Experimental Studies of the Divergence of Pre- and Postcopulatory Phenotypes in Male *Drosophila*

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ABSTRACT

A major focus in biology is understanding the diversification of life and the processes that cause it. Much of this diversity is in the form of phenotypic variation among populations and species. In this thesis, I investigate two separate aspects of such phenotypic divergence. The first is the divergence of male mate preferences and their potential contribution to precopulatory sexual isolation and speciation. The second is the divergence of postcopulatory phenotypic divergence in the form of seminal fluid protein expression.

With respect to the first aspect, in two separate experiments I investigated the contribution of male mate preferences to sexual isolation between two closely related fruit fly species experiencing differential costs to hybridization, *Drosophila recens* and *Drosophila subquinaria*. Male mate preferences are of particular interest because of their potential contribution to sexual isolation, a form of reproductive isolation which can contribute to speciation in sexually reproducing species. In the first experiment, I test for the presence of male mate preferences in each of the two species and whether the relative strength of the preference is concordant with the cost of hybridization. I found that that *D. subquinaria* males indiscriminately courted both their own (i.e. homospecific) females and heterospecific *D. recens* females. While *D. recens* from allopatry showed a similar pattern, those from sympatry courted their own females more than heterospecific females, indicating a pattern of reproductive character displacement. In the second experiment I test the role of learning in the context of these male mate preference in *D. recens,* and whether learning also showed a pattern of reproductive characteristic. I did not find evidence of learning in that *D. recens* males did not reduce their courting intensity towards heterospecific females after experiencing rejection by similar females. Consequently, I did not find an indication of reproductive character displacement.

Finally, with respect to postcopulatory phenotypic divergence, I studied differences in seminal fluid protein expression between experimental populations of *D. melanogaster* experiencing one of three mating environments allowing for differing opportunities of mate competition and the environment in which it took place. These three mating environments include one in which mate competition was absent ($MC_{absent,}$), one in which mate competition occurred in a small, structurally simple environment (MC_{simple}), and one in which mate competition occurred in a larger, somewhat more complex environment ($MC_{complex,}$). Male seminal fluids are of particular interest due to their ability to mediate postcopulatory competition between males and, therefore, can be used to manipulate females to a male's own fitness benefit, potentially at her expense (i.e. sexual conflict). I investigated divergence in one particular seminal fluid protein implicated in sexual conflict, sex peptide (Acp70A). Whereas, gene expression levels among males from the three-mating treatment did not differ on average, relative stored quantities did, with $MC_{complex}$ males carrying significantly less sex peptide than either of MC_{absent} or MC_{simple} males (which did not differ from one another). This result suggests that mate competition and the environment in which it occurs play a significant role in the divergence of sex peptide phenotypes.

ABSTRAIT

Un objectif majeur de la biologie est de comprendre la diversification de la vie et les processus qui la provoquent. Une grande partie de cette diversité se présente sous la forme de variations phénotypiques entre les populations et les espèces. Dans cette thèse, j'étudie deux aspects distincts d'une telle divergence phénotypique. Le premier est la divergence des préférences des mâles et leurs contributions potentielles à l'isolement sexuel pré-copulatoire et à la spéciation. Le second est la différence de la divergence phénotypique post-copulatoire sous la forme de l'expression des protéines du liquide séminal.

En ce qui concerne le premier aspect, dans deux expériences distinctes, j'ai étudié la contribution des préférences de compagnon mâle à l'isolement sexuel entre deux espèces de mouches des fruits étroitement liées subissant des coûts différentiels d'hybridation, *Drosophila recens* et *Drosophila subquinaria*. Les préférences des mâles sont particulièrement intéressantes en raison de leurs contributions potentielles à l'isolement sexuel, une forme d'isolement reproductif qui peut contribuer à la spéciation des espèces se reproduisant sexuellement. Dans la première expérience, je teste la présence de préférences de compagnon mâle dans chacune des deux espèces et si la force relative de la préférence est concordante avec le coût de l'hybridation. J'ai constaté que les mâles de *D. subquinaria* courtisaient sans discernement à la fois leurs propres femelles (c'est-à-dire homospécifiques) et les femelles hétérospécifiques de *D. recens*. Alors que *D. recens* de l'allopatrie a montré un modèle similaire, ceux de la sympatrie courtisaient leurs propres femelles plus que les femelles hétérospécifiques, indiquant un modèle de déplacement du caractère reproducteur. Dans la deuxième expérience, je teste le rôle de l'apprentissage dans le contexte de ces préférences de compagnon masculin dans *D. recens*, et si l'apprentissage a également montré un modèle de caractéristique de reproduction. Je n'ai pas

trouvé de preuve d'apprentissage dans la mesure où les mâles *D. recens* ne réduisaient pas leur intensité de fréquentation envers les femelles hétérospécifiques après avoir été rejetés par des femelles similaires. Par conséquent, je n'ai pas trouvé d'indication de déplacement du caractère reproducteur.

