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Copulatory plugs inhibit the reproductive success of rival males

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Abstract

Ejaculated proteins play important roles in reproductive fitness. In many species, seminal fluid coagulates and forms what has been referred to as a copulatory plug in the female's reproductive tract. In mice, previous work demonstrated that knockout males missing a key seminal fluid protein were unable to form a plug and less successful at siring litters in *noncompetitive* matings (one female, one male), probably the result of reduced sperm transport or insufficient stimulation of the female. Here, we extend these previous studies to *competitive* matings (one female, two males) and make two key insights. First, when first males were unable to form a plug, they lost almost all paternity to second males to mate. Thus, the copulatory plugs of second males could not rescue the reduced fertility of first males. Second, we showed that the copulatory plug of first males effectively blocked fertilization by second males, even if first males were vasectomized. Taken together, our experiments demonstrated that first males lost almost all paternity if they never formed a plug. We discuss our results in the context of natural populations, where in spite of the strong effects seen here, pregnant female mice regularly carry litters fertilized by more than one male.

Introduction

Characterizing the function of ejaculated proteins is critical to a comprehensive understanding of reproductive health and evolutionary fitness. In many sexually reproducing organisms, ejaculated proteins affect fertilization (Price *et al.*, 1999; Poiani, 2006; Wigby *et al.*, 2009), through a variety of functions that includes the balancing of pH (Asari *et al.*, 1996; Arienti *et al.*, 1999), protection and nutrition of sperm (Chen *et al.*, 2002; Wai-Sum *et al.*, 2006; Kawano *et al.*, 2014), modification of sperm physiology (Peitz, 1988; Zhu *et al.*, 2006; Kawano & Yoshida, 2007) and the induction of behavioural modifications in females (Chen *et al.*, 1988; Heifetz *et al.*, 2000; Wolfner, 2002).

A male's ejaculate coagulates to form a copulatory plug inside the female reproductive tract in many different taxa, including nematodes (Abele & Gilchrist, 1977; Barker, 1994; Palopoli *et al.*, 2008), arachnids (Austad, 1984; Masumoto, 1993; Uhl *et al.*, 2010), insects (Parker, 1970; Dickinson & Rutowski, 1989; Orr & Rutowski, 1991; Rogers *et al.*, 2009), reptiles (Devine, 1975; Herman, 1994; Friesen *et al.*, 2013) and mammals (Devine, 1975; Martan & Shepherd, 1976; Voss, 1979; Williams-Ashman, 1984; Dixson & Anderson, 2002). In rodents, this coagulation occurs when a prostate-derived transglutaminase, encoded by the gene *transglutaminase IV* (*Tgm4*), cross-links seminal vesicle-derived secretions upon mixing in the female's reproductive tract (Notides & Williams-Ashman, 1967; Williams-Ashman *et al.*, 1972; Fawell & Higgins, 1987; Esposito *et al.*, 1996; Lundwall, 1996; Lundwall *et al.*, 1997; Lin *et al.*, 2002; Tseng *et al.*, 2008, 2009; Dean, 2013; Kawano *et al.*, 2014).

Although the copulatory plug has been characterized in a diversity of taxa, its function remains incompletely understood. Schneider *et al.* (in press) recently reviewed experiments aimed at characterizing plug function, which generally fall into one of two categories. One category includes experimental removal or reduction of plugs soon after formation; these experiments generally demonstrate that first males lose paternity to second males when their plugs are compromised (Martan & Shepherd, 1976; Dickinson & Rutowski, 1989; Sutter & Lindholm, 2016; Sutter *et al.*, 2016). Consistent with an interpretation that plugs evolved to

