



High opportunity for postcopulatory sexual selection under field conditions

Biz R. Turnell^{1,2} and Kerry L. Shaw^{3,4}

¹Cornell University, W343 Seeley G. Mudd Hall, Ithaca, New York 14853

²E-mail: ert28@cornell.edu

³Cornell University, W317 Seeley G. Mudd Hall, Ithaca, New York 14853

⁴E-mail: kls4@cornell.edu

Received March 11, 2015

Accepted June 29, 2015

In polygamous 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 seminatural 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.

KEY WORDS: Cryptic mate choice, nuptial gifts, opportunity for selection, selection gradients, sperm competition, variance decomposition.

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 also on fertilization success, involving postcopulatory processes such as sperm competition (Parker 1970; Birkhead and Møller 1998; Simmons 2001; Bretman et al. 2009) 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. Although 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, although 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). Although 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.

Data archival location: Dryad Digital Repository, doi:10.5061/dryad.9jd86.

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. 2014). 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, because 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 because 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); whereas 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). Although Collet et al. (2012) found evidence of a positive correlation, Pischedda and Rice (2012), Rose et al. (2013), and Pélissié et al. (2014) 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 seminatural 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. 2010), 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 and 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 laboratory (deCarvalho and Shaw 2010).

Laupala 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) to allow for individual

identification and placed in a 2.5 m³ 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) was provided every three days to approximate the hunger status of wild-caught adults based on feeding trials (B.R. Turnell, unpubl. 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, unpubl. 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). 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, and stored at -20°C . Male body length was measured using digital vernier calipers (BioQuip Products, Rancho Dominguez, CA) and dry weight was measured after 24 h in an oven at 60°C . All data on mating events

and individual traits are archived in the Dryad Digital Repository (doi:10.5061/dryad.9jd86).

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). 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). The five markers were amplified in a 10 μl multiplex PCR containing 1 \times PCR DyNAzyme II buffer and 0.2 U DyNAzyme II (Thermo Fisher Scientific, Waltham, MA); 0.15 mM dNTPs (New England Biolabs, Ipswich, MA); 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) 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) 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 $RS = MS \times FS \times N$, 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):

$$\text{var}(\text{RS}) \approx \text{var}(\text{MS}) + \text{var}(\text{FS}) + \text{var}(\text{N}) + \text{covariances},$$

where the covariances are equal to $2 \text{ cov}(\text{MS}, \text{FS}) + 2 \text{ cov}(\text{MS}, \text{N}) + 2 \text{ cov}(\text{FS}, \text{N})$.

Following Pélissié et al. (2014), variance in relative fertilization success can be further decomposed into variance caused by factors specific to the mating event itself ($\text{FS}_{\text{mating}}$), such as mating order, and residual variance ($\text{FS}_{\text{residual}}$), which is influenced by male-specific factors such as sperm competitiveness. The approximation thus becomes:

$$\begin{aligned} \text{var}(\text{RS}) \approx & \text{var}(\text{MS}) + \text{var}(\text{FS}_{\text{mating}}) + \text{var}(\text{FS}_{\text{residual}}) \\ & + \text{var}(\text{N}) + \text{covariances}, \end{aligned}$$

where the covariances are equal to $2 \text{ cov}(\text{MS}, \text{FS}_{\text{mating}}) + 2 \text{ cov}(\text{MS}, \text{FS}_{\text{residual}}) + 2 \text{ cov}(\text{MS}, \text{N}) + 2 \text{ cov}(\text{FS}_{\text{mating}}, \text{N}) + 2 \text{ cov}(\text{FS}_{\text{residual}}, \text{N})$. 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 $\text{FS}_{\text{mating}}$ and in $\text{FS}_{\text{residual}}$.

To determine $\text{FS}_{\text{mating}}$ and $\text{FS}_{\text{residual}}$, 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 nonvirgin, whether the male mated on the previous day, and the number of micros transferred during the mating (micro number). A quasi-Poisson 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 $\text{FS}_{\text{mating}}$ and $\text{FS}_{\text{residual}}$ (Pélissié et al. 2014).

