SEXUAL SELECTION IN THE FIELD: PRE- AND POSTCOPULATORY DYNAMICS IN A HAWAIIAN CRICKET

A Dissertation

Presented to the Faculty of the Graduate School

of Cornell University

In Partial Fulfillment of the Requirements for the Degree of

Doctor of Philosophy

by

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February 2016

SEXUAL SELECTION IN THE FIELD: PRE- AND POSTCOPULATORY DYNAMICS IN A HAWAIIAN CRICKET

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Cornell University 2016

When females mate multiply, male reproductive success depends not only on mating success but also on fertilization success, which is mediated by postcopulatory processes like sperm competition and cryptic female choice. Although postcopulatory sexual selection has the potential to be a major force in driving evolution, very few studies have estimated its strength in the wild, or measured it in such a way as to enable a quantitative comparison with precopulatory sexual selection. Likewise, though polyandry is widespread across taxa and is the focus of a growing body of research, estimates of natural female mating rates are still limited in number.

I used extensive behavioral observations of a semi-natural population of Hawaiian swordtail crickets, *Laupala cerasina*, combined with molecular paternity assignment, to quantify pre- and postcopulatory selection in this population (Chapter 1). The opportunity for postcopulatory selection was over four times as great as for precopulatory selection. To corroborate the patterns I found in this experiment, I also genotyped the sperm stores and offspring of a group of wild adult females, estimating the number of males each female mated with, the number of males that sired her offspring, and the paternity skew among these sires (Chapter 2). Both of these studies revealed that postcopulatory selection is strong in this population, supporting the hypothesis that such selection is a major component of overall sexual selection.

Chapter 3 concerns strategic sperm allocation, a form of postcopulatory mate choice whereby males differentially allocate their sperm based on female quality or

mating status in order to maximize fitness. Many theoretical models of sperm allocation make assumptions that limit their applicability, such as the common assumption that females mate only twice. Furthermore, many empirical tests of these models fail to make *a priori* predictions, since the species-specific values of the model parameters that dictate these predictions (e.g., female mating rate and sperm precedence pattern) are often unmeasured. I designed a broadly applicable model that is appropriate for multiply, sequentially mating animals. The model's predictions were met in *L. cerasina* and in the two other species for which all the relevant data have been published.

BIOGRAPHICAL SKETCH

Biz Turnell was born in Cleveland, OH, and raised in Shaker Heights. His early love of nature and animals was fostered during childhood summers spent exploring the woods and beaches of eastern Massachusetts. The Cape Cod Bay was also the site of his first experience both with scientific research and with field work: assisting in a population study of horseshoe crabs for his high school senior project.

As a college student at Yale University, Biz was introduced to the fields of animal behavior and entomology through courses taught by his advisor, Dr. Marta Martínez Wells. He pursued these interests during summer internships at the Cleveland Museum of Natural History, where he studied invertebrate colonization of amphibian breeding ponds, and at the Woods Hole Marine Biological Laboratory, where he investigated mate choice behavior in zebrafish. He also conducted a senior research project on the genetics of malarial mosquitoes.

After graduating in 2004 with a BS in biology, Biz spent three years as a research and field assistant, studying *Wolbachia* infections in leaf beetles at the Smithsonian Tropical Research Institute in Panama; coyote, fox, and wolf behavior and ecology in wintertime Yellowstone National Park; and cooperative behavior in meerkats at the Cambridge-run Kalahari Meerkat Project in South Africa. He then began his graduate studies in the Department of Neurobiology and Behavior at Cornell University, working with Dr. Kerry Shaw on sexual selection in Hawaiian crickets. He defended his PhD in August 2015.

ACKNOWLEDGMENTS

Thanks to my advisor, Kerry Shaw, for her constructive criticism throughout my graduate career. Thanks also to my committee members: Kern Reeve, who introduced me to theoretical modeling; Tom Seeley, who always reminded me to imagine the world from the perspective of the study organism; and Rick Harrison, who brought a valuable outside viewpoint. Lunch Bunch attendees and members of the behavior journal club provided much useful feedback and intellectual dialogue, for which I am grateful. I'm also indebted to the postdocs and grad students of the Shaw lab: Dan Fergus, Chris Wiley, Chris Ellison, Kevin Oh, Nick Ratterman, Mingzi Xu, Glenn Stamps, Jon Lambert, and Qionghua Gao.

The NB&B administrative staff provided extremely helpful logistical support, especially Terri Natoli, Saundra Anderson, Dawn Potter, Stacey Coil, and Sara Eddleman. Françoise Vermeylen and Jay Barry at the Cornell Statistical Consulting Unit were generous with their time and their expertise. I received funding from the following sources: the National Science Foundation, the Animal Behavior Society, Sigma Xi, the Andrew W. Mellon Foundation, the Cornell University Graduate School, and the Department of Neurobiology and Behavior.

Huge thanks to everyone who made NB&B such a great place to work and Ithaca such a great place to live, especially Jessie Barker, Caitlin Stern, Julian Kapoor, Holly Summers, Julie Miller, Jude Scarl, and Lynn Fletcher. Thanks as well to my family for their unfailing support and encouragement throughout this whole process. Finally, thanks to all the crickets who were involved in this research, and who made it all possible.

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Chapter 1: High opportunity for postcopulatory sexual selection under field conditions[1]

Abstract

In polyandrous systems, male fitness is determined not only by mating success but also by fertilization success. Despite the growing interest over the past several decades in postcopulatory sexual selection, its relative importance compared to precopulatory sexual selection remains a subject of debate. Here, we use extensive behavioral observations of a semi-natural population of Hawaiian swordtail crickets, *Laupala cerasina*, and molecular paternity assignment to measure the opportunities for pre- and postcopulatory selection. Because postcopulatory selection has the potential to operate at multiple stages, we also separately attribute its effects to factors specific to mating events versus factors specific to males. We find that variance in postcopulatory success is over four times as great as variance in precopulatory success, with most of it unexplained by male mating order or the number of nuptial gifts given. Surprisingly, we also find that male singing effort is under postcopulatory selection, suggesting that males who sing more frequently also have more competitive ejaculates. Our results are consistent with the hypothesis that high polyandry levels promote greater relative postcopulatory selection. They also highlight the need for detailed behavioral observations under conditions as natural as possible when measuring mating and reproductive success.

Introduction

It has become clear over the past several decades that, in species where males and females mate multiply, male reproductive success depends not just on mating success but

[1] Published in Evolution (2015) 69: 2094-2104

also on fertilization success, involving postcopulatory processes such as sperm competition (Parker 1970a; Birkhead and Møller 1998, Simmons 2001a, Bretman et al. 2009a) and cryptic female choice (Thornhill 1983; Eberhard 1996; Manier et al. 2013). Because polyandry is widespread across taxa (Birkhead and Pizzari 2002; Simmons 2005), the consequences of differential postcopulatory success are broadly relevant. While it is evident that postcopulatory selection can contribute to overall sexual selection (Eberhard 2009; Birkhead 2010), the magnitude of this contribution remains unclear.

Precopulatory sexual selection, which operates on mating success, has been well quantified, and appears to be generally stronger than selection on survival (Kingsolver et al. 2001; Siepielski et al. 2011). In contrast, postcopulatory sexual selection, though it has been extensively studied, has seldom been measured in such a way as to enable a quantitative comparison with precopulatory selection (Shuster et al. 2013). While some researchers have argued that precopulatory selection is likely to be the more important of the two in driving evolution (Hosken and House 2011; Shuster et al. 2013), evaluating their relative roles requires a measurement of the relative opportunities for pre- and postcopulatory selection. In other words, it is necessary to measure the variance in mating success and in fertilization success among males in a group of freely interacting individuals, which few studies have yet done.

The studies that have done so suggest that the opportunity for postcopulatory selection does in fact rival the opportunity for precopulatory selection. Fertilization success was found to vary as much as mating success in a semelparous laboratory population of *Drosophila melanogaster* (Pischedda and Rice 2012) and to vary twice as much as mating success in both red junglefowl (Collet et al. 2012) and a hermaphroditic snail species (Pélissié et al. 2014a). The proportion of the variance in reproductive success explained by mating success may decline, and the proportion explained by fertilization success rise, with the degree of polyandry, since multiple males can share the same increase in mating success by mating with the same female but must divide their

resulting increases in reproductive success. This hypothesis, supported experimentally by Collet et al. (2012), may account for the fact that mating success varied more than any other component of female reproductive success in the sex-role reversed Gulf pipefish, in which males mated only once (Rose et al. 2013).

Postcopulatory success can be affected by factors specific to the mating event, such as male mating order, and by factors inherent to the male, such as sperm quality. Additionally, mating-specific factors themselves can potentially be influenced by male-specific traits, for example if some males tend to have more favorable positions in the mating order by efficiently locating virgin females, or tend to minimize the number of postcopulatory competitors by preventing females from remating. To evaluate what types of male traits are likely to be under selection, variance in fertilization success can be decomposed into mating-specific and residual variance. The contribution of mating order to variance in fertilization success should increase with the strength of sperm precedence, since a strong precedence effect will eclipse any male-specific variation in sperm competitiveness. This hypothesis was supported by Pischedda and Rice's (2012) finding that mating order explained virtually all of the variance in fertilization success in a *D. melanogaster* population characterized by extreme last male sperm precedence.

Another unanswered question regarding postcopulatory selection, aside from its relative importance, is whether it amplifies or dampens the actions of precopulatory selection (Mautz et al. 2013; Shuster et al. 2013). A positive relationship between mating and fertilization success is expected if pre- and postcopulatory traits are both condition dependent (e.g., Helfenstein et al. 2010), while a negative correlation may result if, as generally dictated by life history theory, a tradeoff exists between investment in different traits (Roff 2002; but see Devigili et al. 2012). Though Collet et al. (2012) found evidence of a positive correlation, Pischedda and Rice (2012), Rose et al. (2013), and Pélissié et al. (2014a) found no correlation; none of these studies assessed the condition dependence of pre- or postcopulatory traits or success.

Here we present a quantitative analysis of pre- and postcopulatory selection in the Hawaiian swordtail cricket *Laupala cerasina*, using a semi-natural field enclosure, exhaustive behavioral observations over the course of six weeks, and molecular paternity assignment. We first partition variance in male reproductive success into variance in mating success and in fertilization success. We measure female mating rate to determine whether our findings are consistent with the hypothesis that the degree of polyandry is positively associated with the contribution of fertilization success to variance in male reproductive success. We further divide the variance in fertilization success between factors specific to the mating, including male mating order and nuptial gift number, and the residual portion, which includes factors specific to the male. To determine whether our findings are consistent with the hypothesis that the level of sperm precedence is positively associated with the contribution of mating order to variance in fertilization success, we also analyze sperm use patterns. Finally, we evaluate the relationship between pre- and postcopulatory selection acting on a trait commonly associated with mating success in Orthopterans, time spent singing (Simmons 1986a; Cade and Cade 1992,Rodríguez-Muñoz et al. 2010a), by measuring the corresponding univariate selection gradients, and determine whether any positive correlation between the two stages of selection may be due to condition dependence.

Material and methods

Mating observations

In *L. cerasina*, males sing during the day, year round, to attract females. Courtship lasts for several hours and involves the transfer of a series of spermless microspermatophores ("micros"), followed at the end of the day by a single sperm-filled macrospermatophore ("macro") (Shaw & Khine 2004). Because the macro is transferred during a fixed window of time relative to sunset, males and females engage in a

maximum of one mating per day. Micros are transferred according to a predictable temporal rhythm (deCarvalho et al. 2012) and are externally visible both before the transfer, on the male's genitalia, and after the transfer, external to the female and attached via an internal sperm tube. Following each transfer, the female removes and consumes the micro or macro after a variable period of time. The transfer of micros has been shown to increase the chance of sperm uptake (deCarvalho and Shaw 2010). Both males and females have been shown to mate multiply in the lab (deCarvalho and Shaw 2010).

L. cerasina were collected at Kalopa State Park on the island of Hawaii, USA, (20°2' N, 155°27' W, elevation 610 m) in September and October 2012. Twenty adult males and 20 adult females were marked on their femurs and pronotums with Sharpie paint pens (Sanford, Oak Brook, IL, USA) to allow for individual identification and placed in a 2.5 m^3 hexagonal pop-up mesh enclosure (Frikon Industries Ltd., Mississauga, Canada). The resulting population density was similar to levels found in some areas of this field site (B.R. Turnell, pers. obs.). Thirty-six shelters made of 9 cm square plastic flowerpots cut in half diagonally were attached to the walls. Food (Fluker's cricket chow, Port Allen, LA, USA) was provided every three days to approximate the hunger status of wild-caught adults based on feeding trials (B.R. Turnell, unpublished data). To ensure their virginity, females were collected as late-instar nymphs and held in a separate enclosure until adulthood. The six oldest females (two to three weeks post final molt at the start of the experiment) were replaced five days into the experiment with younger virgin females (less than two weeks post final molt) for the purposes of a parallel investigation of female mating behavior not discussed here. All females were used in the analyses unless otherwise specified. To maintain a constant population density and sex ratio, dead or missing individuals were replaced over the course of the experiment, either with wild-caught adult males (n = 5) or in one case with an initially virgin female that had been previously removed.

Mating behavior was observed daily by one person (B.R.T.) over the course of six

weeks (October-December 2012). All matings that occurred in the enclosure during this time were recorded. Six weeks was judged to be enough time for most mating activity to cease, based on a 2011 pilot experiment conducted at the same location in which less than 9% of all matings occurred in the final two weeks of the eight week experiment (Turnell, unpublished data). Our measures of mating and reproductive success are thus intended to approximate lifetime measures. A census was performed each hour from 9:00 to 15:00 to ensure that all mating pairs were located. During these censuses, males were categorized as singing, not singing, or involved in courtship with a female. Mating pairs were observed closely to ensure that all micro and macro transfers were recorded, as well as the timing of females' first attempts to remove the macro and of successful macro removal. The highly regular timing of micro production and transfer (deCarvalho et al. 2012) made it possible to observe all micro transfers even if many pairs were mating on a given day (the maximum was nine pairs). At the end of each day when all matings had been completed (typically around 17:00-18:00; sunset was around 17:45), females were placed in individual cups within the enclosure and provided with oviposition substrate (moistened Kimwipes, Kimberly-Clark Corporation, Irving, TX, USA). Kimwipes were collected each morning at 09:00 when the females were again released into the enclosure (sunrise was around 6:30). After six weeks, all individuals were preserved in 95% EtOH, transported to Cornell University in Ithaca, NY, USA, and stored at -20°C. Male body length was measured using digital vernier calipers (BioQuip Products, Rancho Dominquez, CA) and dry weight was measured after 24 hours in an oven at 60°C.

