The evolutionary consequences of mate competition: adaptation, purging and sexual conflict in *Drosophila melanogaster*

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Thesis submitted to the University of Ottawa
in partial Fulfillment of the requirements for the
Doctorate of Philosophy degree in Biology

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O! the warmth! O! the hum! O! the colours in the dark!

O! the gauzy wings of golden honey-flies!

O! the music of their feet - of their dancing goblin feet!

O! the magic! O! the sorrow when it dies.

J. R. R. Tolkien, *Goblin Feet*, 1915.

This work is dedicated to God the Almighty, “the Lord of the Sciences”, Who has been my refuge and strength (Psalm 46:1), as well as to the myriads of creatures counted strange and/or weird, thanks to being plagued with an inordinate amount of unsatisfiable curiosity.

Table of Contents

List of Tables and Equations

List of Figures

Abstract

Mate competition is a widespread feature of sexual reproduction that can have diverse evolutionary consequences. By generating sexual selection, competition for mates can favour healthier and more vigorous males, potentially promoting adaptation and the purging of deleterious alleles. However, traits that increase male reproductive success can also reduce female fitness as a by-product, generating sexual conflict and male-imposed harm. These evolutionary consequences are not fixed because both the potential benefits and costs of mate competition may vary depending on how mating interactions unfold, the environment in which they occur, and the evolutionary history of the populations involved.

Drosophila has been a model system for studying sexual selection, sexual conflict, and male harm. Much of this work has been conducted using laboratory-adapted populations maintained and assayed in highly simplified environments. Because both sexual selection and male harm can influence adaptation, purging, and population fitness, environmental effects on mate competition can have broad evolutionary consequences. Yet we know relatively little about how mating environments alter the strength of selection and the expression of male harm, or how these effects should influence our interpretation of past work. In this thesis, I examined how the physical mating environment and population evolutionary history shape selection through males and the expression of male harm in *Drosophila*.

In my first experiment (Chapter 2 of this thesis), I tested whether selection through *D. melanogaster* males was stronger in a larger, structurally complex mating environment than in smaller, structurally simple environment (standard *Drosophila* vials) by comparing the fitness of

inbred and outbred flies. Selection against inbred males was strong, but the evidence that this selection was stronger in complex environments was inconclusive: one experiment showed an effect in this direction that approached, but did not achieve, statistical significance, whereas a second experiment found no difference between environments. In my second experiment (Chapter 3), I examined which features of the mating environment reduce the expression of male harm in *D. melanogaster*. By separately manipulating fly density and structural complexity, I showed that structural complexity alone, and not density, reduced the expression of male harm; indeed, harm was essentially eliminated in the structurally complex treatment. In my third experiment (Chapter 4), I investigated whether male harm differed between lab-adapted and nature-derived populations in two species of *Drosophila*. Across three comparisons, lab-adapted males generally imposed greater fitness costs on females than did nature-derived males. This suggests that long-term adaptation to simplified laboratory environments may increase the expression of male harm, although the strength of this effect sometimes depended on species and male–female population combination.

Taken together, my results show that the evolutionary consequences of mate competition can be context-dependent because both the physical environment and a population's evolutionary history can shape selection and sexual conflict. Mate competition may contribute to adaptation and purging, but it can also generate male harm, and the balance between these outcomes depends on the conditions under which mating occurs and the populations involved. Standard *Drosophila* laboratory assays may also overestimate the magnitude of male harm, particularly when they use long-term lab populations in structurally simple environments. A fuller understanding of sexual selection and sexual conflict will therefore require greater attention to

mating environment, population history, and the extent to which laboratory findings reflect processes occurring in more natural contexts.

Résumé

La compétition pour l'accès aux partenaires sexuels est une caractéristique répandue de la reproduction sexuée qui peut avoir diverses conséquences évolutives. En générant de la sélection sexuelle, cette compétition peut favoriser les mâles plus sains et plus vigoureux, contribuant ainsi potentiellement à l'adaptation des populations et à l'élimination des allèles délétères. Toutefois, les caractères qui augmentent le succès reproducteur des mâles peuvent également réduire la valeur adaptative (« fitness ») des femelles comme effet secondaire, engendrant ainsi un conflit sexuel et des préjudices imposés par les mâles. Ces conséquences évolutives ne sont pas fixes, puisque les avantages et les coûts potentiels associés à la compétition pour l'accouplement peuvent varier selon le déroulement des interactions reproductives, l'environnement dans lequel elles se produisent et l'histoire évolutive des populations concernées.

Les drosophiles constituent un système modèle largement utilisé pour l'étude de la sélection sexuelle, du conflit sexuel et des préjudices causés par les mâles. Une grande partie de ces travaux a été réalisée à l'aide de populations adaptées aux conditions de laboratoire, maintenues et évaluées dans des environnements fortement simplifiés. Comme la sélection sexuelle et les préjudices causés par les mâles peuvent tous deux influencer l'adaptation, la purge génétique et la valeur adaptative des populations, les effets de l'environnement sur la compétition pour l'accouplement peuvent avoir des conséquences évolutives considérables. Pourtant, nous connaissons encore relativement peu les mécanismes par lesquels l'environnement d'accouplement modifie l'intensité de la sélection et l'expression des préjudices causés par les mâles, ainsi que les répercussions de ces effets sur l'interprétation des travaux antérieurs. Dans cette thèse, j'ai examiné comment l'environnement physique d'accouplement et l'histoire

évolutive des populations influencent la sélection s'exerçant sur les mâles et l'expression des préjudices causés par ceux-ci chez les drosophiles.

Dans ma première expérience (chapitre 2 de cette thèse), j'ai testé l'hypothèse selon laquelle la sélection chez les mâles de *D. melanogaster* est plus forte dans un environnement d'accouplement de grande taille et structuralement complexe que dans un environnement de petite taille et structurellement simple (les flacons standards utilisés pour l'élevage des drosophiles), en comparant la valeur adaptative de mouches consanguines et exogames. La sélection contre les mâles consanguins était forte, mais les données permettant de conclure qu'elle était plus intense dans les environnements complexes sont demeurées équivoques : une expérience a révélé un effet allant dans cette direction et s'approchant du seuil de signification statistique sans toutefois l'atteindre, tandis qu'une seconde expérience n'a mis en évidence aucune différence entre les environnements. Dans ma deuxième expérience (chapitre 3), j'ai examiné quelles caractéristiques de l'environnement d'accouplement réduisent l'expression des préjudices causés par les mâles chez *D. melanogaster*. En manipulant séparément la densité de population et la complexité structurelle de l'environnement, j'ai montré que seule la complexité structurelle, et non la densité, réduisait l'expression de ces préjudices ; en effet, ceux-ci étaient pratiquement éliminés dans le traitement présentant une structure complexe. Dans ma troisième expérience (chapitre 4), j'ai étudié si les préjudices causés par les mâles différaient entre des populations adaptées au laboratoire et des populations issues du milieu naturel chez deux espèces de drosophiles. Dans l'ensemble des trois comparaisons effectuées, les mâles adaptés au laboratoire imposaient généralement des coûts de valeur adaptative plus élevés aux femelles que les mâles issus de populations naturelles. Ce résultat suggère qu'une adaptation prolongée aux

environnements simplifiés du laboratoire peut accroître l'expression des préjudices causés par les mâles, bien que l'ampleur de cet effet dépende parfois de l'espèce considérée et de la combinaison des populations mâles et femelles.

Pris dans leur ensemble, mes résultats montrent que les conséquences évolutives de la compétition pour l'accouplement dépendent du contexte, puisque tant l'environnement physique que l'histoire évolutive des populations peuvent influencer la sélection et le conflit sexuel. La compétition pour l'accouplement peut contribuer à l'adaptation et à l'élimination des allèles délétères, mais elle peut aussi engendrer des préjudices causés par les mâles, et l'équilibre entre ces effets dépend des conditions dans lesquelles les accouplements ont lieu ainsi que des populations impliquées. Les protocoles expérimentaux standard utilisés chez les drosophiles pourraient également surestimer l'ampleur des préjudices causés par les mâles, particulièrement lorsqu'ils reposent sur des populations maintenues en laboratoire pendant de longues périodes et évaluées dans des environnements structurellement simples. Une compréhension plus complète de la sélection sexuelle et du conflit sexuel nécessitera donc une attention accrue portée à l'environnement d'accouplement, à l'histoire des populations et à la mesure dans laquelle les résultats obtenus en laboratoire reflètent les processus se produisant dans des contextes plus naturels.

Acknowledgments

It seems impossible to mention every single person who has been of notable help to me in the journey of surviving a Doctorate thesis. Thus, I will mention whom I can, and ask the rest for their forgiveness, when my oversight is called out.

First, I would like to profoundly thank my supervisor, Howard Rundle, and (unofficial) co-supervisor, Aneil Agrawal. Aneil, firstly because, for some reason I haven't yet identified, he chose to be interested in a "geriatric" potential student ("geriatric" is my self-description; I was always the oldest student in the department while I was there), and incited Howard's interest in me. His advice, suggestions, insights and contributions have been invaluable as well. Howard, because his was the greater task of handling me; apparently how to handle dozing off on multiple occasions in your supervisor's office isn't mentioned in the grad student handbook. For all my quirks, he has always been kind to me. Also, his constant having of time for me despite his stuffed schedule, his direction, feedback (which I dreaded and looked forward to at the same time), and ferrying me around to get a chair and a winter coat, will ever be appreciated. I would also like to thank all the members of my TAC, past and present: Rees Kassen, Julie Lee-Yaw, Tom Sherratt and Vincent Careau, for their invaluable guidance and advice. Many thanks to Jessica Forrest for helping with translation of my abstract.

Next, I would like to thank the grad students I met in the Rundle Lab: Will Jarvis, Cameron Kendrick, Tristan Ducharme, Callum Levett and Mark Jin. My interactions with them have sharpened me in one way or another. Mark, in particular, as well as Jamie Wilson, Aseel Sabar,

Chloe Pelletier, Matthew Osborne, and a number of other undergrad students helped me a great deal in the exciting drudgery of fly experiments and maintenance.

I have built community outside my more academic theater as well, some of whom I would like to celebrate. Calvary Fellowship Ottawa, the Intervarsity Grad and Faculty Group, the "GLASSCHIMP" Next Level Men's Group, St. Joe's Supper Table (Gerry, your flies were really well-behaved!) are all groups that poured into me in many ways, and encouraged me at times I felt too weak to continue. My many friends, especially Matt and Nikki Nagy-Wilson, and Keaton Sinclair were a constant source of cheer and delight to me.

Finally, I'm not sure I'd have been here if not for my immediate family. To my Dad and Mum, thank you both for believing in me, for your countless prayers, care, encouragement and support. To my elder brother; Tola, thank you for being that grounding presence and encouragement, and your belief that I could get this done. Finally, to my clone (or am I his clone?), my shield-brother and forever partner in crime, Taiwo, I don't think I'd have gotten to this point without those mutual venting periods, the inside jokes that none except only us both would understand, the encouragement and support from the other side of the pond. And finally, Timilehin Bankole, my brother from another mother, I see you, man.

To those I did not mention here, it is not because I do not appreciate you, but because there isn't enough space to relay my gratitude. But to you all, mentioned or not, I will be forever grateful. Fare you well, wherever you fare.

Chapter 1: General Introduction

Darwin (1859) recognized that males often engage in intense competition for the acquisition of mates. It has since been established that mate competition is persistent, widespread, and often intense in sexual species, and that it can occur not just over access to mates (i.e. pre-copulatory) but also post-copulation over access to gametes. The evolutionary consequences of mate competition arise, in part, from the sexual selection it generates. Selection arises due to the existence of variation among individuals in a population, and the effects these variations have on the fitness of individuals relative to others (Darwin 1859). One key effect of mate competition is sexual selection; sexual selection can be defined as selection that arises from differences in fitness associated with non-random success in competition for access to gametes for the purpose of fertilization (Shuker and Kvarnemo, 2021). Darwin opined that mate competition, which is usually a struggle between males for the acquisition of females, is the key factor on which sexual selection depends (Darwin, 1859). Victors in this struggle were ensured large numbers of offspring, while the less fortunate would have few, or no, offspring. Males may compete with each other, both directly and indirectly, via intra- and intersexual processes (e.g., male-male competition and female mate choice), and those that are more successful at securing mates and fertilizing their gametes pass more copies of their genes to the next generation than those that are less successful. Through the sexual selection it generates, mate competition underlies the evolution of extravagant sexual display traits and weapons of intrasexual combat (Andersson, 1994), but it can also have broader evolutionary consequences than just the evolution of sexual traits (Searcy, 1982; Whitlock and Agrawal, 2009).

Aside from its implication in the evolution of secondary sexual characters, sexual selection can also influence fundamental evolutionary processes like adaptation and purging when male reproductive success is condition-dependent. Sexual selection can aid non-sexual selection if the males with highest non-sexual fitness (i.e., the most vigorous and healthiest males) also have greatest reproductive success (Darwin, 1859). Since most alleles in the genome likely contribute to an individual health and vigour, sexual selection is expected to favor the same alleles that are favored by natural selection, leading to alignment of sexual, and non-sexual fitness (Rowe and Houle, 1996; Whitlock and Agrawal, 2009; Dugand et al., 2019; Parrett et al., 2022). For example, if the effect of a specific allele was to increase foraging ability, and thus improve survival, it would be favoured by natural selection. Individuals carrying such an allele are likely to gain more resources and better nutrition, thus being healthier and more vigorous, and hence better competitors for access to mates and their gametes. Hence, sexual selection will also favour this allele.

However, experimental evidence has presented varying results concerning this seemingly straightforward hypothesis, suggesting that the consequences of mate competition may be complex. For example, a review by Whitlock and Agrawal (2009) notes that, while a number of experiments (e.g. Fawcett and Johnstone, 2003; Lorch et al., 2003) provide indirect evidence that sexual selection can assist in the purging of deleterious mutations from the gene pool, there is little direct evidence to support this claim. Also, a meta-data analysis by Cally et al (2019) revealed that, while sexual selection had positive (but relatively small) effects on direct measures of population fitness, it weakened immunocompetence, and mostly favored traits less closely linked to population fitness. The lack of clarity in results of tests aimed at determining the alignment of

sexual and non-sexual selection could be due to the additional impacts mate competition may have beyond the generation of sexual selection (Rowe and Rundle, 2021).

Despite the potential for mate competition to improve the fitness of a population, it can also have detrimental consequences when traits that evolve because they increase male reproductive success also reduce female fitness as a side effect. This is a form of sexual conflict. Sexual conflict occurs when the differing reproductive interests of males and females generate sex-specific selection on shared traits like mating rate, and mate competition is a key reason why selection differs between the sexes (Parker, 1979). For instance, traits which are strongly sexually selected for in males, because of their advantage in securing females or fertilizing their gametes, can also cause a decrease in female fitness as a by-product (Arnqvist and Rowe, 2005). The existence of such traits in males generates selection in females to avoid or mitigate male harm, creating the potential for ongoing sexually antagonistic coevolution (Chapman et al., 2003).

Sexual conflict arises when selection on a shared trait differs between males and females. This conflict is termed intralocus when the same locus affects expression of the trait in each sex, such that selection can be sexually antagonistic on individual alleles. Sexual conflict is interlocus when different loci control the trait in each sex (Chapman et al., 2003). When monogamy is not enforced, interlocus sexual conflict can arise because selection often favours males that invest in acquiring additional fertilizations, whereas females typically invest more heavily in offspring production and may benefit less from additional mating (Parker, 1979; Trivers, 1972; Arnqvist and Rowe, 2005). In many cases, this results in the evolution of traits in males that, while increasing

their chances of reproductive success, may inflict harm on females, leading to a reduction in female productivity (Rowe and Rundle, 2021). This harm, usually imposed on females by males during interactions between sexes, has been studied extensively (discussed by Rowe and Rundle, 2021; more recent studies on male harm in *Drosophila* include Filice and Long, 2016; Yun et al., 2017, 2021; MacPherson et al., 2018; Colpitts et al., 2022).

This thesis examines two potentially contrasting consequences of mate competition: its role in generating selection on males that may promote adaptation and the purging of deleterious mutations, and its capacity to generate sexual conflict through male harm. *Drosophila* has been a central model system for studying both of these. For example, classic work in *D. melanogaster* has demonstrated that exposure to males and excessive mating can reduce female fitness (Fowler and Partridge, 1989; Chapman et al., 1993; Rice, 1996; Wigby and Chapman, 2005; Barnes et al., 2008), with evidence implicating both pre- and post-copulatory mechanisms of male harm. At the same time, *Drosophila* has featured prominently in tests of whether sexual selection can improve population fitness by promoting adaptation or purging deleterious alleles (e.g., Promislow et al., 1998; Whitlock and Bourguet, 2000; Hollis et al., 2009; Singh et al., 2017; Cally et al., 2019), with mixed results. Together, these studies show that mate competition can have both beneficial and detrimental evolutionary consequences, making *Drosophila* an appropriate system in which to ask how the mating environment changes the balance between them. A key unresolved question, and the central focus of this thesis, is how the physical context in which mating occurs, and the evolutionary history of the populations involved, affects the balance between these outcomes.

My research builds on recent long-term evolution experiments in *D. melanogaster* that manipulated both the opportunity for mate competition and the physical environment in which mating occurred (Colpitts et al., 2017; Yun et al., 2017; 2018). These studies showed that mate competition did not have fixed evolutionary consequences. Instead, its effects depended strongly on the mating environment: adaptation to novel larval environments and the purging of deleterious alleles were enhanced when mating occurred in a larger, lower-density, structurally complex environment, but not when mating occurred in standard, structurally simple laboratory vials.

In these experiments, the “simple” environment was a standard fly vial, whereas the “complex” environment was a larger container with multiple food patches and added physical structures. These labels are useful shorthand, but the treatments differed in several (and hence confounded) ways, including volume, fly density, spatial structure, and the distribution of food and oviposition sites. These differences were expected to affect how males and females interacted, including the extent of male harm and the opportunity for selection to act through males and females.

Fig. 1.1. Simple (above) and complex (below) mating environments as used in Yun et al. (2017, 2018, 2019, 2021).

There is evidence (Yun et al., 2017) to suggest that the complex environment reduced male harm and strengthened selection through females, providing a potential explanation for why adaptation and purging were enhanced in that context. However, several questions remain unresolved. In particular, it is unclear whether the complex environment also strengthened selection through males, which specific features of the environment were responsible for reducing male harm, and whether laboratory-adapted populations maintained in simplified

environments provide representative estimates of male harm in nature. These questions form the basis of the three data chapters of this thesis.

My thesis covers three experiments (data chapters) that examine topics relevant to our understanding of the evolutionary consequences of mate competition. In the first experiment (Chapter 2), I followed up on some past work in the Rundle lab showing that the environment in which mating occurs can have profound effects on the consequences of mate competition for adaptation and purging. In brief, via a long-term evolution experiment it was shown that mate competition did not promote adaptation or purging in populations in which mating occurred in high density and structurally simple environments (i.e. standard *Drosophila* lab conditions) (Yun et al., 2018). However, mate competition did promote adaptation and purging in populations in which mating occurred in slightly larger and structurally more complex environments. There is evidence that natural selection through females is strengthened in the complex environment (Yun et al., 2017). I investigated whether total selection through males is also strengthened in the complex environment, seeing as sexual selection has been proposed to cause stronger overall selection in males than in females (Mallet and Chippindale, 2011; Sharp and Agrawal, 2013).

My second experiment attempted to examine which components of environmental complexity were responsible for reducing male harm in complex, versus simple, environments, since male harm was observed to have been reduced in more complex environments (Yun et al., 2017). The two components of the complex environment I investigated are population density and environmental complexity in the form of 3-D structure and resource distribution.

For my third experiment, I investigated whether evolutionary history, particularly long-term adaptation to laboratory conditions, influences the expression of male harm. Natural environments differ significantly from that of the lab, and populations maintained under these different conditions may therefore evolve differences in traits affecting sexual conflict. Previous work (e.g. Yun et al., 2017, MacPherson et al., 2018, Yun et al., 2021) reveal that small changes in mating environments drastically alter the expression and evolution of male harm. Much of what we know about sexual conflict comes from laboratory studies using populations adapted to highly unusual environments (i.e. the lab). If small changes to these lab environments have such dramatic effects on the evolution of male harm, it suggests that lab studies may present an exaggerated view of the importance of male harm in wild populations. I investigated this directly by comparing male harm in wild- vs lab-adapted populations of several *Drosophila* species.

References

Andersson, M. (1994). *Sexual selection*. Princeton University Press.

Arnqvist, G., & Rowe, L. (2005). *Sexual conflict* (Vol. 27). Princeton University Press.

Barnes, A. I., Wigby, S., Boone, J. M., Partridge, L., & Chapman, T. (2008). Feeding, fecundity and lifespan in female *Drosophila melanogaster*. *Proc. R. Soc. B: Biological Sciences*, *275*(1643), 1675-1683.

Cally, J. G., Stuart-Fox, D., & Holman, L. (2019). Meta-analytic evidence that sexual selection improves population fitness. *Nat. Commun.*, *10*(1), 2017.

Chapman, T., Hutchings, J., & Partridge, L. (1993). No reduction in the cost of mating for *Drosophila melanogaster* females mating with spermless males. *Proc. R. Soc. B*, *253*(1338), 211-217.

Chapman, T., Arnqvist, G., Bangham, J., & Rowe, L. (2003). Sexual conflict. *Trends Ecol. Evol.*, *18*(1), 41-47.

Colpitts, J., Williscroft, D., Sekhon, H.S. & Rundle, H.D., 2017. The purging of deleterious mutations in simple and complex mating environments. *Biol. Lett.*, *13*(10), p.20170518.

Colpitts, J., Jarvis, W. M., Agrawal, A. F., & Rundle, H. D., 2022. Quantifying male harm and its divergence. *Evolution*, *76*(4), 829-836.

Darwin, C. (1859). *On the origin of species, 1859*. Routledge.

Dugand, R. J., Tomkins, J. L., & Kennington, W. J. (2019). Molecular evidence supports a genic capture resolution of the lek paradox. *Nat. Commun.*, *10*(1), 1359.

Fawcett, T. W., & Johnstone, R. A. (2003). Mate choice in the face of costly competition. *Behav. Ecol.*, *14*(6), 771-779.

Filice, D. C., & Long, T. A. (2016). Genetic variation in male-induced harm in *Drosophila melanogaster*. *Biol. Lett.*, *12*(4), 20160105.

Fowler, K., & Partridge, L. (1989). A cost of mating in female fruitflies. *Nature*, *338*(6218), 760-761.

Hollis, B., Fierst, J. L., & Houle, D. (2009). Sexual selection accelerates the elimination of a deleterious mutant in *Drosophila melanogaster*. *Evolution*, *63*(2), 324-333.

Long, T. A., Pischedda, A., Stewart, A. D., & Rice, W. R. (2009). A cost of sexual attractiveness to high-fitness females. *PLoS Biol.*, *7*(12), e1000254.

Lorch, P. D., Proulx, S., Rowe, L., & Day, T. (2003). Condition-dependent sexual selection can accelerate adaptation. *Evol. Ecol. Res.*, *5*(6), 867-881.

MacPherson, A., Yun, L., Barrera, T. S., Agrawal, A. F., & Rundle, H. D. (2018). The effects of male harm vary with female quality and environmental complexity in *Drosophila melanogaster*. *Biol. Lett.*, *14*(8), 20180443.

Mallet, M. A., & Chippindale, A. K. (2011). Inbreeding reveals stronger net selection on *Drosophila melanogaster* males: implications for mutation load and the fitness of sexual females. *Heredity*, *106*(6), 994-1002.

Parker, G. (1979). Sexual selection and sexual conflict. *Sexual Selection and Reproductive Competition in Insects*, 123–166.

Parrett, J. M., Chmielewski, S., Aydogdu, E., Łukasiewicz, A., Rombauts, S., Szubert-Kruszyńska, A., ... & Radwan, J. (2022). Genomic evidence that a sexually selected trait captures genome-wide variation and facilitates the purging of genetic load. *Nat. Ecol. Evol.*, *6*(9), 1330-1342.

Promislow, D. E., Smith, E. A., & Pearse, L. (1998). Adult fitness consequences of sexual selection in *Drosophila melanogaster*. *Proc. Natl. Acad. Sci.*, *95*(18), 10687-10692.

Rice, W. R. (1996). Sexually antagonistic male adaptation triggered by experimental arrest of female evolution. *Nature*, *381*(6579), 232-234.

Rowe, L., & Day, T. (2006). Detecting sexual conflict and sexually antagonistic coevolution. *Phil. Trans. R. Soc. B.*, *361*(1466), 277-285.

Rowe, L., & Houle, D. (1996). The lek paradox and the capture of genetic variance by condition dependent traits. *Proc. R. Soc. B*, *263*(1375), 1415-1421.

Rowe, L., & Rundle, H. D. (2021). The alignment of natural and sexual selection. *Annu. Rev. Ecol. Syst.*, *52*, 499-517.

Searcy, W. A. (1982). The Evolutionary Effects of Mate Selection. *Annu. Rev. Ecol. Evol. Syst.*, *13*, 57–85.

Sharp, N. P., & Agrawal, A. F. (2013). Male-biased fitness effects of spontaneous mutations in *Drosophila melanogaster*. *Evol., Int. J. Org. Evol.*, *67*(4), 1189-1195.

Shuker, D. M., & Kvarnemo, C. (2021). The definition of sexual selection. *Behav. Ecol.*, *32*(5), 781-794.

Singh, A., Agrawal, A. F., & Rundle, H. D. (2017). Environmental complexity and the purging of deleterious alleles. *Evolution*, *71*(11), 2714-2720.

Trivers, R. L. (1972). Parental investment and sexual selection. In *Sexual selection and the descent of man* (pp. 136-179). Routledge.

Whitlock, M. C., & Agrawal, A. F. (2009). Purging the genome with sexual selection: reducing mutation load through selection on males. *Evolution*, *63*(3), 569-582.

Whitlock, M. C., & Bourguet, D. (2000). Factors affecting the genetic load in *Drosophila*: synergistic epistasis and correlations among fitness components. *Evolution*, *54*(5), 1654-1660.

Wigby, S., & Chapman, T. (2005). Sex peptide causes mating costs in female *Drosophila melanogaster*. *Curr. Biol.*, *15*(4), 316-321.

Yun, L., Bayoumi, M., Yang, S., Chen, P. J., Rundle, H. D., & Agrawal, A. F. (2019). Testing for local adaptation in adult male and female fitness among populations evolved under different mate competition regimes. *Evolution*, *73*(8), 1604-1616.

Yun, L., Chen, P. J., Kwok, K. E., Angell, C. S., Rundle, H. D., & Agrawal, A. F. (2018). Competition for mates and the improvement of nonsexual fitness. *Proc. Natl. Acad. Sci.*, *115*(26), 6762-6767.

Yun, L., Chen, P. J., Singh, A., Agrawal, A. F., & Rundle, H. D. (2017). The physical environment mediates male harm and its effect on selection in females. *Proc. R. Soc. B*, *284*(1858), 20170424.

Yun, L., Agrawal, A. F., & Rundle, H. D. (2021). On Male Harm: How It Is Measured and How It Evolves in Different Environments. *Am. Nat.*, *198*(2), 219-231.

Chapter 2: Environmental complexity and the strength of selection through males

Abstract

There is a long history of experiments in *Drosophila* that seek to understand the evolutionary consequence of competition for mates. In the vast majority of this work, sexual interactions and mating occur in small, high-density and structurally simple environments (e.g., standard fly vials). However, the environment in which mating interactions occur may play a key role in determining how selection acts and the opportunity for sexual conflict and male harm. For instance, mate competition can alter natural selection on females if females of differing intrinsic quality experience differential male harm, and the physical environment in which mating occurs has been shown to mediate this. The mating environment may also alter sexual selection on males. For example, it may be more challenging for males to secure matings in larger, complex environments, strengthening sexual selection compared to that in standard, and highly simple, lab environments. There are limited data that quantify whether and how sexual selection varies through males in different mating environments. To test this, we performed two experiments. In the first, we used lines from the *Drosophila* Genetic Reference Panel to compare the reproductive success of inbred vs. outbred males by separately competing them with marked males to mate and fertilize the eggs of common females. We did these assays in small/structurally simple and large/structurally complex mating environments. Paternity was inferred by offspring marker status, providing estimates of inbred and outbred male reproductive fitness. The second was a little similar to the first in structure, but we used a different marker, and focal flies were of the same ancestral population as their competition. While the average inbred male fitness was considerably lower than outbred, the strength of sexual selection in the complex environment

was not significantly higher than that in the simple environment. Power may have been limited by the extremely low reproductive success of the inbred males in the first experiment, and by lack of sufficient replication, as well as possible sources of noise in the second.

Note: The work in this chapter involves two experiments, the first was spearheaded by myself and the second by Mark Y. Jin as his honors thesis. The chapter is written in the form of a manuscript for submission to a primary research journal, hence the use of first-person plural pronouns throughout. In addition to conducting the first experiment, I participated in the second experiment through training Mark in necessary techniques and helping with data collection. I also took the lead in merging the experiments, as well as in writing and data analyses, with input from Mark and my supervisor.

Introduction

In sexual species, for males to successfully reproduce they must compete, directly and/or indirectly, for access to females and their gametes. This mate competition can play out in various ways. For instance, males may have to search for and locate females, or otherwise attract them to their location, and they may interact directly (e.g., territorial defense) or indirectly (e.g., scramble competition) with other males in doing so. Once a female is found, a male may have to overcome other males to gain access to her (e.g., contest competition). Males may also have to convince or otherwise coerce a female to mate and, following mating, they may need to guard her from the attention of rival males. Internally, a male's sperm may face further scrutiny (e.g., cryptic female choice) and might also have to compete with that of previous and/or subsequent suitors to fertilize the female's gametes.

Variation among males in their success at mate competition generates sexual selection, which can be persistent and often strong. It has been suggested that, if healthier and more vigorous (i.e. higher condition) males gain more matings and fertilizations, and most genes in the genome affect impact an individual's health or vigor, then sexual selection will align with natural selection across most of the genome, promoting adaptation and the purging of deleterious alleles (Darwin, 1859; Rowe and Houle, 1996; Rowe and Rundle, 2021; Whitlock and Agrawal, 2009). It is easy to imagine that how the various forms of mate competition play out may be sensitive to the environment in which intra- and intersexual interactions occur, and that this could alter the strength of sexual selection, its alignment with natural selection, and hence net selection through males. For instance, in some environments male sexual fitness may depend more strongly on an individual's

health and vigour, and hence be influenced by a larger swath of the genome. This could be because reproductive success is more selective (i.e. less luck-based) in some contexts than others, potentially because some environments enhance the contribution of traits that are highly condition-dependent or require males to compete in additional ways, causing more traits to impact their reproductive success. For example, in Túngara frogs male acoustic calls are condition-dependent (Wilhite & Ryan, 2024), but female preferences for certain call characteristics break down in noisier environments (Coss et al., 2021). And in guppies (*Poecilia reticulata*), sexual selection on both pre- and postmating traits was weaker in urban than non-urban sites (Marques et al., 2022). It has also long been recognized that the ability of males to monopolize females for reproduction is likely to depend on the social and ecological environment, for instance being easier when resources (and hence females) are clumped (Emlen & Oring, 1977).

