Spermathecal variation in temperate Opiliones

Article type: Symposium Article

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Abstract

Most arachnid fertilization occurs internally, allowing for a variety of post-copulatory mechanisms to take place. Females are expected to exert some level of control over sperm fate when 1) the point of gametic fusion is particularly distant from the point of oogenesis, 2) the time of syngamy is significantly later than the time of mating, 3) sperm are non-motile, and/or 4) the morphology of females allows for selective containment of sperm. Many of these conditions are met in Opiliones (a.k.a. "harvesters," "harvestmen," or "daddy-longlegs"), where we have evidence of sexual antagonism, multiple mating, and delayed oviposition for a number of species. We used confocal laser scanning microscopy to capture and analyze images of harvester spermathecae, structures within the genitalia of female arthropods that store and maintain sperm after copulation. Spermathecal morphology may have critical function in controlling seminal movement. We anticipated that species with previously identified traits associated with sexual antagonism would also have thicker and/or relatively more complex spermathecae. We examined spermathecal morphology in thirteen species of *Leiobunum* and one species of *Hadrobunus*, which were collected from North America and Japan. Our results show that eight species had structures consisting of a single chamber with no or partial invagination, and the remainder had multiple cuticular invaginations producing 2-3 lumina within the spermathecae. Using phylogenetic multivariate comparative methods, we estimated a trend towards cross-correlation between conflict and spermathecal traits. Some, but not all, of the species with thicker, more complex spermathecae had morphological traits associated with sexual

conflict (larger body size, thicker genital muscle). In conclusion, we discuss methods to elucidate spermathecal mechanism and sperm precedence in these species. Confocal microscopy allowed us to visualize internal structures difficult to interpret with two-dimensional brightfield microscopy, a technique that could be applied to the characterization of internal reproductive structures in other arthropods.

Running title: Opiliones spermathecal variation

Keywords: Spermathecae, sexual conflict, Opiliones, phylogenetic comparative methods

Introduction

Many arthropod species mate multiply (Arbuthnott et al. 2015; Ridley 1990), leading researchers to hypothesize that sperm competition plays a primary role in any subsequent gametic fusion (Proctor 1998). However, the resulting focus on male interactions in sperm competition may have led researchers to overlook the important role females play in making post-copulatory decisions during sperm competition (Tang-Martínez 2016). Certain reproductive characteristics or conditions may permit female control of sperm usage after mating (Firman et al. 2017). Spermatozoa in a large number of arthropod species are not flagellated, and do not appear to be motile (Dallai et al. 2016). This means that after copulation, females take sole control over gametic movement prior to syngamy. Moreover, in a number of arthropods, syngamy does not occur until oviposition, which may take hours, days, or months (particularly in Hymenoptera; Gotoh et al. 2017, Paynter et al. 2017). In many arthropods, spermatheca are used for storage and preservation of spermatozoa (Mayhew & Merritt 2013; Wolfner 2011) until females oviposit (Schnakenburg et al. 2012). In polygynandrous species, this time allotment during sperm storage may provide females with opportunities to selectively manipulate spermatozoa. Data suggesting the heritability of selective spermatozoan ejection after secondary matings also indicates female choice over sperm fate (Eberhard 1996) may ultimately have significant consequences for paternal fitness (Lüpold et al. 2013).

Leiobunine Opiliones, also called "harvesters" or "daddy-longlegs," are arachnids common to the temperate regions of the United States and Japan (Burns et al. 2012). They have reproductive systems unique among Arachnida: females have an annulated, muscular ovipositor (Fig. 1) covered in sensory hairs, within a pregenital chamber formed by the sternum and genital operculum (Macías-Ordóñez et al. 2010). This chamber may be sclerotized, forming pregenital barriers in some species (Burns et al. 2013). Males have a cuticular penis that is capable of delivering prying forces to the female barrier (Burns et al. 2013), and in some species reinforcement of the penis with fulturae and thickened protractor muscles covaries with the dimorphic development of the male pedipalps, which are used in clasping females during mating (Burns & Shultz 2015; Burns & Tsurusaki 2016). In North American leiobunine harvesters, the female barrier and male antagonistic traits have previously been shown to covary,

suggesting mating system transition towards increasing sexual conflict. Pedipalpal dimorphism is also seen in the Japanese leiobunines *Leiobunum globosum* and *L. manubriatum*, in which females are facultatively asexual and may be experiencing a related transition towards sexual antagonism due to reproductive mode switching (Burns et al. 2017). In all species, the penis is equipped with a flexible glans stylus that is presumably used to deliver aflagellate spermatozoa (Macías-Ordóñez et al. 2010; Moya et al. 2007) to the female's spermathecae.

