Journal of Insect Behavior
https://doi.org/10.1007/s10905-018-9702-0

Patterns of Sperm Transfer Behavior in a Pholcid Spider with Two Distinct Copulatory Phases

Franco Cargnelutti[1,2] · Lucia Calbacho-Rosa[1,2] · Alex Córdoba-Aguilar[3] · Alfredo V. Peretti[1,2]

Received: 9 October 2017 / Revised: 27 September 2018 / Accepted: 11 November 2018
Published online: 21 November 2018

Abstract

Sexual selection is the responsible force for the evolution and maintenance of genital diversity and function. This is the case for example, of genital movements performed by males during mating and copulation duration. Spiders perform ritualized copulations whereby males carry out different types of movements using their pedipalps with varying duration. The function and duration of these pedipalp movements is unclear. In the pholcid spider, *Holocnemus pluchei* males that copulate with virgin females perform two copulatory phases: phase I in which the pedipalps move and phase II in which pedipalps remain motionless. Using *H. pluchei* as a study species, our study aims were: 1) to assess if sperm transfer occurs when pedipalps move or are still and quantify the number of sperm in male bulbs and in the female uterus externus after copulation; and, 2) to determine if amount of sperm transferred to females is associated with duration of each copulatory phase. Two experimental groups (i. e. complete copulation and interrupted copulation) were established in which the amount of sperm remaining in the male bulbs and the amount of sperm stored by females were determined. Our results show that sperm transfer occurs during phase I, that males transfer almost all sperm from their bulbs while the females store only 20% of that male amount. There was no relation between the amount of sperm transferred or stored and the duration of the copulatory phases. These results support the hypothesis that while both phases may serve a copulatory courtship, only phase I (when pedipalps move) serves for sperm transfer.

Keywords Mating · sperm transfer · spermatozoa · *Holocnemus pluchei* · sexual selection

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s10905-018-9702-0) contains supplementary material, which is available to authorized users.

✉ Alfredo V. Peretti
aperetti@unc.edu.ar

Extended author information available on the last page of the article

Introduction

Aside from sperm transfer and storage, the vast diversity of genitalic form and function has been largely attributed to a sexual selection process (Hosken and Stockley 2004; Eberhard 2010; Simmons 2014; Kelly and Moore 2016). According to this, male genital morphology and movements have evolved and are currently maintained by the competition for fertilization (reviewed by Leonard and Córdoba-Aguilar 2010). This has been illustrated, for example, by the male removal ability of rival sperm by spiny aedeagal structures in damselflies and dragonflies (Cordero-Rivera 2017), the stimulatory movements during male genital insertion that could elicit fertilization responses as observed in several animal taxa (reviewed by Eberhard 1996), and the use of copulatory devices that impede female re-copulation (e.g. mating plug) in several insect families (Simmons 2001).

The study of copulation indicates that this process serves functions other than sperm transfer (Elgar 1995). In broad terms, copulation can be used by males to stimulate females to use the mating male's sperm for fertilization (Eberhard 1996) and/or to decrease the probability that females use the sperm of competing males. Examples of both are sperm removal (e.g. Kamimura 2005; Calbacho-Rosa et al. 2013), mating plugs (e.g. Simmons 2001; Eberhard 2004; Uhl et al. 2010, 2014), mate-guarding (e.g. Schöfl and Taborsky 2002; Calbacho-Rosa et al. 2010) or transfer of substances that reduce female receptivity (e.g. Cordero 1995; Chapman and Davies 2004; Aisenberg and Costa 2005). In some species, copulation is highly ritualized, and different copulatory stages have been identified based on male behavior (Weldingh et al. 2011).

One taxon where ritualized copulation occurs is that of spiders. These animals can be classified according to their genital morphology into entelegynae and haplogynae. Entelegynes tend to insert the embolus of their copulatory organs -the pedipalps- inside the female spermathecae's ducts sequentially. In contrast, some haplogynes such as pholcids, insert both palps simultaneously (Foelix 2011; Herberstein and Wignall 2011; Huber 2014). Furthermore, there are examples of ritualized copulations in spider types. For example, in the entelegynae spider *Linyphia triangularis* (Liniphiidae) copulation can be divided into three stages. First, copulation may occur with no sperm transfer (which is technically referred as pseudocopulation). Second, the male performs the first sperm induction and inserts his pedipalps to transfer the sperm. And third, the male dismounts and carries out a second sperm induction and inserts his pedipalps in the female genital opening again (Weldingh et al. 2011). In the wolf spider *Schizocosa malitiosa*, two pedipalp patterns, "PI" and "PII", have been identified during copulation. The PI pattern consists of multiple pedipalp insertions in one of the female genital opening followed by a change of spermathecae and pedipalp. The second insertion, pedipalp pattern PII, consists of simple insertions with each pedipalp alternately. Experiments of interrupted copulation in this species have shown that both patterns (PI and PII) produce offspring in similar numbers and are related to sperm transfer (Costa 1979; Costa and Toscano-Gadea 2003; Albo and Costa 2017).

