Sexual conflict in *Drosophila serrata* across populations and environments

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Abstract

Earlier studies implicate interlocus sexual conflict as having important evolutionary consequences in *Drosophila serrata* but it has never been directly studied. I quantify sexual conflict and its divergence between two laboratory stocks using a full factorial manipulation of male population, female population, and level of male exposure to track longevity and fecundity of individual females. Evidence of strong sexual conflict, as well as divergence of male harm and female resistance between populations, was found. The relationship between environmental complexity and sexual conflict was then explored using a combination of behavioural observations and a factorial experiment to again track longevity and fecundity of individual females. Increased complexity was associated with decreased sexual activity and increased lifespan in females, although effects on fecundity were less clear. Overall, my studies combine to provide initial data for characterizing sexual conflict in *Drosophila serrata* and shed light on important considerations for its study.

Résumé

Des études passées impliquent que les conflits sexuels interlocus ont des conséquences importantes sur l'évolution des *Drosophila serrata*. Ce lien n'a été jamais étudier directement. Je quantifier le conflit sexuel et sa divergence entre les deux stocks de laboratoire en utilisant une manipulation factoriel complet de la population masculine, la population féminine, et le niveau d'exposition des mâles pour suivre la longévité et la fécondité des femelles individuelles. Les indices du conflit sexuel ont été forts, ainsi que la divergence de dommage mâle et femelle résistance entre les populations a été trouvé. La relation entre la complexité de l'environnement et le conflit sexuel a ensuite été explorée en utilisant une combinaison d'observations comportementales et une expérience factorielle de suivre à nouveau la longévité et la fécondité des femelles individuelles. Complexité accrue a été associée à l'activité sexuelle diminué et augmenté la durée de vie chez les femelles, avec des effets sur la fécondité étant moins claire. Pris ensemble, mes études se combinent pour fournir des données initiales pour caractériser le conflit sexuel chez la *Drosophila serrata* et d'introduire des considérations importante pour l'étude de celui-ci.

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Thesis Overview

My thesis is the result of original research conducted at the University of Ottawa using a species of fruit fly endemic to Australia, *Drosophila serrata*. My goal was to quantify sexual conflict and investigate its divergence between two related laboratory stocks, in addition to quantifying how sexual behaviours and their fitness costs are altered across two levels of environmental complexity. The results of my research were first written as a standalone manuscript for publication in a scientific journal. As such, the introduction was relatively brief and assumed background knowledge of the subject. I therefore wrote a broader, more general introduction that I had planned on using as Chapter 1, with the draft manuscript becoming Chapter 2. However, initial attempts at doing so resulted in substantial overlap between Chapter 1 and the introduction to Chapter 2. For this reason, and in consultation with my supervisor, I instead opted to combine everything into a single chapter with an expanded introduction that provides additional background information on the effects and implications of sexual conflict, particularly in *Drosophila* species, while minimizing redundancy.

Chapter 1

Sexual conflict in *Drosophila serrata* across populations and environments

1.1 *Introduction*

Evolution is the culmination of a number of natural processes, each with their individual effects and interactions. Two of the best known mechanisms contributing to the evolution of sexually reproducing organisms are natural and sexual selection. Natural selection results from the differential survival and fertility of individuals in relation to their phenotype and was famously conceptualized by Charles Darwin in the 1800s. Natural selection often leads to adaptation whereby organisms become better suited to perform well in their environment (Darwin 1871). Natural selection is also thought to be responsible for the purging of deleterious mutations that continually arise each generation.

However, it became apparent early on that natural selection could not be the only process affecting evolutionary change. In particular, Darwin (1871) was the first to identify sexual selection as a potential explanation for the evolution of phenotypes that appeared costly to nonsexual fitness (e.g., survival) and thus could not be explained by natural selection alone. Sexual selection arises from variation in reproductive success and can result in the evolution of traits such as resource-intensive sexual displays or armaments that appear maladaptive outside the context of mating (Andersson 1994; Arnqvist and Rowe 2005). Although Darwin recognized the two separate processes, he viewed them as largely harmonious because sexual selection would ensure that only the most robust, well adapted individuals would be chosen to mate. Sexual selection should thus align with, or otherwise aid natural selection by often favouring the same traits (Darwin 1871).

Although this alignment has been supported in some studies (e.g. Whitlock and Agrawal 2009), the benefits of sexual selection have been called into question of late due to the effects of sexual conflict (Long et al. 2009; Arbuthnott and Rundle 2012; Chenoweth et al. 2015).

When sexually reproducing organisms mate, there is often an imbalance of parental investment. In fact, the sexes are defined by their difference in mating strategy (Parker 1979; Bonduriansky 2001), and in almost all cases, the cost of reproduction for females is significantly higher than that of males, regardless of parental investment in the rearing of young. Simply put by Bonduriansky (2001), females generally produce few costly gametes "designed to develop into offspring", while males produce many cheap gametes "designed to parasitise the resources of females." This difference in mating strategy leads to a difference in the ideal outcome of mating interactions; while the male strategy leads to a tight correlation between mating rate and number of offspring produced – and therefore overall fitness – the same is simply not true for females. A perfect correlation of male and female lifetime fitness is only expected if there is random mating combined with lifetime monogamy (Rice 2000).

Any deviation from monogamy and the reproductive interests of males and females can lead to sexually antagonistic selection over a shared trait (Parker 1979 ; Arnqvist and Rowe 2005). This process is known as sexual conflict and can be broken down into two forms. When the genetic basis of the trait is shared between the sexes, an allele that alters its expression may increase fitness in one sex but decrease it in the other, generating what is known as intralocus sexual conflict (Chapman et al. 2003). Alternatively, if the shared trait is the result of an interaction between the sexes with the outcome being determined by different traits, and hence loci, in each sex, it is known as interlocus sexual conflict (Chapman et al. 2003).

Intralocus sexual conflict has been described as an evolutionary tug of war (Bonduriansky and Chenoweth 2009). The male and female of a given species share the majority of their genome, and therefore alleles at a given locus can be in competition with one another depending on whether they appear in the male or female "environment" (Chippindale et al. 2001). Indeed, one genome can have drastically different, and often opposite, fitness effects depending on the sex of the individual. In *Drosophila melanogaster* (Chippindale et al. 2001), for example, it was found that the fitness of different genomes did not differ between the sexes during the larval stages of development, when little to no sex-specific selection is expected to occur. However, the most fit genetic combinations in males were seen to have drastic fitness consequences for females, and vice versa, in the adult stage when the interests of the sexes differ (Chippindale et al. 2001). Intralocus sexual conflict is resolved by the evolution of mechanisms that reduce the genetic correlation between the sexes for the shared trait, thereby allowing each sex to approach or achieve its sex-specific optima and resulting in the evolution of sexual dimorphism. Since sexual dimorphism is pervasive, it suggests that intralocus sexual conflict has been important in the past, but the extent to which it persists is still being explored (e.g., Bonduriansky and Chenoweth 2009; Ingleby et al. 2014).

Interlocus sexual conflict, on the other hand, can occur over a variety of interactions where the optimal outcome differs between males and females, with mating rate being the classic example (Chapman et al. 2003) as mentioned above. For instance, Parker (1979) describes the drowning of female yellow dung flies (*Scathophaga stercoraria*) as males struggle for access to those females. Such male behaviours likely evolved from selection arising from male-male competition, thereby generating selection in females for traits that reduce the risk of death or damage during courtship (Chapman et al. 2003). Increased female resistance or avoidance behaviour would then in turn

strengthen selection in males to increase mating rate and so on, thus leading to a coevolutionary arms race between the sexes and potentially rapid, unpredictable evolutionary change (Chapman et al. 2003). My focus here is on interlocus sexual conflict and although there are many examples of traits that increase male reproductive success at a cost to females, and traits that increase female resistance to male manipulation or harm (Chapman et al. 2003; Arnqvist and Rowe 2005), a great deal of our understanding of sexual conflict has come from studies using the fruit fly, *Drosophila melanogaster*.

Drosophila species are well-known and widely studied model organisms. The fruit fly model has a long history of being used in the laboratory, particularly in the study of genetics, and its extensive use has led to a wide array of techniques and impressive base of knowledge about the genetics, development, and behaviour of these species (Beckingham et al. 2007). The fruit fly is a complex multi-cellular organism with similar fundamental behavioural, genetic and cellular patterns as many other species, including humans (Beckingham et al. 2007). Additionally, it is easy and inexpensive to maintain and manipulate in a laboratory setting, and its relatively short life cycle allows experimentation on a reasonable time scale with relatively large population sizes. As with any model organism, it is the hope that insight into the biology of one species can provide a greater understanding of the biology of a multitude of other species. In the same way, studying laboratory populations is done in the hopes of gaining a better understanding of naturally occurring processes and phenomena. Using populations which are well adapted to the laboratory to make inferences about natural populations has been equated with the use of island populations to gain insight into large-scale patterns of evolution (Rice et al. 2005). In this way, the use of *Drosophila* in the laboratory setting has been expanded to many areas of biological science, including evolutionary ecology.

Studies in *Drosophila melanogaster* have revealed many striking examples of interlocus sexual conflict in action. For example, in 1989 Kevin Fowler and Linda Partridge published work indicating that increased exposure to males decreases the lifetime fitness of females. It may be intuitive to believe that a decrease in lifetime fitness for females could be caused by the stress of resource allocation; should male persistence push a female past her mating rate optimum, it could cause her to spend resources unsustainably on offspring production in the short-term, limiting her future capacity to reproduce. However, Fowler and Partridge (1989) revealed that lifetime fitness was decreased by a reduction in female lifespan alone; females exposed to high mating rates died more quickly than those exposed to low mating rates, but egg-laying rate was unaffected, meaning frequently-mating females had lower lifetime fecundity (Fowler and Partridge 1989).

Similar results have been found since this research. For example, when females were minimally exposed to males, a 20% increase in lifespan (Rice et al. 2006) and a 17% increase in fecundity (Linder and Rice 2005) was found compared to females experiencing "normal" levels of exposure. Remating rate has also been linked to female damage in several studies (e.g. Chapman et al. 2003; Kuijper et al. 2006). It has therefore been suggested that male-induced harm in *D. melanogaster* may come from physical injury and/or seminal fluid components (Fowler and Partridge 1989). The contribution of each of these has received much attention (Chapman 2001).

Drosophila seminal fluid contains a cocktail of proteins that have significant fitness consequences for both males and females through their effects on sperm transfer and storage, female receptivity to subsequent matings, ovulation and oogenesis (Chapman 2001). Female fruit flies are able to store sperm each time they mate, using it to fertilize their eggs at will. It has been proposed that seminal fluid proteins, the composition and genetic make-up of which are highly variable (Chapman 2001), may be toxic in an attempt to kill sperm from other donors present in a

female's sperm storage organ, with negative side effects being felt by the female herself (Kuijper et al. 2006). Indeed, remating has been shown to be one of the most significant causes of damage to females by male *Drosophila*. Not only is there a decrease in female longevity with each additional mating, the decline in lifespan rapidly accelerates as the number of rematings increases (Kuijper et al. 2006).

In addition to toxic seminal fluid, the presence of males has also been shown to reduce female lifespan even in the absence of copulation. For example, using sterile males Partridge and Fowler (1990) showed that harm is brought upon females simply by the sexual attention they receive in the presence of males even in the absence of mating. Selection in males has therefore resulted in the evolution of both pre- and postcopulatory traits that while benefitting males, inflict measurable harm on females.

Aside from issues concerning the cost to females associated with interlocus sexual conflict, the underlying process of sexually antagonistic coevolution between the sexes has been suggested to act as a driver of population divergence and ultimately speciation (Arnqvist et al. 2000). The idea is that the strong and persistent selection imposed by each sex on the other can produce rapid evolutionary change in traits related to mating and reproduction such as courtship behaviours and morphological traits like genitalia (Arnqvist 1998), and that the divergence of these traits may generate reproductive isolation. The particular phenotypes that evolve in males to enhance their reproductive success in competition with other males, and that evolve in females to resist the incidental harm these traits can cause, are thought to be subject to chance events like the identity of the particular mutations available in a given population and the population's unique evolutionary history. The result is that different populations may follow unique coevolutionary trajectories driven by interlocus conflict, even in the absence of ecological differences (Arbuthnott

et al. 2014). Unlike ecologically-driven speciation in which divergent natural selection arises from differences in environment (Schluter 2009), in theory populations maintained under the same conditions may therefore diverge under the influence of interlocus sexual conflict.

Consistent with a role for interlocus conflict in divergence and speciation, genitalia, sexual displays, and other secondary sexual traits are unusually diverse among taxa (Arnqvist & Rowe 2005), often serving as the basis of species identification in the absence of other phenotypic differences. In addition, a study by Arnqvist and colleagues (2000) compared related groups of insects with little opportunity for postcopulatory sexual conflict (i.e. where females mate only once) with those where females mate repeatedly. It was found that when the opportunity for postcopulatory sexual conflict exists, speciation rate was up to four times higher than in related groups where females mate only once (Arnqvist et al. 2000). However, the early stages of divergence in conflict-related traits like male harm and female resistance has received limited attention in the sexual conflict literature and it is unclear how rapidly such differences may occur.

In addition, the traits involved in interlocus conflict are likely targets of natural selection and although differences in ecology are not necessary, how ecology and the resulting natural selection on these traits contributes to their divergence via sexual conflict is unclear. For populations in similar environments for instance, ecological selection could constrain divergence by limiting trait exaggeration or the evolution of novel phenotypes. In other words, despite the potentially random nature of trait evolution under sexually antagonistic coevolution, divergence may be constrained by selection on these traits within an ecological context (Arbuthnott et al. 2014). Alternatively, ecological selection may drive trait evolution on its own, or in combination with antagonistic coevolution, such that conflict-related traits may evolve in parallel among populations inhabiting similar environments (Arbuthnott et al. 2014). Therefore, the dynamics of

the early stages of divergence in independent populations is an empirical question of interest in the sexual conflict literature.

