

## Morphometric Analysis of Intraspecific Shape Variation in Male and Female Genitalia of *Phyllophaga hirticula* (Coleoptera: Scarabaeidae: Melolonthinae)

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**ABSTRACT** The diversity of male genitalia observed among many disparate arthropod taxa has stimulated a number of studies investigating which evolutionary factors contribute to this phenomenon. Much of this research, however, is focused on testing patterns of selection on male genitalia only. Because copulatory processes are a joint act between males and females, detailed study of the selection pressures on female morphology seems equally appropriate. In the current analysis, I used geometric morphometrics to quantify intraspecific shape variation of male and female genitalia in the scarab beetle species *Phyllophaga hirticula* (Knoch). Characters under sexual selection are predicted to be more phenotypically variable than nonsexual characters because of the persistent tendency of sexual selection to eliminate bouts of stabilizing selection. Thus, this analysis tested whether phenotypic variation of male and female genitalia is equivalently higher than shape variation of a nonsexual character, the right elytron. Shape variation was analyzed in SHAPE, a program that transforms two-dimensional outline data into elliptic Fourier descriptors to be analyzed in a principal components analysis. Data from this study supported the hypothesis that male and female genitalia were significantly more variable than the right elytron. In addition, *P. hirticula* female genitalia were significantly more variable than male genitalia. Comparable phenotypic variation of male and female genitalia suggests that like male genitalia, female genitalia also may be subject to sexual selection and should be considered when testing hypotheses of genitalic evolution.

**KEY WORDS** intraspecific variation, postcopulatory sexual selection, genitalic evolution, *Phyllophaga hirticula*, morphometrics

TWO HALLMARKS OF SEXUAL selection include rapid divergence of elaborate and complex sexual characters (Darwin 1871; Eberhard 1985, 1993, 1996) and increased intraspecific variation of sexual versus nonsexual traits (Pomiankowski and Møller 1995). These two characteristics are seemingly ubiquitous throughout many groups of arthropods with diverse and often wildly outlandish male genitalia. Historically, such diversity has been explained by lock-and-key mechanisms and was thought to prevent interspecific mating. However, convincing evidence for postcopulatory sexual selection as a divergent force in the evolution of male genitalia is accumulating from comparative studies and fertilization success analyses (Arnqvist 1998, Arnqvist and Danielsson 1999, Arnqvist et al. 2000, Eberhard 2004).

Postcopulatory sexual selection occurs when males experience differential paternity success when mated to polyandrous females because of sperm competition, and/or female sperm storage and unequal allocation (Parker 1970; Eberhard 1985, 1996). Although there is general agreement for the role of postcopulatory sexual selection in producing genitalic diversity, direct support for the causal mechanism influencing paternal

variation remains unresolved. Many research programs testing hypotheses of postcopulatory sexual selection such as sexual conflict, female choice, and sperm precedence focus on the influence of variations in male morphology and behavior on paternity success (Simmons et al. 1999, Arnqvist and Danielsson 1999, Tadler et al. 1999, Edvardsson and Arnqvist 2000). However, sexual selection hypotheses, such as sexual conflict, rely on male and female counteradaptations to generate an evolutionary arms race. For these adaptations to occur, there must be sufficient demonstrable variation of both male and female morphology and/or behavior on which selection can act (Mayr 1963). Despite this prerequisite, and considerable evidence for female-mediated sperm storage and usage (Bloch Qazi et al. 1998, Hellriegel and Bernasconi 2000, Bloch Qazi 2003, Córdoba-Aguilar 2003, Fedina and Lewis 2004, Heifetz and Wolfner 2004), there are only a handful of studies that address intraspecific variation of female morphology. In one such study, Fedina and Lewis (2004) provided evidence for an association between size variation of spermathecal morphology, and paternity success of the female's multiple partners. This study highlights the impor-

tance of analyzing intraspecific variation in both male and female characters, because both are likely to play a role in differential fertilization success.

