

# Do the size and age of mating plugs alter their efficacy in protecting paternity?

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**Abstract** An obvious means to secure paternity is the production of a mating plug that blocks the female genital opening after mating. Although the mechanical efficacy and persistence of plugs on/in the female genital openings are key traits that determine the degree of paternity protection, these factors have hardly been explored. We therefore investigated the influence of the size of the amorphous plug material (experimentally terminated mating duration as a proxy) and age of the mating plug (time interval between copulations with two successive males) on the efficacy of the plug by analysing the mating success of subsequent males in the dwarf spider *Oedothorax retusus* (Linyphiidae: Erigoninae). Overall, subsequent males attempted to mate in 82 % of trials but only 32.5 % of these resulted in copulation, demonstrating that the plugs are effective safeguards against remating. Remating probability was significantly higher after previous short copulations (~small plug size) compared to long copulations (~large plug size). In the small plug group, fresh plugs (short remating intervals) were significantly less effective compared to older plugs. In the large plug group, remating probability was similarly low over all remating intervals. The observed copulations, however, do not necessarily result in sperm transfer, since sperm masses were found on the plugged female genital area. Our study on *O. retusus* shows that mating plugs are a powerful mechanical safeguard whose efficacy varies with plug size and age. We discuss these findings in the light

of theoretical considerations on the evolution of effective mating plugs.

**Keywords** Mating plug · Sexual selection · Sperm competition · Monopolization · Paternity protection · Dwarf spider · Erigoninae

## Introduction

In polyandrous species, post-copulatory male-male competition occurs if sperm of different males are stored within the female genital tract (Parker 1970). Polyandry is widespread in various animal taxa and has led to numerous behavioural, physiological and morphological male adaptations against sperm competition (Birkhead and Møller 1998; Parker 1998; Simmons 2001; Eberhard 2009). Adaptations can be offensive by reducing mating success of previous males or defensive by impeding or reducing further matings of the female (Parker 1970). Offensive adaptations are, for example, when rival sperm are scraped out of the female genital tract (Simmons 2001), displaced and/or replaced (e.g. Gack and Peschke 1994) or sealed off with a hardening ejaculate (Diesel 1990). A classical defensive male adaptation against multiple female mating is the production of a mating plug. Mating plugs are structures that mechanically block female genital openings after mating and reduce female remating probabilities or impede further matings, thereby securing paternity of the plug-producing male (Simmons 2001). Mating plugs are known from virtually all animal groups (Birkhead and Møller 1998). However, material on or in the female genital openings is often assumed to function as a mating plug without investigating whether it actually limits remating probability of the female. The materials may serve other functions than paternity protection such as preventing sperm leakage and may even be produced by the female (e.g. Aisenberg and Barrantes 2011).

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Moreover, even if the materials were demonstrated to serve as paternity protection devices, the conditions and constraints that shape their efficacy are still largely underexplored.

Supposed mating plugs were found in many spider families (Austad 1984; Uhl et al. 2010). The genital morphology of entelegyne spiders seems to promote the occurrence of mating plugs since females possess three genital openings—two are used for copulation and one for oviposition (Uhl et al. 2010). This allows the male to obstruct the copulatory ducts without impeding oviposition. Further, spiders possess a high potential for sperm competition since females frequently mate with several males, and sperm can be stored over long periods of time in the female genital tract (Elgar 1998). Supposed mating plugs in spiders consist primarily of amorphous material but can also consist of parts of or complete male pedipalps, the secondary copulatory organs (Uhl et al. 2010). Recently, genital fragments were shown to be effective plugs in the orb-weaver *Argiope keyserlingi* (Herberstein et al. 2012). However, in many species, several male fragments can be found in the female genital system, demonstrating that subsequent matings are possible (Uhl et al. 2010).

Here, we focus on amorphous materials on/in the female genital opening since this type of potential mating plug is particularly widespread in the animal kingdom including spiders (Uhl et al. 2010). When the material is transferred from the male to the female, it must be transferred in a liquid state and has to solidify to a certain degree if it is meant to withstand penetration or removal by a rival male. We therefore hypothesize that plugs are not as effective immediately after their placement compared to after curing. Since a plug only helps to secure paternity if it remains functional until oviposition, the degree of persistence of the material is a further crucial aspect that determines paternity success. Furthermore, plug efficacy may strongly depend on plug size if large amounts of material are more difficult to remove compared to small amounts (e.g. Masumoto 1993; Polak et al. 2001). In the butterfly *Cressida cressida*, large mating plugs even present lifelong chastity belts (Orr and Rutowski 1991).

