



Intraspecific and interspecific variation of female genitalia in two species of watersnake

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The morphological differences in female genitalia within and between species are little studied and poorly understood, yet understanding patterns of variation in female genitalia can provide insights into mechanisms of genital evolution. The present study aimed to explore the patterns of intraspecific and interspecific variation in female genitalia in two sister taxa of watersnake (*Nerodia sipedon* and *Nerodia fasciata*) that have similar genital shape. We used a geometric morphometric (GM) approach to study variation in shape of the vagina between and within two sister species. We examined genital shape in female watersnakes ranging from small, sexually immature females to large reproductive females that had recently given birth. We found that shape variation of genitalia is strongly correlated with body size, where larger but not smaller females have a bifurcation in the vagina. However, we also found significant shape variation in the structure of the vagina between the two species, where *N. fasciata* has narrower genitalia with more prominent bifurcation, whereas *N. sipedon* has wider genitalia with less marked bifurcation. Using GM allowed us to detect significant differences in genital shape that were not apparent upon visual examination alone, suggesting that shape variation in female genitalia may be greater than previously assumed. Additional study of morphological differences in male reproductive organs for these species would help to determine whether there has been genital co-evolution, and potentially mechanical reproductive isolation, in these two closely-related and occasionally sympatric species. © 2013 The Linnean Society of London, *Biological Journal of the Linnean Society*, 2014, **111**, 183–191.

ADDITIONAL KEYWORDS: genital evolution – geometric morphometrics – shape analysis – snake reproduction – vagina – vaginal pouch.

INTRODUCTION

Studies of genital evolution centre on understanding morphological variation of male copulatory structures, and more recently on how they interact and co-evolve with female reproductive structures (Hosken & Stockley, 2004; Brennan *et al.*, 2007; Eberhard, 2010). However, morphological differences in female genitalia within and between species are

generally little studied and poorly understood. Although discriminating among hypotheses of genital evolution based on morphological studies alone is difficult, examining patterns of variation in female genitalia can provide insights into evolutionary mechanisms. For example, if there is little variation in the female genitalia of closely-related species, it is possible to rule out the lock and key hypothesis of genital evolution because, in the absence of interspecific differences, mechanical isolation would not be possible during speciation (Eberhard, 1985; Shapiro & Porter, 1989). Typically, female genitalia

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are considered less variable than male genitalia and, for this reason, lock and key has been deemed unlikely to be of widespread importance (Eberhard, 1985).

By contrast, hypotheses of genital evolution by sexual selection and sexual conflict have received widespread support (Hosken & Stockley, 2004). The predictions of sexual selection hypotheses vary according to the proposed mechanism but, unlike lock and key, they do not explicitly predict differences in genitalia among closely-related species. Sexual selection and sexual conflict hypotheses suggest that there should be some evolutionary correlation between male and female genitalia (Hosken & Stockley, 2004), implicitly suggesting that female genital variation should correlate with male genital variation within a species. For example, the 'one size fits all' hypothesis of cryptic female choice predicts, in a similar fashion to the lock and key hypothesis, that there should be little intraspecific variation in female and male genitalia because males are selected to fit the average female genital structure and are under stabilizing selection (Eberhard *et al.*, 1998). However, actual measures of shape or size variation in female genitalia are lacking in most taxa.

Genital variation among male snakes is often remarkable. Snake hemipenes can be so variable that their morphologies have been used for decades to distinguish and describe species (Cope, 1896; Camp, 1923; Dowling & Savage, 1960; Dowling, 1967; Myers & Cadle, 2003). The portion of female genitalia that receives the male intromittent organ in snakes has been called the vagina, cloaca, urodeum, oviduct, and pouch, or has simply been ignored (for a historical summary of oviducal nomenclature in snakes, see Siegel *et al.*, 2012). More recently, female genitalia have been examined in studies looking at a structure known as 'the pouch' or 'vaginal pouch', which has recently been extensively described (Siegel *et al.*, 2011, 2012). The potential for female pouch variation to be used in taxonomic studies has been recognized (Sánchez-Martínez, Ramírez-Pinilla & Miranda-Esquivel, 2007; Siegel *et al.*, 2011, 2012). Determining the extent of the pouch requires a histological approach and so, in the present study, we refer to female genitalia as the vagina. The vagina in vertebrates is the region of the female reproductive tract that is located between the outside of the body (cloacal opening or entrance of the copulatory canal) and the lower portion of the uterus/shell gland. Functionally, it receives the penis or hemipene during copulation and distends to aid the birthing/egg laying process. Very little is known about patterns of intraspecific and interspecific morphological variation of female genitalia in snakes.

