

Copulatory plugs in the Iberian Rock Lizard do not prevent insemination by rival males

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Summary

1. Iberian Rock Lizard (*Lacerta monticola*, Boulenger 1905) males produce copulatory plugs that harden and adhere firmly inside the female cloaca immediately after copulation and occlude both oviductal openings.
2. To determine whether plugs reduce the chance of females being inseminated by rival males, two hypotheses were tested: (i) that plugs reduce female attractiveness and/or receptivity to rival males; and (ii) that plugs function as ‘chastity belts’.
3. There was no evidence to support either hypothesis. Neither male initiation of copulations and female termination of male copulation attempts, nor male insemination success differed significantly between trials in which males were presented with females without a plug and with females with a plug deposited by a different male 1, 3 and 6 or more hours earlier, nor between females without and with a plug.
4. Several hypotheses for the function of Iberian Rock Lizard plugs are discussed. Since rival males were frequently able to displace a plug in the female cloaca, our results are consistent with a ‘sperm protection’ function of male postcopulatory mate guarding in squamate reptiles, but this remains to be tested.

Key-words: Chastity belt, female attractiveness and receptivity, postcopulatory mate guarding, reptile, sexual selection

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Introduction

Sperm competition arises when ejaculates from more than one male overlap within the female reproductive tract and compete for fertilization of the eggs (Parker 1970). The expected result from sperm competition is an evolutionary arms race, both between male mechanisms that allow males to secure fertilizations, driven by male–male competition, and female mechanisms that may counter male adaptations to sperm competition, as these may restrict female interests (Birkhead & Møller 1998). A variety of behavioural, morphological and physiological male traits are best understood as adaptations to sperm competition (Smith 1984; Birkhead & Møller 1992, 1998). Copulatory plugs (sometimes referred to as mating or vaginal plugs) by which males occlude the female reproductive tract may be an example of a trait that has evolved under the selective pressure of sperm competition (reviewed by Shine, Olsson & Mason 2000a). Plugs have been suggested to increase a male’s chance of fertilization by ensuring that enough sperm to outcompete rival sperm reaches

the fertilization site, and/or by preventing sperm from rival males from reaching it (Parker 1970; Devine 1975; Voss 1979).

Copulatory plugs have been described in seven snakes (Devine 1984) and 36 Lacertidae from the West Palearctic (In den Bosh 1994). The plug matrix is composed of secretions produced by the renal sex segments (RSS), which seem to be delivered after insemination, as sperm is denser in the most anterior region of garter-snake and Lacertidae plugs (Devine 1975; In den Bosh 1994). Experimental work aiming to address the function of plugs in reptiles has been limited to garter-snakes (*Thamnophis* sp.) (Shine *et al.* 2000a). Gartersnake plugs are large and gelatinous and firmly occlude the female cloaca for a few days. They have therefore been suggested to function as ‘chastity belts’ (Noble 1937; Devine 1975, 1977, 1984; Shine *et al.* 2000a). However, gartersnake attractiveness and receptivity to males decrease after copulation (Blanchard & Blanchard 1942; Whittier, Mason & Crews 1985; Whittier & Crews 1986; Ross & Crews 1977, 1978; Shine *et al.* 2000a) and the hypothesis that plugs prevent rival male insemination by physically blocking the female reproductive tract has not yet been tested. Anecdotal field observations of gartersnakes that copulated while having a plug in

place (Whittier & Crews 1986), and reports of females with two or more plugs in the cloaca (Shine *et al.* 2000a), indicate that plugs may not function as 'chastity belts' (Whittier & Tokarz 1992). Ross & Crews (1977, 1978) suggested that gartersnake plugs provide pheromonal cues to discourage courtship by rival males or to decrease female receptivity, but Mason (1994) and Shine *et al.* (2000a) have rejected this hypothesis. The source of the anti-aphrodisiac pheromones was found to be a secretion expressed by the male, the female or by both during the act of copulation (i.e. copulatory fluids) rather than by the plug *per se* (Mason 1994; Shine *et al.* 2000a). The function of gartersnake plugs and that of any other reptile therefore remains to be established.