It is tempting to infer that certain environments may consistently promote diverse forms of mate competition that depend on male health or vigor, making reproductive success more condition-dependent, increasing the opportunity for sexual selection and hence strengthening net selection through males. The size and complexity of the environment may be one of these, and this may be particularly the case in laboratory model systems; these are of particular interest because they have played a central role in tests of the alignment of sexual with natural selection (Cally et al., 2019; Rowe and Rundle, 2021). For instance, in lower density environments with patchy resources, *Drosophila* males can become territorial (Hoffmann and Cacoyianni, 1990) and this may impact their reproductive success through different condition-dependent traits than those targeted by female mate preferences (e.g., body size vs. contact pheromones) (White and Rundle, 2015). And compared to standard lab rearing conditions (e.g., small, highly simplified vials or

bottles), larger environments with more widely spaced resources may make the ability of *Drosophila* males to locate females important, and this may strengthen sexual selection against deleterious mutations (MacLellan et al., 2009).

However, one could argue the opposite in terms of the effects of space and complexity. For instance, in *Drosophila* it is possible that sexual selection is particularly strong in simple, high-density cultures (e.g., via intense sperm competition), whereas at low density or in structured environments, chance encounters may play a substantial role in reproductive outcomes. Limited attention has been given to quantitative comparisons of net selection on males in different environments, despite potential impacts for fundamental evolutionary processes including adaptation and purging. That is our primary goal here, and we do so by quantifying and comparing selection through *Drosophila melanogaster* males in two separate manipulative experiments in which sexual interactions and mating occur either in small, highly simplified lab environments (i.e. standard *Drosophila* vials) or larger, and hence lower density, containers that include discrete resources patches and added physical structure. For simplicity, we refer to these as 'simple' vs 'complex' mating environments respectively, recognizing that they differ in several ways.

A secondary motivation for our experiments is to provide insights into earlier results, in particular Yun et al. (2018) who used these same two mating environments in an evolution experiment. Yun and colleagues found that purging was enhanced, and adaptation to three different novel larval environments occurred more rapidly, when mate competition took place in the complex rather than the simple mating environment. This result was well-replicated (42 independent evolved populations were involved in the simple vs. complex comparison), and two separate experiments

also demonstrated faster purging of individual deleterious alleles in similar, but slightly different, versions of the complex compared to the simple mating environments (Colpitts et al., 2017; Singh et al., 2017a). These results invite the question of why adaptation and purging were consistently enhanced in a complex environment? For the environments of Yun et al. (2018), there is evidence that natural selection on females was stronger in the complex compared to the simple mating environment, which would contribute to faster adaptation and more efficient purging (Yun et al., 2017; MacPherson et al., 2018). But whether selection on males also differed between these mating environments has not been addressed.

We quantified net selection through males in two experiments by estimating the fitness of inbred vs. outbred focal males, from which the selective coefficient (*s*) against inbreeding can be calculated, separately when mate competition occurred in the simple vs. complex mating environments. The two experiments differed in the extent of inbreeding and in the nature of mate competition: inbred and outbred males separately competed against males carrying a visible genetic marker in the first experiment, whereas they competed directly with each other in the second.

Materials and Methods

We quantified the strength of selection through males, while manipulating the environment in which sexual interactions and mating took place, in two separate *D melanogaster* experiments. Both experiments used the same two mating environments, essentially mirroring those used in Yun et al. (2017, 2018, 2019, 2021). These environments varied in volume (and hence density of

the flies), as well as structural complexity (i.e. the distribution of food and the presence of physical structure). The simple environment used standard fly vials identical to those used during population maintenance (28.5 mm × 95 mm vials with 10 mL cornmeal-yeast media), while the complex used larger volume 1.65 L cylindrical plastic Ziploc food storage containers within which there were five separate food patches (three 3 oz 'Dixie cups' with 20 mL cornmeal-yeast media and two 1 oz plastic 'portion cups' containing 10 mL cornmeal-yeast media) and two loosely coiled pipe cleaner protruding from the lid into the interior. The food surface of the three Dixie cups was supplemented with 20- 30 dry yeast pellets. The only difference from past studies in the design of these environments (e.g., Yun et al., 2018) was a change in the volume of media in the complex environment food patches: 25 and 7.5 mL in Yun et al. (2018) compared to 20 and 10 mL here. Figure S1 of Yun et al. (2017) provides a photograph of the complex environment.

Experiment 1

In the first experiment, we quantified the strength of selection through males using 30 inbred lines sourced from the *Drosophila* Genetic Reference Panel (DGRP). The DGRP is a resource of 192 fully sequenced inbred *D. melanogaster* lines, originally collected from the wild in Raleigh, North Carolina (Mackay et al., 2012). In brief, sets of inbred and outbred males were created by crosses within and between 15 unique pairs of DGRP lines. Selection was quantified by comparing the performance of the inbred and outbred male offspring from these crosses when they were separately competed against marked competitor males from an outbred, lab-adapted population homozygous for the recessive *bw* mutation causing brown-eyes. Focal males (inbred or outbred)

competed with *bw* males to mate and fertilize the eggs of *bw* females over a 6 day 'interaction phase', with the fitness of the focal males determined by the proportion of offspring they sired. The interaction phase took place in either the simple or complex mating environment, with conditions (i.e., exposure duration, density and handling of flies) matching that in past studies (Yun et al., 2017, 2018, 2019, 2021). Females were subsequently moved to new vials to lay eggs, and offspring were phenotyped (*bw* vs wild-type eyes) to determine the fitness of the focal inbred or outbred males.

The experiment was run in 15 blocks, each involving one pair of unique DGRP lines (i.e., each of the 30 lines was used only once). All combinations of males and females from a given pair of lines (designating the lines as L1 and L2) were separately crossed to yield four genotypes of offspring: two highly inbred (L1 males x L1 females, L2 males x L2 females), and two outbred (L1 males x L2 females, L1 females x L2 males). All flies used for crossing were collected from standard vials containing ~100 eggs per vial to minimize intraspecific competition due to overcrowding.

For each of the four cross-types, ten males and ten virgin females from the appropriate DGRP line(s) were placed in a vial and allowed to mate and lay eggs for 24 hours; within-line crosses producing inbred offspring were left longer because preliminary trials indicated reduced offspring viability, so more eggs were needed to obtain a similar number of adults as the outbred crosses. There were eight vials per cross-type. One day after setting up the DGRP crosses, 20 vials, each containing ten male and ten female *bw* flies, were set up, ensuring that the *bw* and DGRP offspring eclosed at approximately the same time.

Adult virgin offspring were collected following eclosion using light CO_2 anesthesia and were stored separately by sex and cross-type in holding vials (10 flies/vial), with a light sprinkling of live yeast pellets in each vial. Adult virgin *bw* flies were collected and stored in the same way. Approximately 48 h later, flies from the holding vials were used to set up replicate 'containers' (i.e. simple or complex mating environments) for the interaction phase. A given replicate consisted of 10 focal males of a particular cross-type, along with 25 competitor *bw* males and 35 *bw* females. This matches the densities used in past studies (Yun et al., 2017, 2018, 2019, 2021). Three replicates of each of the four cross-types were set up for each mating environment, yielding: 4 cross-types x 2 mating environments x 3 replicates = 24 replicate containers for a given pair of DGRP lines.

Following previous studies (e.g., Yun et al., 2018), flies spent six days in their mating environment and the food was changed on the third day (to prevent mortality due to flies getting stuck in wet food). On the sixth day, flies were anaesthetized with CO_2, sorted based on sex, and the numbers of surviving flies was recorded by sex and eye color. Males were subsequently discarded. In each replicate, surviving females were divided as equally as possible among three fresh food vials, recording the number of females per vial. Females were allowed to lay eggs for ~4 h, after which they were discarded.

Vials were left until eclosion appeared complete, at which point all offspring were collected, separated by eye color (*bw* vs. wild-type, *wt*), and then counted. This was repeated two days later to ensure all offspring were obtained. For a given DGRP line-pair, the fitness of inbred males (W_{inbred}) was calculated as the proportion of offspring sired (i.e., number of *wt* offspring divided by the number of wild-type plus *bw* offspring), where the number of *wt* and *bw* offspring were

summed across all vials in which females laid eggs for all six replicates of the two inbred cross types (i.e. three L1 x L1 replicates and three L2 x L2 replicates). The fitness of outbred males (W_{outbred}) for the same line-pair was similarly calculated as the proportion of offspring sired where the number of *wt* and *bw* offspring were summed across all vials in which females laid eggs for all six replicates of the two outbred cross types (i.e., three L1 x L2 replicates and three L2 x L1 replicates).

To compare total selection through males in each mating environment, for each DGRP line-pair in each mating environment we calculated the selection coefficients (*s*) quantifying the cost of being inbred as follows:

$$s = \frac{(W_{outbred} - W_{inbred})}{W_{outbred}}$$ Eqn. 2.1. Selection coefficient formula for inbreeding cost

Variation in *s* was analyzed using a general linear mixed model with mating environment as a fixed effect and line-pair as a random effect via the *lmer* function in the *lme4* package (Bates et al., 2015) in R v4.3.3 (R Core Team, 2024). Type III tests of fixed effects employed *lmerTest* (Kuznetsova et al., 2017).

Experiment 2

In the second experiment, we compared the strength of selection through males when inbred and outbred males competed directly with one other for access to females and their gametes in either the simple or complex mating environments. Inbred males were also less inbred than in

Experient 1. In brief, as a marker to infer siring success we used a single locus autosomal dominant mutation, *DsRed*, that codes for the expression of a protein with no visible phenotypic effect under standard lighting, but that emits red-orange light when excited under ~545 nm light (Lambert, 2019). Potential fitness effects of this marker were balanced by conducting the assays reciprocally such that *DsRed* inbred males competed against outbred *wt* males in half of the trials, and *wt* inbred males competed against *DsRed* outbred males in the other half.

Both the *DsRed* and *wt* populations were derived from the common genetic background of a single, outbred and lab-adapted stock population that was segregating the *DsRed* allele. This population was created in 2015 by introgressing the *DsRed* mutation into an outbred lab stock (Yun et al, 2018) originally collected in 2005 from the Similkameen Valley, British Columbia, Canada (Yeaman et al., 2010). This population had been maintained on a two-week schedule (non-overlapping generations) in 16 fly bottles at 25 °C, 12 h light:12 h dark photoperiod, and ~50% environmental humidity) for ~10 years prior to its use. A *wt* population was created from this stock by establishing 100 pairs of F0 *wt* virgin females and *wt* males. F1 offspring of families producing only *wt* offspring were pooled and redistributed to 40 standard fly vials to create a *wt* outbred stock population. Similarly, around 300 separate pairs of F0 *DsRed* virgin females and *DsRed* males were created. Because *DsRed* is dominant, pairs that generated any *wt* F1 offspring were discarded to eliminate families with two heterozygous parents. For the remaining families, F1 offspring were mated with their siblings and families that produced any *wt* F2 offspring were discarded, thereby ensuring both F0 parents were homozygote *DsRed*. The remaining families were pooled and re-distributed to 40 standard vials to create a *DsRed* outbred stock population. Both populations started with >20 separate male-female pairs. Given how they were created, the

genetic background of these two populations was largely shared except for the presence/absence of the *DsRed* marker.

In creating the *DsRed* population as described above, it is possible that *wt* alleles could still be present in a given family, the probability of which depends on the number of F2 offspring phenotyped. We used only families that produced >60 offspring. Nevertheless, the *wt* allele was observed to be segregating at a low frequency in the final *DsRed* population. Only *DsRed* males from this population were used in the mate competition assay, although some of these may have been heterozygous, which would cause approximately half of their offspring to be misidentified as being sired by competitor *wt* males. However, because the experimental design was balanced (i.e., an equal number of trials were performed in each mating environment when *DsRed* was associated with inbred vs. outbred males), male fitness estimates were not biased. The *wt* and *DsRed* populations were maintained under the same environmental conditions as the original stock in 40 vials each.

To obtain males for use in the mating assays, replicate female-male virgin pairs were created from both the *wt* and *DsRed* populations. Each family then underwent two generations of inbreeding. First, using the F1 offspring of a family, three separate pairs of full siblings were created. Second, using the F2 offspring, groups of 10 males × 10 females were created for each of the pairs. The resulting F3 offspring therefore varied in relatedness from full siblings to first cousins. Finally, for each family, inbred and outbred F4 offspring were generated by mating five F3 males from that family with either five F3 virgin females from the same family or five virgin females from the appropriate stock population from which the line was derived (i.e. *wt* or *DsRed*).

For the mate competition assays, a 'set' of four replicates was created from a given pair of randomly chosen *wt* and *DsRed* families (i.e. the inbred and outbred F4 offspring from one *wt* family and from one *DsRed* family). For a given set, the two combinations of inbred and outbred males (i.e. inbred *wt* with outbred *DsRed* and inbred *DsRed* with outbred *wt*) separately competed in the simple and in the complex mating environments for reproducing with *wt* virgin females from the *wt* stock. Each set therefore had four replicate inbred vs. outbred mate competition trials in a 2 × 2 factorial cross of mating environment (simple vs. complex) and *DsRed* carrier status (inbred vs. outbred; Fig. S2.1). 27 of these sets were performed across three experimental blocks (10, 6, and 11 sets, respectively) over the course of five months, with unique families created for each block.

Due to the availability of F4 offspring, the number of inbred and outbred males competing in a given trial differed slightly from Experiment 1 and previous studies, all of which had 35 males with 35 females (Yun et al., 2017, 2018, 2019, 2021). In the current experiment, in all cases the number of inbred and outbred males were always equal within a given container, and in blocks 2 and 3 all four replicates of a given set had the same number of inbred and outbred males; in block 1 the number of inbred and outbred males differed among containers (but always a 1:1 ratio within a container). An average of 10.9 ± 4.2 SD (range 3-15) males of each type (inbred and outbred) were used in a given trial, with the appropriate number of *wt* outbred females to yield a 1:1 sex ratio.

As in Experiment 1 and past studies (Yun et al., 2017, 2018, 2019, 2021), flies spent six days in their mating environment with the food being changed on day 3. After day 6, flies were anaesthetized with CO_2, sorted by sex and counted, and males were discarded**.** Females were

transferred into 3-6 fresh vials, with 3-5 females each, to oviposit. The females were discarded after 24 h and offspring were counted as in Experiment 1. For each set, the fitness of inbred (W_{inbred}) and outbred (W_{outbred}) males in a given environment was calculated by summing the count of inbred and outbred offspring respectively across all laying vials. This was done separately by marker status (i.e. when inbred males were *wt* vs. *DsRed*) because preliminary analyses showed a substantial marker effect on male fitness; because the design was not perfectly balanced with respect to this (due to two missing replicates), we wanted to correct for it statistically. For each set, selective coefficient (s) against inbreeding were then calculated for each mating environment and marker status set using Eqn. 2.1. Variation in s was analyzed using general linear mixed model following experiment 1, with set instead of line-par as a random effect and with the addition of a fixed effect of marker status.

Results

In Experiment 1, selection against inbreeding was strong in both mating environments: s_{simple} = 0.835; s_{complex} = 0.886 (Fig. 2.1). Selection was 6.11% stronger in the complex compared to the simple environment, a difference that approached, but did not achieve, statistical significance ($F_{1,14}$ = 3.38, $P_{\text{two-tailed}}$ = 0.087). In Experiment 2, in which inbred males were less inbred than in Experiment 1, selection against inbreeding was also present but was not as strong: s_{simple} = 0.354; s_{complex} = 0.354 (least-square means; Fig. 2.2). However, there was no evidence that its strength varied by mating environment ($F_{1,103}$ = 0.00, $P_{\text{two-tailed}}$ = 0.989). There was a substantial effect of marker status, however, with selection against inbreeding being significantly stronger ($F_{1,103}$ =

7.52, $P_{two\text{-}tailed}$ = 0.007) when inbred males also carried the *DsRed* marker compared to when outbred males carried this marker: s_{DsRed} = 0.496, s_{wt} = 0.213; least-square means).

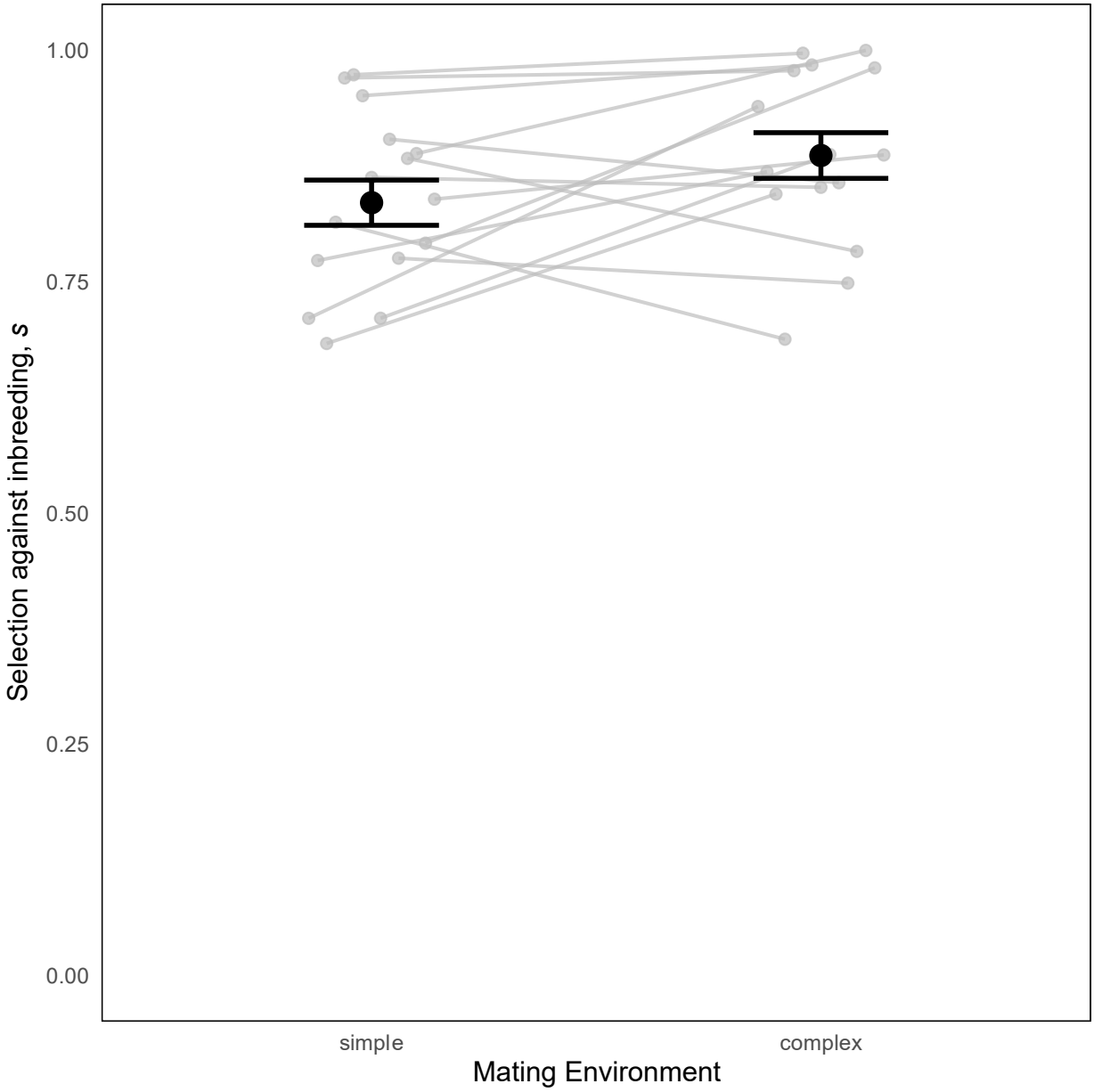

Fig. 2.1. Selection against inbred males, *s*, from Experiment 1, calculated as the relative reduction in fitness following Eqn. 2.1. Dark points are means ± 1 SE, grey points show individual line-pairs.

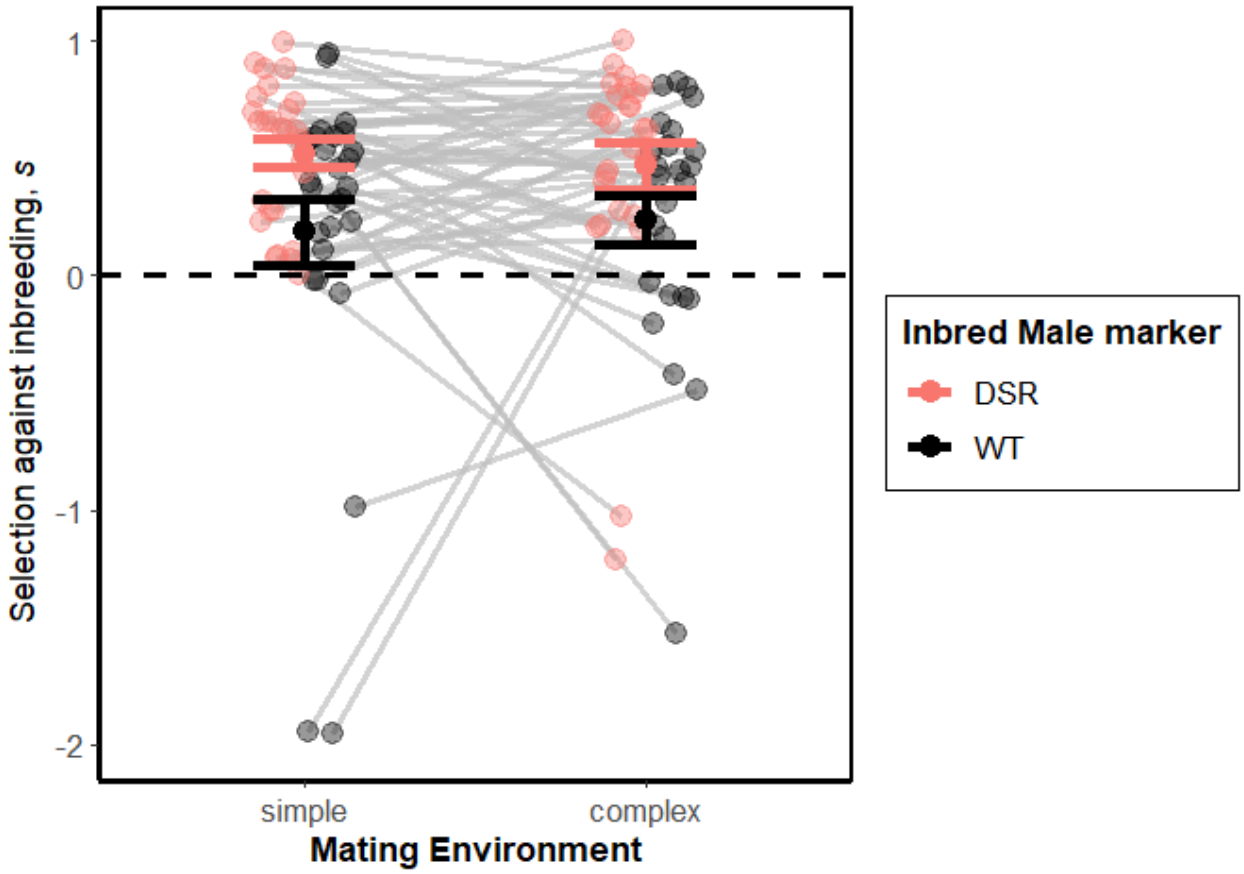

Fig. 2.2. Selection against inbred males, *s*, from Experiment 2, calculated as the relative reduction in fitness following Eqn. 2.1 while taking inbred male marker status into consideration. Larger/darker points are means ± 1 SE separately by inbred male marker status; lighter points show values for individual sets.

Discussion

The strength of selection is likely to vary in different environments and there have been attempts to investigate patterns in this regard, for example whether selection tends to be stronger in more stressful environments (Agrawal & Whitlock 2010) or at higher population densities (Sharp and Agrawal 2008). Sexual selection can be persistent and strong and is thought to play a major role in shaping overall selection through males, with potential impacts on adaptation and the purging of deleterious mutations (Darwin, 1859; Rowe and Houle, 1996; Rowe and Rundle, 2021; Whitlock and Agrawal, 2009). One might therefore also ask more specifically whether selection through males varies consistently in different environments, i.e. do some environments act as a stronger selective sieve on males than others? Reasonable arguments can be made, for example, as to why selection through males might be stronger on average in simpler, higher density environments, or alternatively in those that are more structurally complex and have lower densities. Empirical data on the subject, however, are quite limited. This is of general interest and is also relevant to the interpretation of past lab results demonstrating a stronger alignment of sexual with natural selection in several experimental evolution studies in *Drosophila* (e.g., Colpitts et al., 2017; Singh et al., 2017; Yun et al., 2018).

Here, we conducted two separate experiments to test this whether the strength of selection through *D. melanogaster* males differed in a simple vs. complex lab mating environments. The first revealed a pattern in which selection was 6.11% stronger in the complex compared to the simple environment (s_{simple} = 0.835; $s_{complex}$ = 0.886); this difference approached, but did not achieve, statistical significance ($P_{two\text{-}tailed}$ = 0.087). The second experiment, however, showed no

such trend, with the average strength of selection being essentially the same in the two mating environments (s_{simple} = $s_{complex}$ = 0.354). Selection against inbreeding was stronger when inbred males carried the *DsRed* marker compared to when outbred males carried *DsRed* (s_{DsRed} = 0.496, s_{wt} = 0.213), indicating fairly strong selection against the marker itself. We decided to perform the second experiment in light of the borderline results of the first, and because the fitness of inbred males was so low in Experiment 1 (the selection coefficients reveal a more than 80% reduction in the fitness of inbred relative to outbred males; Fig. 2.1) that we were concerned it may have reduced the ability to detect an effect of mating environment (e.g., if there was little scope for any further decreases in the fitness of inbred males in one environment relative to the other). We reasoned that a second experiment, in which inbred males were less inbred and where they competed directly with outbred males, would provide additional data via an improved design. Replication, however, was lower in the second experiment (total number of offspring phenotyped: Experiment 1, *n* = 68,861; Experiment 2, *n* = 49,960), and noise was added to the estimates of selection due to the fact that the *wt* allele was segregating at low frequency in the *DSred* population (although the balanced design prevented this from biasing estimates of selection; see Methods). The power of the second experiment to detect significance of a difference in selection of 6.11% (i.e., that observed in Experiment 1), based on the replication and observed sampling variation of Experiment 2, was only ~11% (i.e. post-hoc power analysis for a specific effect size). Experiment 2 therefore does not provide strong evidence against an effect of the magnitude observed in Experiment 1, and the overall result of our studies is thus inconclusive. Additional work will be needed to determine whether selection through male *D. melanogaster* is stronger in the complex compared to a simple mating environment. The best approach to doing

this would be to employ the design of Experiment 2, allowing direct competition between outbred and modestly inbred males, while employing greater replication and using a population in which the phenotypic marker is fixed; it may also be worth exploring the use of an alternative marker that does not have such a strong impact on male fitness.

Selection may differ between environments for the reasons we have previously discussed, and stronger selection through males in the complex environment would help explain the faster adaptation and more efficient purging that has been observed in similar environments in earlier experiments (e.g., Colpitts et al., 2017; Singh et al., 2017; Yun et al., 2018). Also consistent with such a difference, MacLellan et al. (2009) found that, for a set of nine deleterious mutations in *D. melanogaster*, selection through males was significantly stronger, on average, in larger mating environments. However, we have not considered reasons why selection through males may *not* differ between these environments, and we do so here for completeness because our results suggest this could be the case. There are two possibilities. First, selection may have favored the same phenotypes in males in the simple and complex environments, or in other words, it did not differ between these environments. Given the results of past work, this seems unlikely: there is evidence that the expression of male sexual behaviors, such as courtship rate and the harm they impart on females, differ in these environments (Yun et al., 2017), and that males that evolved in these environments diverged in both their harmfulness to females (Yun et al., 2021) and in their reproductive fitness (Yun et al., 2019).

A second possibility for why selection may not differ is that it did target different phenotypes, and hence different sets of loci, in males in the two environments, but its overall strength was similar.

Consistent with such a possibility, Singh and Agrawal (2022) used simple and complex mating environments that were similar (but not identical) to ours and found no difference in the strength of selection on *D. melanogaster* male condition, although statistical power may have been limited and condition was manipulated environmentally (via larval rearing density) as opposed to genetically (e.g., inbreeding, as in the current study). There is evidence suggesting that environmental manipulations of condition may not well mimic the effects of genetic variation in condition (Bonduriansky et al., 2015). The absence of any difference would also echo similar findings that the average fitness effect of allelic variants often does not differ across environments (Martin and Lenormand 2006; Agrawal and Whitlock 2010). Finally, although MacLellan et al. (2009) did find that selection through males was significantly stronger, on average, in larger mating environments, their results were heterogenous (only three mutations were individually significant and the trend was reversed in another), and the mutations they used had substantial and visible effects, with six of them affecting eye phenotypes. Such mutations could be particularly important to reproductive success in a large/low density environments, and there may have been other loci on which selection was weaker in this environment. Sharp and Agrawal (2008) did not detect any difference in the average strength of selection on eight visible mutations in flies interacting in environments with different population densities.

Regarding the secondary motivation for this study, concerning in particular the results in Yun et al. (2018) which showed faster adaptation and better purging of deleterious mutations in the complex environment, we wanted to explore whether selection through males may have contributed to this. Our results do not resolve whether this was the case. Earlier studies have shown that selection through females was stronger in the complex environment (Yun et al., 2017;

MacPherson et al., 2018), suggesting this explains, at least in part, the improved adaptation and purging that was observed. There are also other potential contributors that have not been investigated, such as changes in assortative mating by fitness. A positive correlation in the fitness of combining gametes can promote adaptation and purging by increasing the variance in fitness and hence the efficiency of natural selection (Fisher, 1919; Whitlock & Agrawal, 2009). While assortative mating by various phenotypic traits is common in many populations, whether this translates into a positive correlation in the fitness of combining gametes has received limited attention, and the result of the few studies, all in *Drosophila*, are mixed (see Sharp and Agrawal, 2009; Sharp and Whitlock, 2019; Talagala et al., 2024). Whether this varies by mating environment has also not been addressed.

To conclude, additional studies are needed to determine whether selection through males strengthens in complex environments, and whether this may have contributed to earlier results by promoting adaptation and purging. If selection on males is found to be stronger, the roles of size/density and complexity of mating environment would be of interest. While MacLellan et al. (2009) found increased selection in larger/lower density mating cages, Osijo et al. (2026) found that differences in complexity, but not density, were responsible for changes in the harmfulness of males. Of course, environments can vary in more ways, including other axes of complexity, and the generality of any results in other contexts (e.g., Malek & Long, 2019), as well as species, would also be important to address.