As reviewed above, much of our understanding of sexual conflict is derived from studies using *D. melanogaster*, and the vast majority of these use laboratory populations. As previously stated, in using laboratory populations the hope is to gain a clearer understanding of this important evolutionary process so that it can be applied more broadly, including to naturally occurring populations. Although it has been asserted that the study of laboratory populations can indeed shed light on the processes governing natural populations, and should certainly not be discounted (Rice et al. 2005), there is a distinct possibility that the extent of sexual conflict uncovered in laboratory studies is exaggerated relative to that occurring in natural populations due to the highly unusual environment in which these populations are maintained.

Laboratory stocks of most species, *Drosophila* species included, are usually maintained in highly simplified and spatially restricted environments compared to those experienced by the species in nature (Calisi and Bentley 2009). As a result, behaviour is likely to be altered (Calisi and Bentley 2009). With respect to sexual conflict, if space and complexity are limited, the ability of females to remove themselves from situations of unwanted male attention is likely to be lessened or entirely removed. For example, in wild ringlet butterflies, it has been found that females significantly alter their behaviour following mating to avoid further courtship attempts (Wiklund 1982). Prior to mating, females actively solicit courtship from males; however, when copulation is complete and females are no longer receptive, they adopt avoidance behaviours including fleeing courting males (Wiklund 1982). In captivity, such avoidance behaviours are simply less effective as space to flee and locations to hide are extremely limited. In *Drosophila melanogaster*, copulation is not the only means by which male harm occurs (Partridge and Fowler 1990), so even

if females are able to resist males' coercion, their inability to escape courtship and other forms of male persistence may have severe consequences for their fitness. These costs may be easily avoided in the wild by dispersing or hiding.

Due to these fundamental differences between lab maintained and natural populations, there is significant interest in understanding how increasing space and/or complexity of the environments in which laboratory stocks are maintained may alter the effects of sexual conflict. Quantifying sexual conflict across different levels of environmental complexity may help shed light on how large of a grain of salt, so to speak, with which laboratory results should be taken when making inferences about natural populations. Therefore, in addition to studying the dynamics of the early stages of divergence in conflict-related traits, I also quantify male sexual behaviour and its fitness costs when the structural complexity of the environment is manipulated. I address these issues of population divergence and environmental complexity via a series of experiments using *Drosophila serrata*.

Drosophila serrata, a species of fruit fly native to the east coast of Australia, is a good species to address questions of population divergence in the presence of sexual conflict and the effects of environmental complexity. *D. serrata* has primarily been a model system for the study of sexual selection on sexual displays composed of contact pheromones (Chenoweth and Blows 2005; Rundle et al. 2008; Rundle and Chenoweth 2011)**,** the evolutionary genetic consequences of this selection on these traits (e.g. Hine et al. 2011, 2014; Delcourt et al. 2012), and population divergence and speciation (e.g. Higgie et al. 2000; Rundle et al. 2005; Higgie and Blows 2007).

There is indirect evidence of significant male harm in a laboratory population of this species (Rundle et al. 2006) and sexual conflict was recently implicated as hampering adaptation to a novel environment in a laboratory evolution experiment (Chenoweth et al. 2015). When

populations were allowed to adapt to a novel food source, fitness was greatest when natural selection was permitted in the absence of sexual selection, but was lowest in replicate populations where both natural and sexual selection were permitted (Rundle et al. 2006). This hampering effect of sexual selection on adaptation is expected to occur if males preferentially court larger, more fecund females that would otherwise be most fit under natural selection alone (Long et al. 2009). Those females are disproportionately exposed to male-induced harm and therefore perform more poorly than their less attractive counterparts, thus decreasing the frequency of well adapted alleles in the next generation (Chenoweth et al. 2015). Despite the inferences that have been made by such findings, sexual conflict has never been directly quantified in *Drosophila serrata*. The goal of my thesis is therefore to quantify male harm and female resistance in the laboratory population used in these past studies, and to test whether introducing some structural complexity to the mating environment alters this. In addition, by taking advantage of a recent duplication of this stock in two independent labs, I also test for divergence in male harm and female resistance in the absence of any known ecological differences.

1.2 *Materials and Methods*

Stock Populations

In 2003, a stock population of *Drosophila serrata* was created by combining six laboratory populations originating from wild caught individuals from different locations along the east coast of Australia (Rundle et al. 2006). This stock was maintained at the University of Queensland as a large, outbred population under constant conditions (25˚C, 60% relative humidity, 12 hour light/dark cycle) on a standard yeast-agar medium (see Rundle et al. 2005 for ingredients) with non-overlapping generations on a two week schedule. In 2006, a large (>1,000 individuals) sample of individuals was transferred to the University of Ottawa (hereafter referred to as the 2006 stock). A second copy of the University of Queensland stock was again transferred to the University of Ottawa in 2013 and maintained as a separate population from the first copy (hereafter referred to as the 2013 stock). Both stocks at the University of Ottawa were maintained on the same food and following the same maintenance protocols as the University of Queensland stock.

Quantifying sexual conflict and its divergence

To quantify sexual conflict and its divergence between these two stocks, we measured the longevity and lifetime productivity (number of adult offspring produced) of females held under conditions of high vs. low male exposure using 2006 or 2013 University of Ottawa stock males and 2006 or 2013 University of Ottawa stock females. All combinations of male exposure (high vs. low), male stock (2006 vs. 2013), and female stock (2006 vs. 2013) were performed, yielding a 2 × 2 × 2 factorial design involving eight unique combinations of treatment levels. In the high exposure treatment, females were housed in the continuous presence of males, whereas females in

the low exposure treatment were given access to males for a 6 h period every Tuesday and Friday throughout their adult lives.

Sexual conflict is inferred when increased male exposure reduces female fitness (longevity and fecundity), indicative of male-induced harm. Evolved differences in male-induced harm would generate a main effect of male stock or a male stock × exposure interaction, indicating that one type of male reduced female fitness to a greater extent than the other, independent of the type of female (see Discussion for further details). Evolved differences in female resistance to male harm would generate a female stock × exposure interaction, indicating that the fitness of one type of female was reduced less than the other, independent of the type of male. A female stock × male stock × exposure interaction would indicate effects of male harm and/or female resistance that depended on whether males and females had co-evolved (i.e. were from the same population) or not.

Flies for use in the assay were collected from both stocks as virgins 13 days after eggs were laid, separated by sex using light CO_2 anaesthesia, and then stored separately by sex in vials in groups of 10 individuals. Following virgin collection (Day 15), females were marked using red formaldehyde-free fluorescent pigment (Brilliant Group, LLC, San Francisco, CA) for ease of identification. On Day 17, 60 replicate vials per combination were arranged for a total of 480 vials over two equal sized blocks. Each block consisted of 30 replicate vials from each of the eight treatment combinations with the second block starting two weeks after the first. Escaped females were excluded from analysis. One virgin female and six males from the appropriate stock populations were added to each vial. For the low exposure treatment, males were removed and discarded 6 h following introduction to females, while males in the high exposure condition remained with females in their respective vials.

On day 19, females were transferred to new vials with 10mL of fresh media. Males from the high exposure condition were discarded and new males were added along with females in the new vials, while in the low exposure condition new males were placed with females for 6 hours before once again being discarded. All males were selected randomly from the appropriate stock populations throughout the experiment. Transfers like this were conducted every 3/4 days (alternating) until the last female died. Vials were checked at least once daily for dead flies; female deaths were recorded to track longevity, and dead males were replaced with live ones. Following transfer of females to new vials, the old vials were retained and the number of adult offspring was counted 14 days later to determine female productivity.

All analyses were conducted using R version 3.0.2 (The R Foundation for Statistical Computing, 2013). Survival analyses were conducted on longevity data with interval censoring using the survreg function. AICc values of a fully specified model including the terms (and all interactions of) exposure condition, male identity, female identity and block, were employed to determine the best error distribution, along with graphical exploration of the data. When plotted, the data appeared to follow a Weibull distribution, but formal distribution selection revealed a loglogistic distribution to fit best (see Results). Although results of analyses using either the Weibull or loglogistic distributions were qualitatively similar (J. Colpitts, unpublished data), the discrepancy between visual inspection and distribution selection prompted me to further explore the data. This exploration suggested that patterns of mortality differed between the two male exposure treatments, resulting in different best-fit error distributions (Weibull in the low exposure and loglogistic in the high; see Results). Subsequent survival analyses were therefore conducted separately by male exposure treatment, employing the appropriate error distribution. While this prevents a direct test of the male stock × exposure, female stock × exposure and female stock x

male stock x exposure interactions that may be indicative of divergence in male harm and female resistance, lifetime fecundity is the better measure of female fitness (differences in longevity is one reason why fecundity may vary) and these effects can be tested for using this response variable (see below).

For each male exposure level, the best model was determined using a backward selection approach. Starting with the fully specified model, the highest order interactions were removed one by one (in this case, the 4-way interaction on shape and scale). The resulting simplified model was compared to the more complex model using a likelihood ratio tests with the anova function in R ("stats" package). The simplified model with the highest P-value was then used for model selection in the next round. Subsequent rounds of selection removed the remaining highest-order interactions one by one (3-way interactions on both parameters in this case) and compared them to the best model from the previous round. Again, the simplified model with the highest likelihood ratio test P-value was carried through to the next round of selection, where lower order interactions (2-way interactions followed by main effects) were removed. In R, the shape parameter is determined by the strata argument for which only a single term can be fit in any given model. As such, interactions were collapsed into a single factor for use in the strata argument (i.e. the interaction of two factors with two levels each became a single factor with four levels representing each of the four unique combinations of these two factors), so tests of additive effects on the shape parameter were not possible. After many rounds of selection, only significant terms remained in the model. Any time an interaction was present, all of the lower-order interactions and main effects involving these terms were also included in the model. Each term in the best model was removed and these simpler models compared to the best model using likelihood ratio tests to generate the P-values presented here. Once all non-significant terms were eliminated by way of backwards

selection, a forward selection process was conducted to generate the remaining P-values. Each term of interest not present in the best model was individually added to the best model and again compared using likelihood ratio tests.

Lifetime fecundity counts, generated by the sum of offspring produced during all transfers, were analysed using a zero-inflated negative binomial distribution (package pscl in R) because fecundity counts were significantly overdispersed relative to a Poisson distribution (overdispersion test, 'AER' library in R, $p < 0.001$). AICc values of fully specified models including the terms (and all interactions of) exposure treatment, male identity, female identity and experimental block were employed to determine whether a generalized linear model with a negative binomial distribution was sufficient for further model selection or if a zero-inflated model was necessary. Model selection was again performed using likelihood ratio tests, employing the previously described backward and forward selection procedures. To investigate potential survival-reproduction trade-offs, a second round of model selection was conducted for the fecundity data with longevity as a covariate. Again, zero-inflated negative binomial models were employed using backward and forward model selection procedures to determine whether any fecundity effects could be explained by patterns of mortality.

Aside from the observed versus expected mortality rate plots (generated in R), all figures were generated in JMP 10.0.0 (SAS Institute Inc., 2012) using the Graph Builder function.

Effect of environmental complexity on sexual activity

To test whether increased environmental complexity alters sexual interactions in *Drosophila serrata*, behavioural observations were conducted that compared the amount of sexual attention directed towards a female in a simple vs. complex environment. Single, non-virgin

females were randomly selected from the 2006 laboratory stock population and placed together with four males in individual vials. Simple vials contained 10 mL standard yeast-agar media sprinkled with live yeast and were plugged with a foam stopper. Complex vials also contained 10 mL standard yeast-agar media that was sprinkled with live yeast, but had two flat plastic sheets (2 cm × 3 cm), inserted upright into the food at right-angles to one another (fitting together via a vertical slit cut into the center of each), that created an x-shaped barrier when viewed from above. These barriers protruded approximately 1.5 cm from the surface of the food and formed four discreet cells of food. A 15 cm length of crafting pipe cleaner was also twisted to create a tube-like coil that was secured to the bottom of the foam stopper to hang down into the vial from above.

Flies were added to 100 replicate vials of each complexity level and these were then placed inside an incubator at 25˚C and 60% relative humidity on a 12 h dark/light cycle and left for 24 h. Following this period, vials were observed with as little disturbance as possible for 30 s each once every 2-4 h from 09:00 to 17:00 over three consecutive days for a total of 11 observations per vial. During each observation period it was noted whether the female was receiving any sexual attention from a male. Following past studies (Long et al. 2009; Spieth 1974; Greenspan and Ferveur 2000), sexual attention was defined as situations in which the male was (i) within approximately 5 mm of the female and oriented towards her, (ii) actively courting the female, including wing displays, pursuing her, or attempting to mount her; or (iii) mating with the female.

The number of occurrences for each form of sexual attention were pooled across the 11 observation periods for each female, producing a non-Gaussian left-skewed distribution. Differences between the two environments in the total amount of sexual attention a female received was therefore tested using a Wilcoxon signed rank test, treating vials as replicates, while a

Pearson's Chi square test was employed to compare the frequency of female deaths between the two environments over the course of the observation period.

Effect of environmental complexity and male exposure on female fitness

To test the effect of environmental complexity on the extent of sexual conflict, we measured the longevity and lifetime productivity of females held under conditions of high vs. low male exposure in either a simple or complex environment. All combinations of male exposure (high vs. low) and environment (simple vs. complex) were performed, yielding a 2 × 2 factorial design involving four unique combinations of treatment levels. In the high exposure treatment, females were again housed in the continuous presence of males, whereas females in the low exposure treatment were given access to males for a 6 h period every Tuesday and Friday. Again, sexual conflict is inferred when increased male exposure reduces female fitness (longevity and fecundity), indicative of male-induced harm. An increase in either longevity or productivity in the complex environment relative to the simple environment would indicate some increase in female performance, regardless of exposure condition. An environment × exposure interaction would be generated if the effect of environment is dependent on the opportunity for male-induced harm, such as if the opportunity to escape male-induced harm has a larger effect when male exposure is high.

