Asymmetric and Spiraled Genitalia Coevolve with Unique Lateralized Mating Behavior

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Asymmetric genitalia and lateralized mating behaviors occur in several taxa, yet whether asymmetric morphology in one sex correlates or coevolves with lateralized mating behavior in the other sex remains largely unexplored. While lateralized mating behaviors are taxonomically widespread, among mammals they are only known in the harbor porpoise (Phocoena phocoena). Males attempt copulation by approaching a female exclusively on her left side. To understand if this unusual lateralized behavior may have coevolved with genital morphology, we quantified the shape of female and male harbor porpoise reproductive tracts using 2D geometric morphometrics and 3D models of the vaginal lumen and inflated distal penis. We found that the vaginas varied individually in shape and that the vaginas demonstrated both significant directional and fluctuating asymmetry. This asymmetry resulted from complex 3D spirals and vaginal folds with deep recesses, which may curtail the depth or direction of penile penetration and/or semen movement. The asymmetric shapes of the vaginal lumen and penis tip were both left-canted with similar angular bends that mirrored one another and correspond with the left lateral mating approach. We suggest that the reproductive anatomy of both sexes and their lateral mating behavior coevolved.

Left- or right-bias in morphology and behavior in otherwise bilaterally symmetrical animals manifests in diverse biological phenomena such as mating, foraging, predation, predator defense, and communication\textsuperscript{1,2}. Asymmetries in genital morphology are known in several animal taxa\textsuperscript{3–5}, and although lateralization in courtship behavior is found across animal taxa\textsuperscript{6}, it was unknown in mammals until recently\textsuperscript{7}. While some morphological and behavioral asymmetries related to mating have been identified, the relationship between genital asymmetry and lateralized mating behaviors, as well as their evolutionary significance, has rarely been examined. Questions remain about how asymmetry in one sex influences the behavior or morphology of the other sex and whether asymmetries arise from adaptive (directional evolution) or non-adaptive (genetic drift) mechanisms.

Instances in which one or both sexes have asymmetries in both mating behavior and genitalia may be more common than is currently recognized. For example, lateralized mating behavior occurs in male poeciliid fish, which angle their gonodopodium (intromittent organ) to either the left or right side and are restricted to mating with females that have a genital opening on the same side\textsuperscript{8–10}. Similarly, male tree swallows tend to copulate from the left, perhaps because the female's one active oviduct is on the left side\textsuperscript{11}. Male earwigs with paired penises preferentially use their right penis during copulation, which may be driven by female genital morphology\textsuperscript{12}. Male and female waterfowl have asymmetric genitalia that spiral in opposite directions and females have evolved behavioral strategies to influence control over insemination\textsuperscript{13–15}. However, mating behaviors are not lateralized in either waterfowl sex.

Female cetartiodactyls (cetaceans and even-toed ungulates) are unusual in possessing vaginal folds, protrusions of the vaginal wall into the vaginal lumen, which may function in sexual selection, among other

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Photographs were taken of the open reproductive tracts in a standardized bird’s-eye view using digital cameras with a minimum resolution of 10.1 megapixels. The focal plane of the camera was positioned parallel to the ventral surface exposed. The reproductive tract is oriented horizontally (cranial-caudal) in the female’s body. As vaginal folds are most prominent on the dorsal side of the vagina, incisions were made on the ventral side to visualize the shape of internal structures19. A single incision was made down the ventral midline of each female’s vaginal opening7. The complexity of their genital morphology and their lateralized mating behavior make harbor porpoises an exemplary taxon to explore the potential relationship between reproductive morphology and mating behavior. To characterize and quantify genital shape and assess the influence of asymmetry on overall genital shape, we used two-dimensional geometric morphometrics (2DGM) and three-dimensional models of male and female harbor porpoise genitalia. Asymmetry has been previously reported in female genitalia in a wide range of taxa, but can be inconspicuous when present3–5,13,24. Therefore, we examined the pattern of asymmetry in both 2D and 3D by generating models of the vaginal lumen, in addition to assessing gross morphology. This allows for a better understanding of the drivers of sexual patterns in both cetaceans and the wide variety of animals that have asymmetric genitalia with potentially previously unrecognized lateralized mating behaviors.