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block fertilization of competitor males, plugs are more prominent in species inferred to have higher levels of sperm competition (Hartung & Dewsbury, 1978; Dixson & Anderson, 2002) and in such species, males develop relatively large seminal vesicles (Ramm *et al.*, 2005). In gorillas, male dominance reduces the risk of sperm competition, and males have lost the ability to make plugs (Dixson, 1998; Jensen-Seaman & Li, 2003; Kingan *et al.*, 2003; Carnahan & Jensen-Seaman, 2008). However, plugs do not always inhibit paternity of second males (Dewsbury, 1988); many plug-forming species show evidence of insemination by multiple males (Birdsall & Nash, 1973; Schwartz *et al.*, 1989; Searle, 1990; Eberle & Kappeler, 2004; Dean *et al.*, 2006; Firman & Simmons, 2008); and some monogamous species still form a copulatory plug (Foltz, 1981; Baumgardner *et al.*, 1982; Gubernick, 1988; Ribble, 1991), suggesting the plug functions outside the context of mate guarding.

Another category of studies relied upon surgical removal of accessory glands required for normal formation of copulatory plugs; these experiments generally demonstrate that plugs promote migration of the ejaculate through the female's reproductive tract and are necessary for normal fertility (Lawlah, 1930; Blandau, 1945a,b; Pang *et al.*, 1979; Peitz, 1988; Cukierski *et al.*, 1991; Carballada & Esponda, 1992). However, removal of accessory glands is likely to alter many features of the ejaculate, not just the ability of a male to form a plug.

The modern genetics era has enabled a third category of less invasive and more powerful approaches to be applied to studies of copulatory plugs (Dean, 2013; Kawano *et al.*, 2014). Male mice missing a functional copy of *transglumatinase IV* (*Tgm4*) failed to form a copulatory plug but showed normal gross morphology, reproductive anatomy, sperm count and sperm motility (Dean, 2013). In *noncompetitive* matings, *Tgm4* knockout males sired a litter in about 57% of crosses, compared to 82% of successful litters born to females mated with wild-type males. This subfertility was correlated with a reduction in the amount of ejaculate that migrated past the cervix and into the uterus and oviducts (Dean, 2013). In spite of this reduced ejaculate migration, *Tgm4* knockout males still fertilized an average of six oocytes, roughly equivalent to the average litter size sired by wild-type males. This pattern suggested that the fertility defects of *Tgm4* knockout males arise after fertilization, for example, if their embryos were less likely to implant. Therefore, it remains unknown whether *Tgm4* knockout males also show reduced fertility under *competitive* matings, where one female mates with two males in succession. One reason they might show normal fertility is if the plug of the second male somehow rescues fertility, for example, if second males provide copulatory stimulation required by the female to accept implantation of embryos.

Here, we perform two main experiments to better understand the function of the copulatory plug. In Experiment 1, we showed that when first males could not form a copulatory plug, they lost almost all paternity to second males to mate. In contrast, when first males could form a plug, they fertilized almost all embryos. Thus, second males were able to fertilize oocytes before the first male when the first male failed to form a plug. In Experiment 2, we vasectomized first males to mate and showed that if first males could form a plug, they completely blocked second male paternity even though they contributed no sperm. Our study provides direct and noninvasive quantification that the plug biases paternity towards first males to mate, and this bias is not simply due to reduced sperm transport when first males cannot form a plug. We discuss these results in the context of natural mating ecology of house mice, where multiply inseminated females are surprisingly common.

Materials and methods

Study organisms

All husbandry and experimental methods, as well as all personnel involved, were approved by the University of Southern California's Institute for Animal Care and Use Committee, protocols #11394 and #11777.

Four strains of mice were used in this study: (i) wild-type males of the C57BL/6N strain (6N wild type); (ii) *Tgm4* knockout (6N knockout) mice acquired from the Knockout Mouse Project (Austin *et al.*, 2004; Testa *et al.*, 2004), which are genetically identical to 6N wild type except for a ~7 kb 'knockout first' cassette that spans exons 2–3 of *Tgm4*; (iii) a closely related strain, C57BL/10J (10J), acquired from Jackson Laboratories (Bar Harbor, Maine); and (iv) the highly fecund FVB/NJ (FVB).