Copulatory adjustment, a close fit between the male hemipene and the female vagina during copulation,

has been described in several snakes (Pope, 1941; Edgren, 1953; Pisani, 1976), although it is not universal (Siegel *et al.*, 2012). Female genitalia in snakes have been shown to vary at least among families (Siegel *et al.*, 2011) and, in some species, this variation closely matches hemipene shape (Pope, 1941; Edgren, 1953; Pisani, 1976). Such a close fit suggests a co-evolutionary process that could result from intersexual selection (Böhme & Ziegler, 2009), sexual conflict (King *et al.*, 2009), or lock and key mechanisms (Pope, 1941).

In the present study, we use a geometric morphometric approach (GM) (Rohlf & Marcus, 1993; Adams, Rohlf & Slice, 2004) to quantify the degree and compare the major axes of shape variation in female genitalia within and between two closely-related *Nerodia* species: *Nerodia fasciata*, the banded watersnake, and *Nerodia sipedon*, the northern watersnake. Landmark-based geometric methods provide new insights into patterns of biological shape variation that cannot be evaluated by traditional methods (Adams *et al.*, 2004). We examined the change in morphology of the vagina across different size classes that reflect an ontogenetic trajectory. We expected that an ontogenetic examination of the morphology would reveal which aspects of vaginal shape may be important for copulatory and reproductive function in general, given that some morphological traits may be absent in sexually immature juveniles and become developed in reproductive adults. We also examined differences between these two species in the shape of their vagina. Their shape is very similar superficially, although we expected that using a quantitative approach would show whether the shape of the genitalia was indeed indistinguishable in these two species. Finally, we examined whether either of these two species or different reproductive stages within a species showed evidence of greater morphological diversity. Morphological diversity or variation within each species and maturity stage (reproductive versus nonreproductive) was measured using Foote's disparity measure (Foote, 1993). Because selection on genital shape should be stronger when females are reproductive, we expected that reproductive females would have less morphological variation than nonreproductive females in their genital shape, whereas we did not expect any differences in morphological diversity between species overall including both reproductive and nonreproductive individuals.

MATERIAL AND METHODS

STUDY SPECIES

Nerodia fasciata, and *N. sipedon* are closely-related sister species that have at times been treated as a

single species. However, *N. fasciata* was eventually elevated to species status based on consistent differences in external characters throughout much of its range compared with *N. sipedon* (Conant, 1963). The two species meet in contact areas along the Mississippi River Valley and eastward along the Fall Line that separates the Coastal Plain and Piedmont physiographical provinces as far north as North Carolina (Mebert, 2010). Extensive molecular research has concluded that they are both valid species (Alfaro & Arnold, 2001), and they are on independent evolutionary trajectories despite hybridization in some sympatric zones (Mebert, 2010). *Nerodia fasciata* is a lowland species, present in a subtropical climate, whereas *N. sipedon* is found much farther north in more temperate climate. Little published information exists on the mating system of *N. fasciata*, although *N. sipedon* is known to form mating aggregations with both males and females mating with multiple partners. Paternity of clutches is often mixed and the opportunity for sexual selection is greater in males than females as a result of the lack of paternity success of many courting males (Prosser *et al.*, 2002).

SPECIMEN COLLECTION

Nerodia fasciata specimens were collected in 2010 from an introduced population established in Los Angeles County, CA, since at least the late 1960s (Bury & Luckenbach, 1976). They were collected from Machado Lake, a shallow man-made lake approximately 16 ha in size in Ken Malloy Harbor Regional Park. We trapped the snakes using a mix of plastic (<http://www.gatorbuckets.com>) and metal (Gee traps) aquatic minnow traps as described in Rose, Miano & Todd (2013). All captured snakes were removed upon capture and returned to the laboratory where they were humanely euthanized on their day of capture via overdose of inhaled isoflurane anaesthetic gas. Specimens were then frozen whole for later dissection and analysis. Trapping was conducted under California Department of Fish and Wildlife collecting permit #802046-02 to Robert N. Reed of the US Geological Survey. Trapping and euthanasia was approved by the Institutional Animal Care and Use Committee of the USGS Fort Collins Science Center.