In this study, we follow up on findings in the dwarf spider *Oedothorax retusus* (Linyphiidae: Erigoninae) in which an amorphous mass is found on the female copulatory opening after mating (Uhl and Busch 2009). Mating plugs are significantly larger after longer matings which allows using copulation duration as a proxy for plug size (Uhl and Busch 2009). When experimentally terminating matings after 1 or 3 min, small mating plugs proved to be ineffective but large plugs prevented remating of the female in 93 % of cases. In this previous study, all remating trials were staged within 15 min. However, in order to understand the full potential and limitations of mating plugs, we need to assess how plug size, plug age and their interaction determine plug efficacy.

We hypothesize that mating plug efficacy in *O. retusus* depends (i) on first mating duration (short, long) which can

be used as a proxy for plug size and (ii) on plug age taken as the time interval between first copulation and the second mating trial (15 min; 1 h; 1 day; 3 days after mating; after oviposition). Since cleaning behaviour by the female may potentially alter plug presence and size, we observed if females cleaned the genital region during 1 h post-mating. When subsequent males attempted or succeeded in remating, we investigated the condition of the mating plugs under the SEM. In order to test if female receptivity and attractiveness to males may depend on plug size and age, we further registered aggressive behaviour by females towards males and male courtship probability.

## Material and methods

Study species: *O. retusus*

The dwarf spider *O. retusus* belongs to the Erigoninae (Miller and Hormiga 2004) which is the largest group within the Linyphiidae. We used males and females that were raised in the laboratory from egg sacs of females caught along the banks of the river Rhine south of Bonn, Germany (50° 43' 00.26" N, 7° 08' 25.11" E–50° 42' 57.40" N, 7° 08' 36.70" E). Adult spiders were kept individually in 25 ml plastic containers equipped with a moist layer of gypsum at a temperature of 23/17 °C (day/night) and 70 % humidity. They were fed weekly with four to six fruit flies, *Drosophila melanogaster*. Spiderlings were fed *ad libitum* on springtails, *Sinella curviseta*, until the subadult stage (Kunz et al. 2012).

### Courtship and copulation in *O. retusus*

Courtship in *O. retusus* starts with the male vibrating his opisthosoma. As soon as the male achieves the contralateral mating position, he stretches one of his pedipalps (secondary copulatory organs) towards the external female genital structure (epigyne) and tries to insert into one of two female copulatory openings. Copulation starts when the male successfully inserts a pedipalp into the corresponding female copulatory opening and the hematodocha (membranous part of the sperm transfer organ) inflates. The insertion mode is ipsilateral, meaning that the right pedipalp is inserted into the right female copulatory opening, which is typical for entelegyne spiders (Huber and Senglet 1997). A mating plug in *O. retusus* appears as a transparent mass shortly after copulation that transforms into solid opaque material (Kunz and Witthuhn, personal observations).

### Influence of plug size and age on paternity protection

We investigated plug efficacy of *O. retusus* by staging remating trials that varied in plug size as determined by first male copulation durations (Uhl and Busch 2009) and plug age

as determined by latencies between first and second matings. To make sure that both first and second males used the same of the two female genital openings, one of the two pedipalps of each male was amputated on the same side. As a consequence, both males of a mating trial were restricted to using either the right or the left pedipalp.

Courtship and mating behaviours of males and females were observed as described in Kunz et al. (2012). The observation period started when the first male was placed in the container of a virgin female and ended with copulation or after 1 h. Copulations were terminated by the experimenter after 1 or 3 min in order to create the two treatment groups with a high probability of small (1 min) and large (3 min) mating plugs (Uhl and Busch 2009). Undisturbed copulations with one pedipalp last on average 3.21 min in *O. retusus* (Richter 2006). In the following, we use the terms “small plug group” for those remating trials in which the female previously had experienced a 1-min copulation and “large plug group” for those trials in which the female had experienced a 3-min copulation. Subsequent mating trials were staged after five different intervals: 15 min, 1 h, 1 day, 3 days after mating, or after the female had oviposited (same day or day after). These intervals serve as categories for plug age. When copulations occurred in remating trials, they were not terminated by the experimenter. Second males were removed after copulation or after 1 h of observation time. In 19.9 % of cases (38 out of 191), second males did not try to assume mating position and were replaced by third males. In the following, second or third males whose pedipalps were in contact with the female genital area are treated as “second mating partners”. All males were used for a single mating only.