Table 1. Sex, reproductive state, and body sizes of the post-mortem harbor porpoises. * Specimen positioned in ventral recumbency, unlike the other female specimens.

| ID in Fig. 3 | Specimen ID | Sex | Reproductive State | Body Length (cm) | Body Weight (kg) |
|--------------|-------------|-----|-------------------|------------------|-----------------|
| 1            | C-434       | Female | Lactating       | 174              | 68              |
| 2            | C-358       | Female | Pregnant       | 158              | Not Measured    |
| 3            | C-383       | Female | Pregnant       | 169              | Not Measured    |
| 4            | C-392       | Female | Lactating       | 163              | Not Measured    |
| 5            | C-399       | Female | Resting       | 153.5            | 67              |
| 6            | C-403       | Female | Pregnant       | 146              | 51              |
| 7            | C-405       | Female | Resting       | 164              | 72              |
| 8            | C-415       | Female | Pregnant       | 164              | 55              |
| 9            | C-431       | Female | Resting       | 172              | 53              |
| N/A          | C-441*      | Female | Resting       | 143              | 41              |
| N/A          | C-485       | Male | N/A            | 153              | 59              |
| N/A          | C-494       | Male | N/A            | 90               | 61.5            |
| N/A          | C-496       | Male | N/A            | 147              | 61.5            |

Hypotheses16,37. Additionally, among mammals, asymmetric penises are most common in cetartiodactyls4, which have a fibroelastic penis that maintains a turgid state and is resistant to bending16. Both male and female genital morphology in cetaceans is particularly complex. The penis tip of cetaceans is pliable and may be under voluntary control19. Female harbor porpoises (Phocoena phocoena) have one of the most complex vaginal morphologies and the largest number of vaginal folds described in any cetacean species15. Male harbor porpoises have a large blunt knob at the terminal end of the relatively long penis shaft (among the proportionally longest in cetaceans), which connects to a thin distal tip that bends away from the midline19. During copulation, the blunt knob may be obstructed from penetrating the cranial vagina by the largest vaginal fold, as suggested by ex vivo testing22. The thin distal penis tip has been hypothesized to enable passage between the remaining cranial vaginal folds to deposit sperm close to the cervix22. The vaginal folds may also dampen biomechanical forces to surrounding tissues during copulation22. The close biomechanical interaction and fit between male and female genitalia in general during copulation suggests that reproductive morphology may coevolve15.

Harbor porpoises are currently the only known mammal with lateralized mating behavior7. Although asymmetries in marine mammal behavior tend to be right-biased (e.g., feeding23), recent documentation of 142 copulation attempts by male harbor porpoises in San Francisco Bay showed they were all performed on the female’s left side7. Copulatory attempts consisted of rapid (1–4 seconds), high-energy, precision-timed approaches, during which the males often breached out of the water with their penis fully extended and attempted to drive their penis into the female’s vaginal opening7. The complexity of their genital morphology and their lateralized mating behavior make the harbor porpoise an exemplary taxon to explore the potential relationship between reproductive morphology and mating behavior. To characterize and quantify genital shape and assess the influence of asymmetry on overall genital morphology, we used two-dimensional geometric morphometrics (2DGM) and three-dimensional models of male and female harbor porpoise genitalia. Asymmetry has been previously reported in female genitalia in a wide range of taxa, but can be inconspicuous when present3–5,13,24. Therefore, we examined the pattern of asymmetry in vaginal folds in both 2D and 3D by generating models of the vaginal lumen, in addition to assessing gross morphology. This is the first study to address the question of whether lateralized mating behaviors affect genital morphology in mammals, and allows for a better understanding of the drivers of sexual patterns in both cetaceans and the wide variety of animals that have asymmetric genitalia with potentially previously unrecognized lateralized mating behaviors.

Methods
Female (n = 10) and male (n = 3) reproductive tracts were obtained from fresh or moderately decomposed deceased sexually mature harbor porpoises that died of natural causes or fisheries interactions in the San Francisco Bay Area. Specimens were frozen and shipped from The Marine Mammal Center to necropsy facilities at either Mount Holyoke College or the National Oceanic and Atmospheric Administration’s Southwest Fisheries Science Center. Specimens were obtained under a National Marine Fisheries Service salvage permit to D.N.O. or S.L.M. Female reproductive tracts consisted of the external genital opening through to the uterine horns and ovaries25. Male reproductive tracts included the entire penis tip and shaft to the pelvic bones. Sperm was found in the testes of all male specimens, confirming their sexual maturity at the time of death. Specimen information (i.e., body length, weight, and female reproductive state) was provided by The Marine Mammal Center (Table 1).

