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Variance of Female Genital Shape in Spiny Dogfish, *Squalus Acanthias*, a
2-D and 3-D Approach

by

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ABSTRACT

This study aimed to investigate the presence and significance of morphological variation in the internal female genitalia of spiny dog sharks, *Squalus acanthias*. We used traditional linear morphometrics to examine the relationship between the allometry of the vagina and the allometry of the pectoral fin; however due to small sample size we did not detect an allometric trend. We used traditional 2-D and relatively new 3-D geometric morphometrics (GM) to look for variations in genital shape correlated with changing reproductive state (Visibly Pregnant vs. reproductive but Not Visibly Pregnant). Surprisingly, only the 2-D analysis detected significant shape variation associated with reproductive state, however it was only significant after two sets of error replicates were included. This information suggests that further geometric morphometric analyses should include two sets of error models to better understand and compare the different degrees of variation detected by 2-D and 3-D methods. The data from both the 2-D and 3-D GM analyses did show that vaginal size is significantly correlated with shape—supporting the idea that as vaginal size increases the shape of the vagina becomes wider and more open, likely to accommodate for birth.

INTRODUCTION

The evolution of non-genital features is usually driven by biotic and abiotic interactions that occur prior to copulation, while the evolution of genital features and their morphology is usually driven by interactions during and after copulation (Brennan, 2016). There is a significantly greater degree of variation in the shape of genital features in comparison to non-genital features. This variation includes both size and shape components, but the complexity of the variation suggests that "shape", as opposed to size, should be of primary concern in studies focusing on genitalia and their morphological variation (Rowe & Arnqvist, 2012; Brennan, 2016). However, most genital studies have primarily examined size.

Both sexual and natural selection can influence variation in genitalia (Eberhard, 1985; Hosken & Stockley, 2004; Brennan & Prum, 2015). Sexual selection pressures refer to the selective forces that influence individuals' ability to procure mates and reproduce, including: male – male competition, female choice, and sexual conflict (Brennan & Prum, 2015). In male – male competition the males compete with one another for the ability to mate with available females. In the context of genital evolution, male genital features may evolve to outcompete other males during insemination. Female choice refers to the sexual selective preference of the females for specific male traits that help determine their mate choice. For example, females may prefer males with certain penile traits. Sexual conflict occurs when both sexes have a different preferential reproductive method which results in a conflicting competition between the two

sexes for a greater fitness and degree of control in reproductive methods. Males can evolve penile traits that harm females, but give them an advantage in mating (for example penile spines), while females evolve to counteract male harm through genital adaptations (for example thicker vaginal walls) (Arnqvist & Rowe, 2005; Eberhard, 2011; Brennan & Prum, 2015).

Natural selection can increase genital variation through a mechanism known as the “lock and key”, in which males act as “keys” that must fit the correct “locks” of the females during speciation (Shapiro & Porter, 1989; Masly, 2012). Therefore genital variation helps to avoid mating with individuals of the wrong species. This “lock and key” selective pressure limits certain individuals by mating constraints associated with the shape of their genitalia (Brennan & Prum, 2015).

Despite our knowledge of the types of pressures that can influence the degree of variation in genitalia, there is currently not a good general understanding as to which pressure or pressures are the primary drivers of variation. This lack of general understanding is partly related to the relatively few studies that have looked at shape variation in genitalia. As a result, the full range of morphological diversity remains unexplored. The information gained from these studies on genital morphological variation is then limited to their species of study.

The information gained from most genital studies is also frequently limited by the historic male preferential bias often held by past researchers, to

study and search for the significance of morphological variation in only the male genitalia. This bias developed from the belief that the male genitalia were the sole drivers of change and sexual selection in species (Ah-King et al. 2014; Brennan, 2016). This belief affected the “lock and key” hypothesis which, despite being particularly popular among entomologists, was not originally accepted as a driving mechanism of genital evolution due to the common untested assumption that female genitalia did not have the high degree of variability in shape that would be necessary to serve as effective “locks”, despite the fact that the male “keys” seem to be quite variable (Eberhard, 1985; Brennan, 2016). Modern day studies refute this male biased opinion, and some even argue that the coevolution of genitalia shape is biased toward the female structure, which dictates what the male part must conform to in order to successfully reproduce (Hosken & Stockley, 2004; Showalter et al. 2013; Brennan & Prum, 2015).

The current gap in our knowledge concerning the pressures that influence morphological variation in genitalia, as well as the significance of this variation, is not only due to a research bias, but also partially due to the difficulty in studying the internal shape of female genitalia. Determining the shape and detecting variation in the visible external genitalia of males is much easier than in the internal genitalia of females (Brennan & Prum, 2015). New technologies and methods must be utilized in order to see and quantitatively analyze the genital variation in the female internal morphology.

There is growing evidence that the internal genitalia of females varies significantly, supporting the idea that the shape of female genitalia plays an important role in both the evolution within species and of new species. A recent geometric morphometrics study on female watersnakes quantitatively measured internal vaginal pouch shape in the two species, *Nerodia sipedon* and *Nerodia fasciata*, and the results showed significant differences in shape that had not been recognized with the previous qualitative observations (Showalter et al. 2013; Brennan, 2016). Even a geometric morphometrics study on the shape of genital plates in *Drosophila* using SEM and TEM, detected variation between females of different species comparable to that found between different species of males (Yassin & Orgogozo, 2013; Brennan, 2016). Before these new methods had been implemented, studies had suggested that *Drosophila* female genitalia did not vary between species (Eberhard & Ramirez, 2004; Jagadeeshan & Singh, 2006; Brennan, 2016).

The relatively new information on the significant variation in shape of female genitalia led us to want to investigate the nature of variability in the internal vaginal shape of sharks. This requires that we establish methods of detecting and analyzing this variation. A significant gap in our knowledge of shark reproduction revolves around the development and functionality of the female genitalia. There is very little information regarding the relationships between the different genital parts, or the presence of morphological changes during maturation as well as in response to aspects of sexual intercourse,

development, and birth. The potential influence of sexual selection on genital morphology also has yet to be investigated.

For this research we selected the spiny dogfish, *Squalus acanthias*, as our study specimen. Characterized by a defensive spine behind the anterior and posterior dorsal fin, *S. acanthias* is a familiar shark species found off the coasts of New England (“Atlantic States Marine Fisheries Commission, Spiny Dogfish”, 2018). They are the most abundant shark in the Western North Atlantic and comprise a substantial fishery resource (Compagno, 1984; Stehlik, 2007).

Spiny dogfish sharks are small, and are generally considered slow swimmers, partly due to their internal caudal structure (Domenici et al. 2004; Stehlik, 2007). The species is sexually dimorphic—females grow to maximum lengths of approximately 108 to 125 cm, while males only reach maximum lengths of approximately 86 cm, in the Northwest Atlantic Ocean (Fowler et al. 2004; Northeast Fisheries Science Center, 2006). Their small body size and average slower swimming speeds, are rather misleading when considering their adept migration skills up and down the East Coast with a range extending from Labrador, Canada, to Florida, USA (Rago et al. 1998, Stehlik, 2007).

The genus *Squalus*, falls within a basal group of gnathostomes—the elasmobranchs. The elasmobranchs comprise all species of skates, rays and sharks. Our species, *S. acanthias* belongs to the order Squaliformes, which appeared in the early Cretaceous, although major diversification occurred more

recently between 25—75 Ma, from the Late Cretaceous to Paleogene (Sorenson et al. 2014).

Although the elasmobranchs are basal in some aspects, they have rather derived reproductive characteristics, notably that some are viviparous (Parsons & Grier, 1992). Some elasmobranchs are oviparous—they release fertilized eggs into the environment in a protective egg casing in which the embryos develop during a time period ranging from approximately two months to more than a year, depending on the species (Compagno, 1990; Wourms, 1977; Conrath, 2005).

In contrast, the aplacental viviparous *S. acanthias* females retain their eggs and the embryos develop within the mother sustained by individual yolk sacs (Wourms & Lombardi, 1992; Conrath, 2005). The gestation for viviparous species is considerably longer, ranging from six months to more than two years (Compagno, 1990). *S. acanthias* has one of the longest gestation periods of any living vertebrate—up to 24 months (Ketchen, 1972; Nammack et al., 1985; Jones, 2001). (Placental viviparity occurs when the yolk sac joins with the uterine wall, effectively creating a placenta through which nutrients can be passed from the mother to the developing embryo (Hamlett et al. 1985; Conrath, 2005).) These reproductive strategies are rare in bony fishes (Parsons & Grier, 1992).

