MULTIPLE MATING AND *SEX-RATIO* DRIVE IN *DROSOPHILA NEOTESTACEA*

by

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(Under the Direction of KELLY DYER)

ABSTRACT

Selfish genetic elements (SGEs) bias their own transmission into the next generation, often at the expense of the fitness of its carrier. SGEs are a major source of intragenomic conflict, and may have consequences for sexual selection and mating systems, evolutionary change and innovation, and population size and growth rate. X-chromosome meiotic drive, also known as *sex-ratio* (SR) drive, occurs when an X-chromosome causes Y-bearing sperm to die during spermatogenesis such that only female offspring are produced. Theoretically, its large transmission advantage may allow SR to spread very rapidly and ultimately cause its host population to go extinct due to a lack of males. Despite many decades of study, it is currently unknown how SR is maintained as a stable polymorphism in the absence of any costs to female carriers or of genetic suppressors. To better understand how this may occur, we use the SR drive system in the mushroom-feeding fruit fly *Drosophila neotestacea*. The prevalence of SR chromosomes exists between 0 – 30% throughout the species range and this polymorphism has been stable for at least 20 years. Here we investigate the relationship between female remating in the wild and SR prevalence, evaluate the fertility cost that SR males bear relative to standard males, and examine sperm competitive ability of SR males. We find that male fertility costs of *sex-ratio* drive and rates of female remating may be important factors involved in maintaining this selfish genetic element in nature.

INDEX WORDS: Selfish genetic element, intragenomic conflict, meiotic drive, X-chromosome, mating system

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M.S., University of Central Florida, 2010

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A Dissertation Submitted to the Graduate Faculty of The University of Georgia in Partial Fulfillment of the Requirements for the Degree

DOCTOR OF PHILOSOPHY

ATHENS, GEORGIA

2014

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August 2014

ACKNOWLEDGEMENTS

First and foremost I would like to thank my partner, Scott Paterson. Only with his support through some very tough times, was I able to achieve this goal.

I would like to thank my adviser Kelly Dyer, and committee members Shumei Chang, Dave Hall, Daniel Promislow, and John Wares for all of their hard work. I am appreciative for their constructive feedback and professional support throughout this dissertation.

My peers have proven to be excellent teachers and company during this time, including Emily Bewick, Louisa Carter Staton, Amanda Kenney, Jenna Hamlin, Caitlin Conn, and so many others. My lab mates have been essential to helping get tasks done that were at times too big for one person, giving honest feedback on talks, papers, and proposals, and keeping me sane during the day to day grind: thanks current lab members- Brooke White, Nick Arthur, Katie Pieper, Devon Gaydos, Devon Humphreys, Alicia Schmidt, Kathryn Clark, and Randall English; and former lab members- Michael Bray, Agy Gaertig, Jesse Lopez, Rachel Webster, and especially Emily Fawcett for all of her help and always positive attitude. Many thanks to all the staff in the Genetics office, Eddie Mae Lay, James, Jeanice, Carmen, and everyone at UGA Genetics who always brightens my day with every kind interaction.

Finally, I'd like to extend my gratitude to the members of the Athens community, especially at the UGA health center and Rubber Soul Yoga. You've taught me how to live more skillfully and give my energy back to others, and for these lessons I am forever grateful.

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Selfish genetic elements (SGE) are harmful to their host organism, other parts of their host's genome, or the host's population, yet spread rapidly due to their selfish nature (reviewed in [1, 2]). For example, cancer is considered an SGE that is harmful to its host's cells. The spread of SGEs can cause genetic conflict because their spread can be costly to other portions of the genome. Finally, SGEs can be detrimental to organisms by decreasing survival and fertility through changes in things like sex ratio, genetic incompatibilities, and genome structure (reviewed in [3-5]).

There are diverse ways that SGEs function and spread through populations. For example transposable elements, also known as genome parasites, copy themselves and reinsert into other places of the genome [3,4]. Reproductive parasite SGEs spread by altering host reproduction so that they are spread more frequently, such as cytoplasmic male sterility (CMS) and sex chromosome meiotic drive. Cytoplasmic male sterility takes place when the mitochondria (which is passed maternally) increase female fertility at the expense of pollen production, so that they are passed on more frequently. Sex chromosome meiotic drive can occur on the X chromosome, also known as *sex-ratio* (SR) drive, which prevents the development of Y sperm in males. In most species, females have XX sex chromosomes and males have XY. All female eggs have one X chromosome, and males have sperm such that 50% have an X chromosome and 50% have a Y.

So when a male has the SR X-chromosome, he only produces female offspring (reviewed in [3, 4]). Therefore this SGE is harmful to the host's cells (Y sperm), causes genetic conflict (detrimental to genes on the Y and any non-sex, or autosomal chromosomes that are found along with Y, and causes competition with normal X chromosomes), and can be harmful to populations: if SR spreads the population can go extinct due to a lack of males [6].

SR drive should spread very rapidly because of its transmission advantage. Under laws of Mendelian Inheritance, we expect genes to be passed on to 50% of a parent's offspring (in the case of male sex chromosomes in sperm, 50% X and 50% Y). However, SR drive is non-Mendelian because it is passed on 100% of the time instead. Because of the strong genetic conflict SR drive creates, some genomes have evolved suppressors that cancel out or modify the effects of this SGE [7-9]. At a population level, it is very advantageous for these suppressors to spread to all individuals, allowing them to avoid the negative effects of SR drive. If a population has not been able to evolve suppressors for any reason, we might expect to see local extinction where SR was introduced, or if it spreads to enough individuals across many populations, that it could even cause the extinction of the entire species [6]. Therefore, one would not expect to see any active SR drive SGEs in nature.

However, active SR drivers do exist in several species and have been around for a long time without spreading to high frequency and thus causing extinction (reviewed in [5, 10]). A well-studied group of animals that have SR drive are fruit flies. If one goes out into the nature and collects wild fruit flies, it is straightforward to identify if there is a *sex-ratio* driving chromosome present. By placing a female on food, and letting her offspring emerge, if she mated with an SR male then she will have 100% female offspring, where other females who mated with standard (ST) males will have the normal 50% female offspring. In the laboratory, fruit flies can

be maintained very easily. If we collect many fruit flies from a single place, we can take wild-caught males and place them with females raised in the laboratory (that are normal: ST/ST). This way, we can get an estimate of the prevalence of SR in the population by counting the number of males that produced all daughter offspring (these carry SR), the number of males that had normal offspring (these carry ST), and seeing what proportion of the total of the males carry SR. We can do this in many different locations and find out how common SR chromosomes are across a species.

We study a specific species of fruit fly called *Drosophila neotestacea*, which feeds on mushrooms instead of fruit. They live in North America in cooler, moist forests where mushrooms are found. SR prevalence has been estimated across much of its range, and it varies from 0 – 30% depending on where it is [11, 12], with colder climates tending to have higher SR prevalence [11]. Interestingly, these frequencies have been largely stable for over 20 years. Considering that we expect SR to spread very rapidly, it is unknown why its prevalence has been stable over time at low to moderate frequencies [6].

There are many reasons why SR may not spread as fast as expected. One reason is if individuals with one or two SR chromosomes have lower survival, or are less fit (see [13]). This however does not appear to be the case in *D. neotestacea*, as females do not have reduced viability [14]. A second way is if females choose to mate with ST males instead of SR males. Evidence for this pre-mating choice has not been found in this species [14], but we will reevaluate this possibility in this dissertation. Lastly, another way is if SR individuals have lower reproductive fertility, than ST individuals [15]. Females in this species have similar fertility regardless of SR status [14]. However, may be fertility costs for males especially after multiple mating. Since SR males lose half their sperm during spermatogenesis, they may have a

disadvantage against normal males due to their inability to keep up with sperm production. In the same vein, female mating rate is also important as it is closely associated with male multiple mating: if females mate often then males will also mate often and differences in SR male fertility will become magnified. If SR males cannot keep up in sperm production and get depleted more easily, due to just the difference in the number of SR sperm, differences in SR and ST male fertility will be greater after many matings, and it is possible that SR males may be depleted for longer periods of time than ST males. Finally, sperm competitive ability may be reduced in SR males and could further reduce their fertility, if SR sperm sire fewer offspring than expected given the proportion of sperm they pass [16]. In many species with SR, several studies have shown that SR males are poor sperm competitors [17-20]. If this were true in *D. neotestacea*, this could even further reduce the transmission advantage of SR, and possibly explain why we see SR at intermediate frequencies in natural populations.

The populations of *D. neotestacea* are have little genetic differentiation, and there is a lot of migration across its range [11, 21]. The means SR have the potential to move freely among populations, yet in the face of this gene flow, SR frequencies are stable. This suggests that local selection underlies the patterns we observe in nature [11]. In addition to multiple mating by both females and males, local population conditions may play a role in regulating SR prevalence. In this dissertation, first we investigate the potential for the female remating rate to affect SR dynamics in natural populations. In the laboratory, we found that females from populations where SR is rare mate significantly more often than females from populations where SR is common. We also found that only when males mate multiply that the average fertility of SR males decrease to a level that can prevent SR from spreading. Differences in the female mating rate among populations may contribute to SR dynamics in the wild, and the outcome of this

genetic conflict. A similar pattern has subsequently been identified in *D. pseudoobscura* [22]. Finally, we also find evidence for a localized population crash due to SR, which may be due to habitat degradation as well as the reduced female mating rate.

Next, in this dissertation we investigate the effect of *sex-ratio* drive on male fertility during multiple mating and on male sperm competition. We multiply mated either SR or ST males with up to ten females on one day, and up to two on a second day. First, we investigate pre-mating differences between SR and ST males. Consistent with earlier findings in *D. neotestacea* and results from *D. pseudoobscura*, we find no difference in mating success between SR and ST males [14, 23], unlike there is in stalk-eyed flies [24]. Therefore, post-mating processes may be more important in this system. For post-mating selection, we find a decline in SR male fertility that is steeper than ST males after several bouts of mating, which is a result similar to other species with SR [17, 20, 25, 26]. SR males tend to become sperm depleted more quickly, and after a whole day of mating, do not replenish their sperm stores as quickly as ST males. To investigate sperm competitive ability, we mated females first to a SR or a ST male; then secondly we mated the same female to the opposite type of male. We determined the proportion of offspring sired by each father based on the number of sex of the offspring (SR males produce 100% females, and ST males produce ~50:50 males and females). We found that regardless of mating order, SR males always sire fewer offspring when a female also mated with an ST male, but these data are consistent with random sperm mixing: SR sires ~1/3 and ST sires 2/3% which is consistent with the number of sperm they pass (SR has 50% fewer). In stark contrast to other SR drive systems, therefore we do not find evidence for SR males being poor sperm competitors [18-20]. Therefore our results suggest that post-mating selection is important in regulating SR frequency through reduced male fertility after multiple mating.

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CHAPTER 2

ASSOCIATION OF POLYANDRY AND SEX-RATIO DRIVE IN NATURAL POPULATIONS OF *DROSOPHILA NEOTESTACEA*[1]

1 Pinzone, C.A. and K.A. Dyer 2013. *Proc R Soc B*. 280, 20131397.

Abstract

Selfish genetic elements bias their own transmission to the next generation, even at the expense of the fitness of their carrier. Sex-ratio (SR) meiotic drive occurs when an X-chromosome causes Y-bearing sperm to die during male spermatogenesis, so that it is passed on to all of the male's offspring, which are all daughters. How SR is maintained as a stable polymorphism in the absence of genetic suppressors of drive is unknown. Here, we investigate the potential for the female remating rate to affect SR dynamics in natural populations, using the fly *Drosophila neotestacea*. In controlled laboratory conditions, females from populations where SR is rare mate more often than females from populations where SR is common. Furthermore, only when males mate multiply does the average fertility of SR males relative to wild-type males decrease to a level that can prevent SR from spreading. Our results suggest that differences in the female mating rate among populations may contribute to SR dynamics in the wild, and thus also affect the outcome of this intragenomic conflict. In line with this, we also present evidence of a localized population crash due to SR that may have resulted from habitat fragmentation along with a reduced mating rate.

Introduction

Intragenomic conflict occurs when selection acts in opposing directions on elements within a genome. This type of conflict can be a potent source of evolutionary change and innovation, with consequences for the evolution of sex, recombination, and mating systems (reviewed in [1, 2]). An important cause of intragenomic conflict is selfish genetic elements (SGEs), which promote their own transmission into the next

generation, and as a result can spread through a population even if they are harmful to their carriers (reviewed in [3]). In this study, we focus on a classic SGE, X-chromosome meiotic drive, which occurs when a driving X-chromosome prevents the maturation of Y-bearing sperm during male spermatogenesis [4]. A male that carries an unsuppressed driving X-chromosome will thus transmit it to all of his offspring, rather than the usual 50%, and all of his offspring will be female. This sex-ratio (SR) drive is an especially interesting SGE because a secondary effect of this biased transmission is that it can cause the population-level sex ratio to become female biased in away that may not be adaptive for the host population. In theory, SR can change the effective population size and growth rate [5] and the direction or strength of sexual selection in a population [6], and if left unchecked, it is expected to drive the host population to extinction owing to a lack of males [7].

SR males are expected to produce half the number of sperm as wild-type males, but if an SR male transfers more sperm to the female than are necessary to fertilize all of her eggs, this may not result in a reduction in the male's fertility. As long as SR males produce at least half the number of offspring as wild-type males, an SR chromosome is expected to increase in frequency in a population as long as there are no other fitness effects [7]. However, in natural populations, SR is often maintained at low frequencies that are stable in both space and time (reviewed in [4]). It is straightforward to account for a stable SR polymorphism when there are suppressors of drive and/or when SR has severe pleiotropic effects on the fitness of female carriers [8-11]. However, we understand much less about how SR can be maintained as a polymorphism when there are no suppressors of drive present. Because in most species, females cannot discriminate

against mating with SR males (but see [12]), in these situations post-mating mechanisms of selection may be key for SR dynamics. For instance, because SR males produce fewer sperm than wild-type males, they may be at a disadvantage in conditions of sperm competition or when males mate frequently [13, 14]. This has been demonstrated empirically in several SR systems [15-17].

