

The evolution of female-biased genital diversity in bedbugs (Cimicidae)

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Abstract

Rapid genitalia evolution is believed to be mainly driven by sexual selection. Recently, noncopulatory genital functions have been suggested to exert stronger selection pressure on female genitalia than copulatory functions. In bedbugs (Cimicidae), the impact of the copulatory function can be isolated from the noncopulatory impact. Unlike in other taxa, female copulatory organs have no function in egg-laying or waste-product expulsion. Males perform traumatic mating by piercing the female integument, thereby imposing antagonistic selection on females and suspending selection to morphologically match female genitalia. We found the location of the copulatory organ evolved rapidly, changing twice between dorsal and ventral sides, and several times along the anteroposterior and the left–right axes. Male genital length and shape varied much less, did not appear to follow the positional changes seen in females, and showed no evidence for coevolution. Female genitalia position evolved 1.5 times faster than male genital length and shape and showed little neutral or geographic signals. Instead, we propose that nonmorphological male traits, such as mating behavior, may drive female genitalia morphology in this taxon. Models of genitalia evolution may benefit from considering morphological genital responses to nonmorphological stimuli, such as male mating behavior or copulatory position.

Keywords: genital evolution, sexual selection, sexual conflict, traumatic insemination, cryptic female choice, mating behavior

Introduction

Male genitalia and other male molecular, cellular, organ, and behavioral traits related to reproduction evolve rapidly by sexual selection (e.g., Arnqvist, 1998; Brand et al., 2022; Brennan & Plum, 2014; Eberhard, 1985, 1996; Firman et al., 2017; Hosken & Stockley, 2004; Klaczko et al., 2015; Morgan & Hart, 2019; Simmons & Fitzpatrick, 2019; Tanabe & Sota, 2014)—a consensus supported by experimental evidence (e.g., Cayetano et al., 2011; House et al., 2013; Masly & Kamimura, 2014; Simmons & Fitzpatrick, 2019; Simmons & Garcia-Gonzalez, 2011). Most models agree that male–male (or sperm) competition or (cryptic) female choice selects for male genital traits that then impact genital evolution in females. The resulting joint male–female changes in genitalia morphology are, therefore, seen as a result of coevolution (Eberhard, 1985, 1996; Firman et al., 2017; Hosken & Stockley, 2004; Simmons, 2014). However, origins other than sexual selection for male or female variation in genitalia have been proposed (Reinhardt, 2010; Simmons & Fitzpatrick, 2019) and the notion that genitalia coevolution necessarily arises from male and female variation also is not universally supported (Brennan & Plum, 2014; Langerhans et al., 2016).

Antagonistic selection in the form of male harm to females also occurs and so models of sexually antagonistic coevolution

also apply to genitalia. If male genital traits produce costs in females, female defensive traits will be rapidly selected, a dynamic that can result in an arms race between the sexes if females defend by resistance to male traits (Brennan & Plum, 2014; Gavrilets, 2014; Gavrilets & Waxman, 2002; Holland & Rice, 1998; Hosken & Stockley, 2004; Pennell et al., 2016). If the female defense is by resistance, that is, producing fitness costs to males, it will select for trait exaggeration in males (Gavrilets & Waxman, 2002; Holland & Rice, 1998). Across species, males and females are then at different stages of this arms race and different species show different intermediate character states. Female defense to male harm can also occur by tolerance, where there are no costs to males (Gosden & Svensson, 2009; Lessels, 2006; Michels et al., 2015; Reinhardt et al., 2014; Svensson & Råberg, 2010) in which case coevolution over a trait pair comes to a halt. Sexual selection continues and filters novel traits from the male genome (Rice, 2000) and interspecific genital variation may become male-biased, on average, in the sense that males possess more traits.

Sexual antagonism can also lead to a situation captured by the Buridan's ass model (Gavrilets & Waxman, 2002). In this model, male harm causes disruptive selection on females leading to the evolution of female, but not male, polymorphism.

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Males can then be condemned to low mating success with either of the diverged female mating morph (Gavrilets, 2014; Gavrilets & Waxman, 2002). If such female morphs persist across speciation events, interspecifically there would be greater female than male variation. Phylogenetically, one would observe evolutionary nodes with a change of two or more character states. However, a previously untested outcome of this model is that both diverging sister taxa should show a character change, rather than one taxon showing a change in two states. This is necessary because if only one female morph evolves, males are not caught “in the middle.” Alternatively, or under different conditions, or over time, males may diverge and adapt to the two female genital morphs and thereby form sympatric species (Gavrilets, 2014; Gavrilets & Waxman, 2002; Reinhardt et al., 2007), rather than being caught “in the middle.” Under this form of sympatric speciation, one would observe interspecific codiversification (e.g., Iversen et al., 2019). Finally, empirical data show that sexually antagonistic mating morphologies can evolve frequently (Brand et al., 2022; Reinhardt et al., 2014). Interestingly, Brand et al. (2022) showed that the same antagonistic trait repeatedly evolved in males, and a similar response repeatedly evolved in females, resulting in reduced genital diversity despite fast evolutionary rates.

Sexual selection is therefore generally predicted to (a) increase interspecific male genital diversity, or the coevolution to (b) a morphological “fit” if females rapidly respond to changes in males. Antagonistic coevolution might involve (c) higher interspecific female diversity because of the persistence of female-limited polymorphism, (d) sexual codiversification but not necessarily a morphological genital fit, or (e) cosimplification by canalization. These models, and several others, and the body of evidence have been extensively reviewed. The reader is referred to these reviews for further reference (Brennan & Plum, 2014; Eberhard, 1985, 1996; Firman et al., 2017; Hosken & Stockley, 2004; Langerhans et al., 2016; Reinhardt, 2010; Simmons, 2014).

Despite the wealth of data and hypotheses available in this research field, various aspects of genitalia evolution remain poorly understood. First, out of biological necessity, most studies concerned species where female genitalia have critical functions alongside copulation, such as in egg-laying, birth, waste-product disposal, as well as sperm or spermatophore storage. This situation creates a largely intractable obstacle to interpreting whether male and female variation in genitalia arises from copulatory or noncopulatory traits. For example, genital traits involved in egg-laying but not in copulation underwent evolutionary simplification (Brand et al., 2022). As demonstrated in *Drosophila*, natural selection on female noncopulatory traits can be strong, drive male variation (Muto et al., 2018) and so may constrain what is at the heart of models of genital evolution–copulation.

Second, the current diversity of genitalia may not accurately reflect which sex drives diversification. Some experimental and comparative studies show that female genital variation can evolve first, or faster, thus driving male variation and fueling genital evolution (Fritzsche et al., 2014; Genevicius et al., 2020; Huber et al., 2007; Simmons & Fitzpatrick, 2019; Tanabe & Sota, 2014). Actual rates of trait changes have rarely been quantified (but see Simmons & Fitzpatrick, 2019) but lower diversity is not necessarily correlated to slower evolution (Brand et al., 2022).