Flies for use in the assay were collected from the 2006 stock as virgins 13 days after eggs were laid, separated by sex using light CO_2 anaesthesia, and then stored separately by sex in vials in groups of 10 individuals. Females were immediately marked using red formaldehyde-free fluorescent pigment (Brilliant Group, LLC, San Francisco, CA) for ease of identification. The experiment consisted of two equal-sized blocks separated by two weeks. Each block consisted of 60 replicate vials for each of the four treatment combinations, yielding a total of 480 replicates.

Escaped females were excluded from analysis. On Day 14, one virgin female and six males were added to each vial. For the low exposure treatment, males were removed and discarded 6 h following introduction to females, while males in the high exposure treatment remained with females in their respective vials.

On day 18, the same transfer procedure as outlined above was employed. All females were moved to new vials (with or without added structural complexity) with six new males; males were removed 6 h later in the low exposure treatment. Again, vials were checked at least once daily for dead flies and old vials were retained to count the number of adult offspring as a measure of female productivity.

Data analysis followed the same approach as in the first assay. Visual inspection of the data again suggested a Weibull error distribution and this was supported by a formal distribution selection procedure (see Results). Both the low and high exposure data were therefore analysed together using a Weibull error distribution. Fecundity data were again zero-inflated and overdispersed compared to a standard Poisson distribution ($p < 0.001$) and analysed using a zero-inflated negative binomial distribution, both with and without longevity as a covariate.

1.3 *Results*

Quantifying sexual conflict and its divergence

Female longevity was substantially reduced under high as compared to low male exposure, regardless of female population of origin or the males to which they were exposed (Figure 1). Consistent with such differences, initial model selection revealed that the mortality rate for the two male exposure levels followed different error distributions (Table 1; Figure 2). Under conditions of high male exposure, a loglogistic distribution fit best with all other distributions having substantially less support (Table 1). The loglogistic is a two parameter distribution which allows for a unimodal mortality rate when the shape parameter is greater than one (i.e. mortality increases rapidly with age then subsequently declines in later life). When the shape parameter is less than or equal to one, mortality declines monotonically with age. The scale parameter is equal to the median longevity.

In contrast, in the low male exposure treatment mortality rates followed a more typical Weibull distribution, although the exponential distribution was also supported (difference in AICc of 0.619; Table 1). The Weibull is a two parameter distribution in which mortality changes monotonically over time. In this case, the scale parameter is governed by the data's dispersion and represents the 63.2 percentile of the data; larger values indicate a stretching of the data to the right. The shape parameter again governs how mortality rate changes with age. When shape is less than 1, mortality rate decreases over time, when it is equal to 1 mortality rate is constant, and if it is greater than 1 mortality rate increases with time. The exponential distribution is a special case of the Weibull distribution in which the shape parameter is fixed at a value of 1. Due to the difference in mortality rate between the low and high male exposure conditions (Figure 2), data from these treatment levels were analysed separately to ensure the use of the most appropriate error

distribution. Although this prevented a direct statistical comparison of mortality between these two treatments, the notably shorter longevity of high (median female lifespan, lower - upper confidence limits = 3.88 days, 3.52 - 4.23) compared to low exposure females (median female lifespan, lower - upper confidence limits = 13.00 days, 10.88 - 15.12; and see Figures 1 and 2) is indicative of extremely strong sexual conflict in both of the studied stock populations of *Drosophila serrata*.

Survival analysis of the high exposure treatment data using a loglogistic distribution revealed the best model to include a two-way interaction of male identity by experimental block as well as a main effect of female identity on the scale parameter (i.e. median lifespan), and a main effect of male identity on the shape parameter (Table 2), indicating differences in how mortality rate changed over time when females were exposed to the 2006 vs. 2013 stock males. The male x block interaction on median lifespan (i.e. scale) reflected a reduced effect of 2006 males on median female lifespan that was present in block 2 but was not detected in block 1 (median female lifespan, lower - upper confidence limits: block 1/2006 males = 3.75 days, 3.29 - 4.20; 2013 = 3.75 days, 3.21 - 4.29; block 1/2013 males = 3.75 days, 3.21 - 4.29; block 2/2006 males = 5.88 days, 4.90 - 6.85; block 2/2013 males = 4.00 days, 3.60 - 4.40). Under high male exposure, females therefore tended to die more quickly in the presence 2013 males than 2006 males, although the effect was not consistent across blocks. The main effect of female identity on median lifespan (i.e. scale) reflected greater longevity of 2013 compared to 2006 females (median female longevity, lower - upper confidence limits: 2006 females = 3.75 days, 3.34 - 4.16; 2013 females = 4.88 days, 4.30 - 5.45). Under conditions of high male exposure, 2013 females therefore tend to live longer than 2006 females, irrespective of the males to which they were exposed. Under high male exposure, the male identity effect on the shape parameter reflected a greater rise in mortality when females

were exposed to 2013 as compared to 2006 males (shape parameter estimates: 2006 males = 3.91; 2013 males = 4.96), irrespective of female stock of origin.

Survival analysis of the females from the low male exposure treatment level using a Weibull distribution revealed the best model to include main effects of male identity, female identity and experimental block on the scale parameter, or 63.2 percentile of the distribution, with no significant treatment effects on shape (Table 3). As in the high male exposure treatment level, female longevity under low male exposure was greater when they were housed with 2006 as compared to 2013 males (median female lifespan, lower - upper confidence limits: 2006 males = 17.13 days, 14.00 - 20.5; 2013 males = 11.13 days, 8.67 - 13.58). Also consistent with the high exposure data, median longevity in the low exposure treatment level was greater overall for 2013 as compared to 2006 females (median female lifespan, lower - upper confidence limits: 2006 females = 10.38 days, 7.63 - 13.12; 2013 females = 17.13 days, 13.72 - 20.29). Finally, females lived longer overall in block 1 than in block 2 (median female lifespan, lower - upper confidence limits: block 1 = 17.38 days, 14.00 - 20.75; block 2 = 10.88, 8.34 - 13.41), although this did not interact with male or female population.

Lifetime fecundity was modelled using a zero-inflated negative binomial distribution because many females produced no offspring (Figure 3 Figure 4). A zero-inflated negative binomial distribution separates the data into two parts: the probability of producing zero offspring and the distribution of values given that offspring were produced. Variation in the probability of producing no offspring was best described by an exposure by block interaction and a male identity by block interaction, while the number of offspring produced was best modelled by a three-way interaction between exposure condition, male identity and block (Table 4).

When individuals failed to produce offspring, the exposure x block interaction arose because individuals in the low male exposure treatment were more likely to produce offspring than their high male exposure counterparts in the second experimental block, but the reverse was true in the first experimental block (percentage of females that failed to produce offspring: block 1/low exposure = 27%; block 1/high exposure = 42%; block 2/low exposure = 32%; block 2/high exposure = 22%). The male identity x block interaction reflected an increase in the probability of producing offspring in the presence of 2013 males in block 1 but 2006 males in block 2 (percentage of females that failed to produce offspring: block 1/2006 males = 43%; block 1/2013 males = 26%; block 2/2006 males = 20%; block 2/2013 males = 34%).

Variation in non-zero lifetime fecundity was best explained by a 3-way interaction of male exposure, male identity and block. On average, fecundity was higher when females were held with 2006 as compared to 2013 males, and under conditions of low as compared to high male exposure, with the exposure x male identify interaction reflecting a greater difference between male exposure levels (i.e. low vs high) for females exposed to the 2006 as compared to 2013 males (Figure 5). This pattern was qualitatively the same in the two blocks, but differences in median fecundity were greater in block 1 than block 2 (data not shown), generating the 3-way interaction of male exposure, male identity and block.

When longevity was introduced as a covariate, the best fit model was simplified (Table 5). The probability of producing zero offspring was best described by a model that includes effects of female longevity, male exposure treatment, and a male identity x block interaction, while the negative binomial portion of the model was best described by a longevity x exposure treatment interaction.

For the zero-inflation portion of the data, the longevity effect reflects a greater probability of producing no offspring for individuals with shorter life spans (data not shown). The male exposure effect reflects an increased probability of producing no offspring for individuals that experienced high as compared to low male exposure levels (32% vs. 29% of females with zero offspring respectively). The male x block interaction was the same as previously described in the model without a longevity covariate, with an increased probability of producing offspring in the presence of 2013 males in block 1 but 2006 males in block 2.

Variation in non-zero fecundity was best explained by an interaction between longevity and male exposure treatment. Total offspring production and longevity were positively correlated among females under conditions of both high and low male exposure, but the slope of the relationship was steeper under high as compared to the low male exposure (Figure 6), indicating a more rapid increase in fecundity with age, or a greater per-day reproductive rate, for females continually exposed to males.

Effect of environmental complexity on sexual activity

When individual females were housed continuously in the presence of males and sexual activity was observed, those housed in simple vials were exposed to significantly more sexual attention across all observation periods than were females housed in the complex vials (Figure 7; Wilcoxon sign ranked test $P < 0.0001$, $n = 200$ individuals). In addition, over the 3 day observation period significantly more females died in the simple as compared to complex vials (37% vs. 17% respectively; $\chi^2 = 7.41$, df =1, $P = 0.007$).

Effect of environmental complexity and male exposure on female fitness

When females were held in either a simple or complex environment and under conditions of low or high exposure to males, mortality was best described by a Weibull distribution (Table 6; Figure 8). Again, this is a two parameter distribution, with scale representing the 63.2 percentile and shape dictating how mortality rate changes over time. Survival analysis using a Weibull distribution revealed the best fit model to include two-way interactions of male exposure by experimental block, environment by experimental block, and male exposure by environment on the scale parameter, as well as a male exposure by experimental block effect on the shape parameter (Table 7).

Median female longevity (i.e. scale) was substantially reduced under conditions of high compared to low male exposure and was also lower in the simple than complex environment (Figure 8). Both of these effects on the scale parameter varied in magnitude between blocks, generating the significant exposure x block and environment x block interactions, but in both cases the direction of the effect was consistent across blocks. In particular, the exposure x block interaction reflected significantly greater reduction in median lifespan induced by the high male exposure in block 2 than in block 1 (median female lifespan, lower - upper confidence limits: block 1/high exposure = 5.81 days, 5.11 - 6.51; block 1/low exposure = 20.77 days, 17.53 - 24.01; block 2/high exposure = 7.83 days, 7.13 - 8.54; block 2/low exposure = 40.71 days, 37.53 - 43.88), and the environment x block interaction reflected a significantly greater reduction in median lifespan caused by the simple environment in block 1 than in block 2 (median female lifespan, lower - upper confidence limits: block 1/simple environment = 7.38 days, 4.45 - 10.30; block 1/complex environment = 10.97 days, 8.16 - 13.78; block 2/simple environment = 13.16 days, 9.31 - 16.98; block 2/complex environment= 14.58, 11.10 - 18.06). While both high male exposure and a simple

environment reduced longevity, female lifespan was quite variable under conditions of low male exposure, rendering the difference in median longevity between environments nonsignificant in this case and thereby generating the male exposure x environment interaction on the scale parameter (median female lifespan, lower - upper confidence limits: high exposure/simple environment = 5.88 days, 5.25 - 6.50; high exposure/complex environment = 8.77 days, 8.03 - 9.50; low exposure/simple environment = 32.80 days, 29.17 - 36.43; low exposure/complex environment = 35.56 days, 32.29 - 38.84). With respect to changes in mortality with age (i.e. the shape parameter), the male exposure x block interaction reflected a more pronounced rise in mortality with age in the high compared to low exposure treatment in block 1 that was slightly reversed in block 2 (shape estimates: low exposure/block 1 = 1.47; high exposure/block 1 = 2.31; low exposure/block 2 = 2.53; high exposure/block 2 = 2.31).

Variation in female fecundity was again analyzed using a zero-inflated negative binomial distribution due to the high frequency of females producing no offspring (Figure 9). The best fit model included effects of male exposure, environment, and experimental block on the probability of producing zero offspring, and main effects of male exposure and experimental block on the number of offspring produced (given that any offspring were produced; Table 8).

The main effect of male exposure on the probability of producing zero offspring reflected a higher probability in the low compared to the high exposure treatment levels (17% vs. 8% of females with a fecundity of zero respectively, Figure 9). Individuals held in the simple environment were also more likely to have a fecundity of zero compared to those held in the more complex environment: (18% vs. 8%; Figure 9), thereby generating the significant environment effect. Finally, females from block 1 were much more likely to produce zero offspring than those in block 2 (20% and 5% respectively).

For females that did produce offspring (Figure 10), fecundity was significantly greater under conditions of low as compared to high male exposure (median offspring production, lower - upper confidence limits: low exposure = 245.50, 218.72 - 272.27; high exposure = 80.00, 37.83 - 122.17). Block 2 females also tended to be more fecund than block 1 females (median offspring production, lower - upper confidence limits: block 1 = 89.50, 59.75 - 119.25; block 2 = 181.00, 148.31 - 213.69).

When female longevity was introduced as a covariate, a 3-way interaction between longevity, environment and exposure treatment best described the probability of producing offspring, with a longevity x exposure interaction and main effect of block on the number of offspring produced (given that any offspring were produced; Table 9). This indicates that the increased likelihood of block 2 individuals to produce offspring can be explained by their longer lifespan.

When looking at individuals that failed to produce offspring, there was an interaction between lifespan, exposure treatment and environment. In all treatment combinations, the probability of producing zero offspring was lower for individuals with longer lifespans. The probability of producing zero offspring declined more rapidly in the high compared to the low male exposure treatments, and in the complex compared to the simple environment, particularly in the low exposure treatment (Figure 11). The probability of producing zero offspring was greatest at all ages for individuals in the simple environment under the high exposure treatment.

