Function of copulatory plugs in house mice: mating behaviour and paternity outcomes of rival males

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Abstract

Polyandry is widespread across animal taxa, and subjects males to intense post-copulatory sexual selection which favors adaptations that enhance a male's paternity success, either by decreasing the risk of sperm competition and/or by increasing the competitiveness of the ejaculate. Copulatory plugs deposited by males are thought to have evolved in the context of sperm competition. However, experimental studies that assess the function of copulatory plugs remain scarce. Moreover, most studies have used unnatural manipulations, such as ablating plug-producing male glands or interrupting copulations. Here, we investigated whether repeated ejaculation affects plug size in a mammalian model species, the house mouse. When males experience short periods of sexual rest we found that plug size decreased over repeated ejaculations so that time since last ejaculation can be applied as an approximation for plug size. We induced natural variation in plug size arising from variation in male sexual restedness, and investigated the behavior and paternity success of rival males. Male behavior in the offensive mating role (second) was influenced, albeit not significantly, by the sexual restedness of the first male-to-mate, and therefore the size of his plug. However, second males sired a significantly greater proportion of embryos when competing against a male that had recently mated compared to a male that had not. This supports a potential role of the plug in promoting a male's competitive fertilization success when remating occurs, which could be mediated both by delaying female remating and by ensuring efficient sperm transport through the female reproductive tract.

Key words: polyandry, sperm competition, copulatory behavior, sperm depletion, *Mus musculus domesticus*

Lay summary:

Mating plugs increase a males' paternity share in competition against rival males. In many animals males plug the female reproductive tract after mating, supposedly to prevent females from

42 remating. We show that male mice are strongly limited in plug producing ejaculate components.
43 Variation in plug size did not predict female remating, but influenced competing males'
44 competitive fertilization success when remating occurred.
45

Introduction

When females mate with multiple males during a single reproductive cycle, sperm will often be forced to compete for fertilization (Parker 1970). Sperm competition is recognized as a strong evolutionary force that selects for males to maximize their reproductive success through increased production of higher quality sperm (Simmons 2001). Moreover, post-copulatory competition favors behavioral adaptations that optimize ejaculate allocation among available females (reviewed by Wedell et al. 2002) or that decrease the risk of sperm competition, through the manipulation of female mating behavior (Gillott 2003) or mate guarding (Parker 1970). Copulatory plugs have evolved independently in many different animal taxa, including insects (Matsumoto and Suzuki 1992), spiders (Masumoto 1993), reptiles (Devine 1975) and mammals (Hartung and Dewsbury 1978; Dixson 1998), and are thought to obstruct rival males and prevent or delay subsequent inseminations (Parker 1970).

Support for a role of post-copulatory competition in favoring the evolution of copulatory plugs has received experimental support from studies adopting a variety of methodologies and performed on a broad range of taxa (insects: e.g. Orr and Rutowski 1991; Polak et al. 2001; arachnids: e.g. Masumoto 1993; Kunz et al. 2014; snakes: Shine, Olsson, and Mason 2000; rodents: Martan and Shepherd 1976). For example, indirect support comes from comparative studies that have found that plug size correlates negatively with female mating frequency among butterflies (Simmons 2001) and that relative seminal vesicle size (the accessory glands that produce the proteins that coagulate to form the plug) varies with mating system among primates (Dixson 1998). Further support comes from studies that show associations between the rates of evolution of coagulating semen components and both relative testes size among rodents (Ramm et al. 2009) and mating system among primates (Dorus et al. 2004). In contrast, several within species studies suggest that the presence of the copulatory plug does not affect female remating behavior or the outcome of sperm competition (nematodes: Timmermeyer et al. 2010; lizards: Moreira and Birkhead 2003; Moreira et

al. 2007; snakes: Friesen et al. 2014; deer mice: Dewsbury 1988a). However, such findings need not counter the hypothesis that copulatory plugs have evolved in response to selection via sperm competition. Given the many potential benefits of polyandry (Jennions and Petrie 2000), females are expected to counteract male attempts to prevent remating (Stockley 1997), generating sexual conflict over plug efficacy. Moreover, we should also expect to see complex co-evolutionary dynamics between male defensive and offensive adaptations for plugging and plug displacement respectively (Fromhage 2012). Intra- and intersexual conflict are expected to generate considerable variation in plug efficacy across taxa at any point in time.

When considering rodent species, previous researchers have concluded that the mating plug is most likely an adaptation arising from post-copulatory competition (reviewed in Voss 1979). It was noted that (i) many rodent species do not form strong pair bonds and females mate polyandrously (Voss 1979), (ii) copulatory plugs are formed exclusively by males, suggesting a potential conflict of interest between the sexes (Koprowski 1992), (iii) rodent plugs are usually very hard, tightly adhering to the vaginal epithelium and thus difficult to remove (Voss 1979), and (iv) plug tenure in the female reproductive tract typically exceeds the time span over which the ova can be fertilized (Voss 1979). Indirect support for a function of the copulatory plug in rodent sperm competition comes from a phylogenetically controlled comparative study, which showed that the relative size of seminal vesicles covaries positively with testes size relative to body weight, a widely utilized proxy for the level of sperm competition (Ramm et al. 2005). Within species studies offer contrasting findings. While in the guinea pig (*Cavia porcellus*) the copulatory plug was found to be 100% effective at preventing subsequent mates from siring offspring (Martan and Shepherd 1976), experimental plug removal did not affect paternity share in the deer mouse *Peromyscus maniculatus* (Dewsbury 1988a).

The ejaculate represents a substantial reproductive investment by males (Dewsbury 1982), and males can become sperm limited when matings occur frequently or in quick succession (Wedell et

al. 2002). However, while sperm depletion over consecutive ejaculations has been investigated in a number of rodents (Huber et al. 1980; Dewsbury and Sawrey 1984; Austin and Dewsbury 1986; Pierce et al. 1990), reduction in plug-producing ability has not been widely studied. Many male rodents produce large copulatory plugs that occupy the entire vaginal lumen, and thus likely represent a costly investment (Baumgardner et al. 1982). In laboratory rats (*Rattus novegicus*) the size of the copulatory plug decreases across the first three ejaculations, despite the fact that sperm numbers remain consistently high (Austin and Dewsbury 1986; but see Tlachi-López et al. 2012 for an opposite effect at the 8th ejaculation). A reduction in plug size across successive matings highlights the potential for the effectiveness of the copulatory plug in preventing subsequent inseminations to vary, dependent on male mating status.

Male house mice produce large copulatory plugs from coagulating proteins that are secreted from both the seminal vesicles and the coagulating glands (Gotterer et al. 1955; Rugh 1968). Early studies in mice concluded that plug formation was neither necessary nor by itself sufficient for pregnancy (McGill et al. 1968; McGill 1970), but that stimulation by the male's ejaculatory reflex, prolonged by the copulatory plug, increases the likelihood of pregnancy (McGill and Coughlin 1970; Leckie et al. 1973). Pang et al. (1979) suggested that the contents of the seminal vesicles and the associated volume of the ejaculate, rather than the plug *per se*, were crucial to ensure normal fertility. Unfortunately, however, many of the early studies used males whose accessory glands had been removed, making it impossible to rule out pleiotropic effects associated with surgical gland removal. More recently, Dean (2013) demonstrated that females mated to males with a knockout of the *transglutaminase IV* gene, and hence unable to form a copulatory plug, showed a dramatic reduction in uterine sperm numbers and pregnancy rates. This could be indicative of potential sperm reflux immediately after ejaculation and possibly of reduced vaginal stimulation (Dean 2013). These results suggest that the copulatory plug is necessary to ensure fertility in mice even in the absence of post-copulatory competition. Nevertheless, depositing a small plug might be sufficient to

