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Copulatory plugs do not assure high first male fertilisation success: sperm displacement in a lizard

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Abstract Sperm competition selects for opposing male defensive and offensive reproductive traits, and its outcome may be determined by the effectiveness to which one trait has evolved to out-compete the other. We tested the effectiveness of a first male plug physical interference with a second male insemination (defence) vs the effectiveness of plug and associated sperm displacement by a second male (offence) on the outcome of sperm competition in Iberian rock lizards. We conducted a double mating experiment where we compared the proportion of eggs per clutch fertilised by the same second males (against the same first males) when they copulated with females 30 min (first male plug adhered firmly inside the female cloaca) and 4 h (first male plug loosely adhered or shed from the female cloaca) after first males. We found that second males fertilised the majority of the eggs per clutch in the 30-min treatment, whereas fertilisations were equally shared between the two males in the 4-h treatment. These results show that plugs have little defensive effectiveness, and thus, do not assure high first male fertilisation success. Instead, sperm displacement appears to be associated with plug displacement. That is, because sperm embedded in first male plugs, and displaced from competition for fertilisations by second males, is expected to increase in number with decreasing time allowed for female sperm transport, second males thus enjoy higher fertilisation success. This study shows that offensive plug displacement out-competes plug defensive role in Iberian rock lizards. Moreover, it reveals sperm displacement as a novel sperm competition mechanism in reptiles.

Keywords Copulatory plug · Iberian rock lizard · Offensive and defensive traits · Sperm competition · Sperm displacement

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Introduction

Competition between sperm from different males for fertilisation of a female's set of ova is widespread in animals and selects for male reproductive traits that increase a male's fertilisation success at the expense of rivals (Parker 1970; Smith 1984; Birkhead and Møller 1998; Simmons 2001). Sperm competition favours (1) defensive traits that confer high fertilisation success to males copulating with non-mated females ('first males'), such as those that prevent females from remating, and (2) offensive traits that confer high fertilisation success to males copulating with previously mated females ('subsequent males', or 'second males' in the case of doubly mated females), such as those that allow for insemination of sperm in a more favourable position for fertilisation and those that allow for displacement of sperm already inside the female reproductive tract (Parker 1970, 1984; Simmons 2001). Opposing defensive–offensive traits are predicted to co-evolve antagonistically because evolution of more efficient defence selects for improved offence and vice-versa (Parker 1970, 1984). The outcome of sperm competition, frequently

measured in terms of the proportion of ova fertilised by the second (P_2) of two competing males, may be determined by the effectiveness to which one trait has evolved to outcompete the other (Parker 1970, 1984).

Males of many organisms deliver accessory gland secretions during copulation which coagulate and form a copulatory (mating) plug that occludes the female reproductive tract (Birkhead and Møller 1998; Simmons 2001). Plugs may be sperm competition defensive traits by providing a physical barrier to subsequent male genital intromission and insemination ('chastity belt' function; Parker 1970; Devine 1975; Voss 1979), as documented in some butterflies (Dickinson and Rutowski 1989; Matsumoto and Suzuki 1992) and spiders (Masumoto 1993). However, in many species, males copulate with females that carry a plug from a previous mating inside the reproductive tract and displace it, either before or concomitant with delivery of a new plug (Mosig and Dewsbury 1970; Hartung and Dewsbury 1978; Jia et al. 2002; Parga 2003). In addition, when plugs play a role in sperm transport, as in rodents (Matthews and Adler 1977, 1978; Carballada and Esponda 1992, 1993), plug displacement by subsequent males may disrupt the transport of previously inseminated sperm (Adler and Zoloth 1970; Mathews and Adler 1977). The outcome of sperm competition when first male plug defensive role is opposed by second male offensive plug displacement is little studied; plugs assured high first male fertilisation success in one (Martan and Shepherd 1976) but not in another rodent (Dewsbury 1988).