Variation in non-zero fecundity was best described by the model that included a longevity x exposure interaction and a main effect of block (Table 9). Greater per-day reproduction was again seen in the high exposure treatment relative to the low exposure treatment, as illustrated by the steeper slope of the high exposure treatment (Figure 12). Although it is true that the longer a

female lives the more offspring she produces, this longevity by exposure treatment interaction indicates a difference in reproductive rate not explained by lifespan alone. The block effect is the same as previously described (when longevity is excluded as a covariate), with block 2 females tending to be more fecund than block 1 females.

1.4 *Discussion*

Quantifying sexual conflict and its divergence

Despite its relevance to the body of work on sexual selection in *Drosophila serrata*, sexual conflict has never before been quantified in this species. My results suggest that sexual conflict is strong, at least in these particular populations, with female lifespan being 30% longer under conditions of low compared to high male exposure and similar effects on lifetime fecundity. Published values for *D. melanogaster* show similar, if not slightly smaller, changes of approximately 20% when employing similar treatments (Rice et al. 2006). In my analyses, exposure condition was present in the best model of fecundity whether longevity was used as a covariate or not, and appeared in both the zero inflated and negative binomial portions of the model. In other words, there are fitness consequences for females when exposure to males is high and this arises not only indirectly from the effect of exposure on longevity, but also from direct effects on fecundity itself.

The need for separate survival analyses under high vs. low male exposure is in itself a notable result. The Weibull distribution is most typically used for biological applications of survival analysis (Mudholkar et al. 1996) and can accommodate systems where mortality rate decreases with time, as in the case of high initial offspring mortality, or increases over time as in the case of senescence. Here the Weibull distribution was the best descriptor of mortality in the low exposure treatment and all shape estimates were greater than one, indicating an increased mortality rate with age that is consistent with senescence in performance. The loglogistic distribution, in addition to its economic applications, is also commonly used for survival analysis (Gupta et al. 1999). Due to the rapid increase in mortality rate, followed by a decline when the

shape parameter is greater than one, the fit of the data to the loglogistic distribution is likely indicative of selective disappearance of females under high male exposure. When a group of individuals is subjected to stress, genetic or condition-dependent variation will cause some individuals to be more resistant to that stress than others (Hämäläinen et al. 2014). Those that are more susceptible will feel the effects of that stress – death, in this case – more quickly than those that are more resistant. After a short time, only more resistant individuals will remain and mortality rate will slow. This could explain why the loglogistic distribution best fit the high male exposure data. However, it does not directly explain why that same effect was not seen for low male exposure individuals. It is likely that the amount of stress imposed on the females in each male exposure condition plays a key role. Since median lifespan was so short for the high exposure condition, it is evident that constant exposure to males imposes substantial stress on females (Partridge and Fowler 1990). If mortality rate can be explained by selective disappearance, even the most resistant individuals in the high male exposure condition died sooner than many of the more susceptible individuals from the low exposure treatment. With individuals under less stress in the low exposure treatment, selective disappearance was likely gradual enough that other processes, such as senescence (Hämäläinen et al. 2014), were able to overshadow it.

According to theory, divergence of traits under antagonistic sexual selection can occur in the absence of environmental differences, although divergence may be constrained or directed by environmental factors that generate ecological selection on the traits, thus leading to similar evolutionary trajectories (Arbuthnott et al. 2014). The methods used here enable the detection of divergence in traits related to sexual conflict (i.e. male harm and female resistance) between two separate populations reared and maintained under the same conditions for approximately eight years since being split from a common ancestor. Detection of a main effect of female identity on

either longevity or fecundity may indicate changes in resistance to male harm, but may be due to other non-conflict related differences as well. For example, the tendency of 2013 females to live longer and be more fecund than 2006 females could arise if 2013 females were less inbred than their 2006 counterparts, or more vigorous for some reason unrelated to their history of sexually antagonistic selection. One cannot therefore unambiguously infer divergence in female resistance from a main effect of female population alone.

An interaction between female population and male exposure treatment provides more conclusive evidence for divergence in female resistance since, if it were only factors unrelated to male exposure causing differences in fitness, those differences should be consistent regardless of the level of male exposure. When interpreting the effects of male population, this same issue does not exist. Males are harmful to females overall, as shown by the strong effect of male exposure, and differences in female fitness caused by being held with 2006 vs. 2013 males are therefore indicative of differences in male harm. If males differ in how harmful they are, and these effects accumulate with increased exposure, a male population x exposure interaction may result. Alternatively, males may differ in harm but even low exposure is sufficient for this difference to be manifested, with no subsequent accumulation under increased exposure, generating a main effect of male identity.

The results of longevity shown here indicate differences between the two stocks, with 2013 females living longer than 2006 females, and females from both stocks tending to live longer in the presence of 2006 males than 2013 males. Again, due to the two-part analysis, I cannot test for interactions of male and/or female stock with exposure condition. Forcing both treatment levels into a single analysis where at least half of the data are poorly described by the chosen error distribution may produce spurious results given the poor fit of the model.

However, inferences can still be made via qualitative comparisons. Despite the differences in error distributions used for each exposure treatment, the same factors were found to be significant in both treatments. The interactions varied, but it was found that in both cases male identity, female identity and block contributed significantly to variation in female lifespan. Reassuringly, the direction of these findings was the same for both exposure conditions, with 2006 males being associated with longer female lifespan and 2013 females living longer. As described earlier, this provides evidence for divergence in male harm between the two populations, and may or may not indicate differences in female resistance. In addition to the main effect of male identity on longevity providing evidence of divergence, there was a notable difference in the effect of male identity between the two exposure treatments. Male identity described the fit of the shape parameter of the high male exposure data but not the low male exposure data. In other words, the effect of male identity on female mortality rate was greater in the high exposure condition than the low exposure condition, inferring the interaction previously described as being characteristic of divergence in male-induced harm, and that harm being cumulative. This cumulative effect of male exposure has been described in *D. melanogaster* and has been attributed to precopulatory harm (Partridge and Fowler 1990), the effects of repeated mating (Kuijper et al. 2006), and subsequent increased exposure to seminal fluids (Chapman 2001). The presence of a block effect in both exposure conditions suggests that factors beyond what were manipulated in this experiment (i.e. male and female population of origin and levels of male exposure) are able to mediate the strength of sexual conflict in *D. serrata*.

Patterns of fecundity provide additional support for divergence in conflict-related traits between the two stocks. Fecundity data were not separated by exposure treatment, allowing direct testing of its main effect and interactions. Male exposure treatment interacted with male identity

(the magnitude of which varied between the two blocks) to explain non-zero fecundity. Males from the 2006 stocks were again associated with reduced harm compared to 2013 males. The greater difference between the stocks within the low exposure treatment compared to the high exposure treatment may result because rapid death under high exposure reduces the power to detect population differences. It could also arise if even very low exposure to 2013 males – possibly even a single mating – was sufficient to cause substantial harm such that further exposure had little detectable effect. The results analyzing variation in the probability of having zero lifetime fecundity were inconsistent between blocks, suggesting that any treatment effects varied with other unidentified and uncontrolled factors, hampering interpretation.

Although fitness costs are often inferred by decreases in lifespan in the absence of measures of fecundity (Arbuthnott and Rundle 2012), I argue that the strongest approach is one that incorporates both longevity and fecundity when addressing fitness costs. For example, the effect of male identity on fecundity was no longer significant when lifespan was corrected for in this experiment, indicating that the effect of male identity on fecundity was indirect and arose from its effect on lifespan. In contrast, the significant interaction between longevity and exposure condition on lifetime fecundity provides further insight into the effects of sexual conflict not captured by each fitness measure alone. Females consistently exposed to males produced offspring at a higher rate for a shorter period of time than those minimally exposed to males. This provides some evidence for a trade-off between reproductive rate and longevity, but compensation is incomplete since longer-lived females still reproduced more overall. Additionally, short-term increase in the reproductive rate of females confers a fitness advantage for males; rapid egg laying immediately following mating, when a particular male's sperm is most likely to be used by the female, increases the number of offspring he is able to sire. There are several likely reasons for this change in

reproductive rate, but it illustrates that under certain conditions and time scales, completely different pictures of fitness costs can emerge.

It is possible that females in the high exposure treatment are producing offspring at a faster rate than their low exposure counterparts due to the increased exposure to male seminal fluids which are known to affect several aspects of reproduction including female receptivity, ovulation and oogenesis (Chapman 2001). Reproduction has long been associated with increased senescence (e.g. Partridge 1987; Kirkwood and Rose 1991). Increased exposure to males, and therefore increased opportunity to mate and reproduce, is a likely cause of both the rapid mortality rate and rapid rate of early-life reproduction seen here. Previous studies have found a decrease in female longevity with no effect on age-specific progeny production in *D. melanogaster* (Fowler and Partridge 1989) but it is possible the results shown here reflect direct effects of remating on increased offspring production in *Drosophila serrata*. In contrast, the extended lifespan of the low exposure individuals likely contributes to the appearance of a slow rate of reproduction. Female *Drosophila* experience reproductive senescence and their egg laying rate slows as they age (Grotewiel et al. 2005). For the high male exposure group of females, death was far more likely to occur before reproductive rate slowed than for their low male exposure counterparts. Additionally, sperm limitation may have affected reproduction in the low exposure treatment. A study of six *Drosophila* species revealed that sperm limitation can occur between 1.1 and 14.9 days following mating depending on the species (Markow et al. 2012); unfortunately, this timeline is unknown for *D. serrata*.

Effect of environmental complexity on sexual activity

Past *Drosophila* research on the topic of sexual conflict has been conducted almost exclusively in a simplified laboratory environment. The assumption is that inferences can be made from lab findings to natural populations, but the relationship between sexual activity and environmental complexity has, to my knowledge, never been tested explicitly. In my second experiment, substantial reductions in the sexual attention females received from males, and in female mortality, were seen when only a small amount of structural complexity was introduced into the vials where flies were held. In theory, differences in sexual activity and female survival between the two environments could have resulted from abiotic differences between these treatment levels, but care was taken to try to avoid this. For example, both levels of environmental complexity used vials which were identical in shape, size, and material, the food in each vial was identical, and all vials were housed under the same ambient conditions. The volume of the containers and the food they contained also did not differ. Therefore, my behavioural observations and survival results strongly imply that the intensity of sexual conflict can be mediated by ecological factors such as complexity, and thus suggest that the evolutionary importance of sexual conflict in nature may be overestimated by past *Drosophila* studies that have been conducted in highly simplified environments.

Effect of environmental complexity on female fitness

When fitness measures are analysed to give a broader picture of the effects of environmental complexity, a main effect of male exposure on either longevity or fecundity would again be indicative of sexual conflict. A main effect of environment on either fitness measure could be interpreted as a difference in abiotic environment, but as previously described, this appears

unlikely. Instead, a main effect of environment is likely a reflection of differences in male harm mediated by the ability of females to escape/hide, as demonstrated above. An interaction between environment and male exposure level would indicate that sexual conflict is mediated by environmental complexity to a different degree depending on the opportunity for such conflict.

There was once again strong evidence for sexual conflict overall in this species as revealed by the significant reduction in both female longevity and fecundity when male exposure was greater. A main effect of exposure (the magnitude of which differed by block in some cases) was found on both the scale and shape parameters of the best fit mortality model, and was present in both the zero-inflated and non-zero portions of the best model of fecundity.

An interaction between male exposure treatment and environmental complexity was seen in the longevity data. In this case, increased environmental complexity was associated with longer lifespans when male exposure was high, but female longevity did not differ significantly between the two environments when male exposure was low. This pattern is consistent with environmental complexity allowing females to better escape or avoid male harm, with this effect being greatest when male exposure is highest. If sexual activity is infrequent enough in the low exposure treatment, females may not require or make use of the increased opportunity to avoid such attention in the more complex environment. In addition, particularly if sperm limitation is a factor for low exposure individuals, females in either environment may be equally receptive to mating during the six hour periods during which they are exposed to males. In other words, avoidance behaviour by females may be unnecessary, and possibly even detrimental, in the low exposure treatment, nulling the effect of environmental complexity in that treatment. In addition, the results tended to be more variable in the low exposure condition, as evidenced by the significant interaction with block. Again, this may be due to the decrease in sexual activity; with less pressure on females and longer

overall lifespans, the low exposure treatment is likely subject to more noise from external factors than the high male exposure treatment. A main effect of environmental complexity was found on the scale parameter in the best fit mortality model, where individuals housed in more complex vials lived longer than those in simple vials (mediated by block). As previously described, this main effect likely reflects an increased ability for females to avoid or escape unwanted sexual attention in the presence of structural complexity.

A main effect of environmental complexity was also found on the zero-inflation portion of the best fecundity model. Females in the simple environment were more likely to produce zero offspring than those in the complex environment, suggesting there may be some fitness advantage to a more complex environment. Perhaps added structural complexity allows for greater undisturbed egg laying as it increases the likelihood of a female being on the food surface outside the line of sight of a male. In addition, the nature of male induced harm may render females infertile without killing them. Individuals in the simple environment are perhaps more likely to experience harm-induced infertility, consistent with the assumption that increased complexity reduces male induced harm. The previously described increase in longevity afforded to females in the more complex environment did not, however, produce a detectable difference in the number of offspring produced by females, given that any offspring were produced. One reason for this may be that for longer lived individuals, later life fecundity was sufficiently low that it was offset by the higher early-life reproduction rate of shorter lived individuals. Overall, the fitness consequences of increased longevity are unclear given an effect only on the likelihood of a female producing offspring, but not on the number of offspring produced given that she produced any.

