

Cryptic female choice via sperm dumping favours male copulatory courtship in a spider

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Abstract

Males of many animals perform ‘copulatory courtship’ during copulation, but the possible reproductive significance of this behaviour has seldom been investigated. In some animals, including the spider *Physocyclus globosus* (Pholcidae), the female discards sperm during or immediately following some copulations. In this study, we determined which of several variables associated with copulation correlated with paternity success in *P. globosus* when two males mate with a single female. Then, by determining which of these variables also correlated with sperm dumping, we inferred which variables may affect paternity via the mechanism of sperm dumping. Male abdomen vibration (a copulatory courtship behaviour) and male genitalic squeezing both correlated with both paternity and sperm dumping; so, these traits may be favoured by biased sperm dumping. Biased sperm dumping may also be the mechanism by which possible cryptic female choice favours another male trait that was the subject of a previous study, responsiveness to female stridulation.

Introduction

Competition between males with respect to sperm that have been introduced into a female’s body can be intense (Parker, 1970; Eberhard, 1996; Birkhead & Møller, 1997; Simmons, 2001). One way a male may be capable of prevailing in this competition is by courting the female during copulation (copulatory courtship) (Eberhard, 1991, 1994) and thereby inducing her to use his sperm. Males of hundreds of species of animals perform non-genital behaviour during copulation that appears to be courtship; this behaviour includes biting, tapping, rubbing, squeezing, shaking, vibrating, singing to and feeding the female (Eberhard, 1991, 1994, 1996). Such behaviour has been thought to function to induce responses in female reproductive behaviour and physiology that increase the chances that the performing male’s sperm will fertilize her eggs, rather than sperm from other males (Eberhard, 1996). This interpretation has depended, however, almost exclusively on

deductions based on the design of the male behaviour (Eberhard, 1994). There have been few studies that directly tested the possibility that copulatory courtship affects paternity. Demonstrated effects in insects include decreased female mobility during copulation (Humphries, 1967), increased dumping of sperm from previous males (Otronen, 1990; Otronen & Siva-Jothy, 1991), increased copulation duration (Hoikkala & Crossley, 2000; Hoikkala *et al.*, 2000), increased sperm precedence (Edvardsson & Arnqvist, 2000), relaxation of bursal muscles allowing the male to penetrate deeper and to transfer sperm (Tallamy *et al.*, 2003), increased resistance to subsequent mating (King & Fischer, 2005) and increased oviposition (Barbosa, 2009). Cuatianquiz & Cordero (2006) found a possible effect on oviposition, whereas an incomplete survey of possible female responses failed to reveal any effects in a water strider (Arnqvist & Danielsson, 1999).

One possible mechanism of cryptic female choice (CFC), physical ejection of sperm by the female (sperm dumping), has been observed in numerous species (summary Eberhard, 1996; additional cases in Pizzari & Birkhead, 2000; Snook & Hosken, 2004). Sperm dumping occurred in 25% of 53 species of insects and spiders in which it was specifically searched for

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(Eberhard, 1994). In some species the discarded sperm were from previous males (Otronen & Siva-Jothy, 1991; Snook & Hosken, 2004; Burger, 2007), whereas in others they were from the copulating male (summary in Eberhard, 1996; see also Huber & Eberhard, 1997; Pizzari & Birkhead, 2000; Rodríguez *et al.*, 2004).

If sperm dumping is simply be due to the male having transferred a larger ejaculate than can be accommodated within the female's reproductive tract, as suggested by Rehfeld & Sudhaus (1985) and Hass (1990), it may not have reproductive consequences for the male. Alternatively, sperm dumping may result in a reduction in the male's chances of paternity, if his success in fertilization depends on the amount of his sperm that remains within the female. Most previous studies of sperm dumping have assumed that there is a negative effect of sperm dumping on paternity, without supporting data (for exceptions, see Otronen & Siva-Jothy, 1991; Rodríguez *et al.*, 2004; Snook & Hosken, 2004; in all of these the effect of dumping was negative, as expected).

If sperm dumping affects paternity, it can only exercise sexual selection by CFC if two additional conditions are fulfilled: the proportion of sperm discarded must be correlated with a male trait (such as copulatory courtship); and females must normally sometimes mate with more than a single male. Convincing associations of sperm dumping with particular male traits have been established in only four species; the traits include male social dominance (Davies, 1992; Pizzari & Birkhead, 2000) and the sizes and movements of genital sclerites (Rodríguez *et al.*, 2004; Córdoba-Aguilar, 2006). Reduced sperm dumping may also be associated with female orgasm in humans, which in turn may be influenced by traits of the male (Baker & Bellis, 1995), but some doubt remains (Birkhead, 2000) and paternity data are lacking. The reproductive consequences of sperm dumping for the male were established directly in only one of these species, the tortoise beetle *Chelymorpha alternans*. To date there are no studies documenting the effects of nongenital male copulatory courtship behaviour on sperm dumping.