In reptiles, copulatory plugs have been described in colubrid snakes (Devine 1984) and lacertid lizards (Bosh 1994). Plugs in garter snakes (*Thamnophis* sp.) may prevent copulation by subsequent males because they physically prevent hemipenis intromission (Devine 1975, 1977; Shine et al. 2000) or because they contain chemicals that inhibit female attractiveness (Ross and Crews 1977, 1978 but see Shine et al. 2000). In contrast, plugs in Iberian rock lizards (*Lacerta* (=*Iberolacerta*) *m. monticola*; Mayer and Arribas 2003) do not reduce female attractiveness and receptivity to subsequent males or hinder the success of subsequent intromissions of the hemipenis and deliverance of new plugs (Moreira and Birkhead 2003, 2004). Previous studies on Iberian rock lizards did not measure the fertilisation success of competing males and could not exclude the possibility that a first male plug, despite not physically preventing the delivery of a new plug close to the female oviductal openings, might interfere physically with second male insemination (e.g. through effects on male sperm transfer), thus lowering second male fertilisation success. However, it is more likely that second males enjoy high fertilisation success through plug and associated sperm displacement (Moreira and Birkhead 2003). In fact, the delivery of a new plug close to the oviductal openings is associated with displacement of a previous plug to the back, and often out, of the female cloaca seemingly by a mechanism of volume displacement (Moreira and Birkhead 2003, 2004; see Takami 2007 for a similar displacement mechanism in the ground beetle *Carabus insulicola*). Moreover, plugs were found to be embedded with most of the inseminated sperm, thus indicating that plugs constitute reservoirs from where sperm is transported into inner regions of the female reproductive tract after copulation (Moreira, unpublished data). The plugs adhere firmly inside the female cloaca upon delivery, loose adherence to the cloaca and shed an average of 8 daytime hours after copulation (range=1–21 h; Moreira 2002). Accordingly, it is possible that displacement of a previous plug by a subsequent male includes displacement of sperm that remains embedded in the plug, thus leading to a higher fertilisation success by second males.

Using Iberian rock lizards, we tested the effectiveness of a first male plug physical interference with a second male insemination (defence) vs the effectiveness of plug and associated sperm displacement by a second male (offence) on the outcome of sperm competition. If first male plugs interfere physically with second male inseminations, we expected that fertilisations gained by second males should increase with increasing time between first and second male copulations due to first male plugs losing adherence to the female cloaca and shedding. In contrast, if plug displacement by second males is associated with sperm displacement, we expected an opposite variation in fertilisations gained by second males because first male sperm number embedded in the plug should decrease with increasing time allowed for female sperm transport. To test these predictions, we conducted a double mating experiment where non-mated females copulated once with each of two males in sequence and according to two treatments: 30 min (first male plug adhered firmly inside the female cloaca) and 4 h (first male plug loosely adhered or shed from the female cloaca) between first and second male copulations. The number of eggs per clutch fertilised by each male was determined by paternity assignment of female eggs using microsatellite loci.

Materials and methods

Study animals and breeding conditions

Iberian rock lizards are small lacertids that inhabit high mountains and northwest coastal regions of the Iberian Peninsula and comprise several species and subspecies (Mayer and Arribas 2003). *L.* (*I.*) *m. monticola* occurs at the Serra da Estrela Mountain, Portugal, where yearly activity lasts from March–April to October–November. Adult males (snout–vent length, SVL, about 70–85 mm) emerge from

winter hibernation 1–2 weeks before adult females and start pursuing copulations after shedding the skin. The mating season takes place in April–June. Adult females (SVL about 65–95 mm) copulate about four to eight times with different males within 1 week of sexual receptivity, and then ovulate the entire clutch synchronously. Females go through parturition, with eggs remaining in the oviducts, and lay a single clutch of 2–12 eggs per year in June–July (Moreira 2002).

Adult males and females were captured at the highest altitudes of Serra da Estrela in 5–27 April 2005, soon after emerging from hibernation. Males had not shed the skin, and females did not have copulation scars, thus indicating that they had not copulated in the current season. We measured SVL to the nearest 1 mm and selected adult males and females with intact or fully regenerated tails of the 'green' colour morph; this morph is more abundant than the 'blue' (comprises 75 to 95% of the individuals according to altitudes; Moreira 2002).

Lizards were maintained at 7°C from the date of capture to June 22 (males) and 29 (females). Males (in groups of four during the first 10 days and separately after becoming intra-sexually aggressive) and females (continuously in groups of four) were housed indoor in 50×25×25 cm glass cages; detailed cage characteristics, lighting and heating (from 0900 to 1900 h), and feeding conditions are as described in Moreira et al. (2006). Cages were screened from each other using cardboard. After males shed the skin and females developed ovarian egg follicles detectable by abdominal palpation (the onset of sexual activity), we conducted copulation trials from June 11 to 18, between 1030 and 1630 h and according to the experimental design (see below).

