Published in final edited form as:

Behav Ecol Sociobiol. 2013 April ; 67(4): 529–540. doi:10.1007/s00265-012-1472-7.

Sigma virus and male reproductive success in *Drosophila melanogaster*

Clare C. Rittschof,

Department of Biology, University of Florida, P.O. Box 118525, Gainesville, FL 32611-8525, USA

Department of Entomology and Institute for Genomic Biology, University of Illinois, 505 S

Goodwin Avenue, Urbana, IL 61801, USA

Swetapadma Pattanaik,

Department of Biology, University of Florida, P.O. Box 118525, Gainesville, FL 32611-8525, USA

Laura Johnson,

Department of Biology, University of Florida, P.O. Box 118525, Gainesville, FL 32611-8525, USA

Luis F. Matos,

Department of Entomology and Nematology, University of Florida, P.O. Box 110620, Gainesville, FL 32611-0620, USA

Department of Biology, Eastern Washington University, 258 Science Building, Cheney, WA 99004, USA

Jérémié Brusini, and

Department of Biology, University of Florida, P.O. Box 118525, Gainesville, FL 32611-8525, USA

Marta L. Wayne

Department of Biology, University of Florida, P.O. Box 118525, Gainesville, FL 32611-8525, USA

Clare C. Rittschof: ccr22@illinois.edu

Abstract

The risk of disease transmission can affect female mating rate, and thus sexual conflict. Furthermore, the interests of a sexually transmitted organism may align or diverge with those of either sex, potentially making the disease agent a third participant in the sexual arms race. In *Drosophila melanogaster*, where sexual conflict over female mating rate is well established, we investigated how a common, non-lethal virus (sigma virus) might affect this conflict. We gave uninfected females the opportunity to copulate twice in no-choice trials: either with two uninfected males, or with one male infected with sigma virus followed by an uninfected male. We assessed whether females respond behaviorally to male infection, determined whether male infection affects either female or male reproductive success, and measured offspring infection rates. Male infection status did not influence time to copulation, or time to re-mating. However, male infection

Correspondence to: Clare C. Rittschof, ccr22@illinois.edu.

Communicated by S. Cremer

Electronic supplementary material The online version of this article (doi:10.1007/s00265-012-1472-7) contains supplementary material, which is available to authorized users.

did affect male reproductive success: first males sired a significantly greater proportion of offspring, as well as more total offspring, when they were infected with sigma virus. Thus viral infection may provide males an advantage in sperm competition, or, possibly, females may preferentially use infected sperm. We found no clear costs of infection in terms of offspring survival. Viral reproductive success (the number of infected offspring) was strongly correlated with male reproductive success. Further studies are needed to demonstrate whether virus-induced changes in reproductive success affect male and female lifetime fitness, and whether virus-induced changes are under male, female, or viral control.

Keywords

Seminal peptide; Co-evolution; Mate choice; Polyandry; Maternal effect

Introduction

Males and females often have divergent reproductive interests, which can result in sexual conflict and a reproductive arms race (Birkhead et al. 1993; Alatalo et al. 1996; Barta et al. 2002; Ball and Parker 2003; Chapman et al. 2003). Female multiple mating is one common source of sexual conflict (Rowe et al. 1994): because of sperm competition, a male's reproductive success declines with increased numbers of mates per female, while females often benefit directly or indirectly from mating with more than one male (Alcock 1994; Arnqvist and Nilsson 2000; Ball and Parker 2003; Zeh and Zeh 2003). Over evolutionary time, this antagonism has led to a remarkable variety of behavioral and physiological male strategies that inhibit female re-mating with additional males. Male strategies include mate guarding, use of copulatory plugs, and transfer of pheromones and seminal proteins (Barker 1994; Rowe et al. 1994; Chapman 2001; Arnqvist and Rowe 2002; Andersson et al. 2004; Grafe and Bitz 2004). Females, in turn, frequently evolve counter-adaptations to overcome these constraints and mate at an optimum rate, which depends on many factors, but often exceeds one partner (Zeh and Zeh 2003; Perampaladas et al. 2008; Radhakrishnan et al. 2009).

A variety of factors influence optimal female mating rate, including risk of disease transmission from a male to a female or her offspring (Borgia and Collis 1989, Able 1996). Optimal female mating rate may decrease if risk of infection increases with mating frequency (Thrall et al. 2000). Alternatively, female mating rate may increase if, for example, the disease negatively affects sperm viability, and a female must re-mate to achieve maximum fertility. If females evolve the ability to detect male infection status, females may choose against infected males if the costs of foregoing copulation are not prohibitively high (Burley and Foster 2006). However, if costs of passing up a mating opportunity are substantial, females may mate with an infected male but then re-mate with additional males to foster competition between infected and uninfected sperm as a way to increase mean offspring fitness (Simmons 2005). Because females in a population may respond to the presence of disease by altering their choice criteria or mating rate, disease presence is a factor that can intensify or reduce sexual conflict.

The disease-causing agent (e.g., a virus, bacterium, or parasite) is itself under selection to maximize its fitness. It may selfishly influence male or female reproductive behavior in order to increase the rate at which it spreads to new individuals in the population (Knell and Webberley 2004). As a result, disease presence may alter sexual conflict, but with no benefit to either sex. Alternatively, parasites may act cooperatively with one or both sexes when reproductive interests of either sex align with that of the parasite. When interpreting the evolutionary significance of disease-associated changes in male and female reproductive behavior and fitness, it is important to account for the disease itself as a third player in the reproductive arms race.

Here we address whether a common virus influences male and female reproductive success and female mating behavior in *Drosophila melanogaster*. In this species, males transfer seminal proteins that limit female receptivity to subsequent mates, which suggests that female mating rate is a source of sexual conflict (e.g., Chapman et al. 1995; Chapman 2001). *D. melanogaster* is host to a common, vertically transmitted, non-lethal rhabdovirus (negative single-stranded RNA virus) called sigma (L'Héritier and Teissier 1945). This virus is ubiquitous worldwide in natural populations of *D. melanogaster* (Fleuriet 1988). Negative effects of the virus on the host (i.e., virulence) include decreased egg to adult viability (Fleuriet 1996; Wayne et al. 2011), increased development time (Fleuriet 1996), and reduced fitness in crowded conditions (Yampolsky et al. 1999). Interestingly, the virus is transmitted by both sexes: from female to offspring through the eggs, and from father to offspring through individual sperm cells (Fleuriet 1996). Because sigma virus is an intracellular parasite, transferred through individual sperm and not the seminal fluid, only offspring resulting from fertilization by an infected sperm can contract the virus (Brun and Plus 1980).

In this study, we exposed virgin females to two potential male mates in succession in no-choice trials: in one treatment, the first male was infected with sigma virus; and in the other, the first male was uninfected. In both treatments, the second potential mate was uninfected. This ordering allowed us to assess two hypotheses about female response to infection: (1) females avoid copulating with infected males, and (2) because foregoing copulation in a no-choice situation is prohibitively costly, females mate indiscriminately with the first male regardless of his infection status, but respond to infection by mating with an uninfected second male with a shorter latency compared to females whose first mate was uninfected. We assessed female mating behavior, and scored total offspring number and first male paternity to determine whether male viral infection affects either male or female reproductive success. Finally, we analyzed male viral titer (using quantitative polymerase chain reaction [qPCR]) and the rate of viral transmission from sire to offspring to estimate viral reproductive success, and determine whether viral interests align with or diverge from the interests of either sex. We discuss how the effects of sigma virus on host reproductive success may impact the fitness of each of the three co-evolving agents.

