Functional aspects of genital differences in *Leucauge argyra* and *L. mariana* (Araneae: Tetragnathidae)

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Abstract.   Morphological studies have documented the tendency for male genitalia to diverge rapidly compared to other body parts in many animal groups, including spiders. But documentation of how differences in genital structures of closely related species correlate with differences in the behavior of their genitalia during copulation is rare. This study describes how the genitalia of the spider *Leucauge argyra* (Walckenaer 1841), a species in which both male and female have unusual derived structures, are used during copulation and compares their sexual behavior with previous descriptions of genital behavior in the congener *L. mariana* (Taczanowski 1881) and the genital morphology of other *Leucauge* species. Males of *L. argyra* have two prominent derived genital structures, both of which interact directly with the female; one of them apparently locks against a modified female structure, while the other is inserted into the female atrium. On the other hand, the most prominent derived female structure does not lock against or receive any male structure and may serve to sense movements of the male palp, perhaps to trigger deposition of a strong copulatory plug by the female. The female atrium is unusual in that it receives insertions of both the male's conductor and his cymbial hook. Both derived male structures of *L. argyra* may have evolved to stabilize the male's genitalia during intromission, perhaps in response to violent and dangerous female resistance or to perforate the strong plug that is probably produced or at least moved into place by the female. The rotating and projecting movements executed by male genitalia in *L. argyra*, which as in other spiders are presumably produced by the hydraulic unfolding of complex membranes in the palp, are quite different from the movements of the male genitalia of *L. mariana*. We speculate that in spiders in general, changes in palpal sclerites are often accompanied by changes in the movements of the sclerites, and thus by changes in the unstudied internal membranes of the palp.

Keywords: Copulatory plugs, genital movements, genital evolution

Animal genitalia, especially those of males, frequently show especially rapid divergent evolution compared with other body parts, and they often present relatively complex morphologies (Tuxen 1970; Eberhard 1985; Leonard & Córdoba 2010). Despite the abundant documentation of these two morphological patterns in the taxonomic literature of many groups of animals, much less is known about how the rapidly diverging structures of males and females behave during copulation and the evolutionary origins of the diversity. Web-building spiders are a rewarding group in which to study genital behavior, because they can often be induced to copulate with their ventral sides upward under a dissecting microscope, where their genitalia and their movements are easily visible (Eberhard 2004). In addition, most male structures remain outside the female genitalia during mating, where their movements and the coupling mechanisms can be observed.

Although the male and female genitalia of spiders in the tetragnathid genus *Leucauge* White 1841 are not particularly complex compared with those of many other areneoids, they have nevertheless diverged relatively rapidly compared to other structures, as testified by the fact that they are often diagnostic for distinguishing related species (Hormiga et al. 1995; Levi 1980, 2007, 2008; Tso & Tanikawa 2000; Yoshida 2009; Álvarez-Padilla & Hormiga 2011). Previous studies (Eberhard & Huber 1998; Méndez 2004; Aisenberg 2009; Aisenberg & Eberhard 2009) described the movements and physical interactions between the male and female genitalia of *L. mariana* (Taczanowski 1881) during copulation, and how copulatory plugs are deposited and removed (for a general review of copulatory plugs, see Uhl 2010). The present study describes similar details in a second species, *L. argyra* (Walckenaer 1841), which differs strikingly in both male and female genital morphology (Levi 2008; Álvarez-Padilla & Hormiga 2011). We will show that, contrary to expectations, some apparently derived features of the male and female genitalia in *L. argyra* do not interact directly with each other during copulation, raising interesting questions regarding their functions and how they evolved.

METHODS

Field samples.—We collected *L. argyra* from September through November 2009 in plantations of African oil palm (*Elaeis guineensis*) in Parrita, Puntarenas Province, Costa Rica (09°30′N, 84°10′W; elevation 10 m), and observed them at the Escuela de Biología, Universidad de Costa Rica, San José Province, Costa Rica (9°54′ N, 84°03′ W; elevation 1200 m). We observed and photographed each adult female under a Wild model M3Z dissecting microscope (Wild, New York, USA) to check for the presence of copulatory plugs on the epigynum. Cephalothorax lengths were measured on specimens in ethyl alcohol. We photographed the genitalia of *L. argyra*, as well as those of *L. mariana* (collected near San Antonio de Escazú, Costa Rica), and *L. venusta* (Walckenaer 1841) (collected near Baton Rouge, Louisiana, USA.) with a Hitachi Model S-570 scanning electron microscope (SEM).

Copulatory plugs and spermathecae.—We removed thirty six copulatory plugs from *L. argyra* epigyna using a sharp thin needle and mounted each one on a microscope slide. We stained them with acetocarmine, which stains DNA red but does not stain the plug matrix, to check for sperm. We photographed the preparations under a Leica DME light microscope.

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To check sperm stores in adult females captured in the wild, we dissected the epigyna of 31 adult females with a copulatory plug and 32 females without a copulatory plug. Dissections were performed one to five days after the spiders were collected. We removed both spermathecae of each female and mounted them on a microscope slide in a drop of saline solution. Then we placed a cover slip on top and pressed, causing the sperm to emerge from the thin-walled spermathecal chamber I (Quesada & Triana unpubl.). Sperm were active in the saline solution, facilitating detection of both active and inactive cells.

Genitalia and sexual coupling.—We flash-froze two mating pairs of *L. argyra* with ethyl chloride during palpal insertion. The male genitalia did not remain coupled to the female and were preserved in ethyl alcohol; the basal hematodocha collapsed somewhat, but we were nevertheless able to determine the approximate positions of palpal sclerites during copulation. We obtained additional details by clearing two palps in 10% KOH, by dissecting two others, and by making plasticine models of genital structures. We also made video recordings of 12 matings using a SONY DCR TRV50 digital video camera (SONY, San Diego, California, USA) equipped with +4 close-up lenses, and of two additional pairs under a dissecting microscope in which the camera recorded through the ocular and was focused on the epigynum in posterior and slightly ventral view (the view varied somewhat when the animals moved slightly). Specimens were prepared for viewing with SEM using standard procedures.

Data are presented as median ± quartile when we used non-parametric tests and mean ± SD when we used parametric tests. The statistical analyses were performed with Past Palaeontological Statistics, version 1.18 (Hammer et al. 2003), NCSS 2001 (Copyright 2000 Jerry Hintze). Descriptions of genital behavior use the female's body as reference; thus, a ''medial'' movement of the male palp refers to its orientation with respect to the female's rather than with respect to the male's body. Voucher specimens were deposited in the Museo de Zoología of the Escuela de Biología in the Universidad de Costa Rica.

RESULTS

Field samples.—We captured a total of 210 adult females and 98 males of *L. argyra*. Five females laid an egg sac during transportation from the field to the laboratory. Two of these five females had a copulatory plug in the epigynum. Of the other 205 adult females, 113 (55.1%) had copulatory plugs (Fig. 1A). Twenty-four of these plugs (21%) were drawn into one or more thin threads (Fig. 1B): these are indicative of male pedipalp adhesion to the newly formed plug, coinciding with a previous report on captive specimens (Aisenberg & Barrantes 2011).