Pholcids are well studied haplogyne spiders whose different copulatory stages can be distinguished by the type of male pedipalp movements. Pholcid copulation is characterized by rhythmic and continuous movements with both pedipalps while inserted into the female genital opening (Huber and Eberhard 1997; Schäfer and Uhl 2002; Peretti et al. 2006; Calbacho-Rosa et al. 2013). For example, in the cellar spiders

Pholcus phalangioides and *Physocyclus globosus,* the pattern of pedipalp movements changes during copulation. The frequency of the rhythmic movement increases during the very first minutes but then decreases and remains low during the last minutes of copulation (Huber and Eberhard 1997; Schäfer and Uhl 2002). However, the function of such pedipalp movement changes and its duration is unclear (Calbacho-Rosa and Peretti 2015). One interesting model species to unravel such function is *Holocnemus pluchei* as there is previous information regarding its copulatory behavior and mechanisms. Calbacho-Rosa and collaborators (2013) described that when *H. pluchei* males copulate with non-virgin females, males first move their pedipalps alternately and with no clear pattern (which is associated with the removal of sperm transferred by previous males) and then they move them simultaneously yet constantly until the end of copulation. However, when males copulate with virgin females, they perform simultaneous movements with both pedipalps (Calbacho-Rosa et al. 2013). When mating with virgin females, frequency and duration of pedipalp movements are high at the beginning of copulation and then decrease progressively (copulatory phase I - see Online Resource 1-) while in the second part of copulation the pedipalps remain motionless while inside the female genital opening (copulatory phase II - see Online Resource 2-) (Calbacho-Rosa et al. 2013). There is not a conclusive explanation about the function of the copulatory phases, and their associated genital movements. However, Calbacho-Rosa and collaborators (2013) propose that one function for the pedipalp movements during phase I is to transfer sperm, (in addition to female stimulation as has been suggested in other Pholcids [Huber and Eberhard 1997]), so when the pedipalps are not moving, males would not be expected to transfer sperm. Keeping this in mind our working hypothesis in this paper is that sperm transfer occurs during copulatory phase I, so that males who perform both copulatory phases will not transfer more sperm than those males that only perform the copulatory phase I. Thus, males with a longer duration of copulatory phase I will transfer more sperm, while we do not expect a relationship between the duration of phase II and sperm transfer. To test this, here we experimentally investigated the occurrence of sperm transfer and associated pedipalp movements in both copulatory phases using *H. pluchei*. Thus, our aims were: 1) to assess if sperm transfer occurs when pedipalps move or are still and quantify the number of sperm in male pedipalp bulbs (from now on, bulbs) and in the female uterus externus after copulation; and, 2) to determine if amount of sperm transferred is associated with duration of copulation (of each phase).

Material and Methods

Study Species

H. pluchei (Pholcidae) is a spider native of central Europe (Porter and Jakob 1990; Jakob 1991; Huber 2014) that was introduced in South America (Huber 2000) and is now very common in central Argentina (Laborda and Simó 2008). It inhabits urban and natural areas where it builds irregular webs that contain from 1 to 15 spiders of different age, size, and sex (Jakob 1991; Kaster and Jakob 1997). Males actively move between the webs, so chances of multiple matings and sperm competition are likely high (Jakob 1991; Kaster and Jakob 1997; Calbacho-Rosa et al. 2010). Females can mate with

several males before oviposition and show last-male sperm precedence (Kaster and Jakob 1997; Calbacho-Rosa et al. 2010). As a consequence, males perform short-term, post-copulatory mate-guarding to prevent females from remating with other males (Calbacho-Rosa et al. 2010).

Collecting and Rearing

Individuals (subadult females and adult males) were collected from the Universidad Nacional de Córdoba campus, as well as from the local Zoological garden (Zoo Córdoba, Córdoba, Argentina), between September 2012 and March 2014. Each individual was placed in a plastic container (8 × 15 cm height), covered inside with paper to provide surface for web building and water, and left with a photoperiod of 12/12 h light:dark. Penultimate juvenile females were maintained to obtain virgin females for experimentation. Males were used 15 days after their capture to reduce effects of potential differences in mating histories that can affect sperm production. Individuals were fed weekly with *Drosophila melanogaster* adults.

General Observation Conditions

All interactions were recorded using a stereo-microscope equipped with a Logitech QuickCam ® pro 9000 digital camera to allow close-up views of male pedipalp movements during copulation. Adult females were placed individually in containers (8 × 12 cm height) 24 h before males so that the former can build their webs. After copulation, males and females were euthanized by hypothermia (−20 °C) and kept under these conditions for a period that did not exceed 15 days, to then proceed with sperm quantification (Gabel and Uhl 2013). Events were transcribed from digital videos using J Watcher ® 0.9 (Blumstein et al. 2000). Finally, the length of the patella-tibia segment of the first pair of legs was measured in both sexes as an indicator of body size. For this measurement, a picture of the patella-tibia segment was taken under a dissecting microscope (Nikon SMZ 1500) for each individual which were later measured using Image-J image software®. All specimens were deposited in the spider collection of the Laboratorio de Biología Reproductiva y Evolución, Instituto de Diversidad y Ecología Animal (IDEA), Universidad Nacional de Córdoba, Argentina.