Methods

Experimental flies

First male mates—In order to be able to generalize our results across genotypes, the first male mates in our experiment included males from five host genotypes. To generate these

genotypes, we crossed males and females from five uniquely derived, highly inbred laboratory lines (UCD 27, UCD 58, UCD 81, UCD 89, and UCD 134, gift of S.V. Nuzhdin) in a round robin design (e.g., 27×58, 58×81, 81×89, 89×134, 134×27; female parent × male parent). Hereafter, we refer to these round robin crosses as genotypes 1–5. Prior to these crosses, we infected some individuals from each inbred laboratory line with sigma virus, and maintained some individuals as uninfected in separate populations, such that we had an infected and uninfected version of each inbred line. Thus the parental round robin crosses were performed twice, once with all infected parents to generate the infected genotypes and once with all uninfected parents to generate the uninfected genotypes. Three-day-old virgin F₁ heterozygous male progeny from the round-robin crosses (either infected or uninfected) were used as the first male mates in our experiment. Using outcrossed males minimized possible deleterious effects of inbreeding on reproduction and survival. In addition, with an infected and uninfected version of each starting inbred line, we were able to have replicable genotypes across treatments, which allowed us to attribute any treatment effects to virus presence or absence, unconfounded with host genetic differences.

Preparation of virus-infected fly lines—In order to generate infected inbred fly lines, we prepared a viral inoculum for injection from 1,000 sigma-infected flies (600♀+400♂) collected from 12 infected isofemale lines that were previously generated from inseminated, infected females collected in Athens, GA (following Clark et al. 1979). Briefly, we homogenized the infected flies in buffer (HB: 0.005 M Tris–HCl, 0.25 M sucrose, pH 7.5). We centrifuged the homogenate at 1,200×g for 15 min at 2 °C. We centrifuged the supernatant at 6,000×g for 10 min at 2 °C, and then filtered the resulting supernatant through a 0.45-µm filter. We centrifuged the filtered product at 19,500×g for 1 h. We suspended the pellet in 2 ml of HB, aliquoted, and stored at –80°C (Clark et al. 1979).

We injected 1- to 2-day-old female flies from each laboratory line at the second abdominal tergite (i.e., in the vicinity of the ovary) with 0.1 µl inoculum using MINJ-PD Microinjector (Tritech Research, Los Angeles, CA) equipped with a 100-µl syringe and Narishige GD-1 (Serial #08NG1025) needle blanks, which we pulled with a Narishige PC-10 dual-stage puller (East Meadow, NY). Prior to injection we anesthetized the flies on ice. We filled the microinjector tubing, needle holder, and the needle with physiological grade mineral oil (Sigma M8410-100ML; lot #019 K0125) and back-filled the needle with inoculum prior to injection.

After injection, we deposited each fly in a standard food vial along with a male of the same genotype (males were not inoculated). We allowed the flies to recuperate, copulate, and oviposit under standard rearing conditions. Ten days after the injection date, we transferred the offspring from these flies to fresh food. Two weeks after transfer, we haphazardly divided the offspring from each vial into two groups. We exposed one group to CO₂ for 5 min at room temperature and scored for CO₂ sensitivity, which is indicative of infection by sigma virus (L'Héritier and Teissier 1945). Animals who are not infected recover from this anesthesia with virtually zero mortality, while infected animals either die or are paralyzed. If vials contained sensitive flies, we placed the remaining half of the progeny in male–female pairs in fresh vials, under the assumption that some of these flies would carry the virus just as the other half of the sibship. We discarded all progeny from vials where offspring did not

show characteristic CO₂ sensitivity. We continued this process of selection for approximately 3 months, at which point lines produced 100 % infected progeny. We crossed males and females collected from these lines in our round-robin design to generate the infected F₁ males for our experiment.

Prior to the round-robin crosses, we verified infection status for the inbred parental strains. We exposed flies to a stream of CO₂ for 5 min in empty shell vials, and then assessed mortality and/or paralysis following a 30-min recovery period. As expected, lines that we had inoculated with sigma virus showed almost 100 % mortality, verifying that these lines were infected with sigma virus.

Females and second male mates—We used the X-linked recessive phenotypic marker *yellow* (*y*) to assess paternity in the mating experiment. To do this, all females used in our experiment were homozygous for *yellow*, and second male mates were hemizygous for *yellow* (first male mates were wild type for *yellow*). No *yellow* flies were infected with sigma virus. This design only allowed us to assess the effects of viral infection relative to a single genotype of second males and females. It is possible that abnormal characteristics of *y* female or *y* second male behavior explain some patterns of reproductive success in our experiment. However, any such effects are unconfounded with treatment, because we use an identical design in both the uninfected and infected treatments.

The *y* line is a laboratory mutant line (gift of J. V. Fry). These animals were reared at constant density (five females plus five males per vial and allowed to oviposit for 5 days) for at least two generations prior to the experiment. Experimental females were 1-day-old *yellow* (*y*) virgins. Second male mates (3-day-old virgins) were uninfected *y* males. We excluded any males or females with visible wing or leg damage from the experiment, and arbitrarily assigned *y* males and females to treatment categories.

Experimental set-up

Experimental observations took place in the laboratory at consistent temperature and humidity (average 26.2 °C and 55 % relative humidity). We initiated all experiments at 0830 h to control for any effects of circadian rhythm. Prior to the start of the experiment, the virgin *y* females were anesthetized with CO₂ and moved to individual 2.5×9.5 cm polypropylene shell vials containing food, dry Baker's yeast, and paper where they were allowed to acclimate for about 45 min. As expected for uninfected flies, all females recovered from this treatment. After 45 min, the first virgin male of the appropriate infection status and genotype was added to each single female vial. Males were anesthetized on ice (~5 min) prior to transfer to the vials. It took a total of 5–7 min to add all males to the appropriate experimental vials. We initiated mating observations as soon as the final first male was added.

We arranged vials in an arbitrary order and scanned the vials for copulation every 3 min. Because average copulation duration in *D. melanogaster* is on the order of 15 min (Bretman et al. 2010), this scanning time frame was sufficient to allow us to capture copulation behavior. Vials were scanned in a consistent order for the duration of the experiment. We observed experimental vials for 4 h after male introduction, or until the male successfully

copulated with the female, in order to assess whether male infection with sigma virus affected female propensity to mate. We allowed males 20 additional hours with the females to give males more than enough time to successfully copulate with the female so that we could assess possible post-copulatory effects of sigma virus on male and female reproductive output. However, because we did not observe males and females for the entire 24 h, our design did not allow us to determine whether infected or uninfected males copulated for different amounts of time, which could have affected the amount of seminal fluid transferred. During the 20 h following the 4-h observation period, experimental vials were housed in incubators so that we could control day length, temperature, and humidity. Flies were kept at a 12-h/12-h light/dark cycle and constant temperature (25 °C).