Of all staged mating trials, 45.4 % (191 out of 421) were used for the analysis (total  $N=99$  for the 1-min group: 15 min  $N=20$ ; 1 h  $N=19$ ; 1 day  $N=20$ ; 3 days  $N=20$ ; oviposition  $N=20$ ; and total  $N=92$  for the 3-min group: 15 min  $N=19$ ; 1 h  $N=17$ ; 1 day  $N=19$ ; 3 days  $N=20$ ; oviposition  $N=17$ ). During mating trials, we recorded aggressive behaviour of females towards males (attacking and chasing away of the potential mate, Kunz et al. 2012) to detect potential effects of plug size and age on female receptivity. Further, females were observed for 1 h after their first mating in order to detect potential cleaning activity of the genital region that may influence the presence and state of the mating plug. Females of short remating intervals were observed for cleaning activity during their second mating trial.

We controlled for male and female age calculated as days elapsed from the final moult. Females of the small plug group were on average  $26.4\pm 4.6$  days, and those of the large plug group were on average  $25.9\pm 3.5$  days old (Mann-Whitney  $U$  test:  $N=99$ ,  $Z=-0.01$ ,  $p=0.361$ ). Males of the small plug group were  $26.4\pm 4.5$  days old, and males from the large plug group  $26.0\pm 3.8$  days old (Mann-Whitney  $U$  test:  $N=92$ ,  $Z=-0.89$ ,  $p=0.376$ ).

## Scanning electron microscopy

Female opisthosomata ( $N=143$ ) of those trials in which remating attempts or rematings occurred were fixed in Dubosq-Brazil solution (Mulisch and Welsch 2010) in order to investigate the condition of the mating plug. Seventy females were investigated from the small plug group (15 min  $N=12$ ; 1 h  $N=14$ ; 1 day  $N=18$ ; 3 days  $N=14$ ; oviposition  $N=12$ ), and 73 females from the large plug group (15 min  $N=15$ ; 1 h  $N=12$ ; 1 day  $N=17$ ; 3 days  $N=15$ ; oviposition  $N=14$ ). After a minimum of 2 days of infiltration, the samples were dehydrated in an ascending ethanol series, chemically dehydrated with 1,1,1,3,3,3-hexamethyldisilazane (HMDS), sputter-coated for 200 s with gold/palladium (80/20) (Polaron SC7640, Fisons Instruments) and investigated under a SEM EVO LS10 (Zeiss) at the Imaging Centre of the University of Greifswald. We inspected the female genitalia for presence, location (right/left copulatory duct), size category (small/large) and whether spermatozoa were visible in the plug material.

## Statistical analysis

Statistical analyses were performed using IBM SPSS Statistics version 20. Normally distributed data are given as arithmetic mean  $\pm$  standard deviation (mean  $\pm$  SD). Non-normally distributed data are given as median, interquartile range (IQR), and are ln-transformed for GLM analysis. GLM analyses were performed with the parameters “plug size” (1-min group and 3-min group), “plug age” (15 min, 1 h, 1 day, 3 days, oviposition) and “interaction between size and age” and are given as Wald  $\chi^2$  or likelihood ratio  $\chi^2$  (LR  $\chi^2$ ) in the case of transformed data. For binomial data, we used GLM with binomial distribution and logit link function. None of the interactions were significant (all  $p>0.178$ ). We further explored significant parameters from the GLM analysis using  $\chi^2$  and Mann-Whitney  $U$  test.