Enfin, en ce qui concerne la divergence phénotypique post-copulatoire, j'ai étudié les différences dans l'expression des protéines du liquide séminal entre les populations expérimentales de *D. melanogaster* connaissant l'un des trois environnements d'accouplement, permettant différentes possibilités de compétition de compagnon et l'environnement dans lequel elle a eu lieu. Ces trois environnements d'accouplement incluent un environnement dans lequel la compétition entre partenaires était absente ($MC_{absent,}$), un dans lequel la compétition entre partenaires se produisait dans un petit environnement structurellement simple (MC_{simple}) et un dans lequel la compétition entre partenaires se produisait dans un environnement plus grand et un peu plus complexe ($MC_{complexe,}$). Les fluides séminaux mâles sont particulièrement intéressants en raison de leur capacité à négocier la compétition post-copulatoire entre les mâles et, par conséquent, peuvent être utilisés pour manipuler les femelles dans l'intérêt de la forme physique d'un mâle, potentiellement à ses dépens (c'est-à-dire conflit sexuel). J'ai étudié la divergence dans une protéine du liquide séminal particulière impliquée dans un conflit sexuel, le peptide sexuel (Acp70A). Alors que les niveaux d'expression génique chez les mâles du traitement à trois accouplements ne différaient pas en moyenne, les quantités relatives stockées le faisaient, les mâles $MC_{complexe}$ portant significativement moins de peptide sexuel que les mâles MC_{absent} ou MC_{simple} (qui ne différaient pas les uns des autres). Ce résultat suggère que la compétition de partenaire et l'environnement dans lequel elle se produit jouent un rôle important dans la divergence des phénotypes des peptides sexuels.

PREFACE

Chapter 2 is a modified version of the following submitted paper, co-authored by Dr. Kelly A. Dyer (Department of Genetics, University of Georgia) and Dr. Howard D. Rundle (Department of Biology, University of Ottawa), which is currently under review at the journal *Evolution*. Dr. Dyer collected and identified the flies (except from Algonquin Park). Chris Angell collected the flies from Algonquin Park. All authors contributed to experimental design and interpretation of results. I performed the experiments and, together with Dr. Rundle, analyzed the data. Dr. Rundle and I wrote the manuscript with input from Dr. Dyer.

A subset of the same flies collected by Dr. Dyer and Chris Angell and used in Chapter 2 were used in the work of Chapter 3. Dr. Reuven Dukas collected and identified the *D. simulans* flies. The *D. melanogaster* population was maintained by Dr. Mathieu Videlier. I performed the experiments and, together with Dr. Rundle, analyzed the data and interpreted the results.

The evolved *D. melanogaster* populations used in Chapter 4 were created and maintained by Li Yun. Dr. J. Rouhana prepared the antibody-capture oligonucleotide-signal oligonucleotide complex described in Appendix 1, and developed the original Immuno-Q-PCR protocol. I reoptimized and modified Dr. Rouhana's Immuno-Q-PCR protocol and performed the experiment. Together with Dr. Rundle, I analyzed the data and interpreted the results with input from Dr. Tracey Chapman and Dr. Aneil Agrawal.

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Dedication

This thesis is dedicated to a good man

Dr. Howard D. Rundle

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I would like to acknowledge that this Ph.D. was completed at an institution built on the unceded territory of the Algonquin people. In particular, I would like to thank John Ward and Brenda Macdougall for their insight and guidance.

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And to Allyssa Haight.

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Chapter 1

General Background

A key objective in evolutionary biology is to understand the processes underlying the origins of biological diversity. A major component of this diversity is the phenotypic differences that exist among populations and species. My thesis aims to address two aspects of this. The first is the divergence of male mate preferences that may contribute to sexual isolation and hence speciation (the process by which new species arise). The second is the more cryptic aspect of divergence of post-copulatory phenotypes in the form of male seminal fluid protein expression.