Sperm Counting

We adapted a sperm counting technique that has been successfully applied in spiders (Albo and Peretti 2015; Albo and Costa 2017). According to this technique, male bulbs were isolated from the pedipalps under a dissecting microscope (Nikon SMZ 1500). Each bulb was placed individually in a centrifuge tube with 75 μl of the sperm counting solution. The bulbs were crushed using tweezers to release sperm into the solution without affecting sperm structure. The sample was vortexed for 30 s and then centrifuged at 4000 rpm for 10 min. Vortexing and centrifuging was repeated twice. The sample was finally vortexed for 10 s. Note that a similar procedure was carried out after trials, for the female's uterus externus which was removed under a dissecting

microscope, with one difference: after obtaining the sample it was then vortexed and centrifuged five times for 30 s and 4000 rpm for 10 min respectively to facilitate the separation of sperm as these are usually grouped by the female's gland substances (Cargnelutti F, Calbacho-Rosa L and Peretti AV, unpub. data). Finally, the sample was vortexed for 30 s. For sperm count, 10 μl of the sample was taken and placed in a Neubauer chamber under a phase contrast microscope (Nikon Eclipse 50i). Finally, by knowing the number of sperm in 10 μl, we estimated the total number of sperm from the initial 75 μl by using the following equation: Total number of sperm = (75 μl * N° of count sperm)/ 0.4 μl.

Experimental Design

Occurrence of Sperm Transfer and Pedipalp Movements

To determine whether sperm transfer occurs during copulatory phase I (i.e. when pedipalps move) and/or II (i.e. when pedipalps are still), we had two groups. A first group of individuals were allowed to copulate without any interruption (from now on, the "complete copulation group") ($n = 18$). A second group of individuals whose copulation was interrupted by gently touching the couple with a fine paintbrush 1 min after the beginning of the copulatory phase II (from now on, the "interrupted copulation group") ($n = 10$). Using these two groups we compared the spermatozoa number located both in the female uterus externus and male bulbs after copulation. Notice that these two experimental groups were used for sections 2 and 3 below. It is important to highlight that when comparing the amount of remaining sperm after copulation between males of both groups, the sum of sperm in both bulbs was used, since no significant asymmetry in the amount of remaining sperm between both bulbs was found (Wilcoxon signed-rank test for paired data, Complete copulation males: $v = 56$, $p = 0.842$; Interrupted copulation males: $v = 17$, $p = 0.554$). On the other hand, it is also important to mention that there are no significant differences in the sizes of males and females among the experimental groups (Male size: $F_{1,26} = 0.844$, $p = 0.367$; Female size: $F_{1,26} = 1.864$, $p = 0.184$).

Number of Sperm in Male's Bulbs and Female Uterus Externus After Mating

The number of remaining spermatozoa in the bulbs and the number of spermatozoa stored by females in the uterus externus after copulation was estimated in the group's complete copulation and interrupted copulation. We also quantified sperm number stored in both bulbs in a third control group consisting of non-copulating males ($n = 11$). The percentage of sperm transferred by males was calculated as the inverse of the percentage of sperm remaining in the bulbs after copulation. Using the following equation: 100 - (Average number of remaining sperm * 100 / Average number of spermatozoa in control males), we estimated the percentage of sperm transferred by the male. On the other hand, the percentage of sperm stored by the females after copulation was calculated by the following equation (Average number of stored spermatozoa * 100 / Average number of spermatozoa in control males).

Copulation Duration and Sperm Transfer Quantity

To determine if the duration of copulatory phase I and II (in seconds), had an influence on the quantity of sperm that males transfer and that the female stores, such duration was correlated with the number of sperm in the bulbs and female uterus externus, after copulation, of complete copulation and interrupted copulation groups. Notice that for the interrupted copulation group we only tested whether the duration of the copulatory phase I was related to the quantity of sperm transferred as the interruption itself impeded a copulatory phase II.

Statistical Analyses

For aim 1, we compared the sperm number in both the female uterus externus and male bulbs from complete copulation and interrupted copulation groups using two generalized linear models (GLM) with negative binomial distribution using the R package "MASS". GLM analyses were selected once we established graphically (using the R package "Fitdistrplus") and analytically (using Akaike information criterion) that the binomial negative distribution was the distribution that better fit our sperm count data.

In both GLM analyses we included female and male size as covariates. The interactions between the variables were not included in the final model since they lacked statistical significance. For aim 2, we used a Spearman partial correlation with the R "ppcor" package to assess the relationship between the number of sperm stored by females and the sperm remaining in bulbs, with duration of copulatory phase I and II in complete and interrupted copulation groups. In the partial correlation analyses, we used female size as covariate since female size predicts the amount of sperm stored by female in both experimental groups (see results below). R Version 3.3.2 (R Core Team 2016) was used for all analyses described above.

