Copyright page: I have given permission for public access to my thesis and for copying to be done at the discretion of the archives' librarian and/or the College library.

Signature

Date

Analysis of Interspecific Genital Shape Variation in Watersnakes of the Genus *Nerodia* and the Potential for Female-driven Species Divergence

by

Juliet F. Greenwood

A Paper Presented to the

Faculty of Mount Holyoke College in

Partial Fulfillment of the Requirements for

the Degree of Bachelor of Arts with

Honor

Department of Biological Sciences

South Hadley, MA 01075

May 2021

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

ACKNOWLEDGEMENTS

I would like to sincerely thank Dr. Patricia Brennan for granting me the opportunity to participate in such exciting and unique biological research. I am grateful to have been inspired by her mentorship and curiosity over the last two years and will forever utilize the skills and ways of thinking she has introduced to me. I would also like to thank Dr. Brandon Hedrick for his help with statistical analyses, as well as Genesis Lara Granados for being my partner in learning throughout this experience and helping with the process of data collection. Lastly, thank you to Maya Freed-Barlow for her help with Adobe Photoshop and Savita Diggs for her constant encouragement.

Funding for this Senior Thesis was provided by the Mount Holyoke Lynk Universal Application Funding (Lynk UAF) and a Biology Faculty Grant to Dr. Patricia Brennan.

TABLE OF CONTENTS

Page

List of Figures

Page

List of Tables

ABSTRACT

Genital evolution can be driven by a multitude of selective pressures, including the pressure to avoid harm and hybridizations. Recent studies reveal how rapidly evolving female genital structures may drive speciation under selective processes like sexual conflict and reinforcement. This study explores whether the vaginal morphologies of watersnake sister species are diverging more rapidly than male intromittent organs. We used 2D and 3D geometric morphometrics to quantitatively compare the complex shapes of vaginal pouches and hemipenes across the three species *N. sipedon*, *N. fasciata*, and *N. rhombifer*. Previous work examining genital shape differences in female *N. sipedon* and *N. fasciata* revealed significant morphological differences. Similar male genital shape within these two species would support the phenomenon of rapidly evolving female genitalia driving speciation. By also including *N. rhombifer* in the analysis, we were able to compare samples from a morphologically distinct relative. With the new additions to our sample, our results reinforced previous findings that show significant differences in vaginal pouch shape across species. As we predicted, pairwise comparisons showed that the vaginal pouch shape of *N. rhombifer* was more distinct from *N. fasciata* and *N. sipedon* than they were from each other. Male shape was also significantly different across all species, and the same pattern emerged in the pairwise comparisons, which does not support our prediction that hemipene morphology is indistinct across *N. fasciata* and *N. sipedon*. Although this result does not provide sufficient data to support our initial hypothesis, it does not rule out female-driven species divergence. Future steps include using larger samples from *Nerodia* to explore intraspecific coevolutionary patterns and using 3D geometric morphometrics for female specimens.

INTRODUCTION

THE STUDY OF GENITAL DIVERSITY

Genitalia display diverse ornamentation and shape features across taxonomic groups, revealing a fascinating evolutionary pattern for internally reproducing species (Eberhard 1985; House & Simmons 2005). Unlike most organs, genitalia are known to evolve at a rapid rate. As a result, male external genitalia have been used for centuries to easily differentiate species that are otherwise phenotypically similar (Eberhard 1985; Sloan and Simmons 2019). The apparent diversity amongst male intromittent organs has been researched in depth and is thought to be highly influenced by modes of sexual selection such as intrasexual competition, sexually antagonistic coevolution, and cryptic female choice (Brennan and Prum 2015; Eberhard 2010). Although sexual selection is thought to be the dominant driving force acting on genital diversification, hypotheses of species isolation and pleiotropy which also aim to explain this diversity have not been ruled out (House and Simmons 2005).

Sexual reproduction often involves a male intromittent organ and internal female genitalia, which are expected to coevolve due to their close mechanical interaction during copulation. Therefore, it seems most logical to examine both of these structures when experimentally testing evolutionary theories of genital diversity. Despite the widely accepted notion that sexual organs coevolve, research dedicated to female genital diversity has remained fairly limited

(Brennan and Prum 2015). Only within the last fifteen years has research revealed how the female genital structures of certain species can also be highly variable (Puniamoorthy, Kotrba, and Meier 2010; Simmons and Fitzpatrick 2019; Orbach et al. 2018). One explanation for the disproportionate research bias towards male genitalia has been the limited methodologies for conducting shape analysis. Compared to internal structures, external genitalia have been easier to measure, are made of harder tissue, and are more available to the naked eye (Simmons and Fitzpatrick 2019; Sloan and Simmons 2019).

Another reason why female genitalia have not been studied extensively relates to the way their diversity was framed by the work of William Eberhard. Within his influential research on the role of cryptic female choice in genital evolution, Eberhard made an assumption that female genitalia of closely related species display little to no interspecific variation compared to males (Eberhard 1985; Showalter et al. 2014). This claim has since been contradicted by research showing evidence of rapidly evolving female genital structures and the adoption of more advanced modes of shape analysis (Brennan and Prum 2015; Showalter et al. 2014; Simmons and Fitzpatrick 2019; Puniamoorthy, Kotrba, and Meier 2010).

The neglect towards researching female genital diversity and copulatory behaviors to the same extent as males has led to an incomplete understanding of what drives genital diversity (Ah-King et al. 2014). Fortunately, an increasing recognition of this discrepancy is pushing researchers of genital evolution to analyze the reproductive structures of both sexes. Such an approach more accurately captures microevolutionary mechanisms such as copulatory mechanics and sexually antagonistic coevolution within species. It also more accurately informs our understanding of macroevolutionary patterns across species such as reproductive isolating mechanisms that drive speciation (Simmons 2014).

SEXUAL SELECTION

It is thought that most genital diversity can be attributed to sexual selection; a mode of natural selection where traits are favored if they increase the likelihood that an individual will gain an advantage during reproduction (Hosken and Stockley 2004). Modes of sexual selection vary, some taking place before copulation (precopulatory) and others during or after the fact (postcopulatory). Males and females are under different types of selective pressures depending on factors like the mating system, the gametes they produce, harm during copulation, and the amount of existing competition for a mate (Brennan 2010). As a result of misaligned reproductive fitness strategies, males and females may engage in sexual conflict (Arnqvist and Rowe 2005; King et al. 2009).

The three widely investigated modes of sexual selection thought to drive genital diversity are sperm competition, cryptic female choice, and sexually antagonistic coevolution (Eberhard 2019; Simmons 2014). Sexual selection by sperm competition favors male genital traits which aid in the blocking or removal of rival sperm located in the vaginas of polyandrous females (Eberhard 2019; Simmons 2014). Sexual selection by cryptic female choice involves postcopulatory processes controlled by females like biased sperm storage or decreased copulation time and is thought to influence the evolution of female genitalia (Eberhard 2009; Sloan and Simmons 2014). Sexually antagonistic coevolution places both male and female genital structures under sexual selection due to the conflicting reproductive interests of the sexes. This imbalance resulting from sexual conflict can lead to a coevolutionary arms-race where both sexes evolve antagonistic genital traits as they compete for reproductive control (Brennan and Prum 2015).

SEXUAL CONFLICT

Recent research dedicated to sexual conflict has framed it as the primary driving force of female genital diversity (Arnqvist and Rowe 2005; Brennan and Prum 2015). As mentioned previously, patterns of sexual conflict arise out of misaligned reproductive fitness strategies of males and females, resulting in the gradual integration of antagonistic traits meant to target or defend against the other sex (Arnqvist and Rowe 2005; King et al. 2009). The calcified spines on the snake intromittent organ are an example of an antagonistic trait that helps males anchor themselves into female vaginal tissue. This is a mode of sexual conflict in which male snakes have evolved a trait that increases their reproductive fitness at the cost of the females. As a result, copulation duration increases and females are less likely to remate, increasing the likelihood of that male passing on its genes (Friesen et al. 2014; King et al. 2009). This perforation of female tissue during copulation places a selective pressure on females to counter-adapt protective mechanisms against this form of tissue damage, such as a thickening of vaginal lining (Arnqvist and Rowe 2005; Dougherty et al. 2017). What arises out of these conflicting adaptations is an intraspecific coevolutionary mechanism which may reinforce genital divergence and eventually lead to speciation events (Arnqvist and Rowe 2005).

THE LOCK-AND-KEY HYPOTHESIS

A famous theory of genital diversity that William Eberhard's research prematurely deemed unlikely is the lock-and-key hypothesis, which aims to explain some genital diversity as the product of natural selection. Coined by Dufour in the 19th century, "lock" represents the internal female genitalia that has an external male "key" to match (Dufour 1844). The theory explains a mode of mechanical reproductive isolation in which mismatched genitalia act as the barrier mechanism blocking hybridization between closely related species (Brennan and Prum 2015). The hypothesis predicts that rapid genital divergence is reinforced under natural selection, and reproductive character displacement occurs for genital morphology. This leads to insufficient sperm transfer and unsuccessful copulation leading to a speciation event (Eberhard 2011). The outcome of this mismatch is distinct differences in genitalia between recently diverged populations even at the early stages of speciation (Anderson and Langerhans 2015; Greenway et al. 2019).

More recent inquiries on the driving forces of genital diversity suggest both sexual selection and natural selection simultaneously act on genital traits (Arnqvist and Rowe 2005). This idea was recently explored by Greenway et al. (2019) in diverging populations of *Poecilia mexicana*. These Poeciliid fishes show evidence of both sexual conflict and rapidly divergent genitalia which act as a mechanical barrier to interpopulation gene flow. Although evidence of a lock-and-key mechanism within this system is still uncertain, the study provides an example of the overlapping effects of sexual selection and reinforcement on genital divergence (Greenway et al. 2019). Simmons (2014) proposes sexual selection and lock-and-key should not be viewed as separately occurring mechanisms, but rather be viewed on a continuum of female choice. Therefore, we must consider how speciation that arises from a mechanical isolation mechanism may also be a consequence of sexual conflict.