We have a good general understanding of the reproductive features involved in *S. acanthias* copulation; however, more research needs to be conducted in order to determine if the features exhibit variations in shape and then consequently develop a better understanding of the relationship between male and

female copulatory organs. Male *S. acanthias*, like all other sharks, have two claspers, modified pelvic fins that have evolved into reproductive appendages, that extend caudally from the posterior base of the two pelvic fins (Fishbeck & Sebastiani 2015; Hamlett, 2005). The primary male and female reproductive organs, the testes and ovaries, are located dorsal to the liver, near the cranial dorsal body wall of the organism (Fishbeck & Sebastiani 2015).

During fertilization, the male inserts one or both claspers into the female cloacal opening. Then sperm is ejaculated from the male urogenital papilla into a groove on one of the claspers; the sperm flows along this groove into the female (Compagno, 2003; Hamlett, 2005). Each clasper has a spine located on the distal, lateral surface, which is used to aid in the stabilization of the clasper within the female (Hamlett, 2005). It is possible that the clasper spine attaches to the female genital papillae, which is composed of thicker tissue and therefore may be less susceptible to damage; however, this has yet to be investigated (Brennan, Pers. comm).

Female *S. acanthias* have two oviducts, which run posteriorly along the dorsal body wall and continue in a caudal direction, turning into vaginal tissue that continues to the cloacal opening (Fishbeck & Sebastiani 2015). The urinary papilla, through which urine is excreted, is located in the middle of the cloaca, and slightly protrudes from the cloacal opening (Fishbeck & Sebastiani 2015).

In this study we aimed to explore three specific questions. By using traditional linear morphometrics we examine the relationship between the

allometry of the vagina, and the allometry of the pectoral fin. We expect that as females get larger their vagina and fins will get larger, but if vaginas are sexually selected, then vaginas may have a steeper allometric relationship than pectoral fins, meaning they get larger faster than pectoral fins. This pattern has been found before in insects (Eberhard et al. 1998). This study allows us to identify possible variation in the evolutionary natural and sexual selective driving forces and their degree of influence on the developing anatomy of female *S. acanthias*.

We use 2-D and 3-D geometric morphometrics (GM) to determine whether there are differences in vaginal shape that are correlated with reproductive stage (Visibly Pregnant vs. reproductive but Not Visibly Pregnant females), and explore the natural variation in shape found in our sample. We expect that Visible Pregnant females will likely be stretching their reproductive tract to accommodate the embryos, and therefore their vaginal shapes may be different from Non Visibly Pregnant females. The third question concerns the comparison of the results of these two GM methods to establish whether 2-D or 3-D is the best approach to study these structures. 2-D is easier and requires less computational intensity, but it necessarily neglects some components of shape, while 3-D incorporates the functional complex shape, but requires a more difficult analysis. If 2-D and 3-D give the same answers, then 2-D would be a better option moving forward, but if they give different answers, then we would need to explore how the results differ and what they may mean for future studies of genital shape. 3D geometric morphometrics have never been used to study shape

variation of female genitalia, so the impact of applying this technique for the first time to vaginal shape is important.

METHODS

DISSECTION:

A total of 22 female specimens obtained from the National Oceanic and Atmospheric Administration (NOAA), were measured and dissected to remove female reproductive tracts for this study. 22 specimens were included in the ventral 2-D picture analyses, and 25 specimens were included in the 3-D analyses of silicone models made from the lumen of the vagina. All specimens were collected off the coast of Rhode Island during late August/early September of 2017. One vagina, from specimen SA25, ripped during the removal of the silicone mold so we were unable to capture a 2-D photograph. We included one 2-D image and three 3-D silicone molds obtained from previous dissections of *S. acanthias* in our 2-D and 3-D analyses.

LINEAR MORPHOMETRICS:

Linear measurements of the shark body, pectoral fins, oviducts, genital papillae, sperm glands, egg diameter, and length of each embryo were recorded with a ruler to the nearest mm; the cloacal slit was measured with a dial caliper to the nearest half mm (Figure 1). To determine the allometry of the pectoral fin we measured the distal and proximal length as well as width for both pectoral fins. To determine the allometry of the vagina we measured the cloacal slit length, the length of both oviducts, and the length and width of both sperm glands. For this research, we define the vagina as the region starting at the change from oviduct

tissue to vaginal tissue that extends and includes the cloacal slit. We identify oviduct tissue as thin, red-colored tissue with a wrinkled vein-covered surface, and vaginal tissue as thicker, light-pink to white colored tissue with a smooth surface (no veins visible) (Figure 4).

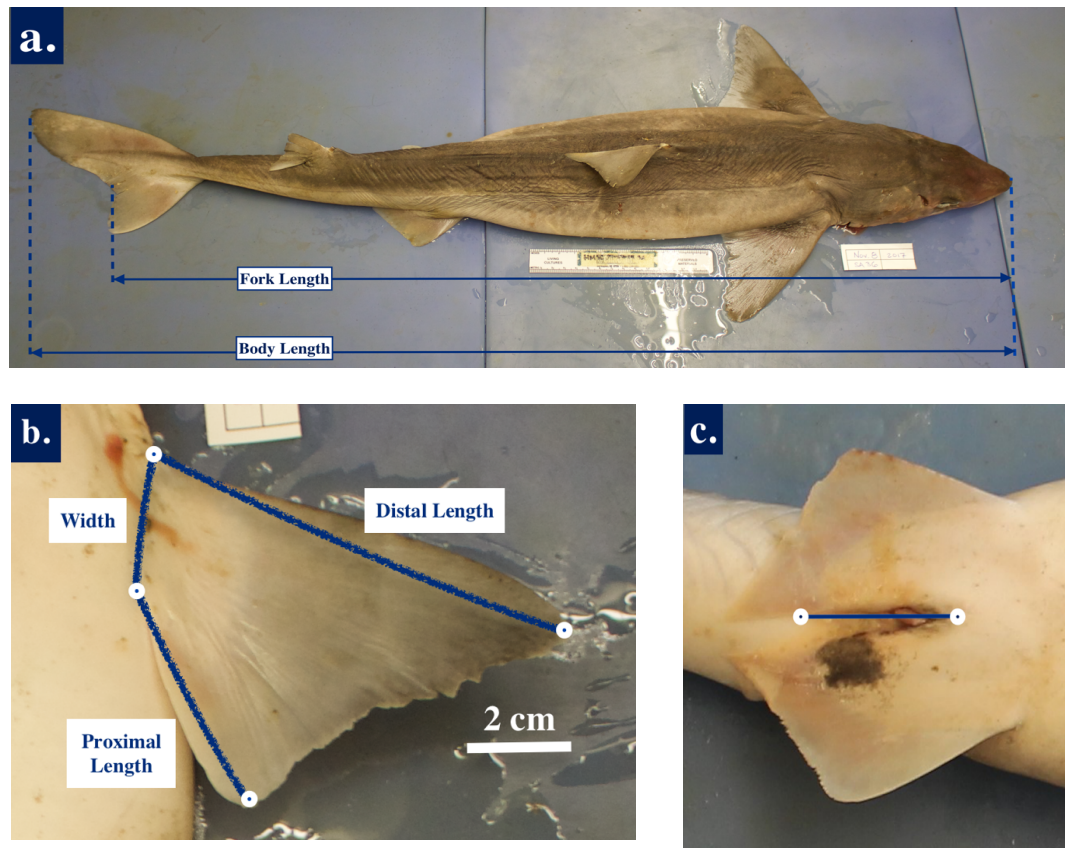


Figure 1: Pictures of *S. acanthias* female specimens, blue lines represent the measured distances for (a.) fork length and body length, (b.) the left pectoral fin distal length, proximal length and width, and (c.) the cloacal slit length.

We recorded the distal length, proximal length, and width for both of the left and right pectoral fins. The cloacal slit measurement was defined by the anterior origin of the opening to the furthest extent of the boundary where the smooth reproductive tissue meets the rough dermal scales. We also recorded the body length of each developing baby found within the pregnant specimens.

During dissection, I determined the reproductive status of each female as belonging to one of three categories: Visibly Pregnant—specimens which had babies or visible embryos within their oviducts, Not Visibly Pregnant but reproductively mature—specimens which did not have babies or visible embryos but did have eggs or egg yolk in their oviducts, and Non Reproductive—specimens that did not have babies, visible embryos, eggs, or egg yolk within their oviducts.