ensure pregnancy. The benefits of producing a large plug are not well understood and might only be revealed when selective forces arising from competition between males are considered. Multiply sired mouse litters have been documented in nature (Dean et al. 2006; Firman and Simmons 2008a; Lindholm et al. 2013; Thonhauser et al. 2014) and from sperm competition trials performed in the laboratory (Firman and Simmons 2008b; Thonhauser et al. 2013; Manser et al. 2014; Sutter and Lindholm 2015). These studies suggest either that plugs are not always deposited, or that plugs are ineffective as a chastity enforcement mechanism. Nevertheless, the copulatory plug could benefit its producer if it affected a subsequent competitors' copulatory behavior in such a way as to delay ejaculation and ensure their rival's sperm reach the fertilization site at a sub-optimal time (Parker 1970; Ramm et al. 2005). Hence, males that ejaculate at the optimal timing while delaying their competitor's ejaculation via a copulatory plug could benefit from an increased paternity share (e.g. Coria-Avila et al. 2004; but see Klemme and Firman 2013 for a contradicting finding in house mice). Notably, in house mice, the first male to mate sires the majority of offspring, even when the copulatory plug is experimentally removed (Levine 1967; Firman and Simmons 2008b), most likely because males mating in this position ejaculate closest to the time that the ova are released (Gomendio et al. 1998).

Here, we used an experimental approach to assess the role of the copulatory plug in sperm competition in house mice. We used controlled experimental matings to investigate variation in copulatory plug size across repeated ejaculations, and its influence on both the mating behavior of rival males and the outcome of sperm competition. By doing so, we assessed multiple mechanisms by which the copulatory plug could affect male fitness, from preventing sperm competition altogether, to altering rival male mating behavior and paternity share.

Materials and Methods

Source populations and experimental animals

Male (N=77) and female (N=88) lab-born house mice (*Mus musculus domesticus*) were fourth to fifth generation outbred descendants of wild mice caught on three islands located off the coast of Western Australia (Boullanger Island, Whitlock Island and Rat Island; see Firman and Simmons 2008a for details). These populations had previously been shown to differ in levels of multiple paternity (between 17% and 71% of litters) that were correlated with relative testes sizes (Firman and Simmons 2008a). The mice were kept in standard mouse boxes (groups: 25 x 40 x 12 cm; individuals: 16 x 33 x 12 cm) on a reversed light-dark cycle (14:10 hours) with a temperature of 24°C and food (Rat and Mouse Pellets, Specialty Feeds) and water provided *ad libitum*. For all three populations, breeding pairs were housed together until the female was visibly pregnant. Before parturition, mice were separated and housed individually. At three weeks of age, litters were weaned and kept in sibling groups (females) or individually (males). For the first experiment, we used sexually experienced mice between 12 and 14 weeks of age (mean body weight +/-SE males: 21.0g +/-0.5, females: 19.1g +/-0.4). For the second experiment, we used the offspring of the mice from the first experiment when they were 7-12 weeks old (mean body weight +/-SE males: 17.0g +/-0.2, females: 14.3g +/-0.3). Females were all virgins and males were sexually naïve at the start of the experiment.

Plug size over consecutive ejaculations

In the first experiment we investigated whether the copulatory plug decreased in size across successive ejaculations. We chose pro-estrous and estrous females based on the appearance of their vagina (Byers et al. 2012), and placed them in a male's cage. Depending on our appreciation of the stage of estrous, females were then checked for a copulatory plug approximately every two hours. Copulatory plugs were removed using a blunt probe (Firman & Simmons 2008b) and weighed to the nearest 0.1mg. A second receptive female was given to the male and again checked every two hours for a copulatory plug. Upon detection, these plugs were again removed and weighed. If no

second ejaculation was achieved within 3 days, the pair was separated and the male rested for at least 7 days before starting new mating trials with different females. We obtained the weights of first and second plugs for 27 of the 30 males that were included in our paired design.

Effect of plug size on copulatory behavior and paternity outcome

In the second experiment, we assessed whether sexual restedness influenced rival copulatory behavior and paternity share (P_2; Figure 1). In each trial, a first sexually naïve male (n = 27) was allocated a sexually receptive female (based on vaginal appearance; Byers et al. 2012) who was checked every two hours for the presence of a copulatory plug. After ejaculation, the copulatory plug was left intact and female A was paired with a second male A. The first male, now sexually unrested, was allocated a different female B which was again checked every two hours for the presence of a plug. Pairs that had not mated were separated at the end of the light cycle and were re-paired at the beginning of the next light cycle. Upon detection of a copulatory plug produced by the first male, female B was paired with second male B. Thus, we used time between ejaculation with female A and female B as a measure of a first male's sexual restedness. It is important to note that when males are sexually rested for a short period of time, they may become depleted with respect to both sperm and copulatory plug material. To investigate potential mechanical effects of the plug on female remating, we recorded and assessed the mating behavior of the second males to mate (see below). However, paternity success is likely to be a function of the relative number of sperm in the female reproductive tract (Gomendio et al. 1998), and thus may be influenced by both sperm and copulatory plug depletion.

Matings performed by the second males were observed remotely via filming with a video camera (Sony DCR-SR40) to obtain behavioral data and to ensure that the males had ejaculated (i.e., ejaculation by a second-male-to-mate cannot be confirmed by the presence/absence of a copulatory plug as the first male's plug is already present). To facilitate remote observation, we transferred

second males and soiled bedding from their own cage into transparent boxes (11 x 18 x 12 cm) immediately before the beginning of the mating trial. Overall, 52 females mated with a first male and were subsequently paired with a second male. After successful mating trials, females were housed individually and provided with nesting material. Females were euthanized by intraperitoneal injection of Euthal 12-14 days *post-coitum*, and embryos were resected and stored in 100% ethanol.

Copulatory behavior

Copulatory behavior of male mice is characterized by initial mounts, a variable number of mounts with intromission (during which the male inserts his penis and performs pelvic thrusts), and ejaculation including the deposition of the copulatory plug (McGill 1962). Ejaculation is characterized by an increase in thrust frequency, a final 'shudder' and a phase of immobility, during which the pair often tip over onto their sides (McGill 1962). One copulatory series includes all mounts and intromissions, and ends with an ejaculation. The copulatory behavior of second-to-mate males was scored from the video recordings. We collected detailed behavioral data from the first copulatory series of second males on (i) the latency from introduction of the female until the first mount, (ii) the latency (from first mount) to the first intromission, (iii) the number of copulatory bouts (mounts and intromissions) until ejaculation, (iv) the latency to ejaculation (from the first mount), (v) and the duration of genital contact during ejaculation. Because males sometimes perform two full copulatory series with the same female (Estep et al. 1975; Preston and Stockley 2006; Ramm and Stockley 2014; Sutter and Lindholm 2015), we also recorded (vi) the total number of ejaculations.

Paternity share

Only 19 of the 52 females were pregnant 12-14 days *post-coitum.* Tissue samples were taken *post mortem* from all embryos, their mothers and their potential sires. DNA was extracted using the

EDNA HISPEX extraction kit (Fisher Biotec, Subiaco, Western Australia). For paternity assignment we scored 12 microsatellites spread across 10 autosomes (D3Mit278, D4Mit227, D5Mit122, D5Mit352, D6Mit139, D6Mit390, Chr8_3, D10Mit230, D11Mit90, D14Mit44, D16Mit139, and Chr19_17). Marker and PCR reaction details are described elsewhere (Bult et al. 2008; Teschke et al. 2008; Lindholm et al. 2013). Paternity analysis using the known mother and the two candidate fathers was performed using the software CERVUS (Kalinowski et al. 2007) and a genotyping error rate of 0.01 (Lindholm et al. 2013). Paternity assignments were accepted at a confidence level of 95% with a single or no mismatch between offspring and assigned father.