The internal morphology of opilionid spermathecae have not received much study except for one manuscript (de Graaf 1882) regarding the widespread, multiply mating species *Phalangium opilio* (Willemart et al. 2006), although some technical drawings have been made (Hunt & Cokendolpher 1991; Cokendolpher 1984). The spermathecae of this species are described as bilobed, open sacs posteriorly contiguous with the vagina, without apparent sclerotization of tissue. It is unclear from this description how sperm precedence is assigned, but syngamy occurs only upon oviposition in both *Phalangium opilio* and the leiobunine harvesters (Macías-Ordóñez et al. 2010; Willemart et al. 2006). Thus, we have multiple points of evidence suggesting that female harvesters may retain the capacity to control the fate of non-motile spermatozoa through physical manipulation of these cells by the spermatheca.

The purpose of our study was to evaluate the morphology of spermathecae of a number of leiobunine Opiliones, and examine the relationship between variation in spermathecal traits and other key reproductive structures associated with sexual conflict. We hypothesized that North American and Japanese leiobunines species with armaments would also have spermathecal characteristics that would allow for greater female control over sperm selection, such as thicker and/or more complex spermathecae. Females of species with antagonistic mating are more likely to encounter coerced mating (Brennan & Prum 2012), presenting selective pressure for mechanisms to control spermatozoan output via spermathecal movement. Because spermathecae are embedded in the ovipositor musculature (Fig. 1a), we theorized that thicker spermatheca would require additional force beyond the movement of ova through the oviduct to manipulate spermatozoa held within the organ (Fig. 1), and would have greater ability for spermatozoan retention. We additionally supposed that internal structures may provide females with a

means to manipulate sperm as seen in other arthropods (e.g. Lüpold et al. 2013). Complexity is a subjective measure; we operationalized our definition of complexity for the spermathecae by classifying species based on the number of internal tissue invaginations we found within the organs, which form partially separated posterior lumina.

To visualize spermathecal morphology in Opiliones, we used confocal laser scanning microscopy to image the autofluorescence signal of the spermathecal tissue within the ovipositor. The use of confocal microscopy to capture the autofluorescence of arachnid internal reproductive structures was pioneered by Chetverikov (e.g. 2014) in the study of mites, but has also been used to examine external ovipositor structure in certain Opiliones (Dreszer et al. 2015; Clouse 2012). Here we also capitalize on the intrinsic fluorescence of sclerotized reproductive structures, which removes the need for additional dye labeling. We used confocal microscopy instead of conventional fluorescence microscopy because it provides greater detail and the ability to take optical sections. Optical sectioning allowed us to image spermatheca within the ovipositor tissue to maintain the integrity of the internal structures, as well as reconstruct spermathecal morphology in three dimensions.

After examining the spermathecae of fourteen leiobunine species, we found all had sclerotized spermathecae; all were lined with a dense, dark material, visible in brightfield microscopy, that we surmised to be cuticle. Spermathecae were enmeshed in muscle fibers of the ovipositor. The majority of species examined had spermathecae with a single chamber with some cuticular invagination (Fig.2b-d), forming partially separated lumina in some species. Using multivariate phylogenetic comparative methods, we developed principal or canonical components for the species traits previously shown to covary with sexual conflict in Burns et al. (2015), and regressed these against spermathecal complexity and thickness. We found that spermathecal thickness was the primary variable associated with increasing sexual conflict. We conclude with a discussion of future methods designed to examine the connection between sperm precedence and spermathecal mechanics.