My research addresses the first aspect via two separate experiments that both use replicate populations of two native N. America species of *Drosophila*: *D. subquinaria* and *D. recens*. In the first experiment I test for the existence of, and divergence in, male mate preferences, and in the second experiment I explore the role of learning in male mate preferences and I test for divergence in this. Male mate preferences are of interest as they can contribute to sexual isolation, a form of reproductive isolation that is thought to play a central role in speciation and hence the origins and maintenance of biological diversity (Coyne & Orr 2004).

With respect to the divergence in the expression of a male seminal fluid protein, my work uses a set of experimental *D. melanogaster* populations that have evolved while experiencing different mating treatments (i.e. opportunities for mate competition and the environment in which this occurs). Male seminal fluid proteins are of interests because they can mediate postcopulatory competition among males for fertilizing a female’s eggs, and they are a means by which males can potentially manipulate females to the male’s advantage. This may, however, come at a cost to the female, thereby generating the potential for sexual conflict. I measure the relative expression of a seminal fluid protein implicated in sexual conflict, sex peptide (Acp70A), between environments offering varying opportunities for mate competition.

Below I provide some general background with respect to the first aspect of my thesis concerning male mating preferences, sexual isolation, and the *D. subquinaria – D. recens* species pair, because this is common to the subsequent two experimental chapters. Background concerning male seminal fluid proteins and their effects on females, including the potential for sexual conflict, is presented in Chapter 4 as it is specific to that study.

Male Mating Preferences in *D. subquinaria* and *D. recens*

Mayr's (1942) "Biological Species Concept" defines a species as a group of actually or potentially interbreeding populations that are reproductively isolated from other groups. Though this definition only applies to sexually reproducing species, it has the advantage of providing a straightforward research approach to studying speciation in sexually reproducing organisms by focussing effort on understanding the origins of reproductive isolation (Coyne & Orr, 2004). Reproductive isolation can be classified based on when isolation occurs: either before or after mating (i.e. pre- and postmating isolation), or before or after the formation of the zygote (i.e. pre- and postzygotic isolation). Sexual isolation (sometimes termed 'behavioural isolation') occurs when behavioural differences cause individuals from different populations to be less attracted to one another as potential mates, thereby reducing or completely eliminating courtship and/or copulation between them. Sexual isolation is thought to arise from the divergence of sexual signals or displays, and mate preferences for them (Coyne & Orr, 2004). Sexual isolation can evolve as a by-product of divergent natural selection adapting populations to different environments or niches (i.e. "ecological speciation"; Schluter 2009), or due to differences between populations in the identity and/or order of new mutations that cause them to follow unique evolutionary trajectories (i.e. mutation-order speciation; Schluter, 2009). Differences in sexual selection between populations is sometimes cited as a third mechanism of speciation, but divergence in such models is due ultimately to ecological or mutation-order processes (Schluter, 2000; Rundle & Rowe 2018)

I focus here on sexual isolation because evidence suggests that its evolution is important to both the early and late stages of the speciation process (Coyne & Orr 2004). With respect to the initiation of speciation, studies indicate that sexual isolation tends to evolve early and its presence will reduce gene flow, permitting the evolution of other barriers (i.e. genetic incompatibilities) that may eventually complete speciation (Coyne & Orr 1989; 1997; 2004; Kawata & Yoshimura 2000). With respect to the late stages, much attention has been given to the hypothesis of reinforcement. Reinforcement is the process by which sexual isolation is strengthened between sympatric populations in response to reduced hybrid fitness (i.e. partial or complete postmating isolation; Paterson, 1982; Harper & Lambert, 1983). The idea is that if hybrids are of lower fitness than homospecific offspring, individuals that avoid heterospecific mating will tend to leave more fit offspring. Therefore, traits that decrease the likelihood of heterospecific mating will be favoured by selection and will spread throughout the population, thereby reinforcing existing reproductive isolation (Butlin, 1987; Coyne & Orr, 2004; Hoskin et al., 2005). If populations of either species exist in allopatry, then the result may be a pattern of reproductive character displacement whereby the strength of sexual isolation, and the traits underlying it, are more divergent in sympatry than in allopatry (Coyne & Orr, 2004; Rice & Pfennig, 2010; Rundle & Dyer, 2015).