Nerodia sipedon specimens were collected in 2011 from an introduced population established in Placer County, CA since at least 2007 (Balfour *et al.*, 2007). They were collected from a small wetland approximately 6 ha in size that feeds Dry Creek, a tributary of the Sacramento River. We trapped the snakes using a mix of plastic and metal aquatic minnow traps as described above (Rose *et al.*, 2013 for additional site and capture information). All captured snakes were removed upon capture and returned to the laboratory

where they were humanely euthanized on their day of capture via overdose of inhaled isoflurane anaesthetic gas. Specimens were then frozen whole for later dissection and analysis. Trapping was conducted under California Department of Fish and Wildlife collecting permit #SC-11197 to B. D. Todd. Trapping and euthanasia was approved by UC Davis Institutional Animal Care and Use Committee protocol #2011-16553.

MORPHOLOGICAL MEASUREMENTS

We obtained mass (g) and snout–vent length (SVL) (mm) of 47 female *N. fasciata* (*Nf*) and 54 *N. sipedon* (*Ns*). We dissected all females to count the number of eggs or embryos and determine their developmental stages. We removed the cloaca and genital tract of each female and clipped the oviduct 3–5 cm from the vagina. The excised organ was rinsed with deionized water, fixed in formalin for 30 min, and then cleaned of connective tissues, muscles, and large intestine. We laid the cleaned vagina carefully to prevent any changes in shape, and we used pins as necessary to make the shape of the structure clearly visible. Using a blunt probe, we identified the outer edge of an arch on the cloacal lip (an unnamed structure present in all specimens) and placed paper arrows on these points to accurately identify these landmarks in all photographs (Fig. 1A, B). We then photographed the ventral aspect of the cloaca next to a ruler for scale. The females were categorized into four groups according to their size (G1: up to 350 mm SVL; G2: 351–377 mm; G3: 378–530 mm; and G4: 530–756 mm) and we tried to include a similar number of specimens for each size class (G1: *Nf* = 10, *Ns* = 11; G2: *Nf* = 12, *Ns* = 16; G3: *Nf* = 12, *Ns* = 10; G4: *Nf* = 20, *Ns* = 10).

Although GM methods are typically used in hard tissues, we were interested in determining whether this method could be useful to detect shape differences in soft tissues, and as such we adapted the most common procedures to answer this question. We performed all our digitalization and preliminary analyses using TPS software (<http://life.bio.sunysb.edu/morph/>). Using tpsDig version 2.0, we digitized six landmarks for each structure that could be identified reliably in every specimen measured. These included the outer edges of the cloacal arches, the midpoint between the cloacal arches just below the ventral cloacal scale, the midpoint of the connection between the oviduct and the vagina, and the midpoint between the two oviduct connections (Fig. 1). These landmarks are structurally analogous in all specimens. We consider that this approach is justified given that we are simply describing the axes of shape variation and differences within and between two species. We also used 22 semi-landmarks along six curves bounded by landmarks (Fig. 2). The use of semi-landmarks is

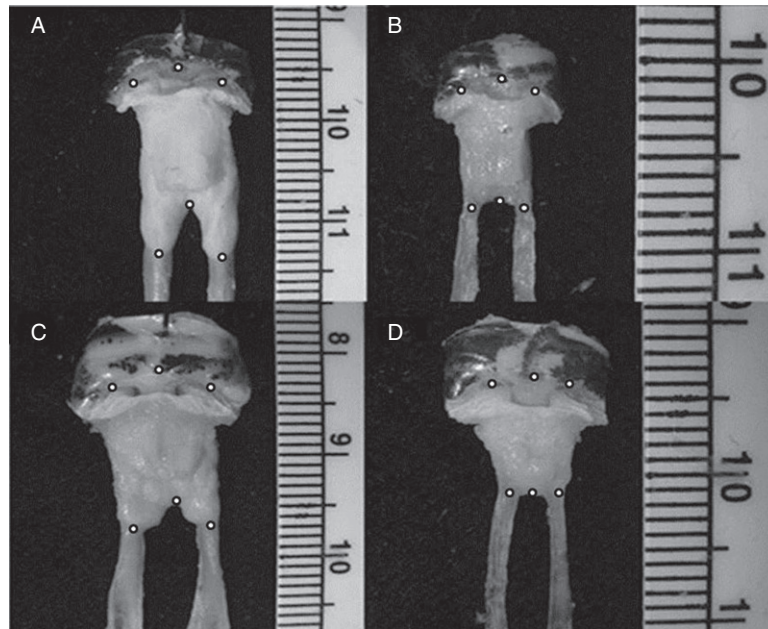


Figure 1. Ventral view of cloaca and vagina of *Nerodia fasciata* (A, B) and *Nerodia sipedon* (C, D). Bifurcation of the vagina is observed in mature females on the left (A, C) and there is a lack of bifurcation in younger females on the right (B, D). The position of six landmarks is shown in (A) and (B).