Copulatory plugs and spermathecae.—Of the 36 copulatory plugs we stained, 12 lacked sperm and were formed exclusively by a matrix of unknown composition, eight consisted mainly of matrix (ca. 95%) with very low numbers of decapsulated sperm, and 16 consisted mainly of a matrix (ca. 95%) that contained low numbers of both encapsulated and decapsulated sperm (Fig. 2). Of the 63 females collected in the field and checked for sperm in their spermathecae, all of the 31 females with copulatory plugs had sperm, and 75% of the 32 females
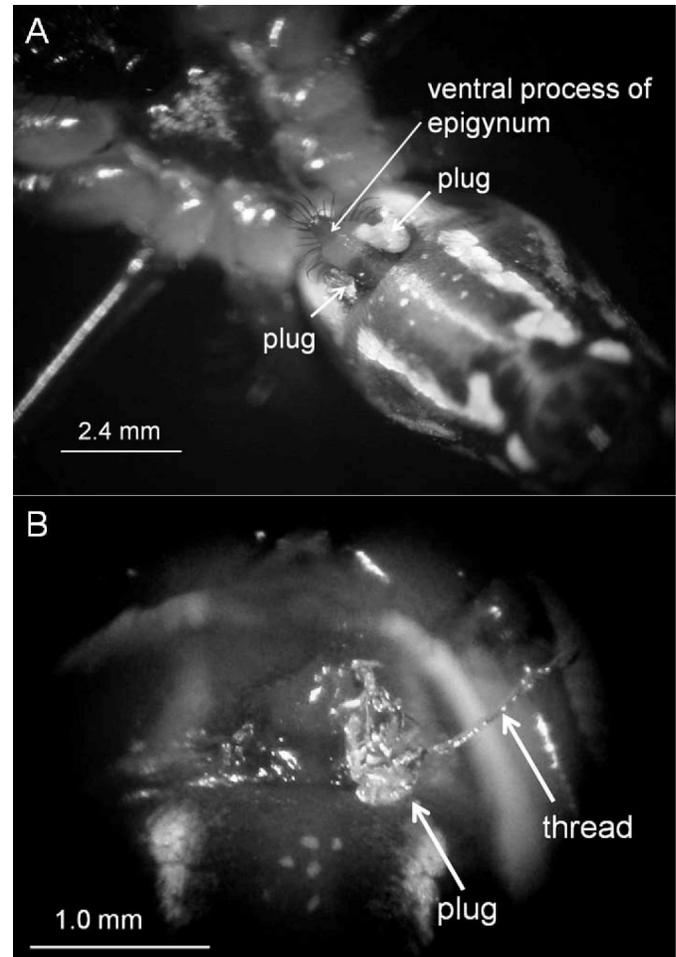

Figure 1.—A) Posterior-ventral view of the ventral epigynal process and its long setae of a mated female, with copulatory plugs of different sizes (arrows) covering the two atria; B) a copulatory plug with a long thread on the left opening of an insemination duct. Such threads are formed when the cymbial hook of the male's palp becomes stuck to the plug material and the male pulls his palp away (ventral-posterior view).

without a copulatory plug had sperm. In all cases, the sperm were abundant (probably hundreds or thousands).

Genital morphology.—One of the most pronounced differences between the male genitalia of *L. argyra* and those of other *Leucauge* species is the large, dorsally directed hook on the antero-dorsal margin of the cymbium (Levi 2008; Figs. 3, 4A) (hereafter the ''cymbial hook''; this is the ''huge macroseta'' mentioned by Álvarez-Padilla & Hormiga 2011 for this species). The hook is apparently a modified seta, as it has an apparent socket at its base (Fig. 4B), and it also broke off easily as a unit in specimens preserved in ethyl alcohol. Its distal exterior surface is covered with many small, distally directed teeth (Figs. 4C, 5A). No aperture was visible near the tip of the hook (Figs. 4A, 5C), nor in the specimen figured by Álvarez-Padilla & Hormiga (2011). There is also a smaller, tooth-like process on the margin of the cymbium (hereafter the ''cymbial tooth'') with a small indentation near its base (Fig. 4C; this is the ''cymbial dorso-basal process'' figured for *L. argyra* by Álvarez-Padilla & Hormiga 2011). Because we
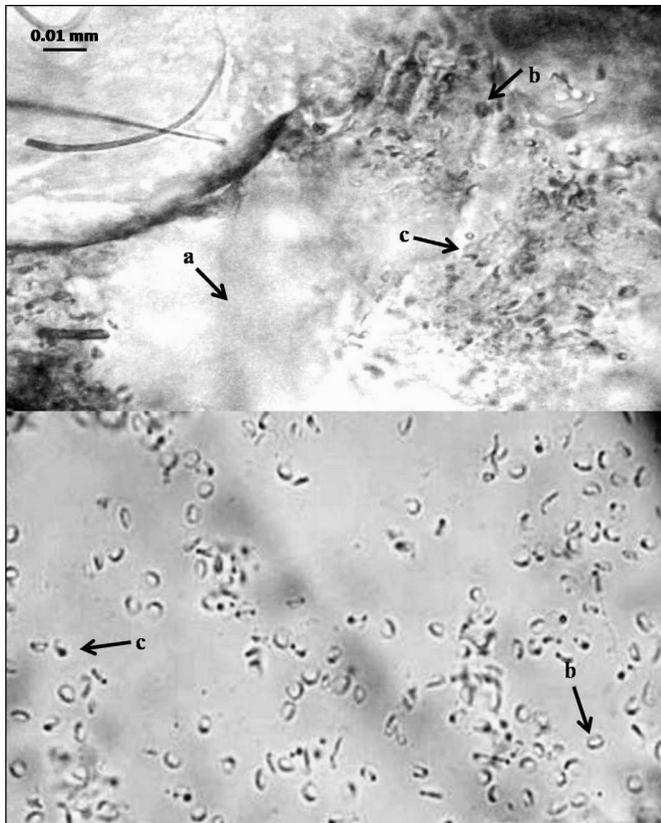
Figure 2.—Above. Copulatory plug stained with acetocarmine, indicating a) the matrix material, b) encapsulated sperm and c) decapsulated sperm; below: sperm that has emerged in saline solution from the spermathecal chamber I of a field-collected female, showing encapsulated sperm and decapsulated sperm.
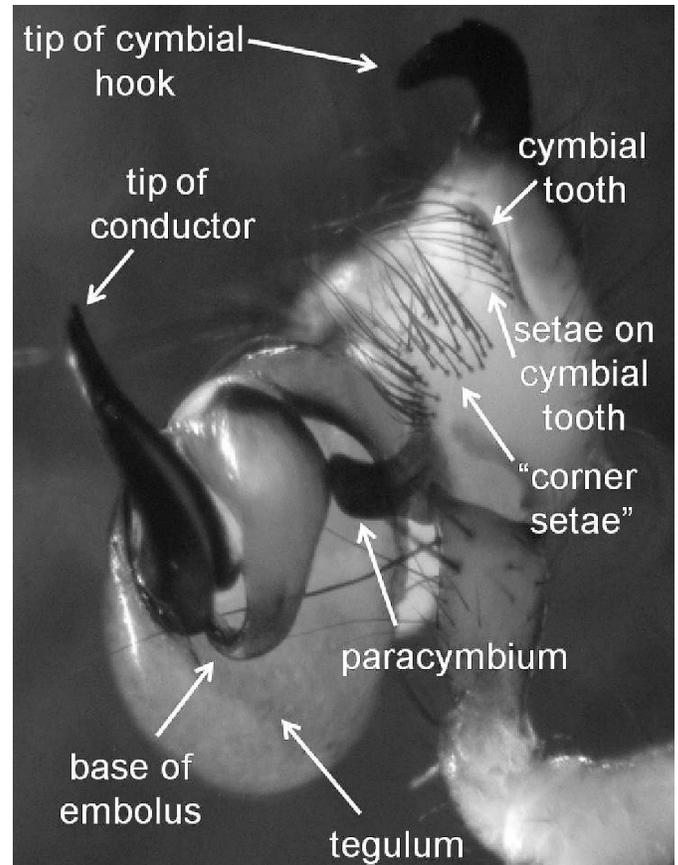

Figure 3.—A photograph of an expanded pedipalp as it would be seen in latero-anterior view during copulation. The relative positions of the conductor and the cymbial hook are somewhat more natural than those in Fig. 4A, as the specimen had not been dehydrated.

are uncertain of homologies, we use only descriptive names here. The outer surface of the tooth bears approximately six long setae in an approximate row (hereafter "tooth setae") (Fig. 4). There is another area with a concentration of long setae at the basal corner of the approximately triangular cymbium (hereafter "corner setae") (Fig. 3).