When females became distended with eggs, their cages were provided with plastic boxes (12×9×6 cm) with incubation medium (1:10 ml demineralised water/vermiculite size '2') for oviposition. Females were inspected twice daily and, upon laying (18–27 days after copulations), clutches were immediately collected and eggs examined for fertility by candling (as in Olsson and Shine 1997). Out of a total of 126 eggs, only one was judged infertile and was preserved in ethanol 70%. Fertile eggs were incubated at 28°C in an incubator (Precision Scientific; model 815) inside 27×15×4 cm plastic boxes with incubation medium and provided with airtight transparent lids. Incubation boxes were divided into 18 equally sized chambers, and we placed one egg per clutch (covered with about 5 mm of incubation medium) in a different box and in a randomised chamber position. Eggs were inspected weekly, and those that died (collapsed) were removed from incubation and preserved separately in 70% ethanol. Towards the end of the incubation period, eggs were inspected daily. Offspring (hatched 29–35 days after oviposition) could not move between incubation box chambers and could be ascribed to their mothers. They were immediately collected, marked individually and thereafter reared under similar conditions as adults.

The infertile egg and the fertile eggs that died during incubation were opened under a dissecting microscope. We separated the embryos from the egg yolk and preserved embryos separately in 70% ethanol. No embryos were found in the egg judged infertile and in one of the eggs initially judged fertile. Tissue samples (about 10 mm of the tail tip) were collected from all adults and offspring and preserved separately in 70% ethanol. Later, we genotyped all adults, offspring and embryos (see below). Adults and offspring were released at capture sites after the study.

Experimental design

To test whether the fertilisation success of second males is affected by the time between first and second male copulations, we conducted an experiment where non-mated females copulated once with each of two males according to two treatments: 30 min and 4 h between copulations. First male plugs were adhered firmly inside the female cloaca 30 min after copulation and had started to loose adherence to the cloaca and shed 4 h after copulation (see "Results"). To control for differences in male intrinsic fertilising ability (e.g. related to sperm quantity and/or quality) on fertilisation patterns between treatments, we used males within a short SVL range (74–80 mm, n=20) and followed an experimental design where first and second males underwent both treatments in a randomised order, while matched on the number of previous copulations. Males were randomly paired (ten male pairs) with a male partner that was not from its initial home cage to avoid familiarity effects (second males might recognise first male scents on the female body; Moreira et al. 2006), and each male per pair was randomly assigned as first or second male. Male SVL did not differ significantly between first and second males (t_{18}=0.36, P=0.72). When both males per pair copulated for the first time in the season, we conducted 30-min (four cases) and 4-h treatments (six cases) using ten non-mated females. When the same males copulated for the second time, we conducted the alternate treatments; 30-min (six cases) and 4-h (four cases) treatments using another ten non-mated females. We allowed 1-day rest between consecutive male copulations to avoid sperm depletion effects; nonetheless, males that copulated once daily did not inseminate decreasing sperm number per copulation along 8 days (Moreira, unpublished data). To minimise effects of female body size and clutch size (clutch size correlates positively with female SVL; Moreira 2002) on fertilisation patterns between treatments, we used females within a short SVL range (75–82 mm, n=20).

Moreover, females were randomly assigned to treatments and male pairs, and female SVL did not differ significantly between treatments (t_{18}=0.88, P=0.39).

For conducting copulation trials, males were placed separately in trial cages (same dimensions as home cages) provided with a 60-W light-spot for thermoregulation and a clean plastic mesh for substrate. After 5 min for male acclimatisation, females were placed in corresponding male trial cages, and copulations were observed. Males and females were returned to their home cages after copulations or after 15 min when copulations did not occur. In the latter case, trials were repeated the following day. In trials involving a female first copulation, and after individuals separated (copulation completed), we confirmed that inseminations were successful by slightly parting the female anal scales and recording a plug in the cloaca. In trials involving a female second copulation, females were examined immediately before starting trials to record whether the first male plug was still inside the cloaca. That was the case for all females in the 30-min treatment and for five (50%) of the ten females in the 4-h treatment (i.e. 4 h after the first male copulation plugs had started to loose adherence to the cloaca and shed). For female second copulations, we also observed copulations from below the cage, through the mesh substrate, and recorded whether a plug came out of the female cloaca during second male copulations. After copulations, we examined whether another plug was in the female cloaca, which was always the case, thus indicating that all second males produced a plug. We collected the plugs that came out of the female cloaca and recorded whether they were whole or half plugs (plugs are bilobed, and one or both lobes may come out of the female cloaca; Moreira and Birkhead 2004). Copulation events were timed with a digital watch. The time between completion of first male copulations and the start (establishment of cloacal contact) of second male copulations was a mean±SD of 32.0±2.91 min (range=26.8–36.1 min) and 4.1±0.13 h (range=3.9–4.4 h) according to treatments.