necessary because the shape of the vagina consists of several curves. Procrustes superimposition was performed using tpsUtil and tpsRelw to minimize differences among individuals based on position, rotation, and scale (Adams *et al.*, 2004). The resulting aligned coordinates for landmarks and semi-landmarks were used for further shape analyses in MorphoJ (Klingenberg, 2011). Visualization of the morphospace separating the specimens by species and size class was done in MorphoJ as well as generating the principal components (PCs). We exported the PC files and added information on the SVL and species for each specimen, and used these for the statistical analysis in R, version 2.13.0 (R Core Development Team). We performed a multivariate analysis of variance on species and maturity (reproductive versus nonreproductive) or size class (1–4) to compare multivariate means across the groups (manova(cbind(pc1,pc2,pc3)~species+maturity (or size class)). We corrected *P*-values for multiple comparisons using the function p.adjust.methods (methods 'holm').

We also measured differences in morphological disparity, or the extent of variation in each group, using IMP software package (<http://www.canisius.edu/~sheets/morphsoft.html>). We used DisparityBox6 to generate Foote's disparity (Foote, 1993), which measures the distance of the centroid of each group to the centroid of all groups, and provides a measurement of which particular group is using more morphospace as

an estimate of morphological diversity. We calculated the within group disparity for each group using the bootstraps within group disparity option (2500 bootstraps) to generate a 95% confidence interval. We used PairDisparity to test whether the disparity of one group was significantly different from the disparity of another group. We compared disparity between species and within reproductive stages of each species (reproductive versus nonreproductive, classified according to the size of the largest egg in the oviduct). We performed pairwise comparisons between species and reproductive status using the permutation analysis with 1000 iterations. If the actual disparity difference between the two original groups is greater than the upper bound of the permutation, then the differences in disparity are considered significant.

RESULTS

The consensus shape of all specimens is shown in Figure 2. The largest morphological changes are in both the degree of bifurcation of the vagina (shown as the longer lines at the bottom of Fig. 2), and the aspect ratio of the vagina itself (shown as the longer lines at the top right and left of Fig. 2). The results of morphospace plots of the vagina separated by species and age class are shown in Figure 3. We found that both ontogeny (size class) and species (*N. fasciata* and *N. sipedon*) significantly affected vagina morphology in these sister taxa (Fig. 3A). PC1 explained 49.47%

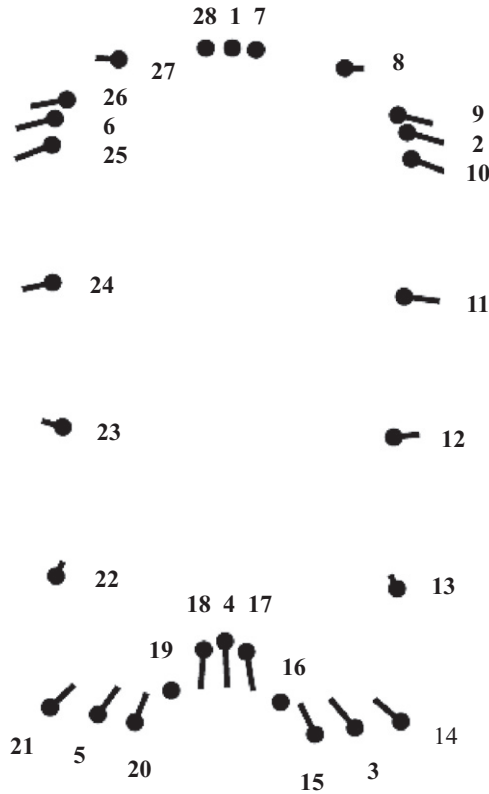


Figure 2. The consensus shape of the vagina in *Nerodia sipedon* and *Nerodia fasciata*. Each dot represents a landmark (1–6) and semi-landmark (7–28) and the length of the lines shows the magnitude and directionality of the shape changes between and within the species.

