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Sexual behavior of *Diplura macrura* and *Ischnothele annulata* (Araneae: Mygalomorphae): further evidence of diplurid polyphyly

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Abstract. The Dipluridae (Araneae: Mygalomorphae) is a recently redefined spider family of funnel-web spinners. Diplurid biology is poorly studied, especially regarding their sexual behavior, which is largely unknown for neotropical species. *Diplura macrura* (C.L. Koch, 1841) (Dipluridae) and *Ischnothele annulata* Tullgren, 1905 (Ischnothelidae) are medium-sized traditional diplurids from Brazil. We describe the courtship and mating behavior of these two species in detail, based on seven mating events under laboratory conditions observed for *D. macrura* and 10 for *I. annulata*. Both sexes of both species present courtship behaviors, and two novel spider sexual behaviors are described for *I. annulata* males (insertion lifting and post-brushing). Evidence for copulatory courtship is further observed for *I. annulata*. These sexual behaviors are analyzed and their importance is discussed in a phylogenetic context. Observed behaviors support the hypothesis of diplurid polyphyly, with Dipluridae closely related to Crassitarsae (Theraphosidae, Barychelidae, Nemesiidae, Cyrtaucheniidae and Microstigmatidae) and phylogenetically distant from Ischnothelidae. Additional biological information on both species is provided.

Keywords: Avicularioidea, Bipectina, copulation, ethology.

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Spiders present specific sexual behaviors of potentially high utility for taxonomic and phylogenetic research, but such comparative studies are rare (Coddington 1990; Wenzel 1992; Ferretti et al. 2013; Costa-Schmidt et al. 2017). In particular, the sexual behavior of spiders of the infraorder Mygalomorphae is poorly studied, as entire clades lack mating descriptions, descriptions usually only exist for relatively few species, and many descriptions are often simple or brief (Jackson & Pollard 1990; Ferretti et al. 2013). In a review of the state of knowledge of reproductive behavior in Mygalomorphae, Ferretti et al. (2013) presented the first proposal of evolutionary patterns of sexual behavior for the group and a standard terminology for sexual behavior units, with detailed descriptions.

Among mygalomorphs, in its traditional configuration, the family Dipluridae (*s. l.*) comprised spiders ranging from small to medium size, characterized by their long posterior lateral spinnerets and bi-pectinate tarsal claws (Raven 1985). Up until recently, Dipluridae contained 201 valid species in 26 genera. Four subfamilies within Dipluridae were recognized: Diplurinae (with four genera), Ischnothelinae (five genera), Masteriinae (four genera), and Euagrinae (11 genera) (Raven 1979; Coyle 1995; World Spider Catalog 2019), with two genera not placed in any subfamily. However, phylogenetic studies by Goloboff (1993), Hedin & Bond (2006) and Bond et al. (2012) all showed that, as currently defined, the family likely constitutes a polyphyletic group. While recent molecular phylogenetic studies by Bond et al. (2012), Hamilton et al. (2016), Pérez-Miles et al. (2017) and Opatova et al. (2020) all pointed to the monophyly of the diplurid subfamilies (except for Masteriinae, which wasn't included in the analyses), they similarly suggest that Diplurinae, Euagrinae and Ischnothelinae represent phylogenetically unrelated clades. Opatova et al. (2020) recovered Ischnothelinae and Euagrinae as being separate early-diverging mygalomorph lineages, outside the clade Bipectina, and Diplurinae as an independent clade belonging to Crassitarsae (within the Bipectina) and erected each as a family, stablishing Dipluridae *s. s.*, Euagridae and Ischnothelidae. Therefore, as already hypothesized for some mygalomorph clades by Ferretti et al. (2013), Dipluridae *s. s.* could be expected to share homologous reproductive behaviors with Crassitarsae species, and differ from other diplurids, reflecting their phylogenetic relationships.

Only seven species of Dipluridae *s. l.* have been studied for their reproductive behavior, and all presented similar vibratory signaling: *Microhexura montivaga* Crosby & Bishop, 1925 (Euagridae), three *Euagrus* spp. (Euagridae), *Australothele jamiesoni* Raven, 1984 (Euagridae), *Phyxioschema suthepium* Raven & Schwendinger, 1989 (Euagridae) and *Thelechoris striatipes* (Simon, 1889) (= *Thelechoris karschi* Bösenberg & Lenz, 1895) (Ischnothelidae) (see Coyle 1985, 1986; Raven 1988; Raven & Schwendinger 1989; Coyle & O'Shields 1990; Ferretti et al. 2013). There are no studies for members of Diplurinae or Masteriinae; and for Ischnothelidae, only one species has been studied (Coyle & O'Shields 1990). General aspects of the biology of South American diplurids and ischnothelids are especially poorly understood (Pérez-Miles & Perafán 2017), and their reproductive biology is completely unknown (Ferretti et al. 2013).

Diplura macrura (C. L. Koch, 1841) is a diplurid commonly found in semi-deciduous Atlantic Forest in south-eastern Brazil, and *Ischnothele annulata* Tullgren, 1905 inhabits areas of the Brazilian Cerrado in dense populations (Coyle 1995). They build webs between or beneath rocks and fallen logs, spreading irregularly in the form of a sheet web, with a silken tube inside the web with one or more openings (Coyle 1995; Brescovit et al. 2004) (Figs. 1I-K, 4C-I).

In the present work, we describe and analyze the sexual behavior of *D. macrura* (Dipluridae *s. s.*) and *I. annulata* (Ischnothelidae), and discuss the results in a phylogenetic context. We also provide additional natural history data on development, phenology and retreat characterization for both species, as these aspects can be linked to reproductive behavior (Ferretti et al. 2013) and represent a contribution to the overall knowledge of neotropical diplurid biology.