2-D GEOMETRIC MORPHOMETRIC ANALYSIS

We dissected out the entire vaginal pouch including at least 2 cm of oviduct tissue on both sides of the structure. This dissection included the cloacal slit and the posterior region of the oviducts. We removed connective tissue to better visualize the entire structure, and trimmed the cloacal slit to a narrow band. We aligned and photographed the specimen in a ventral up position to capture their 2-D shape. We photographed all specimens and models with the Canon EOS Rebel T5i, and we included a ruler for scale in all photographs taken for the 2-D analyses. To reduce specimen placement error due to the ambiguity of placing soft tissues on a surface, I photographed the image from the first vagina we dissected and cut out and laminated the silhouette to be used as a template for placement of all subsequent samples (Figure 2). The template set a fixed angle for the distance between the two oviducts and helped ensure that any changes in shape between the specimens was due to natural variation.

We included 22 vaginas in the 2-D analysis, each of which we photographed 3 times. We photographed the first specimen placement twice, first with small pins on the specimen marking the location of the 12 landmarks and then again without the pin-markers (Figure 3). The first set of non-pinned photographs we used for the main natural variation analysis, and we used the following two sets of photographs, Set 2 and Set 3, to calculate landmarking error. Before each consecutive photo I picked up the entire vagina and gently swirled it in a beaker of water and then reoriented it on the template to ensure that any

variation from the act of rearranging the soft tissue would not be identified as natural variation. I edited and landmarked each set of photographs on a separate day to account for possible error in my placement of the landmarks. I utilized the Notepad program to monitor and double-check the progress of the editing.



Figure 2: Laminated template (left) used to position specimens in the 2-D analysis and one of the specimens (SA35) (right) which we oriented with the template.



Figure 3: Photograph with pin-markers identifying the location of the 12 landmarks (left) and the photograph without pin-markers (right). The first set of non-pinned photographs was used for the main 2-D natural variation analysis, and the set of photographs with pin-markers was used as a guide for identifying landmarks on the images within the computational TPS geometric morphometrics software, tpsDig.

I uploaded and edited each set of photographs in the TPS geometric morphometrics software by F. James Rohlf, tpsUtil and tpsDig (Rohlf, 2007). First, I created a TPS file by uploading one set of photographs into tpsUtil under the operation: “build a TPS file”. Then I opened this raw TPS file of photographs in the program, tpsDig. In tpsDig I set a scale bar and identified and marked the 12 landmarks for each photograph—using the photographs with pin markers as a guide. Landmarks were added to each image in the tpsDig program by first selecting the crosshairs icon and then clicking once on the image to place a landmark—we used the arrow tool to readjust the placement of existing landmarks as needed; it was very important that the same sequence in which all landmarks were identified and marked, stayed the same for every specimen.

Landmarks are features that can be reliably found on all specimens and that capture at least some points of variation across specimens. Landmarks form a “map” of the shape of the genitalia and variance is measured by the degree of change in the landmarks’ relative location to one another. The twelve landmarks we selected for the 2-D analysis are shown in Figure 4.

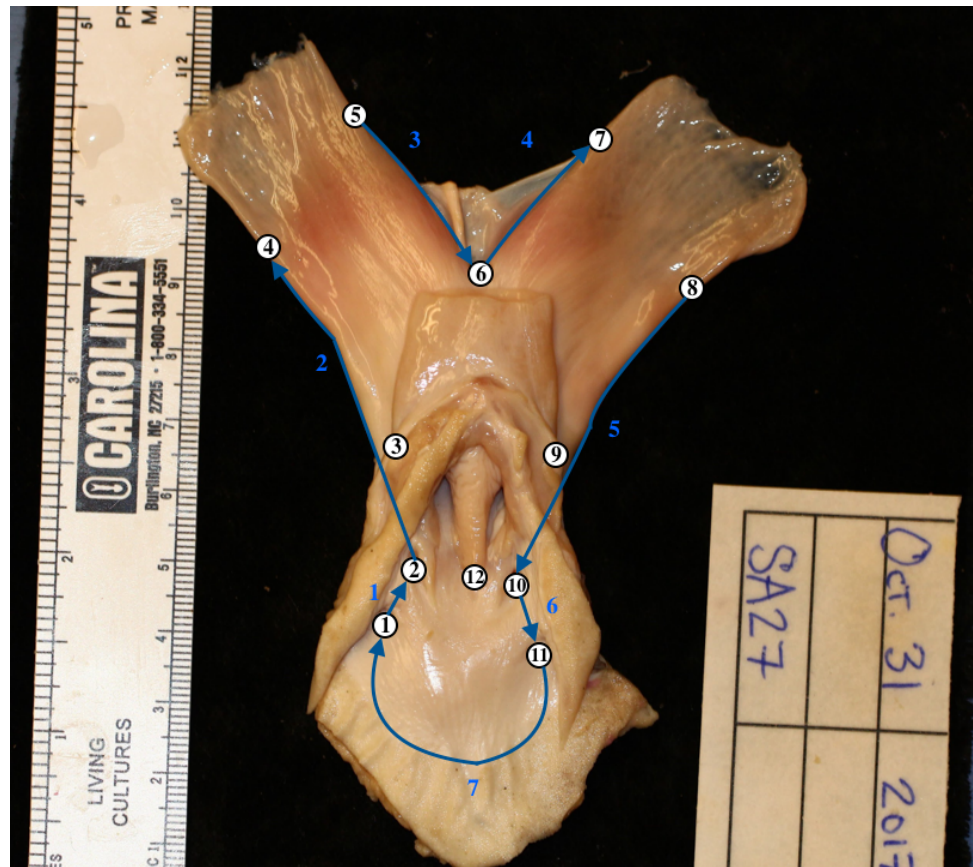


Figure 4: 2-D Ventral image of *S. acanthias* vagina with 12 Landmarks and 7 semi-landmark curves (blue lines) each numbered in the order in which they were identified and marked in the program. Each landmark represented a distinguishable feature: ① right lower papilla ② right abdominal pore ③ right point of connection between intestine and vaginal tissue, ④ farthest extent of vaginal tissue on the right edge of the right oviduct, ⑤ farthest extent of vaginal tissue on the left edge of the right oviduct, ⑥ center of notch at the start of oviduct bifurcation, ⑦ farthest extent of vaginal tissue on the left edge of the left oviduct, ⑧ farthest extent of vaginal tissue on the right edge of the left oviduct, ⑨ left point of connection between intestine and vaginal tissue, ⑩ left abdominal pore, ⑪ left lower papilla, ⑫ tip of urinary papilla.

After I landmarked all photographs in one set, I drew 7 curves with semi-landmarks by selecting the Pencil icon and left clicking the image to place the

semi-landmarks along the predetermined curve boundaries—these curves outline the shape of each vagina. Semi-landmarks are used to capture the contour of a structure and are typically placed between two landmarks. Each curve started and ended at the center of one of the 12 anchored landmarks. I edited each curve to have a set number of semi-landmarks equidistant from one another; first I highlighted the curve, then right clicked and selected the function "resample curve : by length", then entered the number of semi-landmarks that would make up each curve. For the 2-D analyses: Curve 1 and Curve 6 each had 5 semi-landmarks, Curve 3 and Curve 4 each had 7 semi-landmarks, and Curve 2, Curve 5, and Curve 7 each had 15 semi-landmarks (Figure 4).

I saved the landmarked and curved TPS file in tpsDig and then uploaded the file into tpsUtil with the function: "append tps curve to landmarks"; under this function every semi-landmark now operated like a landmark. I then re-inputted the new appended-curve TPS file back into tpsUtil under the function: "delete reorder landmarks", and then selected and deleted all semi-landmarks that overlapped the anchored landmarks. The deletion of these overlapping points prevented some points from having more degrees of freedom than others. I deleted two semi-landmarks from every curve—the starting point and the ending point. Under the "delete reorder landmarks" function I was able to apply the deletion of all overlapping semi-landmark points to all photographs in the set.

Then I uploaded the new curve-reduced TPS file into tpsUtil under the function: "make sliders file"; in this function I re-identified and selected the points

that were the semi-landmarks or the points that will “slide” and move in relation to the anchored landmarks. After marking all points that were semi-landmarks the “make sliders file” function applied the changes to all photographs in the set and created a new NTS file. We then uploaded the curve reduced TPS file and NTS sliders file to R. Further 2-D analyses were performed by Dr. Brandon Hendrick, a postdoctoral fellow at Harvard University. The details of the statistical analysis can be found in the appendix, as written by Dr. Hendrick.