## Results

### Mating plugs as a mechanical safeguard?

We analysed if mating plug efficacy depends on plug size (1 and 3 min during first copulation) and/or plug age (time interval between first and second copulation) by investigating copulation attempts and remating success. One hundred fifty-seven out of 191 (82.2 %) second mating partners performed copulation attempts with their single pedipalp on the plugged female copulatory opening. The probability of copulation attempts was not different between the plug size groups (GLM: copulation attempts yes/no: small plug group, 78/21 (78.8 %); large

plug group, 79/13 (85.9 %); Wald  $\chi^2=0.00$ ,  $df=1$ ,  $p=0.999$ ) but significantly different between plug age classes (copulation attempts yes/no: 15 min, 36/3 (92.3 %); 1 h, 35/1 (97.2 %); 1 day, 34/5 (87.2 %); 3 days, 33/7 (82.5 %); oviposition, 19/18 (51.4 %); Wald  $\chi^2=13.72$ ,  $df=4$ ,  $p=0.008$ ). The lower probability of copulation attempts in the oviposition group accounted for this significant difference. When excluding the oviposition interval, there was no significant effect between the remaining plug age classes (Wald  $\chi^2=0.19$ ,  $df=3$ ,  $p=0.980$ ) (see Table 1 for data per size and age groups). The number of copulation attempts a male performed was marginally significantly different between plug size groups (GLM: number of copulation attempts: small plug group ( $N=78$ ) 16, IQR 41; large plug group ( $N=79$ ) 24, IQR 57; LR  $\chi^2=3.51$ ,  $df=1$ ,  $p=0.061$ ), whereas the number of copulation attempts differed significantly between plug age classes (number of copulation attempts: 15 min ( $N=36$ ) 17, IQR 42; 1 h ( $N=35$ ) 24, IQR 85; 1 day ( $N=34$ ) 24, IQR 49; 3 days ( $N=33$ ) 24, IQR 65; oviposition ( $N=19$ ) 6, IQR 19; LR  $\chi^2=12.27$ ,  $df=4$ ,  $p=0.015$ ). The significant effect can be attributed to the low number of copulation attempts in the oviposition group. When removed from the analysis, the number of copulation attempts was not significantly different between plug age classes (LR  $\chi^2=4.09$ ,  $df=3$ ,  $p=0.252$ ) (see Table 1 for data per size and age groups).

Altogether 32.5 % of males (62 out of 191) copulated with a mated female. The overall probability of remating was significantly higher for the small plug group (GLM: remating yes/no: small plug group, 45/54 (45.5 %); large plug group, 17/75 (18.5 %); Wald  $\chi^2=14.39$ ,  $df=1$ ,  $p<0.001$ ). Likewise, plug age classes overall revealed significantly different female remating probabilities (remating yes/no: 15 min, 22/17 (56.4 %); 1 h, 15/21 (41.7 %); 1 day, 13/26 (33.3 %); 3 days, 7/33 (17.5 %); oviposition, 5/32 (13.5 %); Wald  $\chi^2=17.17$ ,  $df=4$ ,  $p=0.002$ ). In the small plug group, plug age class had a highly significant effect on remating probabilities (Table 1; Fig. 1, light grey bars). Only the 15-min age class accounted for the significant effect since the other age classes (1 h to after oviposition) did not differ significantly when compared without the 15-min age class ( $\chi^2=6.53$ ,  $df=3$ ,  $p=0.088$ ). Differences between plug size groups remained significant after excluding the 15-min interval from the analysis (GLM: remating yes/no: small plug group, 28/51 (35.4 %); large plug group, 12/61 (16.4 %); Wald  $\chi^2=7.18$ ,  $df=1$ ,  $p=0.007$ ). In the large plug group, overall remating probabilities were low and not significantly different between plug age classes (Table 1; Fig. 1, dark grey bars).

The copulation duration of subsequent males was not significantly different between the plug size groups (GLM: copulation duration in seconds: small plug group ( $N=45$ ), 154.8±96.2; large plug group ( $N=17$ ), 126.5±103.4; Wald  $\chi^2=1.52$ ,