Third, one important genital character appears to resist investigation as well as diversification—the position on the body. Males of many species have their genitalia located ventrally at the end of the abdomen, even in noncopulating taxa where sperm transfer is via spermatophores (hermaphrodites—Michiels, 1998, insects—Proctor, 1998; Heming, 2003, Bilateria—Mann, 1984). Exceptions exist in worm-like taxa, fish, or marine mammals, where genital openings are ventral but at a more central position, or in the Odonata and spiders where males have evolved secondary copulatory organs at more anterior segments. Female genitalia are also often situated at the ventral end of the abdomen, including in species where females take up spermatophores indirectly from the ground (Proctor, 1998), where male genitalia are at different parts of the body (such as the above-mentioned Odonata and spiders). Evolutionary stasis is not predicted by any of the sexual hypotheses and so this character represents a challenge to the general explanation of morphological (co) variation. However, along this evolutionary stasis of morphology, dramatic evolutionary changes occur in male mating behavior, male genitalia symmetry, or male mating position (Huber et al., 2007). It remains an open question to what degree nonmorphological traits affect the evolution of genital morphology.

For several reasons, bedbugs (Cimicidae) appear to be a uniquely suited system to address open questions in genitalia evolution. (a) A robust and dated phylogeny (Roth et al., 2019) exists, allowing for a reconstruction of the evolutionary trajectories of male and female genitalia, and the calculation of evolutionary rates. (b) Bedbug males mate by traumatic insemination (Reinhardt et al., 2014; Usinger, 1966) whereby they penetrate the integument of females to transfer sperm. Cimicid males seem to benefit from penetrating the integument at a specific site (because all do it) but nevertheless, this form of mating does not require a morphological match to female genitalia. Whilst imposing functional constraints in some way (Eberhard, 2006), it also removes an important evolutionary constraint—the morphospace that male genitalia can explore. Current models of genital evolution would, therefore, predict that male genitalia evolve more rapidly in bedbugs than in taxa where male genitalia need to match female genitalia. (c) Female cimicids have evolved the spermalege, an organ that solely functions in copulation (Morrow & Arnqvist, 2003; Reinhardt et al., 2003; Usinger, 1966) but have retained a fully functional reproductive tract used for egg-laying and waste expulsion. So, uniquely we can examine the evolution of the copulatory organ in bedbugs in isolation from the selective effects of noncopulatory functions. If variation in female genitalia evolutionarily follows variation in male genitalia, as predicted (Eberhard, 1985, 1996; Firman et al., 2017; Hosken & Stockley, 2004; Simmons, 2014), and if female copulatory function is not constrained by other processes, cimicids are predicted to show particularly tight coevolution between male and female genital morphology. Specifically, evolutionary changes in male genitalia are expected to predict evolutionary changes seen in female genitalia. (d) Bedbugs belong to the true bugs (Heteroptera) that ancestrally have symmetrical male and female genitalia at the ventral end of the abdomen. The asymmetry of male genitalia, and some female copulatory organs, in the Cimicidae (Usinger, 1966) therefore indicate gross evolutionary changes. Finally, (e) the spermalege’s location on the body (Usinger, 1966) varies to a degree that seems unmatched in the animal kingdom. The expression of the organ

at different sites of the body (heterotopy sensu West-Eberhard, 2003) is associated with all body axes (Usinger, 1966): The spermalege appears on the dorsal or ventral side, symmetrically, or asymmetrically, on the left or right side or centrally on the abdomen, depending on species. It can be associated with any segment between the second and the seventh, occur as a single organ or in multiples, and be confined to one segment or span across several (Figure 1, Supplementary Figure S1; Supplementary Table S1), again depending on species. The spermalege additionally shows large variation in external and internal anatomical complexity, ranging from being entirely absent as an organ, to being a simple cuticular invagination, a duplicated structure, or even an epithelium-lined paragenital tract or conduit (Usinger, 1966), and was even expressed in males of one species where males inseminate other males (Reinhardt et al., 2007).

Here we focus solely on the location of the spermalege on the female body and analyze its interspecific variation. We then ask whether two important male genital morphological traits, genital length, and shape, could have driven the heterotopy of the female copulatory organ. We reconstruct the evolution of male genital length and shape, and the heterotopy of the female genitalia and find faster evolutionary rates in female than male copulatory organs. We show the location of the spermalege was under weak selection by male genitalia, that is, not driven by male genital length or shape. Instead, we hypothesize that (sexual) selection by nonmorphological male traits, such as mating behavior, may contribute to shaping the evolution of female genitalia, with the important implication that a lack of morphological coevolution between the sexes may be insufficient to conclude for a general lack of coevolution.

Materials and Methods

Material and trait classification

We analyzed all 35 species of Cimicidae for which robust molecular phylogenetic data are available, one third of known species, two thirds of genera from 5 out of 6 currently recognized subfamilies (Roth et al., 2019). Morphological characters were mainly taken from published drawings in Usinger (1966) (Supplementary Table S1). We also checked field-collected (see Roth et al., 2019) and embedded museum specimens (Supplementary Figure S1). Samples not identified as species were excluded from all analyses. The male copulatory organs are hard, needle-like chitinous structures. Their length relative to the last abdominal segment length and their gross shape are unlikely to differ with the preservation method. Nevertheless, we aimed to avoid overly precise quantifications because of the heterogenous origin of specimens and so determined coarse categories of the paramere length and shape (Supplementary Figure S1). Paramere length was classified into one of four gross categories: shorter than the length of the ninth segment (short), reaching the anterior margin of the eighth segment (medium), reaching the middle of the eighth segment (long), and reaching beyond the eighth segment (extra-long). We used the relation to segment length rather than absolute length to account for overall body size differences. We classified parameres as either straight, faintly S-shaped, convex, or concave (Supplementary Figure S1, Supplementary Table S1). One species with an exceptional shape was classified as “strongly concave” (Supplementary Figure S1, Supplementary Table S1). These categories were unambiguous. The judgment of shape and length classes derived from the drawings in Usinger (1966) differed in