We employed a serial mating design where one female mated in succession to two males. The first male to mate was always either a 6N wild-type male, which can form a copulatory plug, or a 6N knockout male, which cannot. The second male to mate was always a 10J male, and the females were always FVB. The second male (10J) was chosen because it is genetically similar to the first male (6N knockout or 6N wild type), thus minimizing the Bruce effect, which occurs when females block implantation upon exposure to genetically distinct males (Bruce, 1960). We were still able to determine paternity since 10J is genetically distinguishable from 6N (McClive *et al.*, 1994; Keane *et al.*, 2011).

To breed experimental mice, sire and dam were paired for 2 weeks, then separated so that the dam could give birth in isolation. Males and females were weaned at 21–28 days post-partum. Females were weaned with up to three individuals per cage and were used in experiments at 6–8 weeks of age. Males

were housed singly to avoid dominance interactions (Snyder, 1967) and used in experiments at 60–90 days of age. The colony was kept at 14 : 10 h of dark:light and provided food *ad libitum*.

Experiment 1: Serial mating

At 6–8 weeks of age, individual FVB virgin female mice were induced into oestrus with an intraperitoneal injection of 5 U pregnant male serum gonadotropin, followed approximately 48 h later with an intraperitoneal injection of 5 U human chorionic gonadotropin (hCG), which induces ovulation approximately 12 h later (Nagy *et al.*, 2003). Approximately 14 h after the hCG injection, each female was placed in a randomly assigned male cage with either a 6N knockout or 6N wild type for 4 h. *In vitro* fertilization occurs within about 4 h (Dean & Nachman, 2009), and *in vivo* fertilization is likely to take longer given the addition of behavioural and physiological interactions between male and female. Therefore, females were kept with first males for 4 h, then removed and placed in a 10J male's cage for another 4 h. When 6N wild-type males were first to mate, successful mating of the first male could be scored by visual inspection for a copulatory plug. Successful mating of the second male could not be confirmed because any plug deposited by the second male would be indistinguishable from the first. We note, however, that this does not compromise the interpretation of our experiment, as biasing paternity towards first males to mate could take the form of reduced mating attempts of the second male. When 6N knockout males were first to mate, we could not confirm successful mating visually as they do not form a copulatory plug. Therefore, for a subset of these crosses, we observed the first mating for the entire 4-h period. Ejaculation was confirmed from characteristic behaviours, including increasing intromissions, which slow down closer to ejaculation, and a final shudder of the male and phase of immobility during which the pair often fall over onto their sides (McGill, 1962; McGill *et al.*, 1978). The subset of confirmed ejaculations by 6N knockout males is presented in Table 1, and our conclusions remain unaltered if we confine the analyses to this subset of crosses.

Table 1 Distribution of pregnancies sired by first or second males. Numbers in parentheses indicate confirmed ejaculations (see text). Multiply sired pregnancies are assigned to the male that sired the minority of the embryos.

1st male to mate	Successful matings	1st male paternity	2nd male paternity
6N wild type	26	19	1
6N knockout	40 (18)	0 (0)	25 (13)

Experiment 2: Serial mating, first male vasectomized

The above experiment showed that first males achieved nearly all paternity when they could form a plug. It remained unclear whether this paternity bias occurred because first males fertilized all the oocytes by the time the second male mated, or if the plug *per se* somehow blocked access of the second male. To investigate this question, we repeated the above experiment with vasectomized 6N knockout and 6N wild-type males. Vasectomized males do not transfer sperm but still transfer seminal fluid, and vasectomized 6N wild-type males produce normal copulatory plugs. If the plugs themselves blocked access, then we predict that second males cannot impregnate females when vasectomized 6N wild-type males mate first, but will impregnate females if vasectomized 6N knockout males mate first.

Mice were vasectomized using the 'scrotal entry' technique, initially anesthetizing them with 4–5% mg kg^{-1} isoflurane followed by ~1.5% for maintenance during surgery (Nagy *et al.*, 2003). Briefly, once the mouse was anesthetized, a small incision was made in the scrotum, and approximately 3 mm of the vas deferens was removed. Second male paternity was scored as the proportion of times that a female became pregnant and confirmed with genetic assays as described next.