When longevity is introduced as a covariate for fecundity, the resulting 3-way interaction between longevity, male exposure treatment and environmental complexity that best describes the

probability of producing offspring may provide further evidence for sperm limitation in this experiment. The probability of producing zero offspring declines as an individual's lifespan increases, and this decline is more rapid when individuals are held in conditions of high as compared to a low male exposure. It is expected that individuals in the low exposure treatment are more likely to be sperm limited than those in the high exposure condition due to the reduction in mating opportunities, and that the time to first mating will be longer when mates are rare. Additionally, the absolute probability of producing zero offspring was greatest when male exposure was high in the simple environment. As described above, this may be indicative of male harm causing non-fatal sterility more often in the simple environment, and under high male exposure, than in any other combination of experimental conditions.

The interaction between longevity and fecundity showing an increased reproductive rate in the high exposure condition compared to the low exposure condition is again likely caused by increased exposure to seminal fluids in the high exposure treatment and/or sperm limitation in the low exposure treatment.

Summary and future work

I provide clear empirical evidence that sexual conflict is occurring in two closely related laboratory stocks of *Drosophila serrata.* I also present convincing evidence for divergence in male harm between these stocks after eight years of isolation under the same maintenance procedures. My results are also consistent with divergence in female resistance, although general differences in performance/fitness of these two populations outside the context of sexual conflict could underlie these results.

Increased structural complexity of the environments in which flies are housed was associated with decreased sexual activity, with clear benefits to female lifespan and some evidence of an increased likelihood of producing offspring. Although the direct fitness effects of environmental complexity are still unclear, the results outlined here suggest further exploration of the subject to be warranted. In particular, the impacts of ecological factors on sexual conflict should be explored in greater detail and broadened across additional species and extents of structural complexity. In addition to structural complexity, the effects of enclosure size are also of interest as more spacious environments may provide more opportunity to escape harm. This way, it would be possible to address whether increased ability to hide from unwanted sexual activity (i.e. increased structure) and the ability to actively flee courting males (i.e. increased space; Wiklund 1982) cause similar effects. Structural complexity and space may also conceivably interact, with the former becoming more effective in the presence of the latter. The outcome of such research could help researchers better understand the implications of their laboratory work on sexual conflict, and/or improve their maintenance practices to better reflect natural levels of sexual conflict.

Although there is still much debate surrounding the subject, recent laboratory work in *D. melanogaster* has shown the net effect of sexual selection to be negative with respect to the purging of deleterious mutations (Arbuthnott and Rundle 2012). Similar results have also been shown in *D. serrata* with respect to adaptation to a novel environment, where there is genomic evidence of sexual selection impeding the increase in frequency of alleles beneficial to nonsexual fitness (Chenoweth et al. 2015). The interpretation of these data, supported by additional behavioural assays, has focussed on a cost of sexual attractiveness arising from the combination of male mate preferences and male harm (i.e. sexual conflict; Long et al. 2009). The idea is that if males

preferentially court and mate intrinsically better females (larger, more fecund females in the case of *Drosophila* species), these females will experience the brunt of male-induced harm, thereby lowering their realized fitness. The difference in relative fitness between intrinsically good (large) and bad (small) females may therefore be reduced, eliminated, or even reversed (Long et al. 2009). However, if sexual conflict is overestimated in the simplified laboratory environment, as my results suggest may occur, it could mean that this cost of sexual selection is overestimated, or is potentially entirely an artefact of laboratory studies. As a consequence, the question of environmental complexity versus lab simplicity and its effect on sexual conflict is important to our understanding of the relationship between natural and sexual selection.

The results and procedures outlined here also pave the way for other avenues of future research. One such direction is to better understand the true fitness costs of sexual conflict on these populations. In the case of *Drosophila* stocks, maintenance is often done with non-overlapping generations (e.g. Arbuthnott and Rundle 2012), meaning adults are afforded a very limited period of time to lay eggs before being discarded. In the case of the *D. serrata* stocks used here, individuals live for up to 5 days as adults before being discarded. This type of schedule imposes strong selection on early life performance and none on later life (as there is no late life). As a result, the negative effects of sexual conflict, such as decreased lifespan, may not be manifest in the period of time that is relevant to female fitness in these stocks. If lifespan is sufficiently short due to the maintenance schedule, high male exposure may actually be beneficial to females and they may have evolved to take advantage of short-term male-induced increases in their fecundity. The populations I used have evolved under conditions of high male exposure for hundreds of generations, and it is therefore possible that females perform suboptimally under low exposure conditions. Although our protocol involving measures of lifetime fecundity mirrors that in past

studies of sexual conflict (e.g. Partridge and Fowler 1990; Pitnick and Garcia-Gonzalez 2002; Arbuthnott et al. 2014; but see Rice et al. 2005; Rice et al. 2006), the conditions under which these stocks have evolved could explain, for example, why environmental complexity affected the probability of producing offspring but had no effect on the number of non-zero offspring produced. If females have evolved extremely high resistance to male coercion under conditions of high male exposure, that resistance may persist even when males are rare, causing females to refuse mating attempts despite being sperm limited. Unfortunately our assays were not designed to measure fitness on the short timescale that may be relevant to the recent evolutionary history of these populations. For example, in the first experiment testing for divergence in male harm and female resistance, the complexity of the setup prevented me from capturing the 3-5 day window of early life. In future studies, it would be useful to measure both early and lifetime fecundity using populations that have evolved under conditions of high and low male exposure, and short and long generation times, to more fully characterize how these factors may interact.

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Table 1. AICc values of a fully specified model that included the 3-way interaction of male identity, female identity and experimental block on both the scale and shape parameters where applicable, for various error distributions. Data were analyzed separately by male exposure level (i.e. high and low).

Data set	Error Distribution	AICc	ΔAICc
High exposure treatment	Loglogistic	1084.431	0
	Loggaussian	1090.827	6.396
	Logistic	1123.129	38.698
	Weibull	1126.507	42.076
	Rayleigh	1141.679	57.248
	Exponential	1339.975	255.544
Low exposure treatment	Weibull	1949.878	0
	Exponential	1950.497	0.619
	Loggaussian	1972.812	22.934
	Loglogistic	1976.058	26.180
	Logistic	2057.222	107.344
	Rayleigh	2070.493	120.615

Table 2. P-values generated using likelihood ratio tests against the best-fit model with a loglogistic distribution for the high male exposure treatment data: Longevity = Male x Block + Female + Male where scale = Male x Block + Female and shape = Male. P-values were determined by either removing terms present in the best fit model, or by adding terms not already present.

Parameter	**Term**	**P-value**
Scale	Male x Block	**2.468^{-08}**
	Female	**4.554^{-04}**
	Male x Female	0.213
	Female x Block	0.671
	Male x Female x Block	0.362
Shape	Male	**0.035**
	Male x Female	0.062
	Male x Block	0.067
	Male x Female x Block	0.084

Table 3. P-values generated using likelihood ratio tests against the best-fit model with a Weibull distribution for the low male exposure treatment data: Longevity = Male + Female + Block where all terms describe scale. P-values were determined by either removing terms present in the best fit model, or by adding terms not already present.

Parameter	**Term**	**P-value**
Scale	Male	**0.002**
	Female	**0.003**
	Block	**0.003**
	Male x Female	0.488
	Male x Block	0.521
	Female x Block	0.495
	Male x Female x Block	0.792
Shape	Male	0.892
	Female	0.279
	Block	0.974
	Male x Female	0.746
	Female x Block	0.701
	Male x Block	0.066
	Male x Female x Block	0.280

Table 4. P-values generated using likelihood ratio tests against the best-fit model for lifetime fecundity: Total Lifetime Fecundity = Exposure x Male x Block | Harassment x Block + Male x Block, where the first set of terms represents the negative binomial distribution and the second set of terms represents the zero-inflation portions of the model. P-values were determined by either removing terms present in the best fit model, or by adding terms not already present.

Model Portion	**Term**	**P-value**
Zero Inflation	Exposure x Block	**0.012**
	Male x Block	**0.003**
	Female	0.074
	Male x Female	0.194
	Male x Exposure	0.857
	Female x Block	0.203
	Female x Exposure	0.124
	Male x Female x Block	0.437
	Exposure x Male x Block	0.838
	Exposure x Female x Block	0.190
	Exposure x Female x Male	0.505
	Exposure x Male x Female x Block	0.644
Negative Binomial	Exposure x Male x Block	**0.017**
	Female	0.209
	Male x Female	0.435
	Female x Block	0.075
	Exposure x Female	0.442
	Exposure x Female x Block	0.253
	Exposure x Female x Male	0.785
	Male x Female x Block	0.061
	Exposure x Male x Female x Block	0.277

Table 5. P-values generated using likelihood ratio tests against the best-fit model for lifetime fecundity with a longevity covariate: Total Lifetime Fecundity = Longevity x Exposure | Longevity + Exposure + Male x Block, where the first set of terms represents the negative binomial distribution and the second set of terms represents the zero-inflation portions of the model. P-values were determined by either removing terms present in the best fit model, or by adding terms not already present.

Model Portion	**Term**	**P-value**
Zero Inflation	Longevity	**4.091^{-35}**
	Exposure	**4.154^{-10}**
	Male x Block	**0.037**
	Exposure x Block	0.582
	Female	0.871
	Longevity x Exposure	0.086
	Longevity x Male	0.529
	Longevity x Female	0.722
	Longevity x Male x Female	0.963
	Longevity x Exposure x Male	0.365
	Longevity x Exposure x Female	0.400
	Longevity x Exposure x Male x Female	0.814
Negative Binomial	Longevity x Exposure	**6.816^{-12}**
	Exposure x Male x Block	0.514
	Female	0.215
	Longevity x Male	0.584
	Longevity x Female	0.411
	Longevity x Male x Female	0.824
	Longevity x Exposure x Male	0.584
	Longevity x Exposure x Female	0.542
	Longevity x Exposure x Male x Female	0.995

Table 6. AICc values of the fully specified model including a 3-way interaction of exposure treatment, environmental complexity and experimental block on both the scale and shape parameters where applicable for various error distributions.

Error Distribution	**AICc**	**ΔAICc**
Weibull	3388.532	0
Loglogistic	3406.408	17.876
Rayleigh	3414.1	25.568
Loggaussian	3420.802	32.270
Logistic	3434.274	45.742
Exponential	3699.378	310.846

Table 7. P-values generated from the best-fit survival analysis model with a Weibull distribution: Longevity = Exposure x Environment + Exposure x Block + Environment x Block + (Exposure x Block), where terms in parentheses describe shape. Presented terms are either added to or subtracted from the best model.

Parameter	Term	P-value
Scale	Exposure x Block	**0.003**
	Environment x Block	**0.016**
	Exposure x Environment	**0.008**
	Exposure x Environment x Block	0.441
Shape	Exposure x Block	**1.519^{-07}** [a]
	Block	**4.617^{-05}** [a]
	Exposure	**6.516^{-06}** [a]
	Environment x Exposure x Block	0.101

a. The exposure x block interaction, modelled as a single collapsed factor with 4 levels, was a better fit than a model with either main effect on its own; this collapsed factor was therefore used in the best fit model.

Table 8. P-values generated using likelihood ratio tests against the best-fit model for lifetime fecundity, Total Lifetime Fecundity = Exposure + Block | Exposure + Environment + Block, where the first set of terms represents the negative binomial distribution and the second set of terms represents the zero-inflation portions of the model. Presented terms are either added to or subtracted from the best model.

Model Portion	**Term**	**P-value**
Zero Inflation	Exposure	$\mathbf{2.168^{-193}}$
	Environment	$\mathbf{5.125^{-193}}$
	Block	$\mathbf{7.674^{-197}}$
	Exposure x Environment	1
	Exposure x Block	1
	Environment x Block	1
	Exposure x Environment x Block	1
Negative Binomial	Exposure	$\mathbf{1.236^{-209}}$
	Block	$\mathbf{2.540^{-195}}$
	Environment	1
	Exposure x Environment	1
	Exposure x Block	1
	Environment x Block	1

Table 9. P-values generated using likelihood ratio tests against the best-fit model for lifetime fecundity with a longevity covariate, Total Lifetime Fecundity = Longevity x Exposure | Longevity x Exposure x Environment, where the first set of terms represents the negative binomial distribution and the second set of terms represents the zero-inflation portions of the model. Presented terms are either added to or subtracted from the best model.