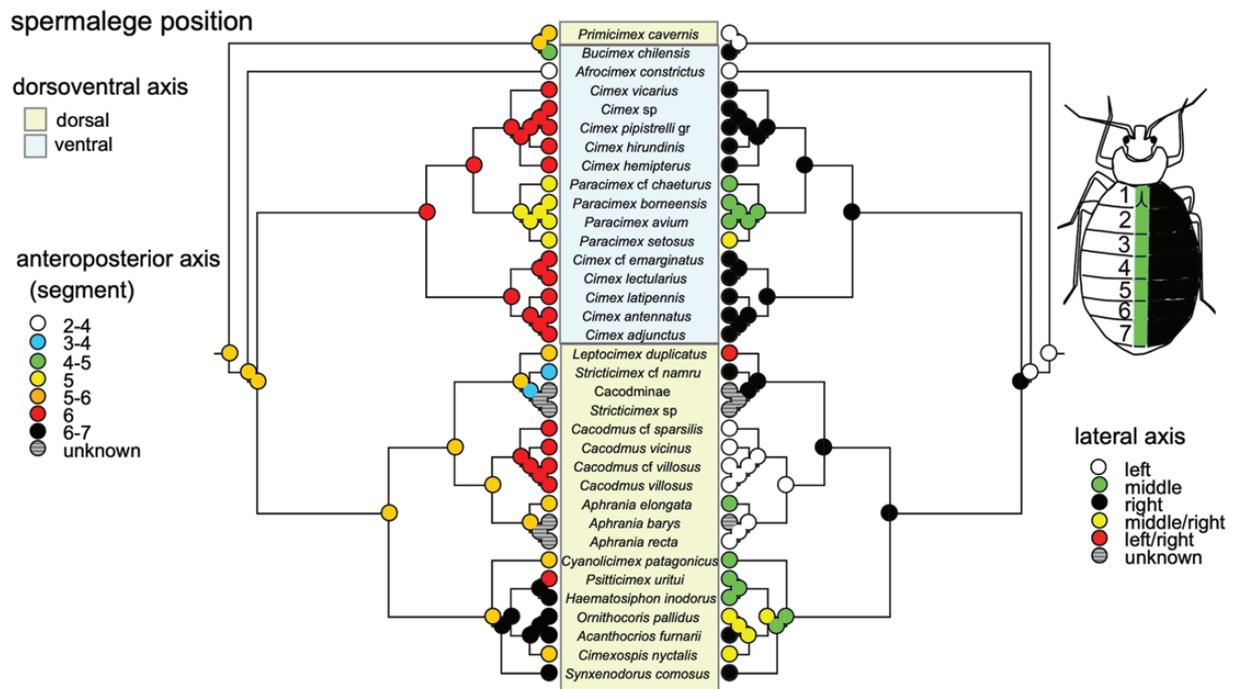


Figure 1. Evolution and diversity of the female copulatory organ, the spermalege, in bedbug species (Cimicidae). The spermalege is a copulatory defense organ against traumatic insemination and is situated on the dorsal or ventral surface of the abdomen (central panel), on the left, right, or central (mid panel) positions of the body (right panel) and positioned along the cephalo-caudal axis (left panel). *Primicimex* lacks a spermalege but the corresponding segments of intromission are indicated. Data are taken from Usinger (1966) and own observations (Supplementary Table S1). Ancestral character states were reconstructed with parsimony in PAUP (Swofford, 2003) on a Bayesian consensus tree (Roth et al., 2019).

five cases between two independent observers (SR and KR) (Supplementary Table S1) and in two cases between the drawings and our own material (Supplementary Figure S1). In all cases, we gave priority to our own material to resolve the controversy (Supplementary Figure S1). Raw data and their respective sources are provided in Supplementary Table S1.

The location of the spermatheca on the body was classified as being either dorsal or ventral; being situated along the left-right axis (five evolutionary states: right, right-middle, middle, left-middle, left); or being situated along the antero-posterior axis (seven states: segments 2–4, segments 3–4, segments 4–5, segment 5, segment 5–6, segment 6, segment 6–7) (Figure 1). There was no case of disagreement between two observers (K.R. and S.R.) in classifying any of the states based on Usinger's (1966) drawings, and no disagreement between our own material and Usinger's drawings.

Ancestral character state reconstruction

Methods

Taxon sampling, phylogenetic analyses, and molecular dating followed Roth et al. (2019). We mapped the characters on the dated tree for ancestral state reconstruction using Mesquite version 3.2 (Maddison & Maddison, 2017). We pruned the outgroup taxa from the tree and collapsed zero-length terminal branches. Ancestral states of nodes were estimated with maximum likelihood using the “trace ancestral character” function and a simple one-parameter Markov model (Lewis, 2001). We explored parsimony with ordered and unordered states, and equal and unequal weights for evolutionary transitions of female and male characters, using Mesquite (Maddison & Maddison, 2017) and PAUP (Swofford, 2003).

As a second method, we used Bayesian ancestral character state analysis (BACA) to explore character states at each node. We used MrBayes version 3.2.7 (Ronquist et al., 2012), specifically the application MBASR in R (Heritage, 2021). MBASR was run with the multiple traits function set for unordered characters in RStudio (RStudio Team, 2022) with R-version 4.2.1 (R Core Team, 2022).

Male and female genitalia

Evolutionary states of males were considered categorical characters because the developmental mechanism underlying male variation is unknown. Considering adjacent character states as more likely evolutionary transitions (by weighting the transition probabilities according to the distance from the ancestral state) did not result in more parsimonious solutions. The same was true for female characters. Therefore, we used the unordered and equal-weighted (transitions between all states equally likely) character states for paramere length and shape and spermatheca location.

Bayesian ancestral state analysis estimates the probabilities of each possible state at each node in a tree (Supplementary Figure S3a–d). Some reconstructions produced low probabilities for any of the alternative states on a node and some of the associated state change events were accordingly weakly supported. Therefore, we relied on parsimony approaches when identifying ancestral states. When recognizing only those state changes resulting in a (highly probable) single state on the nodes (Supplementary Figure S3a–d), both approaches returned a similar number of changes in male and female characters, including the number of simultaneous changes, even though the nature of the trait combinations may differ (see Supplementary Figure S4, Supplementary Table S3).

Geography and hosts. Following Roth et al. (2019), we coded the extant taxa into seven discrete geographical regions: Oriental Region (A), Eurasia (B), Nearctic (C), Neotropics (D), sub-Saharan Africa (E), Oriental-Malaysia (F), and Saharo-Arabian (G). Ancestral distributions of the cimicid lineages were estimated using RASP v.4 (Yu et al., 2015, 2020). As input to RASP, we imported 2,000 post-burn-in trees from the set of 10,000 tree samples and their consensus tree generated with BEAST (Suchard et al., 2018) as published earlier (Roth et al., 2019). We reduced the 82 terminal branches to 35 by pruning branches of all outgroups and duplicate copies of ingroup taxa. With RASP, we sampled 500 random trees from the resulting tree set and used the implemented DIVALIKE model of BioGeoBears (Matzke, 2013) to infer the ancestral range with probability estimates. We chose DIVALIKE because hierarchical vicariance and island models (Ronquist & Sanmartín, 2011) assume each lineage is present in only one area at one time. DIVA and its likelihood version allow for vicariance in more widespread lineages. We kept the number of unit areas in the ancestral distribution to 2 to allow for founder event dispersal and to reduce the tendency to infer more widespread distributions toward the root of the tree (Kodandaramaiah, 2009; Matzke, 2014).

To estimate the effect of dispersal, we designated a time-stratified area exchange matrix that reflects the putative connectivity of the continents within the time periods 0–6.6, 6.61–13.1, 13.11–26.2, 26.21–52.3, 52.31–104.5 MYA. The matrix assumes potential connectivity between all geographic regions in all periods except 6.61–13.1 million MYA. We set a constraint (0.5) on an exchange between Eurasia and the Neotropics (B to D), and between the Neotropics and Oriental-Malaysia (D to F) in the period 6.61–13.1 million MYA because of the wider tectonic separation of these ranges at that time (e.g., Torsvik & Cocks, 2013). We also ran the analysis without area exchange constraints (1.0) for all time periods and obtained similar results except for node 49 (Supplementary Figure S5, Supplementary Table S2).

Parsimony character-change reconstruction allowed us to count the number of branches with simultaneous changes in morphological character state, geographic area, and hosts. Changes along branches were counted from the node they derived (Supplementary Table S2). This approach was possible because the robustness of the topology of our phylogenetic tree provided a solid backbone for between-taxon comparisons.