Paternity assignment

For both experiments, females were euthanized at 12–14 days post-coitum (gestation in mice lasts approximately 21 days), and embryos were dissected for genetic assignment of paternity. DNA was extracted from a small piece of embryonic tissue and purified using a MasterPure Complete DNA extraction kit (Epicentre, Madison, WI, USA). We only performed paternity analysis on fully formed embryos to avoid resorption sites (as shown below, there was no difference in the frequency of resorption across treatments). Each embryo had a known mother (FVB) and was sired by either the first male (either 6N knockout or 6N wild type) or the second male (10J). Using published genomes of these three strains (McClive *et al.*, 1994; Keane *et al.*, 2011; Wong *et al.*, 2012), we designed genotype-specific PCRs to distinguish between the three genotypes. A 6N-specific forward primer (5′-CCACAGACATTGAGAGTGTCAGCA-3′) amplified a 346-bp fragment, or a 10J-specific forward primer (5′-AGACACCAGGAGAGCCAACAGTCCC-3′) amplified a 230-bp fragment, when used with a common reverse primer (5′-CAGCAGAATGTTCCCAGATACCCT-3′). The latter primer pair also amplified a fragment from the maternal (FVB) DNA. Therefore, paternity was assigned to the 6N male if two bands were observed (one band indicating 6N, the other indicating FVB) whereas paternity was assigned to the 10J male if one band was

observed (10J and FVB fragments of the same length). All three primers were added to each PCR.

As the band produced by FVB and 10J genotype were indistinguishable, we designed a 10J-specific forward primer (5′-GCTAGAGAGGCCCCATGGGAG-3′) that amplified a 116-bp fragment and a FVB-specific forward primer (5′-AAGGACAGGGAGAAGGGCCC-3′) that amplified a 220-bp fragment, when used in combination with a common reverse primer (5′-CCTGACTTTGCT CTGTCCTTC-3′). All three primers were added to each PCR. We tested a subset of reactions where we observed a single band in the first PCR and always observed two bands in this second PCR as expected.

DNA was amplified with the same parameters for all primer sets: 11 cycles of denaturation (94 °C, 30 s), annealing (65 °C, 30 s, lowered 1 °C every cycle) and extension (72 °C and 60 s) followed by 19 cycles of denaturation (94 °C, 30 s), annealing (52 °C, 30 s) and extension (72 °C and 60 s). All PCRs used Fermentas 2X master mix. PCR products were scored on a 3% agarose gel.

Statistical analysis

Across all experiments, 54 pregnancies were collected. Fifty-one of these were sired entirely by one male. For the other three, we either (i) excluded them, (ii) assigned them to the male that sired more individual embryos in those pregnancies or (iii) assigned them to the male that sired the minority of the embryos (which increased our chance of losing statistical significance). Our conclusions remained the same regardless of which strategy we employed; we present the third strategy as it is the most conservative. We performed a Fisher's exact test (FET) to determine whether the number of pregnancies sired by the second male depended on the first male's ability to form a copulatory plug. In Experiment 1, we excluded all resorption sites from tests of paternity, but this is only justified if resorption sites are randomly distributed among treatments. To test this, we tested the distribution of resorption sites using a generalized linear model, with first male as fixed effect, and the number of embryos vs. number of resorption sites per litter as the two-vector response variable, using the function GLM of the R package MASS (Venables & Ripley, 2002).

Results

We performed 96 successful crosses across both experiments (Table S1).

Experiment 1: First males achieved nearly all paternity when they could form a plug

Twenty-six females successfully mated with 15 6N wild-type males and were subsequently paired with a 10J male, resulting in 20 pregnancies (Table 1). Of these 20 pregnancies (average number of embryos = 12.55 ± 7.19), 19 were sired exclusively by the 6N wild-type male. One pregnancy included nine embryos sired by the 6N wild-type male and one embryo sired by the 10J male. These 20 pregnancies were sired by 13 different individual males (some males were used more than once, Table S1).