Statistical analyses

All statistical analyses were performed in R, version 3.1.0 (R Core Team 2015). In the first experiment, we explored variation in plug size after repeated ejaculation and variable sexual restedness. We assumed that replenishment of the seminal vesicles that produce the majority of constituents of the copulatory plug would follow an asymptotic function. We analyzed differences between first and second plugs as a function of time difference between a male's two ejaculations using a three-parameter asymptotic function with the asymptote of the difference between two consecutive plugs fixed to 0 (full replenishment over time). Thus, we estimated only two of the three parameters using the nls function in R: the response when time delay is 0, and the rate constant of the asymptotic growth (see Wilson et al. 2014). We compared the asymptotic model against a null model where plug size remains constant over time (i.e. intercept model) based on the Akaike Information Criterion corrected for small sample sizes (AICc).

In the second experiment, we investigated whether sexual restedness of first males affected the copulatory behavior and paternity success of second males. As a predictor variable, we used variation in sexual restedness of the first male, measured as time since his last ejaculation. However, our males were initially sexually inexperienced so that restedness was maximal and could

not be quantified as time rested. Based on the trajectory of plug size differences from the first experiment and on sperm replenishment in a recent experiment using these house mouse populations (Firman et al. 2015), we assumed that copulatory plug fluid reserves would be fully replenished after a week and assigned the maximum value of seven days sexual restedness to sexually naïve males and to males rested for more than a week.

Copulatory behavioral traits of second males were correlated and therefore were reduced using a principal components analysis (PCA). We transformed variables to approach normality using log(x+1) transformation, with the exception of 'the number of copulatory bouts', which was transformed using sqrt(x+1). We tested for an effect of sexual restedness of the first male (applied here as a proxy for plug size) on the copulatory behavior of second males with Linear Mixed Models (LMMs), using the function lmer implemented in lme4 (Bates et al. 2014). Males that did not mount the female (n = 11) and that did not ejaculate despite mounting (n = 8) could not be included in the PCA due to missing data. For these males, we analyzed the occurrence of mounting and of ejaculation by the second male with binary Generalized Linear Mixed Models (GLMMs) using the function glmer in the package lme4 (Bates et al. 2014), including time since previous ejaculation of the first male as a fixed effect and the identity of the first male as a random effect to account for our paired design. Copulatory behavior is likely influenced by a range of parameters, and using significance thresholds to remove predictor variables can lead to biased estimates (Forstmeier and Schielzeth 2011). We thus used an information-theoretic approach to incorporate uncertainty in parameter estimates as well as in model selection uncertainty, while retaining our focus on the effect of the copulatory plug. We fitted full models including either the first or the second principal component of copulatory behavior as the dependent variable, time since previous ejaculation of the first male, the second male's body weight, and population origin as fixed effects. To account for our paired design and to avoid pseudoreplication, the identity of the first male was included as a random effect. We followed the recommendations of Grueber et al. (2011) for model

averaging based on AICc. Using the dredge function in the MuMIn package (Bartoń 2013), we ran a full submodel set and selected all models within a range of four AICc units and averaged across models, using Akaike weights. Because of our interest in the effect of sexual restedness of the first male, we used the natural average method (Grueber et al. 2011).

We analyzed paternity share of the second male (P_2) with GLMMs, using the function glmer. The number of embryos sired by the second male was included as the dependent variable and the number of offspring genotyped as the binomial denominator. Paternity outcome is likely determined by a complex interaction of different effects. However, due to the small sample size for paternity share caused by pregnancy failure, we fitted simple models that included only a few covariates to avoid model overfitting. In the full model, time since previous ejaculation of the first male, and the two first principal components for copulatory behavior of the second male were included as fixed effects. To avoid pseudo-replication, we included identity of the first male as a random factor. Similar to the analyses on copulatory behavior, we ran a full submodel set and selected models within four AICc units for natural averaging (Grueber et al. 2011). Dispersion parameters of the GLMMs were <1. Means ± SE are presented.

Ethical statement

This research was conducted in accordance with the Australian Code of Practice for the Care and use of Animals for Scientific Purposes, and approved by the UWA Animal Ethics Committee (approval number: RA/3/100/1306).

Results

Variation in plug size across successive copulations

In the first experiment, we investigated plug weights when males had ejaculated twice, between two and 56 hours apart (n = 27). Three males produced one plug but failed to ejaculate a second time

296 within three days, and so were only included in the analyses of first plugs. The weight of first plugs
297 was significantly associated with male body weight, but relative plug size did not differ according
298 to source population (ANOVA: body weight $F_{1,26}$= 5.62, p = 0.026; population origin $F_{2,26}$= 1.05, p
299 = 0.334). Populations differed in the time difference between two ejaculations, with Rat Island
300 males being most likely to ejaculate twice on the same day (Rat Island 8/10, Boullanger 3/8,
301 Whitlock 2/9; χ^2 = 6.85, df = 2, p = 0.033). First plugs were larger than second plugs (1^{st} plugs 44.5
302 ± 3.3 mg, 2^{nd} plugs 25.3 ± 2.3 mg; paired t-test, t_{27} = 5.66, p < 0.001), and the difference between
303 first and second plug weight tended to decrease with increasing time between the two ejaculations
304 (Figure 2), although the asymptotic model obtained only a marginally better AICc support than the
305 null model (asymptotic model: AICc = 229.7, intercept model: AICc = 229.9). Time since last
306 ejaculation only explained a small proportion of the variation in plug size differences (quasi-R^2 =
307 0.1). As such, time since last ejaculation was a weak predictor for the size of the second plug. When
308 we omitted males that had produced two plugs during the same dark cycle (up to 7h time
309 difference), there was a smaller but still significant difference in plug size (mean difference 11.1 ±
310 3.9 mg; paired t-test, t_{13} = 2.88, p = 0.013).
311
312 *First male sexual restedness and second male copulatory behavior*
313 In the second experiment, we used two consecutive ejaculations of first males to investigate the
314 effect of male mating status, and consequently plug size, on the copulatory behavior of second
315 males to mate. Fifty-two females mated with a first male and were subsequently paired with a
316 second male. In 79% of the trials, the second male attempted to mate with the female, as evidenced
317 by at least one mount. Eleven trials were omitted from further analyses because we could not
318 ascertain that the female was still sexually receptive as evidenced by mounting. There was no effect
319 of time since previous ejaculation of the first male on the probability of mounting by the second
320 male (GLMM: 52 trials, 27 first males, z = 0.74, p = 0.457, *b [95% CI]* = 0.09 [-0.16,0.35]). We

then omitted trials in which the second male mounted the female but did not ejaculate (8/41 trials). The probability of ejaculation by the second male was not influenced by time since previous ejaculation of the first male (GLMM: 41 trials, 26 first males, z = -0.70, p = 0.485, *b [95% CI]* = -0.39 [-1.53, 0.75]).