Nine female reproductive tracts were dissected in dorsal recumbency (the specimen lying on its dorsal side with the ventral surface exposed). The reproductive tract is oriented horizontally (cranial-caudal) in the female’s body. As vaginal folds are most prominent on the dorsal side of the vagina25, incisions were made on the ventral side to visualize the shape of internal structures19. A single incision was made down the ventral midline of each reproductive tract, from the internal bifurcation of the uterine horn through the clitoris25. Measurements of the thickness of the largest vaginal fold were collected with digital calipers (Mitutoyo 500-171 Digital Calipers). Photographs were taken of the open reproductive tracts in a standardized bird’s-eye view using digital cameras with a minimum resolution of 10.1 megapixels. The focal plane of the camera was positioned parallel to the
midline of the reproductive tract, with the long axis of the vaginal canal oriented such that the vaginal opening was at the bottom of the image. Scales were positioned in both transverse and coronal (frontal) planes.

Two-dimensional geometric morphometrics were employed to quantify variation in the shape of the cervix, first large cranial vaginal fold, large main vaginal fold, and vaginal entrance. The cranial and caudal bounds of each region were outlined using both landmarks (n = 16) and semi-landmark curves (n = 20 curves, 240 semi-landmarks) in TPSDig2 (Fig. 1a)26. All landmarks were positioned by one author (D.N.O.) to eliminate inter-observer error5,17,27,28. As positioning of landmarks in soft tissue can be challenging, they were applied to each image three independent times to quantify intra-observer measurement error. Landmark configurations were imported into R29, where a Generalized Procrustes Analysis (GPA) was performed to rescale, translate, and rotate all landmark configurations into the same shape space 30 using the geomorph package31. Semi-landmark curves were slid to minimize the bending energy between semi-landmarks 32. Landmark configurations were then subjected to principal component analysis (PCA) to visualize the major axes of shape change among the specimens and to ensure that measurement error triplicates plotted more closely to one another than to other individuals. Although total length did not vary substantially within the harbor porpoises (146–174 cm), the effect of size on the shape data was tested using the Regression Score metric with log-transformed total length as a proxy for the size of each individual33,34.

To quantify 2D asymmetry, a multi-factor ANOVA was used with individuals (symmetric variation), sides (directional asymmetry), and the interaction between individuals and sides (fluctuating asymmetry) as factors35,36. Directional asymmetry captures asymmetry where one side is consistently shaped differently from the other (e.g., fiddler crab claws37). Fluctuating asymmetry captures random right-left perturbations from symmetry that are a result of developmental or environmental factors36. The proportion of total variation explained by each individual factor was calculated using the $\eta^2$ effect size metric38.

To capture 3D asymmetry of the vaginal canal, endocasts were made of the lumens prior to dissections. One endocast was made from a female collected from San Francisco Bay and four endocasts were made from adult females obtained from other populations along the Pacific coast of the United States. Female reproductive tracts were suspended and filled with Mold Star® 16 FAST silicone. The endocasts were carefully removed to prevent damage to the molds or tissue and to identify the corresponding ventral and dorsal sides of the female. 3D models of the endocasts were then generated with photogrammetry. The complete circumference of each endocast was photographed using a Canon EOS Rebel T5i camera with a 100 mm lens and was illuminated with four LED lights. Three-dimensional models were reconstructed in 3DF Zephyr lite (3Dflow SRL, Verona, Italy) and scaled and bisected in sagittal and lateral planes using Autodesk® ReCap Photo (v.22.0. San Rafael, CA: Autodesk; 2018).

![Figure 1. (a) Landmarks (numbered) and semi-landmark curves (black dots) of a representative adult harbor porpoise vagina with her ventral side exposed (dorsal recumbency). Semi-landmark curves were placed to capture the cervix (yellow), first large cranial fold (green), and major caudal fold (blue). Scale = 100 mm. 3D model of the silicon mold of the vaginal lumen depicted on the (b) ventral side, and (c) left lateral side (ID: C-434). Scale = 5 cm.](image-url)
The different planes were qualitatively compared to determine the degree of symmetry. The pathway from the vaginal opening to the cervix was digitally superimposed onto the image (Fig. 2b).

The penis tips and shafts of three male harbor porpoises were filled to distention with melted Vaseline (Unilever) to recreate the likely shape attained during erection (Table 1). Vaseline was injected into the corpora cavernosa and corpora spongiosa tissues with an 18-gauge needle connected to a syringe until turgidity and resistance to further distension was achieved (e.g.21,39). The artificially inflated penises were engorged to similar dimensions as observed in several photographs of free-swimming harbor porpoises with erections (Fig. 2d). 3D models were made using photogrammetry with the same methods described above for the vaginal endocasts.