After the 24 h copulation period, we used cold anesthesia in order to remove the male. Males were then frozen immediately in individual 1.5-ml tubes at –80°C for later qPCR analysis to determine male viral titer. We returned females to the mating vials and placed the vials back into the incubator for a 24-h oviposition period. Following this period, females were transferred (without anesthesia) to fresh vials of the same dimensions and contents as the originals. We transferred females to new vials prior to the introduction of the second males to insure that chemical cues from the first male did not interfere with the female's assessment of the second male. We then anesthetized the second males (virgin *y* males) on ice and added them to the vials, following the same protocol for male introduction and behavioral observations as used for the first males. Again, vials were returned to the incubator after the 4-h observation period, and males were allowed 20 additional hours to mate with the female. Following the 24-h mating period, males and females were anesthetized on ice, males were removed from the vials, and females were returned to their vials where they remained for 48 h more to lay eggs. After this 48-h period, females were removed, and offspring were left to develop in their vials in the incubator.

After eclosion, we transferred offspring to vials with new food and allowed them to age for 1 day prior to analysis. We transferred offspring from the first vial to new food 10, 12, and 14 days after the start of the experiment, and offspring from the second vial on days 11, 13, and 15. Following the 24 h aging period, all offspring from the infected treatment group were assayed for CO₂ sensitivity, and scored as “alive” or “dead,” an assessment of viral transmission rate. Following this, we tallied offspring sexes and phenotypes to determine first and second male paternity share.

We determined male paternity share by scoring the body color of the female progeny. Because *yellow* is an X-linked recessive gene, all males were hemizygous for *yellow* and therefore uninformative for paternity analysis. Female progeny of first males are wild type (heterozygous for *y*) and female progeny of second males are yellow (homozygous for *y*). Thus, first male paternity share is calculated as the number of wild-type female offspring divided by the total female offspring. Because first and second male matings (and subsequent oviposition) took place in separate vials, we scored paternity using data from vial 2 only. However, we also evaluated total offspring number by summing offspring from vials 1 and 2. Finally, in addition to evaluating male paternity share as a proportion, we evaluated the total number of offspring sired by the first male (first male total share). All vial 1 offspring are attributable to the first male, because the female was singly mated at all times

while in this vial. Total first male offspring from vial 2 is equal to the first male's paternity share from vial 2 (wild-type females/total females) multiplied by the total offspring number in vial 2 in order to account for male offspring. This scaling is necessary because all male progeny exhibit the *yellow* phenotype and thus cannot be unequivocally assigned to either father. However, viral transmission rate (number of infected offspring/total offspring) is an unambiguous indicator of first-male paternity share, and among infected trials we found a tight positive correlation (regression analysis, $R^2=0.92$, $P<0.0001$) between transmission rate to daughters (number of dead females/total females) and transmission rate to sons (number of dead males/total males), which suggests that paternity share is indeed similar across the sexes.

As the observations for the mating experiment were time-intensive, and there were a large number of different genotypes and treatment groups, we performed experimental replicates across three blocks to achieve adequate sample sizes, and to insure that major results were robust across time. Each treatment $\times$ genotype combination was replicated four times within each block.

Viral titer analysis

We determined the viral titer of first male mates in order to verify that males from inoculated lines showed significant levels of viral infection, and to determine whether the degree of infection affects male reproductive success or the rate of viral transmission to offspring.

RNA purification—We performed total RNA purification after randomizing the flies from the three different blocks. Prior to homogenization, we added 200 μ l of cold Trizol reagent to each frozen fly. We then homogenized flies individually using a GenoGrinder with a 96 tube format for 35 s at 1,740 strokes/min. We then incubated the homogenate at room temperature for 5 min. Following the incubation, we added 20 μ l of chloroform to the tubes, and then vortexed the tubes for 15 s. Next, we centrifuged the tubes for 35 min at $3,220\times g$ at 2 °C. We placed the aqueous phase (80 μ l) in a new tube and added 100 μ l of isopropyl alcohol. We gently mixed samples for 1 min and then incubated samples overnight at -20 °C. After this, we centrifuged tubes for 35 min at $3,220\times g$ at 2 °C. We washed pellets with 70 % cold ethanol and then centrifuged them for an additional 15 min at 3,220 rcf and 2 °C. We poured off the ethanol and repeated the 70 % ethanol wash. We allowed tubes to air dry. We re-suspended the dry pellets in 30 μ l of RNAase-free water and then assessed RNA concentration using a NanoDrop Spectrophotometer (ThermoScientific, Wilmington, DE, USA). We stored samples at -80 °C prior to qPCR analysis.

First-strand synthesis—Briefly, 500 ng of total RNA was used for first-strand synthesis in 20- μ l reactions. Viral genomic RNA was selectively reverse-transcribed using a tagged “sense” (forward) primer to the sigma N gene (FplusTag: gcagtatcgtgagttcgagtgatgccgatgacgtgccgtaact, 22 bp of non-sigma sequence followed by 20 bp of sigma-specific sequence). Reactions consisted of 1 μ l FplusTag primer (10 mM), 1 μ l dNTP mix, sample (0.5–11 μ l depending on concentration), and a variable volume of diethylpyrocarbonate treated water to bring the volume to 13 μ l. Reverse transcription was initiated by incubating at 65 °C for 5 min immediately followed by 1 min on ice. Next, 1 μ l

(200 U) of SuperScript III RT, 1 μ l of 10 mM DTT, 1 μ l (40 U) of RnaseOUT, and 4 μ l of 5 $\times$ reverse transcriptase buffer was added to make a final volume of 20 μ l. All reagents were from Invitrogen (San Diego, CA, USA). Reactions were incubated at 55 $^{\circ}$ C for 50 min; the enzyme was then inactivated by incubation at 70 $^{\circ}$ C for 15 min.

qPCR—We ran 15- μ l reactions in triplicate using *TaqMan* (Applied Biosystems, Foster City, CA, USA). For each sample, we mixed 25 μ l of *TaqMan* Universal PCR Master Mix, 0.9 μ l of forward primer, 0.9 μ l of reverse primer, 1.25 μ l of *TaqMan* probe, 11.95 μ l of RNase-free water, and 2 μ l of cDNA. We included a negative control, which contained all the above ingredients except for the cDNA (an additional 2 μ l of RNase-free water were substituted). For plates included in the data analysis, the negative controls showed no amplification. The forward primer matched the tag sequence from the reverse transcription primer (tag, the first part of the FplusTag primer above: gcagatcgtgagttcgagtgt), while the Taqman probe (T probe: catgagatggaggaaactttctctcca) and the reverse (R: gagtcgcagcttggagttc) primer were specific to the sigma N gene. The total length of the amplicon was 156 bp.

Standard curve—We used absolute quantification with gel-purified PCR product of the N gene amplicon. We estimated the concentration of the purified product using the NanoDrop Spectrophotometer (see above). We constructed a standard curve using a five-point 10-fold serial dilution, ranging from 10^6 to 10^3 .

Data analysis

In total, we produced 12 replicates for each genotype combination (five) and infection status (two), for a total of 120 replicate females in the experiment. Seven replicates were omitted from all offspring analyses: (1) for two replicates, females escaped prior to the end of the experiment (but after the behavioral observations); (2) for four replicates, there were dead *yellow* female offspring. Sigma virus is transferred in the sperm itself (Brun and Plus 1980), and all *yellow* female offspring were sired by uninfected *yellow* males, suggesting that these offspring were scored incorrectly; and finally (3) for one uninfected replicate, there were many dead offspring (we assume that this data entry was incorrect). In addition, we plotted male viral titer by infection status to assess whether there were suspicious points in the qPCR data (Fig. 1). In the uninfected category, this revealed four outliers where males were uninfected but contained an appreciable amount of viral RNA, suggesting an error in sample labeling during randomization for the RNA extraction or qPCR procedures. We omitted these four points from analyses involving viral titer data, but included these replicates in all other analyses. Inclusion or exclusion of these four replicates did not qualitatively affect the results described below. All sample sizes are listed in the text, tables, and figures.