Methods

Specimen Collection and Maintenance

We acquired 52 female specimens of fourteen species of leiobunine harvesters through hand collecting or dry pitfall trapping between August 2017 and October 2019. Eleven of these species are native to North America, and the remaining three were collected in Japan. Adult specimens were preserved in 95% ethanol, and juveniles collected in 2019 were maintained singly with food and water *ad libitum* until adult moulting was complete and species identification was possible using Shultz's key for species of Maryland (2018). These specimens were subsequently preserved in 95% ethanol.

Specimen Preparation, Imaging and Analysis

Ovipositors were dissected and incubated in 1ug/ml DAPI for 24 hours to stain sperm cells, if present. Following staining, ovipositors were rinsed with 1X PBS buffer and mounted on slides using 100% glycerol. Confocal laser scanning microscopy was carried out in the UMBC Keith R. Porter Imaging Facility using a Leica SP5 or a Zeiss LSM 900. Optical sections (i.e. z-stacks) were collected in sequential scan mode using a 405 nm laser with a typical emission window of 400-562 nm for the blue channel (DAPI signal) and a 488 nm laser with a typical emission window of 562-700 nm for the green channel (spermathecal autofluorescence).

Imaris 9.5 (Oxford) was used to visualize Z-stack data for the examination of spermathecal internal complexity. We used the "Section" viewing mode that enables simultaneous transverse, coronal and sagittal cross-sections. The "Snapshot" function was used to capture representative images.. Measurements of posterior spermathecal wall thickness and width of the prosoma between coxae II and III (as a proxy of body size) were made using the line tool on select Z-stack slices in Fiji software (Schindelin et al. 2012). Average thickness was taken from three locations of the posterior wall of image slices approximating the center of the spermatheca (Fig. 1b,c), and corrected for body size by dividing by prosomal width and $\log_{10}$-transforming the result.

Phylogenetic Comparative Methods

We compiled phylogenetic trees and quantitative data associated with sexual conflict from published sources for each species (Fig. 4d). For Japanese species, we used the male variables of penis length, and thickness of the pedipalpal tibia (Burns & Tsurusaki, 2016). We studied North American leiobunine traits previously associated with sexual conflict included male body size, estimated penile flexion and protraction force, and thickness of the penial fulturae; female traits included body size and genital operculum levation force (Burns & Shultz 2015). All traits were $\log_{10}$-transformed. We conducted a phylogenetic principal components analysis (Revell 2012) of male conflict traits for Japanese species and regressed these data against a phylogenetic PCA of spermathecal traits for the three species. Because our sample size of North American taxa was larger, we ran a phylogenetic canonical correlation analysis (Revell & Harrison 2008) to assess the covariance of male and female conflict traits (Table 1) against spermathecal thickness and complexity. Phylogenetic signal was accounted for using the Pagel's lambda metric, where $\lambda = 1$ indicates trait variance is correlated to species relatedness, whereas $\lambda = 0$ means trait variance is not explained by the phylogeny (Molina-Venegas & Rodríguez 2017).

Results

Spermathecal Image Analysis

We examined the spermathecae of 52 specimens. All species had paired spermathecae; however we typically imaged and acquired measurements from only one side. Based on the relatively darker color of spermathecal tissue observed in brightfield views and the brighter autofluorescence signal of spermathecae relative to the surrounding muscle tissue in confocal images, we surmised that spermathecae were entirely sclerotized, including the external wall and internal structures.

Posterior thickness of the external spermathecal wall (Table 1) was more variable in Japanese (JPN) species (mean 8.685 ± 6.638 standard deviation um) as compared to North American (NA) species (Fig. 1b, 1c; 7.879 ± 2.948 um), but Japanese species have thicker external spermathecal walls on average after accounting for female body size (Table 1; NA: $1.963 \times 10^{-3} \pm 7.056 \times 10^{-4}$; JPN: $4.013 \times 10^{-3} \pm 2.661 \times 10^{-3}$) as well.

Spermathecal internal structure complexity was coded on a scale of 0-3, where:

0 = open pit spermatheca with no bifurcation, as in de Graaf's 1882 description of *P. opilio*

1 = spermathecae with a single bifurcation, often asymmetric

2 = spermatheca has a pair of bifurcations, forming two posterior lumina

3 = spermatheca has two pairs of bifurcations, forming three posterior lumina

Eight out of fourteen species examined had a complexity rank of 0 or 1, indicating spermathecae with no or one cuticular bifurcation (Fig. 2a, 2b; Table 1), the latter of which yields a U-shaped lumen (Fig. 2b). The remaining six species had one or two pairs of cuticular invaginations (arranged similarly to the single or double handles of a shopping bag), yielding two to three lumina (Fig. 2c, 2b; Table 1).