Our casual observations on the Iberian Rock Lizard (*Lacerta monticola*, Boulenger 1905) showed that males initiated copulation with females with a plug. By staining the plug in the female cloaca, we found that rival males were able to place their own (unstained) plug underneath the previous (stained) one (i.e. closer to the oviductal openings). These observations challenged the idea that plugs reduce the chance of females being inseminated by rival males. The aim of this study was therefore to test the hypotheses that: (i) plugs reduce female attractiveness and/or receptivity to rival males, and (ii) plugs function as 'chastity belts'.

Materials and methods

STUDY SPECIES

Lacerta monticola is a small insectivorous lizard endemic to the Iberian Peninsula (Pérez-Mellado 1997). In Portugal, it is restricted to a single high mountain population, located at Serra da Estrela, which covers about 57 km². The population was estimated in 1995 to comprise between 400 000 and 700 000 individuals (Moreira *et al.* 1999). Iberian Rock Lizards at Serra da Estrela emerge from winter hibernation between March and May and are active until October to November. The copulation season, between April and June, lasts for 2–4 weeks and both sexes are highly promiscuous. Males and females reach sexual maturity at 64–70 mm snout–vent length (SVL), when they are 1–2 years of age at a study site near Lagoa Comprida lagoon (altitude 1580 m) and 3–4 years at Torre, at the top of the mountain (altitude 1993 m). Females produce a single clutch per year between June and July, with 2–11 eggs, and reproduce annually once mature. Offspring hatch between August and September. Iberian Rock Lizard longevity exceeds 10 years at Serra da Estrela (Moreira *et al.* 1999).

HYPOTHESES AND PREDICTIONS

If plugs reduce female attractiveness and receptivity, respectively, rival males are predicted to initiate fewer copulations when presented with plugged females and

plugged females are predicted to prevent male copulation attempts more frequently. On the other hand, if plugs function as 'chastity belts' their presence in the female tract is predicted to decrease male insemination success. In addition, the efficiency of a plug as a 'chastity belt' is predicted to decrease with time, if females have evolved mechanisms for expelling them. These hypotheses were tested by comparing the frequency of male initiation of copulations, male copulation attempts terminated by females, and successful inseminations, in trials in which males were presented with females without a plug and with females with a plug deposited by a different male 1, 3 and 6 or more hours earlier, and between females without and with (pooled data) a plug.

REARING CONDITIONS

Three groups of lizards were reared in captivity in 1999. Group 1 was captured in the field near Lagoa Comprida lagoon at the start of the copulation season between 9 and 24 May (i.e. when males were shedding the skin and females with copulation scars first started to appear). As in other reptiles (Bauwens, Van Damme & Verheyen 1989; Olsson, Madsen & Shine 1997), male skin shedding in the Iberian Rock Lizard signals the onset of male reproduction (Moreira 2002). Group 2 was captured near Torre also at the start of the copulation season between 24 May and 16 June. Animals in groups 1 and 2 were taken to indoor terraria installed at the field site where they resumed reproduction. Group 3 was captured near Torre at sites where the snow had only recently melted and the lizards had just emerged from hibernation and were still covered in mud, between 29 May and 11 June. Animals in group 3 were immediately placed in plastic boxes with humid vermiculite in the refrigerator at 6–8 °C, simulating the resurgence of winter conditions. Males were removed from this extended period of hibernation on 17 August and females 7 days afterwards. In this way, we created three groups of breeding lizards in captivity, spanning the periods May–June, June–July and August–September, respectively. Only lizards with intact or fully regenerated tails were used and individual SVL was measured to the nearest 1 mm. Lizards were released at their sites of capture after the study.