PRESENT STUDY

The co-occurrence between sexual conflict and lock-and-key is relevant in the present study, which examines how sister-species within the *Nerodia* genus may have diverged by such a mechanism. Current literature dedicated to genital evolution has yet to simultaneously examine male and female genital morphologies of watersnakes to explore hypotheses of genital diversity. In this study, we used a geometric morphometric approach (Rohlf and Marcus 1993; Adams, Rohlf, and Slice 2004) to investigate the degree of variation in genital shape amongst three species from the Genus *Nerodia*. These include sister species *N. fasciata* and *N. sipedon* and their more distant relative *N. rhombifer* (Rohlf and Marcus 1993; Adams, Rohlf, and Slice 2004). The sister species have been observed occupying the same regions of North America and sharing the same springtime mating period, which rules out both geographic and temporal reproductive isolation (Lorenz et al. 2011; Jellen and Aldridge 2013). The sharp basal spines on the hemipenes are likely to deter females from remating, which may have driven the interspecific divergence of vaginal morphology between the *N. sipedon* and *N. fasciata* (Showalter et al. 2014). Therefore, sexual conflict may be acting as a driving force for divergent female genitalia, diverting the two species in different evolutionary trajectories (Arnqvist and Rowe, 2005). *N. fasciata* and *N. sipedon* have been identified in hybrid zones where they coexist but occupy separate ecological niches and occasionally produce inferior hybrid genotypes (Merbert 2008), hence our prediction that there is selection for a species isolation mechanism to operate in this system.

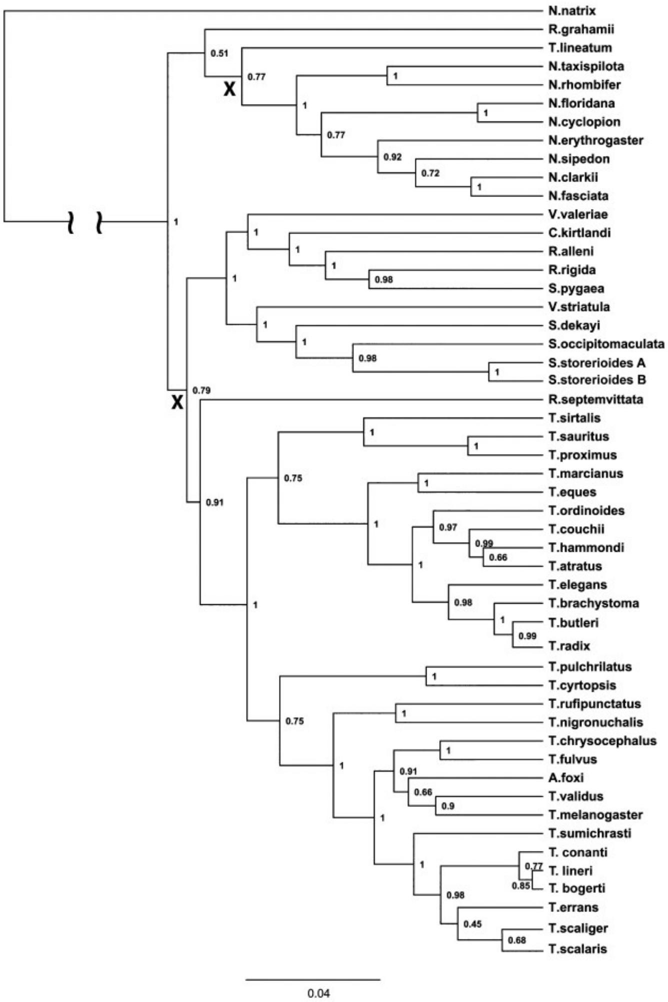

Figure 1. Multilocus Bayesian estimate of phylogeny for *Thamnophiini* including a depiction of the genetic relatedness between sister-species *Nerodia sipedon, Nerodia fasciata*, and *Nerodia rhombifer* (McCay, Flores-Villela, and Carstens 2015).

To answer this question of female-driven divergence, we conducted comparative analyses within sex categories using 2-dimensional (2D) shape data for females and 3-dimensional (3D) shape data for males. A portion of genitalia were acquired from a previous study which found that there were significant shape differences between mature *N. sipedon* and *N. fasciata* females (Showalter et al. 2014), while all *N. rhombifer* samples were collected for the present study. Although quantifying vaginal morphologies in female snakes is difficult due to the elasticity and variation in vaginal width among individuals, we are still able to compare the specimens in 2D to look for significant variation in structurally analogous locations. We expect to find significant differences in vaginal shape between species, with *N. sipedon* and *N. fasciata* being more similar to each other than to *N. rhombifer*, reflecting their evolutionary differences. This result would reinforce previous findings that revealed distinct shape differences between reproductively mature vaginas of *N. sipedon* and *N. fasciata* (Showalter et al. 2014).

The differences between the genitalia of the males in *Nerodia* has not been tested. The hemipenes from each species display a similar bilobed structure and display large basal spines (Showalter et al. 2014). Considering their status as sister-species, we assume that the *N. sipedon* and *N. fasciata* male genitalia are diverging into more distinct morphologies in order to keep up with the rapidly evolving female genitalia (Eberhard 2011; Showalter et al. 2014; Lawson 1987). The incorporation of *N. rhombifer* within the analysis allows us to compare the degree of morphological similarity between *N. sipedon* and *N. fasciata* who more recently share a last common ancestor than *N. rhombifer* (Lawson 1987; McCay, Flores-Villela, and Carstens 2015).

Since the vaginal pouch is known to be different between *N. fasciata* and *N. sipedon*, female genital variation may be enough to drive mechanical isolation, in which case we might find that the variation in hemipene shape between males is less than the variation between females. Alternatively, the finding that variation between males is much greater than between females would suggest a male driven process of speciation. We expect no significant shape variation between males of the two species in 3D. This result would support the hypothesis that sexual conflict drives female choice, which in turn drives the coevolution of male and female genitalia within the two species.

STUDY SYSTEM

Nerodia is a genus made up of aquatic viviparous Natricinae snakes native to North America. Consisting of 10 species (Figure 1), *Nerodia* are known to reproduce seasonally and copulation is heavily influenced by a rise in temperature (Lorenz et al. 2013; Aldrige et al. 1995). Males have been observed traveling during the mating period to ensure they can find a female or multiple females to mate with so they may increase their chances of delivering sperm. The motivation for female movement is unclear, but Brown and Weatherhead (2004) suggest female movement is determined by their avoidance of sexual conflict. Females are known to leave a trail of pheromones in their path during their mating period, which facilitates males locating them (Jellen and Aldridge 2013). Mating with multiple males is also common in female *Nerodia*, leading to increased sperm competition amongst males. The smaller size of mature males compared to mature females may also be explained by sperm competition since males allocate energy towards producing sperm instead of increasing body size (Weatherhead et al.

1995), and females are under selection for increased fecundity, leading to larger body sizes. In order to inseminate females, males must line up their cloacas by intertwining their tails. Observations of copulatory behavior in watersnakes note the aggressive behaviors of males towards females including forceful jerking of the passive female and surrounding males (Weatherhead et al. 1995). The internal genital mechanics during copulation in this genus are understudied, but the sharp basal spines on the male hemipenes suggest copulation involves perforation of the vaginal tissue (Showalter et al. 2014).

Snakes belonging to the Colubridae family have diverse genitalia across species. Before advanced genotyping technologies were used to differentiate species, colubrid snakes were classified based on their morphologically distinct male copulatory organs, which were the only structure differentiating multiple species (Showalter et al. 2014). Male genitalia of squamates called hemipenes are widely considered to be species-specific (Brennan and Prum 2015; Andonov et al. 2017). The hemipenes, plural of hemipenis, are two intromittent organs that become evaginated and erect during copulation. The process of hemipenis evagination is controlled by a muscle called the *m. retractor penis magnus* by relaxation and contraction (Porto et al. 2013). *N. sipedon*, *N. fasciata*, and *N. rhombifer* are Colubridae and share common morphological traits in their hemipene such as a shaft, a sulcus spermaticus, and a bilobed tip. All three species also display sharp calcified basal spines (Andonov et al. 2017). The three snake species within this study exemplify the two categories of “simple” (*N.*

rhombifer) or "bilobed" (*N. fasciata* and *N. sipedon*) hemipene shape that explain the observed variation of this structure (King et al. 2009). Ontogenetic studies of hemipenis morphology reveal how the simple or sub-cylindrical hemipene shape is likely ancestral to the bilobed shape (Jadin and King, 2012). The less-studied female copulatory organ in snakes is referred to as the vaginal pouch. The cloacal opening on females leads to a vaginal pouch which branches off into two oviducts that house the female ova. The vaginal pouch is where female snakes receive an individual hemipenis during copulation and also plays a functional role in giving birth (Showalter et al. 2014).

Pope was the first to introduce the idea of copulatory adjustment in snakes, which suggests a morphological coevolution of hemipenes and vaginal pouches based on copulatory mechanics. The bilobed shape of each hemipene allows for direct intromission of sperm into the diverging female oviducts, while the basal spines of hemipenes anchor into the vaginal tissue and prevent dislodging (Edgren 1953; Pope 1941). This known coevolutionary relationship acting on snake genitalia, which also involves male harm, provides additional support for the potential role of sexual conflict in driving a lock-and-key mechanism in recently diverged *Nerodia* (Showalter et al. 2014).

GEOMETRIC MORPHOMETRICS

The method of geometric morphometrics became relevant in the late 1990s and continues to be utilized today in a range of scientific disciplines including geology, paleobiology, and evolution. The most common application of this method involves the Procrustes superimposition of landmarks. This is the arrangement and alignment of all shapes within a sample onto a common Cartesian coordinate system. Procrustes superimposition disregards size, orientation, and position, which are all considered non-shape variables (Adams, Rohlf, and Slice 2004; Lawing and Polly 2009). The ability to quantitatively measure the complex shapes of 2D or 3D objects can be very informative in evolutionary research, especially when examining the covariation of shape with other variables such as species or habitat to answer questions about the emergence of morphological traits (Adams, Rohlf, and Slice 2004).