Model Portion	**Term**	**P-value**
Zero Inflation	Longevity x Exposure x Environment	**0.022**
	Block	0.184
	Longevity x Block	0.401
Negative Binomial	Longevity x Exposure	**1.556^{-16}**
	Block	**8.443^{-05}**
	Environment	0.408
	Longevity x Environment	0.392
	Longevity x Block	0.135
	Longevity x Exposure x Environment	0.407

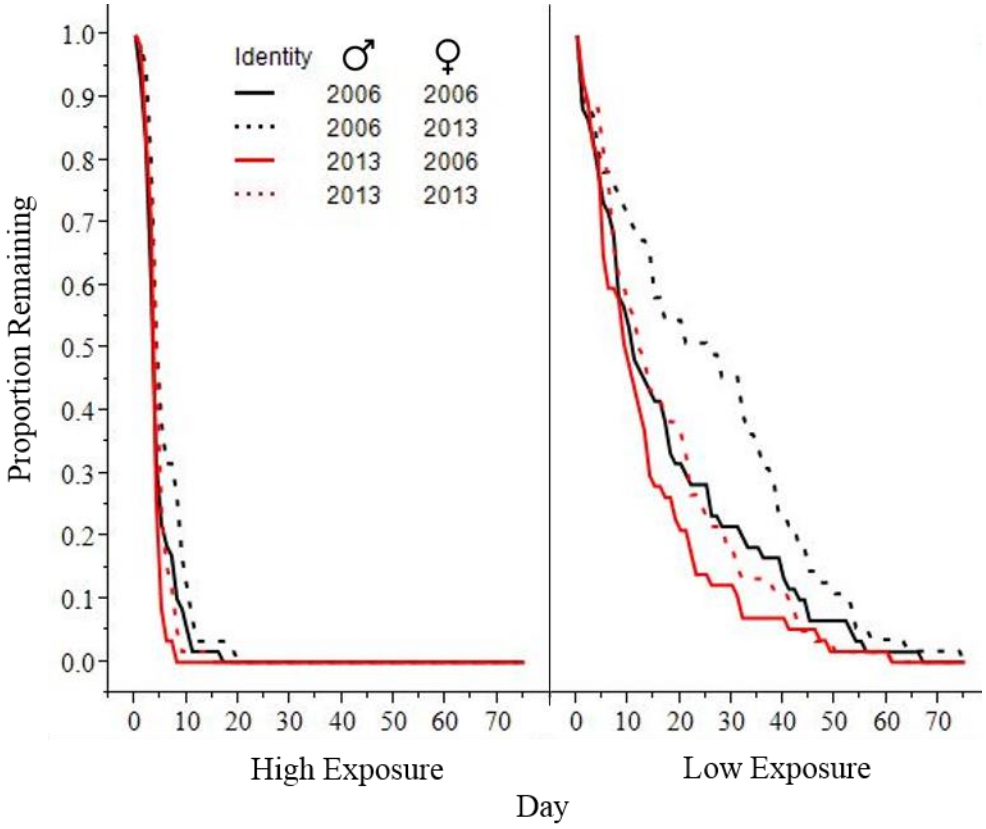

Figure 1. Adult longevity (in days) of *Drosophila serrata* females from two stock populations when exposed to males of the same or different stock under conditions of either low (right) or high (left) exposure to males. Proportion remaining indicates the number of females from each experimental condition alive at the end of the day divided by the starting number of females in each condition, combined across two experimental blocks (n = 53-60 females total per treatment combination). Solid lines indicate females from the 2006 stock while dashed lines indicate females from the 2013 stock. Those held in exposure treatments with 2006 males are shown in black and those exposed to 2013 males are shown in red.

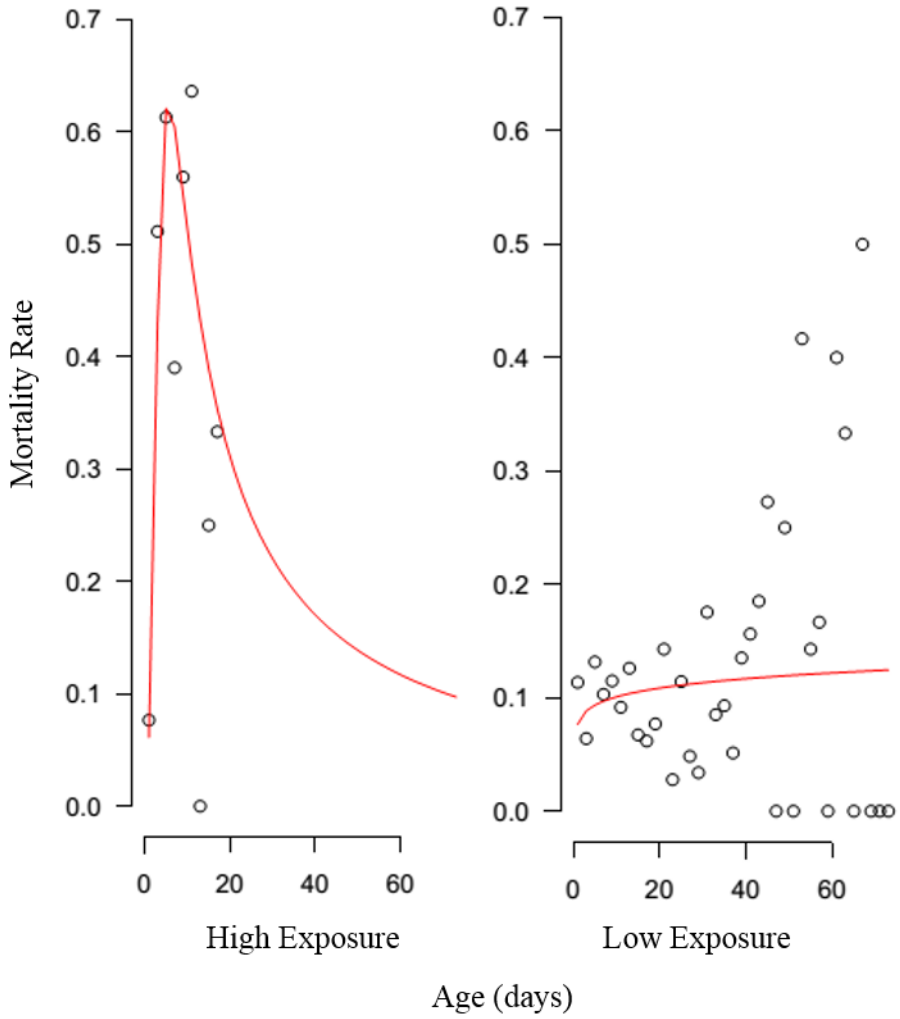

Figure 2. Observed mortality rate of females (open circles) when exposed to males in either a high exposure (left) or low exposure (right) treatment. A loglogistic distribution best fits the data from the high exposure treatment and a Weibull distribution best fits the data from the low exposure treatment; survival functions of each distribution are shown in red.

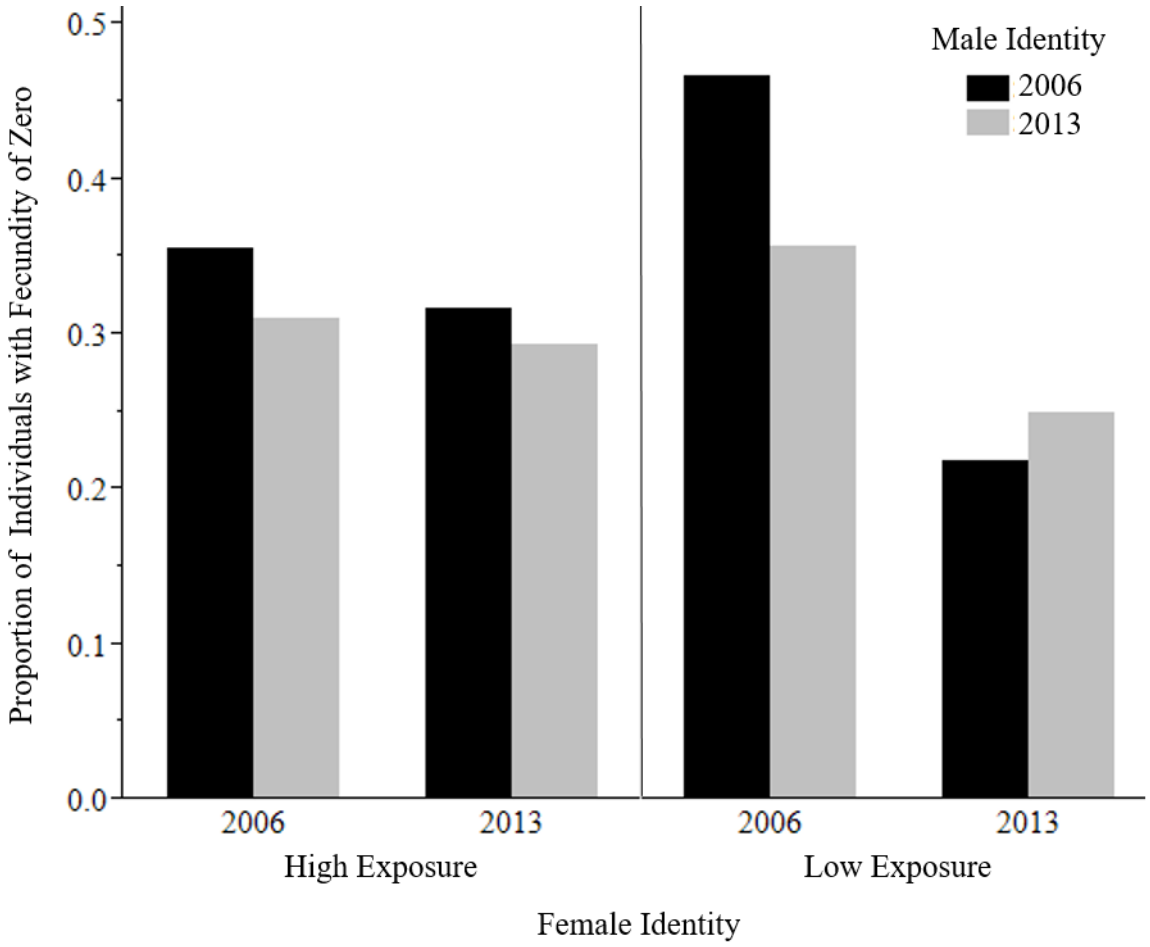

Figure 3. Proportion of females from each of combination of factors (n = 53-60 females per treatment combination) with a lifetime fecundity of zero, as estimated by offspring counts. Females were held in either a high (left) or low (right) male exposure treatment, originated from either the 2006 or 2013 stock population, and were exposed to males from either the 2006 (black bars) or 2013 (grey bars) stock population.

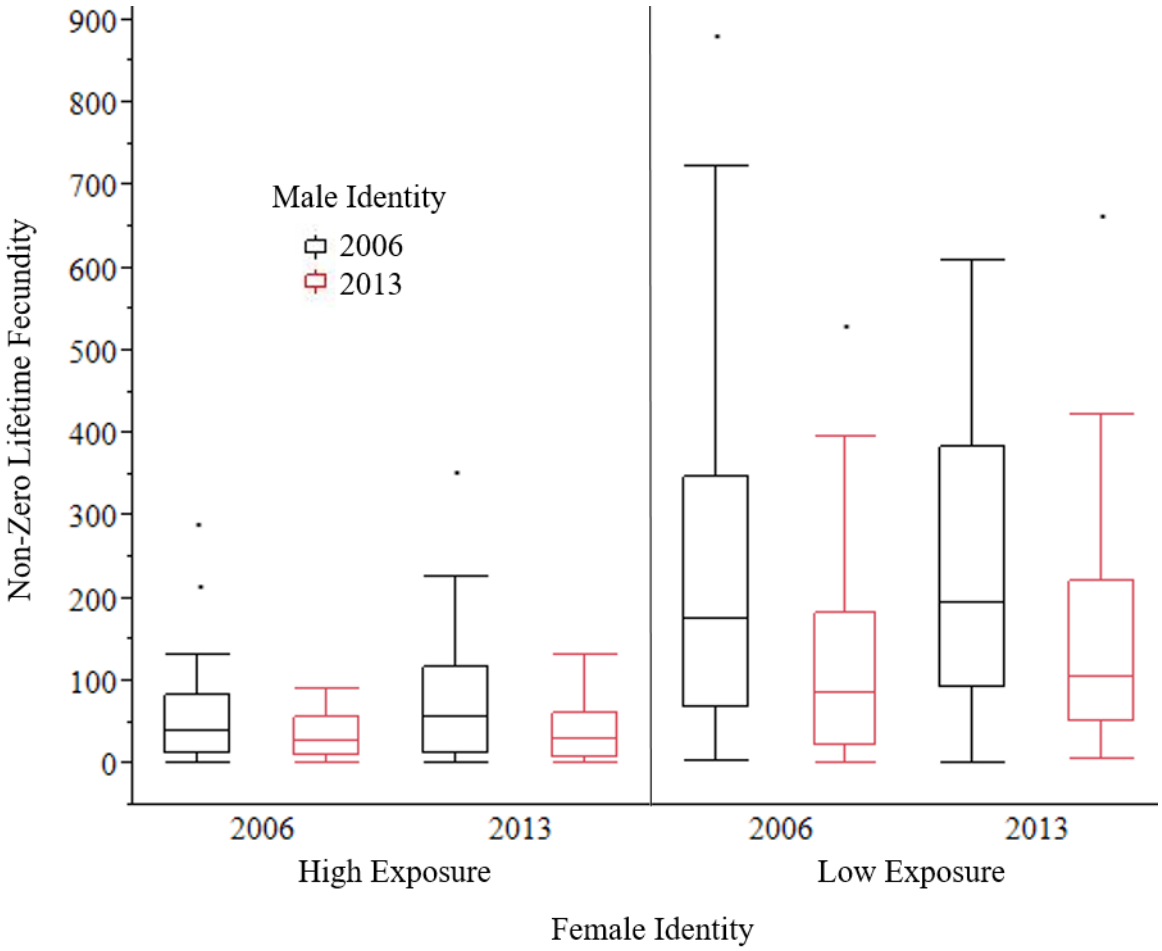

Figure 4. Non-zero Lifetime fecundity as estimated by offspring counts for females originating from two different *Drosophila serrata* populations when housed with males from the same stock or different stock in either a low (right) or high (left) male exposure treatment, pooled across two experimental blocks (n = 32-45 females total per treatment combination). Black lines indicate fecundity of females exposed to males from the 2006 stock while red lines show fecundity of females exposed to males from the 2013 stock. The horizontal line within each box shows median while the ends of the box show the 1st and 3rd quartiles, respectively; whiskers indicate 95% confidence interval.