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 $\text{FS}_{\text{mating}}$

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, because 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 macro-spermatophore 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 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 (\pm 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 19 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 (Fig. 1). Variance in overall FS explained 62% (30–94%) of the variance in reproductive success whereas 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, although there was a trend for FS_{mating} to be positively associated with both MS and N ($\text{cov}(\text{MS}, FS_{\text{mating}}) = 0.026$, $P = 0.053$ and $\text{cov}(FS_{\text{mating}}, N) = 0.017$, $P = 0.061$). 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: $\text{var}(\text{RS}) = 0.44$, $\text{var}(\text{MS}) = 0.09$, $\text{var}(\text{FS}) = 0.39$, $\text{var}(N) = 0.01$. The opportunities for total and for precopulatory sexual selection for the 19 included females were $\text{var}(\text{RS}) = 0.22$ and $\text{var}(\text{MS}) = 0.19$.

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, although 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 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 ($\Delta \text{AICc} > 2$; 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 Figure 2A and B, respectively.

FACTORS AFFECTING FS_{mating}

The final mating-specific model predicting fertilization success included female mating status (virgin or nonvirgin), male mating order, and the number of micros transferred, but excluded whether the male had mated on the previous day ($\Delta \text{QAICc} = 2$; 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

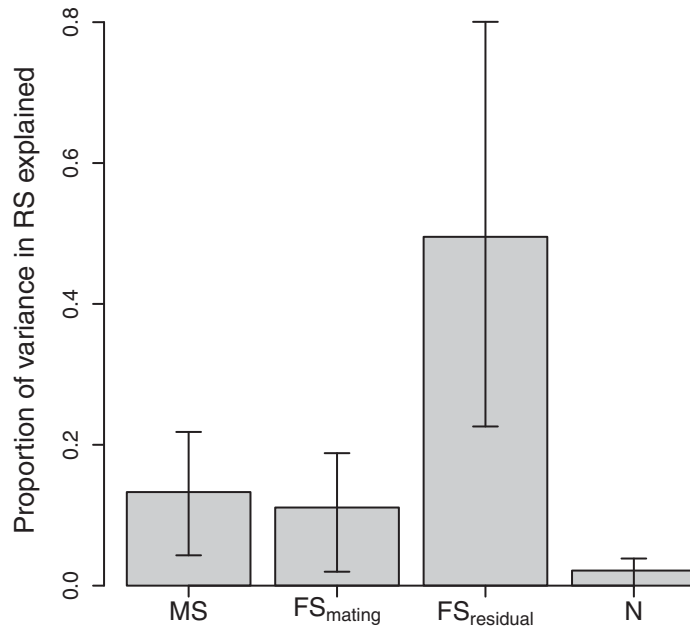


Figure 1. Proportion of the variance (with bootstrapped 95% CIs) in relative male reproductive success (RS) explained by variance in the relative values of mating success (MS), mating-specific fertilization success (FS_{mating}), residual fertilization success (FS_{residual}), and average female fecundity (N). Proportions explained by MS and total FS correspond to the opportunities for precopulatory and postcopulatory sexual selection, respectively.

was both because fewer matings with virgins than with nonvirgins 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 nonvirgin 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 whereas 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 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 nonvirgins, Kruskal–Wallis $P = 0.027$) and with male mating order (more micros were transferred by later males, Spearman’s rank $P = 0.031$, although there was no such effect among matings with nonvirgins 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} (Fig. 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 (Fig. 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