There is growing evidence that suggests that reinforcement may be common in the latter stages of speciation (Butlin, 1987; Howard, 1993; Rundle & Schluter, 1998; Coyne & Orr, 2004; Ortiz-Barrientos et al., 2009). In nature, reinforcement reduces hybridization when incipient species come into secondary contact. The importance in emphasizing the development of pre-zygotic isolation as a driving force for speciation is illustrated by Coyne & Orr (1989, 1997) in which they perform a comprehensive examination of the time course of speciation in *Drosophila* as it relates to pre- and post-zygotic reproductive isolation. One major finding in this study was that strong mate discrimination behaviours evolved prior to offspring sterility or inviability in sympatry, but not in allopatry. This suggests that sexual isolation may be reinforced when allopatric populations become sympatric.

While reinforcement is commonly thought of as a mechanism for the evolution of sexual isolation late in speciation, a role for reinforcement in initiating speciation has also been more recently suggested. This idea, which is referred to as “cascade reinforcement”, is that incipient sexual isolation may arise between populations within a species as a by-product of reinforcing selection with another species (Howard 1993, Humphreys et al., 2016). Consider the case of partial secondary contact between two incipient species such that the region of sympatry does not encompass the entire range of at least one of the two species, leaving some populations in allopatry. Reinforcing selection in sympatry may cause the traits underlying sexual isolation to evolve and thus diverge between sympatric and allopatric populations of a given species, creating a pattern of reproductive character displacement. As a side effect of this evolution in sympatry, sexual isolation may arise between these individuals and their own allopatric populations (Ortiz-Barrientos et al., 2009; Hoskin & Higgie, 2010; Bewick & Dyer, 2014). Unlike classic reinforcement, this sexual isolation can arise even in the absence of any postmating isolation between the populations because the source of selection is the reduced fitness of the between-species hybrids. Several studies suggest that cascade reinforcement can occur in nature (Jaenike et al., 2006; Hoskin et al., 2005; Kozak et al., 2015; Humphreys et al., 2016), although its frequency is unclear.

As discussed above, while much progress has been made in understanding the evolution of sexual isolation in nature, there are various mechanisms by which this can occur. In fact, multiple mechanisms are likely to be important, and potentially interact, in any given situation. There are therefore few species for which we can claim to have a reasonably comprehensive understanding of how sexual isolation between them has evolved. With respect to reinforcement in particular, while evidence strongly suggests that it occurs, the majority of studies focus on male sexual traits and female preferences for them; with less attention given to male mate choice and the role of learning in this.

Drosophila subquinaria and *Drosophila recens* are one example of a species-pair in which past and ongoing work has provided important insight into the evolution of sexual isolation, but for which many unanswered questions remain. Both species are generalist feeders on *basidiomycete* mushrooms and they are morphologically identical with the exception of internal male genitalia (Wheeler 1960). In sympatry, they have been observed on the same mushroom at the same time. *D. subquinaria* and *D. recens* occur in western and eastern North America respectively, with an approximately 1200 km wide region of geographical overlap from western Alberta to western Manitoba (Jaenike et al. 2006). Their current ranges suggest that the species were geographically isolated during the last glacial maximum and have since come into secondary contact at the end of the Wisconsin glaciation within the last 12,000 years.
These species can produce viable hybrid offspring, indicating incomplete reproductive isolation (both pre- and postmating). However, the resulting offspring suffer from cytoplasmic incompatibility (CI) due to wide-spread *Wolbachia*-infection in *D. recens*. Due to the

asymmetric effect of this bacterial infection, in a female *D. subquinaria* × male *D. recens* cross there is an approximately 90% reduction in the number of offspring produced (Shoemaker et al. 1999), whereas in the reciprocal cross (i.e. female *D. recens* × male *D. subquinaria*), *Wolbachia*-infected hybrids are produced with no CI-induced mortality. Female hybrids that survive from this cross are viable and able to mate with either species, although the heterogametic male hybrids of crosses in both directions are sterile (Shoemaker *et al*., 1999), thereby adhering to Haldane's Rule (Haldane, 1922; Coyne & Orr, 2004). For this reason, hybridizing results in substantial fitness loss for parents of both sexes in both species, although the effects are much more severe for *D. subquinaria* females than *D. recens* females, and for *D. recens* males relative to *D. subquinaria* males.

The existence of postmating isolation may generate selection to reinforce premating isolation in sympatry in response to the creation of low fitness hybrids. In these species, attention to date has focused largely on sexual isolation via female mate choice. Consistent with reinforcement, a pattern of reproductive character displacement has been observed in which *D. subquinaria* females from sympatry discriminate against mating with *D. recens* males more strongly than do *D. subquinaria* from allopatry (Shoemaker et al. 1999; Jaenike et al. 2006; Giglio & Dyer 2013; Curtis et al. 2013). Interestingly, a similar pattern of reproductive character displacement between sympatric and allopatric *D. recens* has not been observed. Instead, an intermediate level of mating discrimination against *D. subquinaria* males has been observed in both sympatric and allopatric *D. recens* populations (Jaenike *et al*., 2006).