of the variance, whereas PC2 explained 21.57%, and PC3 explained 6.08%. For our analysis, we plotted PC1 versus PC2. The PC1 axis describes the variation in bifurcation, with negative values describing a vagina with bifurcation, and positive values describing a vagina without distinct bifurcation. The smallest individuals in both species had a nonbifurcated vagina (the right quadrants of Fig. 3), whereas the bifurcation becomes evident as individuals become larger, and it is greatest in adult reproductive females (on the left quadrants of Fig. 3). The PC2 axis describes morphological variation in the aspect ratio of the vagina, with positive values illustrating a longer, narrower vagina, and negative values showing a shorter, wider vagina. Although there is overlap between *N. sipedon* and *N. fasciata*, there is a region of morphospace that is occupied by each species and not the other. The left upper quadrant of morphospace is occupied exclusively by large (groups 3 and 4) *N. fasciata*, whereas the lower right quadrant is occupied primarily by small (groups 1 and 2) *N. sipedon*. Generally, *N. fasciata* have a narrower vagina with more prominent bifurcation whereas

Table 1. Foote’s disparity for each species and reproductive (R) versus nonreproductive (NR) stages

	Foote’s disparity	95% confidence interval	SE
Fasciata all	0.0141	0.0119–0.0160	0.0010
Sipedon all	0.0189	0.0154–0.0211	0.0015
Fasciata NR	0.0125	0.0092–0.0148	0.0014
Fasciata R	0.0113	0.0092–0.0129	0.0008
Sipedon NR	0.0131	0.0088–0.0159	0.0017
Sipedon R	0.0153	0.0117–0.0173	0.0013

Table 2. Pairwise comparisons between disparity indexes using permutation (1000 iterations) (R, reproductive; NR, nonreproductive; S, *Nerodia sipedon*; F, *Nerodia fasciata*)

Comparison	Observed disparity difference	Permutation 95%	P
Sipedon: R versus NR	0.00221	0.0044	0.37
Fasciata: R versus NR	0.00115	0.0032	0.49
Nonreproductive: S versus F	0.00061	0.0047	0.81
Reproductive: S versus F	0.00398	0.0031	0.001*

*The only significant disparity difference was in the comparison between reproductive females.

N. sipedon have a wider vagina with less bifurcation. These results are supported by the multivariate analysis of variance (MANOVA) (corrected for multiple comparisons, all results identical regardless of method) (reproductive versus nonreproductive: $F_{3,96} = 34.9$, corrected $P < 0.001$; species: $F_{3,96} = 23.1$ corrected $P < 0.001$). We also conducted a MANOVA with size class (1–4) instead of reproductive stage with the same highly significant results (size $F_{3,96} = 75.1$, corrected $P < 0.001$; species: $F_{3,96} = 25.1$, corrected $P < 0.001$).

The Foote disparity values and confidence intervals are reported in Table 1. The pair comparison analysis showed that there were significant differences in disparity between reproductive individuals of both species, with reproductive *N. sipedon* occupying a significantly larger morphospace than reproductive *N. fasciata* reflecting greater morphological diversity in the vagina of reproductive *N. sipedon* females (Fig. 3B, Table 2). By contrast to our expectation, there were no disparity differences between reproductive and nonreproductive stages within species or between nonreproductive individuals of both species (Table 2).