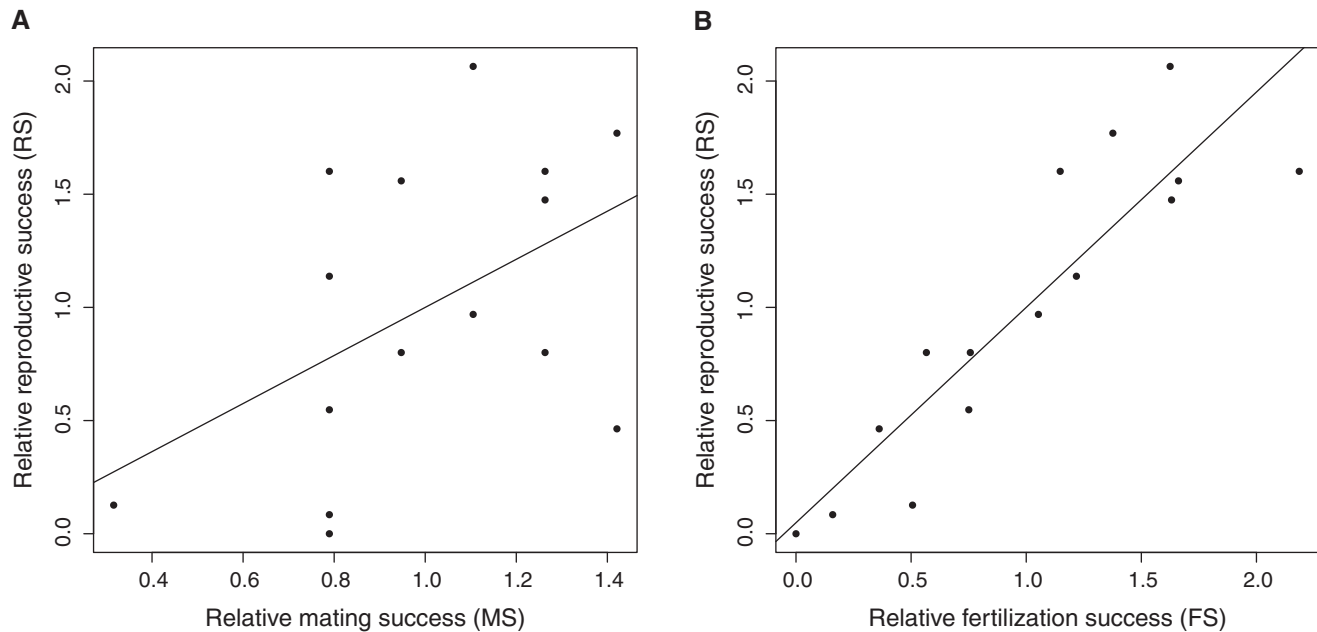


Figure 2. Regression of relative male reproductive success (RS, or reproductive success divided by the mean reproductive success of the population) on (A) relative mating success (MS, or mating success divided by the mean mating success of the population), that is, the male Bateman gradient, and (B) 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.

Table 1. Variance in mating-specific fertilization success explained by the terms in the mating-specific effects model ($n = 99$ matings, pseudo- $R^2 = 0.21$).

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

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%, Fig. 1; Fig. 2B vs. A). 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 (Pélissier et al. 2014), in which fertilization success varied twice as much as mating success, and taken together these results make a 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

