doi:10.1111/evo.13423

Modeling strategic sperm allocation: Tailoring the predictions to the species

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Received January 23, 2016
Accepted December 14, 2017

Two major challenges exist when empirically testing the predictions of sperm allocation theory. First, the study species must adhere to the assumptions of the model being tested. Unfortunately, the common assumption of sperm allocation models that females mate a maximum of once or twice does not hold for many, if not most, multiply and sequentially mating animals. Second, a model's parameters, which dictate its predictions, must be measured in the study species. Common examples of such parameters, female mating frequency and sperm precedence patterns, are unknown for many species used in empirical tests. Here, we present a broadly applicable model, appropriate for multiply, sequentially mating animals, and test it in three species for which data on all the relevant parameter values are available. The model predicts that relative allocation to virgin females, compared to nonvirgins, depends on the interaction between female mating rate and the sperm precedence pattern: relative allocation to virgins increases with female mating rate under first-male precedence, while the opposite is true under later-male precedence. Our model is moderately successful in predicting actual allocation patterns in the three species, including a cricket in which we measured the parameter values and performed an empirical test of allocation.

KEY WORDS: **Evolutionarily stable strategy, female mating frequency, polyandry, sperm competition, sperm precedence, strategic sperm allocation.**

Strategic sperm allocation theory predicts the optimal numbers of sperm that males should provide to different females under different conditions. This field of study emerged several decades ago (Parker 1970b, 1982; Charnov 1982) after researchers began to recognize both the prevalence of sperm competition (Parker 1970a) and the costs of sperm production (Dewsbury 1982; Nakatsuru and Kramer 1982; Van Voorhies 1992). The many theoretical models developed since then (reviewed in Parker and Pizzari 2010) have been accompanied by a wealth of empirical studies on sperm allocation in different mating contexts (reviewed in Wedell et al. 2002; delBarco-Trillo 2011; Kelly and Jennions 2011). These studies have yielded extensive information on sperm allocation patterns in a range of taxa, and it is now evident that males in many species do facultatively adjust the size of their ejaculates.

Most of these studies, however, have two major limitations as effective tests of specific sperm allocation models. First, there is often a discrepancy between the assumptions of the model being tested and the reproductive characteristics of the species being used. As a result, the model may be inappropriately applied to the study system. Second, the model's parameters, which dictate the model's predictions and that may include such factors as the female mating rate and the pattern of sperm precedence, are rarely measured in the focal species. As a result, the model's predictions may be inappropriately applied even when the species follows the assumptions of the model.

The first of these two problems, a mismatch between the model's assumptions and the study species, is especially common in empirical tests of one major class of sperm allocation models, the risk model. Risk models (e.g., Parker 1990a,b) assume that females mate with a maximum of two males, generating a certain level of risk that the ejaculate of the focal male will compete with one other ejaculate. However, in most species, many females mate with more than two males under natural conditions (for a global

analysis see Taylor et al. 2014; for examples see Emery et al. 2001; Bretman and Tregenza 2005; Simmons et al. 2007; Frentiu and Chenoweth 2008; Simmons and Beveridge 2010; Hurtado et al. 2013; Smith 2014; Turnell and Shaw 2015b). Another major class of models, the intensity model (Parker et al. 1996; Ball and Parker 1997), assumes that males can assess the number of total competitors they will face postcopulation. Although this assumption is met in the group spawning species for which the model was originally designed, it is likely to be violated in sequentially mating species (but see Thomas and Simmons 2009).

The second limitation of many tests of sperm allocation models is the lack of empirical measurements of the factors, such as female mating rate and sperm precedence patterns, that constitute the parameters of the model. Because the values of these parameters determine the model's predictions, it is impossible to effectively test predictions without first knowing the parameter values. For example, the risk model, which generally predicts increased sperm allocation as the risk of sperm competition increases, can favor greater sperm allocation to virgin females or to mated females depending in part on whether there is first male sperm precedence (Ball and Parker 2007). As noted in a recent meta-analysis of strategic sperm allocation (Kelly and Jennions 2011), "many studies do not provide this background information and fail to make strong a priori predictions" regarding sperm allocation patterns.

Measuring the relevant parameters for the species in question is not a trivial undertaking. Determining the natural female mating rate requires either intensive observations in the field (e.g., Rodríguez-Muñoz et al. 2010; Turnell and Shaw 2015a) or the genotyping of sperm stores to estimate the number of contributing males (e.g., Bretman and Tregenza 2005; Simmons et al. 2007; Simmons and Beveridge 2010; Turnell and Shaw 2015b). Sperm precedence patterns have been measured in the lab in many species, particularly insects (Simmons and Siva-Jothy 1998), but almost all of these measures come from females that were mated just twice. Patterns of sperm use can change significantly when females are allowed to mate with additional males (Zeh and Zeh 1994).

Here, we present (1) a broadly applicable model of sperm allocation that is appropriate for species mating multiply and sequentially and (2) an empirical test of the model in one such species. Our model was inspired by that of Engqvist and Reinhold (2006), which also aimed to predict optimal sperm allocation based on female mating status. Like our model, Engqvist and Reinhold's does not limit the number of mating per female to two or assume that males can assess the total number of postcopulatory competitors. However, our model differs from theirs in several key respects, most importantly by allowing more flexibility in the distribution of female mating frequency and by incorporating two parameters representing the positive effect of multiple mating on female fecundity, one accounting for female sperm limitation and the other accounting for the positive effects of the ejaculate or of nuptial gifts on offspring production. We test our model in the Hawaiian swordtail cricket *Laupala cerasina*, a species for which we have measured the relevant theoretical parameters, including female mating rate, sperm use patterns, and the effect of multiple mating on offspring production. We also compare the actual and optimal sperm allocation strategies in two additional species for which all of the relevant empirical data have been published: the field cricket *Teleogryllus oceanicus* and the katydid *Requena verticalis*.

