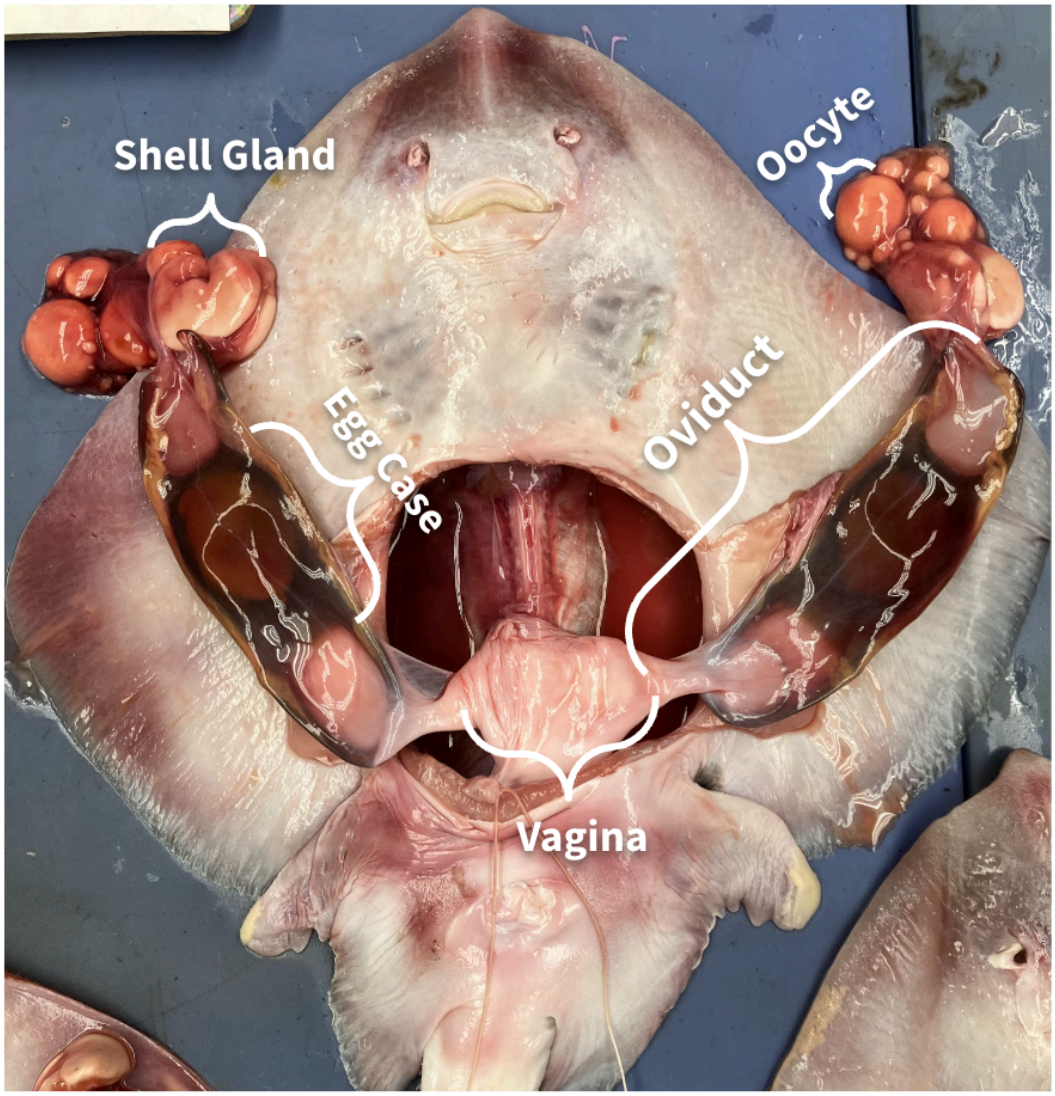


Figure 1. Female Little Skate with reproductive tract dissected out and developing egg cases still in the oviducts.

A.  B. 

Figure 2. A. Open Little skate clasper with two visible hooks; B. Open Winter skate clasper with no hooks present.

Previous work in the Brennan lab had identified the presence of a vaginal structure at the terminal end of the oviducts and before the opening of the cloaca connecting both structures in Little and Winter skates (Schleining et al 2019). This structure is where the claspers are inserted and sperm is released, and is where the eggs pass through the oviduct to eventually be laid by the female. This structure appears to function as a vagina because it directly interacts with the male intromittent organ. Thus, I will proceed in the rest of this thesis referring to this structure as a vagina, even as we seek to describe it and differentiate it from the male cloaca. Therefore, the first question I will study in this thesis is whether there is a vagina in the Little and Winter skate that is distinct from the cloaca, by

examining the cloaca of males and females and determining how they differ. If there is indeed a vagina, we will use the same framework that is used by the Brennan lab to examine morphological variation of genital shape addressing questions related to shape and size variation. Specifically, we will answer the following questions:

1) If there is a vagina in the Little and Winter skates, do they have significant differences in shape?

2) Does vaginal shape vary according to body size within each species, so that larger females have different shaped vaginas?

3) What is the pattern of allometry of the vagina of the Little and Winter skates?

Previous research in the Brennan lab had described the existence of a thick pad present in the dorsal aspect of the vagina in both skate species. Histological examination revealed that the vaginal wall was constructed of a layer of stratified epithelium, with a disorganized layer of collagen and loose connective tissue bundles below. In addition, the pad of the Little skate had very dense connective tissue that was less dense in the Winter skate. They hypothesized therefore that a denser vaginal pad tissue in Little skates provides some protection to the female tissue if the hook of the Little skate clasper attaches to it (Schleining et al 2019). Here we wanted to determine if we could replicate these results, comparing the

density of collagen in the vaginal wall, as well as the thickness of different layers of the vagina across species and regions of the vagina. Recent work on bats (Orr et al 2023) has shown that the thickness of the vaginal wall was significantly associated with the appearance of penile spines, although collagen percentage and the presence of penile spines were not found to be associated. This paper posits the theory that the thickness of the vaginal wall was a reaction to puncture or scraping trauma from the penile spines to help recuperate costs of copulation. With the Little skate males having an additional hook on their claspers and the previous histology work appearing to have similar variation in vaginal thickness to the bat study, we wanted to test if our examinations aligns with this study, at least with an initial non-quantitative probe.

We examined differences between the vaginas of juveniles and adults of each species to see if there are ontogenetic differences that may reveal functionality; for example, if some features are present in the adults but not in the juveniles, that would suggest that the changing features have a role in reproduction.

We lastly examined the histology slides to study and measure the mucosa, lamina propria, and muscularis layers. These layers are present in a particular fashion in vaginal tissue, so finding and examining these layers are essential for determining if the structure we are looking at is, indeed, a vagina. The mucosa layer is the first part of the squamous epithelial layer, and helps to keep the vagina moist to protect it from chafing and disease (Cui et al. 2022). The lamina propria

is a dense layer below the mucosa made up of elastic fibers and blood vessels; it also has a lymphatic supply, as one of the important functions of the lamina propria is to help prevent infection (De Landsheere et al. 2013). The last layer is the muscularis, which has smooth muscle bundles contained within connective tissue like collagen, and functions to add strength and stability to the contractions needed for the vaginal walls (De Landsheere et al. 2013).

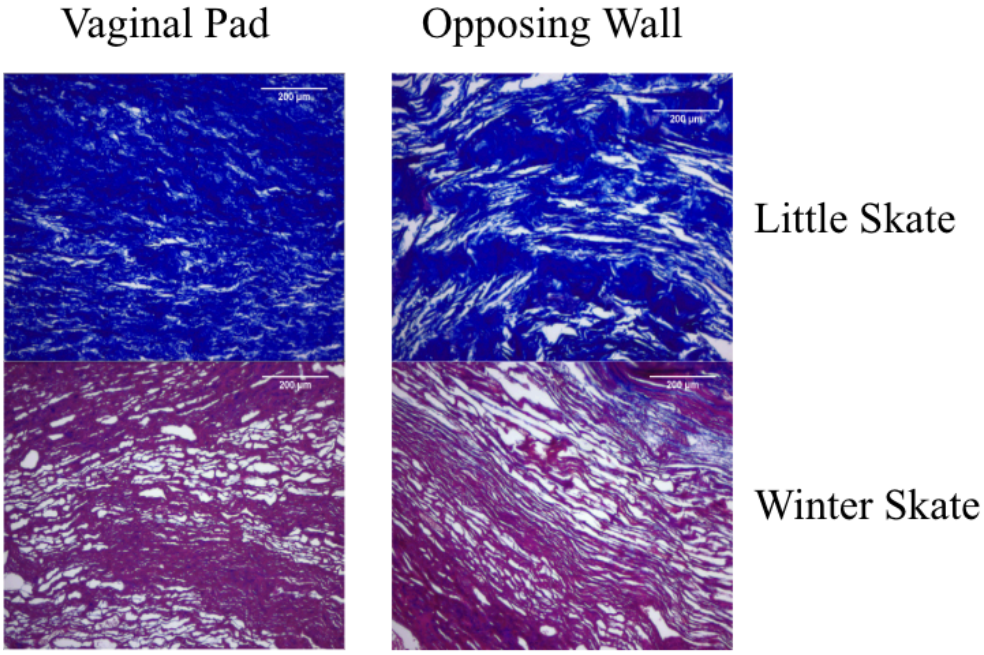


Figure 3. Masson's trichrome stained histology slide comparison of the vaginal pad and opposing vaginal wall in Little and Winter Skates from 2019.

METHODS AND MATERIALS

Dissection

Members of the Brennan lab and I dissected 21 male and 27 female Little Skates, and 29 male and 31 female Winter Skates, for a total of 108 specimens altogether. Many of these specimens were dissected throughout the 2024 summer session by the Brennan Lab, and some have been dissected over varying years from 2018 to 2024. I finished the dissections of the last 29 skates with the assistance of Dr. Brennan and members of the Brennan lab from early September to early October.

Our specimens were provided by the Industry-Based Biological Sampling program run by NOAA Fisheries (sampling request “program_code 34”, Woods Hole Field Office) and arrived frozen. Before dissection, specimens were thawed in water until sufficiently defrosted. Once this was achieved, all skates had various bodily measurements taken, including measurements of the SVL (Snout Ventral Length, measured from the cloacal opening to the most cranial part of the skate), the total length (TL), the length of the vagina for female specimens (Table 9), and measurements of the length of the claspers for male specimens. Other measurements from the pelvic fins, oviducts, and testes were taken for possible

future research beyond this thesis. For this project, both females and males were opened abdominally, and all other organs besides reproductive organs were removed and discarded.

The intestines in both sexes were tied off as close to the cloaca as possible, and dental silicone was used to create an impression of both male cloacas and the female vaginas, to determine if males and females had different cloacal structures (see Endocasts). After the silicone models were made, the pelvic fins of both sexes were removed with the pelvic girdle, with the claspers and pelvic fins either removed separately or kept together, depending on the period collected, and frozen for future projects. The pelvic girdle was removed in a way that left the vagina behind in the females to be able to measure the oviducts, which were either cut off and preserved for other researchers, or discarded. The vaginas were either frozen or preserved in 10% formalin and set aside in vials for histology. While the testes were measured, they were not collected and instead discarded. The rest of the body was placed in a biohazard bag and discarded along with the unused internal organs according to laboratory safety protocol.

Of the 108 skate specimens processed by the lab, 17 skates were determined to be non-reproductive juveniles based on lack of oocytes and size of the vaginas: 2 female Little Skates, and 15 Winter skates (8 male and 7 female). The 91 remaining specimens were determined to be of reproductive age by looking at the development of oocytes in the females.

Endocasts

We made endocasts of both male and female cloacas, using dental silicone, which were set aside for 3D scanning for the morphological analysis. The silicone used was Zhermack Dental elite HD+ vinylpolysiloxane (a-silicone) that was inserted into the cloacal opening by using a silicone gun with a plastic tip. The vagina needed to be cleaned out of any mucus with a paper towel to make sure the silicone modeled the vagina itself without interference from the mucus. The entire vaginal structure was filled with silicone, with some overflow left outside the cloaca to confirm that the entire structure was filled and to make removal of the mold easier (Figure 3). This process did not typically create molds of the oviducts, as they tended to be closed.

For the Little skates, usually less than one canister of silicone was needed; however, with some of the larger Winter skate females, more than one canister was needed. The silicone only took a few minutes to cure, and about two molds were made per specimen, where they were rinsed off of any remaining mucus and placed in a Ziplock bag along with the identifying tag.



Figure 4. Little skates with dissected-out oviducts and curing silicone.

Computational 3D modeling

To be able to take calculations and quantitative results from the models made, we scanned the silicon models with the Einscan Pro Laser Scanner (Version 3.7.3.0) in fixed scan mode using a rotating table. The silicone model was propped up by a needle stuck into the overflow portion of the model in a Play-Doh™ base, and placed on the rotating table in front of the laser scanner. Once the 3D scan was made, a Mesh Model was made, and all holes were

auto-filled by the software. All endocasts were scanned in the same way and saved as an .obj file.

These .obj files were uploaded to MeshMaker (Autodesk Version 3.5.474) software, where the base of these 3D models was edited using the plane cut to achieve a standardized base, and to remove any of the leftover intestine sections that were sometimes cast along with the vagina. To do so, I would find the anterior ventral point of the intestine overflow, and the posterior dorsal point of the cloacal opening where the silicone overflow began, making an angular cut that removed any leftover intestine and silicone overflow, and this process was repeated as the standard procedure for each model. After all edits were done, the model was converted to a solid projection. These finalized edits were saved to an .obj file that was transferred to a computer that ran automated landmarking software (Auto3DGM) to quantify the shape variation in the vagina (Boyer, Moore 1977).

Geometric Morphospace Computations

Auto3DGM, or Auto 3-Dimensional Geometric Morphometrics, is an automated landmarking software that places thousands of semi-landmarks on a set of digital 3D models so that statistical calculations can be made from the comparison of shapes. It sets points on each model to allow the computer to read the shape, find alignments in the landmarks, and have a numeric

three-dimensional point for each landmark. We used 200 landmarks for the first alignment and 3000 landmarks for the final alignment in each analysis.

Traditional geometrics uses a two-dimensional model to place landmarks on a 3D object; however, the amount of variation in vaginas and other reproductive structures as well as the full three-dimensionality of these organs makes it incredibly difficult to get the full variation in these tissues using a 2D model. By using the automated 3D landmarking, we can get the entirety of the variation of these structures without the need to flatten the structure. This leads to higher accuracy, as well as a more in-depth examination of the genitalia (Hendrick 2019).

After running the dataset, the software produces an alignment visualization that can be rotated to ensure accuracy, a .csv file of the unscaled landmarks that automatically organizes the landmarks in a spreadsheet by specimen ID and the axis of the three dimensional plot each landmark fits, and a morphologika .txt file, which includes the raw data of every single landmark on every single specimen. This file is then used in the statistical analyses (see Geometric Morphometrics).

There is occasionally an issue with the Auto3DGM software where, instead of accurately aligning the 3D models in the same position, the software will “flip” the model upside down. This issue arose with several of the Winter skate models that were wider at the base than at the tip, so to counteract this issue, the models were edited in Blender (Version 3.5.1), where asymmetric crosses

were placed on the bottom of each specimen to force the same alignment (Figure 5). These crosses allow for definitive positioning to be determined by the program, and fix the flipping issue.