Results

Occurrence of Sperm Transfer and Pedipalp Movements

There was no difference between the amount of sperm in bulbs after copulation between complete and interrupted copulations ($df = 1$, Deviance = 0.056, $p = 0.812$). However, we found a significant difference in the amount of sperm in the female uterus externus between complete and interrupted copulation ($df = 1$, Deviance = 4.383, $p = 0.036$). Nevertheless, only female size explained the amount of sperm storage by females in both experimental groups ($df = 1$, Deviance = 4.061, $p = 0.044$). Neither female size nor male size explained the amount of remaining sperm in bulbs in complete and interrupted copulation ($df = 1$, Deviance = 0.043, $p = 0.835$; $df = 1$, Deviance = 0.214, $p = 0.643$).

Number of Sperm in Male Bulbs and Female Uterus Externus after Mating

The average number of spermatozoa stored by females as well as the average number of spermatozoa remaining in the bulbs after copulation in both complete and interrupted copulation groups, is shown in Fig. 1 and Table 1. In addition, the average number of

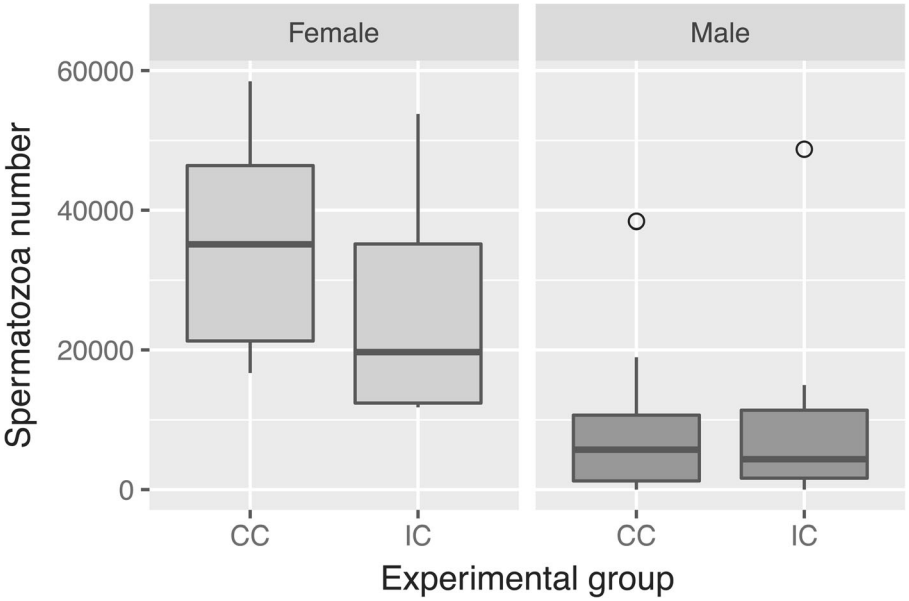

Fig. 1 Sperm number of experimental male bulbs as well as in the external uterus of females with complete and interrupted copulation. Each box plot shows the median (the second quartile) ± one quartile; whiskers show data range. CC=Complete copulation and IC=Interrupted copulation

sperm of the bulbs in non-copulating males is shown in Table 1. Maximum and minimum values of sperm number in male bulbs and female uterus externus in complete and interrupted copulation groups are shown in Table 2. These results indicate that when males copulate with virgin females, they transfer around 95% of their sperm in complete copulations as well as in interrupted copulations, and that females store around 23% of the sperm transferred in complete and 17% in interrupted copulations.

Copulation Duration and Sperm Transfer Quantity

As for the complete copulation group, there was no correlation between the number of sperm in the female uterus externus and the duration of copulatory phase I ($r_s = 0.085$, $p = 0.753$) and phase II ($r_s = -0.289$, $p = 0.296$). A lack of a significant correlation was

Table 1 Sperm number in male bulbs as well as in the uterus externus of females of complete and interrupted copulation groups

	Complete copulation (mean ± STD)	Interrupted copulation (mean ± STD)	Control group (non-copulating) (mean ± STD)
Spermatozoa number in male bulbs	8411 ± 9500 ($N = 18$)	9637 ± 14,641($N = 10$)	164,812 ± 79,608($N = 11$)
Spermatozoa number stored in female uterus externus	36,915 ± 14,175 ($N = 18$)	28,012 ± 14,641 ($N = 10$)	

also detected for sperm number in the bulbs and the duration of copulatory phase I (r_s = 0.085, p = 0.753) and phase II (r_s = 0.362, p = 0.168).

As for the interrupted copulation group, there was no significant relation between duration of copulation and sperm number in the female uterus externus (r_s = −0.211, p = 0.585) and in the bulbs (r_s = 0.161, p = 0.678). Thus, these results show that the number of sperm transferred by males and stored by females does not depend on the duration of the copulatory phases (I or II). Statistics of duration of phases I and II are shown in Table 3.