We observed DAPI-stained cell bodies within two specimens (*L. formosum* and *L. globosum*), which allowed us to visualize where these putative spermatozoa are stored within the spermathecal lumen (Fig. 3). Spermatozoa were ovoid in shape and flagella were not seen. However, DAPI primarily stains the cell nucleus (unlike tubulin-staining elements, which more clearly stain spermatozoan axonemes) and may not have been applied in a concentration sufficient to stain flagella (Hirono & Yoda 1997).

Comparative Analysis Results

Estimates of phylogenetic signal for spermathecal thickness and complexity in United States leiobunines were not significantly different from zero ($\lambda = 6.611 \times 10^{-5}$, log-likelihood = -16.411, p = 1), indicating variance in spermathecal morphology is not explained by shared evolutionary history. Nevertheless we

used phylogenetic multivariate measures to explore correlation of traits associated with sexual conflict and spermathecal morphology in Opiliones.

Analysis of Japanese leiobuine species indicated spermathecal complexity and mean thickness were strongly colinear, necessitating use of principal components analyses to assess cross-correlation of spermathecal and traits related to sexual conflict (Burns & Shultz 2015). The species *L. globosum* and *L. manubriatum*, sister taxa with facultatively asexual females and males with morphological traits associated with sexual antagonism, had significantly thinner, less complex spermathecae than the obligate sexual *L. hiraiwai* (Fig. 4a). This result was in spite of the fact that the male conflict traits used produced loadings of equal strength and opposite signs (pedipalpal tibia thickness = -0.998, penis length = 0. 998).

In a phylogenetic canonical correlation analysis of all analyzed traits in North American leiobunine harvesters (Fig. 4b), we found that most, but not all, species with female pre-genital barriers all had high values for spermathecal traits as well as traits associated with sexual conflict (r=0.748), although this correlation was not statistically significant based on a chi-squared test ($\chi^2 = 6.597$, p=0.581 for the 1st canonical axis). Females of species with the highest scores on the canonical axes had thicker spermatheca (CA1 y-coefficient: 0.704) and greater levator muscle force (CA1 x-coefficient: 0.588). However, these species did not always have complex spermathecae (e.g. *L. formosum* had a complexity ranking of 1, but high spermathecal and conflict scores) or female pre-genital barriers (e.g. *L. ventricosum* females have no pregenital barrier but high spermathecal and conflict scores, while *H. maculosus* has female pregenital barriers but comparatively low spermathecal scores) (Fig. 4b). The canonical loading for spermathecal thickness is likely contributed to by the largely invariant thickness seen in taxa with pregenital barriers, as the relationship between the 1st canonical axis for conflict traits and spermathecal thickness is actually significantly negative (Fig. 4c) (m = -1.158; $F_{1,4} = 9.079$, $p < 0.05$).

Discussion

We expected leiobunine species with traits associated with increasing sexual antagonism, such as female pre-genital barrier presence, thicker male pedipalps, and larger estimated genital muscle forces, to additionally have thicker and/or more complex spermathecae. For both leiobunines of Japan and North America, this hypothesis was not supported. Significantly, we found that spermathecae in all species were sclerotized and cuticular, which may limit the female's control over the organ. Our consequent interpretation of the mechanism of the spermathecae in the leiobunine Opiliones will necessarily change with additional data, which we discuss in this section.

Japanese parthenogens: antagonistic traits and simple spermathecae

For the Japanese facultative parthenogens, we had data on conflict traits solely for males. Two of the three species examined have facultatively parthenogenetic females, and past publications have focused on the antagonistic morphologies found in rare males of these species (Burns & Tsurusaki 2016). Subsequent papers have discussed the uniquely manifest nature of sexual conflict in facultatively parthenogenetic systems (Burke & Bonduriansky 2018; Gerber & Kokko 2016), where we might otherwise expect relaxed selection on males (Jalinsky et al. 2019; Schwander et al. 2013) given the biases towards females in populations (Burns et al. 2017). While males of facultatively parthenogenetic species have larger pedipalps than expected given their body size (Burns & Tsurusaki 2016), females of these species did not have significantly larger or more complex spermatheca than a closely related sexual species.