DNA extraction and paternity analysis

Offspring were collected on emergence and stored as nymphs at -20°C. DNA was extracted from adult hind legs and whole nymphs using DNeasy Blood & Tissue Kits (Qiagen Inc., Valencia, CA, USA). Individuals were screened at five microsatellite loci previously identified in a congener, LKH-004_A16_R, LKH-002_G24_R, EH630969,

EH635281, and EH632048 (Ellison and Shaw 2010), using primers labeled with NED, 6-FAM, VIC, PET, and 6-FAM, respectively (Applied Biosystems, Foster City, CA, USA). The five markers were amplified in a 10 μL multiplex PCR containing 1 X PCR DyNAzyme II buffer and 0.2 U DyNAzyme II (Thermo Fisher Scientific, Waltham, MA, USA); 0.15 mM dNTPs (New England Biolabs, Ipswitch, MA, USA); forward primers in the following respective amounts and reverse primers in the same amounts: 75 nM, 175 nM, 75 nM, 200 nM, and 125 nM; and approximately 20-80 ng DNA. PCRs were run on a Dyad DNA Engine peltier thermalcycler (Bio-Rad Laboratories, Inc., Hercules, CA, USA) under the following conditions: 95° for 2 min; 33 cycles of 95° for 30 s, 56° for 30 s, and 58° for 1 min; and 58° for 5 min. PCR products were diluted 1:10 with HiDi formamide and GeneScan -500 LIZ size standard (Applied Biosystems) and fragment analysis was performed on an ABI 3730xl DNA Analyzer at the Cornell University Biotechnology Resource Center. Alleles were called using GeneMarker (SoftGenetics LLC, State College, PA, USA) and verified by eye.

Paternity analysis was performed using Cervus 3.0 (Kalinowski et al. 2007). Cervus uses parentage simulations to determine logarithm of odds (LOD) scores, representing the natural log of the likelihood ratio of one candidate parent versus another. The simulation parameters were as follows: 10,000 offspring, 22 candidate fathers, 95% of candidate fathers sampled, 96% of loci typed, 1% of loci mistyped.

Partitioning pre- and postcopulatory selection

A male's reproductive success is determined by the number of females he mates with (mating success), the proportion of each of those females' offspring he sires (fertilization success), and the average total number of offspring each of those females produces (average female fecundity). Dividing each of these terms by their population means gives relative reproductive success (RS), relative mating success (MS), relative fertilization success (FS), and relative female fecundity (N), such that RS = MS × FS ×

N, plus an error term. Variance in relative reproductive success, defined by Arnold and Wade (1984) as the opportunity for selection, is given by the following approximation (Bohrnstedt and Goldberger 1969; Webster et al. 1995; Collet et al. 2012):

$$\mathrm{Var(RS)} \approx \mathrm{Var(MS)} + \mathrm{Var(FS)} + \mathrm{Var(N)} + \text{covariances}$$

where the covariances are equal to 2Cov(MS, FS) + 2Cov(MS, N) + 2 Cov(FS, N). Following Pélissié et al. (2014a), variance in relative fertilization success can be further decomposed into variance caused by factors specific to the mating event itself (FS_{mating}), such as mating order, and residual variance ($FS_{residual}$), which is influenced by male-specific factors such as sperm competitiveness. The approximation thus becomes:

$$\mathrm{Var(RS)} \approx \mathrm{Var(MS)} + \mathrm{Var}(FS_{mating}) + \mathrm{Var}(FS_{residual}) + \mathrm{Var(N)} + \text{covariances}$$

where the covariances are equal to $2\mathrm{Cov}(MS, FS_{mating}) + 2\mathrm{Cov}(MS, FS_{residual}) + 2\mathrm{Cov}(MS, N) + 2\ \mathrm{Cov}(FS_{mating}, N) + 2\ \mathrm{Cov}(FS_{residual}, N)$. We calculated the percent of variance in RS explained by variance in MS, in overall FS, and in N, as well as the covariances between these terms, and then repeated the process after partitioning the variance in FS between variance in FS_{mating} and in $FS_{residual}$.

To determine FS_{mating} and $FS_{residual}$, we started with a generalized linear regression in which the response variable was the number of a given female's offspring sired by the male after a given mating event and the effects were male mating order (i.e., the male's place in the female's mating sequence), whether the female was virgin or non-virgin, whether the male mated on the previous day, and the number of micros transferred during the mating ("micro number"). A quasipoisson distribution was used to account for overdispersion, and the log of the total number of offspring produced by the female was included as the offset variable. Because 11 out of 99 matings included in the analysis

were between a male and a female that had already mated with each other, we used an estimation-maximization algorithm to judge how many of that pair's total offspring resulted from each of the two matings (Do and Batzoglou 2008). Paternity values from both matings were considered as separate data points in the measures of FS, but the matings were not counted twice in the measures of MS or N.

We took a model selection approach based on QAICc scores to determine which factors should be retained in the FS model (Burnham and Anderson 2002). For each male, the number of offspring sired predicted by the final regression and the observed minus the predicted values were separately averaged across mating events to calculate his mating-specific and residual fertilization success. These were divided by the mean overall fertilization success to generate the standardized values of FS_{mating} and $FS_{residual}$ (Pélissié et al. 2014a).

Because the abundance of virgin females in the enclosure at the start of the experiment may have biased our measures by decreasing the initial variance in male mating success, if some receptive females settled for less attractive males when the more attractive males were already taken, we measured whether variance in male mating success changed over time.

In addition to partitioning the variance in relative reproductive success, we also used multivariate linear regression to calculate the standardized multivariate selection gradients on mating success, fertilization success, and average female fecundity (Collet et al. 2014). Each of the variables was standardized to have a mean of zero and a standard deviation of one. We used AICc scores to determine the best model.

Factors affecting FS_{mating}

To partition the variance in fertilization success among the mating-specific factors of the FS model, we used partial regression (Legendre and Legendre 2012) based on the pseudo-R^2 values of the generalized linear model. In addition, since micro number was

retained in the model (see Results), we examined the relationship between this variable and two potential mechanisms by which it may increase fertilization success: increasing macrospermatophore attachment time and delaying female remating. We tested for sperm precedence based on male mating order by comparing the fertilization success we observed to that which would be expected if all males to mate with a given female had equal success.

We treated micro number as a mating-specific effect rather than a male-specific effect because micro number depends strongly on the time of day that the mating pair is established, with more micros generally being transferred the earlier courtship begins (Shaw and Khine 2004). To confirm this pattern, we regressed micro number on latency to the start of courtship and found a strong inverse effect ($R^2 = 0.58$, $p < 0.0001$). To rule out the possibility that some males tend to produce more micros than others, for example by attracting females earlier in the day, by producing micros at a faster rate, or by producing the macro later, we regressed micro number on the latency to the first micro transfer with and without male identity as a random effect and found that the model fit better without male identity (likelihood ratio test, $p < 0.0001$). Furthermore, the intraclass correlation coefficient (ICC) values measuring within-male repeatability in micro number, in the latency to the first micro transfer, and in the residuals of the regression of micro number on latency to first micro transfer were all low (0.03, -0.05, and 0.13, respectively).

Selection on singing effort

To determine the univariate selection gradients on male singing effort (equivalent to the selection differential; Arnold and Wade 1984; Collet et al. 2012), we regressed RS, MS, FS, FS_{mating}, $FS_{residual}$, and N on the average proportion of time per day a male spent singing, based on the hourly censuses and exclusive of the time he was actively involved in courtship with a female. Matings with all females were considered in this analysis. We

also examined the relationship between singing effort and male condition, defined as the residual of dry weight on body length, as well as between condition and all measures of success.

All statistical analyses were conducted using R version 3.1.1 (R Development Core Team 2014). Reported confidence intervals were calculated using the basic bootstrap interval (Davidson and Hinkley 1997).

Results

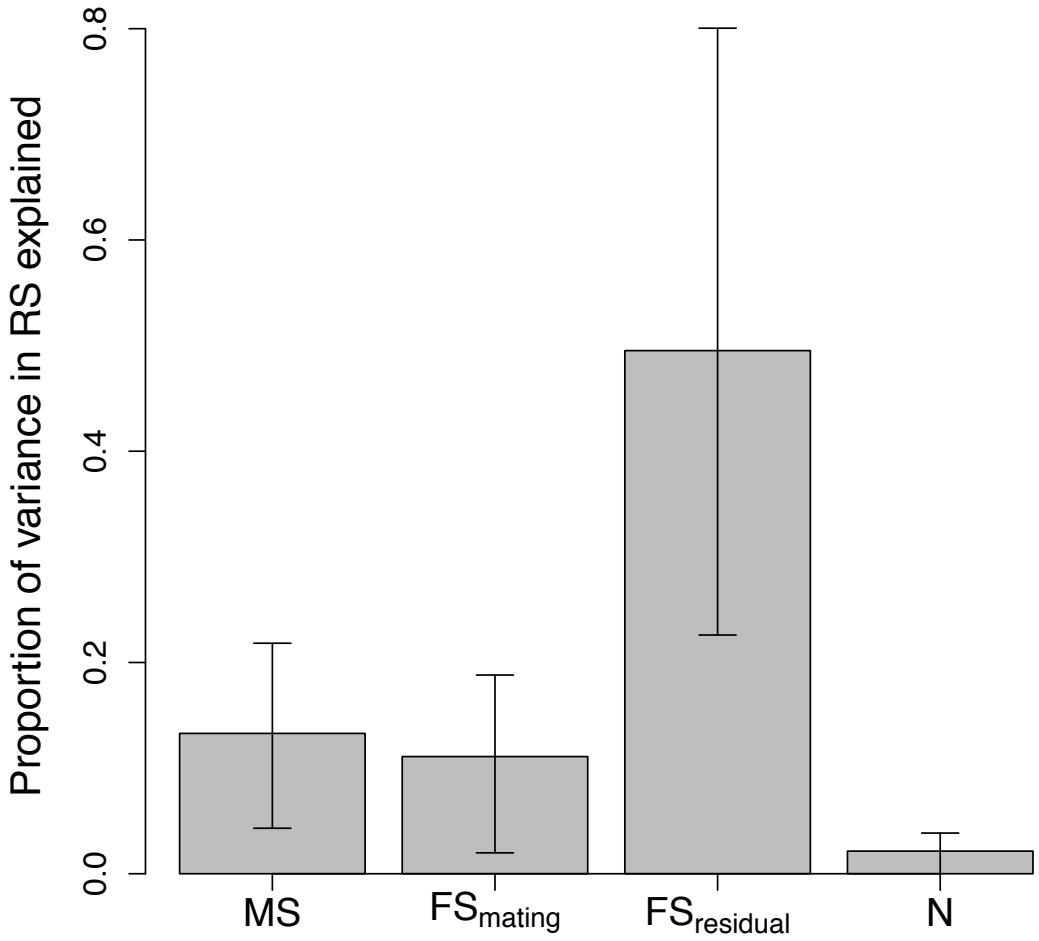
Mating observations and paternity analysis

Fifteen out of the 20 original males were present in the enclosure for the full duration of the experiment, and only these individuals were used in the analyses. The average (± standard deviation) number of mates per male was 6.33 ± 1.91, with all 15 males mating at least once. The average number of mates for the nineteen females present in the enclosure for at least five weeks was 5.37 ± 2.36. Not all matings resulted in fertilizations: on average, males sired the offspring of only 61% (3.87 ± 2.45) of the females they mated with. The average number of micros transferred was 3.98 ± 1.44, and the average proportion of time spent singing was 0.28 ± 0.06. Of 513 eggs laid by the 22 females that mated with the 15 included males, 478 offspring emerged and 428 were assigned paternity at a trio likelihood (likelihood of father given known mother) confidence level of at least 95%. All but three females completed all of their matings before starting to lay eggs. Matings with three of the females were not included in subsequent analyses unless otherwise specified: one female whose offspring (n = 2) could not be assigned paternity, and two females that were removed after five days and that mated with just one male each.

Partitioning pre- and postcopulatory selection

Variance in MS explained 14% (95% bootstrap CI: 4%-23%) of the variance in reproductive success (Figure 1.1). Variance in overall FS explained 62% (30%-94%) of the variance in reproductive success, while variance in female fecundity explained just 2% (0%-4%). When fertilization success was separated into its mating-specific and residual components, FS_{mating} and $FS_{residual}$ explained 11% (3%-18%) and 50% (23%-81%), respectively, of the variance in reproductive success. None of the covariance terms differed significantly from zero, though there was a trend for FS_{mating} to be positively associated with both MS and N ($Cov(MS, FS_{mating}) = 0.026$, $p = 0.053$ and $Cov(FS_{mating}, N) = 0.017$, $p = 0.061$). When the variance partitioning was reanalyzed to incorporate the average hatch rate for each male's mates, variance in hatching success explained less than 0.2% of the total variance in RS (0%-0.3%) and did not covary significantly with any of the other terms. The opportunities for selection, precopulatory sexual selection, postcopulatory sexual selection, and selection on mate fecundity were as follows: $Var(RS) = 0.44$, $Var(MS) = 0.09$, $Var(FS) = 0.39$, $Var(N) = 0.01$. The opportunities for total and for precopulatory sexual selection for the nineteen included females were $Var(RS) = 0.22$ and $Var(MS) = 0.19$.

Variance in male mating success did decrease over time: the coefficient of variation (CV) within each week ranged from 0.45-0.58 for the first half of the experiment, when 83% of the matings occurred, to 1.39-1.72 for the second half of the experiment (regression of CV on week number, $R^2 = 0.70$, $p = 0.038$). We therefore repartitioned the variance in reproductive success considering only the matings that occurred in the second half of the experiment, when females presumably had a full range of precopulatory choice and variance in mating success was not depressed. During this period, variance in fertilization success still explained 39% of the total variance compared to the 12% explained by variance in mating success, though the confidence intervals overlapped (FS: 10%-72%, MS: 7%-18%), probably due in part to a small



sample size (n = 17 matings). Furthermore, a male's overall mating success did not

Figure 1.1. Proportion of the variance (with bootstrapped 95% CIs) in relative male reproductive success (RS) explained by variance in the relative values of mating success (MS), mating-specific fertilization success (FS_{mating}), residual fertilization success ($FS_{residual}$), and average female fecundity (N). Proportions explained by MS and total FS correspond to the opportunities for precopulatory and postcopulatory sexual selection, respectively.

predict the date of his matings (generalized linear mixed model [GLMM] with male ID as a random effect, $p = 0.81$), indicating that less attractive males did not mate disproportionately earlier in the experiment.

Mating and fertilization success were retained in the regression on reproductive success, but average female fecundity was not (delta AICc > 2; we arrived at the same model using backward stepwise regression). The standardized multivariate selection

gradients on mating and fertilization success were $\beta = 0.31$ and 0.85 respectively ($p = 0.005$, $p < 0.001$). For illustrative purposes, the univariate regressions of MS and FS on RS are shown in Figures 1.2 A and B, respectively.

