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ORIGINAL ARTICLE

Female remating decisions and a shorter inter-mating interval diminish last-male sperm precedence

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Abstract
Highly variable within and across species, patterns of sperm use not only are often driven by post-copulatory sexual selection but can also be impacted by experimental design. In investigations of paternity bias using competitive double matings, the inter-mating interval is a temporal factor that can affect sperm use patterns if the first male's sperm is used or lost at an appreciable rate between matings or if its viability or relative competitiveness is influenced by the time since ejaculation. Rapid loss of first-male sperm within the female after mating has been established in the seed beetle (*Callosobruchus maculatus*), a model system in sperm competition studies. However, our understanding of sperm precedence in this species, which disproportionately favors the last (second) male to mate, is based on long inter-mating intervals. Here, I determine the effect of a shortened inter-mating interval on second-male paternity (P_2) and, importantly, the extent to which females in this species are willing to remate immediately. I find that P_2 is significantly reduced when females remate immediately than when they remate 24 or 48 h after the first mating and that immediate remating is common, indicating that there is a substantial potential for female remating decisions to influence the intensity of sperm competition within species. To understand the variation in inter-mating intervals from a female perspective, I further identify key differences between females that did and did not remate at three inter-mating intervals (0, 24, and 48 h after the initial mating) and discuss potential mechanisms for the observed variation in female refractoriness.

Significance statement
Females often mate with multiple males, and in insects it is common for the last male to mate to obtain the greatest share of paternity. One possible explanation for this bias towards last-male sperm use is the passive loss of sperm from the first male with time since mating. Here, I examine the effect of different intervals between matings in a seed beetle species to determine if first-male paternity is increased when females remate sooner. My results show that first males do achieve higher paternity when females remate sooner, which is more common than expected in this species. I also show the differences between females that do and do not remate, which can help explain potential causes for variation in female remating behavior. Together, these results show the significant impact that female remating decisions can have on male competition and, ultimately, their reproductive success.

Keywords Polyandry · Sexual selection · Paternity · Female remating behavior · Sperm precedence · Sperm competition

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Introduction

Female multi-male mating within a single reproductive cycle is the rule, not the exception, across animal taxa (Walker 1980; Thornhill and Alcock 1983; Eberhard 1996). A commonly observed outcome of this female behavior in species with internal fertilization is sperm precedence, the phenomenon

of non-random fertilization success among consecutive mates (Lewis and Austad 1990; Simmons 2001). Such fertilization bias may be examined within a particular species by observing the average proportion of eggs fertilized by the last (second) male to mate in competitive double matings, denoted as P_2 (Boorman and Parker 1976). Previous empirical studies have not only documented species averages for P_2 but have also revealed a high degree of variation in these values both within and across species, with sperm precedence patterns either apportioned equally according to numerical sperm representation among the males (but see Manier et al. 2013) or disproportionately favoring either the first or, more commonly among insects, the last male to mate (Parker 1970a; Lewis and Austad 1990).

The variation in sperm use patterns has been attributed to a number of different factors (Table 1), but non-mutually exclusive sources for this variation are thought to have arisen via an arms race in which female and male interests are expressed via post-copulatory inter- and intra-sexual selection. In the former case, runaway processes such as those involved in cryptic female choice for the preferred male's sperm or stimulating genital morphologies are thought to be important (Eberhard 1996); in the latter case, male traits and/or behaviors that function in offense or defense of sperm competition are thought to be important (Parker 1970a; Simmons 2001). While these two post-copulatory mechanisms and their relative importance in driving sperm precedence patterns are difficult to disentangle and are likely species-specific, studies attempting to elucidate these causes can be further complicated by factors in experimental design that also lead to intra-specific variation in sperm use patterns (Danielsson 1998).

In investigations of paternity bias using competitive double matings, the inter-mating interval is a temporal factor that has been demonstrated to affect sperm precedence (Birkhead and Møller 1992). While most post-copulatory sexual selection studies control for the inter-mating interval, studies in which two distinct intervals were imposed or the interval was allowed to vary naturally based on female remating decisions have exposed changes in sperm precedence patterns with varying intervals between matings (Dickinson 1988; Yamagishi et al. 1992; Arnaud et al. 2001; Drnevich 2003; Blyth and Gilburn 2005). Whereas some studies have revealed a reduction in P_2 with increased time between matings (Radwan 1997), many have revealed an increase in P_2 with an increase in the inter-mating interval (reviewed in Simmons 2001). Results from these studies illustrate that the inter-mating interval is a critical component of paternity outcomes and that by controlling it to draw conclusions about species-specific sperm precedence patterns, we may be masking important variations in P_2 (Eberhard 1996). A more informative approach may be to highlight the variation in sperm precedence values that exists within species and across contexts, which may allow us to elucidate mechanisms for sperm precedence patterns in the process (Eberhard 1996; Cook et al. 1997).