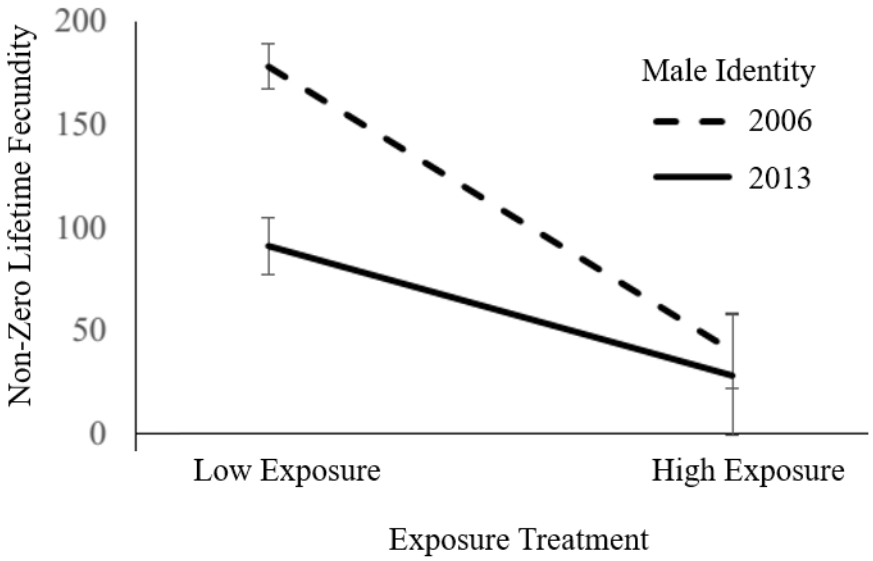

Figure 5. Median non-zero lifetime female fecundity in the presence of 2006 vs 2013 males, under either a high (left) or low (right) male exposure. 95% confidence limits are shown (n = 75-81 per treatment combination).

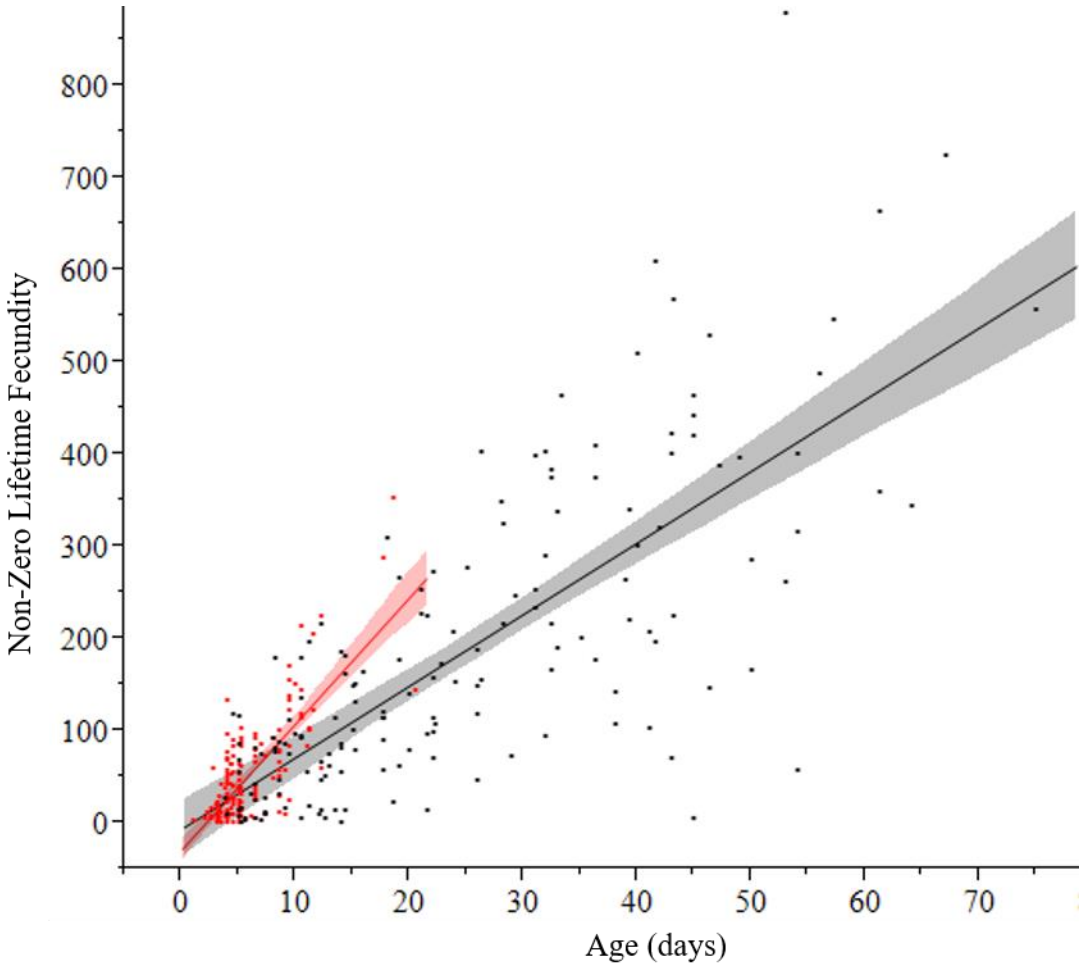

Figure 6. Variation among individuals in non-zero lifetime fecundity as estimated by offspring counts in relation to their longevity for female *Drosophila serrata* from two different stocks, under either a high (red) or low (black) male exposure treatment. Lines of fit and 95% confidence are shown.

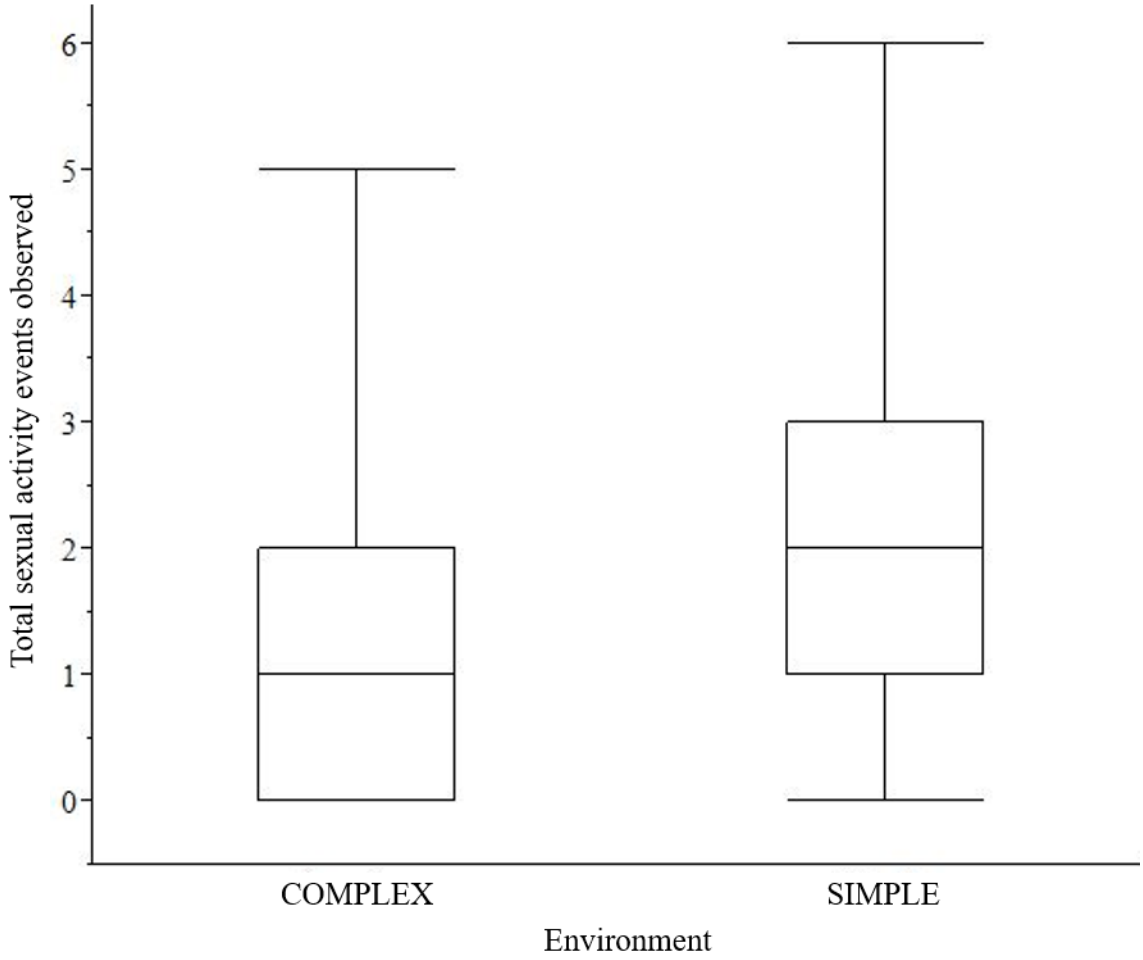

Figure 7. Total sexual activity directed towards individual females (n = 200) over a 3 day observation period when held in either standard vials (right) or those with added structure (left). Sexual activity included orientation of the male toward the female, courtship of the female, and actual mating (see Methods). The horizontal line within each box shows median while the ends of the box show the 1st and 3rd quartiles, respectively; whiskers indicate 95% confidence interval.

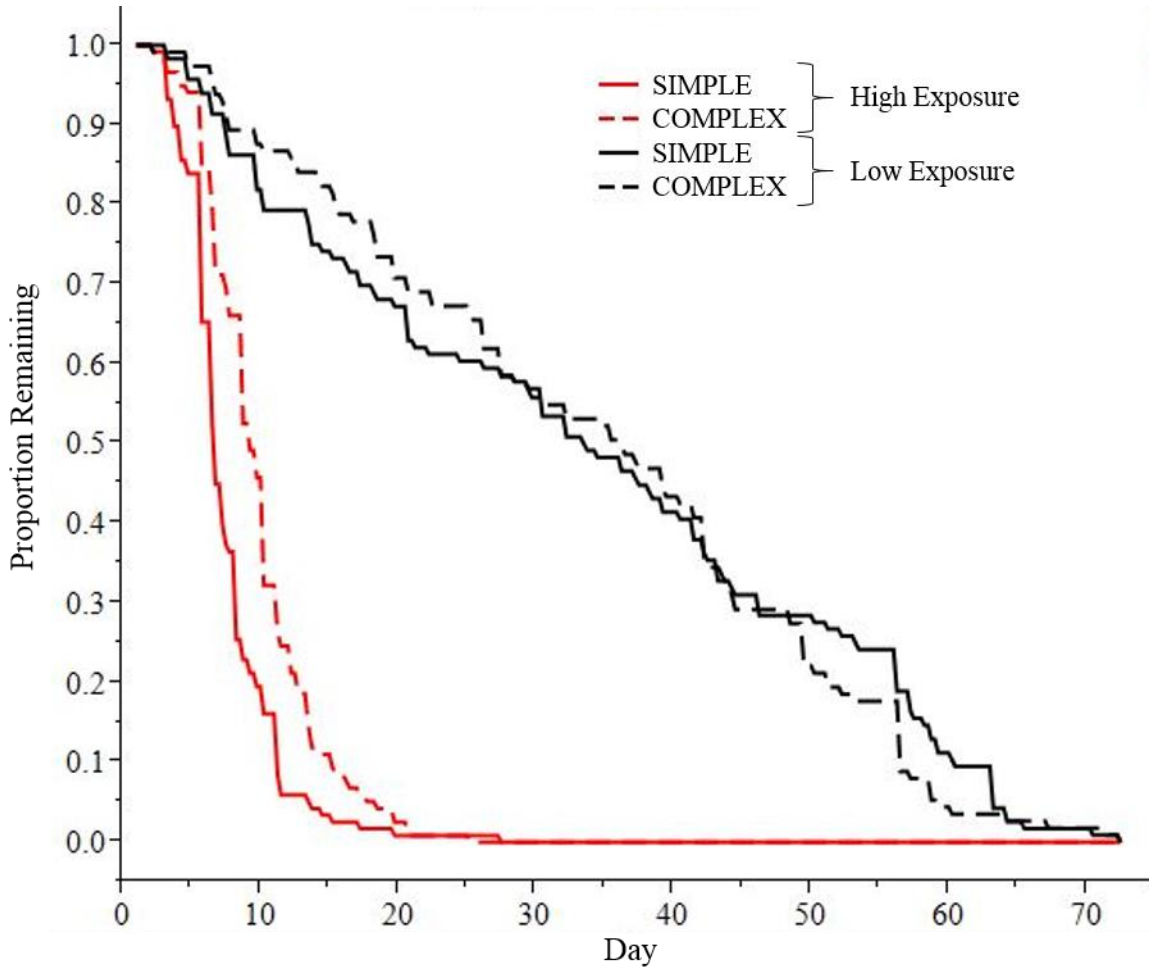

Figure 8. Longevity (in days) of adult female *Drosophila serrata* when held under either low (black) or high (red) male exposure treatments in either a simple (solid) or complex (dashed) environment. Proportion remaining indicates the number of females from each treatment combination alive at the end of each quarter-day divided by the starting number of females in each condition. Results are combined across two experimental blocks (n = 113-118 total females per treatment combination).