We performed statistical analyses using JMP 8.0.2 (SAS Institute, Cary, NC, USA). To determine whether the infection status of a female's first mate affected her time to copulate with a second male, we conducted a Cox Proportional Hazards Analysis (Allison 1995). We used a Wilcoxon sign-ranked test in order to compare viral titers among males, chi-square tests to compare occurrence of copulations across treatments, and two-way ANOVAs to

assess the effect of infection status on first male paternity share, total offspring number, and total first male offspring.

Results

Viral titer analysis

We successfully extracted RNA for males from 94 out of 120 replicates. We averaged triplicate values to derive a mean viral titer for each male. Mean viral titer among infected first males was highly variable (Fig. 1, Fig. S1), although unsurprisingly, overall titer was significantly higher for males in infected versus males in uninfected trials ($Median_{infected}=275.13$ ($N=46$), $Median_{uninfected}=0.52$ ($N=48$); Wilcoxon test, $Z=4.0$, $P<0.0001$; Table 1). Median titer among infected males was similar across genotypes with the exception of genotype 5, where median titer was low and similar to that of uninfected males (Table 1, Fig. S2).

We estimated viral transmission by counting the number of dead offspring after exposure to CO₂. Excluding one discarded replicate (see “Data analysis”), all offspring from uninfected trials survived the assay. Across infected trials, 74 % of replicates had at least some infected offspring. Mean transmission rate (defined as dead wild-type females/total wild-type females) + SE was 44 % +3.3 %. Pooling across blocks, there were significant effects of genotype combination on transmission rate, and similar to the viral titer results, it appears that this effect was driven primarily by a low rate of transmission for genotype 5 (Fig. S3). Because infected males from genotype 5 showed both low viral titers and low transmission rates, genotype 5 was excluded from all further analyses, as the most parsimonious interpretation of these data is that males of this genotype were not infected with sigma virus. Once genotype 5 was excluded, 91 % of infected trials had at least some dead offspring, and mean transmission rate + SE was 55 % +3.5 %, which is in good agreement with past analyses of male transmission rate for sigma virus (Fleuriet 1996; Wayne et al. 2011).

Behavioral observations

We monitored copulation for the first 4 h after male introduction, and within this time frame, 65.6 % of first males successfully copulated ($N=96$; all replicates except genotype 5). A significantly lower percentage of second male mates copulated within 4 h (24 %, chi-square test, $\chi^2=34.8$, $N=96$, $P<0.0001$). First male infection status did not affect whether the first male or second male copulated within the observation window: 63 % of uninfected first males copulated versus 69 % of infected first males ($N_{uninfected}=48$, $N_{infected}=48$; chi-square test, $\chi^2=0.42$; $P=0.52$). In trials with uninfected first males, 29 % of second males copulated versus 19 % of second males in trials with infected first males ($N_{uninfected}=48$, $N_{infected}=48$; chi-square test, $\chi^2=1.4$, $P=0.23$). Across treatments, second males were equally likely to sire at least one offspring ($N_{uninfected}=34$, $N_{infected}=37$; excluding 18 replicates with no offspring in vial 2 and seven other replicates (see Data Analysis), chi-square test, $\chi^2=1.7$, $P=0.19$), which suggests that even outside of our observation time window, second males mated successfully at similar rates across treatments. Finally, the proportional hazards analysis showed that first male infection status did not affect time to copulation for either first or second male mates (Table 2).

Reproductive success

We assessed reproductive success for 89 replicates across both treatments (genotype 5 and seven other suspicious data points were discarded; see “Data analysis”).

Number of offspring—In blocks one and three, total offspring number was higher for trials with infected versus uninfected first males, while in block two there was no difference (Fig. 2). A two-way ANOVA revealed significant effects of block and block $\times$ infection status on total offspring number, but no main effect of first male infection status (Table 3).

Paternity share—We assessed paternity share using only the second of two mating vials because, due to experimental design, the first male sired all offspring in the first vial. In 86 of 89 replicates, females laid at least some offspring in the first vial. In the three cases where females failed to lay offspring in the first vial, offspring were found in the second vial. However, for 18 replicates, there were offspring in the first vial, but not in the second vial, and as a result paternity share could not be determined. Whether or not there were offspring in the second vial did not differ between treatments (trials with no offspring in second vial: infected $N=6$, uninfected $N=12$; chi-square test, $\chi_1^2=2.1$, $P=0.15$).

Pooling across both treatments, first male mates sired a significantly greater percentage of offspring compared to second male mates (Mean difference between first and second male paternity + SE = 35.1 % + 9.3 %, $N=71$ pairs, paired Wilcoxon sign-rank test, $Z=-664.5$, $P<0.0001$). Because not all replicates showed evidence of second male paternity (i.e., 58 % of replicates from the uninfected treatment and 43 % of replicates from the infected treatment had *yellow* female progeny; no difference between treatments, “First male mated” is whether or not the first male was observed mating. The χ^2 value is the result of a likelihood ratio effect test chi-square test, $\chi_1^2=1.7$, $P=0.19$), we assessed first male paternity share in two ways, first including all replicates with offspring in vial 2, and second including only replicates with evidence of second male paternity in vial 2. (1) A two-way ANOVA including all replicates suggested that first male paternity share was higher when the first male was infected as opposed to uninfected with sigma virus (uninfected $N=34$; infected $N=37$ across three blocks; Table 3), although there was a significant block by infection status interaction. 2) When we analyzed male paternity share including only replicates showing evidence of second male paternity, we found that the data trends resemble those of the full analysis (Fig. 3, top right panel versus top left panel). However, likely due to low sample sizes (uninfected $N=20$; infected $N=15$ across three blocks), the overall model does not explain a significant amount of the variation in paternity share (two-way ANOVA, $F_{5,30}=2.3$, $P=0.07$; Table S1).

We assessed the overall effect of infection on male fitness by summing up total first male paternity share. We found that first male mates sired significantly more offspring if they were infected versus uninfected with sigma virus (Table 3), and this pattern is consistent across blocks (Fig. 3, bottom left panel). A similar analysis of first male reproductive success in vial 1 (i.e., offspring laid prior to second male mating) showed a non-significant trend in the same direction (two-way ANOVA, Table 3).

Similar to the paternity analysis, we re-analyzed the effect of infection on total first male paternity share including only replicates for which there was evidence of second male paternity. The model failed to explain a significant amount of variation in total first male offspring number (two-way ANOVA, $F_{5,30}=1.6$, $P=0.19$), although again, the data trends resemble those of the full analysis (Fig. 3, bottom right panel; sample sizes are listed in Table S1). Taken together, these results suggest that sigma virus infection generally results in increased male reproductive success.

Effects of viral titer

Because first male infection affects paternity share and to some degree total offspring number, we assessed whether viral titer (i.e., the severity of male infection) is associated with greater reproductive success. Despite the general advantage of infection (Table 3, Fig. 3), offspring number was not correlated with male viral titer (regression analysis; total offspring: $r^2=0.007$, $F_{1,29}=0.19$, $P=0.66$; total first male share $R^2=0.00002$, $F_{1,29}=0.006$, $P=0.98$), nor was first male paternity share (regression analysis; $R^2=0.03$, $F_{1,24}=0.83$, $P=0.37$).