We discovered one specimen of the facultative parthenogen, *L. globosum*, had spermatheca that were shown to contain spermatozoa (as in Fig. 3). This is a novel result, because *L. globosum* males are exceedingly rare and absent in most populations (Burns et al. 2017; Tsurusaki 1986). Moreover, flow cytometrics support *L. globosum* males and females to be entirely tetraploid (Burns et al. 2017), and gynandromorphs of the species have been collected (Tsurusaki 2001). That spermatozoa were found in the spemathecae of one specimen does not ultimately discount the possibility of hybrid mating, but suggests that sexual behavior in the species is still maintained, even though male density in most populations is very low.

North American leiobunines: What drives spermathecal complexity?

Past work on sexual conflict in North American leiobunines indicated that the variance of some morphological traits (e.g. body size) was strongly predicted by phylogeny. This was not shown to be the case with spermathecal traits, which had near zero measures of phylogenetic lambda. For example, some closely related species (Burns et al. 2012), which we would expect to have similar values of complexity if spermathecal morphology was evolutionarily conserved, had extreme differences in complexity (e.g. *L. calcar* and *L. euserratipalpe*; Table 1). Many of our species sample sizes were small, and we lack sufficient methods to incorporate intraspecific trait variance into our multivariate phylogenetic comparative methods. It is possible that improved sampling would reveal intraspecific variability that would cause our measures of spermathecae to overlap among closely related species.

We hypothesized that thicker spermathecae would be beneficial to females in species where mating antagonism is common, signaled by the presence of the female pregenital barrier. This is because the deformation of the cuticle necessary for movement of non-motile spermatozoa within would be more difficult, potentially providing females with an additional mechanism for exerting mate preference. We found that some North American species with thicker spermathecae also had pregenital barriers (Fig. 4b), but this was not a consistent effect, as spermathecal thickness was not particularly variable for barrier-present species (Fig. 4c, open circles). An alternative interpretation for this finding is that there may be an upper limitation to spermathecal thickness, controlled by the volume of the spermathecal lumen, or the necessary flexibility of the cuticle, that mediates evolutionary increases in spermathecal wall thickness. Spermathecal sclerotization may respond to dual selective pressures for control and functionality, suggesting a more complex relationship between spermathecae and mating system than may be depicted in the linear regression of Fig. 4c. Moreover, although spermathecae are embedded within the vermiform musculature of the ovipositor (Macías-Ordóñez et al. 2010), we are unaware of potential species-level differences in muscle covering the spermathecal cuticle.

Spermathecae with more complexity (e.g. valvular, fully separated chambers, multiple openings, etc.) were hypothesized to be more common in species with frequent mating antagonism, because of the adaptive advantages to females conferred through the ability to selective contain and maintain spermatozoa of certain mates. In species with intense sexual conflict, we expect a general loss of female choice (Brennan & Prum 2012), although the two mechanisms of sexual selection are not necessarily mutually exclusive (Kokko & Jennions 2014). Although we found significant internal diversity in spermathecal morphology, no species had fully differentiated spermathecal lumen to be classified as chambers. Moreover, though many species had cuticular invaginations that partially separated (frontal sections, Figs. 2b,c,d) the single lumen, we did not observe multiple openings to these spermathecae (e.g. Figs 3a,b).

Although the first canonical axes we computed for North American spermathecal traits and traits associated with sexual conflict explained nearly 75% of the variance in these data categories, this model was not statistically significant. Through this result we acknowledge that the adaptive benefits of selective control over spermatozoa via the spermathecae would not necessarily be limited to females of species with rampant sexual antagonism. Such a mechanism for selective sperm containment would be massively advantageous for female fitness (Eberhard 1996), and would be difficult to be overcome by male morphology. Perhaps such a mechanism would drive the evolution of manipulative ejaculates, as seen in *Drosophila melanogaster* (Sirot et al. 2011).