Forty females were paired with 6N knockout males first then successfully mated with a 10J male. Of these 40 females, 25 resulted in pregnancies, 23 of which were sired by the second male (average number of embryos = 10.53 ± 6.55). Two pregnancies were multiply sired; one had 17 embryos sired by 6N and one sired by 10J, and the other had seven sired by 6N and five sired by 10J. First males sired significantly more litters when they could form a plug (Table 1, FET = 10^{-12}).

There was no statistical difference in the number of embryos per pregnancy between the two treatments (Wilcoxon's rank-sum test, $P = 0.42$), nor a difference in the proportion of crosses that resulted in pregnancy among treatments ($\chi^2 = 0.92$, d.f. = 1, $P = 0.34$). Furthermore, the number of resorption sites was randomly distributed among treatment ($P = 0.80$). In sum, only the distribution of paternity, and no other characteristics of litter size or fertilization success, varied according to the first male to mate.

It has been reported previously that 6N knockout male mating behaviour does not differ from 6N wild type, they have normal sperm count and sperm motility, and they are able to fertilize oocytes (Dean, 2013). Nevertheless, we repeated our analysis after only including crosses where ejaculation by 6N knockout males was visually confirmed. Eighteen females were paired with 6N knockout males first, where ejaculation was visually confirmed. Of these 18 females, 13 resulted in pregnancies, 11 of which were sired by the second male (average number of embryos = 10.3 ± 6.0). The two multiply sired pregnancies were from this subset of data. There was no statistical difference in the number of embryos per pregnancy between the two cross types (Wilcoxon's rank-sum test = 0.56), nor a difference in the proportion of crosses that resulted in pregnancy ($\chi^2 = 0$, d.f. = 1, $P = 1$). First males sired significantly more litters when they could form a plug (numbers in parentheses of Table 1, FET = 10^{-8}). In sum, first males achieved nearly 100% paternity if they could form a plug, but nearly 0% paternity if they were unable to form a plug, even when ejaculation was visually confirmed in the latter case.

Experiment 2: Paternity skew remained even when first males were vasectomized

Thirteen females successfully mated with a vasectomized 6N wild-type male, as confirmed by the presence of a copulatory plug, and were subsequently placed with a 10J male. None of these females were pregnant 14 days post-coitum. Seventeen females were

paired with vasectomized 6N knockout males then subsequently placed with a 10J male, of which nine resulted in a pregnancy (average number of embryos = 8.11 ± 5.06), all of which were sired by the second male (as expected since the first male was vasectomized). The ability of a second male to impregnate a female was significantly correlated with the first male's ability to form a plug (FET = 0.003).

Discussion

Here, we employed a knockout mouse model to further characterize the functions of the copulatory plug in mice. First males sired almost all embryos if they could form a plug, but sired almost none if they could not. Therefore, the previously observed reduction in ability of *Tgm4* knockout males to sire litters (Dean, 2013) cannot be rescued by second male plugs. Perhaps most importantly, the skew in paternity remains even if first males were vasectomized, indicating the absence of the plug (and not just reduced fertility of *Tgm4* knockout males) allowed second males to gain paternity.

The blocking effect of the copulatory plug could arise through physical prevention of mating (Parker, 1970; Devine, 1975; Voss, 1979; Shine *et al.*, 2000), or by discouraging second males from mating through visual or olfactory cues (Ramm & Stockley, 2014). In mice, the first male to mate has an advantage (Levine, 1967), which may select for males to avoid mating with females that have already mated (Ramm & Stockley, 2014). Along with indirect evidence that mating and ejaculation are energetically costly for males (Drickamer *et al.*, 2000, 2003; Gowaty *et al.*, 2003; Pizzari *et al.*, 2003; Lüpold *et al.*, 2011; Friesen *et al.*, 2015), males may be selected to conserve ejaculates when possible (Parker, 1998; Ramm & Stockley, 2007).

