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IDENTIFYING THE INFLUENCE OF SELECTIVE EPISODES AND MECHANISMS ON REPRODUCTIVE SUCCESS IN TRIBOLIUM CASTANEUM AND DROSOPHILA MELANOGASTER

Elizabeth Metta Droge-Young
Syracuse University

Abstract: Parsing out what makes some individuals more reproductively successful than others is a key pursuit in evolutionary biology. While reproductive success can ultimately be defined as the number of offspring produced over an individual's lifetime, there are many selective episodes that shape this outcome. Because the majority of animals have multiple mates, achieving matings is but one influence on reproductive success. After copulation occurs, sperm from multiple males compete within the female reproductive tract to fertilize eggs, while females morphologically or behaviorally bias fertilization to preferred males, further shaping reproductive success. Additionally, the act of mating itself may influence parental lifespan or rate of offspring production. Here, I investigate the influence of multiple selective episodes on different aspects of reproductive success in two insects: the fruit fly, *Drosophila melanogaster* and the red flour beetle, *Tribolium castaneum.* Established genomes in both systems enabled the generation of transgenic, fluorescently labeled lines: green fluorescent protein (GFP) driven by a ubiquitin promoter in *D. melanogaster* to look at paternity in eggs, and GFP or red fluorescent protein (RFP) tagged protamines to identify different male's sperm by head color in *T. castaneum.* I investigated relationships between different episodes of reproductive success in *D. melanogaster* and found positive correlations between sperm competitive success and offspring viability; offspring viability itself was influenced by a male × female interaction on hatching success. In *T. castaneum*, I explored potential drivers of their extremely promiscuous mating system and how that system influences mechanisms of postcopulatory reproductive success. I found that repeated receipt of a complete ejaculate directly benefits female reproductive success, but comes at a longevity cost to males. This direct benefit of remating to females

may explain why I found that the proportion of different male's sperm in the main chamber of the female reproductive tract, and not the specialized sperm storage organ as in *D. melanogaster* and many other arthropods, determine the proportion of offspring sired by each male. The great differences in postcopulatory mechanics between *D. melanogaster*, found previously, and *T. castaneum*, found here, illustrate the importance of mating system in shaping aspects of reproductive success.

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by

Elizabeth M. Droge-Young

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Covariance among premating, postcopulatory and viability fitness components in *Drosophila melanogaster* and their influence on paternity measurement

Elizabeth M. Droge-Young[1]*, Mollie K. Manier[1], Stefan Lüpold[1], John M. Belote[1] and Scott Pitnick[1]

[1] Department of Biology, Syracuse University, Syracuse, NY 13244

* Author for correspondence:

email: emdroge@syr.edu

phone: 315-443-7248

fax: 315-443-2012

Author contributions: experiments designed by EMD, MKM, SP; experimental material created by EMD, SL, JMB; experiments performed by EMD, MKM, SL, SP; data analyzed by EMD; manuscript written by EMD, SP.

Abstract

In polyandrous mating systems, male fitness depends on success in premating, postcopulatory, and offspring viability episodes of selection. We tracked male success across all of these episodes simultaneously, using transgenic *Drosophila melanogaster* with ubiquitously expressed green fluorescent protein (i.e., GFP) in a series of competitive and non-competitive matings. This approach permitted us to track paternity-specific viability over all life stages and to distinguish true competitive fertilization success from differential early offspring viability. Relationships between episodes of selection were generally not present when paternity was measured in eggs, however positive correlations between sperm competitive success and offspring viability became significant when paternity was measured in adult offspring. Additionally, we found a significant male × female interaction on hatching success and a lack of repeatability of offspring viability across a focal male's matings, which may underlie the limited number of correlations found between episodes of selection.

Introduction

Natural and sexual selection shape male traits that influence male mating success (Andersson, 1994), competitive fertilization success (Birkhead & Møller, 1998) and the ability to produce high-quality offspring (Roff, 2002). Consequently, premating sexual selection, postcopulatory sexual selection and offspring viability selection constitute three, potentially discrete, or interacting, episodes of selection that determine reproductive success. How selection shapes male phenotypes will depend on trait covariance and the relationships between traits and fitness across episodes (Møller & Alatalo, 1999; Neff & Pitcher, 2005).

Investigations to date reveal that fitness relationships across selection episodes may vary depending upon the nature of the traits and the study organism. For instance, males that are preferred by females or that otherwise experience relatively high mating success have been shown to further benefit from disproportionately high competitive fertilization success in the fruit flies *Drosophila simulans* (Hosken et al., 2008) and *D. melanogaster* (Bretman et al., 2009; Fricke et al., 2010), the red flour beetle *Tribolium castaneum* (Lewis & Austad, 1994) and the guppy *Poecilia reticulata* (Evans et al., 2003; Pilastro et al., 2004; Locatello et al., 2006, but see Evans, 2010). Similarly, males that excel in either episode of sexual selection have been demonstrated to produce offspring of superior quality in *D. melanogaster* (Taylor et al., 1987; Gilchrist & Partridge, 1997) and *P. reticulata* (Evans et al., 2004), as well as in the yellow dung fly *Scathophaga stercoraria* (Hosken et.al., 2003), the field cricket *Gryllus bimaculatus* (Wedell & Tregenza, 1999), the house cricket *Acheta domesticus* (Head et. al., 2005) and the marsupial *Antechinus stuartii* (Fisher et al., 2006).

These relationships are consistent with the "good genes" hypothesis (reviewed in Andersson, 1994) or, in the case of relationships with fertilization success, the "good sperm" hypothesis (Yasui, 1997). The latter model suggests that overall male condition determines sperm competitive success, resulting in higher condition males achieving both increased postcopulatory success and production of superior quality offspring. Conversely, males that are better at obtaining copulations have been found to be disadvantaged in competing for fertilization in the water strider *Gerris lacustris* (Danielsson, 2001) and to have less competitive ejaculates in one study of *P. reticulata* (Evans, 2010). Likewise, a male's success in sexual selection can be at odds with offspring reproductive success (e.g., *D. melanogaster*; Pischedda & Chippindale, 2006) and offspring viability selection (e.g., seed beetle *Callosobruchus maculates*; Bilde et al., 2009), these patterns being attributable to sexual conflict.

Our understanding of the relationships among episodes of selection is further complicated by methodological challenges inherent in avoiding confounding offspring viability effects when assessing fertilization success. For example, competitive fertilization success is frequently measured as the proportion of adult progeny sired by a given male in a competitive mating. Differential genetic or parental effects on early offspring viability are known to exist (e.g., Gilchrist & Partridge, 1997; Barber et al., 2001; Wedekind et al., 2001; Evans et al., 2007; García-González & Simmons, 2007, 2011) and can confound estimates of sperm competitive success (Gilchrist & Partridge, 1997; García-González, 2008). Parsing out the effects of these selective forces is largely constrained by the inability to

nondestructively assay the paternity of eggs, which is a more accurate representation of competitive fertilization success.

Here, we present two experiments designed to discern relationships among episodes of selection as well as to identify male and female influences on offspring quality. In the first experiment, we evaluated male and female influences on variance in offspring quality through non-competitive isoline crosses. In a second experiment, we evaluated the performance of individual male *D. melanogaster* across all three episodes by mating each focal male to multiple females from a standard genetic background, while competing against a genetically standardized competitor male. We overcame the technical constraint of discriminating episode-specific fitness by using standard competitor males from a transgenic line of *D. melanogaster* expressing a ubiquitous green fluorescent protein marker (hereafter Ub-GFP). Ubiquitin is expressed in all *D. melanogaster* tissues at all life-stages (Handler & Harrell, 1999), enabling us to track offspring paternity in competitive matings from egg to adult.

Materials and Methods

Experiment 1: male and female influence on offspring viability

In order to establish male and female effects on offspring viability, non-competitive crosses were conducted for flies from a total of twelve isolines (i.e., six isolines per gender). Eleven lines of experimental flies originated from a genetically variable, outbred laboratory stock (LHm) maintained with overlapping generations in population cages with > 1000

individuals on standard cornmeal-molasses-agar medium supplemented with yeast. The isolines were generated by first backcrossing fluorescent markers into the LHm population. Flies were then subjected to six generations of full-sibling matings, where founders for each isoline were randomly selected from the RFP or GFP marked LHm base populations. Six female-source isolines carried a sperm-specific RFP marker (protamine-RFP, Manier et al., 2010), whereas five experimental male-source isolines carried the Ub-GFP marker and a sperm-specific GFP marker (protamine-GFP, Manier et al., 2010). The Ub-GFP transgenic line was created by germline transformation of the pB[PUbnlsEGFP] vector using methods described in Manier et al. (2010). The pB[PUbnlsEGFP] and pBΔSac helper plasmids were kindly provided by A. Handler (USDA-Agricultural Research Service, Gainesville, FL; Handler & Harrell, 1999). A sixth male line carrying a ubiquitously expressed RFP marker (Ub-RFP) was created by germline transformation of w^{1118} flies, with P-element insertions and a w;Sco/CyO balancer (Tran Van and Joseph Lipsick, unpublished) and were generously provided by the Lipsick laboratory (Stanford University, Stanford, CA). All flies were reared at low density, achieved by moving parental pairs to new vials daily. All flies were collected shortly after eclosion under CO_2 anesthetization and housed by gender at a density of 20 flies per vial until mating. Flies were 2-4 days old for initial matings.

Each male was mated once to a non-experimental LHm virgin female on the day preceding the experiment to avoid any effects related to male virgin status (e.g., Bjork et al., 2007); these females were discarded after mating. Isoline crosses were replicated up to four times with a total of 124 successful matings across all lines with copulation duration quantified to the nearest minute. Following mating, each female was transferred by

aspiration to a new oviposition vial with standard cornmeal-molasses-agar medium supplemented with live yeast once a day for two days.

Eggs in each vial were counted after females were transferred to new vials to determine female fecundity. Unhatched eggs were counted the following day to calculate the proportion of oviposited eggs that hatched (i.e., hatching success). Vials were checked daily for eclosion and egg-to-adult development time was recorded. Posthatching viability was also calculated by dividing the number of eclosed adults by the number of hatched eggs. Females that did not lay any eggs were dissected and their reproductive tracts observed for presence of sperm. Three females lacking stored sperm, indicating unsuccessful copulations, were excluded from analyses.

Statistical analysis of experiment 1

All data were analyzed with R 2.12.2 (R Development Core Team, 2011). Male and female influence on early offspring viability was analyzed with two-way ANOVAs for fecundity, hatching success, posthatching viability and development time. Two crosses failed to produce any adult offspring among all replicates. These missing cells were replaced by line-specific mean values of posthatching viability or development time calculated using viability values from both male and female lines. For some oviposition vials, slightly more offspring were counted as adults than as eggs (n = 7 out of 104 vials), resulting in proportional hatching success and posthatching viability measurements in excess of one. Because the eggs in any vial had an equal chance of being undercounted, these data were analyzed as is. Reported values are mean ± standard deviation, unless otherwise noted.

Experiment 2: reproductive success and offspring viability

Focal male flies originated from the outbred LHm population. Standard competitor males originated from a Ub-GFP isofemale line that was used in an effort to limit genetic variation among competitor males (e.g., Bjork et al., 2007). Females originated from an inbred line of LHm created by three generations of full-sibling mating to reduce genetic variation in mate preference (e.g., Bjork et al., 2007).

Outbred focal males (n = 60) were collected upon eclosion from LHm culture bottles. Inbred experimental females and standard competitor males were reared at a standard density of 50 larvae per 8-dram vial to minimize larval competition and phenotypic expression of inherent variance in condition. These rearing conditions significantly reduced phenotypic variation in thorax length of standard competitor males as compared to focal males (mean ± SD; competitors: 0.94 mm ± 0.03, focal: 0.89 mm ± 0.06; Fligner-Killeen non-parametric test for homogeneity of variance $\chi^2_1 = 33.40$, $p < 0.0001$). Eclosing flies were collected as described in experiment 1 and males were mated once prior to experimental crosses.

To assay contributions to variation in reproductive success among focal males in traits relevant to precopulatory sexual selection, postcopulatory sexual selection and viability selection, each focal male was experimentally mated to four different LHm females, with no more than one mating opportunity per day over six successive days. Subsequent to an initial mating to avoid virgin effects, focal males were subjected to test

matings I, II, and III, where the order of matings II and III was randomized among males in a fully balanced design:

(I) single mating – virgin female singly mated to focal male to assay (i) male attractiveness to a virgin female, (ii) single-mating productivity, (iii) hatching success, (iv) posthatching viability and (v) development time

(II) focal male first – virgin female mated to focal male, then remated to standard competitor male to assay (i) male attractiveness to a virgin female, (ii) induced female refractoriness (i.e., mating latency with standard competitor male), (iii) first-male fertilization success, (iv) hatching success, (v) posthatching viability and (vi) development time

(III) focal male second – virgin female mated to standard competitor, then remated to focal male to assay: (i) male attractiveness to a nonvirgin female, (ii) second-male fertilization success, (iii) hatching success, (iv) posthatching viability and (v) development time

As an index of male "attractiveness" (see Hosken et al., 2008) to virgin females (test matings I and II), we quantified to the nearest minute the time elapsed from introduction of the focal male to the start of copulation (i.e., mating latency). Mating latency with nonvirgin females (III) and ability to induce refractoriness to remating in mates (II) was quantified as day of remating (females provided 6-hour long opportunities to remate on each of three consecutive days following initial mating). Copulation duration was quantified as in experiment 1. As an index of body mass, the thorax length of each fly was measured after completion of mating or oviposition.

Following the conclusion of their final mating, each female was transferred by aspiration to a new oviposition vial or plate once a day for two days to quantify female fecundity. Treatment I females oviposited in vials with standard cornmeal-molasses-agar medium supplemented with live yeast. Treatments II and III females oviposited on plates with apple juice-agar medium supplemented with live yeast because the auto-fluorescence of standard medium interferes with paternity assignment of eggs by fluorescent markers. After hatching was complete, first-instar larvae and the associated apple juice-agar medium were gently transferred from plates to vials with standard medium to allow for offspring development.

Offspring viability and fertilization success associated with each focal male was calculated by quantifying the number of offspring sired by the focal male at multiple stages of offspring development. For treatments II and III, eggs and eclosed adult offspring were counted under fluorescence to determine paternity at egg and hatching stages; eggs fertilized by the focal male were unlabeled and eggs fertilized by the competitor male fluoresced green. This enabled the quantification of paternity-specific offspring viability variables (i.e., hatching success, posthatching viability, and egg-to-adult development time) and the proportion of progeny sired by the focal male for each cross.

Statistical analyses of experiment 2

Univariate correlation or linear regression was conducted to determine relationships between precopulatory, postcopulatory, and offspring viability variables (Table 1.1). In addition to mating latency, precopulatory success included male size due to large-male advantages in gaining copulations (e.g., Partridge, 1988). Because paternity was

determined at multiple offspring life stages, references to proportion of progeny sired by focal males follows García-González (2008): paternity calculated from eggs is referred to as F_1 and F_2, indicating fertilization success, whereas paternity as determined in adult offspring is designated with the traditional P_1 and P_2. Analyses involving paternity were separately conducted using both methods of calculating paternity. Multivariate tests of relationships (e.g., canonical correlation) had prohibitively low power because focal males missing one or more variable would require exclusion (e.g., focal male mated in all treatments, but was lost prior to body size measurement). Complete data sets across the three treatments were gathered for only 12 males. As such, relationships between pre- and postcopulatory episodes, or sexually selected (i.e., pertaining to both precopulatory and postcopulatory variables) and viability selected (i.e., pertaining to offspring viability variables) episodes, were assessed with stepwise elimination of non-significant variables in linear models. Model fitting and simplification followed Crawley (2007). Full models consist of each postcopulatory variable predicted by both precopulatory variables, or each viability variable predicted by all sexually selected variables. In a few cases, residuals of minimal linear models remained heteroscedastic despite variable transformations. Because all such minimal models retained only one predictor variable, significance was tested with Spearman's rank correlations. One outlier for treatment I hatching success and one outlier for treatment II F_2 altered retention of minimal model terms; the following results are presented without these values. Variables in univariate correlations were transformed for normality or analyzed with Spearman's rank correlation. Cross-episode correlations and regressions were tested for significance with alpha values corrected for the False Discovery

Rate (Benjamini & Hochberg, 1995) using 15 tests for fertilization success or paternity success.