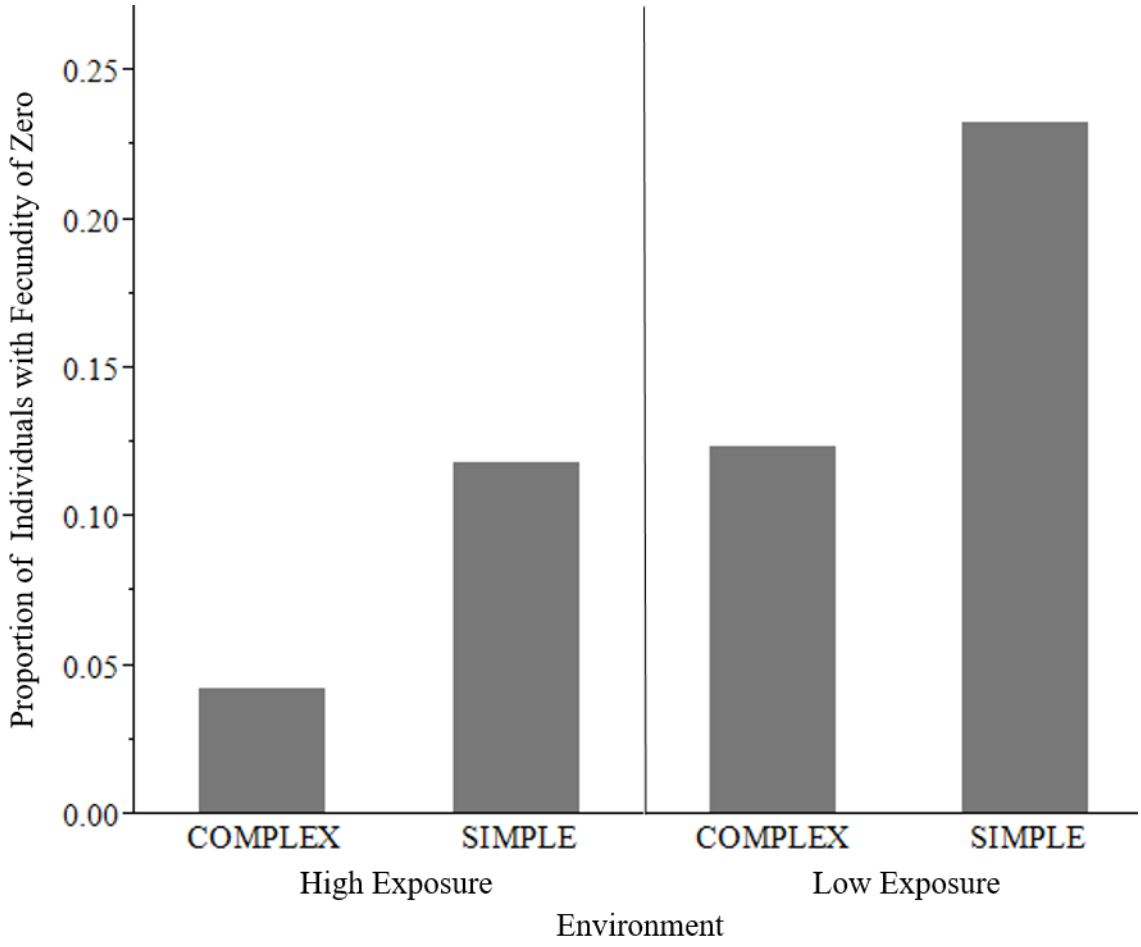

Figure 9. Proportion of females from each combination of factors (n = 113-118 total females per treatment combination) with a lifetime fecundity of zero, as estimated by offspring counts. Females were held in either a high (left) or low (right) male exposure treatment, in a simple or complex environment.

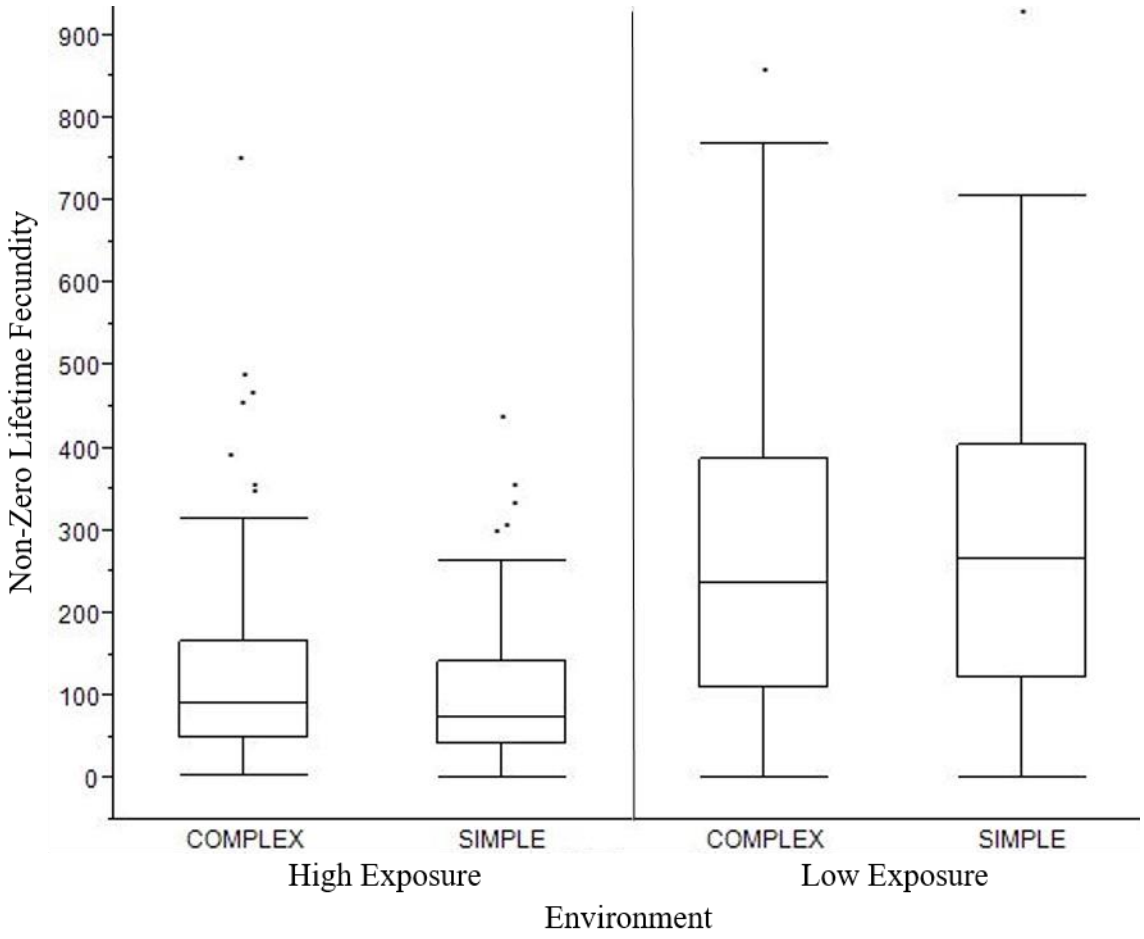

Figure 10. Non-zero lifetime fecundity as estimated by offspring counts for female *Drosophila serrata* populations when held in either a low (right) or high (left) male exposure treatment and a simple or complex environment, pooled across two experimental blocks (n = 89-113 total females per treatment combination). The horizontal line within each box shows median while the ends of the box show the 1st and 3rd quartiles, respectively; whiskers indicate 95% confidence interval.

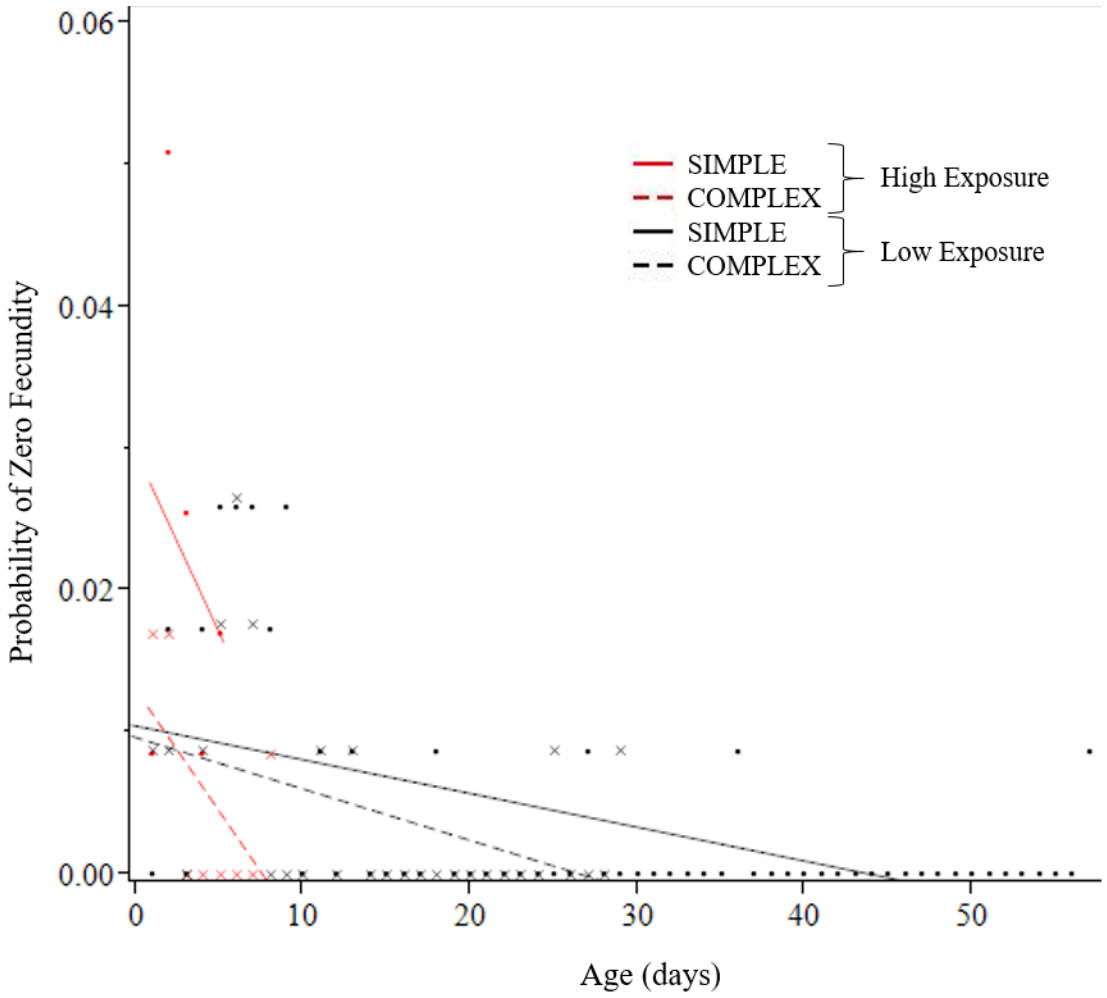

Figure 11. Probability of producing zero offspring in relation to age of death in days for female *Drosophila serrata* under conditions of high or low male exposure in one of two environments (n=113-118 total females per treatment combination). Points represent the daily probability of producing zero offspring; ex symbols represent the complex environment while closed circles represent the simple environment. Black trend lines and symbols are shown for the low male exposure condition, red trend lines and symbols are shown for the high male exposure treatment.

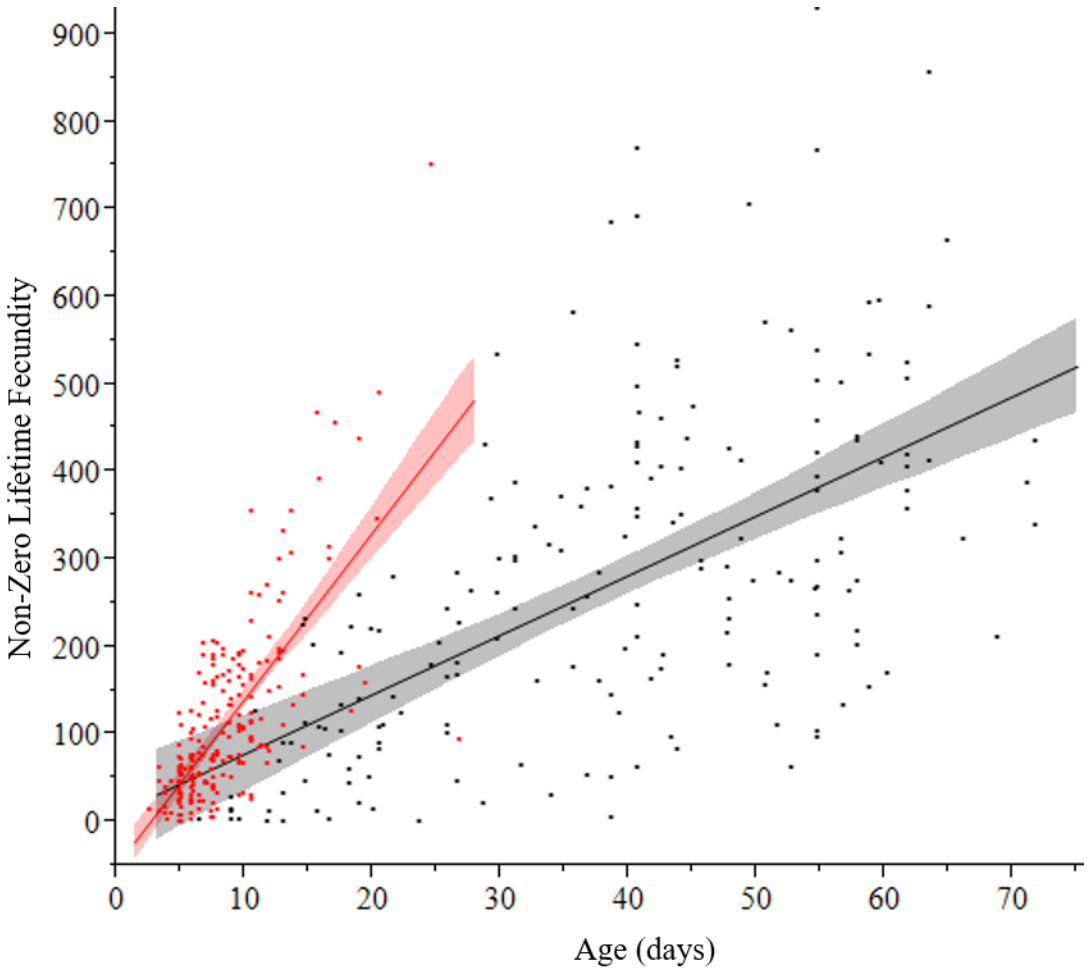

Figure 12. Variation among individuals in non-zero lifetime fecundity as estimated by offspring counts of female *Drosophila serrata* in relation to their longevity when held in two different levels of environmental complexity. Lines of fit and 95% confidence is shown for individuals under high male exposure (red) and low male exposure (black).