The inter-mating interval is likely to be salient for species in which sperm viability declines rapidly after copulation or over time while in storage within the female, the relative competitiveness of males' sperm is influenced by the time since ejaculation, or the first males' sperm are used or lost at an appreciable rate between matings. Indeed, one proposed explanation for the observed bias favoring last-male sperm use is

Table 1 Factors that have been found to affect P_2 in a number of disparate taxa

Factor affecting P_2	Species	Source
Male size	Yellow dung fly (*Scatophaga stercoraria*) and small white butterfly (*Pieris rapae*)	Simmons and Parker (1992) (but see Dickinson (1988); Eady (1994a)); Wedell and Cook 1998
Male mating status	Cowpea weevil (*C. maculatus*) and small white butterfly (*P. rapae*)	Eady (1995); Wedell and Cook (1998)
Number of sperm inseminated by the second male	Cowpea weevil (*C. maculatus*)	Eady (1995)
Spermatophore size	Rattlebox moth (*Utetheisa ornatrix*) and small white butterfly (*P. rapae*)	LaMunyon and Eisner (1994); Wedell and Cook (1998)
Shape of male genitalia	Water strider (*Gerris lateralis*)	Arnqvist and Danielsson (1999)
Copulation duration	Yellow dung fly (*Scatophaga stercoraria*), milkweed leaf beetle (*Labidomera clivicollis clivicollis*), damselfly (*Mnais pruinosa pruinosa*), and water strider (*G. lateralis*)	Parker (1970b); Dickinson (1986); Siva-Jothy and Tsubaki (1989) (but see Eady (1994a)); Arnqvist and Danielsson (1999)
Copulatory courtship behavior	Red flour beetle (*Tribolium castaneum*)	Edvardsson and Arnqvist (2000)
Timing of copulation relative to oviposition	Cowpea weevil (*C. maculatus*) and fruit fly (*Drosophila melanogaster*)	Eady (1994a); Pitnick et al. (2001a)
Female genotype	Cowpea weevil (*C. maculatus*)	Wilson et al. (1997)
Quantity and/or location of sperm in storage	Fruit fly (*D. melanogaster*)	Lüpold et al. (2012)

the "passive sperm loss" hypothesis, which posits that as the interval between matings increases, passive loss of the first male's sperm from the female reproductive tract leads to a disproportionate use of the second male's sperm (Lessells and Birkhead 1990; Birkhead and Biggins 1998). Thus, a prediction of this hypothesis is that P_2 should increase as the inter-mating interval is increased, as shown in numerous species (reviewed in Simmons 2001).

The seed beetle, *Callosobruchus maculatus*, is a widely used model system in sperm competition studies, and previous investigations of its sperm precedence patterns have revealed high (83%) last-male sperm precedence (Eady 1991). However, the conclusion of last-male sperm precedence and other conclusions about sperm competition and relative male fertilization success in this species are disproportionately based on inter-mating intervals of 24 or 48 h after the initial mating (Eady 1991, 1995; Eady et al. 2004; Hotzy and Arnqvist 2009). Previous empirical work in this species has revealed not only that sperm are rapidly reduced in both long-term and short-term sperm storage sites (i.e., the spermatheca and bursa copulatrix) within the female reproductive tract but also that this passive loss of first-male sperm occurs as early as 4 h after the initial mating (Eady 1994b). Thus, first-male sperm are likely to be so greatly reduced at longer inter-mating intervals that they are unable to numerically compete for fertilizations and, as is observed, second males' sperm will be disproportionately favored. Whether this sperm precedence pattern is upheld when the time between matings is decreased below 24 h has never been established.

Here, I use the seed beetle to test an indirect prediction generated by the passive sperm loss hypothesis that last-male sperm precedence, measured as the proportion of offspring sired by the second male to mate (P_2), will be reduced with a shorter inter-mating interval. Using competitive double matings, I measured P_2 at three inter-mating intervals (0, 24, and 48 h after the initial mating) to examine how the timing of female remating influences sperm use patterns. Importantly, I ask how prevalent female remating behaviors are at a shorter inter-mating interval (i.e., when females remate immediately) in comparison to longer inter-mating intervals (i.e., when females wait 24 or 48 h to remate). Lastly, to better understand inter-mating intervals from a female perspective, I identify key differences between females that did and did not remate at each inter-mating interval to reveal potential mechanisms for variation in female remating decisions. If the inter-mating interval is a critical determinant of P_2 and there is a significant variation in female propensity to remate, then most studies of sperm competition in this model system have likely led to an overestimation of last-male sperm precedence and an underappreciation of the females' role in governing sperm use patterns and, ultimately, sperm competition intensity. Furthermore, examining the effect and prevalence of a short inter-mating interval for the first time in a system with previously established last-male sperm precedence patterns can help us infer female decision rules about remating so that we may functionally interpret the adaptive evolution of male and female traits or behaviors involved in reproduction (Eberhard 1996).

Methods

Study system

All seed beetles used in this study originated from southern India and were provided by Dr. Frank Messina of Utah State University. Detailed descriptions of their culturing and maintenance of stock cultures and family lines may be found in the electronic supplementary material ("Methods" section). Briefly, all seed beetles used in this experiment were reared in a laboratory growth chamber at Cornell University under constant conditions of 26 ± 1 °C, 10–50% RH, and a 12:12 light/dark cycle. All matings in the study were conducted between May and November of 2014, resulting in the use of beetles across multiple generations. To ensure that relatives did not mate, matrilines were initiated by isolating randomly infested seeds from a large, outbred population. Virgin males and females reared from these seeds were randomly paired for mating and assigned a unique matriline. The offspring from these matings served as focal individuals in the present study, were reared individually from a single seed to ensure that they were virgins and, upon their adult eclosion, were provided a unique identification number (ID), which included their generation and matriline. Moreover, their egg lay date, eclosion date, death date, sex, and group ID (based on the time of the year in which the matings were conducted) were recorded.