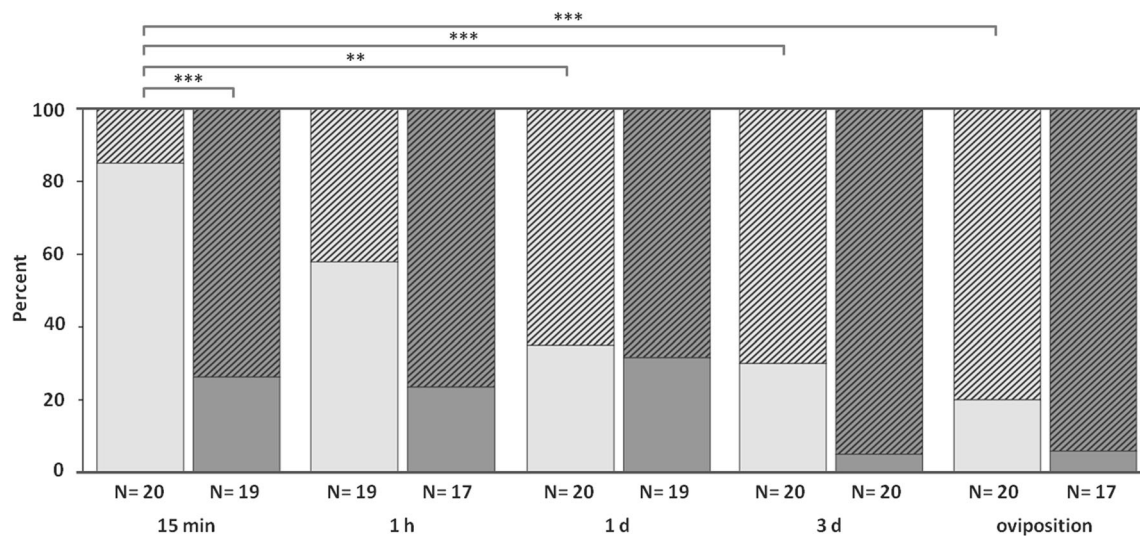
**Table 1** Courtship and mating parameters of *Oedothorax retusus* in mating trials with females bearing small plugs or large plugs. Data are given for five plug age classes (intervals between first and second copulation: 15 min, 1 h, 1 day, 3 days, after oviposition). Data are given as events (proportions), mean ± standard deviation, and median, interquartile range (IQR). Statistical analysis was performed using  $\chi^2$  test, ANOVAs ( $F$ ) and Kruskal-Wallis tests. Significant  $p$ -values are shown in bold. Overall GLM results for the parameters plug size and plug age plus interaction are given in the text

	Plug	15 min		1 h		1 day		3 days		Oviposition		$F^a, \chi^2$	$p$
		Events	Mean/median	Events	Mean/median	Events	Mean/median	Events	Mean/median	Events	Mean/median		
Copulation attempts (yes/no)	Small	20	17/3 (85.0 %)	19	18/1 (94.7 %)	20	18/2 (90.0 %)	20	15/5 (75.0 %)	20	10/10 (50.0 %)	14.95	<b>0.005</b>
	Large	19	19/0 (100.0 %)	17	17/0 (100.0 %)	19	16/3 (84.2 %)	20	18/2 (90.0 %)	17	9/8 (52.9 %)	21.44	<b>&lt;0.001</b>
Copulation attempts [no.]	Small	17	10, IQR 27	18	14, IQR 33	18	32, IQR 90	15	17, IQR 60	10	11, IQR 20	5.55 <sup>b</sup>	0.235
	Large	19	36.4±35.1	17	69.8±65.6	16	34.3±32.9	18	45.2±43.9	9	12.2±12.1	3.00 <sup>a</sup>	<b>0.024</b>
Remating (yes/no)	Small	20	17/3 (85.0 %)	19	11/8 (57.9 %)	20	7/13 (35.0 %)	20	6/14 (30.0 %)	20	4/16 (20.0 %)	21.84	<b>&lt;0.001</b>
	Large	19	5/14 (26.3 %)	17	4/13 (23.5 %)	19	6/13 (31.6 %)	20	1/19 (5.0 %)	17	1/16 (5.9 %)	7.43	0.115
Copulation duration [s]	Small	17	156.6±95.6	11	164.0±72.1	7	130.6±119.9	6	154.2±117.2	4	165.3±126.8	0.14 <sup>a</sup>	0.967
	Large	5	161.2±138.6	4	110.0±93.4	6	123.3±98.5	1	147.0±0.0	1	17.0±0.0	0.39 <sup>a</sup>	0.813

<sup>a</sup> ANOVAs ( $F$ )

<sup>b</sup> Kruskal-Wallis tests





**Fig. 1** Percentages of copulations (full bars) and unsuccessful copulation attempts (striped bars) in *Oedothorax retusus* mating trials with mated females that bear small (light grey bars) or large mating plugs (dark grey bars). Data are given for five plug age classes (interval between first