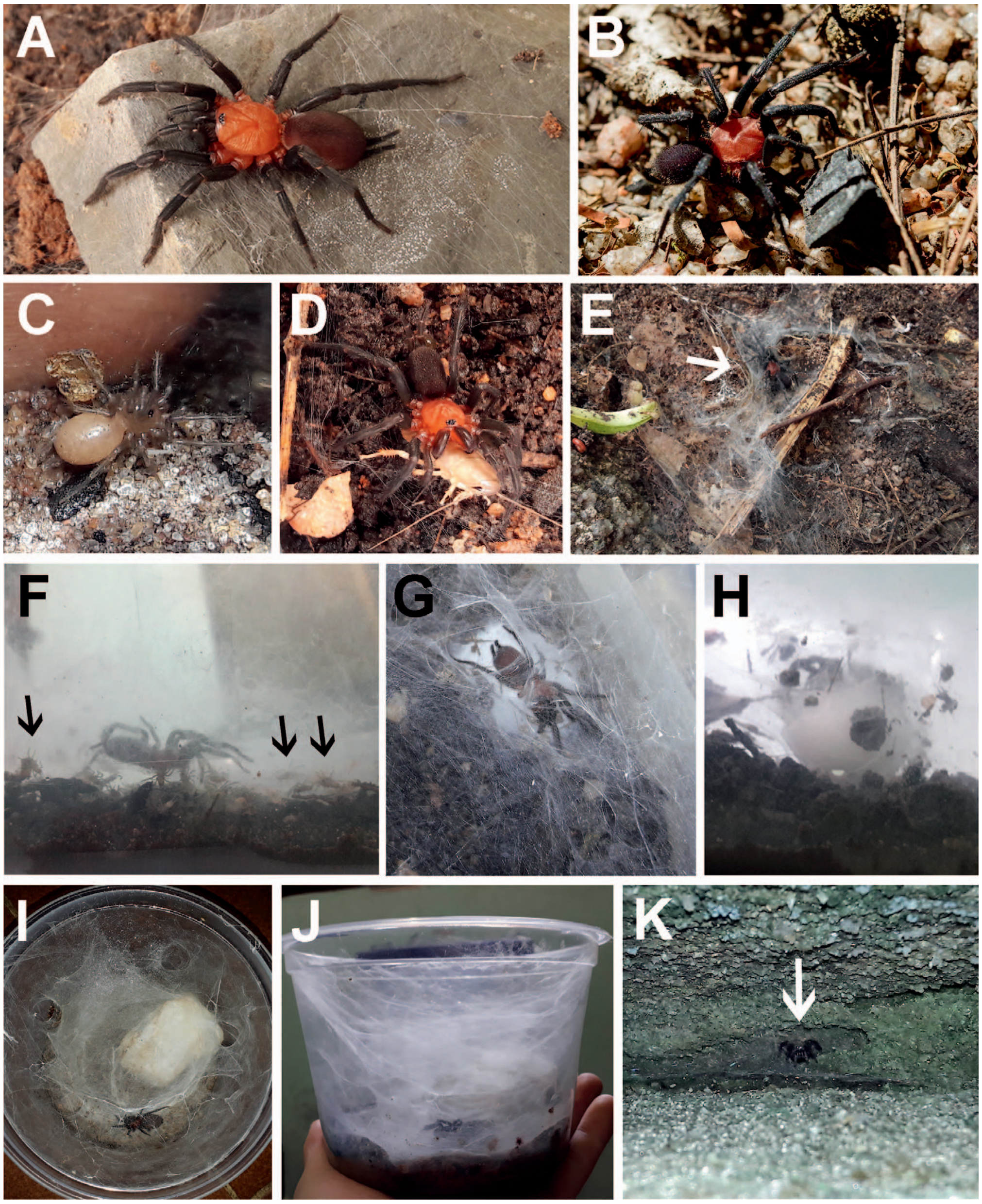

Figure 1.—A–K. *Diplura macrura*. A. Adult female from Campinas, dorsal habitus. B. Adult male *in situ*, Campinas, dorsal habitus (photo courtesy: Pedro Alvaro Barbosa Aguiar Neves). C. Second instar juvenile, from Campinas, dorsal habitus. D. Juvenile from Campinas, preying on cockroach, note the insertion of chelicerae on the back of the large prey. E. Silk of a subadult in nature, under lifted stone in Campinas. F. Female, the same from picture 2A, with newly hatched first instar spiders in its web. G–H. Same female from previous picture, guarding her fixed egg sac. I–J. Web in captivity of an adult female from Campinas. K. Juvenile lurking *in situ*, in crevices at a rock formation in P.E. Ibitipoca.

METHODS

The spiders used in this study were kept in captivity under laboratory conditions at UNESP - Rio Claro, Brazil. *Diplura macrura* were obtained by collecting juveniles and adults in their natural habitat, at four separate sites: (i) altitudinal semi-deciduous Atlantic Forest in Campinas, São Paulo, Brazil (22.905337°S, 46.823532°W), in March 2016 (six adult pairs); (ii) semi-deciduous Atlantic Forest in Itapetininga, São Paulo, Brazil (23.557552°S, 48.054387°W), in April 2017 (one adult pair); (iii) altitudinal semi-deciduous Atlantic Forest in Parque Estadual do Ibitipoca, Conceição de Ibitipoca, Minas Gerais, Brazil (21.711299°S, 43.896000°W), in April 2017 (one adult pair); and (iv) semi-deciduous Atlantic Forest in Pains, Minas Gerais, Brazil (20.388070°S, 45.650214°W), in April 2017 (one adult male). *Ischnothele annulata* were obtained by captive breeding, from an adult pair collected in November 2014, in Barreiras, Bahia, Brazil (12.081222°S, 44.995053°W), in Cerrado (savannah), and also by rearing five juveniles collected in September 2016 in Pirenópolis, Goiás, Brazil (15.841258°S, 48.956166°W), in Cerrado. *Ischnothele annulata* spiders born in captivity were separated after the third molt and reared individually, resulting in 14 adult males and 21 adult females.

The spiders were kept at natural lighting and temperature conditions, in plastic containers with small holes for air circulation, measuring at least 7 cm x 7 cm x 5 cm (length x width x height), with moist cotton or soil for water supply, and stones to provide fixing points for silk spinning and retreats. Cockroaches *Phoetalia pallida* (Brunner von Wattenwyl, 1865) were offered as prey every week. Only limited phenological data and information on retreat characterization for both species, plus a single observation of a male *D. macrura* courting were obtained in the field, from direct observation of the specimens and their retreats; all other data are the result of observations in captivity. For first pair encounters in the laboratory, only virgin females (i.e., those that molted in captivity) were selected, and each male only encountered a single female. At least seven days prior to encounters, most adult females were put in large (20 cm x 20 cm x 9 cm or bigger) glass enclosures, either without substrate, or with soil and stones, providing space for webbing and for interaction with the male, and fed weekly. Upon natural death, individuals were preserved in 70% ethyl alcohol. Voucher specimens from each collected site are deposited in the arachnid collection at Instituto Butantan, São Paulo, Brazil.

Mating encounters took place in the laboratory. Pairs were randomly selected and, in order to start the encounters, a male was placed in the enclosure of its respective female, away from the female's retreat. Ten encounters for *D. macrura* and 13 for *I. annulata* occurred, and each female encountered only one male, except for four *I. annulata* females, each exposed to two males a few days apart, and one *D. macrura* exposed to two males on the same day, plus a third the next day. The behavior of each spider was observed and recorded with a digital camera (Samsung J7 cellphone). Each encounter lasted until the end of the copulatory process, which sometimes consisted of more than one copulation event (defined as: clasping, palpal bulb insertion and unclasping), or after 25 minutes of female inactivity. At the end of encounters, males were removed from female cages. A copulatory process was considered ended if the spiders no longer moved after 15 minutes of the last physical contact between them, or if the male had distanced himself from the female, or if one of the individuals was injured by the other. The observed behavioral units were then analyzed and described according to the terminology of Ferretti et al. (2013).

RESULTS

Natural history (*D. macrura*).—Specimens of *D. macrura* (Fig. 1) were recorded for the first time in the State of São Paulo, representing the southern-most record for the species (Pedroso et al. 2016). Males became adults from February to April, and mating occurred from March to May. A female mated in captivity on 4 May 2016, and built an egg sac fixed to the web in a large tube above the soil on 8 August 2016 (Figs. 1F–H). The female remained close to the egg sac until approximately 90 spiders hatched on 19 September 2016, each measuring around 0.5 cm in body length. The offspring remained in the maternal retreat for five days, before dispersing through the cage, with some adopting their own retreats. By December 2018, all of the offspring had reached maturity, each having molted around 10–12 times.

The web of *D. macrura* (Figs. 1I–K) consists of a mesh, denser in some points, spreading irregularly or in the form of a sheet, which tapers into one or more tubes. Webs are built close to or under rocks, logs or crevices, or to the cage wall in captivity.

Encounters (*D. macrura*).—Nine successful copulations were obtained, eight with pairs from Campinas, and one with a pair from Itapetininga. Three of these resulted from encounters involving the same female, but with different males. The only unsuccessful trial occurred with a pair from different populations: the male, from Pains, started courtship but the female, from Conceição do Ibitipoca, didn't respond and chased him out of her cage.