The influence of sexually and viability selected traits on male fitness was assessed with general linear models, where each mating treatment (i.e., I, II or III) was analyzed individually. Rate-sensitive fitness scores were calculated by dividing the number of each male's eclosed offspring by his offspring's average development time. Our fitness metric included a rate component because larvae develop in a substrate that diminishes in quality over time, suggesting that faster development time may be beneficial (Roff, 2002). Separate models were fit using our rate-sensitive fitness metric, as well as number of offspring or development time as single response variables. Predictor variables were the same as in the cross-episode analyses. Full models included all main effects and any significant interactions from preliminary models as explanatory variables. Copulation duration and female body size were also included as covariates. Although results reflect models fit with untransformed paternity values, arcsine-square root transformation of paternity scores yielded minimal models retaining the same main effects. Relative contribution of minimal model terms to overall variance in male fitness was calculated by partitioning variance based on partial correlation coefficients following Legendre & Legendre (2000).

Repeatability (Lessells & Boag, 1987) of offspring viability variables across the three matings was calculated to further explore male influence on offspring viability. Specifically, repeatability of fitness, fecundity, hatching success and posthatching viability were calculated using ANOVA with mating treatment (i.e., I, II, III) nested within male identity. Male *D. melanogaster* have been shown to differentially transfer sperm (Lüpold et al., 2011)

and amounts of seminal peptides that induce egg production (Sirot et al., 2011) to virgin versus previously mated females. Because differential ejaculate investment may influence fitness and fecundity in our virgin (i.e., treatments I and II) versus non-virgin (i.e., III) mating treatments, repeatability was calculated with standardized response variables (i.e., mean = 0, s.d. = 1) for these two variables.

Because we could not distinguish whether observations where focal males achieved 100% paternity were due to complete sperm-competitive success of the focal male or failure of standard competitors to transfer sperm (focal male F_1 = 1 for 1 of 47 females in treatment II; focal male F_2 = 1 for 12 of 36 females in treatment III), analyses were completed with and without these data. As in experiment 1, slightly more offspring were counted as adults than as eggs in some oviposition vials (n = 13 out of 165 vials) and these data were analyzed as is.

Results

Experiment 1: male and female influence on offspring viability

Significant male × female interactions explained variance in both fecundity and hatching success, as well as a female line main effect on fecundity and a male line main effect on hatching success (Table 1.2).

Experiment 2: reproductive success and offspring viability

(a) Impact of traits on male fitness

In minimal linear models for rate-dependent fitness (Table 1.3) and offspring number (Table 1.S1), variance in response variables were significantly influenced by female fecundity, hatching success, posthatching viability, and fertilization success for the competitive matings. Main effects unique to individual models and interaction terms are discussed in more detail below.

For the single mating treatment (I), a few interactions significantly influenced variance in male fitness (Table 1.3) and total number of eclosed offspring (Table 1.S1). Two-way interactions between fecundity and hatching success or posthatching viability reflect their synergistic influence on numbers of eclosed offspring. At higher fecundity levels, the same proportional increase in hatching success or posthatching viability leads to a larger increase in the absolute number of eclosed offspring and in fitness. In the fitness model, the hatching success × posthatching viability interaction is due to increases in posthatching viability positively influencing fitness at low levels, but negatively impacting it at high hatching success (Figure 1.S1c). This may be explained by larval competition reducing fitness where more offspring successfully hatch. Indeed, a trade-off exists between the number of hatched offspring and survival for this treatment (ρ_{49} = -0.31, p = 0.03). No explanatory variables remained in the minimum adequate model for variance in development time.

In treatment II, variance in male fitness (Table 1.3) and total number of focal male's eclosing progeny (Table 1.S1) were explained by female size in addition to the main effects common to all treatments. A number of synergistic interactions were also present in minimal models explaining variance in fitness (i.e., F_1 × posthatching viability) and

offspring number (i.e., hatching success × posthatching viability, F_1 × posthatching viability, and F_1 × hatch). A female size × F_1 interaction arose in both treatment II models and appeared to also be due to a synergistic relationship, with increases in F_1 benefiting males more when they were mated to a larger female, although this pattern was less clear for offspring number (Figure 1.S1a, b). The minimal model explaining variance in development time was not significant (R^2 = 0.22, $F_{4,33}$ = 2.384, p = 0.07).

In addition to the main effects shared by all models for male fitness (Table 1.3) and number of eclosed offspring (Table 1.S1), variance in male fitness in treatment III was also significantly influenced by copulation duration and male size. Variance in offspring number was explained by the common model terms as well as synergistic interactions of female fecundity with hatching success and posthatching viability. The minimal model on development time of a focal male's progeny included copulation duration, male size and female size (Table 1.S1). A single interaction, male size × copulation duration, was present in models for both male fitness and development time; the mechanism underlying these interactions is unclear (Figure 1.S1d, e).

Including cases of 100% paternity resulted in retention of many of the same main effects in most minimal models (Table 1.S2). In a few cases, new main effects were included (treatment II number of offspring: female refractoriness; treatment III number of offspring: male size and copulation duration) or minimal models were non-significant (treatment III development time).

(b) Relationships between episodes of selection

No significant relationships were found between precopulatory and postcopulatory sexual selection after exclusion of one male with low F_2 (0.56, all other values > 0.78). Spearman's rank correlations between variables associated with postcopulatory selection were non-significant (F_1 and F_2: $\rho_{16} = 0.23$, $p = 0.38$; F_1 and refractoriness: $\rho_{45} = -0.27$, $p = 0.07$). A positive relationship was found between F_1 and hatching success after the removal of a single observation with unusually low hatching success ($r = 0.34$, $t_{39} = 2.24$, $p = 0.03$). Inclusion of cases with 100% paternity yielded qualitatively similar results, with the exception of the relationship between F_2 and male size becoming statistically significant ($r = 0.42$, $t_{33} = 2.63$, $p = 0.01$). Although 95% confidence intervals around the linear model coefficient estimates (i.e., effect size) for both relationships did not include 0 (F_1 and hatching success: coefficient estimate = 0.28, 95% CI = 0.07 – 0.49 ; F_2 and male size: coefficient estimate = 0.42, 95% CI = 0.15 – 0.70), neither relationship remained significant after FDR correction.

It is possible that our inability to distinguish among eggs fertilized by the focal male and unfertilized eggs could influence the above relationships. If unfertilized eggs had been scored as focal male progeny, fertilization success (i.e., F_1) would be artificially inflated, but offspring hatching success would decrease, weakening the strength of the correlation. A comparison of hatching success of focal males and standard competitors across treatments suggests this may be the case (focal hatching success = 0.79 ± 0.13, competitor hatching success = 0.98 ± 0.20, one sided Wilcoxon rank sum test, $W_{71} = 484$, $p < 0.0001$). As such, the results discussed in this section are conservative estimates of these cross-episode relationships.

Notably different results were obtained when relationships between precopulatory and postcopulatory variables were evaluated using P_1 or P_2 as opposed to F_1 or F_2. Relationships became stronger between first-male paternity and hatching success ($r = 0.40$, $t_{38} = 2.72$, $p = 0.01$; outlier and two high leverage points excluded) and appeared between paternity and posthatching viability (P_1: $r = 0.54$, $t_{38} = 4.08$, $p = 0.0002$; P_2: $\rho_{21} = 0.56$, $p = 0.005$), as well as between second male paternity and male size ($r = 0.48$, $t_{21} = 2.51$, $p = 0.02$). Paternity relationships remain significant after FDR correction, except for that between P_2 and male size.

(c) Repeatability of offspring viability across matings

Repeatability of fitness, fecundity, hatching success or posthatching viability across the three test matings in the experiment 2 was not significant (all repeatabilities < 0.11, $F < 1.35$, $p > 0.23$).

Discussion

We found that variance in male fitness was significantly influenced by both sexual (i.e., fertilization success) and offspring viability selection (i.e., hatching success and posthatching viability), the latter was particularly influential when focal males were first to mate against a standard competitor (Table 1.3; experiment 2, treatment II). Interestingly, viability selection may explain the differences we found in presence and strength of cross-episode relationships when "fertilization success" was measured at different points of offspring development. In three cases, relationships became statistically significant after FDR correction when paternity was measured in adult offspring (i.e., with P_1 or P_2) rather

than eggs (i.e., with F_1 or F_2), results we interpret as arising from differential offspring viability influencing P_1 and P_2.

This result reinforces previously identified concerns about the interpretation of cross-episode relationships with paternity (Gilchrist & Partridge, 1997; García-González, 2008). Where studies do not control for offspring viability differences methodologically (e.g., as controlled in Danielsson, 2001; Evans et al., 2003; Fisher et al., 2006; Bilde et al., 2009) or statistically (e.g., as controlled in Hosken et al., 2003; Pischedda & Chippindale, 2006), correlations with paternity may at least partially represent differential offspring viability. Where relationships have been found between P_2 and male attractiveness (Lewis & Austad, 1994; Hosken et al., 2008; Bretman et al., 2009; Fricke et al., 2010), presence of significant differential viability would reduce support for a "good sperm" interpretation (e.g., as suggested by Lewis & Austad, 1994; Hosken et al., 2008) in favor of a model that incorporates offspring viability (e.g., genetic compatibility or good genes).

The lack of repeatability of offspring viability across a focal male's matings in experiment 2 further suggests that caution is warranted when applying correction factors from a separate, single mating to P_1 or P_2 values to account for differential offspring viability. To illustrate this point, when treatment I egg-to-adult viability was used to correct treatment II and III paternity values (i.e., paternity calculated from adult offspring and multiplied by treatment I viability) the same statistically significant relationships were found as when investigating relationships with raw paternity scores (results not shown). This suggests that when viability of offspring is not repeaTable 1.across matings the application of viability correction factors may not adequately control for the influence of

differential offspring viability on paternity measurements. Previous studies have weighted paternity on the population (e.g., Chang, 2004), or genetic isoline level (e.g., Clark et al., 1999; Fricke et al., 2010), as opposed to the individual male level, as in the present study. Because repeatability of viability within lines is rarely reported, it is difficult to determine whether applying correction factors at this level more adequately controls for confounding effects.

Two cross-episodic trends were found when using fertilization success, which did not remain significant after correction for the False Discovery Rate. Positive relationships between fertilization success and either hatching success (treatment II) or male size (treatment III) would be consistent with two models. First, under the "good sperm" model of polyandry, fertilization success is influenced by overall male condition (Sivinski, 1984; Madsen et al., 1992; Yasui, 1997), where high-condition males (e.g., larger) achieve increased postcopulatory success and also produce more robust offspring (e.g., with improved embyo viability). Second, cryptic female choice theory posits that females bias fertilizations in favor of preferred males, which includes more attractive or genetically compatible mates (Eberhard, 1996).

In order to avoid complex male × male × female interactions inherent to competitive matings (e.g., in sperm competitive success; Bjork et al., 2007), it was necessary to limit genotypic and phenotypic variation in females and standard competitor males in our reproductive success experiment. It is possible that the limited number of significant relationships we found, particularly between sexually selected episodes, was due to reduced phenotypic variance through inbreeding of standard competitors and females. We

consider this unlikely for most of the examined traits due to the presence of appreciable variation in most variables (Table 1.1, but see limited variation in development time and treatment III mating latency). Moreover, although some studies using outbred *Drosophila* have found relationships between pre- and postcopulatory success (e.g., between P_2 and mating latency: Hosken et al., 2008; Bretman et al., 2009; Fricke et al., 2010), others have found no correlation between these episodes (Pischedda & Rice, 2012).

Our finding that differential offspring viability significantly explains variance in male fitness reinforces previous cautions about interpreting relationships with paternity as measured in adult offspring (Gilchrist & Partridge, 1997; García-González, 2008). Moreover, the lack of repeatability in offspring viability among focal males' matings and a significant male × female effect on hatching success and posthatching viability illustrates the complexity of quantifying reproductive success, as well as identifying relationships between episodes of selection.

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Table 1.1. Mean and standard deviation of all study variables from first experiment, investigating relationship of reproductive success and offspring quality. Number of males successfully completing each treatment reported in column headers; deviations from these sample sizes noted as applicable. Note, cases of paternity = 1 for treatments II and III have been omitted.

	mating treatment		
	I – single mating n = 55 males	**II – P_1 n = 46 males**	**III – P_2 n = 24 males**
Precopulatory variables			
Focal male thorax length (mm)	0.89 ± 0.06 (n = 50)	0.89 ± 0.06 (n = 42)	0.88 ± 0.06
Mating latency	18.69 min ± 34.11	16.52 min ± 32.00	1.08 days ± 0.28
Postcopulatory variables			
F_1 or F_2	-	0.62 ± 0.15	0.92 ± 0.10
P_1 or P_2	-	0.44 ± 0.17	0.87 ± 0.15 (n = 23)
Refractoriness (days)	-	1.41 ± 0.50	-
Offspring viability variables			
Average development time	12.56 ± 0.37	11.58 ± 0.38	11.38 ± 0.35

(days)			
Female fecundity	36.16 ± 16.73	99.39 ± 24.95	93.25 ± 22.33
Hatching success	0.86 ± 0.13	0.74 ± 0.14	0.85 ± 0.11
Posthatching viability	1.04 ± 0.17	0.71 ± 0.24	0.84 ± 0.15

Table 1.2. Results of Model II analysis of variance, analyzing female fecundity and hatching success[3] (transformed to approach normality) among isoline crosses. Type III sums of squares were used to account for unbalanced data.

source of variance	**SS**	**df**	***F***	***p***
a) female fecundity				
female line	12704	5	4.09	0.01
male line	5471	5	1.76	0.16
female line × male line	15526	25	2.12	0.01
error	24864	85		
b) hatching success				
female line	0.87	5	1.94	0.12
male line	1.22	5	2.73	0.04
female line × male line	2.23	25	1.8	0.03
error	3.37	68		

Table 1.3. Minimal general linear models of effect of sexual and viability selected variables on focal male fitness (i.e., number of offspring eclosed/average development time) and partitioned variance among minimal model terms for competitive matings. All observations with 100% paternity have been excluded. Note: *variation common to multiple terms* quantifies the overlap of variance in the response variable explained by multiple predictor variables (Legendre & Legendre 2000), which is not equivalent to unexplained variance (i.e., residual variance).

source of variance	**estimate**	**S.E.**	***t***	***p***	**percent variance explained**
a) Treatment I - single mating					
Focal male fitness (n = 50 males, minimal model $R^2 = 0.996$, $F_{6,43} = 1669$, $p < 0.0001$)					
Hatching success	-2.32	0.63	-3.68	< 0.0001	0.14%
Posthatching viability	-2.24	0.36	-6.31	< 0.0001	0.43%
Female fecundity	-0.07	0.01	-4.93	< 0.0001	0.26%
Posthatching viability x female fecundity	0.08	0.01	11.54	< 0.0001	1.46%
Posthatching viability x hatching success	2.28	0.36	6.33	< 0.0001	0.43%
Female fecundity x hatching	0.07	0.01	5.85	< 0.0001	0.37%

success

Variation common to multiple terms					96.42%
Residual variance					0.49%

b) Treatment II – competitive mating, focal male mates first

Focal male fitness (n = 38 males, minimal model $R^2 = 0.977$, $F_{7,30} = 182.2$, $p < 0.0001$)

Proportion progeny (F_1)	-30.1	10.75	-2.8	0.01	0.62%
Hatching success	3.77	0.25	15.13	<0.0001	20.83%
Posthatching viability	-2.51	0.53	-4.7	<0.0001	1.93%
Female fecundity	0.03	0	15.11	<0.0001	20.78%
Female size	-14.74	5.96	-2.35	0.03	0.41%
F_1 x female size	25.49	10.18	2.42	0.02	0.44%
F_1 x posthatching viability	10.33	1.02	10.09	<0.0001	9.21%
Variation common to multiple terms					42.93%
Residual variance					2.83%

c) Treatment III – competitive mating, focal male mates second

Focal male fitness (n = 21 males*, minimal model $R^2 = 0.991$, $F_{7,13} = 206.4$, $p < 0.0001$)

Focal male copulation duration	0.76	0.13	6.06	<0.0001	4.47%
Proportion progeny (F_2)	5.13	0.58	8.77	<0.0001	7.41%
Hatching success	5.74	0.44	12.94	<0.0001	17.48%
Posthatching viability	5.71	0.35	16.28	<0.0001	25.20%
Female fecundity	0.06	0	17.96	<0.0001	35.96%
Focal male size	23.24	3.67	6.27	<0.0001	4.63%
Focal male size x focal male copulation duration	-0.91	0.15	-6.08	<0.0001	4.59%
Variation common to multiple terms					-3.11%
Residual variance					3.37%

*One high leverage point removed, its inclusion retains posthatching viability × female fecundity.