Females of the spider *Physocyclus globosus* (Pholcidae) often emit a mass containing sperm (Fig. 1) (hereafter the discarded mass) during or immediately following copulation (Huber & Eberhard, 1997). As in other spiders, each individual sperm cell is encapsulated when transferred to the female, and the encapsulated cells are in a liquid seminal matrix. In another spider of this family, *Pholcus phalangioides*, the female produces a second matrix in which the encapsulated sperm are embedded during or soon after transfer to the female (Uhl, 1994), but such a matrix was absent in sections of a *P. globosus* female (B. Huber & W. Eberhard, unpublished data). A study of genital morphology and copulation behaviour (Huber & Eberhard, 1997) indicated that sperm dumping is not simply removal by the male, and instead almost certainly involves active participation by

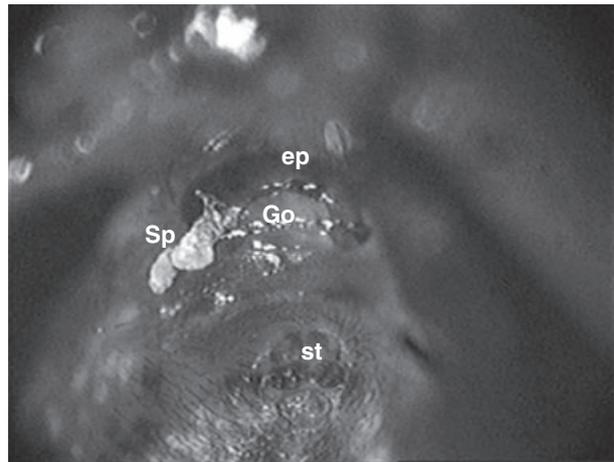


Fig. 1 Ventral surface of abdomen of female *Physocyclus globosus*, showing the sperm mass (Sp) that was expelled from her gonopore (Go); also labelled are her genital plate or epigynum (ep) and her spinnerets (st).

the female (Huber & Eberhard, 1997): the positions and movements of the male's genitalia within the female with respect to the site in the female where sperm are stored are not appropriate to remove stored sperm; male genitalia are not withdrawn during copulation (as in other groups such as odonates – Waage, 1984) and thus cannot pull sperm out of the female during the time period when much sperm is dumped; sperm are emitted in copulations with virgin females; sperm ejection involves active expelling movements of the female's genital opening; and sperm ejection sometimes occurs 60 s or more after copulation has ended. Removal of sperm by the female is morphologically feasible because the male apparently deposits his sperm in a liquid matrix, as indicated by the thin tubular design of his transfer structure, the embolus (Huber & Eberhard, 1997). Contractions of the vaginal walls similar to those that are presumably used during oviposition could move this liquid or viscous mass of material out through the female's gonopore.

Genital behaviour during copulation in *P. globosus* is complex, and includes strong squeezing and twisting movements ('palpal squeezes' hereafter) of the massively muscled, paired male palps. Palpal squeezes also result in thrusting movements of a strong, rod-like process, the procurus. The two palpal procuri couple mechanically so that the thrusting force of one is transferred to the pointed tip of the other; their combined force thrusts this tip against the valve at the inner end of the female's vagina leading to the common oviduct (Huber & Eberhard, 1997). The valve is slightly 'upstream' of the site in the female's reproductive tract where sperm are thought to be stored (Uhl, 1994). These powerful squeezing movements also compress the portion of the female body that lies between the dorsal wall of the

female's vagina and the epigynum on the ventral surface of her abdomen, squeezing it between the male's procurci (which are inside the vagina) and the elaborately sculptured anterior surfaces of his chelicerae (which press against the outer surface of the epigynum) (Huber & Eberhard, 1997). The procurcus is not connected with the sperm duct (Huber & Eberhard, 1997); so, although these movements almost surely stimulate the female (Peretti *et al.*, 2006), they are not directly involved in sperm transfer. Lateral movements of other palpal sclerites (including the embolus) also spread the walls of the vagina.

Polyandry of female *P. globosus* in nature is suggested by several observations. Males in the field change sites frequently, and generally stay with a particular female for only 1 day (Eberhard, 1992; Eberhard *et al.*, 1993). Females are visited by multiple males in the field, live many months in captivity (mature females of the congeneric *P. simonii* live for several years – Bristowe, 1958). Assuming that visits from multiple males result in females mating multiply, as seems likely in the light of the high level of receptivity to remating observed in this study (58 of 60 females remated when presented with a second male), females probably mate repeatedly in nature. When a female in captivity mated with two males, paternity averaged approximately 50% for each (Eberhard *et al.*, 1993), but percentages varied widely; in a sample of only 11 females the values for the first male ranged from 0% to 100%. In agreement with the lack of consistent sperm precedence favouring first or last males, males in the field showed no tendency to prefer to associate with females that were about to oviposit, and only a weak tendency to associate with penultimate female nymphs about to moult to maturity as occurs in some other spiders (Eberhard *et al.*, 1993).

Two biases in sperm use have been demonstrated in *P. globosus*: males that relaxed their palpal squeezes more consistently in response to female stridulation during copulation achieved on average about three times greater paternity; and greater numbers of palpal squeezes correlated with greater paternity (Peretti *et al.*, 2006). The mechanism by which these biases were achieved was not determined. A qualitative measure of sperm dumping showed no relation to paternity (Peretti *et al.*, 2006), but dumping was not analysed quantitatively. In the present study, we determine which of several variables associated with copulation affect paternity in *P. globosus*; then, by determining which of these variables were also associated with larger discarded masses with sperm, we infer which variables probably affected paternity via the CFC mechanism of sperm dumping.