Behavioral units (male *D. macrura*).—See Figs. 2 & 3, Table 1, Supplementary Video 1 (online at https://doi.org/10.1636/JoA-S-19-006.s1).

Body vibration. All legs, mainly I and II, flex while touching the substrate, and then contract, having the effect of pulling the web slowly towards the center of the body and then rapidly moving it slightly outward again (sometimes causing the body to rise slightly), causing a vibration. Flexing movements usually follow or are preceded by pauses, and are sometimes followed by palpal drumming. The frequency is almost one vibration per second (see Table 1), with single vibrations lasting approximately one second. In one instance, body vibration was also observed during insertion.

Palpal drumming. Alternate or simultaneous up and down movements for touching the substrate are performed with slightly flexed palps, varying greatly in frequency, each of up to 3 seconds in duration. In one of the nine encounters, the male did not perform palpal drumming.

Brushing (Fig. 2C). With legs I extended, tarsi I repeatedly touch the female, very quickly moving down and back up, similar to scraping. Brushing is sometimes accompanied by slow palpal up and down movements, touching or not touching the substrate, and these movements sometimes precede palpal boxing after brushing is finished.

Clasping (Figs. 2D, F–H). After brushing and slightly before palpal boxing, and while the female raises her front region, the male fits both of his leg I tibial apophyses between the female palps and chelicerae on each side, holding and pulling the female while approaching to position himself slightly under her. Clasping continues until insertion is finished and the spiders separate.

Palpal boxing (Fig. 2F). Positioned slightly underneath the female, and while clasping, the male quickly extends and flexes the palps repeatedly, beating the female sternum or anterior coxae in rapid movements with the dorsum of the cymbium/tibia, (see Table 1). Small pauses occur between beats.

Hugging (Figs. 2G,H). Accompanied by a pull prior to insertion, the male 'hugs' the female with legs II, wrapping the female prosoma between her legs II and III, thus holding her while insertion occurs.

Insertion (Figs. 2G,H). The male, pulling the female towards him, alternately inserts the embolus of each palp into the female genitalia, for sperm transfer.

Behavioral units (female *D. macrura*).—See Figs. 2 & 3, Table 1, Supplementary Video 1 (online at https://doi.org/10.1636/JoA-S-19-006.s1).

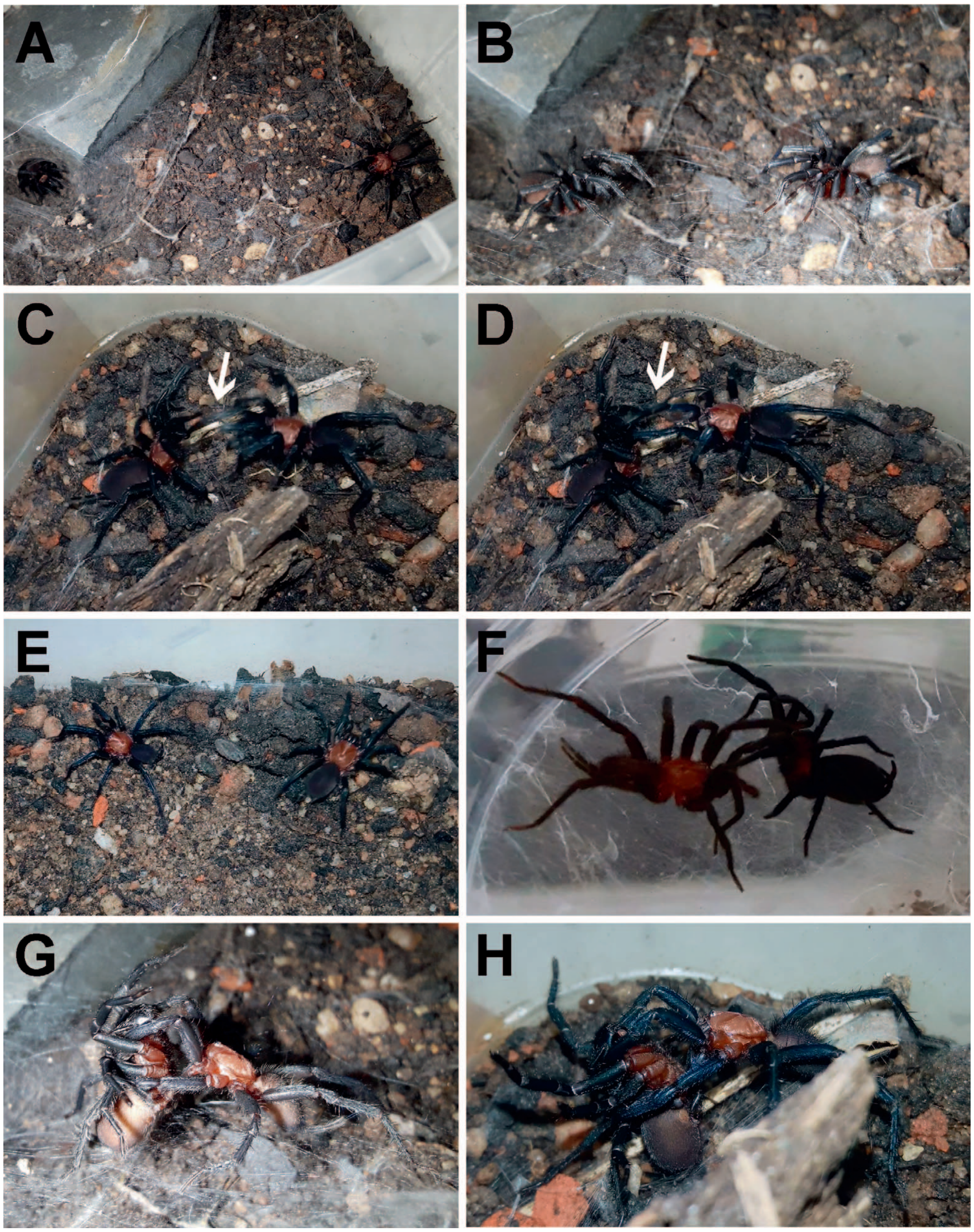

Figure 2.—A–H. Reproductive behavior of *Diplura macrura*. A. Male performing courtship, female at entrance of retreat. B. Male and female getting closer, male courting and female responding. C. Male brushing, female self-raising. D. Male starting clasping and palpal boxing after brushing. E. Female courting the male, with an advance followed by leg tapping (the male had previously retreated). F. Palpal boxing. G–H. Two different encounters with female in catalepsis and male performing insertion, clasping (leg I) and hugging (leg II). Note palp and legs movement (blurred parts) in C, D, E, and F.

Body vibration. The female vibrates her body with her legs, similar to the male. This behavior always causes a male response (mostly body vibration with advancing). It was observed in only two encounters.

Leg tapping. The female beats her flexed palps and leg I (sometimes also leg II) against the substrate, repeatedly, alternately or not, with relatively high frequency and intensity (see Table 1). In a few observations, the movement was also performed very slowly.

Catalepsis (Figs. 2G,H). The female remains motionless and inactive throughout copulation, until the pair separate.