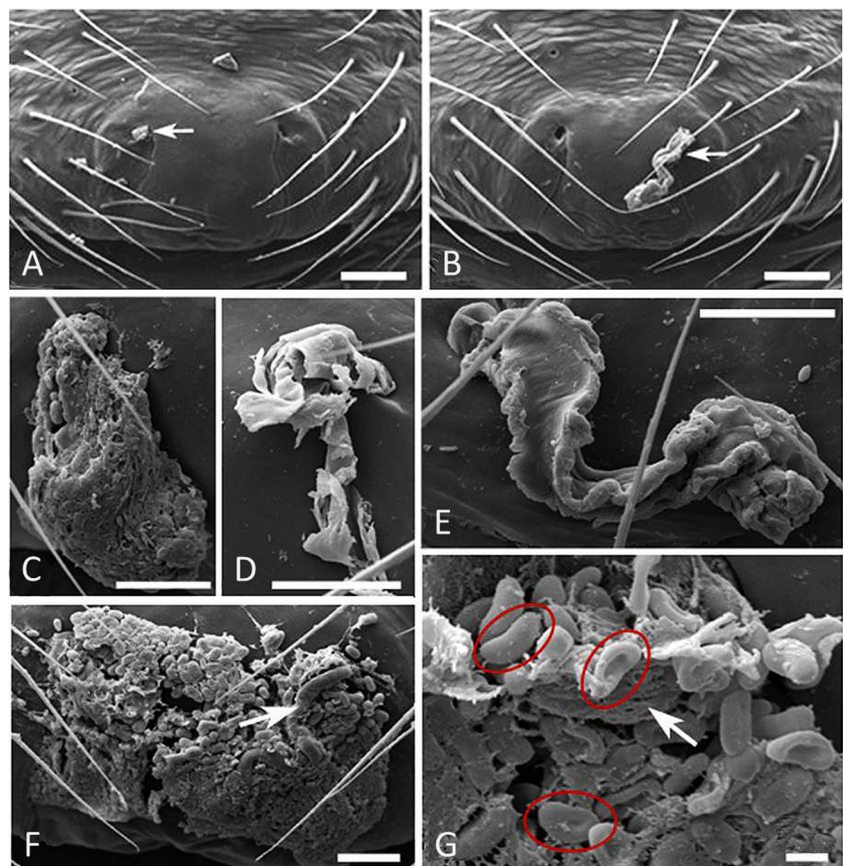
mating and second mating trial: 15 min, 1 h, 1 day, 3 days, after oviposition). Asterisks mark significant differences in pairwise  $\chi^2$  tests after sequential Bonferroni correction (\*\* $0.001 \leq \alpha \leq 0.01$ , \*\*\* $\alpha < 0.001$ )

$df=1$ ,  $p=0.218$ ) nor between plug age classes (15 min ( $N=22$ ),  $157.6 \pm 103.1$ ; 1 h ( $N=15$ ),  $149.6 \pm 78.7$ ; 1 day ( $N=13$ ),  $127.2 \pm 106.1$ ; 3 days ( $N=7$ ),  $153.1 \pm 107.0$ ; oviposition ( $N=5$ ),  $135.6 \pm 128.3$ ; Wald  $\chi^2=1.78$ ,  $df=4$ ,  $p=0.777$ ) (see Table 1 for data per size groups and age classes).

#### State of the mating plug after remating

In those cases in which remating occurred, the plug material contained spermatozoa (Fig. 2) with significantly higher probability than in cases in which only copulation attempts were

**Fig. 2** SEM photographs of the genital regions of mated *Oedothorax retusus* females. **a** Small mating plug (arrowhead) in the right side of the female genital opening typical for the 1-min group (once mated female). **b** Large mating plug (arrowhead) in the left side of the female genital opening typical for the 3-min group (once mated female). **c** Large, inhomogeneous mating plug (once mated female). **d** Damaged mating plug (second male performed 67 copulation attempts). **e** Large, loopy, homogenous mating plug (once mated female). **f** Plane, previously loopy (arrowhead) mating plug including spermatozoa from a subsequent mating. **g** Spermatozoa ( $\sim 3 \mu\text{m}$ , red circles) and amorphous secretion (arrowhead) after remating. Scale bars: **a–f**=20  $\mu\text{m}$ , **g**=2  $\mu\text{m}$



observed (sperm yes/no: successful remating, 27/11 (71.1 %); unsuccessful remating, 14/91 (13.3 %);  $\chi^2=45.45$ ,  $df=1$ ,  $p<0.001$ ). This result holds for the small plug group where most rematings occurred (sperm yes/no: successful remating, 21/8 (72.4 %); unsuccessful remating, 3/38 (7.3 %); Fisher's exact test:  $p<0.001$ ) as well as for the large plug group (sperm yes/no: successful remating, 6/3 (66.7 %); unsuccessful remating, 11/53 (17.2 %); Fisher's exact test:  $p=0.004$ ).