Factors affecting FS_{mating}

The final mating-specific model predicting fertilization success included female mating status (virgin or non-virgin), male mating order, and the number of micros transferred, but excluded whether the male had mated on the previous day (delta QAICc = 2; we arrived at the same model using backward stepwise regression). Female virginity, male mating order, and micro number each accounted for roughly the same amount of variance in FS_{mating} at 23%, 19%, and 24%, respectively (Table 1). Multiple terms explained 26% of the variance, and the remainder was residual. Fertilization success was significantly higher than expected by chance for males mating with virgins (t-test, $p = 0.027$), with first males siring an additional 14% of offspring beyond the 21% expected based on equal representation for a total of 35%. This was both because fewer matings with virgins than with non-virgins resulted in no fertilizations (13% vs. 42%, Fisher's exact test, $p = 0.045$) and because, among matings that did result in fertilizations, matings with virgins resulted in significantly higher fertilization success than expected (t-test, $p = 0.009$). An increase in male mating order was associated with a decrease in fertilization success ($R^2 = 0.10$, $p = 0.001$). However, the difference between observed and expected fertilization success was not affected by mating order among matings with non-virgin females ($R^2 = 0$, $p = 0.80$), indicating that this decrease was due not to sperm precedence but to the fact that later males had more competitors on average than earlier males (e.g., some second males were also the last males to mate and thus competed with only one other male, while all sixth males competed with at least five others).

Micro number, which was positively associated with fertilization success, had a significantly positive effect on female remating latency ($R^2 = 0.07$, $p = 0.016$, with

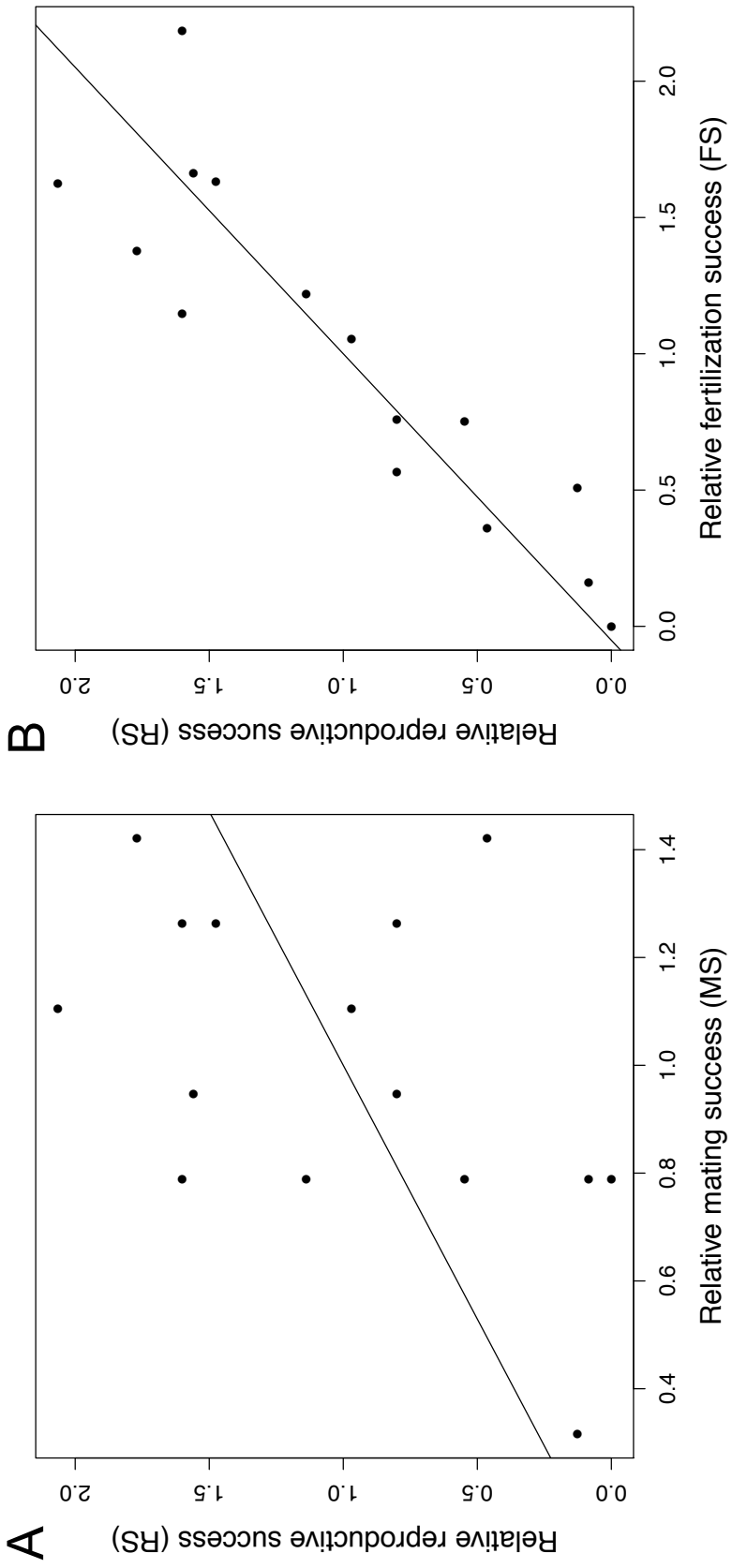

A
Relative reproductive success (RS)
0.0
0.5
1.0
1.5
2.0
0.4
0.6
0.8
1.0
1.2
1.4
Relative mating success (MS)
B
Relative reproductive success (RS)
0.0
0.5
1.0
1.5
2.0
0.0
0.5
1.0
1.5
2.0
Relative fertilization success (FS)

Figure 1.2. Regression of relative male reproductive success (RS, or reproductive success divided by the mean reproductive success of the population) on (A) relative mating success (MS, or mating success divided by the mean mating success of the population), i.e., the male Bateman gradient, and (B) relative fertilization success (FS, or fertilization success divided by the mean fertilization success of the population). Note that a male's fertilization success refers to the average proportion of offspring sired across all of his mates. See text for the standardized multivariate selection gradients.

Term	**Estimate**	**SE**	**t**	**p**	**Variance explained (%)**
Female is virgin	0.7011	0.2936	2.388	0.0189	22.69
Male mating order	-0.1298	0.0613	-2.117	0.0369	19.08
Number of micros	0.1898	0.0775	2.447	0.0162	24.06
Multiple terms					26.15
Residual variance					9.01

Table 1.1. Variance in mating-specific fertilization success explained by the terms in the mating-specific effects model (n = 99 matings, pseudo-R^2 = 0.21).

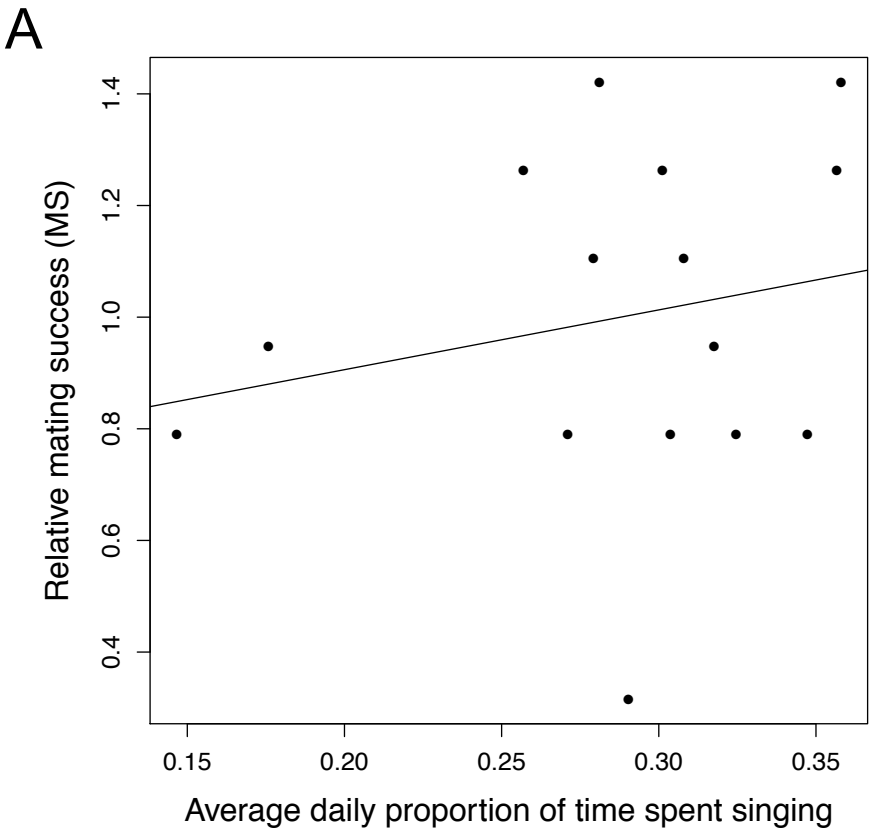
female remating latency log-transformed to normalize the residuals) and on the time between macro transfer and the female's first attempt to remove the macro (R^2 = 0.07, p = 0.010), but not on the time to successful macro removal (R^2 = 0.02, p = 0.16, with both measures of macro attachment time square-root transformed to normalize the residuals). Micro number was significantly associated with female mating status (more micros were transferred to non-virgins, Kruskal-Wallis p = 0.027) and with male mating order (more micros were transferred by later males, Spearman's rank p = 0.031, though there was no such effect among matings with non-virgins only, Spearman's rank p = 0.32). However, we did not detect any problematic multicollinearity from the variance inflation factors when we ran the model as a simple linear regression (all VIFs < 1.5).

Selection on singing effort

The average proportion of time a male spent singing per day predicted $FS_{residual}$ (Figure 1.3A, $R^2 = 0.38$, $p = 0.014$), overall FS ($R^2 = 0.34$, $p = 0.023$), and RS ($R^2 = 0.31$, $p = 0.031$), but not MS (Figure 1.3B, $R^2 = 0.04$, $p = 0.45$), FS_{mating} ($R^2 = 0$, $p = 0.81$), or N ($R^2 = 0.01$, $p = 0.40$). The corresponding standardized selection gradients, calculated by regressing each measure of success on time spent singing when all measures were standardized to have a mean of zero and a standard deviation of one, were $\beta = 0.62$ for residual fertilization success, $\beta = 0.58$ for overall fertilization success (i.e., the univariate postcopulatory selection gradient), $\beta = 0.56$ for reproductive success (i.e., the univariate selection gradient), $\beta = 0.21$ for mating success (i.e., the univariate precopulatory selection gradient), $\beta = 0.07$ for mating-specific fertilization success, and $\beta = 0.24$ for average female fecundity. Male condition did not predict time spent singing ($R^2 = 0.06$, $p = 0.36$), RS ($R^2 = 0.07$, $p = 0.33$), overall FS ($R^2 = 0.07$, $p = 0.34$), $FS_{residual}$ ($R^2 = 0.09$, $p = 0.28$), or MS, FS_{mating}, or N ($p > 0.50$ for all).

Discussion

Although research on postcopulatory processes has proliferated over the past several decades, the opportunity for postcopulatory sexual selection has rarely been quantified, and the magnitude of its contribution relative to precopulatory sexual selection remains unclear. In this study, we found that this contribution can be quite large: in *L. cerasina*, variance in fertilization success explained over four times as much of the variance in reproductive success as did variance in mating success (62% vs. 14%, Figure 1.1; Figure 1.2B vs. 1.2A). To our knowledge, this is the highest measure yet reported of the opportunity for postcopulatory selection relative to precopulatory selection. Our findings are qualitatively similar to those of recent studies in junglefowl (Collet et al. 2012) and hermaphroditic snails (Pélissié et al. 2014a), in which fertilization success varied twice as much as mating success, and taken together these results make a


A
Relative mating success (MS)
0.4
0.6
0.8
1.0
1.2
1.4
0.15
0.20
0.25
0.30
0.35
Average daily proportion of time spent singing

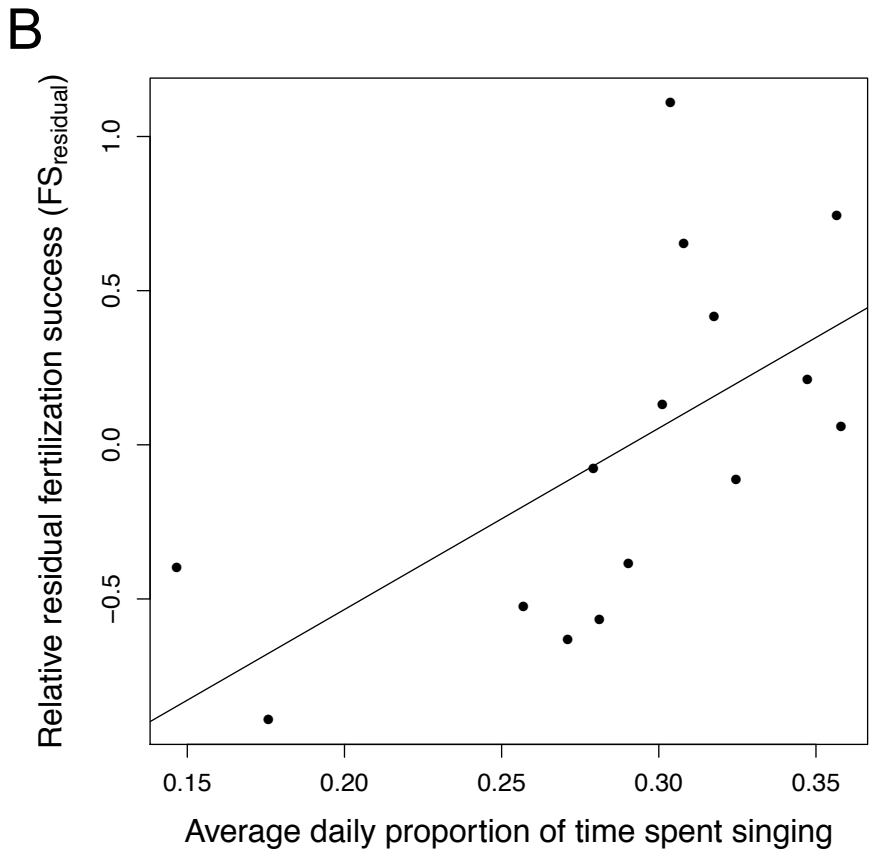

B
Relative residual fertilization success ($FS_{residual}$)
−0.5
0.0
0.5
1.0
0.15
0.20
0.25
0.30
0.35
Average daily proportion of time spent singing

Figure 1.3. Effect of the average proportion of time spent singing per day by males on (A) relative mating success (MS, $R^2 = 0.04$, $p = 0.45$) and (B) relative residual fertilization success ($FS_{residual}$, $R^2 = 0.38$, $p = 0.014$). See text for the standardized univariate pre- and postcopulatory selection gradients on male singing effort.

strong case for the potential of postcopulatory sexual selection to shape the evolution of reproductive traits across taxa.