The specific goal of the current study was to quantify the amount of intraspecific variation of male and female genitalic characters, relative to a nonsexual character, in a taxon with species-specific genitalia. I used a morphometric approach to quantify two-dimensional shape variation of sclerotized portions of the male and female genitalia in the polygamous scarab beetle *Phyllophaga hirticula* (Knoch). Members of the genus *Phyllophaga* are most noted for the stark contrast between the evolutionarily conserved external morphology and diverse, species-specific male and female genitalia (Smith 1888). Thus, by default, this group satisfies the first prediction of sexual selection—that sexual characters are diverging faster than nonsexual characters (Darwin 1871). I focused on the second prediction of characters under sexual selection posed by Pomiankowski and Møller (1995) and investigated patterns of intraspecific shape variation of male and female genitalia, compared with variation in a nonsexual trait. In systems with species-specific male and female genitalia, it is difficult to explain inter- and intraspecific diversity of male genitalia by using postcopulatory sexual selection hypotheses without also suggesting these processes are acting to produce species-specific female genitalia. Thus, comparable variation of male and female genitalia, that is significantly greater than a nonsexual character, would suggest that postcopulatory sexual selection is acting on female as well as male reproductive morphology.

### Materials and Methods

**Study Organism.** *P. hirticula* is one of  $\approx 250$  *Phyllophaga* species in America North of Mexico, and it is associated with the *fraterna* species complex in the subgenus *Phyllophaga* s. str. (Böving 1942; M.P., unpublished data). This species is common throughout its extended east coast distribution and is distinguished from closely related species by the male and female genitalia. *Phyllophaga* beetles emerge in early spring, although commencement and duration of the breeding season is species and region dependent. In areas of high diversity, it is commonplace to collect between six and eight species per night at a given location. Females are polyandrous and attract males by using species-specific pheromones (Ochieng et al. 2002, Robbins 2005). Observation of *P. hirticula* mating behavior reveals a system where the male hangs suspended in the air attached to the female by his genitalia only. Specimens for this study were collected from Connecticut, Pennsylvania, Virginia, South Carolina, and Georgia, representing males from five populations and females from three populations.

**Materials and Measurements.** Male ( $n = 39$ ) and female ( $n = 30$ ) genitalia were extracted and cleared in 5% KOH and digitized using a Leica MZ16 dissecting scope equipped with Automontage syncroscopy software (Synoptics Ltd., Cambridge, United King-

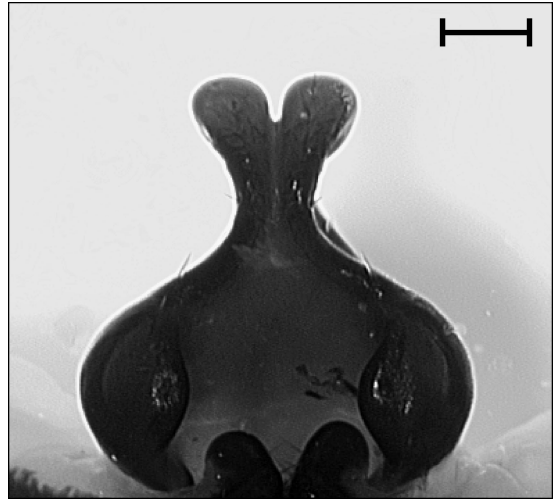


Fig. 1. Ventral view of female pubic process. Scale bar, 1 mm.

dom). To analyze variation of the female genitalia, I separated the membranous internal reproductive system from the sclerotized pubic process (Fig. 1). The female pubic process was then flattened between two cover slides and imaged from a ventral perspective. The orientation of the pubic process within the female beetle is vertical and points dorsally above the entrance to her bursa copulatrix. Although the function of the female pubic process during copulation is currently unknown, this structure is species-specific among members of the genus *Phyllophaga*.

Male genitalia are asymmetrical such that the right paramere is reduced in size relative to the left paramere (Fig. 2). A lateral view of the left male paramere was used to quantify intraspecific shape variation. This portion of the male genitalia is inserted into the female during copulation and is hypothesized to “hook” into the female pygidial hemisternite (Eberhard 1993; personal observation) (Fig. 3). To evaluate relative variation of male and female reproductive morphology, I also analyzed shape variation of the right elytron in an equal number of males and females from a subset of the study specimens ( $n = 20$ ). Including an external morphological character such as the right elytron allows for comparison of intraspecific variation in sexual versus nonsexual characters. This external character was chosen because it was possible to consistently capture images from the same perspective.