The most distinctive trait of the epyginum of *L. argyra* is the large conical ventral process of the central posterior portion of the epigynum (hereafter the "ventral process"). It is provided with dense, long setae on its anterior surface, especially near its tip and around its base, but is naked on its posterior surface (Figs. 1A, 6). A second, much less conspicuous feature is a small ridge along the lateral and antero-lateral margin of the epigynum (Fig. 6B) (hereafter the "epigynal ridge"). The atrium is located on the base of the ventral process, just posterior to the anterior (setose) surface (Fig. 6). The opening of the insemination duct is on the medial side of the atrium, and the duct is directed more or less medially.

Sexual coupling and genitalia.—As in *L. mariana* (and other tetragnathids – see Crome 1954; Huber & Singlet 1997; Álvarez-Padilla & Hormiga 2011), the female faced the male and grasped his sexually dimorphic chelicerae with hers just prior to genitalic coupling, following an exchange of courtship vibrations (Aisenberg 2009; Aisenberg & Eberhard 2009). Cheliceral coupling occurred after the female spread her chelicerae and extended her fangs; the male then inserted the distal portion of the basal segment of each of his chelicerae into the space between the female's open fang and her basal cheliceral segment. The female then closed her fangs to clamp the male, and he immediately extended one pedipalp anteriorly toward her genitalia and attempted palpal insertion.

Genital coupling consisted of two stages – insertion of the cymbial hook into the ipsilateral atrium, followed by insertion of the conductor and embolus into the other, contralateral atrium. At the beginning of the first stage, the male extended his palp toward the female's epigynum with its distal portion rotated medially about 90° so that his cymbial hook was directed toward her epigynum (Fig. 7). The male moved the entire bulb laterally (e.g., left and right) back and forth across the epigynum from one to three times in this rotated position, apparently searching to contact the epigynum with the cymbial hook. The basal haematodocha was not perceptibly inflated at this stage, and the palp moved as a unit. On one occasion a favorable viewing angle allowed us to see that the cymbial hook snagged briefly on the ventral process of the epigynum, with its tip on the posterior surface of the process. On the next pass, the bulb contacted the epigynum, and the cymbial hook contacted the atrium of the epigynum; the male immediately re-positioned the hook slightly as it penetrated deeper into the ipsi-lateral atrium. As soon as the cymbial hook was inserted in the atrium, the basal haematodocha
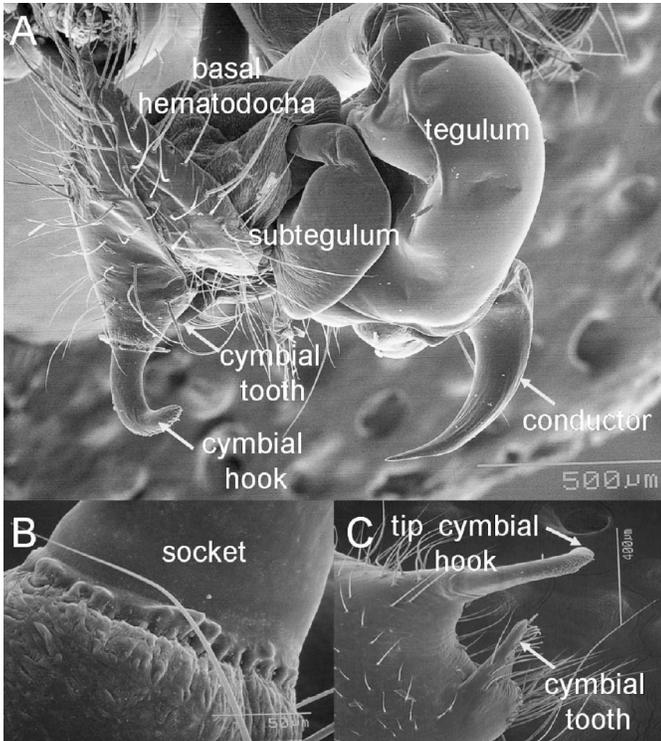
Figure 4.—SEM photos of male palps. A) A partially expanded palp seen as it would be in a posterior view of a copulating pair, illustrating the tong-like positions of the conductor and the cymbial hook. The basal hematodocha is partially collapsed, and the cymbial hook has twisted somewhat toward the viewer. B) Close up view of the base of the cymbial hook. C) Close up view of cymbial tooth showing its setae and also the teeth on the tip of the cymbial hook.

inflated rapidly. This expansion produced a ventromedial rotation of the tegulum, the conductor and the embolus away from the cymbium. This brought the medially directed, curved conductor into the contralateral atrium of the epigynum (Fig. 7). Thus the tips of the cymbial hook and the conductor pressed in approximately opposite directions, each into an atrium (Figs. 4A, 7), with each tip directed medially and slightly posteriorly. Although the tips of the conductor and the cymbial hook were not perfectly opposed, the overall mechanical effect was to pinch the female's epigynum as if with a pair of tongs. The cymbial tooth and the small

indentation at its base were not visible in the posterior views in our video recordings, so it was not possible to observe their mesh with the female directly. Nevertheless, the recordings, SEM photographs that provided approximate scales, and manipulation of plasticine models allowed some deductions. The tooth and the indentation did not mesh with sides of the ventral process (confirmed by direct observation in the videos). They also did not mesh with the rim of the atrium, because the indentation was too far from the cymbial hook and the rim was too close (in addition, the orientation of the tooth was inappropriate – it was nearly parallel to the rim, rather than perpendicular to it). One further possibility was that the tooth and the indentation hooked onto the epigynal ridge when the cymbial hook was inserted into the atrium (Fig. 7). This area was never directly visible in recordings that provided sufficient magnification (two made through the dissecting microscope); but manipulation of the models showed that if the cymbial hook was inserted deeply and the insemination duct was directed slightly anteriorly, the tooth and its indentation would have been positioned exactly over the epigynal ridge. One further detail favoring this hypothesis was that in this position the setae on the cymbial tooth would have been directed toward the female epigynum; they would thus have been in position to function, allowing the male to sense the presence of the ridge and thus orient his palp.