In addition to increased mating discrimination against male *D. recens*, sympatric female *D. subquinaria* also discriminate against mating with their own (*i.e*. *D. subquinaria*) males from allopatry, even when presented with no other mating options (Jaenike *et al*., 2006). This mating discrimination behaviour has not been observed in allopatric females (*i.e*. allopatric *D. subquinaria* females readily mate with both sympatric and allopatric *D. subquinaria* males), suggesting that selection to avoid mating with *D. recens* has altered mate preferences in sympatric *D. subquinaria* females. Jaenike *et al*. (2006) did not detect evidence of any postzygotic isolation in the F1 or F2 generation offspring of allopatric × sympatric *D. subquinaria* crosses. This suggests that incipient sexual isolation has evolved solely as a by-product of reinforced isolation against *D. recens*, and that this may be initiating a secondary speciation event between sympatric and allopatric *D. subquinaria* populations via “cascade reinforcement” (Jaenike *et al*., 2006; Kozak *et al*., 2015; Hoskin *et al*., 2005). A detailed understanding of the nature and evolution of sexual isolation in these species may therefore shed light on both the early and late stages of separate speciation events (*i.e*. between sympatric and allopatric populations within *D. subquinaria*, and between *D. subquinaria* and *D. recens*).

At least some of the traits underlying mate choice, and hence that are important to sexual isolation, have been identified in these species. Via a series of sensory ablations, Giglio and Dyer (2013) showed that *D. subquinaria* females did not rely on visual cues when selecting mates. However, removal of the female antennae, which affects olfaction, reduced homospecific (*i.e*. within-population) mating rates by 30% in *D. recens*, 95% in allopatric *D. subquinaria*, and 100% (of n = 60, 24-hour mating trials) in sympatric *D. subquinaria*. Removal of the antennae also impairs a fly’s ability to detect auditory cues, due to the loss of the aristae which are located on the ends of the antennae. However, the removal of the aristae alone had no significant effect, indicating that mate discrimination behaviour in both species is strongly linked to olfactory cues and thus suggesting that pheromones are important. Dyer *et al*. (2014) provided further evidence for this in a manipulative study in which they perfumed allopatric *D. subquinaria* males and *D.*

recens males, with male sympatric *D. subquinaria* pheromones. In both cases they observed an increase in mating rates between the perfumed males and female *D. subquinaria* (which normally discriminate against mating with both of these male types). Specifically, they observed a two-fold increase in mating rate between sympatric *D. subquinaria* females and allopatric *D. subquinaria* males, and a 3.4% mating rate (increased from 0%) between sympatric *D. subquinaria* females and *D. recens* males. Taken together, these results provide strong evidence that contact pheromones are key to mate choice and species isolation in *D. subquinaria* and *D. recens*, although other traits are likely also important. Contact pheromones have been similarly implicated in mate choice and species discrimination in other species of *Drosophila* as well (e.g., *D. serrata* and *D. birchii*; Higgie *et al.*, 2000; Howard *et al.*, 2003; Higgie & Blows, 2007; Rundle *et al*., 2008).

Contact pheromones in many insects, including *Drosophila*, consist of a suite of long-chain, largely non-volatile fatty-acid derived hydrocarbons and their derivatives that are collectively referred to as cuticular (or epicuticular) hydrocarbons (i.e. CHC's). CHC's function to increase desiccation resistance as they form a waxy layer that helps waterproof the cuticle. They are thought to have been co-opted as a means of chemical communication important to mate-choice within populations and sexual isolation between species (Ivy et al. 2005; Mullen *et al*., 2007; Curtis *et al*., 2013; Dyer *et al*., 2014; Rundle & Dyer, 2015). CHC's are phenotypically plastic and individuals can alter their expression in response to abiotic and biotic environmental variables, including social conditions. These changes have been shown to occur quickly, within minutes in *D. serrata* (Gershman *et al*., 2014; Gershman & Rundle, 2016). Gershman *et al*., (2014) also demonstrated that in *D. serrata*, CHC expression exhibits a circadian pattern, which itself is modified by social environment including, for males, the presence/absence of females.