Images were converted to black-and-white bitmaps in Adobe Photoshop 5.5 (Adobe Systems, Mountain View, CA) and imported into the program SHAPE (Iwata and Ukai 2002). This program uses elliptic Fourier descriptors (EFDs) to analyze shape variation of two-dimensional outline data. Because male and female genitalia are highly three-dimensional, shape analysis of two-dimensional data are likely to provide a conservative estimate of intraspecific variation because variation provided by the  $z$ -axis, or third dimension, is not included. Thus, estimates of shape variation

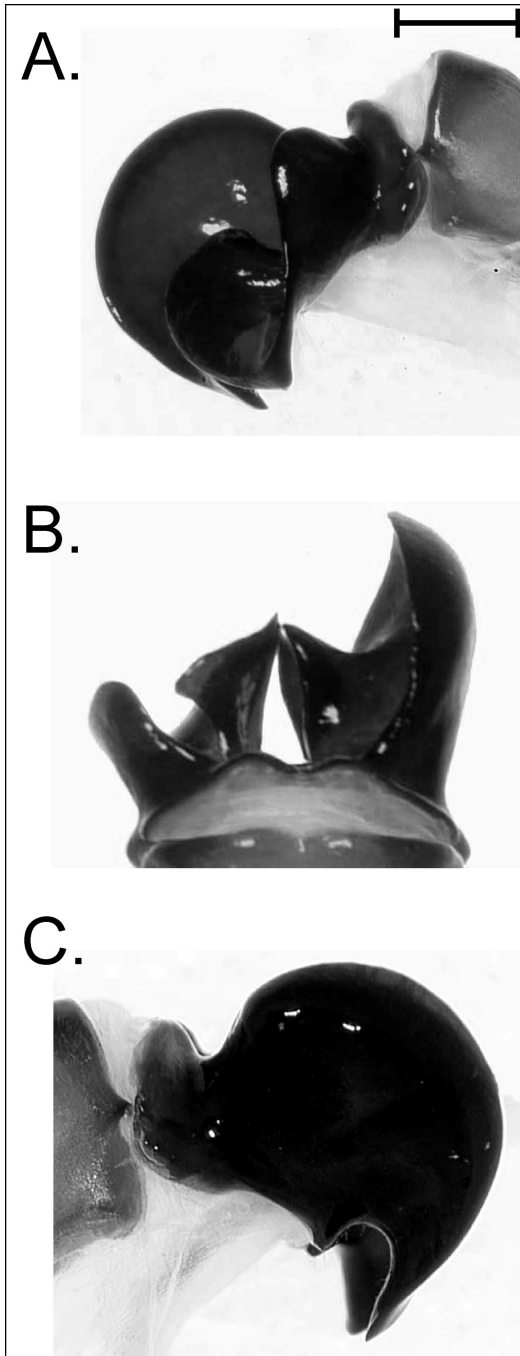


Fig. 2. Varying views of male parameres. (A) Right lateral. (B) Ventral. (C) Left lateral. Scale bar, 1 mm.

from this analysis should be considered an underestimate of total variation. Elliptic Fourier analysis using SHAPE involved three programs. The first program, Chaincoder, translated the outline of the focal character into chaincode, a series of integers between 1 and 7. The second program, Chc2Nef, accepted the transformed Chaincoder files and calculated normal-

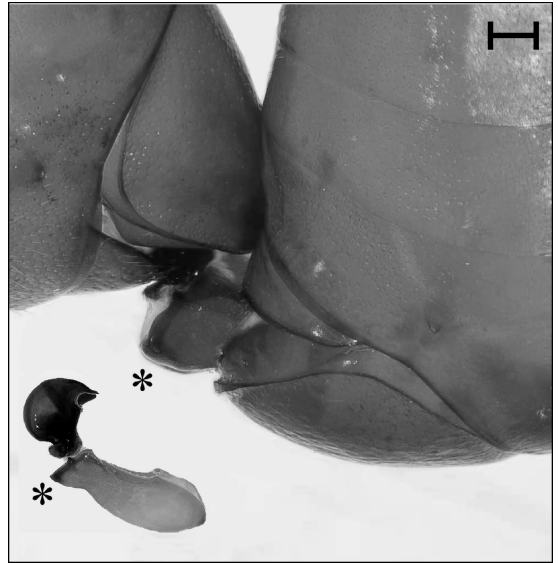


Fig. 3. Left lateral view of *P. hirticula* mating. The male is on the right and the female on the left. The asterisks mark the same location on the male genitalia inlay and on the mating pair. The entire left paramere is "hooked" within the pygidium of the female. Scale bar, 1 mm.

ized EFDs based on the first harmonic ellipse. Last, PrinComp performed a principal component analysis using the variance-covariance matrix from the EFD coefficients, providing a visual and numerical analysis of shape variation. By default, PrinComp will use the first 20 harmonics of the EFD coefficients in the principal components analysis. When analyzing variation of male and female genitalia and elytra, I used the first 30 harmonics to increase the number of EFD coefficients used to describe variation. Data from these analyses were saved in tabbed text format and easily imported into additional statistical software as needed.