Rhythmic inflation of the haematodochae and palpal sclerite movements.—Once the tip of the conductor was inserted into the atrium, the palp executed a stylized sequence of movements (0.97 ± 0.11 s, $n = 11$) that rhythmically withdrew and then reinserted the cymbial hook into the atrium. The sequence began with a partial collapse of the basal haematodocha. As the haematodocha gradually collapsed, the edge of the cymbium was displaced medially toward the tegulum, and the cymbial hook was lifted out of the epigynal atrium (Fig. 7B). This movement brought the medial edge of the cymbium near or sometimes slightly past the middle of the female's ventral process (Fig. 7B). At the same time, the tegulum moved slightly toward the cymbium (the movement of the tegulum was much smaller than that of the cymbium). Toward the end of the collapse, the embolus base moved away from the atrium over about 0.39 ± 0.11 s ($n = 11$) (visible only with certain viewing angles and indicated by the arrows in Fig. 7), indicating that the tip of the embolus was retracted gradually. The tip of the conductor remained inside the atrium.
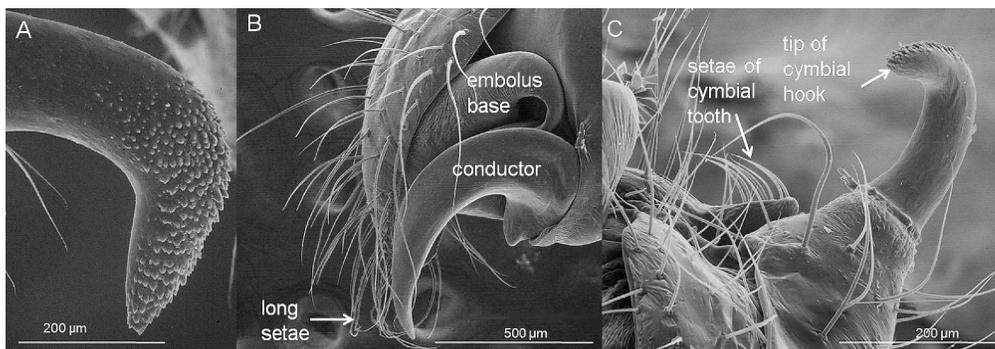

Figure 5.—The pedipalp of a male *L. argyra*. A) Closeup of the teeth on the tip of the cymbial hook; B) long setae (arrow) close to the tip of the conductor in an unexpanded palp; C) long setae on the cymbial tooth.
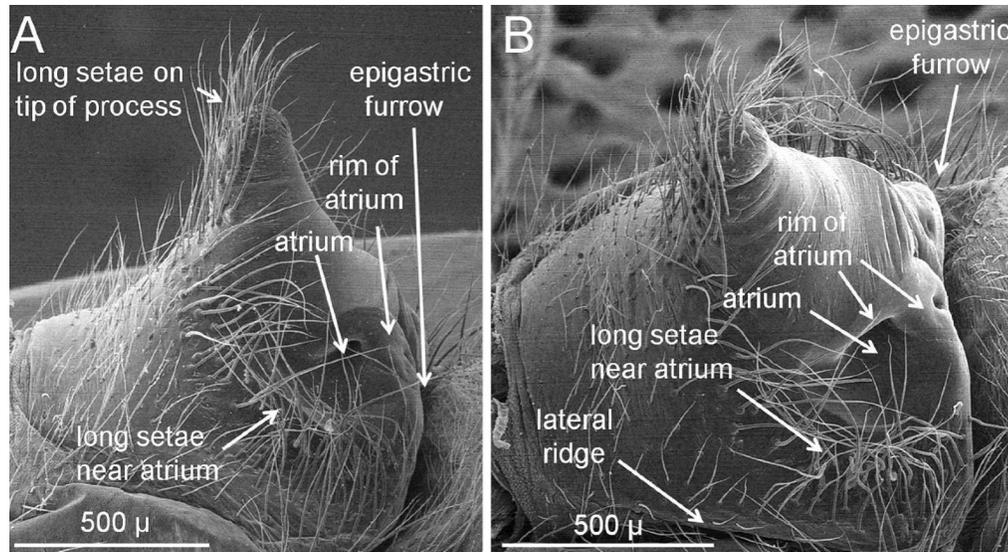
Figure 6.—Epigynum of *L. argyra*. A) in lateral view; and B) in latero-ventral view, showing the wide atrium and long setae anterior and lateral to the atrium.

The second portion of the sequence began with reinflation of the basal haematodocha. The inflation was more abrupt (lasting a mean of 0.28 ± 0.05 s, $n = 11$) than the collapse (lasting a mean of 0.64 ± 0.10 s, n = 11). Inflation reversed the movements just described. Approximately 0.20 s (± 0.02, $n = 11$) after expansion began, the cymbial hook moved rapidly back into the epignal atrium (and small cymbial tooth and its accompanying indentation may have hooked on the epignal ridge). In some cases, as much as the distal half of the cymbial hook disappeared into the atrium. At the same time the tegulum moved slightly away from the cymbium. After the haematodocha had begun to reinflate, the embolus began to move into the insemination duct 0.034 ± 0.009 s ($n = 11$), basically at the same time that the hook began to descend to the atrium; it took 0.15 ± 0.02 s ($n = 11$) for the base of the embolus to disappear out of sight (Fig. 7C).

It is very likely that the movements of the cymbium bring it or its setae (and perhaps also the tegulum) into contact with at least one set of the especially long setae that are associated with the ventral process of the epigynum – those at its base, near the atrium. The in-and-out movements of the cymbial hook must inevitably deflect setae at the edge of the atrium (Fig. 6). The area of the cymbium near the base of the cymbial hook likely deflected setae on the anterior surface of the ventral process. The especially dense group of long, ventrally directed setae near the tip of the ventral process (Fig. 6A) was not clearly contacted by the male palp, however, though it is possible that they were touched by setae on the cymbium.

Comparisons of sexual behavior and copulatory plugs between *L. argyra* and *L. mariana*.—Data on several aspects of the sexual behavior of *L. argyra* can be compared with those of *L. mariana* (data on *L. mariana* are taken from Aisenberg 2009; Aisenberg & Eberhard 2009). The males of *L. argyra* are relatively larger (relative to conspecific females) than are those of *L. mariana*. Comparing the degree of sexual dimorphism in cephalothorax length (male/female), the respective medians and quartiles were 0.98 ± 0.16 (0.59–1.25), $n = 26$) for *L. argyra*; and 0.87 ± 0.13 (0.61–1.17), $n = 43$ for *L. mariana*

(Student t Test: $t = 3.09$, $df = 67$, $P = 0.003$). The relatively larger size of *L. argyra* males may be related to the greater danger that females represent for males in this species, in which the frequency of sexual cannibalism was greater (11.1% of 45 pairs in *L. argyra*, 0% of 62 in *L. mariana* ($\chi^2 = 7.23$, $df = 1$, $P = 0.007$). Vigorous struggles associated with copulations, in which the female appeared to try to grasp the male and the male appeared to try to escape, but which did not end in cannibalism, were also more common in *L. argyra* (10 out of 12 copulations with previous virgins and five out of five copulations with mated females); no such struggles occurred in 43 pairs of *L. mariana* with virgin females ($\chi^2 = 43.8$, $df = 1$, $P = 0.0001$), or in 18 pairs of *L. mariana* with non-virgin females ($\chi^2 = 23.0$, $df = 1$, $P = 0.0001$). These data are all from matings in captivity, but we also saw attacks on males by female *L. argyra* in the field.