References

Agrawal, A. F., & Whitlock, M. C. (2010). Environmental duress and epistasis: How does stress affect the strength of selection on new mutations? *Trends Ecol. Evol.*, *25*(8), 450–458. https://doi.org/10.1016/j.tree.2010.05.003

Bates, D., Mächler, M., Bolker, B., & Walker, S. (2015). Fitting Linear Mixed-Effects Models Using **lme4**. *J. Stat. Softw.*, *67*(1). https://doi.org/10.18637/jss.v067.i01

Bonduriansky, R., Mallet, M. A., Arbuthnott, D., Pawlowsky-Glahn, V., Egozcue, J. J., & Rundle, H. D. (2015). Differential effects of genetic vs. Environmental quality in *Drosophila melanogaster* suggest multiple forms of condition dependence. *Ecol. Lett.*, *18*(4), 317–326. https://doi.org/10.1111/ele.12412

Cally, J. G., Stuart-Fox, D., & Holman, L. (2019). Meta-analytic evidence that sexual selection improves population fitness. *Nat. Commun.*, *10*(1), 2017. https://doi.org/10.1038/s41467-019-10074-7

Chippindale, A. K., Gibson, J. R., & Rice, W. R. (2001). Negative genetic correlation for adult fitness between sexes reveals ontogenetic conflict in *Drosophila*. *Proc. Natl. Acad. Sci.*, *98*(4), 1671–1675. https://doi.org/10.1073/pnas.98.4.1671

Colpitts, J., Jarvis, W. M. C., Agrawal, A. F., & Rundle, H. D. (2022). Quantifying male harm and its divergence. *Evolution*, *76*(4), 829–836. https://doi.org/10.1111/evo.14471

Colpitts, J., Williscroft, D., Sekhon, H. S., & Rundle, H. D. (2017). The purging of deleterious mutations in simple and complex mating environments. *Biol. Lett.*, *13*(10), 20170518. https://doi.org/10.1098/rsbl.2017.0518

Coss, D. A., Hunter, K. L., & Taylor, R. C. (2021). Silence is sexy: Soundscape complexity alters mate choice in túngara frogs. *Behav. Ecol.*, *32*(1), 49–59. https://doi.org/10.1093/beheco/araa091

Darwin, C. (1859). *On the origin of species by means of natural selection, or, The preservation of favoured races in the struggle for life*. John Murray, Albemarle Street. https://doi.org/10.5962/bhl.title.82303

Emlen, S. T., & Oring, L. W. (1977). Ecology, Sexual Selection, and the Evolution of Mating Systems. *Science*, *197*(4300), 215–223. https://doi.org/10.1126/science.327542

Fisher, R. A. (1919). XV.—The Correlation between Relatives on the Supposition of Mendelian Inheritance. *Earth Environ. Sci. Trans. R. Soc. Edinb.*, *52*(2), 399–433. https://doi.org/10.1017/S0080456800012163

Hoffmann, A. A., & Cacoyianni, Z. (1990). Territoriality in *Drosophila melanogaster* as a conditional strategy. *Anim. Behav.*, *40*(3), 526–537. https://doi.org/10.1016/S0003-3472(05)80533-0

Lambert, T. J. (2019). FPbase: A community-editable fluorescent protein database. *Nat. Methods*, *16*(4), 277–278. https://doi.org/10.1038/s41592-019-0352-8

Mackay, T. F. C., Richards, S., Stone, E. A., Barbadilla, A., Ayroles, J. F., Zhu, D., Casillas, S., Han, Y., Magwire, M. M., Cridland, J. M., Richardson, M. F., Anholt, R. R. H., Barrón, M., Bess, C., Blankenburg, K. P., Carbone, M. A., Castellano, D., Chaboub, L., Duncan, L., … Gibbs, R. A. (2012). The *Drosophila melanogaster* Genetic Reference Panel. *Nature*, *482*(7384), 173–178. https://doi.org/10.1038/nature10811

MacLellan, K., Whitlock, M. C., & Rundle, H. D. (2009). Sexual selection against deleterious mutations via variable male search success. *Biol. Lett.*, *5*(6), 795–797. https://doi.org/10.1098/rsbl.2009.0475

MacPherson, A., Yun, L., Barrera, T. S., Agrawal, A. F., & Rundle, H. D. (2018). The effects of male harm vary with female quality and environmental complexity in *Drosophila melanogaster*. *Biol. Lett.*, *14*(8), 20180443. https://doi.org/10.1098/rsbl.2018.0443

Malek, H. L., & Long, T. A. F. (2019). Spatial environmental complexity mediates sexual conflict and sexual selection in *Drosophila melanogaster*. *Ecol. Evol.*, *9*(5), 2651–2663. https://doi.org/10.1002/ece3.4932

Marques, P., Zandonà, E., Amaral, J., Selhorst, Y., El-Sabaawi, R., Mazzoni, R., Castro, L., & Pilastro, A. (2022). Using fish to understand how cities affect sexual selection before and after mating. *Front. Ecol. Evol.*, *10*. https://doi.org/10.3389/fevo.2022.928277

Martin, G., & Lenormand, T. (2006). The fitness effect of mutations across environments: a survey in light of fitness landscape models. *Evolution*, *60*(12), 2413–2427. https://doi.org/10.1111/j.0014-3820.2006.tb01878.x

Osijo, K., Agrawal, A. F., & Rundle, H. D. (2026). On the expression of male harm in *Drosophila melanogaster*: Impacts of density and structural complexity of the mating environment. *Evolution*, qpaf245. https://doi.org/10.1093/evolut/qpaf245

R Core Team. (2024). *R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing. https://www.R-project.org/* (Version 4.3.3) [Computer software]. https://www.R-project.org/

Rowe, L., & Houle, D. (1996). The lek paradox and the capture of genetic variance by condition dependent traits. *Proc. R. Soc. B*, *263*(1375), 1415–1421. https://doi.org/10.1098/rspb.1996.0207

Rowe, L., & Rundle, H. D. (2021). The Alignment of Natural and Sexual Selection. *Annu. Rev. Ecol. Syst.*, *52*(Volume 52, 2021), 499–517. https://doi.org/10.1146/annurev-ecolsys-012021-033324

Sharp, N. P., & Agrawal, A. F. (2008). Mating density and the strength of sexual selection against deleterious alleles in *Drosophila melanogaster*. *Evolution*, *62*(4), 857–867. https://doi.org/10.1111/j.1558-5646.2008.00333.x

Sharp, N. P., & Agrawal, A. F. (2009). Sexual Selection and the Random Union of Gametes: Testing for a Correlation in Fitness between Mates in *Drosophila melanogaster*. *Am. Nat.*, *174*(5), 613–622. https://doi.org/10.1086/605960

Sharp, N. P., & Whitlock, M. C. (2019). No evidence of positive assortative mating for genetic quality in fruit flies. *Proc. R. Soc. B*, *286*(1912), 20191474. https://doi.org/10.1098/rspb.2019.1474

Singh, A., & Agrawal, A. F. (2022). Sex-Specific Variance in Fitness and the Efficacy of Selection. *Am. Nat.*, *199*(5), 587–602. https://doi.org/10.1086/719015

Singh, A., Agrawal, A. F., & Rundle, H. D. (2017). Environmental complexity and the purging of deleterious alleles. *Evolution*, *71*(11), 2714–2720. https://doi.org/10.1111/evo.13334

Talagala, S., Rakosy, E., & Long, T. A. F. (2024). Sexual selection and the nonrandom union of gametes: Retesting for assortative mating by fitness in *Drosophila melanogaster*. *Evolution*, *78*(1), 26–38. https://doi.org/10.1093/evolut/qpad191

White, A. J., & Rundle, H. D. (2015). Territory defense as a condition-dependent component of male reproductive success in *Drosophila serrata*. *Evolution*, *69*(2), 407–418. https://doi.org/10.1111/evo.12580

Whitlock, M. C., & Agrawal, A. F. (2009). Purging the genome with sexual selection: reducing mutation load through selection on males. *Evolution*, *63*(3), 569–582. https://doi.org/10.1111/j.1558-5646.2008.00558.x

Wilhite, K. O., & Ryan, M. J. (2024). Condition dependence in the sexual communication system of the túngara frog. *Behav. Ecol. Sociobiol.*, *78*(5), 54. https://doi.org/10.1007/s00265-024-03470-7

Yeaman, S., Chen, Y., & Whitlock, M. C. (2010). No effect of environmental heterogeneity on the maintenance of genetic variation in wing shape in *Drosophila melanogaster*. *Evolution*, *64*(12), 3398–3408. https://doi.org/10.1111/j.1558-5646.2010.01075.x

Yun, L., Agrawal, A. F., & Rundle, H. D. (2021). On Male Harm: How It Is Measured and How It Evolves in Different Environments. *Am. Nat.*, *198*(2), 219–231. https://doi.org/10.1086/715038

Yun, L., Bayoumi, M., Yang, S., Chen, P. J., Rundle, H. D., & Agrawal, A. F. (2019). Testing for local adaptation in adult male and female fitness among populations evolved under different mate competition regimes. *Evolution*, *73*(8), 1604–1616. https://doi.org/10.1111/evo.13787

Yun, L., Chen, P. J., Kwok, K. E., Angell, C. S., Rundle, H. D., & Agrawal, A. F. (2018). Competition for mates and the improvement of nonsexual fitness. *Proc. Natl. Acad. Sci.*, *115*(26), 6762–6767. https://doi.org/10.1073/pnas.1805435115

Yun, L., Chen, P. J., Singh, A., Agrawal, A. F., & Rundle, H. D. (2017). The physical environment mediates male harm and its effect on selection in females. *Proc. R. Soc. B*, *284*(1858), 20170424. https://doi.org/10.1098/rspb.2017.0424

Chapter 2 Supplementary Material

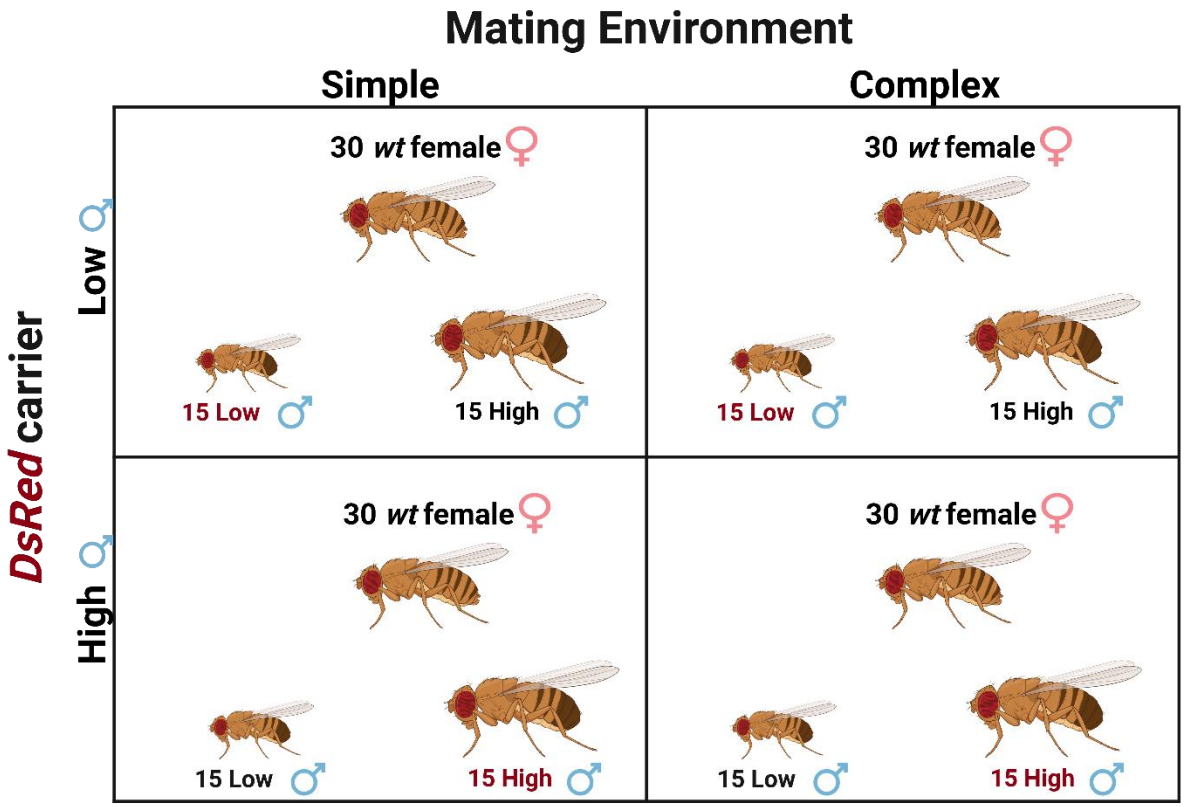

Fig. S2.1. Visual illustration of the four treatment combinations tested in a given competition set, in Experiment 2. Low- and high-condition males from appropriate families were paired to directly compete to sire 30 *wt* virgin females in either a simple or a complex mating environment. *DsRed* markers were present in either the low- or the high-condition male to account for potential marker effect. In a given set, male count always matched and female count was equal to total number of males in a given container (expect for block 1, n = 9 out 27 sets).

Chapter 3: On the expression of male harm in *Drosophila melanogaster*: impacts of density and structural complexity of the mating environment

Abstract

Male harm occurs when traits in males that increase their reproductive success incidentally reduce female fitness. In *Drosophila melanogaster*, many lab studies have revealed the presence of male harm, but recent work has shown that its expression can be dramatically reduced, even eliminated, when sexual interactions and mating occur in an environment that differs from traditional lab rearing vials. Here we follow up on this to separately test the effect of fly density and structural complexity of the mating environment in mediating the expression of male harm. We performed separate two-way factorial assays that measured the fitness of females while manipulating their exposure to males and the density of flies or the structural complexity of the environment during exposure. Male harm, quantified as the relative reduction in female fitness under increased male exposure, was not affected by density, but was significantly reduced – essentially eliminated– by increased structural complexity. Our results demonstrate that seemingly simple choices, like the environment used in a laboratory model system, can have profound impacts on the expression of harm and hence views on the prevalence of sexual conflict. This is noteworthy because conflict can shape other fundamental evolutionary processes including adaptation, purging, and speciation.

Note: Chapter 3 is a very slightly modified version of the following published article:

> Osijo, K., Agrawal, A. F., & Rundle, H. D. (2026). On the expression of male harm in *Drosophila melanogaster*: impacts of density and structural complexity of the mating environment. *Evolution*, *80*(2), 496-503.

I have retained first-person plural pronouns for simplicity. All three authors contributed to the design of the experiment; I collected all the data and took the lead role in analyses and manuscript preparation, with input from my co-authors.

Introduction

Male harm arises when traits that have evolved to increase a male's success when competing for mates incidentally reduce female fitness (Parker, 1979, 2006; Rice & Holland, 1997; Morrow et al., 2003; Arnqvist & Rowe, 2005; Lessells, 2006). Male harm is common across species and can result from both pre- and postcopulatory sexual interactions (Arnqvist & Rowe, 2005). The existence of male harm generates selection in females for traits that minimize this harm, even if this reduces male fitness as a side effect (Rice, 1996). This evolutionary conflict of interest between males and females, a phenomenon termed sexual conflict, can drive bouts of sexually antagonistic selection that can play an important role in the coevolution of the sexes and how they interact (Arnqvist & Rowe, 2002; Ortigosa & Rowe, 2002; Mueller et al., 2007). It has even been suggested that this may foster population divergence and, in some situations, drive the evolution of reproductive isolation (Parker & Partridge, 1998; Arnqvist et al., 2000; Gavrilets, 2000; Martin & Hosken, 2003; but see Barerra et al., 2024). Due to the importance of male harm

in generating sexual conflict, and the resulting evolutionary consequences this conflict can have, we are interested in developing a comprehensive understanding of factors that can affect the expression of harm.

In sexual species, males are necessary for females to reproduce. Harm can reasonably be considered to exist when female fitness declines with increased exposure to males beyond the minimum required to fertilize her eggs. The existence of male harm can thus be demonstrated by comparing female fitness under varying levels of male exposure (Partridge & Fowler, 1990; Yun et al., 2021; Colpitts et al., 2022). Following Yun et al. (2021), male harm (*H*) can be quantified as the proportional reduction in the average fitness of females under normal (e.g., high) exposure to males compared with what it is under minimal (e.g., low) exposure:

$$H = \frac{\bar{W}_{low\ exposure} - \bar{W}_{high\ exposure}}{\bar{W}_{low\ exposure}}$$ Eqn. 3.1. Quantification of male harm.

H is a dimensionless quantity, allowing the magnitude of male harm to be compared between situations (e.g., environments) in which the absolute fitness of females may vary.

Drosophila melanogaster has been an influential model system in the study of male harm. Pivotal work first revealed costs of exposure to, and mating with, males that could not be explained via the physiological costs of reproduction itself (Fowler & Partridge, 1989; Partridge & Fowler, 1990).

Chapman et al. (1995) subsequently showed that the seminal fluid of males includes compounds that manipulate female oviposition rates and their willingness to remate, but that also increase female mortality. A few years later, Holland and Rice (1999) demonstrated that, by enforcing monogamy and hence aligning the reproductive interests of the sexes, there was an evolutionary decrease in male harm and female resistance compared to populations maintained under polygamy (where conflict can occur). Notably, these changes under monogamy benefitted females, which had higher reproductive output when exposed to, and mating with, males evolved under monogamy compared to polygamy. Building on these seminal studies, there has been much research on the evolution, expression, and consequences of male harm in *D. melanogaster* (e.g., Pitnick & García-González, 2002; Wigby & Chapman, 2004; Rice et al., 2006; Long et al., 2009; Arbuthnott et al., 2012; Maklakov et al., 2013; Filice & Long, 2016; Sepil et al., 2016; Londoño-Nieto et al., 2023).

The abundance of evidence for male harm, most notably in *D. melanogaster*, has sometimes been interpreted as suggesting that harm is ubiquitous. However, as Holland and Rice (1999) demonstrated, its evolution will depend on the extent of mate competition, as harm is the incidental outcome of selection for traits that increase a male's reproductive competitiveness; taxa in which mate competition is weak are thus likely to have little harm. However, even in non-monogamous species, the expression of harm can vary with other factors. For example, if females have greater control over sexual interactions, the opportunity for male harm may be reduced (Zuk et al., 2014). This may be the case in species in which sexual interactions and mating occur in

habitats that are distinct from other activities, like feeding (e.g., Wiklund et al., 1993; García-González & Simmons, 2005; Edvardsson, 2007).

Variation in the environment in which sexual interactions and mating occur may affect the control females have, and hence the expression of male harm. Byrne et al. (2008) demonstrated that the addition of a refuge, accessible only to *D. melanogaster* females, significantly reduced mating rates compared to standard lab conditions, although this did not alter male harm in their study. On the other hand, Yun et al. (2017, 2019) showed that, in a lab population of *D. melanogaster*, sexual interactions and mating were less frequent in a mating environment that was larger, lower density, and structurally more complex than standard *Drosophila* culture vials. Consequently, males that were otherwise harmful to females in the standard (i.e. simple) environment were much less so in the lower density, more complex one (Yun et al., 2017, 2021). And when replicate populations were maintained across many generations in these two mating environments, males from the lower density, more complex environment evolved to be much less harmful to females compared to males from the higher density, simple environment (Yun et al., 2021).

The feature(s) of these environments that altered the expression of harm are not known. The so-called “simple” vs. “complex” environments differed in volume (and thereby fly density), as well as structural complexity. Effects of density on mating behaviors have been documented in other taxa. For example, Vercken et al. (2007) demonstrated that population density affected the attention males paid to females of different color patterns in lizards, Gosden and Svensson (2009) showed that mating attempts were more frequent at high densities in damsel flies, and Winkler

et al. (2023) demonstrated that increasing group size reduced the number of copulation attempts, and copulations per partner, in both male and female red flour beetles. But Yun et al.'s (2017, 2021) mating environments also differed in structural complexity, with multiple separate food patches, allowing females a choice of where to feed and lay eggs. There was also added physical structure in the form of pipe cleaners, potentially providing more places for females to hide or to perch while avoiding the attention of males that tend to congregate around the food (males can exhibit a 'territory defense' mating system in this species; Hoffmann, 1987). This contrasts with the standard, and highly simplified, lab environments for *Drosophila* that usually consist of a small vial or bottle with food media at the bottom, a stopper at the mouth, and nothing else.

Here, we test the effects of fly density and the structural complexity of the mating environment on the expression of male harm in separate experiments. This is of value for interpreting past results showing that simple vs. complex environments affected mating rates and the expression (and evolution) of male harm (Yun et al., 2017, 2019, 2021). It is also of interest more broadly because studies have also shown that these environments altered the consequences of mate competition for fundamental evolutionary processes. For example, adaptation and purging of deleterious alleles were improved in the complex compared to the simple environment (Colpitts et al., 2017; Singh et al., 2017; Yun et al., 2018), and this was due, at least in part, to how the environments mediated the opportunity for male harm and the selection it generated (Yun et al., 2017; MacPherson et al., 2018). The complex environment also permitted the evolution of reproductive isolation in response to ecologically divergent selection, while the simple environment appeared to constrain this (Barerra et al., 2024). Understanding whether and how

key aspects of *Drosophila* lab maintenance protocols alter harm is therefore relevant to the use of this species as a model system for the study of diverse evolutionary processes.

Materials and Methods

Our experiments used an outbred, lab-adapted population of *D. melanogaster* that was created by combining 15 populations from the long-term evolution experiment described in Yun et al. (2018, 2021; see Supplementary materials). In brief, these 15 populations had been evolving via a life cycle that each generation involved a 6 d "adult interaction period", in standard culture vials, in which males and females interacted and mated, after which males were discarded and females laid eggs in fresh vials for ~24h. Previous work indicated that males from these populations could cause substantial harm to their females (Yun et al., 2021). After pooling, the new population was maintained for 27+ generations on cornmeal-yeast food under the same life cycle before being used in the current experiments.

Previous experiments on the evolution and expression of male harm compared, as mating environments during the adult interaction period, standard *Drosophila* culture vials vs. larger, 1.65 L cylindrical plastic Ziploc® containers that had multiple food patches and added structural complexity via two coiled pipe cleaners inside (Yun et al., 2017, 2021). Here we wanted to quantify the separate effects of density and structural complexity of the mating environment on the expression of harm while minimizing confounds, including the volume of the mating environment. This necessitated having the same container in all treatments. To facilitate this, we used intermediate sized 473 mL cylindrical polypropylene 'deli' containers (16 oz 'Heavy Duty Deli

Cups', Uline Canada). For ventilation, and to facilitate the addition/removal of flies, each container had two ~1.5 cm circular holes cut opposite one another in the side wall and two in the lid, each of which was plugged with a foam stopper (Fig. S3.1). Approximately 200 mL of cornmeal-yeast food was added to each container, the surface of which was generously sprinkled with live yeast pellets. The interaction period in these containers was shortened to four days, minimizing the handling of the flies because they no longer needed to be transferred to fresh containers half-way through (as required in past studies with 6-day interaction periods to deal with liquification of the food by developing larvae).

Density experiment

We tested the effect of fly density on male-induced harm to females by comparing the fitness of females in a two-way factorial experiment that manipulated their exposure to males (intermittent vs. continuous) and the number of flies (low vs. high density) during the four-day interaction period (Fig. S3.2). In the low density treatments, we used 15 males and 15 females per replicate container, while in the high density treatments we used 60 males and 60 females per replicate. The experiment was performed in four blocks, with each block consisting of 18 replicates of each of the male exposure treatments that experienced low fly densities, and 12 replicates of each of the male exposure treatments that experienced high fly densities.

Male exposure was manipulated following Yun et al. (2021). In the intermittent exposure treatment, females were held together with males for two 4 h periods, one at the start of the four-day interaction period and the second at its end, just prior to transferring females to fresh

vials for egg laying. These male exposure periods involved 15 males and 15 females together in a vial, yielding one/ four vial(s) for a given low/high density replicate, respectively. During the intervening time, males were removed and stored separately (in groups of ~15), while females were held together in a single deli container. In the continuous male exposure treatment, females experienced the same 4 h exposure to males in vials (15 males and 15 females per vial) both at the start and the end of the interaction period, but males were also present between these periods when the flies were held in a deli container (i.e. 15/60 males together with 15/60 females for a given low/high density replicate, respectively). For both intermittent and continuous male exposure treatments, the deli containers mirrored in design those used in simple treatment of the complexity experiment below (i.e., no added structural complexity). Males that died during the interaction phase were replaced from a pool of extra males any time a transfer occurred (i.e., into or out of the 4 h exposure vials); females that died were not replaced. After the second male exposure at the end of the interaction period, males were discarded and groups of 3-5 females were transferred to fresh vials for egg laying for 15-20 h, after which they were discarded. Adult offspring were counted 10-11 days later following eclosion, with a second count 2-3 days after that to ensure none had been missed.

Complexity experiment

We tested the effect of structural complexity of the mating environment on male-induced harm to females by comparing the fitness of females in two-way factorial experiment that manipulated their exposure to males (intermittent vs. continuous) and the environment in which this occurred (simple vs. complex) during the four-day interaction period (Fig. S3.3). The experiment was

performed in three blocks in which each block included 15 replicates each of the four treatment combinations. Each replicate involved 15 females and 15 males, and male exposure was manipulated as in the density experiment above, with intermittent exposure involving two 4 h exposures in vials at the start and end of the interaction phase, with males absent or present (intermittent vs. continuous male exposure, respectively) during the intervening time when the females were held in the same deli containers as in the density experiment. 'Simple' replicates had only food (with abundant live yeast sprinkled on top) within the deli container, while the 'complex' replicates also had a thin, white, polypropylene barrier consisting of six radial arms inserted into the food, and a pipe cleaner that protruded from the lids into the interior of the container (Fig. S3.1). After the second male exposure, males were discarded and groups of 3-5 females were transferred to fresh vials for egg laying for 15-20 h, after which they were discarded. Eclosed adult offspring were subsequently counted as in the density experiment.

Statistical analyses

For a given replicate, female fitness was estimated as the product of the average survival of females (i.e. proportion alive after the 4-day interaction period) and the average number of offspring produced per surviving female. Variation in female fitness was analyzed, separately for the density and complexity experiments, using a general linear model fit via least squares in *R* (v4.4.2; R Core Team 2024). Male exposure (intermittent vs. continuous), experimental treatment (low vs. high density or simple vs. complex mating environment), their interaction, and block were included as categorical fixed effects. Type III (i.e. partial) tests were performed via the *car* package

(Fox & Weisberg, 2019). Contrasts for categorical variables were specified as sum-to-zero via the *contr.sum* statement.

Relative male harm (*H*) was estimated separately for the low and high density treatments, and for the simple and complex treatments, as the proportional reduction in female fitness under continuous compared to intermittent exposure to males (Eqn. 3.1). In doing so, $\bar{W}_{intermittent\ exposure}$ and $\bar{W}_{continuous\ exposure}$ were the average fitnesses of females from all replicates of that exposure level in a given density or complexity treatment level. 95% confidence intervals (CI's) for *H* were estimated via 1,000 bootstrap replicates, sampling with replacement within each combination of experimental treatment, male exposure, and block.

Results

Density experiment

Increased exposure to males significantly reduced average female fitness (main effect of male exposure, Table S3.1), indicating the presence of male harm (Fig. 3.1A). Average female fitness was also significantly higher in the low- compared to the high-density mating environment (main effect of density), but there was no significant interaction between density and male exposure. Examining the components of female fitness separately reveals that male harm arose through impacts on the fecundity, but not the survival, of females, whereas density impacted both female fecundity and survival (Fig. S3.5).

Relative male harm, *H* (the proportional reduction in female fitness caused by increased exposure to males), was 0.108 (95% CI: 0.028, 0.181) in the high-density mating environment and 0.141 (0.095, 0.185) in the low, both of which had 95% CI's that excluded zero (Fig. 3.1C), indicating the presence of male harm. The resulting difference in *H* ($H_{\text{high density}}$ - $H_{\text{low density}}$ = -0.033) had a 95% CI that overlapped zero (-0.118, 0.05), providing no evidence of a significant effect of density in altering male harm.

Complexity experiment

Increased exposure to males significantly reduced average female fitness (main effect of male exposure, Table S3.2), again indicating the presence of male harm (Fig. 3.1B). Average female fitness was also significantly higher in the complex compared to the simple mating environment (main effect of environmental complexity; Table S3.2). However, there was a significant interaction between male exposure and complexity (Table S3.2) such that increased exposure to males reduced female fitness more in the simple compared to the complex mating environment (Fig. 3.1B). Examining the component of female fitness separately revealed that all of the above arose from impacts on female fecundity and not survival (Fig. S3.7).

Relative male harm, *H*, was 0.170 (0.135, 0.207) in the simple mating environment, with a 95% CI that did not overlap zero, compared to 0.013 (-0.027, 0.054) in the complex, with a 95% CI that overlapped zero (Fig. 3.1D). The resulting difference in *H* (H_{simple} - H_{complex} = 0.156) had a 95% CI

that excluded zero (0.107, 0.212), indicating significantly less male harm in the structurally complex compared to simple mating environment.

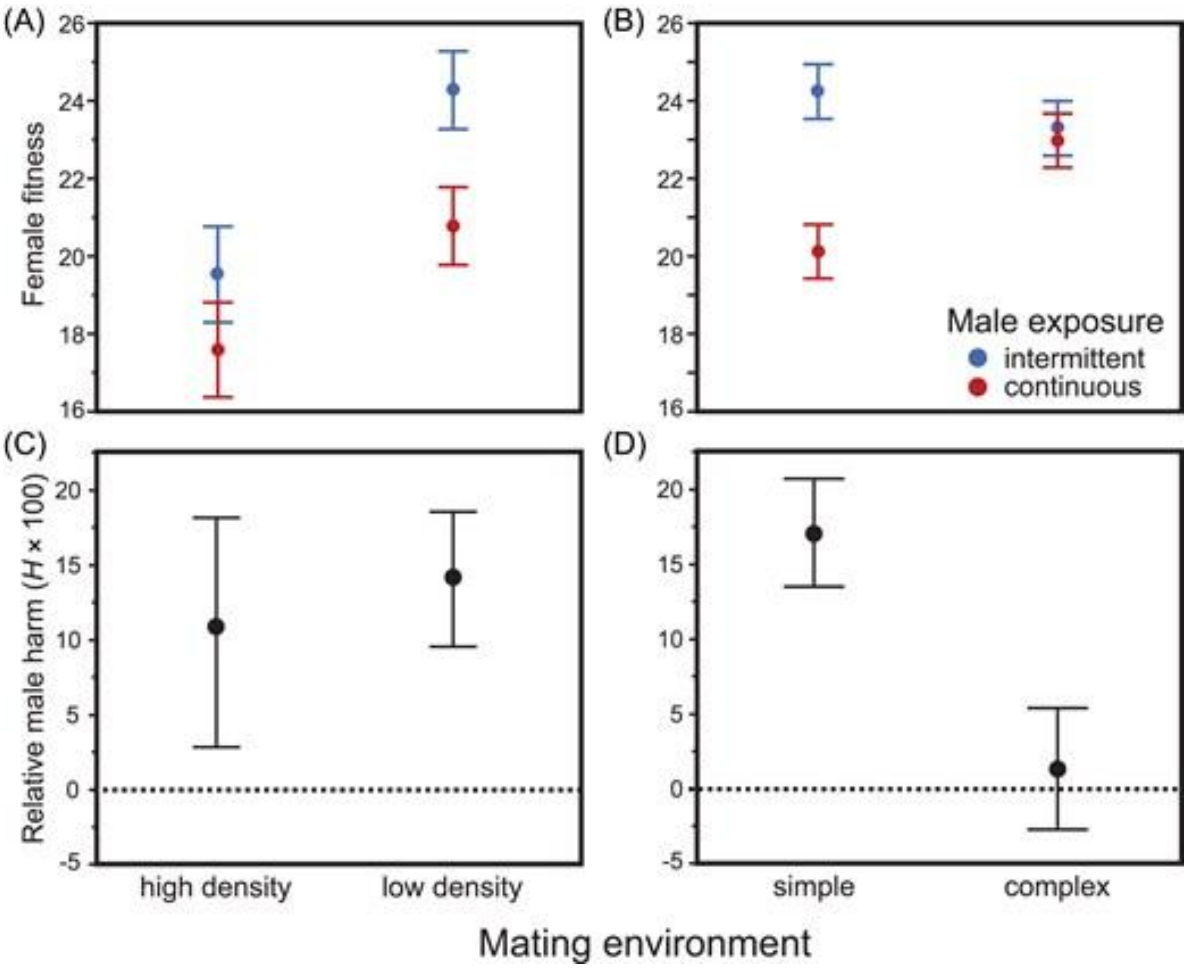

Fig. 3.1. Fitness of females under intermittent vs. continuous exposure to males in a mating environment that was either A) high vs. low density, or B) structurally simple vs. complex (i.e., low vs. high complexity, respectively); values are least square means (± 1 SE) from the linear models in Tables S3.1 and S3.2, respectively. Variation in relative male harm (*H*; see Eqn. 3.1) experienced by females in mating environments that were either C) high vs. low density, or D) structurally simple vs. complex, expressed as a percentage with 95% bootstrap confidence intervals.