Up to four females were placed in 50 × 25 × 25 cm³ glass terraria with a single male. A 14D : 10L light cycle was supplied by full-spectrum lamps and heating was provided by one 60-W light bulb placed over one end of the terraria. During the light period, temperature ranged from *c.* 28 °C, in the coolest end, to *c.* 35 °C below the light bulbs (selected body temperature range of this species is 29.8–33.9 °C; Martín & Salvador 1993). Terraria were supplied with a substrate of pine bark chips and cardboard pieces. Lizards were fed *ad libitum* on mealworm (*Tenebrio* sp.) and crickets (*Achaeta* sp.) dusted with multivitamin and calcium supplements, and water was always provided. Copulations were observed directly or inferred from fresh copulation

Table 1. Outcome of trials in which sexually active and rested males were presented with sexually active females without a plug and with a plug deposited by a different male 1, 3 and 6 or more hours earlier, and results regarding all the 214 trials performed

	Plug treatment				Total
	Without	1 hour	3 hours	6 hours	
Lizards sexually active and males rested	39	20	6	13	78
Copulation attempts	31	16	5	8	60
Cloacal contact not achieved	5	1	0	0	6
Cloacal contact lost	0	3	0	1	4
Copulation achieved	26	12	5	7	50
Successful insemination	23	10	5	7	45
All trials	115	47	26	26	214
Copulation attempts	54	23	12	11	100
Cloacal contact not achieved	7	1	0	1	9
Cloacal contact lost	2	3	0	1	6
Copulation achieved	45	19	12	9	85
Whole plug not inseminated	2	3	1	0	6
Part of the plug not inseminated	2	0	0	0	2
Successful insemination	41	16	11	9	77
Whole previous plug expelled	–	8	5	7	20
Part of previous plug expelled	–	4	5	1	10
Previous plug not expelled	–	4	1	1	6
Previous plug seen being expelled	–	12	8	8	28
Previous plug adhered to hemipenis	–	4	3	3	10
Plug transparent tips expelled	–	6	0	0	6

scars (Bauwens & Verheyen 1985) and copulatory plugs. Females were examined three or four times a day at regular intervals so that the history of copulations of all lizards in captivity could be established.

PLUG FUNCTION EXPERIMENTS

Males of standard SVL (78–82 mm) were presented with females in 50 × 25 × 25 cm³ glass terraria (heating and temperature gradient as before) with a substrate consisting of a plastic mesh that allowed us to observe copulations from below. Plugs in the female cloaca were marked before the trials by applying a vital stain (Cresil blue) to its emergent face with a cotton bud. Males were placed in the trial terraria 15 min before females and trials were terminated if males had not attempted to copulate within 15 min. Trials were filmed from above with an 8-mm video camera and observations from below (e.g. previous plugs being expelled from the female cloaca) were recorded. After copulations, females were examined and inseminations were considered successful when a new (unstained) plug was well inserted in the cloaca. In copulations involving plugged females, this meant that a new plug was deposited underneath the previous (stained) one and had projections into both oviductal openings. This was confirmed by extracting new plugs with forceps immediately after copulation, which also allowed us to ascertain the position of previous plugs. Male hemipenes were examined after copulation for plug remains.

A total of 214 trials was performed, out of which males attempted copulation in 100 cases. However, males failed to achieve cloacal contact in nine cases, as females bit and/or escaped from them. In six cases

males lost cloacal contact soon after assuming the copulatory posture because the female escaped (Table 1). When males did not attempt to copulate, females were presented to another male, either immediately for the same plug treatment or later for a different plug treatment. However, and in order to test whether plugs influenced female attractiveness and/or receptivity to rival males, only the first trial using that female was considered. Moreover, for testing these hypotheses only trials involving males and females that were *a posteriori* confirmed to be within their sexually active period (i.e. after their first and before their last copulation in captivity) and rested males (i.e. more than 6 h since the last copulation) were considered, totalling 78 trials (Table 1). In order to test the 'chastity belt' hypothesis, all 85 trials in which males achieved copulation were considered. Thirty-one males and 31 females were used in the 85 trials, but only four male–female pairs were repeated per treatment (two in the without plug, one in the 1-h and one in the 3-h treatments). Data from the three breeding events were pooled in the analyses, as there were no significant differences in the results between breeding events (data not shown).

Results

Copulatory plugs in Iberian Rock Lizards are similar to those described in Lacertidae by In den Bosh (1994). They are visible in the female cloaca after copulation by parting the anal scales. Plugs consist of a portion that fills the urodaeum and two projections into both oviductal openings (Fig. 1). Most of the plug material resembles dense white foam, but at the tip of the oviductal projections its appearance is more like transparent gel.