Discussion

H. pluchei males exposed to complete and interrupted copulations transfer almost all their stored sperm in their pedipalps bulbs, while virgin females store approximately a quarter. It is important to highlight that there are no previous studies that investigate sperm counts in male and female haplogynae spiders in the Synspermiata clade. Hence, we compare our data with those of entelegynes which have been analyzed in detail. For example, Snow and Andrade (2004) found that the amount of sperm transferred in *Latrodectus hasselti* (Theridiidae) depended on whether they were cannibalized (90% of sperm supply) or not (78% of sperm supply). Bukowski and Christenson (1997) observed a similar pattern in *Micrathena gracilis* (Araneidae), whose complete insemination is produced by two consecutive copulations separated when the male dismounts from his female mate. The latter authors found that males transfer 85% of the sperm stored in the bulbs, while females only stored 20% of that quantity when only one spermathecae is inseminated (females with one copulation). If the female copulates once again (thus, securing insemination for her second spermathecae), the quantity of sperm stored in the first spermathecae increases by 60% compared to the proportion stored after the first copulation. Thus, *M. gracilis* males transfer a percentage of sperm that is considerably high when copulating with virgin females, which is expected in spiders with first male sperm priority. Similarly, *H. pluchei,* males may transfer almost all their sperm to virgin females to ensure a higher percentage of paternity. This great inversion may be used to compensate the likely sperm removal by males in subsequent female matings so that some sperm of the first male still remain in the spermathecae (Calbacho-Rosa et al. 2013). Still, it is not clear why females store around 20% of the total transferred sperm. Interestingly, Bukowski and Christenson (1997) found that a second copulation facilitates the storage of sperm initially transferred by a first male by

Table 2 Maximum and minimum values of sperm number in male bulbs as well as in the uterus externus of females with complete and interrupted copulation

	Complete copulation (Max-Min)	Interrupted copulation (Max-Min)	Control group (non-copulating) (Max-Min)
Spermatozoa number in male bulbs	38,437–0	48,750–0	276,750–18,563
Spermatozoa number stored in female uterus externus	58,500–16,687	53,812–11,812	

Table 3 Duration (in min) of phase I in the complete and interrupted copulation groups, and phase II of the complete copulation group

	Mean ± STD	Max	Min
Duration of phase I (complete copulation)	13.11 ± 5.70	30.07	4.58
Duration of phase II (complete copulation)	14.28 ± 10.24	38.66	2.18
Duration of phase I (Interrupted copulation)	13.38 ± 3.02	18.95	9.82

means of a potential stimulation. This could facilitate the transport of sperm (transferred during the first copulation) from the sperm duct to the spermathecae. Conversely, male stimulation would avoid the loss of sperm from the spermathecae. However, it is not possible that stimulation by *H. pluchei* males induces a female to move the sperm to the storage cavity, since unlike entelegyne spiders, *H. pluchei* males have access and deposit their sperm directly into the female uterus externus (Huber 1995; Calbacho-Rosa et al. 2013). On the other hand, it is less feasible that male stimulation prevents a sperm retreat from the female storage cavity. The reason for this is that uterine gland's secretions may be involved in preventing the loss of sperm from the female genitalia as it has been documented in *P. phalangioides* (Uhl 1994, 1996).

It is possible that the low percentage of female storage in *H. pluchei* are due to other causes. First, once the copulation ends, part of the sperm transferred could be lost when the male disengages from the female genitalia. In fact, sperm have been observed on the male pedipalps (e.g. over the procursus and apophysis) after both sexes decouple in our study species (Cargnelutti F, Calbacho-Rosa L and Peretti AV, unpub. data) as well as in *Physocyclus* species, such as *P. globosus* and *P. dugesi, Mesabolivar globulosus* and *Psilochorus conjunctus* (Peretti AV unpub. data). Conversely, females can use several mechanisms to manipulate the sperm they store (for a detailed list of this mechanism see Eberhard 1996). In fact, Albo and Costa (2017) found that in the wolf spider *Schizocosa malitiosa* (Lycosidae) females stored less than 20% of the sperm transfer by males, which led authors to suggest that in these species such storing pattern would be explained by female control over spermatozoa during or after mating. However, the precise mechanism of such female control remains unclear.

The simultaneous pedipalp movements made by *H. pluchei* males during copulatory phase I include strong squeezes and torsion movements (Huber 1995). Calbacho-Rosa and collaborators (2013) proposed that one function of simultaneous pedipalp movements implies sperm transfer. Our observations support this idea, since our results show no differences in the number of sperm in the bulbs after copulating between complete and interrupted copulation. Given this, we can conclude that sperm transfer - when copulating with virgin females - occurs during the simultaneous pedipalp movements (copulatory phase I) but not when pedipalps are still (copulatory phase II). Sperm transfer in spider could be produced by the entrance of fluid in the lumen of the sperm duct (secreted by glands in the bulbs). However, this explanation is incomplete (Eberhard and Huber 2010). Also, females that copulated with males that performed both copulatory phases (complete copulation group) stored more sperm than those females belonging to the interrupted copulation group. One possibility is that males use copulatory phase II to stimulate females and when this stimulus is not perceived, females cryptically discard

the sperm of such males. In other pholcids like *P. globosus* the rhythmic pedipalp movements could be related to cryptic female choice by stimulation rather than with sperm transfer (Huber and Eberhard 1997) and there is evidence that *P. globosus* females eject sperm depending on their copulatory courtship (Peretti and Eberhard 2010). Another possibility would be that by interrupting copulation before it ends naturally, part of the sperm is removed by the male's pedipalps. More studies are needed to establish the exact mechanism by which this difference would occur in the amount of sperm stored by the females in the two experimental groups. Interestingly, we found that larger females in both experimental groups stored more sperm than smaller females. A possible explanation is that the uterus externus of *H. pluchei* is not a rigid structure so that it is possible that in larger females it may expand more than in smaller females, allowing for greater storage of sperm.