The overall frequency of intromission attempts that failed ("flubs") was lower in *L. argyra* than in *L. mariana* ($U = 63$, $n_1 = 11$, $n_2 = 43$, $P = 0.0002$). However, in *L. mariana* flubs were less frequent with long than short intromissions, and *L. argyra* performed only long intromissions (the mean number of long, cymbial insertions per mating in 12 copulations was 2.54 ± 1.13, while the number of hematodochal inflations per insertion was 83.3 ± 56.9), so this comparison may not be appropriate. When we took this difference into account by creating an index of the number of flubs/the number of insertions for each mating, and using only long insertions in *L. mariana*, the respective means of the indices were still statistically different (0.52 ± 0.69 in *L. argyra* and 5.4 ± 7.2 in *L. mariana*; $U = 70.5$, $n_1 = 43$, $n_2 = 12$, $P = 0.0004$). In fact, the flubs of *L. argyra* were limited exclusively to the preliminary attempts to first insert the cymbial hook into the atrium; once this engagement occurred, the insertion attempts with the conductor that followed, resulting from hematodochal expansion (i.e., the movements homologous to insertion attempts in *L. mariana*) never failed.

Copulatory plugs are formed during copulation or in the following hours in both *L. argyra* and *L. mariana* (75% of 12
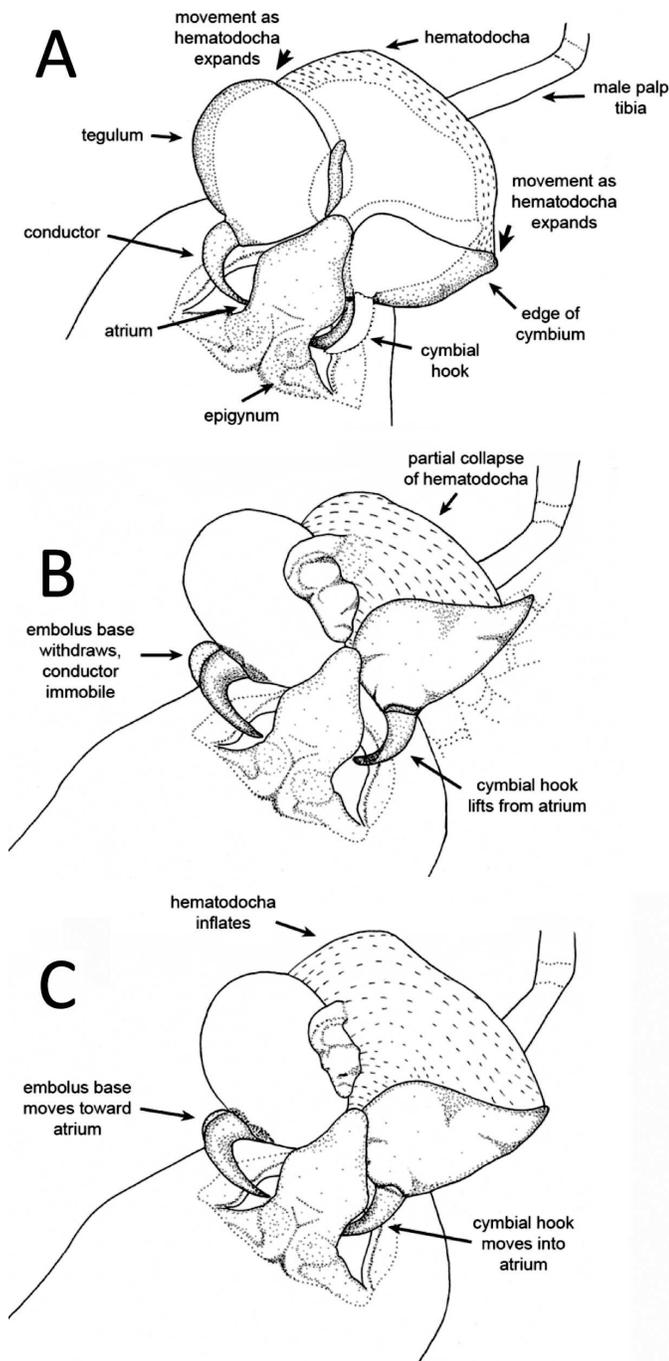
Figure 7.—Partially schematic posterior-ventral view of female and male genitalia during palpal insertion (A) and movements of the sclerites of the left male bulb during collapse and inflation of the haematodochae (B) and (C). The dotted lines in (A) indicate positions when the hematodocha is collapsed.

first copulations for the female in *L. argyra*, 44.2% of 43 in *L. mariana*) (χ^2 = 3.56, df = 1, P = 0.06). In both species the plug is sometimes but not always effective in preventing intromission by a second male (71% of 7 cases in *L. argyra*, 53% of 19 in *L. mariana* (χ^2 = 0.19, df = 1, P = 0.46). Testing the consistency of plugs of field-collected females by probing them with a fine needle indicated that those of *L. argyra* are harder and adhere more tenaciously to the epigynum; when female

setae were embedded in a plug of an *L. argyra*, the plug could usually only be pulled away from the female by breaking off the setae, which remained embedded in the plug (N. Caballero & A. Aisenberg unpubl.). We did not carefully test whether the consistency of *L. argyra* plugs changed over time; they remained hard once they had solidified, because the plugs in females collected in the field and kept for multiple days afterward were hard.

Comparisons of *L. argyra* genital morphology with that of other *Leucauge* species.—Our observations in the SEM of the male and female genitalia of *L. argyra*, *L. mariana* and *L. venusta* permit comparisons of many details. In the epigyna of all three species (Figs. 6, 8, 9) there is a setose anterior region, and a naked posterior region, where, at least in *L. argyra* and *L. mariana,* the palpal sclerites press against the female. The setose anterior region of *L. mariana* ends abruptly at the shallow wall that marks the anterior edge of the naked region (Fig. 8), while that of *L. venusta* extends posteriorly, forming a hood that partially covers the naked posterior region. This hood has a pair of depressions with a knobby internal surface whose functional significance remains to be determined (Fig. 9). The anterior surface of the large ventral projection of *L. argyra* is setose and is thus apparently an extension of the anterior region, if one can use setae as markers for these regions; the posterior surface of the projection is completely naked (Fig. 6B).

Some of the epigyneal setae of *L. argyra* are relatively longer than those of either *L. mariana* or *L. venusta* (Figs. 6, 8, 9). The longest setae occur near the tip of the epigyneal projection and along the lateral margins near the atria, toward which they project. The setae are denser in the areas where they are longest. The epigynal setae of the other two species are more uniform in distribution and length, although *L. venusta* has small patches of setae that project toward the naked area on the lateral portions of the posterior edge of the hood (Fig. 9A).

The entrances of the insemination ducts of *L. argyra* are relatively exposed compared with those of *L. mariana* and *L. venusta,* which are somewhat protected by epigynal structures – the lateral plates of *L. mariana* (Fig. 8), and the hood of *L. venusta* (Fig. 9). As far as we know, the atrium of *L. argyra* is unique among spiders in receiving insertions by two different male palpal structures, the conductor and the cymbial hook. The lateral epigynal ridge of *L. argyra,* which may engage the male cymbial tooth, is apparently absent in the other two species.