Multiple mating may allow for a balanced polymorphism of SR through frequency-dependent selection [13,14]. High male mating rates may inhibit the spread of SR: as the rate of male mating increases, SR males may transfer fewer sperm to the female compared to their wild-type counterparts, and they would be expected to have reduced relative fertility. In the absence of any other fitness effects, a balanced polymorphism could occur at the SR frequency where SR males sire half as many offspring as wild-type males [13]. In addition, sperm competition between SR and wild-type males, which is expected to occur when SR is at low-to moderate frequency in a population, can be sufficient to stabilize an SR polymorphism if the success of SR sperm decreases with an increased male mating rate [14]. Because of these effects, it has been proposed that variation in female multiple mating, or polyandry, may affect the invasion and maintenance of SR and other SGEs (reviewed in [18]). If a female mates multiple times, she is more likely to mate with a non-SR male than if she only mates once, and thus is more likely to produce sons [19]. In addition, increased female mating rates may also intensify sperm competition between SR and wild-type males, further reducing SR male fertility [14, 20, 21]. Empirical evidence for a potential role of polyandry in SR dynamics has come from experimental evolution in *Drosophila pseudoobscura*, where enforced female multiple mating slowed the rate of extinction due to SR relative to

population cages where females were only allowed to mate once [22, 23].

Female mating rate varies within and among species and has a genetic component [24, 25]. Many factors may affect the evolution of this trait in the wild; for instance, the mating rate may respond to selection from environmental factors, demographic variables, the risk of inbreeding and even the presence of SR drive [6, 26-28]. If polyandry is important for SR dynamics, then differences in the multiple mating rate may explain why some species are especially prone to invasion by SR. Furthermore, if the strength and direction of selection pressures on the mating rate also vary among populations within a species, differences in the local level of polyandry may contribute to the among-population variation in SR prevalence that is seen in several systems (reviewed in [4]). A first step towards understanding the role of polyandry for SR dynamics in natural populations is to ask whether natural populations that differ in SR prevalence also vary in the level of polyandry. Here, we investigate multiple mating in natural populations of the fly *Drosophila neotestacea*. This fly is a non-cosmopolitan, mushroom-feeding species that inhabits temperate and boreal forests across North America. It exhibits a stable cline in SR frequency, ranging from 0 to 30% across populations [29, 30]. There is no evidence for any active genetic suppressors or pleiotropic effects of drive in females [29, 31, 32]. Because levels of gene flow across the species range are moderate to high, natural selection probably maintains the geographical distribution of SR drive prevalence [29]. The selective force(s) that maintains this cline in SR frequency remains unknown.

In this study, we test for the potential of polyandry to contribute to the maintenance of the cline in SR in *D. neotestacea*. First, we ask whether the fertility of SR males is decreased relative to wild-type males, indicating whether fertility selection

against SR males can occur in *D. neotestacea*. Second, we use flies derived from five different natural populations to test for the presence of genetic variation in female polyandry. Finally, we ask whether the variation in polyandry we observe in the laboratory is correlated with the local prevalence of SR. We hypothesize that populations with a higher rate of polyandry would have stronger fertility selection against SR males, and thus a lower prevalence of SR.

Materials and methods

Fly collections, fly maintenance, and sex-ratio prevalence

We collected wild adult *D. neotestacea* in 2010 and/or 2011 near Edmonton, Alberta (AB), Coeur d'Alene, Idaho (ID), Missoula, Montana (MT), Portland, Oregon (OR) and Seattle, Washington (WA) (table 1 and the electronic supplementary material, table S1). We created isofemale lines from wild-caught females, and maintained lines for at least seven generations before using them in mating experiments. We maintained cultures on instant *Drosophila* medium (Carolina Biological Supply) with a piece of commercial mushroom (*Agaricus bisporus*) on a 14 L : 10 D cycle at 20°C. Virgins were collected within 24 h of emergence using light CO2, housed 10–15 flies per vial and were 5–10 days old when initially used in an experiment. Air aspiration was used during all mating assays, which commenced within 1 h of the incubator lights turning on.

To estimate the SR frequency in each population, we genotyped wild-caught flies at two X-linked microsatellite markers that are in linkage with SR drive [29]. Based on previous data, Dn8377 and Dn8385 (accession nos. EF199832 and EF199836, respectively) together have 94% accuracy in assigning an X-chromosome as SR or

standard (ST) based on different size fragments. Methods for microsatellite genotyping and fragment analysis were as described previously [31]. Estimates of SR prevalence from OR and WA were also obtained by mating wild-caught males or F1 sons of wild-caught females to laboratory females; those that produced greater than or equal to 10 offspring, of which were greater than or equal to 90% females, were considered to carry SR. We tested for an association of SR prevalence and population sex ratio using a linear regression, weighted by the number of flies collected. Unless otherwise noted, statistical analyses were performed in JMP v. 10 (SAS Institute, Cary, NC, USA).

Effect of sex-ratio on male fertility

To assess SR male fertility relative to ST, wild-type males, we used laboratory stocks from Rochester NY established in the early 1990s by J. Jaenike, which maintains the SR and ST X-chromosomes on the same genetic background (for crossing scheme see [29]). We paired a single 7-day-old virgin SR or ST male with a single virgin wild-type (ST/ST) female for 1 h, and then transferred each male that mated to a vial that contained 10 additional virgin wild-type (ST/ST) females. After 24 h, we discarded the male from each vial and allowed the females to oviposit individually in food vials, transferring each female to fresh food after 5 days and discarding them after 10 days. We counted the offspring produced from each first and subsequent mating from 24 SR and 28 ST males, and used Wilcoxon rank sum tests to compare the number of offspring produced by SR and ST males.

Variation in female polyandry

To assess variation in female remating behavior across different locations, we used five isofemale lines from WA; six isofemale lines each from AB, ID and MT, and nine isofemale lines from OR, which several generations earlier had been combined together to make three stocks each comprised three isofemale lines. The lines we used did not carry SR, which we verified by scoring offspring sex ratios of a sample of males. On day 1 of the assay, we combined one virgin female with 10 virgin males from the same population. The line identity of the female was recorded, and the males used were chosen randomly from the lines within each population. We checked each vial every 5 min and recorded the number of times each female mated. In *D. neotestacea*, copulations last about 15 min. After 12 h, we aspirated out each female that successfully mated and allowed each to recover alone overnight. Females that did not mate on the first day were discarded and not included in any analyses. The following morning, we placed each of the mated females with 10 new males from the same population, and recorded whether each female remated within 2 h.

First, we investigated differences among populations in the total number of female copulations on day 1 only and across both mating days. We used a Wilcoxon rank sums test and the Steel–Dwass method for multiple comparisons [33, 34], as we were unable to transform the data to normality. Second, we tested for differences among populations in the proportion of females that remated on the second day of the assay, using a contingency analysis with a likelihood ratio test, and an analysis of means for proportions. Finally, we asked whether there was an association between the level of female polyandry and the population prevalence of SR. We performed Spearman rank

correlations between the frequency of SR and the different measures of polyandry, including the number of copulations and proportion of females that remated on the second day. We determined significance using statistical tables for small sample sizes.

Genetic differentiation among populations

To test whether the variation we observe in polyandry may be due to the effects of drift rather than local selection, we surveyed the level of population variation and differentiation at five autosomal microsatellite loci. We genotyped wild-caught individuals from each of the five populations at Neo6003, Neo6429, Neo7013,Neo8380 and Neo8394 from Dyer [35] using methods described previously [29]. We tested for the presence of null alleles, linkage disequilibrium between pairs of loci, and departures from Hardy–Weinberg equilibrium in GENEPOP v. 4.0.10 [36]. We calculated allele richness, observed and expected heterozygosity, and measures of population differentiation (F_{ST} and R_{ST}) using ARLEQUIN v. 3.5 [37], with significance determined from 1000 permutations. To infer the number of genetic clusters (K), we used STRUCTURE v. 2.3 [38]. We used a model that assumed no admixture and correlated allele frequencies, used the collecting location as a prior, and ran the program five times at each $K = 1$ through to $K = 5$, with a burn in of 150,000 steps and a run length of 200,000 steps. We determined the most probable value of K using the highest log-likelihood of the posterior probability of the data across values of K [38] and also via the ΔK method of Evanno et al. [39].

Results

Sex-ratio prevalence and population-level sex ratio

Using SR-linked microsatellite loci, we estimated the SR frequency to range between 4 and 50% across the five populations (Table 1 and electronic supplementary material, Table S1). The prevalence of SR at the three locations that were sampled previously, AB, ID and MT (0.06, 0.15 and 0.20, respectively), were similar to the frequencies found in the 2001–2002 collections that were based on offspring sex ratio of wild-caught males (0, 0.12 and 0.18, respectively [29]). The OR and WA populations were not sampled previously, and both estimates of SR prevalence (0.50 and 0.47, respectively) are higher than ever observed in *D. neotestacea* [29,30]. These estimates are consistent with the SR frequencies we obtained using the offspring sex ratio from wild-caught flies, suggesting that they are not artifacts (OR: SR = 0.56, 95% CI 0.30–0.80, n = 16; WA: SR = 0.47, 95% CI 0.33–0.60, n = 58; electronic supplementary material, figure S1).

Of the five populations, three had a significantly female-biased population-level sex ratio (table 1 and electronic supplementary material, table S1). While caution should be used interpreting these estimates, as females and males may be attracted at different rates to baits, we find in this sample that higher SR prevalence is associated with a more female-biased population-level sex ratio ($R^2 = 0.83$, $F_{1,3} = 14.90$, $P = 0.03$; electronic supplementary material, Figure S2). We note that the observed population sex ratio of the WA population is significantly higher than expected based on its SR frequency: with a SR frequency of 0.47 (95% CI 0.40–0.54), the expected population SR is 73.5% female (95% CI 70–77%), the bounds of which are lower than the 95% CIs of the observed

population sex ratio (91% female, 95% CI 86–95%). The observed population-level sex ratio of the other four populations is within the expected range given the sample size and observed SR frequency.

Effect of sex-ratio on male fertility

Overall, SR males produce significantly fewer offspring than ST males. From the initial mating, SR males produced 64% as many offspring as ST males (Wilcoxon rank sum test $\chi^2_1 = 4.7$, $P = 0.030$; figure 1). This fertility effect is magnified upon repeated matings of the male: summed over the 10 subsequent females, SR males produced about 30% as many offspring as ST males (Wilcoxon rank sum test $\chi^2_1 = 13.1$, $P = 0.0003$; figure 1). This is owing to a lower number of females that produced offspring (SR: 1.3 ± 0.3, ST: 2.6 ± 0.2 (mean ± s.e.); Wilcoxon rank sum test $\chi^2_1 = 11.0$, $P = 0.0009$). Because the number of offspring produced by each of these females is not significantly reduced for SR males, this suggests that SR males run out of sperm faster than ST males (SR: 28.0 ± 5.8, ST: 41.5 ± 3.7; Wilcoxon rank sum test $\chi^2_1 = 2.1$, $P = 0.15$). Summed over all 11 potential mates, SR males produced 44% as many offspring as ST males (Wilcoxon rank sum test $\chi^2_1 = 16.2$, $P < 0.0001$; figure 1). Using these fertility estimates, we estimate that after 2.8 matings in a 25 h period using the mean number of offspring and after 2.2 matings using the median number of offspring the relative fertility of SR males is expected to drop below 50% of ST males. Thus, if the only aspect of host biology that is affected by SR is male fertility, we expect that SR could invade a population if males mated only once per day, but would not invade a population where males mate more than two to three times per day. These estimates assume SR and ST males are equally likely to

obtain copulations and also do not consider the consequences of sperm competition.

Variation in female remating behavior

First, we find evidence for variation among populations in polyandry. We find no significant effects within populations on mating rate due to a line or day effect, so therefore we combine data across lines and days for all analyses (Wilcoxon rank sums tests all $P > 0.05$ using a Bonferroni correction). There is significant variation among populations in the number of copulations on the first mating day ($\chi^2_4 = 21.10$, $P = 0.0003$; electronic supplementary material, figure S3a). Using the Steel–Dwass method of multiple comparisons, there are significant differences between two geographically overlapping groups (AB, ID) and (WA, ID, MT, OR). There is also significant variation in the total number of copulations a female engaged in over the 2 days of the mating assay ($\chi^2_4 = 30.07$, $p < 0.0001$; electronic supplementary material, figure S3b), with significant differences between populations falling into three overlapping groups (AB, ID), (WA, ID, MT) and (WA, MT, OR). Results of an analysis of variance were consistent, though the data violated the assumption of normality (results not shown). There is also significant variation among populations in the fraction of females that remated on the second day ($\chi^2 = 11.2$, $P = 0.0249$; electronic supplementary material, figure S4), with fewer females from OR remating relative to the other populations ($P < 0.05$). Second, the prevalence of SR correlates with estimates of female polyandry. Females from populations with a lower SR prevalence tended to mate more often than those from higher prevalence populations, when considering either the number of matings on the first assay day only ($\rho_3 = 20.9$, $p < 0.05$; electronic supplementary material, figure

S5) or combined across both days of the assay (ρ_3 = -1.0, P = 0.01; figure 2a). We also find that females from populations with a lower SR prevalence were more likely to remate on the second day (ρ_3 = -1.0, P < 0.01; figure 2b). Summed together, these results indicate that higher levels of polyandry are associated with lower levels of SR prevalence in natural populations of *D. neotestacea*.

Genetic differentiation among populations

Consistent with previous studies in *D. neotestacea* [29,31], we find substantial levels of genetic diversity within populations and high gene flow among the populations we surveyed in this study. No pairs of loci showed evidence for a signature of linkage disequilibrium (all P > 0.05), and none showed consistent evidence for an excess of homozygotes or inbreeding within populations (all P > 0.05; electronic supplementary material, table S4). Across populations there is no correlation of either the average observed heterozygosity or the average number of alleles per locus with geographical distance, the prevalence of SR or the population-level sex ratio (all P > 0.3). None of the pairwise estimates of genetic differentiation among populations were significantly greater than zero (F_{ST} and R_{ST} all P > 0.05; electronic supplementary material, table S3). Using the program STRUCTURE, the most probable number of genetic clusters (K) was K = 1 using the highest averaged likelihood across runs (average lnL = -2704.32), and K = 3 using the ΔK method of Evanno et al. [39] (average lnL = -2715.32; electronic supplementary material, table S4). For K = 3, there is no assignment probability of a population to a cluster of more than 0.6 (see the electronic supplementary material, table S5), and this weak clustering is also evident in the assignment of

individuals by population into the genetic clusters (see the electronic supplementary material, figure S6). Concordantly, if the locations are not used as prior information, none of the populations had clear support for any cluster assignment. Thus, consistent with the results of a previous study in *D. neotestacea* [29], which included some of the same populations but used samples collected 10 years earlier, there appears to be moderate to high levels of gene flow among populations, indicating differences in polyandry are probably owing to selection rather than drift.