Significance tests of equal variance of shape in male and female genitalia and elytra were performed in Minitab 13.1 (Minitab, Inc., State College, PA). In addition, standard linear regression analyses were done to determine whether shape variation was correlated with size of male and female beetles.

Analyses of measurement consistency were done on a subset of male individuals to determine how much of the observed variation was because of measurement error. Males ( $n = 15$ ) were reimaged, edited, and analyzed in SHAPE. A test of equal variance and a repeated measures analysis were performed in Systat (Systat Software, Inc., Point Richmond, CA) to determine whether the two separate analyses provided consistent results.

## Results

Because the majority (>50%) of the shape variation of male and female genitalia was described by the first two principal components, all subsequent analyses were carried out on PC1 and PC2 for each character

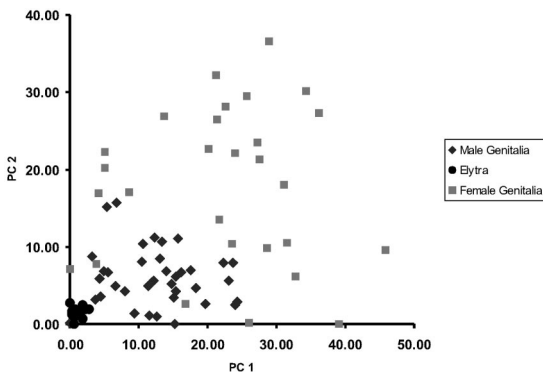
**Table 1.** Amount of variation described by principal component one and two for each morphological trait

	Proportion of total variance (%)
Female genitalia	
PC 1	44.60
PC 2	33.72
Total	78.32
Male genitalia	
PC 1	54.93
PC 2	17.31
Total	72.24
Right elytron	
PC 1	31.61
PC 2	22.80
Total	54.41

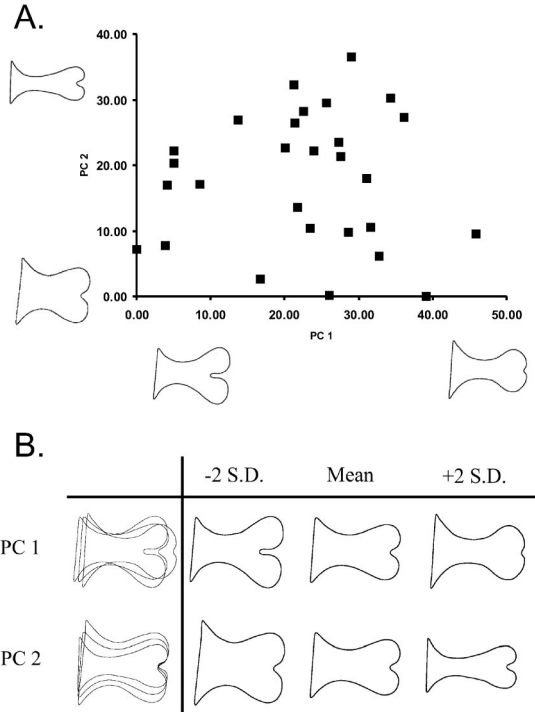
quantified (Table 1). In addition, principal component scores were transformed by adding the minimum score to all values in each analysis so that all scores would be greater than or equal to zero.

Shape variation of the male and female genitalic outline was significantly greater than shape variation of the right elytron by using an F-test of equal variances (PC1:  $F = 0.006$ ,  $P < 0.001$ ; PC2:  $F = 0.007$ ,  $P < 0.001$ ) (Fig. 4). This result was significant when data from males and females were pooled or tested separately. In addition, female genitalia were significantly more variable than male genitalia (PC1:  $F = 3.011$ ,  $P = 0.002$ ; PC2:  $F = 7.225$ ,  $P < 0.001$ ) (Fig. 4). Shape variation of the right elytron described by the first principal component was not significantly different between males and females so the data were combined in all analyses ( $F = 1.092$ ,  $P = 0.897$ ).

Reconstructed shape contours from PrinComp allowed for visualization of the shape variation described by individual principal components (Figs. 5–7). The first column in each reconstruction figure overlays the mean shape with the positive and negative standard deviation. The following rows show these same shapes individually. Thus, nonoverlapping lines in the first column depict regions that are the most variable. Reconstructed contours from the anal-



**Fig. 4.** Scatter plot comparing variation of the first two principal components for all morphological characters analyzed.