The male genitalia of *L. argyra* differ from those of the other species with respect to both the cymbium and the palpal bulb. There were no signs of either projections or indentations on the cymbia of *L. mariana* or *L. venusta* that might correspond to the cymbial hook or the cymbial tooth of *L. argyra*. The form of the paracymbium is similar in all three species; its position in the expanded palp of *L. argyra* (Fig. 3) suggests that it functions not to contact the female but to push against the embolus base, as occurs in *L. mariana* (Eberhard & Huber 1998).

Comparisons are also possible with recent published (Tso & Tanikawa 2000; Yoshida 2009) and digitally published (Levi 2007) taxonomic drawings of the genitalia of 44 other *Leucauge* species beside the ones we studied (42 species of females, 15 species of males). Less detail is available because
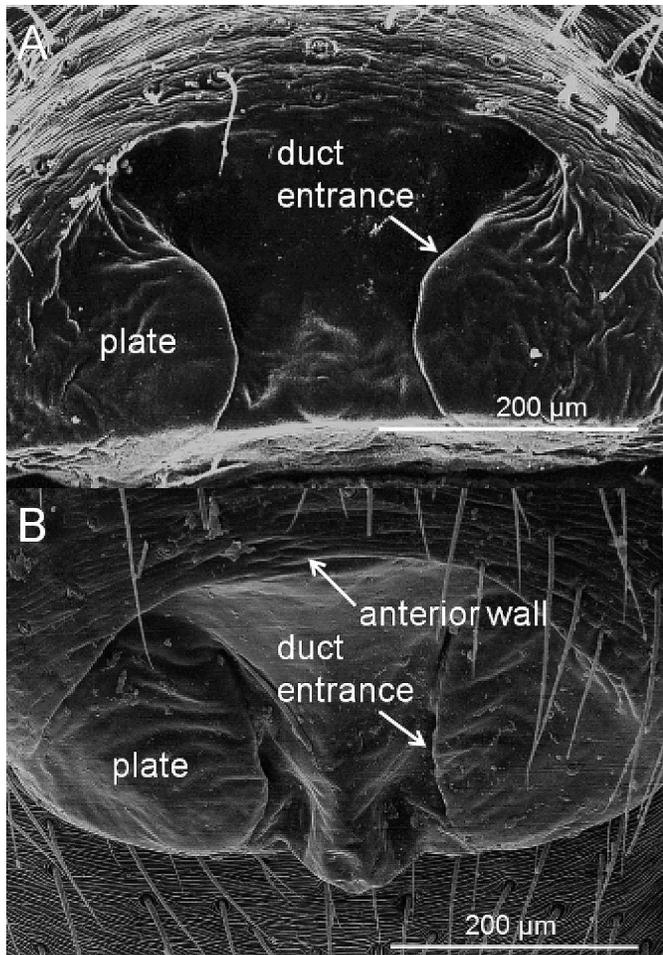
Figure 8.—SEM images of unplugged epigyna of *L. mariana* seen in A) ventro-posterior view, and B) latero-posterior view. The small hood over the anterior wall of the atrium, the lateral plates, and the entrance to the insemination duct are visible. The rear edge of the epigynum in B is tilted more strongly away from the ventral surface of the abdomen than is that in A.

not all structures are visible in all of the drawings, and because the setae are omitted in nearly all cases. In none of the 42 species in which the epigynum was drawn is there any elevation similar to or even suggestive of the ventral process of *L. argyra*, nor do any have such exposed, unprotected atria as those of *L. argyra*. There was also no epigynal ridge in any other species, but this structure is cryptic, so it could have been omitted from drawings. In only one of the 15 species of males, *L. ocellata* (a junior synonym of *Metabus ocellatus* Platnick 2013) is there a cymbial hook. This hook is approximately the same size as that of *L. argyra* and in a similar position on the cymbium, except that it is located less basally (about one-third of the distance to the distal tip). There is no cymbial tooth or indentation visible in *M. ocellatus* or in any other species. The atrium of *M. ocellatus* is relatively unexposed and is more similar to that of *L. mariana* than that of *L. argyra*.

DISCUSSION

Sperm plugs and their origins.—Our observations of *L. argyra* agree in some respects with the previous conclusion of Aisenberg & Barrantes (2011) that the copulatory plugs of this species are usually if not always produced by the female rather than by the male. The plug material usually lacked sperm completely, had at most only a small fraction of sperm, and sometimes had unencapsulated sperm, all indicative of a female origin for the plug. The contents of the sperm duct of *L. mariana* were densely loaded with sperm, all of which were encapsulated (Méndez 2004). Nevertheless, it is not impossible that male material transferred to the female in *L. argyra* lacks sperm, so this is not conclusive evidence. Much of the wall of the soft chamber I of the spermatheca of *L. argyra* is apparently glandular, and it could be the source of the plug material (Quesada & Triana, unpubl.) (spider spermathecae in general often have associated glands, however, so this is also inconclusive evidence – see Eberhard & Huber 2011).

It is not clear, however, how a female, having just received an ejaculate of spermatozoa that largely fills the lumen of chamber I of her spermatheca, can then move gland products produced by the walls of this chamber to the external surface of her epigynum without the gland products becoming mixed with the sperm that the male has just deposited near the spermathecal entrance. It would seem that there must have been sperm in chamber I when the plug formed on the surface of her epigynum; the sperm are not eliminated or moved elsewhere soon after copulation, because we frequently observed sperm in the spermathecal chamber I in females several days after they were collected in the field and isolated from males.

This puzzle could be explained if the plug material were derived from the male rather than the female. If it were transferred after the sperm were transferred and did not mix with the sperm inside the female, it would be possible to explain the observation that some plugs appear only hours after the end of copulation (Aisenberg & Barrantes 2011). One problem with this hypothesis is that the sizes of some sperm plugs seemed too large to be housed, along with the sperm volume that is stored in chamber I, in the male's palp. Perhaps the plug material includes instead a combination of male and female products as in *L. mariana* and is formed when some female component of the plug material crosses the walls of the insemination duct to mix with the male product, and then the combination emerges onto the surface of her epigynum. The long delays between copulation and plug formation (often many hours), and the direct observations of plug material welling up from inside the female and then hardening (Aisenberg & Barrantes 2011) make it difficult to believe that there is not some sort of active female participation in the process.

Evolution of new genital structures.—The phylogeny of species in *Leucauge* is not known; to our knowledge, this large genus has never even been revised. Many details of evolutionary transitions thus cannot yet be determined. Nevertheless, some prominent structures in *L. argyra* are apparently unique to this species, so it is possible to use the behavioral and experimental data from this and other studies to make some preliminary deductions.