The PCA on copulatory behavior of males copulating to ejaculation yielded two principal components with eigenvalues larger than one. The first component (PC1) explained 46% of the variation in copulatory behavior. PC1 was negatively loaded by the number of mounts/intromissions and ejaculation latency, and positively loaded by the number of ejaculations (Table 1). The second component (PC2) explained 21% of the variation and was positively loaded by mount latency and negatively loaded by intromission latency. Given the positive loading of the number of ejaculations and the negative loading of latency to first ejaculation, PC1 can be interpreted as ejaculatory ease, with males obtaining high PC1 values reaching ejaculation sooner and more often than males with low PC1 values. For PC2, long latencies to the first mount coincided with short latencies to the first mount with intromission. PC2 can thus be interpreted as copulatory delay, with higher scores indicating a long latency to the onset of copulation. We used PC1 and PC2 for further analyses. Model selection and effect sizes from model averaging indicated that ejaculatory ease of the second male (PC1) tended to decrease with sexual restedness of the first male (Figure 3). The model including only sexual restedness obtained the best AICc support, although the null model obtained similar support (ΔAICc = 0.72; Table 2). The effect size of sexual restedness on ejaculatory ease was negative. However, the 95% confidence interval overlapped zero (*b [95% CI]* = -0.64 [-1.34, 0.06]). Variation in PC2 was most strongly influenced by body weight of the second male to mate, with heavier males showing shorter copulatory delay (standardized effect size *b [95% CI]* = -1.10 [-1.86, -0.34]). Sexual restedness of the first male did not have an effect on PC2 (*b [95% CI]* = 0.10 [-0.61, 0.82]).

345

346 *First male sexual restedness and second male paternity share*

347 Of 52 females that received an ejaculation by at least one male, only 19 had implanted embryos at
348 the time of dissection. Pregnancy was not associated with female body weight at the time of mating
349 (GLMM: 52 trials, 27 first males, z = 0.53, p = 0.596, *b [95% CI]* = 0.31 [-0.86,1.48]), sexual rest
350 of the first male (z = -0.09, p = 0.929, *b [95% CI]* = -0.05 [-1.22,1.11]) or with whether the second
351 male ejaculated (z = 1.15, p = 0.250, *b [95% CI]* = 0.72 [-0.54,1.99]). Of the 19 pregnant females,
352 we excluded five from trials during which the second male had not ejaculated. Thus, our final
353 sample size for paternity share analyses was 14 trials where both males had ejaculated. The
354 corresponding number of implanted embryos was 99 (mean per female = 7.1, range 5-9), of which 8
355 embryos (8%) could not be assigned a father. The rate of multiple paternity was 57%, with six
356 females having all embryos sired by a single male (in four cases by the first male). Second males
357 sired a smaller proportion of offspring than first males (mean P_2: 0.33 ± 0.09), in agreement with a
358 first male advantage previously described for house mice (Firman and Simmons 2008b). In a
359 univariate analysis, sexual restedness of the first male had a significant negative effect on P_2
360 (GLMM: 14 trials, 11 first males, z = -2.52, p = 0.012, *b [95% CI]* = -1.96 [-3.65, -0.28]), showing
361 that first males who had recently mated had a lower paternity share than first males that had not
362 mated recently. After incorporating additional variables, model comparison revealed that the model
363 with the lowest AICc value included sexual restedness of the first male and ejaculatory ease (PC1)
364 of the second male, but a model including only ejaculatory ease obtained an AICc value that was
365 only 1.5 units larger (Table 3). Effect sizes after model averaging indicated that ejaculatory ease
366 had a strong positive effect on P_2 (*b [95% CI]* = 3.86 [1.55, 6.17]), while sexual restedness of the
367 first male had a negative but non-significant effect on P_2 (*b [95% CI]* = -1.67 [-3.33, 0.01]; Figure

4). Sexual restedness and ejaculatory ease showed only weak collinearity (variance inflation factors < 1.3).

Discussion

Copulatory plugs are deposited by males at mating in a large variety of taxa and have been posited to be an adaptation to post-copulatory competition, providing fitness benefits through the avoidance of or engagement in sperm competition. Here we show that male house mice produced smaller plugs when ejaculating after a shorter period of sexual rest, and thus appear to be significantly limited in producing seminal fluids that result in plug formation. We assume that sexually rested males may also have been able to produce ejaculates containing more sperm. We found only weak support for the hypothesis that plugs represent a physical barrier to sperm competition rivals. Although larger plugs tended to be associated with later ejaculation by second males this effect was not statistically significant. Males in the second-to-mate role obtained a lower paternity share when competing against sexually rested males, which were able to produce a large plug. This is possibly due to effects of the plug on both ejaculation latency and sperm retention. Our experimental design did not allow us to disentangle the effects of plug size and ejaculate size, but a reduction in plug size may accentuate a reduction in ejaculate size, if large plugs promote sperm retention in the female reproductive tract.

Constraints on plug production

When males ejaculated twice over a period of a few days, the copulatory plug they deposited was smaller at the second ejaculation. We did not experimentally manipulate the time difference between two ejaculations but attempted to get second ejaculations as soon as possible and opportunistically explored the resulting variation. While a large proportion of males used in this experiment ejaculated twice on the same day (13/30 = 43%), some males had a longer time

393 difference between their two ejaculations and for three males we did not obtain two plugs within
394 three days. The time difference between the two ejaculations was associated with the level of sperm
395 competition in the populations from which the mice were originally derived (Firman and Simmons
396 2008a). Males from the population with the most intense sperm competition (Rat Island) exhibited
397 the shortest time difference between two ejaculations. It is plausible that the high level of sperm
398 competition on Rat Island has selected for a higher mating potential in these males (Linklater et al.
399 2007). In accordance with sperm competition theory, Rat Island population males have also been
400 found to produce greater numbers of sperm compared with males from the other two populations
401 (Firman et al. 2013; Firman et al. 2015). However, we cannot rule out that the observed pattern was
402 due to other factors, such as differences in female estrous length, receptivity, or in our ability to
403 detect receptivity based on vaginal appearance (Byers et al. 2012) among these populations.
404 First plugs were positively correlated with male body weight, but relative plug weight did not
405 differ between mice from populations with different histories of sperm competition intensity. This is
406 in agreement with previous reports that sperm competition cues in the social environment or in the
407 immediate mating context do not influence plug size (Ramm and Stockley 2007; Klemme and
408 Firman 2013). The size difference between two consecutively produced plugs tended to decrease
409 over time, indicating the need for seminal fluid replenishment between matings. Thus, when males
410 ejaculated twice on the same day, the plug produced at their second ejaculation was reduced in size
411 on average by 50% (-24 mg), but one or two days later this reduction in plug size was only 19% (-
412 11 mg). There was large among male variation in the difference in size between first and second
413 plugs, which we could not explain. Given the low sample size, large individual variation and the
414 limited variation in the time difference between two ejaculations, our data do not fully support
415 recovery of plug size over time. However, our data show that males are significantly plug limited
416 after a recent ejaculation, and full recovery likely takes place in sexually mature males when given
417 sufficient time. Thus, even though our findings do not allow an estimation of the rate of recovery,

418 our results suggest that full recovery of a male's plug producing capacity may take up to three days
419 and that males are significantly plug limited after a recent ejaculation. These findings enabled us to
420 use time since last ejaculation as a broad proxy for plug size in exploring plug function.
421
422 *Is the plug a barrier to copulations by rival males?*
423 In our second experiment, we investigated how variation in plug size, as estimated by the duration
424 of sexual rest among first males, affected the copulatory behavior of a second male and his paternity
425 outcome. We found no evidence for an association between the extent to which a first male had
426 been sexually rested and the second male's sexual interest or likelihood of ejaculation. However,
427 experimental difficulties with reducing the length of sexual restedness of first males call for
428 prudence in interpreting these results. Only 16/27 (59%) first males copulated with two different
429 females within three days, out of which only two ejaculated twice on the same day. Our data from
430 the first experiment showed that plug size reduction was substantial when males were rested for less
431 than a day and that plug size was largely restored after this time. Thus, average plug size differences
432 between sexually naïve and variably sexually rested males might have been too small to represent
433 large differences in terms of physical resistance that would affect sexual interest or ejaculation
434 likelihood.
435 Overall, the rate of female remating was high and was not influenced by the sexual restedness of
436 first males (33/41 second males ejaculated). This is in agreement with other laboratory studies in
437 house mice that found evidence for high rates of multiple mating without experimental plug
438 removal (20/21 in Rolland et al. 2003; at least 57/78 in Sutter & Lindholm 2015). Moreover, as
439 found here and in previous studies (Estep et al. 1975; Preston and Stockley 2006; Ramm and
440 Stockley 2014; Sutter and Lindholm 2015), males occasionally ejaculate more than once with the
441 same female, supposedly removing their previously deposited copulatory plug before their second
442 ejaculation. This provides further indications that the plug does not prevent subsequent copulations.