What is interesting is that a pattern of reproductive character displacement has been found in male *D. subquinaria* CHC's between sympatric and allopatric populations (Rundle & Dyer 2015).

In addition to the perfuming and manipulative studies described above, further evidence that CHC's are important to mate choice in *D. subquinaria* and *D. recens* comes from a series of female choice mating trials within each species. Analyzing the results of these trials, Curtis *et al*., (2013) found that variation in male CHC's explained 13.5% of the variance in mating success within *D. subquinaria,* and 25.6% within *D. recens*. Finally, also consistent with CHC's being important to sexual isolation is the fact that in *D. subquinaria*, we observe a pattern of reproductive character displacement not only in CHC's but also in female preferences for them. Rundle & Dyer (2015) quantified population-level (*i.e*. average) female mate preferences among populations of *D. subquinaria* and showed that these differed between sympatry and allopatry, echoing the similar pattern of reproductive character displacement seen in CHC patterns of the same sympatric and allopatric populations.

As the above discussion attests, substantial effort has gone into understanding sexual isolation in this species pair including patterns in it, the traits underlying it, and how these traits vary between sympatry and allopatry. However, other aspects of sexual isolation remain unexplored in this system. My thesis aims to add to our understanding of sexual isolation between *D. subquinaria* and *D. recens*. First, I test for the presence of sexual isolation arising from male mating preferences in both *D. subquinaria* and *D. recens* and determine whether there is character displacement in this and evidence that reinforcement has occurred. Second, I test for a role of learning in male mating preferences and again ask whether this differs between sympatric and allopatric populations.

During my Ph.D. I also conducted two additional experiments, the first designed to test for plasticity in CHC expression in *D. subquinaria* males in response to the presence and absence of *D. subquinaria* females and *D. recens* males, and the second to test whether this varied between sympatry and allopatry (which would suggest that the plastic response may itself have been a target of reinforcing selection). After performing both of these experiments I discovered that the sympatric stocks of *D. subquinaria* I used had been contaminated via gene flow from allopatric *D. subquinaria* populations (prior to my receiving them in the Rundle lab). Sympatric and allopatric *D. subquinaria* are morphologically identical but differ in both CHC profiles and sexual behaviour. However, mixed sympatric – allopatric stocks lose their distinct patterns of mating discrimination. Behavioural changes were confirmed in our sympatric populations in a follow-up assay. Results of both experiments are therefore uninformative and are not presented here.

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Chapter 2

Asymmetrical Reinforcement of Sexual Isolation Between *Drosophila recens* and *D. subquinaria*

Introduction

Reinforcement is the process by which prezygotic barriers to gene exchange between populations are strengthened by natural selection in response to reduced hybrid fitness (Dobzhansky 1937; Coyne and Orr 2004). Though previously thought to be rare or unlikely, evidence has been building over the past few decades that reinforcement is possible and has occurred in at least some species in nature, although its overall frequency is uncertain (Coyne and Orr 1989, 1997; 2004; Servedio and Noor 2003; Yukilevich 2012). Empirical evidence from natural populations has focused on demonstrating enhanced prezygotic isolation between sympatric relative to allopatric populations/species, a pattern sometimes referred to as reproductive character displacement (Howard 1993). Inferring the evolutionary cause of such a pattern can be challenging, however, as processes other than reinforcement can produce the same outcome (Rundle and Schluter 1998; Coyne and Orr 2004; Hopkins 2013).

A particularly strong form of evidence for reinforcement comes from species in which postzyogtic isolation is asymmetrical such that hybrid fitness is reduced more in one direction of the cross than in the reciprocal direction. Such asymmetries are common and have been referred to as Darwin’s corollary to Haldane’s rule (Turelli and Moyle 2007). Reinforcement theory predicts that selection for prezygotic isolation is stronger when hybridization is more costly such that, when postzygotic isolation is asymmetric, the strength of prezygotic isolation should show a matching asymmetry (Yukilevich 2012). Consistent with this, evidence from *Drosophila* (Yukilevich 2012), parasitic wasps (Bordenstein et al. 2000), angiosperms (Tiffin et al. 2001), and spadefoot toads (Pfennig & Simovich 2002) show the expected concordant asymmetries in the strength of pre- and postzygotic isolation in sympatry but not in allopatry, strongly inferring reinforcement.