Discussion

That *D. melanogaster* males can be harmful to females is well established, but less attention has been given to factors impacting the expression of such harm. A few studies have shown reduced mating frequencies (Byrne et al., 2008; Malek & Long, 2019; Yun et al., 2019) and enhanced female fitness (Yun et al., 2021; Talagala et al., 2024) when mating interactions occur at lower density and/or in structurally more complex environments. Yun et al. (2017, 2021) provided direct estimates of changes in the magnitude of male harm, showing that it was reduced in a mating environment that was both lower density and more structurally complex compared to standard lab conditions. Here we independently tested the impact of density and structural complexity on the expression of male harm. This is of interest in interpreting past results, but also because evidence indicates that changes in the expression of harm can alter broader evolutionary and demographic processes (e.g., Colpitts et al., 2017; Singh et al., 2017; Yun et al., 2018; Flintham et al., 2023; Gómez-Llano et al., 2023; Barerra et al., 2024; Berger & Liljestrand-Rönn, 2024). *D. melanogaster*'s value as a model system is enhanced by knowledge of how seemingly simple aspects of laboratory maintenance may impact fundamental evolution processes. Our results reveal that the expression of harm was quite sensitive to relatively minor changes in structural complexity, but not to a four-fold change in fly density.

Why might structural complexity, but not density, have impacted male harm? *Drosophila* females go to the surface of the larval medium (the 'food') to feed on live yeast and to lay eggs. In a structurally simple environment, males may easily be able to locate females simply by staying at these locations (i.e. on or near the surface of the food). Over the relatively small surface area of

standard rearing vials or bottles, or even our somewhat larger deli containers, reduced density may be of little help; females arriving at the food may be rapidly detected by one or more males. But when the surface of the food is divided into separate patches, there is likely to be variation in local male density (i.e. patches with more or fewer flies). This, together with sufficient space and added structural complexity to give places for females to escape or avoid unwanted sexual attention when not feeding or laying eggs, may allow females to reduce their exposure to harmful male sexual attention. Consistent with this, Yun et al. (2017) found that females fed more often in their complex compared to simple mating environment.

Male *Drosophila* are known to defend territories, presumably because doing so provides access to females that are attracted by the resources on those territories (i.e., food and egg-laying substrates; Hoffmann, 1987; White & Rundle, 2015). The social and/or physical environment during mating may also impact such male-male interactions. For instance, Hoffmann and Cacoyianni (1990) found that males were less likely to establish and defend a territory at higher densities. The effect of structural complexity on male territoriality has not been addressed, to the best of our knowledge, and we are not aware of any data on how changes in territoriality impact the expression of male harm. If territoriality reduces male harm (perhaps by decreasing the likelihood of multiple-male courtship), and territoriality is more common in structurally complex environments (and less common at high density), this could help explain our results. How mating environments alter male-male interactions and whether this impacts harm is a topic that would benefit from future investigation.

Although harm was not reduced at our lower density, there are likely densities sufficiently low for this to occur (e.g., if males are so rare that that females can feed and lay eggs with little harassment). Our choice of densities was motivated by Yun et al. (2018, 2021), with similar low-density treatment levels being used (0.023 vs. 0.050 males/mL available volume in Yun et al. vs. the current study). However, because we wanted to minimize confounding differences between density treatments, including the volume and design (e.g., shape, structure) of the container housing the flies during the 'adult interaction period', our high-density treatment was less than that in much smaller standard fly vials of past experiments (1.16 vs. 0.22 males/mL in Yun et al. vs. the current study). In theory, the absence of a density effect on male harm, as we observed, could therefore arise if both densities were too low for harm to occur. Our results are not consistent with this, however, because we detected significant harm even at our low density.

While there are likely densities low enough for harm to be diminished, this may be especially so in larger and/or more complex environments. In other words, density and complexity may interact. Our current results do not speak to this because we manipulated these factors in separate experiments. We did this, in part, because we were unsure whether we could achieve sufficiently high density for harm to be manifested while controlling for the container used (i.e. when using 437 mL 'deli' containers for both low- and high-density treatment levels, while Yun et al. (2018, 2021) detected harm only in standard fly vials of almost an order of magnitude smaller volume). We therefore first manipulated density to establish this, and then we used these results to help design the complexity test. Exploring potential density × complexity interactions would be an interesting avenue for future work.

In this study, the low density, low complexity environment could be viewed as a baseline context, which was present in both our experiments. Both experiments show clear evidence of male harm in this context, with remarkably similar point estimates (Fig. 3.1 C & D). A four-fold increase in density reduced female fitness (Fig. 3.1A) but it did not alter the relative effect of male harm (*H*; Fig. 3.1C). However, the significant harm observed at the low density was eliminated by the addition of structural complexity (Fig. 3.1D).

Our experiments focused on the lab context, both to shed light on past lab results and because *D. melanogaster* is a model lab system in evolution and genetics. Nevertheless, naturally occurring variation in local male density (or sex ratio) is common in many species (e.g., Lawrence, 1986; Rowe, 1992; Krupa & Sih, 1993; Gwynne et al., 1998; Croft et al., 2003), although the impact this has on male harassment (and presumably harm) may differ substantially depending on the unique biology the species in question (e.g., Rowe, 1992; Cordoba-Aguilar, 2009). It has also long been recognized that ecological factors like population density, competition, predation, and resource availability, can impact sexual conflict, for instance by changing costs and benefits of sexual interactions, via behavioral plasticity, and by altering sexual selection (Perry & Rowe, 2018). With respect to structural complexity in particular, differences in habitat complexity are known to alter mate-location behavior in a forest butterfly (Bonte & Van Dyck, 2009), and lab manipulations simulating naturally occurring habitat variation in the two-spotted goby showed that increased complexity decreased female encounter rates with males and resulted in fewer cases of multiple-male courtship (Myhre et al., 2013). Female water striders are also known to use structurally complex vegetated refuges to avoid male harassment in the wild (Rowe, Krupa,

and Sih, 1996). In *D. melanogaster*, natural habitats are likely more complex than standard lab environments, and the expression of harm in wild populations might thus differ substantially from that in lab environments. There is considerable spatio-temporal variation in the habitats this species encounters in nature, and this almost certainly generates variation in the realized level of harm females experience.

While we focused on the impacts of density and structural complexity, there are other ways in which the environment can alter the expression and/or evolution of male harm. For instance, male harmfulness has been shown to vary in response to the temperature of the mating environment (Londoño-Nieto et al., 2023). In a different vein, in *Drosophila* (Arbuthnott et al., 2014) and in seed beetles (Fricke et al., 2010), male harm has been shown to evolve in correlation with environment, suggesting that ecological selection can limit or otherwise bias the evolution of traits underlying it. Understanding the varied impacts of environment on harm is important because harm can have broad evolutionary and demographic consequences. For instance, how harm is allocated to different types of females (thereby affecting selection) has been shown to alter adaptation to novel environments and the purging of deleterious mutations (e.g., Arbuthnott & Rundle, 2012; Singh et al., 2017, Yun et al., 2018). By altering female productivity, harm can also decrease population size and increase the risk of extinction (e.g., Flintham et al., 2023; Gómez-Llano et al., 2023; Berger & Liljestrand-Rönn, 2024). Continued exploration of how diverse environmental factors shape male harm will be essential for building a more comprehensive understanding of its evolutionary and ecological consequences.

References

Arbuthnott, D., & Rundle, H. D. (2012). Sexual selection is ineffectual or inhibits the purging of deleterious mutations in *Drosophila melanogaster*. *Evol.*, *66*(7), 2127-2137.

Arbuthnott, D., Dutton, E. M., Agrawal, A. F., & Rundle, H. D. (2014). The ecology of sexual conflict: ecologically dependent parallel evolution of male harm and female resistance in *Drosophila melanogaster*. *Ecol. Lett.*, *17*(2), 221-228.

Arnqvist, G., & Rowe, L. (2002). Antagonistic coevolution between the sexes in a group of insects. *Nature*, *415*(6873), 787-789.

Arnqvist, G., & Rowe, L. (2005). *Sexual conflict*. Princeton University Press.

Arnqvist, G., Edvardsson, M., Friberg, U., & Nilsson, T. (2000). Sexual conflict promotes speciation in insects. *Proc. Natl. Acad. Sci. U.S.A*, *97*(19), 10460-10464.

Barerra, T. S., Sattolo, M. L., Kwok, K. E., Agrawal, A. F., & Rundle, H. D. (2024). Mating environments mediate the evolution of behavioral isolation during ecological speciation. *Evol Lett*, *8*(3), 448-454.

Berger, D. & Liljestrand-Rönn, J. (2024). Environmental complexity mitigates the demographic impact of sexual selection. *Ecol. Lett., 27*, e14355.

Bonte, D., & Van Dyck, H. (2009). Mate-locating behaviour, habitat-use, and flight morphology relative to rainforest disturbance in an Afrotropical butterfly. *Biol J Linn Soc, 96(4)*, 830–839.

Byrne, P. G., Rice, G. R., & Rice, W. R. (2008). Effect of a refuge from persistent male courtship in the *Drosophila* laboratory environment. *Am Zool*, *48*(2), e1-e1.

Chapman, T., Liddle, L. F., Kalb, J. M., Wolfner, M. F., & Partridge, L. (1995). Cost of mating in *Drosophila melanogaster* females is mediated by male accessory gland products. *Nature*, *373* (6511), 241-244.

Colpitts, J., Jarvis, W. M., Agrawal, A. F., & Rundle, H. D. (2022). Quantifying male harm and its divergence. *Evol.*, *76*(4), 829-836.

Colpitts, J., Williscroft, D., Sekhon, H. S., & Rundle, H. D. (2017). The purging of deleterious mutations in simple and complex mating environments. *Biol Lett*, *13*(10), 20170518.

Cordoba-Aguilar, A. (2009). A female evolutionary response when survival is at risk: male harassment mediates early reallocation of resources to increase egg number and size. *Behav. Ecol. Sociobiol*., 63, 751-763.

Croft, D. P., Albanese, B., Arrowsmith, B. J., Botham, M., Webster, M., & Krause, J. (2003). Sex-biased movement in the guppy (*Poecilia reticulata*). *Oecologia*, *137*, 62-68.

Edvardsson, M. (2007). Female Callosobruchus maculatus mate when they are thirsty: resource-rich ejaculates as mating effort in a beetle. *Anim. Behav.*, *74*(2), 183-188.

Filice, D. C., & Long, T. A. (2016). Genetic variation in male-induced harm in *Drosophila melanogaster*. *Biol Lett*, *12*(4), 20160105.

Flintham, E. O., Savolainen, V., & Mullon, C. (2023). Male harm offsets the demographic benefits of good genes. *Proc. Natl. Acad. Sci. U.S.A*, 120 (10), e2211668120.

Fowler, K., & Partridge, L. (1989). A cost of mating in female fruitflies. *Nature*, *338*(6218), 760-761.

Fricke, C., Andersson, C. & Arnqvist, G. (2010). Natural selection hampers divergence of reproductive traits in a seed beetle. *J. Evol. Biol.*, 23, 1857–1867.

García-González, F., & Simmons, L. W. (2005). Sperm viability matters in insect sperm competition. *Curr. Biol.*, *15*(3), 271-275.

Gavrilets, S. (2000). Rapid evolution of reproductive barriers driven by sexual conflict. *Nature*, *403*(6772), 886-889.

Gómez-Llano, M., Faria, G.S., García-Roa, R., Noble, D. W. A., & Carazo, P. (2024). Male harm suppresses female fitness, affecting the dynamics of adaptation and evolutionary rescue, *Evol Lett*, 8(1), 149–160.

Gosden, T. P., & Svensson, E. I. (2009). Density-dependent male mating harassment, female resistance, and male mimicry. *Am. Nat.*, *173*(6), 709-721.

Gwynne, D. T., Bailey, W. J., & Annells, A. (1998). The sex in short supply for matings varies over small spatial scales in a katydid (*Kawanaphilanartee*, Orthoptera: Tettigoniidae). *Behav. Ecol. Sociobiol*., *42*, 157-162.

Hoffmann, A. A. 1987. A laboratory study of male territoriality in the sibling species *Drosophila melanogaster* and *Drosophila simulans*. *Anim. Behav.* 35:807–818.

Hoffmann, A. A., & Cacoyianni, Z. (1990). Territoriality in *Drosophila melanogaster* as a conditional strategy. *Anim. Behav.*, *40*(3), 526-537.

Holland, B., & Rice, W. R. (1999). Experimental removal of sexual selection reverses intersexual antagonistic coevolution and removes a reproductive load. *Proc. Natl. Acad. Sci. U.S.A*, *96*(9), 5083-5088.

Krupa, J. J., & Sih, A. (1993). Experimental studies on water strider mating dynamics: spatial variation in density and sex ratio. *Behav. Ecol. Sociobiol*., *33*, 107-120.

Lawrence, W. S. (1986). Male choice and competition in *Tetraopes tetraophthalmus*: effects of local sex ratio variation. *Behav. Ecol. Sociobiol*., *18*, 289-296.

Lessells, C. K. M. (2006). The evolutionary outcome of sexual conflict. *Philos. Trans. R. Soc. B, Biol. Sci.*, 361(1466), 301-317.

Londoño-Nieto, C., García-Roa, R., González, P., & Carazo, P. (2023). Thermal phenotypic plasticity of pre-and post-copulatory male harm buffers sexual conflict in wild *Drosophila melanogaster*. *ELife*, *12*, e84759.

Long, T. A., Pischedda, A., Stewart, A. D., & Rice, W. R. (2009). A cost of sexual attractiveness to high-fitness females. *PLoS Biol.*, *7*(12), e1000254.

MacPherson, A., Yun, L., Barrera, T. S., Agrawal, A. F., & Rundle, H. D. (2018). The effects of male harm vary with female quality and environmental complexity in *Drosophila melanogaster*. *Biol Lett*, *14*(8), 20180443.

Maklakov, A. A., Immler, S., Løvlie, H., Flis, I., & Friberg, U. (2013). The effect of sexual harassment on lethal mutation rate in female *Drosophila melanogaster*. *Proc Biol Sci*, *280*(1750), 20121874.

Malek, H. L., & Long, T. A. (2019). Spatial environmental complexity mediates sexual conflict and sexual selection in *Drosophila melanogaster*. *Ecol Evol*, *9*(5), 2651-2663.

Martin, O. Y., & Hosken, D. J. (2003). The evolution of reproductive isolation through sexual conflict. *Nature*, *423*(6943), 979-982.

Morrow, E. H., Arnqvist, G., & Pitnick, S. (2003). Adaptation versus pleiotropy: why do males harm their mates? *Behav. Ecol.*, *14*(6), 802-806.

Mueller, J. L., Page, J. L., & Wolfner, M. F. (2007). An ectopic expression screen reveals the protective and toxic effects of *Drosophila* seminal fluid proteins. *Genetics*, *175*(2), 777-783.

Myhre, L. C., Forsgren, E., & Admundsen, T. (2013). Effects of habitat complexity on mating behavior and mating success in a marine fish. *Behav. Ecol., 24*(2), 553–563.

Ortigosa, A., & Rowe, L. (2002). The effect of hunger on mating behaviour and sexual selection for male body size in *Gerris buenoi*. *Anim. Behav.*, *64*(3), 369-375.

Parker, G. A. (1979). Sexual selection and sexual conflict. In *Sexual selection and reproductive competition in insects*. Blum, M. S., & Blum, N. A. (Eds.) (pp. 123-166). Academic Press (N. Y.).

Parker, G. A. (2006). Sexual conflict over mating and fertilization: an overview. *Philos. Trans. R. Soc. B, Biol. Sci.*, *361*(1466), 235-259.

Parker, G. A., & Partridge, L. (1998). Sexual conflict and speciation. *Philos. Trans. R. Soc. B, Biol. Sci.*, *353*(1366), 261-274.

Partridge, L., & Fowler, K. (1990). Non-mating costs of exposure to males in female *Drosophila melanogaster*. *J Insect Physiol*, *36*(6), 419-425.

Perry, J. C., & Rowe, L. (2018). Sexual conflict in its ecological setting. *Philos. Trans. R. Soc. B, Biol. Sci.*, *373*(1757), 20170418.

Pitnick, S., & García–González, F. (2002). Harm to females increases with male body size in *Drosophila melanogaster*. *Proc Biol Sci*, *269*(1502), 1821-1828.

Rice, W. R. (1996). Sexually antagonistic male adaptation triggered by experimental arrest of female evolution. *Nature*, *381*(6579), 232-234.

Rice, W. R., & Holland, B. (1997). The enemies within: intergenomic conflict, interlocus contest evolution (ICE), and the intraspecific Red Queen. *Behav. Ecol. Sociobiol*., *41*, 1-10.

Rice, W. R., Stewart, A. D., Morrow, E. H., Linder, J. E., Orteiza, N., & Byrne, P. G. (2006). Assessing sexual conflict in the *Drosophila melanogaster* laboratory model system. *Philos. Trans. R. Soc. B, Biol. Sci.*, *361*(1466), 287-299.

Rowe, L, Krupa, J. L., & Sih, A. (1996) An experimental test of condition-dependent mating behavior and habitat choice by water striders in the wild. *Behav. Ecol., 7*(4), 474–479.

Rowe, L. (1992). Convenience polyandry in a water strider: foraging conflicts and female control of copulation frequency and guarding duration. *Anim. Behav.*, *44*, 189-202.

Sepil, I., Carazo, P., Perry, J. C., & Wigby, S. (2016). Insulin signalling mediates the response to male-induced harm in female *Drosophila melanogaster*. *Sci. Rep.*, *6*(1), 30205.

Singh, A., Agrawal, A. F., & Rundle, H. D. (2017). Environmental complexity and the purging of deleterious alleles. *Evolution*, *71*(11), 2714-2720.

Talagala, S., Rakosy, E., & Long, T.A.F. (2024) Beyond simple vs. complex: exploring the nuanced and unexpected effects of spatial environmental complexity on mating patterns and female fecundity. *J. Evol. Biol.,* 37(9), 1043–1054.

Vercken, E., Massot, M., Sinervo, B., & Clobert, J. (2007). Colour variation and alternative reproductive strategies in females of the common lizard *Lacerta vivipara*. *J. Evol. Biol.*, *20*(1), 221-232.

White, A. J., & Rundle, H. D. (2015). Territory defense as a condition-dependent component of male reproductive success in *Drosophila serrata*. *Evolution*, *69*(2), 407–418. https://doi.org/10.1111/evo.12580.

Wigby, S., & Chapman, T. (2004). Female resistance to male harm evolves in response to manipulation of sexual conflict. *Evolution*, *58*(5), 1028-1037.

Wiklund, C., Kaitala, A., Lindfors, V., & Abenius, J. (1993). Polyandry and its effect on female reproduction in the green-veined white butterfly (*Pieris napi* L.). *Behav. Ecol. Sociobiol*., *33*, 25-33.

Winkler, L., Eilhardt, R., & Janicke, T. (2023). Population density affects sexual selection in an insect model. *Funct. Ecol.*, *37*(10), 2734-2747.

Yun, L., Agrawal, A. F., & Rundle, H. D. (2021). On male harm: How it is measured and how it evolves in different environments. *Am. Nat.*, *198*(2), 219-231.

Yun, L., Bayoumi, M., Yang, S., Chen, P. J., Rundle, H. D., & Agrawal, A. F. (2019). Testing for local adaptation in adult male and female fitness among populations evolved under different mate competition regimes. *Evolution*, *73*(8), 1604-1616.

Yun, L., Chen, P. J., Kwok, K. E., Angell, C. S., Rundle, H. D., & Agrawal, A. F. (2018). Competition for mates and the improvement of nonsexual fitness. *Proc. Natl. Acad. Sci. U.S.A*, *115*(26), 6762-6767.

Yun, L., Chen, P. J., Singh, A., Agrawal, A. F., & Rundle, H. D. (2017). The physical environment mediates male harm and its effect on selection in females. *Proc. Biol. Sci.*, *284*(1858), 20170424.

Zuk, M., Garcia-Gonzalez, F., Herberstein, M. E., & Simmons, L. W. (2014). Model systems, taxonomic bias, and sexual selection: beyond *Drosophila*. *Annu. Rev. Entomol.*, *59*(1), 321-338.

Chapter 3 Supplementary Material

An outbred, lab-adapted population of *D. melanogaster* was created by combining 15 populations from the long-term evolution experiment described in detail in Yun et al. (2018, 2021). These 15 populations had been evolving for over 170 non-overlapping generations in a treatment (termed MC_{simple} in previous papers) in which mate competition occurred each generation in a structurally simple and low-volume mating environment (i.e. standard *Drosophila* culture vials). In brief, for a given population the life cycle each generation involved four groups of 35 males and 35 females being placed together in standard *Drosophila* culture vials (28.5 mm × 95 mm, ~177 mL total volume, containing 10 mL of cornmeal-yeast medium with live yeast pellets sprinkled on top) for a six day 'interaction phase', with flies transferred to fresh vials after three days to avoid deaths caused by flies getting stuck in liquifying food. Following this, flies were anaesthetized, males were discarded, and 105 of the surviving females were evenly distributed among seven to lay eggs for ~24h, after which they were discarded. These populations also experienced one of three novel rearing environments each generation (five populations in each rearing environment) that altered both the food medium and temperature regime they experienced as larvae.

The 15 populations were pooled by first combining each of the five populations raised in a specific larval environment to make one population, resulting in three populations, each one having been reared on a different larval environment. Next, flies from each of the combined populations were pooled and, from these flies, 280 males and 280 females were randomly selected and used to found the new 'pooled 'population. Local adaptation to the different larval environments was not of interest with respect to the current experiments so the new pooled population was reared on

a standard cornmeal-yeast-based medium (matching that of the ancestor of the evolution experiment), at a constant 25 C, but via a life cycle that mirrored that of all the MC_{simple} populations from which it was created, including the mating environment for the adults, but doubling the population size. For the pooled population, each generation eight groups of 35 males and 35 females were placed together in standard *Drosophila* culture vials for the 6 d interaction phase, after which males were discarded and 225 of the surviving females were randomly chosen and evenly allocated among 15 fresh vials for egg laying. Offspring that eclosed from these vials were used to set up the interaction phase for the next generation.

After 27+ generations, flies for use in the current experiments were obtained from this population by placing groups of ten males and ten females into vials for 1-2 hours to lay eggs (to avoid larval overcrowding). Approximately nine days later, virgins were collected from these vials within eight hours after eclosion, using light CO_2 anesthesia, and were stored separately by sex in vials of 15 flies/vial. The experiments were performed in blocks and the flies used in each block were all collected within a 36 h window to minimize variation in age.

The four-day interaction phase of the current experiments was carried out in 473 mL cylindrical polypropylene 'deli' containers (16 oz 'Heavy Duty Deli Cups', Uline Canada). A volume of about 90 mL of cornmeal-agar fly food was dispensed into each container, leaving ~380 mL of air space in both fly density experiment treatments, and the simple environmental complexity treatment (i.e. replicates without the complexity inserts included). The internal surface area of each container was ~295 cm^2. The internal surface area of each of the complex complexity treatment

containers (i.e. treatments with the complexity insert included) was ~425 cm^2. Each insert's surface area was ~243 cm^2 (when not inserted into the food). Air space volume in each complex complexity treatment (with food and complexity insert included) container was ~328 mL.

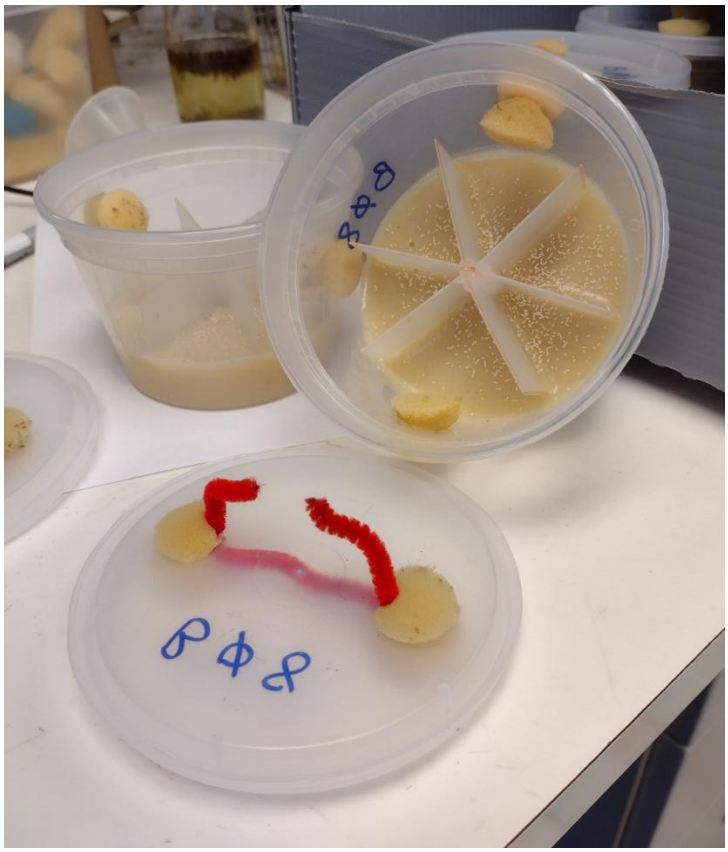

Fig. S3.1. Example of a container used during the 4-day interaction phase for a structurally complex mating environment. It includes a 6-way ‘divider’ inserted into the surface of the food and a pipe cleaner protruding from the lid into the interior space. The structurally simple mating environment lacked both of these, having only a ‘lawn’ of food. The simple version of these containers was also used for both low and high density treatment levels in the density experiment.

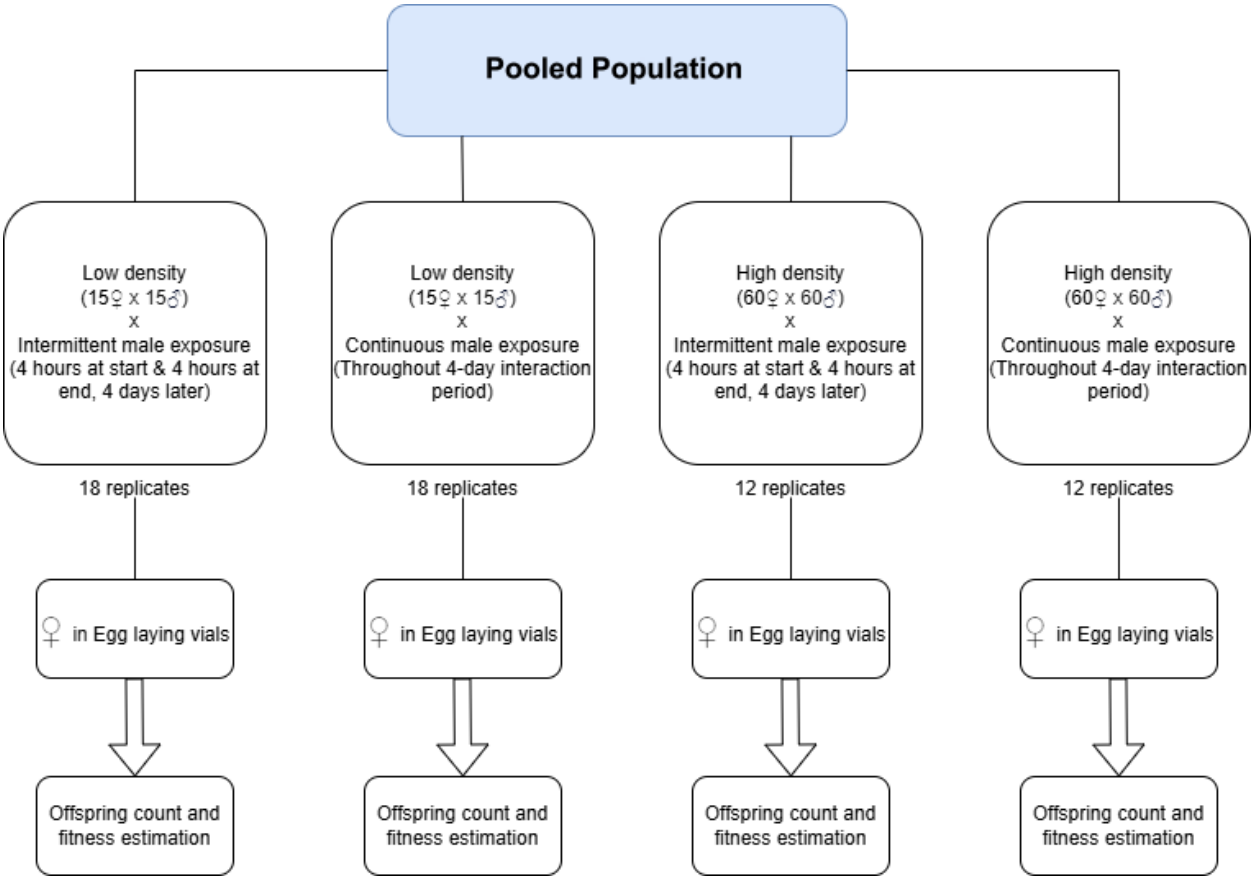

Fig. S3.2. Schematic of the design of the density experiment.