Materials and methods

Each of 60 virgin females was raised to maturity in captivity and mated to two males that were also raised to maturity in captivity. Each animal was kept at room

temperature in a separate plastic cup (11 cm diameter) and fed twice a week with adult *Drosophila melanogaster*. Mating followed the protocols of Huber & Eberhard (1997) and Peretti *et al.* (2006). The first mating occurred 5–7 days after the female's final moult, and 24 h after she had built a web in the mating arena. The first male was removed soon after mating, and a second male was introduced the next day. All females accepted the first mating, and only two (which were excluded from this study) refused the second male. Only males that had moulted to maturity < 150 days previously were used, as the copulation behaviour of males > 250 days old changed in another pholcid, *P. phalangioides* (Schäfer & Uhl, 2002; Schäfer *et al.*, 2008). Male age during these first 150 days had no effects in one-by-one Spearman tests of correlation with the male behaviour variables, and was not included in the analyses. The sizes of males and females were estimated later by measuring the width of the cephalothorax in 80% ethanol under a dissecting microscope. Some (40) of the pairs in this study formed part of a previous study (Peretti *et al.*, 2006), whereas 20 additional pairs are included here (of the 68 pairs of the previous study, the volume of discarded sperm masses was measured in only 40). All mean values are followed by ± 1 SD, and all statistical tests employed $\alpha = 0.05$.

Each copulation was taped in its entirety using a video camera coupled to a dissecting microscope, allowing close-up views of movements of the male's pedipalps in the female's genital opening, his abdomen and of at least one of the female's pedipalps. Male behaviour patterns recorded during copulation included his long, rhythmic twisting squeezes of the female's genital area with his pedipalps, and bursts of rapid dorso-ventral vibrations of his abdomen (Huber & Eberhard, 1997). Events were transcribed from video tapes using ETHOLOG 2.2 (© 1999 E. B. Ottoni), and JWATCHER 0.9 (© 2000 D. T. Blumstein, C. S. Evans and J. C. Daniel). The female was observed for several minutes following copulation to check to post-copulatory emission of sperm. We judged whether the male or the female initiated termination of copulation by determining which animal began to pull away from the other first as copulation ended.

Paternity was determined by mating each female with one fertile male and one male sterilized with 50 Gy (5 krad) radiation, a dosage previously found to be appropriate (Eberhard *et al.*, 1993). Each male was used only once, and only the female's first clutch was used; the order of mating (sterile–fertile) alternated for successive females. Males were not virgin, but all previous copulations occurred at least 20 days prior to the copulations in this study. The fertility of the eggs in the clutch was checked > 10 days after oviposition, when fertile eggs had transformed to pre-embryos. Clutches that were analysed for paternity contained a mean of 30.6 ± 10.5 eggs. Control females which had mated only with a fertile male laid nearly all fertile eggs (98.2% of 277 eggs in 11 clutches; maximum number of infertile eggs/clutch = 1);

the eggs of females mated only with a sterilized male were all infertile (215 eggs in 9 clutches). Paternity success was therefore quantified directly as the fraction of fertile or infertile eggs. As in previous studies of this species (Eberhard *et al.*, 1993; Peretti *et al.*, 2006), there was no significant difference in the paternity success of fertile and sterilized males ($51.7 \pm 35.6\%$ vs. $48.1 \pm 39.2\%$; Mann–Whitney *U*-test: $Z = 0.721$, $P = 0.34$, $N_1 = N_2 = 58$). Female stridulation was quantified as in previous studies as the number of bursts of stridulatory movements of her palps against her chelicerae (Peretti *et al.*, 2006). A male's 'responsiveness' to female stridulation was quantified as the fraction of the times that he relaxed his squeeze on the female's genitalia with his pedipalps within 2 s after the female began to stridulate.

Estimates of the volume of sperm dumped by the female employed a technique used in studies of other animals (Siva-Jothy & Hooper, 1995; Córdoba-Aguilar, 1999, 2006). The discarded mass was transferred with fine forceps to a glass slide where it was covered and partially squashed with a cover slip; small pieces of 0.10-mm-thick glass placed previously on the slide kept the cover slip 0.10 mm from the slide. The mass was then photographed under a compound microscope, and IMAGE TOOL 2.0 was used to determine its area in mm^2 ; multiplying this area by 0.10 mm gave the mass's estimated volume ($1 \mu\text{L} = 1 \text{mm}^3$). Each mass was stained with acetocarmine, a DNA stain, to verify that it contained sperm, but sperm densities were too high for accurate cell counts. In the descriptions below, the phrase 'volume of the discarded sperm mass' refers to the volume of the entire mass.

Statistical analyses

The volume of the discarded sperm mass was analysed as a continuous variable. Multiple linear regressions (MLR) of log-transformed data (in which normal distributions were confirmed) were employed, selecting the variables in the final model that best explained the variation. The robustness of the model was tested using a forward and backward stepwise selection procedure, with the inclusion probability set to 0.05 and the exclusion probability set to 0.10 (Schäfer *et al.*, 2008). The program removed variables one by one on the basis of low *F*-values, then checked to see if another variable could be included in the resulting model. Because of the large number of independent variables, three independent multivariate analyses were performed after variables had been grouped, mostly according to biological similarity (e.g. morphological variables; behavioural characteristics of the first male; behavioural characteristics of the second male) (see the Results section). Paternity values (% of offspring sired) were arcsine transformed for all analyses. As in previous studies of this species (Eberhard *et al.*, 1993; Peretti *et al.*, 2006), there was no effect of mating

order on paternity (first male 0.55 ± 0.37 , second male 0.47 ± 0.34 , $Z = -0.91$, $P = 0.34$ with Mann–Whitney *U*-test); so, mating order was not included in the analyses.