Our results are consistent with the hypothesis that high levels of polyandry predict a relatively large contribution of variance in fertilization success to variance in reproductive success. This effect was demonstrated experimentally by Collet et al. (2012), who found that raising the female mating rate increases the relative contribution of fertilization success by decreasing variance in mating success. The females in our enclosure mated over five times on average, a number that is substantially higher than reported in the other recent studies discussed here and is thus in line with the correspondingly greater role of fertilization success we found.

Our results are also consistent with the hypothesis that the degree of sperm precedence associates positively with the contribution of mating order to variance in fertilization success. The sperm precedence we detected in *L. cerasina* was only moderate, with first males gaining more fertilizations than expected but siring on average only about one-third of the offspring. Correspondingly, most of the variance in fertilization success we measured was not due to mating order or other mating-specific factors but rather was residual or male-specific. This residual variance explained nearly five times as much of the variance in reproductive success as did variance caused by the mating-specific effects of male mating order, female virginity, and number of micros transferred (50% vs. 11%). Here again our results are qualitatively similar to, but more exaggerated than, those of Pélissié et al. (2014a), who found that first-male precedence was mild and that variance in residual fertilization success was nearly twice as great as the variance in mating-specific (in their study, order-adjusted) fertilization success. In contrast, in the experimental population of *D. melanogaster* used in Pischedda and Rice's

study (2012), last-male precedence was roughly 80%, creating a strong mating order effect that swamped out any male-specific, order-independent variability in sperm competitiveness. (Their measure of variance in order-adjusted fertilization success, which accounted for less than 2% of total variance in reproductive success, may be an underestimate, since mating order was inferred by paternity outcomes rather than directly observed.)

Mating-specific factors such as mating order and nuptial gifts can themselves be affected by male traits, for example if some males are consistently the first or last male to mate or provide more or better gifts. Depending on the particular mechanisms involved, the male traits affecting these mating-specific factors may be under either pre- or postcopulatory selection, blurring the distinction between the two (see Pélissié et al. 2014). Our data show that in *L. cerasina*, males increase their mating-specific fertilization success by mating with virgin females, by mating earlier in the mating sequence (which results in fewer competitors on average if there is any variation in female promiscuity), and by transferring more microspermatophores (Table 1.1). The ability to attract virgin females may plausibly be under precopulatory selection in some species. Perhaps some males compete more strongly for virgin females or prefer them to non-virgins. Alternatively, virgin females may be more choosy (e.g., Ligout et al. 2012), so that in some species males with more attractive or frequent song benefit not only by mating with more females but by mating with proportionally more virgins. Aside from mating with virgin females or earlier in the mating sequence, males may also reduce the number of competitors by minimizing the number of times a female will remate. This ability seems highly likely to be under postcopulatory rather than precopulatory selection; for example, in many insects, males transfer substances in the ejaculate to manipulate female remating behavior (Avila et al. 2011).

The transfer of micros also seems most appropriately categorized as a postcopulatory factor since it has been shown to facilitate sperm transfer (deCarvalho and

Shaw 2010). In this study we found that micro number delays a female's remating as well as her first attempt to remove the sperm-filled macrospermatophore. Delaying a female's remating may improve the male's fertilization success by increasing the chances of her ovipositing before accepting sperm from a competing male; however, this mechanism seems unlikely to play a major role in *L. cerasina*, since most females in this study completed all of their matings before starting to lay eggs. Delaying the removal of the macrospermatophore should increase a male's fertilization success by extending the potential duration of sperm uptake (e.g., Sakaluk 1984; Simmons 1986b). Interestingly, we only found a relationship between micro number and the time to the first macro removal attempt, not the total macro attachment time, suggesting that females may exert some control over the evacuation of sperm during macro attachment. We also found that males give more micros to non-virgins, perhaps because they are certain to face sperm competition in matings with non-virgin females. Consistent with previous work (Shaw and Khine 2004), we found that micro number was strongly predicted by the time of day that the mating pair was established rather than by male identity, though it is nonetheless possible that there is across-male variation in micro production too small to detect in this study. In other species, nuptial gifts have been found to increase male reproductive success in a variety of other ways, such as by preventing female remating entirely, which would affect a male's mating-specific fertilization success, or by increasing offspring number or viability, which would affect his average mate fecundity level (see Vahed 1998, Gwynne 2008 for reviews).

We found no evidence that postcopulatory selection either works in concert with or opposes precopulatory selection. As in Pischedda and Rice (2012), Rose et al. (2013), and Pélissié et al. (2014a), the correlation between mating and fertilization success in our study was not significantly different from zero. We did, however, find that time spent singing, a trait commonly associated with mating success in the Orthoptera, predicted both overall and residual fertilization success (Figure 1.3B), though surprisingly not

mating success (Figure 1.3A). Singing effort thus appears to be under postcopulatory selection, but in this study there was no evidence of precopulatory selection. Given previous work in other crickets showing that calling song predicts mating success (Simmons 1986a; Cade and Cade 1992,Rodríguez-Muñoz et al. 2010a), our failure to find such an effect may be due to a relatively small sample size. Alternatively, the population density and thus the encounter rate in our enclosure may have been high enough to eliminate any mating advantage to be gained by singing more often.

The positive relationship we found between singing effort and residual fertilization success suggests that some males have a greater ability than others both to sing more often and to produce a larger or better ejaculate. The high energetic costs of singing have been quantified in other species of cricket – calling can more than double the rate of oxygen consumption (Hoback & Wagner 1997; Hack 1998) and can even require anaerobic metabolism (Mowles 2014) – and sperm production also has demonstrated costs in a range of taxa (Voorhies 1992; Gage & Cook 1994; Olsson 1997; see Wedell et al. 2002). Theoretically, males in better condition should be able to allocate more resources to many traits at once (van Noordwijk and de Jong 1986; Andersson 1994), but we found no relationship between male condition and time spent singing, nor between condition and any component of reproductive success. It is possible that our measure of condition was not reflective of males' actual energy resources (see Cotton et al. 2004). Alternatively, the link between singing effort and fertilization success may be mediated not by condition dependence but through a genetic correlation (e.g., Hosken et al. 2008; though see Evans 2010, Simmons et al. 2010, and Engqvist 2011 for evidence of negative correlations), or by cryptic female choice (Edvardsson and Arnqvist 2000; Tallamy et al. 2002; Peretti and Eberhard 2010; Manier et al. 2013).

Our results join others in highlighting the importance of direct behavioral observations when attempting to accurately measure mating and reproductive success. Using reproductive success to infer mating success can lead to overestimates of

precopulatory sexual selection: matings that do not result in offspring (40% of all matings in this experiment) will not be detected, and thus mating success will seem to influence reproductive success more than it actually does (Anthes et al. 2010; Pélissié et al. 2012; Collet et al. 2014). We also advocate making these behavioral observations under conditions as natural as possible. Our study is unique in its high number of freely interacting males and females (20 each) and in its long period of mating opportunities (six weeks). Data from a separate experiment suggest that the level of polyandry observed in our experimental enclosure is reflective of the level in the natural population: a group of adult females that were collected at the time of this experiment and allowed to oviposit had roughly the same number of sires contributing to their offspring as did the females in the enclosure (B.R. Turnell, unpublished data). Furthermore, mating behavior in our enclosure was greatly reduced after about three weeks, and we think it is unlikely that our experimental females would have mated many more times if given the opportunity.

The high mating rate during the first half of the experiment did correspond to a lower variance in male mating success compared to the second half of the experiment, possibly due to some of the initially virgin females accepting males that they would have rejected had the more attractive males been available. However, even during the second half of the experiment, variance in fertilization success explained over three times as much of the total variance in reproductive success as did variance in mating success. One factor we did not take into account in our partitioning of the variance in reproductive success is adult lifespan. Many studies have examined the relative roles of mating success, fecundity, and lifespan in lifetime reproductive success (e.g., see Clutton-Brock 1988), and considering fertilization success as well in future such studies would provide a more complete picture of how selection operates.

In conclusion, we found the greatest relative opportunity for postcopulatory sexual selection yet reported, supporting recent findings that such selection plays a major

role in overall sexual selection. Our results corroborate the finding of Collet et al. (2012) that a high degree of polyandry is likely to be associated with a high opportunity for postcopulatory selection relative to precopulatory selection. They also support the hypothesis that low or moderate sperm precedence should be associated with a low or moderate contribution of mating order to variance in fertilization success. In addition, we provide further evidence that precopulatory traits may predict postcopulatory success (Mautz et al. 2013). Ours is the latest of several recent studies attempting to partition variance in reproductive success into pre- and postcopulatory elements. Hopefully, as such experiments are conducted in more species and mating systems, we will develop a more detailed understanding of what factors – level of polyandry, degree of sperm precedence, operational sex ratio, population density – affect the relative contributions of these two components.

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Chapter 2: Polyandry and postcopulatory sexual selection in a wild population[1]

Abstract

When females mate multiply, postcopulatory sexual selection can occur via sperm competition and cryptic female choice. Although postcopulatory selection has the potential to be a major force in driving evolution, few studies have estimated its strength in natural populations. Likewise, though polyandry is widespread across taxa and is the focus of a growing body of research, estimates of natural female mating rates are still limited in number. Microsatellites can be used to estimate the number of mates represented in females' sperm stores and the number of sires contributing to their offspring, enabling comparisons both of polyandry and of two components of postcopulatory selection: the proportion of males that mate but fail to sire offspring, and the degree of paternity skew among the males that do sire offspring. Here we estimate the number of mates and sires among wild females in the Hawaiian swordtail cricket *Laupala cerasina*. We compare these estimates to the actual mating rates and paternity shares we observed in a semi-natural population. Our results show that postcopulatory sexual selection operates strongly in this species: wild females mated with an average minimum of 3.6 males but used the sperm from only 58% of them. Furthermore, among the males that did sire offspring, paternity was significantly skewed. These patterns were similar to those observed in the field enclosure, where females mated with an average of 5.7 males and used the sperm from 62% of their mates, with paternity significantly skewed among the sires.

Introduction

Polyandry, or multiple mating by females with different males over a single

[1] Published in Molecular Ecology (in press)

reproductive cycle, generates postcopulatory sexual selection. Mediated by sperm competition (Parker 1970a; Birkhead and Møller 1998,Simmons 2001a) and by cryptic female choice (Thornhill 1983; Eberhard 1996), postcopulatory selection can be as influential as precopulatory sexual selection in driving evolution (Collet et al. 2012; Pischedda and Rice 2012, Pélissié et al. 2014b; Turnell and Shaw 2015). The level of polyandry and the resulting potential for postcopulatory selection has implications not just for male (Hosken and Ward 2001; Simmons and García-González 2008; Firman and Simmons 2010) and female (Manier et al. 2013) reproductive traits, but also for genomic traits (Price et al. 2008; Mank et al. 2013; Wedell 2013), the maintenance of genetic variation (Chesser and Baker 1996; Zeh et al. 1997; Holman and Kokko 2013), and the nature of sexual conflict (Parker and Birkhead 2013).

Measuring postcopulatory selection is essential to understanding overall sexual selection because it can either amplify or dampen the effects of precopulatory selection. If males that are more successful at mating are also more successful at gaining fertilizations once they mate (Lewis and Austad 1994; Evans et al. 2003; Hosken et al. 2008; Mautz et al. 2013), measuring mating success alone will underestimate total sexual selection. Conversely, if males that mate more often are disadvantaged at the postcopulatory stage (Danielsson 2001; Preston et al. 2001; Demary and Lewis 2007; Simmons et al. 2010, Engqvist 2011b), mating success will overestimate total sexual selection. Decades of research on precopulatory selection have yielded a wealth of empirical estimates of mating success and of the strength of precopulatory selection as it operates in nature (Kingsolver et al. 2001; Siepielski et al. 2011). In contrast, despite the prevalence of polyandry across taxa (Birkhead and Pizzari 2002; Simmons 2005; Taylor et al. 2014) and a growing interest in the evolutionary implications of multiple mating (e.g., Pizzari and Wedell 2013), relatively few natural measures of postcopulatory selection exist.

The strength of postcopulatory sexual selection is affected by two factors:

whether a male sires any of a female's offspring after he mates with her, and how many of those offspring he sires compared to her other mates. To incorporate both of these factors, it is necessary to determine both the number of mates and the number of sires per female in a population. An increasing number of studies have estimated the number of sires per female and the number of offspring per sire in a range of taxa, providing valuable information about the degree of paternity skew in these populations (Emery *et al*. 2001; Frentiu & Chenoweth 2008; Price *et al*. 2011; Hurtado *et al*. 2013; Smith 2014; for a review see Taylor *et al*. 2014). However, only a handful of studies (Bretman & Tregenza 2005; Simmons *et al*. 2007; Simmons & Beveridge 2010; see also Demont *et al*. 2011) have also estimated the number of mates per female, thereby incorporating into their measures of postcopulatory selection those males that succeed in mating but fail to sire any offspring.

Here, we measure the level of polyandry and the intensity of postcopulatory sexual selection in a wild population of the Hawaiian swordtail cricket *Laupala cerasina*. There is significant potential for postcopulatory selection in *L. cerasina*, both through cryptic female choice and through sperm competition. 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). Females mate multiply in the lab (deCarvalho and Shaw 2010) and may exercise cryptic choice by removing the macro earlier or later (Turnell and Shaw 2015). Micros enhance sperm transfer (deCarvalho and Shaw 2010) and predict siring success (Turnell and Shaw 2015), and males provide more micros to non-virgin females (Turnell and Shaw 2015), possibly to increase the amount of sperm transferred under a high risk of sperm competition (Parker 1998).

In this study, we use microsatellites to genotype the sperm stores and offspring of two groups of wild females, thereby estimating the number of mates and the number of

sires per female, as well as the degree of paternity skew. We compare these estimates to the corresponding measures taken from a field enclosure in which all matings were directly observed and all offspring were assigned paternity, enabling an analysis of both mating and sperm use patterns within individual females. Though the overall degree of postcopulatory selection in this semi-natural population has been reported elsewhere (Turnell and Shaw 2015), details on female mating rates and the level of paternity skew are reported here for the first time.

Methods

Field collection and mating observations

Crickets were collected at Kalopa State Park on the island of Hawaii, USA, (20°2' N, 155°27' W, elevation 610 m) between September and December 2012. *L. cerasina* females mate and lay eggs year round. A total of 83 adult females were collected, of which 36 were sacrificed immediately and stored in 70% EtOH at -20°C. The remaining 47 were transported to Cornell University in Ithaca, NY, USA, and allowed to oviposit for eight weeks or until their deaths. They were housed in individual specimen cups with oviposition substrate (moistened Kimwipes, Kimberly-Clark Corporation, Irving, TX, USA) under a 12:12 light:dark cycle at 20°C and provided *ad libitum* with food (Fluker's cricket chow, Port Allen, LA, USA). Nymphs were collected upon hatching and stored at -20°C.