Supplemental materials

Figure S1.1

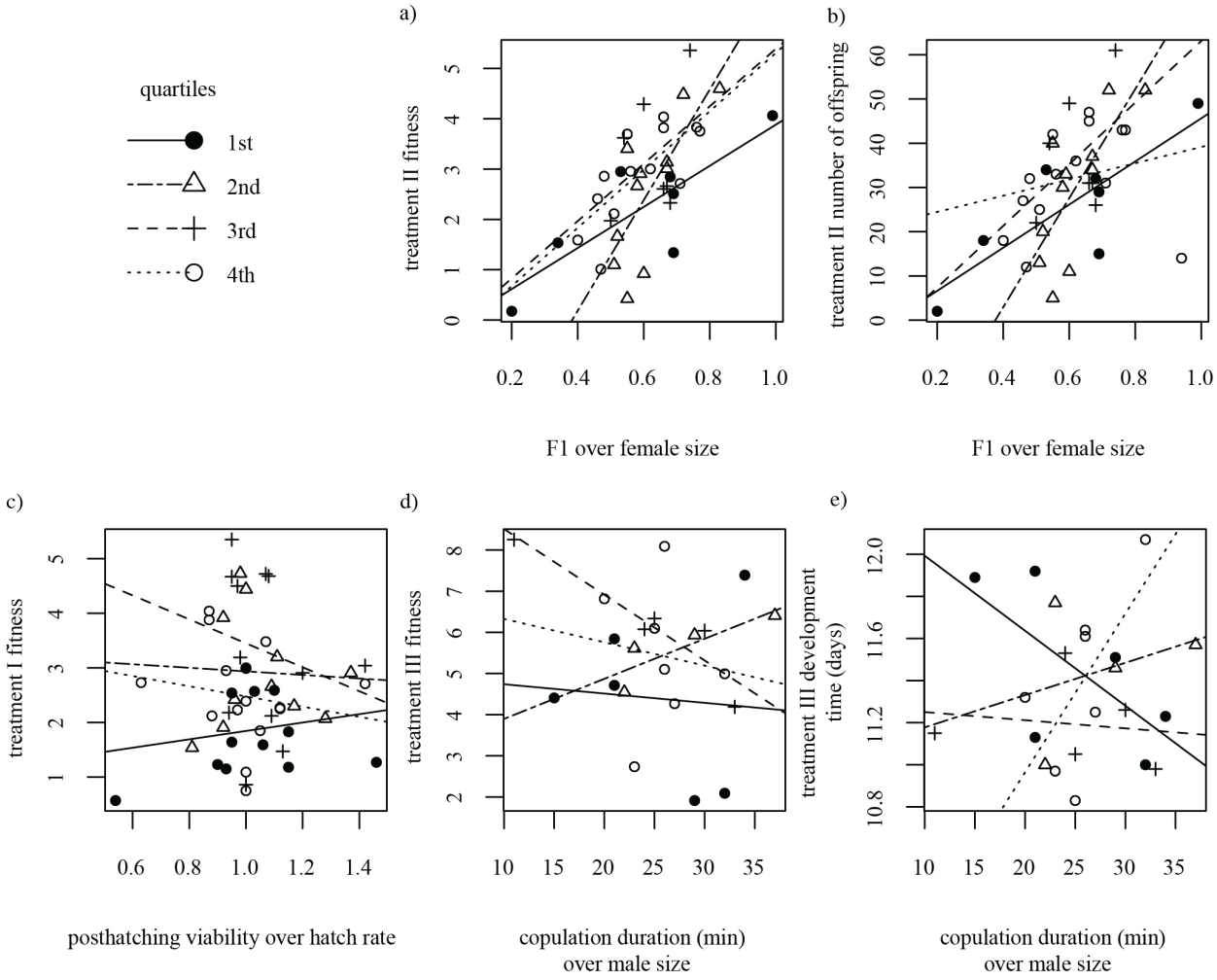

Figure S1.1 Non-synergistic interactions significant in minimal linear models. Each panel illustrates the influence of two interacting, explanatory variables on fitness (a, c, d), number of offspring (b) or development time (e). The first interacting variable is plotted on the x-axis of each panel and quartiles of the second interacting variable are indicated with different symbols. The slope of the relationship between the first explanatory variable (e.g., "F_1" in panel a) and the response variable is plotted over each quartile of the second explanatory variable (e.g., "female size" in panel a), with each quartile indicated by unique symbol and line styles (top left panel).

Table S1.1 Minimal general linear models of effect of sexually and viability selected variables on number of offspring sired by the focal male as well as offspring development time. Observations with 100% paternity have been excluded. Inclusion of high-leverage points retains *posthatching viability × hatching success or †F_2 × posthatching viability.

source of variance	**estimate**	**S.E.**	***t***	***p***
a) Treatment I - single mating				
Number of eclosed offspring (*two high leverage points removed**; n = 49 males, minimal model R^2 = 0.996, $F_{4,44}$ = 2521, p < 0.0001)				
Hatch	30.92	1.39	22.3	<0.0001
Posthatching viability	2.08	2.63	0.79	0.4330
Female fecundity	0.08	0.08	1.05	0.2980
Posthatching viability x female fecundity	0.83	0.08	10.79	<0.0001
b) Treatment II – competitive mating, focal male mates first				
Number of offspring (n = 39 males, minimal model R^2 = 0.99, $F_{9,29}$ = 288.5, p <				

0.0001)				
Proportion progeny (F_1)	-330.97	79.35	-4.17	0.0003
Hatch	-37.65	14.02	-2.69	0.0119
Posthatching viability	-53.59	8.25	-6.5	<0.0001
Female fecundity	0.33	0.01	25.67	<0.0001
Female size	-1.58	0.55	-2.84	0.0081
F_1 x female size	2.75	0.88	3.11	0.0042
Posthatching viability x F_1	110.69	8.05	13.75	<0.0001
Hatch x F_1	79.92	18.35	4.36	0.0002
Hatch x posthatch	43.32	10.81	4.01	0.0004

c) Treatment III – competitive mating, focal male mates second				
Number of offspring from "F2" mating (*four leverage points removed*†; n = 18 males, minimal model R^2 = 0.997, $F_{6,11}$ = 711.6, $p < 0.0001$)				
Proportion progeny (F_2)	71.13	4.56	15.61	<0.0001
Hatching success	4.46	27.97	0.16	0.8762
Posthatching viabiltiy	9.94	10.21	0.97	0.3511
Female fecundity	-0.65	0.29	-2.25	0.0462
Posthatching viability x fecundity	0.75	0.11	6.86	<0.0001
Hatching success x fecundity	0.84	0.31	2.71	0.0202
Development time of offspring (n = 22 males, minimal model R^2 = 0.5427, $F_{4,17}$ = 5.04, p = 0.007)				
Copulation duration	-0.51	0.15	-3.29	0.004

Female size	0.06	0.02	3.20	0.005
Male size	-0.20	0.06	-3.43	0.003
Male size x copulation duration	0.01	0.002	3.28	0.004

Table S1.2 Minimal general linear models of effect of sexually and viability selected variables on focal male fitness, including cases of 100% paternity. *Inclusion of high-leverage points retains male size × copulation duration.

source of variance	**estimate**	**S.E.**	***t***	***p***
a) Treatment II – competitive mating, focal male mates first				
Focal male fitness (R^2 = 0.9791, $F_{7,31}$ = 207.9, $p < 0.0001$)				
Proportion progeny (F_1)	-20.31	7.90	-2.57	0.0152
Hatching success	3.83	0.25	15.42	<0.0001
Posthatching viability	-2.50	0.54	-4.63	<0.0001
Female fecundity	0.03	0.00	15.40	<0.0001
Female size	-0.12	0.06	-1.92	0.0636
F_1 x female size	0.20	0.10	2.05	0.0489
F_1 x posthatching viability	10.28	1.04	9.92	<0.0001
Posthatching x	0.07	0.02	3.44	<0.0001

fecundity				
Hatch x fecundity	0.06	0.02	2.38	0.0270
Number of offspring (*three way interactions omitted for improved fit of residuals**; n = 40 males, R^2 = 0.9947, $F_{10,29}$ = 548.7, p < 0.0001)				
Refractoriness	-1.08	0.45	-2.42	0.0223
Proportion progeny (F_1)	-43.36	5.21	-8.33	<0.0001
Hatch	-4.61	6.44	-0.72	0.4793
Posthatching viability	-79.16	6.62	-11.96	<0.0001
Female fecundity	-0.23	0.07	-3.40	0.0020
Female size	0.38	0.12	3.25	0.0029
Posthatching viability x fecundity	0.32	0.04	7.25	<0.0001
Posthatching viability x F_1	65.74	9.08	7.24	<0.0001
Fecundity X F_1	0.50	0.07	7.38	<0.0001
Hatch x	69.26	8.78	7.89	<0.0001

posthatch				
b) Treatment III – competitive mating, focal male mates second				
Focal male fitness (n = 33 males, R^2 = 0.985, $F_{9,23}$ = 168, p < 0.001)				
Copulation duration	0.30	0.10	3.00	0.0063
Proportion progeny (F_2)	4.27	0.60	7.14	<0.0001
Hatching success	-1.10	1.98	-0.56	0.5828
Posthatching viability	1.89	1.27	1.49	0.1500
Female fecundity	-0.05	0.02	-2.57	0.0170
Male size	0.12	0.04	2.99	0.0066
Male size x copulation duration	-0.004	0.001	-3.06	0.0056
Posthatching viability x fecundity	0.05	0.02	3.25	0.0035
Hatching	0.07	0.02	3.41	0.0024

success x fecundity				
Number of offspring (n = 33 males, R^2 = 0.992, $F_{9,23}$ = 317.8, p < 0.0001)				
Copulation duration	1.80	0.83	2.16	0.0414
Proportion progeny (F_2)	51.18	4.92	10.40	<0.0001
Hatching success	-6.88	16.32	-0.42	0.6772
Posthatching viability	10.72	10.45	1.03	0.3155
Female fecundity	-0.54	0.15	-3.69	0.0012
Male size	0.65	0.32	2.03	0.0538
Posthatching viability x fecundity	0.68	0.13	5.31	<0.0001
Hatching success x fecundity	0.75	0.18	4.30	0.0003
Male size x	-0.03	0.01	-2.12	0.0449

copulation duration				

Extreme ecology and mating system: discriminating among direct benefits models in red flour beetles

Elizabeth M. Droge-Young[1]*, John M. Belote[1], Anjalika Eeswara[1], and Scott Pitnick[1]

[1] Department of Biology, Syracuse University, Syracuse, NY 13244

* Author for correspondence:

email: emdroge@syr.edu

phone: 315-443-7248

fax: 315-443-2012

Author contributions: experiments designed by EMD, SP; experimental material created by EMD, JMB; experiments performed by EMD, AE; data analyzed by EMD; manuscript written by EMD, SP.

ABSTRACT

We address the adaptive significance of female remating in the red flour beetle, *Tribolium castaneum*, a model system with an extreme mating system of little-to-no premating discrimination and rapid remating. In light of their specific ecology: the occupation of dried grain stores with no use of liquid water, we tested predictions of four non-mutually exclusive hypotheses addressing direct benefits that females may receive from mating: (1) topping off of sperm, (2) oviposition-stimulating seminal plasma, (3) ejaculate-derived nutrition or (4) hydration by the ejaculate. By examining the female fitness consequences of exposure to differing humidity and nutrition environments and exposure to males manipulated to deliver different ejaculate products during mating, we found strong support only for the ejaculate hydration hypothesis. We also investigated the effects of promiscuity on males and found evidence that providing moisture in the ejaculate is costly. This is in contrast to the frequently found pattern of sexual antagonism in which males benefit from an elevated mating rate at a cost to female fitness. We found no evidence that short-term exposure to different humidity conditions influences either female remating behavior or male competitive fertilization success. We consider the role of *T. castaneum's* ecology and mechanisms of postcopulatory sexual selection on the evolution of its mating system.

INTRODUCTION

Female remating behavior is a principal determinant of the intensity of sexual selection (Kvarnemo and Simmons 2013; Shuster et al. 2013). When females frequently remate with different males (i.e., display polyandry), both premating and postcopulatory sexual selection can be intense (e.g., Holland and Rice 1999; Martin and Hosken 2004; Wigby and Chapman 2004; Pai et al. 2007; Simmons and García-González 2008; Giardina et al. 2011; Debelle et al. 2014; Firman et al. 2015). Understanding female remating behavior is thus foundational to our understanding of sexual selection and its role in the diversification of reproductive characters.

There is no shortage of theoretical and empirical studies addressing and documenting the putative costs and benefits of mating multiple times or to multiple males (e.g., Ryan 1990; Sheldon 1994; Clutton-Brock and Parker 1995; Keller and Reeve 1995; Zeh and Zeh 1996, 1997; Yasui 1997; Jennions 1997; Holland and Rice 1998; Arnqvist and Nilsson 2000; Jennions and Petrie 2000; Hosken and Stockley 2003; Arnqvist and Rowe 2005; Neff and Pitcher 2005; Hosken et al. 2009; Pizzari and Wedell 2013 and references therein). Remating beyond what is necessary to acquire adequate sperm can benefit female fitness directly through increased lifetime reproductive success or through indirect genetic benefits, which enhance offspring quality. Alternately, repeated female mating can arise or be maintained by sexual antagonism where males benefit from elevated mating rates, but females experience costs. More recently research has suggested that polyandry is an appropriate null model for mating systems and that multiple mating may simply be reflective of mate encounter rates (Kokko and Mappes 2013). Indeed, a vast majority of

species are recognized as being polyandrous (Birkhead and Møller 1998; Taylor et al. 2014). However, cases of extreme promiscuity suggest females may remate adaptively, our understanding of which remains incomplete for most species.

When resources are difficult to acquire males may provide nuptial gifts of accumulated resources to females in exchange for mating (Vahed 1998). Whereas there is some evidence suggesting that male-contributed materials may manipulate (e.g., hormonally) female reproduction in ways that benefit the male to the detriment of female fitness (Vahed 2007), the balance of studies indicate that females benefit from receipt of these materials. This result supports the hypotheses that nuptial gifts represent male paternal investment and/or mating effort (Gwynne 2008). As such, nuptial gifts should have net fitness benefits for males, but also may be demonstrably costly for males to provide.

As one of the most common pests of stored grain worldwide, the red flour beetle, *Tribolium castaneum*, has become a model system for studies of ethology and sexual selection (Sokoloff 1974; Fedina and Lewis 2008). This species has a polygynandrous mating system characterized by extreme promiscuity by both sexes. Females can mate multiple times an hour with no precopulatory courtship or competition and apparently very limited premating sexual selection in general (Sokoloff 1974; Fedina and Lewis 2008; Fedina and Lewis 2015). In contrast, the ejaculates of numerous males can coincide within the female reproductive tract leading to very intense postcopulatory sexual selection (e.g., Lewis and Jutkiewicz 1998; Arnaud et al. 2001; Pai and Yan 2003; Michalczyk et al. 2011b; Droge-Young et al., unpublished).

Ecology is known to influence mating system evolution (Emlen and Oring 1977; Badyaev and Hill 2003), and the ecology of *T. castaneum* is notably extreme. They live, feed, mate, oviposit and develop in a dark, dry, three dimensional matrix of tunnels in stored, cracked grain (Sokoloff 1974). Because females do not aggregate and are continually surrounded by unlimited foraging and oviposition opportunities, there is little environmental potential for males to monopolize females or the resources they require to reproduce (Emlen and Oring 1977). Males also lack pronounced clasping devices and do not appear capable of forcing copulation (Fedina and Lewis 2008; Fedina and Lewis 2015). Indeed, females appear to be able to easily reject males simply by walking away or rarely by dislodging males that attempt mating (Wojcik 1969; Pai and Yan 2003), suggesting that females do not remate to avoid the costs of rejection.

Indirect benefits of polyandry for offspring viability and son reproductive success have been found in *T. castaneum* (Pai and Yan 2002; Pai et al. 2005; Michalczyk et al. 2011b; but see Pai and Yan 2003 for no effect and Bernasconi and Keller 2001; Pai and Yan 2002 for offspring costs). Direct benefits of polyandry have been investigated less frequently, but multiple mating has been shown to guard against infertility when males are sperm-limited (Pai et al. 2005).