Behavioral sequence (*D. macrura*).—An ethogram is depicted in Fig. 3, summarizing sexual behavior for *D. macrura*. Mating starts with male courtship after contacting the female web, either immediately or after a few seconds. Copulation occurs either on soil, slightly covered by web, or on the web mesh above the soil. Courtship behaviors are often interrupted by pauses. The male performs body vibrations, and sometimes palpal drumming, followed by pauses and/or advances. Females respond positively by advancing or leg tapping, which seems to encourage males to advance or to continue courting. With mutual advancement, when reaching the

Table 1.—Data on frequency (bouts per seconds, b/s), with standard deviation (±SD) and sample size (*n*), duration in seconds (s) and seconds per palpal insertion (seconds/palpal ins.) with SD and sample size, for sexual behavior observed and measured in *I. annulata* and *D. macrura*. Female leg tapping in *D. macrura* frequency is naturally varying and increased due to alternate participation of legs I, II and palps.

	Ischnothele annulata		*Diplura macrura*	
Behavior	Frequency (b/s)	Duration (s)	Frequency (b/s)	Duration (s)
Body vibration ♂	1.7 ±0.73 (*n* = 14)	1 – 20	0.8 ±0.24 (*n* = 11)	0.5 – 5
Palpal drumming ♂	5.3 ±2.14 (*n* = 6)	2 – 5	6.8 ±4.25 (*n* = 3)	0.5 – 1.5
Brushing ♂	11.8 ±6.75 (*n* = 8)	2.5 – 20	20 ±6.18 (*n* = 4)	1.5 – 4.5
Palpal boxing ♂	13.6 ±5.26 (*n* = 7)	2 – 8	20 ±2.13 (*n* = 4)	1.2 – 2
Post-brushing ♂	8.1 ±3.75 (*n* = 5)	4 – 15	—	—
* *Seconds/palp ins.* ♂	10 ±3.5 (*n* = 10)	17-200	33 ±10 (*n* = 4)	150-574
Body vibration ♀	1.5 ±0.64 (*n* = 2)	0.5 – 8	—	—
Leg tapping ♀	6 ±3.35 (*n* = 10)	1 – 7	11.6 ±6.37 (*n* = 6)	2 – 5.7

* Note the different unit of measurement (duration of each palpal insertion).

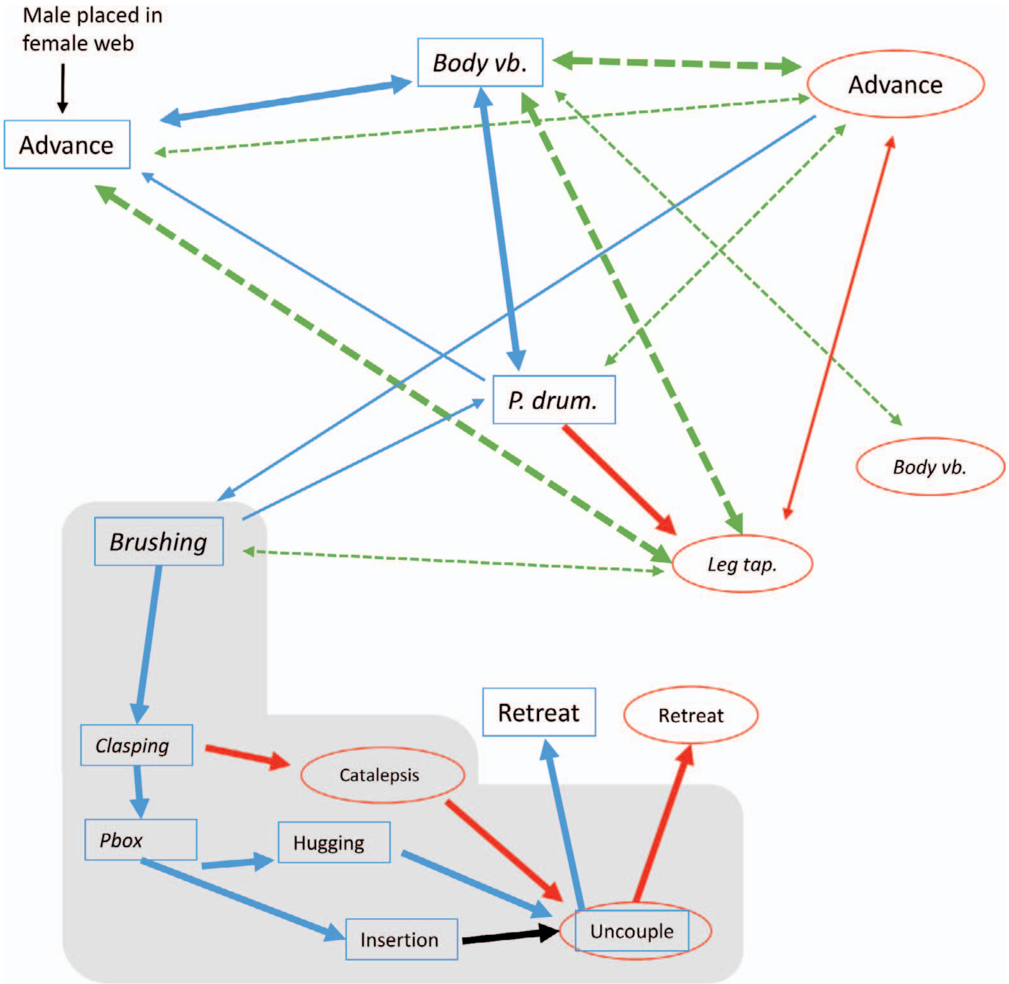

Figure 3.—Ethogram of *D. macrura* sexual behavior, based on the analysis of seven copulatory processes. Male behavior: blue rectangles; female: red ellipses. Arrows indicate behavioral sequences. Red arrows = female response; blue arrows = male response; green dashed arrows = responses by both male and female, in both possible directions. The most frequent behavioral sequences are indicated by thicker arrows. Body vb = body vibration; P. drum = palpal drumming; leg tap = leg tapping; Pbox = palpal boxing. Shaded area = male and female in contact (copulation).

Figure 4.—A–I. *Ischnothele annulata*. A. Adult female, from Barreiras. B. Adult pair (female on left) with first instar offspring on female web, from Pirenópolis. C. Fixed egg sac on the female web (female is hidden beneath the web), in captivity, from Barreiras. D. First instar spiders sharing the web of the maternal female in captivity, from Barreiras. E. Juvenile in sheet-web, in captivity, from Pirenópolis. F. Adult female web in captivity, from Barreiras. G. Juvenile web in captivity, from Pirenópolis. H. Adult female and her web *in situ*, with first instar offspring, Barreiras (note the occasional presence of an adult female *Dolichothele exilis* Mello-Leitão, 1923). I. Adult female and web *in situ*, with first instar offspring, Barreiras.

female, the male stretches his legs to perform brushing. Upon brushing, the male either retreats, or proceeds to finalize brushing, leading to insertion. Males often quickly resume courtship after a retreat. The brushing reaches the female's anterior legs. The female, in response, quickly rises, lifting her body, mostly the anterior part, in an apparently threatening position, causing the brushing to touch her chelicerae, coxae, sternum or trochanters I and II. During brushing, the female slightly moves her flexed legs I or II, for only a few times and to a lesser intensity than the movement of the male legs, briefly before and while raising. The male then fits his tibia I clasping spurs into the correct position as the female's anterior part rises, and proceeds to palpal boxing. The female stops moving after clasping is established, when the male performs hugging. Shortly before insertion, the female genital opening is slightly protruded, leaving the genital opening exposed.