Discussion

The consequences of SGEs for host genome evolution and reproductive biology are significant [3]. For an SGE such as X-chromosome drive, males produce fewer sperm overall as a result of the mechanism to bias transmission, which can make them vulnerable to reduced fertility and sperm competition under conditions of multiple mating. Thus, polyandry has been proposed as a mechanism to counteract the spread of SR (reviewed in [4,18]). *Drosophila neotestacea* is an ideal system to test this in the wild, because populations exhibit stable differences in SR prevalence, and there are no confounding factors such as suppressors of drive or known pleiotropic effects on female fitness [29,31,32]. Importantly, *D. neotestacea* females mate multiply—in this study, females mated up to four times in one day in the laboratory, and others have noted that *D. neotestacea* females also remate readily in the wild (J. Jaenike 2013, personal communication). This sets the stage for fertility selection against SR males, and for sperm competition between SR and ST males to occur.

Can multiple mating prevent the invasion of sex-ratio?

When there are no pleiotropic fitness effects on survival or female fecundity, SR is expected to increase in frequency in a population when SR males produce at least half as many offspring as wild-type males [7]. In *D. neotestacea*, we estimate that the relative fertility of SR males drops below 50% of ST males after two to three matings in a 25 h period, which means that if males mate at least this much SR may not be able to invade a population. These relative fertility estimates are similar to other mushroom-feeding *Drosophila* species that harbor SR [13]. However, to determine whether multiple mating is important to SR dynamics in the wild, we must consider not only the relative fertility of SR males but also the amount that females actually mate. To take an extreme example, if it takes 10 matings in a day for the relative fertility of SR males to fall below 50% of ST males, but females only ever mate once a day, then it is unlikely fertility selection due to multiple mating plays a substantial role in SR dynamics. In our assays, populations varied in how many times females mated per day, with average values between 1.6 and 2.4 copulations in a 26 h period (figure 2). The upper range, for example as is seen in the AB population, overlaps with number of times males must mate for fertility selection to prevent the spread of SR. At the lower end of our observed polyandry values, for example in OR and WA, this is not likely to be the case. Sperm competition in *D. neotestacea* has not been investigated, but may further intensify fertility selection against SR. Severely reduced fertility of SR males in conditions of sperm competition have been shown to occur in several other SR systems, for example *Drosophila simulans*, D. pseudoobscura and stalk-eyed flies [15, 40, 41]. In addition, even in populations where females mate less often, the spread of SR may be inhibited once the population sex ratio

is female biased enough such all males mate several times even if females do not [13].

Can polyandry maintain a cline in sex-ratio prevalence?

Our data support the scenario hypothesized by others and demonstrated in laboratory cage populations, where low levels of polyandry allows SR to increase in frequency, and higher polyandry may reduce SR frequency or prevent its invasion due to fertility selection and sperm competition against SR males [13,22,23]. *Drosophila pseudoobscura* also exhibits a similar cline in SR frequency; in this system, there are also differences in polyandry among natural populations of *D. pseudoobscura*, such that higher polyandry populations also have lower SR prevalence (T. Price 2013, personal communication). Additionally, a similar situation has also been inferred to explain lower than expected frequencies of the t-haplotype segregation distortion system in the mouse [42]. By contrast, this pattern does not seem to be the case in stalk-eyed flies, but owing to segregating suppressors the SR dynamics may be much more complicated in this system [43]. In theory, in the absence of suppressors or pleiotropic effects on female fitness, multiple mating and sperm competition alone can maintain a stable equilibrium of SR in a population, though the conditions are very restrictive [14]. This also assumes that populations are isolated entities, with SR dynamics operating independently within each population to maintain each at a stable equilibrium. However, populations of *D. neotestacea* show high levels of gene flow, which should homogenize the prevalence of SR among populations. Because we see substantial differences in SR prevalence across populations that appear to be stable over time, we suggest that the cline in SR frequency is more likely maintained by migration–selection balance via local differences in female

mating rate. In populations such as AB, female mating rate is high, which may result in strong selection against SR alleles and the observed low frequency of SR. However, where remating is lower, such as in OR, there may be less of a fertility disadvantage for SR males, which may allow SR to spread to a higher prevalence. The constant migration of SR and/or ST alleles into a population may prevent it from ever fixing or eliminating SR. Thus, fertility selection against SR alleles within populations appears to be balancing the high rates of gene flow among populations.

Why might polyandry vary among populations?

We found that polyandry differs among natural populations under laboratory conditions and in the absence of SR, which suggests that there is a genetic basis to this variation. There are several reasons why selection may favor different levels of multiple mating among populations. First, polyandrous behavior may evolve as a mechanism for inbreeding avoidance, for example, by increasing the diversity of the offspring when there is a cost to inbreeding [44]. While this could occur in some species, we do not think this is the case in *D. neotestacea*. In our study, the mixed isofemale lines from OR may be less inbred than the isofemale lines from the other populations and we do find the lowest level of multiple mating in this population; however, if we exclude the OR population we still find substantial variation in polyandry among the remaining populations. Furthermore, *D. neotestacea* harbors substantial genetic diversity (this study, [29,31]) and does not show evidence for recent inbreeding, as no population had an excess of homozygotes or showed evidence of reduced genetic diversity as SR prevalence increased. Finally, in nature we do not expect a high risk for sibling-mating for this

species, as a mushroom host is decomposed by the time that flies emerge from it, and thus each generation of flies must find a new mushroom to mate and oviposit on.

Second, the presence of SR in a population may select for more promiscuous female mating behavior. For instance, in *D. pseudoobscura* population cages, the rate of polyandry increased in the presence of SR relative to controls, suggesting that SR selected for increased polyandry [26]. Furthermore, a study in stalk-eyed flies found that a species with SR drive had a higher rate of multiple mating than a closely related species without drive, although there was no association of SR prevalence and mating rate among populations within species [43]. If the presence of SR selects for increased polyandry, we would expect that populations with higher polyandry would also have higher SR prevalence. However, this is the opposite pattern to what we observe in *D. neotestacea*, as well as to what is found in *D. pseudoobscura* (this study; T. Price 2013, personal communication). Furthermore, if there is a cost to female polyandry, as is seen in many species [45-47], we predict that populations would fluctuate in SR frequency through time. This is because once higher mating rates eliminated SR there would be no selective force to maintain the increased polyandry, and thus it would return to lower levels that would allow the re-invasion of SR. However, in many systems, including *D. neotestacea* and *D. pseudoobscura*, the frequencies of SR in natural populations have been remarkably stable through time. Thus, we suggest that selection due to SR probably does not explain the variation in female multiple mating in *D. neotestacea*. It would be useful to quantify empirically the costs associated with female multiple mating in *D. neotestacea* and other species with SR drive as well as to model theoretically how fast the SR dynamics would be expected to change under this scenario.

This could also shed light on the more general question of the conditions that permit polyandry to be favored specifically to combat SGEs [18].

Finally, and we argue most likely, the selective agent may be an environmental or demographic variable that differs among populations but which may not be directly related to the presence of SR. Environmental factors may vary among populations and cause the female mating rate to evolve in the wild; for example, it is conceivable that the local population density, resource availability, day length and temperature could each affect the strength of selection for multiple mating [48-50]. The SR cline in *D. neotestacea* correlates with local temperature, and there are many environmental factors related to temperature that could affect selection on mating rate. If other species that are sympatric with *D. neotestacea* but that do not harbor SR show a similar geographical pattern in polyandry this may indicate that selection is a result of the local environment and is not species specific. In addition, these variables, as well as the presence of SR, may affect aspects of the local mating system such as the operational sex ratio and the intensity of sexual selection and sexual conflict, which may alter local selection on the female mating rate (reviewed in [27, 51]). At this point, we can only hypothesize about why the mating rate varies among populations, and it is clear that much work remains to determine what causes differential selection on the female mating rate in this and other species. Comparative studies will also be useful to determine whether certain ecological or demographic variables or aspects of the mating system are common among species that harbor SR versus those that do not.

A potential extinction event due to sex-ratio

Prior to this study, SR chromosomes in *D. neotestacea* had been found at a maximum of about 30% across the geographical range in North America and have been stable for at least 20 years [29]. This is comparable to SR clines in *D. pseudoobscura* and *D. subobscura*, in which SR frequencies also range between 0 and 30% [52-54]. Two of the sites we sampled in 2010, OR and WA, harbored an extremely high prevalence of SR, between 40 and 50%, which is higher than that has ever been observed in any species where SR drive is unsuppressed (reviewed in [4]). These SR frequencies may not be typical for this region, as 10 years prior in other locations in Oregon and in British Columbia the SR prevalence was approximately 25–30% [29]. Both of these populations also had highly female-biased population sex ratios and the lowest rates of polyandry in our assays (figure 2 and table 1). Interestingly, it appears that the WA population crashed the year after we collected there. In 2010, we collected 380 *Drosophila*, including 175 *D. neotestacea*, of which 91% were female (see the electronic supplementary material, table S1). We returned to the same site in 2011 at the same time of year and for the same length of time, and out of 249 *Drosophila*, only three were *D. neotestacea*, all of which were female. Of these three flies only one was inseminated, and all carried SR, one of which was an SR/SR homozygote. This is a strikingly smaller proportion than the year before (2010, 46.1%; 2011, 1.2%; Fisher's exact test, $P < 0.0001$), and may indicate the occurrence of a localized SR-induced extinction. To our knowledge, this is the first time an SR drive-induced population crash may have been witnessed in a natural population.

Both the WA and OR sites are large parks surrounded by an expanse of urban development, whereas the other populations we have sampled in this and other studies

were from more continuously forested areas. Based on our population genetic analyses, the WA and OR populations do not appear to be highly differentiated from the other populations in this study, nor do they harbor a lower level of genetic diversity. However, if gene flow has been suppressed only recently, these signatures may not be evident yet. We suggest that a reduced rate of immigration along with a low remating rate may have been sufficient to permit SR to drive the WA population to extinction. In support of this, we note that WA and MT had similar rates of polyandry, but there was a large difference in SR prevalence between these populations (figure 2 and table 1). While this is a limited sample, it highlights the potential for habitat fragmentation to affect the population dynamics of SGEs. It also suggests that SR drive and other SGEs may persist for longer periods of time in species with high levels of gene flow among populations [55].

Conclusion

There is still much debate as to the proximate and ultimate causes of multiple mating [27, 56]. Recently, multiple mating has been proposed as a force to protect a host against SGEs [18], and here we show that higher polyandry is associated with lower SR prevalence in natural populations. We do not find evidence, however, that multiple mating has evolved in response to the presence of the SGE. Instead, we suggest it is more likely that the mating rate is affected by local ecological or demographic factors, which vary among populations. However, the selective forces that underlie the observed differences in polyandry remain unknown, and await further study. Nevertheless, the rates of multiple mating we observe are right in the range of plausible values that can prevent or permit SR from spreading in a population, and further theoretical

investigations will determine whether selection from polyandry alone can explain the differences we observe in SR prevalence in nature. Furthermore, models can be extended to ask if, and how, multiple mating affects SR drive dynamics when populations are polymorphic for suppressors of drive, as is seen in many SR systems. It will also be of interest to use paternity studies of wild-caught females to infer whether the patterns of polyandry we find in wild-derived flies occur in the wild, which has found to be the case in other *Drosophila* [57]. In summary, we suggest that the interaction between host ecology, mating system, and SGEs has the potential to affect both the population dynamics on an ecological scale as well as the long-term evolution of SGEs. This may have consequences not only for the prevalence of SGEs within a species, but also for the distribution of SGEs across a broader diversity of taxa.

Acknowledgements

We are grateful to B. White, M. Bray, J. Lopez and E. Fawcett for laboratory assistance; E. Bewick, T. Brekke and N. Greening for assistance with fieldwork and W. Anderson, D. Hall, J. Jaenike, K. Pieper, D. Promislow and A. Sweigart for useful discussion and/or comments on the manuscript. This work was funded by an SSE Rosemary Grant Award, NIH Training grant fellowship (T32GM007103), and UGA Bishop Award to C.A.P., and by grants from the Ellison Medical Foundation and National Science Foundation to K.A.D.

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Table 1. Summary of populations used in this study. *Sex-ratio* (SR) frequency is based on SR-linked microsatellites, with 95% confidence intervals (CI) calculated using a binomial sampling distribution. The population sex ratio is calculated as the proportion of females out of the total wild flies collected, with 95% confidence intervals (CI) calculated using a binomial sampling distribution. Significance from 50:50 was determined using a χ^2 test, where * and ** indicate $p < 0.05$ and $p < 0.001$, respectively.

abr.	population location	*sex-ratio* frequency (±95% CI)	population sex ratio (±95% CI)
AB	Edmonton, Alberta	0.06 (0.03-0.10)	0.51 (0.46-0.56)
WA	Seattle, Washington	0.47 (0.40-0.54)	0.91 (0.86-0.95)**
ID	Coeur d'Alene, Idaho	0.15 (0.08-0.25)	0.45 (0.35-0.54)
MT	Missoula, Montana	0.20 (0.11-0.34)	0.72 (0.56-0.85)*
OR	Portland, Oregon	0.50 (0.41-0.59)	0.77 (0.69-0.83)**

FIGURE LEGENDS

Figure 1. Fertility of *sex-ratio* (SR) (n=24) and standard (ST) (n=28) males. Shown is a box plot of the total number of offspring produced from the first and subsequent matings and summed over all the females each male had the opportunity to mate with. The box indicates the quartiles, with the median depicted as the horizontal line within each box.

Figure 2. Association of *sex-ratio* (SR) prevalence in natural populations with the rate of female polyandry. Shown is the relationship between SR prevalence and A) the mean number of female copulations over the two days of the mating assay, and B) the fraction of females that remated on the second day. Population abbreviations are adjacent to the respective data point. Standard errors (SEM) are indicated for copulation number, and the 95% confidence intervals (CI), calculated with a binomial distribution, are indicated for *SR* prevalence and fraction remated.

Figure 1.