Evidence that plug-forming species show multiple paternity

In apparent contrast to the very strong effects we observed in this study, 10–70% of pregnant female house mice carry offspring sired by more than one male in natural populations (Dean *et al.*, 2006; Firman & Simmons, 2008). Many other plug-forming species also show high rates of multiple paternity (Birdsall & Nash, 1973; Schwartz *et al.*, 1989; Searle, 1990; Møller, 1998; Eberle & Kappeler, 2004; Uhl *et al.*, 2010). Several hypotheses could reconcile the strong inhibitory effects of the plug observed here with the common observation of multiple paternity in nature.

We did not vary several aspects of mating ecology that might influence paternity, such as the number of males a female encountered during a single fertile period. The opportunities for multiple mating may not be confined to two males presented in succession, as occurred in our experiments, if the females actively pursue multiple mating in natural populations. For example, multiple paternity is more common in relatively dense populations (Dean, 2013), and we would not have captured such variation in our serial mating design. Furthermore, we did not vary the time each male spent with the female. Here, the first male was left with the female for 4 h. Based on our watched subset, mating occurred on average of 2.5 h after crossing (Table S1). The sooner a second male is able to dislodge a first male's plug, the more effective he is at gaining paternity (Wallach & Hart, 1983; Sutter & Lindholm, 2016; Sutter *et al.*, 2016) as the copulatory plug promotes sperm transport (Matthews & Adler, 1978; Toner *et al.*, 1987; Dean, 2013). It is possible that our 4-h time window biased paternity towards the first male, and this was amplified when the first male could form a plug.

In the present study, ovulation and behavioural oestrus were experimentally controlled through hormonal manipulation, and copulation was likely to have occurred very close to ovulation. In nature, the time between copulation and oestrus is likely to be more variable. For example, if first males mate 'too early' relative to a female's ovulation, their plug might not survive long enough to inhibit later, better-timed matings of second males (Coria-Avila *et al.*, 2004; Firman & Simmons, 2009; Breedveld & Fitze, 2016). Copulatory plugs get smaller and less adhered to the vaginal–cervical canal over time (Mangels *et al.*, 2015), suggesting that the efficacy of the plug decreases over time. Furthermore, not all matings result in a well-formed and properly seated copulatory plug (Hartung & Dewsbury, 1978; Matthews & Adler, 1978; Masumoto, 1993). In fact, females may exert some control over the fate of the copulatory plug.

Females may prevent proper orientation of the copulating male (Matthews & Adler, 1978) or remove deposited plugs (Koprowski, 1992; Parga, 2003). In our study, such females may have been excluded, because crossing to 6N wild-type males was deemed successful only after observing a well-formed plug. By potentially excluding females that had already removed plugs, we might be overestimating success of 6N wild-type males.

Subsequent males may also affect the efficiency of the plug by actively removing the copulatory plug before copulation (Parga, 2003; Parga *et al.*, 2006) or dislodging the copulatory plug through multiple intromissions before ejaculation (Mosig & Dewsbury, 1970; Milligan, 1979; Dewsbury, 1981; Wallach & Hart, 1983; Sutter & Lindholm, 2016; Sutter *et al.*, 2016). We observed instances where the second male removed the copulatory plug of the first male, which may be interpreted as the second male attempting to gain fertilization. However, these trials did not result in second male paternity.

Lastly, we did not vary male or female genotypes in the current study. It is possible that the 6N wild-type males used here make especially strong plugs; that the 10J males (always second to mate) were simply unable to remove previously deposited plugs; or that the FVB

females were especially resistant to remating if a plug-forming male was first to mate. Mangels *et al.* (2015) showed that different male genotypes vary in both the size and survival of copulatory plugs they form.

Conclusions

Our study provides direct, noninvasive quantification of the copulatory plug's role in inhibiting second male paternity, at least in a laboratory setting. When first males cannot form a plug, they lose almost all paternity to second males, and this cannot simply be due to reduced sperm transport of the first male. It is important to recognize that our study does not reject the role of the copulatory plug in other aspects of fertility. Future studies should place lessons learned here in the context of the many variables encountered in nature, to better understand the ecology and evolution of the copulatory plug.

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Supporting information

Additional Supporting Information may be found online in the supporting information tab for this article:
Table S1 Raw data from serial mating experiments

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