**Fig. 5.** Shape variation of the female genitalia described by the first two principal components. (A) Scatterplot demonstrating range of variation. (B) Reconstructed contour of shape variation.

ysis of female genitalia revealed shape differences in the width and height of the “stem” portion as well as the shape of the bulbous region distal to the stem (Fig. 5). Reconstructed contours of male genitalia demonstrated that variation of the left paramere was because of differences in the rounded antero-dorsal portion, the region of the hook, and where the paramere articulates with the rest of the genitalic capsule (Fig. 6). The shape contours of the male and female genitalia were contrasted with that of the elytra, where lines of the mean and positive and negative standard deviation were largely overlapping (Fig. 7).

Neither of the first two principal components describing variation of male and female genitalia was correlated with body size (PC1 male:  $r^2 = 4.0\%$ ,  $P = 0.372$ ; PC2 male:  $r^2 = 0.7\%$ ,  $P = 0.715$ ; PC1 female:  $r^2 = 1.9\%$ ,  $P = 0.629$ ; PC 2 female:  $r^2 = 1.9\%$ ,  $P = 0.623$ ).

The consistency of male measurements was assessed in two ways. The first analysis examined differences in the amount of variation described by both data sets by using an F-test of equal variance, and revealed no significant differences (PC1:  $F = 0.644$ ,  $P = 0.439$ ; PC2:  $F = 1.092$ ,  $P = 0.994$ ), suggesting the duplicate data described the same amount of variation as the original data set. In addition, a repeated measures analysis was implemented comparing variation within subjects, from the two different measurements, and variation between subjects. This test also demonstrated that there was no significant difference be-

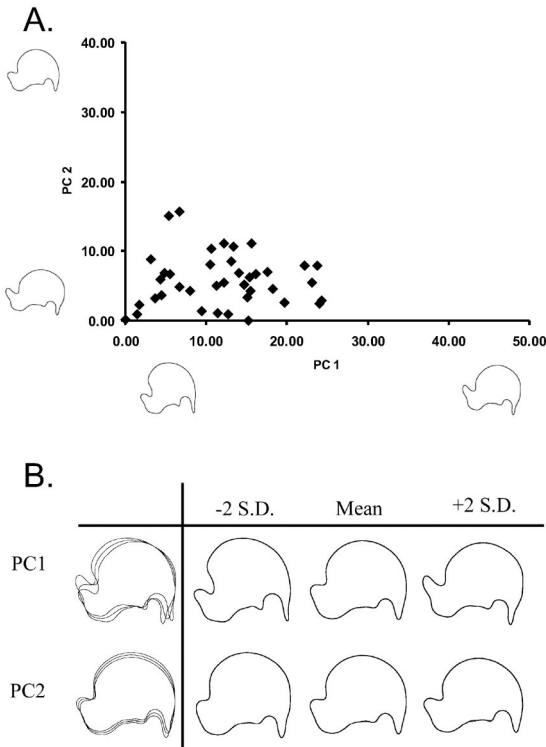


Fig. 6. Shape variation of the male genitalia described by the first two principal components. (A) Scatterplot demonstrating range of variation. (B) Reconstructed contour of shape variation.

tween the two sets of measurements for the first two principal components (PC1:  $F = 0.093$ ,  $P = 0.765$ ; PC2:  $F = 0.000$ ,  $P = 0.988$ ).

### Discussion

Results from this study provide evidence that the male and female genitalia of *P. hirticula* are significantly more variable than the right elytron, a nonsexual character. A higher degree of intraspecific variation in sexual characters is hypothesized to reflect an evolutionary history with constant directional selection resulting from sexual selection processes (Pomiankowski and Møller 1995). This hypothesis is supported by experimental analyses providing evidence for a correlation between variation of male genitalia and paternity success (Arnqvist 1998, Arnqvist and Danielsson 1999, Arnqvist et al. 2000, Eberhard 2004). However, the current analysis suggests that female genitalia also may be subject to sexual selection, underscoring the importance of including female characters in fertilization success analyses testing for postcopulatory sexual selection.