3-D GEOMETRIC MORPHOMETRIC ANALYSIS

We used Elite HD+ Super Light Body dental Silicone TM to make 3-D molds of the complete vaginal structure for each specimen (Figure 5). Each silicone mold was photographed on a rotating stool completely around its circumference, so that at least 70 photos were obtained per mold to be used in model reconstruction. We used the photos to generate a 3-D computerized model using the photogrammetry program, 3DF Zephyr lite.

I uploaded the 3-D models into Autodesk R Recap Studio and set a scale bar for each model, then uploaded the scaled 3-D models into Landmark Editor (IDAV) and placed 16 landmarks on each of the models, making sure to cover areas all around the 3-D structure. First, I uploaded the 3-D model into the Space A or Space B region, selected the add-landmark symbol function: a red box with a yellow arrow (on the top of the screen it is the fourth icon from the left), and then by holding down the shift key while clicking once on the model I was able to

place a landmark on a specific location; the hand symbol could be selected and used to adjust the placement of existing landmarks. Just as in the 2-D analysis, landmarks had to be placed in the same sequence for every 3-D model in order to accurately capture the shape and analyze for variation (Wiley et al. 2002-2006). We used the 3-D silicone models as a guide for identifying the specific location of the landmarks on the computerized 3-D models.

These landmarks worked the same way as the 2-D analysis, so that we could map and graph the morphological variation in the vagina. We then drew a total of 36 curves with intermediate landmark points in the regions between the landmarks to outline the shape of each vagina. Intermediate landmark points were drawn by selecting the semi-landmark curve function: represented by a line through a red, white and green dot (on the top of the screen it is the fifth icon from the left). After the semi-landmark curve function was selected we then again held down the shift key and clicked on the image to place the appropriate semi-landmarks. By outlining the complex shape in 3DF Zephyr lite we could computationally wrap and analyze a grid around the 3-D specimen in order to measure variation in shape. The 16 landmarks and 36 curves used in the 3-D analyses are shown in Figure 6. These 3-D computerized models were then morphologically analyzed for variation in shape by Dr. Brandon Hendrick (see appendix for details of statistical analyses written by Dr. Hendrick).

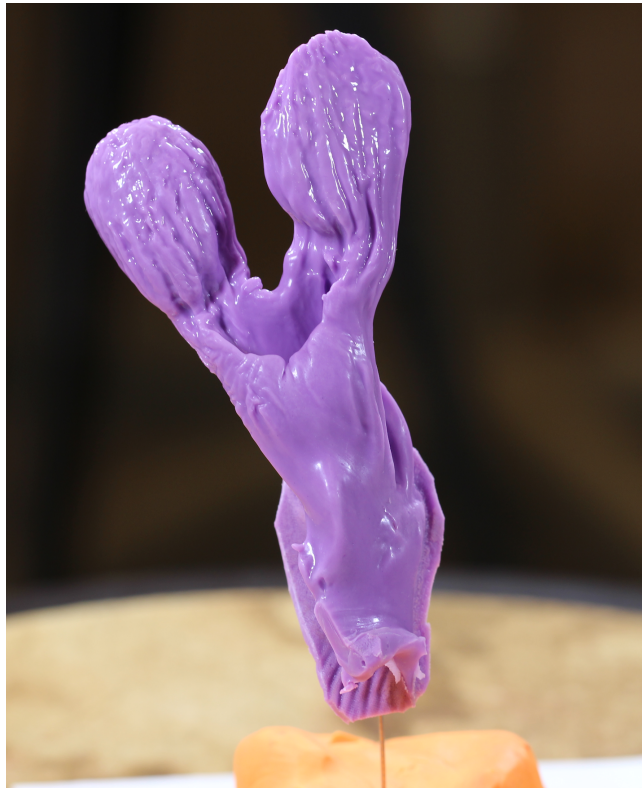


Figure 5: Silicone internal mold of female *S. acanthias* genitalia. We positioned each silicone mold upright on a rotating stool and photographed the entire circumference in order to create a computerized 3-D model from the collection of photos.