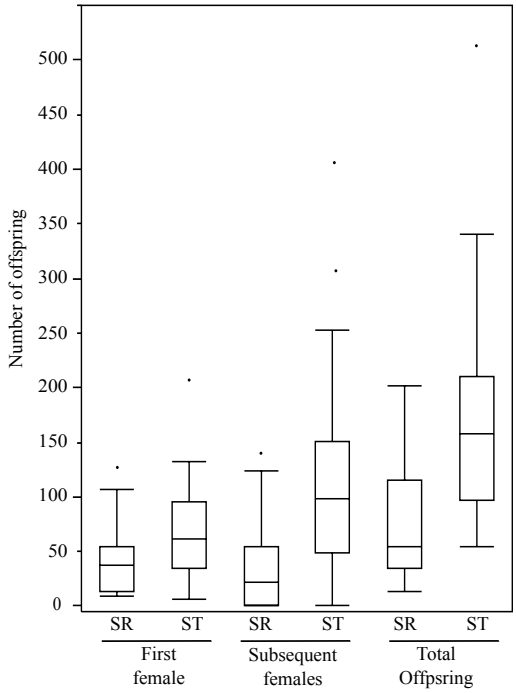

Number of offspring
500
450
400
350
300
250
200
150
100
50
0
SR
ST
SR
ST
SR
ST
First female
Subsequent females
Total Offpsring

Figure 2.

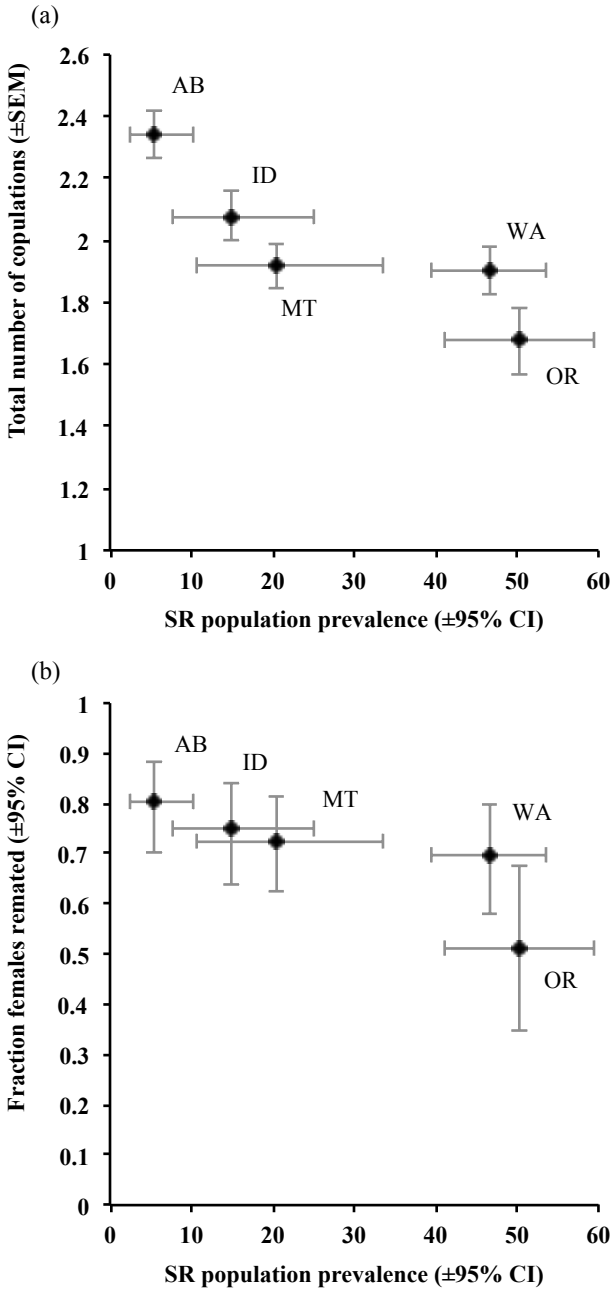

(a)
Total number of copulations (±SEM)
2.6
2.4
2.2
2
1.8
1.6
1.4
1.2
1
AB
ID
MT
WA
OR
0
10
20
30
40
50
60
SR population prevalence (±95% CI)
(b)
Fraction females remated (±95% CI)
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
AB
ID
MT
WA
OR
0
10
20
30
40
50
60
SR population prevalence (±95% CI)

Table S1. Full descriptions of populations sampled in this study. Locations are listed by latitude from north to south with the abbreviation, year of collection, GPS coordinates of each field site, frequency of *sex-ratio* (SR) carrying males estimated from X-linked microsatellites, number of flies assayed at microsatellite loci, the population sex ratio estimated from the proportion of females out of the total wild flies, and the total number of wild-caught flies.

Abr.	Population location	Date	Latitude	Longitude	*Sex-ratio* frequency (±95% CI)[1]	N alleles at X-linked microsatellites	Population sex ratio (±95% CI)[1,2]	N wild flies
AB	Edmonton, Alberta	2010	53.51	-113.54	0.06 (0.03-0.10)	163	0.51 (0.46-0.56)	462
WA	Seattle, Washington	2010	47.73	-122.26	0.47 (0.40-0.54)	202	0.91 (0.86-0.95)**	175
ID	Coeur d'Alene, Idaho	2011	47.60	-116.66	0.15 (0.08-0.25)	74	0.45 (0.35-0.54)	112
MT	Missoula, Montana	2011	46.93	-113.97	0.20 (0.11-0.34)	54	0.72 (0.56-0.85)*	43
OR	Portland, Oregon	2010	45.52	-122.84	0.50 (0.41-0.59)	123	0.77 (0.69-0.83)**	149

[1] Based on microsatellites, confidence Intervals (CI) calculated using a binomial sampling distribution.

[2] Significance from 50:50 using a χ^2 test, with * and ** to indicate $P < 0.05$ and $P < 0.001$, respectively.

Table S2. Variability measures for each of the five microsatellites within each population and across all populations. Shown are the number of sampled alleles (N), the number of different alleles (A), the observed heterozygosity (H_O), and the expected heterozygosity (H_E). H_O values in italics show a deficiency of heterozygotes at $P < 0.001$. Populations are listed from north to south, in the same order and with the same abbreviations as in Table 1.

Locus		AB	WA	ID	MT	OR	Mean	s.d.	Total
	N	64	50	66	60	46	57.2	8.79	286
6003	A	14	14	15	13	13	13.80	0.84	16
	H_O	0.88	0.65	0.77	0.89	0.76	0.79	0.10	*0.80*
	H_E	0.91	0.92	0.93	0.92	0.91	0.92	0.01	0.92
6429	A	12	17	10	14	12	13.00	2.65	23
	H_O	*0.76*	0.74	0.68	0.78	0.62	0.72	0.06	*0.72*
	H_E	0.89	0.93	0.82	0.90	0.90	0.89	0.04	0.91
7013	A	11	10	14	10	10	11.00	1.73	19
	H_O	0.85	0.71	0.86	0.79	0.82	0.80	0.06	0.81
	H_E	0.82	0.88	0.84	0.84	0.83	0.84	0.02	0.85
8380	A	11	6	-	-	8	5.00	4.90	14
	H_O	0.57	0.71	NA	NA	0.60	0.38	0.35	0.62
	H_E	0.57	0.66	NA	NA	0.62	0.37	0.34	0.61
8394	A	12	11	12	12	12	11.80	0.48	17
	H_O	0.83	0.64	0.64	0.63	0.67	0.68	0.08	*0.69*
	H_E	0.86	0.84	0.87	0.86	0.89	0.86	0.02	0.86
Mean all loci	A	12.00	11.60	10.20	9.80	11.00	10.92	0.92	17.80
	s.d.	1.22	4.16	6.02	5.67	2.00	3.82	2.15	3.42
	H_O	0.78	0.69	0.59	0.62	0.69	0.67	0.07	0.73
	s.d.	0.12	0.04	0.34	0.36	0.09	0.19	0.15	0.08
	H_E	0.81	0.85	0.69	0.71	0.83	0.78	0.07	0.83
	s.d.	0.14	0.11	0.39	0.40	0.12	0.23	0.15	0.13

Table S3. Pairwise population differentiation at five autosomal microsatellite loci. FST is above the diagonal, and RST is below the diagonal. All *P* values were greater than 0.05, based on 1000 permutations. Populations are listed from north to south, in the same order and with the same abbreviations as in Table 1.

	AB	WA	ID	MT	OR
AB		0.004	-0.089	-0.083	0.010
WA	0.009		-0.087	-0.067	0.008
ID	-0.028	0.011		-0.019	-0.091
MT	-0.033	-0.014	0.016		-0.067
OR	-0.011	-0.015	0.012	-0.031	

Table S4. Proportion of membership for each population into three clusters, as defined by the Structure analysis. One of five runs of *K*=3 is shown here; the results were consistent among runs. Populations are listed in the rank order of the probability of each cluster assignment. N is the number of individuals genotyped at each microsatellite locus. The number in bold type for each population is the cluster with the highest representation.

Population	*N*	Cluster 1	Cluster 2	Cluster 3
WA	25	0.365	**0.596**	0.039
AB	32	0.390	**0.492**	0.118
ID	33	0.242	**0.431**	0.327
OR	23	**0.449**	0.414	0.137
MT	30	0.260	**0.396**	0.344

Table S5. Results of the Structure analyses of autosomal microsatellites. Table of means and standard deviations of the natural logarithm of the likelihood of the posterior probability of the data (ln[Pr(X|*K*)]) for a given number of genetic clusters (*K*), as well as the second-order rate of change (Δ*K*) in ln[Pr(X|*K*)] with respect to *K*. The values in bold type indicate the *K* values with the highest support using the likelihood alone and the Δ*K* method of Evanno (2005).

K	Mean ln Pr(X|*K*)	SD of ln Pr(X|*K*)	Δ*K*
1	**-2704.32**	0.476	
2	-2724.5	12.777	2.30
3	-2715.32	6.095	**43.97**
4	-2974.1	162.877	1.78
5	-2942.36	102.107	0.31

Figure S1. Distribution of the offspring sex ratio of wild-caught X-chromosomes from Seattle, WA and Portland, OR. Wild-caught males or a single F1 son of a wild-caught female were used to sire offspring. Males were only included if they sired at least 10 offspring. Males are assigned as carrying an SR chromosome if they produce at least 10 offspring, of which at least 90% were female. Seattle, WA is shown in dark grey and Portland, OR in light grey.

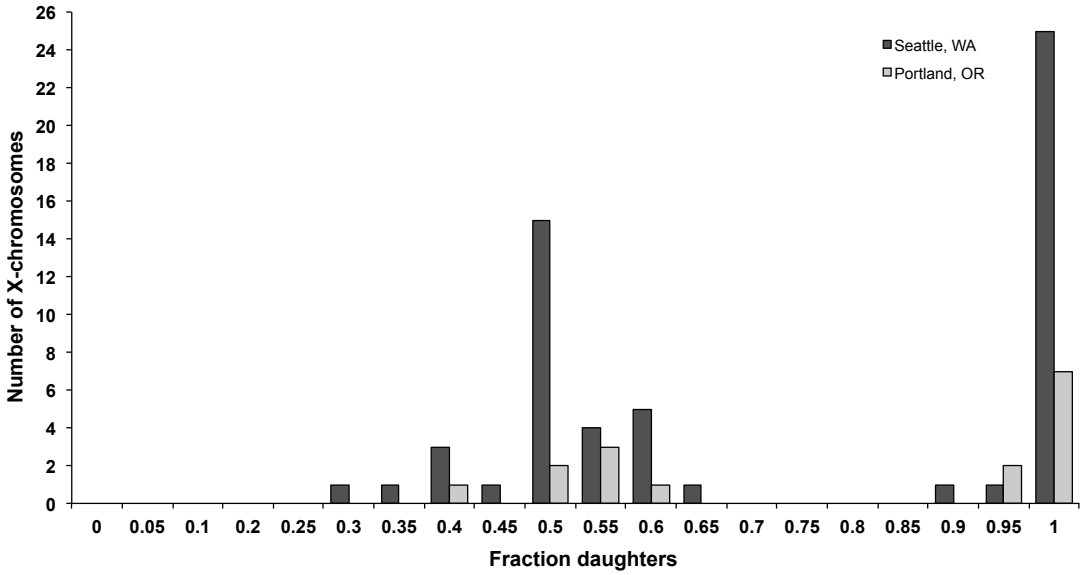


Figure S2. Association between the population sex ratio (Table S1) and the prevalence of *sex-ratio* (SR). There is a statistically significant positive association ($R^2 = 0.83$, $F_{1,3} = 14.90$, $P = 0.03$).

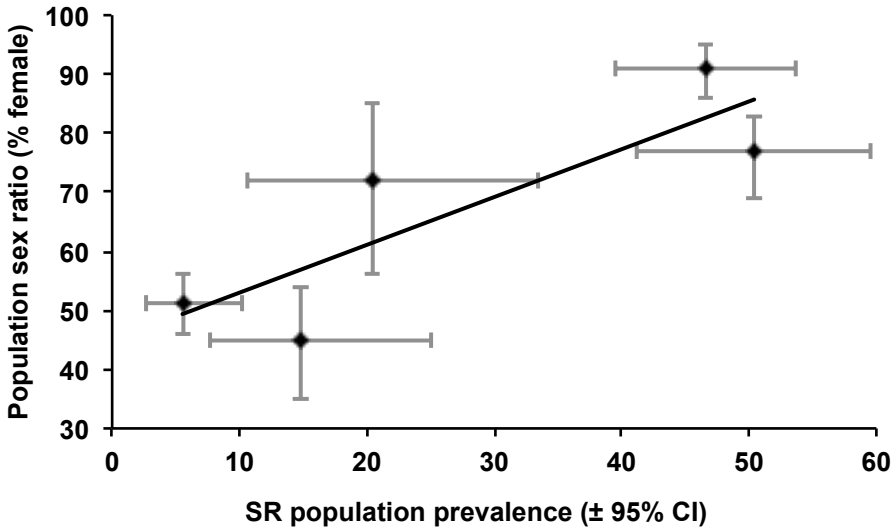
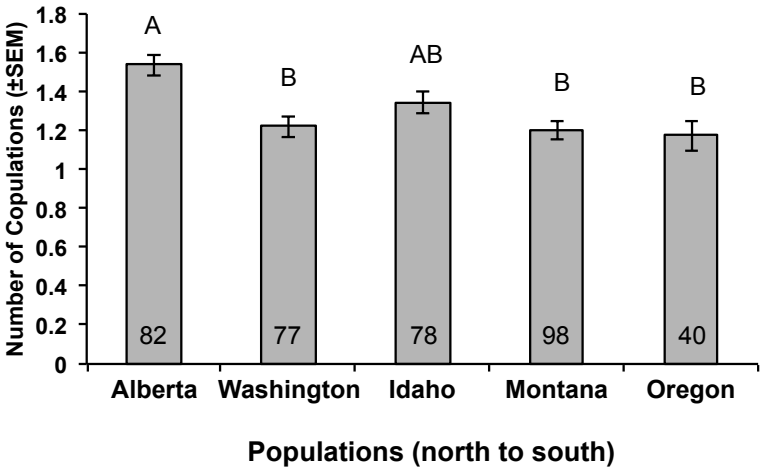


Figure S3. Total number of copulations by females from each population. A) Day one of the mating assay and B) Days one and two of the assay. Grouping of populations using the Steel-Dwass method is denoted using letters above each bar, where each letter denotes a group. Sample sizes are noted within bars, and standard errors (SEM) were used to estimate error.