Methods

SPERM ALLOCATION MODEL

Our model assumes that all females mate at least once and mate n additional times with a frequency following a Poisson distribution $P(n)$ with a mean of M:

$$P(n) = \frac{e^{-M} M^n}{n!} \tag{1}$$

Males are assumed to have the ability to distinguish between virgin and nonvirgin females. There is a tradeoff between sperm allocation to virgin females (S_V) and sperm allocation to nonvirgin females (S_{NV}) such that

$$S_V \cdot p_V + S_{NV} \cdot (1 - p_V) = 1 \tag{2}$$

where p_V is the likelihood that a mating female is virgin. Because a female will be virgin in one out of all her matings, an average of $M + 1$ in total, this likelihood is

$$p_V = \frac{1}{M + 1} \tag{3}$$

Our tradeoff differs from the typically assumed tradeoff between the number of sperm ejaculated per mating and the number of matings achieved (e.g., Parker 1990a), but is similar to the premise of Fryer et al. (1999) where males have a fixed amount of sperm to allocate between two rounds of mating. In our model, males have a fixed amount of sperm to allocate to all of their matings, with the number of matings being the same for all males. (Assuming an equal sex ratio, this male mating rate will be equal to the average female mating rate, $M + 1$.) We chose this tradeoff function because we are interested not in absolute ejaculate size, which is relevant for across-species tests of sperm allocation theory, but rather in the relative amount of sperm allocated to virgins versus to nonvirgins, which is relevant for within-species tests.

The sperm of males mating with nonvirgin females is weighted by a factor of r, where $0 < r < \infty$. If $r = 1$ there is a fair raffle (Parker 1990a) and males mating with a multiply mating female are each expected to gain a paternity share

proportionate to the number of sperm they allocate. If $r < 1$ there is first male sperm precedence, and if $r > 1$ there is later male sperm precedence. (Note that we do not multiply each successive male's sperm by a factor of r; c.f. Engqvist and Reinhold 2006). We accounted for possible female sperm limitation by including the term ε, representing the fraction of an average ejaculate required to fertilize 50% of a female's eggs (Mesterton-Gibbons 1999; Ball and Parker 2000; note that since the average ejaculate size in our model is 1 [see eq. (2)], this is equivalent to ε_D *sensu* Ball and Parker 2000. Higher ε values correspond to stronger sperm limitation).

We also discounted the fitness a male gains by mating with a singly mating female by a factor of α, where $\alpha \leq 1$. Females that choose to mate once may be inherently less fecund than other females (Arnqvist and Nilsson 2000), and in addition nonsperm components of the ejaculate or of nuptial gifts may increase the fecundity of multiply mating females (South and Lewis 2011), for example by providing nutrition or stimulating oviposition (Gwynne 2008; Avila et al. 2011), or even by rescuing the viability of embryos sired by other males (García-González and Simmons 2007). While multiple mating is also likely to entail costs for females, the benefits of mating multiply versus just once appear to outweigh these costs, at least in insects (Arnqvist and Nilsson 2000).

The expected number of male matings taking place with females that mate $(n + 1)$ times overall is equal to the proportion of such females in the population, $P(n)$, times the number of times they mate with males, $(n + 1)$. The proportion of male matings with such females is equal to this term divided by total number of male matings, which is simply the sum of all these $P(n)*(n + 1)$ terms. This sum is in fact $M + 1$, which equals the mean number of female matings, as expected. Thus, the likelihood that a male's female mating partner mates exactly n other times is

$$\frac{P(n) \cdot (n+1)}{M+1} \tag{4}$$

(see also Parker et al. 1997, section 2(a); Engqvist 2012, Appendix B). A male's fitness relative to that of other males in the population is equal to the likelihood of mating with a female that mates exactly n additional times, which does not differ between males, multiplied by the proportion of that female's offspring he sires, and summed across all values of n. Note that because our tradeoff is between allocation to virgins versus to nonvirgins rather than between total sperm allocation and number of matings (see eq. (2)), all males are assumed to mate an equal number of times. Therefore, male mating rate does not appear in the fitness function (eq. (5), below). The fitness function thus represents the average relative fitness a male gains from all of his matings rather than his absolute fitness.

Let S_V* and $S_{NV}*$ be the optimal sperm allocation under the evolutionarily stable strategy (ESS, Maynard Smith 1982). The relative fitness of a mutant male allocating S_V and S_{NV} in a population allocating S_V* and $S_{NV}*$ will be equal to

$$\begin{aligned} W = {} & \frac{P(0)}{M+1} \cdot \alpha \cdot \left[\frac{S_V}{S_V + \varepsilon} \right] \\ & + \sum_{n=1}^{N} \frac{P(n) \cdot (n+1)}{M+1} \cdot \left[\frac{1}{n+1} \cdot \frac{S_V}{S_V + S_{NV}^* \cdot r \cdot n} + \varepsilon \right. \\ & \left. + \frac{n}{n+1} \cdot \frac{S_{NV} \cdot r}{S_{NV} \cdot r + S_V^* + S_{NV}^* \cdot r \cdot (n-1) + \varepsilon} \right] \end{aligned} \tag{5}$$

where N denotes the maximum number of rematings per female, which was set to 100 to simplify calculations. The first expression in this equation represents the fitness a male gains from mating with a virgin female who does not go on to mate with any other males ($n = 0$). The second expression represents the fitness a male gains from mating with a nonvirgin female who mates with a total of n other males, where $n = 1$ to N. In a fraction $1/(n + 1)$ of those cases, the focal male will be the first of the female's $n + 1$ total mating partners, and so his sperm allocation will be S_V. In the remaining cases, a fraction $n/(n + 1)$, the focal male will not be the first of the female's $n + 1$ total mating partners, and so his sperm allocation will be S_{NV}. Note that because S_{NV} is a function of S_V (see eq. (2)), the fitness function can be solved in terms of S_V alone. The ESS is found by setting

$$\begin{aligned} \left. \frac{\partial W}{\partial S_V} \right|_{S_V = S_V^*} &= 0 \\ \left. \frac{\partial^2 W}{\partial S_V^2} \right|_{S_V = S_V^*} &< 0 \end{aligned} \tag{6}$$

ESTIMATION OF THE MODEL PARAMETERS FOR *L. CERASINA*

To estimate the female remating frequency in *L. cerasina*, represented in the model by the Poisson distribution $P(n)$, we used mating data from a previously published experiment (Turnell and Shaw, 2015a). In that study, 20 males and 20 initially virgin females were allowed to mate freely for six weeks in a large field enclosure, after which their offspring were genotyped and assigned paternity using Cervus 3.0 (Kalinowski et al. 2007). To corroborate the female mating rate observed in the field enclosure, we also genotyped the sperm stores of 34 adult females collected at the same time and location and estimated the number of mates per female (see Turnell and Shaw 2015b for details). To estimate the level of sperm precedence in this species, represented by the parameter r, we combined the paternity data from the field enclosure experiment with the empirical sperm allocation values estimated in the current experiment to find the

value of r that minimized the sum of the squared differences between the observed and expected paternity shares.