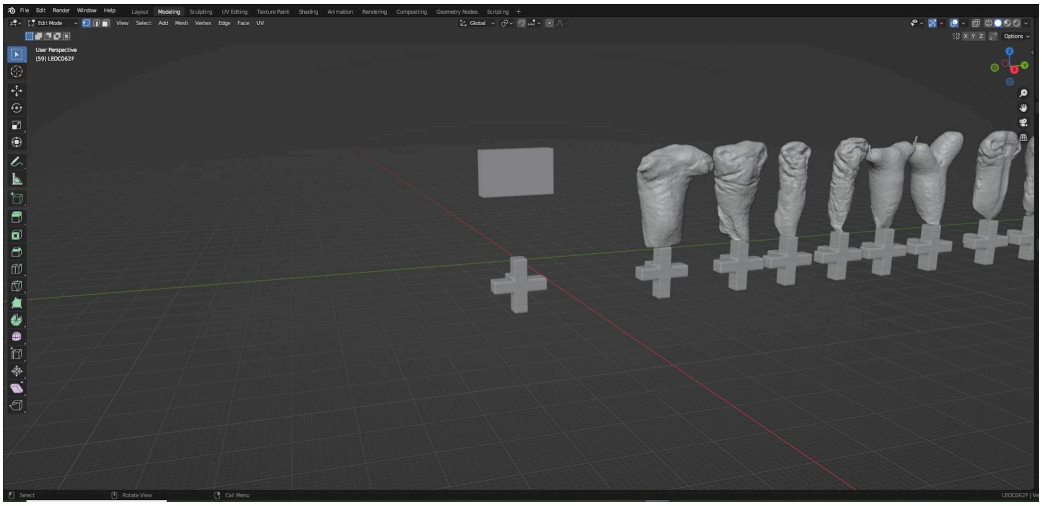


Figure 5. Blender window of the template for crosses placed on specimens.

We ran three separate alignments with our 3D models: The first included all females of both species to check for differences between species. The second was solely the females from the Little skates (Figure 6), and the third was solely the females from the Winter skates (Figure 7).

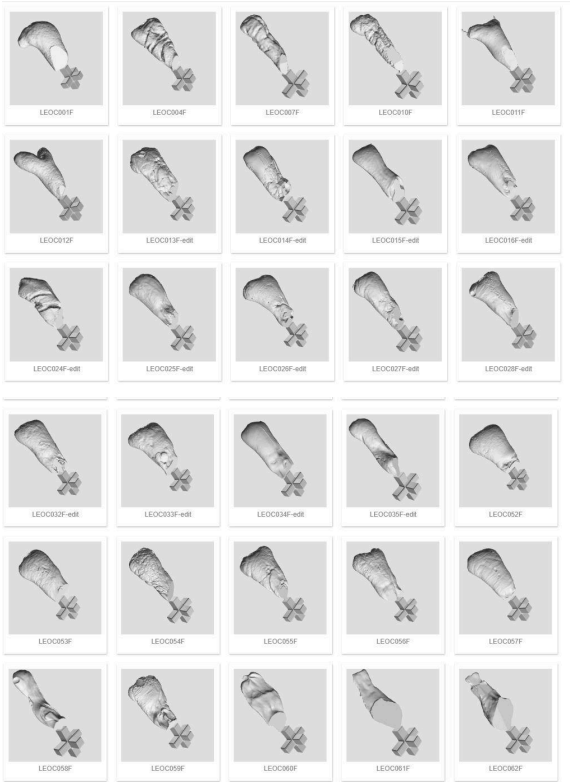


Figure 6. Auto3DGM projected models of Winter skate vaginas

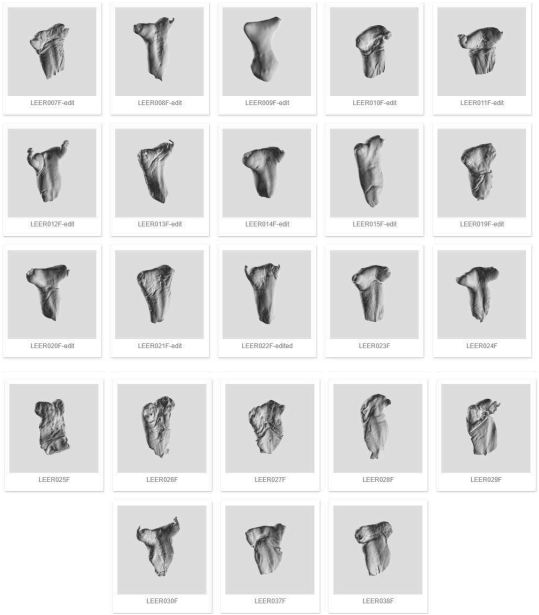


Figure 7. Auto3DGM projected models of Little skate vaginas

Geometric Morphometrics

To quantify shape variation in the vagina, the landmark data and a .csv file that included the specimen ID, the species, the TL (Total Length), and the volume of the vagina (calculated in Blender from the 3D models) was uploaded into R Studio Version 2024.12.1+563. Packages *Geomorph*, *Ape*, *Phytools*, *geiger*, *calibrate*, *dplyr*, *Morpho*, and *nlme* were used to run the script.

To best analyse the shape data, the geomorph package was used to run a Generalized Procrustes Analysis (GPA) in order to do a Principal Component Analysis (PCA). The GPA is named after the Greek myth of Procrustes as it takes the shape data, superpositions it, and stretches it so that all the vagina models are all of equal size, while maintaining the proportions of the 3D models. This allows for variation to be examined and removes any confounding variables caused by size. Size is then separately measured using the calculated volume (extracted from Blender) so that this variable can be added back in when needed comparisons are run.

The Principal Component Analysis (PCA) takes this GPA and pulls its coordinates in order to measure the distance between the 3,000 landmarks of the Auto3DGM analysis and calculates the variation between each distance of every landmark in every specimen. Through this analysis, it will calculate the largest distance between differing points on different models and add each run to a table. The PCA organizes them from most important to least important, with Principal Component 1 indicating the highest proportion of variance that is explained by

that PC, and the last numeric PC number having the least significant proportion of variance. Typically, only components 1 or 2 will have a proportion of variance that is biologically relevant (explaining over 10% of the variance); this is what is used to plot the PCA figures. From this information, we can take the most significant PC values and generate a visual point cloud model that shows the extreme shape variation in the negative plot and the positive plot for each of our separate analyses.

The Procrustes data is then used to run an Analysis of Variance (ANOVA) of the data, which allows us to run a statistical comparison to see if the difference between two groups is more than that of the distance between the individuals in the group when considering multiple independent variables. These ANOVAs will produce a p-value that determines if the data are statistically significant. We ran an ANOVA for the combined data, with species as the independent variable and vaginal shape as the dependent variable, to find if the variation in the shape of the vagina is species specific. Three different ANOVAs were run for only the Little skate data and only the Winter skate data. The first ANOVA had the total length as the independent variable and the vaginal shape as the dependent variable, in order to find if the size of the animal affected the variation in shape. The second ANOVA had the total length as the independent variable and the vaginal volume as the dependent variable to see if the size of the animal affected the volume of the vagina. The last ANOVA had the vaginal volume as the independent variable and the vaginal shape as the dependent variable, to see if the size of the vagina

affected the variation in the vaginal shape. While the latter three were done for just the Little data and just the Winter skate data, these variables cannot be used for the combined data. This is due to the Winter skate species having a much larger overall size than the Little skate, meaning that any data that could be gained from the combined species would be unclear due to the size difference.

Usually, SVL is used for comparison of the data collected from Auto3DGM, but there were six specimens that did not have recorded SVL data. Instead, Total Length (TL) needed to be substituted, as there were only two specimens with a missing TL. SVL is used most often as it tends to be a more accurate assessment, especially in certain animal specimens where the tails arrive either broken, missing, or otherwise damaged. However, most, if not all, of the skate specimens had intact tails, so using TL was determined to be a satisfactory substitute for SVL in our data.

The last of the morphometrics data that I examined was the allometry of the total length and vaginal volume for each species, which is performed as a regression. Allometry is a specific type of statistical integration used for developmental biology. It tests if the occurrence of anatomical covariance in body parts is affected by overall developmental size factors (Hallgrímsson et al. 2019). The specific type of allometry that I used for this study was static allometry, which tests for the covariation of shape and size of individuals of the same age category, adults in this case. This is why I used the total length variable, as this represents the overall body size of the animal, and the vaginal volume variable, as

this represents the size of the vagina. This test will determine if the size of the vagina appears to be in proportion to the overall size of the animal. To measure this, I ran a General Length Squares (GLS) and obtained confidence intervals to determine the slope of the regression. Usually, a confidence interval is used for a value of 1 for one-dimensional space, but due to us using volume rather than a linear measurement, the confidence interval is determined by a factor of 3. This type of test was only used for the individual species data.

Histology

After dissection, frozen vaginas were thawed and fixed in 10% formalin for a minimum of 48 hours before sectioning would occur. Nine vaginas (6 Winter skate and 3 Little skates) were cut along the sagittal and transverse planes, with transverse being labeled alphabetically and sagittal being labeled numerically. Once sectioned out, the samples were placed into plastic cassettes and held in ethanol until the dehydration procedure. The tissue needs to be dehydrated first for the cells to be able to be preserved in paraffin without damage. This was done according to protocol (Table 10-13) with a series of increasingly concentrated ethanol solutions, cleared with histoclear, infiltrated with paraffin, and fixed into wax blocks. These blocks were then sliced with a microtome (5-7μ), mounted onto glass slides, and stained with Masson's trichrome, hematoxylin (H&E), to view muscle cells, nuclei, and collagen arrangement. Masson's trichrome is a stain used to differentiate between

musculature and connective tissue; muscle cells stain red or pink, and connective tissue stains dark blue. H&E, or hematoxylin and eosin, is used to differentiate cells from disorganized protein structures; it stains nuclei a dark purple, and cytoplasm and other tissues stain a lighter pink.

Some existing histology slides were created in 2018 by Brennan Lab members Schleinig P. E. and Tanner L. V. Later slides and paraffin blocks were created in 2023 and in the summer of 2024 by Maeesha Tasnim Naomi. The existing histology slides were created from four Winter skate and two Little skate specimens, with each having a range of 10- 40 histology slides per specimen, with paraffin blocks in storage that I used to make additional slides. I also added more slides to the collection in January of 2025 from the specimens prepared in the fall, which include two juvenile Winter skates and an adult Little skate vagina. This equaled about 90 total usable slides, not including discards or slides that came out poorly. All the histological slides are listed in Table 14.

ImageJ Analysis

For the analysis of the histological differences between the two skate species, two adults and one juvenile per species were selected. Slides that were made from the area that contained the vaginal pad and the dorsal and ventral wall were selected.

In order to take pictures of the slides, I used brightfield microscopy. At Mount Holyoke, brightfield microscopy is done on an Olympus BX51

microscope, and photos are edited and processed in OLYMPUS cellSens Standard 4.2.1 software, where the image is saved as a .tiff file with the scale bar burned into the photo.

Table 1. Skate specimens and histological tissues sections used for Brightfield microscopy photos.

	Little Skate			Winter Skate		
	LEER009 F VPC	LEER048 F VP3	LEER008 F 1B	LEOC012 F C	LEOC061 F VAG	LEOC011 F C
Adult		X	X	X		X
Juvenile	X				X	
Top Ventral wall	X	X	X	X	X	X
Mid Ventral wall	X	X	X	X	X	X
Bottom Ventral wall	X	X	X	X	X	X
Top Dorsal wall	X	X	X	X	X	X
Mid Dorsal wall	X	X	X	X	X	X
Ventral Dorsal wall	X	X	X	X	X	X
Image Focus	10x	10x	10x	10x	10x	10x
Massons	X			X	X	X
H&E		X	X	X		X

These photos were then imported to ImageJ (version 1.54g Java 1.8.0_345 (64-bit)), to find the average density of histological tissue samples from the dorsal and ventral vaginal walls to determine if there were obvious differences between species and between ontogenetic stages of the same species. I took a screenshot of the photograph to exclude the scale bar and any white space outside of the tissue being measured, so that only the white space of the image would be measured and not any extraneous objects. This screenshot was uploaded to ImageJ, where the image type was changed from color to 8-bit, which turns the image grayscale for thresholding. The image is adjusted for the threshold, which determines what shades of the grayscale are considered part of the white background. At first, I would adjust the threshold for each individual image; however, after viewing the results, it was clear that repeated measures resulted in differences in the density values. To minimize measurement error, I redid the thresholding with a set threshold of 215 and 255 for all slides to eliminate white space. This method appeared to produce much less variation in the image results, so this was the method that was used for the final thresholding. After the threshold was set, a black and white image with no grayscale was created. The tool Analyze Particles was used to outline the white spaces and calculate the pixel area that each white space represented. The percentage of white space was calculated by using the pixel area of the white space divided by the overall pixel area of the image. This percentage was then inverted to find the tissue density of the image. Unit scales

were not necessary for this conversion, as we were not measuring a specific unit of density, only percentage of coverage.

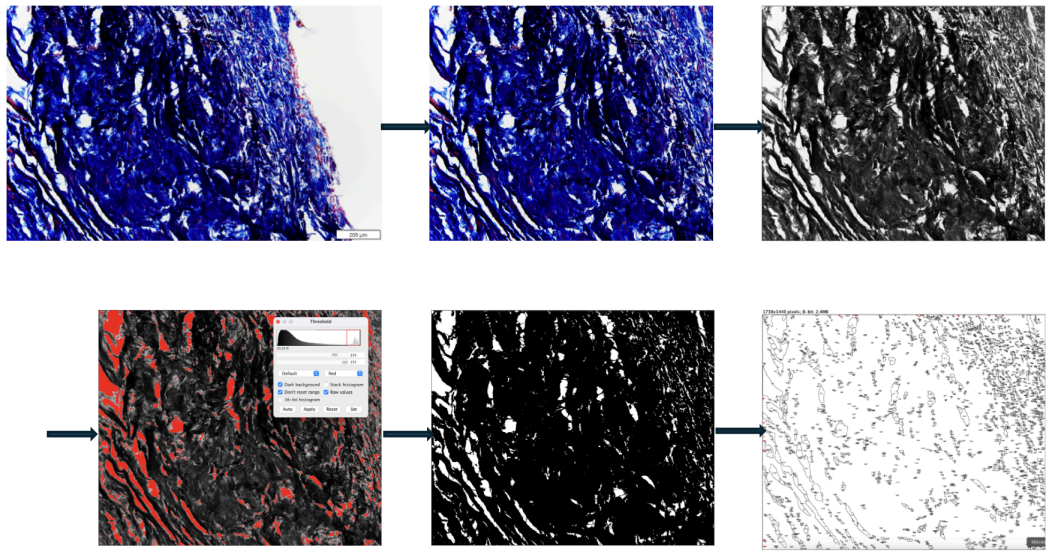


Figure 8. ImageJ photo procedure.

Measurements were taken in ImageJ of the mucosa, lamina propria, and muscularis in the histology photos (Table 16). These layers can be distinguished by their cell structure and position; the mucosa is the proximal most layer to the lumin and has noticeable bubble-like cells; the lamina propria is a layer of connective tissue under the mucosa that is rich in collagen; the muscularis is the deepest layer and contains the highest concentration of muscle bundles (Figure 9). The images were uploaded to ImageJ and the pixel length of the 200 µm scale bar was measured and set in the software. Measurements were taken by drawing a

line and measuring the length of each section of tissue, repeating this for each specimen. One adult and one juvenile from each species was used for this.

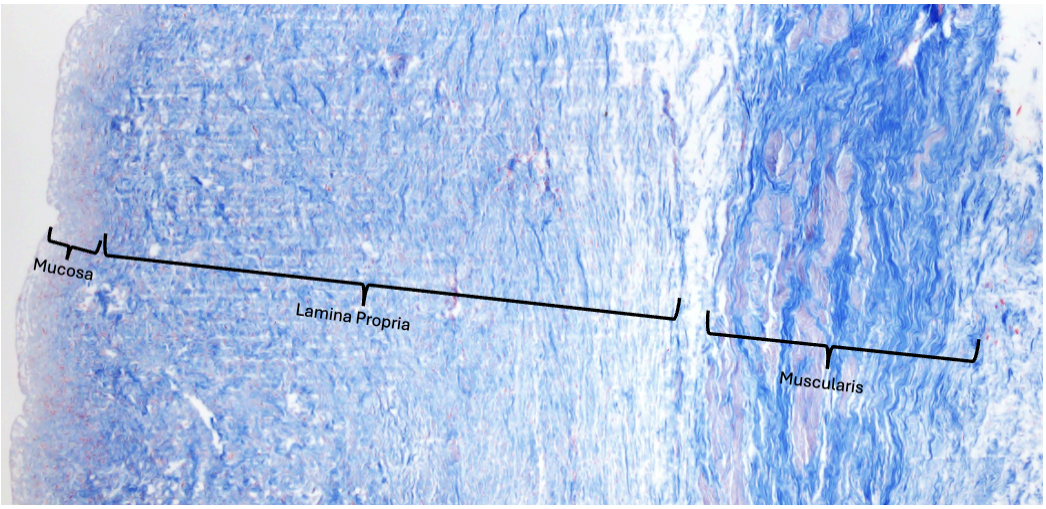


Figure 9. Diagram of measurements made for the mucosa, lamina propria (LP), and muscularis.