Asymmetries in postzygotic isolation generate sex-specific consequences of hybridization: if ♀A × ♂B produces fewer fertile offspring compared to the reciprocal cross (♀B × ♂A), then females from species A experience a greater cost of hybridization than do females of species B, while males of species B experience a greater cost of hybridization than do males of species A. All else being equal, such asymmetric costs should cause sex-specific reinforcing selection for enhanced prezygotic isolation. Sexual (or behavioral) isolation, which occurs when individuals from different populations are less attracted to one another as potential mates (i.e. they have different mating preferences; Mendelson et al. 2018), is recognized as a particularly important form of reproductive isolation in speciation (Mayr 1963; Coyne and Orr 2004) and has been a focus of studies of reinforcement in particular (Coyne and Orr 1989; 1997; Noor 1995; 1997). In the case described above, reinforcing selection may not only strengthen sexual isolation in females of species A, but also in males of species B.

Not all may be equal, however, because females typically invest more in each reproductive event then do males such that females are expected to be choosier and males less discriminating (Trivers 1972). Given this, costs of hybridization are generally thought to be lower in males relative to females and hence attention has historically focused on the contribution of female mating preferences to speciation. However, more recent theory indicates that there are conditions under which male preferences can evolve (Servedio and Lande 2006; Fitzpatrick and Servedio 2018) and empirical studies have detected male preferences in a range of taxa including birds (Jones and Hunter 1993), fish (Amundsen and Forsgren 2001), and insects (Bonduriansky and Brooks 1998; Bonduriansky 2001; Bertram et al. 2017). Furthermore, male mating preferences can contribute to sexual isolation (Espinedo et al. 2010; Edward and Chapman 2011; Shahandeh et al. 2018) and can evolve independently of female preferences

(Yukilevich and Peterson 2019). The reinforcement of male mating preferences has received much less attention than their female counterpart, although theory suggests that it can occur (Servedio 2007) and empirical results provide evidence consistent with this in several taxa including fish (Gregorio et al. 2012; Martin and Mendelson 2016; Schlupp 2018), mice (Latour et al. 2013), and fruit flies (Shahandeh et al. 2018). However, a recent meta-analysis of 66 *Drosophila* species-pairs found that female and not male mating preferences were responsible for cases of enhanced sexual isolation in sympatry (Yukilvich and Peterson 2019). Investment in reproduction and hence the cost of hybridization, along with other factors affecting the strength of reinforcing selection (e.g., the relative frequency of the two species in sympatry; Yukilevich 2012), are likely to vary among species. Thus, it remains an open question as to how often sexual isolation by males is reinforced in sympatry.

Here we quantify sexual isolation arising from male mating (i.e. courtship) preferences for the species pair *Drosophila subquinaria* and *Drosophila recens*. *D. subquinaria* and *D. recens* occur in western and eastern N. America, respectively. Both species are generalists on basidiomycete mushrooms and they are nearly morphologically identical. It is thought that the two species were geographically isolated during the last glacial maximum and came into secondary contact with the retreat of the glaciers ~12,000 years ago such that they now share a ~1200 km wide zone of sympatry east of the Rocky Mountains (Wheeler 1960; Jaenike et al. 2006; Dyer et al. 2018). Genetic incompatibilities cause F1 hybrid males to be sterile in both directions of the cross, but postzygotic isolation is further strengthened asymmetrically due to a *Wolbachia* infection in *D. recens* that greatly reduces the survival of female (and male) hybrids produced by *D. subquinaria*, but not *D. recens*, females (Shoemaker et al. 1999). Sexual isolation by *D. subquinaria* females is enhanced in sympatry, whereas sexual isolation by *D.*

recens females is weaker and does not differ between allopatry and sympatry (Jaenike et al. 2006; Curtis et al. 2013; Dyer et al. 2018). This asymmetric sexual isolation in females is concordant with the differential cost of hybridization for females, implying reinforcement as the underlying cause.

The existence of male mating preferences in this system has not been addressed. Here we quantify sexual isolation arising from male mating (i.e. courtship) preferences in *D. subquinaria* and *D. recens,* and compare its strength between multiple sympatric and allopatric populations of both species. The cost of hybridization is greater for *D. recens* males than *D. subquinaria* males, so a concordant asymmetry in enhanced sexual isolation in sympatry (i.e. greater enhancement in *D. recens* than *D. subquinaria*) would provide strong evidence for the reinforcement of male mating preferences.