Here we consider the role that *T. castaneum*'s extreme ecology has on its correspondingly extreme mating behavior. *Tribolium* beetle longevity and other life history traits are known to be influenced by both environmental conditions (Howe 1956) and exposure to the opposite sex (Spratt 1980), although the combined roles of ecology and mating rate have received little attention (but see Grazer and Martin 2011; Grazer et al.

2014). In a series of experiments, we examine fitness consequences (i.e., initial reproductive success, lifetime reproductive success and life span) for females by varying both environmental conditions and the materials delivered by males during mating. The results permit discrimination among four alternative hypotheses for direct benefits underlying the evolution of extreme female promiscuity in *T. castaneum*. Our design also permits testing of the hypothesis that females suffer a fitness cost from male-derived materials, which would be indicative of female promiscuity through male coercion. We also investigate the costs of extreme promiscuity for males. The identified costs and benefits of promiscuity for both sexes are then considered in the context of recent advances to our understanding of sperm precedence mechanisms in this species (Droge-Young et al., unpublished).

MATERIALS AND METHODS

Experimental populations and culturing

Experimental females originated from the cSM line (Wade 1976) and carry a homozygous, naturally arising, semi-dominant mutation resulting in black body color, which allows easy identification of beetles by sex. Males came from one of four lines, the first being the outbred wild type population that has been maintained at large population sizes since their collection in 2008, referred to here as “wild type” (provided by Mike Wade,University of Indiana, Bloomington, IN; see Drury et al. 2009 for WLIN collection details). The second line, E12808: hereafter referred to as “spermless” (provided by Jochen Trauner, University

of Erlangen-Nuremberg, Erlangen, Germany) was discovered during the GEKU transgenic screen (see Trauner et al. 2009 for details) in which males homozygous for the insertion fail to produce sperm due to a disruption in a dynein intron (*personal communication*, Trauner, J). The last two lines were from newly generated transgenic lines bearing sperm marked with green or red fluorescent protein-tagged protamines, a protein specific to DNA packaging in sperm heads. These transgenic lines enable identification of individual male's sperm by color after transfer to the female reproductive tract.

Beetle stocks were cultured in quart jars filled with standard yeast-enriched flour medium of 95% whole wheat flour, 5% yeast by weight, supplemented with 0.0003% Fumagillin to prevent microbial infection in a dark and humid growth chamber. All lines were maintained with overlapping generations since their arrival to the Pitnick lab. Populations of beetles were moved to fresh media every two months with initial population densities of approximately 1 beetle/1g medium. Experimental beetles were sexed as pupae and maintained separately by sex to ensure virginity. Beetles were 1 – 2 weeks old at the initiation of the experiments.

Sex-specific, direct effects of multiple mating

Female effects

We investigated four non-mutually exclusive hypotheses for mechanisms by which females might directly benefit from high mating rates: 1) sperm replenishment, 2) oviposition stimulants transferred in the ejaculate, 3) nutritive substances in the ejaculate and/or 4) moisture in the ejaculate (note, *T. castaneum* live in dry granaries and do not drink;

Sokoloff 1974), together with a fifth hypothesis: that females suffer a direct cost of materials delivered by males during mating. To test discrete predictions of these hypotheses (Table 2.1), we quantified female life span, initial (i.e., first 6 days) reproductive success and lifetime reproductive success while experimentally varying culture humidity, medium quality, and the substances transferred by males during mating. These three factors varied as follows:

1) two humidity treatments: beetles maintained under either control (58.3 ± sd 12.8%) or low relative humidity (21.4 ± sd 14.0%), where the weekly humidity difference between chambers was 40.1 ± sd 12.3%,
2) two media quality treatments: beetles maintained in either standard yeast-supplemented flour ("control medium"), or 1 part standard medium mixed with 4 parts non-nutritive cellulose ("low quality medium"), and
3) three experimental male treatments: unmanipulated wild type males (hereafter "control males"), wild type males with genitalia ablated with microscissors (hereafter "ablated males"), or spermless males. Control males transfer a normal ejaculate contained in a spermatophore, ablated males mate normally, but transfer nothing (*personal observation*) and spermless males transfer a sperm-free spermtophore and are expected to transfer the normal complement of seminal plasma (*personal communication*, J. Trauner).

Test females were randomly assigned to experimental treatments, which followed a partially factorial 2 x 2 x 3 design. Specifically, humidity level, media nutrition, and control vs. ablated male treatments were fully factorial (n = 30 females/treatment, with 11 of the

360 females lost throughout the experiment). Because spermless males were only used to test the sperm replenishment and the oviposition stimulant hypotheses, and no environment x male treatment interactions were predicted by those hypotheses, spermless males were only tested under the control humidity and the control media conditions (n = 30 females per treatment, with 2 of the 60 females lost throughout the experiment).

Each week female mortality was recorded and live females were moved to a fresh vial with 10g of medium and paired with a control male for one day for *ad libitum* mating to enable offspring production (*T. castaneum* produce fertilized eggs for more than a month following a single mating; Bloch Qazi et al. 1996). Females were then paired with their assigned experimental male type for the remainder of the week. Previous week's vials were transferred to control humidity conditions for offspring development, with reproductive success assayed as the number of adult offspring eclosing.

Potential non-fecundity influences on reproductive output

To ensure that offspring counts in the female benefits experiment were not biased by media quality, we investigated the effect of media on egg cannibalism, egg-to-adult viability, and egg investment.

Because *T. castaneum* is known to cannibalize eggs (Sokoloff 1974), a behavior influenced by diet quality (Via 1999), we tested whether cannibalism rates differed in low quality versus control media. Pairs of black females and wild type males were placed in vials replicating experimental conditions of 5g of control medium or 10g low quality medium along with 10 eggs (equivalent to one day's oviposition; Fedina and Lewis 2008)

dusted with green fluorescent dye to distinguish test eggs from newly laid eggs (n = 20 vials / medium treatment). After six days, the maximum time that a pair of experimental beetles would be in a vial during the main experiment, the vial was sifted and number of green eggs was counted.

Because environmental conditions can influence offspring viability, we tested whether our experimental environmental conditions would impact egg-to-adult viability. Black females were singly mated to a wild type male and then transferred to vials replicating experimental conditions of the control, low humidity, and low quality medium (n = 30 vials/environmental treatment). After 24 hours, females were removed and number of eggs counted. Number of offspring was quantified at maturity.

We also investigated potential differences in egg investment by females in the control versus low humidity treatments by measuring the size of up to ten eggs from black females singly mated to control males in the three environmental treatments to examine potential trade-offs between offspring size and number (n = 30 females / treatment). Size was measured as area of digitized images of eggs under 40x magnification using Image J.

Male mating motivation

In the event that observed female benefits were due to reduced harm from certain male treatments mating less frequently, we tested for differences in mating motivation among the three experimental males. Pairs of experimental males and virgin black females were observed in mating arenas of 35 mm petri dishes lined with filter paper for one hour

(n = 30 males/experimental type). Number of mounts, including those not leading to copulations, and copulation durations were recorded.

Male effects

Results from the female effects experiment directed our focus on the consequences of varying humidity on male life span and reproductive success. In addition to varying humidity, we also controlled the opportunity for male multiple mating by varying weekly duration of female exposure. We thus conducted a second, complementary experiment using a fully factorial 2 x 2 x 3 design in which test males, randomly assigned to one of two manipulations (control or ablated), were exposed to one of two humidity treatments (control or low humidity), and one of three female exposure treatments (exposed to a female zero, one or seven days a week) (n = 28 males per treatment, with n = 26 of 336 males lost throughout the experiment). Every week for 20 months, males were provided with fresh control medium and new females as indicated by treatment. Mortality was recorded weekly. Males remaining alive at 20 months were designated as having died at the longest time point. Note that only control males with zero female exposure were alive after 20 months, thus making results conservative.

Effect of humidity on the intensity of postcopulatory sexual selection and paternity outcomes

Based on results from the experiments described above, we proceeded to investigate two potential influences of humidity on the intensity and outcome of postcopulatory sexual

selection: (1) the timing of remating and (2) the impact of male exposure to different humidities on competitive fertilization success, specifically the proportion of progeny sired by the second male (i.e., P_2)

Remating timing

Effect of humidity on time of remating was examined in two ways, with experimental beetles assigned to either low or control humidity chambers for one week prior to the experiment. First, to examine female effects, all virgin females were observed during an initial mating to a control humidity RFP male. Following first matings, groups of 30 low or control humidity females were moved to treatment-specific bottles containing 30 virgin, control humidity, GFP males and 30 g of control medium. Beetles were left to interact and mate *ad libitum* until one of five freezing times: 0.5, 0.75, 1, 1.5, or 2 hours after the first mating (n = 30 females x 5 freezing times x 2 humidity treatments; n = 300 females total). Females were thawed and dissected to check for presence of GFP sperm, which would indicate that females had remated at least once within the treatment's time frame. Second, a similar procedure was followed to determine effects of male humidity environment on female remating, where first mating RFP males were housed in either control or low humidity for one week prior to matings; all females and GFP males were housed in control humidity. Remating and freezing followed as above.

Paternity outcomes

The effect of humidity on paternity outcomes was tested by quantifying the proportion of progeny produced by the second male in competitive matings where each female mated to a control GFP male first and then to an RFP male previously housed in control or low humidity, beginning one week prior to the experiment (n = 24 males per treatment). All matings were observed and copulation durations were recorded. Latency, measured as the time from pair introduction to mating arena until copulation began, was also recorded for the second, experimental matings. Paternity was determined by observing mature sons' testes for the inherited RFP or GFP tag and observing grandson testes from F1 daughters that were isolated as virgins and mated to unmarked wild type males.

Statistical analyses

All analyses were performed in R 2.12.2 (R Core Team 2011). Sex-specific influences of remating were analyzed with ANOVA unless otherwise noted. Body size of experimental beetles was included as a covariate in initial analyses, but was never significant and was thus removed from presented models. Two metrics of female offspring production were tested: first week initial reproductive success (hereafter, IRS) and lifetime reproductive success (hereafter, LRS), where IRS provides information during offspring production peak and LRS incorporates post-peak production and mortality. Humidity effects on female remating behavior were analyzed with generalized linear modeling with a binomial error distribution to account for binary responses. Full models included interacting predictor variables of freeze time, GFP sperm presence, and first male body size. Model simplification

followed Crawley (2007). Humidity effects on paternity outcomes were analyzed with ANOVA. Reported values are means ± standard errors.

RESULTS

Sex-specific, direct effects of multiple mating

Female effects

IRS was significantly influenced by the main effects of environment (i.e., medium quality and humidity) and experimental male treatment, as well as their interaction (Table 2.2). A Tukey's test of *post hoc* honestly significant differences (Tukey HSD) indicates that low quality medium or being paired to a spermless male significantly reduced IRS (Figure 2.1a). The significant interaction term can be explained by the differential effect of control and ablated males in control versus low humidity environments (Figure 2.1a). When females were paired to a control male there was no significant difference in IRS between low and control humidity environments (Tukey HSD = -7.03, $p = 0.86$). In contrast, when females were paired to an ablated male, they suffered significantly lower IRS in the low versus the control humidity environment (Tukey HSD = -25.67, $p < 0.0001$).

LRS was also significantly reduced by non-control environments or by being paired to spermless males (Table 2.1, Figure 2.1b). Although the interaction term was not significant, a Tukey's test indicates a greater reduction in LRS when females were in low humidity versus control humidity and were also paired to an ablated male versus a control male (Tukey HSD low versus control humidity with control male = -228.33, $p = 0.016$; low

versus control humidity with ablated male = -370.57, $p < 0.0001$), suggesting that the detrimental effect of low humidity was compounded by not receiving ejaculates.

Female longevity was only influenced by the main effect of environment (Table 2.1), where females died sooner in low humidity versus control or low quality medium (Figure 2.1c). There was a marginally non-significant interaction attributable to the reduction in female life span being greater in the low versus control humidity environment when females were paired to ablated males (Tukey HSD low versus control humidity = -33.76, $p = 0.0502$).

Potential non-fertility influences on reproductive output

Offspring numbers were not differentially influenced among treatments due to cannibalism (ANOVA: $F_{1,28} = 0.99$, $p = 0.33$), nor was egg-to-adult viability influenced by low humidity or low quality media (ANOVA: $F_{2,86} = 0.80$, $p = 0.46$). Egg size was significantly influenced by female environment (ANOVA: $F_{2,83} = 15.05$, $p < 0.0001$) with females from the low humidity condition producing larger eggs than other environmental treatments (control: 0.13 ± 0.002 mm^2, low humidity: 0.14 ± 0.001 mm^2, low quality media: 0.12 ± 0.001 mm^2). However, no trade-offs between size and number of eggs were observed (Pearson correlation: $t_{85} = -1.54$, $p = 0.13$).

Male mating motivation

Males from the three experimental lines differed in the number of mounts (ANOVA: $F_{2,87} = 7.748$, $p < 0.0001$) and average copulation duration (ANOVA: $F_{2,87} = 3.53$, $p = 0.04$),

where control followed by spermless males mounted less frequently (ablated: 19.27 ± 0.34, control: 10.80 ± 0.06, spermless: 1.30 ± 0.03). Ablated followed by spermless males had shorter average durations of copulation (ablated: 44.97 ± 0.44 sec, control: 75.68 ± 0.93 sec, spermless: 24.69 ± 0.40 sec).

Male effects

Male longevity was significantly influenced by male treatment, environment and female exposure main effects, as well as by an interaction between male treatment and female exposure, and by the three-way interaction between male treatment, female exposure and environment (Table 2.3).

Predictions of the ejaculate moisture hypothesis were strongly, but not universally, supported. First, males lived significantly longer in the control versus the low humidity environment (Figure 2.2A, Table 2.3), indicating that low humidity poses a survival challenge for males. However, there was no significant interaction between environment and female exposure as predicted (Fig. 2A, Table 2.3). Also contrary to prediction, control males lived significantly longer than ablated males under all conditions tested, which may be due to harm inflicted by the ablation process. By the same token, the strongest support for the ejaculate hydration hypothesis comes from the combination of a significant main effect of female exposure and a significant interaction between female exposure and male treatment on male longevity. Specifically, increasing exposure to females resulted in an increasing decline in male longevity in control males, but had no influence on longevity in

ablated males (Fig. 2B; Tukey HSD in seven versus zero day female exposure in control males = -95.04, $p = 0.0001$; in ablated males = 16.84, $p = 0.97$).

The significant three-way interaction between female exposure, male treatment, and humidity comes from the highest female exposure reducing male longevity, but only in control males and under control humidity conditions (Figure 2.2; Tukey HSD in seven versus zero day female = -156.21, $p = 0.00002$). Highest female exposure did not influence male lifespan when ablated males were in control humidity (Tukey HSD in seven versus zero day female = 30.08, $p = 0.99$), or when control males were in low humidity conditions (Tukey HSD in seven versus zero day female = 1.10, $p = 1$). Variance in longevity was reduced as female exposure increased for control males, and under either control or low humidity treatments (Levene's test: all $F_{2,152 \text{ or } 156} > 3.49$, $p < 0.03$), but not for ablated males (Levene's test: $F_{2,152} = 0.11$, $p = 0.90$).

Effect of humidity on the intensity of postcopulatory sexual selection and paternity outcomes

Remating timing

When females were exposed to control or low humidity the week prior to remating trials, the minimal model explaining variance in remating time retained only time of freezing as a significant explanatory variable ($z_{1,153} = 3.18$, $p = 0.001$), indicating no difference in remating time among humidity treatments. The minimal model predicting remating time when males were exposed to different environments retained no significant variables.

Paternity outcomes

Out of 48 mating trios, 20 females successfully mated to both males. Whether second males were exposed to control or low humidity the week prior to remating trials did not significantly influence second-male paternity (ANOVA: $F_{1,18} = 0.48$, $p = 0.50$).

DISCUSSION

We found that female *T. castaneum* reproductive success was directly benefited by repeated matings, particularly when in low humidity environments. Additionally, we found that having constant remating opportunities was costly to male lifespan. Taken together our results suggest a less commonly observed form of sexual conflict, where instead of males coercing females to mate at a rate above their fitness optimum (Arnqvist and Rowe 2005), females appear to influence males to copulate beyond what is beneficial for male fitness. We consider the role that *T. castaneum*'s extreme ecology and unique postcopulatory processes may play in this unexpected relationship.