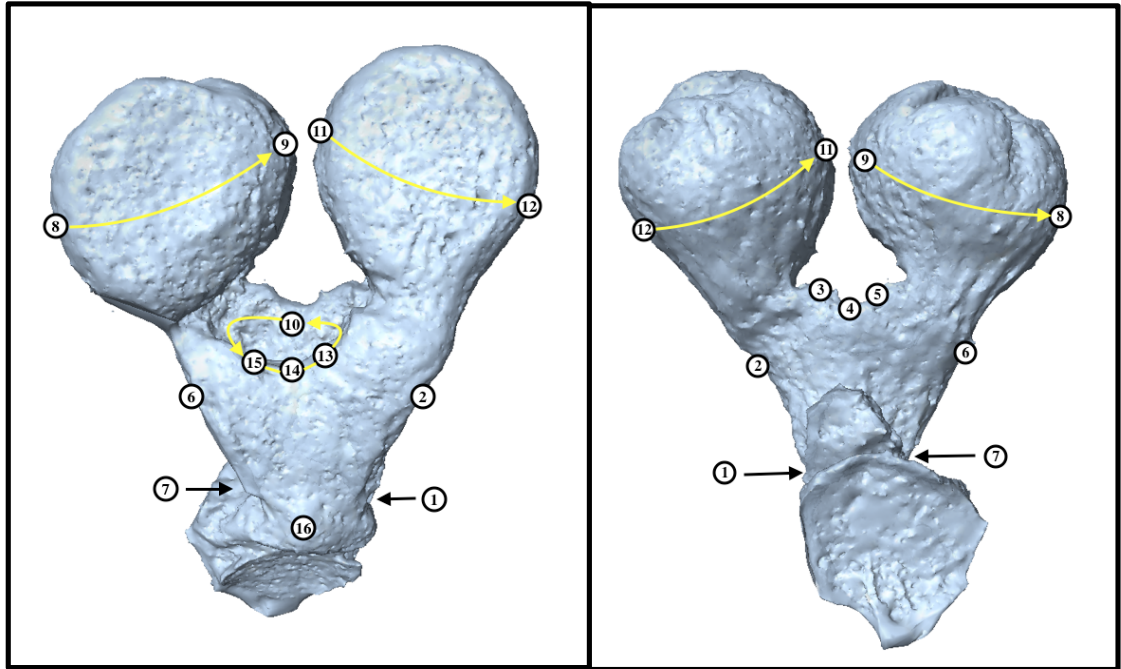


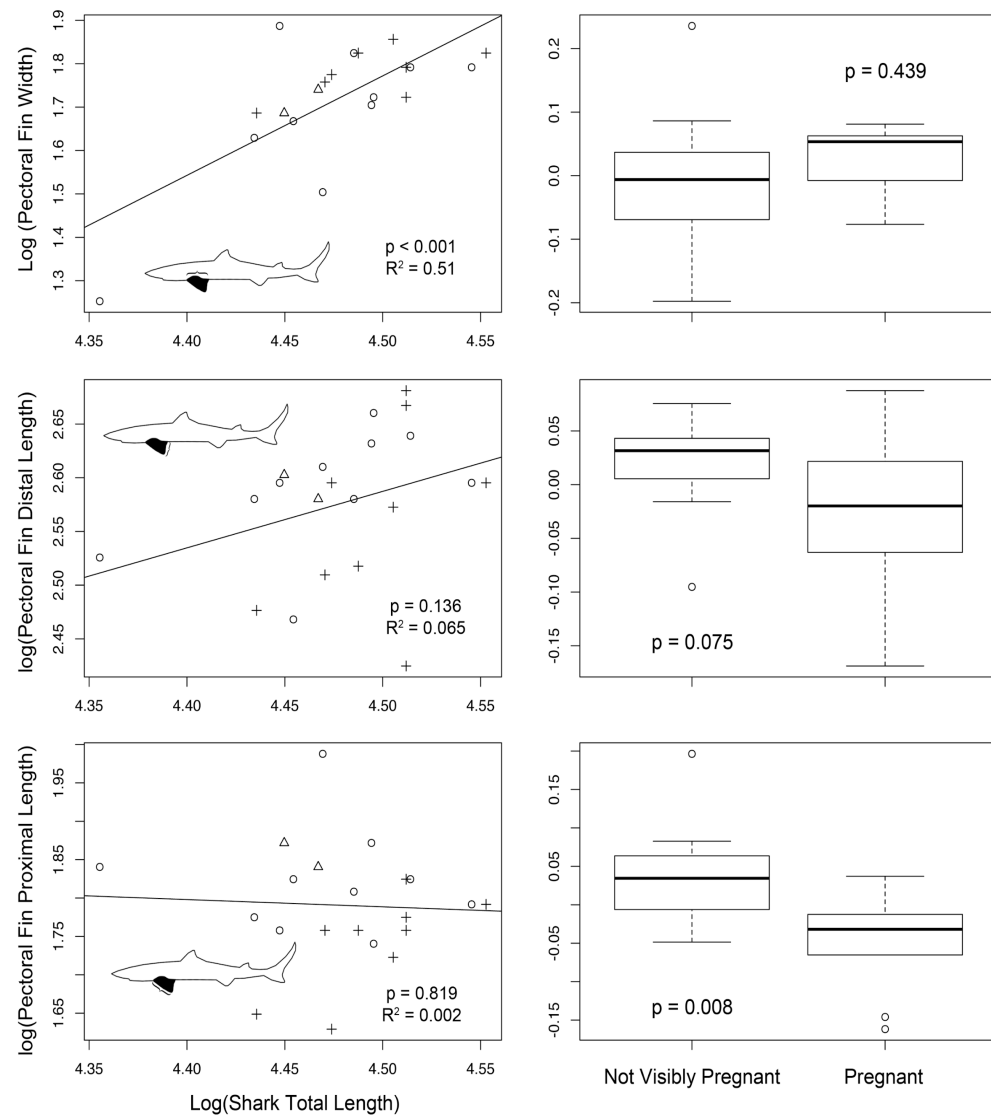
Figure 6: 3-D computerized model of *S. acanthias* vagina (specimen SA35) with 16 Landmarks and 33 semi-landmark curves (yellow lines) each numbered in the order in which they were identified and marked in the program. Each landmark represented a distinguishable feature: ① right intestine termination point, ② location of hymen on right side of right oviduct, ③ location of hymen on left side of right oviduct, ④ center of notch at start of oviduct bifurcation, ⑤ location of hymen on right side of left oviduct, ⑥ location of hymen on left side of left oviduct, ⑦ left side intestine termination point, ⑧ left side of left oviduct connection between vaginal and oviduct tissue, ⑨ right side of left oviduct connection between vaginal and oviduct tissue, ⑩ highest point in genital papilla space, ⑪ left side of right oviduct connection between vaginal and oviduct tissue, ⑫ right side of right oviduct connection between vaginal and oviduct tissue, ⑬ point of termination of hymen on dorsal right, ⑭ center notch in genital papilla space, ⑮ point of termination of hymen on dorsal left, ⑯ highest point on dorsal raised protrusion.

RESULTS

LINEAR MORPHOMETRICS:

We were looking for allometric differences in vagina length and pectoral fin length (or width), and also for differences in the length of the vagina from Visibly Pregnant vs. reproductive but Not Visibly Pregnant females. We found no significant differences in any of these patterns (Figure 7).

7a.



7b.

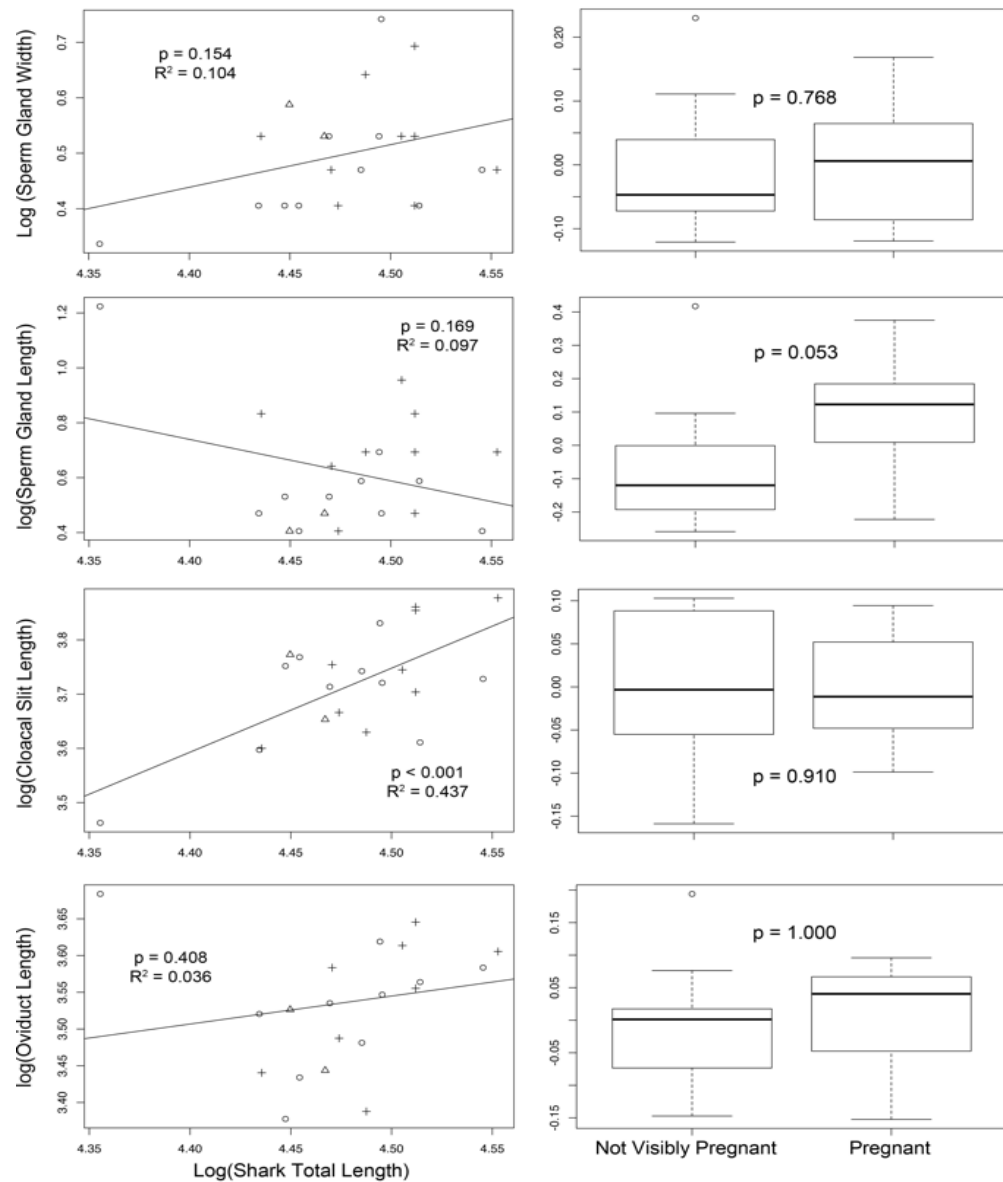


Figure 7: Graphs of allometric relationships between Not Visibly Pregnant and Pregnant specimens for pectoral fin measurements (7a.) and reproductive characters (7b.). The fin characters used for the allometric analysis were (a.) the pectoral fin width, (b.) pectoral fin distal length, and (c.) pectoral fin proximal length. The reproductive characteristics were (a.) sperm gland width, (b.) sperm gland length, (c.) cloacal slit length, and (d.) oviduct length. Each shape represents one shark; triangles are Non-Reproductive, circles are Not Visibly Pregnant, and pluses are Visibly Pregnant specimens. All p values were not significant (except for the relationship between pectoral fin width and body size, and cloacal slit length and body size) and all R values were small.

2-D GEOMETRIC MORPHOMETRICS

We used both 2-D and 3-D geometric morphometric analyses to see if there are differences in vaginal shape correlated with changes in the reproductive state, specifically between Visibly Pregnant vs. Not Visibly Pregnant females. The results of the ANOVAs are summarized in Table 1. Using only the raw data, it is difficult to describe and visualize what the shape differences are between visible pregnant and non-visibly pregnant females when examining the whole morphospace as shown in Figure 8a, where is clear that there is almost full overlap in the 2D shapes of the two reproductive states within the morphospace. In other words, Visibly Pregnant females do not cluster in a particular region of the morphospace away from Not Visibly pregnant females in the 2-D analysis.

Table 1: Results of the ANOVAs for the 2-D GM analysis, the 2-D GM symmetric component of shape variation analysis, and the 3-D GM analysis.