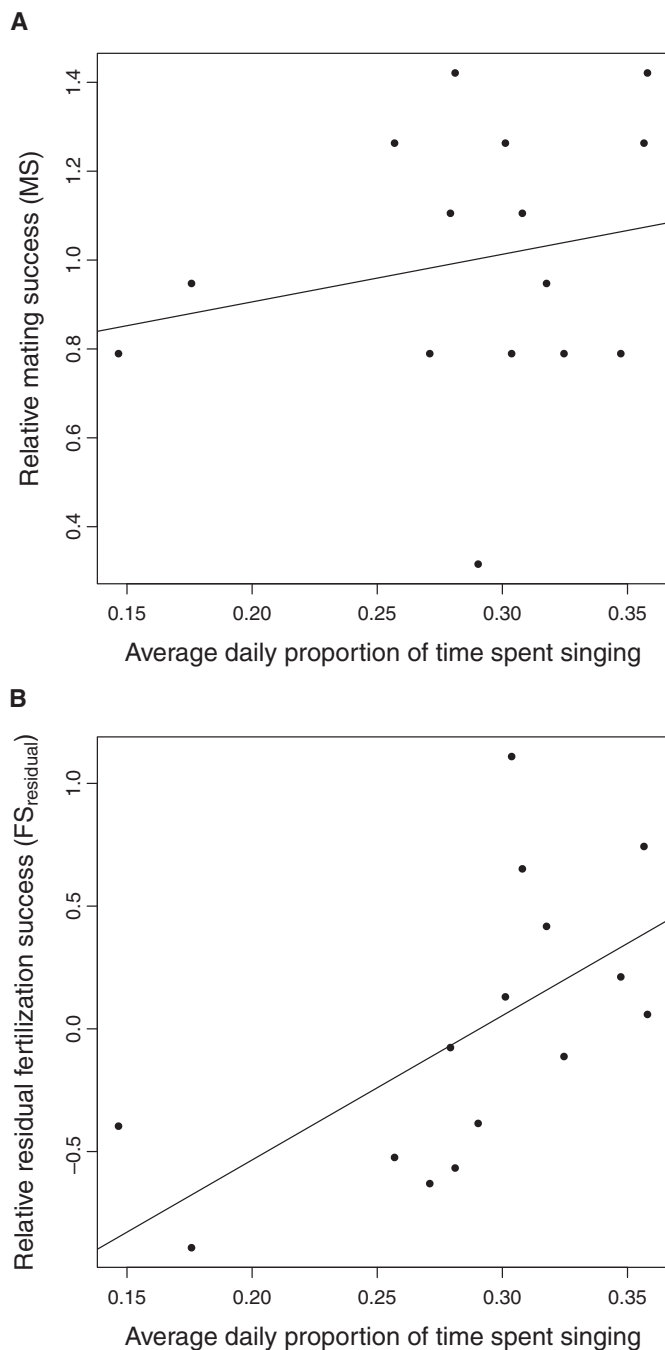


Figure 3. Effect of the average proportion of time spent singing per day by males on (A) relative mating success (MS, $R^2 = 0.04$, $p = 0.045$) and (B) relative fertilization success (FS_{residual} , $R^2 = 0.38$, $p = 0.014$). See text for the standardized univariate pre- and postcopulatory selection gradients on male singing effort.

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. 2014, 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 because 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). 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 nonvirgins. 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 because 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* because 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 nonvirgins, perhaps because they are certain to face sperm competition in matings with nonvirgin 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, although 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 Péliissié et al. (2014), 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 (Fig. 3B), although surprisingly not mating success (Fig. 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. 2010), 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 and 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 and 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 re-

productive 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; although 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; Péliissié 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 (Turnell, unpubl. 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 life span. Many studies have examined the relative roles of mating success, fecundity, and life span 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.

ACKNOWLEDGMENTS

Thanks to M. Xu, N. Ratterman, J. Collet, and an anonymous reviewer for comments on earlier versions of this manuscript. Thanks also to J. Booth and the Cornell Statistical Consulting Unit for help with the estimation-maximization algorithm. This work was funded by a National Science Foundation Doctoral Dissertation Improvement Grant to BRT.

DATA ARCHIVING

The data are archived in the Dryad Digital Repository (doi:10.5061/dryad.9jd86).