Of the four examined hypotheses addressing putative direct benefits to female *T. castaneum* accrued through multiple mating: (1) sperm replenishment, (2) oviposition stimulation, (3) nutrition, and/or (4) hydration, we only found strong support for the ejaculate hydration hypothesis, along with weak support for the sperm replenishment hypothesis (Table 2.1):

(1) Sperm replenishment: significant declines in both the IRS and LRS of females paired to spermless males in the control environment supports a prediction of the sperm

replenishment hypothesis. We interpret this result with caution, however, given that female productivity in the spermless treatment was reduced beyond that of females paired to ablated males. This result does not fit with the sperm replenishment hypothesis since females received no sperm in either treatment. We conjecture that, because spermless males did still transfer a spermatophore whereas ablated males did not, it is possible that the repeated transfer and ejection by females of spermless spermatophores may have displaced many resident sperm in the bursa, making fertilization of eggs less likely (Droge-Young et al., unpublished).

(2) Oviposition stimulation: The lack of any detrimental effect on female IRS or LRS by females paired to ablated males in the control environment fails to provide support for the oviposition stimulation hypotheses.

(3) Ejaculate nutrition: Although low quality medium reduced IRS and LRS, this hypothesis predicted a "rescue" of reproductive success when females were paired to control versus ablated males in low quality medium, which was not observed. We do note that it is possible that females preferentially consumed flour over cellulose in this mixed medium, which would lessen detrimental effects of the low nutrition medium. However, because IRS and LRS were indeed reduced by low nutrition medium we suggest that preferential consumption of flour was limited.

(4) Hydration: When females were exposed to low humidity, the receipt of normal ejaculates (i.e. from control versus genital ablated males) significantly "rescued" the detrimental effects on reproductive success.

Finally, because in no circumstance did we find that female fitness increased when paired with ablated males, there is no experimental support for the hypothesis that females are harmed by the receipt of male ejaculates or physically by the male aedeagus.

We did observe significant mating behavior differences in experimental males. The increased copulation frequency of ablated males and reduced frequency of spermless males would not, however explain the relationships found with offspring production or longevity in response to environment and male type. If male harassment or the act of repeatedly copulating were costly to females we would expect to see the highest female values for life history traits when paired with spermless males and the lowest for females paired with ablated males. Our results did not meet this prediction; rather, in control environmental conditions, lowest offspring production was seen when females were paired with spermless males and equivalent offspring production was seen when females were paired with ablated or control males (Figure 2.1).

The lack of female harm from repeated mating in this study is somewhat in contrast to another *T. castaneum* study examining the influence of sex ratio on female mating costs (Michalczyk et al. 2011b). Michalczyk et al. (2011b) found that females from experimental evolution lines with a female-biased sex ratio suffered reduced fitness when exposed to increasing numbers of males. It is of note, however, that no cost of polyandry was observed in experimental lines evolved under male biased sex ratios. The lack of harm to females observed in this study might thus be due to our stock collections being maintained at a natural sex ratio (untested in Michalczyk et al. 2011b), as opposed to being strongly female biased.

Whenever food is limiting, nuptial gifting may arise through selection on females, as a means of foraging and/or for assessment of relative male quality, and through selection on males, as a means of increasing reproductive success (Parker and Simmons 1989; Wedell 1993; Vahed 1998; Vahed 2007; Gwynne 2008). Nuptial gifting in the form of ejaculate donation is expected to be evolutionarily labile and widespread, irrespective of selective causation (Parker and Simmons 1989; Vahed 2007; Gwynne 2008). Ejaculate donation in the form of moisture rather than nutrients has not been widely investigated, but we predict it to be widespread in desert-dwelling species, those inhabiting other dry environments and when liquid water is not consumed by adults (both of the latter two conditions apply to *T. castaneum*; Sokoloff 1974). It has been convincingly demonstrated in the seed beetle, *Callosobruchus maculatus* (Arnqvist et al. 2005; Edvardsson 2007; Ursprung et al. 2009) and the decorated cricket, *Gryllodes sigillatus* (Ivy et al. 1999); it may also occur in another seed beetle, *Callosobruchus chinensis* (Harano 2012) and in the almond moth, *Ephestia cautella* (Ryne et al. 2004; but see McNamara et al. 2008). Contrary to the studies with *Callosobruchus* (Edvardsson 2007; Harano et al. 2012), however, we found no evidence for behavioral plasticity in female remating in response to changes in humidity. We similarly found no evidence that males modulate their ejaculates based on short-term humidity exposure, at least not in ways that influenced mating frequency or reproductive outcomes.

Results of a meta-analysis by Arnqvist and Nilsson (2000) suggest that females of most animal species will maximize fitness by mating at some intermediate rate that balances the costs and benefits of multiple mating. However, in those exceptional cases

where the marginal benefits of remating exceeds the costs, female fitness should increase monotonically with mating rate, resulting in a high mating rate. Our results and previous studies suggest that the extreme remating in *T. castaneum* is consistent with this model. Females reap the benefit of a hydrating spermatophore delivered in each mating, and there is little-to-no discernable harm inflicted by males during the brief copulations. That is, we found no decline in any female fitness assay associated with the receipt of full ejaculates. Note, however, that among-population (and strain) variation in both remating frequency and its fitness consequences has been reported in *T. castaneum* (Nilsson et al. 2002; Pai et al. 2007; Michalczyk et al. 2011a). Such variation could be attributable in part to the evolutionary histories of strains differing with regard to humidity (i.e., a differential benefit of remating).

The high remating frequency of females does however appear to have potential negative fitness consequences for *T. castaneum* males. Male longevity significantly declined with increasing exposure to females. Because no decline was observed in ablated males, this pattern reflects a cost of ejaculate production and transfer (perhaps due to moisture loss) rather than a cost of courtship and other interaction with females. Ejaculate production is known to be costly in invertebrates, and we have no indication that the cost we observed in male *T. castaneum* is excessive relative to other species (e.g., Partridge and Farquhar 1981; Dewsbury 1982; Van Voorhies 1992; Martin and Hosken 2003; Kotiaho and Simmons 2003; Paukku and Kotiaho 2005).

Note, however, that male longevity was significantly reduced by an interaction between low humidity, ablation, and female exposure. Zero versus seven days of exposure

to females significantly reduced male lifespan, but only if males were both in control humidity conditions and in the control, non-ablated treatment. Interestingly, 7 days of female exposure under control conditions was as costly to longevity as low humidity in all female treatments (Tukey HSD: low humidity / 0, 1, or 7 day female exposure vs control humidity / 7 day female exposure $p > 0.11$). Control males lived significantly longer than ablated males under all conditions tested. We speculate that this pattern is attributable to direct harm caused by the ablation process or indirectly to health consequences of ablated males being incapable of voiding the products of spermatogenesis.

As an apparent consequence of the moisture content of spermatophores, *T. castaneum* females benefit from extreme polyandry while males suffer a cost. This pattern is notably the inverse of that observed in most investigations of sexual conflict (Arnqvist and Rowe 2005). In particular, the pattern of conflict we found in *T. castaneum* is somewhat different than that of *C. maculatus*, another stored product pest that lives in arid environments. Females *C. maculatus* benefit from receiving moisture from a male's ejaculate (Edvardsson 2007), but are also physically harmed during copulation by the male's spiny aedeagus (Crudgington and Siva-Jothy 2000). The combination of direct costs and benefits in this system lead to highest female fitness being achieved by high or low, but contrary to predictions (Arnqvist and Nilsson 2000), not intermediate mating frequencies (Arnqvist et al. 2005). This difference among species occupying similar environments suggests additional factors, such as presence of male harm, also influence female remating behavior.

We here speculate as to why male *T. castaneum* have not evolved potentially less costly adaptations to postcopulatory sexual selection (i.e., smaller spermatophores containing a lower water content), either by limiting remating by their mates or investing in sperm quality over quantity. First, we contend that mate guarding has likely not been a viable evolutionary option because females do not clutch eggs and because it would prove difficult for males to effectively guard in the three-dimensional cracked grain environment, particularly if females were not keen on having their acquisition of additional spermatophores restricted. We cannot speculate as to why non-contact guarding adaptations such as copulatory plugs or anti-aphrodisiacs have not arisen (South et al. 2011), as these have been described in other beetle species (e.g., Takami et al. 2008; Schlechter-Helas et al. 2011). Alternatively, males could limit sperm competition by physically removing rival sperm from the female. There is some evidence for this in *T. castaneum* (Haubruge et al. 1999) but its effectiveness appears limited (Tigreros et al. 2009). More specific insights, however, can be drawn from consideration of the recently resolved mechanisms of competitive fertilization success in this species (Droge-Young et al., unpublished).

The design of the female reproductive tract (i.e., morphology, physiology and biochemistry) can influence the intensity and nature of postcopulatory sexual selection on males (Eberhard 1996; Pitnick et al. 2009). Specialized sperm-storage organs (typically "spermathecae") are presumed to house the "fertilization set," which is the pool of sperm that directly compete to fertilize each ovum, although this has only been empirically confirmed in a few species (Simmons et al. 1999; Bretman et al. 2009; Manier et al. 2010;

Manier et al. 2013; Holman et al. 2011; Lüpold et al. 2012; Tyler et al. 2013; but see Siva-Jothy and Hooper 1995). As a striking exception to this expectation, the fertilization set in *T. castaneum* constitutes not those sperm occupying the spermatheca, but rather those in the main chamber of the reproductive tract, the bursa copulatrix, into which sperm are inseminated and where fertilization also occurs (Droge-Young et al., unpublished). Despite some displacement of resident sperm from the bursa following each mating, a given male's sperm can remain relevant across many subsequent rematings. Sperm are then used for fertilization in direct proportion to their representation (Droge-Young et al., unpublished).

Under these conditions, males may be evolutionarily constrained to transfer the greatest number of sperm possible, with the requisite amount of high-moisture seminal fluid similarly constrained. Further investigation of the sex-specific economics of extreme polyandry in *T. castaneum* are required, but this may represent a rare case of males being manipulated by female to mate more frequently than is in their best evolutionary interests.

This study exposed opposing sex-specific effects from repeated matings in *T. castaneum*. We found strong support for the hypothesis that female *T. castaneum* benefit from receiving a full ejaculate from males in stressful, low humidity conditions. Conversely, providing males with constant mating opportunities reduces longevity under benign environmental conditions. We did not, however, find any evidence that males or females modulate their reproductive behavior in response to proximate humidity conditions. These sex-specific direct effects of mating, taken with their characteristically extreme ecological conditions, provide insight into *T. castaneum's* extreme promiscuity. This study highlights

the importance of considering environmental conditions to better understand the evolution of extreme reproductive behaviors.

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FIGURE LEGENDS

Figure 2.1

Influence of experimental male treatment and environment (● control, ❍ low quality medium, △ low humidity) on female (a) six-day initial reproductive rate, (b) longevity, and (c) lifetime reproductive success.

Figure 2.2

Influence on male longevity of female exposure level, experimental male treatment and environment (● control humidity and control male, ❍ control humidity and ablated male, ▲ low humidity and control male, △ low humidity and ablated male).

Figure 2.1

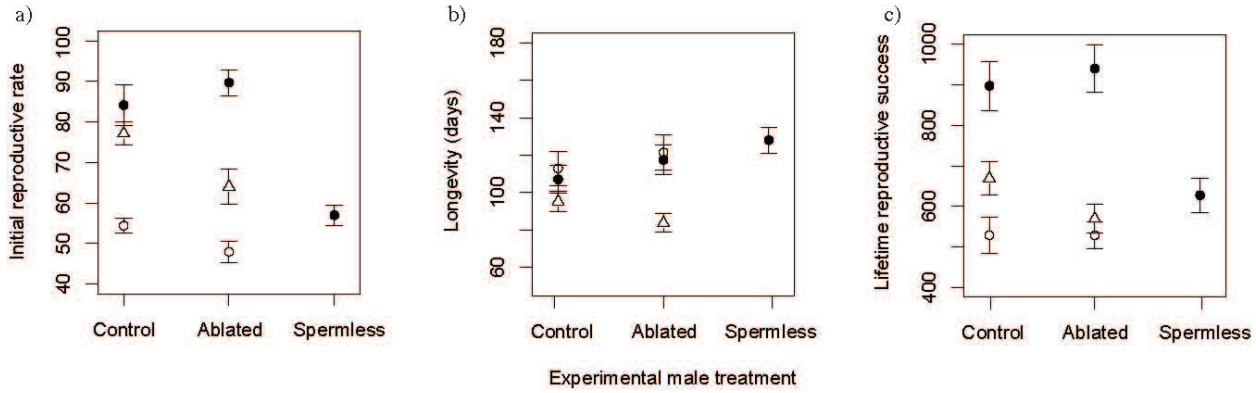
a)
Initial reproductive rate
40
50
60
70
80
90
100
Control
Ablated
Spermless
b)
Longevity (days)
60
100
140
180
Control
Ablated
Spermless
Experimental male treatment
c)
Lifetime reproductive success
400
600
800
1000
Control
Ablated
Spermless

Figure 2.2

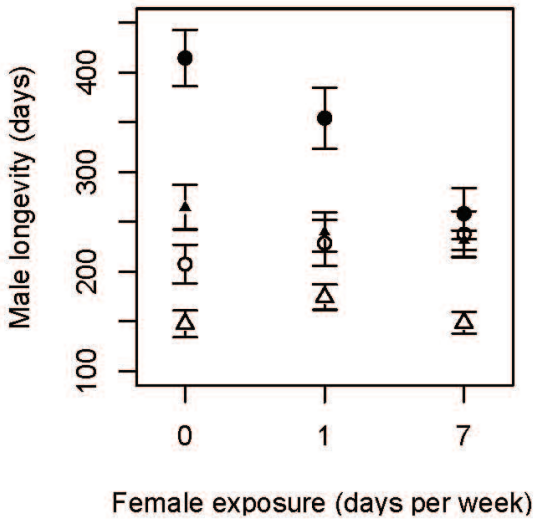
Male longevity (days)
100
200
300
400
0
1
7
Female exposure (days per week)

Table 2.1

Treatment effects on offspring production predicted by four alternative direct benefit hypotheses and the male coercion hypothesis, which predicts direct costs.

hypothesis	**predicted treatment effects on offspring production**	**support from experiment**
Sperm replenishment	In all environments: intact males > ablated or spermless males (spermless comparison in control environment only)	Mixed support: IRS with intact and ablated males > spermless
Oviposition stimulant	In all environments: intact and spermless males > ablated males (spermless comparison in control environment only)	No support: IRS with intact males and ablated males > spermless males
Moisture donation	In low humidity: control male > ablated male	Strong support: IRS in low humidity control male > ablated male
Nutrition donation	In low medium: control male > ablated male	No support: low medium uniformly lowered reproductive success across male treatments
Male coercion	*If coercion is due to male-transferred proteins*: In control environment: ablated	No support: control and ablated males > spermless males

males > control or spermless males	
If coercion is due to male behavior:	
No observable effect	n/a

Table 2.2

Results of ANOVAs identifying the contributions of experimental environment (control, low humidity, or low medium) and experimental male (control, ablated, or spermless) on variance in three female life history traits.

sources of variance	**d.f.**	**SS**	**MS**	***F***	***p***
female initial reproductive rate (IRS)					
environment	2	24509	12255	36.58	< 0.0001
experimental male	2	18900	9450	28.21	< 0.0001
environment x male	2	2674	1337	3.99	0.02
female lifetime reproductive success (LRS)					
environment	2	3389684	1694842	26.69	< 0.0001
experimental male	2	1714381	857191	13.50	< 0.0001
environment x male	2	159955	79977	1.26	0.29
female longevity					
environment	2	30046	15023	9.43	0.0001
experimental male	2	5052	2526	1.59	0.21
environment x male	2	4101	2050	1.29	0.28

Table 2.3

Results of ANOVAs identifying the contributions of experimental environment (control or low humidity), male treatment (control or ablated) and female exposure (0, 1, or 7 days per week) on variance in male longevity.

sources of variance	**d.f.**	**SS**	**MS**	***F***	***p***
environment	1	491507	491507	41.85	< 0.0001
male treatment	1	848169	848169	72.21	< 0.0001
female exposure	2	89097	44548	3.79	0.02
environment x male	1	19329	19329	1.65	0.20
environment x exposure	2	26749	13374	1.14	0.32
male x exposure	2	166921	83461	7.11	0.001
environment x male x exposure	2	83810	41905	3.57	0.03

Resolving mechanisms of competitive fertilization success in the red flour beetle

Elizabeth M. Droge-Young*, John M. Belote, Giselle S. Perez, and Scott Pitnick

Department of Biology, Syracuse University, Syracuse, NY 13244

* Author for correspondence:

237 Life Sciences Complex

107 College Place

Syracuse University,

Syracuse, NY 13244 USA

email: emdroge@syr.edu

Author contributions: experiments designed by EMD, SP; experimental material created by EMD, JMB; experiments performed by EMD, GSP, SP; data analyzed by EMD; manuscript written by EMD, SP.