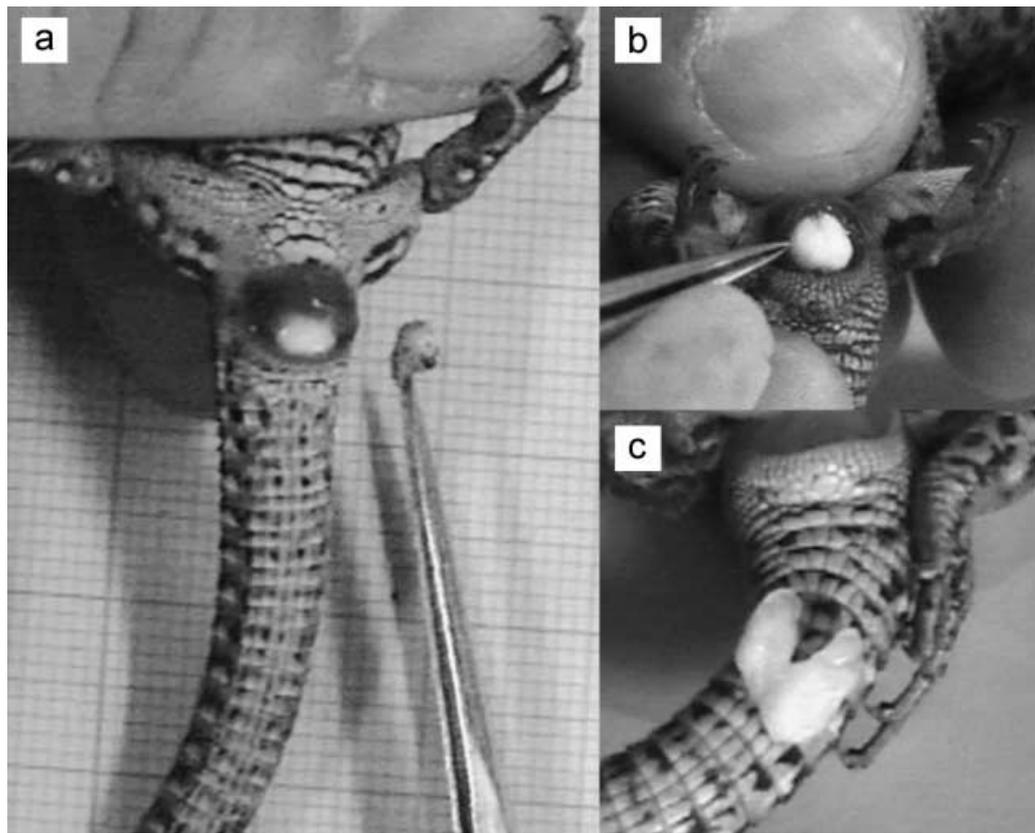


Fig. 1. Iberian Rock Lizard plugs deposited in the female cloaca (a and b), expelled during copulation by a rival male (at the tips of the forceps, a), and extracted from the female cloaca immediately after copulation (c).

Our study did not aim to quantify sperm within plugs, but preliminary microscopic observations suggested that sperm were dense in the transparent tips. Plugs harden and adhere firmly to the cloacal walls immediately after

copulation, which makes them difficult to remove with forceps. However, plugs eventually lose their adherence to the cloacal walls and are ultimately expelled. Expelled plugs are about the same dimensions as fresh plugs. In captivity, females expelled plugs a mean of 8 daytime hours (range 1–20) after copulation (Moreira 2002).