Mating observations

All mating trials were performed in the afternoon and were staged within the females' 35-mm Petri dish. Copulation in seed beetles begins with the male drumming the female's back with his antennae prior to mounting her; once he has successfully inserted his aedeagus, he leans back and remains relatively motionless while he transfers his ejaculate. Sperm are transferred to the female via a spermatophore, which first gets deposited in the bursa copulatrix before the sperm migrate to and enter the spermatheca, which reaches capacity approximately 0.5 to 2 h after insemination (Eady 1994a, 1995). At some interval after the onset of copulation, the female begins kicking the male, at which point a struggle ensues until his aedeagus is successfully dislodged from her genitalia. *Kicking latency* was calculated as the time the male leaned back until the time the female began to kick, and *kicking duration* was calculated as the time the female started to kick until the pair was separated. *Copulation duration* was calculated as the time

from when the male leaned back to when he removed his aedeagus (i.e., pair separation). Each of these mating behaviors was recorded within the nearest 10 s. All individuals were weighed immediately before and after mating using a Sartorious MP1601 microbalance to the nearest ±0.1 mg. Individuals were weighed twice and the resulting weights were averaged; values that differed by more than 0.1 mg were re-measured, and the average weight was taken using all three measurements. Male ejaculate size was calculated by subtracting his post-mating weight from his pre-mating weight.

Mating trial protocol

The mean (±SE) age of focal females was 2.7 ± 0.1 days, and the mean age of all focal males was 3.2 ± 0.1 days. Focal females (n = 115) were weighed, randomly paired with a virgin male, and continuously observed until they successfully mated. Females that failed to mate by 20 min after introduction were discarded from the study (n = 2, 1.7%). Females that did successfully mate were re-weighed and immediately provided the opportunity to mate with a new virgin male. Females that were unwilling to remate at this time point exhibited several resistance behaviors that prohibited successful copulation—they ran away, kicked, or moved their abdomen to make their genitalia inaccessible to approaching males. The proportion of females remating within 25 ± 10 min was recorded to measure receptivity. Females that did not remate within this time frame were separated from the male and provided a single seed for oviposition until the next mating opportunity. Because resource availability in this species has been shown to affect both female remating propensity and P_2 (Eady et al. 2004), females were given only a single seed to attempt to constrict their use of first-male sperm and minimize the number of eggs laid between matings. Further mating opportunities were provided to focal females 24 h after the initial mating and again 48 h after the initial mating. The same male was used in each subsequent mating trial to remove variation in female remating due to preferences for particular males. Females that failed to remate within the trial that occurred 24 h post mating were provided the same single seed for oviposition, while females that failed to remate within the trial that occurred 48 h of their first mating were recorded but excluded from paternity analyses since they only mated once (n = 44, 38.9% of all females that mated once in the trial). Hence, female remating propensity was allowed to vary in this experiment, and this variation corresponded to an inter-mating interval of 0, 24, or 48 h after the initial mating. Successfully double-mated females (n = 69) were transferred to a new Petri dish containing clean seeds as oviposition substrate every 24 h until their natural death. The seed quantities provided each day were changed mid-experiment (from 50, 25, 15, and 10 to 40, 20, 10, and 5 provided each day, respectively) but still exceeded average daily oviposition rates and did not affect the numbers of eggs laid by females. Female IDs were used so that eggs could be blindly counted and scored to determine sperm precedence without knowledge of the inter-mating interval or sterilization order.

Sperm precedence

The sterile male technique was used to assign paternity. Only one of the two males was randomly selected for sterilization by exposure to 70 Gy of gamma radiation from a cesium source at Cornell University. A balanced design was used to control for sterilization order; thus, approximately half of all matings were NR matings, in which the first male to mate was normal (N) and the second male to mate was sterile (R), and half were RN matings. Using the Boorman and Parker (1976) formula to correct for rates of hatch failure due to natural infertility and incomplete sterilization (see Electronic supplementary material, Table S1), P_2 was calculated for each focal female. The proportions obtained from this formula were multiplied by the number of eggs laid after the second mating and then rounded to a whole number to quantify the absolute number of eggs fertilized by each male sire. Females that laid an unusually low number of eggs (<10) after the second mating (n = 2) were removed from the analysis. Moreover, one case in which P_2 was 100% and eggs laid between matings could not be quantified was excluded from the paternity analysis to remove the possibility of an unsuccessful mating (e.g., no sperm transferred or male infertility in the first mating). Hence, sperm precedence was analyzed for 66 females in total.

Female remating propensity

To examine how prevalent female remating behaviors are at shorter compared to longer inter-mating intervals, I determined the proportion of females that successfully remated at each inter-mating interval.

Key differences between females that did and did not remate

To compare females that did and did not remate, all focal females were marked as either remated ("1") or not ("0") at each inter-mating interval. Females that did not remate at 0 h (n = 87) were given additional opportunities at 24 h or, if necessary (n = 62), 48 h after the initial mating. The ages and weights of focal females and second males were documented at the start of each interval since these varied with time. The amount of weight lost between matings was also calculated for females with an inter-mating interval >0 h.

Statistical analyses

All statistical analyses were conducted using R version 3.1.2 (R Development Core Team 2014). All means are presented as ±1 SE.