ABSTRACT

Postcopulatory sexual selection occurs when sperm from multiple males occupy a female's reproductive tract at the same time and is expected to generate strong selection pressures on traits related to competitive fertilization success. However, knowledge of competitive fertilization success mechanisms and characters targeted by resulting selection is limited, partially due to the difficulty of discriminating among sperm from different males within the female reproductive tract. Here, we resolved mechanisms of competitive fertilization success in the promiscuous flour beetle *Tribolium castaneum*. Through creation of transgenic lines with fluorescent-tagged sperm heads, we followed the fate of focal male sperm in female reproductive tracts while tracking paternity across numerous rematings. Our results indicate that a given male's sperm persist and fertilize eggs through at least seven rematings. Additionally, the proportion of a male's sperm in the bursa (the site of spermatophore deposition), which is influenced by both timing of female's ejecting excess sperm and male size, significantly predicted paternity share. Contrary to expectation, proportional representation of sperm within the female's specialized sperm-storage organ did not significantly predict paternity. We address the adaptive significance of the identified reproductive mechanisms in the context of *T. castaneum's* unique mating system and ecology.

1. INTRODUCTION

Postcopulatory sexual selection, which includes sperm competition to fertilize eggs (Parker, 1970) and cryptic female choice among competing ejaculates (Eberhard, 1996), is credited with generating rapidly evolving (reviewed in Swanson and Vacquier, 2002) and highly divergent traits (reviewed in Pitnick *et al.*, 2009a). Such selection consequently impacts variation in reproductive success within populations (Pizzari and Parker, 2009) and reproductive isolation between populations and species (e.g., through conspecific sperm precedence; Howard *et al.*, 2009; Manier *et al.*, 2013a). Resolving the processes underlying variation in competitive fertilization success, along with the sex-specific targets of accompanying selection, is thus important for our understanding of biodiversity. Yet, such knowledge is presently limited.

Mating system differences among species, including female remating frequency and mate number, determine sperm competition intensity for males and the potential for choice among ejaculates for females. These mating system characteristics in turn influence the evolution of traits that determine variation in competitive fertilization success, including sperm and other ejaculate traits (Pizzari and Parker, 2009; Snook, 2005) and female reproductive physiology, morphology and behavior (Eberhard, 1996; Lüpold et al., 2013). Both sperm quantity (Parker and Pizzari, 2010) and sperm quality (e.g., *Malo et al., 2006;* Pattarini *et al*., 2006; Lüpold *et al.*, 2012), as well as morphological relationships between sperm and female sperm-storage organs (García-González and Simmons, 2007; Miller and Pitnick, 2002), have been found to influence competitive fertilization success in different systems. Fertilization success can also be influenced by female behaviors, such as

the timing or quantity of sperm ejected after copulations (e.g., Pizzari and Birkhead, 2000; Bussière *et al.*, 2006; Lüpold *et al.*, 2013), differential storage of preferred sperm (e.g., Pilastro *et al.*, 2004; Bretman *et al.*, 2009), or altering oviposition behavior (Bretman et al., 2006). Ultimately, it is a species mating system that will determine the relative importance of these or other reproductive adaptations to postcopulatory sexual selection.

When discerning mechanisms underlying patterns of competitive fertilization success, it is critical to identify the subset of sperm that are used to fertilize eggs (i.e., the "fertilization set" *sensu* Parker *et al.*, 1990). Not all locations that sperm can occupy within the female reproductive tract have an equal probability of supplying sperm for fertilization (Manier et al., 2013c; Pitnick et al., 2009b). Few studies have identified the fertilization set as well as the more general spatiotemporal dynamics of sperm storage and use following competitive matings (but see LaMunyon and Ward, 1998; Bussière *et al.*, 2010; Manier *et al.*, 2010, 2013b; Holman *et al.*, 2011; Lüpold *et al.*, 2012, 2013) due to the difficulty of distinguishing sperm from different males within the female reproductive tract (but see for unique methodological solutions: Otronen and Siva-Jothy, 1991; Otronen *et al.*, 1997; Schärer *et al.*, 2007; Bussière *et al.*, 2010; Manier *et al.*, 2010).

Finally, whereas the majority of investigations of characteristics that influence paternity outcomes have employed a standard experimental design using virgin females that are then mated with two males, paternity estimates from field collected specimens suggest that mating with more than two males is common in many species (e.g., Zeh *et al.*, 1997; Bretman and Tregenza, 2005; Simmons *et al.*, 2007; Demont *et al.*, 2011). Relatively few studies have investigated the dynamics of postcopulatory success when three or more

males compete for fertilization (but see Radwan, 1991, 1997; Zeh and Zeh, 1994; Cooper *et al.*, 1996; Eady and Tubman, 1996; Lewis and Jutkiewicz, 1998; Arnaud *et al.*, 2001; Drnevich, 2003; Lewis *et al.*, 2005; Bjork *et al.*, 2007). Because mating system, including remating frequency, is expected to influence mechanisms of postcopulatory sexual selection, it is critical to understand the persistence of a focal male's sperm in the female reproductive tract throughout multiple rematings.

The red flour beetle, *Tribolium castaneum,* has unusually high mating rates as compared to other internally fertilizing model systems commonly used to study postcopulatory sexual selection (e.g. fruit flies, dung flies, bruchid beetles, crickets, and birds), with male and female flour beetles observed to mate multiple times an hour (Fedina and Lewis, 2008). Due to the expected influence of mating system on traits important to competitive fertilization success, unique mechanisms are predicted to underlie variance in postcopulatory success in *T. castaneum* than have been identified in other systems (e.g. proportion of focal male sperm in specialized storage organs in *Drosophila* (Lüpold et al., 2012; Manier et al., 2013c, 2010) and *Gryllus* crickets (Bretman et al., 2009)).

Here, we used transgenic lines of the red flour beetle, *Tribolium castaneum*, featuring males that produce sperm that have heads tagged with green (GFP) or red fluorescent proteins (RFP) to address the persistence of focal male sperm through multiple rematings and the sperm's continued relevance to fertilization. These fluorescently labeled lines enable the tracking of a focal male's ejaculate through multiple matings as well as connecting patterns of sperm storage to patterns of paternity. A greater understanding of postcopulatory sexual selection is particularly desirable in this species due to extreme

female promiscuity, a general absence of precopulatory mate choice (Sokoloff, 1974) and substantial, yet largely unexplained (but see Edvardsson and Arnqvist, 2000) variation in competitive fertilization success (Lewis and Austad, 1990).

2. MATERIALS AND METHODS

2.1 EXPERIMENTAL SYSTEM AND CULTURING

During mating, male *T. castaneum* transfer a spermatophore to the female that rapidly everts to release sperm into the bursa copulatrix (henceforth "bursa") (Fedina, 2007). Following spermatophore eversion, sperm move to storage in the spermatheca, are retained in the bursa (Figure 3.1), or are ejected from the female reproductive tract along with remnants of the spermatophore. Sperm remain viable for fertilization for many months after mating (Bloch Qazi et al., 1996). The female's last mate generally sires a majority of offspring (i.e., displays "last male sperm precedence"; but see (Edvardsson and Arnqvist, 2000; Fedina and Lewis, 2004)).

All experimental females are from the WLIN (West Lafayette, Indiana) population that has been maintained at large population sizes since their collection in 2008 (see Drury et al. 2009 for collection details). Unless otherwise noted, experimental males are from transgenic lines bearing sperm marked with GFP or RFP tagged protamines, a protein specific to DNA packaging in sperm heads, enabling identification of individual male's sperm after transfer to the female reproductive tract. The transgenic protamine lines are referred to as GFP and RFP hereafter. Preliminary experiments also used Blk males, which carry a homozygous, naturally arising, semi-dominant mutation causing black body color.

The WLIN and Blk lines were generously provided by Dr. Mike Wade (University of Indiana, Bloomington IN), whereas the GFP and RFP lines were created by the authors.

Beetle stocks were cultured in quart jars filled with standard yeast-enriched flour medium of 95% whole wheat flour, 5% yeast by weight, supplemented with 0.0003% Fumagillin to prevent microbial infection in a dark and humid growth chamber. All lines were maintained with overlapping generations since their arrival to the Pitnick lab. Populations of beetles were moved to fresh media every two months with initial population densities of approximately 1 beetle/1g medium. Experimental beetles were sexed as pupae and maintained separately by sex to ensure virginity. Prior to experimental matings, males were marked with a small dot of non-toxic acrylic paint for sex identification during matings. Additionally, each male was isolated for a minimum of 24 hours prior to experiments to prevent any male-male matings, which are common in this species (e.g., Levan et al. 2009).

2.2 CONSTRUCTION OF THE GFP- AND RFP-TAGGED *TRIBOLIUM* PROTAMINE GENES AND GERMLINE TRANSFORMATION

The sequence of the *Drosophila melanogaster* ProtamineA protein (also known as Mst35Ba-PA) was used to query the translated *T. casteneum* genome sequence using the tblastn search function of the NCBI BLAST resource. A gene named LOC663849 (referred to here as TcProtamine-1) was identified with significant match (E value of 4e-08). Genomic DNA was isolated from beetles of the GA-2 wild-type strain, and PCR performed to amplify a 2.0 kb fragment containing this gene, using primers: Tc2 =

AGCACATCAAAATCTATAAGATAGAATCGG and Tc4 = CAGTTAGCTTCGGTCCGAAATGATGTAAAC. The product was cloned into pCR2.1 using the TOPO TA cloning kit (Invitrogen, Grand Island, USA) and sequenced. The 2.0 kb fragment was then excised using *Eco*RI and ligated into pBS/2xAsc, a modified pBlueScript-KS+ plasmid (Stratagene) in which two *Asc*I sites, flanking the Multiple Cloning Site, had been created by site directed mutagenesis. A unique *Nde*I site was created at the C-terminus of the TcProtamine-1 coding region by site directed mutagenesis using mutagenesis primers Tc2-4NdeMUTS = AGGAGCGGCTCCCGAAGCATATGCTACAGCTATTAAATTG and Tc2-4NdeMUTAS = CAATTTAATAGCTGTAGCATATGCTTCGGGAGCCGCTCCT. GFP and RFP tagged fusion genes were made by inserting the eGFP or mCherry coding sequences, in frame with the TcProtamine-1 coding sequence, as *Nde*I cassettes. These cassettes were created by PCR amplification of plasmids pEGFP and pmCherry (Invitrogen, Grand Island, USA).

The TcProtamine-1-GFP or TcProtamine-1-mCherry constructs were subcloned into the *Asc*I site of the transformation vector pBac3xP3-EGFPaf (Horn and Wimmer 2000; provided by E. A. Wimmer; Georg August University, Göttingen, Germany) to give pBac{3xP3-EGFP, TcProtamine-1-GFP} or pBac{3xP3-EGFP, TcProtamine-1-mCherry}, respectively. *Tribolium* germline transformation was carried out essentially as described in Berghammer et al.(1999), using the white-eyed *pearl* mutant strain as host (provided by Dr. Richard Beeman; USDA, Manhattan, KS, USA). The helper plasmid was phspBac (Handler and Harrell 1999; provided by Dr. Alfred Handler; USDA, Gainesville, FL, USA). Four independent Protamine-1-GFP, and one Protamine-1-RFP, transformed lines were

obtained; only one of each was used in this experiment. The RFP transformation is homozygous lethal, likely due to the construct's insertion location, so heterozygous individuals bearing the dominant marker were selected as pupae prior to each experiment. It is important to note that all sperm from RFP males is marked, but not all progeny will inherit the RFP marker.

2.3 PRELIMINARY EXPERIMENTS

A series of preliminary experiments were performed to determine appropriate remating intervals, timing of reproductively relevant events, and equivalence of transgenic lines. For all observed matings, a single female and male were introduced to a 35 mm x 10 mm petri dish, lined with filter paper to provide traction. Copulations were observed under low light, with copulation durations recorded to the nearest second.

2.3.1 Determination of remating intervals

An initial experiment was conducted to determine female remating rates under simulated natural conditions. Initial copulations between virgin female and RFP male pairs (n = 34) were observed. Groups of six singly-mated females and six GFP males were placed in vials of flour at a density of 2 beetles / g medium under standard stock keeping conditions. Vials were frozen at one of five timepoints from four hours to three days after the initial copulation. Females were then dissected and their reproductive tracts were observed under fluorescent illumination for presence of GFP sperm, indicating at least one remating had occurred. The reproductive tracts in a majority of females in the four-hour

treatment contained GFP and RFP sperm (n = 18 of 19), which we chose as the intermating interval for subsequent double mating experiments.

2.3.2 Onset of oviposition

In order to select biologically relevant freezing points for double matings, we conducted a preliminary experiment to determine the time elapsed from the conclusion of a second mating to the onset of oviposition – an event that may affect sperm competition by influencing the number of sperm present in the female reproductive tract. Females (n = 40 females) were mated to an RFP male followed by a GFP male and promptly transferred to an oviposition dish with 1g of finely sifted media. Dishes were checked for presence of eggs by sifting media and transferring females to new oviposition dishes approximately once an hour for a total of 5 hours. The first egg was observed 53 minutes after a second mating (n = 35 females, average oviposition onset = 1.92 ± 0.8 hours).

2.3.3 Confirmation of transgenic line equivalence

Another preliminary experiment was conducted to identify any differences in transgenic versus WLIN male performance in postcopulatory success or offspring viability. To compare postcopulatory success of transgenic males, females were mated first to Blk males. Four hours later, females were remated to one of three males: GFP, RFP, or WLIN (n = 30 females / second male) and were then transferred to standard medium to oviposit individually for four days. Paternity was assigned by body color 40 days after copulations when offspring reached maturity, where progeny with dark brown bodies were sired by Blk first males and progeny with red bodies were sired by second males. Females that produced only dark brown offspring, potentially indicating a failure of the second male to

transfer sperm, were dissected and observed under fluorescence using an Olympus BX50 microscope (Olympus, Center Valley, USA) for the GFP and RFP treatments to determine second male sperm transfer success, or excluded for analysis in the Blk treatment. Females producing only red offspring, indicating a potential failure of the first male from transferring offspring were excluded for analysis. No difference in last male precedence was found between the lines (Kruskal-Wallis $\chi^2_2 = 1.44$, $p = 0.49$), nor did GFP and RFP lines differ in success in transferring a spermatophore ($\chi^2_2 = 2.11$, $p = 0.35$).

Offspring quality was determined following single matings with females and GFP, RFP, or WLIN males (n = 40 females / male line). Number of eggs laid over four days in 5g of finely sifted media and number of eclosing progeny after 40 days were quantified to obtain metrics of male fertility as well as egg-to-adult viability. For vials where no progeny eclosed, females were dissected to check for successful sperm transfer and were excluded from analyses if the reproductive tract contained no sperm. Sperm was never transferred in matings under 33 seconds. Egg number significantly differed between treatments (Kruskal-Wallis $\chi^2_2 = 7.52$, $p = 0.02$), as did egg-to-adult viability (Kruskal-Wallis $\chi^2_2 = 20.70$, $p < 0.0001$). However *post hoc* Tukey comparisons between treatments show that both of the significant results are due to differences only between GFP and WLIN offspring (Tukey HSD egg number: $p = 0.02$, viability: $p = 0.003$), whereas there were no statistically significant comparisons between GFP and RFP lines (all Tukey $p > 0.2$). This absence of transgenic line-specific effects indicates estimates of paternity in competitive matings between GFP and RFP males is not confounded by differential offspring viability (Gilchrist and Partridge 1997).

It is of note that GFP males used in the experiment were significantly smaller than focal RFP males (GFP = 2.28 mm ± 0.005 mm, RFP x = 2.51 ± 0.007 mm; Wilcoxon rank sum test, $W = 78574$, $p < 0.0001$).

2.4 MAIN EXPERIMENTS

Two complementary experiments were performed to 1) identify spatiotemporal dynamics of sperm transfer, storage, and ejection in doubly mated females and 2) evaluate the persistence of a focal male's ejaculate in the female reproductive tract through up to seven additional rematings. Both experiments also connected variance in sperm location and non-ejaculate traits (e.g. body size) to patterns of paternity. Together, these experiments enabled identification of the fertilization set as well as sources of variance in postcopulatory success. All experimental copulations were observed and size of all beetles was quantified by measuring the length of the elytron using a stereomicroscope and converting to mm. Sperm numbers of each male stored by females were separately quantified for the bursa and spermatheca using an Olympus BX50 microscope with fluorescent illumination (Olympus, Center Valley, USA). Egg or spermatophore presence in the bursa was also noted.