Figure 2. Shape of harbor porpoise penis and pathway through the vaginal lumen. (a) Dorsal view of 3D model of inflated portion of the penis angled in a coronal plane with the tip facing cranially (ID: C-485). Straight black lines with numbers demarcate angular changes calculated in Table 2. (b) 3D model of the silicon mold of the vaginal lumen depicted on the dorsal side. Straight black lines and numbers correspond to major qualitative changes in orientation of the vaginas (ID: C-434). (c) Female reproductive tract with dorsal side exposed (ventral recumbency) with an incision made down the dorsal midline (ID: C-441). Straight black lines show the pathway from the vaginal opening to the cervix around the complex vaginal folds. Numbers represent the major qualitative changes in orientation of the vagina. (d) Erect penis of free-swimming harbor porpoise immediately after a copulation attempt depicting angles of the penis.
which was set at 0 degrees (Fig. 2a). Major changes in penis angles were calculated relative to the preceding angles using the angle tool in ImageJ (v.1.52k40).

Table 2. Angles of the inflated penis tips of three harbor porpoises. The first angle is relative to the long axis of the penis shaft such that 0° angles parallel to shaft in the cranial direction and the subsequent angles are relative to the preceding angle. The four angles show marked directional changes in the penis tip (Fig. 3a). Prior to measurement, penises were angled in a coronal plane with the tip facing cranially.

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Significance statement. We explored the coevolution of genital morphology and mating behavior asymmetry between male and female harbor porpoises, the only mammal known to demonstrate exclusive lateralized mating behavior. By comparing 2D and 3D shapes of excised male and female reproductive tracts, we found that genital asymmetry is likely functional. Male and female genital shapes are congruent in angular bends and a
left-sided sexual approach by males is obligatory to overcome physical obstructions inside the female reproduc-
tive tract. Our results suggest male copulatory behavior and morphology conform with female morphological
modifications to achieve fertilization in a system which may undergo an evolutionary arms race.

Results
We found substantial variation in vaginal shape in 2D using PCA. Principal component (PC) 1 (26.06% of total
shape variation) was driven by the relative size of the lower vagina (caudal to the largest vaginal fold; Fig. 3a),
PC2 (21.96% of total shape variation) was driven by a left- or right-biased curvature of the entire reproductive
tract (Fig. 3a), and PC3 (18.6% of total shape variation) was driven by the relative widths of the cervix and vagina
(Fig. 3b,c). Vaginal shape was not significantly correlated with total body length (p = 0.757; Fig. 3d). Based on
ANOVA effect sizes, individual variation accounted for 52.6% of total shape variation, directional asymmetry
for 8.3% of total shape variation, and fluctuating asymmetry for 37.1% of total shape variation. All three of these
factors were significant (p < 0.001; Fig. 3e). Measurement error, by contrast, only comprised 2% of the total shape
variation, demonstrating that the landmarking process did not generate a substantial error component.

The internal vaginal lumen is highly asymmetric due to both complex 3D spirals and vaginal folds with deep
recesses (Fig. 1b,c). The largest vaginal fold had a mean thickness of 19.6 mm (S.D. = 4.7 mm, n = 8). The pathway
through the vaginal lumen to the cervix had an overall bend and spiraled to the left in all five endocasts when
positioned in ventral recumbency, as found during mating under natural conditions (Fig. 2b). This bend, and
two additional moderate bends in the vaginal lumen, are generated by the vaginal folds. When viewed dorsally,
the tips of all three inflated penises originate on the left side of the blunt knob and cant to the left in a series of
four consecutive bends (Table 2; Fig. 2a). Specifically, relative to the midline of the long axis of the shaft, the penis
angles markedly to the right slightly proximal to the left and downwards toward the shaft, followed by subler bends to the right then left near the distal tip. These bends parallel the shape of the internal vaginal lumen (Figs. 1c and 2h) and accurately reflect the penis shape of free-swimming harbor porpoises in San Francisco Bay (Fig. 2d) when males attempt copulation. A video of a copulatory attempt shows the body orientation of males relative to females (Supplemental Video 1).