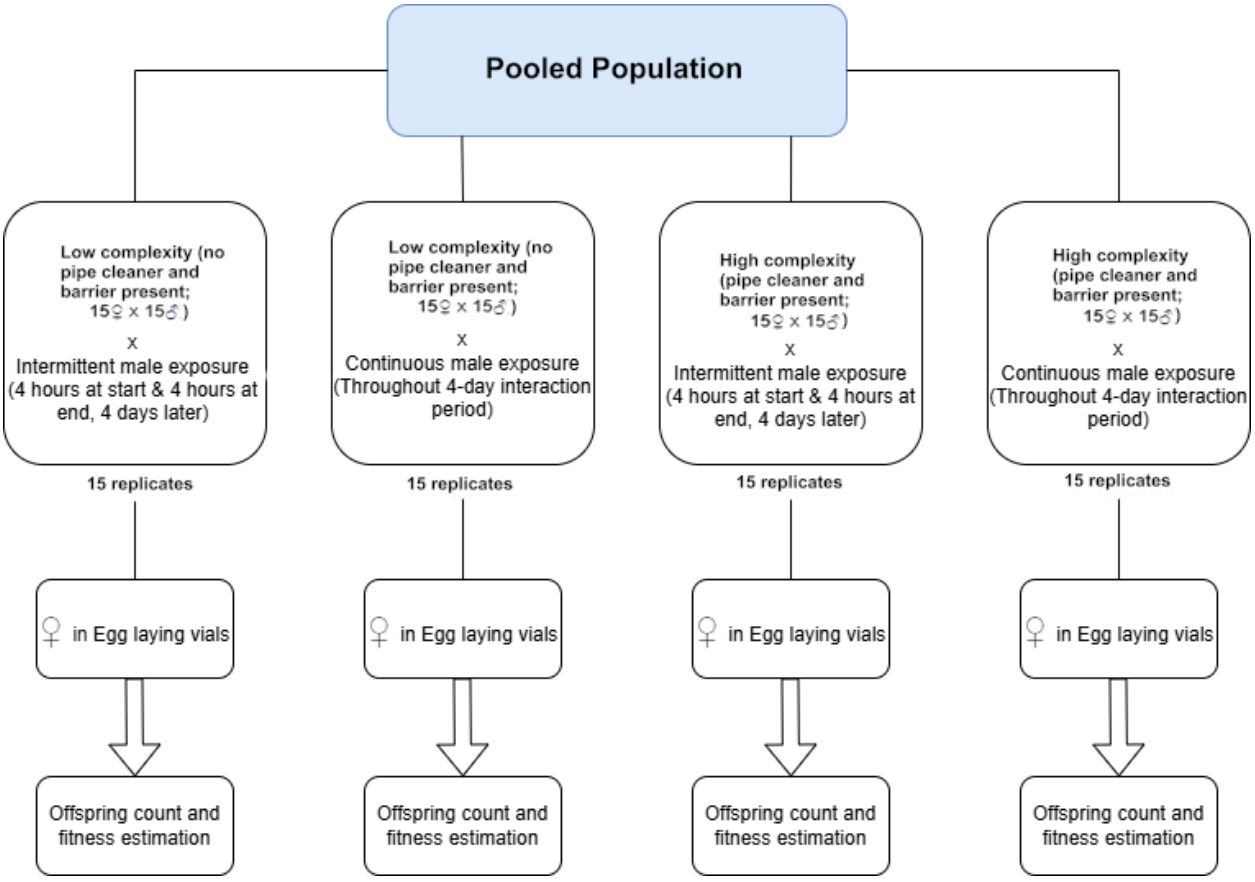

Fig. S3.3. Schematic of the design of the complexity experiment.

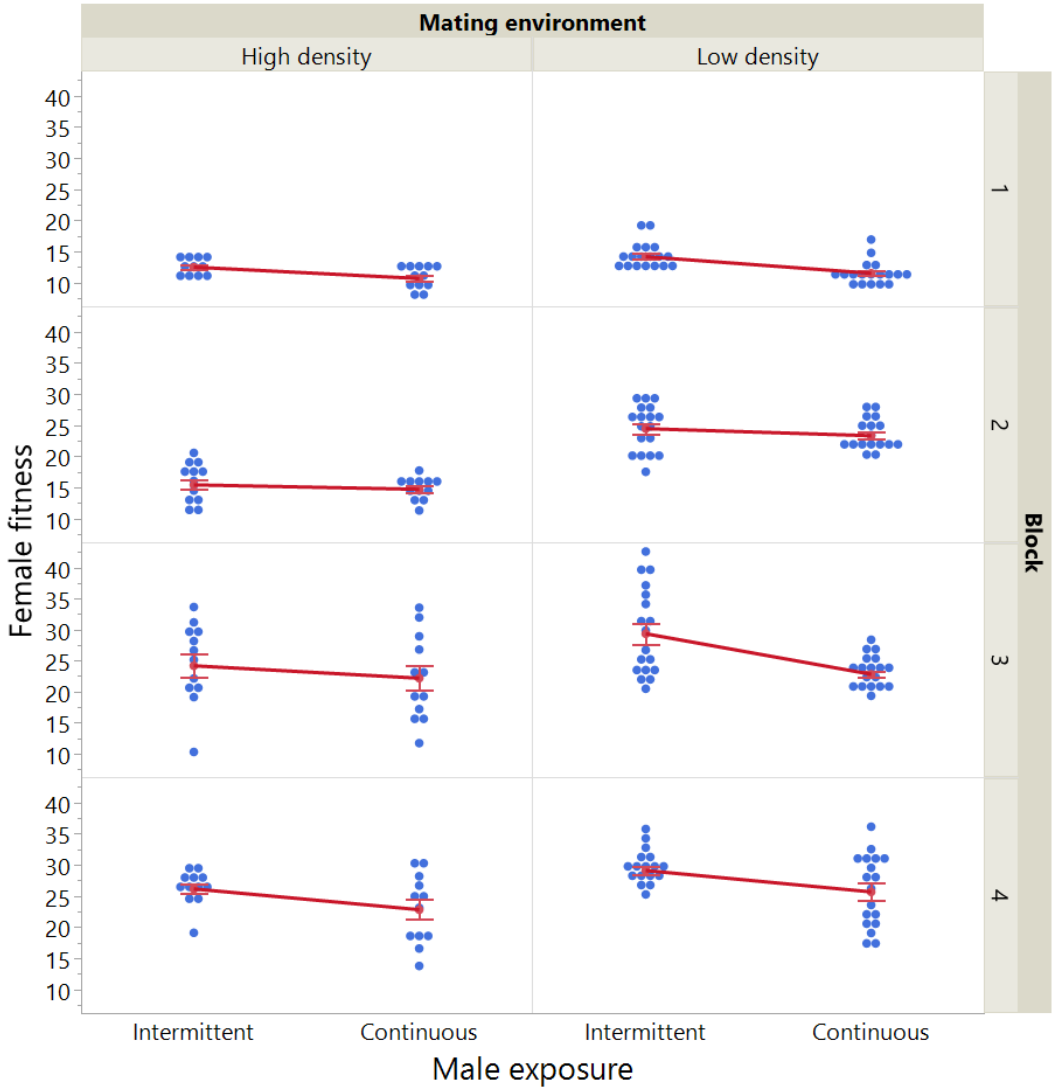

Fig. S3.4. Fitness of females under intermittent vs. continuous exposure to males in either a low or high density mating environment. Points are independent replicates and are plotted separately by block. The line connects the average female fitness (± 1 SE) under intermittent vs. continuous male exposure for a given block and mating environment.

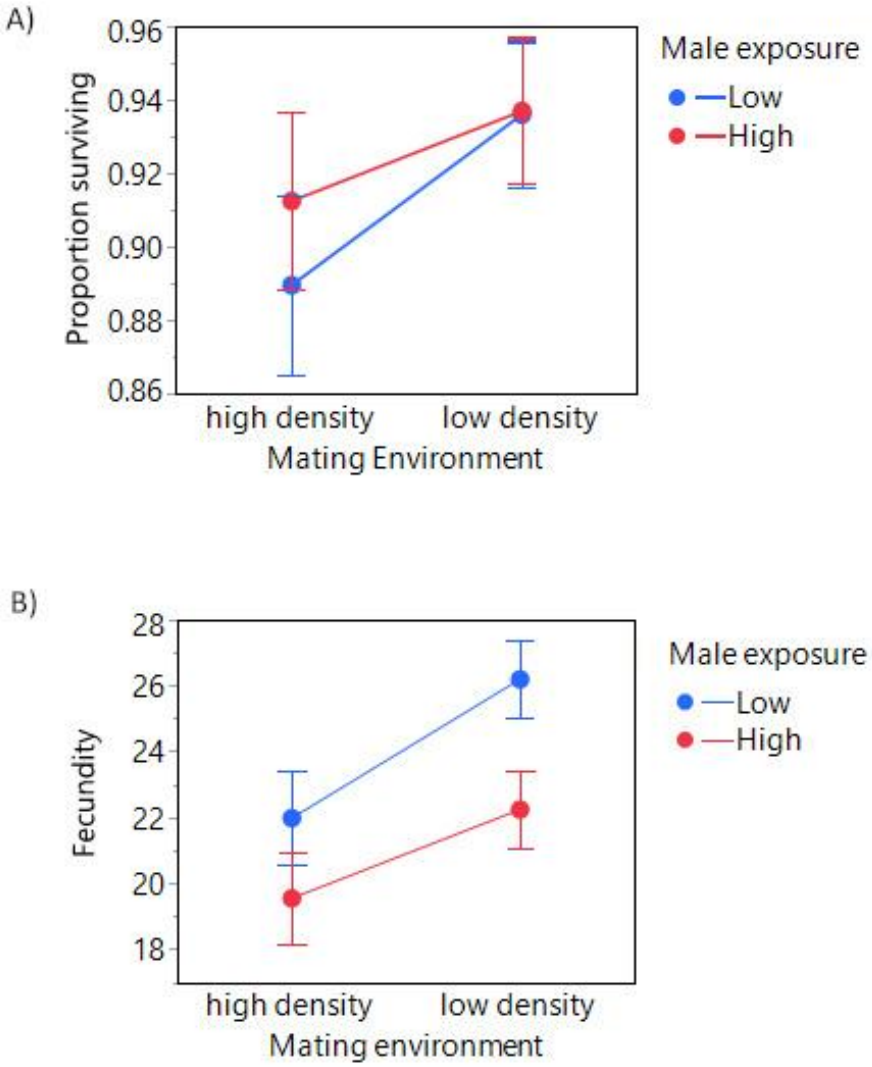

Fig. S3.5. Average A) survival and B) fecundity of females under intermittent vs. continuous exposure to males in either a high or low density mating environment. Values are least square means (± 1 SE) from separate linear models that included density of the mating environment, male exposure, their interaction, and block, as fixed effects. Fecundity was estimated as the number of adult offspring produced by surviving females.

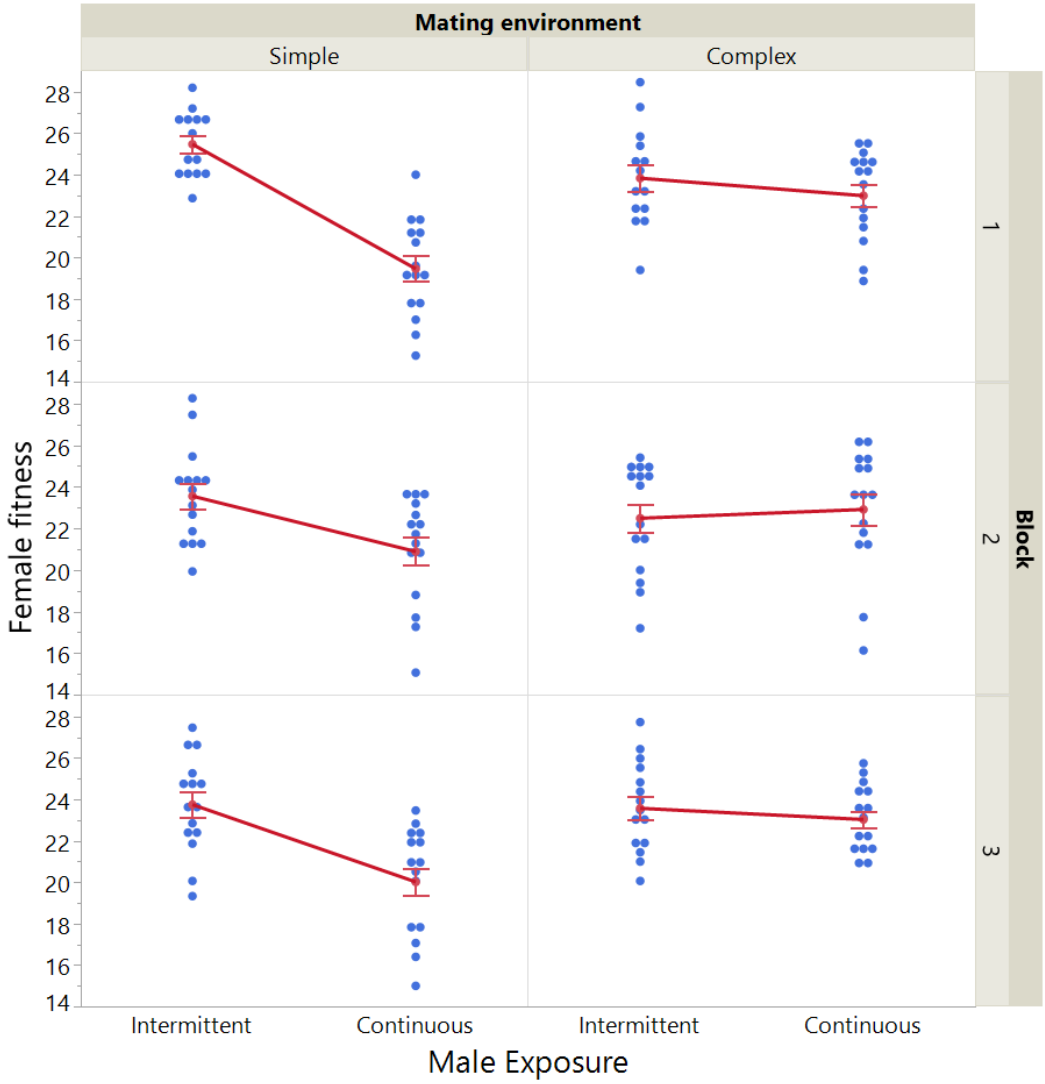

Fig. S3.6. Fitness of females under intermitted vs. continuous exposure to males in either a structurally simple or complex mating environment. Points are independent replicates and are plotted separately by block. The line connects the average female fitness (± 1 SE) under intermitted vs. continuous male exposure for a given block and mating environment.

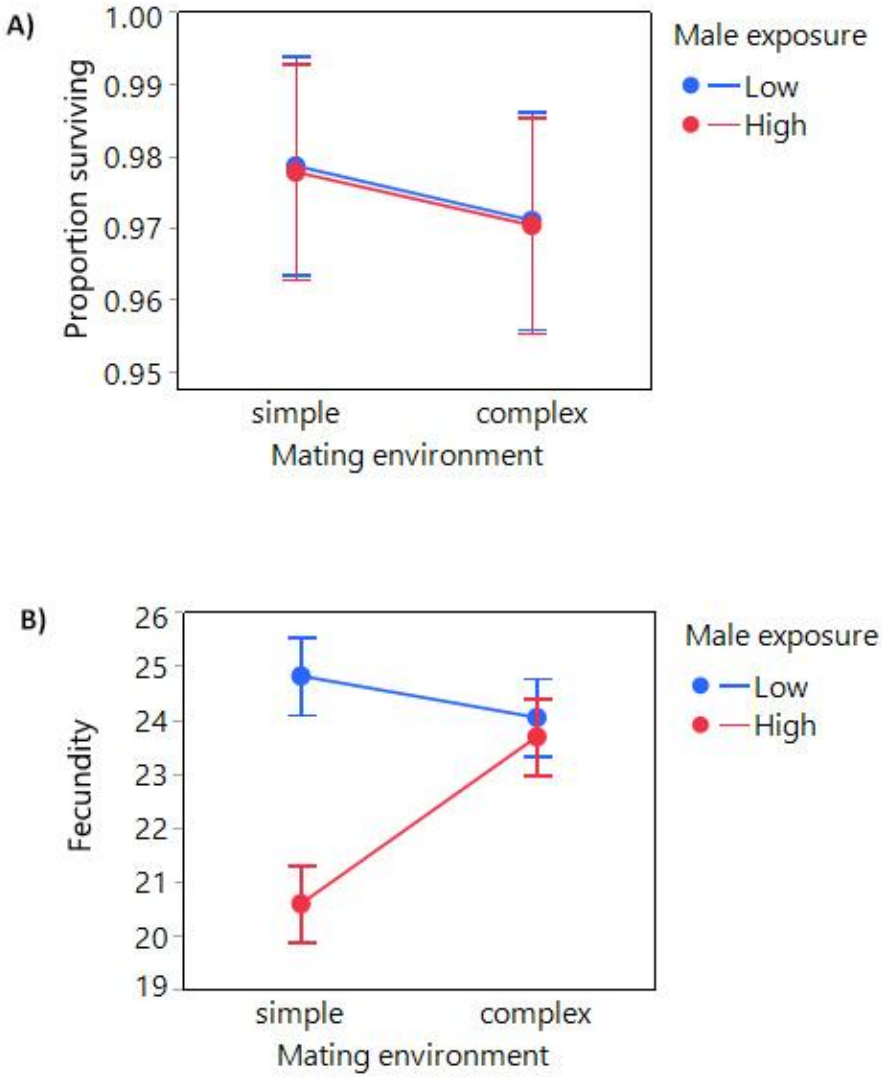

Fig. S3.7. Average A) survival and B) fecundity of females under intermittent vs. continuous exposure to males in structurally simple or complex mating environments (i.e., low vs. high complexity, respectively). Values are least square means (± 1 SE) from separate linear models that included structural complexity of the mating environment, male exposure, their interaction, and block, as fixed effects. Fecundity was estimated as the number of adult offspring produced by surviving females.

Table S3.1. Results from a general linear model analyzing variation in female fitness under treatments varying male exposure and fly density. Bold denotes significant effects ($P < 0.05$).

Effect	**DF**	**F**	**P**
Density	1, 230	48.71	**< 0.0001**
Male exposure	1, 230	22.91	**< 0.0001**
Density × Male exposure	1, 230	1.88	0.172
Block	3, 230	127.1	**< 0.0001**

Table S3.2. Results from a general linear model analyzing variation in female fitness under treatments varying male exposure and the structural complexity of the mating environment. Bold denotes significant effects ($P < 0.05$).

Effect	**DF**	**F**	**P**
Environmental complexity	1, 172	7.29	**<0.0076**
Male exposure	1, 172	39.15	**<0.0001**
Environmental complexity × Male exposure	1, 172	28.79	**<0.0001**
Block	2, 172	0.57	0.565

Chapter 4: A comparison of male harm in lab-adapted vs nature-derived populations in two species of *Drosophila*

Abstract

Male harm occurs when traits that increase male reproductive success impose fitness costs on females as a by-product. Much of our understanding of male harm in *Drosophila* comes from long-term laboratory populations adapted to, and assayed under, highly simplified conditions. Recent work shows that male harm can be reduced when laboratory populations interact in, or adapt to, larger and more structurally complex mating environments, raising the possibility that adaptation to simplified laboratory conditions favours more harmful males. Here, we tested whether long-term lab-adapted males differ in harmfulness from nature-derived males in three comparisons involving two species of *Drosophila*: one in *D. serrata* and two in *D. melanogaster*. In each experiment, we manipulated male population, female population, and female exposure to males, quantifying harm as the proportional reduction in female fitness under increased male exposure. Lab-adapted males generally imposed greater fitness costs on females than did nature-derived males. This pattern was clearest in both *D. melanogaster* comparisons, where lab-adapted males caused greater reductions in female fitness across female backgrounds. In *D. serrata*, harm depended more strongly on the specific male–female population combination: lab-adapted males reduced the fitness of both female types, whereas nature-derived males harmed lab-adapted females but not nature-derived females. These results suggest that laboratory adaptation can alter the expression of male harm in *Drosophila*, at least when assayed under lab

conditions. More broadly, together with previous work, they show that male harm is not fixed, but depends on population history, female background, and assay environment.

Note: This chapter has been written in the style of a manuscript for submission to a peer-reviewed journal. It therefore uses first-person plural pronouns to reflect co-authorship with Howard Rundle and Aneil Agrawal. All three of us contributed to the design of the experiments, while I collected all of the data. I also took the lead role in data analyses, with input from Aneil and Howard, and in the writing, with input from Howard.

Introduction

Drosophila has been a model system for the study of sexual conflict ever since pivotal experiments by Partridge and Fowler demonstrated the existence of pre- and post-copulatory male harm (Fowler & Partridge, 1989; Partridge & Fowler, 1990). These studies, as well as subsequent work by Chapman et al. (1995), Holland & Rice (1999) and others (e.g. Johnstone & Keller, 2000; Chippindale et al., 2001; Stewart et al., 2005), have suggested that sexual conflict is prevalent and often strong such that exposure to males, beyond that needed to provide sufficient sperm, adversely affects female fitness. As a body of work, this *Drosophila* research has been influential; considered alongside a growing number of studies demonstrating male harm in other taxa (Arnqvist & Rowe, 2005), sexual conflict is now often perceived as being widespread, strong, and evolutionarily important, to the extent that male harm is expected whenever mate competition occurs.

But is male harm an inevitable outcome of mate competition in non-monogamous taxa? In some species, females appear to have a high degree of control over sexual interactions and this may limit the extent, or even opportunity, for harm (Zuk et al., 2014). For example, female *Callosobruchus* beetles mate in different habitats from which they subsist. These 'mating habitats' are usually guarded by males, which must be sedentary to control these spaces and, as such, cannot pursue and harass females. Rather, it is the females that approach males for mating, limiting the harm males can inflict via precopulatory harassment and elevated mating rates (Edvardsson, 2007). Ecological context may also limit the opportunity for male harm. For example, damselflies are generally characterized by intense male harassment and strong sexual conflict

(Gosden & Svensson, 2007, 2009). But in the banded demoiselle, *Calopteryx splendens*, Gomez-Llano et al. (2018) showed that female survival was higher under conditions that reduced male mating success, including lower density and the presence of heterospecific males, the interpretation being that these ecological conditions were limiting male harassment.

The ecological context of mating, and how this impacts male harm, has been explored in laboratory populations of *Drosophila*. Most research on male harm in *Drosophila* has been performed using the same vials or bottles commonly used to propagate lab populations; these environments are spatially restricted (and hence often high density) and feature a single patch of food (fly media at the base of a vial or bottle) with no physical structure beyond the container walls (Stocker & Gallant, 2008). However, several recent studies have shown that altering the density and/or the complexity of the mating environment can reduce mating rates (Byrne et al., 2008; Malek & Long, 2019; Yun et al., 2019) and improve female fitness (Yun et al., 2021; Talagala et al., 2024). With respect to harm itself, Yun et al. (2017, 2021) showed that males that were harmful in standard vials were no longer so when sexual interactions and mating occurred in a larger (and hence lower-density), more complex environment that featured multiple food patches and added physical structure in the form of pipe cleaners. Moreover, males maintained in the more complex environment across generations evolved to be less harmful than those maintained in the high-density, simple environment (Yun et al., 2021), and this came at no apparent cost to their sexual fitness when competing for mates in either environment (Yun et al., 2019). Following up on this, Osijo et al. (2026) showed that it was structural complexity, and not density, of the mating environment that appears to underlie differences in the expression of male harm.

The work of Yun et al. (2017, 2019, 2021) was motivated by an interest in the effects of increased space and structural complexity because these are common features of natural environments. The strong effects of structural complexity observed in these studies raise the question of how reliably male harm, as estimated in standard laboratory assays, reflects its expression more generally. One concern is that standard laboratory assays are ecologically simplified: they are spatially restricted, typically high density, and lack the structural complexity that may allow females to avoid or control sexual interactions. A second, less explored concern is that the populations used in such assays have themselves experienced many generations of adaptation to these same simplified laboratory conditions. If long-term laboratory maintenance favours male behaviours or reproductive strategies that elevate female costs, then estimates of male harm from lab-adapted populations may exaggerate its strength and importance compared to that in natural populations.

Experimental-evolution studies show that male harm can evolve in response to the mating environment. For example, males maintained in structurally complex environments evolved to be less harmful than males maintained under standard, structurally simple conditions, demonstrating that recent evolutionary history can alter the expression of harm (Yun et al., 2021). However, these studies do not directly address whether the long-term laboratory populations commonly used in sexual-conflict research are representative of populations derived from nature. This distinction is important because much of the evidence shaping views of male harm in *Drosophila* comes from populations with extended histories of laboratory maintenance. If such populations are consistently more harmful than nature-derived populations, then laboratory studies may provide a biased view of the magnitude, and perhaps the generality, of male harm.

This issue is important not only because *Drosophila* has helped shape views of sexual conflict, but also because *Drosophila* are a model system for studying fundamental evolutionary processes such as adaptation to new or changing environments and the purging of deleterious mutations. Several studies have shown that male harm can affect these processes in important ways. For instance, Yun et al. (2019) demonstrated that the opportunity for mate competition was associated with more rapid adaptation and more efficient purging when mating interactions occurred in a complex, but not a simple, laboratory environment. In separate experiments tracking the frequencies of deleterious mutations, Colpitts et al. (2017) and Singh et al. (2017) likewise showed faster purging on average in populations experiencing more complex, compared to simpler, mating environments. Additional work has linked these effects of mating environment to changes in male harm that, in turn, alter selection through females. We therefore have evidence that mating environment can affect male harm, and that male harm can influence important population-genetic processes. If lab adaptation systematically alters male harm, then conclusions drawn from laboratory populations may also affect how we interpret the evolutionary consequences of mate competition more broadly.

Here, we test whether long-term laboratory adaptation is associated with elevated male harm by comparing harm in three independent population pairs involving two species of *Drosophila*: one pair of *D. serrata* and two pairs of *D. melanogaster*. We chose these species because past work has shown substantial male harm in laboratory populations of both (e.g. Fowler & Partridge, 1989; Partridge & Fowler, 1990; Colpitts et al., 2022). In each case, we compared males from a long-term laboratory-adapted population with males from a recently collected or otherwise nature-derived population. Females from both populations within each pair were included so that

differences in male harmfulness were not confounded with female background, population matching, or recent sex-specific coevolution. Following past studies, we manipulated female exposure to males to quantify harm as the proportional reduction in female fitness under increased male exposure (Yun et al., 2021; Colpitts et al., 2022). This design allows us to ask whether lab-adapted males are consistently more harmful than nature-derived males, and therefore whether reliance on lab-adapted populations may bias inferences about male harm in *Drosophila*. It also provides insight into whether the expression of harm depends on female background and on the specific male–female combination, as might be expected under sex-specific coevolution.

Materials and Methods

We compared the harmfulness of three independent population/species pairs: 1) an outbred, laboratory stock population of *D. serrata* vs. a population of *D. serrata* recently collected from the wild; 2) an outbred, laboratory stock population of *D. melanogaster* vs. a population of *D. melanogaster* recently collected from the wild; and 3) a different outbred, laboratory stock population of *D. melanogaster* vs. an outbred population of *D. melanogaster* created by pooling a set of inbred lines (the *Drosophila* Genetic Reference Panel or DGRP; Mackay et al., 2012) originally founded from wild-collected flies and hence representing standing genetic variation sampled from nature (see details below concerning all three pairs). For convenience, we refer to these two population classes as ‘lab-adapted’ and ‘nature-derived’, respectively; we use these labels as shorthand only: ‘lab-adapted’ denotes outbred populations with an extended history of

maintenance under laboratory conditions, whereas 'nature-derived' denotes populations either recently collected from nature or reconstructed from naturally sampled standing genetic variation. For each population pair, male harm was quantified in a manipulative lab experiment that used a 2×2×2 factorial design, with male population (lab-adapted vs. nature-derived), female population (lab-adapted vs. nature-derived), and male exposure (intermittent vs. continuous) as the factors.

Manipulating male exposure as a means of demonstrating male harm is a common approach in past studies (e.g., Partridge & Fowler, 1990; Yun et al., 2021; Colpitts et al., 2022; Osijo et al., 2026). Following Yun et al. (2021), male harm (*H*) can be quantified as the proportional reduction in the average fitness of females under increased exposure to males:

$$H = \frac{(\bar{W}_{intermittent} - \bar{W}_{constant})}{\bar{W}_{intermittent}}$$ Eqn. 4.1. Quantification of male harm.

This recognizes that harm is present when additional exposure to males, beyond that necessary for reproduction, causes a decrease in female fitness. As a dimensionless metric, the magnitude of *H* can be compared between contexts in which absolute female fitness may vary, like here when using females from different populations.

We included females from both the lab-adapted and nature-derived populations when assaying harm to avoid confounding differences in male harmfulness with differences arising from the

female background used. Had we used females from only one of the two populations, one population of males would necessarily have been tested with females from its own population, whereas the other would have been tested with females from a different population. This would confound the comparison of lab-adapted vs. nature-derived males with effects of population matching, shared history, and/or coadaptation between the sexes. In principle, this issue could be reduced by instead assaying all males against females from a third, independent population with no recent history of coevolution with males from either focal population; however, such a population would necessarily be either more or less lab-adapted, so a potential confound would remain. By assaying males with both female types, our design provides a more balanced test of male harm across female genetic backgrounds, increasing the generality of inference beyond females from a single population and providing insight into male harm, the effects of female background on its expression, and whether harm depends on the specific male-female combination, as might be expected under sex-specific coevolution.

Experiment 1

An outbred, stock population of *Drosophila serrata* was acquired from the University of Queensland, Australia, in the early spring of 2024. This population was originally created in 2003 by pooling six outbred lab populations that had been originally collected several years earlier from six sites along the east coast of Australia, ranging from Cooktown, Queensland, in the north to Wollongong, New South Wales, in the south (Rundle et al., 2006). Given a 2-week generation time during normal stock maintenance in this species, this population had therefore been adapting to

lab conditions for over 500 generations. Since arriving at uOttawa, the populations were maintained at relatively high densities (50-100 per bottle), with 16 bottles per generation. This was carried out in a non-overlapping two-week generation cycle on standard agar-yeast media (referred to as 'ancestral environment' in Rundle et al., 2005) at an ambient temperature of 25°C and relative humidity of 50% prior to the use in the experiment, closely matching previous maintenance conditions.

In the spring of 2025, wild female *D. serrata* were collected in Brisbane, Australia and used to found 26 isofemale lines. F2 offspring of these lines were shipped to uOttawa where they were maintained as isofemale lines to limit adaptation to the lab environment, being kept under the same conditions as the lab-adapted population above. Two generations later, an outbred population was made by pooling approximately 40 virgin females and 40 males from all 26 isofemale lines and then randomly distributing them into egg-laying vials.

Flies for use in the experiment were collected from both populations as virgins using light CO_2 anesthesia; the nature-derived populations were the F1 descendants of the offspring of the pooled isofemale lines. All flies were held in vials on fresh food for 3 days (30 flies/vial) prior to use in the experiment. For the experiment, replicates consisted of groups of 15 males and 15 females in a vial with fresh food for a 4-day interaction phase. Exposure was manipulated by allowing males and females to remain together throughout this period ('continuous' male exposure), or by separating males and females from each other after 4 hours of interaction on the first day, and then putting them back together 4 hours before the end of the interaction phase on the 4^{th} day ('intermittent' male exposure). Between these periods, the 15 females were held

together in their vial in the absence of males. When not together with the females, the males were stored separately in vials; immediately prior to the second exposure dead males were replaced (using extra males collected alongside the original ones) such that 15 males were returned to each interaction vial. To ensure similar handling, all flies in both exposure treatments were anesthetized with CO_2 when the flies in the intermittent treatment were being separated after 4 hours of interaction. Both treatments were also anesthetized and transferred to fresh food for the final 4-hour interaction phase; this also served to reduce the number of flies that could die from being stuck in the liquefied food (due to larval activity). For each replicate, at the end of the interaction phase the number of surviving females was recorded, males were discarded, and the females were divided as equally as possible among three egg-laying vials. These females were discarded after 20-24 hours (times being constant within a block). Egg-laying vials were kept until most of the offspring had eclosed, at which time they were removed and counted. A second count was performed two days later to ensure that all surviving offspring were recorded.