Results

Description of sperm dumping

Sperm emerged in an irregular white mass from the female's genitalia (Fig. 1) near the end of some copulations or during the minute or so immediately following. The mass remained there briefly, and eventually either fell, or adhered to the lateral surface of one of the male's palps and was then removed and subsequently fell to the ground when the palp was withdrawn. Mention below of sperm discarded 'during' copulation refers to sperm discarded both during and immediately following copulation. Sperm was discarded in fewer first copulations (25.9%), than in second copulations (51.7%; $\chi^2 = 8.17$, d.f. = 1, $P = 0.004$). Discarded masses in first copulations seemed to usually have a more liquid consistency than those in second copulations, but both types squashed readily when mounted on slides. Discarded masses that emerged during first copulations were smaller than those discarded during second copulations (mean values were 0.0055 ± 0.0048 and $0.019 \pm 0.014 \mu\text{L}$ respectively; Mann–Whitney *U*-test: $U = 66.5$; $Z = -2.383$, $P = 0.01$). There was no relation between the sizes of the first and second masses discarded by the same female ($r_s = -0.079$, $P = 0.67$).

Variables associated with paternity

The first male's paternity was weakly reduced when the size of the discarded sperm mass in his copulation was larger, and also when the size of the discarded mass in the second copulation was larger (Table 1). Furthermore, paternity for the first male was also lower when the total size of the sperm masses that were discarded in both copulations was greater. Thus, the sizes of discarded sperm masses correlated with paternity. In addition, first male's paternity was reduced when the first copulation was longer relative to the second copulation, when the number of bursts of abdomen vibration by the second male was greater, and when the number of palpal squeezes was relatively smaller in the first compared with the second copulation (Fig. 2a,b, Table 1).

Although male responsiveness did not show a relation with paternity in the general analysis (Table 1), analysis of females in which there was an especially strong difference in the responsiveness of the two males with which they mated showed a positive relation between responsiveness and paternity. In the subset of pairs of males in which the female stridulated in both the first and second copulations and the two males differed in responsiveness by more than 25% (the criterion used in a previous study of male responsiveness, Peretti *et al.*,

Table 1 Summary of generalized linear model (GLM) ANOVA and multiple linear regression analyses of the effects of differences in the variables on the paternity success of the first male.

Variables	GLM ANOVA		Multiple linear regression		
	T-value	Prob. level	$\beta \pm SE$	T-value	P-value
Male size					
First	-1.35	0.16	-	-	> 0.1
Second	-0.32	0.70	-	-	> 0.1
(First - second)	1.12	0.23	-	-	> 0.1
Male size/female size	-0.98	0.40	-	-	> 0.1
Sperm dump					
First	-1.91	0.037	-0.612 \pm 0.236	-1.891	0.041*
Second	-3.89	0.002	-9.873 \pm 2.10	-3.978	0.001
First + second dump	-2.40	0.014	-4.680 \pm 1.141	-2.321	0.031
Duration of copulation	-3.02	0.003	-8.399 \pm 2.131	-3.701	0.002
Palpal squeezes					
Number	3.89	0.001	1.192 \pm 2.159	4.565	0.0006
Rate (x/min)	-0.17	0.97	-	-	> 0.1
Bursts of male abdomen vibration					
First male:					
Number	1.21	0.30	-	-	> 0.1 (0.3)
Rate (x/min)	0.002	0.98	-	-	> 0.1
Second male:					
Number	-2.51	0.012	-4.761 \pm 1.872	-2.625	0.011
Rate (x/min)	-0.05	0.89	-	-	> 0.1
(First male - second male)					
Number	1.44	0.10	-	-	> 0.1
Rate (x/min)	0.72	0.43	-	-	> 0.1
Bursts female stridulation					
Number	-1.19	0.25	-	-	> 0.1
Rate (x/min)	-0.31	0.75	-	-	> 0.1
Male responsiveness to female stridulation	1.11	0.23	-	-	> 0.1

Almost all variables are only first male minus second male values except for male size, second sperm dump and bursts of male abdomen vibration. Final regression model: F -ratio $F_{6,57} = 4.970$, $R^2 = 0.811$, adjusted $R^2 = 0.632$, $P = 0.006$.

*A one-tailed test is probably more appropriate ($P = 0.021$) because the predicted effect of larger discarded masses was a decrease in paternity for the first male, as observed.

2006), the paternity of the more responsive male was nearly three times that of the other (mean values $74.5 \pm 20.1\%$ vs. $25.5 \pm 20.1\%$; paired t -test = 3.52, $P = 0.001$). This confirms a similar finding in a previous study (Peretti *et al.*, 2006); six of the 19 females in this subset of pairs in the present study were also in the previous study.