Analysis of character covariation

Studying the evolutionary correlation among male and female morphological traits in a phylogenetic context is challenging if multistate traits are discrete (Maddison & FitzJohn, 2015). Here we used Reverse Jump stepping stone MCMC in BayesTraits v4 (Meade & Pagel, 2022; Pagel et al., 2004) that estimates the marginal likelihood by placing a number of “stones” which link the posterior with the prior: “The stones are successively heated, forcing the chain from the posterior towards the prior, providing an effective estimate of the marginal likelihood” (Meade & Pagel, 2022). We first calculated the marginal likelihood of parameters assuming a homogeneous rate of evolution for these characters. Next, we ran the data with the covarion model (Tuffley & Steel, 1998) that allows variable rates of evolution across the tree. This model also allows the trait to change from its current state into any other state over infinitesimally small intervals of time

(a variant of the continuous-time Markov model allowing variable rates of trait evolution within and between branches). If the variation in the rate of evolution of a specific trait depends not only on the variability of the trait itself but also on the variability of other traits in the data set, the resulting association of the traits can be assessed by this approach. We applied the covarion model in pairwise comparisons of coded states for male and female genital structures, geographical distribution, and host type ([Supplementary Tables S4 and S5](#)).

In both the homogenous and the covarion models, we used an exponential hyper-prior with a mean drawn from a uniform distribution with a range of 0–100 for the parameters. Model runs were performed with 200 stones and 2,000 iterations per stone. The marginal likelihoods of the homogenous and the covarion models were compared by Bayes factor estimates (BF). BF quantifies the probability by which the covarion model (i.e., that the variability in the evolutionary rate of a trait depends on the evolutionary rate of another trait) is more likely than the null model (which assumes homogenous evolutionary rates for all traits). By convention, $\log BF < 2$ indicates weak, 2–6 positive, and 6–10 strong, and $\log BF > 10$ is very strong evidence against the null hypothesis of no covariance (Meade & Pagel, 2022).

Estimation of phenotypic evolutionary rates

In the phylogeny (Roth et al., 2019), each branch length represented a time segment. We estimated rates of morphological evolution within each of those segments from the distribution of character states on the terminal branches. We initially used BayesTraits v.3 (Meade & Pagel, 2017) to compute marginal likelihoods in pairwise comparisons of character covariation and tested an equal rates model against a variable rates model for the morphological data using Bayes Factors. Using stepping stone MCMC we generated 1,000 trees with branches that are scaled by the rate of phenetic change as estimated from the multistate character matrix. Extended branches indicate increased rates, and shortened branches decreased rates. Independent runs with the two matrices of coded female and male characters supported the variable rates model over a homogeneous rates model in females ($\log BF$: 4.3) and possibly in males ($\log BF$: 2.15). Both models returned similar results for estimated branch lengths. We used BayesTrees 1.3 (Meade, 2011) to compute consensus trees for the two tree sets. The “male tree” had an estimated mean tree depth of 1467.47; the “female tree” 266.40. Note, that we accounted for the scale differences between the characters we used by adjusting branch lengths by rescaling female and male trait evolution to the same dimension. Scaling was calculated as follows: male characters rate \times female tree depth / (male tree depth + female tree depth), and female characters rate \times male tree depth / (male tree depth + female tree depth), respectively. This procedure standardizes for the different nature, and number, of character states in males and females.

Results

Data are presented from parsimony-based analyses, unless otherwise stated. BACA analyses are presented in the supplement. Character state reconstruction with PAUP returned no ambiguous states on internal nodes (Figure 1, [Supplementary Figure S2](#)). Using Mesquite to trace character state changes returned more than one most parsimonious solution for some

characters. We explored these multiple resulting parsimony scenarios for possible cases of genital covariation (Figure 2). By this approach, we retained clarity and parsimony but maximized the number of possible cases of genital covariation.

Patterns of genital variation

Females

Using parsimony, we reconstructed the ancestral spermalege as situated dorsally between segments 5 and 6 (5/6) on either the right or left (but not central). The ancestral spermalege underwent 1 change toward the ventral position and 20 positional changes along the segmental and other axes (Figure 1; [Supplementary Table S2](#)), 9 on the anteroposterior (segmental) axis, and 11 on the left–right axes (Figures 1 and 2, [Supplementary Figures S1 and S2](#), [Supplementary Table S2](#)). Bedbugs are 3–12 mm long, meaning the changes in spermalege location concerned distances in the order of hundreds of micrometers to millimeters.

There was little evidence of canalization: among the 9 segmental and 11 left-right transitions, only one type of change occurred twice (segment 5/6 to 6 and right to middle), all others were unique ([Supplementary Table S2](#)). Together, all the changes concerned 17 of the 68 branches (25%) (or only 16—[Supplementary Table S2](#)) on our tree. BACA reconstructed the same ancestral state ([Supplementary Figure S3a and b](#)) and slightly more changes for most characters ([Supplementary Figure S6](#), [Supplementary Table S3](#)).

Males

In all species, the genitalia were ventral, at the end of the abdomen, and all were asymmetric—a single paramere pointing left ([Supplementary Figure S1](#)). Parsimony-based reconstruction identified the ancestral paramere as a convex organ of intermediate length ([Supplementary Figure S2](#)). Unlike in females, the changes in paramere length and shape were in the order of tens of micrometres, except for four species with strongly concave or very long parameres ([Supplementary Figure S1](#), [Supplementary Table S1](#)).

We identified 17 paramere character changes, 9 in length, 8 in shape (Figure 2, [Supplementary Figure S2](#), [Supplementary Table S2](#)). Of the nine changes in genital length, three involved an extension, six a shortening (Figure 2, [Supplementary Figure S2](#), [Supplementary Table S2](#)), the latter including both lineages with extralong parameres. Three of the eight shape changes were from straight to concave (Figure 2, [Supplementary Figure S2](#), [Supplementary Table S2](#)) (all in the subfamily Haematosiphoninae), three from convex toward straight ([Supplementary Table S2](#)). Changes occurred on 14 (or only 13—[Supplementary Table S2](#)) of the 68 branches (20.6%) of our tree.

BACA showed the same ancestral state ([Supplementary Figure S3c and d](#)) and slightly more character changes for most character states ([Supplementary Table S3](#)). In addition, the analysis of character covariation showed that interspecific variation in length co-occurred with variation in shape ([Supplementary Table S4](#)).