LITERATURE CITED

- Andersson, M. 1994. Sexual selection. Princeton Univ. Press, Princeton, NJ.
- Anthes, N., P. David, J. R. Auld, J. N. A. Hoffer, P. Jarne, J. M. Koene, H. Kokko, M. C. Lorenzi, B. Péliissié, D. Sprenger, et al. 2010. Bateman gradients in hermaphrodites: an extended approach to quantify sexual selection. *Am. Nat.* 176:249–263.
- Arnold, S. J., and M. J. Wade. 1984. On the measurement of natural and sexual selection: theory. *Evolution* 38:709–719.
- Avila, F. W., L. K. Sirot, B. A. LaFlamme, C. D. Rubinstein, and M. F. Wolfner. 2011. Insect seminal fluid proteins: identification and function. *Annu. Rev. Entomol.* 56:21–40.
- Birkhead, T. R. 2010. How stupid not to have thought of that: post-copulatory sexual selection. *J. Zool.* 281:78–93.
- Birkhead, T. R., and A. P. Møller. 1998. Sperm competition and sexual selection. Academic Press, San Diego, CA.
- Birkhead, T. R., and T. Pizzari. 2002. Postcopulatory sexual selection. *Nat. Rev. Genet.* 3:262–273.
- Bohrnstedt, G., and A. Goldberger. 1969. On the exact covariance of products of random variables. *J. Am. Stat. Assoc.* 64:1439–1442.
- Bretman, A., C. Fricke, and T. Chapman. 2009. Plastic responses of male *Drosophila melanogaster* to the level of sperm competition increase male reproductive fitness. *Proc. R. Soc. B* 276:1705–1711.
- Burnham, K. P., and D. R. Anderson. 2002. Model selection and multimodel inference: a practical information-theoretic approach. 2nd ed. Springer, New York.
- Cade, W., and E. Cade. 1992. Male mating success, calling and searching behaviour at high and low densities in the field cricket, *Gryllus integer*. *Anim. Behav.* 43:49–56.
- Clutton-Brock, T. 1988. Reproductive success. The University of Chicago Press, Chicago.
- Collet, J. M., R. F. Dean, K. Worley, D. S. Richardson, and T. Pizzari. 2014. The measure and significance of Bateman's principles. *Proc. R. Soc. B* 281:20132973.
- Collet, J., D. S. Richardson, K. Worley, and T. Pizzari. 2012. Sexual selection and the differential effect of polyandry. *Proc. Natl. Acad. Sci. USA* 109:8641–8645.
- Cotton, S., K. Fowler, and A. Pomiankowski. 2004. Do sexual ornaments demonstrate heightened condition-dependent expression as predicted by the handicap hypothesis? *Proc. R. Soc. B* 271:771–783.
- Davidson, A., and D. Hinkley. 1997. Bootstrap methods and their application. Cambridge Univ. Press, Cambridge.
- deCarvalho, T. N., D. J. Fergus, R. C. Bell, and K. L. Shaw. 2012. Rhythmic male reproductive behavior controls timing of courtship and mating in *Laupala cerasina*. *Behav. Ecol. Sociobiol.* 66:1333–1340.
- deCarvalho, T., and K. Shaw. 2010. Elaborate courtship enhances sperm transfer in the Hawaiian swordtail cricket, *Laupala cerasina*. *Anim. Behav.* 79:819–826.