An additional 20 females were collected as late-instar nymphs. Upon reaching adulthood they were individually marked on their femurs and pronotums with Sharpie paint pens (Sanford, Oak Brook, IL, USA) and placed in a large field enclosure along with 20 individually marked adult males from the same population, as described in Turnell and Shaw (2015). All matings were observed over the course of six weeks. This was judged to be enough time for most mating activity to cease, based on a 2011 pilot

experiment conducted at the same location in which less than 9% of all matings occurred in the final two weeks of the eight week experiment (B.R. Turnell, unpublished data). Our measures of the number of mates per female are thus intended to approximate lifetime measures.

Females in the enclosure were housed individually at night with oviposition substrate to allow for collection of their eggs. Food (Fluker's cricket chow, Port Allen, LA, USA) was provided every three days to approximate the hunger status of wild-caught adults based on feeding trials (B.R. Turnell, unpublished data). At the end of the observation period, males were stored in 95% EtOH at -20°C and females were transported to Cornell University and allowed to continue ovipositing as described above. All offspring were collected upon hatching and stored at -20°C.

DNA extraction and genotyping

DNA was extracted from adult hind legs and whole nymphs using DNeasy Blood & Tissue Kits (Qiagen Inc., Valencia, CA, USA). Spermathecae were dissected and the DNA extracted from the sperm stores following a protocol modified from Simmons *et al*. (2007). This method involves an initial incubation step that digests only maternal cells, followed by a second incubation with dithiothreitol (DTT), which digests the sperm cell heads by hydrolyzing their disulfide bridges. Because the sperm in this species is highly clumped and sticky, the entire intact spermatheca rather than the dissected sperm store was digested to prevent the adhesion of the stored sperm to the dissecting tools and the potential loss of some of the sperm. Spermathecae were washed with 1 mL of 10 mM Tris-HCl pH 8.0, vortexed, and centrifuged for 30 min at 21,428 *g*, after which the supernatant was removed and the wash step repeated. Samples were placed in 330 µL DNA extraction buffer (50 mM Tris-HCl pH 8.0, 50 mM EDTA, 100 mM NaCl, 1% SDS) and 20 µL proteinase K and incubated overnight at 56°C. The samples were centrifuged for 30 min, the supernatant removed, and the pellet washed twice with 1 mL

of 10 mM Tris-HCl pH 8.0 as described above. The pellet was then incubated for three hours at 56°C in 320 µL DNA extraction buffer, 10 µL proteinase K, and 20 µL DTT. After cooling to room temperature, 150 µL 5M NaCl was added and the samples were vortexed and centrifuged 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 overnight at room temperature in 50 µL TE buffer.

All adults and nymphs were genotyped as described in Turnell and Shaw (2015). Briefly, samples were screened at five microsatellite loci, LKH-004_A16_R, LKH-002_G24_R, EH630969, EH635281, and EH632048 (Ellison and Shaw 2010) using multiplex PCR.

Two replicate, singleplex PCRs were performed for each sperm store. The five markers were amplified separately using a PCR protocol that differed from the multiplex protocol used for the adults and nymphs only in that it called for 650 nM each of the forward and reverse primer and had an extension and final step temperature of 72°C.

Fragment analysis was performed and alleles were called using GeneMarker (SoftGenetics LLC, State College, PA, USA) and verified by eye. The five loci were tested for deviation from Hardy-Weinberg equilibrium and the presence of null alleles using Cervus 3.0 (Kalinowski et al. 2007), MICRO-CHECKER (van Oosterhout et al. 2004), and ML-NullFreq (Kalinowski and Taper 2006). Paternity analysis for the offspring of the females in the field enclosure was performed using Cervus.

Estimation of the number of mates for wild females

Two methods were used to estimate the number of males contributing to the sperm stores of the wild females: allele counting and a probabilistic method based on

population allele frequencies developed by Bretman and Tregenza (2005). For each of these methods, four separate estimates were made of the number of mates per female, ranging from least stringent to most stringent. For the least stringent estimate, all alleles in a given sperm store were included in the analysis. For the two intermediately stringent estimates, alleles were either excluded if they met the first or excluded if they met the second of the following two criteria: (1) the putatively male allele was shared by the female; (2) the allele was present in only one of the two replicate PCRs. For the most stringent estimate, alleles meeting the first and alleles meeting the second criteria were both excluded. Each separate estimate was based on the locus in a given sperm store that was the most polymorphic, considering only the alleles included in that particular analysis.

For allele counting, the greatest number of alleles present at the most polymorphic locus in each sperm store was divided by two to give a minimum estimate of the number of males represented in the sample. The probabilistic method is based on the probability of observing a given set of alleles assuming that the female mated with a certain number of males. The probability of *not* observing a given allele is

$$P_{\text{not}} = [1\text{-}f(a)]^t$$

where $f(a)$ is the allele's frequency and t is the number of attempts at observing the allele, or twice the number of males contributing to the sperm store. The probability of observing a given allele, P_{obs}, is one minus P_{not}, and the probability of observing a given array of alleles is equal to the product of P_{obs} for each of the alleles included in the array and P_{not} for each of the alleles in the population not included in the array. This value was calculated for $t = 2$ to 40, or one to 20 male mates per female, at the locus or loci with the greatest number of alleles for each sample. The value of t giving the highest probability

was divided by two to give the most probable number of males contributing to the sperm store.

Estimation of the number of sires for wild females

Three methods were used to estimate the number of males siring the offspring of the wild females: allele counting, GERUD (Jones 2005), and Colony (Jones and Wang 2010). For allele counting, the greatest number of non-maternal alleles present at any locus for each female's set of offspring was divided by two to give a minimum estimate of the number of sires. GERUD also calculates a minimum estimate of the number of sires contributing to a female's offspring when the female genotype is known, but it uses multiple loci simultaneously resulting in a more accurate measurement (Jones 2005). Colony uses population allele frequencies to calculate the most likely number of sires for a given set of offspring. Population allele frequencies were based on 92 individuals used in this study and in Turnell and Shaw (2015). A short run of very high likelihood precision using the full-likelihood method was conducted. Allelic dropout for each locus was calculated using Cervus and the error rate was set at 0.01. To assess the accuracy of these three methods, each of them was also used to estimate the number of sires contributing to the offspring of the females in the field enclosure, which was known from behavioral observations and paternity analysis.

Paternity skew

Paternity skew, or the sum of the squared proportions of offspring sired by each male (Starr 1984; Pamilo 1993), was also estimated using GERUD and Colony. Both programs use Mendelian probability and population allele frequencies to reconstruct paternal genotypes and assign paternity to the offspring. Colony estimates the single most likely set of sires, while GERUD reconstructs multiple potential combinations of sires and offspring and assigns each one a separate probability score. For the GERUD

estimate, paternity skew for each wild female's progeny was calculated by averaging the skews of each potential sire combination weighted by their likelihood scores. Again, these two methods were assessed by using them to estimate the paternity skew for the females in the field enclosure and comparing these two estimates to the skew values that were calculated from the field enclosure paternity analysis results generated by Cervus. Finally, the average proportion of females' mates contributing to their offspring was calculated by comparing the numbers of mates and sires estimated by the allele counting method.

Results

Number of mates for wild females

Of the 36 spermatheca samples, 34 yielded sperm DNA. Of these, 32 were missing at least one maternal allele at one or more loci, indicating that the exclusion of maternal DNA from the extraction was at least partly effective. However, a given allele was more likely to be present in a sperm store if the female also had that allele than if she did not (77% vs. 39%, X^2 df = 1, n = 2211, $p < 0.001$), indicating that maternal DNA was not entirely removed in every case. Eighty-nine percent of all alleles amplified in both replicate PCRs. All five loci showed substantial polymorphism, and none showed evidence of deviation from Hardy-Weinberg equilibrium or of a significant presence of null alleles (Table 2.1).

The average numbers of mates per female estimated by the allele counting and probabilistic methods according to each of the four levels of conservatism are shown in Table 2.2 and Figure 2.1. The overall average minimum number of mates based on allele counting was 3.64 ± 1.30 SD, with estimates ranging from one to six males. The most probable numbers of mates based on population allele frequencies were much higher, averaging 7.47 ± 4.61 overall and ranging from one to 25 males. According to both

methods, 85-97% of females, depending on the level of conservatism, mated with at least two males.

Locus	*N*	*k*	bp	H_O	H_E	F_{Null}
LKH-002_G24_R	92	18	197-249	0.87	0.896	0.0120
LKH-004_A16_R	89	13	203-241	0.798	0.807	0.0018
EH630969	92	17	217-252	0.815	0.870	0.0291
EH632048	90	11	301-347	0.789	0.793	-0.0020
EH635281	90	15	299-343	0.911	0.837	-0.0468

Table 2.1. Information on the five microsatellite loci used (*N* = number of individuals typed, *k* = number of alleles, bp = fragment size, H_O and H_E = observed and expected heterozygosity, F_{Null} = frequency of null alleles estimated by Cervus).

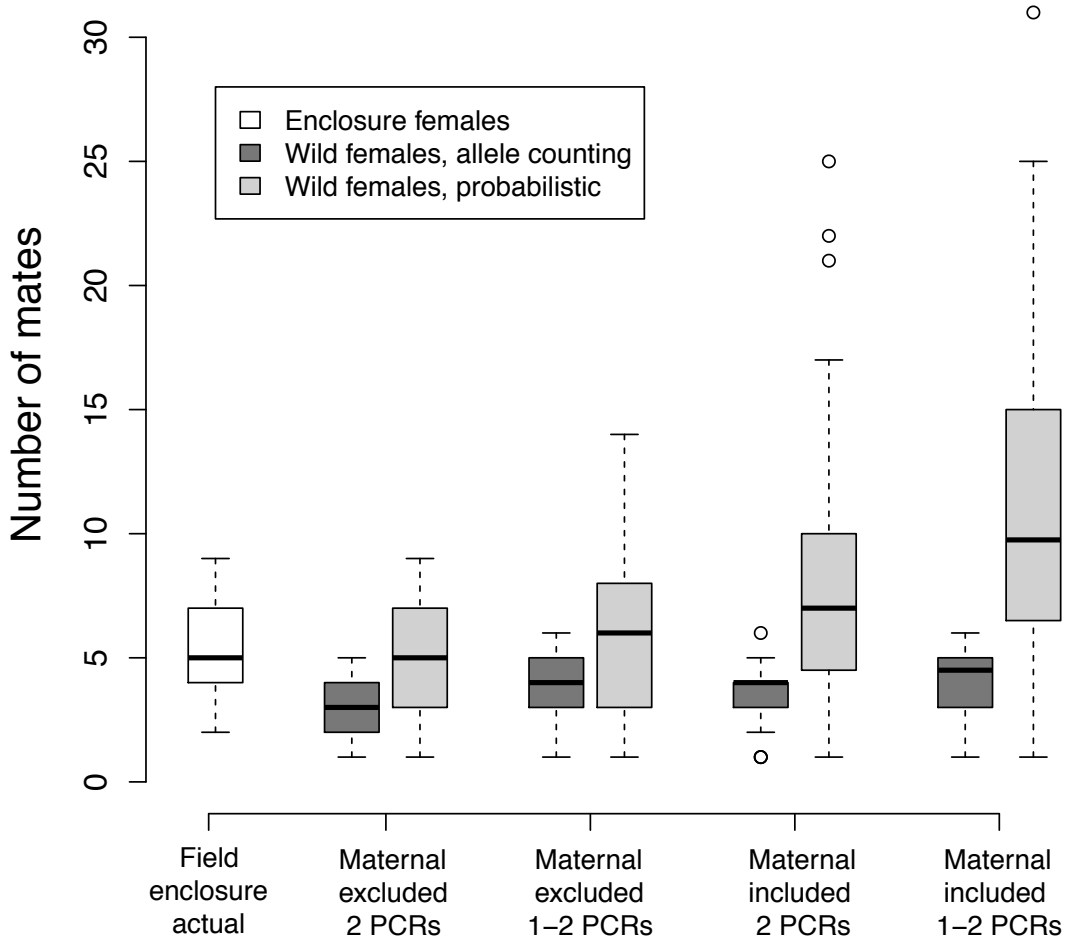

Figure 2.1. Number of actual mates per field enclosure female (white bar) and estimated mates per wild females according to the allele counting (dark gray bars) and probabilistic (light gray bars) methods under the four levels of stringency. The most polymorphic locus was selected after (1) either excluding or not excluding any alleles potentially coming from the mother and (2) either excluding or not excluding any alleles that amplified in only one of the two replicate PCRs.

		Allele counting		**Probability**	
Maternal alleles	**Alleles in at least one or both PCRs**	**Average**	**SD**	**Average**	**SD**
Excluded	Both	3.18	1.29	4.74	2.51
	At least one	3.68	1.30	5.94	3.09
Included	Both	3.56	1.26	8.35	5.94
	At least one	4.15	1.35	10.86	6.91
Average		**3.64**	**1.30**	**7.47**	**2.71**

Table 2.2. Average numbers of mates per wild female (n = 34) estimated by the allele counting and probabilistic methods under the four levels of conservatism. The locus with the most alleles was selected for each female under each of the four levels, ranging from most stringent (maternal alleles excluded, all alleles in both PCRs) to the least stringent (maternal alleles included, alleles in at least one PCR).

Number of sires for wild females

Of the 47 females collected as adults and allowed to oviposit, only ten females laid eggs. These ten females produced a total of 149 offspring (14.90 ± 10.48, range = 4 to 36), of which 142 were successfully genotyped (broken down by female: 4, 5, 5, 9, 10, 13, 12, 19, 29, 36). This was not significantly different from the number of offspring produced by the females in the field enclosure (see below; $t_{25} = 1.46$, p = 0.16). The numbers of sires per female estimated by the three methods, allele counting, GERUD, and Colony, are shown in Figure 2.2. Allele counting and GERUD provided very similar minimum estimates (2.10 ± 0.88 vs. 2.30 ± 1.16), while Colony provided a higher estimate of the most likely number of sires (3.40 ± 2.63) based on population allele frequencies. There was a marginally significant positive correlation between offspring number and minimum sire number (allele counting: $R^2 = 0.35$, p = 0.072; GERUD: R^2 =

0.39, p = 0.056), but not between offspring number and most likely sire number (Colony: $R^2 = 0.04$, p = 0.59).

According to all three methods, 70% of females used sperm from at least two males. Comparing the number of sires to the number of mates estimated by the allele counting method, wild females use the sperm from approximately 58% of their mates (51% to 66%, depending on the level of conservatism used to estimate the number of mates).