The study of morphometrics once relied on basic linear measurements or the naked eye, which hardly capture the pure organic shape of biological structures. This has led to decades of misconceptions about the apparent homogeneity of biological structures that actually have hidden variability, only detected by approaches like a multivariate analysis of landmark data (Lawing and Polly 2009; Showalter et al. 2014). With new technological advancements in morphometrics research, we have the ability to plot individual specimens along axes of shape variation through Principal Components analysis (PCA). This type of data visualization captures dynamic morphological changes by arranging points in a 2D linear morphospace where they are set apart by varying Procrustes distances. The PC scores found on the axes of the morphospace graph can then be used to run multivariate analyses that include the variable “shape” (Adams, Rohlf, and Slice 2004; Lawing and Polly 2009). Geometric morphometrics thus allow biologists to more easily observe shape variation within a sample on the microevolutionary level and macroevolutionary level (Brennan and Prum 2015).

2D geometric morphometrics (2DGM) and 3D geometric morphometrics (3DGM) are used depending on the structures being analyzed as well as the time and technologies available to the researcher. 2D shapes are only approximations of 3D shapes, and therefore going down a 3DGM route is often preferred but not always computationally possible (Hedrick and Antalek-Schreg et al. 2019). In this study, 2DGM of vaginal pouches is expected to capture most of the shape variation in females, while 3DGM analysis of male genitalia is expected to fully capture the shape variation of the hemipene structure.

METHODS

SPECIMEN COLLECTION

N. sipedon and *N. fasciata* genitalia specimens were previously dissected and analyzed by Irina Showalter in 2013 at University of Massachusetts Amherst (Showalter et al. 2014). *N. sipedon* snakes were taken from an established population in Placer County, CA in 2011 where they were trapped from a small wetland under the California Department of Fish and Wildlife collecting permit #SC-11197 to B. D. Todd. Both trapping and euthanasia were performed according to UC Davis Institutional Animal Care and Use Committee protocol #2011-16553. *N. fasciata* were trapped and euthanized in Los Angeles County, CA in 2010 from an established population. These actions were approved through the California Department of Fish and Wildlife collecting permit #802046-02 to Robert N. Reed of the US Geological Survey. All snakes were frozen and preserved for later dissection as outlined in Showalter et al. (2014). *Nerodia rhombifer* specimens were acquired in collaboration with the University of Alabama Department of Biological Science and collected by Stephen Secor under Alabama collecting permit number 2018101271668680. We defrosted and dissected 48 *N. rhombifer* at Mount Holyoke College during the summer months of 2019.

DISSECTION OF WHOLE SPECIMENS

We defrosted whole *N. rhombifer* snakes in a large sink by submerging them in warm water for approximately one hour. Once they were thawed, we recorded measurements of their snout-to-vent length (SVL) with a meter stick to the nearest centimeter and their mass on an electronic scale in grams. On dissection mats, we placed snakes on their dorsal side and used surgical scissors to carefully cut down their midline from their anterior to their posterior. This cut began above their stomach and continued to just above their cloacal opening. We removed all layers of fat in order to access their internal structures and gonads, which gave us information on the specimen's sex and reproductive status when their genitalia were not initially visible. For males, we based reproductive status on whether the specimen had a coiled sperm duct or vas deferens. Coiled vas deferens suggest the snake was producing sperm and is therefore reproductive, while non-coiled vas deferens suggests the opposite. For females, we based reproductive status on the size of the eggs which clued us into their reproductive maturity. Females with yolk-filled or fertilized eggs in their oviducts were considered reproductive, while females with small eggs (<2mm) in their ovary were nonreproductive. In addition to recording their reproductive status, we took measurements to the nearest millimeter of sex-specific structures. For males, we measured the length and diameter of testes, kidney, retractor penis muscle, and the left and right hemipene. For females, we measured the length and diameter of the largest egg. If eggs were fertilized, we measured the length of the germinal disk or embryo depending on the stage of development. After collecting the necessary data and tissue specimens, we disposed of the snake carcass through biohazardous waste facilities.

VAGINAL MOLD MAKING AND PRESERVATION

We used surgical scissors to cut the connective tissue surrounding the vaginal pouch, remove any remnants of the intestines, and cut the oviducts at the point where there is a visible change in tissue composition. We used dental floss to close the oviducts and the intestinal opening. We laid vaginas on their dorsal side and photographed them using Canon EOS Rebel T5i. The full structure depicted in photographs includes the cloacal opening, the vaginal pouch, and two oviducts. We made vaginal molds using dental silicone to capture the negative space and vaginal folds that exist within the vaginal pouch. We made molds while the vaginas were still attached to the specimens to make sure their three-dimensional shape was as accurate as possible. Using a syringe, we injected dental silicone into the female cloacal opening and waited until it cured to remove it. We washed vaginal molds and used them to make models for future 3D analyses. After making molds and dissecting out vaginal pouches, we placed all tissue specimens in saline solution to be frozen.

HEMIPENE INFLATION AND PRESERVATION

To get the most accurate morphological representation of the male reproductive structures, we inflated the left hemipenes of male snakes with warmed petroleum jelly to mimic the way they are everted and engorged during copulation. While the hemipenes were still fully attached to the specimen, we filled a small syringe with melted petroleum jelly and everted and inflated the hemipenes by inserting the needle into the base of the shaft. When the hemipene became fully inflated with liquid petroleum jelly, we waited for it to solidify before cutting the genital structures off of the specimen. To ensure the hemipenes maintained their shape and no petroleum jelly escaped from the bottom, we used dental floss to tie a knot at the base. We then displayed all inflated hemipenes in a way that made their largest basal spine visible so they could be photographed using a Canon EOS Rebel T5i. We preserved the specimens in formalin 10%. Only the left hemipenes of snake specimens were everted and inflated for analysis, while right hemipenes were everted, removed, and frozen in saline without being inflated.

2-D LANDMARK PLACEMENT OF VAGINAL POUCHES

We used the software tpsDig2 (Rohlf 2007) to quantitatively determine the shape of 92 vaginal pouches (*N. rhombifer*; n =29, *N. fasciata*; n =30, *N. sipdeon*; n = 33) using a combination of anchoring landmarks (n=6) and semilandmarks (n= 6 curves). We accessed photographs of *N. sipedon* and *N.*

fasciata females from the data archives previously used by Showalter et al. (2014). *N. rhombifer* were photographed using Canon EOS Rebel T5i immediately after dissection in the Summer of 2019. All photographs depicted vaginal pouches ventral side up to best represent the shape of the vaginal pouch, oviducts, and the opening of the cloaca.

To begin the landmarking process, we created a folder on the desktop that included a photograph of each specimen in the same orientation. We then opened a blank document in the text editor “Notepad” saved under “All Files” and added “.tps” to its file name. We used tpsUtil to build the new TPS file containing our folder of photographs and selected the function “randomly order specimens” to help reduce bias towards species during manual landmark placement. This randomization produced a new text file that we used as the output location for all digitized landmark coordinates within the sample.

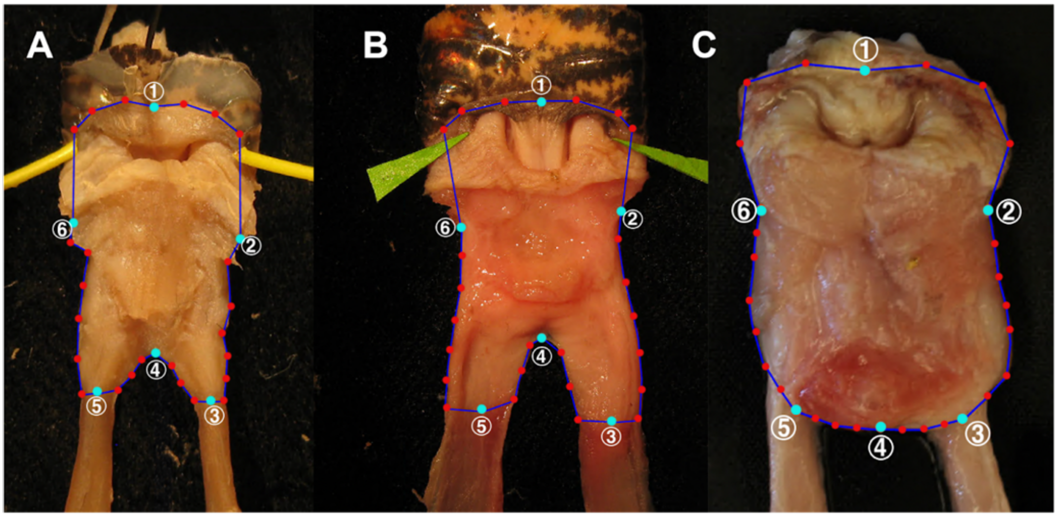

Figure 2. Photographs of *Nerodia* vaginal pouches after landmarking in tpsDig2. Blue points represent homologous anchoring landmark placement and red points represent semilandmark placement on *N. fasciata* **(A)***, N. sipedon* **(B)***, and N. rhombifer* **(C)**. The locations of the anchoring landmarks were; (1) the intersection of the scale and vaginal tissue at cloacal opening, (2) the intersection of vaginal pouch with cloacal opening on the right side, (3) the midpoint where thicker vaginal tissue meets oviduct tissue on the right side, (4) the midpoint in the portion of vaginal tissue between the two oviducts, (5) the midpoint where thicker vaginal tissue meets oviduct tissue on the right side, (6) the intersection of vaginal pouch with cloacal opening on the left side. The dark blue outline represents the consecutive 2D shape based on anchoring landmarks and semilandmarks made up of a total of six curves.

To assign digitized landmarks, we opened the randomized text file in tpsDig2 and placed the same number of anchoring landmarks and semilandmarks on each specimen. We assigned six anchoring landmarks to each specimen in predetermined, distinguishable, and structurally analogous locations. These locations were featured on each vaginal pouch regardless of variables like species, reproductive status, and SVL (Figure 2). We used the blue crosshair function to place the anchoring landmarks, making sure to place them in the same order for each specimen. After completing this process, we placed semilandmarks on each specimen using the pencil tool to create connecting curves. The location of semilandmark curves on each specimen was determined by the outline of vaginal tissue we observed between anchoring landmarks.