The male then positions himself in front of, and slightly below, the female. After palpal boxing, the male inserts one of his palps into the female genital opening, as is standard for Mygalomorphae: the palp is extended, followed by embolus

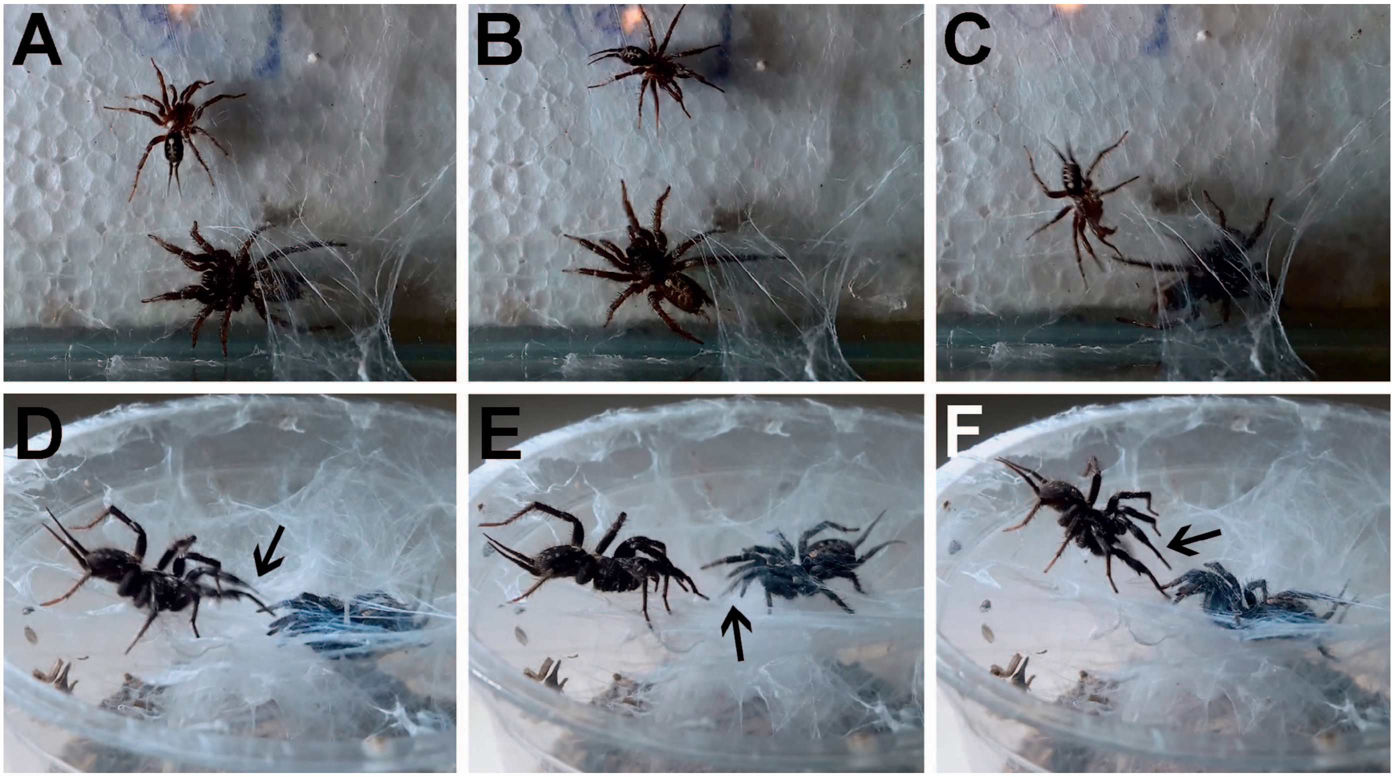

Figure 5.—A–F. Reproductive behavior of *I. annulata*. A–C. Chronological sequence of behaviors. A. Female advanced and male retreated. B. Female courted (leg tapping) and male turns towards her and advances. C. Male courts (palpal drumming) and advances towards female, female retreats. D. Male performing attempt at brushing, frustrated by web. E. Female performing leg tapping. F. Male performing palpal drumming. Note palps and legs movements (blurred parts) in C, D, E and F.

extension, which is rotated approximately 170° towards the body axis, and inserted by further palp extension. The male slowly moves the embolus in and out during insertion, and withdraws after a few seconds, alternating to the other palp. There were eight to 11 insertions per copulation.

After insertions, the male waits a few seconds to quickly unclasp and moves away while the female briefly remains cataleptic. In one mating, a male remained in a copulatory position while meticulously cleaning his embolus using his chelicerae for two minutes before suddenly unclasping. Successful copulatory processes lasted from six to 18 minutes, and 13 minutes on average. Cannibalism was not observed.

Natural history (*I. annulata*).—Males became adults in September and October. Mating occurred from November to February, with offspring hatching approximately one to two months after a mating. One female from Pirenópolis mated on 16 November 2016 and laid a fixed egg sac (Fig. 4C) on 1 December 2016, that hatched on 22 December 2016 bearing 70 spiders. Another egg sac from the same female without further mating, was laid on 19 January 2017 and hatched on 5 February 2017 with approximately 50 spiders. Individuals from Barreiras also made egg sacs in December and January, with less than one month of incubation and producing 50–100 spiders. Offspring remained on the female web (Fig. 4D) for some weeks to few months, sharing prey with each other and with their maternal female. Some siblings that were kept together isolated from the maternal female showed the same behavior of prey sharing. Cannibalism occurred only if they weren't properly fed.

Male and female *Ischnothele annulata* (Figs. 4A,B) are apparently mutually tolerant, as a male in Barreiras was found living with a female in the same web, and they were kept together in the same container for almost three months without agonistic behavior.

Specimens build large irregular webs, which are visible around cracks or holes in rocks, beneath fallen logs, loose rocks or termite nests, or the cage wall in captivity, in parts forming a sheet, with inner silk tubes opening in the web mesh. In captivity, the web almost always spreads irregularly over a large area (Figs. 4E–I).

Encounters (*I. annulata*).—Ten successful copulations were obtained; seven with spiders from Barreiras, two with spiders from Pirenópolis, and one with a male from Pirenópolis and a female from Barreiras. Three of the four females exposed to more than one male a few days apart didn't respond to male courtship, but one of these cases resulted in two successful copulations. Only one case of cannibalism was observed, right after a single successful copulation; following a period of inactivity, the female suddenly chased, killed and ate the male.

Behavioral units (male *I. annulata*).—See Figs. 5–7, Table 1, Supplementary Video 2 (online at https://doi.org/10.1636/JoA-S-19-006.s2).