443 Nevertheless, a plug could benefit its producer by delaying ejaculation by competitor males and
444 enhancing the first male's paternity share. Ramm and Stockley (2014) found that males preferred to
445 mate with unmated females compared to recently mated females, as evidenced by a lower mating
446 success with mated females. Copulating with mated females involved more intromissions and a
447 longer ejaculation latency, potentially due to resistance imposed by the copulatory plug, and thus
448 might be energetically more costly than copulating with unmated females (Ramm and Stockley
449 2014). To look at the effects of plug size variation on copulatory behavior, we reduced variation in
450 the observed behaviors of second males that had achieved ejaculation to two main principal
451 components: ejaculatory ease and copulatory delay. If the copulatory plug represented an effective
452 mechanical barrier to copulation and larger plugs provided higher effectiveness, one might predict a
453 negative effect of first male sexual restedness (i.e. larger plugs) on ejaculatory ease of the second
454 male. Indeed, the negative effect size of sexual restedness of the first male on ejaculatory ease of
455 the second male aligns with the prediction that larger copulatory plugs lead to a longer ejaculatory
456 delay, but the confidence intervals of the effect were broad and overlapped zero. Given the afore-
457 mentioned limitations of our experimental approach, our estimate of the effect of plug size on rival
458 behavior was associated with substantial uncertainty. The size of mouse copulatory plugs does not
459 appear to be adjusted in response to the perceived risk of sperm competition (Ramm and Stockley
460 2007; Klemme and Firman 2013), despite males responding to the immediate risk of sperm
461 competition in other copulatory features (Preston and Stockley 2006; Ramm and Stockley 2007).
462 Moreover, males respond to sperm competition cues in their social environment by increasing
463 sperm production (Firman et al. 2013), but not seminal vesicle size (Ramm and Stockley 2009).
464 Collectively, these findings do not support the hypothesis that the house mouse plug serves a
465 significant function in preventing female remating, but may nonetheless represent a physical
466 obstacle for rival males to overcome. Notably, a recent study found that after monogamous matings,
467 small plugs persisted in the female reproductive tract for longer than large plugs despite being more

susceptible to proteolytic degredation by females (Mangels et al. 2015). The authors suggested that smaller plugs may be more difficult to remove by females, whereas large plugs may be more difficult to remove by competitor males (Mangels et al. 2015), and our study lends some support to the latter hypothesis.

Does the plug influence paternity outcome?

We found that paternity share of second males (P_2) decreased as the time since previous ejaculation of the first male increased. Higher ejaculatory ease of second males, which tended to be associated with short sexual restedness of first males, had a strong positive effect on P_2. Notably, after controlling for the effect of ejaculatory ease of the second male, sexual restedness of the first male still tended to influence P_2, although the 95% confidence interval overlapped zero. The number of ejaculated sperm is a major determinant of paternity success in sperm competition in mammals (Gomendio et al. 1998). Meadow voles respond to an elevated risk of perceived sperm competition through ejaculation of larger sperm numbers without altering ejaculation frequency (Delbarco-Trillo and Ferkin 2004) whereas male house mice have been shown to respond through multiple ejaculations (Preston and Stockley 2006) and increased sperm production (Ramm and Stockley 2009; Firman et al. 2013). Meta-analyses across animal taxa have shown that males respond to an increased risk of sperm competition by allocating more sperm (Delbarco-Trillo 2011; Kelly and Jennions 2011). Our results confirm that repeated ejaculation can confer a fitness benefit through an increase in paternity share, since PC1 (ejaculatory ease) had a strong effect on paternity share and was loaded strongly by the number of ejaculations. However, because of collinearity between the latency to ejaculation and the number of ejaculations, we cannot disentangle the effects of the number of ejaculations and the delay between the two rivals' ejaculations. Likewise, the effect of the first male's sexual restedness on paternity share might be attributable to the number of the first males' sperm in competition, since there was still a trend after controlling for variation in the

493 second male's ejaculation latency and number of ejaculations. Little is known about ejaculate size
494 as a function of time since last ejaculation in mice, but full sperm replenishment in male rodents
495 typically takes up to a week (Ramm and Stockley 2014 and references therein). In humans,
496 ejaculate size increases as a function of time since last ejaculation for at least one week (Baker and
497 Bellis 1993). It is thus plausible that our observed negative effect of first male sexual restedness on
498 P_2 was caused entirely by slow recovery in the number of sperm ejaculated. Interestingly however,
499 in a recent experiment performed on mice from these populations, the number of epididymal sperm
500 did not significantly differ among males that had been sexually rested for two months and males
501 that had mated between 3-5 days prior, although the direction of the effect is consistent with sperm
502 depletion (Firman et al. 2015). Alternatively, a reduction in plug size accompanied by sperm
503 limitation may contribute to the observed sperm competition outcome through decreased sperm
504 retention (Parker 1970). When males ejaculated twice on the same day, uterine sperm numbers were
505 reduced even more drastically (by 80%; Huber et al. 1980) than the copulatory plug in our study
506 (~50% reduction). If small copulatory plugs are deficient in assisting sperm transport into the uterus
507 (Carballada and Esponda 1992; Dean 2013), a reduction in plug size could interact with an
508 underlying decrease in the number of sperm ejaculated, exacerbating the reduction in uterine sperm
509 numbers. Thus, large copulatory plugs could be beneficial in sperm competition by ensuring
510 optimal sperm transfer (Ramm and Stockley 2007).
511 Unfortunately, a substantial proportion of mated females did not become pregnant, greatly
512 reducing the sample size for our paternity analysis. Pregnancy failure was not related to female
513 body weight or sexual rest of the first male, but could be related to the relatively young age of
514 females and their lack of reproductive experience. Alternatively, pregnancy failure could be related
515 to the Bruce effect, the block of pregnancy by exposure of mated females to a non-stud male or his
516 odor (Bruce 1959). However, we did not find the association between female remating and
517 pregnancy (i.e. pregnancy block by females that did not remate) predicted by the Bruce effect.