Between anchoring landmarks 2 and 3 and anchoring landmarks 5 and 6, we placed 8 semilandmarks. Between anchoring landmarks 1 and 2, 3 and 4, 4 and 5, and 6 and 1, we repeated the same process but with 5 semilandmarks. The semilandmarks at the tail ends of each curve overlapped the anchoring landmarks that they were placed between. All semilandmarks formed a total of 6 connecting curves, which together created a unique landmark configuration for each individual (Figure 2). After landmarking, we added a scale bar to each photograph according to the rulers that were featured in the photographs next to the specimens. The total landmark configurations made up of anchoring landmarks and curves were used as quantitative representations of each specimen's "shape" as it is defined in geometric morphometrics. All landmarking of 92 vaginas was completed by Juliet Greenwood to avoid inconsistencies that would arise from having multiple observers.

When all landmark placement and scaling was complete, we re-uploaded the TPS file into tpsUtil and selected the function "append tps curve to landmarks" which converted all semilandmarks into landmarks. We deleted all semilandmarks which overlapped anchoring landmarks by using the function "delete reorder landmarks," resulting in 30 total landmarks per specimen with no instances of overlapping. We saved the reduced curves TPS file to be later uploaded to RStudio for our 2DGM analysis.

LASER SCANNING TO PRODUCE 3D MODELS

We used the EXScan Pro 2X Plus laser scanner and associated software to create 3D object files for the left hemipene of each male specimen. We selected the settings “fixed scan” and “non-texture scan” for each round of scans. To prepare the specimens, we inserted a needle into the solidified petroleum jelly exposed at the base of the inflated hemipene that had been removed from the whole snake. We then placed the opposite end of the needle into a piece of modeling clay to ensure the hemipene remained upright and in place during the scanning process. If there was still dental floss at the base of the specimen, we trimmed it to make sure it did not interfere with the scanning processes.

We used various methods to create an opaque hemipene surface since the transparent genital tissue was often not easily detectable by the scanner. In certain cases, we painted the hemipene with washable green paint or sprayed the specimen with a thin layer of powder. A large portion of *N. fasciata* and *N. sipedon* hemipenes had been inflated with blue petroleum jelly to negate this issue. We placed the specimen on the center of a rotating circular platform labeled with fixed points and aligned the specimen with the plus sign projected by the scanner light. We used the fixed scan setting to scan each specimen at 3 angles; upright, tilted left, and tilted right. These angles allowed the scanner to capture every detail of the complex hemipene shape. The number of rotations we selected ranged between 8 and 12 and was typically higher for small specimens and lower for large specimens. After completing 3 rounds of rotations, we converted the point-cloud output into a "high detail" watertight mesh model that was automatically scaled. After this step, we selected the edit tool "sharpen; low" to slightly emphasize the surface details of the hemipene model. After scanning was complete, we saved and exported each model as an object file.

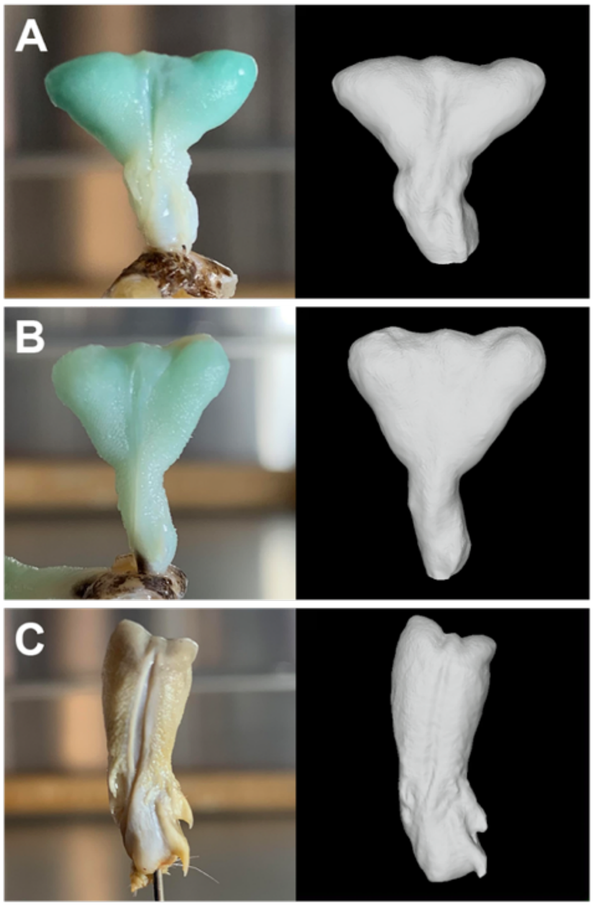

Figure 3. Sulcate view of inflated left hemipenes of *N. fasciata* (A) *N. sipedon* (B) and *N. rhombifer* (C) next to their corresponding 3D models. 3D models are based on laser scans of tissue specimens with the removal of inconsistencies at the basal portion of the model.

We used MeshMixer software to manually correct inconsistencies between the specimen and the 3D model using sculpting and smoothing features. These inconsistencies included the occasional bumps or dents added to the 3D model by the laser scanning software that could be easily corrected with the digital sculpting tools. We also used the “plane cut” feature in MeshMixer to create a flat base for every specimen. Our intention with this editing was to make it easier for the Auto3dgm software to recognize a common flat surface on each hemipene and align them all in the same orientation.

2D GEOMETRIC MORPHOMETRIC ANALYSIS

The TPS file used for the analysis portion was created from the process of digitizing landmarks and semilandmarks onto 92 photographs of vaginal pouches in TPSDig2 (Rohlf 2007). We then installed the *geomorph* package (Adams and Otárola-Castillo 2013) in RStudio (R Core Development Team 2016) and imported the 2D landmark data using the function “readland.tps.” We created a variable called “femaleShape"' from this landmark data and with it ran a Generalized Procrustes analysis (GPA) using the function “gpagen.” The GPA aligned all landmark configurations and scaled them in relation to centroid size (Adams, Rohlf, and Slice 2004).

To explore how variation of shape relates to other variables in our analysis, we created a spreadsheet which listed specimen IDs, species, SVL, and reproductive status for each individual. After uploading this file to R, we created a data frame with the function “geomorph.data.frame” labeled “femaleGDF.” This step was necessary for our later statistical analyses pinpointing relationships between our continuous dependent variable “shape” with the grouped independent variables “species,” and “repro,” or the continuous independent variables “SVL” and “centroid size.” This step also allowed us to keep track of which individual specimens were potential outliers or sat at the extreme ends of PCA axes.

To create a PCA plot comparing species, we first had to encode the vector variable “species” as a factor with three categories; “rhom” (*N. rhombifer*), “sip” (*N. sipedon*), and “fas” (*N. fasciata*) with the R function “factor.” To run the PCA, we analyzed the GPA-aligned shape data through function “gm.prcomp” and labeled it “femalePCA.” This produced the same amount of principal component (PC) variables as there were individuals in the sample. PC1 through PC92 each represented a proportion of shape variance within the sample, starting from largest proportion to smallest proportion. We created a PCA plot comparing PC1 vs. PC2 using the generic R function “plot” and assigned each species a color using the “col” argument. We created deformation grids for specimens representing the extreme ends of the PCA axes using the function “picknplot.shape.” For subsequent analysis we selected only the first two PCs that each explained more than 10% of the variance.

In addition to visualizing differences in shape through PCA, we used a multi-factor MANOVA to assess whether the vaginal pouch shape was significantly affected by the variables species, body length (SVL), and reproductive status. To explore significant shape differences between different pairs of species, we ran the multi-factor MANOVA output through the function "pairwise" from the R package RRPP.

3D GEOMETRIC MORPHOMETRIC ANALYSIS

We uploaded 62 male 3D models (*N. rhombifer*; n =22, *N. fasciata*; n =20, *N. sipdeon*; n = 20) into 3D Slicer as individual object files to be analyzed through Automated 3D Geometric Morphometrics (Auto3Dgm). The Auto3Dgm analysis technique involves the automatic placement of pseudolandmarks on the surface of 3D models followed by the computing of correspondences and differences between individuals within the sample. This method is a precise mode of object analysis that explores variance between 3D forms. The analysis computed pairwise distances between each pseudolandmark on every 3D hemipene specimen (Gunnel et al. 2018).

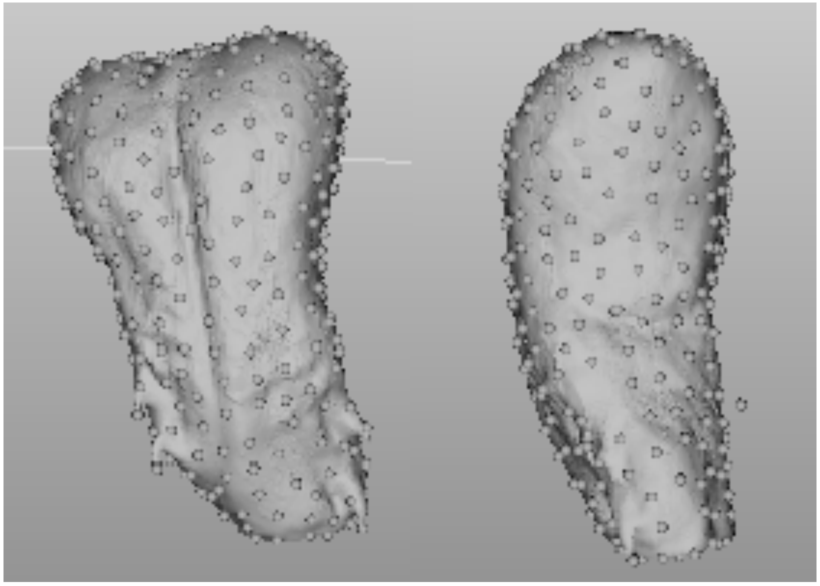

Figure 4. 3D model of a *N. rhombifer* hemipene assigned 400 pseudolandmarks in Auto3Dgm. Example of one specimen from the sample of males with its entire surface covered in pseudolandmarks to be later analyzed using Generalized Procrustes Analysis.