2-D Shark Shape by Reproduction Status (not including replicates, n = 21)							
Shape by Number of Babies							
	df	SS	MS	R ²	F	Z	P.value
Number of Babies	1	0.012449674	0.012449674	0.059512384	1.202286207	1.101219459	0.277
Residuals	19	0.196745006	0.010355				
Total	20	0.20919468					
Shape by Reproductive State							
	df	SS	MS	R ²	F	Z	P.value
Reproductive State	2	0.030619658	0.015309829	0.146369202	1.543199728	1.415731504	0.061
Residuals	18	0.178575022	0.009920835				
Total	20	0.20919468					
2-D Shark Shape by Reproduction Status (including replicates, n = 18•3, 54)							
Symmetric Component of Shape by Reproductive State (Three Reproductive State Categories)							
	df	SS	MS	R ²	F	Z	P.value
Reproductive State	2	0.02644582	0.01322291	0.077376381	2.138572738	1.876658721	0.026
Residuals	51	0.315335742	0.006183054				
Total	53	0.341781563					
Symmetric Component of Shape by Number of Babies							
	df	SS	MS	R ²	F	Z	P.value
Number of Babies	1	0.020941339	0.020941339	0.061271119	3.394055787	2.720697859	0.01
Residuals	52	0.320840224	0.006170004				
Total	53	0.341781563					
3-D Shark Shape by Reproduction Status (n = 22, including NRs)							
Shape by Number of Babies							
	df	SS	MS	R ²	F	Z	P.value
Number of Babies	1	0.03747	0.037471	0.054532	1.1536	1.0213	0.298
Residuals	20	0.64966	0.032483				
Total	21	0.68713					
Shape by Reproductive State							
	df	SS	MS	R ²	F	Z	P.value
Reproductive State	2	0.08373	0.041863	0.12185	1.3182	1.2061	0.193
Residuals	19	0.6034	0.031758				
Total	21	0.68713					

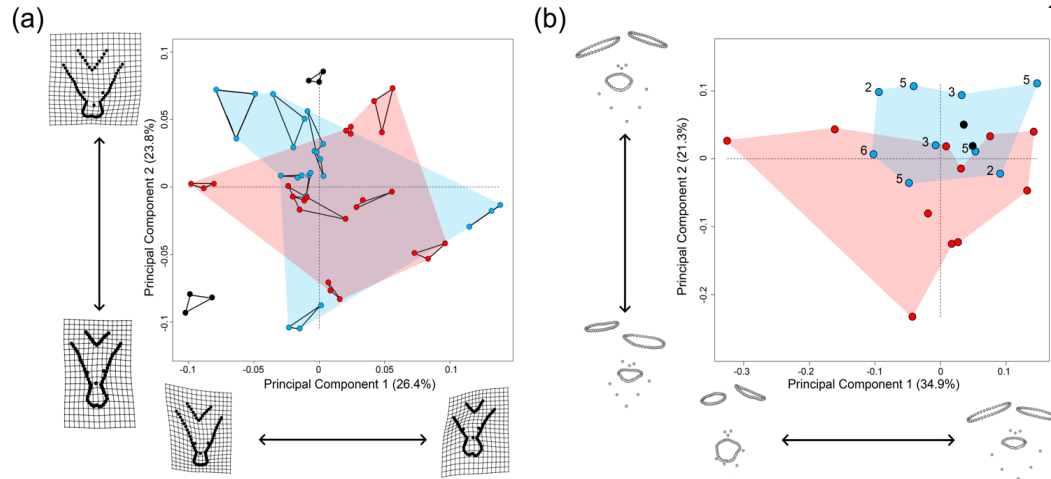


Figure 8: Graphs showing the variation in shape measured by principal components from the 2-D geometric morphometric data (a.) and the 3-D geometric morphometric data (b.) with TPS grids and landmark configurations showing the major shape changes occurring along principal components 1 and 2. In the 2-D GM analysis (b.) the 1st principal component represents a vaginal shape with a narrow center and left asymmetry (left) that transitions to a vaginal shape with a more open center and right asymmetry (right); the 2nd principal component represents a vaginal shape that is long, thin and narrow (bottom) that transitions to a shorter, wider, and more open vaginal shape (top). In the 3-D GM analysis (a.) the 1st principal component represents a long, thin and narrow vaginal shape (left) that transitions to a wider, squatter, and more centrally open vaginal shape (right); the 2nd principal component represented a left asymmetric vaginal shape with a narrow central region (bottom) that transitions to a symmetric, wider vaginal shape with a more open central region (top). Black represents Non-Reproductive, red represents Not Visibly Pregnant, and blue represents Visibly Pregnant specimens. For the 2-D analysis the three points comprising each triangle represents the three photographs we took for each vaginal specimen. The different length of the lines connecting the points of each triangle illustrate the degree of variation in shape from measurement error. The 2-D analysis with replicates detected significant changes in genital shape associated with changing reproductive state, while the 3-D analysis did not.

The 2-D geometric morphometric analysis did not detect significant variation in shape in relation to reproductive state ($p = 0.06$) or number of babies ($p=0.21$), as shown in Figure 8a. However, we noticed that the vagina of spiny dogfish sharks was quite asymmetric, and we examined whether this asymmetry

could change the relationship between shape and reproductive state. The asymmetry analysis compiled all detected variation from the 2-D GM analysis with replicates, and then measured the percent of variation resulting from symmetric variation, individual variation, directional asymmetry, and fluctuating asymmetry. Fluctuating asymmetry, when either side of a feature is larger than the other, rather than one side always being larger than the other (directional asymmetry) is an important component of shape and accounted for 30% of the detected morphological variation. Symmetry accounted for 59% of the total variation, measurement error accounted for 10% of the total variation, and directional asymmetry accounted for <1% of the total variation.

The asymmetry analysis showed that there was a large degree of variation within our 2-D asymmetric specimens and their replicates associated with fluctuating asymmetry. Often studies on morphological variation will conduct an analysis to look for variation without the presence of fluctuating asymmetry because it acts as an overlying noise parameter that can obscure more subtle underlying variations in shape. Fluctuating asymmetry is a measure of the developmental instability or randomness of the shapes to have either side larger than the other; by removing it we are able to better see subtle variations in shape and gain a more accurate understanding of the degrees of influence of certain selective pressures.

We reran all of the 2-D GM analyses on the symmetric component of shape variation. In this analysis we looked for more subtle morphological

variations within the previously detected 59% symmetry variation by dropping fluctuating asymmetry and the original measurement error. The original measurement error was dropped because it was calculated when fluctuating asymmetry was still included in the 2-D GM analysis and also acts as noise. The results of the analysis of the symmetric component of shape variation are summarized in Table 1. This second 2-D GM analysis detected significant variation in vaginal shape by reproductive state ($p = 0.026$, $R^2 = 0.077$), and significant variation in vaginal shape by number of babies ($p = 0.01$, $R^2 = 0.061$).

We conducted further analyses of allometric relationships with the symmetric component of shape variation between both the 2-D vaginal shape and vagina centroid size, and the 2-D vaginal shape and the total length of vagina (Figure 9). The allometric relationship was significant for centroid size ($p = 0.017$, $R^2 = 0.058$), but not total body length ($p = 0.078$, $R^2 = 0.038$). This significant allometric relationship between shape and size supports our original expectation that as the size of the vagina increases the shape of the vagina becomes wider and more open, likely to accommodate for birth.

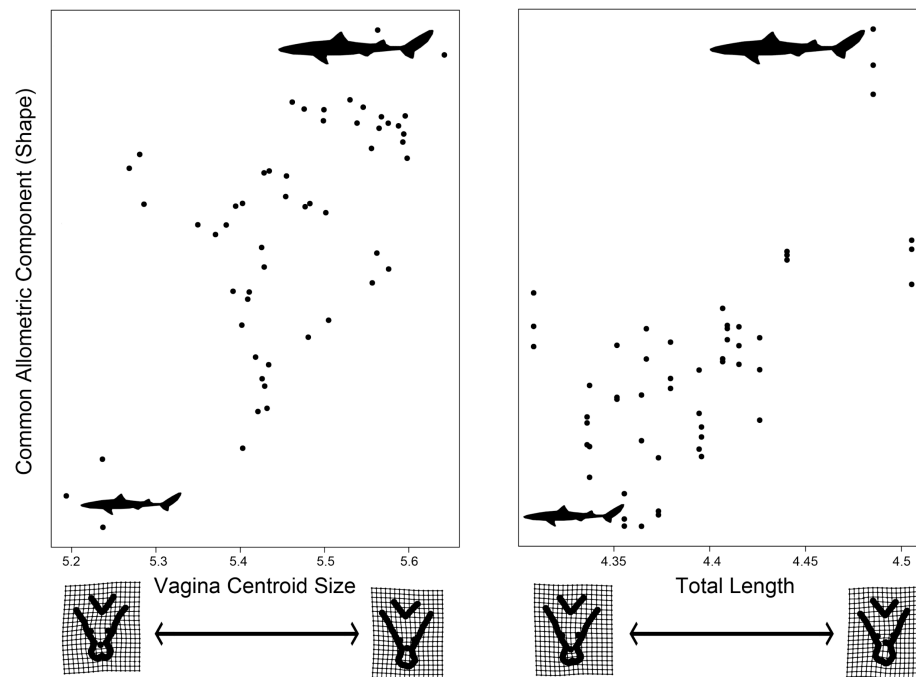


Figure 9: Graphs of allometric relationship for 2-D geometric morphometric data between the common allometric component of shape and vagina centroid size (left graph) and total length (right graph). TPS grids demonstrate shape changes from smaller sharks (left) to larger sharks (right).