Experiment 2

We used an outbred, lab-adapted population of *Drosophila melanogaster* that was created by combining 15 populations from the long-term evolution experiment described in Yun et al. (2018, 2021), the ancestor of which was an outbred, lab-adapted stock population originally collected from the Similkameen Valley, British Columbia, in 2005. Osijo et al. (2026) provide a detailed description of the derivation of the population from these 15 experimental evolution populations. In brief, the 15 populations (termed MC_{simple} in Yun et al., 2018, 2021) had evolved for 170+ non-

overlapping generations via a life cycle that, each generation, involved a 6-day interaction phase in vials of 35 males and 35 females, after which males were discarded and females laid eggs in fresh vials for ~24 hr. After pooling, the new population was maintained for 45+ generations on cornmeal-yeast food under the same life cycle as the evolution experiment before being used in the current experiment. About two weeks before the collection of wild *D. melanogaster*, the lab-adapted flies to be used in the impending experiment were shifted to a two-week generational cycle, similar to the populations used in Experiment 1.

To create the nature-derived population, wild *D. melanogaster* females were collected from two different sites in Ottawa, Ontario, a few km south of the University of Ottawa (45° 23' 54.6" N, 75° 44' 53.9" W and 45° 24' 8.5" N, 75° 40' 22.2" W). 40 isofemale lines were created from wild-collected females and were maintained under the same conditions as the lab-adapted population, with about 20-40 flies per isofemale line per generation. Two generations later, an outbred population was made by pooling virgin females and males from all 40 isofemale lines and then randomly distributing them into egg-laying vials. The male harm assay was performed two generations later. Methods followed that of Experiment 1 above, again with four blocks each consisting of five replicates of each of the eight treatment combinations.

Experiment 3

As an independent, outbred population with a long history of laboratory maintenance, we used the *Ives* population of *D. melanogaster*. This population was founded from flies collected in

Amherst, Massachusetts, in 1975 and has since been maintained on a 14-day discrete-generation culture regime (for details see Mallet & Chippindale, 2011; Kimber & Chippindale, 2013). We obtained a copy of this population in late 2025 and it was maintained under a similar two-week schedule on cornmeal-yeast medium for 4-5 generations prior to assaying male harm.

As an independent nature-derived population, we created an outbred population carrying standing genetic variation sampled from nature by pooling 30 inbred lines from the *Drosophila* Genetic Reference Panel (DGRP). The DGRP lines were derived from mated females collected from a natural population in Raleigh, North Carolina, USA, and were established by 20 generations of full-sib inbreeding of their progeny (Mackay et al., 2012). Lines were obtained in late 2025 from the Bloomington *Drosophila* Stock Centre, Indiana University, and were maintained on cornmeal-yeast media via a two-week non-overlapping generational cycle at the University of Ottawa. After two generations, an outbred population was made from pooling virgin females and males from all inbred lines and then randomly distributing them into egg-laying vials. The male harm assay was performed two generations later. Methods followed that of Experiments 1 and 2, again with four blocks with each block consisting of five replicates of each of the eight treatment combinations.

Statistical analyses

Analyses were conducted separately for each experiment. For a given replicate, female fitness was quantified as the product of the average survival of the females (i.e., proportion alive after

the 4-day interaction phase) and the average number of offspring produced per surviving female. Variation in female fitness was analyzed using a general linear model, fit via least squares in R (v4.5.1; R Core Team, 2024). The model included male population (lab-adapted vs. nature-derived), female population (lab-adapted vs. nature-derived), male exposure (intermittent vs. continuous), the two- and three-way interactions among these, and block as categorical fixed effects. Type III (i.e., partial) tests were performed via the *car* package (Fox & Weisberg, 2019), with contrasts for categorical variables specified as sum-to-zero via the *contr.sum* statement.

Male harm (*H*) was then estimated for each of the four combinations of male and female population following Eqn. 4.1 and using the average fitness of females under intermittent vs. continuous male exposure for each combination, pooling replicates across all blocks. 95% confidence intervals (CIs) for *H* were estimated via 10,000 bootstrap replicates, sampling the original data with replacement within each combination of male population, female population, and male exposure. Block structure was ignored when estimating *H* and its confidence intervals because the experimental designs were near balanced (they were balanced by design although a small number of replicate vials were lost due to chance errors), including or excluding block had no qualitative effect on the terms of interest in the fitness analyses, and replication was insufficient within blocks to permit bootstrapping at this level.

Sperm limitation assay

Results of Experiment 1 indicated a benefit, not a cost, of increased exposure to males for one combination of male and female *D. serrata* population: nature-derived males with nature-derived females (see Results). This could arise if exposure to males in the intermittent treatment was too low to ensure all females had sufficient sperm to fertilize their eggs (i.e. if there was sperm limitation in this low exposure treatment for this particular combination of male and female populations). To gain insight into this, using F2 descendants of the pooled outbred population of the wild *D. serrata* isofemale lines, we created 10 additional replicates using nature-derived females with nature-derived males: five low male exposure and five high male exposure. Details mirrored those of the Experiment 1 except, at the end of the interaction period, the surviving females were distributed individually (not in groups) among fresh vials to lay eggs. We recorded the number of offspring each female produced. The proportion of females producing zero offspring in each replicate can be used as an index of sperm limitation, and this was compared between exposure levels using a two-sample t-test. Absolute female fitness was again quantified, separately by replicate, as the product of female survival and the average fecundity of surviving females, and this was also compared between exposure treatments using a two-sample t-test. A value of *H* was also calculated from the mean fitnesses under low and high male exposure following equation 1.

Results

Experiment 1

In the *Drosophila serrata* population-pair, increased exposure to males significantly reduced average female fitness overall (main effect of 'male exposure': $P < 0.001$; Table 4.1), consistent with the presence of male harm (Fig. 4.1). Lab-adapted females also had significantly higher fitness than nature-derived females overall (main effect of 'female population': $P = 0.003$; Table 4.1). However, the effect of male exposure depended strongly on the combination of male and female population involved, generating a significant three-way interaction (male population × female population × male exposure, $P = 0.010$; Table 4.1), with increased exposure to males decreasing female fitness in all combinations except nature-derived females with nature-derived males, for which it increased female fitness (Fig. 4.1).

This interaction was reflected in the estimates of relative male harm, H (the proportional reduction in female fitness caused by increased exposure to males; Fig. 4.2). Harm was present (i.e. 95% CIs excluded zero) and similar (95% CIs overlapped) among three male-female combinations: lab-adapted males × nature-derived females, $H = 0.384$ (95% CI: 0.174, 0.576); lab-adapted males × lab-adapted females, $H = 0.312$ (0.141, 0.471); nature-derived males × lab-adapted females, $H = 0.284$ (0.099, 0.452). In contrast, for nature-derived males × nature-derived females, 'harm' was significantly negative, indicating a benefit (not cost) to females of increased exposure to males: $H = -0.382$ (-0.526, -0.254), and this value differed significantly from the other three (non-overlapping 95% CIs).

Examination of the components of female fitness separately revealed that increased exposure to lab-adapted males reduced the survival of both types of females, whereas increased exposure to nature-derived males did not (Fig. S4.1). With respect to the fecundity of surviving females, increased exposure to lab-adapted males had little effect on either type of female, whereas increased exposure to nature-derived males had opposite effects, reducing the fecundity of lab-adapted females but increasing the fecundity of nature-derived females (Fig. S4.2).

Table 4.1. Results from a general linear model of Experiment 3, analyzing variation in *D. serrata* female fitness under treatments varying male population (lab-adapted vs. nature-derived), female population (lab-adapted vs. nature-derived), and male exposure (intermittent vs. continuous), with experimental block included as a fixed effect.

Effect	**df**	**F**	**P**
Male population	1, 145	0.56	0.456
Female population	1, 145	9.02	**0.003**
Male exposure	1, 145	13.18	**<0.001**
Block	3, 145	2.62	0.053
Male population × female population	1, 145	43.62	**<0.001**
Male population × male exposure	1, 145	16.47	**<0.001**
Female population × male exposure	1, 145	13.94	**<0.001**
Male population × female population × male exposure	1, 145	6.86	**0.010**

Note. Bold denotes significant effects ($P < 0.05$).

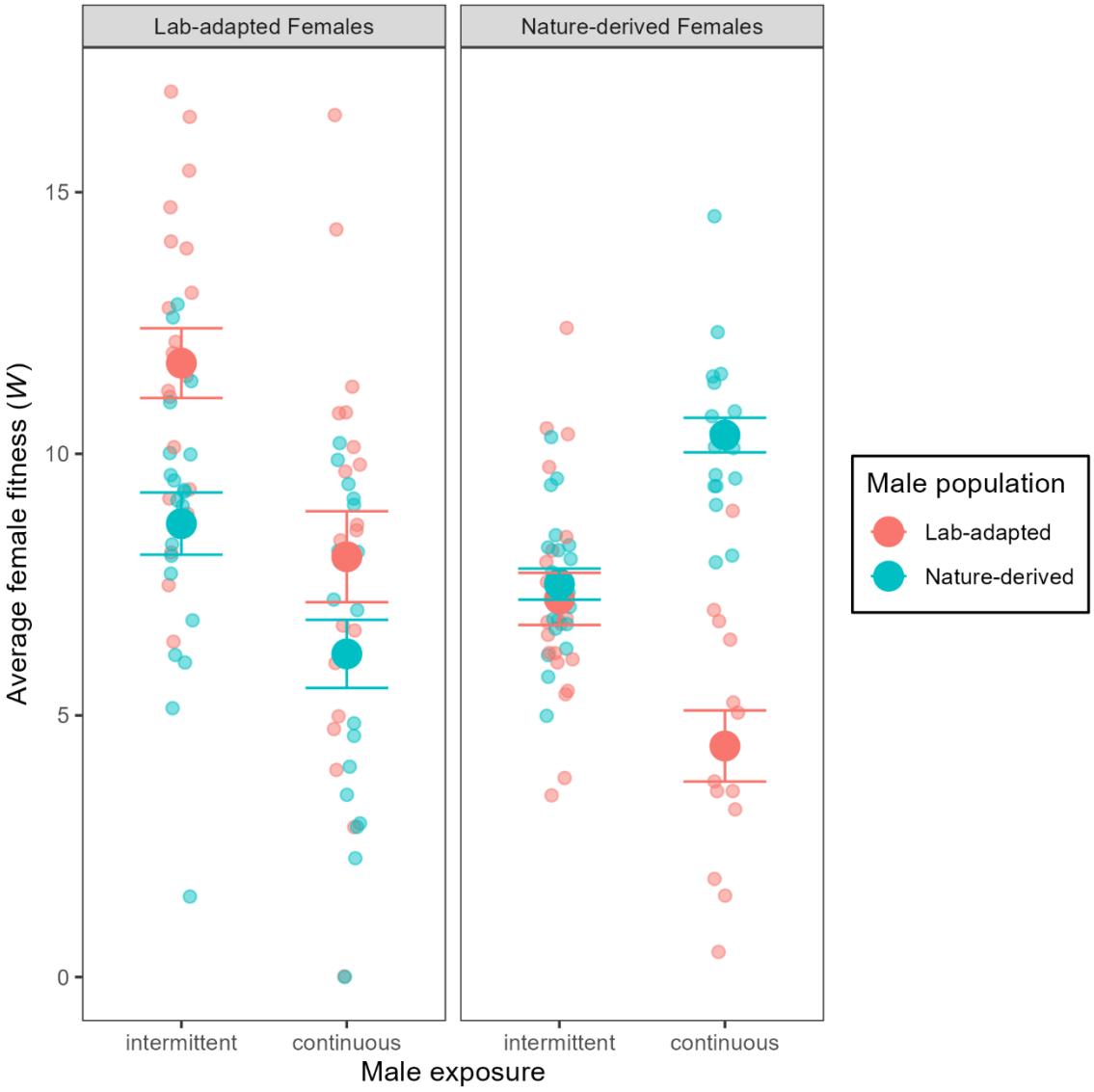

Fig. 4.1. Average female fitness (*W*) as a function of male exposure (intermittent vs. continuous), male population (lab-adapted vs. nature-derived) and female population (lab-adapted vs. nature-derived) in *D. serrata* (Experiment 1). Female fitness is average number of offspring per female, calculated as the product of average female survival and the average fecundity of surviving females. Large points are means (± SE) of treatment combinations; smaller points are values for individual replicates.

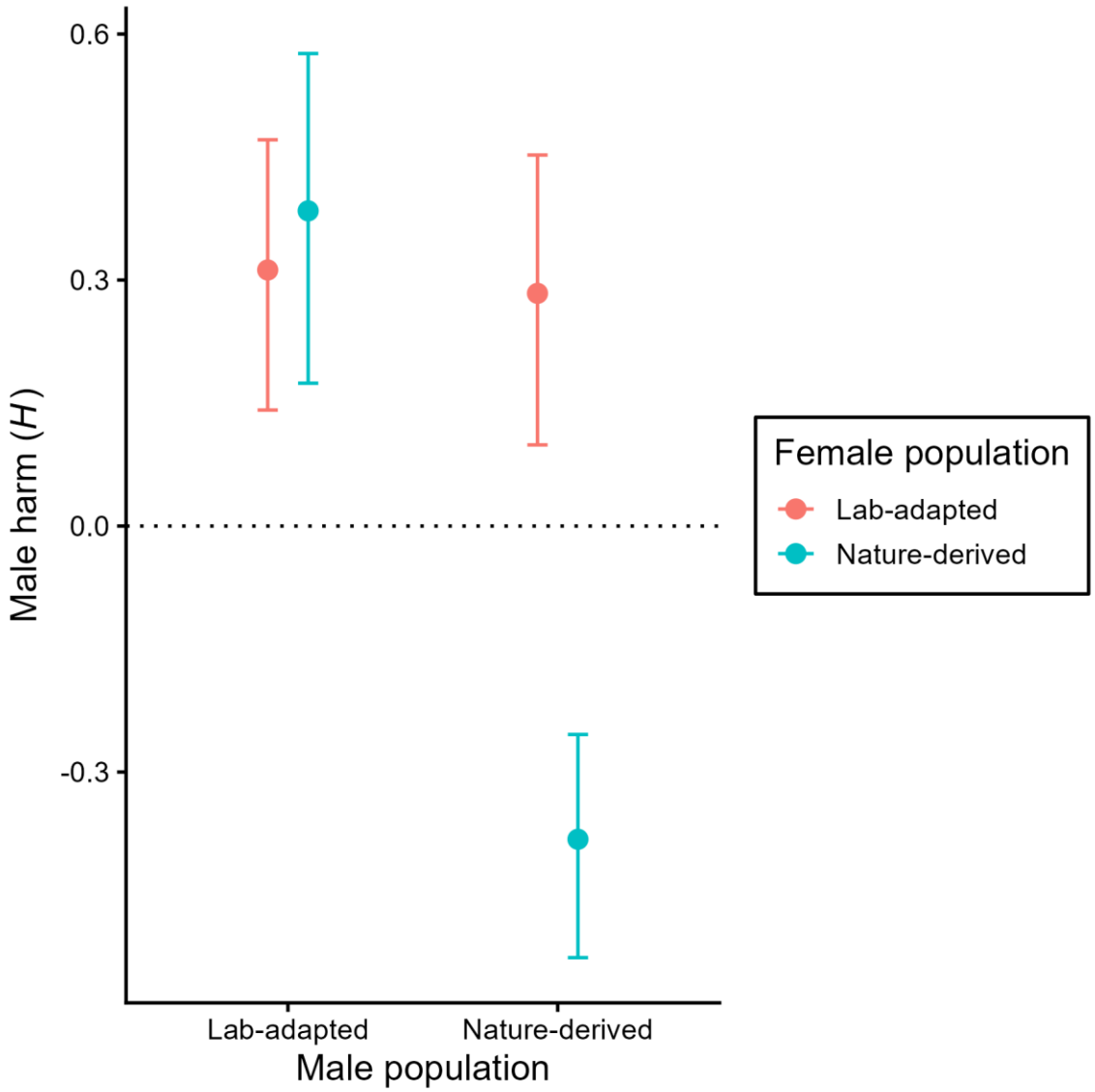

Fig. 4.2. Variation in relative male harm, *H* (the proportional reduction in female fitness under increased exposure to males; see Equation 1) experienced by lab-adapted vs. nature-derived *D. serrata* females in Experiment 1. Females interacted and mated with either lab-adapted or nature-derived *D. serrata* males. Points are values of *H* with 95% bootstrap confidence intervals. Negative values of *H* indicate a benefit (not cost) of increased exposure to males.

Because a benefit to females of increased exposure to males could arise if females were sperm limited in the intermittent male exposure treatment level, but not (or less so) in under continuous male exposure, we conducted an assay to test for this. The assay used only the nature-derived males with nature-derived females under both intermitted and continuous male exposure, with females laying individually post-exposure. The mean proportion of females that produced no offspring was somewhat higher under intermittent than continuous exposure (26.6% vs. 20.6%, respectively; Fig. 4.3A), although the difference was not significant (two-sample t-test: $t_{8\ df} = 1.05$, P = 0.171). Female fitness was again higher in the continuous compared to intermittent exposure treatment (mean offspring/female ± SE: intermittent, 19.1 ± 1.5; continuous, 20.2 ± 1.5; Fig. 4.3B), although the difference was not significant (two-sample t-test: $t_{8\ df} = 0.55$, P = 0.594). The estimate of male 'harm' was thus negative (*H* = -0.061), although replication was not sufficient to estimate a bootstrap CI.

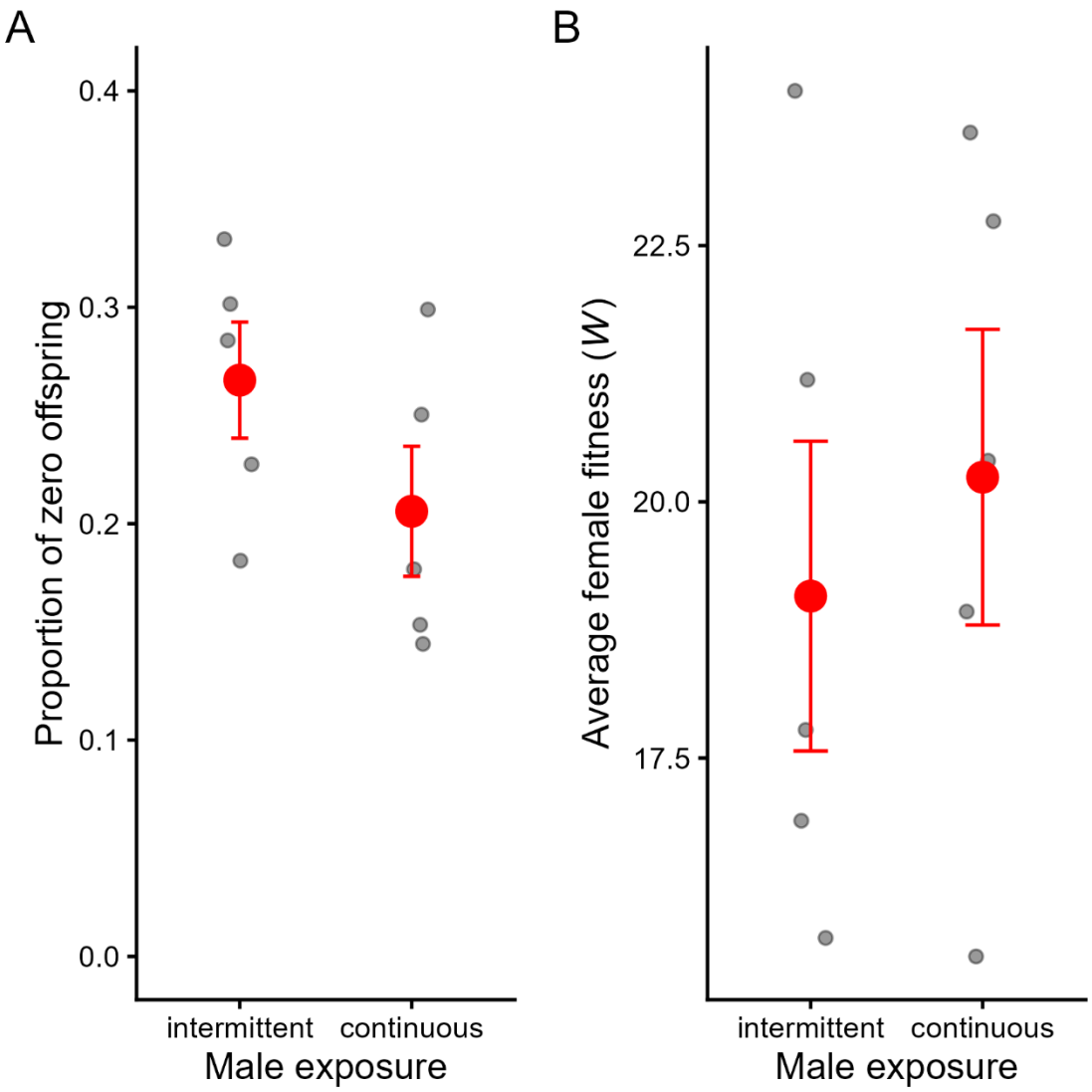

Fig. 4.3. Results from the *D. serrata* sperm limitation assay, including: (A) the proportion of females producing no offspring, and (B) the average fitness of females (*W*) as a function of male exposure (intermittent vs. continuous) for nature-derived males with nature-derived females. Female fitness is average number of offspring per female, calculated as the product of average female survival and the average fecundity of surviving females. Large points are means (± SE); smaller points are values for individual replicates.

Experiment 2

In the first *D. melanogaster* population-pair involving a long-term lab population and a newly collected population from the wild, increased exposure to males again significantly reduced female fitness overall (main effect of 'male exposure': $P < 0.001$; Table 4.2), consistent with the presence of male harm (Fig. 4.4). Females also had higher fitness overall when paired with nature-derived compared to lab-adapted males (main effect of 'male population': $P < 0.001$; Table 4.2). However, there was a significant interaction between these factors (male population × male exposure, $P = 0.001$; Table 4.2), with increased exposure to lab-adapted males decreasing female fitness more than increased exposure to nature-derived males (Fig. 4.4). This effect did not depend on female population (i.e., non-significant three-way interaction; Table 4.2).

Relative male harm, *H*, was highest and significant (95% CIs excluded zero) when females interacted and mated with lab-adapted males, both for nature-derived females, $H = 0.619$ (95% CI: 0.534, 0.698) and for lab-adapted females, $H = 0.517$ (0.423, 0.603), with these estimates not differing significantly from each other (overlapping 95% CIs; Fig. 4.5). Relative male harm was still present (95% CIs excluded zero) when females interacted and mated with nature-derived males, both for nature-derive females, $H = 0.255$ (0.143, 0.350) and lab-adapted females, $H = 0.311$ (0.199, 0.406; Fig. 4.5). These estimates did not differ significantly from each other (overlapping 95% CIs), but they were significantly less than the estimates involving lab-adapted males: the difference in *H* ($\overline{H}_{lab-adapted\ males} - \overline{H}_{nature-derived\ males} = 0.285$) had a 95% CI that did not overlap zero (0.206, 0.364).

Examining the components of female fitness separately reveals that the greater harmfulness of lab-adapted vs. nature-derived males arose through effects on both female survival and fecundity. Increased exposure to lab-adapted males reduced the survival of both lab-adapted and nature-derived females, with a larger effect on the latter, whereas increased exposure to nature-derived males had little effect on the survival of either type of female (Fig. S4.3). With respect to the fecundity of surviving females, patterns were qualitatively the same as those for total fitness (i.e. Fig. 4.4), with increased exposure to lab-adapted males being more harmful to both types of females compared to increased exposure to nature-derived males (Fig. S4.4).

Table 4.2. Results from a general linear model of Experiment 2 analyzing variation in *D. melanogaster* female fitness under treatments varying male population (lab-adapted vs. nature-derived), female population (lab-adapted vs. nature-derived), and male exposure (intermittent vs. continuous), with experimental block included as a fixed effect.

Effect	**df**	**F**	**P**
Male population	1, 149	64.87	**<0.001**
Female population	1, 149	1.17	0.281
Male exposure	1, 149	158.08	**<0.001**
Block	3, 149	5.21	**0.002**
Male population × female population	1, 149	47.73	**<0.001**
Male population × male exposure	1, 149	10.47	**0.001**
Female population × male exposure	1, 149	0.40	0.531
Male population × female population × male exposure	1, 149	0.30	0.586

Note. Bold denotes significant effects ($P < 0.05$).

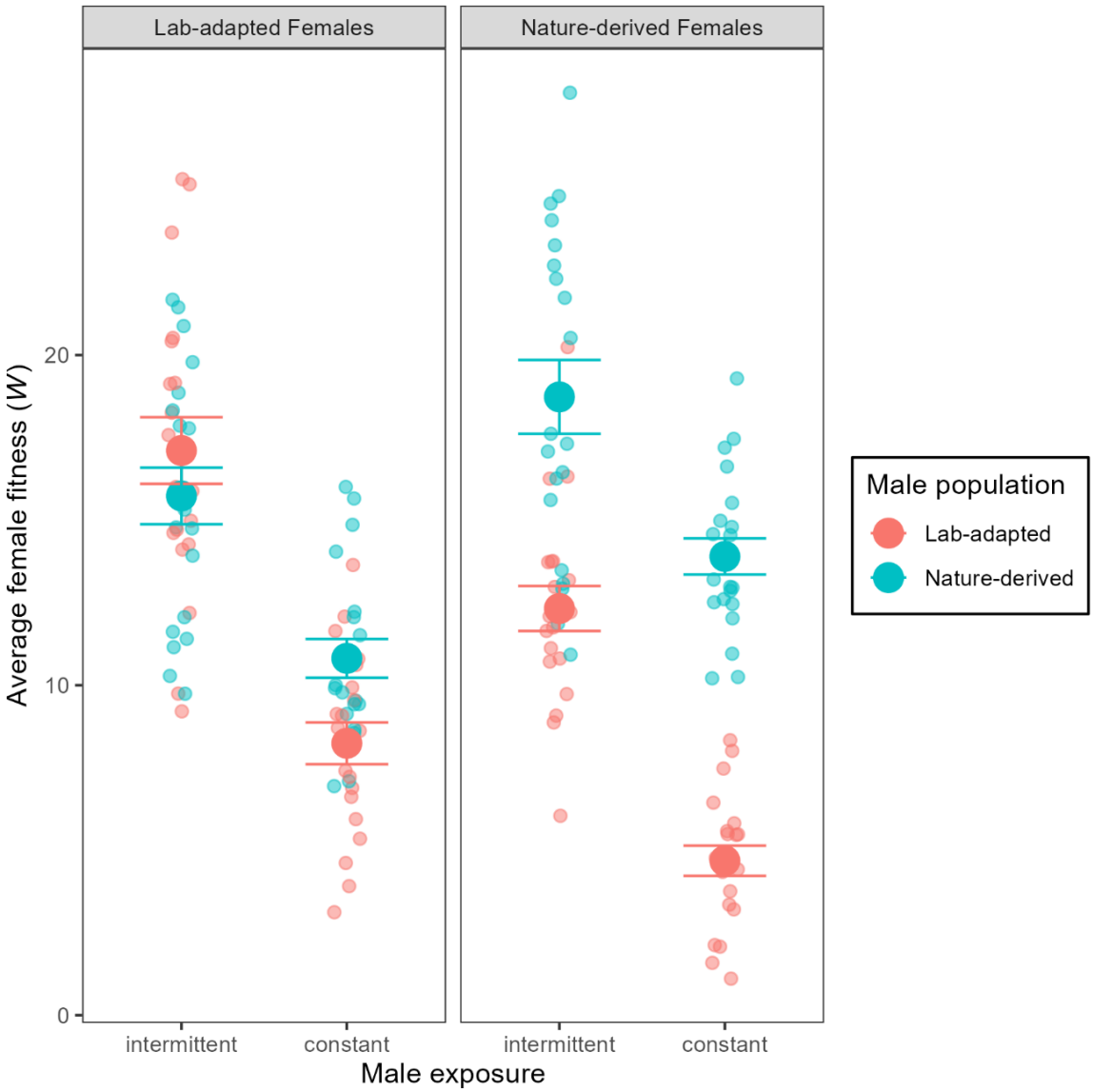

Fig. 4.4. Average female fitness (*W*) as a function of male exposure (intermittent vs. continuous), male population (lab-adapted vs. nature-derived) and female population (lab-adapted vs. nature-derived) in *D. melanogaster* (Experiment 2). Female fitness is average number of offspring per female, calculated as the product of average female survival and the average fecundity of surviving females. Large points are means (± SE) of treatment combinations; smaller points are values for individual replicates.

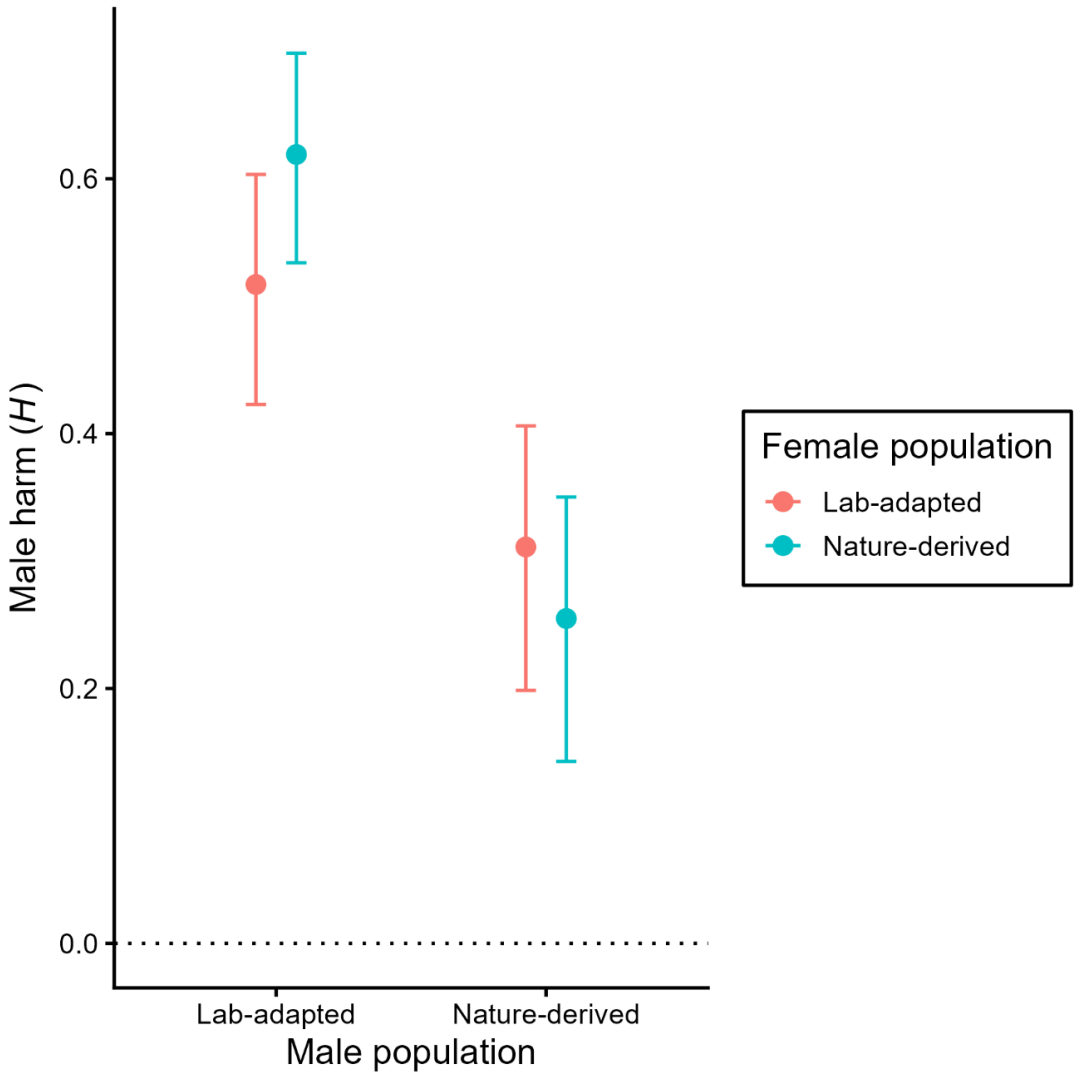

Fig. 4.5. Variation in relative male harm, *H* (the proportional reduction in female fitness under increased exposure to males; see Equation 1) experienced by lab-adapted vs. nature-derived *D. melanogaster* females in Experiment 2. Females interacted and mated with either lab-adapted or nature-derived *D. melanogaster* males. Points are means with 95% bootstrap confidence intervals.