Experimental matings were observed as described in preliminary experiments. Pairs copulating for less than 30 seconds were allowed additional opportunities to mate because in a preliminary experiment no matings under 33 seconds resulted in sperm transfer (see "confirmation of transgenic line equivalence" above).

2.4.1 Dynamics of sperm transfer and storage – double matings

To determine timing of sperm transfer and storage in doubly mated females, each virgin female was mated to a GFP male followed by a RFP male and flash-frozen at the initiation of a second copulation or 0, 0.25, 0.5, 0.75, 1, 2, 4, or 24 hours following the conclusion of a second mating (n = 60 females / time treatment, male order reversed for half of the matings). For treatments longer than 30 minutes, females were transferred to oviposition dishes filled with 1g of finely sifted medium which was sifted following female freezing and observed under a fluorescent stereoscope to identify eggs and ejected spermatophores (readily observable under fluorescence). All collected eggs were transferred to 10 g of standard medium to enable offspring to mature. Paternity was determined in F1 sons by observing testes for fluorescence (i.e., green scored as "GFP", red or unlabeled scored as "RFP"). To increase the sample size for paternity analyses paternity was also assigned to F1 daughters depending on the presence and genotype of brothers (son mean n = 1.07 ± 0.11, with daughters mean n = 2.57 ± 0.16):

1. Only RFP brothers: daughters oviposit alone for three days; production of any F2 sons with GFP testes indicates F1 daughter was sired by GFP father
2. GFP brothers present: daughters paired to WLIN male for one week for ad libitum mating prior to ovipositing alone in fresh media; proportion of F2 GFP sons is used to assign daughter paternity (> 1 sd below mean = GFP, > 2 sd & < 1 sd = uninformative & removed from analysis (n = 10 of 183), < 2 sd = RFP)
3. No brothers: daughter paired with WLIN male as above; any F2 GFP sons indicate daughter was sired by GFP male

To quantify sperm numbers, reproductive tracts were dissected from females in a drop of phosphate-buffered saline. Presence of eggs or spermatophores, which are easily distinguishable by a long, fibrous tail, was recorded. Sperm from the bursa and spermatheca were separately evacuated in 5 μL drops of PBS by separating each organ into multiple pieces with fine forceps. Contents of the spermatheca were diluted to 10 μL and bursal contents to 10 - 40 μL depending on observed sperm density, and gently mixed. Diluted samples were then counted with a hemacytometer under fluorescent illumination to distinguish RFP and GFP sperm.

Male and female size was quantified by measuring elytron length using a stereomicroscope.

2.4.2 Persistence of sperm – multiple matings

We quantified the persistence of sperm transferred in a single mating by a focal RFP male through up to seven rematings with GFP standard competitors, as well as tracked focal male's fertilization success following each remating. Because males are considerably more likely to encounter females with varied mating histories rather than virgin females, females were housed with GFP males for 48 hours for *ad libitum* mating to establish a representative distribution of sperm in the female reproductive tract prior to focal matings. Females were assigned to one of eight experimental treatments: remating with the RFP focal male followed by 0-7 additional GFP standard competitors (n = 25 females/treatment). Each mating was separated by eight hours, with females ovipositing in standard flour medium between matings. Females were frozen at the conclusion of the oviposition period following their final mating. To establish patterns of sperm use over a longer time frame, females in the seven-rematings treatment were moved to fresh vials of

medium every three days for a total of 30 days. All matings to RFP focal males and GFP standard competitors were conducted as described in the double mating experiment. Some GFP males were reused in two matings (n = 195 of 443 total rematings), but were never used with the same female and were rested in isolation for at least 24 hours before use in a second mating.

Paternity determination, sperm counts, and adult size measurements were completed as described in the double mating experiment.

2.5 STATISTICAL ANALYSIS

All data were analyzed in R 2.12.2 (Team, 2011). Unless otherwise stated, changes in sperm distribution within the female reproductive tract, fecundity, or paternity over time were analyzed with linear modeling following Crawley (2007). All reported values are mean ± standard errors.

2.5.1 Double matings

Changes in sperm distribution within the female reproductive tract over hours post-mating were analyzed with general linear modeling with log-transformed hours post-mating and square-root transformed sperm numbers to improve residual normality and heteroscedasticity. All models constructed for the double mating experiment included second male protamine tag color as an explanatory variable in full models, but this term was never retained in minimal significant models. Females that failed to receive sperm from the first male (n = 6 of 60 females) for the freezing time point directly before a second copulation, or that failed to receive sperm from both males for all subsequent time points

(n = 300 of 540 females) were excluded from analysis. Individual model degrees of freedom vary slightly (i.e. n = 276 – 237) due to missing values from a particular model's predictor variables (e.g., no second male body size, copulation duration not recorded).

Identification of variables influencing paternity outcomes was conducted with generalized linear modeling with a binomial error structure and logit link or a quasibinomial error structure for cases of overdispersion. Models predicting proportion of offspring sired by the second male included the following predictors: copulation duration, proportion of second male sperm in the bursa and spermatheca separately, spermatophore or egg presence in the bursa, hours post-mating, and female and male size. Only females that received sperm from both males and produced offspring (n = 95 of 420 females who were transferred to oviposition dishes) were used in paternity modeling. All reported values are mean ± standard error.

2.5.2 Multiple matings

Changes in proportions of focal male sperm or proportions of focal male progeny over remating intervals was analyzed with Kruskal-Wallis ANOVA, due to non-normality of at least one variable. Identification of variables influencing proportions of focal male sperm in the female reproductive tract or variables influencing paternity outcomes was conducted with linear modeling or generalized linear modeling with quasibinomial error structure to account for overdisperion where appropriate. Models predicting proportion of sperm in the female reproductive tract or proportion offspring sired by the focal RFP male included the same variables as in the double mating experiment. In models of proportion of

offspring paternity, only offspring produced in the oviposition period directly prior to freezing were used for the response variable.

One female in the first freezing treatment had no GFP sperm, suggesting a failure to mate to GFP males prior to the experimental matings, and was excluded from the analysis. Any females that failed to mate at any opportunity were excluded from the remainder of the study (n = 3 of 443 total matings).

3. RESULTS

3.1 DYNAMICS OF SPERM TRANSFER AND STORAGE – DOUBLE MATINGS

Sperm dynamics differed between the bursa and spermatheca following a female's second mating. In the bursa, absolute numbers of first and second male sperm significantly declined over time (first male: $R^2 = 0.08$, $F_{1,276} = 25.46$, $p < 0.0001$, second male: $R^2 = 0.05$, $F_{1,238} = 12.42$, $p = 0.0005$, Figure 3.2a). This decline in sperm number was unbiased by male order such that, after the second copulation, proportions of first versus second male sperm did not change over time ($R^2 = 0.0007$, $F_{1,238} = 0.17$, $p = 0.58$). In contrast, proportions of sperm in the spermatheca significantly differed over time ($R^2 = 0.002$, $F_{1,237} = 11.57$, $p = 0.0008$) due to the number of first male sperm remaining consistent ($R^2 = 0.01$, $F_{1,276} = 1.87$, $p = 0.17$, Figure 3.2b), while second male sperm significantly increased over time ($R^2 = 0.09$, $F_{1,237} = 23.86$, $p < 0.0001$).

Copulation durations were significantly longer for second as compared to first matings (1st copulation = 1.77 ± 0.07 min, 2nd copulation = 2.13 ± 0.08 min; paired Wilcoxon rank sum test, n = 509, $V = 47308.5$, $p < 0.0001$). By using only females that

successfully mated to the second male and were frozen directly after the second mating and prior to any sperm ejection, we determined that copulation duration did not significantly affect the number of sperm transferred by the second male (variables square-root or log transformed for normality, respectively, t-test: t_{17} = -1.27, p = 0.22).

When both males mated successfully and females produced offspring (n = 95 of 216 successful double matings where progeny could have been collected), average proportion of offspring sired by the second male ("P_2") was 0.58 ± 0.04. Variance in P_2 was predicted by proportion of second male sperm in the bursa as well as by an interaction between male and female size (Table 1). The body size interaction is explained by small males having higher P_2 when mating to larger females, whereas larger males achieved higher P_2 when mating to smaller females (Figure 3.3). For females in the 24 h treatment that successfully produced offspring, the number of eggs produced was unrelated to female body size (Spearman's ρ_{29} = 0.94, p = 0.12).

Male body size significantly influenced two aspects of postcopulatory success. When mating to a virgin female, larger males were more successful in transferring a spermatophore (Wilcoxon rank sum test W = 423, n = 61, p = 0.04). Additionally, after a successful copulation, a significant interaction between second male body size and hours post-mating (Table 2) suggests that females mated to larger second males retained spermatophores for longer than did females mated to smaller second males (Figure 3.4).

3.2 PERSISTANCE OF SPERM – MULTIPLE MATINGS

Sperm from most focal males persisted through the entire remating period (e.g. n = 15 of 21 females from the six-remating treatment; Figure 3.5a) and continued to be relevant to fertilization for some pairs (n = 2 of 7 females that produced progeny after a sixth remating; Figure 3.5b). Excluding females that only remated to the focal RFP male, there was a significant decrease in proportion of RFP sperm in the bursa over all rematings (Kruskal-Wallis $\chi^2_7 = 48.60$, $p < 0.0001$, Figure 3.5a). However, neither the proportion (Kruskal-Wallis $\chi^2_7 = 11.88$, $p = 0.10$, Figure 3.5a) nor the absolute number (Kruskal-Wallis $\chi^2_6 = 4.61$, $p = 0.60$) of focal, RFP sperm in the spermatheca differed significantly among remating treatments. Similar to the decline in focal male sperm in the bursa, the proportion of focal males' progeny significantly declined over female rematings (Kruskal-Wallis $\chi^2_6 = 22.75$, $p = 0.0008$; Figure 3.5b). However, paternity did not significantly differ across 3-day oviposition periods for the 30 days following the final remating (n = 22 females, focal male paternity = 0.25 ± 0.02, Kruskal-Wallis $\chi^2_9 = 9.58$, $p = 0.39$).

The minimal model predicting proportion of RFP-male sired offspring, using data pooled across all treatments, retained the single predictor of arcsine, square-root transformed proportion of RFP sperm in the bursa (GLIM with quasibinomial error, *pseudo-*$R^2 = 0.47$, *deviance*$_1 = 54.06$, $p < 0.0001$). Surprisingly, the proportion of RFP sperm in the spermatheca did not contribute significantly to the model.

To determine influences on the proportion of RFP sperm in the bursa during the remating period, we constructed a linear model of proportion bursal sperm predicted by copulation duration, presence of spermatophore and eggs in the female reproductive tract, and the total number of offspring produced during this oviposition period, as well as male

and female body size. Proportion RFP sperm in the bursa was significantly predicted by the positive main effects of spermatophore presence and male body size, along with their interaction (Table 3). The interaction may be explained by a uniformly high proportion of RFP sperm in the bursa whenever females retained a spermatophore, but whenever a spermatophore was ejected prior to freezing, there was a positive relationship between male size and the proportion of sperm in the bursa (spermatophore present: proportion RFP sperm = 0.90 ± 0.03, Spearman's ρ_5 = -0.71, p = 0.14; spermatophore ejected: Spearman's ρ_{15} = 0.52, p = 0.04).

Copulation duration significantly increased across remating periods (Kruskal-Wallis χ^2_7 = 61.72, p < 0.0001). However, using data from females mated only to the focal RFP mating, there was no relationship between copulation duration and number of RFP sperm in the female reproductive tract (Spearman's ρ_{24} = 0.08, p = 0.73).

4. DISCUSSION

We found that dynamics of sperm storage and use in *T. castaneum* differ from general expectation based on studies of other insects, specifically in the identification of the bursa as housing the fertilization set. Moreover, sperm from a single male not only persisted within the female reproductive tract, despite numerous rematings, they continued to be relevant to fertilization.

A growing number of studies are moving beyond the "two competing males" experimental paradigm of estimating P_2 (Boorman and Parker, 1976) by examining sperm relevance when females remate to two males following a focal mating (e.g., in

pseudoscorpions (Zeh and Zeh, 1994), damselflies (Cooper et al., 1996), bulb mites (Radwan, 1997), *T. castaneum* (Arnaud et al., 2001; Lewis and Jutkiewicz, 1998; Lewis et al., 2005), spiders (Elgar et al., 2003), tenebrionid beetles (Drnevich, 2003), *D. melanogaster* (Bjork et al., 2007), and snails (Garefalaki et al., 2010)). However, the fate of sperm beyond that point is unknown in controlled mating studies except for *D. melanogaster,* where after three rematings a first male's sperm is no longer used in any fertilizations (Bjork et al., 2007). Note also that sperm-storage organs of wild-caught female insects have sometimes been found to contain more than three individual male's sperm, indicating sperm persistence through a minimum of three matings is possible in the field (e.g., in crickets (Bretman and Tregenza, 2005), yellow dung flies (Demont et al., 2011), and ants (Holman et al., 2011)).

Contrary to expectation (Pitnick et al. 2009b), sperm from the bursa more strongly influenced fertilization success than did sperm from the specialized sperm-storage organ: the spermatheca. We identified sperm occupying the bursa as the fertilization set, the composition of which was significantly influenced both by male size and female sperm ejection behavior.

The dynamics of sperm storage and use differed between the female's bursa and spermatheca. Following double matings, proportions of first and second male sperm in the bursa were consistent over time, suggesting that the second male's ejaculate does not directionally displace resident sperm. Rather, competing sperm evenly mix prior to female ejection of the remains of the spermatophore and of excess sperm. Results from our multiple mating experiment compliment this interpretation by demonstrating that

repeated mixing of new male sperm followed by ejection of excess sperm leads to a gradual yet significant decrease in the proportion of focal male sperm over many matings. Resident sperm in the spermatheca, however, were more resistant to loss than were resident sperm in the bursa. In both experiments neither the absolute number (double matings) nor the proportion (remating) of focal male sperm significantly changed over all matings. The lack of significant displacement of focal male sperm in the spermatheca is surprising and different from experimental evidence of sperm storage in *D. melanogaster*, the best studied model for sperm dynamics in competitive matings, where 26% of stored first male sperm (i.e. sperm in the paired spermathecae or seminal receptacle) is displaced following only one additional remating (Manier et al., 2010).

Although sperm displacement in the spermatheca appeared to be relatively minimal, based on the RFP focal male displacing 30% of resident sperm following the focal mating, we would have expected greater focal male sperm displacement to have occurred over the seven subsequent standard competitor rematings than was observed, assuming constant sperm displacement (Table S1). This pattern suggests that sperm in all successive rematings was not being transferred or stored as successfully as in the focal mating. Considering our observation that larger males were more successful in sperm transfer and spermatophore retention, limited sperm displacement may be due to the significantly smaller GFP males being less likely to successfully transfer spermatophores.

Results of both experiments support the identification of the bursa as the location of the fertilization set. Proportion of focal male progeny after any number of matings was consistently predicted by the proportion of focal male sperm in the bursa as opposed to the

spermatheca. This is in contrast to most other studies identifying a specialized storage organ as housing the fertilization set in a variety of insects (e.g. (Bretman et al., 2009; Holman et al., 2011; Lüpold et al., 2012; Manier et al., 2013a, 2010; Tyler et al., 2013), but see (Siva-Jothy and Hooper, 1995)).

We found multiple influences of male size, as well as an effect of female ejection behavior, on the composition of the fertilization set. Specifically, in the double mating experiment, larger males experienced two benefits: 1) increased success in spermatophore transfer when mating to a virgin female and 2) longer duration of female spermatophore retention when mating last to a female. This second benefit may similarly explain the influence of male size and spermatophore retention on proportion of focal male sperm in the bursa from the multiple mating experiment, where a greater proportion of sperm from larger males was retained in the bursa, but only when the female had ejected excess sperm at an unknown point during their last 8-hour oviposition period. Specifically, had females in the multiple mating experiment retained spermatophores from larger males for longer, as was observed in the double mating experiment, this would have enabled more sperm from larger males to exit the spermatophore or achieve a position that was more resistant to ejection (Fedina and Lewis, 2015). The relationship between spermatophore ejection and paternity outcomes has yet to be investigated in greater detail.