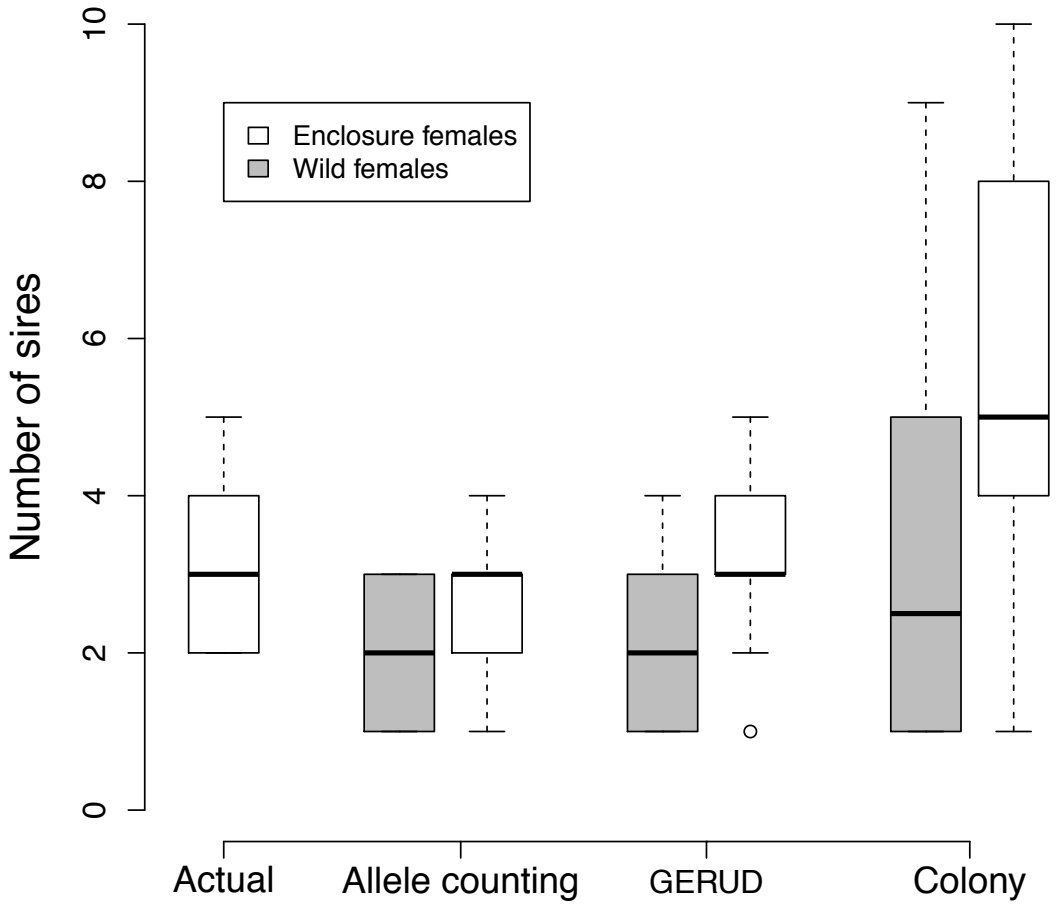

Figure 2.2. Number of sires for the wild (gray bars) and enclosure (white bars) females, estimated by allele counting and GERUD (minimum sires) and Colony (most likely number of sires). The actual number of sires inferred with 95% likelihood for the enclosure females is also shown.

Number of mates and sires for field enclosure females

Of the 19 females that were in the field enclosure for at least five weeks, one produced no offspring and another produced only two, which could not be genotyped. The remaining 17 females produced 333 offspring (19.59 ± 6.32, range = 8 to 29) of which 311 were assigned paternity by Cervus at a trio likelihood (likelihood of father given known mother) confidence level of at least 95%. Because our confidence in these paternity assignments is so high, and for the sake of brevity, we will henceforward refer to the number of sires and the level of paternity skew estimated among the enclosure females as "actual" sire number and skew.

We directly observed that the females in the field enclosure mated with 5.65 ± 2.18 males on average (Figures 2.1 and 2.3), with a range of two to nine. They used the sperm of an average of 3.18 ± 1.07 (62 ± 21%) of those males (Figures 2.2 and 2.3). All females mated with and used the sperm of at least two males. There were nine cases in which a female (n = 6) mated twice with the same male (3 of the 6 females did so with two different males), such that the number of matings per female was 6.35 ± 2.78. The number of sires was only weakly dependent on the number of mates (Figure 2.3; R^2 = 0.18, p = 0.09). Mate number did not vary significantly more than sire number when scaled to have the same mean (Levene's test, $F_{1,32} = 0.10$, p = 0.75). Sire number was underestimated by allele counting (2.71 ± 0.68 sires; paired two-tailed t-test, $t_{16} = 2.22$, p = 0.041) and overestimated by Colony (5.88 ± 2.52 sires, $t_{16} = -5.28$, p < 0.0001; Figure 2.2). The minimum number of sires estimated by GERUD (3.29 ± 1.16) was slightly higher than the actual number of sires due to four families with mismatches between one to five offspring and the assigned sire.

The minimum number of mates per wild female as estimated by allele counting, at each of the four levels of stringency used, was lower than the actual number of mates per female in the field enclosure (Figure 2.1; t-test, $t_{49} = -5.09$ to -3.03, p < 0.0001 to 0.004). While the three most stringent probability-based estimates of the number of mates per

wild female did not differ significantly from the actual number of mates per field enclosure female (Figure 2.1; t_{49} = -1.28 to 1.81, p = 0.73 to 0.08), the least stringent estimate was significantly higher (t_{49} = 2.99, p = 0.004). Females in the field enclosure used or tended to use the sperm from more males than did the wild caught adult females based on all three estimates of sire number (Figure 2.2; allele counting: difference = 0.61 ± 0.30, t_{25} = 2.00, p = 0.056; GERUD: 1.0 ± 0.46, t_{25} = 2.15, p = 0.041; Colony: 2.48 ± 1.02, t_{25} = 2.43, p = 0.023).

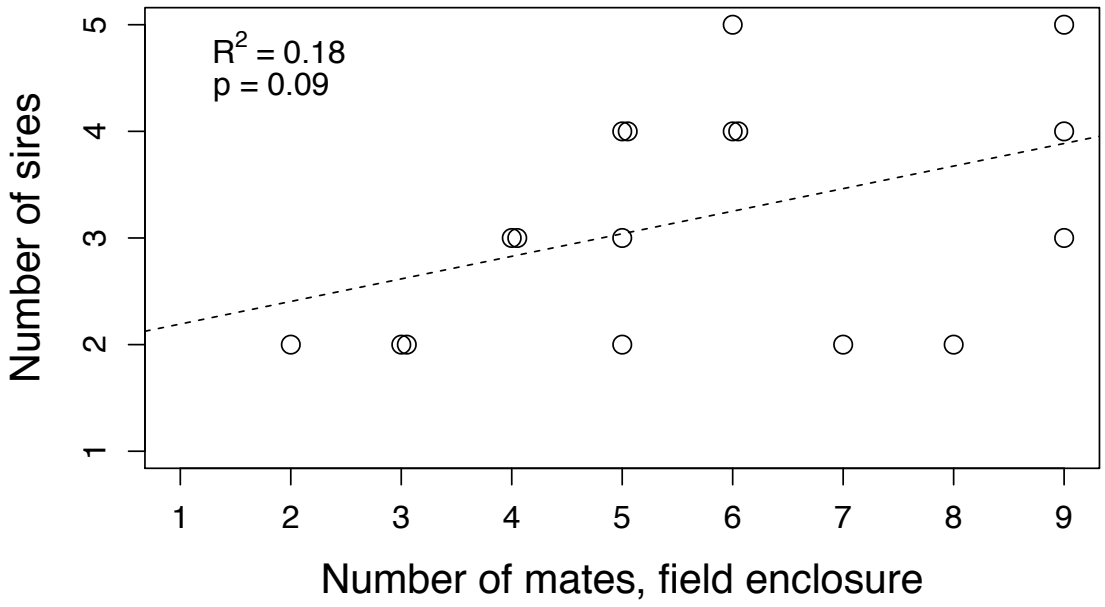

Figure 2.3. Number of mates (directly observed) and sires (inferred with 95% likelihood) for females in the field enclosure (n = 17). Overlapping points are offset for clarity. The number of males contributing a female's offspring is only weakly dependent on the number of males she mates with.

Paternity skew

Paternity skews for the wild females were higher or tended to be higher than would be expected if all males to mate with a female had equal paternity shares (GERUD: observed-expected = 0.06 ± 0.05, two-tailed paired t-test, t_6 = 3.46, p = 0.013; Colony: 0.10 ± 0.12, t_6 = 2.21, p = 0.07). Actual paternity skews among the field

enclosure females were also higher than expected (0.14 ± 0.09, $t_{16} = 6.53$, $p < 0.0001$). GERUD and Colony underestimated the degree of skew among the enclosure females (GERUD $t_{15} = 3.20$, $p = 0.006$, Colony $t_{15} = 5.17$, $p = 0.0001$) and also tended to underestimate the degree to which the observed skew exceeded the expected skew (GERUD: 0.03 ± 0.07, $t_{15} = 1.98$, $p = 0.07$; Colony: 0.05 ± 0.1, $t_{15} = 1.80$, $p = 0.09$).

Expected skew declines nonlinearly as sire number increases (Figure 2.4). Actual skew among females in the field enclosure and estimated skew among wild females also declined with sire number ($R^2 = 0.69$, $p < 0.001$), though the decline was not nonlinear (second degree polynomial fit, $t_{21} = 1.36$, $p = 0.19$). Controlling for sire number, the field enclosure females had higher values of skew than expected (least square means 0.49 ± 0.18 vs. 0.35 ± 0.12, $t_{24} = 2.99$, Tukey adjusted $p = 0.017$; Figure 2.1), though the wild females did not (GERUD estimate, least square means 0.44 ± 0.10 vs. 0.38 ± 0.12, $t_{24} = 0.85$, Tukey adjusted $p = 0.68$). Skew was marginally higher among the field enclosure females than among the wild females ($t_{24} = 2.49$, Tukey adjusted $p = 0.050$). The difference between observed and expected skew did not change with sire number ($R^2 = 0.03$, $p = 0.41$).

Discussion

Polyandry has been described as "arguably the most important agent of evolutionary change" (Kvarnemo and Simmons 2013). Despite this, relatively few studies have measured its intensity, or the intensity of the postcopulatory sexual selection that it generates, in nature. In this study, we found a considerable degree of polyandry and a strong level of postcopulatory selection in a wild population of *L. cerasina*: females mate with a minimum average of 3.6 males (Table 2.2; Figure 2.1), of which an average of 42% fail to sire any offspring, and paternity is unequally distributed among sires (Figure 2.4). These patterns are corroborated by our data from the field enclosure, in which females mated with an average of 5.7 males (Figure 2.1), of which an average of 38%

failed to sire any offspring, and paternity among successful sires was likewise significantly skewed (Figure 2.4).

Because it relies on allele counting, our measure of a natural mating frequency of 3.6 mates per wild female is almost certainly an underestimate. Our study allows us to assess the size of this underestimation by comparing the number of sires per female in the field enclosure as estimated by allele counting vs. that actually inferred with 95% likelihood by Cervus. (This estimation assumes that allele counting underestimates mate

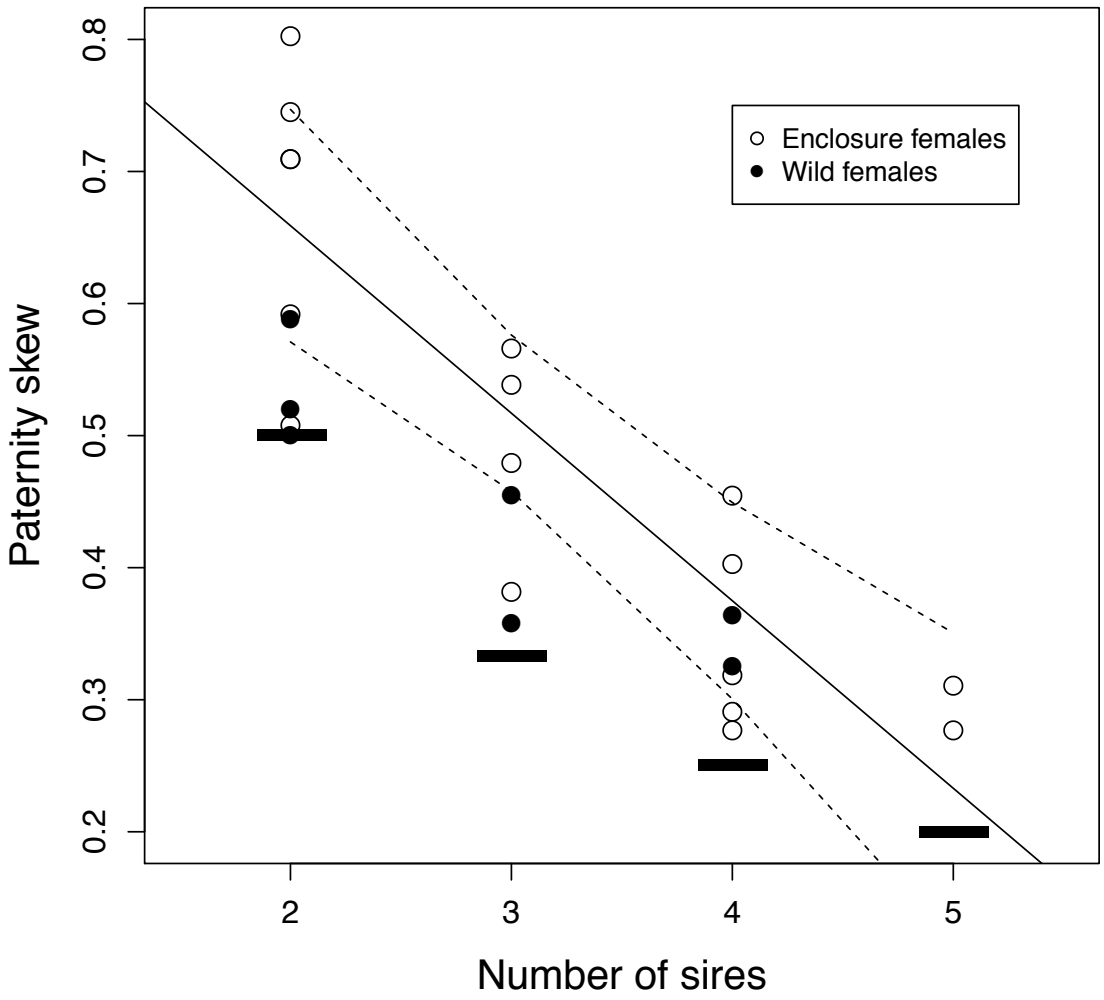

Figure 2.4. Paternity skew for wild females (filled circles) as estimated by GERUD and actual paternity skew for females in the field enclosure (open circles) inferred with 95% likelihood. Black bars show the level of paternity skew expected if all males to mate with a female have equal paternity shares. Regression line and 95% confidence interval are for the field enclosure females only.

number and sire number to an equal degree.) The average number of sires per enclosure female actually inferred was 17% higher than the average number as estimated by allele counting; multiplying our minimum estimate of 3.6 mates per wild female by this correction factor gives an adjusted estimate of 4.2 mates per wild female, as compared to the 5.7 mates per female observed in the field enclosure.