- Devigili, A., J. L. Kelley, A. Pilastro, and J. P. Evans. 2012. Expression of pre- and postcopulatory traits under different dietary conditions in guppies. *Behav. Ecol.* 24:740–749.
- Do, C. B., and S. Batzoglou. 2008. What is the expectation maximization algorithm? *Comput. Biol.* 26:897–899.
- Eberhard, W. 2009. Postcopulatory sexual selection: Darwin's omission and its consequences. *Proc. Natl. Acad. Sci. USA* 106:10025–10032.
- Eberhard, W. G. 1996. Female control: sexual selection by cryptic female choice. Princeton Univ. Press, Princeton, NJ.
- Edvardsson, M., and G. Arnqvist. 2000. Copulatory courtship and cryptic female choice in red flour beetles *Tribolium castaneum*. *Proc. R. Soc. B* 267:559–563.
- Ellison, C. K., and K. L. Shaw. 2010. Mining non-model genomic libraries for microsatellites: BAC versus EST libraries and the generation of allelic richness. *BMC Genomics* 11:428.
- Engqvist, L. 2011. Male attractiveness is negatively genetically associated with investment in copulations. *Behav. Ecol.* 22:345–349.
- Evans, J. P. 2010. Quantitative genetic evidence that males trade attractiveness for ejaculate quality in guppies. *Proc. Biol. Sci.* 277:3195–3201.
- Gage, M. J. G., and P. A. Cook. 1994. Sperm size or numbers? Effects of nutritional stress upon eupyrene and apyrene sperm production strategies in the moth *Plodia interpunctella* (Lepidoptera: Pyralidae). *Funct. Ecol.* 8:594–599.
- Gwynne, D. T. 2008. Sexual conflict over nuptial gifts in insects. *Annu. Rev. Entomol.* 53:83–101.
- Hack, M. A. 1998. The energetics of male mating strategies in field crickets (Orthoptera: Gryllinae: Gryllidae). *J. Insect Behav.* 11:853–867.
- Helfenstein, F., S. Losdat, A. P. Møller, J. D. Blount, and H. Richner. 2010. Sperm of colourful males are better protected against oxidative stress. *Ecol. Lett.* 13:213–222.
- Hoback, W., and W. Wagner. 1997. The energetic cost of calling in the variable field cricket, *Gryllus lineaticeps*. *Physiol. Entomol.* 22:286–290.
- Hosken, D. J., and C. M. House. 2011. Sexual selection. *Curr. Biol.* 21:R62–5.
- Hosken, D. J., M. L. Taylor, K. Hoyle, S. Higgins, and N. Wedell. 2008. Attractive males have greater success in sperm competition. *Curr. Biol.* 18:R553–R554.
- Kalinowski, S. T., M. L. Taper, and T. C. Marshall. 2007. Revising how the computer program CERVUS accommodates genotyping error increases success in paternity assignment. *Mol. Ecol.* 16:1099–1106.
- Kingsolver, J., H. Hoekstra, J. Hoekstra, D. Berrigan, S. Vignieri, C. Hill, P. Hoang, P. Gilbert, and P. Beerli. 2001. The strength of phenotypic selection in natural populations. *Am. Nat.* 157:245–261.
- Legendre, P., and L. Legendre. 2012. Numerical ecology. Elsevier, Amsterdam.