Sperm precedence

Last-male sperm precedence (P_2) was analyzed using a generalized linear mixed model (GLMM) using the glmer function with a logit link function from the "lme4" R package (Bates et al. 2014). The binomial response was the number of eggs fertilized by the second male, and the total number of eggs laid after the second mating was the binomial denominator (n = 66). In the initial statistical model, the residual deviance was observed to be larger than the residual degrees of freedom, which is an indication of overdispersion (Crawley 2013). An observation-level random effect (OLRE) was used as a random factor in all subsequent analyses to control for overdispersion (Harrison 2014). The experimental group and nested matrilines and patrilines within a generation number for all focal individuals were included as random factors in the initial model, but only those effects that contributed to residual variability were included in the final model. These random factors were then used in bivariate analyses for variables that could potentially explain paternity bias, including the females' age and weight and the absolute differences between second and first male demographics and mating variables (age at time of mating, pre-mating weight, ejaculate size, kicking latency, kicking duration, and copulation duration). Predictors that had a p value of 0.20 or below were considered for the final model, and these predictors were screened for collinearity with other significant predictors and removed when collinearity was present so that only the one with greater relative significance was included in the final GLMM. Because the data used in this study came from an experiment meant to test a separate hypothesis (the sexy sperm hypothesis), the mothers of focal males presented here came from different mating backgrounds (monandry or polyandry). Mating order was reciprocally balanced such that half of the double matings were MP matings, in which sons from monandrous (M) females mated first and sons from polyandrous (P) females mated second, and half were PM matings; thus, mating order was included as a predictor in statistical analyses, although it was not directly relevant to the present study. Sterilization order (NR or RN matings) was also considered as a predictor in statistical analyses. Non-significant terms were dropped one at a time, and models were compared using Akaike information criterion (change in AIC < 2). Only the best fitting model is reported here. The final model included female generation and OLRE as random effects; the mating order, inter-mating interval, sterile order, absolute ejaculate size, and copulation duration differences between first and second males were included as fixed effects. Post hoc comparisons were made among the inter-mating intervals using the Tukey HSD adjustments for multiple comparisons with the "LSmeans" R package (Lenth 2016).

Female remating propensity

The number of females that remated at each inter-mating interval was divided by the total number of females to obtain the proportions of females remating at each interval, which were then compared through a proportions test.

Key differences between females that did and did not remate

To investigate the differences between females that did and did not remate at each inter-mating interval, I used separate binomial GLMMs and GLMs with the response being whether focal females remated ("0" for no, "1" for yes) for each inter-mating interval. For the 0-h inter-mating interval, I used a GLMM, and the only random effect that contributed to variation in the response variable was the second male generation. Because none of the random effects could explain a significant amount of the variability in the response variable for the 24- or 48-h inter-mating intervals, I used GLMs instead. Predictors that were considered for all models included female traits (age at the time of first mating, pre- and post-mating weight, and ejaculate uptake), first male traits (age at the time of first mating, pre-mating weight, and ejaculate size), first mating behavioral variables (mating latency, kicking latency, kicking duration, and copulation duration), and second male traits (age, pre-mating weight, and absolute age and weight differences between him and the first male at the remating interval). The female's age at the time of the trial and their weight loss between matings were predictors considered for the 24- and 48-h remating intervals. Additional predictors that were considered for the 24-h inter-mating interval included the number of eggs laid and the proportion of eggs laid (as a function of the total number of eggs laid across the female's lifetime) during the inter-mating interval. This predictor was not considered for the 48-h inter-mating interval, however, since these eggs were not quantified for females that did not remate beyond a 48-h interval. Because females that lay more eggs lose more weight between matings (unpublished data), this factor has been indirectly taken into account by including female weight loss over the 48-h remating interval as a covariate. For the binomial GLMM, a bivariate analysis was conducted separately for each predictor of interest with the second male generation as a random effect. For the binomial GLMs, bivariate analyses were conducted separately for each predictor of interest with no random effects. From these bivariate analyses for the GLMM and GLMs, predictors that had a p value of 0.20 or below were considered for each final model

(with the exception of the sterilization order and mating order, which were included as candidate predictors in all initial models regardless of significance). Pairwise comparisons using a linear model were used to screen all significant candidate predictors for collinearity. Whenever collinearity was present, only the predictor with the greater relative significance was included in the initial model. Non-significant terms were then dropped one at a time, and models were compared using Akaike information criterion (change in AIC < 2). Only the best fitting model is reported here. Fixed effects included within all final models may be found in Table 2.

Results

Sperm precedence

The mean (±SE) P_2 was 0.54 ± 0.05 for a 0-h inter-mating interval, 0.69 ± 0.05 for a 24-h inter-mating interval, and 0.72 ± 0.04 for a 48-h interval. P_2 was significantly lower at an inter-mating interval of 0 h compared to longer inter-mating intervals (binomial GLMM: n = 57; 0–24 h p = 0.007; 0–48 h p = 0.02; Fig. 1, Table 2). Pairwise comparisons adjusted for multiple comparisons using LSmeans show that P_2 did not significantly differ between 24- and 48-h inter-mating intervals (p = 1.00). Fitted values of P_2 using LSmeans were 0.55 ± 0.06, 0.74 ± 0.04, and 0.74 ± 0.05 for 0-, 24-, and 48-h inter-mating intervals, respectively (Fig. 1).

Female remating propensity

Out of 113 focal females, 26 remated immediately (23.0%), 25 remated 24 h after their first mating (22.1%), and 18 remated 48 h after their first mating (15.9%; Fig. 2). In total, 45.1% of females remated within a day of the first mating, and 61.1% of females remated within 2 days of the first mating. Forty-four females (38.9%) failed to remate within 2 days of the first mating. Of the females that did remate, the proportion of females remating did not significantly differ among the inter-mating intervals (proportions test: χ^2 = 1.14, df = 2, p = 0.57). These results suggest that inter-mating intervals that are shorter than the standard intervals typically used in this species (e.g., 24 and 48 h after the initial mating) are prevalent.

Key differences between females that did and did not remate

Females that remated immediately spent a significantly shorter duration of time kicking their first mate than females that did not remate at this time point (Remated = 103.3 ± 15.4 s, Didn't Remate = 232.7 ± 22.7 s; binomial GLMM: n = 108, p = 0.01; Table 3). Females that did and did not remate at this time interval did not significantly differ in their pre-mating age or weight or post-mating weight gain, nor were there any differences in the traits of their first or second mates (i.e., absolute or relative ages and weights of males at the remating interval or the first ejaculate size) or the first mating behavioral variables (i.e., mating latency or copulation duration). The first mating kicking latency did significantly differ between these females, but this trait was collinear with the first-male ejaculate size, the latter of which was included in the final model.