A. Day one only



B. Days one and two

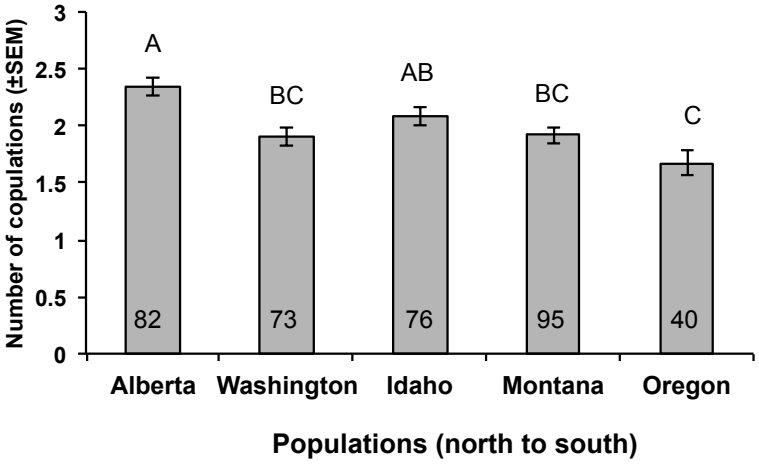


Figure S4. Fraction of females that mated remated on the second day, as well as the fraction of females that mated on the first day for comparison. Populations are listed from north to south. Dark and light grey bars show the fraction of females that mated on days one and two, respectively. Sample sizes for each group are within bars, and 95% confidence intervals (binomial distribution) were used to estimate error. Variation among populations using a contingency analysis detects significant variation on both day one ($\chi^2 = 17.8$, $P = 0.0013$) and day two ($\chi^2 = 11.2$, $P = 0.0249$). Using an analysis of means of proportions, females from Oregon mate less than all other populations, both on day one and on day two.

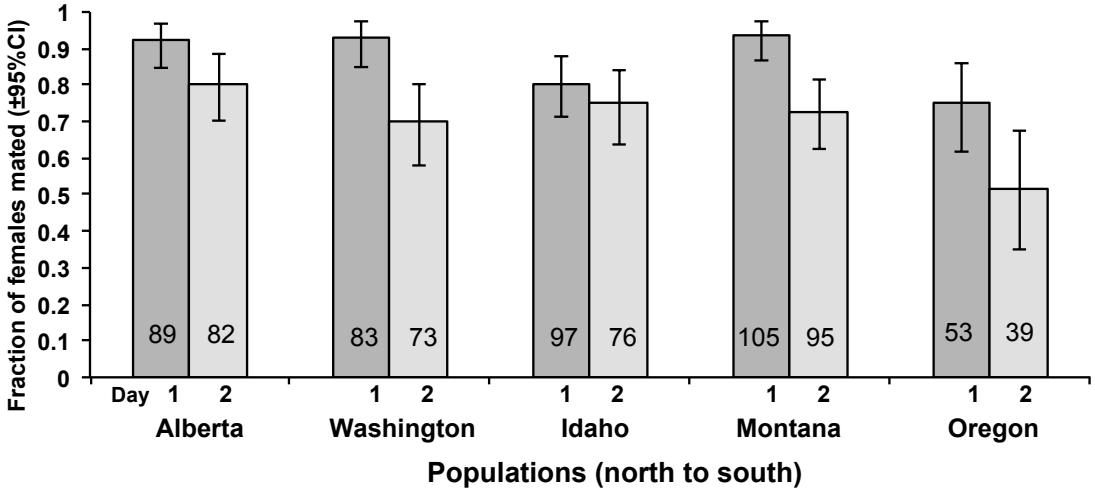


Figure S5. Association of the mean number of copulations on day one of the mating assay and the local SR population prevalence. There is a significant negative association using Spearman correlation ($\rho = -0.9$, d.f. = 3, $0.025 < P < 0.05$). Population abbreviations are adjacent to the respective data point. To estimate error, standard errors (SEM) were used for copulation number, and the 95% confidence intervals for *SR* prevalence (binomial distribution).

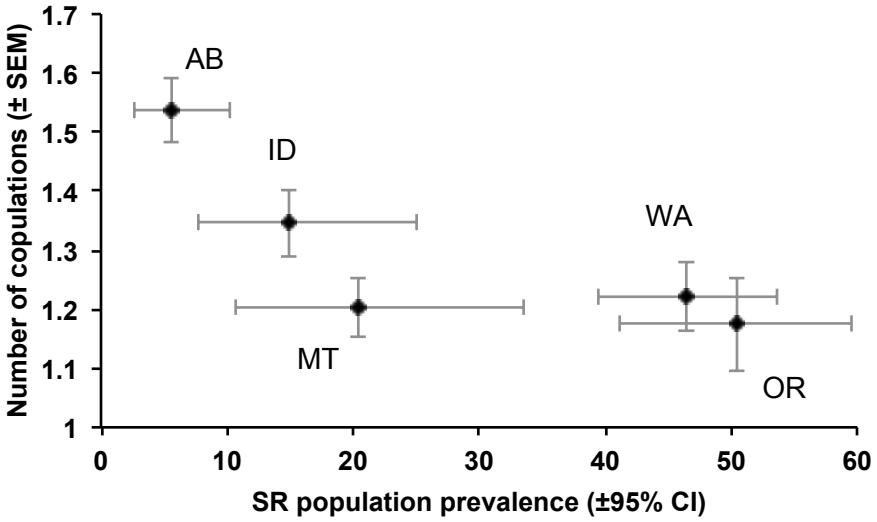
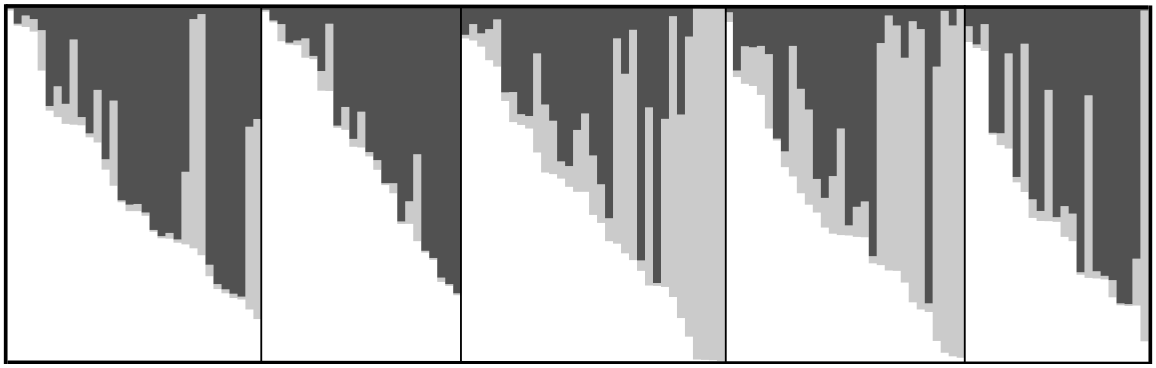


Figure S6: Results of Structure analysis with $K = 3$ populations. Each column depicts an individual, and shows the membership of that individual in each of the three clusters. Individuals are grouped by population, and are sorted by genetic cluster using the abbreviations from Table S1. Individual proportions of membership into each cluster are shown in white, gray, and dark gray.



CHAPTER 3

EFFECT OF *SEX-RATIO* DRIVE ON MALE FERTILITY WITH MULTIPLE MATING AND SPERM COMPETITION IN *DROSOPHILA NEOTESTACEA*

Introduction

Intragenomic conflict is a significant source of evolutionary change and innovation, with potentially far-reaching consequences for the evolution of sex and mating systems [1, 2]. This conflict occurs when selection acts in opposing directions within a single genome, and is often caused by selfish genetic elements (SGEs). SGEs favor their own transmission into the next generation, even if they are harmful to their hosts, and can spread rapidly through a population (reviewed in [3]). *Sex-ratio* (SR) meiotic drive is a selfish genetic element in which a male carries a SR allele on the X chromosome that causes Y-bearing sperm to degrade during spermatogenesis. As a consequence, 100% of his offspring are carriers of the SR allele and all are daughters. Unchecked, SR may spread very rapidly through a population, and may cause extinction due to lack of males [4]. However, many species with SR drive are not extinct in nature and in fact SR alleles are maintained at intermediate frequencies for long periods of time (reviewed in [5]). In the absence of genetic suppression and pleiotropic effects on female fitness, it is unclear why SR does not spread completely through populations. Some proposed forces for keeping SR drive from reaching fixation are pre- and post-copulatory selection, fertility selection, and mating rates [5].

Pre-copulatory selection can occur if there is female choice against SR males that lowers SR male reproductive success. If females can distinguish against SR drive males, the ability for SR to spread may be hindered. Preference for males that do not carry drive can evolve when it is linked to a male ornament [6], or when males carry autosomal suppressors of drive (but only when the frequency of choice is rare; [7]). While there is empirical evidence for female pre-mating choice against driving males in stalk-eyed flies, where females prefer long eye-span males which indicates non-drive [8], many species do not appear to have indicator of drive and thus no other evidence for pre-copulatory choice has been found (see [9]).

Because the mechanism of SR drive manipulates spermatogenesis, it follows that SR male fertility may be negatively affected, since they lose half of their sperm. However, in order for reduced male fertility to inhibit the large transmission advantage of SR, the reduction must be more than 50% [10]. Fertility selection against a driving allele itself can be enough to inhibit its spread in populations [11]. Overall, the fertility of males with SR is reduced in many systems, although some differences only appear after multiple mating (reviewed in [12]). Multiple mating may allow for stable polymorphism of SR through frequency-dependent selection [11, 13]**.** For example high male mating rates may inhibit the spread of SR: as male mating rates increase, SR males may disproportionately suffer from higher levels of reduced fertility. Conversely, higher levels of multiple mating may be necessary to uncover fertility differences because males may pass more than enough sperm than is necessary to fertilize all of an individual female's eggs. If this is the case, only after males begin to become sperm depleted will differences in fertility between SR and ST become evident, assuming no difference in sperm competitive ability. The rate at which male sperm will be depleted is likely to depend on a species' reproductive traits such as male sperm quantity, number of sperm passed during mating, sperm production rates,

and female remating rates. In many SR systems, there is evidence that SR males suffer reduced fertility especially after multiple mating: in *Drosophila pseudoobscura* there was no significant difference in fertility between virgin SR and ST males, but non-virgin SR had 64% the fertility of non-virgin ST males [14]. Similarly, in *D. quinaria*, there was no significant among virgin male types but non-virgin SR males had 69% as much fertility [13]. *D. simulans* virgin SR males had 77% the fertility of normal males, and non-virgins had 50% as much fertility [15]. Similarly, *D. recens* virgin SR males had 72% as much fertility, and non-virgins had only 41% the fertility of normal males [13]. It seems that a reduction in SR male fertility after multiple mating is a common feature in SR drive systems (reviewed in [5]).

During meiosis half of all sperm are destroyed, therefore SR males may be disadvantaged in sperm competition, either due to lesser fertility, or they might suffer even larger effects if X-bearing sperm are also disturbed as a side effect of drive [16, 17]. Sperm competition between driving and non-driving males can be enough to stabilize an SR polymorphism, if SR male fertility decreases with increased male mating rates [11]. Many species with SR show evidence for reduced sperm competitive abilities. *D. pseudoobscura* SR males are poor sperm competitors [18], and *C. dalmanni* SR males suffer reduced sperm competitive ability [19, 20] such that driving male sperm survival in females is lower [21]. *D. simulans* SR males suffer reduced paternity when placed in competition with standard male ejaculates [15], and interestingly, females appear to play a role in the outcome of post-copulatory selection: females preferentially dump sperm from SR males after several days of storage [22]. Poor sperm competitive ability of driving males appears to be a generic feature of drive systems, possibly indicating a general cost of carrying SR [11, 12]. Overall, the outcome of sperm competition can be predictable: for example, although there is considerable interspecific variation in sperm utilization patterns of

insects; in general the last male to mate with a female tends to obtain more fertilizations than the first [23]. Therefore, it is possible to determine whether differences in sperm competitive ability of SR males are attributable to the destruction of half of their gametes, or if the reduction in paternity is even more disproportionately reduced (reviewed in [24]).

When females mate multiply, both SR fertility costs and reductions in sperm competitiveness may become magnified. Theoretical and laboratory studies suggest that if females mate less than twice, *sex-ratio* drive can invade and persist in populations [11, 25], and that female multiple mating can allow for a stable SR equilibrium frequency [11]. Clines in female remating show that higher remating is associated with lower SR prevalence, suggesting that selection in the form of local mating rates may regulate SR frequencies in natural populations [26, 27]. The rate at which females mate is at least partly determined by genetics [28, 29] and also by the population sex ratio, which may be influenced by the frequency of SR itself [27]. If female mating rates are low and there is strong last male sperm precedence, in the wild the majority of a female's offspring should be sired by one male (the last to mate). In *Drosophila*, there is usually last male sperm precedence due to processes like sperm displacement, incapacitation, or other mechanisms [30]. For example, *D. simulans* mates infrequently and shows strong last male sperm precedence in the laboratory [31], and paternity analysis of offspring from wild females shows an average of only 1.4 sires per brood [32]. Many studies of sperm precedence in the lab match estimates of paternity in the wild (reviewed in [33]), however a few studies show mixed or contrary evidence (see [34, 35]). If SR does poorly in sperm competition in the laboratory, it is possible that SR males will also sire a similarly small proportion of offspring in the wild, taking into account the normal level of sperm precedence.