Paternity assignment

Genomic DNA was isolated from tail and embryo samples using a Jetquick Tissue DNA kit (Genomed). Polymerase chain reaction (PCR) microsatellite amplification was performed in a total volume of 25 μl with 2 μl of isolated DNA, 1× buffer (Promega), 1.2 mM $MgCl_2$, 0.1 mM dNTPs, 20 pmol each primer and 0.5 U *Taq* polymerase (Promega) using a GeneAmp PCR System 2700 (Applied Biosystems) and the following profile: initial denaturation at 95°C for 3 min; 30 cycles at 94°C for 30 s; range of temperatures covering the locus-specific annealing temperature (Table 1) for 30 s; 72°C for 30 s; and final elongation at 72°C for 30 min. Fluorescent marked (FAM, NED, HEX) PCR products were genotyped with an ABI Prism 310 automated sequencer (Applied Biosystems), and allele size was established with GeneMapper version 3.7 (Applied Biosystems). We used four microsatellite loci (1 to 4; Table 1) to genotype all adults, offspring and embryos. Among the 40 adults, genotypes did not show heterozygote deficit (Table 1) or linkage disequilibria (P>0.13 for all loci combinations; analyses conducted on GENEPOP; Raymond and Rousset 1995). Two additional loci (5 and 6; Table 1) were used to genotype one female, corresponding two males and embryos whose paternities were not resolved with previous loci. We assigned the paternity of offspring and embryos per female to a single candidate male for 122 eggs (97% of all eggs laid by females; 98% of the eggs that hatched or contained an embryo) by assuming Mendelian inheritance of codominant loci. For two embryos of different clutches, genotypes could not be established, possibly due to deficient PCR amplification.

Data analyses

Paternities of offspring and embryos per female were used to calculate the proportion of eggs per clutch fertilised by second males (P_2). We excluded the two eggs without

Table 1 Microsatellite loci used for genotyping *L.* (*I.*) *m. monticola* from Serra da Estrela (name and GenBank accession number indicated when available)

Name (accession no.)	T_a	Size	Number	H_o	H_e	P value	Reference
1. La-3 (not available)	55–61	164–170	4	0.17	0.18	0.24	Gullberg et al. (1997)
2. Lv-4-alpha (AF100291)	55–58	110–130	10	0.33	0.31	0.82	Boudjemadi et al. (1999)
3. Lv-4-115 (AF100293)	54–63	144–190	10	0.33	0.35	0.15	Boudjemadi et al. (1999)
4. Pb73 (AY545228)	55–58	129–165	9	0.30	0.29	0.41	Pinho et al. (2004)
5. Lvir1 (AJ783621)	55–58	383–403	5	–	–	–	Böhme et al. (2005)
6. Lvir17 (AJ783631)	55–58	296–307	3	–	–	–	Böhme et al. (2005)

Forty adults were genotyped using loci 1 to 4, and one female and two males were additionally genotyped using loci 5 and 6. P values are given for Hardy–Weinberg tests of heterozygote deficit for loci 1 to 4.
T_a Range of temperatures used for PCR and covering the locus annealing temperature (°C), *Size* allele size (bp), *Number* number of alleles detected per loci, H_o and H_e observed and expected heterozygosity, respectively

embryos and the two embryos whose paternities were not established from P_2 calculations. We determined whether P_2 values were normally distributed within treatments (Shapiro–Wilk test W), and that was the case for just one of the treatments. Therefore, we compared P_2 and the frequency of second males that fertilised the majority of the eggs per clutch ($P_2>0.50$) between treatments using non-parametric tests (robust rank order test $\grave{U}$, Fisher exact test P; critical values for the former when $P<0.10$ in Feltovich 2005 and exact P when $P>0.10$ provided by the author). We also analysed whether P_2 varied according to: (1) first male plug present or absent from the female cloaca at the time of second male copulations; (2) whole or half plugs observed coming out of the female cloaca during second male copulations; (3) order of treatments underwent by each male pair; (4) male and female SVL; (5) number of eggs per clutch. We used parametric tests (t test t, product moment correlation r) to analyse normally distributed data and non-parametric tests (Spearman correlation r_s, robust rank order test $\grave{U}$) to analyse non-normally distributed data.