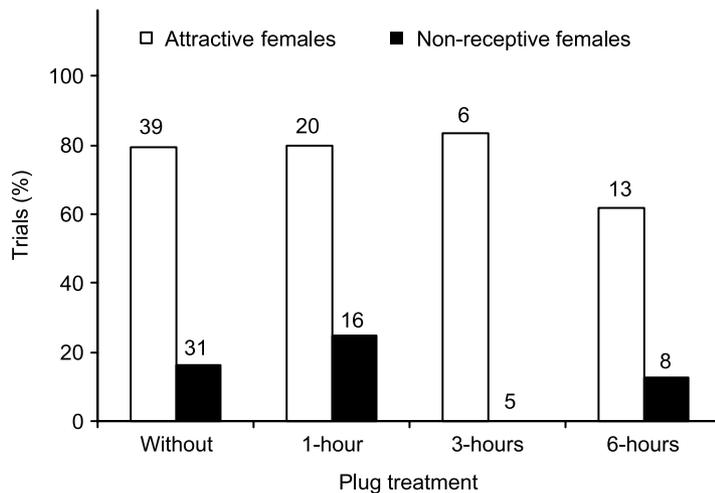


Fig. 2. The proportion of trials in which males attempted copulation (i.e. attractive females, open bars) and non-receptive females terminated male copulation attempts (filled bars) did not differ significantly between the four plug treatments (i.e. females without a plug and with a plug deposited by a different male 1, 3 and 6 or more hours earlier) (attractive: $\chi^2 = 2.1$, $df = 3$, $P > 0.50$; non-receptive: $\chi^2 = 1.9$, $df = 3$, $P > 0.50$) or between females without and with (pooled data) a plug (attractive: $\chi^2 = 0.29$, $df = 1$, $P = 0.59$; non-receptive: $\chi^2 = 0.01$, $df = 1$, $P = 0.91$), suggesting that plugs did not render females less attractive or less receptive to rival males. Sample sizes are indicated.

FEMALE ATTRACTIVENESS AND RECEPTIVITY

Copulatory plugs did not render females less attractive or less receptive to rival males. Males attempted copulation in 60 of 78 trials (77%), and the frequency of male copulation attempts did not differ significantly between the four plug treatments nor between females without and with a plug (Table 1 and Fig. 2). Females terminated 10 of 60 (17%) copulation attempts, but the frequency of copulation attempts terminated by seemingly unreceptive females did not differ significantly between the four plug treatments nor between females without and with a plug (Table 1 and Fig. 2). Females prevented males from achieving cloacal contact in 6 of the 9 (67%) cases in which they bit males during the copulation preliminaries, and broke cloacal contact in 4 of the 17 (24%) cases in which they moved very actively once males had assumed the copulatory posture. The remaining females that appeared to be unreceptive were nonetheless forcibly copulated.

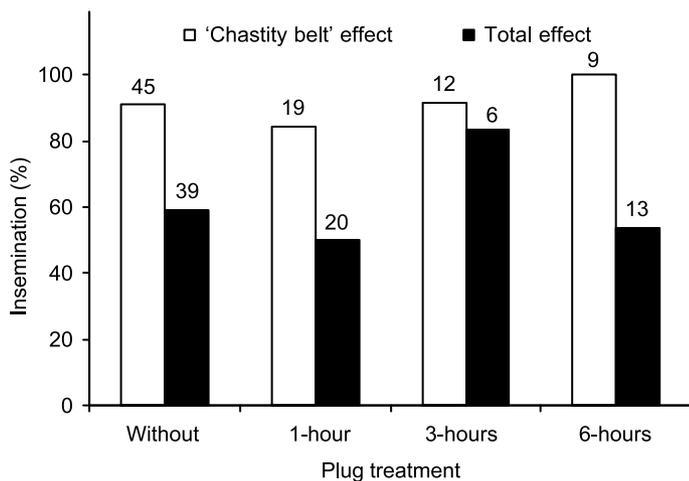


Fig. 3. Insemination success did not differ significantly between the four plug treatments (i.e. females without a plug and with a plug deposited by a different male 1, 3 and 6 or more hours earlier) ($\chi^2 = 1.9$, $df = 3$, $P > 0.50$) nor between females without and with (pooled data) a plug ($\chi^2 = 0.03$, $df = 1$, $P = 0.86$), suggesting that plugs did not function as 'chastity belts' (open bars). The 7% difference in insemination success between females without a plug and with a 1-h plug was also not statistically significant ($\chi^2 = 0.65$, $df = 1$, $P = 0.42$). Considering the sample of rested and sexually active males that were presented with sexually active females (filled bars), insemination success did not differ between the four plug treatments ($\chi^2 = 2.2$, $df = 3$, $P > 0.50$) nor between females without and with (pooled data) a plug ($\chi^2 = 0.05$, $df = 1$, $P = 0.82$), suggesting that plugs did not reduce the overall chance of females being inseminated by rival males. Sample sizes are indicated.