To explore the conditions that keep SR from spreading to fixation (and resulting in extinction) as is expected due to its large transmission advantage, *D. neotestacea* is an excellent study system. *D. neotestacea* is a mushroom feeding fruit fly that inhabits temperate and boreal forests in parts of North America. Populations vary in the local prevalence of SR drive, where 0 – 30% of males carry SR. These frequencies have existed as a stable cline for over 20 years [36, 37], except for a case of a SR-induced crash in an isolated habitat [26]. There is no evidence for pleiotropic effects of drive in females, or for any active genetic suppression [36, 38, 39]. Although a considerable amount of theoretical and empirical work has focused on drive dynamics, little is known about the selective factors that maintain this cline in SR frequency. Previous work in this species finds no evidence of pre-copulatory selection between SR and ST males [39], evidence of reduced SR male fertility especially after multiple mating [26, 39], and evidence of reduced SR fertility from wild-caught males during hypothesized conditions of high multiple mating, which may be due to either reduced SR sperm stores and/or decreased SR sperm competitive ability relative to ST [39]. Depending on the location of origin, females will mate on average about twice (females from some locations average below two times, and in some locations average more than twice) in about 14 hours in the laboratory [26].

In this study, we explore the potential for multiple mating to reduce the transmission advantage of SR in *D. neotestacea*. First, we investigate whether male mating rates differ between SR and wild-type males. Second, we explore the reduction of SR male fertility relative to wild-type after each of several matings, and sperm replenishment ability over multiple days. Third, we sequentially and reciprocally mate SR and wild-type males to test for differences in sperm competitive ability. Finally, we ask if the sperm utilization patterns we observe in the laboratory are consistent with paternity estimates from wild-caught flies. We hypothesize that

there will be no difference in male mating rates between SR and wild-type males, SR males will have lesser fertility especially with multiple mating and will be less able to recover after multiple days of mating, and SR males will do poorly in sperm competition in the laboratory and in the wild.

Materials and Methods

Fly lines and maintenance

Flies were collected in 2010 near Edmonton, Alberta off rotting commercial mushrooms (*Agaricus bisporus*) by sweeping a net above the piles. Five isofemale lines were established and maintained for approximately 3 years in food vials that contain instant *Drosophila* medium (Carolina Biological Supply) with a piece of commercial mushroom (*Agaricus bisporus*) with a 14 L : 10 D cycle at 20°C.

We created a mixed stock using a round-robin crossing design to ensure that genotypes from all lines were represented. We utilized a stock that carries a *sex-ratio* (SR) chromosome extracted from wild-caught flies collected near Eugene Oregon in 2001, and has been maintained using a series of three crosses which maintains SR as a homozygote in females, and produces both SR and standard (ST) males (see [36]). We introgressed ST males from our mixed stock into the SR stock for over 10 generations, so that the vast majority of the genetic background was replaced with the mixed stock except for the X chromosome, which are SR.

Effect of sex-ratio allele on male fertility with multiple mating

To quantify the impact of *sex-ratio* (SR) on male fertility after multiple mating relative to standard (ST) males, we allowed SR and ST males to mate with up to 10 females and counted how many offspring they produced. Males were allowed to recover overnight and were then presented with the opportunity to mate two more times on the second day. We collected virgin standard (ST/Y) and sex-ratio (SR/Y) males, and standard (ST/ST) females, and each sex was housed separately at a density of 20 flies per vial and allowed to age for 7-10 days to become reproductively mature. Approximately 36 hours before the experiment, we aspirated either ten or two females into vials. On the first day of the experiment, we transferred a single male (either SR or ST) to vials with ten virgin females and watched for successful copulations. When a vial was observed to have a copulating pair, we aspirated out the non-mated females and transferred them into a fresh vial. Once the original copulation pair had separated, we removed that male and transferred him into the vial containing the remaining virgin females without replacement (nine females then eight, etc.). We recorded the male genotype as well as the N^{th} female mating order. We allowed males the opportunity to mate for about 8 hours, then removed the males and housed them alone to recover overnight. The following day, we assessed male ability to replenish sperm stores after depletion by transferring the same males to vials containing two additional virgin females and allowed them to mate in the same manner. If males were unsuccessful in mating after about 2 hours, we added 1-3 more females to their vial. Here, we gave males the opportunity to mate for a total of four hours before separating them. Each female was transferred to a fresh food vials within an hour of the end of copulation, where she was housed alone. Females were transferred to new food every four days for a total of twelve days, and then were allowed to remain in the final vial until death. We counted the number of offspring produced by

each female and excluded vials in which females died before the final transfer. We compared the offspring production of 41 SR males with 41 ST males.

We investigated differences between SR and ST males in the number of matings and the number of offspring on each day using Mann-Whitney U tests, as our count data are non-normal [40]. We also investigated the effect of male genotype on the number of offspring from each mating on each day using a generalized linear model (GLM) with a Poisson distribution and log-link function. All statistical analyses were performed in JMP v. 11 (SAS Institute, Cary, NC, USA).

Effect of sex-ratio allele on sperm precedence/competition

To assess the ability of SR males to gain fertilizations when their sperm are in the female reproductive tract with standard (ST) males, we sequentially mated both types of males to females and counted the number of offspring of each sex. We collected virgin standard (ST/Y) and *sex-ratio* (SR/Y) males, and standard (ST/ST) females. Virgins of each sex were housed separately at a density of 20 flies per vial, and allowed to age for 7-10 days to become reproductively mature. Approximately 36 hours before the experiment, females were aspirated onto fresh food, one female per vial. Within one hour of the incubator lights turning on, we aspirated one individual male (either a SR or a ST male) into a vial with a female. We observed and recorded those that mated successfully. Males were removed within 15 minutes after the copulation had ceased. To assess the number of offspring of each sex from SR and ST males alone, we randomly chose a fraction of the females to receive a single mating, and placed them individually in fresh vials immediately after removing the males. We allowed the remainder of

the mated females to recover overnight alone in food vials, and then performed the same experiment again the following morning. Females were randomly placed in double-mated treatments so that they received one male of either the same or a different genotype (1st day then 2nd day: SR then SR, ST then ST, SR then ST, or ST then SR). We watched for copulation, and within 15 minutes of mating ending, the male was removed and the female was transferred to a fresh vial, so that we only counted offspring that emerged from eggs laid after the second mating. If a female in the double-mated treatment did not successfully mate on the second day, she was discarded. We transferred females to fresh vials every four days for a total of 16 days, and then allowed females to remain in the final vial. We excluded any instances in which females were found dead before the final transfer. Once offspring started to emerge, we checked vials every other day and placed the offspring in 70% ethanol. We separated offspring by sex and counted the total number of each sex produced. Our sample sizes were for single matings: SR = 21, ST = 53, and for double matings: SRSR = 20, STST = 22, SRST = 38, and STSR = 41.

We compared the mean numbers of offspring between singly and doubly mated females with the same male genotype (SR vs. ST, SRSR vs. STST), as well as compared doubly mated females with reciprocal male genotypes (SRST vs. STSR) using t-tests (these data were normally distributed) in JMP. We calculated the proportion of offspring of each sex in each male mating treatment, and used the singly mated proportions (SR or ST only) to create an expectation of the relative numbers of each sex that should be produced by each male genotype when mated reciprocally (SRST or STSR). For example, if mating with ST males produces 50% male offspring and mating with SR males produces 100% female offspring, then any male offspring produced by reciprocal matings can be attributed to the ST father. If a total of 100 offspring are produced and 25 are male, then this means that 25 of the female offspring are expected to be

from the ST father (because ST produces 50% male and 50% female offspring) and the SR father sired the remaining 50 females. Therefore, from the proportions of the sex of offspring produced by SR and ST alone, we estimated the percentage of offspring sired by the first male and the second male to mate in the reciprocal crosses, and tested for differences with a 2-sample z-test.

Female multiple mating in the wild

To assess the level at which females multiply mate in nature, we collected flies from the Great Smoky Mountains National Park (GSMNP), in June and September of 2012. We placed wild-caught females singly into vials, and reared their offspring at 20° C on a 14 L: 10 D cycle. We froze the females and her offspring at -80° C. We genotyped individuals at four autosomal microsatellite markers, Dn6003, Dn6429, Dn7013, and Dn8380 (Accession nos. EF199834, EF199835, EF199837, and EF199827), which were found to have high heterozygosity in this population, as well as the two X-linked markers, Dn8377 and Dn8385 (Accession nos. EF199832 and EF199836, respectively), which together have 94% accuracy in assigning an X-chromosome as SR or ST based on different size fragments [26]. The methods for all microsatellite genotyping and fragment analysis were as previously described [41].

We genotyped individuals from 14 families from each collection time (June and September), for a total of 28 families. Most families consisted of 20 randomly chosen offspring, except for seven families that had between 12-19 offspring. Four families did not have a maternal sample. We calculated the minimum number of fathers that explained the genotypes present in each family using Gerud 2.0 [42], which uses an exhaustive algorithm to reconstruct the parental genotypes. We were also able to reconstruct the maternal genotypes of families that

lacked a maternal sample using this program. Additionally we obtained an upper limit estimate of paternity using COLONY 2.0, which uses a full-pedigree likelihood method to infer both sibship and parentage [43]. These data fit a Poisson distribution, so means were compared using the nonparametric Mann-Whitney U test and homogeneity of variances were tested with the Brown-Forsythe test, which is robust to violations in normality [44].

Results

Effect of sex-ratio allele on male fertility with multiple mating

We do not find any evidence of pre-mating differences in the ability for *sex-ratio* (SR) males to gain mates relative to standard (ST) males. On the first day, SR had an average of 3.91 mates and ST had an average of 3.58 (U = -1.59, P = 0.11; Figure 3.1). The maximum number was 10 matings, but only one male mated that many times. On the second day, both SR and ST males had an average of 1.50 matings (U = 0.00, P = 1.00; Figure 3.1), which includes vials in which extra females were added (> 2%).

We found that SR produced fewer offspring than ST. The total mean number of offspring across both mating days for SR males was 503.95 offspring and 561.42 offspring for ST males, which a 10% lower fertility for SR and is a significant difference (U = 2.30, P = 0.02; Figure 3.2). On day one SR males produced about the same number of offspring as ST males (the total mean number of offspring for SR was 425.68 and for ST was 443.43). However, on day two SR males produced about 65% as many offspring as ST males (SR had a total average of 78.26 compared to 117.99 ST). We were unable to detect a statistical difference while considering mating days separately. Additionally, we looked for an effect of male genotype on the number of

offspring by the mating order and mating day using a GLM (for values of number of offspring per day for SR and ST see Figure 3.3). On mating day one, we found significant effects for the first ($\chi^2 = 87.51$, $P < 0.0001$), second ($\chi^2 = 38.45$, $P < 0.0001$), fifth ($\chi^2 = 52.94$, $P < 0.0001$), and sixth mating ($\chi^2 = 64.16$, $P < 0.001$). In each case except for the second mating, ST males produced more offspring than SR males. We did not evaluate the ninth and tenth matings because there were too few males that mated this many times. On the second mating day, we detected a difference between ST and SR for both the first mating ($\chi^2 = 128.84$, $P < 0.0001$) and the second mating ($\chi^2 = 168.17$, $P < 0.0001$), both of which ST males produced more offspring than SR. Overall SR produced fewer offspring than ST, especially after sperm depletion and recovery.

Effect of sex-ratio allele on sperm precedence/competition

Overall females that mated with ST males produced more offspring than those mated with SR males (Figure 3.2). For single matings, the mean number of offspring for SR males was 127.76 and for ST males was 146.93, with a 13% lower fertility for SR that is not significant ($t(72)= 0.97$, $P = 0.33$; Figure 3.4). For double matings, females mated to a SR then another SR had an average of 126.75 and those mated to ST then ST males had an average of 146.92, with a non-significant 14% lower fertility for SR ($t(41) = 0.86$, $P = 0.39$; Figure 3.4). Similarly, we did not detect a difference in the number of offspring between reciprocal male double matings, where SR males first followed by ST produced 132.43 and ST males first followed by SR produced 149.49, with a non-significant 11% lower fertility when SR males mate first ($t(77)= 1.02$, $P = 0.31$; Figure 3.4). Interestingly, SR and SRSR matings and ST and STST matings

produced remarkably similar numbers of offspring. It appears that in this limited set of crosses when a female mates with an SR male she does not suffer a statistically significant reduction in fecundity.

Consistent with what we know about SR drive in this species, SR males produced nearly all (100%) female offspring in single and double matings. Also consistent with our expectations, in both single and double matings, ST males produced nearly half (52%) females (Figure 3.5). Interestingly, when females were mated to both an SR and ST males, either reciprocal mating produced a similar proportion of female offspring (SRST = 66%, STSR = 68%; Figure 3.5). For the SRST treatment, we estimate that SR males sired 29% of the total offspring when mating first and ST sired 71% when mating second. When ST was the first male, they sired 67% of offspring, and SR, the second male to mate, sired only 33% (Figure 3.6). The proportion of offspring sired by SR is significantly lower for SR in both crosses ($z = 39.90$, one-tail $P < 0.0001$). Regardless of mating order, SR males sired ~1/3 of a female's offspring when placed in competition with ST males. This suggests that there is no last male precedence, as has been found in many other *Drosophila* species [30].

Female multiple mating in the wild

We find evidence for female multiple mating in the wild. In both June and September collections, we estimated that females produced offspring from an average minimum of 2.00 fathers ($U = -0.36$, $P = 0.72$; Figure 3.7). The variance in minimum father number was different among collections, as displayed by the distributions of number of fathers by the number of families (Brown-Forsythe: $F_{1,26} = 5.44$, $P = 0.028$: Figure 3.8). Most families in June were

estimated to have a minimum of two fathers, whereas most families in September had a minimum of one father, although several females required a minimum of two, three or four fathers to explain the genotypes of their progeny. We also used likelihood methods to obtain an upper estimate of paternity. We estimated the mean number of fathers in June to be 4.71 and September 4.07, which are not different from one another ($U = 0.79$, $P = 0.37$; Figure 3.7). Most families in June were estimated to have three fathers but one family was estimated to have a maximum of nine fathers. Most families in September were estimated to have two fathers, but one family was also estimated to have a maximum of nine fathers (Figure 3.9). The variances in these estimates were not different (Brown-Forsythe: $F_{1,26} = 0.02$, $P = 0.89$). The minimum and maximum likelihood paternity estimates were significantly correlated with one another ($R^2 = 0.24$, $P = 0.0088$). In sum, we find that most wild-caught females have mated multiply. This also suggests that females use the sperm from multiple males and supports our finding from our laboratory study that there is not strong displacement of all the sperm of previous males in the female's reproductive tract by the last male.