Females that remated 24 h after the initial mating laid a significantly smaller percentage of their total eggs between their matings (Remated = 34.3 ± 2.6%, Didn't Remate = 54.5 ± 5.1%; GLM: n = 32, p = 0.009) than females that did not remate at this time point. These females also weighed significantly less at the time of the second mating trial (Remated = 5.53 ± 0.12 mg, Didn't Remate = 5.82 ± 0.10 mg; GLM: p = 0.02) compared to females that did not remate (Table 3). These females did not differ in any other traits (either her own or her mates') or the first mating behavioral variables, however. Females that remated 48 h after the initial mating were significantly younger (Remated = 4.2 ± 0.2 days, Didn't Remate = 4.9 ± 0.2 days; GLM: n = 48, p = 0.013), weighed significantly more at the time of their first mating (Remated = 6.39 ± 0.22 mg, Didn't Remate = 6.14 ± 0.09 mg; GLM: p = 0.04), and lost more weight

Table 2 Output from binomial GLMM on the effect of the inter-mating interval (0, 24, or 48 h after the initial mating) on the proportion of eggs fertilized by the second male to mate (P_2)

Model term	Second-male paternity, N = 56 (binomial response: second-male eggs, first-male eggs)				
	Beta (SE)	Exp (beta)	95% CI	z	Pr(>|z|)
Intercept	−0.29 (0.27)				
Inter-mating interval (24 h)	0.83 (0.31)	0.70	(0.56, 0.81)	2.70	0.00703
Inter-mating interval (48 h)	0.84 (0.36)	0.70	(0.53, 0.82)	2.33	0.01970
Mating order (PM)	0.53 (0.25)	0.63	(0.51, 0.74)	2.11	0.03489
Sterilization order (RN)	0.38 (0.26)	0.59	(0.47, 0.71)	1.49	0.13688
Ejaculate size difference (male 2 − male 1)	−0.57 (0.65)	0.36	(0.14, 0.67)	−0.88	0.37866
Copulation duration difference (male 2 − male 1)	−0.04 (0.15)	0.49	(0.41, 0.56)	−0.28	0.78287

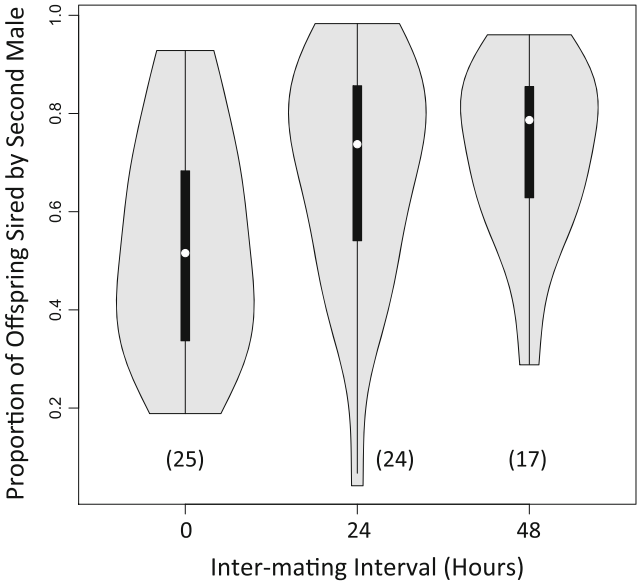

Fig. 1 Proportion of offspring sired by the second male to mate (P_2) across three inter-mating intervals—0, 24, and 48 h after the first mating (sample sizes in *parentheses*); the *white dots* represent the actual median values, the *black bars* represent the inter-quartile range, and the *gray areas* represent the full distribution of the data

between matings (Remated = 1.79 ± 0.10 mg, Didn't Remate = 1.35 ± 0.09 mg; GLM: $p = 0.02$) than females that did not remate at this time point (Table 3). For the 24-h inter-mating interval, females that lost more weight between their matings laid more eggs during this time interval (LM: $F_{1,30} = 13.8$, $p = 0.001$). Thus, it is reasonable to assume the same for females that waited 48 h to remate, which suggests that females that did remate at this time point laid more eggs during the interval between matings

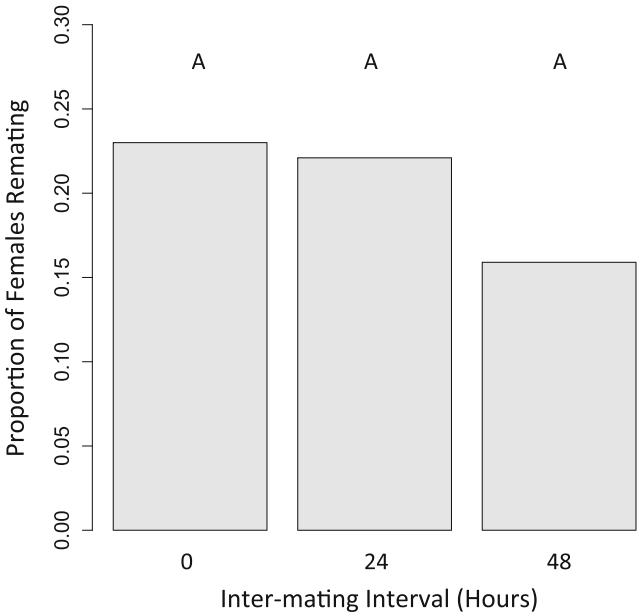

Fig. 2 Proportion of females ($n = 113$) that remated at each of the three inter-mating intervals—0, 24, or 48 h after the initial mating

than females that did not remate. Females that did and did not remate at the 48-h remating interval did not differ in any other traits (either her own or her mates') or in the first mating behavioral variables.