518 Other studies that used a similar competitive mating design did not find high rates of pregnancy
519 failure, suggesting that exposure to more than one male *per se* does not lead to pregnancy failure
520 (Firman and Simmons 2008b; Sutter and Lindholm 2015). Because of the small sample size, we
521 focused on variables that were at the center of interest of our study (first male sexual restedness and
522 second male copulatory behavior).
523
524 *Evolutionary implications*
525 Fromhage (2012) modeled the maintenance of plug efficiency under varying levels of female
526 remating, and found that high rates of polyandry are expected to result in low plug size and
527 efficiency, because as males get mating opportunities, they invest more heavily into sperm
528 production and mating capacity rather than into copulatory plugs. The model assumed that
529 copulatory plugs only affected the likelihood of female remating. Our study supports the notion that
530 a decrease in plug size might also affect the outcome of sperm competition through delaying
531 remating or/and influencing sperm transport. This might provide an evolutionary incentive for large
532 plugs arising from sperm competition even if they are relatively ineffective at preventing female
533 remating (Parker 1970).
534 However, differences between taxa are likely to be important in determining the costs and
535 benefits of copulatory plugs, limiting the generality of our findings. Even among rodents, there are
536 indications for differential plug effectiveness. While the plug was found to be an effective mate
537 guard in guinea pigs (Martan and Shepherd 1976), there was no effect of experimental plug removal
538 on the paternity outcome in deer mice (Dewsbury 1988a). Bank voles increase the size of their
539 seminal vesicles in response to social cues to sperm competition but do not increase sperm
540 production (Lemaître et al. 2011), whereas the inverse pattern was found in house mice (Ramm and
541 Stockley 2009). The effectiveness and maintenance of copulatory plugs as a mating block may be
542 greatly determined by the reproductive biology of the species being considered. For example, costs

543 and benefits of plugging females may depend on the operational sex ratio, sexual size dimorphism,
544 length of female receptivity, level of polyandry, sperm and seminal fluid depletion rates, sperm
545 precedence patterns and plug removal skills (Dunham and Rudolf 2009; Fromhage 2012).
546 Copulatory plugs may also be subject to sexual conflict over female remating (Koprowski 1992;
547 Stockley 1997; Mangels et al. 2015), which could lead to co-evolutionary dynamics between male
548 manipulation and female control over plug efficacy and thus to different levels of plug efficacy
549 among different species that are evolving under very similar selective forces. Currently available
550 data on house mice suggest that the dynamics of copulatory plugs are complex (Mangels et al.
551 2015), that plugs may be necessary for fertility (Dean 2013), and that large plugs may provide
552 fitness benefits to males when engaging in sperm competition.
553
554 *Concluding remarks*
555 Using controlled experimental matings, we show that after a single ejaculation male house mice
556 became limited in the seminal fluids that produce the plug and recover relatively slowly. Although
557 the effect was not significant, the size of a first-to-mate male's copulatory plug tended to delay
558 ejaculation of a second-to-mate rival male. First males that had recently mated obtained a smaller
559 paternity share in sperm competition relative to first males that had been rested. This was probably
560 due to a combination of both small plug and small ejaculate production, resulting in a shorter
561 ejaculation delay for rival males and in fewer sperm being transported to the fertilization site,
562 respectively. Thus, current evidence in house mice suggests that the copulatory plug does not
563 represent a strong barrier to copulation, but might still offer an advantage in sperm competition by
564 delaying remating and ensuring efficient sperm transport.
565
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752

753 **Figure legends:**

754

755 *Figure 1:* Experimental design of the second experiment. A sexually naïve first male was mated to
756 a receptive female A, which was subsequently paired with second male A. The first male was then
757 paired with another receptive female B. After ejaculation female B was paired with second male B.
758 The copulatory behavior of both second males was remotely recorded. Females were sacrificed 12-
759 14 days *post-coitum* and paternity of the embryos was determined using 12 microsatellite markers.
760 We analyzed copulatory behavior and paternity share of second males as a function of sexual
761 restedness of the first male.

762

763 *Figure 2:* Differences in plug weights between males' first and second plugs in experiment 1. Plug
764 weight differences [mg] are shown as a function of time difference between a male's two
765 ejaculations (sexual restedness). Point color indicates the population the mice were derived from,
766 with shading darkness (colour version online) increasing with multiple paternity levels (Firman and
767 Simmons 2008a). The grey line indicates the model prediction from a three parameter asymptotic
768 model (see main text). A pooled version of all differences and the overall mean difference +/-SE is
769 shown in the right panel.

770

771 *Figure 3:* Ejaculatory ease (PC1 of copulatory behavior) of second males to mate as a function of
772 sexual restedness of the first male. Males that did not ejaculate were omitted for the PCA and
773 males that ejaculated twice are indicated in dark grey (color version online: red). Males rested for
774 longer than 7 days were assumed to be fully rested and were pooled. Sexual restedness of sexually
775 naïve first males (triangles) is maximal. For the analyses, we assigned a maximal value of 7 days.
776 The line and shaded area indicate model predictions of the mean effect of sexual restedness ± SEM
777 with body weight and population origin centered. The effect size and unconditional standard error

778 were obtained from model averaging of LMMs. Ejaculatory ease tended to be higher when sexual
779 restedness was short (see main text).
780
781 *Figure 4:* Paternity share of second males (P_2) as a function of restedness of their first competitor.
782 Point size and grayness (color version online: redness) are proportional to PC1 scores. Numbers
783 indicate the number of embryos genotyped. The line and shaded area indicate model predictions of
784 the mean effect of sexual restedness ± SEM for an average PC1 score ± SEM. The effect size and
785 unconditional standard error were obtained from model averaging of GLMMs and back-
786 transformed using the inverse logit. Restedness of the first male to mate tended to negatively affect
787 P_2 and ejaculatory ease of the second male to mate had a strong positive effect on P_2 (see main
788 text).
789

790 **Tables:**

Behavioral trait	***Mean***	***SD***	***PC1***	***PC2***
Time of first mount (mount latency) [s]†	1100	1268	0.316	**0.656**
Latency to first intromission [s]†	280	299	-0.379	**-0.702**
Number of copulatory bouts‡	29	19	**-0.883**	-0.175
Latency to ejaculation [s]†	1833	996	**-0.912**	0.156
In copula duration at first ejaculation [s]†	11.4	4.4	0.520	-0.438
Number of ejaculations	1.2	0.4	**0.795**	-0.324
Eigenvalue	-	-	2.76	1.28
% explained	-	-	46.6%	21.3%

791 † log(x+1) transformed for PCA; ‡ sqrt(x+1) transformed for PCA

792 *Table 1:* Observed copulatory behavioral traits, their variability indices and results from a principal
793 component analysis (PCA). Eigenvectors in bold were interpreted as contributing significantly to
794 the PC.

795

	Intercept	Sexual rest 1st male	Body weight 2nd male	Population: Rat	Population: Whitlock	df	AICc	ΔAICc	*w*
Model 1	0.02	-0.64				4	119.2	0	0.39
Model 2	0.01					3	119.9	0.72	0.27
Model 3	0.02	-0.65	0.08			5	122	2.8	0.10
Model 4	0.00		0.08			4	122.5	3.32	0.07
Model 5	-0.90	-0.61			+	6	122.6	3.49	0.07
Model 6	-1.03				+	5	122.7	3.54	0.07
Model 7	-1.04		0.04		+	6	125.7	6.58	0.02
Model 8	-0.91	-0.62	0.02		+	7	125.9	6.79	0.01
Estimate	-0.17	-0.64	0.08	1.02	1.81				
Unconditional SE	0.61	0.34	0.51	0.90	1.11				
Lower 95% CI	-1.40	-1.34	-0.97	-0.82	-0.48				
Upper 95% CI	1.06	0.06	1.13	2.87	4.10				
Relative importance		0.57	0.18	0.14					