Results

Patterns in the proportion of eggs per clutch fertilised by second males (P_2) differed between treatments (Fig. 1). P_2 was not normally distributed in the 30-min treatment (W=0.78, P=0.009; Fig. 1), while it was normally distributed in the 4-h treatment (W=0.93, P=0.43; Fig. 1). Second males fertilised the majority of the eggs per clutch when they copulated with females 30 min after first males. In this treatment, mean±SD P_2 was equal to 0.73±0.36 (median=0.93) and differed from 0.50, but the results were not statistically significant (t_9=2.05, P=0.07). In fact, eight second males (80%) fertilised more than 50% of the eggs per clutch ($P_2>0.50$), and two (20%) fertilised less than 50% of the eggs ($P_2\leq0.50$; Fig. 1). Whereas five second males (50%) fertilised all the eggs per clutch (P_2=1.00), only one (10%) failed to fertilise any egg (P_2=0.00), and four clutches (40%) showed mixed paternities with P_2 ranging from 0.25 to 0.86. In contrast, fertilisations were equally shared between the two males when copulations were separated by 4 h. In this treatment, mean±SD P_2 was equal to 0.48±0.26 (median=0.44), and it did not differ significantly from 0.50 (t_9=−0.20, P=0.85). In fact, only three second males (30%) fertilised more than 50% of the eggs per clutch ($P_2>0.50$), and seven (70%) fertilised 50% (one case) or less (six cases) of the eggs per clutch ($P_2\leq$ 0.50; Fig. 1). Only one second male (10%) fertilised all the eggs per clutch, none (0%) failed to fertilise any egg, and nine clutches (90%) showed mixed paternities with P_2 ranging from 0.13 to 0.80.

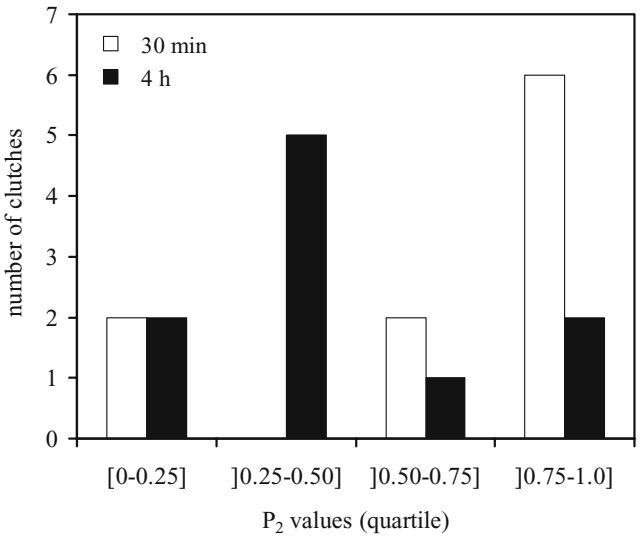

Fig. 1 Proportion of eggs per clutch fertilized by second males (P_2) according to the time between first and second male copulations

When comparing fertilisation patterns between treatments, P_2 was significantly higher in the 30-min than in the 4-h treatment ($\grave{U}$=2.11, P=0.03). Moreover, the number of second males that fertilised more than 50% of the eggs per clutch differed significantly between the 30-min and the 4-h treatment (P=0.035), being greater in the former (80%) than in the latter (30%).

In the 30-min treatment, we observed six whole plugs and four half plugs coming out of the female cloaca during second male copulations. P_2 did not differ significantly according to whether whole or half plugs came out of the female cloaca ($\grave{U}$=−1.10, $P>0.28$). In the 4-h treatment, P_2 did not differ significantly between cases where first male plugs were present or absent from the female cloaca at the time of second male copulations ($\grave{U}$=−0.07, P=1.00). In this treatment, and regarding the five females that remained with first male plugs, we observed two whole and three half plugs coming out of the female cloaca during second male copulations.