Variables associated with the size of the discarded mass

First copulation

Variables related to the size of the male, the size of the female, and male responsiveness to female stridulation showed no relation with the size of the discarded mass in the first copulation (Table 2). The size of the discarded mass correlated negatively with the number of bursts of abdomen vibration (Fig. 2c) and the rate of palpal squeezing; it correlated positively with the frequency of bursts of abdomen vibration (Table 2). Greater male size weakly

correlated with greater numbers of bursts of abdomen vibration ($r_s = 0.44$, $P = 0.025$) but not with greater frequency ($r_s = 0.030$, $P = 0.88$) and strongly correlated with lower rates of palpal squeezing ($r_s = -0.63$, $P = 0.0005$). There was no association between male responsiveness and the volume of the discarded sperm mass in the 19 females with large differences in responsiveness between the two males (t -test = 0.89, $P = 0.47$).

Second copulation

Again, variables related to male and female size, and to male responsiveness to female stridulation showed no relation with the size of the discarded mass in the second copulation (Table 3). However, larger discarded masses correlated with greater numbers of bursts of abdomen vibration by the second male, and also with three comparative variables (in which values for the second male were subtracted from those of the first): relatively shorter second copulations, relatively higher numbers of palpal squeezes by the second male (Fig. 2d) and

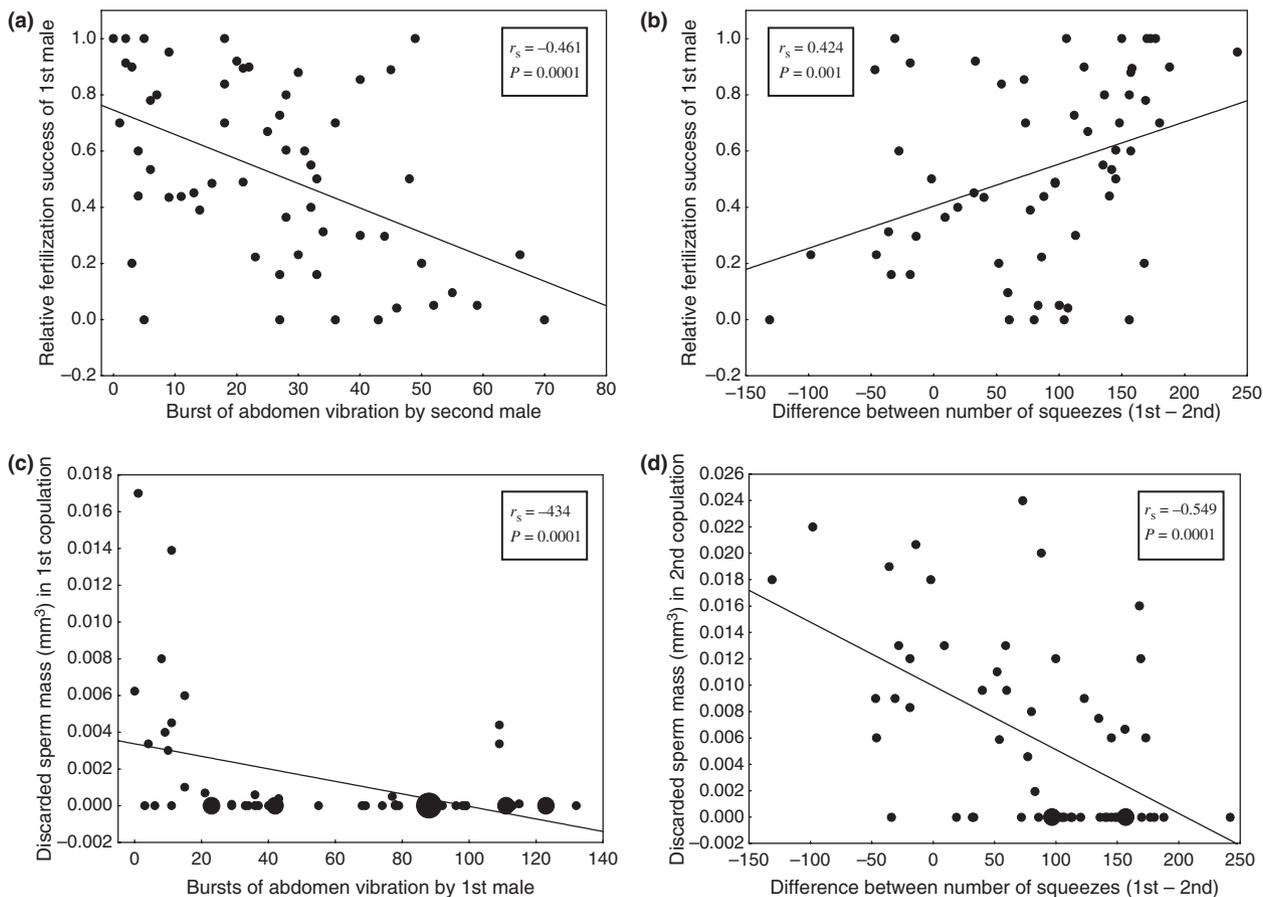


Fig. 2 Relations between male copulatory behaviour and paternity (a, b) and sperm dumping (c, d).

relatively greater numbers of bursts of abdomen vibration by the second male (Table 3).