Body vibration. All legs, mainly I, II and IV, vigorously flex and contract while touching the substrate, thus causing the body to vibrate and move up and down repeatedly. Flexing

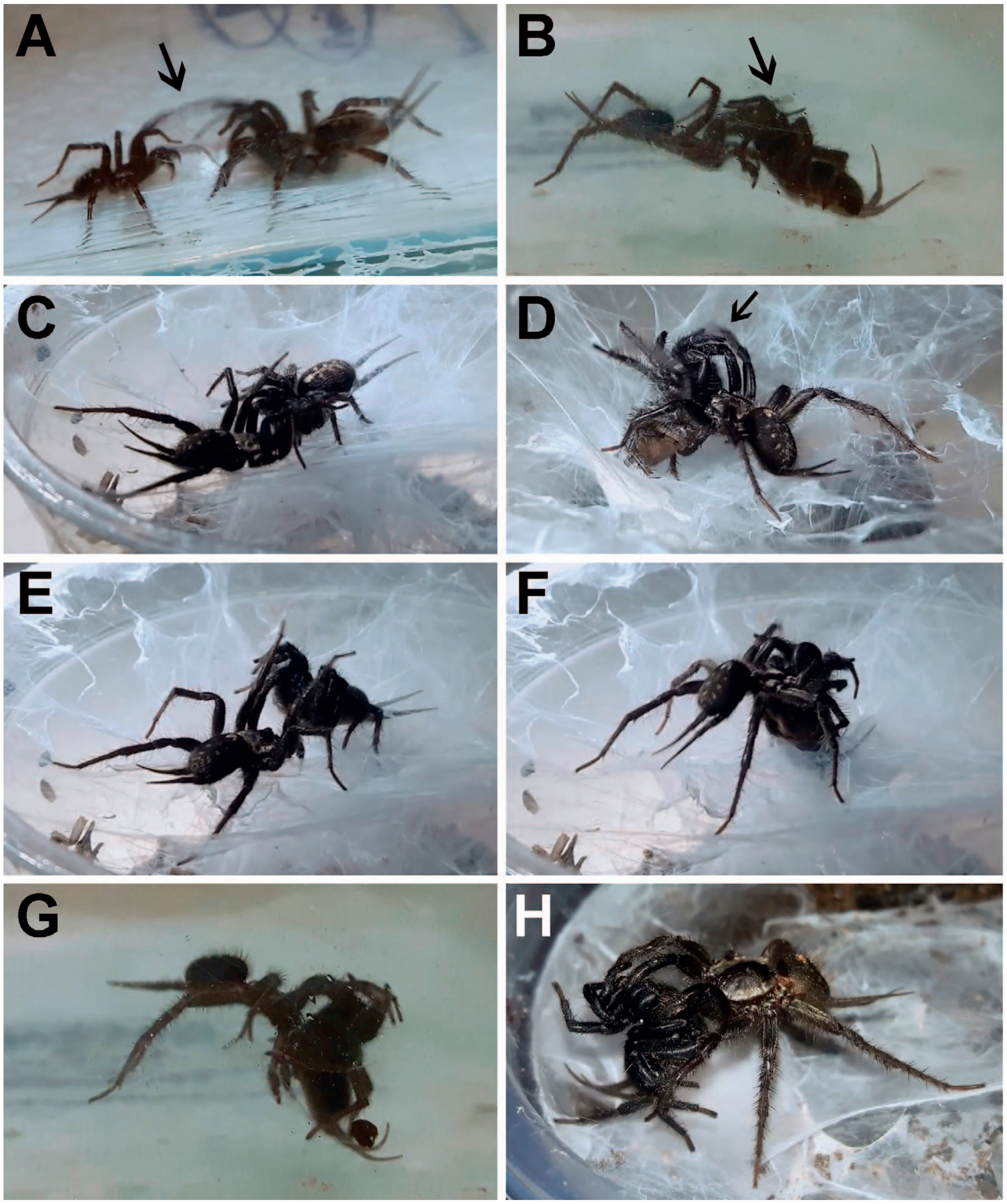

Figure 6.—A–H. Reproductive behavior of *I. annulata*. A. Male performing brushing, with female accompanying with her legs. B. Male performing palpal boxing, together with clasping and post-brushing. C. Male positioned below the female, preparing for palpal boxing and insertion lifting. D. Male performing post-brushing during insertion. E. Male performing post-brushing, palpal boxing, and clasping, preparing for insertion lifting (a fraction of a second before d). F. Shortly after the sudden insertion lift, concomitant with insertion. G. Male inserting his left embolus. H. Male performing insertion, legs I clasping. Note palps and legs movements (blurred parts) in A, B, D–F.

movements are usually performed in a series of subsequent bouts, with short pauses between bouts, however the intensity varies, and slower or less vigorous vibrations are common. Vibrations are usually followed or preceded by pauses, and sometimes followed by palpal drumming, and are often performed along with advancement (towards the female) or palpal drumming. One male was observed performing body vibration during insertion.

Palpal drumming (Fig. 5F). Alternate up and down movements for touching the substrate are performed with the palps slightly flexed, usually at relatively high amplitude and frequency (see Table 1). Palpal drumming is usually preceded by body vibration, and usually followed by a pause, advancing, or even brushing, gradually changing from one movement to another. One particular kind of palpal drumming is performed accompanying brushing and during clasping, in a sequence which progressively becomes palpal boxing.

Brushing (Figs. 5D, 6A). In proximity to the female, the male extends legs I and II and advances to reach the female by repeatedly touching his tarsi on anterior parts of the female's anterior legs, moving his tarsi up and down quickly, similar to scraping. The male's legs I are extended prior to the advance, and legs II start moving only after contacting the female. The beginning of this movement is usually accompanied by body vibration, and is always accompanied by palpal drumming,