PLUGS AS A 'CHASTITY BELT'

Copulatory plugs were not effective as 'chastity belts'. Male insemination success was 84%, 92% and 100% when copulating females with a plug deposited 1, 3 and 6 or more hours earlier, respectively, and 91% when copulating females without a plug (Fig. 3). Insemination success did not differ significantly between the four plug treatments nor between females without (91%) and with (90% overall insemination success) a plug. Insemination success was slightly lower (by 7%) for females with a 1-h plug than for females without a plug, but, again, this difference was not statistically significant (Fig. 3). Of the eight failed inseminations, the entire new plug (six cases) or part of it (two cases) lay outside the female cloaca after copulation (Table 1).

If we consider the overall effect of plugs on female attractiveness and receptivity and as 'chastity belts', considering the 78 trials in which rested and sexually active males were presented with sexually active females, there was no evidence that plugs reduced the chances of insemination (Fig. 3). Differences in insemination success between treatments did not seem to result from the replication of males or females, since eight different males and six different females were involved in the eight failed inseminations. All these males and females participated in other copulations that resulted in successful inseminations.

Plugs were displaced by rival males in 36 of 40 (90%) cases. In 6 cases (17%), previous (stained) plugs remained in the cloaca but on top of the new

(unstained) plug (i.e. away from the oviductal openings). In 20 (57%) and 10 (28%) cases, respectively, they were totally or partly expelled from the female cloaca, the part remaining in the cloaca being on top of the new plug (Table 1). In the two cases in which expelled plugs were not directly seen being expelled during copulation, they were found at the tip of the male hemipenis (Table 1). In fact, 10 expelled plugs (36%, the above 2 plus 8 that were seen being expelled from the female cloaca during copulation) were adhered to the male hemipenes after copulation. However, hemipenes were never seen being retracted at the time of plug expulsion and since they are not spinous (P. L. Moreira & T. R. Birkhead, personal observation) it is unlikely that they function by hooking to and pushing plugs from the female cloaca. Plugs seemed to have simply adhered to hemipenes once expelled.

Displaced plugs were usually highly deformed (Fig. 1) and their most anterior transparent tips were not easily detected. Nonetheless, these tips were confirmed to be present in 6 of the 16 (38%) 1-h displaced plugs. In the remaining cases, it is possible that females had already mobilized the plug tips into the inner regions of the oviducts. There were no significant differences between 1-h, 3-h and 6-h treatments in the proportion of plugs that remained inside the cloaca or that were partially or totally expelled from it ($\chi^2 = 4.48$, $df = 4$, $P > 0.30$), indicating that plugs seem to be equally likely to be expelled as a consequence of rival male copulation irrespective of the duration of their retention by females.

Discussion

Iberian Rock Lizard plugs did not render females less attractive or less receptive to rival males, nor did they function efficiently as 'chastity belts'. It is not clear whether the slight (7%), and statistically non-significant 'chastity belt' effect observed for 1-h plugs, might *per se* select for male plug production. In terms of sperm competition, plugs do not have to be 100% effective to be adaptive (Parker 1970). However, the costs of producing plugs may not be trivial, as RSS secretions seem to be the sole product of the glandular system comprising the RSS and its ducts (Olsson & Madsen 1998). In addition, RSS secretion production seems to be the major determinant of male refractory period in gartersnakes (Shine *et al.* 2000a,b). It is also questionable whether 1-h plugs increase the likelihood of sperm precedence as in Sand Lizards (*Lacerta agilis*) a 24-h interval between two copulations did not result in first male precedence (Olsson, Gullberg & Tegelström 1994).