The duration of copulatory phases I and II are not related to the sperm number present in females and males after complete copulations. Similarly, sperm number present in females and males after interrupted copulations did not show evidence of a relation with respect to copula duration. These results echo those observed in other spiders (Bukowski and Christenson 1997; Bukowski et al. 2001; Snow and Andrade 2004; Danielson-François and Bukowski 2005; Danielson-François 2006; Linn et al. 2007). However, this is not a general rule, and some spiders like *Pisaura mirablis* (Pisauridae) (Albo et al. 2013), *Argiope bruennichi* (Araneidae) (Schneider et al. 2006) and *A. aurantia* (Araneidae) (Assis and Foellmer 2016) show a positive relation between duration copulation and sperm transfer. Considering the results obtained, the question then arises as which other functions could be related to copulation apart from sperm transfer.

From a sexual selection perspective, four hypotheses have been put forward to explain long copulations: 1) sperm displacement (Siva-Jothy 1987; Siva-Jothy and Tsubaki 1989), 2) sperm loading (Dickinson 1986; Parker et al. 1990), 3) mate-guarding (Alcock 1994), and 4) cryptic female choice (Eberhard 1996; Peretti and Aisenberg 2015). First, we can rule out the hypothesis of sperm removal since we used virgin females and when copulating, no patterns of alternated pedipalp movements were observed related to sperm displacement nor the sperm release from the females genital opening (Calbacho-Rosa et al. 2013). Second, the sperm loading hypothesis suggests that the longer the copula, the greater the amount of sperm transferred. Nevertheless, the duration of the copulatory phases is not related to the number of sperm transferred. Third, the mate guarding hypothesis suggests that males prolong copulation to guard the female. There is indeed evidence in *H. pluchei* that males guard females after copulation (Calbacho-Rosa et al. 2010). We cannot discard that male may guard his mating, especially during copulatory phase II when it remains attached to the female genitalia without any noticeable pedipalp movement. Fourth, the cryptic female choice hypothesis suggests that an extended copula duration provides more intense female stimulation by males (Eberhard 1991, 1994, 1996). Szirányi et al. (2005) suggest that after transferring enough sperm to secure fertilization, *Pardosa agrestis* (Lycosidae) males could use the last phase of copulation to court his female mate. In *H. pluchei*, it is possible that the different copulatory phases may serve as a form of copulatory courtship as well. Although pedipalp movements were not observed during phase II, there may be subtle movements of the male genitalia that are not easy to observe which may stimulate the female (Eberhard 1996). In fact, as discussed above,

that the females of the complete copulation group store more sperm than the females of the interrupted copulation group could support the hypothesis that the copulatory phase II is used to stimulate females. However, whether *H. pluchei* males stimulate females as a form of cryptic female choice awaits for a proper test.

Acknowledgements We thank Camilo I. Mattoni, Alejandra Ceballos and Matias Izquierdo for their useful comments to previous versions of this paper. We also thank Fedra Bollatti, Macarena González, David Vrech, Paola Olivero and Natalia Rivetti for their help during collecting trips. We also thank the Jardín Zoológico de Córdoba and the Universidad Nacional de Córdoba for allowing us to collect the samples. Mathias Foellmer and an anonymous referee provided helpful and constructive comments which improved the manuscript. Financial support was provided by the Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Fondo para la Investigación Científica y Tecnológica (FONCYT) and Secretaría de Ciencia y Tecnología de la Universidad Nacional de Córdoba (SECYT).

Compliance with Ethical Standards This study was conducted in compliance with the "Guidelines for the use of animals in research" as published in Animal Behaviour (1991, 41, 183–186), and with the laws of the country where the research was conducted.

Conflict of Interest The authors declare that they have no conflict of interest.

References

Aisenberg A, Costa FG (2005) Females mated without sperm transfer maintain high sexual receptivity in the wolf spider *Schizocosa malitiosa*. Ethology 111:545–558

Albo MJ, Costa FG (2017) Female wolf spiders exert cryptic control drastically reducing ejaculate size. Ethology 123:659–666

Albo MJ, Peretti AV (2015) Worthless and nutritive nuptial gifts: mating duration, sperm stored and potential female decisions in spiders. PLoS One 10:e0129453

Albo MJ, Bilde T, Uhl G (2013) Sperm storage mediated by cryptic female choice for nuptial gifts. Proc R Soc Biol Sci Ser B 280:20131735

Alcock J (1994) Post-insemination associations between male and female insects: the mate-guarding hypothesis. Annu Rev Entomol 39:1–21

Assis BA, Foellmer MW (2016) One size fits all? Determinants of sperm transfer in a highly dimorphic orb-web spider. J Evol Biol 29:1106–1120

Blumstein DT, Evans CS, Daniel JC (2000) JWatcher. http://galliform.psy.mq.edu.au/jwatcher/

Bukowski TC, Christenson TR (1997) Determinants of sperm release and storage in a spiny orbweaving spider. Anim Behav 53:381–395