Evolutionary rates

Given the age of the Cimicidae of about 115 million years (Roth et al., 2019), there was approximately one spermalege segmental change every 12.6 million years, and a left-right change every 10.4 million years, on average. In males,

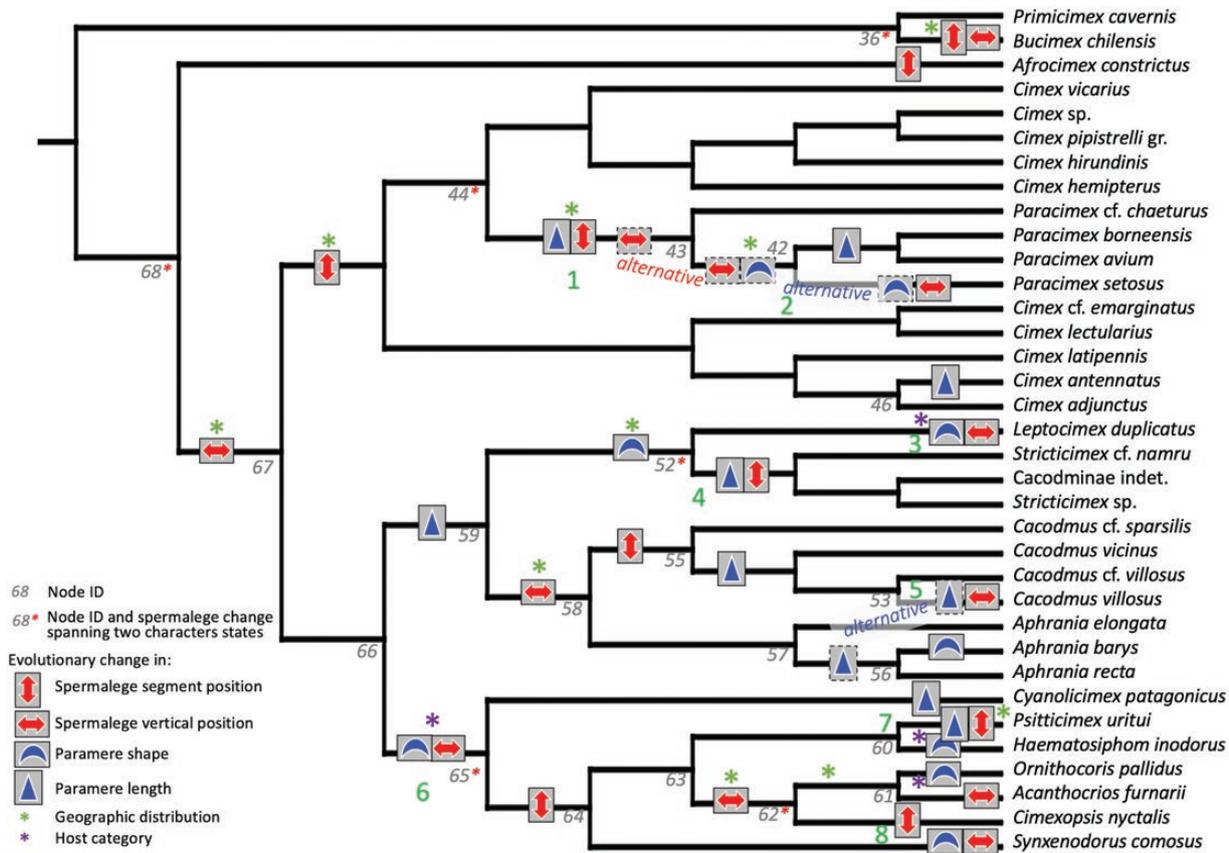


Figure 2. Number and position of evolutionary changes in male (triangle and sickle-shape symbol) and female (arrows) genital structures in the bedbug family (Cimicidae) as inferred from parsimonious ancestral character state analysis (cf. Figure 1, Supplementary Figure S2). Vertical double arrows mark spermalege changes along the segmental axis; horizontal double arrows along the left–middle–right axes (Figure 1). Evolutionary changes in paramere length are denoted by blue vertical triangles, in paramere shape by blue sickle shapes (Supplementary Figure S2). Node IDs in gray next to nodes are only shown if there were evolutionary changes and correspond to those in Supplementary Table S2. Trait changes under different parsimony scenarios are shown in stippled lines. Red asterisks denote nodes with spermalege changes of two character states. Single asterisks mark branches with changes in male or female characters that are accompanied by geographic (green) or host changes (purple). For details of individual trait changes, see Supplementary Table S2.

paramere length changed once every 12.6 million years and shape once every 14.3 million years, on average. Examining changes across the entire Cimicidae, the spermalege location evolved 1.58 times faster than paramere length and shape (Figure 3, Supplementary Figure S6; Supplementary Table S4). This trend was true not only for both the terminal taxa alone (1.61 times faster) but also for the five oldest nodes (1.89 times faster) (Supplementary Table S4). However, there was a slight heterogeneity across subfamilies where the rate bias ranged from 0.97 to 1.38 (Supplementary Table S4). In all three subfamilies, the rate bias was smaller in terminal taxa, being 0.55 to 0.04 times lower and resulting in male-biased ratios in two subfamilies (Supplementary Table S6).

Sexual selection without female harm

Models of these categories predict some degree of genital match (see Introduction). For the species on our tree, there was little evidence of such matching (Usinger, 1966) nor that such matching happened during evolution (Figure 2). Some individual species may show some degree of “morphological fit” (Discussion) but they do not interfere with the overall lack of fit observed here (Figure 2).

The lack of morphological fit and lack of synchronous coevolution do not exclude that female and male character variability is affected by the variability of the other sex. We

then accepted coevolution whenever a male and female genital character changed on the same branch (“simultaneous”) and found several equally parsimonious scenarios. If, of those parsimonious scenarios, we used the one with the largest number of simultaneous male–female changes, we detected such simultaneous changes on six branches (but up to eight for equally parsimonious scenarios) (Figure 2). On our tree, a male genital change (20.6% of branches, see above) and a female genital change (25% of branches) may by chance fall on the same branch with a probability of $0.206 \times 0.25 = 0.052$, or 3.5 branches. Only if all eight, but not six or seven, simultaneous changes are assumed, will the observed changes fall outside the range of chance (χ^2 (Yates correction) = 1.143 to 4.571, $0.285 < p < .032$). Broken down for individual male and female genital characters, left–right positional changes of the spermalege (16.2% of branches) co-occurred with a change in paramere shape (11.8% of branches) more frequently than expected (Supplementary Table S7). Analysis of character covariation confirmed covariation between male and female traits (Supplementary Table S4).

There were no specific male–female trait combinations that evolution converged to (Figure 2; Supplementary Table S2), and no combination appeared twice. Both observations suggest that male and female genital traits may change simultaneously but there is no joint pattern of natural selection for