Analysis of the subset of 19 females in which there were strong differences in the responsiveness of the two

males showed that there was also a positive association for the second of the two males with which the female copulated between the volume of the discarded sperm mass and the male's degree of responsiveness. When the

Variables	$\beta \pm SE$	<i>T</i> -value	<i>P</i> -value
Male size	–	–	> 0.1
Female size	–	–	> 0.1
Male size/female size	–	–	> 0.1
Duration of copulation (min)	–	–	> 0.1
Palp squeezes			
Number	–	–	> 0.1
Rate (×/min)	$-1.64E-03 \pm 4.4E-04$	-3.72	0.001
Bursts of male abdomen vibration			
Number	$-1.01E-04 \pm 3.56E-05$	-2.84	0.009
Rate (×/min)	$2.43E-03 \pm 8.78E-04$	2.76	0.011
Burst of female stridulation			
Number	–	–	> 0.1
Rate (×/min)	–	–	> 0.1
Male responsiveness to female stridulation	–	–	> 0.1

Final regression model: *F*-ratio $F_{3,57} = 4.73$, $R^2 = 0.57$, adjusted $R^2 = 0.45$, $P = 0.003$, power = 0.953.

Table 2 Multiple linear regression report of the effects of morphological traits and behavioural components on the volume of the discarded sperm mass in the first copulation.

Table 3 Multiple lineal regression report of the effects of morphological traits and behavioural components on the amount of sperm dumped in the second copulation.

Variables	$\beta \pm SE$	T-value	P-value
Male size			
First	–*	0.66	0.52
Second	–*	0.052	0.95
(First – second)	–*	–0.27	0.79
Female size	–*	–0.65	0.54
Male size/female size			
First	–*	–0.61	0.55
Second	–*	–0.073	0.94
(First – second)	–*	0.0001	0.99
First sperm dump	–*	0.17	0.86
Duration of copulation (min)			
First	–†	0.38	0.71
Second	–‡	–	> 0.1
(First – second)	2.9E–03 ± 9.5E–04§	3.03	0.008
Palp squeezes number			
First	–†	0.90	0.38
Second	–‡	–	> 0.1
(First – second)	–3.1E–04 ± 1.2E–05§	–2.69	0.01
Rate (x/min)			
First	–†	–0.24	0.81
Second	–‡	–	> 0.1
(First – second)	–§	–	> 0.1
Bursts of male abdomen vibration number			
First	–†	–1.65	0.13
Second	5.7E–04 ± 2.5E–04‡	2.31	0.02
(First – second)	–2.9E–04 ± 1.5E–04§	–2.31	0.014
Rate (x/min)			
First	–†	1.22	0.25
Second	–‡	–	> 0.1
(First – second)	–§	–	> 0.1
Male responsiveness to female stridulation			
First	–†	0.52	0.62
Second	–‡	–	> 0.1
(First – second) (without ties)	–§	–	0.1

*Final regression model (morphology): *F*-ratio $F_{1,59} = 0.126$, $R^2 = 0.099$, adjusted $R^2 = 0.0002$, $P = 0.96$.

†Final regression model (behaviour first male): *F*-ratio $F_{1,58} = 1.55$, $R^2 = 0.56$, adjusted $R^2 = 0.19$, $P = 0.24$.

‡Final regression model (behaviour second male): *F*-ratio $F_{1,58} = 5.357$; $R^2 = 0.219$; adjusted $R^2 = 0.178$; $P = 0.031$.

§Final regression model (difference first – second male behaviour): *F*-ratio $F_{3,56} = 4.932$; $R^2 = 0.387$; adjusted $R^2 = 0.281$; $P = 0.014$.

second male was the more responsive of the two ($N = 11$), the volume discarded during the second copulation was larger (mean $0.011 \pm 0.004 \mu\text{L}$) than when the second male was the less responsive of the two (mean volume $0.0038 \pm 0.0027 \mu\text{L}$, $N = 8$) (*t*-test 2.86 , $P = 0.015$).

Copulation duration

Female termination of copulation (when the female rather than the male initiated the separation that resulted in the end of copulation) occurred in 11 of the 60 first copulations, and in none of the 60-s copulations ($\chi^2 = 16.8$, d.f. = 1, $P < 0.0001$). The first copulations which were terminated by the female were only about half as long (mean 17.9 ± 9.0 min) as those

terminated by the male (31.2 ± 8.3 min; $F = 10.76$, $P = 0.0027$ with one-way ANOVA). Thus, when a female terminated her first copulation, she apparently did so 'prematurely' with respect to when the male would have ended it. Second copulations were much shorter than first copulations (mean 13.1 ± 7.9 vs. 27.3 ± 9.4 min; $t = 7.30$, $P < 0.0001$ with paired *t*-test). Comparing male-terminated first copulations with second copulations, the difference was less striking (17.9 ± 9.0 min vs. 12.9 ± 6.1 ; $t = 1.38$, $P = 0.03$ with two sample *t*-test).

Female as opposed to male termination of the first copulation did not correlate significantly with the volume of the discarded mass (mean values for female- and male-terminated copulations were 0.0012 ± 0.0019 and $0.0020 \pm 0.0043 \mu\text{L}$ respectively; one-way ANOVA

$F = 0.20$, $P = 0.65$). First male's paternity was also not statistically different in female-terminated compared with male-terminated copulations (0.41 ± 0.32 and 0.59 ± 0.38 respectively; one-way ANOVA $F = 1.30$, $P = 0.26$).