This adjusted figure of 4.2 mates per wild female likely remains an underestimate, since at the time of collection some of our wild females may have already used up the sperm of early mates, while some may not yet have completed all of their matings. However, it is possible that the mating rate in the field enclosure was higher than the actual average mating rate in the wild population. While the population density in the enclosure was within the natural range found at the field site (B. R. Turnell, pers. obs.), this density, and therefore the encounter rate between males and females, was higher in the enclosure than in some areas of the field site. Recent theoretical work suggests that the level of polyandry may be a function of encounter rates (Kokko and Mappes 2013).

Because sire number among the females in the field enclosure was known, our study design provides a useful assessment of the relative accuracies of different estimation methods. As expected, allele counting underestimated the number of sires per field enclosure female, by about a third (Figure 2.2). Colony overestimated the number of sires by more than 80% (possibly due to the relatively small number of loci; see Harrison *et al*. 2013), while GERUD's estimate was remarkably accurate. The three methods varied less in their estimates of sire number among the wild females, probably in part because the mean was lower.

Our estimation of the number of sires per wild female is based on a relatively small sample size, since only ten of the 47 females laid eggs. Our previous study (Turnell and Shaw 2015) found that most females complete all of their matings before laying any eggs. It seems likely that some of the 37 females that did not lay eggs had only mated a few times and were awaiting further mating opportunities before starting to oviposit.

Under this scenario, our estimate of sire number would not be biased, since the females that did lay eggs would be representative of uncollected, wild females whose mating activity was complete. In addition, it is possible that some of the 37 females were virgin, though this number would likely be small as nearly all of the females whose spermathecae were dissected had mated.

Alternatively, some of the females may have laid most or all of their eggs before being collected. If this is true, then the ten females that did lay eggs in the lab may have been more fecund on average than those that did not. Such a collection bias could result in an overestimation of sire number, if more fecund females also mated with more males (e.g., Arnqvist & Nilsson 2000; South & Lewis 2011; Slatyer *et al*. 2012). A collection bias such as this seems unlikely to us, however, as *L. cerasina* do not have a seasonal life cycle (Otte 1994). Thus we would not expect our collection to be biased toward a senescent phase of the adult life history period. Regardless, it is worth reiterating that two of the three measures of the number of sires per wild female, those estimated by allele counting and by GERUD, provide minimum rather than most likely numbers. Apart from these possibilities, we have no reason to expect that females who mated many or fewer times were more or less likely to lay eggs in our study.

The levels of polyandry and of postcopulatory sexual selection we found are similar to those reported in previous studies of Orthopterans. In wild populations of the field crickets *Gryllus bimaculatus* (Bretman and Tregenza 2005) and *Teleogryllus oceanicus* and *T. commodus* (Simmons and Beveridge 2010), as well as the katydid *Requena verticalis* (Simmons et al. 2007), females were found to mate with a minimum average of three to five males. Postcopulatory sexual selection was high in the three species of field crickets, with 25% to 40% of a female's mates failing to sire any offspring. Paternity shares were significantly skewed among those males that were successful in *Teleogryllus oceanicus* and *T. commodus*. In contrast, postcopulatory sexual selection appears to be weaker in *R. verticalis*, in which the average number of sires was

virtually equal to the average number of mates, though paternity in this species was also skewed.

Because such a high proportion of a given female's mates fail to sire any of her offspring, females seem to be mating with more males than is necessary to fertilize their eggs. Both the wild and the field enclosure females mated with about 60%-70% more males than ended up siring their offspring, and the number of males siring the offspring of a given female in the field enclosure (a maximum of five) was only weakly predicted by the number of males she mated with (a maximum of nine; Figure 2.3). This raises the question of why females are mating so many times. Despite the many costs of mating (Magnhagen 1991; Crudgington and Siva-Jothy 2000; Innocenti and Morrow 2009), females may benefit both directly and indirectly from polyandry. Indirect, or genetic, benefits include the postcopulatory biasing of paternity towards unrelated males, as has been shown in other species of cricket (Bretman et al. 2004, 2009; Simmons et al. 2006; but see Jennions et al. 2004), towards males that are otherwise more genetically compatible (reviewed in Tregenza and Wedell 2000; Puurtinen et al. 2009), or towards conspecifics (Manier et al. 2013; Tyler et al. 2013). Females that facilitate sperm competition by mating multiply may also benefit if males with more competitive sperm pass this trait on to their sons (the 'sexy sperm' hypothesis, Curtsinger 1991), or produce more viable offspring (the 'good sperm' hypothesis, Yasui 1997; but see Simmons 2003).

Several direct, or non-genetic, benefits of polyandry are also possible in *L. cerasina*. Seminal fluid components such as prostaglandins may stimulate oviposition and increase egg production, as has been demonstrated in other cricket species (Destephano and Brady 1977; Loher 1979; general reviews in Gillott 2003 and Avila *et al*. 2011). Components of a male's ejaculate may also influence the fitness of a female's offspring, even those that are sired by other males (Crean et al. 2014). In *Teleogryllus* crickets, for example, the viability of embryos sired by one male can be rescued by the

paternal effects of another (García-González and Simmons 2007), a phenomenon likely mediated by Acps (accessory gland proteins; García-González & Simmons 2005). Mating with several males can also protect against the consequences of male infertility (García-González 2004; Hasson and Stone 2009), which may occur in *L. cerasina* (one male in the field enclosure mated with five females but failed to sire any offspring, perhaps indicating infertility; Turnell & Shaw 2015).

Another possible direct benefit of multiple mating in this species is nutrition in the form of the microspermatophores transferred to and consumed by the female during courtship. Although micros constitute a fraction of total body mass, with males losing only about 3% of their weight during mating (B.R. Turnell, unpublished data), they are probably costly to produce, as the number of micros produced decreases over consecutive days of mating (Jadin 2009). It is thus possible that micros boost fecundity by contributing significantly to female nutritional status, potentially by transferring a limiting nutrient (e.g., Karlsson 1998; but see Warwick *et al*. 2009). However, although spermatophore consumption has been shown to elevate fecundity in certain species of katydids producing very large spermatophylaxes (Gwynne 1984, 1988; Simmons 1990; though for negative results see Reinhold and Heller 1993; Vahed and Gilbert 1997), consumption of the smaller spermatophores produced by other species of Ensifera has no measurable effect on egg production (Wedell and Arak 1989; Reinhold and Heller 1993; Will and Sakaluk 1994; Vahed 2003). This pattern is in line with the prevailing hypothesis that nuptial gifts function primarily to facilitate sperm transfer rather than to increase offspring number or fitness (Vahed 1998, 2007; but see Gwynne 2008). Indeed, in *L. cerasina*, microspermatophores have been shown to enhance sperm transfer (deCarvalho and Shaw 2010) and to influence a male's paternity success (Turnell and Shaw 2015).

In conclusion, we found both a considerable level of polyandry and a substantial degree of postcopulatory sexual selection operating in a natural population. The mating

and sperm use patterns we estimated among the wild females are similar to those we observed among the females in the field enclosure. Likewise, the natural measures of postcopulatory selection reported here corroborate the high level of postcopulatory selection we found to be operating on the field enclosure males (Turnell and Shaw 2015). This study is the latest in a growing body of work demonstrating the major role played by postcopulatory selection in nature (Emery et al. 2001; Bretman and Tregenza 2005; Simmons et al. 2007; Frentiu and Chenoweth 2008,Rodríguez-Muñoz et al. 2010b; Simmons and Beveridge 2010; Price et al. 2011; Hurtado et al. 2013; Smith 2014). Further studies of natural mating rates and differential sperm use across additional species and taxa would shed more light on the prevalence of postcopulatory selection and its role in driving evolution.

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Chapter 3: Modeling strategic sperm allocation: tailoring the predictions to the species

Abstract

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. If aspects of the study species' biology violate theses assumptions – for example, if a polyandrous species is used to test a model that assumes females mate only once or twice – then the test may not be valid. Unfortunately, sperm allocation models frequently make assumptions that do not hold for many, if not most, multiply and sequentially mating animals. The second challenge of testing sperm allocation theory is that 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. We test the model in the Hawaiian swordtail cricket *Laupala cerasina*, a species for which we have measured all of our model's major parameters. Finally, we also evaluate the model using two additional species for which all of the relevant data have been published. The model's predictions are remarkably well supported in all three species.

Introduction

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; Charnov 1982; Parker 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 which 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 many, if not most, species, females mate with more than two males under natural conditions (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). 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 post-copulation. 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 can predict 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 2015) or the genotyping of sperm stores to estimate the number of contributing males (e.g., Turnell and Shaw, in press; Bretman and Tregenza 2005; Simmons et al. 2007; Simmons and Beveridge 2010). 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 like ours 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 other factors. 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:

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

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

$$S_V \cdot V + S_{NV} \cdot (1 - V) = 1 \quad [2]$$

where V is the likelihood that a mating female is virgin. Because all females are virgin one time out of the $n+1$ times they mate, this likelihood is

$$V = \sum_{n=0}^{100} \frac{P(n)}{n+1} \qquad [3]$$

The maximum number of rematings per female was set at 100 to simplify the calculations. 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.

The sperm of males mating with nonvirgin females is weighted by a factor of p, where $0 < p < \infty$. If $p = 1$ there is a fair raffle (Parker 1982; Parker 1990b) 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 $p < 1$ there is first male sperm precedence, and if $p > 1$ there is later male sperm precedence. 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 equation 2], this is equivalent to the the number of sperm required). 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 non-sperm 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).

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 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. Let S_V^* and S_{NV}^* be 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

$$W = \sum_{n=0}^{0} P(n) \cdot \alpha \cdot \left[\frac{S_V}{S_V + \varepsilon}\right] + \sum_{n=1}^{100} P(n) \cdot \left[\frac{1}{n+1} \cdot \frac{S_V}{S_V + S_{NV}^* \cdot p \cdot n + \varepsilon} + \frac{n}{n+1} \cdot \frac{S_{NV} \cdot p}{S_{NV} \cdot p + S_V^* + S_{NV}^* \cdot p \cdot (n-1) + \varepsilon}\right] \quad [4]$$

The ESS is found by setting

$$\left.\frac{\partial W}{\partial S_V}\right|_{S_V = S_V^*} = 0 \quad [5]$$

$$\left.\frac{\partial^2 W}{\partial S_V^2}\right|_{S_V = S_V^*} < 0$$

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, 2015). 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, in press, for details). To estimate the level of sperm

precedence in this species, represented by the parameter p, 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 p that minimized the sum of the squared differences between the observed and expected paternity shares.

For the sperm limitation parameter ε, we used Ball and Parker's (2000) estimate for yellow dung flies (*Scatophaga stercoraria*). 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 on the number of offspring produced by females mating once with one male vs. twice with two different males (J. Lambert and Q. Gao, unpublished 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 12:12h 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 non-focal 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 non-experimental 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 = 5.45 ± 3.58) was used to reflect natural conditions and to determine if male mating latency differentially affected sperm allocation to virgins vs. nonvirgins. This distribution is similar to that observed in a semi-natural population (4.57 ± 4.57; Turnell and Shaw 2015). For intermating intervals of less than six days, a given male's two intervals differed by a maximum of one day (0.08 ± 0.27 days, n = 41) . For intermating intervals of six days or greater, the maximum difference between a male's two intervals was ten days (2.89 ± 2.64 days, n = 42). Males were three to nine weeks post-final molt, while females were five to 21 days post-final molt. The difference in age between the two experimental females paired with a given male was seven days or less (1.76 ± 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 x 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. 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 3.1 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.

Under fair raffle conditions ($p = 1$), our model predicts that males should allocate more sperm to virgins when females mate with approximately four or more males, and more sperm to nonvirgins at lower mating frequencies. As first male precedence becomes stronger ($p < 1$), 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 $p = 0.1$ (extreme first-male precedence), the ESS is to allocate more sperm to virgins if females mate with approximately two or more males. Conversely, as later male precedence become stronger ($p > 1$), 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 ($p > 1.2$), our model predicts that males should always allocate more sperm to nonvirgins than to virgins.

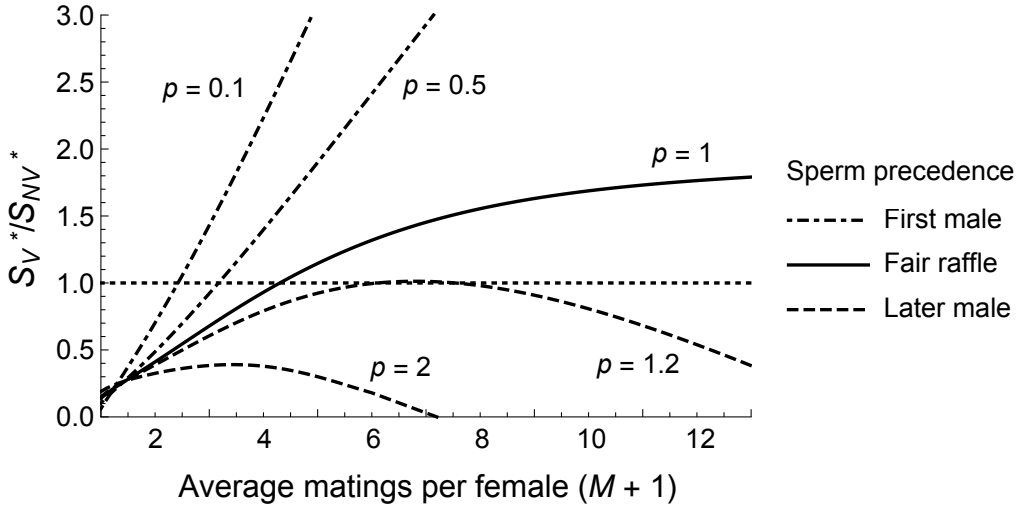

Figure 3.1. ESS sperm allocation to virgins vs. to nonvirgins (S_V^*/S_{NV}^*) plotted against the mean number of matings per female in the population (i.e., the mean number of rematings, M, plus one initial mating). Males should allocate more sperm to virgins above the dotted line and more sperm to nonvirgins below it. The ESS is indicated by the solid line under a fair raffle ($p = 1$), by the dot-dashed lines under first-male precedence ($p < 1$), and by the dashed lines under later male precedence ($p > 1$).