P_2 correlated positively with second male SVL in the 30-min treatment (r_s=0.88, t_9=5.21, P=0.001), but not in the 4-h treatment (r=0.17, t_9=0.49, P=0.64). P_2 did not correlate significantly with first male SVL (30-min r_s=−0.15, t_9=−0.44, P=0.67; 4-h r=0.10, t_9=0.28, P=0.79) or female SVL (30-min r_s=0.29, t_9=0.85, P=0.42; 4-h r=−0.09, t_9=−0.25, P=0.81). Number of eggs per clutch (4–9 eggs, mean±SD=6.3±1.26 eggs, n=20) did not differ significantly between treatments (t_{18}=1.07, P=0.30), and P_2 did not correlate significantly with number of eggs per clutch in any of the treatments (30-min r_s=0.35, t_9=1.07, P=0.32; 4-h r=−0.45, t_9=0.40, P=0.19). P_2 did not differ significantly according to whether male pairs underwent each of the treatments on their first or second copulations (30-min $\grave{U}$=−0.61, P=0.52; 4-h $\grave{U}$=0.72, P=0.48).

Discussion

We found that second males fertilised the majority of the eggs per clutch when they copulated with females 30 min after first males, whereas first and second males had equal chances of fertilising eggs when copulations were separated by 4 h. These results demonstrate that in Iberian rock lizards, plugs do not assure high first male fertilisation success by interfering physically with second male inseminations. Actually, when first male plug defensive role was potentially greater (30-min treatment), the majority of the eggs per clutch were fertilised by second males. This result is consistent with the hypothesis that plug displacement is associated with sperm displacement. That is, sperm from first mating partners remain embedded in the plug and are displaced by second males, and thus, second male fertilisation success increases with decreasing time allowed for female transport of sperm from the first male. Indeed, it appears that, in this system, offensive plug displacement out-competes plug defensive role and confers high fertilisation success to second males that copulate with females within at least 30 min after first males. Moreover, to the best of our knowledge, this study reveals sperm displacement as a novel sperm competition mechanism in reptiles.

Plugs were observed coming out of the female cloaca during second male copulations. We did not attempt to discriminate visually between the nature of these plugs; they may correspond to the displacement of first male plugs from the female cloaca and, less frequently, to the delivery of second male plugs outside the female cloaca (Moreira and Birkhead 2003, 2004). The two types of plugs may be distinguished by, for instance, dying first male plugs before second male copulations, as conducted by Moreira and Birkhead (2003, 2004). In this study, we did not use such methods because dyes might affect sperm quality. Nonetheless, P_2 values indicate that plugs observed coming out of the female cloaca were mostly first male displaced plugs. In fact, in the 30-min treatment, only one second male failed to fertilise any of the clutch eggs, and most second males fertilised the majority of the eggs per clutch. In the 4-h treatment, none of the second males failed to fertilise eggs. In the case of the second male that failed to fertilise any egg, it is possible that the first male plug prevented the second male from delivering its plug inside the female cloaca, thus the plug outside the female cloaca belonged to the second male. In a previous study, subsequent male success delivering a new plug inside the female cloaca and close to the oviductal openings also decreased slightly with decreasing time that a previous plug had been inside the female cloaca (Moreira and Birkhead 2003). However, the present study indicates that the defensive role of recently delivered plugs that adhere more firmly inside the female cloaca possibly does not drive the evolution of plugs. This is because first male plug physical interference with second male insemination is out-competed by second male plug displacement. That is, when a first male plug has been more recently delivered, and its defensive role is potentially greater, second males enjoy high fertilisation success because plug displacement seems to be associated with displacement of larger first male sperm number.

In this study, all females remained with first male plugs in the cloaca 30 min after copulations, indicating that female sperm transport should have been incomplete at that time. In contrast, 4 h after first male copulations, female sperm transport should have been completed, as 50% of the first male plugs had shed, and because we did not find significant differences in P_2 between females that remained or not with first male plugs at the time of second male copulations. Accordingly, differences in first male sperm number remaining embedded in the plug and displaced by second males may explain P_2 differences between treatments. In the 30-min treatment, second males seem to have displaced large first male sperm numbers, and thus, enjoyed high fertilisation success. In contrast, in the 4-h treatment, first male sperm numbers displaced by second males were likely low, and sperm numbers of the two males competing for fertilisations likely similar, so that the two males had equal chances of fertilising eggs.