The SEM analysis of the state of the mating plug after remating revealed that 58 % of mating plugs show areas of broken off plug material (sharp edges) (Fig. 2d) that were not observed in previous investigations on once mated females (Uhl and Busch 2009).

#### Potential influence of plug size and age on female attractiveness and receptivity

To assess the potential effects of size and age of the mating plug on female attractiveness, we analysed courtship behaviour of second mating partners. Courtship probabilities of second males were not different between plug size groups (GLM: courtship yes/no: small plug group, 97/2; large plug group, 92/0; Wald  $\chi^2=0.00$ ,  $df=1$ ,  $p=0.999$ ). There was also no difference in courtship probability between plug age classes (courtship yes/no: 15 min, 39/0; 1 h, 36/0; 1 day, 39/0; 3 days, 39/1; oviposition, 36/1; Wald  $\chi^2=0.00$ ,  $df=4$ ,  $p=1.000$ ). Courtship latency of second males was also not significantly different between the plug size groups (GLM: courtship latency in seconds: small plug group ( $N=97$ )  $124.4\pm 316.6$ ; large plug group ( $N=92$ )  $147.6\pm 385.1$ ; Wald  $\chi^2=0.20$ ,  $df=1$ ,  $p=0.653$ ) nor between plug age classes (15 min ( $N=39$ ),  $115.1\pm 202.7$ ; 1 h ( $N=36$ ),  $120.6\pm 234.8$ ; 1 day ( $N=39$ ),  $159.4\pm 433.1$ ; 3 days ( $N=39$ ),  $216.7\pm 543.6$ ; oviposition ( $N=36$ ),  $59.8\pm 149.3$ ; Wald  $\chi^2=4.22$ ,  $df=4$ ,  $p=0.377$ ). Likewise, the time it took to achieve mating position was not significantly different between the plug size groups (GLM: latency mating position: small plug group ( $N=83$ ) 254, IQR 443; large plug group ( $N=81$ ) 273, IQR 725; LR  $\chi^2=0.72$ ,  $df=1$ ,  $p=0.398$ ) nor between plug age classes (latency mating position: 15 min ( $N=39$ ) 302, IQR 746; 1 h ( $N=36$ ) 239, IQR 537; 1 day ( $N=36$ ) 220, IQR 352; 3 days ( $N=33$ ) 274, IQR 505; oviposition ( $N=20$ ) 399, IQR 1,087; LR  $\chi^2=5.51$ ,  $df=4$ ,  $p=0.239$ ).

We further analysed aggressive behaviour of females towards second mating partners in order to assess the potential effects of mating plug size and age on female receptivity. The probability of female aggressiveness was not significantly different in remating trials between plug size groups (GLM: female aggressiveness yes/no: small plug group, 24/75 (24.3 %); large plug group, 14/78 (15.2 %); Wald  $\chi^2=1.48$ ,  $df=1$ ,  $p=0.224$ ) nor between plug age classes (female aggressiveness yes/no: 15 min, 3/36 (7.7 %); 1 h, 4/32 (11.1 %); 1 day, 10/29 (25.6 %); 3 days,

8/32 (20.0 %); oviposition, 13/24 (35.1 %); Wald  $\chi^2=8.16$ ,  $df=4$ ,  $p=0.086$ ). The number of aggressive interactions was not significantly affected by plug size (GLM: number of aggressive interactions: small plug group ( $N=24$ ) 1, IQR 2; large plug group ( $N=14$ ) 1, IQR 2; LR  $\chi^2=0.31$ ,  $df=1$ ,  $p=0.579$ ) nor by plug age (number of aggressive interactions: 15 min ( $N=3$ ) 0, IQR 0; 1 h ( $N=4$ ) 3, IQR 5; 1 day ( $N=10$ ) 1, IQR 0; 3 days ( $N=8$ ) 1, IQR 1; oviposition ( $N=13$ ) 2, IQR 4; LR  $\chi^2=6.94$ ,  $df=1$ ,  $p=0.139$ ).