Figure 3.2 illustrates separately the optimal levels of sperm allocation to virgins and to nonvirgins. While is S_{NV}^* is minimally affected by either the level of sperm precedence or the female mating frequency (at least above approximately three matings per female), 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 ($p = 0.5$) or half as competitive ($p = 2$). At six matings per female, however, the difference in optimal allocation to virgins is over eightfold. This makes sense given the

model's tradeoff: virgins are quite rare at high mating frequencies, so males can afford to allocate large amounts of sperm to any such females they happen to encounter, and should do so if their sperm will be favored by sperm competition.

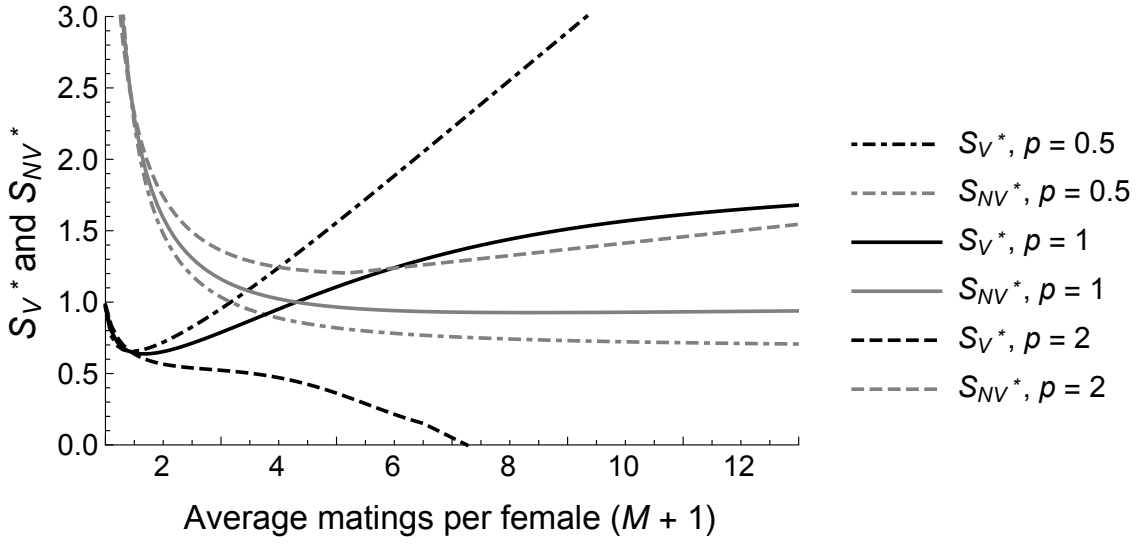

Figure 3.2. ESS sperm allocation to virgins (S_V^*, black lines) and to nonvirgins (S_{NV}^*, gray lines) plotted separately. Optimal allocation to nonvirgins remains steady as female mating frequency increases, while optimal allocation to virgins becomes more extreme in a direction that depends on the sperm precedence pattern (p).

As the average female mating frequency approaches 1, so does the optimal allocation to virgins, while optimal allocation to nonvirgins increases indefinitely (Figure 3.2). This is because males encounter very few receptive, nonvirgin females under these conditions and can afford to allocate more sperm to each of them. As the average female mating frequency increases, so does the encounter rate with receptive nonvirgin females, and males must decrease the amount of sperm they allocate to each of these females. Meanwhile, optimal allocation to virgins will decrease initially as males allocate more total sperm to nonvirgins (that is, as the product of S_{NV}^* and the frequency of nonvirgins

increases), but by approximately two matings per female may increase again depending on the sperm precedence pattern.

Neither the sperm limitation parameter ε nor the fecundity parameter α has a strong effect on optimal sperm allocation. At $\alpha = 0.74$, which corresponds to a 35% fecundity increase for multiply vs. 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 Figures 3.1 and 3.2, $\alpha = 0.74$ 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, in press). 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 p 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).

The dung fly sperm limitation parameter we used as a proxy was $\varepsilon = 0.030$, which is close to the average (± SD) ε value of 0.022 ± 0.019 reported for six animal species (Ball and Parker 2000). Because the ratio of offspring produced by doubly vs. singly mated *L. cerasina* females had overlapping 95% confidence intervals across the two data sets, we 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 difference between the number of sperm that each male allocated to the virgin female vs. to the nonvirgin female was not affected by whether the mating trial was conducted in Fall 2013 or Spring 2014 (t-test, $t_{83} = 0.49$, p = 0.62, n = 28 and 55; all reported t-tests are two-tailed). The data were therefore pooled. Three outliers were excluded (iterative Grubbs test, $p < 0.005$, p = 0.030, p = 0.011). Males allocated more sperm to virgins than to nonvirgins (mean ± SD = 3.71 ± 0.71 x 10^4 and 3.50 ± 0.73 x 10^4, respectively; paired t-test, $t_{82} = 2.53$, p = 0.013). (The DNA concentrations on which these extrapolations are based were 1.449 ± 0.279 ng/µl and 1.369 ± 0.284 ng/µl.) The average value of S_V/S_{NV} across males was 1.059 (95% CI: 0.971, 1.155), while the within-male value was 1.084 (95% CI: 1.032, 1.134; Figure 3.3).

Sperm number was positively associated with male age ($R^2 = 0.14$, $p < 0.001$; generalized linear mixed model with male ID as a random effect, $p < 0.0001$)) and with the number of days since the male's previous mating ($R^2 = 0.14$, $p < 0.001$; GLMM, p = 0.0001), but was negatively associated with female age ($R^2 = 0.13$, $p < 0.001$; GLMM, p

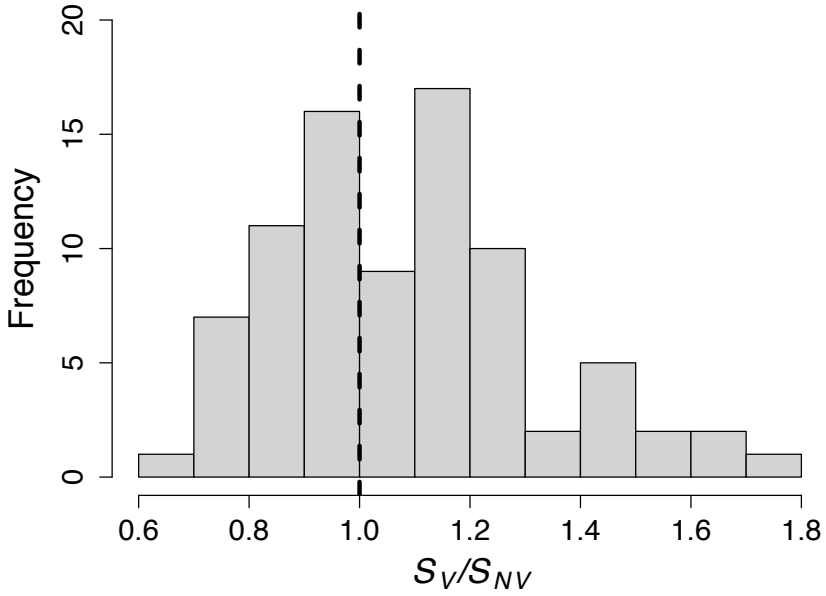

Figure 3.3. Histogram of the amount of sperm allocated by a male during his mating with a virgin vs. his mating with a nonvirgin female (S_V/S_{NV}). Males to the left of the dotted line allocated more to the nonvirgin female, while males to the right of the dotted line allocated more to the virgin female. On average, males allocated more sperm to virgin females (mean = 1.084, 95% CI = 1.033, 1.134; paired t-test, p = 0.013).

= 0.030). Sperm number tended to decline more rapidly with age for virgin than for nonvirgin females ($R^2 = 0.27$, $p < 0.001$ for virgins, $R^2 = 0.04$, $p = 0.06$ for nonvirgins; GLMM interaction term, $p = 0.076$). There was no significant interaction between female mating status and male intermating interval on sperm number (GLMM, $p = 0.71$). Sperm number was negatively associated with female weight, even controlling for female age (GLMM, $p = 0.012$; female weight and age were positively associated, $R^2 = 0.31$, $p < 0.0001$). 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, $t_{82} = 1.17, -0.82$; $t_{79} = -0.97$; $t_{82} = -1.16$; $p = 0.24, 0.40, 0.34, 0.25$).

Males transferred more microspermatophores to virgin females than to nonvirgins (6.51 ± 1.76 vs. 5.92 ± 1.74, paired *t*-test, $t_{75} = 2.29$, p = 0.025). However, the rate of micro transfer was higher for nonvirgins (1.96 ± 0.42 vs. 1.65 ± 0.37 micros per hour, $t_{71} = 5.45$, p < 0.0001). Courtship duration was longer for virgins (253 ± 77 min vs. 194 ± 82 min,,$t_{74} = 4.83$, p < 0.0001), who began mating earlier in the day (by 68 ± 119 min, p < 0.001; though courtship also ended earlier, by 10 ± 42 min, p = 0.037). Older females began mating earlier ($R^2 = 0.15$, p < 0.001; GLMM with male ID as a random effect, p < 0.0001) and so received more micros ($R^2 = 0.18$, p < 0.001; GLMM, p < 0.0001), but controlling for female age there was no relationship between micro number and sperm number (p = 0.84). Macrospermatophore weight was positively associated with sperm number, though only weakly ($R^2 = 0.07$, p < 0.001; GLMM, p = 0.004;).

Predictions vs. results for L. cerasina

The optimal and actual sperm allocation strategies for *L. cerasina* are shown in Figure 3.4. At the empirically measured levels of sperm precedence and female mating frequency, the optimal allocation is $S_V^*/S_{NV}^* = 1.138$, close to the observed within-male value of $S_V/S_{NV} = 1.084$, though just outside of the 95% confidence interval; however, the optimal allocation level does fall within the 95% confidence interval of the across-male estimate. Error bars show the 95% CIs for observed sperm allocation within males and for observed female mating frequency. The ESS across the 95% confidence interval for observed sperm precedence ranges from $S_V^*/S_{NV}^* = 2.14$ at $p = 0.38$ (i.e., allocate most sperm to virgins under strong first-male precedence) to $S_V^*/S_{NV}^* = 0$ at $p = 2.81$ (i.e., allocate all sperm to nonvirgins under strong later-male precedence).

Predictions vs. 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 ($p = 1$) whether females mate with two or with four males (Simmons 2001a; 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 that of dung flies, at $\varepsilon = 0.030$ (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 2.79 ± 0.74 (2.51, 3.06) mates per female (Simmons et al. 2007). Sperm precedence strongly favors the first male at $p = 0.22$ (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 2001a; Ivy and Sakaluk 2005) yields an estimate of the fecundity parameter α of 0.67. Again, the degree of female sperm limitation is assumed to be similar to that of dung flies. 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 3.4. The error bars represent the 95% confidence intervals for the observed sperm allocation strategies and the observed female mating frequencies. The observed value of S_V/S_{NV} overlaps the ESS for both *T. oceanicus* and *R. verticalis*. 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.

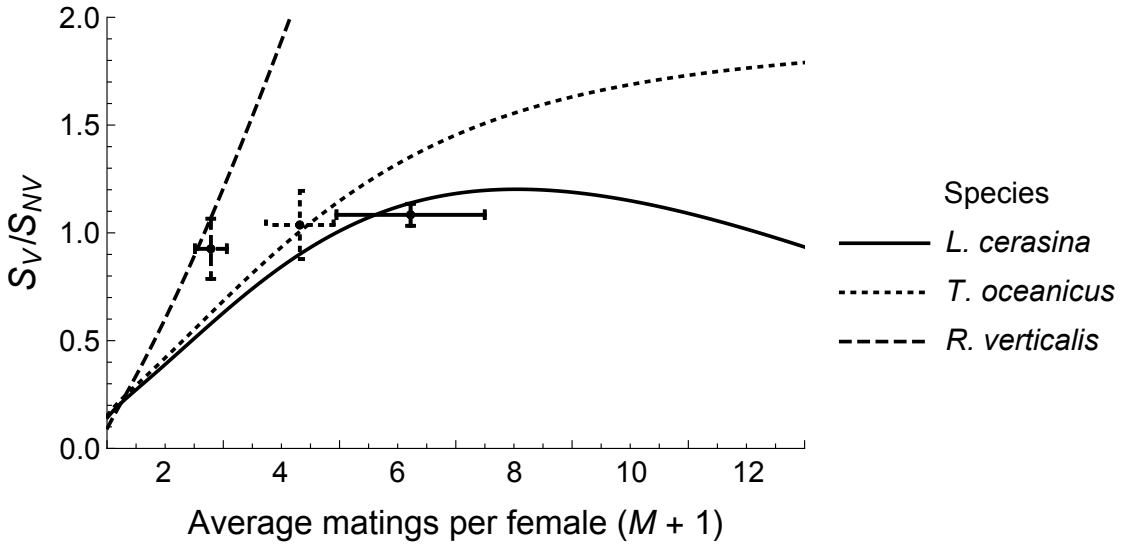

Figure 3.4. Actual and optimal sperm allocation to virgins vs. nonvirgins for three different species. Bars show the 95% confidence intervals for observed sperm allocation patterns and observed female mating frequencies. The confidence intervals overlap the ESS for all three species.

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 (Figure 3.4), our model is so far remarkably accurate 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, the lower the female mating frequency above which allocation to virgins should exceed allocation to nonvirgins (Figure 3.1). 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 (Figure 3.1). Both models also generally predict greater allocation to nonvirgins under conditions of later male precedence. However, the two models differ greatly 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 (Figure 3.2), Engqvist and Reinhold's generally predicts that allocation to the two types of females will converge as female mating rate increases (except under fair raffle conditions).

Given the differences in structure between our two models, the overall similarity in their results is encouraging. Apart from our tradeoff function 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, in press), 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 (Figure 3.1). 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). As for the intensity models of sperm allocation, their predictions can not properly be compared with ours: because they assume that fertilization by all of a female's mates occurs simultaneously, female mating status (i.e., virgin vs. nonvirgin) is meaningless.

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 (Figure 3.1). 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 vs. to nonvirgins should not automatically conclude that the species in question can not discriminate based on female

mating status or is not behaving optimally. By the same logic, given that both female mating frequencies and sperm precedence patterns vary widely 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).

A potential limitation of our model is the formulation of the sperm precedence parameter, which 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 2015), it is unlikely to apply to all species. However, this is also true of the sperm precedence structure most commonly used in other sperm allocation models, 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 (e.g., Parker 1990a). Unfortunately, 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 (for exceptions see Zeh and Zeh 1994; Simmons et al. 2003; Turnell and Shaw 2015).

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 refs therein). Indeed, this variance is quite high in *L. cerasina* (Turnell and Shaw 2015), which helps account for the high uncertainty around our estimate of the sperm precedence parameter in this study.

Another 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 vs. 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, males may also be able to assess the 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 vs. nonvirgins, but not to nonvirgins mated more vs. fewer times, suggests that they may not (Turnell and Shaw 2015). 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; though 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).

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 vs. 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.

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