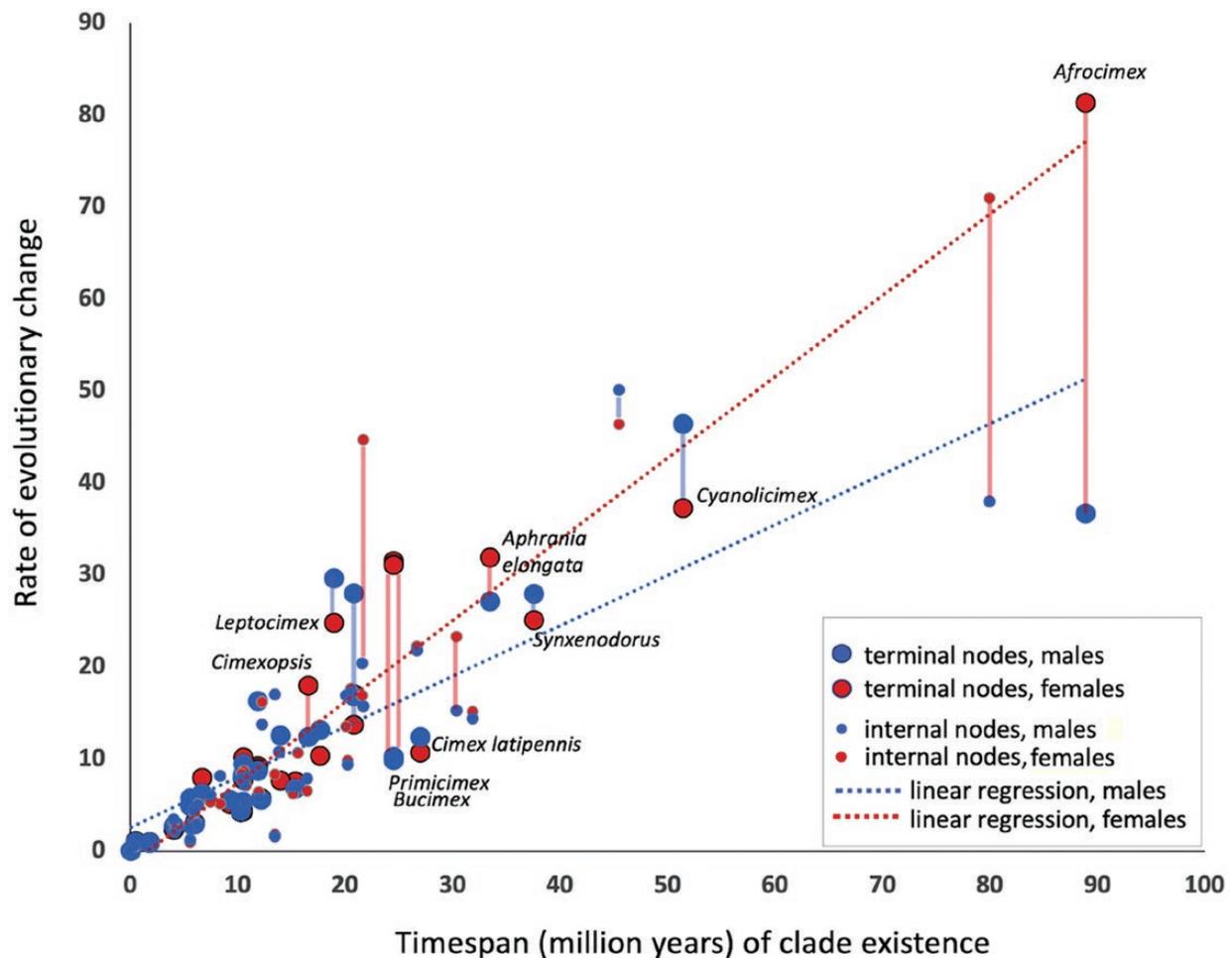


Figure 3. Evolutionary rates of genital characters in relation to node age. Different depths of phylogenetic changes (tree depths) in males and females were accounted for by adjusting branch lengths by rescaling female and male trait evolution to the same dimension (Materials and Methods). Example taxa are indicated by their taxonomic name; red vertical lines indicate faster evolution in females; and blue lines indicate faster evolution in males. For further sex-specific regression analysis, see [Supplementary Table S6](#).

genital matching, or of sexual selection on paramere length or shape to positionally match the spermalege position. The previously mentioned observation that paramere responses are in the micrometer range whereas the spermalege variation was in the millimeter range suggests little genital “tracking” in one sex by the other. The only way we can envisage sexual selection to explain the pattern of cimicid genital variation is by abandoning the view of the strict morphological covariation but assuming instead that female genital morphology may respond to the sexually selected, nongenital male trait(s) (see Discussion).

Sexually antagonistic coevolution

Not all models of this process require a fit between male and female genitalia ([Arnqvist & Rowe, 2002](#); [Gavrilets, 2014](#); [Jandausch et al., 2022](#)) but do not exclude it, even in traumatically inseminating taxa ([Tatarnic & Cassis, 2010](#)). The most prominent model assumes perpetual coevolutionary cycles of morphological escalation and resistance (e.g., [Arnqvist & Rowe, 2002](#); [Gavrilets, 2014](#); [Holland & Rice, 1998](#)). In males, the average node age without a paramere change (33.3 ± 30.7 (mean \pm SD) million years) was similar to that with change (23.3 ± 20.1 million years) ($t = 0.276$, $df = 1$, $p = .828$), suggesting that paramere changes occurred

randomly over time, were not concentrated on a certain time period and so do not contradict that changes are “perpetual.” In females, however, positional changes of the spermalege were not homogenous or random across time but were concentrated toward older nodes: nodes with heterotopy were at 36.9 ± 27.5 MYA (mean \pm SD, $N = 17$), without heterotopy at 18.0 ± 21.4 MYA; $N = 19$ ($t = 2.246$, $df = 1$, $p = .031$) (or even only 13.3 MYA if excluding the oldest split of 103 MYA from the heterotopy group).

In the model where male antagonism is met by female tolerance, rather than resistance, male–female trait pairs stop coevolving but may still facilitate the selection of novel male traits (see [Michels et al., 2015](#) for an explanation). The limited variation in male genitalia suggests that female tolerance could explain variation in female genital morphology only if nongenital traits had diversified and would drive female morphology (see Discussion).

Females responding to male harm by the Buridan’s ass process must diverge in *both* female morphs from the ancestral one (Introduction). We found that the phylogenetic trajectory of the spermalege ([Figures 1 and 2](#)) is incompatible with this model: 6/21 phylogenetic changes in the spermalege (28.6%) resulted in a divergence of two characters states or more ([Figures 1 and 2](#), [Supplementary Table S2](#)), but all

involved two changes in one morph, not one change each in *both* morphs. No paramere change was associated with the two-step character changes (Supplementary Table S2). The sympatric speciation model of sexually antagonistic coevolution (Gavrilets & Waxman, 2002) predicts simultaneous male and female character changes across two character states plus the “extinction” of the ancestral trait but no such change occurred. Assuming that parsimony analyses might not be able to detect such two character changes, we applied BACA but did not detect such change(s) (Supplementary Figure S4).

The role of geography and host species

Because of the poor support by sexual selection models, we examined other hypotheses for genitalia evolution, including neutral, geographic, and host changes. For the latter two types of changes, it is relevant that the common bedbug sexually transmits microbes that originate from the local environment (Bellinvia et al., 2019; Oti et al., 2017; Reinhardt et al., 2005). If this local microbe community impacts on, and selects for, female genital characters, a geographic or host species signal is expected in the spermalege variation. To test for such variation, we first used Bayesian dispersal–vicariance analysis and examined the effect of geography, and parsimony analysis for host type. We reconstructed the geographic origin of the Cimicidae as the land area of the present Nearctic, Neotropics, and sub-Saharan Africa (Supplementary Figure S5). From this state, 18 branches showed a geographic change (26.5%) and eight a host change (11.8%) as derived from Roth et al. (2019).