The sources of variation in postcopulatory success we identified in this study highlight the importance of mating system in determining postcopulatory processes and its potential to shape trait evolution. Extreme promiscuity is supported in this system by a number of factors: (1) beetles aggregate in grain stores (Sokoloff, 1974) and are thus likely

to have high encounter rates with potential mates, (2) *T. castaneum* females directly benefit from mating by receiving moisture in the ejaculates that males transfer (Droge-Young *et al.*, 2016) suggesting females should frequently be receptive to mating attempts, and (3) males lack a strategy to limit female remating (Fedina and Lewis, 2015, 2008). Rapid remating and our finding that sperm are retained in the bursa in substantial numbers 24 hours following matings (14,148 ± 3276.50 total sperm) predict that sperm will routinely be found in the bursa. A consistent reservoir of sperm in the bursa has likely contributed to that organ, as opposed to the expected spermatheca, housing the fertilization set. It is possible that the spermathecal sperm plays a more important role in fertilization in the event that females do not have access to mates, such as during early colonization of a distant grain patch, which warrants further investigation.

The location of the fertilization set in the relatively voluminous and sac-like bursa, as opposed to the narrower and more morphologically constricted spermatheca, has selective implications for male trait evolution. *Tribolium castaneum* fertilization success is likely to be influenced by behaviors, such as successful spermatophore transfer, by the timing of female sperm ejection and by sperm numbers. This is in contrast to a significant role for morphological interactions between sperm and the female reproductive tract that has emerged as a widespread pattern across numerous, diverse taxa. Female reproductive tract morphology, especially that of the sperm-storage organs, evolves rapidly and can vary widely even among closely related species (reviewed by Pitnick *et al.*, 2009b; Puniamoorthy *et al.*, 2010; Higginson *et al.*, 2012), and there is a strong pattern of correlated morphological evolution between sperm and the female sperm-storage organ(s)

across diverse taxa (e.g., Briskie et al. 1997; Pitnick et al. 1999; Presgraves et al. 1999; Morrow and Gage 2000; Minder et al. 2005; Beese et al. 2006; García-González and Simmons 2007), including beetle lineages (Dybas and Dybas, 1981; Higginson et al., 2012; Sasakawa, 2007). There have only been a few direct demonstrations of the interaction mechanisms between sperm and female morphologies and of the resulting influence on competitive fertilization success (García-González and Simmons, 2007; Lüpold et al., 2012; Miller and Pitnick, 2002; Pattarini et al., 2006). Given our unexpected finding that the bursa rather than the spermatheca is the primary source of sperm for fertilization in *T. cataneum*, it is interesting to note that preliminary results from a recent study evaluating relationships across populations of *T. castaneum* with significantly divergent sperm and spermathecal morphologies found no significant correlations among them (M. J. G. Gage, unpublished observation).

Although conjectural, the postcopulatory processes identified here may evolutionarily go hand in hand with the recent finding that *T. castaneum* females accrue hydration benefits through repeated rematings (Droge-Young *et al.*, 2016). That is, selection on females to consistently receive moist spermatophores may underlie use of sperm as a fair raffle from the bursa, along with sperm from any given male remaining relevant to fertilization across numerous rematings. Fair-raffle sperm use evolutionarily sustains selection on males to produce and transfer large spermatophores containing numerous sperm (and, consequently as a constraint, high moisture content). If true, then this system supports Eberhard's (1996) suggestion that female reproductive tract design

and sperm use patterns determine the rules and playing field by which males compete for fertilizations.

4.1 CONCLUSION

We show that *T. castaneum* patterns of sperm storage and use differ from expectations based on previously studied organisms, particularly the more studied *D. melanogaster*. The location of the fertilization set in the bursa rather than the spermatheca has important implications for our understanding of selection on male morphological and behavioral trait evolution. Specifically, the timing of female spermatophore ejection is more likely to generate selection on males than is sperm-storage organ morphology, as seen in other species. The extreme degree of female promiscuity and the unexpected location of the fertilization set may both be influenced by *T. castaneum's* extreme mating system, including hydration benefits to females (Droge-Young et al., 2016). We hope that this study inspires others to investigate mechanisms of postcopulatory sexual selection, particularly across many matings, in a variety of organisms so that we may begin to assemble a comprehensive understanding of the complex relationships between ecology, mating system and the sex-specific targets of postcopulatory sexual selection.

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Table 3.1 Results of the minimally significant generalized linear model with quasibinomial error structure explaining variance in proportion of offspring sired by the second male in the double mating experiment with *T. castaneum* (*pseudo-*R^2 = 0.23, *deviance*$_4$ = 47.04, p < 0.0001).

variance source	**est.**	**se**	***t***	***p***
proportion 2nd male sperm in bursa	2.66	0.68	3.91	0.0002
female size	45.64	17.29	2.64	0.01
male size^2	21.62	8.10	2.67	0.009
female size x male size^2	-8.58	3.25	-2.64	0.01

Table 3.2 Results of the minimally significant generalized linear model with binomial error structure explaining variance in spermatophore presence within the bursa (i.e. spermatophore retained) over hours post-mating from 0.5 – 4 hours after the conclusion of the second copulation in *T. castaneum* (*pseudo-*R^2 = 0.05, *deviance*$_3$= 12.51, *p* = 0.006).

variance source	**est.**	**se**	**t**	**p**
male size	-2.005	2.62	-0.765	0.4442
hours post-mating	-6.702	3.026	-2.215	0.0268
male size x hours post-mating	2.876	1.306	2.202	0.0277

Table 3.3 Results of the minimally significant general linear model explaining variance in proportion of focal male's RFP sperm in the bursa in *T. castaneum* (R^2 = 0.39, $F_{3,18}$ = 3.77, p = 0.03).

variance source	**est.**	**se**	***t***	***p***
male size	0.09	0.03	3.02	0.01
spermatophore presence	8.00	3.50	2.29	0.03
male size × spermatophore presence	-0.10	0.04	-2.26	0.04

FIGURE LEGENDS

Figure 3.1 *Tribolium castaneum* female reproductive tract under fluorescent illumination after mating with an RFP male followed by GFP males. Fluorescently labeled sperm are visible in the main and specialized sperm storage organs (i.e. bursa copulatrix and spermatheca, respectively). Major organs are outlined and labeled. Scale bar represents 200 μm.

Figure 3.2 Numbers of first (open circles) and second male (closed circles) sperm (± se) over hours post-mating in *(a)* the bursa or *(b)* the spermatheca in *T. castaneum*. Average onset of oviposition and ejection indicated by dashed or dotted lines, respectively in *(a)*. Number of females per freezing treatment indicated beneath the x-axis in *(b)*.

Figure 3.3 Relationship between female body size and proportion of second male paternity over quartiles of male body size.

Figure 3.4 Average size of second male to mate (± se) where females had either ejected (open circles) or retained (closed circles) spermatophores until freezing across hours post-mating from 0 to 4 hours after the end of a successful second copulation in *T. castaneum*. Number of females per freezing treatment indicated beneath the x-axis.

Figure 3.5 *(a)* Proportion of focal male's RFP sperm (± se) in the spermatheca and bursa over R1 focal mating and subsequent G1 – G7 GFP rematings and *(b)* proportion of focal male's progeny (± se) over R1 focal mating and subsequent G1 – G6 GFP rematings in *T. castaneum*. Number of females per freezing treatment or oviposition period noted beneath x-axis.

Figure 3.1

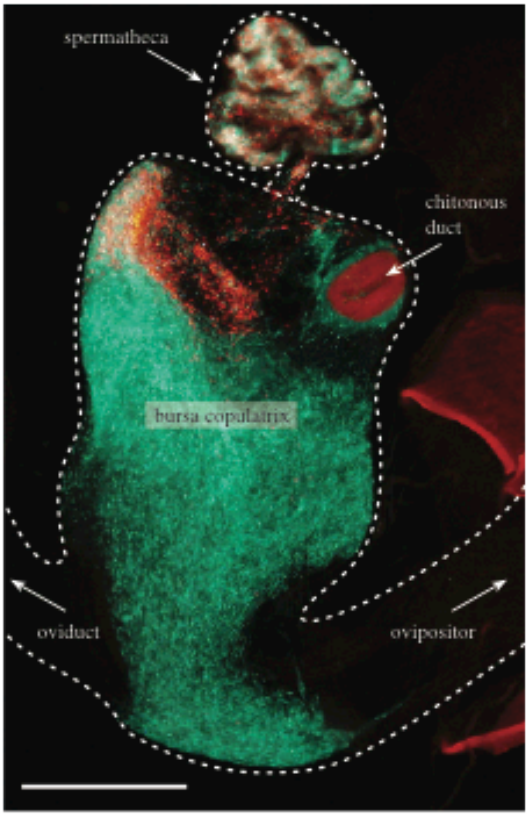

spermatheca
chitonous duct
bursa copulatrix
oviduct
ovipositor

Figure 3.2

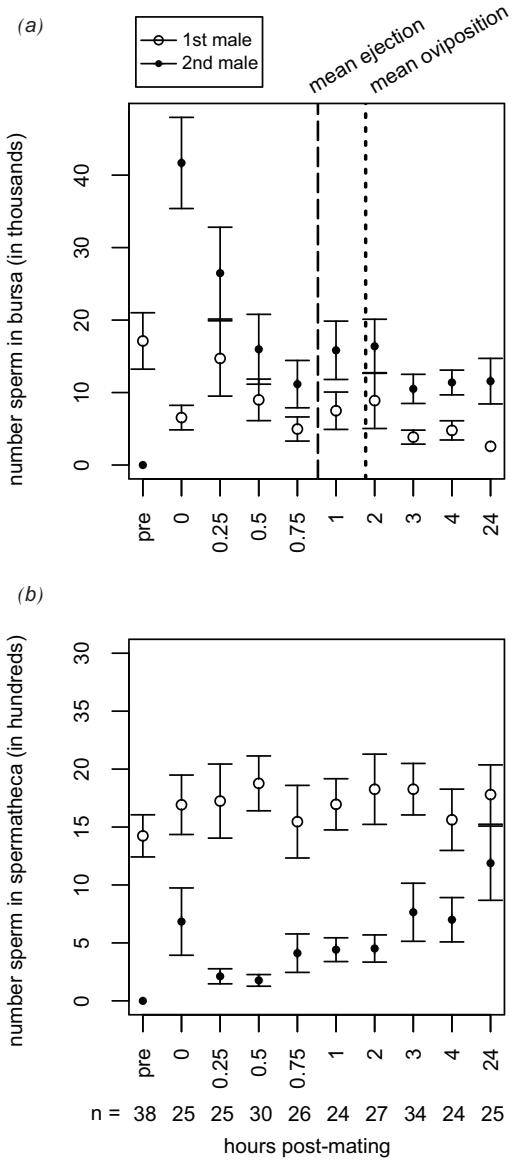

(a)
1st male
2nd male
mean ejection
mean oviposition
number sperm in bursa (in thousands)
0
10
20
30
40
pre
0
0.25
0.5
0.75
1
2
3
4
24
(b)
number sperm in spermatheca (in hundreds)
0
5
10
15
20
35
30
n = 38 25 25 30 26 24 27 34 24 25
hours post-mating

Figure 3.3

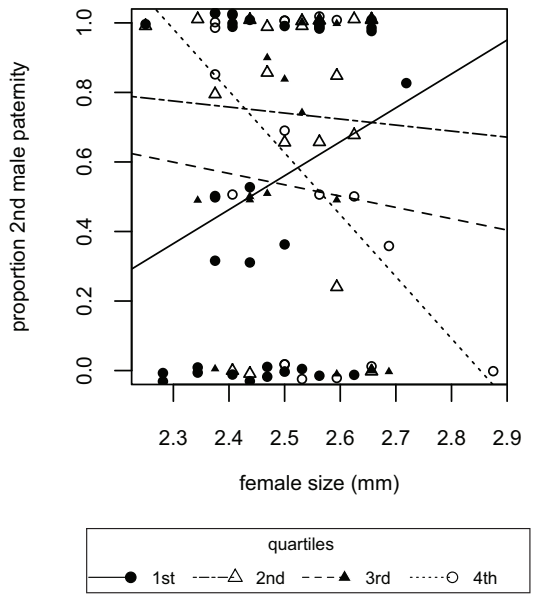

proportion 2nd male paternity
0.0
0.2
0.4
0.6
0.8
1.0
2.3
2.4
2.5
2.6
2.7
2.8
2.9
female size (mm)
quartiles
1st
2nd
3rd
4th

Figure 3.4

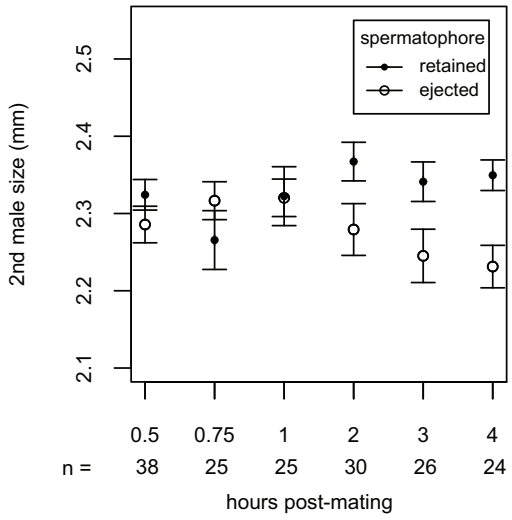
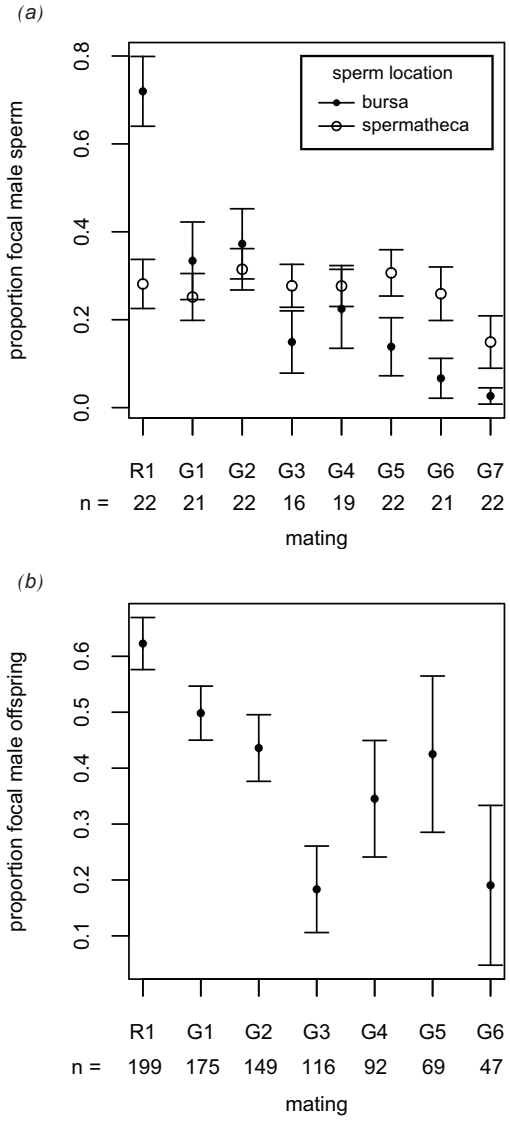

spermatophore
retained
ejected
2nd male size (mm)
2.1
2.2
2.3
2.4
2.5
0.5
0.75
1
2
3
4
n =
38
25
25
30
26
24
hours post-mating

Figure 3.5


(a)
proportion focal male sperm
0.0
0.2
0.4
0.6
0.8
sperm location
bursa
spermatheca
R1 G1 G2 G3 G4 G5 G6 G7
n = 22 21 22 16 19 22 21 22
mating
(b)
proportion focal male offspring
0.1
0.2
0.3
0.4
0.5
0.6
R1 G1 G2 G3 G4 G5 G6
n = 199 175 149 116 92 69 47
mating

VITA

NAME OF AUTHOR: Elizabeth Metta Droge-Young

PLACE OF BIRTH: Denver, Colorado, USA

DATE OF BIRTH: September 23, 1983

GRADUATE AND UNDERGRADUATE SCHOOLS ATTENDED:

Colorado State University, Fort Collins, Colorado, USA

DEGREES AWARDED:

Bachelor of Science in Biology, 2005, Colorado State University

AWARDS AND HONORS:

American Association of University Women: American Dissertation Fellowship

National Science Foundation: Doctoral Dissertation Improvement Grant

Society for the Study of Evolution: Rosemary Grant Award

PROFESSIONAL EXPERIENCE: Teaching Assistant, Department of Biology, Syracuse University, 2010 – 2011, 2012 – 2014, 2015 - 2016