Random terms: 1|male1

796 df = degrees of freedom; *w* = relative model weights

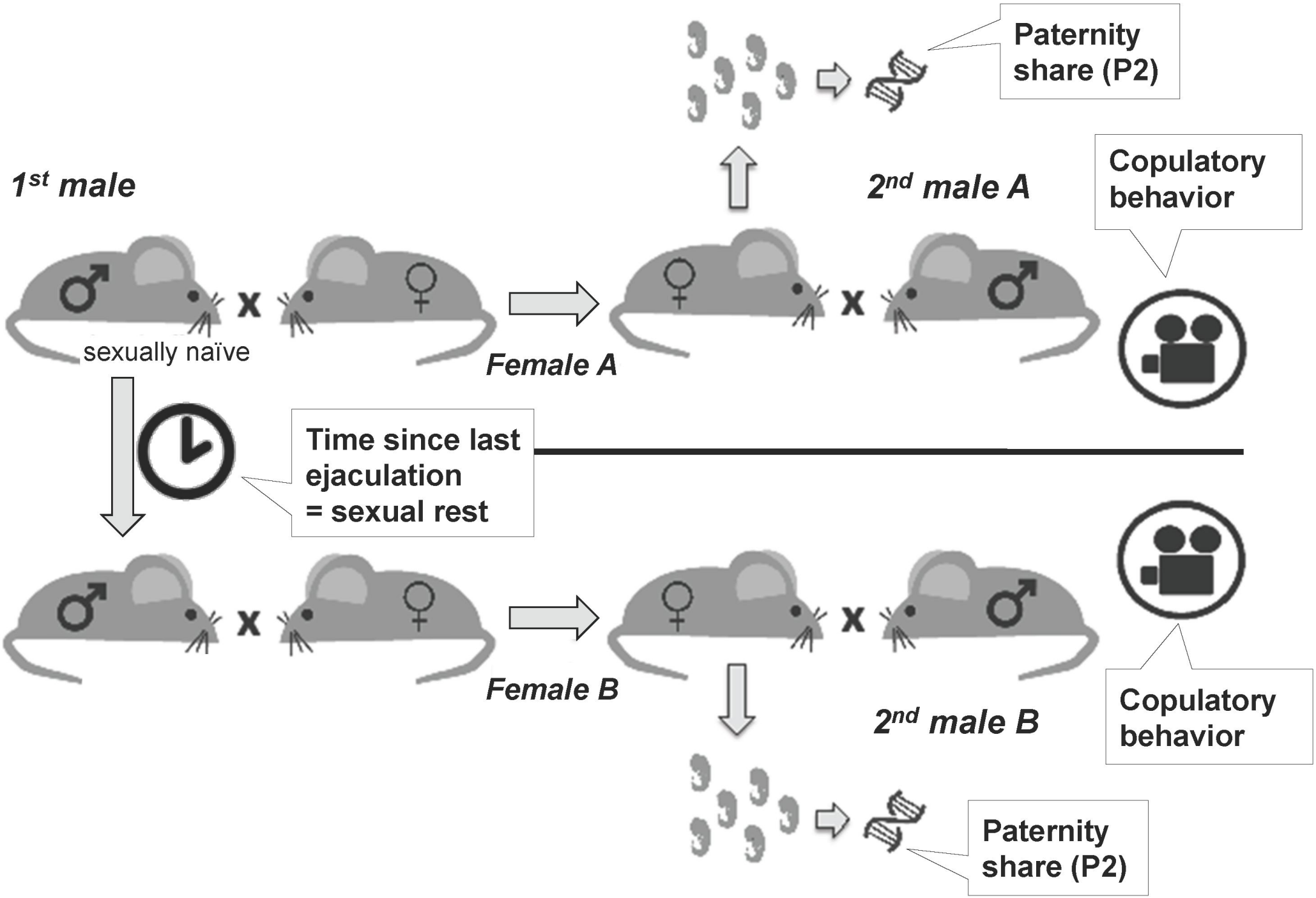
Table 2: Model summary statistics of submodels on ejaculatory ease. The full model included sexual restedness of the first male, body weight of the second male and population origin as fixed effects, and the identity of the first male as a random effect. Models within four AICc units of the best model were used for estimating standardized effect sizes using the natural average.

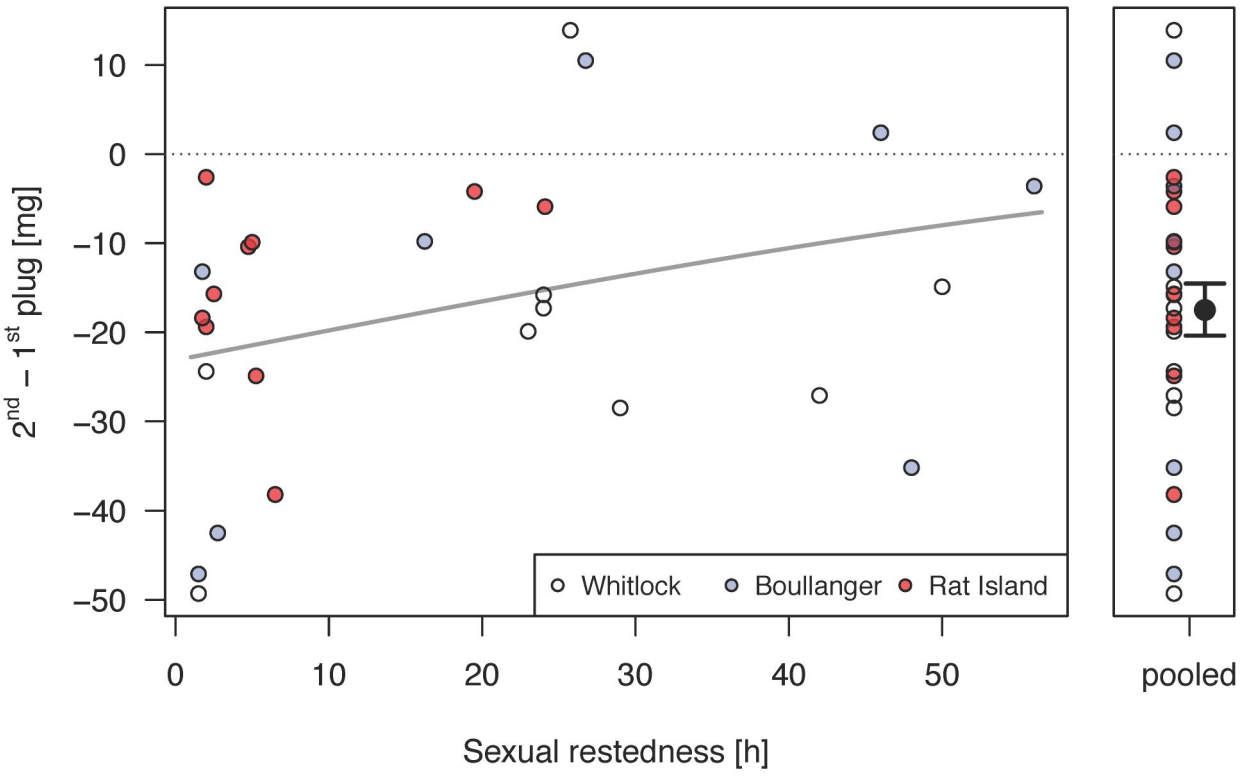
	Intercept	Sexual rest 1st male	Ejaculatory ease [PC1]	Copulatory delay [PC2]	df	AICc	ΔAICc	*w*
Model 1	-0.56	-1.66	4.02	–	4	41.1	0	0.60
Model 2	-0.79	–	3.52	–	3	42.6	1.51	0.28
Model 3	-0.55	-1.64	3.79	-0.47	5	45.4	4.34	0.07
Model 4	-0.77	–	3.39	-0.47	4	45.9	4.87	0.05
Model 5	-1.12	-1.96	–	–	3	55.8	14.78	<0.01
Model 6	-1.32	–	–	–	2	59.2	18.13	<0.01
Model 7	-1.06	-2.06	–	-0.47	4	59.6	18.54	<0.01
Model 8	-1.47	–	–	0.72	3	62	20.97	<0.01
Estimate	-0.63	-1.67	3.86					
Unconditional SE	0.31	0.75	1.04					
Lower 95% CI	-1.32	-3.33	1.55					
Upper 95% CI	0.06	0.01	6.17					
Relative importance		0.68	1					