Male–female covariation

Two (or three—Figure 2) changes in geography and two host changes occurred simultaneously with spermalege + paramere change (Figure 2, Supplementary Table S2). This meets chance expectations of 0.5 branches (χ^2 (Yates correction) = 2.056, $df = 1$, $p = .152$) for geography and 1.1 branches (χ^2 (Yates correction) = 1.743, $p = .189$) for host. BACA found even fewer events of covariation between male and female traits and geography or hosts (Supplementary Table S3). Thus, geography and host use did not explain covariation of male and female genitalia; but did they affect male and female traits separately?

Male variation

Three (or four—Figure 2) of the 17 paramere length and shape changes happened simultaneously with a geographic change (Figure 2, Supplementary Table S2), not different from chance expectation (4.5 branches, χ^2 (Yates correction) = 0.888, $df = 1$, $p = .346$), host and male changes saw four simultaneous events (expected: 1.76, χ^2 (Yates correction) = 1.720, $df = 1$, $p = .190$). BACA found fewer events (Supplementary Table S3) and only weak to some support for paramere changes being associated with geography (Supplementary Table S5), and stronger support for an association with host changes. Note that this analysis includes delayed responses that happen post-branching, that is, also post-spermalege branching if the species vary in spermalege position (Supplementary Discussion).

Female variation

Seven (or eight) branches saw simultaneous changes in spermalege and geography (Figure 2; Supplementary Table S2), falling well within the range of five expected by chance

(χ^2 (Yates correction) = 0.430 or 1.214, $df = 1$, $p = .271$ or $.512$). BACA found slight support for covariation between spermalege and geography (Supplementary Table S5). Geographic changes may include changes because of genetic bottlenecks after dispersal events. However, most spermalege changes were associated with vicariance ($N = 6$), not dispersal changes ($N = 1$) (Supplementary Table S2, for path probabilities > 0.6). The eight host changes on our tree (Roth et al., 2019) were associated with two simultaneous spermalege changes, meeting the chance expectation of 2.2. Analysis of character covariation found stronger support for host covariation with spermalege left–right variation (Supplementary Table S5).

Discussion

Bedbug females have extremely variable copulatory organs and here we restricted ourselves to reconstructing the evolution of their location. We aimed to test whether male genital traits predict that variation because selective pressure on female genitalia by male genitalia is predicted by most sexual selection models, including antagonistic coevolution, but also several nonsexual selection models (Brennan & Plum, 2014; Eberhard, 1985, 2006; Hosken & Stockley, 2004). Our analysis of both individual and joint trajectories of male and female genital traits through evolutionary time, continental distribution, and host use showed that female copulatory organs evolve faster than male ones but did not reveal evidence that male genital traits were responsible for driving this variation. Because these findings were obtained in a system where the effects of sexual selection should be particularly strong, they have implications for female genitalia evolution, as well as for sexual selection models.

Rapid evolution of female copulatory organs

Our and some previous studies (Genevicius et al., 2020; Simmons & Fitzpatrick, 2019) have in common that female genitalia show greater evolutionary rates than male genitalia. Simmons and Fitzpatrick (2019) estimated that a female genital structure evolved three times as fast as a male genital structure. Our overall estimate in the Cimicidae is only half as large (Figure 3, Supplementary Figure S6, Supplementary Table S6). Our study further adds that evolutionary rates differ across the phylogenetic tree for both males and females.

Empirical evidence for faster female phenotypic evolution of genital traits is generally scarce, even though increased research efforts change this scarcity (Fritzsche et al., 2014; Genevicius et al., 2020; Simmons & Fitzpatrick, 2019). The origin of this faster evolution does not seem to be clear. We can exclude for the Cimicidae that natural selection for reproductive processes other than copulation has driven variation in female genitalia (Muto et al., 2018) because the spermalege only functions in copulation. We conclude that copulation, and not any other function of the spermalege, drove its variation. This notion extends to the immune function of the spermalege (Oti et al., 2013, 2017; Reinhardt et al., 2003; Siva-Jothy et al., 2019). Immune activity occurs against sexually transmitted microbes and so as a consequence of copulation, and at the site of it. It seems less plausible that males target a body site by traumatic insemination *because* there is locally high immune activity.

Sexual selection

Three aspects suggest that sexual selection had a small role in the genitalia evolution of bedbugs: the lack of morphological

fit between male and female copulatory organs (Usinger, 1966), the smaller variation in male than female copulatory traits, and the scarce evidence for morphological coevolution shown here. In the light of a morphological fit of genitalia, one may imagine that nongenital, even nonmorphological, stimulation by males might bias fertilization by females toward a specific male genotype (i.e., cryptic female choice—Eberhard, 1996). However, we are unable to propose a realistic scenario of how such stimulation might be related to the dramatic change in the location of the spermalege.

None of the four male genital parameters we looked at, position, asymmetry, length, and shape, differed greatly, nor did they explain variation in females. Why these four male parameters differed relatively little is outside the scope of this study. Selective constraints on genital complexity, associated with the need to penetrate the cuticle may be responsible (Eberhard, 2006) in some parameters. Note, however, that the paramere parameters we used, perhaps with the exception of curvature, would appear to be not much constrained by the mechanics of penetration. Doubtless, fine-scaled geometrical morphometrics, sophisticated 3-D reconstruction of genitalia or multiphoton excited autofluorescence imaging (Matsumura et al., 2020; McPeck et al., 2008; Reinhardt et al., 2017) will expose more variability in male genital traits, such as finer shape variation, different material properties or surface structures of the parameres. It is also plausible that female characters might drive variation in subtle paramere shape or material properties. However, neither of these can currently be reasonably hypothesized to drive mm-range shifts in the position of the spermalege. Based on their findings in dung beetle genitalia, Simmons and Fitzpatrick (2019) argue that female preference traits may evolve more rapidly than male genital traits under female choice. For the spermalege location in cimicids, this suggestion is then plausible only if female preference traits in genitalia would evolve in response to male nongenital traits. Below we provide a suggestion for such a scenario (see “An a posteriori hypothesis to explain female-biased genital variation”).

One may oppose the idea that genital evolution is driven by one single general force across the tree and in principle, every branch on the tree may evolve by a different mechanism. For example, the individual case we looked at (Supplementary Table S2) could show a posterior move of the spermalege associated with either a lengthening or a shortening in parameres. Such phylogenetic heterogeneity could explain the different rates we observed for the different subfamilies. Similarly, only a subset of the species within the genera *Cacodmus* and *Leptocimex* may be classified as showing morphological “fits,” where curved parameres are associated with the evolution of copulatory tubes in females that show similar curvature (Usinger, 1966). Regardless of any heterogeneity in the processes by which the evolutionary changes are driven, no process undermines the overall pattern that female copulatory organs in cimicids are much more diverse and evolve faster than male genitalia, and do generally not fit them.