Discussion
We provide evidence that genital asymmetry has coevolved with lateralized mating behavior in harbor porpoises,
the first example amongst mammals. The vaginal lumen of harbor porpoises is significantly directionally asym-
metric, and its shape corresponds to the leftward cant of the erect penis tip. The left-sided mating behavior of
male harbor porpoises appears to have coevolved with genital shape variation in both sexes so that the penis can
circumvent large and protruding vaginal folds. The close genital correspondence could be the result of several
potential coevolutionary mechanisms, including sexual conflict, female choice, and "lock-and-key". As hybrid-
ization is common among cetaceans, it is unlikely a "lock-and-key" mechanism, and the close correspondence
likely arises as a result of an intersexual evolutionary arms race in harbor porpoises. In the San Francisco Bay pop-
ulation, males initiate all mating events, attempt copulations throughout the year, do not engage in pre-copulatory
courtship, and approach females in all reproductive states. Females may respond to the constant pursuit of males
by allowing copulations that reduce costs of evasion and harassment. However, they have also evolved sophisti-
cated mechanisms that may control paternity including asymmetric genitalia, complex vaginal folds, changes in
body positioning during copulation that may function to misalign the optimal angle of penetration and prevent
the ejaculate from reaching the cervix, and through resistant behaviors (e.g., dives). Both sexes appear to exhibit
both behavioral and anatomical adaptations and counter-adaptations that may control paternity, although it is
possible that an evolutionary arms race has not reached an equilibrium end point (e.g.43). It is clear that penile
penetration into the most cranial regions of the vagina requires a specific angle of entry to bypass the vaginal
folds. By angling their bodies slightly when males approach, females could potentially prevent the tip of the penis
from penetrating past their vaginal folds. Males seem to have evolved complex penile morphology with numer-
ous angles that match the internal shape of the vagina and may increase their chances of successful copulation.
The large relative testes size of harbor porpoises compared to other cetaceans suggests a substantial investment
in post-copulatory sperm competition and there is likely strong selection on females to evolve mechanisms to
control paternity after insemination by multiple males.

Although all the female reproductive tracts examined were asymmetric and complex, we found that vaginal
shape strongly varied among individuals. Female genitalia were previously considered to be less variable than
male genitalia, yet emerging patterns suggest a high level of intra- and inter-specific variation in females, which
highlights the need to continue to explore female genital morphological variation across taxa. Reproductive
state and history could have contributed to this variation, but our sample size was too small to examine this
possibility quantitatively. For example, a study on reproductive tract lengths in common bottlenose dolphins
(Tursiops truncatus) found that pregnant females had longer vaginal lengths than lactating or resting adult
females, although the sample size was also too small for statistical analysis. We qualitatively note that pregnant
harbor porpoises generally had more symmetrical cervices, potentially reflecting stretching, while resting females
had more asymmetrical cervices. Recent assessment of soft vaginal structures in other taxa using 2DGM have
similarly found that vaginal shape is highly variable and influenced by factors such as ontogeny and reproductive
state (beetles, snakes, cetaceans; sharks). However, much of the documented variation remains unexplained.

More than 8% of total shape variation in the harbor porpoise vagina was explained by directional asymmetry.
As directional asymmetry is often functional and all vaginal endocasts canted to the left, the data support a
left-sided constraint on males to approach females only from one direction and body orientation. We found that
fluctuating asymmetry was significant and explained 37% of total variation. This high degree of fluctuating asym-
metry in soft tissue structures is common (e.g., dogfish shark vagina), and may result from less canalization in com-
parison with rigid hard tissues. While genital laterality is to the left, other morphological asymmetries known in
habor porpoises are to the right (e.g., flipper size\textsuperscript{54}; nasal complex asymmetries\textsuperscript{55,56}), and right-dominated asymmetries in non-mating behavior occur in other marine mammals\textsuperscript{29}. This makes it likely that the genital asymmetry is adaptive under the influence of selection, and does not result from pleiotropy in harbor porpoises, which could favor right-sided development concurrently with other right-sided morphological asymmetries.

We have demonstrated the development and coevolution of lateralized sexual behavior and genital morphology in harbor porpoises, which appears to reflect an intersexual evolutionary arms race. It is not yet possible to determine whether the lateralized behavior evolved in response to morphological evolution or vice versa, or how it became fixed in the species. Substantial future work on additional species is necessary to establish the commonality of correlations between lateralized behavior and morphology among vertebrates.

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Author contributions
S.L.M., W.K. and D.N.O. conceived the project idea. D.N.O. and S.L.M. collected the reproductive tract photographs and M.A.W. provided the live animal image. P.L.R.B. and D.N.O. inflated the penises, made the endoscopes, and created the 3D models. D.N.O. landmarked the images. B.P.H. conducted the 2DGM analyses. D.N.O. wrote the manuscript with input from all co-authors.

Competing interests
The authors declare no competing interests.

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