3-D GEOMETRIC MORPHOMETRICS

As mentioned previously, we used both 2-D and 3-D geometric morphometric analyses to see if there are differences in vaginal shape correlated with changes in reproductive state. The more complex 3-D analysis captured the complete internal vaginal shape, allowing us to identify more landmarks and curves to create and analyze a more complex shape (Figure 10). The 3-D geometric morphometric analyses, unlike the 2-D analysis, did not detect a significant difference in vaginal shape associated with the reproductive state of the *S. acanthias* specimens, and yet, there is a clear clustering of Visibly Pregnant females in one region of the morphospace, separate from Not Visibly Pregnant

females (Figure 8b.). It seems that Visibly Pregnant individuals (blue) have more symmetrical, squatter vaginas with wider oviducts, while Not Visibly Pregnant individuals (red) have a wider range in shape from asymmetrical to symmetrical as well as from long and thin to wide and squat vaginal shapes. The two individuals labeled Non-Reproductive were graphed within the region of Visibly Pregnant individuals, indicating that they too had more symmetrical, shorter vaginas with wider oviducts (Figure 8b.).

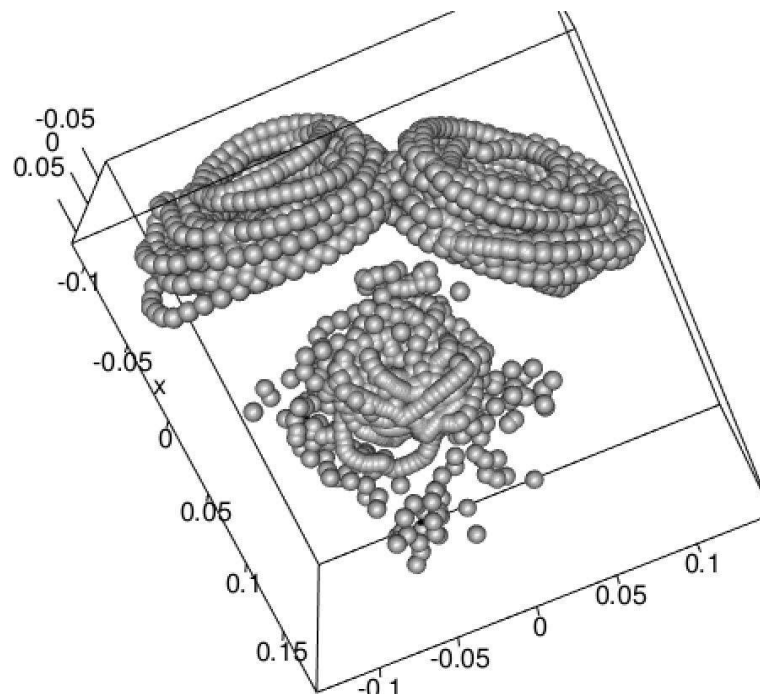


Figure 10: The consensus shape of all landmarks of the 25 specimens included in the 3-D geometric morphometric analysis graphed in the same shape space. The more complex 3-D method captured the complete internal vaginal shape, and this figure illustrates the array of all landmarks in the space.

Figure 11 shows the results of an allometric analyses of the relationship between the 3-D vaginal shape and the vagina size, and the 3-D vaginal shape and

the total length of the vagina. The relationship between the 3-D vaginal shape and the vagina centroid size was significant ($p = 0.025$, $R^2 = 0.10$), but was only marginally significant with shark total length ($p = 0.064$, $R^2 = 0.094$). We found that neither reproductive state ($p = 0.193$) nor the number of babies ($p = 0.298$) was significantly correlated with 3D shape, despite what seemed a clear separation of shape in morphospace as shown in Figure 8b.

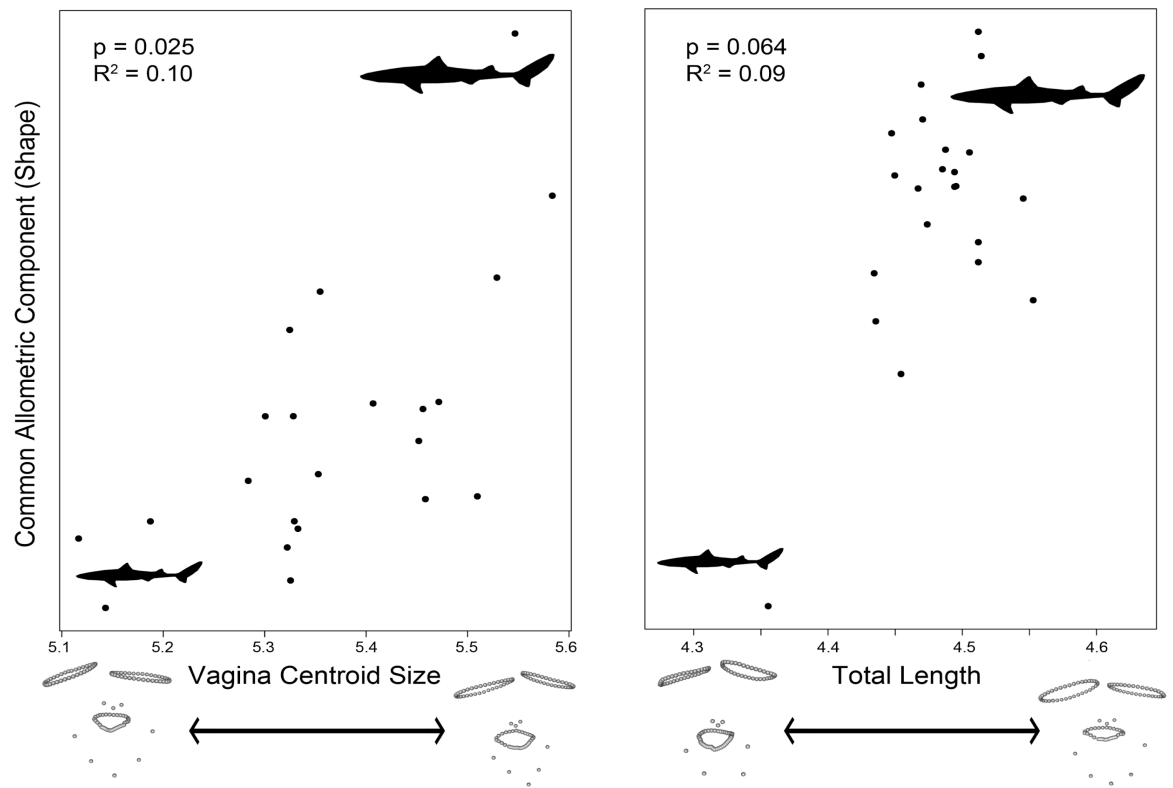


Figure 11: Allometric relationship for 3-D geometric morphometric data between the common allometric component of shape and vaginal centroid size (left graph) and total length (right graph). Landmark coordinates demonstrate shape changes from smaller sharks (left) to larger sharks (right).

DISCUSSION

We expected to see an allometric trend in the fin characters with no significant difference between Not Visibly Pregnant and Pregnant specimens, and an allometric trend in the reproductive characters with a significant difference between Not-Visibly Pregnant and Pregnant specimens, but the data showed no allometric trend. The absence of an allometric trend is likely due to the close range of body sizes of all our specimens. The longest specimen was 94.9 cm (fork length of 84.8 cm) and the shortest specimen was 77.9 cm (fork length of 69.9 cm). The significant allometric relationship between pectoral fin width and body size and cloacal slit length and body size, is likely explained by the fact that we were measuring a feature directly connected to the main body, so that any minor change in body length would change the length of the feature. Future studies with a wider range of body sizes will hopefully be able to shed more light on the allometric relationship of vaginas in comparison to other allometric character relationships. Therefore we could not test the hypothesis that shark vaginas may be under increased sexual selection if they demonstrate positive allometry.