As Iberian Rock Lizard plugs are retained for several hours and block both oviducts, they may function as a medium for storing sperm or they may prevent sperm leakage after copulation (Parker 1970; Devine 1975; Voss 1979). These hypotheses generate different predictions regarding the distribution of sperm within

the plug matrix (Devine 1975), but our study did not aim to address them experimentally. Nonetheless, our observations suggest that plugs may serve other functions. Because plugs appear to be like a foam, they may fill the female cloaca during insemination and aid in building a positive pressure that pushes sperm forward, thereby functioning in sperm transport into the uteri in a similar manner to that documented for the laboratory rat (*Rattus norvegicus*) (Matthews & Adler 1977, 1978, 1979; Toner, Attas & Adler 1987; Sofitikis *et al.* 1990; Carballada & Esponda 1992, 1993, 1997). Being highly adherent to the female cloacal walls immediately after copulation, plugs might also prevent females from exerting any control over paternity through differential ejection of sperm. Sperm ejection following forced copulations has been reported in birds (Birkhead & Møller 1992). In female feral fowl (*Gallus gallus*), it is mediated by male social status (Pizzari & Birkhead 2000). Since Iberian Rock Lizard males appear to be able to force copulations, there seems to be an opportunity for sexual selection to favour female mechanisms of sperm choice and for males to evolve counter mechanisms. Further support for this hypothesis comes from the fact that Iberian Rock Lizard males keep their grasp on the female pelvic region, thereby maintaining the copulation position, after insemination has been achieved (Moreira 2002), suggesting that males ensure that plugs have hardened and adhered to the cloaca before separating from females. On the other hand, rival males were frequently able to displace previous plugs, by depositing their own closer to the oviductal openings. Plugs might therefore have evolved as sperm displacement devices, as RSS secretions might increase the volume and/or viscosity of the ejaculates, allowing them to aid in pushing previous ejaculates out of the oviductal orifices and even out of the female cloaca during ejaculation. Whether Iberian Rock Lizard RSS secretions contain substances that hinder fertilization by rival males is yet to be tested, but Olsson *et al.* (1994) found no evidence for this in Sand Lizards.

Plug displacement in the Iberian Rock Lizard may help to resolve the paradox of male postcopulatory mate guarding in the Sand Lizard, another species that produces plugs (In den Bosh 1994). Olsson *et al.* (1994) found that the duration of postcopulatory mate guarding in Sand Lizards did not ensure first male precedence and they suggested that guarding behaviour might not be the main target of sexual selection. Instead, they suggested that guarding resulted from constraints on male replenishment of sperm. Squamate reptiles are unique among vertebrates in having two independent intromittent organs connected with functionally separate ducts and testes (Crews 1978). They are therefore able to alternate the use of their hemipenes when copulating in quick succession (reviewed in Olsson & Madsen 1998; Shine *et al.* 2000b) and to deliver a normal sized ejaculate from the opposite side (if rested) immediately after copulation (Tokarz & Slovinsky 1990). Under the

'sperm replenishment' hypothesis we might therefore expect the duration of postcopulatory mate guarding to be shorter when males after copulating are still rested from the opposite side. On the other hand, male Sand Lizards, and other squamate reptiles, might be selected to guard females as a means of protecting their sperm investment. The 'sperm protection' function of mate guarding has been put forward in rats since plugs may be displaced (Lisk 1969; Mosig & Dewsbury 1970; Wallach & Hart 1983) and the transport of sperm into the uteri disrupted by intromission from a rival male (Adler & Zoloth 1970; Matthews & Adler 1977). According to the 'sperm protection' hypothesis, postcopulatory mate guarding may be adaptive even if its duration is shorter than the time necessary to ensure sperm precedence. It predicts that the average duration of mate guarding should be equal to the time necessary for females to uptake sperm to regions of their reproductive tract from where it cannot be removed by subsequent copulations. It also predicts that mate guarding duration should not differ according to the level of male sperm depletion.

In conclusion, copulatory plugs in the Iberian Rock Lizard do not seem to prevent female insemination by rival males. This species seems to be a good model system to investigate copulatory plug function and to determine its evolutionary significance. Some of the hypotheses put forward would be worth addressing experimentally.

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