For the sperm limitation parameter ε, we averaged the values reported by Ball and Parker (2000) for six species of mammals, birds, and arthropods. To estimate the effect of multiple mating on female offspring production, represented by the parameter α, we combined this sperm limitation estimate with two sets of data from *L. cerasina* on the number of offspring produced by females mating once with one male versus twice with two different males (J. Lambert and Q. Gao, unpubl. data). Most of the singly mated females were not assigned to that treatment and were thus self-selected to mate fewer times than the other females. Calculations were performed in Mathematica 10.1 (Wolfram Research, Inc., Champaign, IL, USA).

Collection and maintenance

All individuals were first and second generation offspring of adults collected at Kalopa State Park on the Big Island of Hawaii in December 2012 and transported to Cornell University in Ithaca, NY, USA. Nymphs were housed in plastic specimen cups lined with moistened Kimwipes and maintained on a diet of Fluker's Cricket Feed (Fluker Farms, Port Allen, LA, USA) at 20°C on a 12:12 h light/dark cycle. They were separated by sex at approximately their third instar. After reaching adulthood, females were housed individually while males were housed in pairs to simulate natural male–male encounters (male exposure to other males prior to mating has been shown to increase sperm allocation; Gage and Baker 1991; Schaus and Sakaluk 2001). Females were typically checked for maturity every one to five days, while males were typically checked one to two times per week. To enable identification, a spot of paint was placed on each adult male's thorax using a Sharpie paint pen (Sanford, Oak Brook, IL, USA).

MATING TRIALS

Mating trials were conducted from October to December 2013 and May to July 2014. Each male was mated to a virgin female and to a nonvirgin, once-mated female, with the order randomized. Each nonvirgin female had been mated to a nonfocal male on the day before the mating trial (or in two cases, two days before). Prior to being used in the trials, males were paired with nonexperimental females so that all focal males were nonvirgin for both experimental matings. A wide range of male intermating intervals (0 to 16 days, mean ± SD = 4.55 ± 3.59) was used to reflect natural conditions and to determine if male mating latency differentially affected sperm allocation to virgins versus nonvirgins. This distribution is similar to that observed in a semi-natural population (4.57 ± 4.57; Turnell and Shaw 2015a). For intermating intervals of less than three days, a given male's two intervals differed by a maximum of one day (0.08 ± 0.28 days, $n = 36$). For intermating intervals of three days or greater, the maximum difference between a male's two intervals was ten days (2.70 ± 2.60 days, $n = 50$). Males were three to nine weeks postfinal molt, while females were five to 21 days postfinal molt. The difference in age between the two experimental females paired with a given male was six days or less (1.77 ± 1.57 days).

Courtship in this species involves the transfer of a series of spermless microspermatophores ("micros") from the male to the female over the course of several hours, culminating at the end of the day in the transfer of a single sperm-filled macrospermatophore ("macro"; Shaw and Khine 2004). During mating trials, one male and one female were placed inside a mating arena consisting of the two large halves of a 100 × 20 mm plastic petri dish (Becton Dickinson Labware, Franklin Lakes, NJ, USA) taped together. Mating pairs were established at 10:00 and observed continuously until the male produced a macrospermatophore, typically between 15:00 and 17:00. All microspermatophore transfers were recorded. When the male attempted to transfer the macro by backing up underneath the female, approximately one hour after the macro was produced, the male was anesthetized with CO_2 and the macro was collected and placed in a 1.5 mL microcentrifuge tube so that the ejaculate drained onto the side of the tube. The tubes were weighed to the nearest 0.1 mg before and after macro collection. Females were weighed to the nearest 0.1 mg at the end of the mating trials. Macros were stored at –20°C for later DNA extraction.

DNA EXTRACTION

DNA was extracted from the macrospermatophore following a protocol modified from Simmons et al. (2007). Macros were crushed with microforceps and 330 μL DNA extraction buffer (50 mM Tris-HCl pH 8.0, 50 mM EDTA, 100 mM NaCl, 1% SDS), 20 μl dithiothreitol DTT, and 10 μl proteinase K were added to the tube. Samples were incubated for 24 hours at 56°C, and were vortexed and centrifuged every hour for the first three hours to aid in digestion. After cooling to room temperature, 150 μL 5M NaCl was added and the samples were vortexed and centrifuged at 21,428 *g* for 10 min. The supernatant was transferred to a new tube and 500 μL isopropanol and 3.3 μL GlycoBlue coprecipitant (Thermo Fisher Scientific, Waltham, MA, USA) were added and mixed by inversion. The samples were incubated at room temperature for 10 min, then centrifuged for 10 min. The isopropanol was removed and the DNA pellet was washed twice with 70% EtOH. Pellets were air-dried for 20 minutes and resuspended for approximately 90 minutes at 56°C, then overnight at room temperature, in 50 μL TE buffer. DNA concentration was measured using a Qubit 3.0 fluorometer and a dsDNA high sensitivity assay kit (Thermo Fisher Scientific). The concentration in ng/μL was multiplied by 50 μL to get a measure of the total ng of DNA in the macrospermatophore, then converted to an estimate of sperm number using the haploid genome size of *L.*

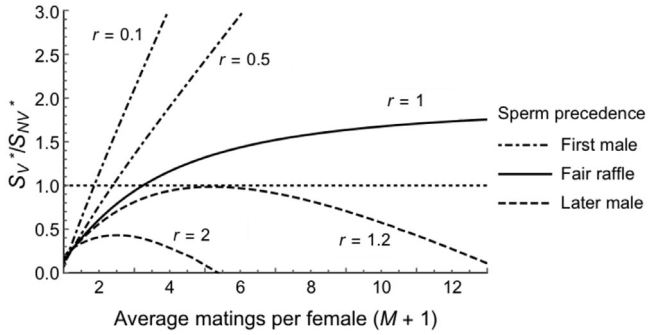

Figure 1. ESS sperm allocation to virgins versus to nonvirgins (S_V*/S_{NV}*) plotted against the mean number of matings per female in the population (i.e., the mean number of rematings, *M*, plus one initial mating; note that the *x*-axis therefore starts at 1 rather than 0). Males should allocate more sperm to virgins above the dotted line and more sperm to nonvirgins below it. The ESS is indicated by the solid line under a fair raffle ($r = 1$), by the dot-dashed lines under first-male precedence ($r < 1$), and by the dashed lines under later male precedence ($r > 1$).