The 2-D analysis with replicates detected significant variation in genital shape associated with changes in the *S. acanthias* reproductive state, and with the number of babies in the oviduct, but only when accounting for fluctuating asymmetry. Surprisingly, the plotted genital shapes of the two main reproductive groups had minimal separation in the morphospace, and therefore this variation is difficult to explain because there is extensive overlap between the Visibly

Pregnant and Not Visibly pregnant shapes (Figure 8). Although the 3-D analysis was more complex and looked at the entire internal vaginal shape, further geometric morphometric analyses should include two sets of error models to better understand the different degrees of variation detected by the 2-D and 3-D methods.

We analyzed the significant variation detected by the 2-D geometric morphometric analysis and found that symmetry accounted for 59% of total variation, directional asymmetry accounted for <1% of total variation, fluctuating asymmetry accounted for 30% of total variation, and measurement error accounted for 10% of total variation. The low directional asymmetry suggests that *S. acanthias* vaginas are not typically right or left dominant, while the considerable degree of fluctuating asymmetry is likely a result of the action of giving birth to offspring that are positioned offset to one another within the two oviducts. The relatively high measurement error is likely a result of working with soft tissue, despite our best efforts to standardize sample position. Future studies of GM with soft tissues should make sure to use these and other techniques to minimize error.

As mentioned previously, the 3-D geometric morphometric analysis did not detect any significant variation in genital shape associated with changes in reproductive state, however the plotted genital shapes of the two main reproductive groups had clear separation in the morphospace (Figure 8b.). The graph does suggest that Visibly Pregnant sharks appear to have more

symmetrical, shorter and wider vaginas with wider oviducts. These results partially support the idea that the vagina and oviducts stretch and change shape in order to accommodate for the growth and eventual birth of the developing young. The Non-Visibly pregnant sharks have a much wider range in vaginal shape, and we suspect that this also because some of these sharks could have been pregnant and given birth in the time before they were caught. It is possible that the Non-Visibly pregnant sharks that have more asymmetrical, longer vaginas with thinner oviducts have never been pregnant, but more research needs to be conducted with a wider range of specimens in order to better understand how the female genitalia shape changes with maturation and changing reproductive state.

Although we originally identified two Non-Reproductive sharks in our study, the two females had body lengths of 85.6 cm (fork length of 76.4 cm) and 87.1 cm (fork length of 79.3 cm) which are comparable and exceed the body sizes of some of the Visibly-Pregnant sharks in our study. Other studies have reported that female *S. acanthias* reach sexual maturity at approximately 75 cm (approximately 12 years of age) (ASMFC: Spiny Dogfish, 2018). This information suggests that the Non-Reproductive sharks were not the youngest specimens in our study and may have been pregnant and given birth prior to being caught.

A study of *S. acanthias* in the Eastern Black Sea found that the egg diameters increase from 10 to 12 months into the pregnancy, but before this

period the diameters of fertilized eggs and unfertilized eggs cannot be distinguished as both their diameters are ≥ 1.5 cm. (Although their study focused on *S. acanthias* in the Eastern Black Sea the authors noted that the reproductive characteristics they discuss are similar to those of populations in the Northern Hemisphere (Demirhan & Seyhan, 2007).) The diameter of the eggs found in the two Non-Reproductive specimens was approximately 2 cm and 0.4 cm, respectively. The egg diameter also suggests that at least one of the two Non-Reproductive sharks might have been reproductively mature and possibly pregnant before it was captured. Additional future research could investigate if early developing sharks start out with more symmetrical genitalia and then get asymmetrical with growth. We are cautious in our speculation on the shape of the Non-Reproductive sharks genitalia due to the minimal number of specimens in our study.

The results of this study suggest that the more complex 3-D geometric morphometric method may not be more effective at identifying variation in shape than the traditional 2-D geometric morphometric method. The lack of significance in 3-D despite the clear separation in morphospace is puzzling, and may have been the result of our relatively small sample size. Repeating the 3-D landmarking scheme two more times, might increase the resolution of our 3-D analyses and make it more comparable to the 2-D analysis, and this component should be added to this research.

We expected that both the 2-D and 3-D methods would detect significant variation, but that the types of variation detected would differ-but this was not the case. By incorporating two sets of error replicates in both the 2-D and 3-D GM methods we can hope to account for any sample size discrepancies, and achieve a more clear comparison between the results of both analyses.

CONCLUSION

The main conclusion of this study is that *S. acanthias* vaginal size is significantly correlated with shape—this result was significant in both the 2-D and 3-D analyses of allometric relationships with the resultant geometric morphometric data. The presence of this significant allometric relationship between shape and size supports our original expectation that as the size of the vagina increases the shape of the vagina becomes wider and more open, likely to accommodate for birth.

The information gained from this research will aid and hopefully prompt further studies on the copulatory behavior and mechanics of the different features of not only *S. acanthias*, but other species as well (Brennan, 2016). Although our results are unexpected, they highlight the current need for more research on the best methods for studying and detecting variation in shape. In utilizing the newer and more complex 3-D GM analysis future studies will likely need to include a larger sample size in order to detect significant morphological variation. Unfortunately, due to the absence of replicates in our 3-D GM analysis we were unable to conduct a parallel analysis between the two different methods, but we expect that the more complex 3-D GM analysis will likely detect significant shape variation if two sets of error replicates are included.

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APPENDIX

Statistical Analyses Methods

Written by Dr. Brandon Hedrick

Traditional morphometric analyses:

To test for allometric differences in naturally selected fin shape, with which to compare to sexually selected characters, we evaluated the relationship between total length, pectoral fin width at the base of the fin, and fin length at the proximal and distal ends with a linear model of log-transformed measurements. Genital characters were also compared with total length including sperm gland width and length, cloacal slit length, and oviduct length. Given significant allometric trajectories for some variables, residuals of each variable's relationship with total length were compared with reproductive state (hypothesis 1). Given that only two sharks were non-reproductive in this sample, non-reproductive sharks were pooled with non-pregnant reproductive sharks.

Geometric morphometric analyses:

Two-dimensional landmarks ($n = 12$) and semi-landmarks ($n = 7$ curves) were digitized on photographs in TPSDig2 (Rohlf, 2006). All landmarks were taken by Patricia A-S to eliminate inter-observer error. Each image was landmarked three separate times to quantify intra-observer measurement error given the difficulty of landmark placement in soft tissues (REF). Landmark data were then imported into R (R Core Development Team, 2016) and opened in the R *geomorph* package (Adams and Otárola-Castillo, 2013). Generalized Procrustes analysis (GPA) was performed translating, rescaling, and rotating the landmark configurations into the same shape space (Zelditch et al., 2012). Sliding semi-landmarks were slid by minimizing bending energy between adjacent semi-landmarks (Perez et al., 2006).

Given that vaginas are biologically asymmetric, we ran a multi-factor ANOVA with individuals (symmetric variation), sides (directional asymmetry), and the interaction between individuals and sides (fluctuating asymmetry) as factors. This allowed separation of the symmetric and asymmetric components of total shape variation (Klingenberg and McIntyre, 1998). We then tested whether asymmetry in the vagina made up a significant portion of total variation and also quantified the magnitude of asymmetric variation compared to the total variation using the η^2 effect size metric (Olejnik and Algina, 2003). To assess whether asymmetry was higher in non-pregnant female vaginas or pregnant females, we re-ran these analyses separating out the non-pregnant and pregnant specimens.

To test for changes in morphospace occupation, we ran a principal component analysis (PCA). We tested the relationship between total shape variation and reproductive state (currently pregnant, not pregnant, but

reproductive, and not reproductive) and the relationship between total shape variation and the number of babies present with Procrustes ANOVAs (Goodall, 1991) (hypothesis 2). Additionally, we examined these relationships with additional Procrustes ANOVAs using only the symmetric component of total shape variation. Finally, the common allometric component of shape data (Mitteroecker et al., 2004; Drake and Klingenberg, 2008) was compared to vaginal centroid size and shark total body length to evaluate the effects of allometry on the data.

Three-dimensional landmarks ($n = 16$ landmarks, 33 semi-landmark curves) were placed on 3D models using Landmark Editor (Wiley et al., 2005). Landmarks were placed into a tps format using custom code written by Andrew Conith and were then subjected to GPA with semi-landmarks slid using the bending energy criterion. Analyses in 3D followed those performed in 2D to allow for a comparison of data quality (hypothesis 4).