#### Can female cleaning behaviour affect plug presence and size?

Female post-copulatory cleaning behaviour may influence plug presence and size. Cleaning after copulation occurred in 6.3 % of females (12 out of 191). Our experimental plug size groups did not significantly differ in cleaning probability (cleaning yes/no: small plug group, 6/93 (6.1 %); large plug group, 6/86 (6.5 %);  $\chi^2=0.02$ ,  $df=1$ ,  $p=0.896$ ). Due to the rare occurrence of cleaning behaviour, plug age classes were not analysed.

## Discussion

Polyandrous mating combined with long-term sperm storage is particularly widespread in insects and spiders (Elgar 1998; Simmons and Siva-Jothy 1998), and mechanical safeguards against polyandrous mating are suspected to have evolved many times independently. The mating plug in *O. retusus* clearly functions as a mechanical obstacle to rival males. Overall, small plugs are less effective than large plugs. Shortly after plug placement, small plugs are least effective suggesting that size and hardening of the material plays an important role. Once plugs are older than a day, they strongly serve to monopolize access to the duct that leads to the female sperm storage organ. However, even if subsequent males are able to mate, at least part of their sperm mass remains outside of the female genital tract, which considerably adds to the efficacy of the mating plug. Consequently, if a male succeeds in plugging both of the females' copulatory openings, his paternity success is expected to be very high.

There is increasing evidence that mating plugs can be produced by the female (Aisenberg and Eberhard 2009; Aisenberg and Barrantes 2011; Kuntner et al. 2012), demonstrating that detailed analysis of the origin of the substance is a basic requirement to understand the adaptive context in which the plug evolved. In our model species *O. retusus*, the mating plug is produced inside of the male copulatory organs, the pedipalps (Uhl et al. 2014) and there is no indication that females participate in plug production or can remove the plug after mating. As shown here, the efficacy of the mating plug is strongly shaped by plug size,

with efficacy greater in large plugs. This suggests that smaller portions of plug material can be more easily removed or overcome by a subsequent male as was also shown for some insects and spiders (Jackson 1980; Matsumoto and Suzuki 1992; Masumoto 1993). Plug age can also determine plug efficacy, although this effect occurred only for small plugs, which became more effective as they aged, suggesting that hardening of the material is required (see also Matsumoto and Suzuki 1992).

It is not clear whether males that copulated with mated females were able to transfer sperm, since sperm were found on the genital area, suggesting that the paternity protection afforded by mating plugs is even greater than that suggested by remating probabilities. Instead of operating as a physical barrier, mating plugs may alter female behaviour and receptivity (Eberhard 2004; Colonello and Hartfelder 2005; Wedell 2005; Avila et al. 2011) or reduce female attractiveness for subsequent males (Parker 1970; Shine et al. 2000; O'Donnell et al. 2004). In *O. retusus*, we cannot disentangle potential effects of mating from those of the mating plug itself, since mating results in a visible plug in 98 % of cases (Uhl and Busch 2009). However, the level of female receptivity did not depend on plug size and age. Only after oviposition, female aggressiveness towards the male was increased, possibly due to a short phase of brood care (Kunz, personal observation). Transfer of antiaphrodisiac pheromones that reduce female attractiveness has been reported for example for beetles and butterflies (Andersson et al. 2003; Schulz et al. 2008; Schlechter-Helas et al. 2011). There were no significant differences in courtship probabilities and latencies of second *O. retusus* males between plug size and age treatments. Thus, we consider it unlikely that plugs contain antiaphrodisiac.

When considering the evolution of mating plugs, the particular morphology of the genitalia system plays a largely neglected but important role. In species with one female genital opening, a mating plug needs to decay before oviposition, whereas in animal groups with separate copulatory and oviposition ducts, such as most Lepidoptera and Araneae, long-lasting plugs applied to the copulatory ducts may easily evolve. However, spiders are special since most species exhibit two copulatory ducts in the female genital tract (Uhl et al. 2010). Females may allow one or two insertions by a particular male thereby allowing him to plug one or two ducts. This important morphological component and the resulting possibilities for female mate choice require being included into models on the evolution of mating plugs (e.g. Fromhage 2011).

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**Conflict of interest** The authors declare that there is no conflict of interest.

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