We assigned 100 landmarks for phase 1 and 800 landmarks for phase 2 and used the phase 2 data for our analyses. The Auto3Dgm output provided us with a .csv file containing the total landmark coordinates of each specimen. We uploaded this file to R using the function read.csv and labeled it "maleShape." To convert these coordinates into a 3D array, we created a matrix out of the values using the function "as.matrix" and followed that up with the function "arrayspecs" to create the array. We then followed the same steps and code as the 2DGM to create a data frame and run a GPA, PCA, multi-factor MANOVA, and pairwise comparisons according to species groups.

To rule out the possibility that within our sample genital shape significantly changes with reproductive state, we compared shape differences between reproductive and nonreproductive males of the group *N. fasciata,* the only species for which we had similar number of males who were reproductive (n =9) and nonreproductive (n=11). Following our combined-species analysis, we ran the *N. fasciata*-only (n=20) analysis using the same number of landmarks per specimen. Due to a limited number of available genital specimens, *N. rhombifer* were disproportionately reproductive and *N. sipedon* were disproportionately nonreproductive and could not be included in our assessment of how shape changes with reproductive state.

RESULTS

SHAPE VARIATION IN VAGINAL POUCHES

The PCA plot comparing PC1 and PC2 (Figure 5) revealed that each species approximately occupies a distinct section of the linear morphospace. *N. rhombifer* forms a separated cloud in the negative end of PC1 mostly remaining between the PCA scores -.02 and 0. Along PC1, *N. sipedon* and *N. fasciata* show overlap between the PC1 scores -.01 and 0.2. The majority of separation between *N. fasciata* and *N. sipedon* happens along PC2. *N. sipedon* are clumped towards the negative end of the axis while *N. fasciata* are located towards the positive values. *N. rhombifer* is mostly concentrated in the negative end of PC2 (Figure 5). Vaginal pouches from N. rhombifer primarily occupy the negative values of PC1 and PC2 and show very little bifurcation. They also have a broader cloacal opening compared to the anterior region of the vaginal pouch. The positive values of PC1 describe an increasing bifurcation, while the negative values of PC1 describe an overall wider pouch shape with less bifurcation. The positive values of PC2 describe increased bifurcation and narrowing of the cloacal opening, while the negative values of PC2 represent less bifurcation and a broader cloacal opening compared to the anterior region of the pouch.

MULTIVARIATE ANALYSIS OF FEMALES

The differences in shape of the vaginal pouch are significant as shown by our multi-factor MANOVA (Table 1a). The analysis revealed a significant effect on shape in relation to species, where shape was significantly variable across species ($p<0.001$). The pairwise comparisons (Table 1b) of species also revealed that significant shape differences existed across all species pairs, however the Z value is smaller for the comparison between *N. sipedon* and *N. fasciata* than for *N. sipedon* and *N. rhombifer* or *N. fasciata* and *N. rhombifer* (Table 1a). The multi-factor MANOVA also revealed a significant effect of reproductive status ($p < 0.05$) and body length ($p < 0.001$) on vaginal pouch shape (Table 1a).

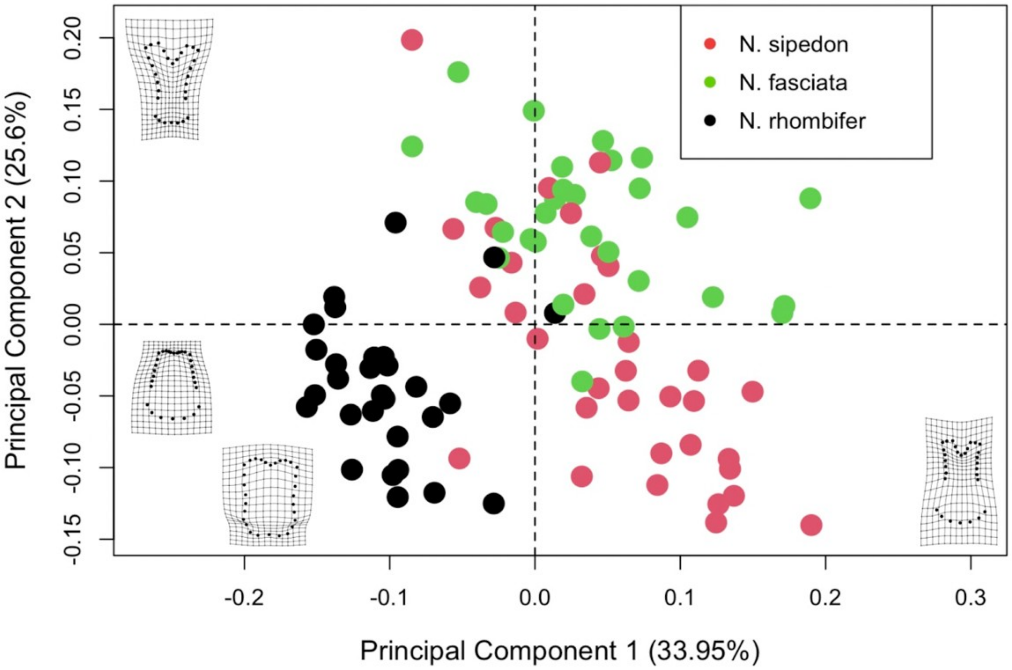

Figure 5. The 2DGM Principal Components Analysis (PCA) depicts changes in vaginal shape across the two largest axes of variation. PC1 accounts for 33.95% of variation and shows the transition from a wider, square pouch (left) to a narrower, curved, bifurcated pouch (right). PC2 accounts for 25.6% of variation and displays a transition from bifurcated, narrow pouch (top) to a wider overall shape particularly at the cloacal entrance, with very little bifurcation (bottom). Deformation grids represent individual specimens located at the extreme ends of each PC.

SHAPE VARIATION IN HEMIPENES

The PCA for all hemipenes (Figure 6) revealed distinct aggregation based on species categories within our sample. *N. rhombifer* are exclusively concentrated in the negative portion of PC1, while *N. fasciata* and *N. sipedon* remain mostly on the positive end of this axis. On the PC2 axis, *N. sipedon* remains mostly within the positive quadrant while *N. rhombifer* clumps towards the middle and *N. fasciata* towards the negative end. The general shape of hemipenes within the negative values of PC1 exhibit a sub-cylindrical shape with a wider base. This wider base likely reflects the position of the large basal spines observed in *N. rhombifer*. The positive values of PC1 are associated with increased bilobation at the apical portion of the hemipene. The more bilobed apical region is accompanied by a narrowing of the base, reflecting small basal spines (Figure 7A). Along the PC2 axis, negative values are representative of a wider overall hemipene shape in both the basal and apical regions as well as from sulcal and lateral views. *N. fasciata* occupies this negative quadrant of PC2 while *N. sipedon* occupies the positive values where overall hemipene shape is narrower (Figure 7B). The PCA examining shape and reproductive state in *N. fasciata* (Figure 8) reveals no aggregation of individuals along PC1 and PC2 based on reproductive state.

MULTIVARIATE ANALYSIS OF MALES

The multi-factor MANOVA examining the effect of species category on hemipene shape revealed a significant relationship ($p < 0.001$) (Table 1a). Pairwise comparisons of shape differences for species pairs were also significant across all species pairs (Table 1b). *N. rhombifer* was more significantly different from *N. fasciata* and *N. sipedon* ($p < 0.01$) than they were from each other ($p < 0.05$) (Table 2b). The multi-factor MANOVA also revealed that shape changes with species and SVL, revealing that body size had a significant effect on hemipene shape ($p < 0.01$) (Table 2a). Our additional single-factor MANOVA

(Table 3) revealed no significant effect of reproductive state on hemipene shape in the *N. fasciata* sample ($p > 0.05$).

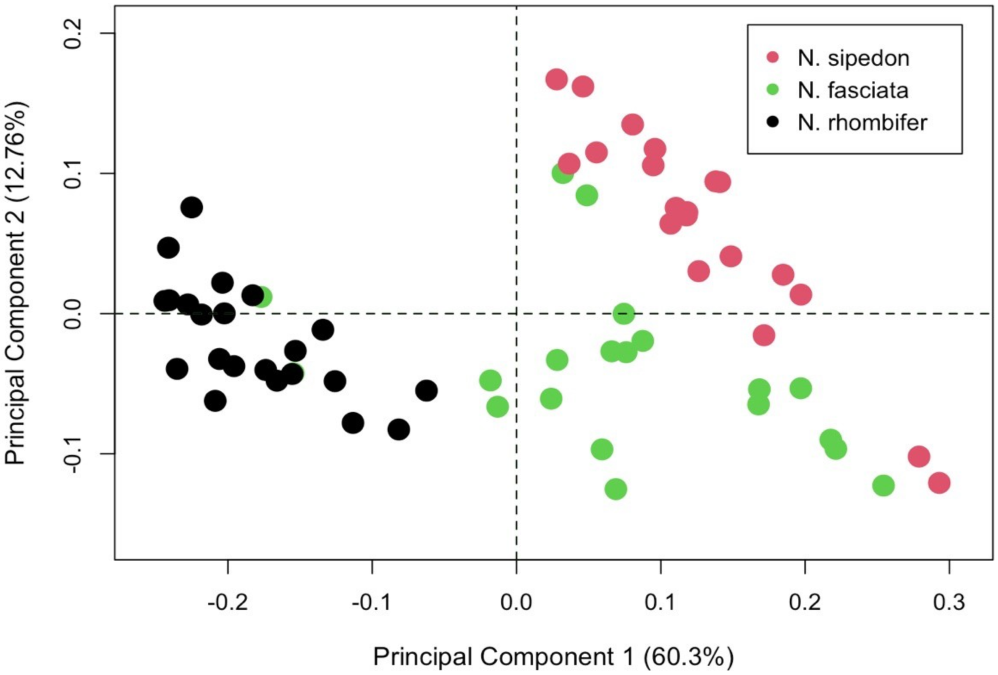

Figure 6. The 3DGM Principal Components Analysis (PCA) depicts changes in hemipenal shape across the two largest axes of variation. PC1 accounts for 60.3% of variation and PC2 accounts for 12.76% of variation of hemipene shape from the geometric morphometric data. Species groups are indicated by colors and show distinct aggregation within the linear morphospace.