RESULTS

Presence of a Vagina in Female Skates

Despite their conspicuous absence from the reproductive literature of skates, we can confirm that skates have a vagina, a structure derived from the cloaca, that is present only in females and not in males. Figure 10 displays what the casting of the male cloaca looks like, showing that males have an incipient structure, but only females have a well differentiated structure that extends beyond the walls of the intestine, and that connects to the oviducts.

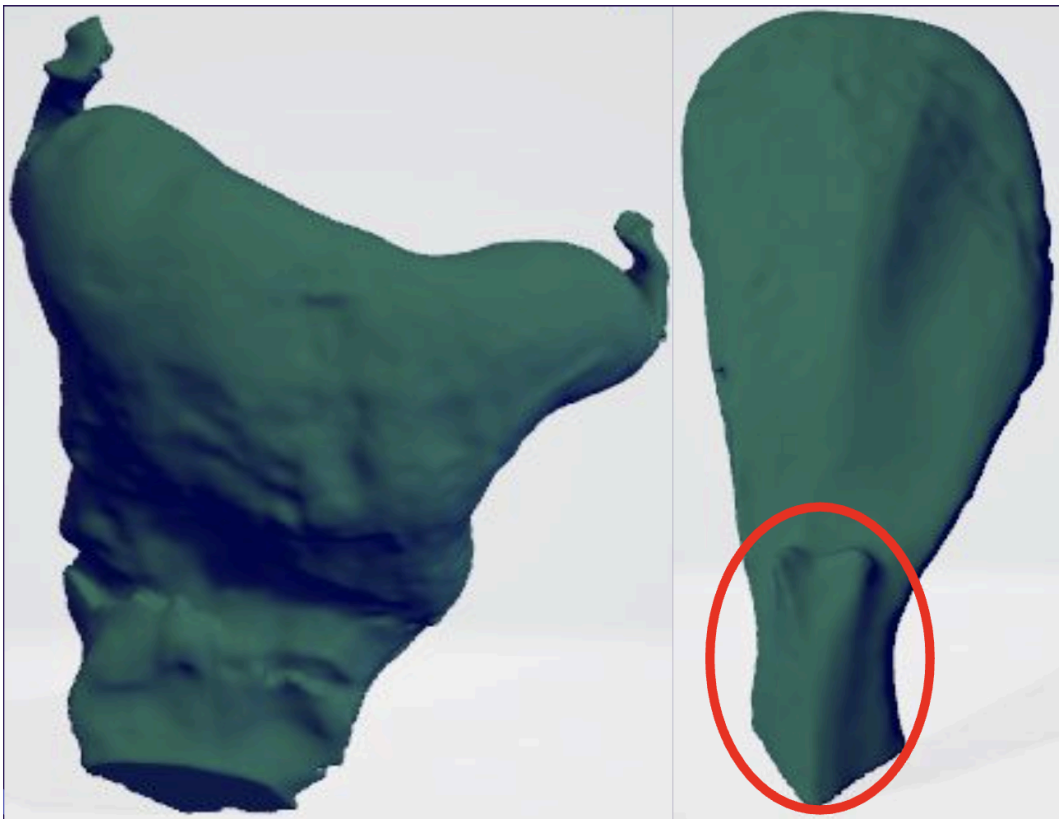


Figure 10. 3D Mesh models of female vaginal mold (Left), and male cloaca/ intestine mold (Right) with incipient structure of the vagina circled in red.

Shape Analysis

Between Species Comparison

We found significant vaginal shape differences between the two species we examined. For the Principal Component Analysis (PCA) for both species data combined, the proportion of variance was 44.9% for Component 1, and 15.8% for Component 2 (Figure 11). Components 3 and above were all less than 10%, and so were not plotted.

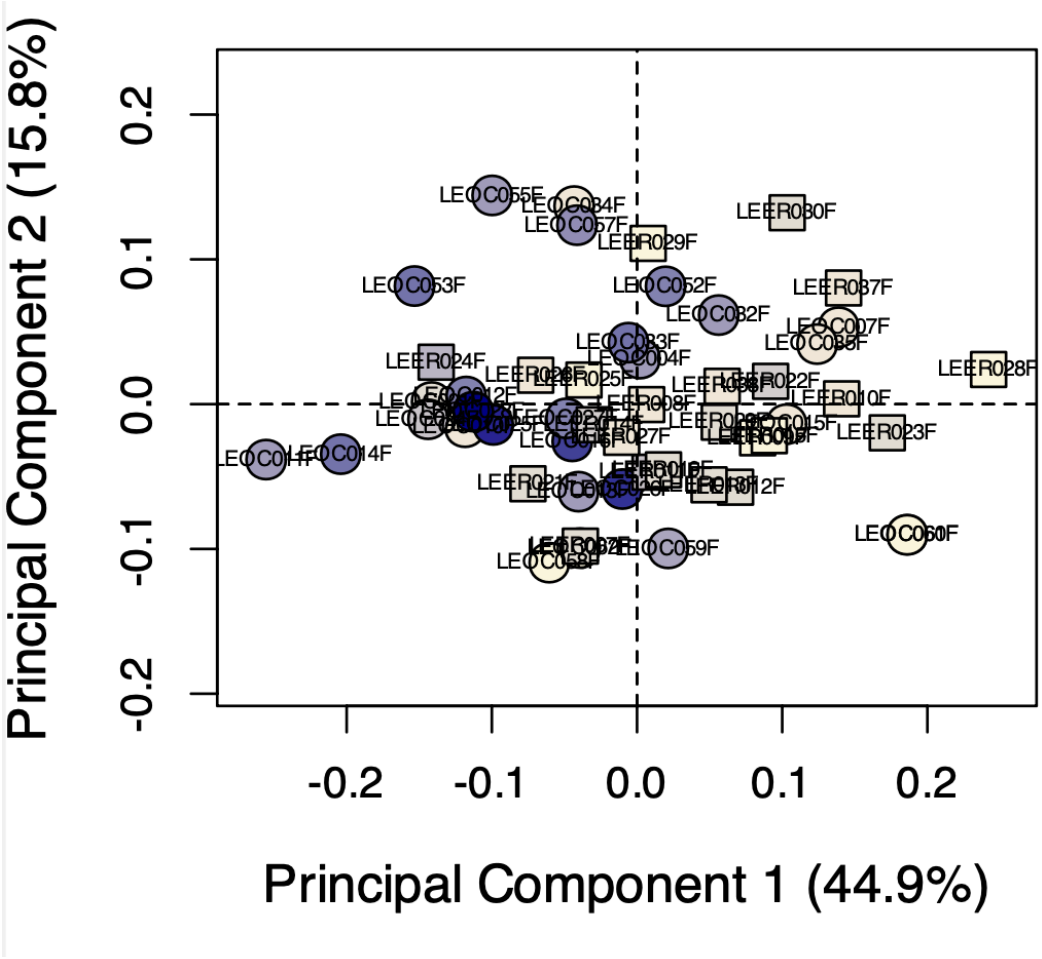


Figure 11. Combined Species Geometric Morphometrics Data plotting Principal Component Analysis 1 and 2 with circles representing Winter skates and squares representing Little skates; dark blue represents larger vaginal shape and yellow represents smaller vaginal shape.

The ANOVA using species as an independent variable was significant ($p = 0.01099$), confirming that the variation in vaginal shape is species-specific (Table 2).

Table 2. Procrustes ANOVA values for Combined Species Geometric Morphometrics Data. Key: Combined: both species; * = p-value < 0.05, ** = p-value < 0.01, *** = p-value < 0.001.

Test	Dependent Variable	Independent Variable	p-Value
ANOVA	Combined Vagina Shape	Combined Species	0.01099*

Figure 12 illustrates the point clouds for the PC1 and PC2 combined species data. The shapes in the positive PC1 are cylindrical, narrow, and longer, whereas the negative PC1 are wider and deeply bilobed. Positive PC1 values are mostly associated with Little skates, whereas negative PC1 values are mostly associated with Winter skates. PC2 showed slightly less variation than PC1, but had similar associations for the Little and Winter skates. The positive PC2 Point cloud is wider than the negative PC2. The negative PC2 point cloud still has a slightly bilobed appearance.

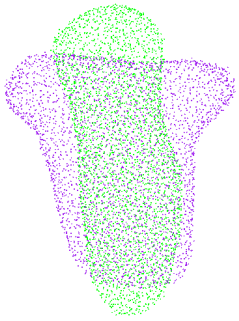
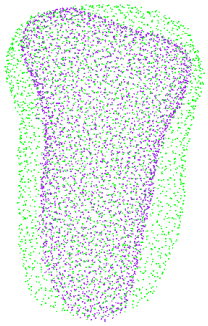
Species	Principle Component 1	Principle Component 2
Combined		

Figure 12. Point clouds of the Combined Species Principle Component Analysis illustrating the shape variation between positive PC1 and PC2 values (green) and negative PC1 and PC2 values (purple) on Figure 11.

Little skates (LEER)

For the Principal Component Analysis for the Little skate data, PC1 explained 45.5% of the variation, and PC2 explained 18.0% (Figure 13). All other PCs were below 10%, and therefore not plotted.

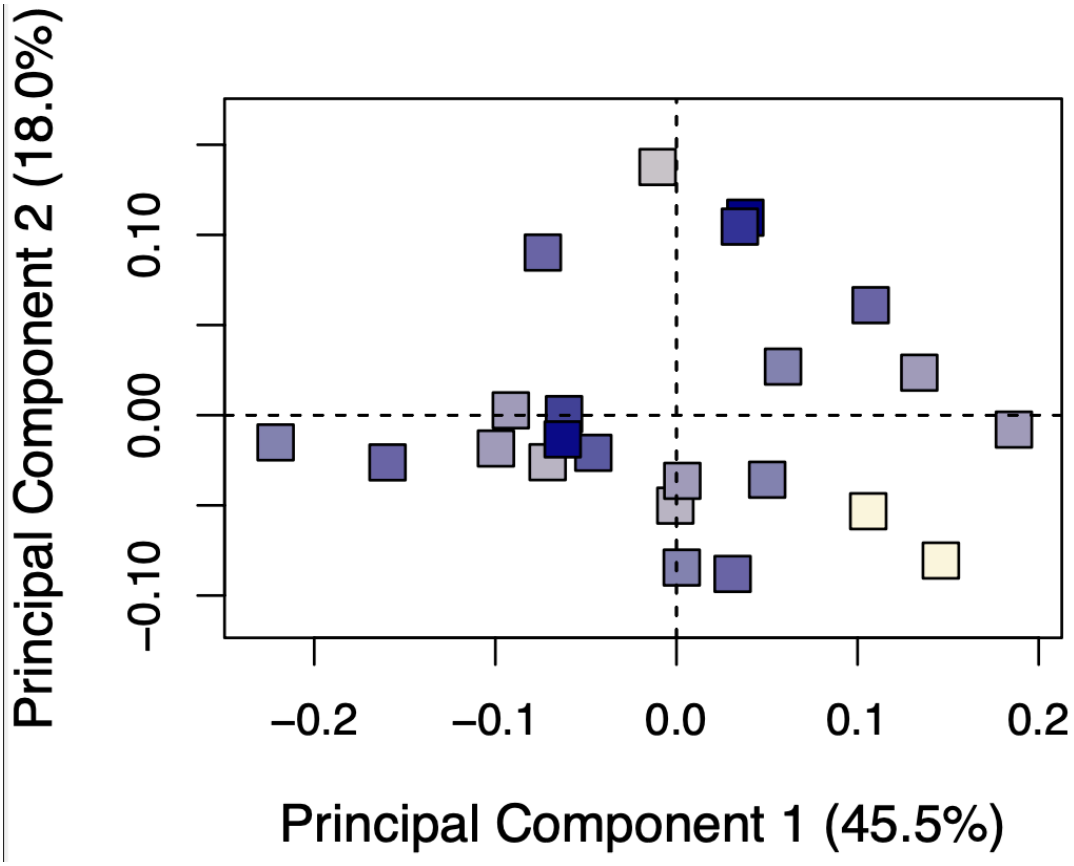


Figure 13. Principal Component plot for the Little skate Geometric Morphometrics Data, with blue representing larger skates and yellow representing smaller skates.

Figure 14 illustrates the point shapes of the PC1 and PC2 for the Little skate data. The shapes in the positive PC1 are rectangularly oblong and longer, whereas the negative PC1 are wider and sharply bilobed. PC2 showed slightly less variation than PC1. The positive PC2 Point cloud has a wider oblong shape than PC1 with some faint bilobed appearance, and seems to be wider than the

negative PC2 apparition. The negative PC2 point cloud still has a slight bilobed appearance, but is more stretched out and has less intense variation in the lobes than PC1.

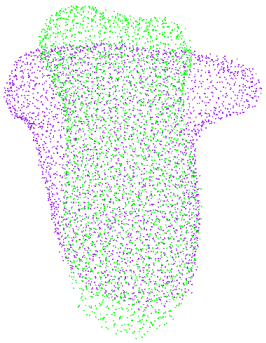
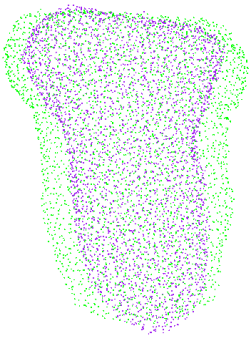
Species	Principle Component 1	Principle Component 2
Little Skate		

Figure 14. Point clouds of the Little skate Principle Component Analysis illustrating the shape variation between positive PC1 and PC2 values (green) and negative PC1 and PC2 values (purple) on Figure 13.

In the Little skates, there was no significant impact of total length on the shape of the vagina, meaning that larger individuals do not have different vaginal shapes than smaller individuals. However, there was a significant impact of total length on the vaginal volume ($p = 0.001998$), meaning that the size of the animal affects the size of the vagina. There was a highly significant impact of the vaginal volume on the vaginal shape ($p = 0.000999$), meaning that larger vaginas have a different shape than smaller vaginas in Little skates (Table 3).

Table 3. Procrustes ANOVA values for Little skate Geometric Morphometrics Data. Key: LEER: Little skate; * = p-value < 0.05, ** = p-value < 0.01, *** = p-value < 0.001.

Test	Dependent Variable	Independent Variable	p-Value
ANOVA	LEER Vagina Shape	LEER Total Length	0.1019
ANOVA	LEER Vagina Volume	LEER Total Length	0.001998**
ANOVA	LEER Vagina Shape	LEER Vagina Volume	0.000999***

Winter skates (LEOC)

For the Principal Component Analysis for the Winter skate data, PC1 explained 53.4% of the variation, and PC2 explained 11.5% (Figure 15). All other PCs were below 10%, and so were not plotted.