Discussion

The purpose of this study was to determine if last-male sperm precedence observed in the seed beetle, *C. maculatus*, is upheld at a shorter inter-mating interval. Because passive loss of first-male sperm in the female reproductive tract is expected to lead to disproportionate use of second-male sperm as the time between matings increases, I predicted that P_2 would be diminished with a shorter time interval between matings. I found that P_2 is significantly reduced when females remate immediately than when they remate 24 or 48 h after the initial mating. Surprisingly, I found no difference in the proportions of females remating at each inter-mating interval, which indicates that short inter-mating intervals are relevant to this species, despite the traditional use of longer inter-mating intervals in experimental designs aimed at examining sperm competition and sperm precedence (Eady 1991, 1995; Eady et al. 2004; Hotzy and Arnqvist 2009). Lastly, I found that the differences between females that did and did not remate changed across the inter-mating intervals. Specifically, females that waited a day to remate were smaller and laid fewer eggs between matings, whereas females that waited two days to remate were younger, larger, and lost more weight between their matings when compared to females that did not remate at these time intervals. Together, these results indicate that female remating decisions and, consequently, sperm precedence are more variable than previously appreciated in this species (but see Eady et al. 2004). More generally, this study highlights the substantial potential for female remating decisions to influence the intensity of sperm competition within species.

The sperm precedence patterns observed with varying inter-mating intervals in the present study are consistent with studies in other taxa that have revealed an increase in P_2 by experimentally increasing the interval between matings (reviewed in Simmons 2001, but see Ala-Honkola et al. 2013). For example, P_2 increased from 83% at a 1-day inter-mating interval to 99% at a 14-day inter-mating interval in *Drosophila melanogaster* (Boorman and Parker 1976). In the milkweed leaf beetle (*Labidomera clivicolli*), the average P_2 was close to 50% when matings were successive but increased to 87% with a 5-day inter-mating interval (Dickinson 1988). Similarly, average P_2 values were close to 50% for successive matings but increased as the inter-mating interval was increased in the melon fly, *Bactrocera cucurbitae* (Yamagishi et al. 1992). Moreover, whereas two separate studies in *C. maculatus* have been used to suggest that P_2 increases from a 24- to 48-h inter-mating interval (Eady 1994a, 1995),

Table 3 Output from the GLMM on the differences between females that did and did not remate immediately after the initial mating and from the GLMs on the differences between females that did and did not remate 24 or 48 h after the initial mating

Inter-mating interval (hours)	Model term	Female remated (binomial response: yes or no)				
		Beta (SE)	Exp (beta)	95% CI	z	Pr(>|z|)
0	Intercept	−0.52 (0.77)				
	Ejaculate size of the first male (mg)	−3.20 (1.84)	0.04	(0.00, 0.60)	−1.74	0.0815
	First mating kicking duration (s)	−1.34 (0.55)	0.21	(0.08, 0.43)	−2.45	0.0142
24	Intercept	38.22 (15.72)				
	Proportion of eggs laid between matings	−19.26 (7.34)	0.90	(0.55, 0.99)	−2.62	0.00873
	Female weight at 24 h (mg)	−5.20 (2.24)	0.38	(0.19, 0.61)	−2.32	0.02031
48	Intercept	−12.85 (6.41)				
	Sterile order (sterile–normal)	1.62 (0.92)	0.84	(0.45, 0.97)	1.76	0.0786
	Female age at 48 h (days)	−1.27 (0.51)	0.22	(0.09, 0.43)	−2.49	0.0128
	Female pre-mating weight (mg)	−1.79 (0.85)	0.86	(0.53, 0.97)	2.10	0.0355
	Female weight loss between 0- and 48-h remating interval (mg)	3.49 (1.44)	0.97	(0.66, 1.00)	2.42	0.0154

this study reveals that P_2 does show an increasing trend in line with these previous studies but ultimately does not significantly differ between these intervals. This result is perhaps unsurprising given that sperm numbers in both the bursa copulatrix and spermathecae most precipitously decline between a 0- and 24-h inter-mating interval but decline less steeply between a 24- and 48-h inter-mating interval (Eady 1994b). Although it was beyond the scope of the present study, it would be interesting to assess sperm precedence patterns on an even finer scale (e.g., every 2 h from 0 to 10 h after the initial mating) to compare the results with what we know about sperm storage and decline within the female reproductive tract over time.

One possible explanation for why paternity is more evenly shared at a 0-h inter-mating interval is that first-male sperm have not yet moved into the spermatheca and so are more numerically and equally represented when being selected to fertilize ova; thus, the mechanism of sperm precedence at this time point does not depend on passive sperm loss. This explanation seems likely given what we know about passive sperm loss in the spermatheca in this species—sperm numbers decline from 6000 to 4000 to 2000 for each 24-h time interval since the first mating (Eady 1994b). Another possibility is that first-male sperm are more equally competitive when females remate immediately, but their relative competitiveness declines with time since ejaculation. While there were several instances in which P_1 was actually higher at longer inter-mating intervals (e.g., P_1 ranged from 2 to 96% for the 24-h inter-mating interval and 4 to 71% for the 48-h inter-mating interval), it was beyond the scope of the present study to assess other sperm traits that might contribute to their relative competitiveness (i.e., length or motility) and so this explanation cannot be ruled out. Another possible explanation is that first-male sperm are more viable when females remate immediately, but sperm viability declines over time while in storage within the female (Orr and Brennan 2015). Whether sperm mortality naturally occurs or can be accelerated by active movement by the female into certain sperm storage sites that increase sperm mortality remains unknown (Lessells and Birkhead 1990; Eberhard 1996). Further empirical studies are warranted to distinguish among these possibilities.