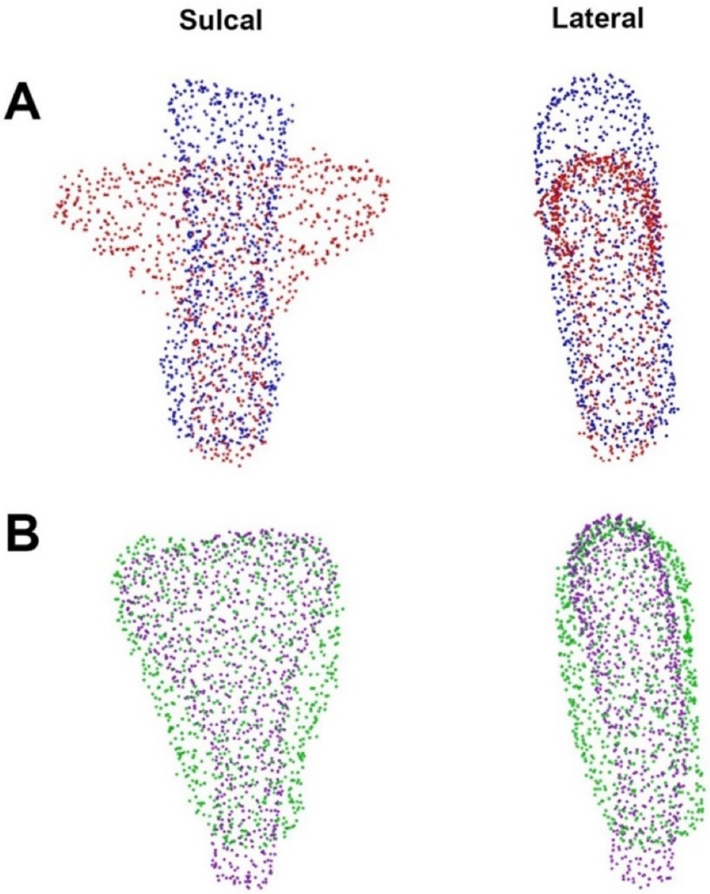

Figure 7. **Deformation grids showing hemipenal shape variation in PC1 and PC2 from sulcal and lateral perspective. (A)** The blue point cloud corresponds with sub-cylindrical hemipene shape at the negative end of PC1. The red point cloud corresponds with bilobed hemipene shape at the positive end of PC1. **(B)** The green point cloud corresponds with the wide base and widened width at the negative end of PC2. The purple point cloud corresponds with the elongated hemipene base shown at the positive end of PC2.

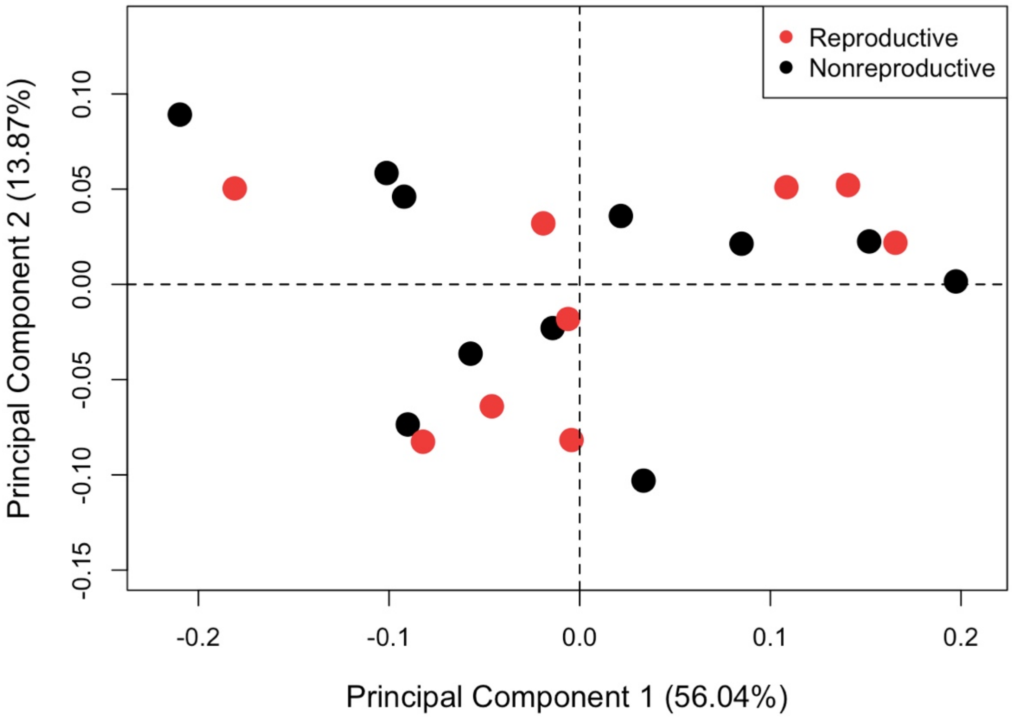

Figure 8. Principal Components Analysis depicts hemipene shape variation in *N. fasciata* (n=20) according to reproductive state. Along the two largest axes of variation, there is no clear aggregation of reproductive and nonreproductive individuals. Reproductive (red) and nonreproductive (black) are located in approximately equal ranges of PC1 and PC2.

Table 1. MANOVA and pairwise comparisons output for all females.

1a) 2D Vaginal Pouch Shape by Species, SVL, and Reproductive State (n=92)							
	Df	SS	MS	R^2	F	Z	P.value
Species	2	0.7002	0.3501	0.31262	24.359	8.8551	0.001 **
SVL	1	0.25875	0.25875	0.11553	18.003	5.0248	0.001 **
Repro. State	1	0.0304	0.0304	0.01357	2.115	1.7935	0.043 *
Residuals	87	1.25044	0.01437	0.55828			
Total	91	2.23979					

1b) Pairwise distances in vaginal pouch shape by species				
	d	UCL (95%)	Z	Pr > d
Rhom vs. Sip	0.1636159	0.05803061	5.302609	0.001
Rhom vs. Fas	0.1807663	0.06079185	5.486593	0.001
Sip vs. Fas	0.1023514	0.05897893	3.507864	0.001

Table 2. MANOVA and pairwise comparisons output for all males.

2a) 3D Hemipene Shape by Species and SVL (n=62)							
	Df	SS	MS	R^2	F	Z	P.value
Species	2	1.31242	0.65621	0.50939	32.5764	4.7668	0.001 **
SVL	1	0.09569	0.09569	0.03714	4.7505	2.514	0.005 **
Residuals	58	1.16834	0.02014	0.45347			
Total	61	2.57645					

2b) Pairwise distances in hemipene shape between species				
	d	UCL (95%)	Z	Pr > d
Rhom vs. Sip	0.3212525	0.1038551	3.398618	0.001
Rhom vs. Fas	0.2581122	0.100572	3.389502	0.001
Sip vs. Fas	0.1289095	0.1023106	2.078794	0.012

Table 3. Single-factor MANOVA for hemipenal shape and reproductive state in *N. fasciata*.

3D Hemipenal Shape by Reproductive State in *N. fasciata* (n=62)							
	Df	SS	MS	R^2	F	Z	P.value
Repro. State	1	0.00703	0.0070332	0.01609	0.2943	-1.6892	0.996
Residuals	18	0.43017	0.0238986	0.98391			
Total	19	0.43721					

DISCUSSION

We predicted that there would be no statistically significant difference in shape between male *N. fasciata* and *N. sipedon* hemipenes compared to females, who were previously found to have significant shape differences using 2DGM (Showalter et al. 2014). With the addition of the distant *N. rhombifer* species to both male and female analyses, we also predicted *N. rhombifer* genital shape would be more distinct from *N. fasciata* and *N. sipedon* than they are to each other. Together, these results would have suggested a lock-and-key mechanism driven by rapidly evolving female genitalia due to sexual conflict. Since the 3DGM shape analysis of males revealed significant shape differences between *N. fasciata* and *N. sipedon* (p=0.012), our hypothesis that speciation is being driven by rapidly divergent female genitalia was not supported. This finding does not rule out the possibility of sexual conflict driving the coevolution of *Nerodia* genital traits, but it could not provide us with the evidence to say females are ahead of males in the coevolutionary arms-race and responsible for driving species divergence.

Our findings, which revealed distinct genital shape across closely related species of *Nerodia*, are consistent with the patterns of species-specific genital diversity often observed in squamates (Brennan and Prum 2015). The data showed significant shape differences across species pairs for both vaginal and hemipenal shape, suggesting how a lock-and-key mechanism may be driving species divergence by reinforcement of genital divergence. Additionally, the similarly changing morphologies of intraspecific males and females supports the potential role of sexually antagonistic genital coevolution in these systems.

The results of our PCA, multi-factor MANOVA, and pairwise comparisons for females aligned with our predictions, showing a pattern of species-specific shape variation. In the negative quadrant of PC1 where *N. rhombifer* was congregated, deformation grids in Figure 5 revealed a more square-like vaginal pouch shape with very little bifurcation where the oviducts begin. This was the shape variable that set *N. rhombifer* apart from *N. sipedon* and *N. fasciata* the most, suggesting the vaginal pouches of more recently diverged species have evolved more bifurcated and elongated genitalia. PC2 is the axis which explains the majority of shape differences between *N. sipedon* and *N. fasciata* females. *N. fasciata* is clumped along the positive end of this axis where deformation grids show prominent bifurcation and narrower pouch shape. The negative end of PC2 where *N. sipedon* is crowded revealed wider cloacal shape and widening of the vaginal pouch. This apparent change in cloacal pouch opening between sister-species could present as a potential female-driven mechanical barrier to hybridization. Considering the cloaca is the point of entrance for the hemipene, this divergence in cloacal shape indicates a possible female-evolved reproductive isolation mechanism to prevent interpopulation mating that is worth investigating in a future study.