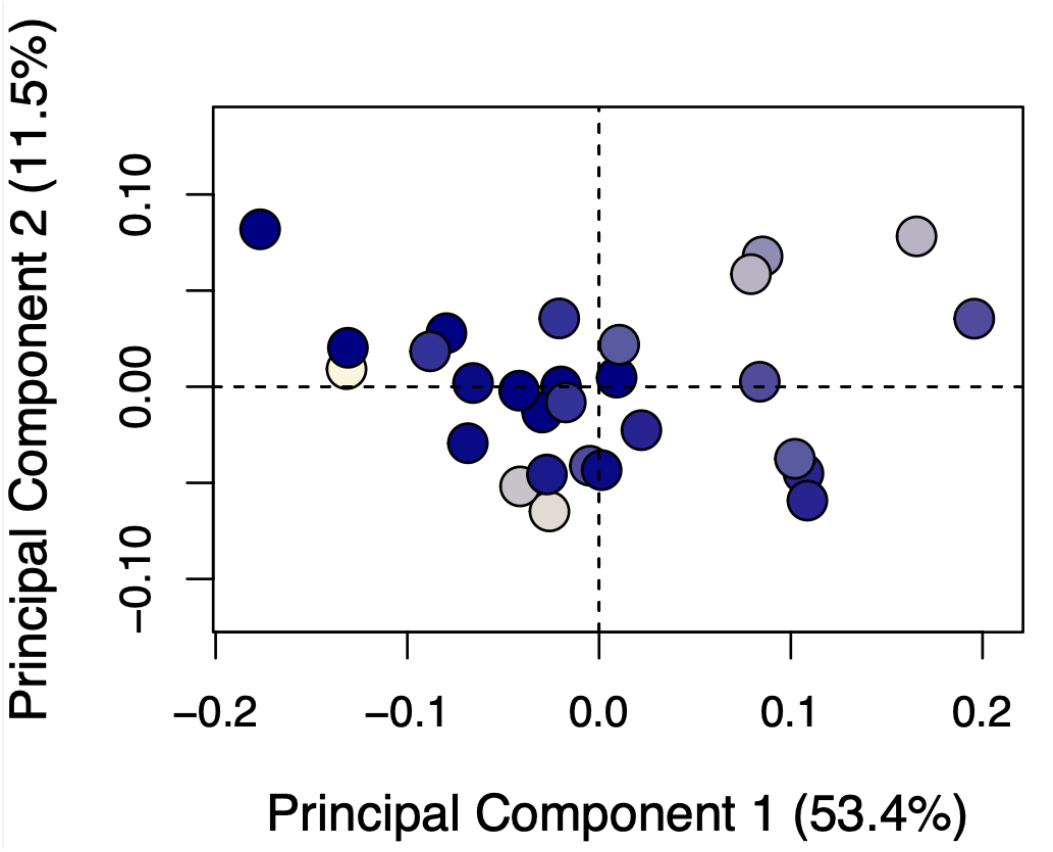


Figure 15. Principal Component plot for Winter skates with larger skates in dark blue and smaller skates in yellow.

Figure 16 illustrates the point shapes of the PC1 and PC2 for the Winter skate data, with the cross we used for alignment visible at the bottom of the point clouds. The shapes in the positive PC1 are cylindrical, narrow, and longer, whereas the negative PC1 are wider and bilobed. The positive PC2 point cloud has a wider, oblong shape. The negative PC2 point cloud still has a slight bilobed appearance.

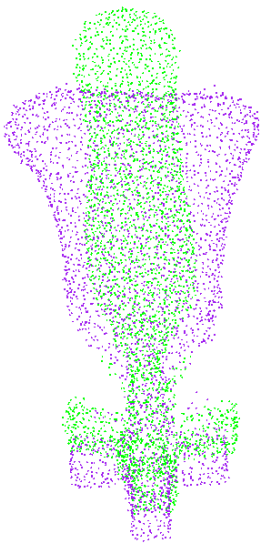
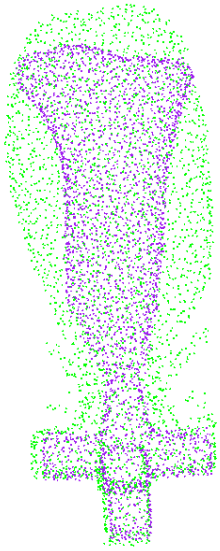
Species	Principle Component 1	Principle Component 2
Winter Skate		

Figure 16. Point clouds of the Winter skate Principle Component Analysis illustrating the shape variation between positive PC1 and PC2 values (green) and negative PC1 and PC2 values (purple) on Figure 15. Crosses are artificial and were created in Blender for Auto3DGM.

The Winter skate ANOVAs were similar to those of the Little skates. In the Winter skates, the ANOVA determined there was no significant impact of total length on the shape of the vagina, meaning that larger individuals do not have different vaginal shapes than smaller individuals. However, there was a significant impact of total length on the vaginal volume ($p = 0.004995$), meaning that the size of the animal affects the size of the vagina. There was a highly significant impact of the vaginal volume on the vaginal shape ($p = 0.000999$), meaning that size is a

determinant in the variance of the shape of the vagina for Winter skates and larger vaginas have a different shape than smaller vaginas (Table 4).

Table 4. Procrustes ANOVA values for Winter skates Geometric Morphometrics Data. Key: LEOC: Winter skate; * = p-value < 0.05, ** = p-value < 0.01, *** = p-value < 0.001

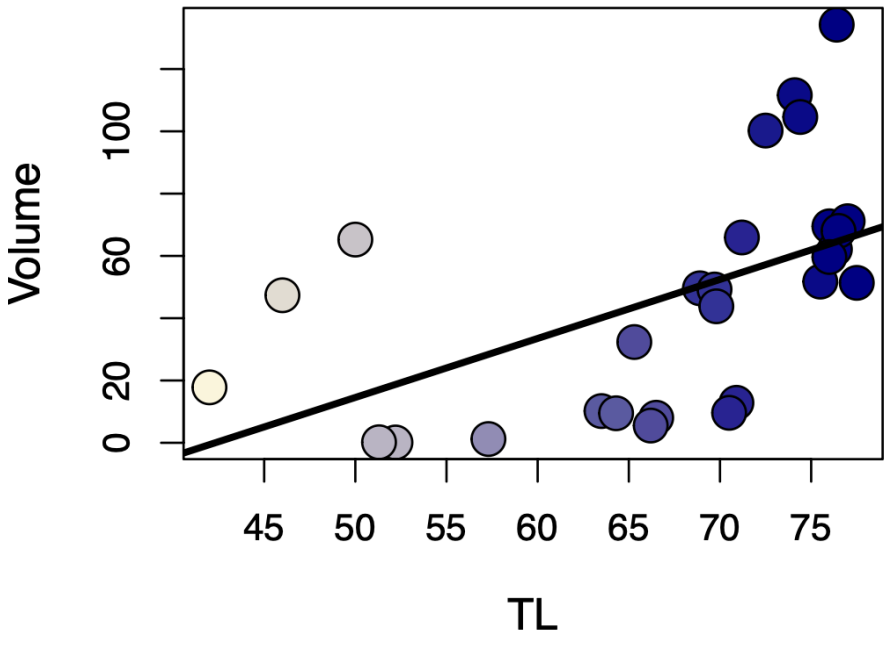
Test	Dependent Variable	Independent Variable	p-Value
ANOVA	LEOC Vagina Shape	LEOC Total Length	0.2218
ANOVA	LEOC Vagina Volume	LEOC Total Length	0.004995**
ANOVA	LEOC Vagina Shape	LEOC Vagina Volume	0.000999***

Allometry

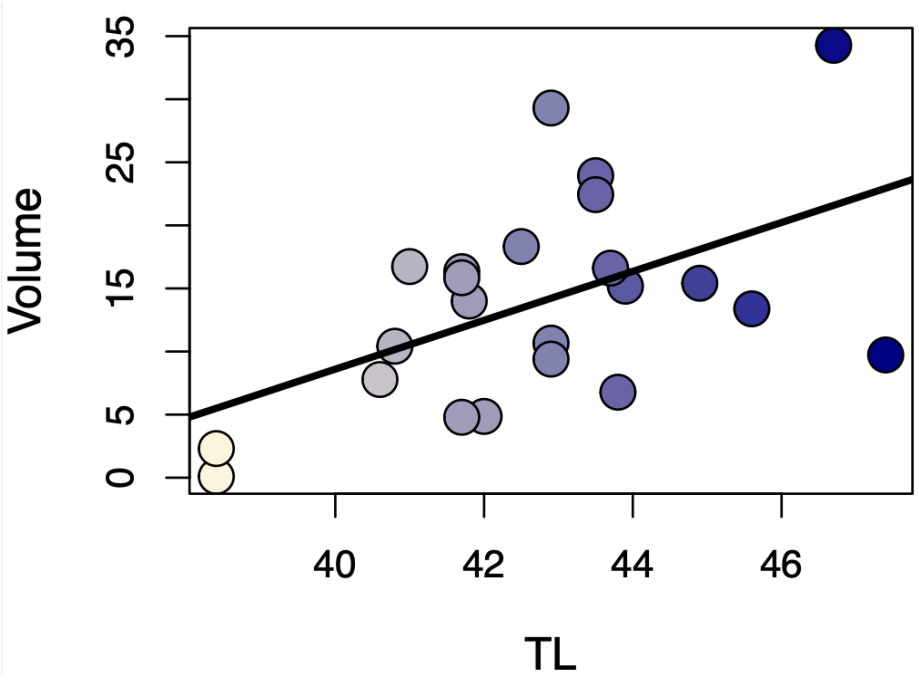
In both the Little and Winter skates, our regression analysis deemed the allometry to be isometric (Table 5) for the increase of total length and volume of the vagina for both Winter and Little skates (Figure 17), so that the vaginal volume increases in proportion to body size.

Table 5. Allometric trajectory confidence intervals.

Species	Confidence Interval	Confidence Interval	Allometry
Little skate	0.5735596	3.301903	Isometric
Winter skate	0.7214535	3.0677029	Isometric



A.



B.

Figure 17. Regression allometry plots for Winter skates (A.) and Little skates (B.).

Gross Dissection

Table 6. Measurements from gross dissection of Little and Winter skate vaginas preserved in 10% formalin.

	Winter Skate Adult		Winter Skate Juvenile		Little Skate Adult		Little Skate Adult	
Secretory structure								
Length (mm)	40.3		14.4		19.4		12.2	
Largest lobe width (mm)	21.8		5.6		7.5		5.7	
Vagina								
Length (mm)	80.0		46.4		46.3		40.0	
Narrowest width (mm)	26.5		13.6		17.0		13.7	
Widest width (mm)	53.3		19.0		44.3		27.6	
Thickness (mm)	11.3		7.7		11.2		10.3	
Vagina Wall								
Wall thickness (mm)	3.0		2.0		3.0		2.5	
Oviduct entrance thickness (mm)	Left	Right	Left	Right	Left	Right	Left	Right
	7.0	7.8	3.0	3.0	5.7	6.2	5.7	5.0
Mid dorsal	0.8		0.8		1.0		1.2	

aspect (mm)				

Following Schlenig et al 2019, we performed a gross dissection of two adult Winter skate vaginas and two Little skate vaginas, one adult and one juvenile. Vaginas were cut along the mid ventral aspect, and then along the mid cranial aspect to open the vagina fully and expose the lumen and the oviduct entrances fully. Here, I describe our general observations.

In both species, we found an odd bladder-like structure cranial and dorsal to the vagina attached to the outside that was full of secretion, that we have temporarily labled a secretory structure. There was a layer of connective tissue around this structure holding it to the vagina that could be removed without puncturing it. There were two lobes that had a thin, non-viscous aqueous substance in the lobes, giving them a balloon-like appearance. The secretion inside the structure could pass between the lobes when slight pressure was applied to one lobe and not the other. There was no outer opening, but upon further examination, we determined that there was a single opening from the structure to the cranial region of the vaginal lumen in between the oviduct openings. In the Winter skates, this opening was underneath the large, transverse folds (Figure 18); however, in the Little skates, the opening was in the mid-dorsal area between the oviducts and was not among any folds. Upon examination of the juvenile vagina, the mucus around the bladder opening was thick and viscous in texture, leading us

to believe that this structure is most likely secreting something, possibly some of the abundant vaginal mucus that we observed in all skate specimens.

In the Winter skate adult, we found some variation in the thickness of the vaginal pad. In the dorsal wall region, it was quite thin to the point of being almost translucent. We found that Winter skate vaginas had visible folds that were latitudinal in the dorsal and ventral folds. However, there were very few folds in the thin dorsal section, and those that did exist were thin. In the cranial region of the vaginas, we found large, thick folds arranged longitudinally, with a concentration of the thick folds around the oviduct openings. There also appeared to be a thickening in the wall surrounding the oviducts (Figure 18).

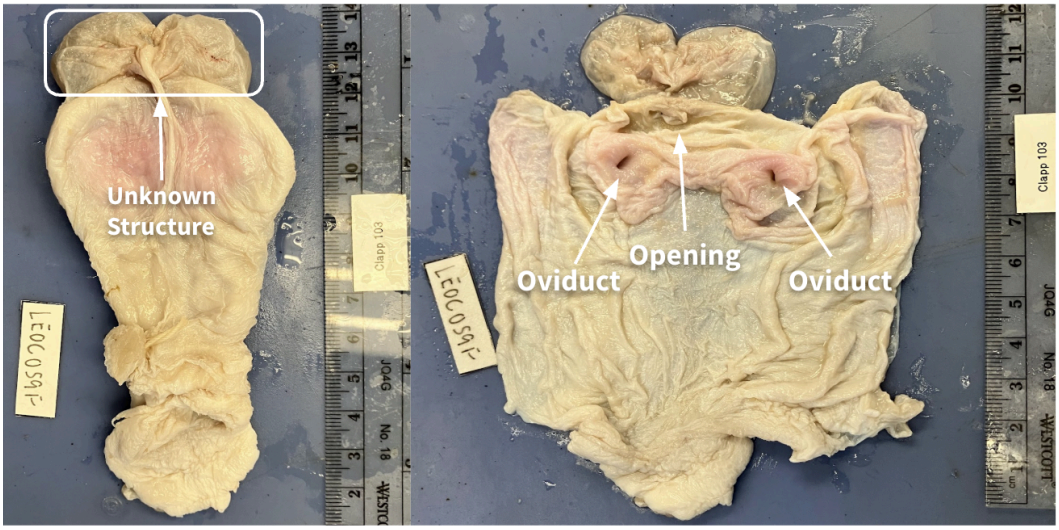


Figure 18. Adult Winter skate vagina pre-dissection (Left) and post-dissection (Right).

This juvenile had fewer folds, with none of the thick cranial folds we observed in the adult. The thickness of the walls felt more uniform than the adult, but they had a similar thin wall in the mid dorsal aspect (Figure 19).

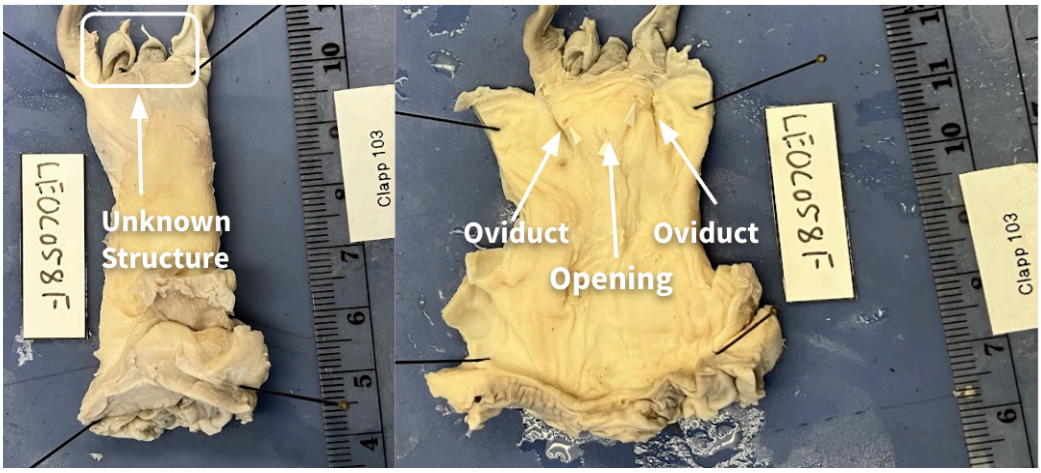


Figure 19. Juvenile Winter skate vagina pre-dissection (Left) and post-dissection (Right).