If the complexity observed in leiobunine harvester spermatheca does not contribute to female control over spermatozoan fate, does it have an adaptive function? In this paper we have outlined a method for imaging of internal morphology, and we have successfully identified spermatozoa within spermathecae (Fig. 3), although we cannot be sure how many matings this result constitutes. Ongoing work will use these methods to systematically observe packing of spermatozoa within spermathecae, by mating virgin females and preserving their genitalia for imaging, potentially through the use of live stains for multiple mates. This would allow for visualization of gamete storage for a known number of reproductive events, assessment of the longevity of stored spermatozoa, and insight into how sperm might

be utilized passively. We observed that most species with cuticular invaginations in their spermathecal lumen had U-shaped cross-sections, suggesting that without any active mechanism, spermatozoa from the first and last matings would be most likely to achieve syngamy. With the number of high-throughput genotyping mechanisms we have developed and employed for Japanese leiobunines (Brown et al. in review), this hypothesis may be easily tested.

Acknowledgements

Funding for this project was facilitated through the NIH-UMBC BUILD program, a UMBC START grant to MB, and a UMBC SURE grant to ZK. Collection assistance was provided by many persons, including Michael Mercado, Rahaf Alhabashi, Harper Montgomery, Tyler Brown, Sarah Stellwagen, James Barklage, and Nobuo Tsurusaki. Ryan Gunnison and Shea Walsh assisted with live specimen maintenance. We furthermore thank two anonymous reviewers for constructive comments provided in revision of the manuscript.

Data Availability

All composite images and R-scripts for analysis available upon request from burnsm@umbc.edu.

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Figure captions

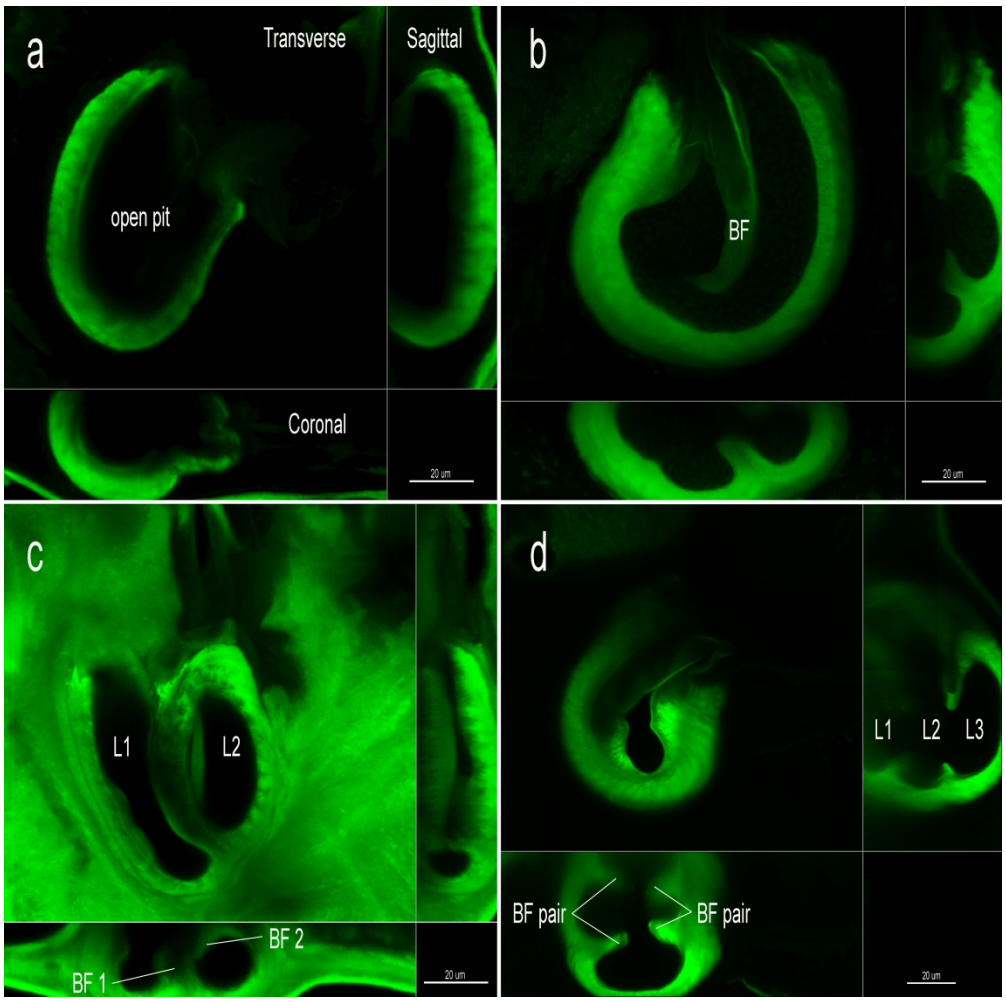
Figure 1. Representative images of an Opiliones ovipositor and spermathecae on opposing ends of the spermathecal external wall thickness scale. a) Maximum intensity projection of distal *H. maculosus* ovipositor showing location of paired spermathecae, vaginal opening, and sensilla; box around left spermatheca corresponds to high magnification image (b). b) Single transverse optical section of *H. maculosus* spermatheca that represents a relatively thin spermathecal wall, posterior thickness measurements represented with dashed lines. c) Single transverse optical section of *L. formosum* spermatheca that represents a relatively thick spermathecal wall, posterior thickness measurements represented with dashed lines. Anterior facing up, scale bar = 15 um.