Discussion

Paternity

The first male was likely to sire a larger fraction of the female's offspring when the duration of the first copulation was relatively short compared with that of the second; when the sizes of the discarded masses in the first and (especially) second copulations were smaller; when the second male made fewer bursts of abdomen vibration; and when the number of palpal squeezes was relatively larger in the first than in the second copulation. In the subset of females in which there were strong differences between the two males in their responsiveness to female stridulation, greater paternity was also associated with greater male responsiveness. This strong effect, combined with the lack of association between male responsiveness and paternity in the general analysis, suggests a threshold effect of responsiveness on paternity, as seen previously (Peretti *et al.*, 2006).

These correlations imply that the sperm discarded during the first copulation were the sperm transferred during that same copulation, whereas the sperm discarded during the second copulation tended to be sperm from the first copulation. They also indicate that selection due to paternity differences favours two potential male copulatory courtship behaviour patterns (abdomen vibration and palpal squeezing), shorter copulations and a male tendency to relax palpal squeezes when the female stridulates. By examining which of these variables that are associated with paternity were also associated with larger volumes of discarded masses, we infer whether or not the mechanism by which a given variable affected paternity was sperm dumping.

Sperm discarded during the first copulation

Larger discarded masses were weakly associated with reduced paternity for the first male; so, at least some of this discarded sperm would probably have otherwise been used to fertilize eggs. Two aspects of first copulations that were associated with paternity success also had strong negative correlations with the volumes of discarded sperm masses: more bursts of abdomen vibration and a higher frequency of palpal squeezes. There was also a weaker positive correlation with higher rates of bursts of abdomen vibration. However, the first male's paternity did not correlate with his abdomen vibration behaviour (only second male abdomen vibration had an effect, Table 1); so, the consequences of this variable are not clear.

Abdomen vibration and palpal squeezing are under male rather than female control, and thus are appropri-

ately taken to be traits of the male favoured by this selection. It is possible, of course, that female stridulation or other unobserved female responses, such as events in her internal genitalia, could induce male responses in these behaviour patterns, and that the male trait under selection involves his responses to the female. Female stridulation, however, had no effect on paternity (Table 1) or sperm dumping (Table 2). On the other hand, copulation duration (termination) was sometimes controlled by the male and sometimes by the female. The significance of early female termination is not clear because interruption by the female had, by itself, no significant effect on paternity or the size of the discarded sperm mass in the first or second copulation. However, the sample of female-initiated terminations was small ($N = 11$); so, this conclusion is only tentative.

Male abdomen vibration is not a physically coercive behaviour, and its effects on sperm dumping seem likely to result from stimulation of the female rather than physical force. Because of this lack of coercion, and because the only obvious pay-off to the female in responding to the male via sperm dumping would be a change in relative paternity, it is likely that the selection favouring male abdomen vibration is CFC rather than sexually antagonistic coevolution (SAC). The pay-off to the female for exercising CFC is improved offspring quality (Eberhard, 1996), something which could indeed correlate with biased sperm usage; by contrast, the female benefit in SAC models explicitly excludes genetic quality of offspring, and instead postulates increased numbers offspring for the female (Chapman *et al.*, 2003, Arnqvist & Rowe, 2005), something that is seemingly unlikely to be affected by selective sperm dumping.

Palpal squeezing, by contrast, is a powerful, potentially coercive movement. Females sometimes apparently attempt to inhibit squeezing (Peretti *et al.*, 2006) and it is possible (although not demonstrated) that squeezing results in damage to setae on the female's epigynum (Peretti *et al.*, 2006). Association of squeezing with CFC rather than SAC might thus seem less certain. It should be noted, however, that the coevolved physical, coercive resistance by the female expected under some versions of SAC (e.g. Alexander *et al.*, 1997; Arnqvist & Rowe 2002a, b) is lacking; females do not oblige males to shorten squeezes, but rather induce responses with noncoercive, strictly communicative stridulation behaviour (Peretti *et al.*, 2006). In addition, as just noted, the likely pay-offs to females from biased sperm dumping are compatible with CFC but not SAC.

Sperm discarded during the second copulation

The strongest positive correlation between variables associated with both paternity and the volume of the discarded mass in the second copulation was with the duration of the first copulation relative to that of the second: relatively longer first copulations were

associated with larger discarded masses in second copulations. Relatively longer first copulations also correlated with increased first male's paternity (Table 1). Other, weaker correlations with the volume of the discarded mass echo relations with the volume of the discarded mass that occurred in first copulations: a positive correlation with higher numbers of bursts of abdomen vibration by the second male; a negative correlation with the difference in the number of bursts of abdomen vibration between the first and second copulations; and a moderately strong negative correlation between the size of the discarded mass and the difference in the numbers of palpal squeezes in the first and second copulations.

If one assumes that the sperm discarded in the second copulation came from the first male (see above), then these correlations would result in selection via sperm dumping on the first male to perform relatively short copulations, more bursts of abdomen vibration and larger numbers of palpal squeezes. In addition, based on the subset of females with large differences in the responsiveness of the first and second males, selection via sperm dumping may favour higher male responsiveness to female stridulation (Peretti *et al.*, 2006).

Copulation duration

The duration of the first copulation showed a strong negative correlation with first male's paternity and a strong correlation with the size of the discarded mass in both the first and second copulations. The duration of the first copulation was sharply reduced when the female rather than the male terminated the copulation. If sperm are transferred gradually, as occurs in some arthropods (Simmons, 2001), it could be that the apparent shortening of copulation by the female would correlate with reduced paternity, as longer copulations might give the male a chance to transfer more sperm. We cannot evaluate this hypothesis, however, as the timing of sperm transfer during copulation is not known in pholcids.