For the two Little skate specimens, we did see the pad that was described by Schleining et al (2019), which was in the dorsal region for both specimens. There were also areas of thicker tissue in the walls, which felt stiffer and harder compared to the thick tissue in the Winter skate specimens. The Little skate vaginas also had less folds across the internal wall, but still had the transverse folds across the cranial region. There was more uniformity in the overall thickness of the wall compared to the Winter skates, and the walls had a smoother appearance than the Winter skates (Figure 20).

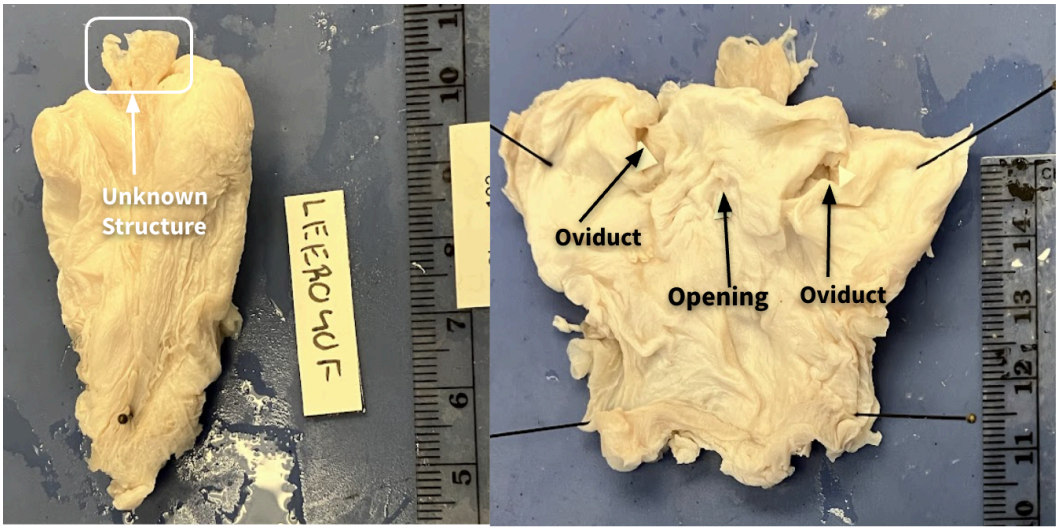


Figure 20. Adult Little skate vagina pre-dissection (Left) and post-dissection (Right).

Histology

Tissue density

Due to the small sample size of histological slides, interpretation of the ImageJ data is not quantitative, but rather descriptive for the purposes of further research; therefore, we cannot state whether these differences are in fact species-specific without more quantification. However, we found generally similar results as those reported by Schleining et al in that the Little Skate appears to have denser collagen in its dorsal vaginal wall. The ImageJ analysis revealed variation in the dorsal wall and the ventral wall of the Little skate vaginas, with the dorsal wall appearing to be slightly denser than the ventral wall. In the mid-ventral wall, the area theorized to contain the pad, the area was incredibly

dense in the Little skate but slightly less dense in the Winter skate vagina. The juveniles appear to have an overall lesser density of the vaginal wall tissues than the adult skates, with the Winter skate juvenile having the least dense tissue of all (Table 7).

Table 7. ImageJ vaginal tissue density calculations for two adult and one juvenile Little and Winter skate specimens.

Adult		Juvenile	
Little skate	Average tissue%	Little skate	Average tissue%
Top Ventral wall	86.4805	Top Ventral wall	88.589
Mid Ventral wall	86.9095	Mid Ventral wall	75.362
Bottom Ventral wall	82.162	Bottom Ventral wall	86.883
Top Dorsal wall	89.887	Top Dorsal wall	81.412
Mid Dorsal wall	99.778	Mid Dorsal wall	91.626
Bottom Dorsal wall	95.885	Bottom Dorsal wall	98.439
Winter skate		Winter skate	
Top Ventral wall	90.429	Top Ventral wall	65.323
Mid Ventral wall	84.015	Mid Ventral wall	52.802
Bottom Ventral wall	87.669	Bottom Ventral wall	49.505
Top Dorsal wall	93.5355	Top Dorsal wall	79.73

Mid Dorsal wall	97.507	Mid Dorsal wall	74.855
Bottom Dorsal wall	95.1945	Bottom Dorsal wall	68.072
Averaged Vaginal Wall Density Values			
Little skate	Average tissue%	Little skate	Average tissue%
Ventral Wall	85.184	Ventral Wall	83.611
Dorsal Wall	95.183	Dorsal Wall	90.492
Winter skate		Winter skate	
Ventral Wall	87.371	Ventral Wall	55.877
Dorsal Wall	95.412	Dorsal Wall	74.219

Wall thickness

The measurements taken from the histology photos seem to show that the lamina propria makes up the thickest portion of the tissue, with the mucosa being the thinnest layer. The Winter skate adult had overall thicker layers; however, when vaginal length is taken into account, the Little skate adult has comparatively thicker vaginal walls. For the juveniles, the Winter skate had a slightly smaller mucosa and lamina propria than the Little skate, but a slightly larger muscularis layer; however, even when vaginal length was taken into account, the Little skate specimen did not have thicker walls than the Winter skate (Table 8).

Table 8. Measurements of histological vaginal wall tissue in μm. Key: LP-Lamina Propria

		Measurements (μm)					
Species	Specimen	Mucosa	LP	Muscularis	Vagina Length (cm)	Wall Thickness (μm)	Wall/ Length
Winter Skate Adult	LEOC011 F C	361.491	883.098	787.645	7.79	2109.71	0.027
Little Skate Adult	LEER048 F VP3	159.431	1004.742	724.914	4.28	3221.509	0.075
Winter Skate Juvenile	LEOC061 F VAG	134.817	1239.076	695.863	1.16	2232.756	0.192
Little Skate Juvenile	LEER009 F VPC	145.727	1251.097	514.898	1.57	1940.967	0.123

DISCUSSION

Skates have a vagina

The endocasts of male and female cloacas revealed that females have a clearly defined and very well developed vagina, and that males have an incipient structure where the vagina develops in females. While some measurements of this structure have been reported previously referring to this structure as part of the cloaca, following a functional definition of a vagina (Keffe and Brennan 2024), it is clear that this structure should be called a vagina since it is the terminal region of the female reproductive tract that interacts with the intromittent organ, is involved in oviposition, and has a defined mucosa, lamina propria, and muscularis. Further works on reproductive biology of skates should refer to this female structure as a vagina and efforts should be made to study how vaginas vary in shape and composition with life history traits such as oviposition and copulation.

Shape analysis

Our shape analysis revealed that there are significant differences in the shape of the vaginas for both Little and Winter skates. If genitalia are species-specific in sister taxa, this generally suggests that these structures are

under selection to perform some function, either during copulation or oviposition. We do not know whether the differences we find here are functional or not. These differences may be associated with the different morphologies of the claspers and with the size of the egg cases of these species, as Winter skates lay larger egg cases than Little skates. Future work on the vagina shape of other skate species will likely reveal that they are also variable, making further study of these structures interesting, and incentivising the study of the association between vaginal shape, copulation mode and oviposition.

The ANOVA confirmed that there is species-specific variation in the vaginal shape, so there is enough variation present between even these two sister taxa to make the vaginas of the two skates uniquely shaped. Since these two species have different reproductive ecologies (Frisk and Miller 2009), future research could examine if the differences in these two skates' reproduction models have any effect on the shape of the vaginas. It could also be posited that this variation in shape could be due to the difference in the claspers, as male Little skates have very large claspers for their body size in comparison to Winter skates, as well as having a hook on their claspers that is not found in the Winter skate males. These findings add to the evidence that female genitalia are widely diverse, and variation should not be assumed even for sister taxa. Keeping the assumptions of little diversity in variation of vaginas closes off massive amounts of possible research into this phenomenon, and studies like this should motivate outside

researchers to continue, or even to start looking at vaginal variation between species to better understand copulation and reproduction.

In Little skates, PC1 and PC2 explained a total of 45.5% and 18.0% of the variance, respectively, while in the Winter skates, PC1 and PC2 explained a total of 53.4% and 11.5% of the variance, respectively. The high PCA percentage variation implies overall variance among the vagina shapes for both species is quite variable, especially between individuals in the same species. The point clouds showed a stark visual contrast in the shape variations, and displayed the wide range of vaginal variations displayed in these skates. This lends further credence to the hypothesis that vaginas are highly morphologically variable organs.

For both Little and Winter skates, the ANOVA found that there was no significant impact of the length of the specimen on the variation in the shape of the vagina. It seems that the shape, while variable, stays within a certain variation despite adult maturation, and that factors other than body size, such as pregnancy or recent oviposition could be influencing vaginal shape. In the future, adding juveniles to the sample for both species may show the effect of maturation on vaginal shape. There was, however, a statistical significance between the size of the vagina and its shape, meaning that larger vaginas have different shapes than smaller vaginas in both species. We theorize that this could be due to possible deformations of the vagina before and after copulation, or before and after oviposition. Further research could be done by creating endocasts from specimens

that have just laid eggs, have never laid eggs, or are pregnant. This could reveal if there are differences in vaginal size during these reproductive moments, and if the variation observed is due to these deformations. Alternatively, some of the shape variation could be nonfunctional, and just the result of random chance. Hedrick et al (2019), reported that in spiny dogfish shark vaginas, about 34% of variation in symmetry of the vagina was due to fluctuating asymmetry. Future work in skates could also evaluate symmetry, especially in Little skates that have lateral lobes in the cranial region of the vagina.

For both the Little and Winter skate individual data, the ANOVA found that there was a statistical significance between the length of the specimen and the volume of the vagina (Little: $p = 0.001998$; Winter: 0.004995). This determined that the size of the vagina is dependent on the size of the specimen, which was important for our allometry examination. For the allometry results, it was not unsurprising that the size of the vagina and the total length were isometric. Recent research has shown that vaginas are typically isometric with respect to body size, meaning that they grow at the same rate as the rest of the body (Keeffe et. al. 2025; Orr et al. 2022).

Gross Morphology and Histology

Examining the endocasts of many of the vaginas, we noticed that there seemed to be a lot of variation in vaginal folds that were present in the models. In

the dissection notes for one of the Little skate specimens, there is a comment about the vagina appearing stretched out, possibly due to having recently laid an egg. Since these skates are oviparous, it would be interesting to do further research on skates post birth to see if there is a recovery period where the shape and smoothness of the vagina is affected. The Brennan Lab has one skate in its possession that was found to be pregnant with two egg cases, and further research could be done with those samples.

Future research could also be done on the claspers of the males for both species, as those samples were collected during dissections. Further 3D modeling and examination should be done to determine if the size or shape of the claspers have an effect on the volume and shape of the vaginas, especially examining differences between juveniles and reproductive aged females and males. Similar research is being done in the Brennan lab with snake hemipenes, vaginal pouches, and hemiclitori, so protocol is already in place to process these samples.

We could not find information on the secretory structure; however, there was a similar structure found years ago in the Brennan Lab during a shark dissection. This structure was instead found flush to the vagina as opposed to the cranial end of the vagina, but had a similar bladder-like appearance with no known equivalence found in anatomy manuals. The abundance of mucus inside this structure suggests that it is specialized for secretion, but further histology work should be done to determine its function.

We found differences in the thickness and amount of vaginal folds of the two species; however, we cannot currently determine if these folds have functionality. Future work should measure the features of a few more vaginas per species to try to quantify differences better.

Many of the histology slides showed that the vaginal walls were mainly collagen material, with some sporadic muscle bundles. It seems that these vaginas are suited for stretching and shape deformation as opposed to contraction.

We were able to confirm previous observations of a difference in collagen density of the Little skate and Winter skate vaginal walls. We believe that the thickness of this dorsal wall is where the vaginal pad we observed in both Winter and Little skates is located, and it was noted that this pad is much more prominent in Little skates. This could be due to the hooked nature of the Little skate claspers, as remarked by Daniel, J. F. 1934 (pp. 309). This hard, collagen rich tissue could be an evolutionary development to protect the vagina during copulation while allowing for elasticity during egg production. However, we do not have enough data yet to quantitatively measure the density of the tissues, so future work should increase this sample size as well.

The relatively lower densities of the juvenile skate vaginas could be due to having not reached reproductive age, and so do not need the hardened tissue to protect themselves from male claspers or to stretch while laying an egg.

Human error is often discussed both at the classroom level and at the academic level, and something that should always be accounted for. I learned this

firsthand when working with ImageJ and figuring out the thresholds and seeing what I believe to be correct by simply eyeing the thresholds, versus taking into account the mechanical standard error of the ImageJ threshold settings. Usually, when this kind of error discrepancy is uncovered, there is a standard protocol to test what the human standard error of an individual person is. If I had been doing a quantitative analysis of the image a material, it would have been best for me to go over the images three times for all images, setting my own threshold each time, noting these values down into a spreadsheet and averaging out each value, then calculating a standard deviation that would become my error. If my error was over 5%, then my human error would be too high to be considered valuable data for submission. This is a common and important practice for procedures like these, where the automation of technology has not quite caught up with what is necessary for human input. It would be interesting for future research to see if there is an automated thresholding procedure that could be used for ImageJ or other computer software.

To help with the ImageJ software, some variations that might be helpful for future research would be to make sure to do the same stain for all the photos, as I had to use a mixture of Mason's and H&E between the specimens used. It would also be best to make sure the lighting is the same on all the photos, as there were some specimens that had some lighting difficulties that made setting the threshold a bit difficult.

The measurements from the histological wall samples showed a notably thick lamina propria layer, with the next thickest layer being the muscularis. The lamina propria being thickest is evidence that this vagina functions more in terms of elasticity rather than tensile strength. Orr et al. 2022 had similar findings in female bat species where the males had penile spines; future research should be done in other females where the males of the species have penile spines to see if there is a trend across multiple groups. Interestingly enough, the lamina propria appeared to be thicker in juveniles rather than adults. A study done on human individuals also found that the lamina propria layer tends to be thicker solely based on age and is not due to birth, gestation, or timing of the menstrual cycle; however, this was a study done solely on adults, with no child/infant samples (Wang et al. 2024). It should also be acknowledged that though the data collected during this thesis is promising, these measurements were only done on a few samples, and a more quantitative review should be done in the future to better understand these findings.

These kinds of studies on the layers of the vagina are not just important for continuing genitalia and reproductive research in the animal kingdom, but also help to provide essential insight into our human lives. A clinical study examining the causes of vaginal prolapse in women found that young adults who experienced a vaginal prolapse had significantly lower fractions of the muscularis layer, and that postmenopausal woman who were not taking estrogen replacement or other hormonal therapies had the most diminished vaginal muscle tissue (Boreham et al.

2002). Neglecting to research vaginas and female genitalia in the animal kingdom based on an assumption that there is just “too little variation” in these structures to be worth studying. This gap in the research has had lasting consequences on our understanding of both the animal world and human society, and needs to continue to be corrected.

APPENDIX

Tables

Table 9. List of specimens with ID number, species name, Total Length (TL), and vagina volume.