Displacement of whole first male plugs (instead of half plugs) out of the female cloaca might be expected to be associated with displacement of larger first male sperm numbers and result in higher P_2 values. However, P_2 in the 30-min treatment did not differ significantly between cases where whole and half plugs came out of the female cloaca during second male copulations. An alternative possibility is that sperm embedded in displaced half plugs that remain inside the female cloaca are not successfully transported by females, as they are pushed to the back of the female cloaca and become separated from the oviductal openings by the new plug (Moreira and Birkhead 2003, 2004). We also observed that P_2 correlated positively with second male SVL in the 30-min but not in the 4-h treatment. This result suggests that larger-sized males are more efficient at displacing sperm from first males, but it is not possible to ascertain the mechanism involved.

In contrast to suggested for the garter snakes (Devine 1975, 1977; Ross and Crews 1977, 1978; Shine et al. 2000), Iberian rock lizard plugs do not reduce the likelihood of successful insemination and egg fertilisation by subsequent males. In this lizard, plugs contain most of the inseminated sperm and appear to function as sperm reservoirs (Moreira, unpublished data), thus providing subsequent males with the opportunity to displace previously inseminated sperm through plug displacement. Females of non-plug-producing reptiles were also reported to remain with sperm in the cloaca for long time periods, and sperm transport is relatively

slow in reptiles (reviewed in Olsson and Madsen 1988). Accordingly, it is possible that sperm displacement in reptiles is more widespread than previously considered. In the insects, sperm displacement exerts strong selective pressures on numerous male reproductive traits (Simmons 2001) and may be responsible for the evolution of male prolonged copulation (Sillén-Tullberg 1981) and post-copulatory mate guarding (Waage 1984; Takami 2007) as behavioural adaptations that prevent sperm displacement by subsequent males. These behaviours are common among male reptiles (Olsson and Madsen 1988), and we suggest that the role of sperm displacement on their evolution should be considered by future studies.

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References

Adler NT, Zoloth SR (1970) Copulatory behavior can inhibit pregnancy in female rats. Science 168:1480–1482

Birkhead TR, Møller AP (1998) Sperm competition and sexual selection. Academic, London

Böhme MU, Berendonk TU, Schlegel M (2005) Isolation of new microsatellite loci from the green lizard (*Lacerta viridis viridis*). Mol Ecol Notes 5:45–47

Bosh in den HAJ (1994) First record of mating plugs in lizards. Amphib Reptil 15:89–93

Boudjemadi K, Martin O, Simon J-C, Estoup A (1999) Development and cross-species comparison of microsatellite markers in two lizards species, *Lacerta vivipara* and *Podarcis muralis*. Mol Ecol 8:518–520

Carballada R, Esponda P (1992) Role of fluid from seminal vesicles and coagulating glands in sperm transport into the uterus and fertility in rats. J Reprod Fertil 95:639–648

Carballada R, Esponda P (1993) Structure of the vaginal plugs generated by normal rats and by rats with partially removed seminal vesicles. J Exp Zool 265:61–68

Devine MC (1975) Copulatory plugs in snakes: enforced chastity. Science 187:844–845

Devine MC (1977) Copulatory plugs, restricted mating opportunities and reproductive competition among male garter snakes. Nature 267:345–346

Devine MC (1984) Potential for sperm competition in reptiles: behavioral and physiological consequences. In: Smith, RL (eds) Sperm competition and the evolution of animal mating systems. Academic, London, pp 509–521

Dewsbury DA (1988) A test of the role of the copulatory plug in sperm competition in deer mice (*Peromyscus maniculatus*). J Mammal 69:854–857

Dickinson JL, Rutowski RL (1989) The function of the mating plug in the chalcedon checkerspot butterfly. Anim Behav 38:154–162

Feltovich N (2005) Critical values for the robust rank-order test. Commun Stat Simul Comput 34:525–547

Gullberg A, Tegelström H, Olsson M (1997) Microsatellites in the sand lizard (*Lacerta agilis*): description, variation, inheritance, and applicability. Biochem Genet 35:281–295

Hartung TG, Dewsbury DA (1978) A comparative analysis of copulatory plugs in muroid rodents and their relationship to copulatory behavior. J Mammal 59:717–723

Jia Z, Duan E, Jiang Z, Wang Z (2002) Copulatory plugs in masked palm civet: prevention of semen leakage, sperm storage or chastity enhancement. J Mammal 83:1035–1038

Martan J, Shepherd BA (1976) The role of the copulatory plug in reproduction of the guinea pig. J Exp Zool 196:79–84