Although the ventral epigynal process of *L. argyra* is large and prominent, there is no sign of any similar structure in any of the other species of *Leucauge* that we checked. This process appears to represent a ventral projection of the posterior area
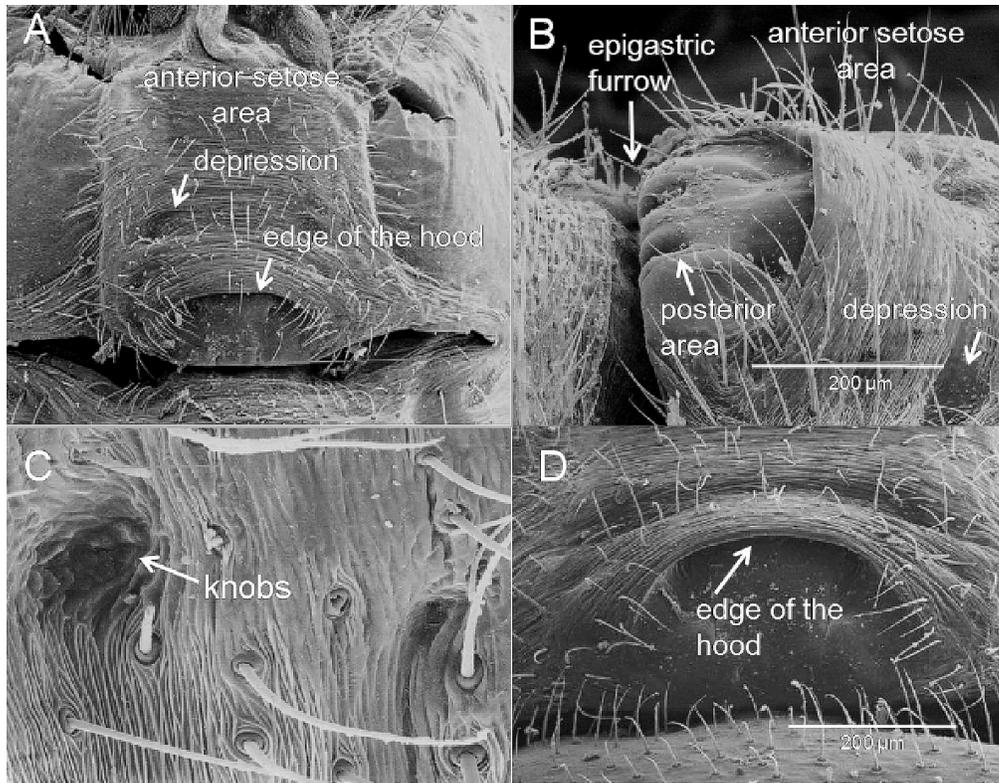
Figure 9.—SEM images of the epigynum of a *L. venusta* seen in A) ventral view, B) posterior-lateral view, C) close up view of depression in the anterior portion and D) posterior view. The epigynum in B) is tilted farther away from the epigastric furrow than is the epigynum in D).

of the anterior, setose region of the epigynum and of the anterior area of the posterior, naked region (see Figs. 6, 8, 9). The epigyna of other leucaugine tetragnathids such as *Chrysometa alajuela* Levi 1986 and *Azilia affinis* O.P.-Cambridge 1893 also have a setose anterior region and a naked posterior region, but lack ventral processes (Álvarez-Padilla & Hormiga 2011).

Functionally, the ventral process of the epigynum and its associated setae apparently serve sensory functions. The process does not mesh mechanically with any male structure during copulation. It has no internal structures such as cuticular projections, muscles or glands of obvious functional significance (Álvarez-Padilla & Hormiga 2011; Quesada & Triana unpubl.). It does not represent an obstacle to the male, as may be the case with a similarly protruding epignyal process of the pholcid *Mesabolivar* sp. —see Huber et al. 2005, because the atria of *L. argyra* are on the sides of the process rather than hidden behind it. Instead, the relatively abundant and elongate setae on the ventral process are contacted by the male's palp in one and possibly two contexts. The most certain contact is between the especially long and dense setae around the base of the ventral process and the atrium (Fig. 6), and the male's cymbial hook and conductor; they lie in the area through which the cymbial hook passes when it is being inserted and withdrawn from the atrium during copulation. The rhythmic in-and-out movements of the cymbial hook must deflect these setae repeatedly.

One other, less dramatic areas of contact may occur between the setae on the anterior surface of the ventral process, especially near its tip, and the surface of the cymbium or its setae; a second possible area of contact is between the setae nearer the base of the ventral process and the corner setae of the cymbium. Our posterior angle of observation prevented us from distinguishing whether the palp did or did not deflect setae on the anterior surface of the epignyal process. The cymbium moves past the setae on the anterior portion of the female epignyal process as the conductor is moved into position for the first insertion and during the rhythmic in-and-out movements of the cymbial hook. This interpretation is not in accord, however, with the orientation of the setae on the distal anterior surface of the process, especially near its tip. Here they project ventrally (distally) rather than anteriorly, as might be expected if they were designed to contact the male's cymbium. These interpretations also leave unresolved the significance of the length of the ventral process. At no point did we see a clear contact between any male structure and the distal portion of the ventral process. We were limited, however, by our angle of viewing, and perhaps also by having close-up recordings of only two copulations.

Experimental immobilization of the epignyal setae on or near the base of the ventral process (which presumably largely prevented their being stimulated by the male) inhibited female production of strong copulatory plugs (N. Caballero & A. Aisenberg, unpubl.). Thus, it may be that by stimulating these setae, the male increases his chances of paternity by affecting cryptic female choice. The morphology of females of *L. venusta* hints that they may also be sensitive to male genital movements, as the epigynum has setae concentrated at the lateral corners of the hood (Fig. 9a). Perhaps elaboration of

the ventral process of *L. argyra* occurred under selection to increase the female's ability to sense male palps and their movements. Further data will be needed to test these ideas.

Cymbial hook and female atrium.—The large cymbial hook of *L. argyra* is apparently unusual in the genus *Leucauge*; this structure is so prominent that it is unlikely to have been overlooked in taxonomic drawings. The hook was inserted into the atrium on the side of the epigynum opposite the atrium into which the conductor and embolus were inserted. The numerous small teeth near the hook's tip (Fig. 5a) presumably serve to increase the friction between the hook's tip and the smooth wall of the atrium or the insemination duct. The mechanical consequence of inserting and anchoring the cymbial hook in one atrium while the conductor and embolus are inserted into the other is that the palpal bulb seizes the female's epigynum as with a pair of tongs. The tong-like grip may anchor the conductor more solidly in the atrium and the insemination duct, perhaps facilitating or stabilizing insertion of the embolus and sperm deposition in the insemination duct.

Insertion of the hook into the atrium also results in the male obtaining a mechanical reference point and thus improving his ability to insert his conductor into the other atrium. This would explain how *L. argyra* avoids the frequent "flubs" made by *L. mariana*. These interpretations do not explain, however, all of the male's behavior, as they do not account for the subsequent rhythmic movements of his cymbial hook into and out of the atrium. In effect, the male rhythmically releases his grip on the female's epigynum and then grasps it again. Two functions for these rhythmic movements occur to us. They may serve to perforate the plug material when the hook grasps an atrium that has a rigid copulatory plug, much as an ice pick is used to break a chunk of ice. An alternative, non-exclusive hypothesis is that the movements serve to stimulate the female. The plug removal hypothesis is in accord with our observation that the conductor, which is the only other male genital structure that is positioned appropriately to perforate plugs in *L. argyra*, is relatively weak and flexible, and seems physically incapable of perforating the hard plug material. On the other hand, it does not explain the long female setae positioned to sense movements of the cymbium (above), nor the distally directed teeth on the tip of the cymbial hook. It would seem that basally directed teeth would be more effective in removing plug material, as in the basally directed spines on the odonate penis that remove sperm (Waage 1983). Perhaps these structures and their movements have both mechanical and stimulatory functions.

The physical coupling of the cymbium with the epigynum prior to insertion of the conductor contrasts strongly with the mechanics of *L. mariana* copulation (Eberhard & Huber 1998). The cymbium of *L. mariana* is not coupled mechanically in any way to the female when the male attempts to insert his conductor and embolus into the epigynum. The rounded "external", setose surface of the male's cymbium is simply placed on the apparently featureless, also sparsely setose surface of the female's abdomen; inflation of the basal hematodocha then causes the conductor and embolus to twist away from the cymbium and the abdominal surface and to roll so that the conductor is driven toward the epigynum; there is no other preliminary contact (Eberhard & Huber 1998). This

movement is apparently homologous with the second stage of insertion in *L. argyra* (following insertion of the cymbial hook).