We evaluated the relationship between male reproductive success and viral fitness as assessed by viral transmission rate (fraction of infected wild-type offspring relative to total wild-type offspring). There was no relationship between mean male titer and transmission rate (regression analysis; $R^2=0.016$, $F_{1,29}=0.47$, $P=0.5$). Viral transmission rate was not affected by total offspring number (regression analysis; $R^2=0.04$, $F_{1,40}=1.84$, $P=0.18$), nor was it related to total offspring sired by the first male (regression analysis; $R^2=0.04$, $F_{1,40}=1.70$, $P=0.20$). First and second male pairings took place in separate vials, and transmission rate did not differ significantly between vials ($N=35$ infected trials with offspring in both vials; Wilcoxon sign-rank test, $Z=24$, $P=0.65$). Thus viral fitness, as measured by viral transmission rate, showed no correlation with male reproductive success or other measured variables.

A second measure of viral fitness is the total number of infected offspring, measured as the number of dead offspring in infected trials following the CO₂ assay. Similar to transmission rate, there was no relationship between mean male titer and the total number of dead offspring ($F_{1,29}=0.014$, $P=0.90$, $R^2=0.0005$). Unsurprisingly, given that transmission rate was consistent across the two vials and that the total number of offspring sired by the first male decreased in vial 2 due to sperm competition, total dead offspring decreased from vial 1 to vial 2 (paired Wilcoxon sign-rank test; $\text{Mean}_{\text{vial 1}}=32.3$, $\text{Mean}_{\text{vial 2}}=18.5$, $\text{SE}=3.5$, $Z=-174.5$, $P<0.0004$), and the total number of dead offspring overall was positively correlated with first male total offspring number ($R^2=0.47$, $F_{1,41}=36.8$, $P<0.0001$). Thus, viral fitness is linearly correlated with male reproductive success.

Discussion

Infected first males sired a significantly greater proportion of offspring compared to uninfected first males (Fig. 3, Table 3). In two out of three blocks, females that mated with an infected first male had more total offspring compared with females who mated with an uninfected first male (Fig. 2), although this trend was not significant by two-way ANOVA

(Table 3). When the effects on paternity share and total offspring number are combined, infected first males sired more total offspring compared to uninfected first males (Fig. 3, Table 3). The total number of offspring infected with sigma virus (based on a CO₂ assay) was strongly correlated with first male reproductive success, while viral transmission rate was not affected by any measured variable. Overall, infection with sigma virus confers a reproductive advantage to males, and male and viral reproductive interests are aligned.

Data for first male reproductive success and total offspring number showed strong block effects in our experiment, driven primarily by block 2. These effects are most appropriately considered to be a form of uncontrolled environmental variation. Although there is no simple explanation for these effects, we note that block effects are common, particularly for behavioral studies (Higgins et al. 2005) and for life history components. One possible source of variation is batch to batch variation in food, which may affect female fecundity or larval condition. Indeed, the ubiquitous nature of block variation is one reason why multiple blocks were included in this study. A single measurement could be misleading, but repeated observations of the same trends across time are more compelling.

Our finding that viral infection improves first male paternity share is unusual. Infection with a virus, bacteria (including *Wolbachia*), or parasite is typically associated with decreased male fertility (Luong and Kaya 2005; de Crespigny and Wedell 2006; Garolla et al. 2011). Within the time frame of our experiment, there were no signs of male fertility costs to infection: viral transmission rate (a measure of the fertilization success rate of infected sperm) was not affected by time elapsed after mating or the presence of rival sperm (i.e., transmission rate was equal between vial 1 and vial 2), which suggests that sperm infected with sigma virus did not show decreased longevity or competitive ability. Our observations are consistent with those of Fleuriot (1981), who noted a slight advantage to infection by sigma virus in male reproductive success. In contrast to some known developmental consequences for the host, here we have shown that infection with sigma virus may positively affect *D. melanogaster* male reproductive success, with consequences for male and female reproductive tactics.

We found no evidence that male infection with sigma virus alters female choice or latency to re-mate (Table 2). Because courtship and copulation behavior did not qualitatively differ across treatments (i.e., we have no reason to believe that infected males forced copulations), our results suggest that females do not respond behaviorally to male infection. It may be that females cannot identify infected males, or simply have not evolved the ability to alter their behavior in response to infection. Another possibility is that male infection with sigma virus has minimal effects on female reproductive success: for example, because sperm infected with sigma virus are as viable and competitive as uninfected sperm, females who copulate with infected males are not sperm-limited (sperm limitation is a common cost of disease presence that is known to affect female mating rates and sexual conflict (Arnqvist and Nilsson 2000)).

Moreover and in contrast to predictions, our results suggest that females who mate with infected males have a marginal advantage in terms of total offspring number (Fig. 2, Table 3), and furthermore they may even benefit by producing infected sons who have a

competitive advantage in the next generation (Fig. 3, Table 3). If the reproductive advantage for infected male progeny offsets offspring viability costs (e.g., Fig. 3; Bartholomew 1970), females may increase their reproductive output to take advantage of offspring infection. Females may even be able to bias fertilization in favor of infected males by altering post-copulatory sperm ejection behavior (Manier et al. 2010), which could explain the higher paternity share of infected sires in our study. If male viral infection imposes only weak costs to females, or is beneficial some of the time, viral presence in males may have no effect on sexual conflict. However, any female strategy characterized by increased reproductive output may come at a cost to future reproduction. Future studies should measure lifetime female reproductive success to definitively assess how viral presence affects female fitness and sexual conflict.

Our study corroborates evidence suggesting that parental origin of sigma virus affects offspring fitness costs. While there is clear evidence that egg to adult offspring viability is lower when the mother is infected with sigma virus (Fleuriet 1981; Wayne et al. 2011), when only the father is infected, there is evidence that viability is unaffected, and may even increase for some genotypes (Fleuriet 1981). In terms of total adult offspring number, females infected with sigma virus produce fewer total offspring relative to uninfected females, while paternal infection (when the mother is uninfected) is associated with slight increases in offspring number (Table 3; Fleuriet 1996). Positive consequences of sigma virus infection in some contexts may explain the maintenance of the virus in populations of *D. melanogaster* over time despite costs of infection.

The relationship between viral or bacterial infection and sexual conflict can be direct or indirect. For example the endosymbiont *Wolbachia* is ubiquitous among arthropods, but it has many different mechanisms to facilitate its invasion into host species populations. Some mechanisms affect reproductive behavior, and as a result have direct effects on sexual conflict. For example, in some species, *Wolbachia* infection causes cytoplasmic incompatibility, which affects female mating rate (de Crespigny et al. 2008). Other beneficial mechanisms that favor invasion, e.g., host nutritional supplementation (Hosokawa et al. 2010; Brownlie et al. 2009) or conferral of viral resistance (Fenton et al. 2011), have only indirect effects on sexual conflict (if any at all). Here we demonstrate that, like *Wolbachia*, sigma virus results in both benefits and costs to the host; however, these effects appear to have only indirect implications for sexual conflict because they do not affect female mate choice or mating rate. Future experiments that examine lifetime reproductive output, male and female reproductive investment, and offspring fitness across multiple generations may clarify how viral presence affects the antagonistic co-evolution between males and females.