Bukowski TC, Linn CD, Christenson TR (2001) Copulation and sperm release in *Gasteracantha cancriformis* (Araneae: Araneidae): differential male behaviour based on female mating history. Anim Behav 62:887–895

Calbacho-Rosa L, Peretti AV (2015) Copulatory and post-copulatory sexual selection in haplogyne spiders, with enphasis on pholcidae and oonopidae. In: Peretti AV, Aisenberg A (eds) Cryptic female choice in arthropods: patterns, mechanisms and prospects. Springer, New York, pp 109–137

Calbacho-Rosa L, Córdoba-Aguilar A, Peretti AV (2010) Occurrence and duration of post-copulatory mate guarding in a spider with last sperm precedence. Behaviour 147:1267–1283

Calbacho-Rosa L, Galicia-Mendoza I, Dutto MS, Córdoba-Aguilar A, Peretti AV (2013) Copulatory behavior in a pholcid spider: males use specialized genitalic movements for sperm removal and copulatory courtship. Naturwissenschaften 100:407–416

Chapman T, Davies SJ (2004) Functions and analysis of the seminal fluid proteins of male Drosophila melanogaster fruit flies. Peptides 25:1477–1490

Cordero C (1995) Ejaculate substances that affect female insect reproductive physiology and behavior: honest or arbitrary traits? J Theor Biol 174:453–461

Cordero-Rivera A (2017) Sexual conflict and the evolution of genitalia: male damselflies remove more sperm when mating with a heterospecific female. Sci Rep 7:7844

Costa FG (1979) Análisis de la cópula y de la actividad postcopulatoria de *Lycosa malitiosa* Tullgren (Araneae, Lycosidae). Rev Bras Biol 39:361–376

Costa FG, Toscano-Gadea CA (2003) Experimental interruption and re-initiation of mating in a wolf spider: an analysis of behavioural patterns and resultant progeny. Ethol Ecol Evol 15:173–181

Danielson-François AM (2006) Natural history of *Glenognatha emertoni* (Araneae, Tetragnathidae): mating behavior and sperm release in a haplogyne. J Arachnol 34:387–398

Danielson-François AM, Bukowski TC (2005) Female mating history influences copulation behavior but not sperm release in the orb-weaving spider *Tetragnatha versicolor* (Araneae, Tetragnathidae). J Insect Behav 18:131–148

Dickinson JL (1986) Prolonged mating in the milkweed leaf beetle *Labidomera clivicollis* (Coleoptera: Chrysomelidae): a test of "sperm-loading" hypothesis. Behav Ecol Sociobiol 18:331–338

Eberhard WG (1991) Copulatory courtship and cryptic female choice in insects. Biol Rev 66:1–31

Eberhard WG (1994) Evidence for widespread courtship during copulation in 131 species of insects and spiders, and implications for cryptic female choice. Evolution 48:711–733

Eberhard WG (1996) Female control: sexual selection by cryptic female choice. Princeton University Press, Princeton

Eberhard WG (2004) Why study spider sex: special traits of spiders facilitate studies of sperm competition and cryptic female choice. J Arachnol 32:545–556

Eberhard WG (2010) Rapid divergent evolution of genitalia: therory and data updated. In: Leonard JL, Cordoba-Aguilar A (eds) The evolution of primary sexual characters in animals. Oxford University Press, New York, pp 40–78

Eberhard WG, Huber BA (2010) Spider genitalia: precise maneuvers with a numb structure in a complex lock. In: Leonard JL, Cordoba-Aguilar A (eds) The evolution of primary sexual characters in animals. Oxford University Press, New York, pp 249–284

Elgar MA (1995) The duration of copulation in spider: comparative patterns. Rec West Aust Mus Suppl 52:1–11

Foelix RF (2011) Biology of spiders, 3rd edn. Oxford Univerity Press, New York

Gabel E, Uhl G (2013) How to prepare spider sperm for quantification. Arachnology 16:109–112

Herberstein ME, Wignall A (2011) Introduction: spider biology. In: Herberstein ME (ed) Spider behaviour, flexibility and versatility. Cambridge Univerity Press, New York, pp 1–21

Hosken DJ, Stockley P (2004) Sexual selection and genital evolution. Trends Ecol Evol 19:87–93

Huber BA (1995) Copulatory mechanism in *Holocnemus pluchei* and *Pholcus opilionoides*, with notes on male cheliceral apophyses and stridulatory organs in Pholcidae (Araneae). Acta Zool (Stockh) 76:291–300

Huber BA (2000) New World pholcid spiders (Araneae: Pholcidae): a revision at generic level. Bull Am Mus Nat Hist 254:1–348

Huber BA (2014) Pholcidae. In: Claps LE, Morrone JJ (eds) Roig-Juñet S. Biodiversidad de artropodos argentinos Editorial INSUE-UNT, San Miguel de Tucumán, pp 131–141

Huber BA, Eberhard WG (1997) Courtship, copulation, and genital mechanics in *Physocyclus globosus* (Araneae, Pholcidae). Can J Zool 74:905–918

Jakob EM (1991) Costs and benefits of group living for pholcid spiderlings: losing food, saving silk. Anim Behav 48:482–484