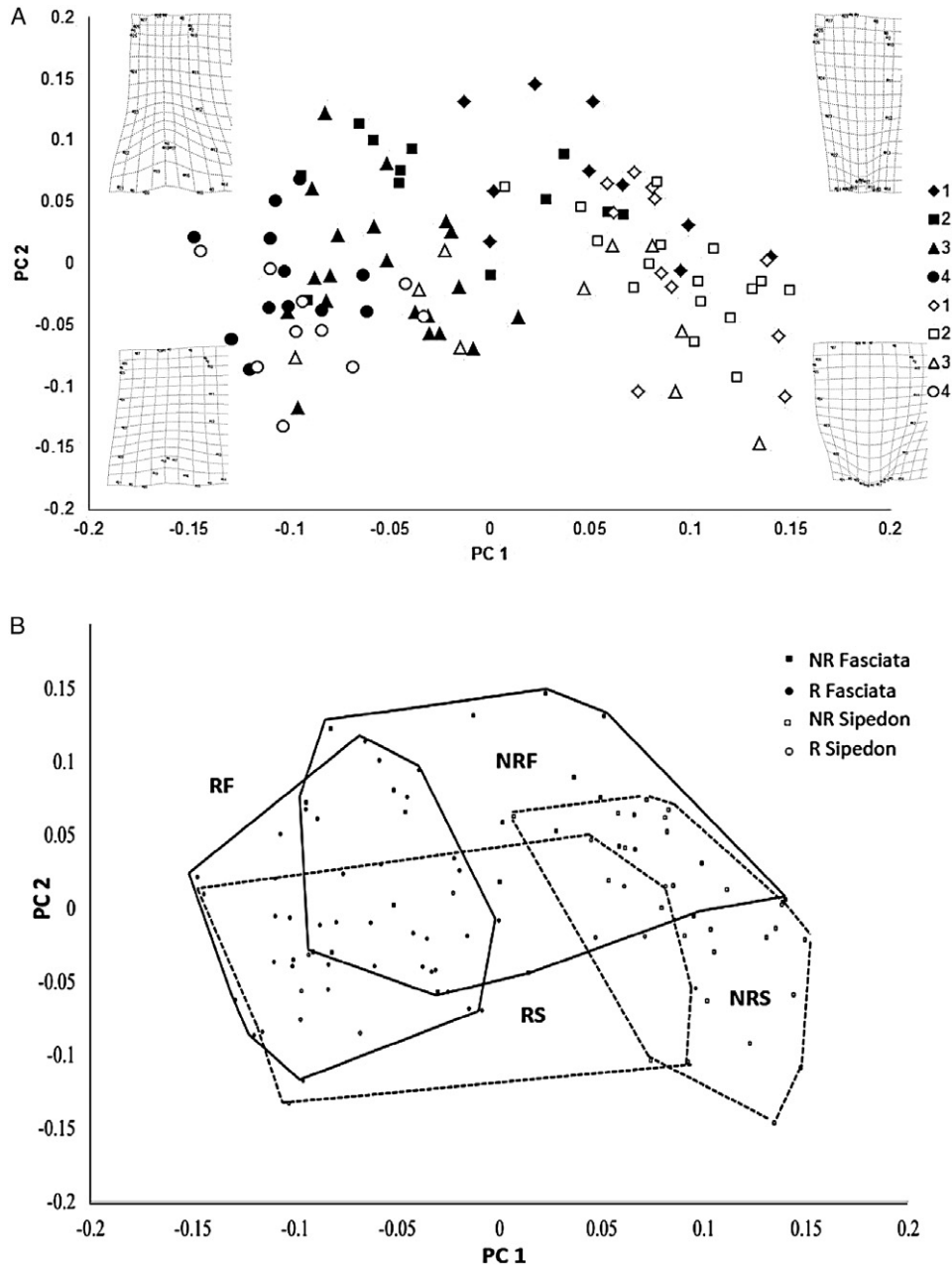


Figure 3. Morphospace of the vagina in two sister taxa of watersnake. Black symbols represent *Nerodia fasciata* and white symbols represent *Nerodia sipedon*. A, data separated by length categories: the smallest females (size category 1) are occupying primarily positive values of PC1 (nonbifurcated) and large females (size category 4) are occupying negative values of PC1 (bifurcated). Although there is overlap, each species occupies some space in separate quadrants of morphospace. B, data separated by reproductive (R) versus nonreproductive status (NR) and species (S, *sipedon*; F, *fasciata*). The only significant differences in disparity were between reproductive individuals of the two species.

DISCUSSION

We found that morphological variation in female vagina is strongly correlated with ontogeny and sexual maturation, and that the vagina is different

in these two closely related species. Bifurcation of the vagina develops when females become sexually mature, suggesting that the bifurcation itself is related to reproductive function: either bearing young or copulating, the two known functions of the vagina