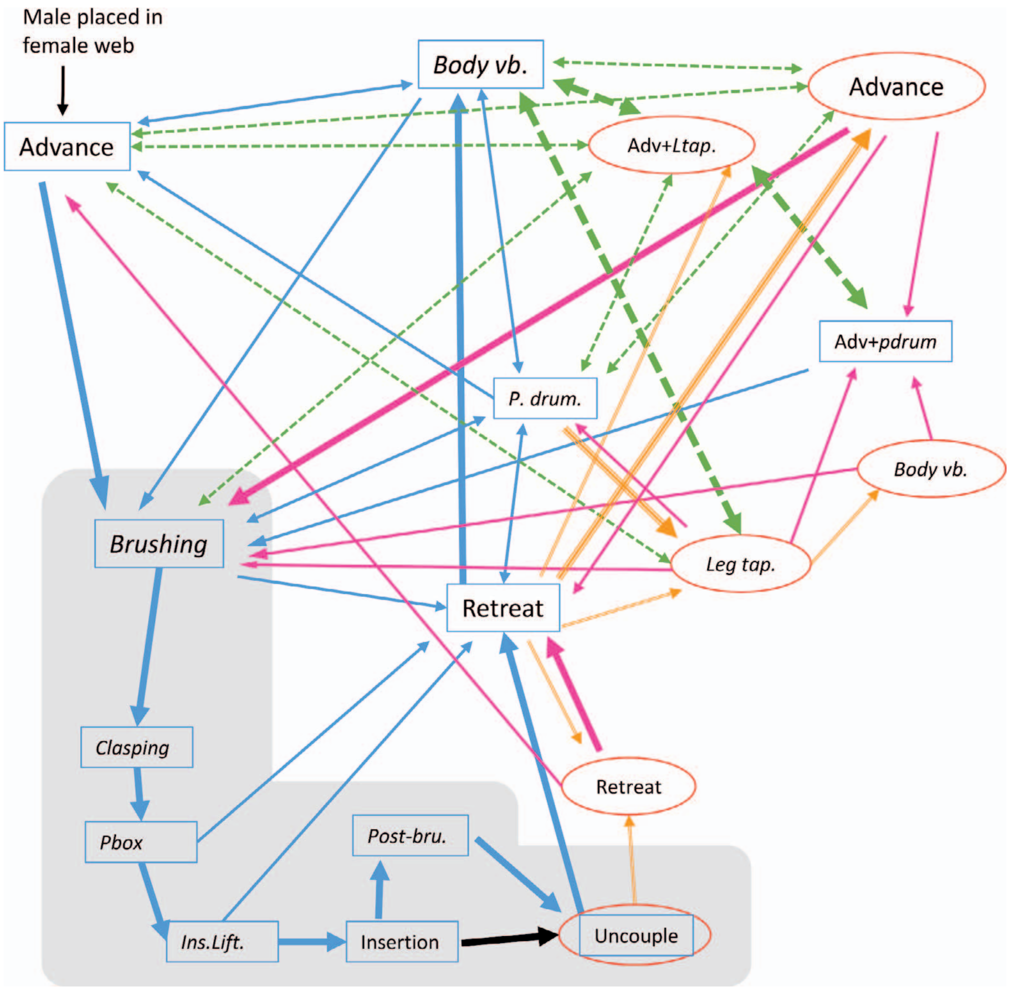

Figure 7.—Summary of the sequence of *I. annulata* sexual behavior, based on the analysis of eight copulatory processes. Male behavior in blue rectangles, female in red ellipses. Arrows indicate behavior sequence. Orange compound arrows = female response; magenta arrows = male response to female behaviors; blue arrows = male sequence behavior; green dashed arrows = responses between male and female, in both possible directions. The most frequent behavioral sequences are indicated by thicker arrows. Body vb = body vibration; P. drum = palpal drumming; Leg tap = leg tapping; Adv+Ltap = advance with leg tapping; Adv+pdrum = advance with palpal drumming; Pbox = palpal boxing; Ins.Lift = insertion lifting; Post-bru = post-brushing. Shaded area = male and female in contact (copulation).

but with the palps more extended in a continuous movement from the beginning of the brushing, until palpal drumming becomes palpal boxing. Brushing is always followed by either palpal boxing and clasping, or retreat.

Post-brushing (Figs. 6B–H). After brushing, the male stretches and directs legs II upwards, moving them quickly up and down, and touching the female's legs I or II, similar to beating. Sometimes upon touching the female, the tarsi are dragged sideways, similar to scraping. This behavior is either performed alternately by each leg or simultaneously, and continues during insertion lifting and insertion, with some pauses. When paused, the male's legs II remain firmly in contact with the female legs. During insertion, the male legs mainly beat or touch the distal parts of the female's legs I and II.

Clasping (Figs. 6B–H). After brushing and slightly before palpal boxing, the male fits his leg I tibial apophyses between the female palps and chelicerae on each side, holding the female, after which he begins to place his body below the female and slightly lifts her, in preparation for insertion lifting. The clasping continues until insertion is completed.

Palpal boxing (Fig. 6B). While clasping, the male quickly extends and flexes his palps repeatedly, as the dorsum of the cymbium/tibia rapidly beats the female sternum or anterior coxae. The male gradually pushes his palps underneath the female while transitioning from palpal drumming to palpal boxing, increasing in speed. Palpal boxing always occurs after brushing and before insertion lifting or a retreat.

Insertion lifting (Figs. 6E,F). Once palpal boxing and clasping are complete, and the male often performing post-brushing, with legs III flexed, and legs IV stretched or flexed, with the female being raised approximately 30° from the substrate. This behavior consists of a very quick, sudden and wide lift, as legs IV become fully extended, and legs III are halfway to fully extended. The female is lifted and raised approximately 90° from the substrate, with her abdomen slightly lifted upwards. While lifting, the male begins inserting the embolus of one of his palps into the genital opening of the female.

Insertion (Figs. 6F–H). From the lifted position during insertion lifting, the male alternately inserts the embolus of each of his palps into the female genitalia, for sperm transfer.

Behavioral units (female *I. annulata*).—See Figs. 5–7, Table 1, Supplementary Video 2 (online at https://doi.org/10.1636/JoA-S-19-006.s2).

Body vibration. Discrete body vibrations are similar to the male, but usually only one contraction or bout per movement are performed at a much lower intensity, as the female's body is not significantly lifted and the web does not vibrate as in the male's body vibrations. Some females vibrated discreetly more than once in a short period of time (5–6 seconds), and this behavior was noted on seven occasions.

Leg tapping (Fig. 5E). Females flex their palps and legs I, and rarely also legs II, to rapidly and repeatedly beat (alternately or not) against the substrate. Leg tapping is frequently performed along with walking (advancing). Sometimes females beat very lightly and slowly, and only with their palps, and this behavior occasionally precedes the usual leg tapping.

Behavioral sequence (*I. annulata*).—An ethogram is depicted in Fig. 7, summarizing the sexual behavior for *I. annulata*. Mating starts with male courtship after contacting the female web, either immediately or after a few seconds. Copulation occurs mostly on the female web mesh, above soil. Courtship behaviors are often interrupted by pauses. During courtship and even between insertions, both the male and female advance toward each other, which sometimes results in short-range retreats (up to six body distances), or in a continuation of courtship, with advances. These retreats are sometimes accompanied by a short chase by the other spider. Soon after retreating, the spiders turn towards their partner and continue the courtship (Figs. 5A–C). This chasing was frequently observed in most of the encounters.

The male performs body vibrations, followed by pauses and/or advances. The female responds positively by advancing and usually leg tapping (rarely with body vibrations, only when the male was distant), which seems to encourage the male to advance and/or continue courtship. Male and female courtship and/or advancement often happen simultaneously. Some females seem to rhythmically advance along with leg tapping. With mutual advancement, once close to the female, the male stretches his legs to start brushing. When the male performs brushing, either he retreats, or he proceeds with brushing, leading to palpal boxing, insertion lifting and clasping, then to insertion. Some rhythmic advances of the male happen along with body vibration and a less intense palpal drumming. It is also common for brushing to occur shortly after body vibrations. Most times during brushing, the female, without lifting the body, moves her legs I or II with the same intensity as the male, accompanying his movement in a mutual brushing (Fig. 6A). From brushing, the male switches to palpal drumming, thus beating the substrate with the tip of the cymbium and also beating the female's chelicerae with the dorsum of the tibia and cymbium, while slightly pushing her up and putting his palps underneath her and increasing beat velocity to establish palpal boxing. Some males were also observed retreating during palpal boxing or after insertion lifting, without proceeding to insertion, but resumed courtship shortly after. Female genitalia were observed to be protruded as in *D. macrura*.

During insertion, the pair are usually positioned as in Figs. 6F–H. Unusual positions occurred in courtships with very brief and repeated insertions, where the male or female moved a lot during and between insertions. The male inserts the embolus in the same manner as described for *D. macrura*, but with much faster insertion movements.

In a few encounters, some discrete behaviors that caused no apparent responses or changes in the courtship were observed: (i) friction of chelicerae, where both males and females rub their own chelicera against the other up and down, usually alternating with body vibrations; and (ii) biting the web, which occurs while performing body vibration and advancing, and usually results in the male tearing the web.

There were one to six insertions per copulation, and three to 12 per complete copulatory process, with a mean of four insertions per copulation (Table 1). After and during insertion, females of *I. annulata* are almost always active and moving, usually retreating soon after uncoupling, even in cases where mating continues shortly after. During insertion, some females keep stretching and flexing the legs, and in some cases even rotate themselves in the web mesh. The constant activity of females during insertion clearly shows the absence of catalepsis behavior. Some males retreat from insertion without rushing, often remaining inside the female's web mesh, and even near her, without signs of aggression. Some individuals of both sexes clean their appendages calmly shortly after copulation, apart from each other. No insertions were observed without previous insertion lifting, but less vigorous liftings that did not always result in insertion were observed in two instances. These copulations were the only ones in which the male was very cautious and agitated, performing multiple insertion attempts and retreating frequently between quick single palpal insertion attempts.