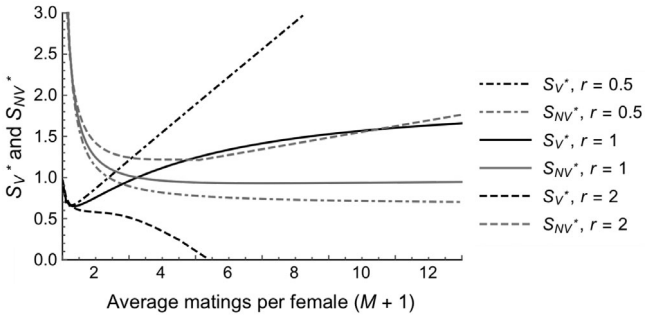

Figure 2. ESS sperm allocation to virgins (S_V*, black lines) and to nonvirgins (S_{NV}*, gray lines) plotted separately. Optimal allocation to nonvirgins remains steady as female mating frequency increases, while optimal allocation to virgins becomes more extreme in a direction that depends on the sperm precedence pattern (*r*).

cerasina (Petrov et al. 2000). Statistical analyses were performed in R version 3.1.1 (R Development Core Team 2014) and JMP version 11.0 (SAS Institute Inc., Cary, NC, USA).

Results

SPERM ALLOCATION MODEL

Figure 1 illustrates the optimal level of sperm allocation to virgin females relative to nonvirgin females (S_V*/S_{NV}*) in relation to sperm precedence and to the average female mating frequency. The horizontal dotted line indicates where S_V*/S_{NV}* = 1; above this line, the ESS is to allocate more sperm to virgin females, while below the line the ESS is to allocate more sperm to nonvirgin females. Note that because our model assumes that all females mate at least once, the *x*-axis starts at 1 rather than 0.

Under fair raffle conditions ($r = 1$), our model predicts that males should allocate more sperm to virgins when females mate with approximately four or more males on average, and more sperm to nonvirgins at lower mating frequencies. As first male precedence becomes stronger ($r < 1$), the threshold mating frequency above which males should allocate more sperm to virgins decreases, and the parameter space favoring greater allocation to virgins expands. For example, when $r = 0.1$ (extreme first-male precedence), the ESS is to allocate more sperm to virgins if females mate with an average of approximately two or more males. Conversely, as later male precedence become stronger ($r > 1$), the threshold mating frequency above which males should allocate more sperm to virgins increases, and the parameter space favoring greater allocation to virgins narrows. Under moderate to extreme later-male precedence ($r > 1.2$), our model predicts that males should always allocate more sperm to nonvirgins than to virgins.

Figure 2 illustrates separately the optimal levels of sperm allocation to virgins and to nonvirgins. As the average female mating frequency approaches 1, so does the optimal allocation to virgins, while optimal allocation to nonvirgins increases indefinitely. Above approximately two matings per female, while S_{NV}* is minimally affected by either the level of sperm precedence or the female mating frequency, S_V* is highly sensitive to both parameters. The more a male is favored by sperm precedence when mating with a virgin (i.e., when he is the first male), the more sperm he should allocate in that role; and this effect becomes more exaggerated as female mating frequency increases. At roughly two matings per female, optimal sperm allocation to virgins does not greatly differ whether first males are twice as competitive as later males ($r = 0.5$) or half as competitive ($r = 2$). At five matings per female, however, the difference in optimal allocation to virgins is over 17-fold.

Neither the sperm limitation parameter ε nor the fecundity parameter α has a strong effect on optimal sperm allocation (see Figs. S1–6). At $\alpha = 0.74$, which corresponds to a 35% fecundity increase for multiply versus singly mating females, the average effect size found in a meta-analysis of 56 arthropod species (South and Lewis 2011), optimal allocation is shifted very slightly to nonvirgins compared to the ESS when $\alpha = 1$ (i.e., no fecundity increase). At $\varepsilon = 0.022$, the fraction of an average ejaculate required to fertilize half of a female's eggs averaged for six species of mammals, birds, and arthropods as reported by Ball and Parker (2000), optimal allocation is shifted very slightly to virgins compared to the ESS when $\varepsilon = 0$ (i.e., no female sperm limitation). (In Figs. 1 and 2, $\alpha = 0.74$ and $\varepsilon = 0.022$). The slight positive effect of sperm limitation on S_V*/S_{NV}* is most pronounced when later male sperm precedence is high and mating frequency is low.

ESTIMATION OF THE MODEL PARAMETERS FOR *L. CERASINA*

Two of the 20 females in the field enclosure did not lay any eggs and were excluded from the mating frequency analysis. The remaining 18 females mated an average (±SD) of 6.22 ± 2.76 times, with the frequency following a Poisson distribution (Goodness of fit test: Kolmogorov's D = 0.101, $P < D = 0.89$). This mating frequency was close to that estimated for the wild females (Turnell and Shaw 2015b). Paternity was assigned at 95% likelihood to 401 of the 423 offspring from 17 of these 18 females and from four females that were in the enclosure for a shorter period of time. Observed paternity shares were compared to those that would be expected given a combination of the sperm allocation patterns measured in the current experiment (see below) and a range of possible sperm precedence levels. The value of the sperm precedence parameter r that minimized the sum of the squared differences between the observed and expected paternity shares was 1.12, close to a fair raffle (95% CI: 0.37, 2.81).

We used the average sperm limitation level reported by Ball and Parker (2000), $\varepsilon = 0.022 \pm 0.019$, as a proxy. For the fecundity parameter α, the two datasets from *L. cerasina* did not differ in the ratio of offspring produced by doubly versus singly mated females ($X^2 = 0.41$, $P = 0.52$). We therefore combined the data. Doubly mated females produced 2.02 times as many offspring as singly mated females (95% CI: 1.53, 2.77; $n = 99$ vs 45 females), corresponding to an α of 0.50.