in this live-bearing group of snakes. Because many snakes do not have a bifurcated vaginal pouch, including both live-bearing and egg-laying taxa (Siegel *et al.*, 2011, 2012), a potential live bearing function does not appear to be as important as a potential role during copulation in explaining the appearance of the bifurcation. We suggest that the bifurcation in the female vagina develops in sexually mature females to allow for a fit with the male hemipenes during mating. The hemipenes of both *N. sipedon* and *N. fasciata* are bilobed (though not deeply) and they have a greatly developed apical portion (Rossman & Eberle, 1977; King *et al.*, 2009). If there is copulatory adjustment in these species (shape covariation between male and female genital shape), we would predict that *N. fasciata* males would have more prominent development of the lobes in their hemipenes than *N. sipedon*, to match the more pronounced bifurcation of the *N. fasciata* female vagina compared to *N. sipedon*. Measuring morphological differences in male hemipenes and correspondence with females would be required to provide evidence supporting genital coevolution and is the subject of future investigation. Despite several reports of copulatory adjustment in snakes (based on shape correspondence), evidence of whether the male hemipenes reach all the way to the cranial end of the vagina during copulation remains scant. Pope (1941) examined *Liophis* killed *in copula* and determined that the male hemipene did indeed reach the most cranial portion of the vagina by examining the pattern of spine imprints left in the female to corroborate his conclusion.

Our specimens were obtained from introduced populations that may have suffered from a bottleneck effect (*N. sipedon* introduced in California the last decade, *N. fasciata* at least four decades ago), and therefore we cannot make any inferences about the mechanism responsible for variation among species. Regardless of the mechanism, the interspecific differences in female genitalia exist and are significant: either differences were generated after their introduction to a novel environment, or the differences were maintained after introduction. This shows that female genitalia can vary significantly even among closely-related species, and this observation is generally consistent with the idea that genital variation may be involved in speciation and reinforcement, via mechanical reproductive isolation. *Nerodia sipedon* and *N. fasciata* have both extremely high and low levels of hybridization depending on the location of their contact zones (Mebert, 2010), and an examination of genitalia of specimens from different contact zones may reveal whether lock and key is potentially involved in their speciation and hybridization. However, divergence in female genital morphology

among closely related species is not inconsistent with either intersexual selection or sexual conflict. The mating aggregations of *N. sipedon* provide ample opportunity for both mechanisms to operate: female choice by biasing fertilization success towards preferred males, or sexual conflict over copulation duration (King *et al.*, 2009).

The disparity measures showed that reproductive *N. sipedon* females have significantly greater morphological diversity in their vagina than reproductive *N. fasciata* females. This is not likely to be an artefact of our data because, for both species, all the females in size categories 3 and 4 were classified as reproductive and, although there were a few more reproductive females in size category 2 in *N. fasciata* than *N. sipedon* (5 versus 2), this was not sufficient to explain the pattern of disparity. It is possible that the increased variation in the vagina of *N. sipedon* reflects relaxed selection that can accompany colonization of a new environment (Collyer, Heilveil & Stockwell, 2011), and that *N. fasciata* is on its way to similarly increasing genital variation in successive generations. A comparison with *N. sipedon* and *N. fasciata* from their native range would help to determine whether this is indeed the case. By contrast to our expectation, there was no difference in morphological diversity between nonreproductive and reproductive individuals within either species. Our criteria for reproductive status was based solely on the size of ova, and the smallest reproductive *N. sipedon* female in the present study had an SVL of 39 cm. However, in the wild, the smallest *N. sipedon* female reported mating had an SVL of 60 cm (Weatherhead *et al.*, 1995), whereas gravid *N. fasciata* are reportedly of 62.7 cm on average (Lorenz *et al.*, 2011). Mating is likely the strongest selective pressure acting on snake genitalia but, if egg maturation occurs much earlier than actual mating, our groups may not have accurately reflected the functional stages and a more appropriate comparison may be between females large enough to mate versus all others. Our sample size did not include sufficient females > 60 cm SVL and so we could not quantitatively examine this alternative.

Morphometric analyses of soft tissues can be difficult, and proper quantification relies on careful dissection and preservation of specimens to ensure that no folds or turns affect the two-dimensional structure, and that all specimens are treated and photographed consistently as we have done so in the present study. Large sample sizes are required so that specimens that are not perfectly preserved, or where the landmarks are not clearly visible, can be eliminated from the analysis.

Our results suggest that differences in genital shape variation in females, albeit subtle, can be

significant. Genital shape in these watersnakes appeared similar superficially but using a quantitative shape analysis method (GM) allowed us to detect significant differences in the shape of the vagina. We suggest that applying this method to the study of female genitalia may reveal previously unappreciated shape variation.

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