Yet another non-mutually exclusive explanation for the observed reduction in P_2 is that females did not oviposit between matings for the shortest inter-mating interval and so had more first-male sperm in storage to use. In a previous study that examined the effects of oviposition on the patterns of sperm use in the seed beetle, females given fewer oviposition sites during 24- and 48-h inter-mating intervals had a lower P_2 when they did remate, which the authors conclude is because they laid fewer eggs between matings and thus used fewer sperm from their first mate (Eady et al. 2004). Because females with longer inter-mating intervals were given only a single seed for oviposition between matings in the present study, they were restricted in their use of first-male sperm. Thus, this explanation for a lower P_2 based on differences of first-male sperm in storage seems unlikely for females remating at different intervals. However, sperm and ova were not directly counted in the present study or in the Eady et al. (2004) study, which is required to definitively test this assumption.

In addition to demonstrating that the inter-mating interval is a critical determinant of P_2, this study surprisingly reveals that immediate remating by females is common in this species, which differs from previous findings (Edvardsson et al. 2008; den Hollander and Gwynne 2009). It is possible that female remating behaviors differ among population strains based on

subtle genetic differences and unintentional selection regimes in the lab. The female inter-mating interval has been shown to be quite variable but significantly heritable in *Drosophila* (Lüpold et al. 2013) and, through artificial selection, was shown to be significantly heritable within several generations in *C. maculatus* (Eady et al. 2004). The current study further demonstrates variation in female remating propensities on which selection can act, which corroborates findings in other seed beetle species (Harano and Miyatake 2005; Maklakov et al. 2005). Although female mating strategies are likely to be complex and depend on a number of species-specific factors (e.g., life history, sperm longevity, female reproductive tract complexity, timing of sperm storage, and mechanism for sperm precedence), what females gain from the timing of their remating decisions should be a point of focus for future studies.

Moreover, a common practice is to use the remating interval as a means for understanding female preferences for certain males (e.g., female choice of the second male relative to the first male). For example, a study in *D. melanogaster* observed whether females adjust their remating interval based on mating opportunities with males of varying sizes and found that females remated more quickly when courted by large (i.e., higher quality) second males (Pitnick 1991). Moreover, two separate studies in *Drosophila simulans* used copulation latency as a measure for assessing female preference and revealed that attractive males preferred by females were more successful in sperm competition when second to mate (Hosken et al. 2008) and that females use their first mate as a basis for evaluating the quality of their second mate (Ala-Honkola and Manier 2016). In the present study, if immediate remating is an indication of female preference for the second male over the first male, then it is unclear why females do so given that it lowers the second male's reproductive success. One possible explanation for this puzzling outcome is that males that are preferred in a pre-copulatory context are not intrinsically better sperm competitors and so perform poorly in a post-copulatory context, as has been observed in *D. simulans* (Ala-Honkola and Manier 2016; but see Hosken et al. 2008). Such a contradictory outcome merits further investigation.

Furthermore, examining key differences between females that did and did not remate at each inter-mating interval reveals potential mechanisms for female refractory behavior, including risk of sperm depletion. Females that remated at a 24-h remating interval laid a significantly smaller proportion of their eggs before their second mating, which may be because they did not have an adequate sperm supply and so remated to obtain more sperm. It cannot be ruled out, however, that these females may have had a lower-quality first mate and waited to lay the majority of their eggs after "trading up" with a second mate (Thornhill and Alcock 1983), which would support female remating based on male quality. Similarly, females that remated at the 48-h remating interval were younger and weighed significantly more prior to their first mating than females that did not. Post hoc analyses reveal a trend of younger and larger females being more fecund, which makes these females more likely to run the risk of sperm depletion. Furthermore, females that remated at 48 h lost more weight between their matings. Given that female weight loss is highly correlated with the number of eggs laid during this time interval, these females necessarily used more of the first males' sperm and so risk sperm depletion. Another intriguing possibility is that it is the presence of sperm itself within the female reproductive tract that inhibits female remating, as has been found in the almond moth, *Cadra cautella* (McNamara et al. 2008). Although quantifying sperm was beyond the scope of this study, an interesting follow-up would be to dissect the spermatheca of females that become receptive again to determine if they are sperm-depleted.

Female remating behavior may also have been based on her condition, since females that remated at a 24-h remating interval weighed significantly less than females that did not remate at that time point, and females that delayed remating by another day (i.e., with a 48-h remating interval) weighed significantly more prior to their first mating than females that did not remate at that time point. However, it is unclear why the female's condition would not have been an important distinguishing factor for females at a 0-h interval. That smaller, less fecund females would be more likely to remate at a 24-h interval is perplexing and contradicts results from previous investigations of female remating propensities in *D. melanogaster* and the almond moth (Pitnick et al. 2001b; McNamara et al. 2008). Further analyses revealed that these smaller females did not differ in the number of eggs laid between matings or the size of the ejaculate obtained from their first mating, both of which make sperm deficiency in these females unlikely. One possibility is that these smaller females were of inherently lower quality and so remated to acquire more resources through a second ejaculate, which supports previous findings in the seed beetle (Edvardsson 2007). This result does seem unlikely, however, given that it was not consistent for all females across remating intervals and females that remated at 48 h were relatively larger prior to mating.