Sexually antagonistic coevolution and female-limited polymorphism

The Cimicidae have served as an example of genital evolution by the theoretically well-developed Buridan’s ass process that results in the evolution of female-limited polymorphism, including the female copulatory organs within a species (Reinhardt et al., 2007). Different morphs in a population may

not necessarily be maintained evolutionarily but can be lost by drift or selection (Blow et al., 2021; Corl et al., 2010). We, therefore, focused on those six cases, where spermalege positional changes spanned two character states. In all of them, the ancestral trait was retained in one branch. The retention of the ancestral morph clearly is impossible under Buridan’s ass. However, we note that the Buridan’s ass idea was developed for sexual conflict before the idea of female tolerance evolution was introduced to sexual conflict (Lessels, 2006; Michels et al., 2015; Svensson & Råberg, 2010). We wonder whether one female morph may evolve resistance to male harm (Rice, 2000), thereby diverging from the ancestral morph, whereas the other may evolve tolerance, thereby remaining unchanged. This is not a parsimonious scenario but would agree with the notion of “one-sided” morph changes in other types of female-limited polymorphism systems (Blow et al. 2020) but also with morph-specific resistance across different populations (Gosden & Svensson, 2009). Future refinements of the sympatric speciation following Buridan’s ass process (Gavrilets & Waxman, 2002) may also include the consideration that males can plastically respond to a divergence of female morphs. The plasticity in the intromission site in cimicids (Supplementary Table S8) but also the behavior of male damselflies toward different female morphs (Blow et al., 2021) show that male plasticity is more than a mere theoretical possibility.

Our data provide a striking dissimilarity to the pattern in a related group of plant bugs from the genus *Coridromius*. Females in this genus, like cimicids, possess a spermalege that is used for copulation. This organ varies somewhat in position and the morphological characters around it (Tatarnic & Cassis, 2010; Tatarnic et al., 2014) but is so strongly associated with interspecific variation in the male intromittent organ that it classifies as morphological fit (Tatarnic & Cassis, 2010; Tatarnic et al., 2014). Whilst in the Cimicidae some individual species might show a genital fit (see below), an important difference between the Cimicidae and *Coridromius* is that the mating is traumatic in all, or almost all, species of Cimicidae, whereas in *Coridromius* evidence for actual traumata (wounds) exists for only a minority of species (Tatarnic & Cassis, 2010; Tatarnic et al., 2014).

Other possible selection pressures

Local microbe effects by host or habitat changes remain a theoretical possibility to explain spermalege variation. If so, we expected host or habitat response to be correlated to geography. A geographic signal may be expected if local environmental microbes are transmitted, affect fitness (Bellinvia et al., 2019; Otti et al., 2013, 2017), and are caused by genital variation (Reinhardt, 2010). We found geography had only a weak ability to explain variation in some aspects of female copulatory morphology. Moreover, microbe variability may drive the evolution of spermalege complexity but it is not clear how it could drive the *position* of the spermalege. Evolution by genetic drift could produce a geographic pattern via founder events. However, our data indicate that geographic changes were not driven by dispersal-related genetic bottlenecks. The reason for the (rather weak) geographic variation in female genitalia remains unclear.

An a posteriori hypothesis to explain female-biased genital variation

In insects, male copulatory position evolves rapidly and is correlated to female variation, especially genital asymmetry

(Huber et al., 2007). In cimicids, mating behavior or position (and, by extension, maybe other nongenital traits) may have been under diversifying sexual selection and driven variation in the intromission site on the female body. The benefits of the spermalege in ameliorating one or several of the different types of harm reported (e.g., Michels et al., 2015) might then have led the spermalege to “follow” the intromission site. Such change in the spermalege site would not reduce male fitness (female tolerance) thus suspending selection on male genital morphology and thereby leading to an (observed) association of low male and large female morphological variation in genitalia. This scenario assumes that the rapid evolution of, and intraspecific variation in, the mating position also exists in cimicids. The observation of interspecific variation in mating position is confirmed, but unfortunately, precise data are only known from four genera (Usinger, 1966). We examined where exactly males penetrate the female body in five species of cimicids, including 35,000 females of *Cimex lectularius* (Supplementary Method), and established inter- and intraspecific variation in the intromission site, and thus plastic male mating behavior: between 0.02% and 85% of females received intromissions outside the spermalege (Supplementary Table S8).

Female choice or sexual conflict can both be selected for altered male mating behavior. However, neither would suspend our hypothesis that nongenital male traits, such as mating behavior, select for female genital morphology. For example, a thickening of the female integument at the intromission site (but see Michels et al., 2015) or female defensive postures may not affect the evolution of genital morphology but alter male behavior to change intromission site and cause selection on female genital morphology. Our hypothesis applies to genitalia, but we note that similar suggestions have been made for the morphological diversification of sperm storage organs in females, where, for example, specific mating behaviors, or multiple mating, selects for multiple, or compartmentalized female sperm storage organs. While our a posteriori explanation for both male stasis and female variation obviously requires testing, we suggest that models seeking to explain genital diversification (Brennan & Plum, 2014; Eberhard, 1985, 2006; Hosken & Stockley, 2004; Michels et al., 2015); meanwhile, benefit from considering male behavior as a possible driver of evolutionary change in genital morphology (see also Brand et al., 2022).

Limitations of our study

We were able to use the unique properties of our system to challenge the generality of some existing hypotheses. However, at the same time, the uniqueness may introduce limitations that we wish to discuss. First, a recent study by Brand et al. (2022) in the plathelminth genus *Macrostomum* found that traumatic insemination repeatedly had a canalizing, rather than a diversifying effect. The repeated simplification included a genital structure not involved in copulation (the antrum) which may have a constraining effect on genital diversification. In our system, we were unable to test the constraining effect of noncopulatory on copulatory traits. This prevented us from examining whether low male variability might arise from constraints imposed by joint developmental pathways of male and female noncopulatory genital traits, such as ovipositor length and width.

Second, our study did not include intraspecific variation. This prevented us from using variance-analytical approaches

(e.g., Denton & Adams, 2015) and we were unable to provide error rates. Third, the absence of a necessity for a morphological fit between male and female genitalia may alter those selection processes that are more than others based on the physical interaction during this fit, such as cryptical female choice. In addition, the spermalege varies largely in complexity (Introduction). It is possible that this variation in complexity may introduce variation in morphological fit, not an entire absence across the family. However, we feel that none of these limitations, in addition to a phylogenetically representative, but low, sample size, is suitable to reject the general conclusions of our study.

Conclusion

Our results suggest that female copulatory organs might evolve more rapidly if freed from the constraints of other genital functions. This idea will be difficult to test empirically but future studies of genitalia evolution may benefit from correcting statistically for the constraints imposed by structures dedicated to egg-laying, birth-giving, or waste expulsion. That few of our data agree with current models of genital evolution is additionally explained by low genital variability in males that does not match the large female variability. To help resolve this dilemma, we provided an a posteriori hypothesis on copulatory behavior. If valid, future studies on genitalia coevolution might benefit from expanding the morphological coevolution analysis to include nonmorphological functions.

Supplementary material

Supplementary material is available online at *Evolution*.

Data availability

All data needed to evaluate the conclusions are present in the article. Additional data related to this article may be requested from the authors. Roth, Steffen (2023), Supplementary Material, Zenodo, <https://doi.org/10.5281/zenodo.10267083>.

Author contributions

S.R., M.S.-J., E.H.M., and K.R. designed the study, S.R., O.B., M.S.-J., E.H.M., and K.R. contributed material, S.R., E.W., and K.R. analyzed the data, S.R., and K.R. wrote the first draft, all authors contributed to revisions.

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