Male size

The clear lack of correlation of male size with either the amount of sperm discarded or with paternity suggests that paternity was not biased on the basis of differences in male size. No significant effects of male body size emerged, even when all other variables except body size measures were excluded from a multiple regression on the size of the first discarded mass, and when all variables in Table 3 were run at once on the size of the second discarded mass.

Possible limitations?

There are several possible limitations in the present study. We standardized two factors that are known to

influence sperm precedence in some arthropods (Simmons, 2001): the number of males that copulated with the female (always two) and the time elapsed between the first and second copulations (always 1 day). Our results may not apply under other conditions. We established only correlations, not cause-effect relations; so, causes remain to be tested. Some of our estimates may also be imprecise. Sperm numbers were not counted directly but were estimated on the basis of the volume of the discarded mass. We verified that discarded masses always contained sperm, but do not know whether sperm density was constant in different masses (it could presumably be affected by amounts of 'matrix' material from either the male or the female). Our use of the volume of material discarded as an estimator of the amount of sperm that remained inside the female also depended on the assumption that the amount of sperm transferred by different males is approximately constant; we have no data on this point. In a distantly related spider, larger males tend to transfer larger ejaculates (Cohn, 1990), but we found no effects in *P. globosus* of male size on paternity, or on the sizes of discarded masses. Other sperm variables, such as swimming abilities after they became dis-encapsulated inside the female and their viability were also assumed to be equal. Finally, our inclusion of many variables in the analyses may have made the analyses less sensitive, and led us to miss some significant effects.

In general, these sources of imprecision in our estimates do not give strong reason to doubt our conclusions with respect to the correlations behaviour and paternity, and between behaviour and sperm dumping. This is because there is no *a priori* reason to expect that they would bias the results in ways causing the correlations we found to be produced. For instance, differences in sperm swimming and viability would seem more likely to introduce noise into the results with respect to the variables we measured, making it more difficult rather than easier to perceive the correlations that we found. Similarly, the imprecisions in our estimates of sperm dumping would not be expected to be biased so as to produce correlations with the male's copulatory courtship behaviour. Our conclusions regarding the reproductive significance of a behaviour would be likely to be affected only if that behaviour was influenced by a factor that correlated with total ejaculate volume, thus altering the reproductive significance of the volume of discarded sperm.

Alternative interpretations

An alternative possibility to active female rejection of gametes in order to influence paternity is that the male broke off or dislodged masses of stored sperm within the female's vagina at an earlier stage, and that the female then simply finished this process by expelling the mass. The repeated, powerful thrusting movements of the

procursus against the inner valve of the vagina (Huber & Eberhard, 1997) could cause the procursus to push into or scrape against the mass of stored sperm in the vagina, perhaps dislodging or breaking off sections of this mass. Perhaps the function of female dumping behaviour was to 'clean up' her vaginal cavity, rather than to influence paternity by discarding sperm. We can neither confirm nor deny this 'clean up' hypothesis for female dumping movements on morphological grounds. However, this hypothesis is only reasonable, if a portion of the mass that breaks away can no longer be stored for future fertilization. Such loss would presumably be more likely if the mass is more rigid and less liquid. Our observations that discarded masses were solid, but soft and malleable, do not give clear support to either alternative. This 'clean up' hypothesis for sperm dumping in second copulations leave unexplained, however, the correlations between the size of the discarded mass and male abdomen vibration behaviour that has no direct physical relation with the sperm.

A second alternative possibility is that the newly transferred sperm itself drives out stored sperm from a previous male. Re-examination of the sections of female genitalia during copulation made in a previous study (Huber & Eberhard, 1997) suggest that the likely answer is no. Substantial portions of the vagina of *P. globosus* were empty, and there were folds in the vaginal walls. The walls are also probably extensible, to permit the passage of eggs during oviposition. It thus seems unlikely that sperm dumping in *P. globosus* is due to simple overflow from an overly full vagina. The active female movements during expulsion and the emission of sperm after the male has removed his palps (substantially decreasing the degree to which the vagina was filled) that were mentioned above also argue against this idea. Nor is it likely that the thrusting or the squeezing movements of the male palps are themselves sufficient to press sperm from the vagina, as has been suggested in the pholcid *P. phalangioides* (Uhl *et al.*, 1995). This idea is contradicted in both species by the fact that all the movements of the male's palps occur *within* the vagina. The palps thus cannot reduce the vagina's volume and squeeze out sperm in either species. The major force exerted by palpal movements in *P. globosus* is to squeeze a portion of the vaginal wall and the female's epigynum between the palp (especially the procursus, which is inside the vagina) and the male's chelicerae (which press on the outside of the female's epigynum) (Huber & Eberhard, 1997).

We conclude that palpal squeezing in *P. globosus* in first copulations induces the female to refrain from removing the first male's sperm, and does not directly remove sperm. This 'genital courtship' interpretation is compatible with comparative data from other pholcid spiders. Palpal squeezing is also rhythmic in other genera, and the rhythms show diverse temporal patterns that involve both twisting and squeezing (Huber & Eberhard, 1997);

diversity of this sort is a common result of evolution under sexual selection.

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