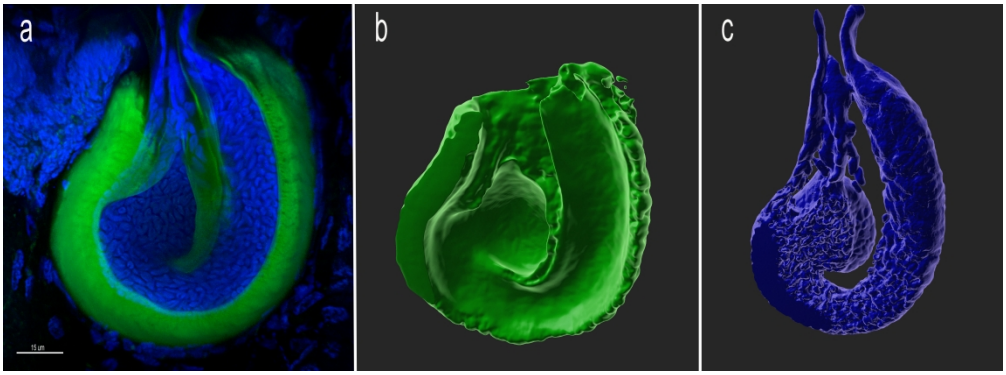
Figure 2. Representative images of spermatheca along the internal complexity scale, with lumina (L) and bifurcations (BF) labeled for complexity scale classification. Five um thick transverse, sagittal and coronal sections of species that represent levels 0, 1, 2, and 3 of spermathecal complexity, respectively a)

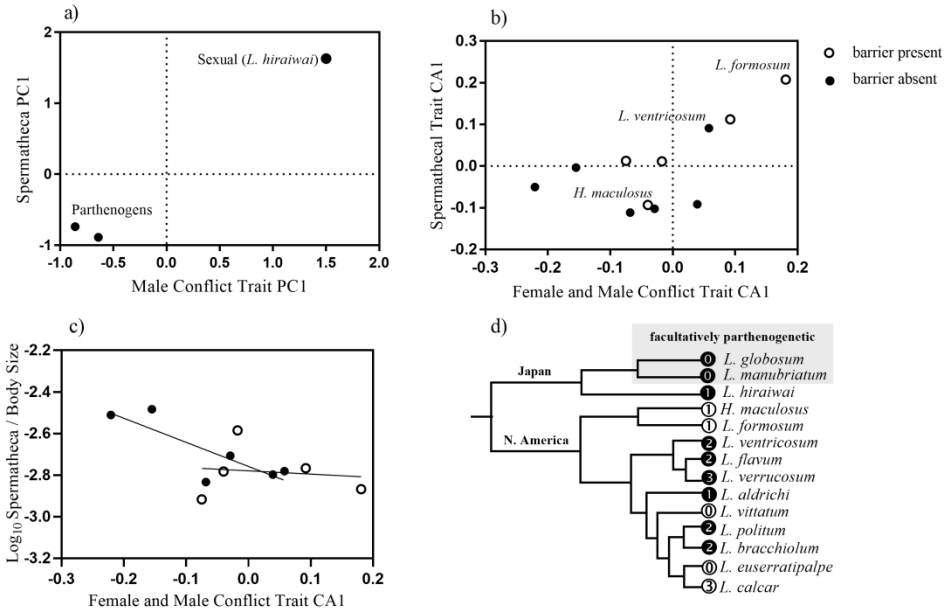
L. euserratipalpe; b) *L. formosum*; c) *L. bracchiolum;* d) *L. verrucosum.* Anterior facing up, scale bars = 20 um.