We ran two PCAs, two multi-factor MANOVAs, and pairwise comparisons that explored hemipene shape variation within our male sample. The first PCA included all males and found that hemipene shape is significantly different across all three species. The PCA allowed us to visualize the way in which *N. sipedon* and *N. fasciata* hemipene shape have evolved in relation to *N. rhombifer* and in relation to each other. PC1 accounted for more than half of the variance, revealing the dynamic shape transition from mostly sub-cylindrical to bilobed when moving from the negative to the positive end of this axis. We saw a large amount of overlap between *N. sipedon* and *N. fasciata* in the positive end of PC1, while *N. rhombifer* remained clumped in the negative region where specimens were more tubular. Considering the evolutionary relationship between *N. rhombifer* and the sister-species, this observation supports the ontogenetic prediction that the sub-cylindrical hemipene is ancestral to the bilobed hemipene (Jadin and King 2012). Similar to the pattern in females, the male data revealed how recently diverged species display dramatically different genital shapes in comparison to the more distant relative. PC2 accounted for less than a quarter of the variance and represents the portion of the morphospace where sister-species *N. sipedon* and *N. fasciata* were separated by shape variation surrounding the shaft, which showed a transition from wide to narrow. Specimens in the negative end of PC2 were wider from the lateral perspective as well. This suggests a potential trade off of length for width in *N. fasciata* that could be explained by male morphology coevolving to keep up with the evolving female (Brennan and Prum 2015).

In comparing all three species, it is interesting to note a lack of hemipene bilobation was associated with a wider base where spines are found. As the definition of the bilobed hemipene shape increases, the degree to which spines contribute to the shape of the base of the hemipene is reduced. This pattern may be explained by the idea that sub-cylindrical hemipenes like those of *N. rhombifer* require a spinier base in order to remain attached to the female during copulation (Friesen et al. 2014; King et al. 2009). It is possible that the shape of bilobed hemipenes may be used for the same anchoring purpose and therefore do not require large spines. If this pattern in *Nerodia* hemipene morphology exists, there is a possibility that sexual conflict is ameliorated in species that display bilobation. Finding evidence for this phenomenon would require closer examination of copulatory mechanics and basal spine morphology across these species.

To account for potential error considering our disproportionately nonreproductive *N. sipedon* sample and our disproportionately reproductive *N. rhombifer* sample, we examined whether reproductive status contributed to shape variation in the males of *N. fasciata*. Our results showed that reproductive status had no interaction effect with shape within the *N. fasciata* sample ($p = 0.996$), allowing us to rule out reproductive status as a confounding variable influencing our main 3-way analysis. In the future, it would be more desirable to have a larger sample size that includes nonreproductive *N. rhombifer* males and reproductive *N. sipedon* males to more effectively analyze the relationship of reproductive status to male hemipene shape.

In addition to reinforcing previous findings in both squamate and general genital evolution studies, this research represents the first attempt to measure hemipene morphology using Automated 3Dgm and the first to run a 3-way comparative analysis of snake vaginal pouch shape in 2D. The structural similarities in *N. sipedon* and *N. fasciata* hemipenes led us to believe they would not have significant shape differences, and although our findings contradicted this prediction, they prove the ability of Automated 3Dgm to capture complex shape variation that is impossible to quantify with the naked eye. This study also proves the reliability of 2D geometric morphometrics since it is a repeated experiment testing morphological variation in *N. sipedon* and *N. fasciata* (Showalter et al. 2014). Using the same methodologies and same specimens as the previous study, our 2DGM results revealed the same difference in bifurcation and elongation of vaginal pouches.

There were a number of limitations in this study which may have contributed to our unexpected findings. The first limitation was our use of 2D geometric morphometrics to measure shape variation of vaginal pouches. Although 2DGM is a reliable mode of shape analysis, there was potential vaginal pouch variation that a 3D model of the internal space would have more accurately captured. Additionally, unlike the automatic placement of landmarks in Auto3Dgm, 2D landmark placement in tpsDig2 relies on the potentially hidden bias of the human eye. In future steps we hope to remove this bias by collecting silicone models for all species of vaginas to analyze them in 3D. The vaginal specimens for these species had been removed and preserved before the beginning of this study, therefore only 2D photographs were available to us. Another potential drawback was that our male samples had uneven variance in regard to reproductive status. The disproportionately nonreproductive specimens in the *N. sipedon* sample could have contributed to the unexpected shape differences between the male sister-species considering our finding that hemipene shape changes with body length.

This study represents an important step in using advanced shape analysis to explain drivers of genital diversity. This study is also one amongst a limited number of studies to measure female snake genital variation and explore male and female genitalia co-currently. Through this research we present a unique insight into genital shape differences amongst species of snakes who diverged at different times from their last common ancestor. Our data quantitatively reveals the phenomenon of rapid genital divergence amongst closely related species, and the results of our shape analysis depict a potential coevolutionary arms-race between interspecific males and females. This research has resurfaced the prematurely rejected lock-and-key hypothesis, hopefully encouraging future examinations of the simultaneous influence of natural and sexual selection on genital diversification.

In future steps we hope to incorporate more species from the Genus *Nerodia* to more closely examine patterns of morphological divergence in relation to divergence time with larger samples. Larger and more equally distributed sample sizes of males and female genital specimens would also allow us to re-test the question of female-driven species divergence with stronger statistical power. We hope to eventually find a way to incorporate the geometric morphometric approach to test shape correspondence between males and females of the same species and test hypotheses surrounding sexually antagonistic coevolution.

LITERATURE CITED

Adams, D. C. and E. Otarola-Castillo. (2013). Geomorph: an R package for the collection and analysis of geometric morphometric shape data. Methods Ecol. Evol. 4:393– 399.

Adams, D. C., Collyer, M. L., Kaliontzopoulou, A., and Baken, E. (2021). Geomorph:Software for geometric morphometric analyses. R package version 3.3.2.

Adams, D.C., Rohlf, F.J., and Slice D.E. (2004). Geometric morphometrics: Ten years of progress following the 'revolution'. Italian Journal of Zoology 71:5-16.

Ah-King, M., Barron A.B., Herberstein, M.E. (2014). Genital evolution: Why are females still understudied? PLoS Biol 12:e1001851.

Aldridge, R.D., Flanagan, W.P., and Swarthout, J.T. (1995). Reproductive biology of the water snake N*erodia rhombifer* from Veracruz, Mexico, with comparisons of tropical and temperate snakes. Herpetologica 51:182-192.

Anderson, C. M., and Langerhans, R. B. (2015). Origins of female genital diversity: Predation risk and lock-and-key explain rapid divergence during an adaptive radiation. Evolution 69:2452–2467.

Andonov, K., Natchev, N., Kornilev, Y.K., and Tzankov, N. (2017). Does Sexual Selection Influence Ornamentation of Hemipenes in Old World Snakes? The Anatomical Record 300:1680–1694.

Arnqvist, G., and Rowe, L. (2005). Sexual Conflict. Princeton, NJ: Princeton UniversityPress.

Brennan, P. L.R., and Prum, R.O. (2015). Mechanisms and Evidence of Genital Coevolution: The Roles of Natural Selection, Mate Choice, and Sexual Conflict. Cold Spring Harb Perspect Biol 7:a017749.

Brennan, P.L.R. (2010). Sexual Selection. Nature Education Knowledge 3(10):79.

Defour, L. (1844). Anatomie generale des Dipteres. Ann. Sci. Nat. 1, 244–264.

Dougherty, L. R., van Lieshout, E., McNamara, K. B., Moschilla, J. A., Arnqvist, G., and Simmons, L. W. (2017). Sexual conflict and correlated evolution between male persistence and female resistance traits in the seed beetle Callosobruchus maculatus. Proceedings of the Royal Society B: Biological Sciences 284:20170132.

Eberhard W.G. (1985). Sexual selection and animal genitalia. Cambridge, MA: Harvard University Press.

Eberhard W.G. (2009). Postcopulatory sexual selection: Darwin's omission and its consequences. PNAS 106: 10025–10032.

Eberhard W.G. (2010). Evolution of genitalia: theories, evidence, and new directions. Genetica 138:5–18.

Eberhard W.G. (2011). Experiments with genitalia: A commentary. TREE 26:17–21.

Eberhard W.G. (2019). Demonstrating sexual selection by cryptic female choice on male genitalia: What is enough? Evolution 73:2415-2435.

Edgren R.A. (1953). Copulatory adjustment in snakes and its evolutionary implications. Copeia 1953:162–164.

Friesen C., Uhrig, E.J., Squire, M.J., Mason, R., and Brennan P.L.R. (2014). Sexual conflict over mating in red-sided Garter snakes *Thamnophis sirtalis* as evidenced by experimental manipulation of genitalia. Proc Biol Sci 281:20132694.

Greenway, R., McNemee, R., Okamoto, A., Plath, M., Arias-Rodriguez, L., and Tobler, M. (2019). Correlated divergence of female and male genitalia in replicated lineages with ongoing ecological speciation. Evolution 76:1200-1212.

Gunnell, G.F., Boyer, D.M., Friscia, A.R., Heritage, S., Manthi, F.K., Miller, E.R., Sallam, H. M., Simmons, N.B., Stevens, N.J., and Seiffert, E.R. (2018). Fossil lemurs from Egypt and Kenya suggest an African origin for Madagascar's aye-aye. Nature Communications 10.1038/s41467-018-05648-w.

Hedrick, B., Antalek-Schrag, P., Conith A.J., Natanson, L.J., and Brennan, P.L.R. (2019).Variability and asymmetry in the shape of the Spiny Dogfish vagina revealed by 2D and 3D geometric morphometrics. Journal of Zoology 308:16-27.

Hosken, D.J. and Stockley, P. (2004). Sexual selection and genital evolution. TrendsEcol. Evol. 19: 87–93.