Experiment 3

In the second *D. melanogaster* population-pair involving a different long-term lab population and a reconstituted sample of standing genetic variation from nature (created by pooling a set of DGRP lines), increased exposure to males again significantly reduced average female fitness overall (main effect of 'male exposure': $P < 0.001$; Table 4.3), consistent with the presence of male harm (Fig. 4.6). Females also had higher fitness overall when paired with nature-derived compared to lab-adapted males (main effect of 'male population': $P < 0.001$; Table 4.3). However, there was a significant interaction between these two factors (male population × male exposure, $P = 0.003$; Table 4.3), with increased exposure to lab-adapted males decreasing female fitness more than increased exposure to nature-derived males (Fig. 4.6). This effect did not depend on female population (i.e., the three-way interaction was non-significant; Table 4.3).

Variation in relative male harm, *H*, followed a pattern very similar to that in Experiment 2. Harm was highest and significant (95% CIs excluded zero) when females interacted and mated with lab-adapted males, both for nature-derived females, $H = 0.593$ (95% CI: 0.513, 0.668) and for lab-adapted females, $H = 0.476$ (0.362, 0.579), with these estimates not differing significantly from each other (overlapping 95% CIs; Fig. 4.7). Relative male harm was still present (95% CIs excluded zero) when females interacted and mated with nature-derived males, both for nature-derived females, $H = 0.294$ (0.192, 0.387) and lab-adapted females, $H = 0.376$ (0.301, 0.448; Fig. 4.7). These estimates did not differ significantly from each other (overlapping 95% CIs), but they are significantly less than the estimates involving lab-adapted males: the difference in *H*

($\overline{H}_{lab-adapted\ males} - \overline{H}_{nature-derived\ males} = 0.199$) had a 95% CI that did not overlap zero (0.100, 0.299).

Examining the components of female fitness separately reveals that the increased harmfulness of lab-adapted vs. nature-derived males arose largely through the greater effects of the former males on female survival (Fig. S4.5), whereas effects on female fecundity differed less (Fig. S4.6).

Table 4.3. Results from a general linear model of Experiment 3, analyzing variation in *D. melanogaster* female fitness under treatments varying male population (lab-adapted vs. nature-derived), female population (lab-adapted vs. nature-derived), and male exposure (intermittent vs. continuous), with experimental block included as a fixed effect.

Effect	**df**	**F**	**P**
Male population	1, 149	26.59	**<0.001**
Female population	1, 149	0.00	0.996
Male exposure	1, 149	227.81	**<0.001**
Block	3, 149	0.89	0.446
Male population × female population	1, 149	13.17	**<0.001**
Male population × male exposure	1, 149	9.13	**0.003**
Female population × male exposure	1, 149	0.03	0.863
Male population × female population × male exposure	1, 149	1.46	0.229

Note. Bold denotes significant effects ($P < 0.05$).

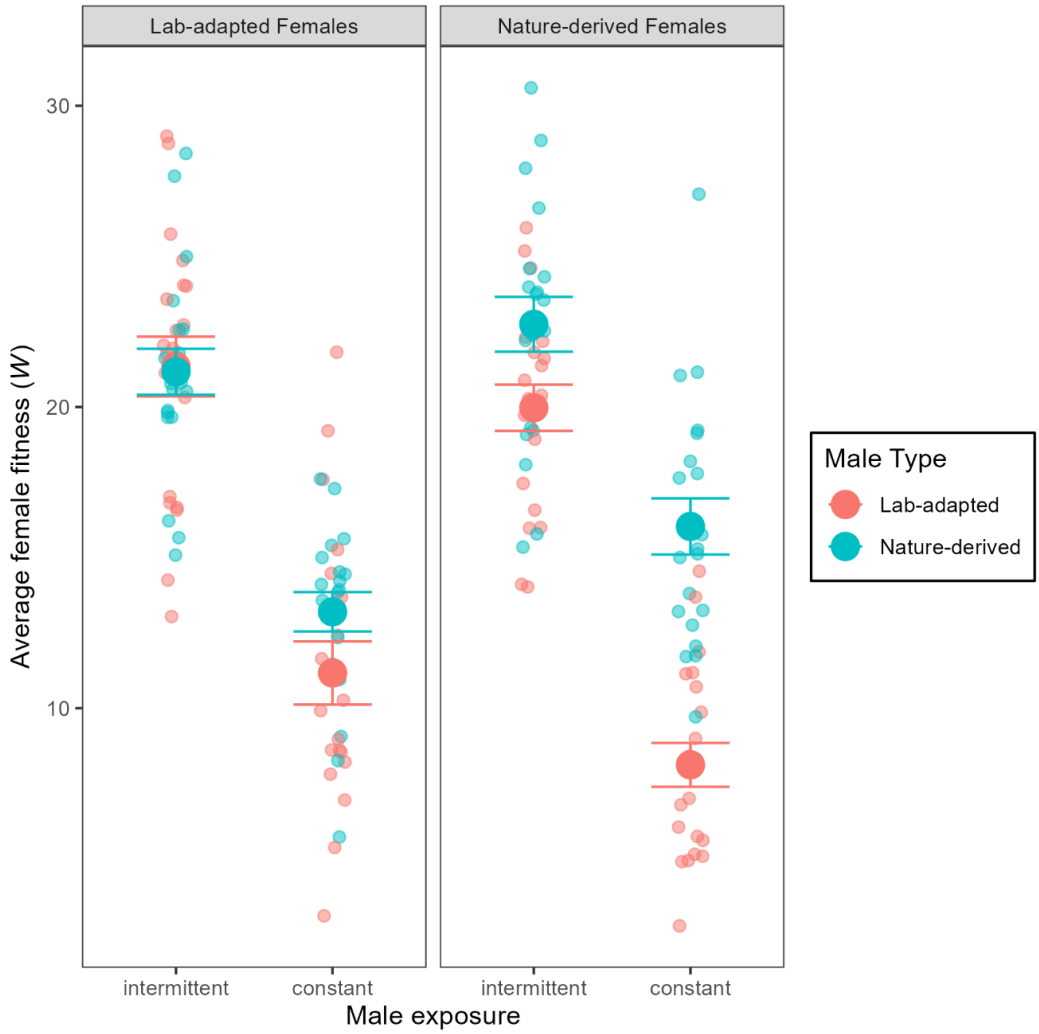

Fig. 4.6. Average female fitness (*W*) as a function of male exposure (intermittent vs. continuous), male population (lab-adapted vs. nature-derived) and female population (lab-adapted vs. nature-derived) in *D. melanogaster* (Experiment 3). The nature-derived population was a reconstituted sample of genetic variation from the wild created by pooling a set of DGRP lines (see Methods). Female fitness is average number of offspring per female, calculated as the product of average female survival and the average fecundity of surviving females. Large points are means (± SE) of treatment combinations; smaller points are values for individual replicates.

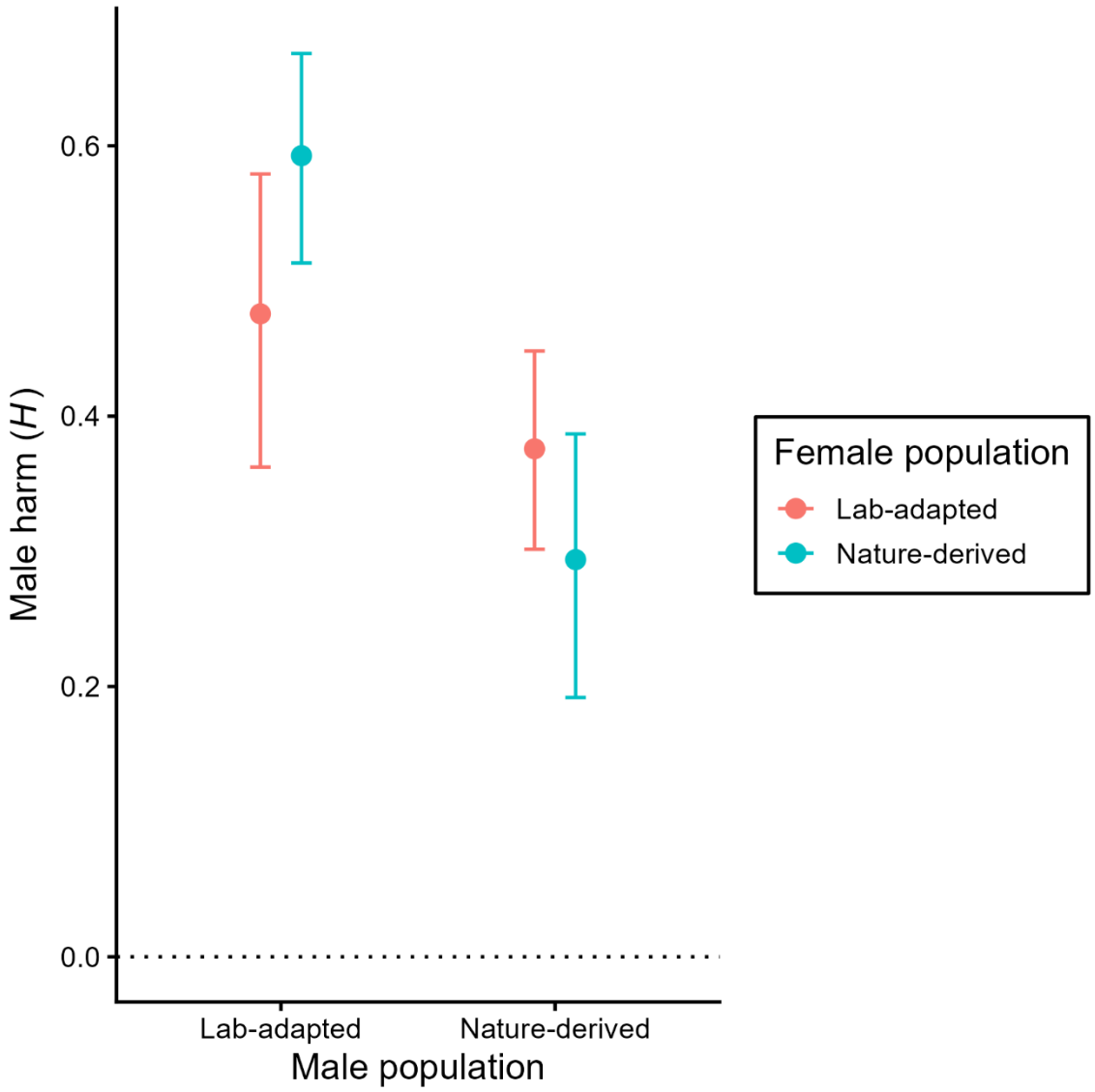

Fig. 4.7. Variation in relative male harm, *H* (the proportional reduction in female fitness under increased exposure to males; see Equation 1) experienced by lab-adapted vs. nature-derived *D. melanogaster* females in Experiment 3. Females interacted and mated with either lab-adapted or nature-derived *D. melanogaster* males. Points are means with 95% bootstrap confidence intervals.

Discussion

Recent studies have shown that both the expression and evolution of male harm in *Drosophila* can depend strongly on the environment in which sexual interactions and matings occur (Yun et al., 2017, 2021), and in particular on the structural complexity and/or distribution of resources (Osijo et al., 2026). Standard laboratory environments are highly simplified and may differ markedly from the more varied and complex natural environments in which flies interact, and lab populations used in sexual-conflict research have generally evolved in, and presumably adapted to, such unusual laboratory conditions. Here we asked whether lab-adapted males are generally more harmful than nature-derived males. Across three independent comparisons involving two *Drosophila* species, our results provide broad support for this. Increased exposure to males reduced female fitness overall in all three experiments, confirming the presence of male harm under our assay conditions. More importantly, lab-adapted males were consistently associated with stronger negative effects on female fitness than were nature-derived males. This pattern was clearest in both *D. melanogaster* population pairs, where lab-adapted males caused significantly greater reductions in female fitness than nature-derived males, and this difference was evident across both female backgrounds. In *D. serrata*, results were more complex because the expression of harm depended on the particular male–female population combination; nevertheless, they were also generally consistent with greater harm by lab-adapted males for which increased exposure reduced the fitness of both types of females, whereas nature-derived males were harmful only to lab-adapted females. Collectively, our results suggest that long-term adaptation to laboratory conditions may elevate male harm, and that estimates of harm from lab-adapted populations may overstate the magnitude of harm expressed by natural populations.

The *D. serrata* results are interesting because they suggest that the expression of male harm can depend strongly on the specific combination of male and female populations involved. Substantial male harm has previously been documented in two lab-adapted populations of *D. serrata* (Colpitts et al., 2022), and our results are consistent with this: increased exposure to lab-adapted males reduced the fitness of both lab-adapted and nature-derived females. By contrast, nature-derived males reduced the fitness of lab-adapted females, but not nature-derived females in which increased male exposure benefitted female fitness, producing a negative estimate of harm. Such male–female population specificity is not unexpected under sexual conflict, where traits mediating male harm and female susceptibility, resistance, or tolerance may co-evolve between the sexes, and where the fitness consequences of mating interactions may therefore depend on the particular male–female combination involved (Arnqvist and Rowe, 2005; Rowe and Day, 2006; Rowe et al., 2005). Consistent with this, reproductive outcomes in *Drosophila* can show strong male × female interaction effects (e.g., Reinhart et al., 2015). This also highlights the value of assaying males against more than one female background: had we used only lab-adapted or only nature-derived females, the interpretation of the *D. serrata* results would have been quite different.

One possible explanation for the benefit of increased exposure in the nature-derived male × nature-derived female combination is sperm limitation under intermittent exposure. If the shorter exposure period was insufficient for some females to mate or acquire enough sperm, then reduced fertilization success in the intermittent-exposure treatment could depress fecundity and thereby make continuous exposure appear beneficial. However, several aspects of the data suggest that sperm limitation is unlikely to fully explain this pattern. First, the follow-up assay

provided, at most, limited evidence for sperm limitation: the proportion of females producing no offspring was higher under intermittent than continuous exposure, but not significantly so and the effect size was modest. Second, in the original experiment, the fecundity of nature-derived females exposed intermittently to nature-derived males was only low relative to the same male-female combination in the corresponding continuous-exposure treatment, but it was not especially low in absolute terms compared with other treatment combinations, including nature-derived females exposed intermittently to lab-adapted males (Fig. 4.1). Thus, the negative harm estimate appears to reflect comparatively high fecundity under continuous exposure to nature-derived males, rather than clear evidence of fecundity depression under intermittent exposure. Finally, decomposing *D. serrata* female fitness into its components shows that variation in survival closely matched the pattern seen in both *D. melanogaster* experiments: female survival was higher overall with nature-derived than lab-adapted males, increased exposure to lab-adapted males substantially reduced female survival, increased exposure to nature-derived males had little or no effect on female survival, and this pattern was similar for lab-adapted and nature-derived females. Because sperm limitation should affect fecundity rather than survival, the survival results are not subject to this concern and thus support the general inference that lab-adapted males were more harmful than nature-derived males, including in *D. serrata*.

The consistency of our results across three independent comparisons provides empirical support for the concern that long-term laboratory maintenance can alter the male phenotypes that give rise to sexual conflict. Lab-adapted males imposed greater costs to females than nature-derived males in all three cases, suggesting that estimates of harm from long-term laboratory populations may overstate the magnitude of harm expressed in natural populations. These results are

consistent with experimental-evolution studies showing that, in lab populations, male harm evolves to be greater in simple as opposed to complex mating environments (Yun et al., 2019, 2021). However, our results cannot definitively infer that laboratory adaptation was the underlying cause of the observed differences in harm because we compared populations with different evolutionary and demographic histories. A direct test of lab adaptation as the mechanism could experimentally evolve replicate populations from a common, recently nature-derived ancestor under laboratory conditions and then compare their harmfulness with populations in which adaptation to those conditions was minimized or prevented.

Why might adaptation to lab conditions increase male harm? One possibility is that standard lab conditions may favour male traits that increase mating success under conditions of high local density, limited space, and minimal structural complexity. In such environments, females may have reduced opportunity to avoid males or to control sexual interactions, potentially increasing the fitness returns to males of persistent courtship, elevated mating attempts, or other behaviours that impose costs on females. Over many generations, adaptation to these conditions could therefore favour males that are more effective competitors in the lab environment, but also more harmful to females. This interpretation is consistent with recent work showing that structural complexity can reduce the expression of male harm, and that populations evolved in more complex environments tend to evolve lower harmfulness than those maintained in standard, structurally simple laboratory environments (Yun et al., 2017, 2021; Osijo et al., 2026). Identifying the traits responsible for this pattern will require linking elevated harm to behavioural and/or physiological mechanisms, such as courtship, harassment, remating, and seminal-fluid effects.

It is also worth noting that male harm was assayed in standard laboratory vials, not under natural conditions — whatever those may be. This matters because the assay environment is part of the phenotype being measured: previous work has shown that the expression of male harm depends on the physical environment in which sexual interactions occur (Yun et al., 2017, 2021). Standard vials are spatially restricted and structurally simple, and can promote the expression of harm relative to more complex environments. Thus, the greater harm expressed by lab-adapted males in our assays does not necessarily imply that the same magnitude of harm would be expressed under more natural conditions. Conversely, the lower harm expressed by nature-derived males, especially in the two *D. melanogaster* comparisons, should not be taken to mean that these males would necessarily impose similarly low costs in natural contexts. Our results identify a potential bias in standard laboratory estimates of male harm, but they do not provide a direct estimate of harm in nature.

Turning to the broader *Drosophila* literature, our results have implications for how male harm is interpreted. Much of the evidence for strong harm comes from long-term laboratory populations assayed in standard, and hence structurally simple, environments. Our results do not undermine this work, but they do suggest that such studies may overestimate the magnitude, and perhaps generality, of male harm in more natural contexts. It has now been shown that both assay environment and population evolutionary history can affect the expression of harm, and that these effects may be systematic. Importantly, nature-derived *D. melanogaster* males were still harmful in both experiments, so harm is not simply an artefact of lab adaptation, but long-term laboratory populations may exaggerate its expression. Because male harm can influence how mate competition affects fundamental evolutionary processes like adaptation, the purging of

deleterious mutations, selection through females, and possibly even the evolution of reproductive isolation (Yun et al., 2017, 2018; Colpitts et al., 2017; Singh et al., 2017; Barerra et al., 2024), such exaggeration may affect broader inferences about the evolutionary consequences of mate competition.

In conclusion, our results support the concern that long-term laboratory maintenance can bias estimates of male harm in *Drosophila*. Across three independent comparisons in two species, lab-adapted males generally imposed greater costs on females than did recently collected or otherwise nature-derived males, although the expression of harm also depended on female background. Together with previous work showing that harm is sensitive to the physical environment in which sexual interactions occur, these results argue against treating male harm as a fixed property of a species or population. Long-term *Drosophila* laboratory populations have been central to the study of sexual conflict, but they may have led us to overestimate the magnitude, and perhaps the generality, of male harm, at least in this group. Whether similar effects of laboratory history occur in other well-studied systems of male harm is an important question for assessing how generally laboratory studies reflect sexual conflict in nature.

References

Arnqvist, G., & Rowe, L. (2005). *Sexual Conflict*. Princeton University Press.

Byrne, P. G., Rice, G. R., & Rice, W. R. (2008). Effect of a refuge from persistent male courtship in the *Drosophila* laboratory environment. *Integr. Comp. Biol.*, *48*(2), e1. https://doi.org/10.1093/icb/icn001

Cally, J. G., Stuart-Fox, D., & Holman, L. (2019). Meta-analytic evidence that sexual selection improves population fitness. *Nat. Commun.*, *10*(1), 2017. https://doi.org/10.1038/s41467-019-10074-7

Chapman, T., Liddle, L. F., Kalb, J. M., Wolfner, M. F., & Partridge, L. (1995). Cost of mating in *Drosophila melanogaster* females is mediated by male accessory gland products. *Nature*, *373*(6511), 241–244. https://doi.org/10.1038/373241a0

Chippindale, A. K., Gibson, J. R., & Rice, W. R. (2001). Negative genetic correlation for adult fitness between sexes reveals ontogenetic conflict in *Drosophila*. *Proc. Natl. Acad. Sci.*, *98*(4), 1671–1675. https://doi.org/10.1073/pnas.98.4.1671

Colpitts, J., Jarvis, W. M. C., Agrawal, A. F., & Rundle, H. D. (2022). Quantifying male harm and its divergence. *Evolution*, *76*(4), 829–836. https://doi.org/10.1111/evo.14471

Colpitts, J., Williscroft, D., Sekhon, H. S., & Rundle, H. D. (2017). The purging of deleterious mutations in simple and complex mating environments. *Biol. Lett.*, *13*(10), 20170518. https://doi.org/10.1098/rsbl.2017.0518

Edvardsson, M. (2007). Female *Callosobruchus maculatus* mate when they are thirsty: Resource-rich ejaculates as mating effort in a beetle. *Anim. Behav.*, *74*(2), 183–188. https://doi.org/10.1016/j.anbehav.2006.07.018

Fowler, K., & Partridge, L. (1989). A cost of mating in female fruitflies. *Nature*, *338*(6218), 760–761. https://doi.org/10.1038/338760a0

Gomez-Llano, M. A., Bensch, H. M., & Svensson, E. I. (2018). Sexual conflict and ecology: Species composition and male density interact to reduce male mating harassment and increase female survival. *Evolution*, *72*(4), 906–915. https://doi.org/10.1111/evo.13457

Gosden, T. P., & Svensson, E. I. (2007). Female sexual polymorphism and fecundity consequences of male mating harassment in the wild. *PLoS ONE*, *2*(6), e580. https://doi.org/10.1371/journal.pone.0000580

Gosden, T. P., & Svensson, E. I. (2009). Density-dependent male mating harassment, female resistance, and male mimicry. *Am. Nat.*, *173*(6), 709–721. https://doi.org/10.1086/598491

Holland, B., & Rice, W. R. (1999). Experimental removal of sexual selection reverses intersexual antagonistic coevolution and removes a reproductive load. *Proc. Natl. Acad. Sci.*, *96*(9), 5083–5088. https://doi.org/10.1073/pnas.96.9.5083

Johnstone, R. A., & Keller, L. (2000). How males can gain by harming their mates: Sexual conflict, seminal toxins, and the cost of mating. *Am. Nat.*, *156*(4), 368–377. https://doi.org/10.1086/303392

Mackay, T. F. C., Richards, S., Stone, E. A., Barbadilla, A., Ayroles, J. F., Zhu, D., Casillas, S., Han, Y., Magwire, M. M., Cridland, J. M., Richardson, M. F., Anholt, R. R. H., Barrón, M., Bess, C., Blankenburg, K. P., Carbone, M. A., Castellano, D., Chaboub, L., Duncan, L., … Gibbs, R. A. (2012). The *Drosophila melanogaster* Genetic Reference Panel. *Nature*, *482*(7384), 173–178. https://doi.org/10.1038/nature10811

Malek, H. L., & Long, T. A. F. (2019). Spatial environmental complexity mediates sexual conflict and sexual selection in *Drosophila melanogaster*. *Ecol. Evol.*, *9*(5), 2651–2663. https://doi.org/10.1002/ece3.4932

Osijo, K., Agrawal, A. F., & Rundle, H. D. (2026). On the expression of male harm in *Drosophila melanogaster*: Impacts of density and structural complexity of the mating environment. *Evolution*, qpaf245. https://doi.org/10.1093/evolut/qpaf245

Partridge, L., & Fowler, K. (1990). Non-mating costs of exposure to males in female *Drosophila melanogaster*. *J. Insect Physiol.*, *36*(6), 419–425. https://doi.org/10.1016/0022-1910(90)90059-O

R Core Team. (2024). *R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing. https://www.R-project.org/* (Version 4.3.3) [Computer software]. https://www.R-project.org/

Reinhart, M., Carney, T., Clark, A. G., & Fiumera, A. C. (2015). Characterizing male-female interactions using natural genetic variation in *Drosophila melanogaster*. *J. Hered.*, *106*(1), 67–79. https://doi.org/10.1093/jhered/esu076

Rowe, L., Cameron, E., & Day, T. (2005). Escalation, retreat, and female indifference as alternative outcomes of sexually antagonistic coevolution. *Am. Nat.*, *165*(S5), S5-S18.

Rowe, L., & Day, T. (2006). Detecting sexual conflict and sexually antagonistic coevolution. *Phil. Trans. R. Soc. B.*, *361*(1466), 277-285.

Rowe, L., & Rundle, H. D. (2021). The alignment of natural and sexual selection. *Annu. Rev. Ecol. Syst., 52, 499–517*. https://doi.org/10.1146/annurev-ecolsys-012021-033324

Rundle, H. D., Chenoweth, S. F., & Blows, M. W. (2006). The roles of natural and sexual selection during adaptation to a novel environment. *Evolution*, *60*(11), 2218–2225. https://doi.org/10.1111/j.0014-3820.2006.tb01859.x

Rundle, H. D., Chenoweth, S. F., Doughty, P., & Blows, M. W. (2005). Divergent selection and the evolution of signal traits and mating preferences. *PLoS Biol.*, *3*(11), e368. https://doi.org/10.1371/journal.pbio.0030368

Singh, A., & Agrawal, A. F. (2022). Sex-Specific variance in fitness and the efficacy of selection. *Am. Nat.*, *199*(5), 587–602. https://doi.org/10.1086/719015

Singh, A., Agrawal, A. F., & Rundle, H. D. (2017). Environmental complexity and the purging of deleterious alleles. *Evolution*, *71*(11), 2714–2720. https://doi.org/10.1111/evo.13334

Stewart, A. D., Morrow, E. H., & Rice, W. R. (2005). Assessing putative interlocus sexual conflict in *Drosophila melanogaster* using experimental evolution. *Proc. R. Soc. B*, *272*(1576), 2029–2035. https://doi.org/10.1098/rspb.2005.3182

Stocker, H., & Gallant, P. (2008). Getting Started. In C. Dahmann (Ed.), *Drosophila: Methods and Protocols* (pp. 27–44). Humana Press. https://doi.org/10.1007/978-1-59745-583-1_2

Talagala, S., Rakosy, E., & Long, T. A. F. (2024). Sexual selection and the nonrandom union of gametes: Retesting for assortative mating by fitness in *Drosophila melanogaster*. *Evolution*, *78*(1), 26–38. https://doi.org/10.1093/evolut/qpad191

Yun, L., Agrawal, A. F., & Rundle, H. D. (2021). On male harm: How it is measured and how it evolves in different environments. *Am. Nat.*, *198*(2), 219–231. https://doi.org/10.1086/715038

Yun, L., Bayoumi, M., Yang, S., Chen, P. J., Rundle, H. D., & Agrawal, A. F. (2019). Testing for local adaptation in adult male and female fitness among populations evolved under different mate competition regimes. *Evolution*, *73*(8), 1604–1616. https://doi.org/10.1111/evo.13787

Yun, L., Chen, P. J., Kwok, K. E., Angell, C. S., Rundle, H. D., & Agrawal, A. F. (2018). Competition for mates and the improvement of nonsexual fitness. *Proc. Natl. Acad. Sci.*, *115*(26), 6762–6767. https://doi.org/10.1073/pnas.1805435115

Yun, L., Chen, P. J., Singh, A., Agrawal, A. F., & Rundle, H. D. (2017). The physical environment mediates male harm and its effect on selection in females. *Proc. R. Soc. B*, *284*(1858), 20170424. https://doi.org/10.1098/rspb.2017.0424

Zuk, M., Garcia-Gonzalez, F., Herberstein, M. E., & Simmons, L. W. (2014). Model systems, taxonomic bias, and sexual selection: Beyond *Drosophila*. *Annu. Rev. Entomol.*, *59*(Volume 59, 2014), 321–338. https://doi.org/10.1146/annurev-ento-011613-162014

Chapter 4 Supplementary Material

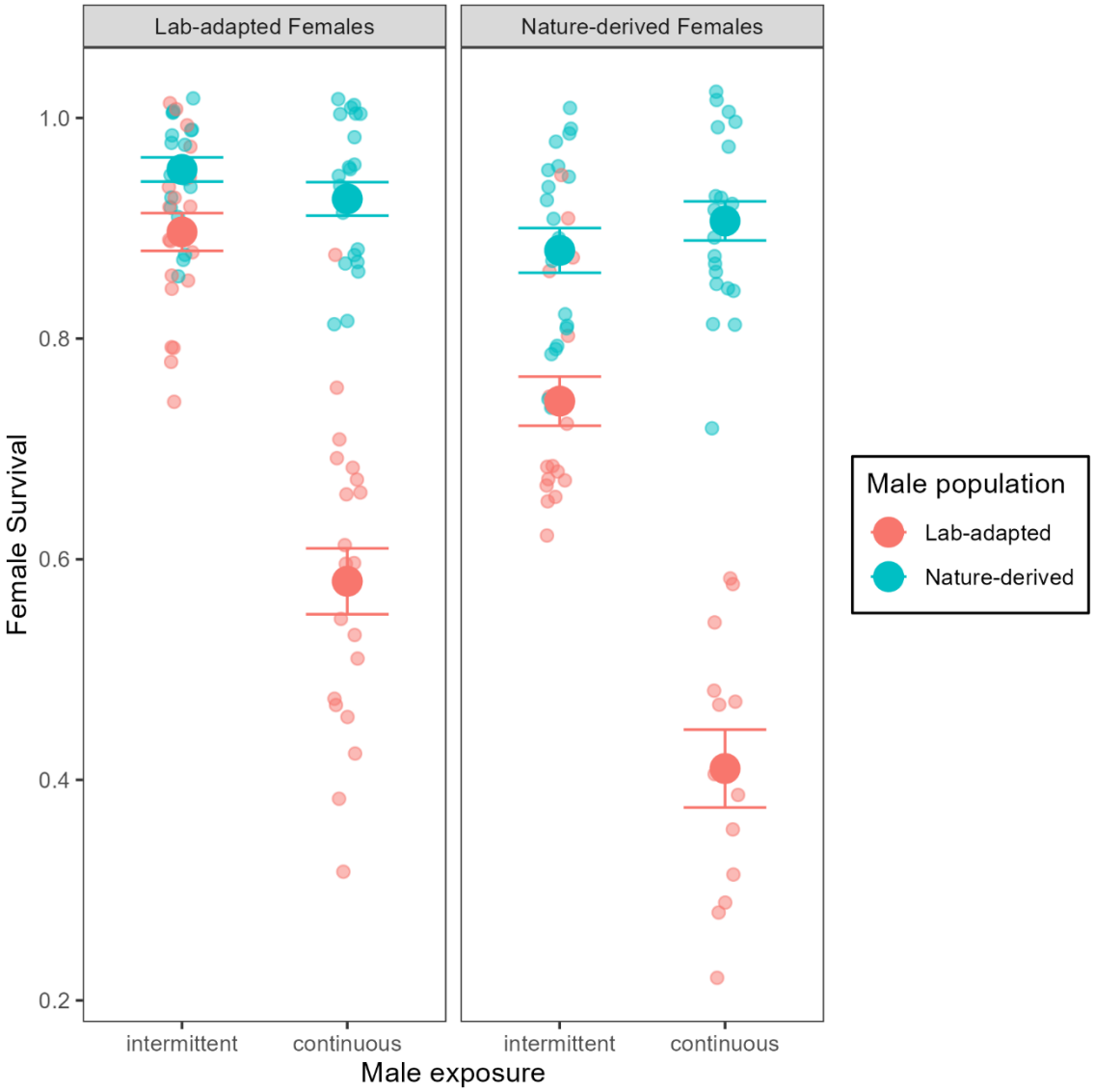

Fig. S4.1. Average survival of females as a function of male exposure (intermittent vs. continuous), male population (lab-adapted vs. nature-derived) and female population (lab-adapted vs. nature-derived) in *D. serrata* (Experiment 1). Large points are means (± SE) of treatment combinations; smaller points are values for individual replicates.