Specimen ID	Species Name	TL (cm)	Vaginal Volume (cm^3)
LEOC001F	LEUCORAJA OCELLATA	42	17.7947
LEOC004F	LEUCORAJA OCELLATA	75.5	51.7441
LEOC007F	LEUCORAJA OCELLATA	70.9	12.839
LEOC010F	LEUCORAJA OCELLATA	70.5	9.61186
LEOC011F	LEUCORAJA OCELLATA	46	47.3624
LEOC012F	LEUCORAJA OCELLATA	50	65.2476
LEOC013F	LEUCORAJA OCELLATA	77.5	51.3454
LEOC014F	LEUCORAJA OCELLATA	76	69.5056
LEOC015F	LEUCORAJA OCELLATA	66.5	8.05078
LEOC016F	LEUCORAJA OCELLATA	72.5	100.244
LEOC024F	LEUCORAJA OCELLATA	65.3	32.3482
LEOC025F	LEUCORAJA OCELLATA	74.1	111.611
LEOC026F	LEUCORAJA OCELLATA	74.4	104.598
LEOC027F	LEUCORAJA OCELLATA	71.2	65.9197

LEOC028F	LEUCORAJA OCELLATA	76.4	134.234
LEOC032F	LEUCORAJA OCELLATA	68.9	49.6046
LEOC033F	LEUCORAJA OCELLATA	77	71.1453
LEOC034F	LEUCORAJA OCELLATA	63.5	10.1808
LEOC035F	LEUCORAJA OCELLATA	64.3	9.47278
LEOC052F	LEUCORAJA OCELLATA	76.3	62.1299
LEOC053F	LEUCORAJA OCELLATA	76.5	68.0404
LEOC055F	LEUCORAJA OCELLATA	69.7	49.2213
LEOC057F	LEUCORAJA OCELLATA	76	59.652
LEOC058F	LEUCORAJA OCELLATA	66.2	5.52725
LEOC059F	LEUCORAJA OCELLATA	69.8	43.8197
LEOC060F	LEUCORAJA OCELLATA	57.3	1.20887
LEOC061F	LEUCORAJA OCELLATA	52.2	0.107128
LEOC062F	LEUCORAJA OCELLATA	51.3	0.174132
LEER007F	LEUCORAJA ERINACEA	41.8	14.0002
LEER008F	LEUCORAJA ERINACEA	42.9	10.6699
LEER009F	LEUCORAJA ERINACEA	38.4	0.0959795
LEER010F	LEUCORAJA ERINACEA	40.8	10.4196
LEER011F	LEUCORAJA ERINACEA	42.9	29.3025
LEER012F	LEUCORAJA ERINACEA	44.9	15.4123
LEER013F	LEUCORAJA ERINACEA	42.5	18.3192

LEER014F	LEUCORAJA ERINACEA	43.5	23.9394
LEER015F	LEUCORAJA ERINACEA	38.4	2.30832
LEER019F	LEUCORAJA ERINACEA	41	16.7338
LEER020F	LEUCORAJA ERINACEA	41.7	16.3341
LEER021F	LEUCORAJA ERINACEA	43.9	15.179
LEER022F	LEUCORAJA ERINACEA	43.5	22.4344
LEER023F	LEUCORAJA ERINACEA	41.7	15.8495
LEER024F	LEUCORAJA ERINACEA	46.7	34.2734
LEER025F	LEUCORAJA ERINACEA	42	4.85685
LEER026F	LEUCORAJA ERINACEA	42.9	9.39746
LEER027F	LEUCORAJA ERINACEA	47.4	9.73033
LEER028F	LEUCORAJA ERINACEA	41.7	4.78919
LEER029F	LEUCORAJA ERINACEA	43.8	6.77491
LEER030F	LEUCORAJA ERINACEA	43.7	16.6131
LEER037F	LEUCORAJA ERINACEA	40.6	7.78128
LEER038F	LEUCORAJA ERINACEA	45.6	13.3853

Table 10. Protocol for setting specimen tissue cassettes in Paraffin blocks.

Dehydration I	70% EtOH	1 hr
Dehydration II	95% EtOH	1 hr
Dehydration III	100% EtOH I	1 hr

Dehydration IV	100% EtOH II	1 hr
Dehydration V	100% EtOH III	4 hr/overnight
Clearing I	1:1 Histoclear+EtOH	30 min
Clearing II	100% Histoclear I	30 min
Clearing III	100% Histoclear II	30 min
Clearing IV	1:1 Histoclear+Paraffin	15 min
Infiltration I	Paraffin I	30 min
Infiltration II	Paraffin II	30 min
Infiltration III	Paraffin III	1 hr

Table 11. Rehydration protocol for staining.

Histoclear I	5 min
Histoclear II	2 min
100% EtOH	2 min
95% EtOH	2 min
70% EtOH	2 min
50% EtOH	2 min
35% EtOH	2 min
dH2O	2 min

Table 12. Masson's Trichrome Protocol.

Masson A	1.5 min
dH_2O rinse	sink
Masson B	1.5 min
Masson C	20 sec
dH_2O rinse	sink
1% Acetic Acid	2.5 min
1:1 Acetic Acid+EtOH	30 sec
100% EtOH rinse	x2 (2min each)
Histoclear rinse	x2 (5min each)

Table 13. H & E (Hematoxylin and Eosin) procedure.

Hematoxylin	20 sec
dH_2O rinse	sink
35% EtOH	2 min
50% EtOH	2 min
Eosin Y	2.5 min
95% EtOH	2 min
100% EtOH (I)	2 min
100% EtOH (II)	2 min
Histoclear (I)	5 min
Histoclear (II)	5 min

Table 14. Usable histology slides created from 2019-2025.

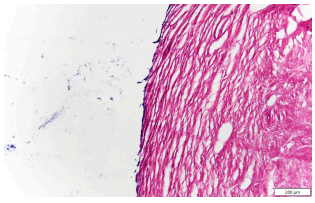
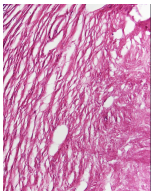
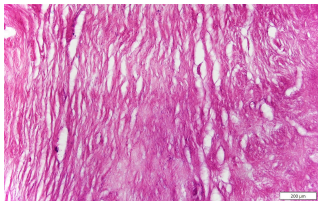
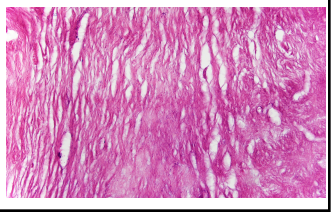
TRANSVERSE			SAGITTAL		
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LEOC007b	X		LEOC007-12	X	X
LEOC007b	X		LEOC007-13	X	X
LEOC007b	X		LEOC007-20	X	X
LEOC007c		X	LEOC007-21	X	X
LEOC007c		X	LEOC007-23	X	X
LEOC007c		X	LEOC007-24	X	X
LEOC007c	X		Sample	Masson's Trichrome	H&E
LEOC007c	X		LEOCO25F VP 1A	X	
LEOC007c	X		LEOCO25F VP 1D	X	
LEOC007c	X		LEOCO25F VP 3A	X	X
LEOC007c	X		LEOCO25F VP 3B	X	X
LEOC007b		X	LEOCO25F VP 4A	X	X
LEOC007d	X		LEOCO25F VP 4B	X	X
LEOC007e	X		Sample	Masson's Trichrome	H&E
LEOC007f		X	LEOC061F VAG	X	X
LEOC007f	X		Sample	Masson's Trichrome	H&E
LEOC007g		X	LEOC062F VAG	X	X
LEOC007g	X		Sample	Masson's Trichrome	H&E
LEOC007h		X	LEER008F VP 1B	X	X
LEOC007h	X		LEER008F VP 2B	X	X

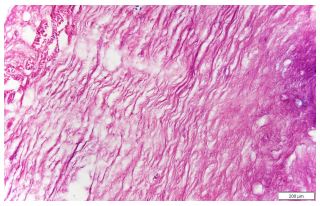
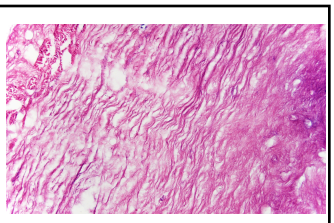
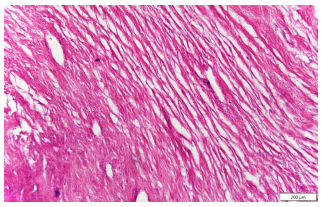
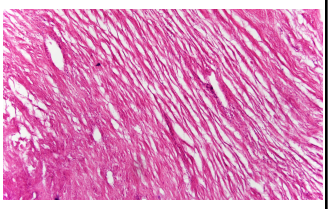
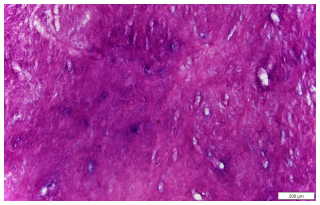
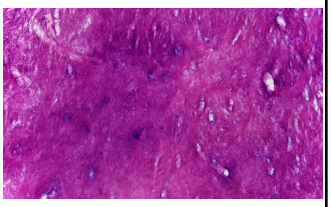
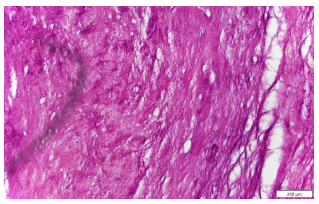
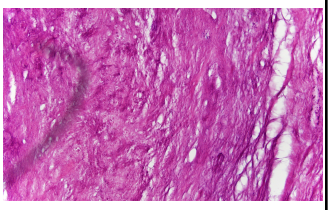
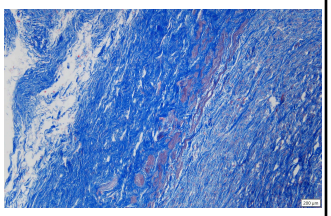
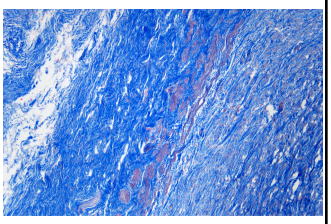
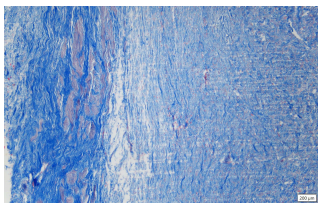
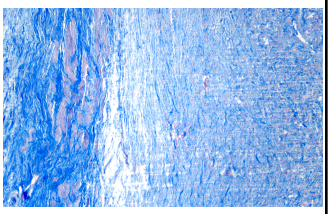
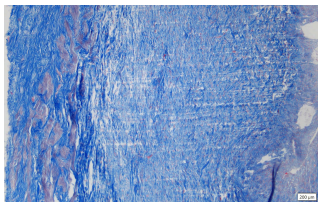
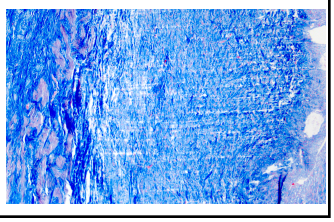
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LEOC007i	X		
LEOC007j-k		X	
LEOC007j-k	X		
LEOC007j-k		X	
Sample	Masson's Trichrome	H&E	
LEOC11Fa	X		
LEOC11Fc		X	
LEOC11Fc	X		
LEOC11Fc	X		
LEOC11Fd	X		
LEOC11Ff		X	
LEOC11Ff	X		
LEOC11Fg	X		
LEOC11Fk	X		
Sample	Masson's Trichrome	H&E	
LEOC012a	X		
LEOC012a		X	
LEOC012bm		X	
LEOC012bm	X		
LEOC012bm	X		
LEOC012C		X	
LEOC012C	X		
LEOC012C	X		
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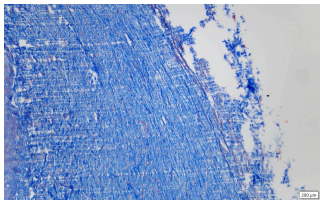
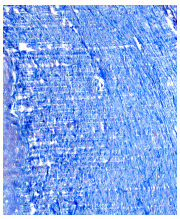
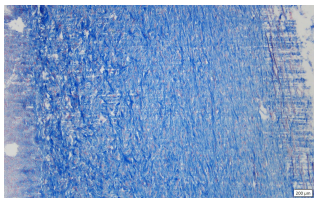
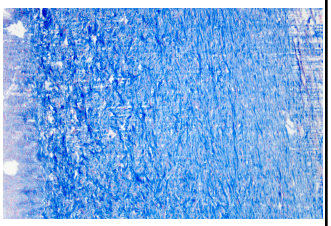
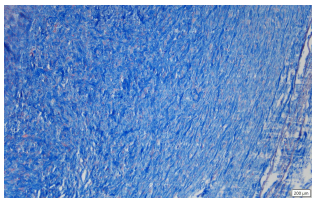
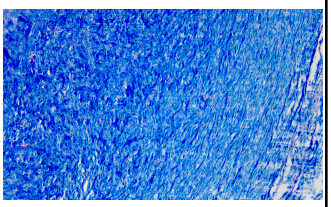
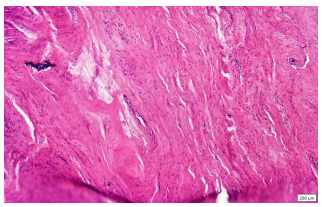
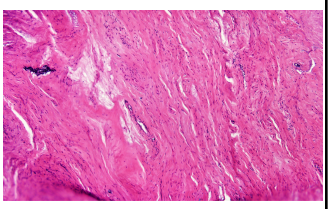
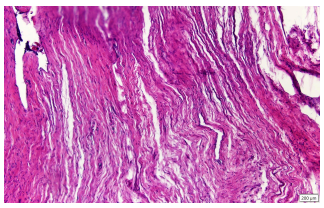
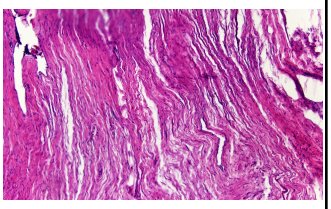
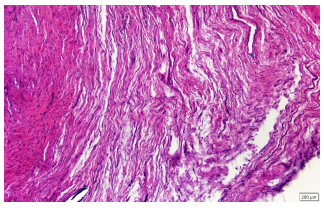
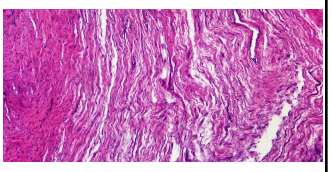
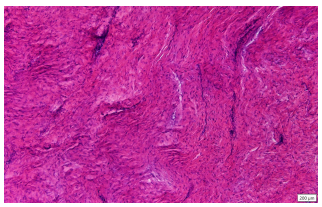
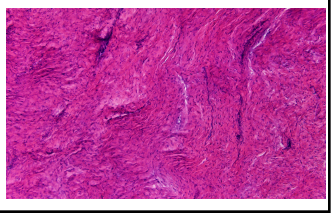
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LEOC012-4j	X		
LEOC012kl	X		
Sample	Masson's Trichrome	H&E	
LEOCO25F VP A1	X	X	
LEOCO25F VP A2	X	X	
LEOCO25F VP B1	X		
LEOCO25F VP B2	X	X	
LEOCO25F VP C	X	X	
LEOCO25F VP D	X		
LEOCO25F VP E		X	
LEOCO25F VP F		X	
LEOCO25F VP G	X		
LEOCO25F VP H	X	X	
LEOCO25F VP I	X	X	
LEOCO25F VP J	X	X	
LEOCO25F VP K	X		
LEOCO25F VP L	X		
LEOCO25F VP M	X		