Kamimura Y (2005) Last-male paternity of Euborellia plebeja, an earwig with elongated genitalia and sperm-removal behavior. J Ethol 23:35–41

Kaster JL, Jakob EM (1997) Last-male sperm priority in a haplogyne spider (Araneae: Pholcidae): correlations between female morphology and patterns of sperm usage. Ann Entomol Soc Am 90:254–259

Kelly DA, Moore BC (2016) The morphological diversity of intromittent organs: an introduction to the symposium. Integr Comp Biol 56:630–634

Laborda A, Simó M (2008) First south American records of *Holocnemus pluchei* (Scopoli, 1763) and *Spermophora senoculata* (Duges, 1836) (Araneae: Pholcidae). Ganaya 72:261–265

Leonard J, Córdoba-Aguilar A (2010) The evolution of primary sexual characters in animals. Oxford University Press, New York

Linn CD, Molina Y, Diffata J, Christenson TE (2007) The adaptive advantage of prolonged mating: a test of alternative hypotheses. Anim Behav 74:481–485

Parker GA, Simmons LW, Kirk H (1990) Analyzing sperm competition data: simple models for predicting mechanisms. Behav Ecol Sociobiol 27:55–65

Peretti AV, Aisenberg A (2015) Cryptic female choice in arthropods: patterns, mechanisms and prospects. Springer, New York

Peretti AV, Eberhard WG (2010) Cryptic female choice via sperm dumping favours male copulatory courtship in a spider. J Evol Biol 23:271–281

Peretti AV, Eberhard WG, Briceño RD (2006) Copulatory dialogue: female spiders sing during copulation to influence male genitalic movements. Anim Behav 72:413–421
Porter AH, Jakob EM (1990) Allozime variation in the introduced spider *Holocnemus pluchei* (Araneae: Pholcidae) in California. J Arachnol 18:313–319
R Core Team (2016) R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL https://www.R-project.org/
Schäfer MA, Uhl G (2002) Determinants of paternity success in the *Pholcus phalangioides* (Pholcidae, Araneae): the role of male and female mating behaviour. Behav Ecol Sociobiol 51:368–377
Schneider JM, Gilberg S, Fromhage L, Uhl G (2006) Sexual conflict over copulation duration in a cannibalistic spider. Anim Behav 71:781–788
Schöfl G, Taborsky M (2002) Prolonged tandem formation in firebugs (Pyrrhocoris apterus) serves mate-guarding. Behav Ecol Sociobiol 52:426–433
Simmons LW (2001) Sperm competition and its evolutionary consequences in the insects. Princeton University Press, New Jersey
Simmons LW (2014) Sexual selection and genital evolution. Austral Entomol 53:1–17
Siva-Jothy MT (1987) The structure and function of the female sperm-storage organs in libellulid dragonflies. J Insect Physiol 33:559–567
Siva-Jothy MT, Tsubaki Y (1989) Variation in copulation duration in *Mnais pruinosa pruinosa* Selys (Odonata: Calopterygidae), I: alternative mate-securing tactics and sperm precedence. Behav Ecol Sociobiol 24:39–45
Snow LS, Andrade MC (2004) Pattern of sperm transfer in redback spiders: implications for sperm competition and male sacrifice. Behav Ecol 15(5):785–792
Szirányi A, Kiss B, Samu F, Harand H (2005) The function of long copulation in the wolf spider *Pardosa agrestis* (Araneae, Lycosidae) investigated in a controlled copulation duration experiment. J Arachnol 33:408–414
Uhl G (1994) Genital morphology and sperm storage in *Pholcus phalangioides* (Fuesslin, 1775) (Pholcidae, Aranae). Acta Zool 75:1–12
Uhl G (1996) Sperm storage secretion of female cellar spiders (*Pholcus phalangioides*; Araneae): a gel-electrophoretic analysis. J Zool 240:153–161
Uhl G, Nessler SH, Schneider JM (2010) Securing paternity in spiders? A review on occurrence and effects of mating plugs and male genital mutilation. Genetica 138:75–104
Uhl G, Kunz K, Vöcking O, Lipke E (2014) A spider mating plug: origin and constraints of production. Biol J Linn Soc 113:345–354
Weldingh DL, Toft S, Larsen ON (2011) Mating duration and sperm precedence in the spider *Linyphia triangularis*. J Ethol 29:143–152

Affiliations

Franco Cargnelutti [1,2] · Lucia Calbacho-Rosa [1,2] · Alex Córdoba-Aguilar [3] · Alfredo V. Peretti [1,2]

[1] Facultad de Ciencias Exactas, Físicas y Naturales, Departamento de Diversidad Biológica y Ecología, Universidad Nacional de Córdoba, Córdoba, Argentina

[2] Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Laboratorio de Biología Reproductiva y Evolución, Instituto de Diversidad y Ecología Animal (IDEA), Córdoba, Argentina

[3] Departamento de Ecología Evolutiva, Instituto de Ecología, Universidad Nacional Autónoma de México, Apdo. Postal 70-275, Ciudad Universitaria, 04510 Distrito Federal, Mexico