Interestingly, females that did not remate immediately kicked for a significantly longer duration of time during the first mating. Corroborating other studies of *C. maculatus*, this unanticipated result has two potential explanations, both of which involve the males' spiny genitalia that have been shown to cause exceptional damage to the female reproductive tract (Crudgington and Siva-Jothy 2000). One supported function of these genital spines is that they increase the rate at which male seminal products are transferred into the female's circulatory fluid, since females that mated with males with longer spines had a larger proportion of the males' ejaculate move from their reproductive tract into their body (Hotzy et al.

2012). From this study, it was suggested that the increased absorption of seminal products benefits males by affecting the pattern of sperm use by females. Although the kicking phase of copulation has been shown to reduce female reproductive tract damage (Crudgington and Siva-Jothy 2000; but see Dougherty and Simmons 2017), results from the present study suggest that the females' reproductive tract may undergo cumulative damage during kicking and that females that kick longer incur more damage. Hence, one hypothesis for why female remating propensity differed based on kicking duration is that females that kicked for a longer duration more quickly absorbed manipulative seminal products (Yamane et al. 2008) that immediately reduced their receptivity. Further studies are warranted to test this hypothesis given these conflicting results with those previously published as well as the known effects of male seminal fluids on sperm precedence, female receptivity, and egg production in *C. maculatus* (Yamane et al. 2015).

Another non-mutually exclusive hypothesis is that females that kick for a longer duration of time wait to remate to avoid the cost of more reproductive tract damage. If damage from kicking is cumulative, then males that can induce female refractoriness by inflicting harm can reduce the benefit to females for remating (Johnstone and Keller 2000). Whether the harm caused by genital spines in male seed beetles is adaptive or "collateral" remains unknown, but such harmful male tactics are suggested to be more likely in species with a greater last-male mating advantage (Johnstone and Keller 2000). Furthermore, why the influence of kicking on female remating would be significant for only the shortest remating interval is unclear, but perhaps it coincides with the time it takes for damaged female tissues to heal or represents the threshold of when the benefits for female remating exceed the costs. One obvious hypothesis that emerges from these results is that the time the female spends kicking during copulation is a primary cause of reproductive tract damage. A recent investigation of female wounding in relation to mating duration and female kicking revealed that significant wounding is present prior to the onset of kicking and yielded no support for the hypothesis that kicking increases the rate of wounding (Dougherty and Simmons 2017). However, because this study assessed wounding at only two time points after kicking but did not do so for uninterrupted matings, we cannot rule out that further wounding occurs with extended kicking durations. Further studies are warranted to investigate these relationships between the female kicking duration, reproductive tract damage and repair, and the timing and extent of ejaculate component absorption within female bodily tissues.

One surprising result is that the ejaculate size itself did not significantly affect female remating at any remating interval. The large ejaculate size in this species is presumed to reduce female propensity to remate (Edvardsson 2007), and previous studies have confirmed this (Eady 1995; Savalli and Fox 1999). However, these latter studies manipulated ejaculate size by varying male mating status, whereas in the present study, male mating status was controlled and ejaculate sizes naturally varied among virgin males. That the results between these studies and the present study differ suggests that there are confounding effects of ejaculate components that likely vary by male mating status rather than ejaculate size. The result that no differences in female remating were observed based on ejaculate size in the present study suggests that that there are no dosage-dependent effects (Eberhard 1996). This result in combination with previous results implicates the importance of male mating status and suggests that virgin males either produce more of these manipulative compounds or their refractory effects are more potent. It also seems plausible that these compounds vary among males, which may be capable of tailoring these compounds to their advantage based on environmental or social contexts (e.g., female mating status and/or male mating role; Sirot et al. 2011). Males are assumed to control female mating frequency through the ejaculate by varying its size and hydration benefits contained within it, thus changing how beneficial remating is for the female (Edvardsson 2007), but the results from the present study do not support this assumption. Future studies should look into the ejaculate components and how these differ among and within males across contexts.

Conclusions that have been made about sperm use patterns in different taxa are likely to be dramatically impacted by experimental design (Danielsson 1998). For example, the last-male sperm precedence pattern found in a species of pseudoscorpion was discredited by a simple change in protocol from two to three matings (Zeh and Zeh 1994). Moreover, as investigators, we often discard females that are unwilling to remate at a designated inter-mating interval (but see Harano et al. 2006). Might we be missing important information here? While it is important to control for variables that can potentially affect sperm use and storage, discarding females unwilling to remate may mean the dataset includes only females that have lower thresholds for remating or lower-quality first mates. If so, then females with high remating thresholds or higher-quality first mates will never be screened for differential sperm use, which necessarily limits our understanding of the consequences of female behavioral decisions on sperm use patterns. Careful consideration is needed in future studies for factors in experimental designs that can influence sperm use patterns.

Conclusions

In summary, I found that P_2 typical in the seed beetle is significantly reduced when females remate immediately than when they wait to remate 24 or 48 h after the first mating. Intriguingly, I also found that immediate remating is common,

indicating that there is a substantial potential for female remating decisions to influence the intensity of sperm competition in this and other species more generally. I further identified key differences between females that did and did not remate at three distinct inter-mating intervals and found support for several mechanisms for female refractoriness, including enhanced direct benefits (i.e., adequate sperm supplies and nutrient acquisition) and reduced costs (i.e., cumulative damage incurred during mating).

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Compliance with ethical standards

Data availability The datasets generated and/or analyzed during the current study are available in the Dryad Digital Repository, [http://dx.doi.org/10.5061/dryad.7h7h3].

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Conflict of interest The author declares that she has no conflict of interest.

Ethical approval All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted.

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