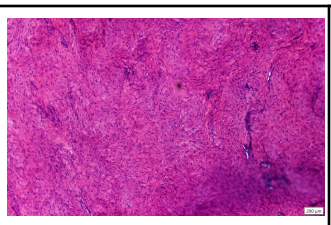
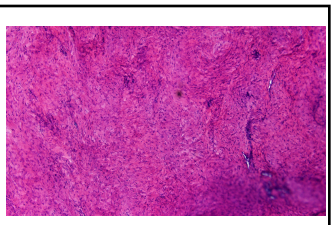
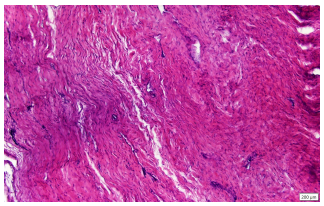
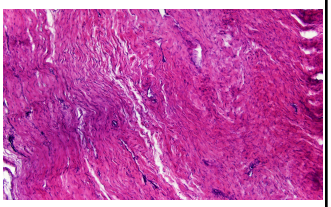
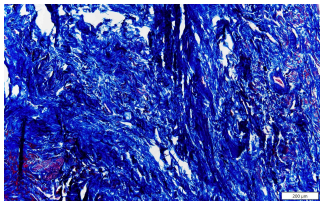
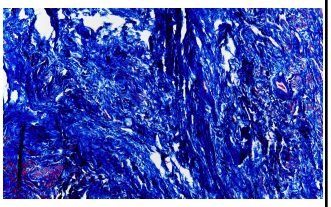
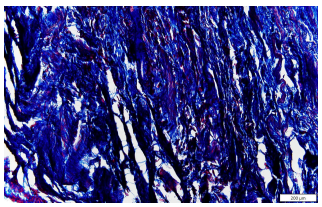
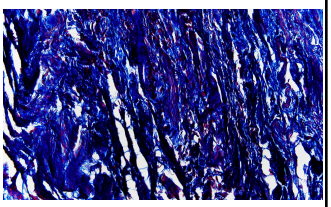
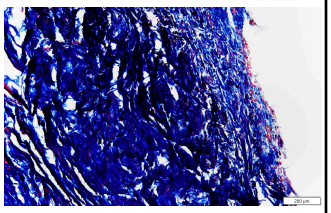
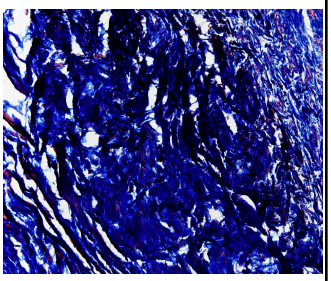
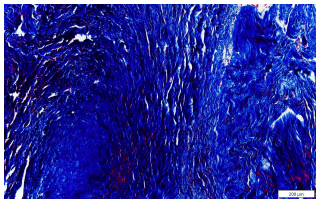
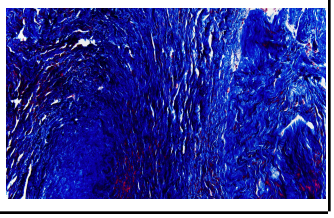
Sample	Masson's Trichrome	H&E	
LEER009F VP A	X	X	
LEER009F VP B	X	X	
LEER009F VP C	X	X	
Sample	Masson's Trichrome	H&E	
LEER048F VP 1	X	X	
LEER048F VP 2	X	X	
LEER048F VP 3	X	X	
LEER048F VP 4	X	X	

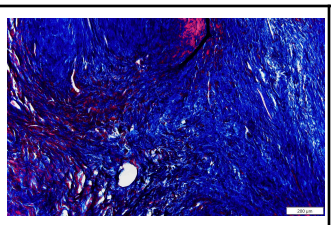
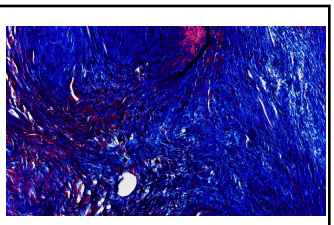
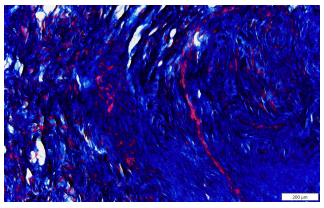
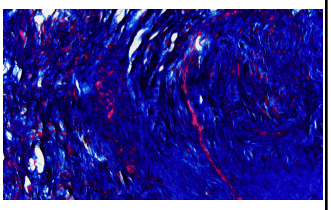
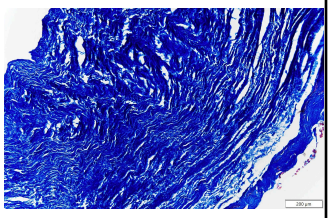
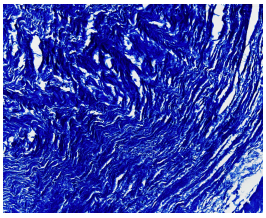
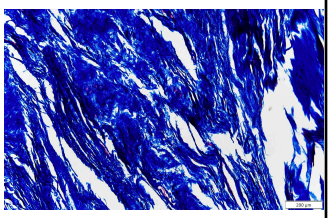
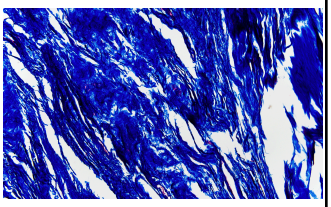
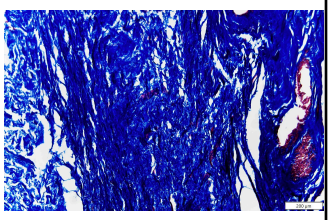
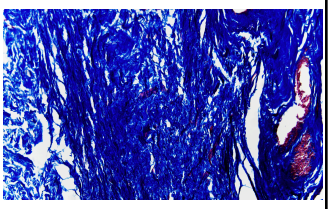
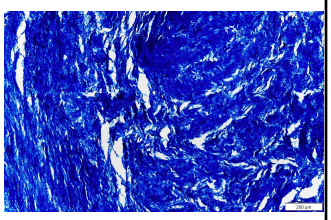
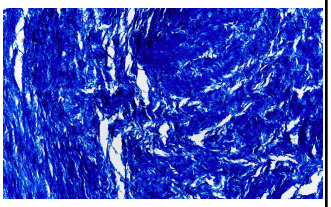
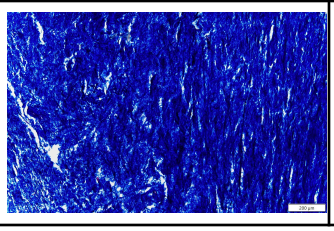
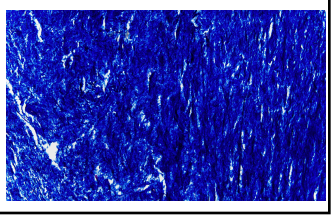
Table 15. Histology slide specimens used for ImageJ analysis.

Specimen	Orientation	Original Photo	ImageJ Screenshot
LEER008F VP1B	Top Ventral wall		
	Mid Ventral wall		

	Bottom Ventral wall		
	Top Dorsal wall		
	Mid Dorsal wall		
	Bottom Dorsal wall		
LEER009F VPC	Top Ventral wall		
	Mid Ventral wall		
	Bottom Ventral wall		

	Top Dorsal wall		
	Mid Dorsal wall		
	Bottom Dorsal wall		
LEER048F VP3	Top Ventral wall		
	Mid Ventral wall		
	Bottom Ventral wall		
	Top Dorsal wall		

	Mid Dorsal wall		
	Bottom Dorsal wall		
LEOC011FC	Top Ventral wall		
	Mid Ventral wall		
	Bottom Ventral wall		
	Top Dorsal wall		

	Mid Dorsal wall		
	Bottom Dorsal wall		
LEOC012FC	Top Ventral wall		
	Mid Ventral wall		
	Bottom Ventral wall		
	Top Dorsal wall		
	Mid Dorsal wall		

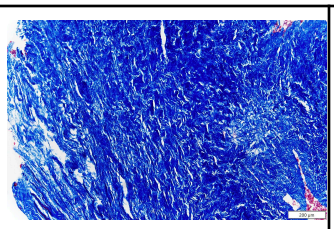
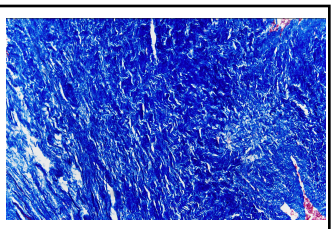
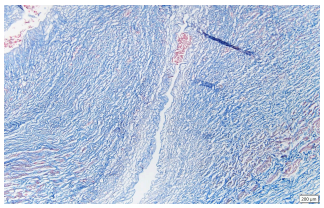
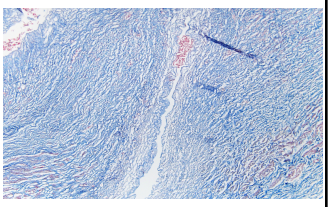
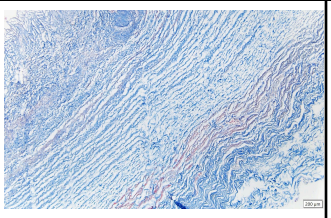
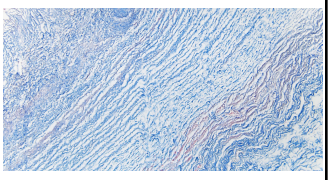
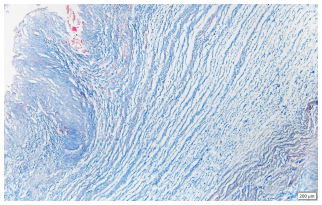
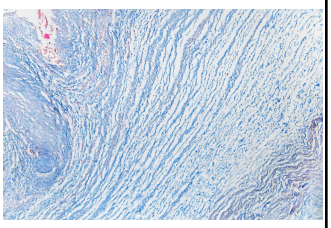
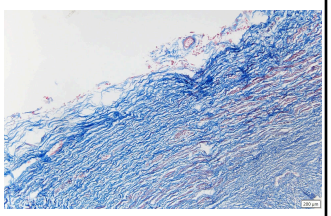
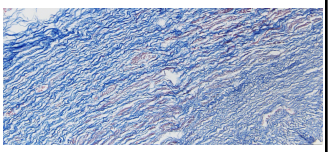
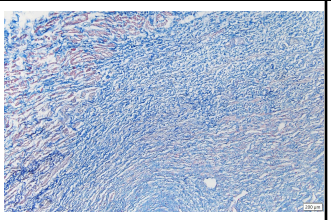
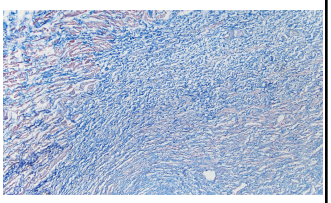
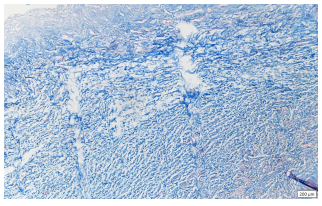
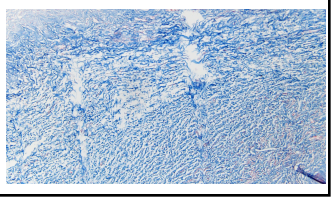
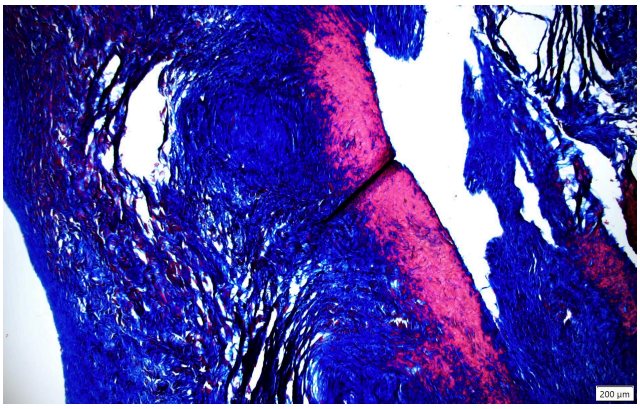
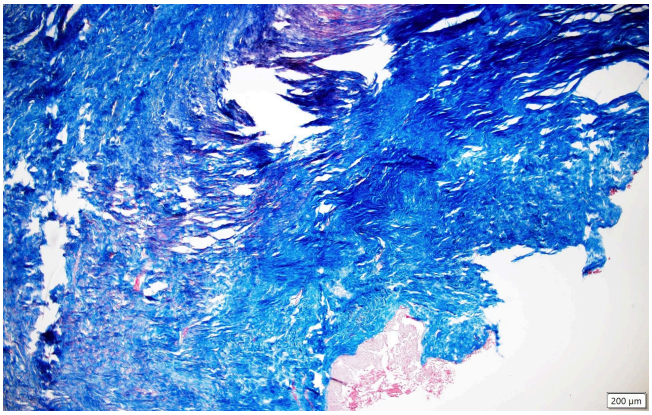
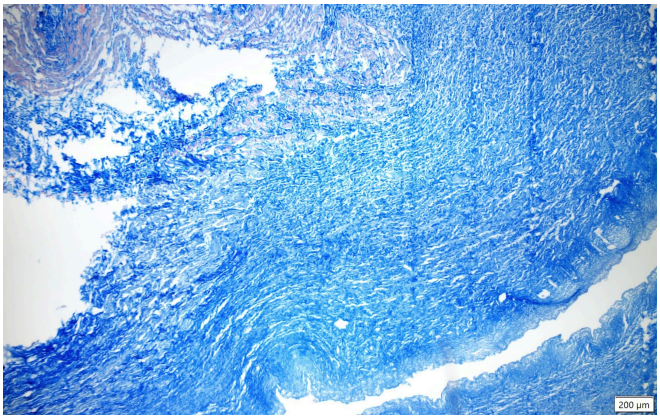
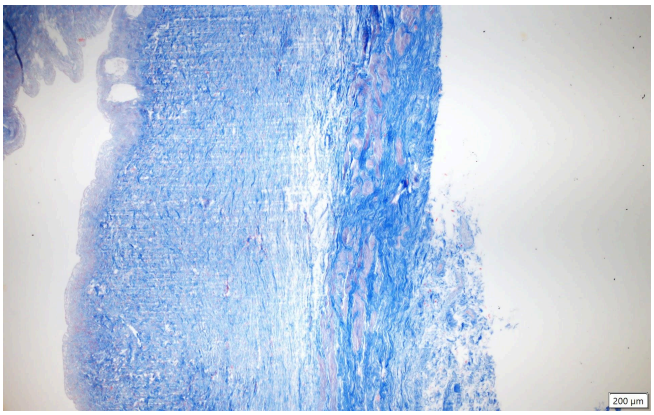
	Bottom Dorsal wall		
LEOC061F VAG	Top Ventral wall		
	Mid Ventral wall		
	Bottom Ventral wall		
	Top Dorsal wall		
	Mid Dorsal wall		
	Bottom Dorsal wall		

Table 16. Histology slide specimens used for mucosa, lamina propria, and muscularis measurements.