SPERM ALLOCATION EXPERIMENT

The ratio of the number of sperm that each male allocated to the virgin female versus to the nonvirgin female was not affected by whether the mating trial was conducted in Fall 2013 or Spring 2014 (Welch's t-test, $t = 0.23$, $P = 0.82$, $n = 32$ and 55; all reported t-tests are two-tailed). The data were therefore pooled. Males allocated a similar number of sperm to virgins as to nonvirgins (mean ± SD = $3.67 \pm 0.82 \times 10^4$ and $3.51 \pm 0.75 \times 10^4$, respectively; paired t-test, $t_{86} = 1.12$, $P = 0.27$; the DNA concentrations on which these extrapolations are based are 1.423 ± 0.319 ng/μL and 1.378 ± 0.291 ng/μL). However, the average value of S_V/S_{NV} was significantly greater than 1 at 1.067, indicating a slight allocation bias toward virgins (95% CI: 1.008, 1.126; Fig. 3).

Sperm number was positively associated with male age (Spearman's rho = 0.36, $P < 0.0001$; generalized linear mixed model, $P < 0.0001$) and with the number of days since the male's previous mating ($r_s = 0.37$, $P < 0.0001$; GLMM, $P < 0.0001$; all GLMMs included male ID as a random effect; unless otherwise stated, each fixed effect was run in a separate model; in models with multiple fixed effects, all interaction terms were included). Sperm number was negatively associated with female age among both virgins ($r_s = –0.38$, $P < 0.001$) and nonvirgins ($r_s = –0.23$, $P = 0.034$; GLMM, $P < 0.001$). There was no significant interaction between female mating status and male intermating interval on sperm number. Sperm number was negatively associated with female weight, even controlling for female age (GLMM, $P < 0.001$; female weight and age were positively associated, $r_s = 0.55 \pm 0.07$, $P < 0.0001$). There was no difference in male age, female age, female weight, or male intermating interval between the virgin and the nonvirgin mating trials (paired t-test, $t_{85} = 0.38$, $t_{85} = –1.10$, $t_{85} = –1.03$, $t_{85} = –1.06$; $P = 0.65$, 0.27, 0.31, 0.29).

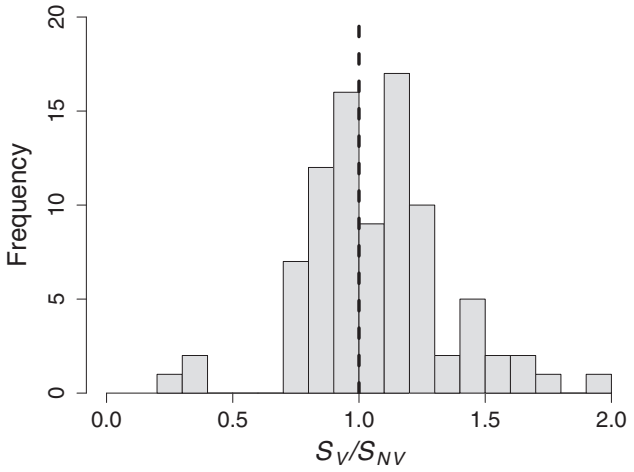

Figure 3. Histogram of the amount of sperm allocated by a male during his mating with a virgin versus his mating with a nonvirgin female (S_V/S_{NV}). Males to the left of the dotted line allocated more to the nonvirgin female, while males to the right of the dotted line allocated more to the virgin female. On average, males allocated more sperm to virgin females (mean = 1.067, 95% CI = 1.008, 1.126).

Males transferred more microspermatophores to virgin females than to nonvirgins (6.42 ± 1.80 vs 5.85 ± 1.74, paired t-test, $t_{79} = 2.29$, $P = 0.025$). However, the rate of micro transfer was higher for nonvirgins (1.96 ± 0.61 vs 1.65 ± 0.37 micros per hour, $t_{76} = 3.58$, $P < 0.001$). Courtship duration tended to be longer for virgins (248 ± 79 min vs 212 ± 151 min, $t_{77} = 1.93$, $P = 0.057$), who began mating earlier in the day (by 65 ± 125 min, $P < 0.0001$; though courtship also ended earlier, by 12 ± 43 min, $P = 0.013$). Older females began mating earlier ($r_s = –0.41$, $P < 0.0001$; GLMM, $P < 0.0001$) and so received more micros ($r_s = 0.42$, $P < 0.0001$; GLMM, $P < 0.0001$). There was no relationship between micro number and sperm number, even controlling for female age. Macrospermatophore weight was positively associated with sperm number, though only weakly ($r_s = 0.26$, $P < 0.001$; GLMM, $P < 0.001$).

PREDICTIONS VERSUS RESULTS FOR *L. CERASINA*

The optimal and actual sperm allocation strategies for *L. cerasina* are shown in Figure 3. At the empirically measured levels of

sperm precedence and female mating frequency, the optimal allocation is $S_V^*/S_{NV}^* = 1.152$, which falls just outside the 95% confidence interval for the observed allocation value of 1.067 (1.008, 1.126). Error bars show the 95% CIs for observed male sperm allocation and for observed female mating frequency. The ESS across the 95% confidence interval for observed sperm precedence ranges from $S_V^*/S_{NV}^* = 3.56$ at $r = 0.38$ (i.e., allocate most sperm to virgins under strong first-male precedence) to $S_V^*/S_{NV}^* = 0$ at $r = 2.81$ (i.e., allocate all sperm to nonvirgins under strong later-male precedence).

PREDICTIONS VERSUS RESULTS FOR OTHER SPECIES

There are two other species for which data on all three of the major variables in our model, female mating frequency, sperm precedence, and sperm allocation, have been published: the field cricket *Teleogryllus oceanicus* and the katydid *Requena verticalis*. Female *T. oceanicus* mate with an average minimum of 4.32 ± 0.74 (95% CI: 3.73, 4.90) males in the field (Simmons and Beveridge 2010). Sperm precedence follows a fair raffle ($r = 1$) whether females mate with two or with four males (Simmons 2001b; Simmons et al. 2003). Mating with multiple males increases hatch rate by 15% in this species (Simmons 2001), which combined with data from two other species of field cricket on the effect of multiple mating on egg production (Simmons 1988; Subramaniam et al. 1988) yields an estimate of the effect of mating on offspring production and a corresponding measure of the fecundity parameter α of 0.63. As with *L. cerasina*, the degree of female sperm limitation is assumed to be similar to the average, at $\varepsilon = 0.022$ (Ball and Parker 2000). The observed sperm allocation strategy in *T. oceanicus* is $S_V/S_{NV} = 1.037$ (0.860, 1.207) (Thomas and Simmons 2007).