We examined the X linked loci of offspring to identify ST and SR alleles from our minimum paternity estimates, because it was straightforward to keep track of the genotypes from each father. We combined data from June and September, as we found they were similar when analyzed separately. We found that the frequency of SR was ~ 25 – 26%, which is similar to estimates of this site from ten years ago (25% by K. Dyer). The proportion of SR fathers was ~ 26 – 27%, which is expected to match SR frequency if there is no pre-copulatory selection against SR. There were four families that had a double mating with one SR father and one ST father. Of the total of 73 offspring produced, SR males sired 27% of the offspring. This is

consistent with the findings of our laboratory reciprocal matings between SR and ST males, and suggests this sperm utilization pattern holds in nature.

Discussion

The effect of SGEs on evolution and reproduction are significant [3]. For the SGE, *sex-ratio* drive, males produce fewer sperm as all Y-bearing sperm die during spermatogenesis, which may make them vulnerable to reduced fertility and poorer sperm competitors during multiple mating. *Drosophila neotestacea* mates often, which allow us to investigate their reproductive biology in relation to *sex-ratio* drive relatively easily. First, we investigate pre-copulatory differences between SR carrying males and standard (ST) males. Consistent with earlier findings in *D. neotestacea* and results from *D. pseudoobscura*, we find no difference in the mating success between SR and ST males [9, 39]. It is possible that there is no female preference or male trait associated with SR drive, as there is in stalk-eyed flies [8]. Therefore, post-copulatory processes may be more important in this system.

We found an effect of multiple mating on SR male fertility, especially on a second day of mating. On the first day and first mating, SR males sired 85% as many offspring as ST males, compared to 64% from a previous study [26]. Lines from different populations were used in this study it is possible that while SR was introgressed into this population genetic background there were different epistatic effects with male fertility. In this study, SR male fertility fell below 50% after the 5th mating, which is enough to counter its transmission advantage [10]. A second day of mating after up to ten mating opportunities on the first day was meant to evaluate sperm

depletion. On this day, after the first mating SR males had 72% of the fertility of ST males, and after the second mating they had 42% as much fertility as ST males, which is closer to what has been estimated previously in this species [26]. While we did find a reduction in SR male fertility on the first day, it is unknown whether enough males will have that many opportunities to mate in one day, in order for this fertility difference to be important. However, it does seem that SR males being less able to replenish sperm stores is plausible, as flies utilize mushrooms that exist for many days at a time as mating substrate, and may stick around to mate with receptive females.

In contrast to other SR drive systems, we do not find evidence for SR males being poor sperm competitors above the effects of sperm count halving [15, 17, 18]. Our data support a sperm-mixing model, where the total number of sperm transferred translates to the number of offspring that will be produced [45]. SR males carry 50% less sperm than ST. If ST has 1,000 sperm (500 X, 500 Y), then SR should have 500 sperm (500 X^{SR}). If sperm mix equally and a female produces 120 offspring then 40 ($1/3^{rd}$) will be from SR (all females), and 80 ($2/3^{rd}$) will be from ST (40 males and 40 females). For example, in our reciprocal double matings, when ST were followed by SR males a total of 6,129 offspring were produced. Of these offspring, 1,970 were males and 4,159 were females. Based on ST single matings we determined that ST fathers produce 48% males and 52% females, so we were able to estimate that 2,147 of the 4,159 females were from ST fathers, and the remaining 2,012 are from SR fathers (~33% of all offspring). This result is consistent with sperm mixing. When SR males mate first, they only gain 29% of fertilizations, which although a small difference, is statistically significant ($\chi^2 = 8.85$, d.f. = 1, $P = 0.003$), which suggests that ST may be slightly better at last male sperm precedence. A limitation of this result is that we do not know what normal levels of sperm precedence are. For

example, if last male sperm precedence is normally strong (~ 66%), which is what we found when ST mated last, it is possible that when SR mates second they only gain ~33% which would then be evidence that SR has poorer sperm competitive ability. Therefore, it would be useful to utilize visible or molecular markers with two ST males to evaluate this possibility. However, we can independently verify our sperm utilization estimates by estimating levels of paternity and multiple mating in nature.

We find evidence for occasionally high levels of multiple mating in a natural population of *D. neotestacea*, which supports the interpretation that last male sperm precedence is not particularly strong. Using our minimum paternity estimates, we detected four families that mated with two different males (one SR and one ST). We found that SR males sired 27% of the 73 total offspring produced by these matings, which is similar to the expectation of 33% consistent with sperm mixing. Females in this species mate on average about twice in 12 hours in the laboratory [26], yet we find up to 4 or 9 fathers per brood (for minimum and maximum likelihood estimates, respectively). *Drosophila buzzati* have a similar average of 3.57 fathers per brood (compared to 2.0 or 4.7 in this study), but high levels of last male sperm precedence as measured in the laboratory (proportion of offspring sired by second male = 0.82; [46]). In *D. buzzati* the ability to detect a large number of fathers despite strong sperm precedence is likely due to extremely rapid rates of female remating: females mate on average 2.15 times in only four hours, compared to *D. neotestacea* which mates up to four times in twelve hours [26, 47].

Alternatively, it has been proposed that because female multiple mating promotes sperm competition, and SR males are often poor sperm competitors, females may evolve a strategy to undermine the transmission of SR by biasing paternity towards non-driving males [48]. An alternative hypothesis that could explain our results is that the proportion of offspring sired by

SR (29-33% regardless of mating order) is due to a mechanism of cryptic female choice. Existing mate choice models apply to pre-copulatory traits and preferences, but might easily translate to post-copulatory mate choice [6]. The hypothetical ejaculate trait that females would cryptically use to distinguish SR sperm would be tightly linked with drive, so that recombination could not break this association, as with other traits [49]. However, we suggest that random sperm mixing is a more parsimonious explanation for our results, but comparative transcriptomics of sperm and seminal fluid-related gene expression between SR and ST males may be able to investigate this alternative further.

Instead of last male sperm precedence, which is common in insects and especially *Drosophila* [30], our data suggest that *D. neotestacea* sperm competition is a fair raffle in which sperm are simply added numerically and mix randomly. It would be interesting to evaluate whether sperm mixing would also take place with more than two matings. In our wild paternity data, only two families mated three times with the two types of males (once with SR and twice with ST males). SR males sired 13% of offspring, compared to the expectation of 20% ($1/5^{th}$) under a sperm-mixing model. It is unknown how often these males have mated previously; it is possible that prior sperm depletion may explain deviation from expectations. Future experiments should quantify the reduction in the proportion of offspring sired by SR males in the laboratory with reciprocal matings after males have mated at least one time previously, and are thus sperm depleted. This will evaluate the effect of sperm depletion on male fertility in more realistic way, likely more closely to conditions that are found in nature.

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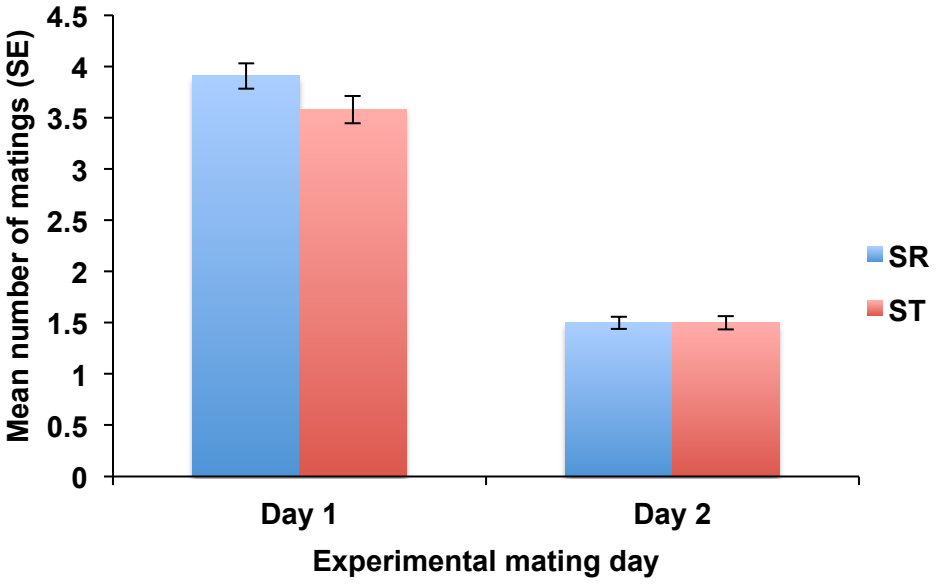

Figure 3.1 Mean number of matings by *sex-ratio* (SR) (N=41, in blue) and standard (ST) (N=41, in red) males across two experimental mating days. Mating day 1 is out of ten matings and mating day 2 is out of two matings. To estimate error, standard errors (SE) were used.

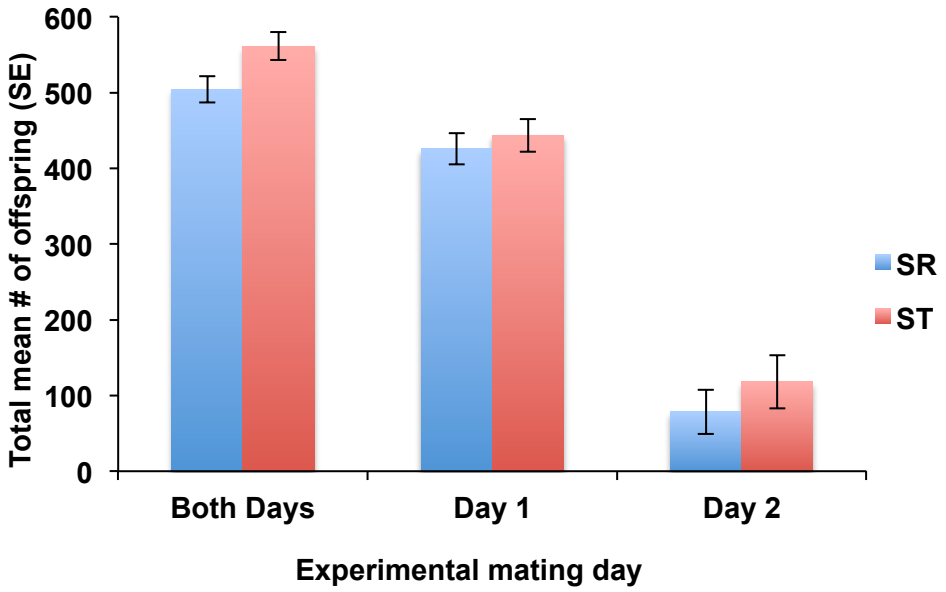

Figure 3.2 Mean number of total offspring produced by *sex-ratio* (SR, in blue) and standard (ST, in red) males across each of two experimental mating days, and both days combined. To estimate error, standard errors (SE) were used.

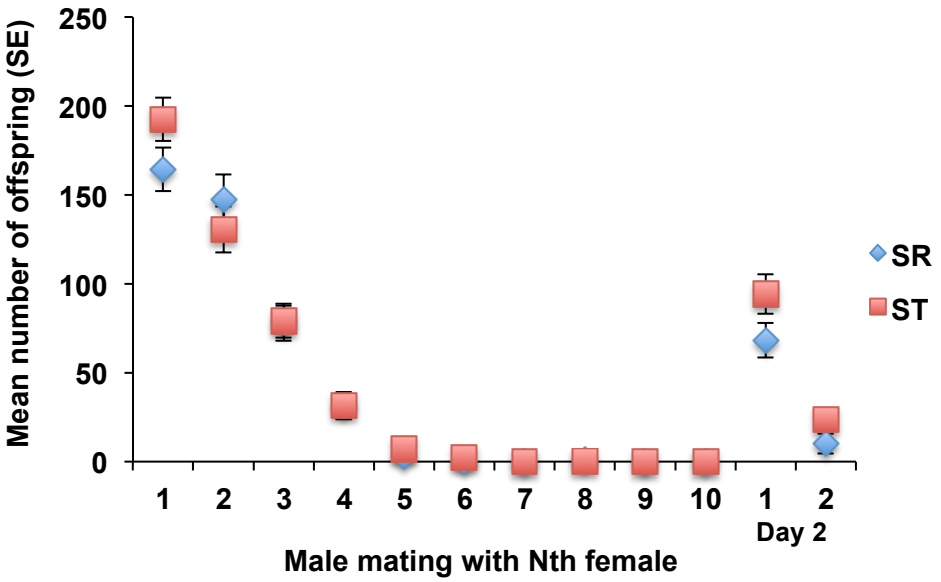

Figure 3.3 The mean number of offspring produced by *sex-ratio* (SR, blue diamonds) and standard (ST, red squares) males, by the Nth mating on day 1 (1-10) and day 2 (1-2). Only males that mated N times were included for each estimate. To estimate error, standard errors (SE) were used.

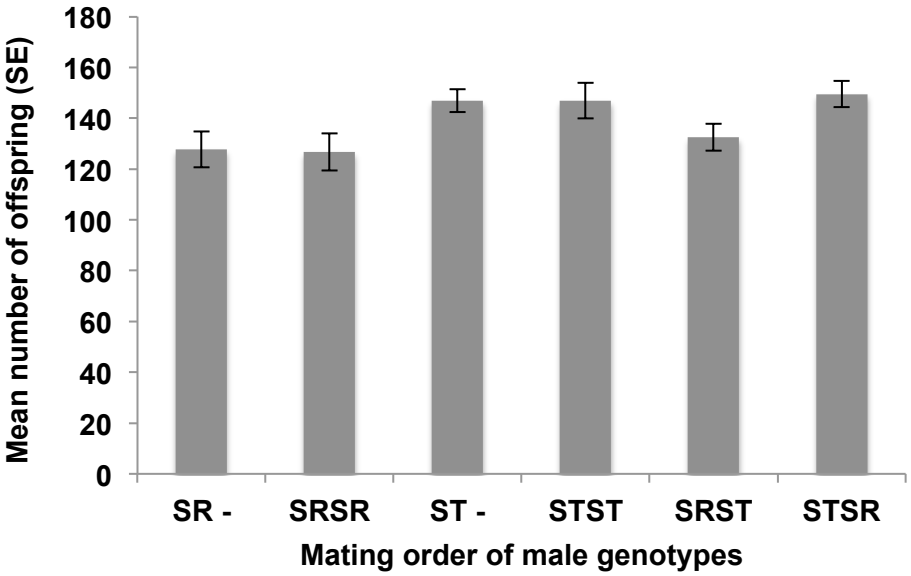

Figure 3.4 Mean number of offspring produced by single or double matings with *sex-ratio* (SR) and standard (ST) males in different mating orders. To estimate error, standard errors (SE) were used.