Studies that target the mechanisms underlying infected male advantage may provide insight into the potential role of sigma virus in sexual conflict. Differences in first and/or second male mating frequency and duration could explain infected male reproductive advantage since copulation duration is correlated with male reproductive success (Gilchrist and Partridge 2000). During our observation period, males mated at similar frequencies across treatments, and there were no differences in observed time to copulation (latency). Furthermore, second males were equally likely to sire at least some offspring across both

treatments, and thus were similarly likely to copulate at least once regardless of first male infection status. However, it is possible that there were unobserved treatment differences in copulation duration during the 24-h copulation period. Given our finding that viral infection has a positive effect on male reproductive success, future studies should control copulation duration in order to detect whether male advantage is the result of treatment differences in copulation duration or some other post-copulatory process.

Infected first male advantage could result from first males transferring more sperm (Manier et al. 2010) and/or transferring a different composition of seminal compounds compared to uninfected males. This hypothesis does not exclude the aforementioned possibility that infected males copulate longer or at a higher frequency compared to uninfected males (and transfer more ejaculate or sperm in doing so). Post-copulatory progression of oogenesis is induced by *sex peptide* (*Acp70a*), which is transferred by males in the seminal fluid (Soller et al. 1999), and may be transferred in higher quantities by infected males. Seminal peptides could also have indirect effects on paternity share, e.g., by increasing fecundity in the short-term and thereby increasing first male paternity (Herndon and Wolfner 1995; Heifetz et al. 2001). Finally, several seminal peptides are implicated in male sperm competition, e.g., *Acp62F* (Mueller et al. 2008).

It is important to note that our findings of first male paternity advantage across both treatments conflicts with the general finding of last male sperm precedence in *D. melanogaster* (e.g., Boorman and Parker 1976). Sperm precedence is strongly affected by the length of the interval between matings as well as genetic and environmental factors (Boorman and Parker 1976; Clark and Begun 1998; Clark et al. 1999; Lupold et al. 2011). Our inter-mate interval of 1 day, which is relatively short, may have contributed to lower second male paternity share relative to other studies. Also, in order to easily determine paternity, we used *yellow* mutant females and *yellow* second male mates. The *yellow* mutation has been shown to affect reproductive behavior and male mating success (reviewed by Drapeau et al. 2006), and thus may contribute to the overall pattern of first male paternity advantage observed in our study. However, our experimental design does not explain our major result, that relative to uninfected first male mates, first male mates infected with sigma virus have increased reproductive success. Future studies should assess the effects of sigma virus infection on first male reproductive success across a wider array of female and second male genotypes, and at different inter-mate intervals.

If infected male reproductive advantage is a consequence of increased investment in ejaculate, this short-term advantage could come as a trade-off to lifetime reproductive success. If infected males gain an advantage by transferring greater quantities of seminal peptides (e.g., Wigby et al. 2009) or more sperm (Reinhardt et al. 2011) compared to uninfected males, they may become depleted of either or both, limiting future mating ability (Parker 1970; Dewsbury 1982; Pizzari et al. 2008). While early reproductive success seems intuitively important in a highly vagile species that is frequently found in growing populations rather than in stable ones, future studies can more holistically address the effect of viral infection by measuring lifetime male reproductive success. Although our study did not address whether infected males suffer a cost of infection at different stages of reproduction, e.g., in direct pre-copulatory competition with other males, or during the

courtship phase, our results suggest that infected males that survive to adulthood may outperform uninfected males in certain measures of male–male competition.

Future studies will investigate mechanisms to clarify whether males, females, or both sexes are involved in altering patterns of fecundity and/or sperm usage in response to male infection with sigma virus. Due to the importance of offspring survival in assessing viral costs to the host, as well as viral fitness, it may be necessary to measure reproductive success and transmission across host lifetimes and/or multiple host generations. Studies that address the mechanistic basis of virus-induced changes in male reproductive success will also provide insight into the costs and benefits that result from the three-way interaction between the male, female, and virus.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Sebastian Vasquez and Lyvie-Sara Sylvestre for help keeping the flies and setting up experiments, and H. Jane Brockmann and L. Sirot for helpful discussions. This work was funded by National Institutes of Health grant GM083192.

References

- Able DJ. The contagion indicator hypothesis for parasite-mediated sexual selection. *Proc Natl Acad Sci USA*. 1996; 93(5):2229–2233. DOI: 10.1073/pnas.93.5.2229 [PubMed: 8700912]
- Alatalo RV, Burke T, Dann J, Hanotte O, Hoglund J, Lundberg A, Moss R, Rintamaki PT. Paternity, copulation disturbance and female choice in lekking black grouse. *Animal Behaviour*. 1996; 52:861–873. DOI: 10.1006/anbe.1996.0234
- Alcock J. Post-insemination associations between males and females in insects — the mate-guarding hypothesis. *Annu Rev Entomol*. 1994; 39:1–21.
- Allison, PD. *Survival analysis using the SAS system: a practical guide*. SAS Institute; Cary, NC: 1995.
- Andersson J, Borg-Karlson AK, Wiklund C. Sexual conflict and anti-aphrodisiac titre in a polyandrous butterfly: male ejaculate tailoring and absence of female control. *Proc R Soc Lond Ser B-Biol Sci*. 2004; 271(1550):1765–1770. DOI: 10.1098/rspb.2003.2671
- Arnqvist G, Nilsson T. The evolution of polyandry: multiple mating and female fitness in insects. *Animal Behaviour*. 2000; 60:145–164. [PubMed: 10973716]
- Arnqvist G, Rowe L. Antagonistic coevolution between the sexes in a group of insects. *Nature*. 2002; 415(6873):787–789. [PubMed: 11845208]
- Ball MA, Parker GA. Sperm competition games: sperm selection by females. *J Theor Biol*. 2003; 224(1):27–42. DOI: 10.1016/s0022-5193(03)00118-8 [PubMed: 12900202]
- Barker DM. Copulatory plugs and paternity assurance in the nematode *Caenorhabditis elegans*. *Animal Behaviour*. 1994; 48(1):147–156. DOI: 10.1006/anbe.1994.1221
- Barta ZN, Houston AI, McNamara JM, Szekely T. Sexual conflict about parental care: the role of reserves. *Am Nat*. 2002; 159(6):687–705. DOI: 10.1086/339995 [PubMed: 18707390]
- Bartholomew GA. A model for the evolution of pinniped polygyny. *Evolution*. 1970; 24(3):546–559.
- Birkhead TR, Moller AP, Sutherland WJ. Why do females make it so difficult for males to fertilize their eggs. *J Theor Biol*. 1993; 161(1):51–60. DOI: 10.1006/jtbi.1993.1039
- Boorman E, Parker GA. Sperm (ejaculate) competition in *Drosophila melanogaster*, and reproductive value of females to males in relation to female age and mating status. *Ecol Entomol*. 1976; 1(3): 145–155. DOI: 10.1111/j.1365-2311.1976.tb01217.x