R. verticalis mate fewer times in the wild than either *L. cerasina* or *T. oceanicus*, with an average minimum of 1.96 ± 0.71 (1.70, 2.23) mates per female when measured during the middle of the breeding season (Simmons et al. 2007). Sperm precedence favors the first male at $r = 0.38$ (95% CI: 0.10, 0.88, data taken from Fig. 1 in Gwynne and Snedden 1995). Females receiving three spermatophylaxes lay 31% more eggs than females receiving only one (Gwynne 1984), which combined with data from two Orthopteran species on the effect of multiple mating on hatching success (Simmons 2001b; Ivy and Sakaluk 2005) yields an estimate of the fecundity parameter α of 0.68. Again, an average degree of female sperm limitation was assumed. The observed sperm allocation strategy in *R. verticalis* is $S_V/S_{NV} = 0.926$ (0.796, 1.075) (Simmons et al. 1993).

The optimal and actual sperm allocation strategies for *T. oceanicus* and *R. verticalis* are shown in Figure 4. The error bars represent the 95% confidence intervals for the observed sperm allocation strategies and the observed female mating frequencies. Note that the mating frequencies shown here represent minimum estimates based on counting the alleles in a female's sperm stores and dividing by two; the actual mating frequencies are likely to be slightly higher (Simmons et al. 2007; Simmons and Beveridge 2010). The ESS for *T. oceanicus*, 1.220, falls just outside of the 95% confidence interval for the observed value of S_V/S_{NV}. The ESS for *R. verticalis*, 0.799, is within the 95% confidence interval for the observed S_V/S_{NV} value. The ESS across the 95% confidence interval for observed sperm precedence in *R. verticalis* ranges from $S_V^*/S_{NV}^* = 0.63$ at $r = 0.88$ (i.e., allocate less sperm to virgins under weak first-male precedence) to $S_V^*/S_{NV}^* = 1.10$ at $r = 0.10$ (i.e., allocate slightly more sperm to virgins under strong first-male precedence).

Discussion

To be effective, empirical tests of sperm allocation models must meet those models' assumptions. Unfortunately, the assumptions of the most frequently tested sperm allocation models, namely that females mate a maximum of twice (risk models) and that males can assess the total number of postcopulatory competitors (intensity models), limit the range of taxa for which those models are appropriate. Our model addresses this problem: explicitly designed for multiply and sequentially mating species, it provides testable predictions for a wide array of animals. Based on results from the three species for which data on the relevant parameter values are available (Fig. 4), our model is moderately successful in predicting sperm allocation patterns.

Our model shares many of the predictions of an earlier model designed for multiply, sequentially mating species by Engqvist and Reinhold (2006). Both models predict greater allocation to virgins than to nonvirgins under conditions of first male precedence and at least moderate female mating frequencies (more than two matings per female). The stronger the first male precedence,

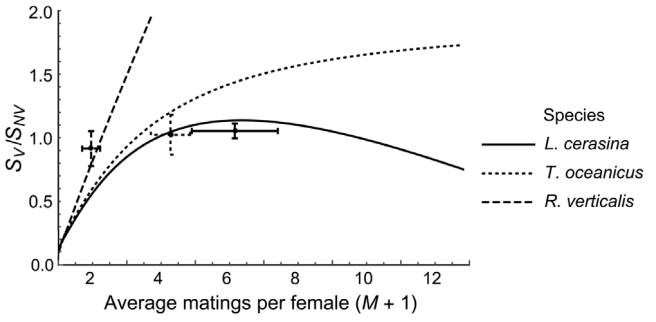

Figure 4. Actual and optimal sperm allocation to virgins versus nonvirgins for three different species. Bars show the 95% confidence intervals for observed sperm allocation patterns and observed female mating frequencies. For information on how the different species' 95% confidence intervals for *r* (sperm precedence pattern) affect their ESS, see text.

the lower the female mating frequency above which allocation to virgins should exceed allocation to nonvirgins (Fig. 1). Under fair raffle conditions, the two models are in close agreement: males should allocate more sperm to virgins if the average number of matings per female exceeds roughly four (Fig. 1). Both models also generally predict greater allocation to nonvirgins under conditions of later male precedence.

The two models differ greatly, however, in their behavior at high female mating frequencies. While our model predicts relatively constant, intermediate allocation to nonvirgins and increasingly extreme (high or low) allocation to virgins (Fig. 2), Engqvist and Reinhold's generally predicts that allocation to the two types of females will converge toward a very low value as female mating rate increases (except under fair raffle conditions). This discrepancy is likely due to the different tradeoffs in the two models. In Engqvist and Reinhold's model, a male's sperm allocation, relative to that of other males, is pitted against his relative success in obtaining matings. At very high female mating rates, males should generally spend fewer resources on sperm and more on securing mates. In our model, sperm allocation is independent of mating success, which does not differ between males. Because our tradeoff is between sperm allocation to virgin and nonvirgin females, rather than between total sperm allocation and relative mating success, optimal allocation remains high even under extreme polyandry.

Given the differences in structure between our two models, the overall similarity in their results is encouraging. Apart from our tradeoff function, the formulation of the sperm precedence parameter, and our inclusion of two terms accounting for the effect of multiple mating on female fecundity, the main structural difference between the two models is in the distribution of female mating frequencies: while we assume a Poisson distribution, Engqvist and Reinhold assumed a geometric distribution, entailing a mode female mating frequency of one mating per female. Based on the available data on minimum female mating frequencies in the field, which has been gathered for species of Drosophila (Frentiu and Chenoweth 2008; Hurtado et al. 2013), dung flies (Demont et al. 2011), crickets (Bretman and Tregenza 2005; Simmons and Beveridge 2010; Turnell and Shaw 2015b), katydids (Simmons et al. 2007), social insects (reviewed in Simmons 2001a), squid (Emery et al. 2001), and swordtail fish (Smith 2014), most females seem to mate more than once (major exceptions are many species of social insects [Strassmann 2001] and of mosquitoes [Yuval 2006]).

Our model's predictions also share similarities with those of the risk models of sperm allocation. At mating frequencies at or below two mates per female, the maximum allowed by risk models, our model predicts greater allocation to nonvirgins under all but the most extreme conditions of first male precedence (Fig. 1). Risk models likewise typically predict greater allocation to nonvirgins (Parker et al. 1997) unless there is a strong first male advantage and significant sperm limitation (Ball and Parker 2007).