A second clear contrast with *L. mariana* was that female *L. argyra* often struggled violently during copulation, and occasionally killed and cannibalized the male (Aisenberg & Barrantes 2011). The more secure mechanical coupling of the palp to the epigynum in *L. argyra* could have evolved to overcome female resistance, or female resistance could have evolved to test the stability of the male's coupling. But, as just mentioned, the subsequent rhythmic in-and-out movements of the cymbial hook do not make sense as attempts to physically overcome female resistance, so there is more than a physical male-female struggle occurring in *L. argyra*. It is also clear that the added mechanical stability provided by the cymbial hook in *L. argyra* does not come without a cost. In some copulations the male's cymbial hook becomes trapped in the sticky plug material produced by the female, and she kills him (Aisenberg & Barrantes 2011).

The female structure with which the cymbial hook interacts is the atrium. Its wide, flaring form, at least in general aspect, shows no modification that is complementary to the hook's form. The atrium of *L. argyra* is large and much more exposed, however, than the atria of any of the other *Leucauge* species for which we have information, making insertion of a cymbial process mechanically easier in *L. argyra* than it would be in the other species. This possible coevolutionary change in the female genitalia of *L. argyra* is appropriate to favor the corresponding male genitalic structure (the cymbial hook), rather than to defend against its use to anchor the palp to the epigynum. Such apparent "selective cooperation" by the female is typical of genital coevolution in many other groups (Eberhard 2004, 2010). It is compatible with females exercising cryptic choice (Eberhard 1996) by favoring males that have hooks, but is not in accord with the sexually antagonistic coevolution hypothesis for genital evolution (Arnqvist & Rowe 2005).

The atria of the epigynum of *M. ocellatus*, the only other related species in our survey with a cymbial hook, are very different. They are hidden from any direct insertion of the cymbial hook, suggesting that the hook in this species probably has a different, unknown function.

The cymbial tooth and its associated indentation.—The small cymbial tooth and the associated indentation in the cymbial margin are also absent in 17 other species of *Leucauge*. These structures are small, however, and could have been overlooked (they are visible, though not emphasized, in Levi's drawing (1980) of *L. argyra*). Functionally, the small tooth and the associated indentation may be associated with insertion of the large cymbial hook into the atrium. The form of the tooth and the indentation seem designed to hook or snag on some protruding structure. More by a process of elimination than by direct observation, we have concluded that the tooth and the indentation may hook the lateral epigynal ridge when the cymbial hook is inserted into the atrium; they may brace the tooth there more securely. Presumably the long setae on the hook serve as sense organs that inform the male regarding whether his tooth is near the lateral ridge of the female. Tentatively we propose that the cymbial hook evolved before the tooth and its accompanying indentation; the tooth

presumably evolved later to improve the mechanical stability of the hook when inserted into the atrium.

The female modification that may match the cymbial tooth and indentation is the ridge on the lateral and anterior margin of the epigynum. There is no similar ridge in *L. mariana* or *L. venusta*. It is not clearly present in any of the other 44 species, but it is an inconspicuous trait, so its absence in taxonomic drawings does not provide certain evidence. The ridge of *L. argyra* seems designed to increase the purchase of the cymbial tooth and thus to increase the firmness of the coupling of the cymbial hook with the atrium. Thus these male and female structures may have co-evolved in *L. argyra,* but this is uncertain speculation because our evidence for the mechanical mesh with the ridge is only indirect, and we lack information for nearly all other *Leucauge* species. If our hypothesis is correct, this design of the female functions to selectively cooperate with males, aiding those males that have an appropriate tooth and indentation forms rather than excluding them. It is thus compatible with the cryptic female choice hypothesis rather than the sexually antagonistic coevolution hypothesis for genital evolution.

Judging by the distance moved by the embolus base during copulation in *L. argyra*, the tip of the male's embolus passes through the relatively short insemination duct and enters the basal portion of the large, soft-walled receptacle (spermatheca chamber I), as also appears to occur in *L. mariana* (Eberhard & Huber 1998). The sclerotization of the lining of chamber I at and around its entrance in *L. argyra* (Quesada & Triana, unpubl.) supports this interpretation. Presumably it protects against friction with the embolus tip. The mechanism by which the embolus is moved (pushed by the paracymbium on the embolus base) is also similar in the two species. The more internal portions of the internal female genitalia differ dramatically, however, in the two species. In *L. mariana* there are two rather than one additional hard-walled chambers with finger-like inward projections of their walls, and both are substantially larger than the single chamber II of *L. argyra* (Quesada et al. 2011). These female structures are never contacted by the male genitalia, and the significance of these differences is unclear.

Hematodocha behavior.—The durations of the insertions of the conductor of *L. argyra* differed from those of *L. mariana*. Copulation in *L. mariana* includes two types of insertion: long insertions with multiple inflations of the basal hematodocha during each insertion, which tended to occur early in a copulation, and were associated with transfer of ejaculate to the female spermatheca; and short insertions with only a single hematodochal inflation, often repeated over and over (associated with deposition of sperm plug material on the surface of the epigynum) (Eberhard & Huber 1998). Only long insertions occurred in *L. argyra*, and males did not obviously transfer copulatory plug material.

There were also sharp differences between *L. argyra* and *L. mariana* in both the patterns of inflation of hematodochae and the sclerite movements that they produced. The tegulum of *L. argyra* first turned about 90° without any perceptible inflation of the basal hematodochae, and only then was the hematodocha inflated to insert the conductor into the atrium. In contrast, inflation of the basal hematodocha rotates the bulb about 180° in *L. mariana* without any prior rotation of the tegulum. In *L. argyra,* subsequent collapses and inflations of the basal

hematodocha caused small movements of the cymbial hook in and out of the atrium while leaving the conductor in place. Similar rhythmic inflations and collapses in *L. mariana* produced two distinct movements, both different from those of *L. argyra*. During short palpal insertions the conductor and embolus of *L. mariana* withdrew entirely from the atrium with each collapse. During long insertions they remained inserted, but the tip of a process on the conductor (absent in *L. argyra*) was rotated to contact the anterior wall of the atrium with each inflation. The median hematodocha caused further movement in *L. mariana,* but it was never seen to be inflated in *L. argyra*. Collapsing the median hematococha in *L. argyra* did not produce exactly the inverse sequence of the movements produced by inflation, as appears to occur in *L. mariana*.

At first glance, the disparity in the ways that the hematodochae of the two species are inflated and in the movements that they produce might seem surprising. On further consideration, however, it seems likely that the evolution of new sclerites and processes in spider pedipalps is often accompanied by new movements to employ these structures. Almost by definition, the use of a new process will involve new movements. Given that spider palps are driven by hydraulic pressure rather than intrinsic muscles (Huber 2004), differences in movements such as those documented here presumably result from differences in the forms of membranes that connect the sclerites within the palp and the ways in which these membranes are folded and twisted. We hypothesize that the frequent evolutionarily rapid changes in sclerites in male spider palps are often accompanied by changes in the internal membranes of the palp, and that these membranes probably often have traits that would be useful characters for distinguishing species.

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