Species	Specimen	Image Used (5x Focus)
Winter Skate Adult	LEOC011F C	
Little Skate Adult	LEER048F VP3	
Winter Skate Juvenile	LEOC061F VAG	

Little Skate Juvenile	LEER009F VPC	

R Code

Combined Species Code

```
rm(list=ls(all=T))
library(geomorph)
library(ape)
library(phytools)
library(geiger)
library(calibrate)
library(RevGadgets)
library(dplyr)
library(Morpho)
library(nlme)

setwd("~/Desktop/Skate Thesis/Data/Combined Data")

#####Read Combined Vagina data#####
```

```
LEVaginaShape <- read.morphologika("combined
morphologika_unscaled.txt") #Load in TPS file
LEVaginaGPA <- gpagen(LEVaginaShape, ProcD = FALSE) #Run
Generalized Procrustes Analysis using bending energy criterion
LEVaginaData <- read.csv("Combined Skate TL Data.csv") #Load in
csv file with SpecimenID, TL (cm), Species, Volume

#####Run Combined Vagina PCA#####

LEVaginaPCA <- gm.prcomp(LEVaginaGPA$coords,
                              phy = NULL)
LEVaginaPCASum <- summary(LEVaginaPCA) #Prints variance and
eigenvalues

components <- LEVaginaPCA[["x"]]
components <- data.frame(components)

#####Create Combined Vagina plots#####
#Set a color palette for the PCA based on Volume where larger
females are yellow and smaller females are blue
pal = colorRampPalette(c("cornsilk", "darkblue"))
LEVaginaGPA$col <-
pal(20)[as.numeric(cut(LEVaginaData$Volume_cm.3,breaks = 20))]

#write plot
LEVaginaPCAPlot <- plot(LEVaginaPCA,
                            xlab = paste("Principal Component 1
(44.9%)"),
                            ylab = paste("Principal Component 2
(15.8%)"),
                            pch =
as.numeric(LEVaginaData$Species),
```

```
                              col = "black",
                              bg = LEVaginaGPA$col,
                              cex = 2,
                              cex.lab = 1.2)

text(LEVaginaPCAPlot$PC.points[ , 2] ~ LEVaginaPCAPlot$PC.points[
, 1],
     labels = LEVaginaData$Specimen_ID, cex= 0.5, col =
c("black"))


### Generating POINT CLOUDS
MLPFprocfemale <- procSym(LEVaginaShape)
plot(MLPFprocfemale$PCscores[,2] ~ MLPFprocfemale$PCscores[,1])

#Plot at ends of PC1 axes, negative is purple and positive is
green, Magnified by 2 to accentuate trends
palette(c("white", "green", "purple"))
posPC1female <- showPC(2*sd(MLPFprocfemale$PCscores[,1]),
MLPFprocfemale$PCs[,1], MLPFprocfemale$mshape)
negPC1female <- showPC(-2*sd(MLPFprocfemale$PCscores[,1]),
MLPFprocfemale$PCs[,1], MLPFprocfemale$mshape)
deformGrid3d(posPC1female, negPC1female, ngrid = 0, lines =
FALSE, size = 1)

posPC2female <- showPC(2*sd(MLPFprocfemale$PCscores[,2]),
MLPFprocfemale$PCs[,2], MLPFprocfemale$mshape)
negPC2female <- showPC(-2*sd(MLPFprocfemale$PCscores[,2]),
MLPFprocfemale$PCs[,2], MLPFprocfemale$mshape)
deformGrid3d(posPC2female, negPC2female, ngrid = 0, lines =
FALSE, size = 0.75)
```

```
# 2 - Stats for Combined
#

#Make a geomorph dataframe for using functions in geomorph
package
LEVaginaData$Species <- factor(LEVaginaData$Species)

LEVaginaGDF <- geomorph.data.frame(shape = LEVaginaGPA$coords,
                                   tl =
log10(LEVaginaData$TL_cm),
                                   vol =
log10(LEVaginaData$Volume_cm.3),
                                   species =
(LEVaginaData$Species))

#Run a Procrustes ANOVA of Combined Shape and Combined TL
LEVaginaFit <- procD.lm(shape ~ tl,
                        data = LEVaginaGDF,
                        iter = 1000)
summary(LEVaginaFit)
### p = 0.02098

#Run a Procrustes ANOVA of Combined shape and Combined Species
LEVaginaFit2 <- procD.lm(shape ~ species,
                         data = LEVaginaGDF,
                         iter = 1000)
summary(LEVaginaFit2)
##p = 0.01099

#Run a Procrustes ANOVA of Combined TL and Combined Vagina size
LEVaginaFit3 <- procD.lm(vol ~ tl,
                         data = LEVaginaGDF,
```

```
                        iter = 1000)
summary(LEVaginaFit3)
##p = 0.000999

#Run a Procrustes ANOVA of Combined shape and Combined Volume
LEVaginaFit4 <- procD.lm(shape ~ vol,
                        data = LEVaginaGDF,
                        iter = 1000)
summary(LEVaginaFit4)
## p = 0.000999

##Allometry
#Assess whether Combined Volume and Combined TL are significantly
correlated
tlDFLEVagina <- data.frame(LEVaginaData$TL_cm,
                              LEVaginaData$Volume)
colnames(tlDFLEVagina) <- c("LEVaginaTL", "LEVaginaVol")
TLVolFitLEVagina <- gls(LEVaginaTL ~ LEVaginaVol,
                           data = tlDFLEVagina)
#Read Combined data
LEVaginaData <- read.csv("Combined Skate TL Data.csv") #Load in
csv file with SpecimenID, SVL (mm)

#Combined Regression Relationship of TL and Volume
TLVolFitLEVagina <- gls(Volume_cm.3 ~ TL_cm,
                        data = LEVaginaData)

#Check if the confidence intervals include 3 to assess allometric
trajectory
summary(TLVolFitLEVagina)
confint(TLVolFitLEVagina)
#conf. int. 1.048611  1.96934
```

```
#Calculate Combined regression line
lmVol_TL_Plot_LEVagina <- lm(LEVaginaData$Volume_cm.3 ~
LEVaginaData$TL_cm)

#Write plot.
LEVagina_TL_Plot <- plot(LEVaginaData$Volume_cm.3 ~
LEVaginaData$TL_cm,
                         data = LEVaginaData,
                         pch = as.numeric(LEVaginaData$Species),
                         col = "black",
                         bg = LEVaginaGPA$col,
                         cex = 2,
                         cex.lab = 1.2,
                         xlab = "TL",
                         ylab = "Volume")
abline(a = -52.63687,
       b = 1.50898,
       lw = 3)
```

Little Skate Code

```
rm(list=ls(all=T))
library(geomorph)
library(ape)
library(phytools)
library(geiger)
library(calibrate)
library(RevGadgets)
library(dplyr)
library(Morpho)
```

```
library(nlme)

setwd("~/Desktop/Skate Thesis/Data/LEER Data")

#####Read LEER Vagina data#####

ERVaginaShape <- read.morphologika("LEER
morphologika_unscaled.txt") #Load in TPS file
ERVaginaGPA <- gpagen(ERVaginaShape, ProcD = FALSE) #Run
Generalized Procrustes Analysis using bending energy criterion
ERVaginaData <- read.csv("LEER Skate TL Data.csv") #Load in csv
file with SpecimenID, TL (cm)

#####Run LEER Vagina PCA#####
ERVaginaPCA <- gm.prcomp(ERVaginaGPA$coords,
                              phy = NULL)
ERVaginaPCASum <- summary(ERVaginaPCA) #Prints variance and
eigenvalues

components <- ERVaginaPCA[["x"]]
components <- data.frame(components)

#####Create LEER Vagina PCA plots#####

#Set a color palette for the PCA based on TL where larger females
are yellow and smaller females are blue
pal = colorRampPalette(c("cornsilk", "darkblue"))
ERVaginaGPA$col <-
pal(20)[as.numeric(cut(ERVaginaData$TL_cm,breaks = 20))]

#write plot
ERVaginaPCAPlot <- plot(ERVaginaPCA,
```

```
                            xlab = paste("Principal Component 1
(45.5%)"),
                            ylab = paste("Principal Component 2
(18.0%)"),
                            col = "black",
                            pch = 22,
                            bg = ERVaginaGPA$col,
                            cex = 2,
                            cex.lab = 1.2)
text(ERVaginaPCAPlot$PC.points[ , 2] ~ ERVaginaPCAPlot$PC.points[
, 1],
     labels = ERVaginaData$Specimen.ID, cex= 0.5, col =
c("black"))

####
### Generating POINT CLOUDS
MLPFprocfemale <- procSym(ERVaginaShape)
plot(MLPFprocfemale$PCscores[,2] ~ MLPFprocfemale$PCscores[,1])

#Plot at ends of PC1 axes, negative is purple and positive is
green, Magnified by 2 to accentuate trends
palette(c("white", "green", "purple"))
posPC1female <- showPC(2*sd(MLPFprocfemale$PCscores[,1]),
MLPFprocfemale$PCs[,1], MLPFprocfemale$mshape)
negPC1female <- showPC(-2*sd(MLPFprocfemale$PCscores[,1]),
MLPFprocfemale$PCs[,1], MLPFprocfemale$mshape)
deformGrid3d(posPC1female, negPC1female, ngrid = 0, lines =
FALSE, size = 0.75)

posPC2female <- showPC(2*sd(MLPFprocfemale$PCscores[,2]),
MLPFprocfemale$PCs[,2], MLPFprocfemale$mshape)
```

```
negPC2female <- showPC(-2*sd(MLPFprocfemale$PCscores[,2]),
MLPFprocfemale$PCs[,2], MLPFprocfemale$mshape)
deformGrid3d(posPC2female, negPC2female, ngrid = 0, lines =
FALSE,  size = 0.75)

# 2 - Stats for LEER

#Make a geomorph dataframe for using functions in geomorph
package
ERVaginaGDF <- geomorph.data.frame(shape = ERVaginaGPA$coords,
                                   tl =
log10(ERVaginaData$TL_cm),
                                   vol =
log10(ERVaginaData$Volume_cm.3))
#Run a Procrustes ANOVA of LEER shape and LEER TL
ERVaginaFit <- procD.lm(shape ~ tl,
                        data = ERVaginaGDF,
                        iter = 1000)
summary(ERVaginaFit)
## p = 0.1019

#Run a Procrustes ANOVA of LEER TL and LEER Vagina
ERVaginaFit2 <- procD.lm(vol ~ tl,
                         data = ERVaginaGDF,
                         iter = 1000)
summary(ERVaginaFit2)
## p = 0.001998***

### Run a Procrustes ANOVA of LEER shape and size
ERVaginaFit3 <- procD.lm(shape ~ vol,
                         data = ERVaginaGDF,
                         iter = 1000)
```

```
summary(ERVaginaFit3)
## p = 0.000999***

###Allometry
#Assess whether Volume and TL are significantly correlated
tlDFERVagina <- data.frame(ERVaginaData$TL_cm,
                                ERVaginaData$Volume_cm.3)
colnames(tlDFERVagina) <- c("ERVaginaTL", "ERVaginaVol")
TLVolFitERVagina <- gls(ERVaginaTL ~ ERVaginaVol,
                            data = tlDFERVagina)

summary(TLVolFitERVagina)

#Read LEER data
ERVaginaData <- read.csv("LEER Skate TL Data.csv") #Load in csv
file with SpecimenID, TL (cm)

#Regression Relationship of TL and Volume
TLVolFitERVagina <- gls(Volume_cm.3 ~ TL_cm,
                        data = ERVaginaData)

#Check if the confidence intervals include 3 to assess allometric
trajectory
summary(TLVolFitERVagina)
confint(TLVolFitERVagina)
##Isometric
#conf. int. 0.5735596  3.301903

#Calculate LEER regression line
lmVol_TL_Plot_ERVagina <- lm(ERVaginaData$Volume_cm.3 ~
ERVaginaData$TL_cm)
```

```
#Write plot.
ERVagina_TL_Plot <- plot(ERVaginaData$Volume_cm.3 ~
ERVaginaData$TL_cm,
                         data = ERVaginaData,
                         pch = 21,
                         col = "black",
                         bg = ERVaginaGPA$col,
                         cex = 2,
                         cex.lab = 1.2,
                         xlab = "TL",
                         ylab = "Volume")
abline(a = -68.90579,
       b =  1.93773,
       lw = 3)
```

Winter Skate Code

```
rm(list=ls(all=T))
library(geomorph)
library(ape)
library(phytools)
library(geiger)
library(calibrate)
library(RevGadgets)
library(dplyr)
library(Morpho)
library(nlme)

setwd("~/Desktop/Skate Thesis/Data/LEOC Data")
```

```
#####Read LEOC vagina data#####

OCVaginaShape <- read.morphologika("LEOC
morphologika_unscaled.txt") #Load in TPS file
OCVaginaGPA <- gpagen(OCVaginaShape, ProcD = FALSE) #Run
Generalized Procrustes Analysis using bending energy criterion
OCVaginaData <- read.csv("LEOC Skate TL Data.csv") #Load in csv
file with SpecimenID, TL (cm)

#####Run LEOC Vagina PCA#####

OCVaginaPCA <- gm.prcomp(OCVaginaGPA$coords,
                              phy = NULL)
OCVaginaPCASum <- summary(OCVaginaPCA) #Prints variance and
eigenvalues

components <- OCVaginaPCA[["x"]]
components <- data.frame(components)

#####Create Vagina plots#####

#Set a color palette for the PCA based on TL where larger females
are red and smaller females are blue
pal = colorRampPalette(c("cornsilk", "darkblue"))
OCVaginaGPA$col <- pal(20)[as.numeric(cut(OCVaginaData$TL,breaks
= 20))]

#write plot
OCVaginaPCAPlot <- plot(OCVaginaPCA,
                            xlab = paste("Principal Component 1
(53.4%)"),
```

```
                              ylab = paste("Principal Component 2
(11.5%)"),
                              col = "black",
                              bg = OCVaginaGPA$col,
                              pch = 21,
                              cex = 2,
                              cex.lab = 1.2)
text(OCVaginaPCAPlot$PC.points[ , 2] ~ OCVaginaPCAPlot$PC.points[
, 1],
     labels = OCVaginaData$Specimen.ID, cex= 0.5, col =
c("black"))

#^last line run so far

### Generating POINT CLOUDS
MLPFprocfemale <- procSym(OCVaginaShape)
plot(MLPFprocfemale$PCscores[,2] ~ MLPFprocfemale$PCscores[,1])

#Plot at ends of PC1 axes, negative is purple and positive is
green, Magnified by 2 to accentuate trends
palette(c("white", "green", "purple"))
posPC1female <- showPC(2*sd(MLPFprocfemale$PCscores[,1]),
MLPFprocfemale$PCs[,1], MLPFprocfemale$mshape)
negPC1female <- showPC(-2*sd(MLPFprocfemale$PCscores[,1]),
MLPFprocfemale$PCs[,1], MLPFprocfemale$mshape)
deformGrid3d(posPC1female, negPC1female, ngrid = 0, lines =
FALSE, size = 0.75)

posPC2female <- showPC(2*sd(MLPFprocfemale$PCscores[,2]),
MLPFprocfemale$PCs[,2], MLPFprocfemale$mshape)
negPC2female <- showPC(-2*sd(MLPFprocfemale$PCscores[,2]),
MLPFprocfemale$PCs[,2], MLPFprocfemale$mshape)
```

```
deformGrid3d(posPC2female, negPC2female, ngrid = 0, lines =
FALSE,  size = 0.75)

# 2 - Stats for LEOC

#Make a geomorph dataframe for using functions in geomorph
package
OCVaginaGDF <- geomorph.data.frame(shape = OCVaginaGPA$coords,
                                   tl =
log10(OCVaginaData$TL_cm),
                                   vol =
log10(OCVaginaData$Volume_cm.3))
#Run a Procrustes ANOVA of LEOC shape and TL
OCVaginaFit <- procD.lm(shape ~ tl,
                        data = OCVaginaGDF,
                        iter = 1000)
summary(OCVaginaFit)
## p = 0.2218

### Run a Procrustes ANOVA of LEOC TL and Vagina Volume
OCVaginaFit2 <- procD.lm(vol ~ tl,
                         data = OCVaginaGDF,
                         iter = 1000)
summary(OCVaginaFit2)
## p = 0.004995**

### Run a Procrustes ANOVA of LEOC shape and size
OCVaginaFit2 <- procD.lm(shape ~ vol,
                         data = OCVaginaGDF,
                         iter = 1000)
summary(OCVaginaFit2)
## p = 0.000999 ***
```

```
###Allometry
#Assess whether Volume and TL are significantly correlated
tlDFOCVagina <- data.frame(OCVaginaData$TL_cm,
                                OCVaginaData$Volume)
colnames(tlDFOCVagina) <- c("OCVaginaTL", "OCVaginaVol")
TLVolFitOCVagina <- gls(OCVaginaTL ~ OCVaginaVol,
                            data = tlDFOCVagina)

summary(TLVolFitOCVagina)

#Read LEOC data
OCVaginaData <- read.csv("LEOC Skate TL Data.csv") #Load in csv
file with SpecimenID, TL (cm)

#Regression Relationship of TL and Volume
TLVolFitOCVagina <- gls(Volume_cm.3 ~ TL_cm,
                        data = OCVaginaData)

#Check if the confidence intervals include 3 to assess allometric
trajectory
summary(TLVolFitOCVagina)
confint(TLVolFitOCVagina)
#conf. int. 0.7214535  3.0677029 ### Isometric

#Calculate regression line
lmVol_TL_Plot_OCVagina <- lm(OCVaginaData$Volume_cm.3 ~
OCVaginaData$TL_cm)

#Write plot. Figure 6A
OCVagina_TL_Plot <- plot(OCVaginaData$Volume_cm.3 ~
OCVaginaData$TL_cm,
```

```
                                data = OCVaginaData,
                                pch = 21,
                                col = "black",
                                bg = OCVaginaGPA$col,
                                cex = 2,
                                cex.lab = 1.2,
                                xlab = "TL",
                                ylab = "Volume")
abline(a = -80.17495,
       b = 1.89458,
       lw = 3)
```

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