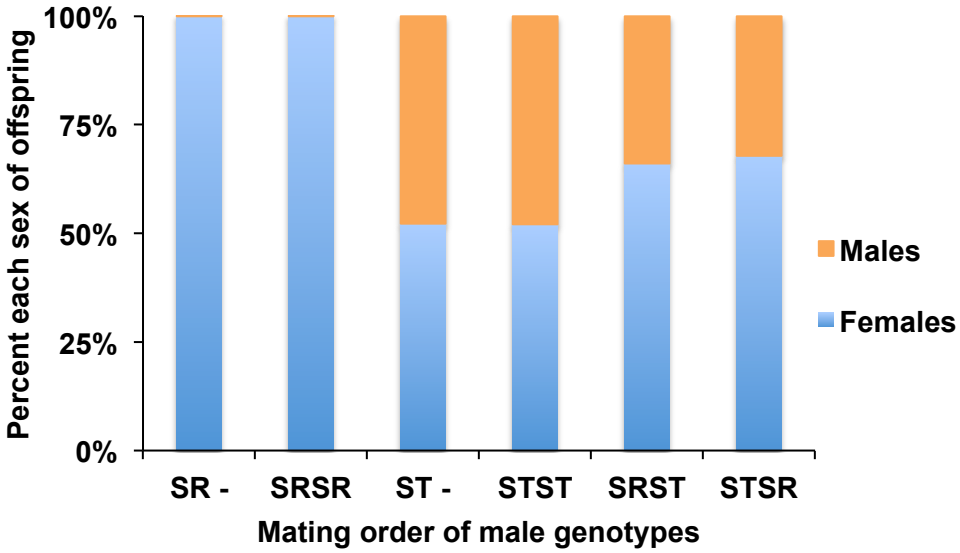

Figure 3.5 The percentage of each sex (females in blue, males in orange) of the offspring produced by single and double matings of *sex-ratio* (SR) and standard (ST) males in different mating order combinations.

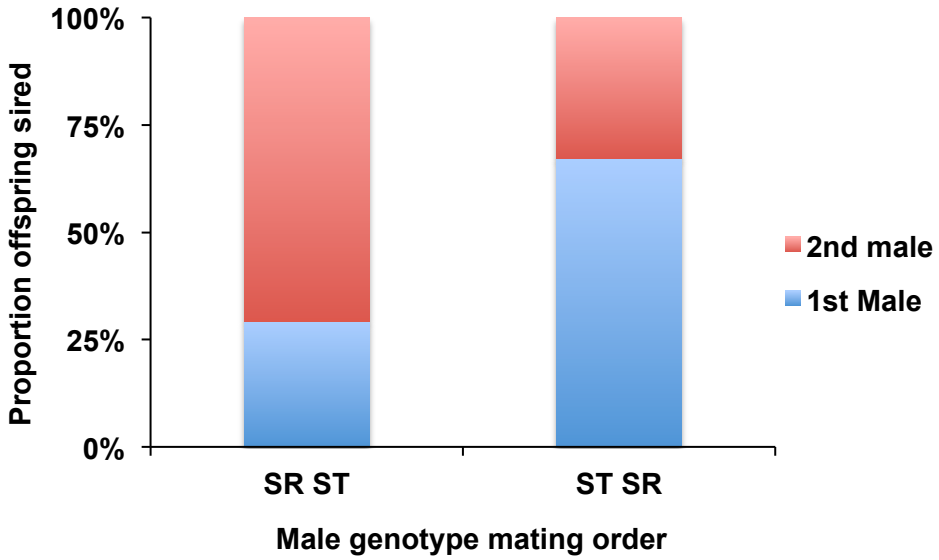

Figure 3.6 The proportion of offspring produced by the 1st (in blue) and 2nd (in red) male to mate after reciprocal double matings with *sex-ratio* (SR) and standard (ST) males. Regardless of mating order, SR males produced fewer offspring (29% and 33%, 1st and 2nd mating respectively).

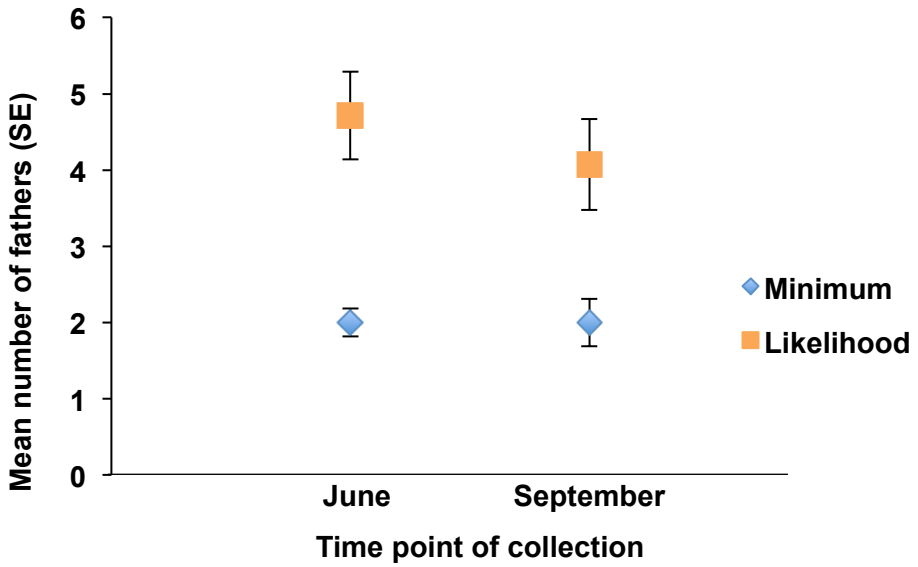

Figure 3.7 The average number of fathers found to produce offspring with wild-caught females from paternity analysis, at two different collection times (June and September). Estimates of the minimum number of fathers to explain offspring genotypes are shown with blue diamonds, and likelihood estimates are shown with orange squares. To estimate error, standard errors (SE) were used.

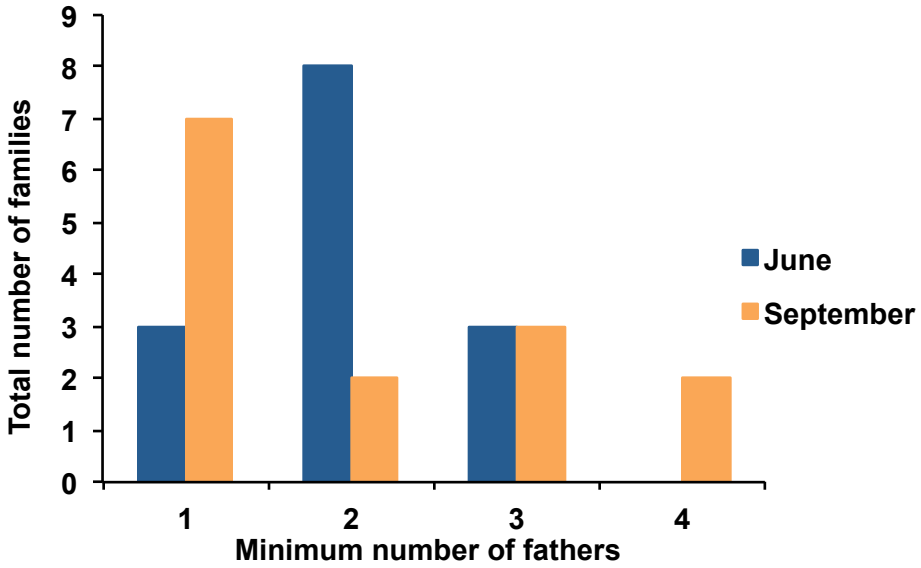

Figure 3.8 The distribution of the total number of families sorted by the minimum number of fathers found by paternity analysis by the month of collection (June in blue, September in orange).

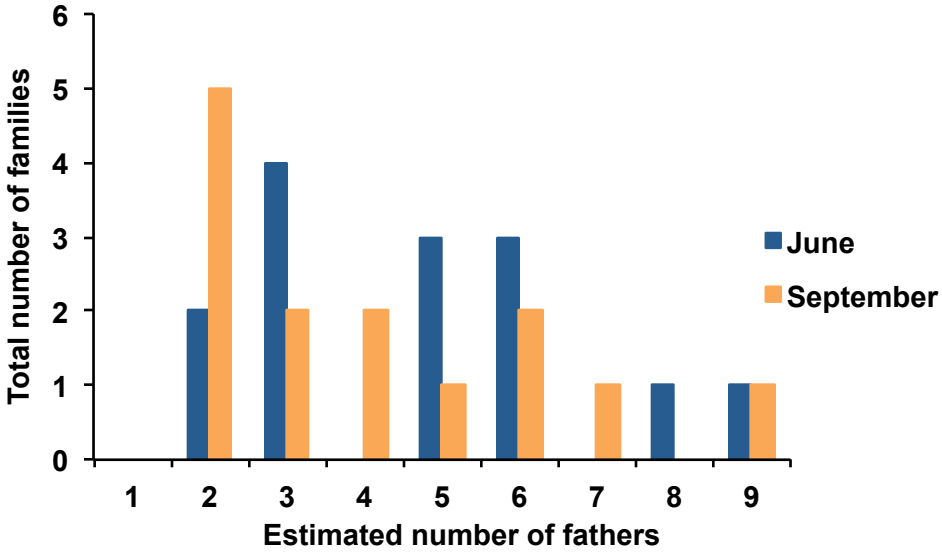

Figure 3.9 The distribution of the total number of families sorted by a likelihood estimate of the number of fathers found by the month of collection (June in blue, September in orange).

CHAPTER 4

SUMMARY AND CONCLUSIONS

Selfish genetic elements (SGEs) can greatly influence their host's genome and reproductive biology (reviewed in [1-3]). For the SGE known as *sex-ratio* (SR) drive, males produce fewer offspring as their Y-bearing sperm degrade during spermatogenesis (reviewed in [4, 5]). Thus, they may be more vulnerable to reduced fertility in situations of multiple mating. This disadvantage may explain why despite their large transmission advantage (~100%), some SRs are observed at intermediate frequencies in nature.

The fruit fly *Drosophila neotestacea* is an ideal species to investigate the role of multiple mating in SR prevalence. Both males and females mate promiscuously and SR exists at stable frequencies across locations through time [6, 7]. We know a lot about their ecology, so we can collect natural populations and easily maintain them in the laboratory to test specific hypotheses through experimentation. We and others have shown that females do not distinguish between SR and standard (ST) males (chapter 3; and [8], therefore male fertility selection may play a role in influencing SR prevalence through both female and male mating rates.

In chapter 2 of this dissertation we investigated whether differences in female multiple mating behavior, also known as polyandry, could help explain the geographic cline in *sex-ratio* prevalence among populations. We showed an association between polyandry and SR prevalence such that females from locations with high SR prevalence mated less often than those from areas with lower SR prevalence, across a geographic cline. Our results support the scenario that high

levels of polyandry may suppress the frequency of SR, and low levels of polyandry may allow SR to increase in prevalence due to fertility selection against SR males. When females mate twice on average, when SR is at low frequency males will also mate on average twice. However, when SR is at higher frequency (which leads to a female-biased population level sex ratio), males will mate even more on average. For example, if SR frequency is 20% males will mate on average three times, if SR is at 50% males will mate six times on average. Therefore, if females mate more than twice, the number of times males mate on average will continue to increase, and may be detrimental to SR fitness due to lesser male fertility. Looking across populations, because there are differences in SR prevalence that are stable over time, it is possible that SR is maintained by migration-selection balance through male fertility selection (via differences in female remating rates), given the substantial levels of gene flow we found across this species. Finally, we found the highest SR prevalence ever reported in this species and in any species without segregating suppressors of drive (45-50%). Additionally, we witnessed a population crash likely due to a combination of high SR prevalence and habitat fragmentation. As far as we know this SR-induced local extinction is the first reported, and was previously only hypothetically predicted [9].

In chapter 3 of this dissertation we investigated the effect of SR on male fertility after multiple mating and in sperm competition. First, we found that overall SR males produced fewer offspring on average than their ST counterparts after several rounds of multiple mating in the laboratory, and that SR males were more sperm depleted on a second day of mating. Second, counter to other species with SR drive [10-12], we found that SR males do not do poorly in sperm competition: they sire ~ 1/3rd of a female's offspring regardless of mating order, which is consistent with them passing $1/3^{rd}$ the amount of sperm (given 50% of their sperm die, compared

to 100% of ST that live). This result suggests that female's utilize sperm with random mixing, rather than last male sperm precedence as is found in many other insect species [13, 14]. Lastly, we found that wild-caught flies showed similar patterns of sperm usage to those we found in the laboratory, supporting our result of a sperm-mixing model. Therefore, in *D. neotestacea* it appears that SR male fertility decline after multiple mating is what differs from standard, wild type males.

In sum, the results of this work suggest that multiple mating, in both males and females are important determinants of SR prevalence in natural populations of *Drosophila neotestacea*. Differences in male fertility after multiple mating, but not poorer sperm competitive ability, may allow selection against SR to inhibit the spread of this chromosome to fixation, and therefore extinction in populations. Local female polyandry may also influence this stable cline in SR frequency observed in natural populations, which is suggested by the presence of a cline in female multiple mating behavior. Interestingly, clines in polyandry of species with unsuppressed SR chromosomes [15, 16] appear to differ from the majority of polyandrous species: usually southern latitudes have more polyandry and northern less [17], but *D. neotestacea* and *D. pseudoobscura* show the opposite pattern. It is possible that more female-biased population sex ratios due to greater SR frequencies may decrease the amount females multiply mate, and thus relax selection on female mating rate. Perhaps instead of SR selecting for increased polyandry, as suggested in other studies [18], SR may select for lower rates of polyandry in populations. Thus, if females mate less that means that males will also mate less, perhaps allowing SR to be stabilized at higher equilibrium frequencies. *Sex-ratio* (SR) meiotic drive is a classic selfish genetic element (SGE) that is a significant source of evolutionary change and innovation [2, 3].

Our findings are important for understanding both the long and short-term maintenance of SR in populations, as well as understanding the importance and evolution of reproductive behavior.

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