Only *I. annulata* was observed to copulate multiple times, i.e., multiple copulation events in a single copulatory process for six of the 10 encounters. In two of the four encounters that resulted in a single copulation event, the female still exhibited courtship behavior after uncoupling. Successful copulatory processes lasted from seven to 33 minutes, and 13 minutes on average.

Insertion lifting and post-brushing behaviors are novel, herein described for the first time in *I. annulata*.

DISCUSSION

Both species in this study presented standard mygalomorph sexual behaviors as well as poorly explored and novel sexual behaviors (Ferretti et al. 2013), that can be analyzed and compared to other groups according to the current knowledge concerning mygalomorph sexual behavior. The mating chronological sequence was peculiar to each species and can also be analyzed in the context of mygalomorph phylogeny.

Before discussing the spiders' sexual behaviors, it is noteworthy to mention the characteristic in *I. annulata* of the offspring remaining on the maternal web, as well as the occurrence of males and females on the same webs, indicating a high tolerance of intraspecific coexistence, which is very unusual for mygalomorphs (Ferretti et al. 2013; Pérez-Miles & Perafán 2017).

Regarding sexual behavior, *I. annulata* showed two interesting novel behaviors, so far exclusive to this species. Post-brushing was frequently observed, and it comprises strong evidence of copulatory courtship, outside the courtship phase, something rare in mygalomorphs (N. Ferretti, pers. comm.). The importance and definition of copulatory courtship as a sexual selection factor was elaborated by Eberhard (1994) and stressed by Ferretti et al. (2012), and occurs when male movements stimulate the female to remain copulating and to keep still during insertion. This function would be compatible with the characteristic of *I. annulata* females being very active and moving a lot during insertion, which constitutes a greater difficulty for the male to fertilize her, and only males that can sufficiently stimulate and hold females would achieve successful fertilization. Copulatory courtship and insertion lifting may also influence female cryptic sexual selection (Eberhard 1996), as they are apparently necessary for insertion accomplishment and apparently require energy and motor skills by the male, which are characteristics often evaluated by females (Barske et al. 2011).

The sexual behavior presented by *I. annulata* is very complex: it has many behavioral units; there is a lot of movement, even during insertion; many behaviors seem to blend, gradually changing from one to another; males and females often court simultaneously; and mating is usually carried out with more than one copulation event. These characteristics, mainly behavioral blending, simultaneous courtship, and multiple copulation events are unusual for Mygalomorphae (Ferretti et al. 2013). Two of these seem to be also present in *T. striatipes*, the only other Ischnothelidae studied so far: behavioral blending, and multiple copulation events, that may be related to lower female aggressiveness (Coyle & O'Shields 1990). Therefore, behavioral blending and multiple copulation events could be a characteristic of ischnothelid sexual behavior.

Female body vibration has already been described for several Mygalomorphae (Ferretti et al. 2013) and it was observed in both species analyzed in the current study. It most likely indicates female receptivity or, in *I. annulata*, willingness to mate again, given the tendency towards multiple copulations in this species. This behavior may also be interpreted as a form of checking the male physical condition, urging him to continue the courtship and demanding further energetic investment (N. Ferretti, pers. comm.).

Diplura macrura presented behaviors that were already observed in other Crassitarsae representatives: male palpal boxing and hugging and female self-raising. Both hugging and female self-raising could be linked to pair stability prior to insertion, as these set the spider's positions during insertion and are mostly present in species that mate on wispily webbed soil (only with flat sustaining points), which is the case for most Crassitarsae (Costa & Pérez-Miles 2002; Bertani et al. 2008; Ferretti et al. 2013). Female self-raising is apparently triggered by male brushing. The female assumes an upright position lifting its body and anterior legs, usually also opening the fangs, just before male clasping. This is clearly distinct from being passively lifted by the male upon clasping. It apparently facilitates male clasping, as the fitting areas become more exposed to the male. This specific movement of females is very common in Theraphosidae (Costa & Pérez-Miles 1992, 2002; pers. obs.), and also present in Microstigmatidae (Ferretti et al. 2012) and Pycnothelidae (R.P. Indicatti, pers. comm.), all of which belong to the Crassitarsae clade. The hugging behavior is already described in the literature as "hug" by Bertani et al. (2008), but not considered as a category in Ferretti et al. (2013). Hugging is present in several Crassitarsae representatives, like many Theraphosidae and Pycnothelidae (Costa & Pérez-Miles 1992, 2002; Bertani et al. 2008; Ferretti et al. 2011; Pérez-Miles et al. 2014), and absent in some non-Crassitarsae groups, like previously studied Euagridae, Ischnothelidae and Mecicobothriidae (Coyle 1985, 1986; Raven 1988; Raven & Schwendinger 1989; Coyle & O'Shields 1990; Costa & Pérez-Miles 1998). Curiously, it is apparently present in Porrhothelidae (see figures in Jackson & Pollard 1990), which is not a member of the Crassitarsae (Bond et al. 2012; Opatova et al. 2020).

Interestingly, palpal boxing was observed in both studied species, and was until now apparently exclusive to Crassitarsae species, and absent in previously studied *s. l.* diplurids. Palpal boxing has been described for Nemesiidae, Pycnothelidae, Microstigmatidae and Theraphosidae (Ferretti et al. 2013). Although the presence of palpal boxing in *D. macrura* was expected since it is a Crassitarsae species (Opatova et al. 2020), its occurrence in *I. annulata* is intriguing, as this is the first non-Crassitarsae to show this behavior. Noteworthy, in *Ischnothele* palpal boxing, the male can retreat without finishing the behavior. In the other groups that perform palpal boxing, including *D. macrura*, it always leads to insertion, resulting in a successful copulation event (Ferretti et al. 2013; pers. obs.). Two previously studied *s. l.* diplurid species present some behaviors related to *I. annulata* palpal boxing: male *T. striatipes* (Ischnothelidae) perform intense palpal drumming on the web, just prior to insertion (Coyle & O'Shields 1990), which is similar to *I. annulata* behaviors that lead to palpal boxing; and male *A. jamiesoni* (Euagridae) alternately beat their palps on the female chelicerae upon clasping (Raven 1988), prior to palpal boxing. These may be primitive states that could have led to palpal boxing as observed in *I. annulata*, which could indicate a recent and independent acquisition of the behavior in Euagridae and Ischnothelidae, groups that are outside Crassitarsae (Bond et al. 2012; Ferretti et al. 2013; Opatova et al. 2020).

Our results corroborate the recent molecular phylogenetic hypothesis that treats Ischnothelidae and Dipluridae *s. s.* as distinct, distantly-related clades (Bond et al. 2012; Opatova et al. 2020), as the behavior of *I. annulata* and *D. macrura* are strikingly different and each presents behavioral similarities to groups more phylogenetically closely related as already discussed above, even though both species possesses similar retreat structures, i.e., a dense web mesh. Both species also have behavioral differences regarding care for their young and mutual tolerance. In general, similarities between *D. macrura* and other Crassitarsae representatives are in the general chronology of male-female behavioral responses, and the hugging, self-raising and classic palpal boxing (always leading to insertion) behaviors, contrasting with *I. annulata* and other ischnothelids and euagrids (Ferretti et al. 2013). These shared behaviors could be behavioral synapomorphies of Crassitarsae.

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SUPPLEMENTARY FILES

Supplementary Video File 1.—*Diplura macrura* sexual behavioural categories. Online at https://doi.org/10.1636/JoA-S-19-006.s1.

Supplementary Video File 2.—*Ischnothele annulata* sexual behavioural categories. Online at https://doi.org/10.1636/JoA-S-19-006.s2.

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