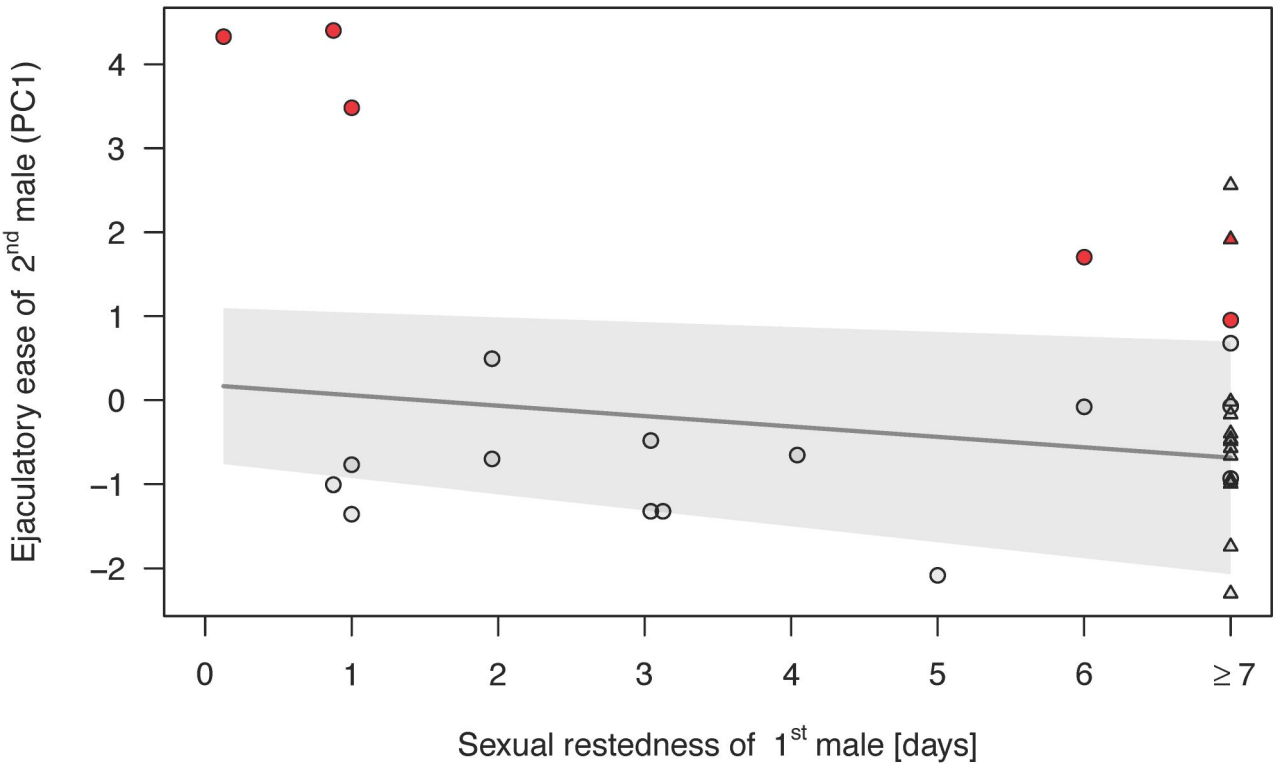
Random terms: 1|male1

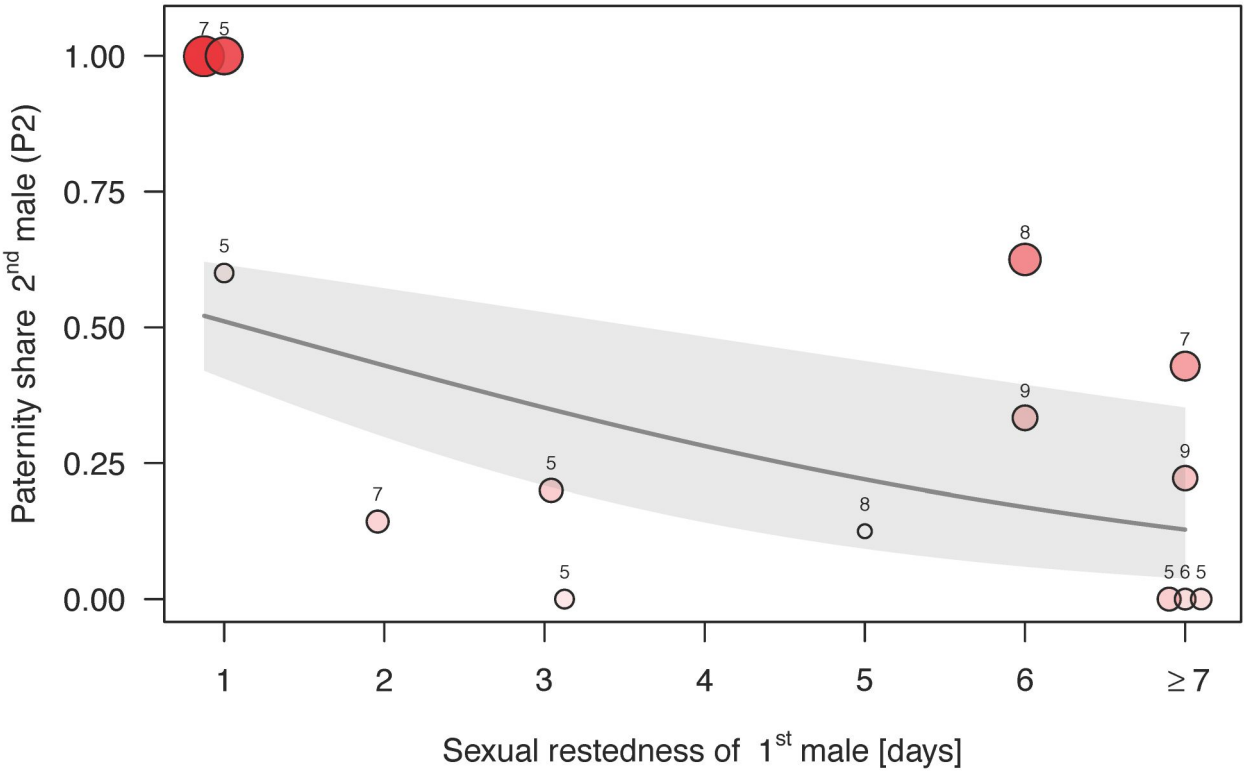
df = degrees of freedom; *w* = relative model weights

Table 3: Model summary statistics of submodels on P_2. The full model included sexual restedness of the first male and both principal components of copulatory behavior of the second male as fixed effects, and the identity of the first male as a random effect. Models within four AICc units of the best model were used for estimating standardized effect sizes using the natural average.


Paternity share (P2)
1st male
2nd male A
Copulatory behavior
sexually naïve
Female A
Time since last ejaculation = sexual rest
Female B
2nd male B
Copulatory behavior
Paternity share (P2)


10
0
−10
−20
−30
−40
−50
2nd – 1st plug [mg]
Whitlock
Boullanger
Rat Island
0
10
20
30
40
50
Sexual restedness [h]
pooled


Ejaculatory ease of 2nd male (PC1)
4
3
2
1
0
−1
−2
0
1
2
3
4
5
6
≥ 7
Sexual restedness of 1st male [days]


Paternity share 2nd male (P2)
1.00
0.75
0.50
0.25
0.00
1
2
3
4
5
6
≥ 7
Sexual restedness of 1st male [days]