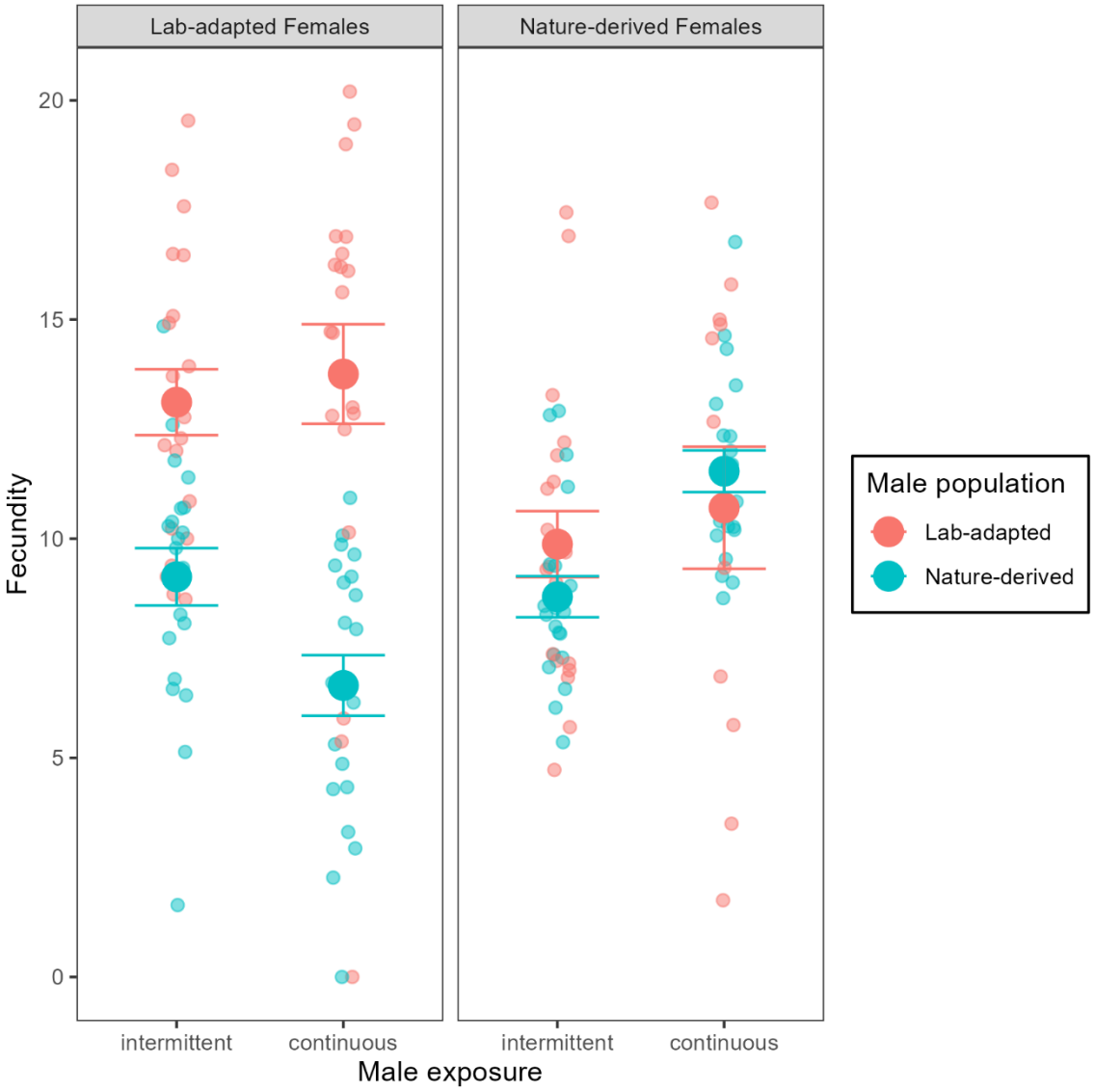

Fig. S4.2. Average fecundity of females as a function of male exposure (intermittent vs. continuous), male population (lab-adapted vs. nature-derived) and female population (lab-adapted vs. nature-derived) in *D. serrata* (Experiment 1). Large points are means (± SE) of treatment combinations; smaller points are values for individual replicates. Fecundity was estimated as the number of adult offspring produced by surviving females.

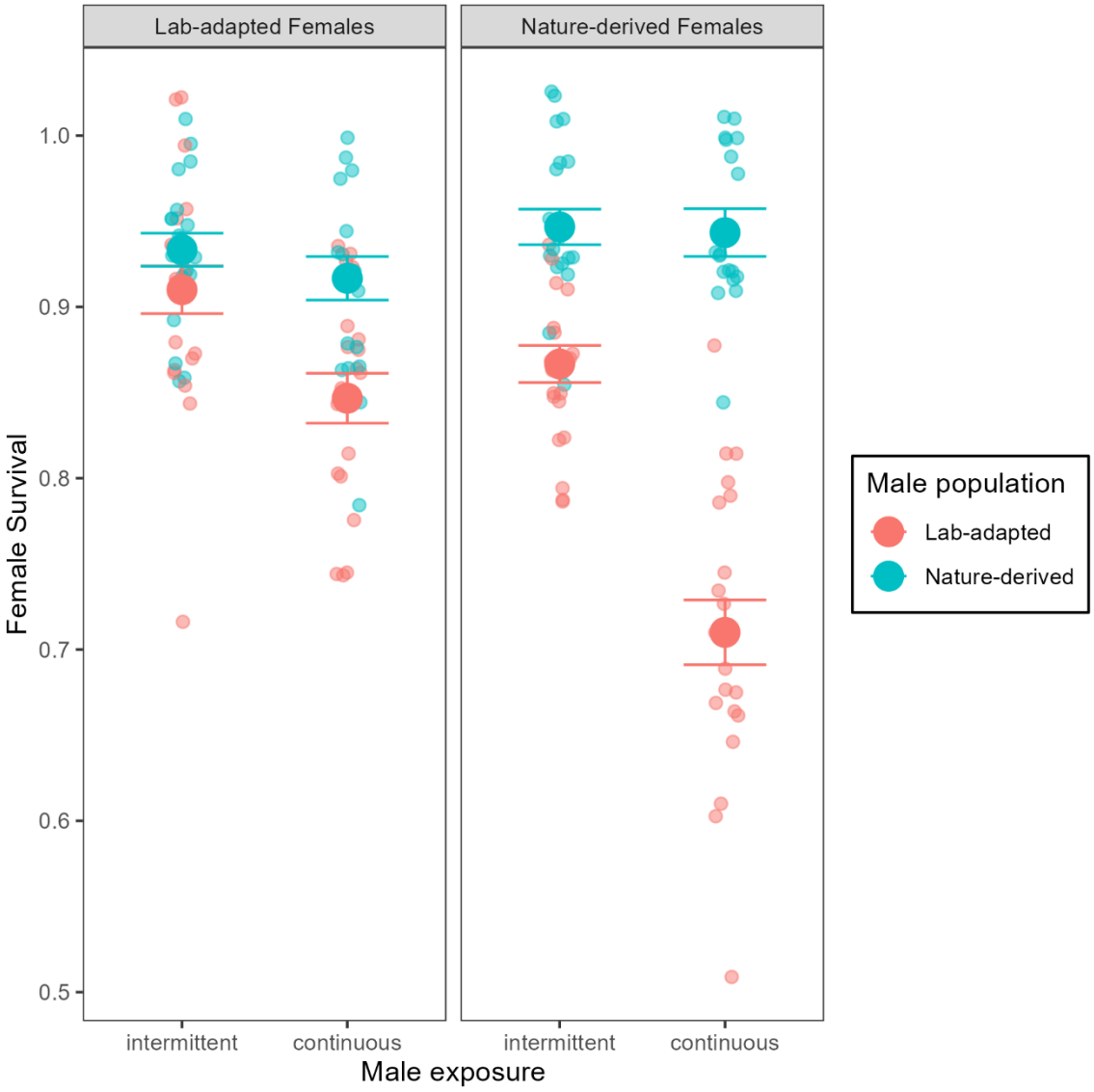

Fig. S4.3. Average survival of females as a function of male exposure (intermittent vs. continuous), male population (lab-adapted vs. nature-derived) and female population (lab-adapted vs. nature-derived) in *D. melanogaster* (Experiment 2). Large points are means (± SE) of treatment combinations; smaller points are values for individual replicates.

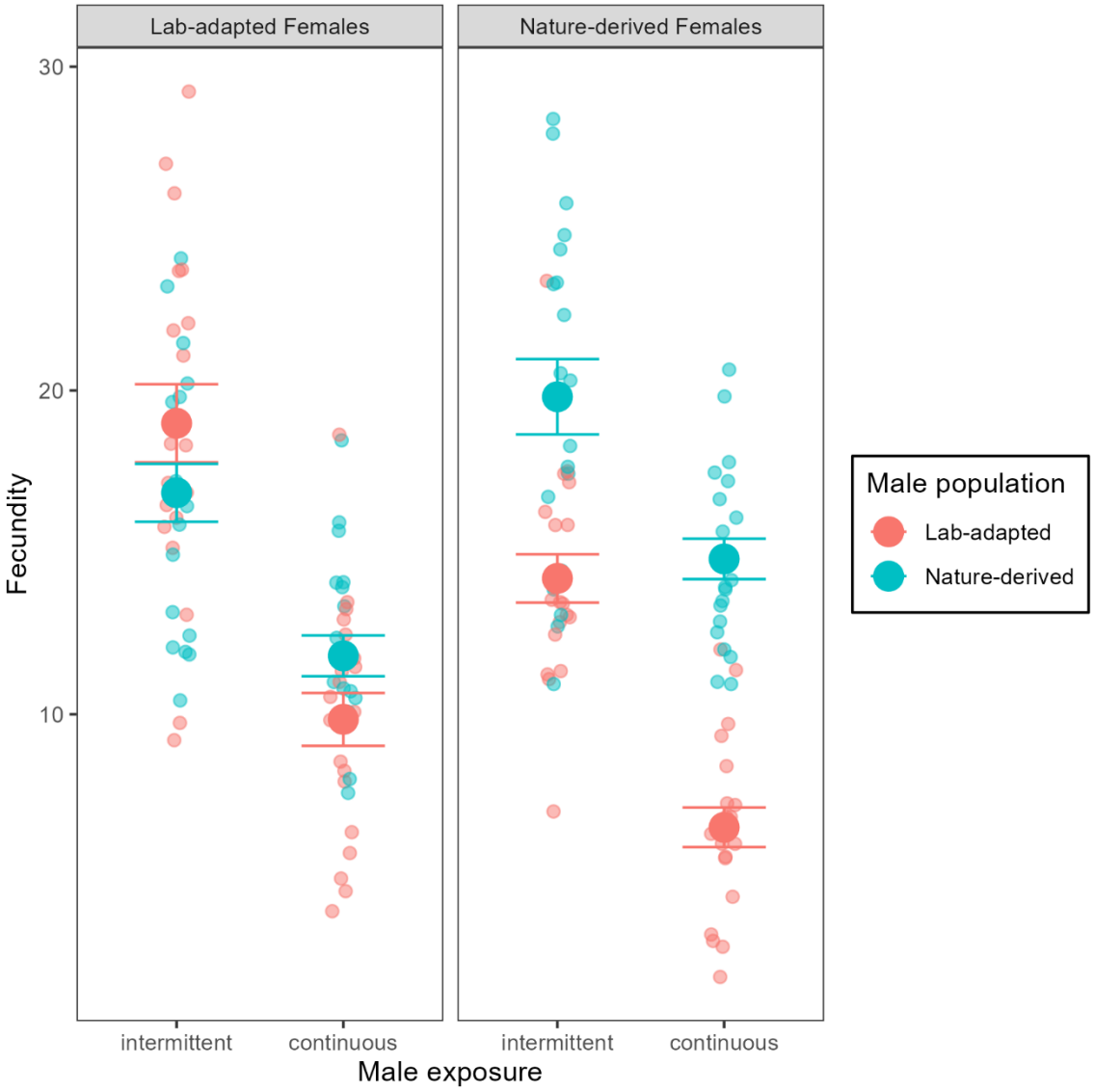

Fig. S4.4. Average fecundity of females as a function of male exposure (intermittent vs. continuous), male population (lab-adapted vs. nature-derived) and female population (lab-adapted vs. nature-derived) in *D. melanogaster* (Experiment 2). Large points are means (± SE) of treatment combinations; smaller points are values for individual replicates. Fecundity was estimated as the number of adult offspring produced by surviving females.

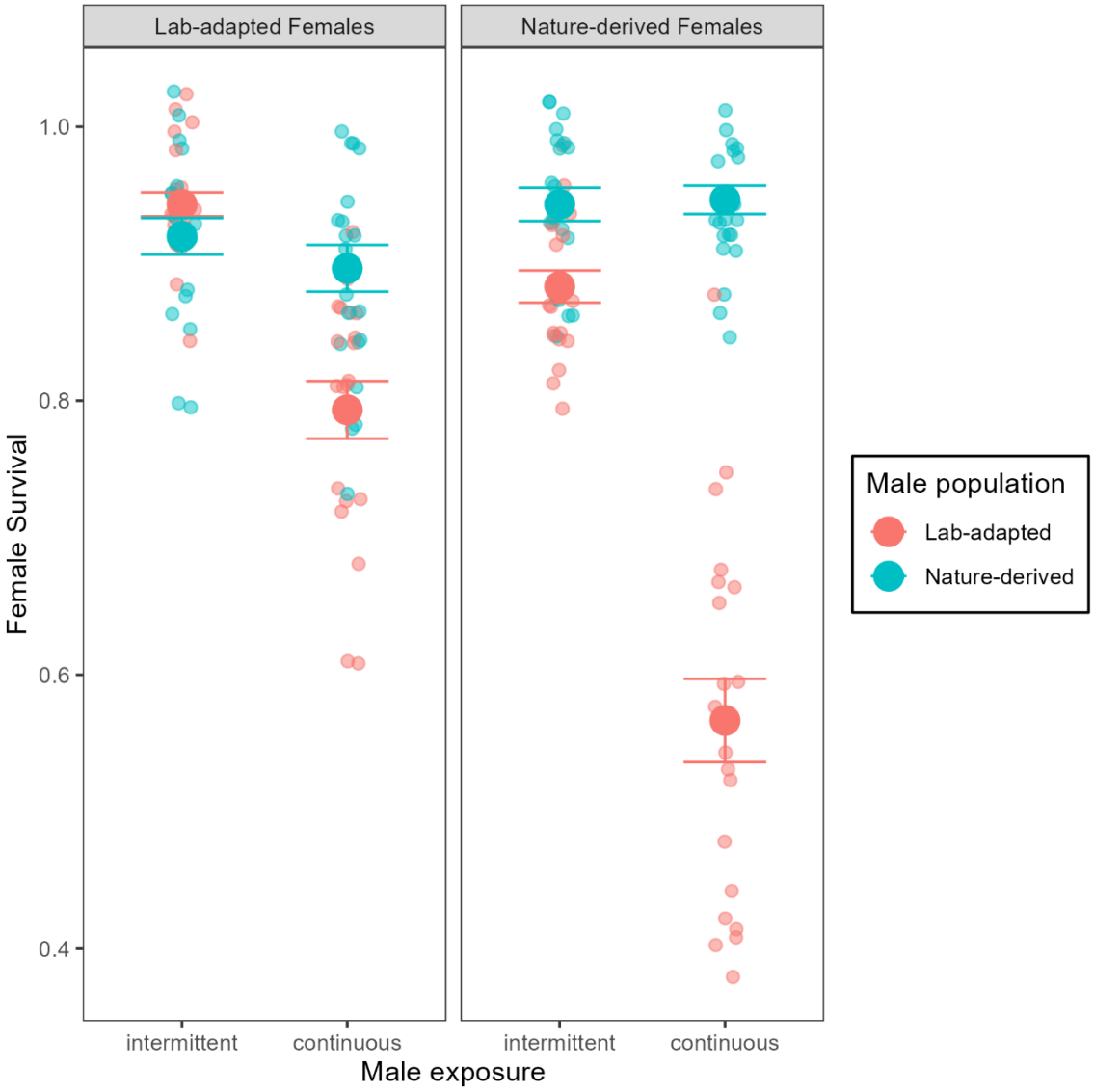

Fig. S4.5. Average survival of females as a function of male exposure (intermittent vs. continuous), male population (lab-adapted vs. nature-derived) and female population (lab-adapted vs. nature-derived) in *D. melanogaster* (Experiment 3). The nature-derived population was a reconstituted sample of genetic variation from the wild created by pooling a set of DGRP lines (see Methods). Large points are means (± SE) of treatment combinations; smaller points are values for individual replicates.

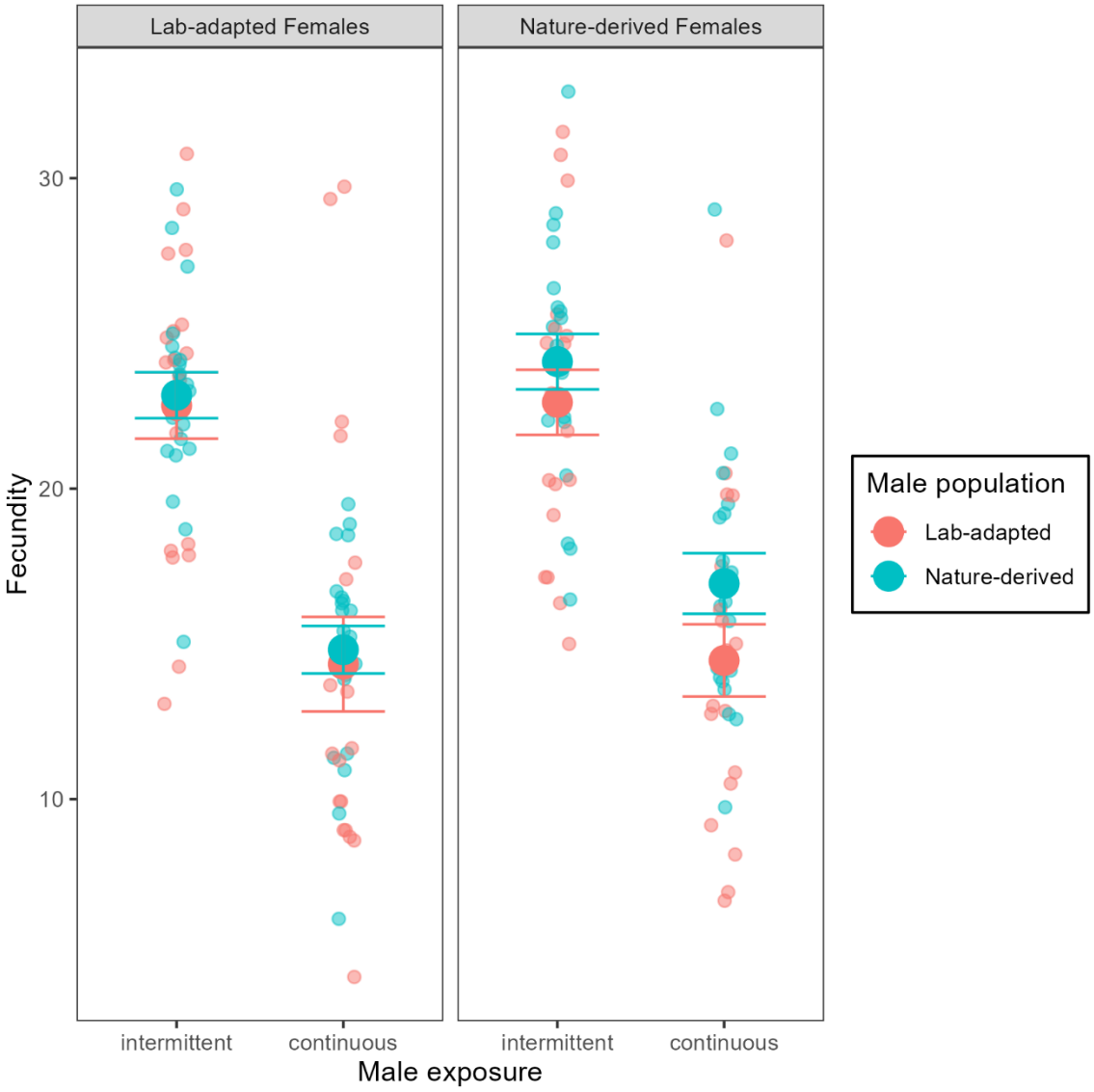

Fig. S4.6. Average fecundity of females as a function of male exposure (intermittent vs. continuous), male population (lab-adapted vs. nature-derived) and female population (lab-adapted vs. nature-derived) in *D. melanogaster* (Experiment 3). The nature-derived population was a reconstituted sample of genetic variation from the wild created by pooling a set of DGRP lines (see Methods). Large points are means (± SE) of treatment combinations; smaller points are values for individual replicates. Fecundity was estimated as the number of adult offspring produced by surviving females.

Chapter 5: General Discussion

My thesis examined how mating environment and population evolutionary history shape selection through males and the expression of male harm in *Drosophila*. These processes are important because they can influence whether mate competition contributes to adaptation and purging, generates sexual conflict, or both. These topics are connected by a broader question: why does mate competition sometimes appear to promote population-level benefits, such as adaptation and purging, while in other cases it generates costs through sexual conflict and male harm? In this chapter, I summarize the findings of the three data chapters and then discuss what they suggest about the roles of mating environment, population history, and laboratory context in shaping the evolutionary consequences of mate competition.

Chapter 2 asked whether the complex mating environment, previously associated with faster adaptation and purging, also imposes stronger selection through males. The results showed strong selection against inbred males, but did not clearly demonstrate that selection was stronger in the complex environment, although results of the first experiment approached significance. This suggests that selection through males may contribute to adaptation and purging in complex environments, but this thesis does not resolve this. There are additional processes that could contribute to differences in adaptation and purging by mating environment, including altered selection through females generated by differences in male harm, and assortative mating by fitness. There is evidence that differences in male harm caused natural selection on females to be stronger in the complex environment (Yun et al., 2017), while assortative mating by fitness has

not been investigated. Future work could address whether complex environments strengthen selection through males using greater replication, direct competition between inbred and outbred males, and a marker system with smaller fitness effects. It would also be useful to test whether assortative mating by fitness varies by mating environment.

Chapter 3 followed up on earlier work showing that male harm was reduced in more complex environments (Yun et al., 2017) by asking which feature of complexity matters most. The main conclusion is that structural complexity, rather than density alone, appears to be the important factor reducing male harm. This suggests that complex environments improve female outcomes not simply because flies are less crowded, but because structural complexity changes male-female interactions — for example, by allowing females to avoid or reduce persistent male attention (Byrne et al., 2008). Still, it is quite possible that, at certain densities, structural complexity and density may interact and investigating this is a potential area of interest for future research. Also, in our set up, structural complexity had two major components: pipe cleaners, and multiple food/egg laying resources. It may be possible to attempt to further partition these factors to determine their effects on the expression of male harm in these environments.

Chapter 4 asked whether males from long-term lab populations are more harmful than recently collected or otherwise nature-derived males. Across three population pairs in two species, increased male exposure generally reduced female fitness, in line with other *Drosophila* studies (Fowler & Partridge, 1989; Partridge & Fowler, 1990; Chapman et al., 1995; Kamimura, 2007) and

confirming that male harm was present under the assay conditions. Lab-adapted males generally imposed greater costs on females than nature-derived males, especially in the two *D. melanogaster* comparisons. These findings suggest that male harm is not simply a lab artefact, but long-term lab populations, especially those maintained under highly simplified conditions, may exaggerate its magnitude. An interesting result from this chapter is that the expression of male harm sometimes depended on female background, most clearly in *D. serrata*, where nature-derived males harmed lab-adapted females but not nature-derived females. This matters because it suggests that male harm is not simply a property of males, but depends on the specific male–female combination involved, potentially reflecting differences in female susceptibility, resistance, or recent coevolutionary history. Future work could explore this by comparing a broader range of male and female population combinations, and determining the behavioural and physiological mechanisms of harm.

Taken together, the three data chapters suggest that the consequences of mate competition can depend on mating environment, female background, and population evolutionary history. The physical environment can alter how males and females interact, changing the expression of male harm and potentially the strength of selection through males (Yun et al., 2017; 2018). Population evolutionary history can also influence the traits that mediate male-female interactions, and hence the costs females experience, as shown by previous work on the evolution of male harm in different mating environments (Yun et al., 2021) and by the population-history effects documented in Chapter 4. Thus, mate competition should not be expected to have a single evolutionary outcome across environments or populations.

A broader question raised by all three data chapters is how general these effects are likely to be. One aspect of this is taxonomic: my experiments focused on *Drosophila*, but mating environments may also influence sexual selection, conflict, and male harm in other species, especially where females vary in their ability to avoid or control sexual interactions. Exploring these effects in other model systems is an important avenue for future work. A second aspect concerns environmental complexity: there are many axes to complexity and hence many ways that two environments may differ. The environments used here, and in the earlier studies motivating my work, differ from standard laboratory environments in several ways, including size, density, spatial structure, resource distribution, and the availability of potential refuges. Understanding how these features alter sexual interactions mechanistically — for example, by changing encounter rates, courtship, harassment, mating frequency, female avoidance, and/or male–male competition — will be important for predicting how other environmental differences may impact harm and selection through males (e.g., see Malek and Long, 2019; Talagala et al., 2024. This, in turn, may help determine when and how environmental differences might influence broader evolutionary processes such as adaptation and purging.

These issues of context and generality have several broader implications. First, with respect to sexual conflict, male harm should not be treated as a fixed property of a species or population. It depends on the physical environment (shown in Chapter 3), male traits, female background, and population history (shown in Chapter 4). Regarding adaptation and purging, my thesis suggests

that the benefits of mate competition in complex environments may involve reduced male harm and altered selection through females, not necessarily only stronger selection through males as traditionally thought (Chapter 2). Regarding experimental design, standard *Drosophila* vial assays are useful, but they represent a highly simplified context; future studies of sexual conflict should consider environmental structure, density, population history, and female background.

Several limitations of this thesis point to useful directions for future work. First, Chapter 2 did not resolve whether complex mating environments strengthen selection through males. Although selection against inbred males was strong, the evidence for stronger selection in the complex environment was inconclusive. As noted above, future work could address this via better replication, direct competition between inbred and outbred males, and by using a marker with smaller fitness effect. Second, Chapter 3 separated density and structural complexity, but there are many more axes by which natural environments differ from lab environments. Subsequent work could explore more of these dimensions of structural complexity, and explore how they alter the expression of male harm to see if generalities exist. Finally, Chapter 4 is consistent with lab adaptation increasing male harm, but because lab-adapted and nature-derived populations differed in many aspects, a direct experimental-evolution test would provide additional evidence to establish causation. Also, I assayed harm under lab conditions, as it is difficult to conceive how one would measure it in nature. Nevertheless, further work could seek to examine the expression of harm in more natural environments.

In conclusion, my thesis shows that the consequences of mate competition can depend strongly on context. Building on previous work showing that mate competition can affect adaptation and purging, my results highlight how the physical mating environment and population history potentially shape selection through males and the expression of male harm. While Chapter 2 did not resolve whether complex environments strengthen selection through males, Chapters 3 and 4 show that male harm depends strongly on structural complexity and population evolutionary history. These findings suggest that mate competition cannot be understood independently of the environments in which mating occurs or the populations being studied.

References

Byrne, P. G., Rice, G. R., & Rice, W. R. (2008). Effect of a refuge from persistent male courtship in the *Drosophila* laboratory environment. *Integr. Comp. Biol.*, *48*(2), e1. https://doi.org/10.1093/icb/icn001

Cally, J. G., Stuart-Fox, D., & Holman, L. (2019). Meta-analytic evidence that sexual selection improves population fitness. *Nat. Commun.*, *10*(1), 2017. https://doi.org/10.1038/s41467-019-10074-7

Chapman, T., Liddle, L. F., Kalb, J. M., Wolfner, M. F., & Partridge, L. (1995). Cost of mating in *Drosophila melanogaster* females is mediated by male accessory gland products. *Nature*, *373*(6511), 241–244. https://doi.org/10.1038/373241a0

Fowler, K., & Partridge, L. (1989). A cost of mating in female fruitflies. *Nature*, *338*(6218), 760–761. https://doi.org/10.1038/338760a0

Kamimura, Y. (2007). Twin intromittent organs of *Drosophila* for traumatic insemination. *Biol. Lett.*, *3*(4), 401–404. https://doi.org/10.1098/rsbl.2007.0192

Malek, H. L., & Long, T. A. F. (2019). Spatial environmental complexity mediates sexual conflict and sexual selection in *Drosophila melanogaster*. *Ecol. Evol.*, *9*(5), 2651–2663. https://doi.org/10.1002/ece3.4932

Partridge, L., & Fowler, K. (1990). Non-mating costs of exposure to males in female *Drosophila melanogaster*. *J. Insect Physiol.*, *36*(6), 419–425. https://doi.org/10.1016/0022-1910(90)90059-O

Talagala, S., Rakosy, E., & Long, T. A. F. (2024). Sexual selection and the nonrandom union of gametes: Retesting for assortative mating by fitness in *Drosophila melanogaster*. *Evolution*, *78*(1), 26–38. https://doi.org/10.1093/evolut/qpad191

Yun, L., Agrawal, A. F., & Rundle, H. D. (2021). On Male Harm: How It Is Measured and How It Evolves in Different Environments. *Am. Nat.*, *198*(2), 219–231. https://doi.org/10.1086/715038

Yun, L., Chen, P. J., Kwok, K. E., Angell, C. S., Rundle, H. D., & Agrawal, A. F. (2018). Competition for mates and the improvement of nonsexual fitness. *Proc. Natl. Acad. Sci.*, *115*(26), 6762–6767. https://doi.org/10.1073/pnas.1805435115

Yun, L., Chen, P. J., Singh, A., Agrawal, A. F., & Rundle, H. D. (2017). The physical environment mediates male harm and its effect on selection in females. *Proc. R. Soc. B*, *284*(1858), 20170424. https://doi.org/10.1098/rspb.2017.0424