Figure 3. DAPI-staining reveals the location of sperm within a *L. formosum* spermatheca. a) Maximum intensity projection of select z-stack slices showing sperm (DAPI/blue channel) inside of spermatheca (autofluorescence/green channel); b) Surface rendering of green channel with dorsal slices showing the transverse spermathecal lumen; c) Surface rendering of blue channel showing the location of sperm cells throughout the spermatheca. Anterior facing up, scale bar= 20 um.

Figure 4. Graphs of (a) spermathecal trait phylogenetic principal component 1 regressed against male conflict trait phylogenetic principal component 1 in Japanese species; (b) Phylogenetic canonical correlation axes of spermathecal traits regressed against male and female conflict traits in North American species; (c) Regression of log-transformed, size-corrected spermathecal thickness by conflict canonical axis 1, separated for species with and without female pregenital barrier. In parts (b) and (c), open circles indicate species where females have pre-genital chamber barriers, and closed circles indicate species without female pre-genital barriers. d) Simplified phylogeny of study species after Burns & Tsurusaki (2016), Burns & Shultz (2015), and Hedin et al. (2012). Facultatively parthenogenetic taxa are indicated within shaded box. Numbering within tip labels signifies our spermathecal complexity ranking for the species, while open circle labeling indicates species where females have pre-genital chamber barriers, and closed circles indicate species without female pre-genital barriers.







240x156mm (300 x 300 DPI)

Table 1. Species examined, collection locations, and data acquired from spermathecal images. Complexity was defined on a discrete scale from 0-3, where a '0' indicated spermathecae examined had no bifurcation, while a '3' had two pairs of bifurcations, forming three posterior pits.

Species	**Collection Location**	**Prosomal width (mm)**	**Mean Spermathecal Wall Thickness (um)**	**Spermatheca Internal Complexity**	**Size-corrected Spermathecal Wall Thickness (um)**
United States					
Hadrobunus maculatus (N = 7)	MD: Baltimore County, UMBC Campus	4.747	5.755	1	0.00121
Leiobunum aldrichi (N = 7)	MD: Baltimore County, UMBC Campus	2.705	3.676	1	0.00136
L. bracchiolum (N = 3)	MD: Baltimore County, UMBC	2.673	5.258	2	0.00197

	Campus				
L. calcar (N = 2)	MD: Baltimore County, UMBC Campus	3.701	11.422	3	0.00309
L. euserratipalpe (N = 2)	MD: Baltimore County, UMBC Campus	5.025	8.296	0	0.00165
L. flavum (N = 4)	AR: Garland, Scott, and Yell Counties, Ouachita Nat'l Forest	4.348	6.387	2	0.00147
L. formosum (N = 1)	MD: Baltimore County, UMBC Campus	3.843	12.628	1	0.00329

L. politum (N = 1)	MD: Baltimore County, UMBC Campus	3.949	6.765	2	0.00171
L. ventricosum (N = 1)	VA: Rockbridge County	4.625	12.027	2	0.0026
L. verrucosum (N = 4)	MD: Baltimore County, UMBC Campus	4.991	7.961	3	0.0016
L. vittatum (N = 8)	MD: Baltimore County, UMBC Campus	3.921	6.497	0	0.00166
Japan					
L. globosum (N = 5)	Aomori Prefecture, Asamushi	2.021	5.341	0	0.00264

	Forest Park				
L. hiraiwai (N = 1)	Nagano Prefecture, Togakushi	2.306	16.329	1	0.00708
L. manubriatum (N = 6)	Saitama Prefecture, Mt. Mitsumine	1.891	4.384	0	0.00232