House, C.M. and Simmons L.W. (2005). The evolution of male genitalia: patterns of genetic variation and covariation in the genital sclerites of the dung beetle Onthophagus taurus. Journal of Evolutionary Biology. 5: 1281-1292.

Jadin, R.C. and King, R. B. (2012). Ontogenetic Effects on Snake Hemipenal Morphology. Journal of Herpetology 46:393–395.

Jellen, B.C. and Aldridge, R.D. (2014). It takes two to tango: female movement facilitatesmale mate location in wild northern watersnakes (*Nerodia sipedon*). Behaviour 151:421-434.

King R.B., Jadin R.C., Grue M., and Walley H.D. (2009). Behavioral correlates with hemipenis morphology in New World natricine snakes. Biological Journal of theLinnean Society 98:110–120.

Lawing, A.M. and Polly, P.D. (2009). Geometric morphometrics: recent applications tothe study of evolution and development. Journal of Zoology. 280:1-7.

Lawson, R. (1987). Molecular studies of the Thamnophiine snakes: 1. The phylogeny ofthe genus *Nerodia*. Journal of Herpetology. 21:140-157.

Lorenz, T.O., Horne, B.D., Anderson, N.J., and Cheek, A.O. (2011). Reproductive physiology of the broad banded watersnake, *Nerodia fasciata confluens*, in southeastern Louisiana. Herpetological Conservation and Biology 6:410−421.

McVay, J.D., Flores-Villela, O., and Carstens, B. (2015). Diversification of North American natricine snakes. Biological Journal of the Linnean Society 116:1-12.

Merbert, K. (2008). Good species despite massive hybridization: genetic research on thecontact zone between the watersnakes *Nerodia sipedon* and *N. fasciata* in the Carolinas, USA. Molecular Ecology. 17:1918-1929.

Orbach, D.N., Hedrick, B.P., Würsig, B., Mesnick, S.L. and Brennan, P.L.R. (2018). The evolution of genital shape variation in female cetaceans. Evolution 72, 261–273.

Pope C.H. (1941). Copulatory adjustment in snakes. Field Mus Nat Hist, Zool Ser 24:249–252.

Porto, M., de Oliveira, M. A., Pissinatti, L., Rodrigues, R. L., Rojas-Moscoso, J. A., Cogo, J. C., Metze, K., Antunes, E., Nahoum, C., Mónica, F. Z., and De Nucci, G. (2013). The Evolutionary Implications of Hemipenial Morphology of Rattlesnake Crotalus durissus terrificus (Laurent, 1768) (Serpentes: Viperidae: Crotalinae). PLoS ONE 8:e66903.

Puniamoorthy, N., Kotrba, M., and Meier, R. (2010). Unlocking the “Black box”: Internal female genitalia in Sepsidae Diptera evolve fast and are species-specific. BMC Evol Biol 101:275.

Rohlf, F.J. and Marcus, L.F. (1993). A revolution in morphometrics. Trends in Ecology and Evolution 8: 129–132.

Rohlf, F. J. (2007). BIOMstat, TPS software. Morphometrics at SUNY Stony Brook.

Showalter I., Todd B., and Brennan P.L.R. (2013). Intraspecific and interspecific variation of the vagina in two species of water snakes. Biol J Linn Soc Lond111:183–191.

Simmons, L.W. (2019). Sexual selection and genital evolution. Austral Entomology 53:1-17.

Simmons, L.W. and Fitzpatrick, J.L. (2019). Female Genitalia Can Evolve More Rapidlyand Divergently than Male Genitalia. Nature Communications 10.1038/s41467- 019-09353-0.

Sloan, N. S. and Simmons, L.W. (2019). The evolution of female genitalia. J. Evol. Biol. 2019:1–18.

Weatherhead P.J., Barry, F.E., Brown G.P., and Forbes M.R. (1995). Sex ratios, matingbehavior and sexual size dimorphism of the northern water snake, *Nerodia sipedon*. Behavioral Ecology and Sociobiology 36: 301–311.

APPENDIX

FEMALE DATA

Specimen ID	Species	SVL (cm)	Reproductive?
NF157	Fas	98	Yes
NF059	Fas	55.1	Yes
NF046	Fas	65.7	Yes
NF158	Fas	72	Yes
NF013	Fas	58.9	Yes
NF202	Fas	35.4	No
NF249	Fas	54	No
NF196	Fas	56.5	No
NF133	Fas	26.8	No
NF045	Fas	63.2	Yes
NF006	Fas	74.4	Yes
NF100	Fas	67.9	Yes
NF193	Fas	58.7	No
NF189	Fas	60.5	No
NF040	Fas	54.4	No
NF084	Fas	31.2	No
NF207	Fas	76.6	Yes
NF242	Fas	67	Yes
NF015	Fas	49.7	No
NF089	Fas	76.6	Yes
NF186	Fas	16.1	No
NF017	Fas	63	Yes
NF081	Fas	37	No
NF088	Fas	69	Yes
NF028	Fas	62.5	Yes
NF058	Fas	58.6	Yes
NF073	Fas	30.3	No
NF261	Fas	53.5	Yes
NF185	Fas	17.3	No
NF211	Fas	38.5	No
NR1813*	Rhom	82	No
NR1819	Rhom	57	No
NR1816	Rhom	72.5	Yes
NR1813	Rhom	54.5	No

NR1800	Rhom	87	Yes
NR1827	Rhom	94.5	Yes
NR1815*	Rhom	81	Yes
NR1801	Rhom	96.5	Yes
NR1370g	Rhom	102	Yes
NR1825	Rhom	65	No
NR1811*	Rhom	60.7	No
NR1811	Rhom	47.5	No
NR1807*	Rhom	102.5	Yes
NR255g	Rhom	66.5	No
NR1812*	Rhom	76	Yes
NR1826	Rhom	101	Yes
NR1810*	Rhom	90	Yes
NR1823	Rhom	82.5	Yes
NR428	Rhom	71.5	No
NR1821	Rhom	74	No
NR1806	Rhom	67	Yes
NR1822	Rhom	89.5	Yes
NR1825*	Rhom	72.5	Yes
NR1316g	Rhom	97	Yes
NR1812	Rhom	80.5	Yes
NR1814*	Rhom	60.5	No
NR1809	Rhom	93	Yes
NR1840	Rhom	43.2	No
NR1820	Rhom	90.5	Yes
NS053	Sip	33.6	No
NS108	Sip	60	Yes
NS106	Sip	34.1	No
NS029	Sip	49	No
NS015	Sip	33	No
NS102	Sip	40.1	No
NS066	Sip	34.1	No
NS049	Sip	72	Yes
NS048	Sip	89	Yes
NS073	Sip	70	Yes
NS044	Sip	27.3	No
NS072	Sip	38.1	No
NS041	Sip	25.6	No

NS070	Sip	33.3	No
NS037	Sip	47.5	No
NS005	Sip	38.4	No
NS023	Sip	72.4	Yes
NS099	Sip	75.6	Yes
NS107	Sip	49.3	Yes
NS076	Sip	39.1	No
NS075	Sip	37.7	No
NS097	Sip	68	Yes
NS017	Sip	34.9	No
NS074	Sip	53	No
NS71	Sip	31.2	No
NS062	Sip	45.7	No
NS101	Sip	68.3	Yes
NS109	Sip	59.4	Yes
NS033	Sip	30.5	No
NS084	Sip	32.2	No
NS014	Sip	36.2	No
NS056	Sip	37.9	No
NS046	Sip	80.5	Yes

MALE DATA

Specimen ID	**Species**	**SVL (cm)**	**Reproductive?**
NF056M	Fas	32.8	No
NF067M	Fas	56.6	Yes
NF075M	Fas	35.2	No
NF085M	Fas	37.3	No
NF087M	Fas	44.8	Yes
NF096M	Fas	58.1	Yes
NF139M	Fas	50.7	Yes
NF144M	Fas	39	No
NF151M	Fas	40.4	No
NF163M	Fas	51	Yes
NF177M	Fas	39.3	No
NF181M	Fas	33.5	No
NF188M	Fas	16.5	No
NF210M	Fas	57	Yes
NF227M	Fas	38.5	No

NF238M	Fas	44.2	No
NF246M	Fas	43	Yes
NF255M	Fas	43.5	Yes
NF256M	Fas	42.2	Yes
NF265M	Fas	44	No
NR100M	Rhom	72	Yes
NR101M	Rhom	57.6	Yes
NR102M	Rhom	57.2	Yes
NR103M	Rhom	48.2	Yes
NR1539M	Rhom	63	Yes
NR162gM	Rhom	57	Yes
NR1804M	Rhom	61	Yes
NR1805M	Rhom	63	Yes
NR1806M	Rhom	60.5	Yes
NR1807M	Rhom	59.5	Yes
NR1808M	Rhom	76.5	Yes
NR1810M	Rhom	73	No
NR1814M	Rhom	78	Yes
NR1815M	Rhom	67	Yes
NR1816M	Rhom	73.3	Yes
NR1817M	Rhom	67.5	Yes
NR1818M	Rhom	79	Yes
NR1820M	Rhom	57	Yes
NR1824M	Rhom	57.5	Yes
NR1828M	Rhom	56.5	Yes
NR1880M	Rhom	69	Yes
NR417aM	Rhom	74	Yes
NS002M	Sip	35.3	No
NS006M	Sip	33.2	No
NS008M	Sip	29.5	No
NS010M	Sip	41	Yes
NS019M	Sip	39.6	Yes
NS020M	Sip	37	No
NS035M	Sip	37.1	No
NS043M	Sip	36	No
NS060M	Sip	34.6	No
NS061M	Sip	34.4	No
NS068M	Sip	46.2	Yes

NS080M	Sip	29.7	No
NS081M	Sip	37.3	No
NS085M	Sip	36.6	No
NS087M	Sip	31.7	No
NS088M	Sip	32	No
NS089M	Sip	35.8	No
NS098M	Sip	42.5	Yes
NS103M	Sip	35.9	No
NS104M	Sip	35.4	No