Masumoto T (1993) The effect of the copulatory plug in the funnel-web spider, *Agelene limbata* (Araneae: Agelenidae). J Arachnol 21:55–59

Matsumoto K, Suzuki N (1992) Effectiveness of the mating plug in *Atrophaneura alcinous* (Lepidoptera, Papilionidae). Behav Ecol Sociobiol 30:157–163

Matthews M, Adler NT (1977) Facilitative and inhibitory influences of reproductive behavior on sperm transport in rats. J Comp Physiol Psychol 91:727–741

Matthews Jr MK, Adler NT (1978) Systematic interrelationship of mating, vaginal plug position, and sperm transport in the rat. Physiol Behav 20:303–309

Mayer W, Arribas O (2003) Phylogenetic relationships of the European lacertid genera *Archaeolacerta* and *Iberolacerta* and their relationships to some other 'Archaeolacertae' (*sensu lato*) from Near East, derived from mitochondrial DNA sequences. J Zool Syst Evol Res 41:157–161

Moreira PL (2002) Sexual selection and sperm competition in the Iberian rock lizard (*Lacerta monticola*). PhD thesis, The University of Sheffield, UK

Moreira PL, Birkhead TR (2003) Copulatory plugs in the Iberian rock lizard do not prevent insemination by rival males. Funct Ecol 17:796–802

Moreira PL, Birkhead TR (2004) Copulatory plug displacement and prolonged copulation in the Iberian rock lizard (*Lacerta monticola*). Behav Ecol Sociobiol 56:290–297

Moreira PL, López P, Martín J (2006) Femoral secretions and copulatory plugs convey chemical information about male identity and dominance status in Iberian rock lizards (*Lacerta monticola*). Behav Ecol Sociobiol 60:166–174

Mosig DW, Dewsbury DA (1970) Plug fate in the copulatory behaviour of rats. Psychon Sci 20:315–316

Olsson M, Madsen T (1988) Sexual selection and sperm competition in reptiles. In: Birkhead TR, Møller AP (eds) Sperm competition and sexual selection. Academic, London, pp 503–577

Olsson M, Shine R (1997) Advantages of multiple matings to females: a test of the infertility hypothesis using lizards. Evolution 51:1684–1688

Parga JA (2003) Copulatory plug displacement evidences sperm competition in *Lemur catta*. Int J Primatol 24:889–899

Parker GA (1970) Sperm competition and its evolutionary consequences in the insects. Biol Rev 45:525–567

Parker GA (1984) Sperm competition and the evolution of animal mating strategies. In: Smith RL (ed) Sperm competition and the evolution of animal mating systems. Academic, London, pp 2–60

Pinho C, Sequeira F, Godinho R, Harris DJ, Ferrand N (2004) Isolation and characterization of nine microsatellite loci in *Podarcis bocagei*. Mol Ecol Notes 4:286–288

Raymond M, Rousset F (1995) GENEPOP (version 1.2): population genetics software for exact tests and ecumenicism. J Heredity 86:248–249

Ross P, Crews D (1977) Influence of the seminal plug on mating behaviour in the garter snake. Nature 267:344–345

Ross P, Crews D (1978) Stimuli influencing mating behavior in the garter snake, *Thamnophis radix*. Behav Ecol Sociobiol 4: 133–142

Shine R, Olsson M, Mason RT (2000) Chastity belts in gartersnakes: the functional significance of mating plugs. Biol J Linn Soc 70:377–390

Sillén-Tullberg B (1981) Prolonged copulation: a male 'postcopulatory' strategy in a promiscuous species, *Lygaeus equestris* (Heteroptera: Lygaeidae). Behav Ecol Sociobiol 9:283–289

Simmons LW (2001) Sperm competition and its evolutionary consequences in the insects. Princeton University, Oxford

Smith RL (1984) Sperm competition and the evolution of animal mating systems. Academic, London

Takami Y (2007) Spermatophore displacement and male fertilization success in the ground beetle *Carabus insulicola*. Behav Ecol 18:628–634

Voss R (1979) Male accessory glands and the evolution of copulatory plugs in rodents. Occ Pap Mus Zool Univ Mich 689:1–27

Waage JK (1984) Sperm competition and the evolution of Odonate mating systems. In: Smith RL (ed) Sperm competition and the evolution of animal mating systems. Academic, London, pp 251–290