- Ligout, S., D. Munier, L. Marquereau, and M. D. Greenfield. 2012. Chronological vs. physiological age as determinants of mating decisions: studies on female choice over lifespan in an acoustic moth. *Ethology* 118:740–751.
- Manier, M., S. Lüpold, J. Belote, W. T. Starmer, K. S. Berben, O. Ala-honkola, W. F. Collins, and S. Pitnick. 2013. Postcopulatory sexual selection generates speciation phenotypes in *Drosophila*. *Curr. Biol.* 23:1853–1862.
- Mautz, B. S., A. P. Møller, and M. D. Jennions. 2013. Do male secondary sexual characters signal ejaculate quality? A meta-analysis. *Biol. Rev.* 88:669–682.
- Mowles, S. L. 2014. The physiological cost of courtship: field cricket song results in anaerobic metabolism. *Anim. Behav.* 89:39–43.
- Olsson, M. 1997. Is sperm really so cheap? Costs of reproduction in male adders, *Vipera berus*. *Proc. R. Soc. B* 264:455–459.
- Parker, G. 1970. Sperm competition and its evolutionary consequences in the insects. *Biol. Rev.* 45:525–567.
- Pélissié, B., P. Jarne, and P. David. 2012. Sexual selection without sexual dimorphism: Bateman gradients in a simultaneous hermaphrodite. *Evolution* 66:66–81.
- Pélissié, B., P. Jarne, V. Sarda, and P. David. 2014. Disentangling precopulatory and postcopulatory sexual selection in polyandrous species. *Evolution* 68:1320–1331.
- Peretti, A. V., and W. G. Eberhard. 2010. Cryptic female choice via sperm dumping favours male copulatory courtship in a spider. *J. Evol. Biol.* 23:271–281.
- Pischedda, A., and W. R. Rice. 2012. Partitioning sexual selection into its mating success and fertilization success components. *Proc. Natl. Acad. Sci. USA* 109:2049–2053.
- RDevelopment Core Team. 2014. R: a language and environment for statistical computing. *Vie nna*, Austria.
- Rodríguez-Muñoz, R., A. Bretman, J. Slate, C. A. Walling, and T. Tregenza. 2010. Natural and sexual selection in a wild insect population. *Science* 328:1269–1272.
- Roff, D. A. 2002. *Life history evolution*. Sinauer Associates, Sunderland, MA.
- Rose, E., K. A. Paczolt, and A. G. Jones. 2013. The contributions of premating and postmating selection episodes to total selection in sex-role-reversed Gulf pipefish. *Am. Nat.* 182:410–420.
- Sakaluk, S. K. 1984. Male crickets feed females to ensure complete sperm transfer. *Science* 223:609–610.
- Shaw, K., and A. Khine. 2004. Courtship behavior in the Hawaiian cricket *Laupala cerasina*: males provide spermless spermatophores as nuptial gifts. *Ethology* 95:81–95.
- Shuster, S. M., W. R. Briggs, and P. A. Dennis. 2013. How multiple mating by females affects sexual selection. *Proc. R. Soc. B* 368:20120046.
- Siepielski, A. M., J. D. DiBattista, J. A. Evans, and S. M. Carlson. 2011. Differences in the temporal dynamics of phenotypic selection among fitness components in the wild. *Proc. R. Soc. B* 278:1572–1580.
- Simmons, L. 1986a. Inter-male competition and mating success in the field cricket, *Gryllus bimaculatus* (de Geer). *Anim. Behav.* 34:567–579.
- Simmons, L. W. 1986b. Female choice in the field cricket *Gryllus bimaculatus* (De Geer). *Anim. Behav.* 34:1463–1470.
- . 2001. Sperm competition and its evolutionary consequences in the insects. Princeton Univ. Press, Princeton, NJ.
- . 2005. The evolution of polyandry: sperm competition, sperm selection, and offspring viability. *Annu. Rev. Ecol. Evol. Syst.* 36:125–146.
- Simmons, L. W., R. M. Tinghitella, and M. Zuk. 2010. Quantitative genetic variation in courtship song and its covariation with immune function and sperm quality in the field cricket *Teleogryllus oceanicus*. *Behav. Ecol.* 21:1330–1336.
- Tallamy, D. W., B. E. Powell, and J. A. McClafferty. 2002. Male traits under cryptic female choice in the spotted cucumber beetle (Coleoptera: Chrysomelidae). *Behav. Ecol.* 13:511–518.
- Thornhill, R. 1983. Cryptic female choice and its implications in the scorpionfly *Harpobittacus nigriceps*. *Am. Nat.* 122:765–788.
- Vahed, K. 1998. The function of nuptial feeding in insects: a review of empirical studies. *Biol. Rev.* 73:43–78.
- Van Noordwijk, A., and G. deJong. 1986. Acquisition and allocation of resources: their influence on variation in life history tactics. *Am. Nat.* 128:137–142.
- Voorhies, W. Van. 1992. Production of sperm reduces nematode lifespan. *Nature* 360:456–458.
- Webster, M., S. Pruett-Jones, D. Westneat, and S. Arnold. 1995. Measuring the effects of pairing success, extra-pair copulations and mate quality on the opportunity for sexual selection. *Evolution* 49:1147–1157.
- Wedell, N., M. J. G. Gage, and G. A. Parker. 2002. Sperm competition, male prudence and sperm-limited females. *Trends Ecol. Evol.* 17:313–320.

Associate Editor: A. Chippindale
 Handling Editor: J. Conner