The predictions of intensity models of sperm allocation are not strictly comparable to ours, since these models assume that fertilization by all of a female's mates occurs simultaneously and so do not distinguish between virgin and nonvirgin females. However, it is worth noting that the general result of such models, that males should allocate less sperm as the intensity of sperm competition increases above two matings per female, is mirrored by our results, at least under first-male precedence and fair raffle conditions. The intensity of sperm competition that a male will ultimately face is necessarily lower, on average, in matings with virgin than with nonvirgin females, since the total number of matings varies across females and nonvirgin females have already mated at least once. Our prediction that allocation to virgins (lower intensity) exceeds allocation to nonvirgins (higher intensity) above two or three matings per female, depending on the sperm precedence pattern, is thus in line with the predictions of intensity models (see also Engqvist and Reinhold 2006).

One interesting result of our model is the wide range of female mating frequencies and sperm precedence patterns at which optimal allocation to virgins and to nonvirgins is roughly equal (Fig. 1). Given that equal allocation can be optimal at many various and biologically plausible combinations of these two parameters, studies that find no difference in sperm allocation to virgins versus to nonvirgins (i.e., $S_V/S_{NV} = 1$) should not automatically conclude that the species in question cannot discriminate based on female mating status or is not behaving optimally. Of course, such studies can also provide support for a "null model" of no differential allocation. Of the species we used to test our model, the 95% confidence intervals for observed S_V/S_{NV} overlaps with 1 for *T. oceanicus* and *R. verticalis*. The observed 95% CI for *L. cerasina* also overlaps with 1 when S_V/S_{NV} is calculated across males rather than within males (i.e., by dividing the average amount of sperm allocated to virgins by the average amount allocated to nonvirgins; 95% CI: 0.969, 1.097). Notably, however, the within-male allocation estimate for *L. cerasina*, which has less associated error than the across-male estimate, differs significantly from equal allocation: males give 7% (95% CI: 1%, 13%) more sperm to virgins. Given this result, as well as the relatively modest differences in optimal allocation predicted by our model across a wide range of parameter values, we advocate using a within-male experimental design when measuring sperm allocation to minimize error and detect potentially small effects.

Another takeaway from our results is that, given the wide variation in both female mating frequencies and sperm precedence patterns across species, researchers should not expect to find a universal pattern of sperm allocation. Indeed, this variation in species-specific reproductive parameters and the corresponding

variation in optimal sperm allocation may account for the failure of two recent meta-analyses to find a general effect of female mating status on sperm allocation (delBarco-Trillo 2011; Kelly and Jennions 2011; the latter study found greater allocation to virgins only when proxy measures like copulation duration were considered, whereas there was no effect of mating status on sperm number itself).

Several aspects of our model may limit its applicability in some species. First, the formulation of the sperm precedence parameter distinguishes between the first male to mate and all subsequent males. While this structure approximates the pattern observed in our study species, *L. cerasina*, in which a male's fertilization success depends largely on whether he is the first to mate with a female (Turnell and Shaw 2015a), it is unlikely to apply to all species. However, this is also true of any other sperm precedence structure, including the one used in the only other model that currently exists for multiply, sequentially mating species, whereby the second male's sperm is offset by a factor of r, the third male's by a factor of r^2, and so on (Engqvist and Reinhold 2006).

Regardless of the structure of the sperm precedence parameter, a major challenge of testing sperm allocation models that involve more than two competing males is the lack of empirical data on sperm precedence patterns in such cases. While numerous measures exist of P1 and P2, or the proportion of a doubly mated female's offspring sired by each of the two males (e.g., see Simmons and Siva-Jothy 1998), very few studies have examined what happens to sperm precedence patterns when a female mates more than twice. (Two of the few studies to do so, Turnell and Shaw (2015a) and Simmons et al. (2003) [see also Zeh and Zeh 1994], provided our sperm precedence estimations for *L. cerasina* and *T. oceanicus*, respectively.) A further complication in modeling sperm precedence is the wide within-species variance in this parameter reported by many studies (e.g., see Lewis and Austad 1990; Harvey and Parker 2000; and references therein). Indeed, this variance is quite high in *L. cerasina* (Turnell and Shaw 2015a), which helps account for the high uncertainty around our estimate of the sperm precedence parameter in this study.

A second potential limitation of our model is its assumption, implicit in the fitness function, that all males mating with a female have the chance to fertilize each one of her eggs. In fact, if females begin ovipositing before they have completed all of their matings, this will not be true. When testing the model in such cases, the appropriate empirical estimate of female mating rate is not the total lifetime number of mates per female, but rather the number of ejaculates competing to fertilize a female's eggs, averaged over the course of her reproductive lifetime. Depending on the relative timing of mating and oviposition, the former measure may overestimate the latter to a greater or lesser extent.

For *L. cerasina*, our assumption that these two measures are identical, or nearly so, is justified: out of 19 females observed for six weeks in a field enclosure study, all but three waited until after their final mating to begin ovipositing (Turnell and Shaw 2015a). For *R. verticalis*, we attempted to minimize any discrepancy between the two measures by using the number of mates per female estimated during the middle of the breeding season rather than at the end (Simmons et al. 2007), by which point females have already laid a substantial fraction of their eggs (e.g., females in the lab have been shown to lay multiple clutches of eggs within 2–3 weeks of their first two matings [Gwynne 1988], whereas the breeding season lasts for 2–3 months [Simmons et al. 2007]). For *T. oceanicus*, as for most species, detailed data on the relative timing of mating and oviposition are not available. Notably, if the estimate of the total lifetime number of mates per female we used for this species (Simmons and Beveridge 2010) does in fact overestimate the "effective" female mating rate, then the sperm allocation strategy observed in *T. oceanicus* is almost certain to fall within the ESS (see Fig. 4; the empirical value will be shifted to the left).

In addition to the formulations of the sperm precedence parameter and of the female mating rate, a third aspect of our model that may limit its applicability to all species is its assumption that males, while they can distinguish between virgin and nonvirgin females, are unable to determine how many times a female has mated. That males in many species are capable of detecting whether a female has mated at all is evidenced by the differential allocation of sperm to virgins versus to mated females that is often observed across taxa (delBarco-Trillo 2011; Kelly and Jennions 2011). This capability may be mediated by chemical cues, as in the bedbug *Cimex lectularius*, in which males detect the presence of a previous male's ejaculate using chemosensors on their intromittent organs (Siva-Jothy and Stutt 2003). In some species, though, males may also be able to assess the actual number of a female's previous mates. For example, in the cricket *T. oceanicus*, male were shown to adjust the viability of their sperm based on the number of different male-derived cuticular hydrocarbon (CHC) profiles applied to the female (Thomas and Simmons 2009). However, such CHCs may provide information about the presence of rivals in the population rather than the females' mating status (Lane et al. 2015).