- Borgia G, Collis K. Female choice for parasite-free male satin bowerbirds and the evolution of bright male plumage. *Behav Ecol Sociobiol.* 1989; 25(6):445–453. DOI: 10.1007/bf00300191
- Bretman A, Fricke C, Hetherington P, Stone R, Chapman T. Exposure to rivals and plastic responses to sperm competition in *Drosophila melanogaster*. *Behavioral Ecology.* 2010; 21(2):317–321. DOI: 10.1093/beheco/arp189
- Brownlie JC, Cass BN, Riegler M, Witsenburg JJ, Iturbe-Ormaetxe I, McGraw EA, O'Neill SL. Evidence for metabolic provisioning by a common invertebrate endosymbiont, *Wolbachia pipientis*, during periods of nutritional stress. *PLoS pathogens.* 2009; 5(4):e1000368. [PubMed: 19343208]
- Brun, G.; Plus, N. The viruses of *Drosophila*. In: Ashburner, M.; Wright, TRF., editors. The genetics and biology of *Drosophila*. Vol. 2. Academic Press; London: 1980. p. 626–672.
- Burley NT, Foster VS. Variation in female choice of mates: condition influences selectivity. *Animal Behaviour.* 2006; 72:713–719. DOI: 10.1016/j.anbehav.2006.01.017
- Chapman T. Seminal fluid-mediated fitness traits in *Drosophila*. *Heredity.* 2001; 87:511–521. [PubMed: 11869341]
- Chapman T, Liddle LF, Kalb JM, Wolfner MF, Partridge L. Cost of mating in *Drosophila melanogaster* females is mediated by male accessory gland products. *Nature.* 1995; 373(6511):241–244. [PubMed: 7816137]
- Chapman T, Arnqvist G, Bangham J, Rowe L. Sexual conflict. *Trends Ecol Evol.* 2003; 18(1):41–47.
- Clark AG, Begun DJ. Female genotypes affect sperm displacement in *Drosophila*. *Genetics.* 1998; 149(3):1487–1493. [PubMed: 9649536]
- Clark MA, McCrady WB, Fieldling CL. Independent production of CO₂ sensitivity by nuclear gene-ly and a transmissible agent from delayed-recovery *Drosophila melanogaster*. *Genetics.* 1979; 92:503–510. [PubMed: 114449]
- Clark AG, Begun DJ, Prout T. Female × male interactions in *Drosophila* sperm competition. *Science.* 1999; 283(5399):217–220. DOI: 10.1126/science.283.5399.217 [PubMed: 9880253]
- de Crespigny FEC, Wedell N. *Wolbachia* infection reduces sperm competitive ability in an insect. *Proc R Soc Lond Ser B-Biol Sci.* 2006; 273(1593):1455–1458. DOI: 10.1098/rspb.2006.3478
- de Crespigny FEC, Hurst LD, Wedell N. Do *Wolbachia*-associated incompatibilities promote polyandry? *Evolution.* 2008; 62(1):107–122. DOI: 10.1111/j.1558-5646.2007.00274.x [PubMed: 18067571]
- Dewsbury DA. Ejaculate cost and male choice. *Am Nat.* 1982; 119(5):601–610.
- Drapeau MD, Cyran SA, Viering MM, Geyer PK, Long AD. A *cis*-regulatory sequence within the yellow locus of *Drosophila melanogaster* required for normal male mating success. *Genetics.* 2006; 172(2):1009–1030. DOI: 10.1534/genetics.105.045666 [PubMed: 16272418]
- Fenton A, Johnson KN, Brownlie JC, Hurst GDD. Solving the *Wolbachia* paradox: modeling the tripartite interaction between host, *Wolbachia*, and a natural enemy. *Am Nat.* 2011; 178(3):333–342. DOI: 10.1086/661247 [PubMed: 21828990]
- Fleuriet A. Comparison of various physiological traits in flies (*Drosophila melanogaster*) of wild origin, infected or uninfected by the hereditary rhabdovirus sigma. *Archives of Virology.* 1981; 69(3–4):261–272. DOI: 10.1007/bf01317341 [PubMed: 6794546]
- Fleuriet A. Maintenance of a hereditary virus, the sigma virus in populations of its host, *Drosophila melanogaster*. *Evolutionary Biology.* 1988; 23:1–30.
- Fleuriet A. Polymorphism of the *Drosophila melanogaster* — Sigma virus system. *J Evol Biol.* 1996; 9:471–484.
- Garolla A, Pizzol D, Foresta C. The role of human papillomavirus on sperm function. *Curr Opin Obstet Gynecol.* 2011; 23(4):232–237. DOI: 10.1097/GCO.0b013e328348a3a4 [PubMed: 21666465]
- Gilchrist AS, Partridge L. Why it is difficult to model sperm displacement in *Drosophila melanogaster*: the relation between sperm transfer and copulation duration. *Evolution.* 2000; 54(2):534–542. DOI: 10.1554/0014-3820(2000)054[0534:wildtm]2.0.co;2 [PubMed: 10937230]
- Grafe TU, Bitz JH. Functions of duetting in the tropical boubou, *Laniarius aethiopicus*: territorial defence and mutual mate guarding. *Animal Behaviour.* 2004; 68:193–201. DOI: 10.1016/j.anbehav.2003.11.003

- Heifetz Y, Tram U, Wolfner MF. Male contributions to egg production: the role of accessory gland products and sperm in *Drosophila melanogaster*. *Proc R Soc Lond Ser B-Biol Sci*. 2001; 268(1463):175–180.
- Herndon LA, Wolfner MF. A *Drosophila* seminal fluid protein, ACP26AA, stimulates egg-laying in females for 1 day after mating. *Proc Natl Acad Sci USA*. 1995; 92(22):10114–10118. DOI: 10.1073/pnas.92.22.10114 [PubMed: 7479736]
- Higgins LA, Jones KM, Wayne ML. Quantitative genetics of natural variation of behavior in *Drosophila melanogaster*: the possible role of the social environment on creating persistent patterns of group activity. *Evolution*. 2005; 59(7):1529–1539. DOI: 10.1554/04-762 [PubMed: 16153038]
- Hosokawa T, Koga R, Kikuchi Y, Meng X-Y, Fukatsu T. *Wolbachia* as a bacteriocyte-associated nutritional mutualist. *Proc Natl Acad Sci USA*. 2010; 107(2):769–774. DOI: 10.1073/pnas.0911476107 [PubMed: 20080750]
- Knell RJ, Webberley KM. Sexually transmitted diseases of insects: distribution, evolution, ecology and host behaviour. *Biological Reviews*. 2004; 79(3):557–581. [PubMed: 15366763]
- L'Héritier P, Teissier G. Transmission héréditaire de la sensibilité au gaz carbonique chez la *Drosophile*. *Publ lab ENS Biologie*. 1945; 1:35–76.
- Luong LT, Kaya HK. Sexually transmitted nematodes affect spermatophylax production in the cricket, *Gryllobates sigillatus*. *Behav Ecol*. 2005; 16(1):153–158. DOI: 10.1093/beheco/arh146
- Lupold S, Manier MK, Ala-Honkola O, Belote JM, Pitnick S. Male *Drosophila melanogaster* adjust ejaculate size based on female mating status, fecundity, and age. *Behavioral Ecology*. 2011; 22(1): 184–191. DOI: 10.1093/beheco/arq193
- Manier MK, Belote JM, Berben KS, Novikov D, Stuart WT, Pitnick S. Resolving mechanisms of competitive fertilization success in *Drosophila melanogaster*. *Science*. 2010; 328(5976):354–357. DOI: 10.1126/science.1187096 [PubMed: 20299550]
- Mueller JL, Linklater JR, Ravi Ram K, Chapman T, Wolfner MF. Targeted gene deletion and phenotypic analysis of the *Drosophila melanogaster* seminal fluid protease inhibitor Acp62F. *Genetics*. 2008; 178:1605–1614. [PubMed: 18245332]
- Parker GA. Sperm competition and its evolutionary consequences in the insects. *Biological Reviews*. 1970; 45:525–567.
- Perampaladas K, Stoltz JA, Andrade MCB. Mated redback spider females re-advertise receptivity months after mating. *Ethology*. 2008; 114(6):589–598. DOI: 10.1111/j.1439-0310.2008.01513.x
- Pizzari T, Dean R, Pacey A, Moore H, Bonsall MB. The evolutionary ecology of pre- and post-meiotic sperm senescence. *Trends Ecol Evol*. 2008; 23(3):131–140. DOI: 10.1016/j.tree.2007.12.003 [PubMed: 18280006]
- Radhakrishnan P, Perez-Staples D, Weldon CW, Taylor PW. Multiple mating and sperm depletion in male Queensland fruit flies: effects on female remating behaviour. *Animal Behaviour*. 2009; 78(4): 839–846. DOI: 10.1016/j.anbehav.2009.07.002
- Reinhardt K, Naylor R, Siva-Jothy MT. Male mating rate is constrained by seminal fluid availability in bedbugs, *Cimex lectularius*. *PLoS ONE*. 2011; 6(7):e22082.doi: 10.1371/journal.pone.0022082 [PubMed: 21779378]
- Rowe L, Arnqvist G, Sih A, Krupa J. Sexual conflict and the evolutionary ecology of mating patterns — water striders as a model system. *Trends Ecol Evol*. 1994; 9(8):289–293. DOI: 10.1016/0169-5347(94)90032-9 [PubMed: 21236857]
- Simmons LW. The evolution of polyandry: sperm competition, sperm selection, and offspring viability. *Annu Rev Ecol Evol Syst*. 2005; 36:125–146. DOI: 10.1146/annurev.ecolsys.36.102403.112501
- Soller M, Bownes M, Kubli E. Control of oocyte maturation in sexually mature *Drosophila* females. *Developmental Biology*. 1999; 208(2):337–351. DOI: 10.1006/dbio.1999.9210 [PubMed: 10191049]
- Thrall PH, Antonovics J, Dobson AP. Sexually transmitted diseases in polygynous mating systems: prevalence and impact on reproductive success. *Proc R Soc Lond Ser B-Biol Sci*. 2000; 267(1452): 1555–1563.