Intraspecific variation of female characters has been found in podismine grasshoppers in an investigation of the covariance between sexual and nonsexual characters among populations (Tatsuta and Akimoto 1998). This study found that patterns of covariance

were more variable for females than males and was hypothesized to result from genetic drift. However, inclusion of fitness measures in a subsequent analysis revealed this pattern also could result from directional sexual selection on female characters (Tatsuta and Akimoto 2000). Furthermore, in a description of the female genitalia in a calopterygid damselfly, Córdoba-Aguilar (2003) suggested that the number of vaginal mechanoreceptive sensilla, used to detect the presence of an egg in the vagina, were highly variable among females. Last, Fedina and Lewis (2004) coupled intraspecific variation in female sexual characters with paternity analyses, providing direct support for postcopulatory sexual selection on female genitalic traits in *Tribolium castaneum* (Herbst). In light of these studies as well as increasing evidence for female mediated sperm storage, it seems the paradigm of the choosy female has prevented further study of postcopulatory sexual selection on female reproductive morphology.

Redirecting our perspective to include selection on female reproductive morphology has the potential to provide additional insight into the postcopulatory processes responsible for rapid genitalic divergence. This more inclusive view increases our ability to interpret observed patterns of diversity by testing additional factors not previously considered. For example, investigating the effect of the interaction of male and female reproductive morphology is rare or unknown in analyses of fertilization success and postcopulatory

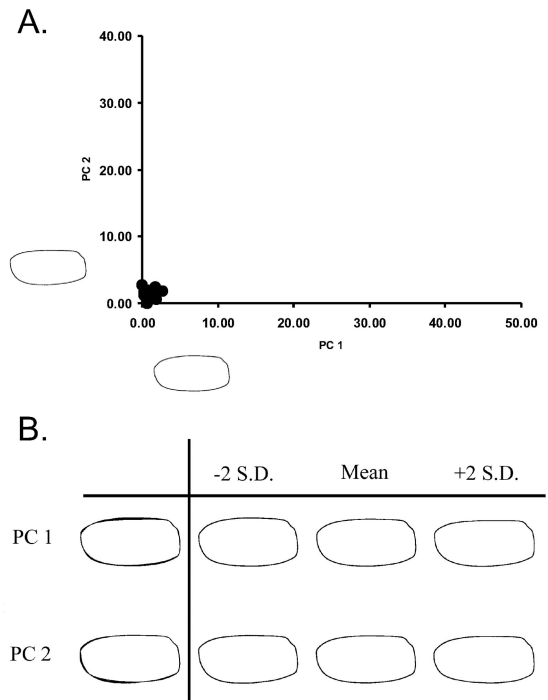


Fig. 7. Shape variation of the right elytron described by the first two principal components. (A) Scatterplot demonstrating range of variation. (B) Reconstructed contour of shape variation.

sexual selection. However, evidence for the role of these interactions in sexual selection processes has been demonstrated from studies of male and female genotypes in *T. castaneum* (Nilsson et al. 2003) as well as in the external fertilization system of the *Heliocidaris erythrogramma* sea urchin (Evans and Marshall 2005). In addition, Miller and Pitnick (2002) found a positive relationship between the interaction of male sperm length and female seminal receptacle length on paternity success in *Drosophila*. These studies provide specific examples of how selection on male-by-female interactions can influence the outcome of postcopulatory processes.

Thus, evidence for phenotypic variation in female reproductive morphology suggests these same types of interactions may occur between male and female genitalia. For example, variation of the left paramere in *P. hirticula* is most pronounced in the distal tip of the paramere, which is known to hook in to the female during copulation (Eberhard 1993). Predictions of current sexual conflict hypotheses would suggest that variation in the hook region allows some males to extend the duration of copulation. Under this view, counter-adaptations of female genitalia would evolve to prevent this type of male manipulation. An alternative explanation is that particular combinations of male and female genitalia interact to prolong copulation and increase fertilization success. This process could equally explain the patterns of diverse, species-specific genitalia in *Phyllophaga* species as well as other arthropod groups. However, because postcopulatory sexual selection on female genitalic characters is largely understudied and misunderstood, these types of hypotheses have not been considered or tested.

Simple studies quantifying intraspecific shape variation of male and female genitalia provide the groundwork for determining which characters may be subject to sexual selection. The current analysis provides support for the hypothesis that like males, females also may be subject to postcopulatory sexual selection. This highlights the importance of evaluating intraspecific variation of female characters and including them in paternity success analyses. Arthropod groups with species-specific male and female genitalia offer an excellent opportunity to investigate these processes because both sexes have divergent, species-specific genitalia, satisfying the first prediction of characters under sexual selection.

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