Engqvist and Reinhold (2006) found that accounting for this possible ability to distinguish between singly and multiply mated females changes the optimal patterns of sperm allocation significantly. According to this scenario, males should give more sperm to singly mated females than to virgins under all conditions of female mating frequency and sperm precedence. Relative allocation to multiply mated females should generally be highest when sperm precedence favors later males and lowest when it favors earlier males. In the future, it would be

interesting to expand our model to allow for males to distinguish between singly and multiply mated females and see whether our predictions match those of Engqvist and Reinhold.

As for *L. cerasina*, it remains to be tested whether males can assess the number of a female's previous mates. Previous work showing that males differentially allocate micros to virgins versus nonvirgins, but not to nonvirgins mated more versus fewer times, suggests that they may not (Turnell and Shaw 2015a). The mechanism by which they apparently assess whether a female has mated at all is also unknown, but given the results from *T. oceanicus*, as well as evidence of sex-specific CHC profiles from at least one other *Laupala* species (Mullen et al. 2007; but see Mullen et al. 2008), this assessment seems likely to be at least partly mediated by the mechanical transfer of cuticular hydrocarbons from the male to the female during mating. Females may also potentially alter their own production of different cuticular compounds after mating, as has been shown in *Drosophila melanogaster* (Everaerts et al. 2010) and flour beetles (Lane et al. 2015).

Our finding of male discrimination against old virgin females but not older mated females has also been shown in the moth *Plodia interpunctella* (Cook and Gage 1995). This pattern may indicate that males perceive such females to be low-quality. Xu and Wang (2009) found that males in another species of Pyralid moth, *Ephestia kuehniella*, also discriminate against older virgins. However, since they did not test for such an effect among nonvirgin females, it is possible that males in this species provide less sperm to older females in general, perhaps because they have a lower residual reproductive value (Williams 1966; for a similar effect among nonvirgin *Drosophila melanogaster*, see Lüpold et al. 2010). In contrast to *P. interpunctella*, male dung flies (*Sepsis cynipsea*) discriminate against older females when mating with nonvirgins, but not when mating with virgins (Martin and Hosken 2002). The authors' interpretation of this finding, that older virgin females represent an elevated risk of sperm competition, is at odds with the apparent ability of males in this species to distinguish between virgin and nonvirgin females.

The negative relationship we found between sperm number and female weight, even when controlling for female age, was surprising, given that body mass is often considered a proxy for fecundity in insects (Bonduriansky 2001). A recent meta-analysis across various taxa (Kelly and Jennions 2011) found that heavier females tend to receive more sperm, though the effect was not significant. It is possible that other traits are better predictors of female fecundity in *L. cerasina*, such as body size or relative abdomen width (Bonduriansky 2001). Indeed, Kelly and Jennions (2011) found that larger females do receive significantly more sperm. However, this still does not explain why heavier females in our study actually received less sperm. Since female weight increases with age, it is possible that males use female weight, potentially evaluated when the female mounts the male during copulation, as a proxy to assess a female's age and thus her residual reproductive value (Williams 1966). Males may also evaluate female age chemically, if CHC profiles change with age as in *D. melanogaster* (Everaerts et al. 2010).

In conclusion, our model generates realistic predictions of optimal sperm allocation for multiply, sequentially mating species. In testing this and other models of sperm allocation, we advocate using parameter values taken from the species being studied, as this is the only way to accurately determine the predictions to be tested. There is also a need for further empirical studies to assess the biological realism of this and other models' parameter and tradeoff structures. In particular, we do not currently have enough data to confidently model sperm precedence across multiple matings. Expanding on our model by modifying the sperm precedence parameter, for example to distinguish between the last male to mate versus all previous males, would reveal how influential the structure of this parameter is in shaping the model's predictions. Allowing for the possible ability of males to assess the number of a female's previous mates would also be an informative extension of our model. We hope that future work will build on ours to generate more widely applicable and testable predictions of optimal sperm allocation.

AUTHOR CONTRIBUTIONS

B.R.T. and H.K.R. designed the model. B.R.T. tested the model, designed the empirical study, carried out the data collection and statistical analysis, and wrote the manuscript. K.L.S. contributed to the writing of the manuscript.

ACKNOWLEDGMENTS

Thanks to Jon Lambert and Qionghua Gao for providing the data on female fecundity in *L. cerasina*. Thanks also to several anonymous reviewers, whose comments greatly improved this manuscript.

DATA ARCHIVING

The doi for our data is https://doi.org/10.5061/dryad.n2048.

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Associate Editor: L-M. Chevin
Handling Editor: Servedio

Supporting Information

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Figure S1. The effect of female sperm limitation on optimal sperm allocation under first-male sperm precedence ($r = 0.5$). Under sperm limitation ($\varepsilon > 0$), males should increase their allocation to virgins. The illustrated sperm limitation values are taken from Ball & Parker (2000) and include the average across six species ($\varepsilon = 0.022$) and the most extreme reported value ($\varepsilon = 0.050$, in zebrafinches). In this and the following two figures, the female fecundity parameter is $\alpha = 0.74$. Note the different scales of the axes.

Figure S2. The effect of female sperm limitation on optimal sperm allocation under a fair raffle ($r = 1$).

Figure S3. The effect of female sperm limitation on optimal sperm allocation under later-male sperm precedence ($r = 2$).

Figure S4. The effect of multiple mating on female fecundity under first-male sperm precedence ($r = 0.5$). When multiply mated females produce more offspring than singly-mated females ($\alpha < 1$), males should increase their allocation to non-virgins. The illustrated sperm limitation values are taken from South & Lewis (2011) and include the average across 56 arthropod species ($\alpha = 0.74$) and the most extreme reported value ($\alpha = 0.15$, in the cat flea *Ctenocephalides felis*). In this and the following two figures, the female fecundity parameter is $\varepsilon = 0.022$.

Figure S5. The effect of multiple mating on female fecundity under a fair raffle ($r = 1$).

Figure S6. The effect of multiple mating on female fecundity under later-male sperm precedence ($r = 2$).