- Wayne ML, Blohm GM, Brooks ME, Regan KL, Brown BY, Barfield M, Holt RD, Bolker BM. The prevalence and persistence of sigma virus, a biparentally transmitted parasite of *Drosophila melanogaster*. *Evol Ecol Res.* 2011; 13:323–345.
- Wigby S, Sirot LK, Linklater JR, Buehner N, Calboli FCF, Bretman A, Wolfner MF, Chapman T. Seminal fluid protein allocation and male reproductive success. *Current Biology.* 2009; 19(9):751–757. DOI: 10.1016/j.cub.2009.03.036 [PubMed: 19361995]
- Yampolsky LY, Webb CT, Shabalina SA, Kondrashov AS. Rapid accumulation of a vertically transmitted parasite triggered by relaxation of natural selection among hosts. *Evol Ecol Res.* 1999; 1:581–589.
- Zeh JA, Zeh DW. Toward a new sexual selection paradigm: polyandry, conflict and incompatibility (Invited article). *Ethology.* 2003; 109(12):929–950.

Fig. 1. Box plot of male viral titer for uninfected and infected males with all genotypes included. Points represent outliers below the 10th percentile or above the 90th percentile, $N=44$ infected, $N=50$ uninfected

Fig. 2.

Mean total offspring +1 SE by block and first male infection status. There was a significant effect of block ($P < 0.004$) and block $\times$ infection status ($P < 0.04$) on total offspring number

Fig. 3.

Top two panels show mean first male paternity share (wild-type female offspring/total female offspring)+1 SE by block and first male infection status. When all trials are included (*left top panel*), there are significant effects of block ($P<0.016$), infection status ($P<0.015$), and their interaction ($P<0.007$) on first male paternity share. When only trials with evidence for second male paternity are assessed (*top right panel*), results are not significant, but trends are similar to the first analysis. Bottom two panels show mean total first male offspring (all offspring from vial 1 + first male paternity share $\times$ total offspring in vial 2) + 1 SE by block and first male infection status. When all trials are included (*bottom left panel*), there are significant effects of block ($P<0.03$) and infection status ($P<0.009$) on first male total offspring. There was no significant effect of block $\times$ infection status. When only trials with evidence for second male paternity are assessed (*bottom right panel*), results are not significant, but trends are similar to the first analysis.

Table 1

Viral titer with 95 % confidence limits divided by genotype (1–5) and male infection status (Uninfected versus Infected). These data exclude four outliers reflecting qPCR error

Genotype (N)	Median titer	Mean titer	Low CI	High CI
1 (7) Uninfected	0.167	54.5	-78.0	187.0
2 (9)	1.000	1.32	0.19	2.5
3 (8)	0.522	0.62	0.22	1.0
4 (9)	0.75	3.66	-3.11	10.42
5 (11)	0.307	12.55	-13.82	38.91
1 (9) Infected	325.3	392.5	140.0	645.0
2 (10)	483.3	505.2	126.3	884.1
3 (8)	240.41	238.0	77.9	398.1
4 (8)	591.85	735.6	127.8	1343.4
5 (11)	0.879	212.3	-48.5	473.2

Table 2

Proportional hazards analyses for latency to mate with the first and second male mate

First male proportional hazards analysis ($N_{\text{uninfected}}=48$, $N_{\text{infected}}=48$)			
Source	<i>df</i>	χ^2	<i>P</i>
Block	2	26.3	0.0001
Infection status	1	2.3	0.13
Second male proportional hazards analysis ($N_{\text{uninfected}}=48$, $N_{\text{infected}}=48$)			
Source	<i>df</i>	χ^2	<i>P</i>
Block	2	7.8	0.02
Infection status	1	1.2	0.27
First male mated	1	2.7	0.10

Table 3

Two-way ANOVA offspring analyses

Total offspring number ($N_{\text{uninfected}}=46$, $N_{\text{infected}}=43$)				
Source	<i>df</i>	Effect size	<i>F</i>	<i>P</i>
Block	2	16.0	5.9	0.004
Infection status	1	3.8	1.0	0.32
Block $\times$ Infection status	2	10.5	3.4	0.04
First male paternity share (V2: WT F/Total F; $N_{\text{uninfected}}=34$, $N_{\text{infected}}=37$)				
Block	2	0.07	4.5	0.016
Infection status	1	0.11	6.8	0.015
Block $\times$ Infection status	2	0.15	5.3	0.007
First male total offspring (all offspring in V1+WT F/Total F in V2 * Total offspring in V2; ($N_{\text{uninfected}}=46$, $N_{\text{infected}}=43$))				
Block	2	6.6	3.6	0.030
Infection status	1	9.0	7.1	0.009
Block $\times$ Infection status	2	1.7	0.3	0.729
Total offspring in vial 1 only; ($N_{\text{uninfected}}=46$, $N_{\text{infected}}=43$)				
Block	2	2.7	1.3	0.27
Infection status	1	3.6	2.3	0.13
Block $\times$ Infection status	2	3.7	2.3	0.10

For Infection status, a positive effect size means that first male infection with sigma virus had a positive effect on the dependent variable, while a negative effect means male infection had a negative effect. Block and Block $\times$ Infection status values represent the average effect, ignoring the direction of the effect. For first male total offspring

V1 vial 1, V2 vial 2