Methods

Experimental Populations

Six experimental populations each of *D. recens* and *D. subquinaria*, three from allopatry and three from sympatry, were created by pooling multiple isofemale lines collected from the wild (Table 2.1). Isofemale lines from a given population were combined and allowed to mate *en masse* for 2-4 generations prior to conducting the experiment. All cultures were maintained with non-overlapping generations at 20°C and 75% relative humidity on a 12-hour light/dark cycle in glass vials (25 × 95 mm). Each vial contained 1.3 g of Instant *Drosophila* Medium (Formula 4-24; Carolina Biological Supply Company, Burlington NC), 6 mL of water, a piece of folded blotting paper as a pupation substrate, and a piece of fresh commercial *Agaricus bisporus* mushroom (approximately 5 × 5 × 10 mm). A small set of no-choice mating trials demonstrated that sympatric *D. subquinaria* females from these populations strongly discriminate against *D. recens* males, as previously observed for other sympatric *D. subquinaria* populations (Jaenike et al. 2006; Fig. 2.1).

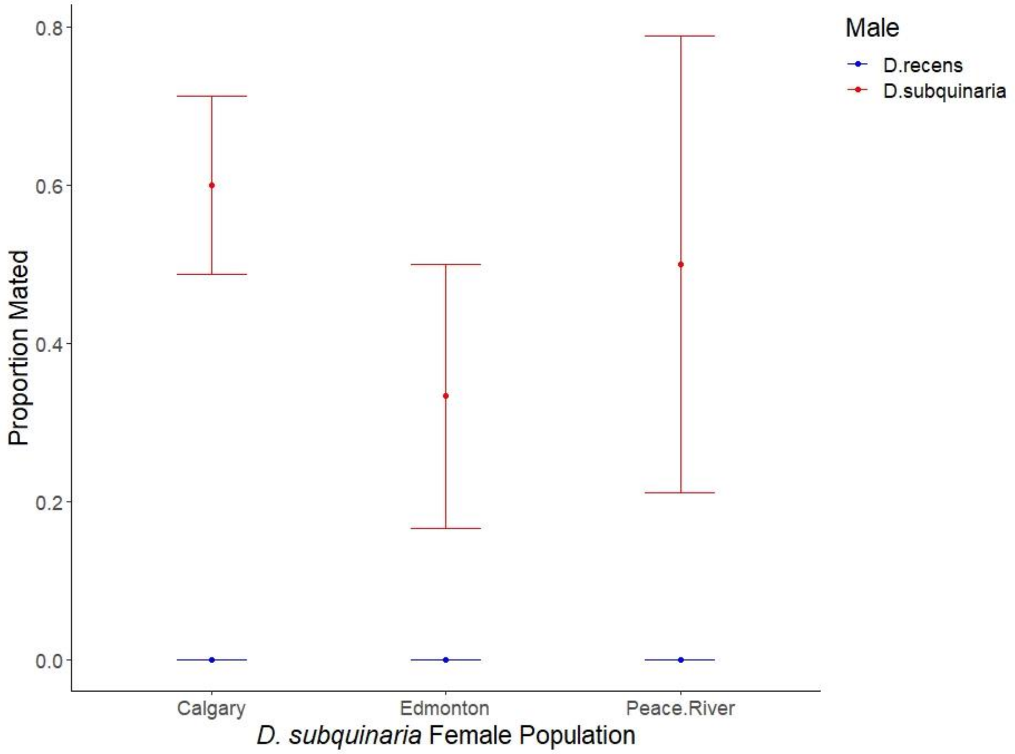

Fig. 2.1 – *The proportion of sympatric D. subquinaria females that mated with either a heterospecific (D. recens) or conspecific (D. subquinaria) male*. Five-day old virgin *D. subquinaria* females from three sympatric populations were held in a standard glass vial containing 7.5mL of mushroom-agar medium. Either a conspecific or a heterospecific male from a different sympatric population were added to each population. Pairs were scored based on whether they mated or not during a one-hour observation period beginning immediately after the addition of the male. Female response between populations did not significantly differ (main effect of female population, $F_{2,62} = 0.855$, $p = 0.43$), but male species did (main effect of male species, $F_{1,62} = 33.85$, $p < 0.0001$).

Assaying Mate Discrimination

We quantified sexual interest of *D. recens* males toward females of both species, and tested for character displacement in this, using a 2 × 2 factorial design. In this assay, courtship behavior of single focal *D. recens* males from sympatry or allopatry was quantified when it was held with two *D. recens* (i.e. homospecific) or two *D. subquinaria* (i.e. heterospecific) females. A given male was only used in a single trial. This design was subsequently repeated two generations later using individual *D. subquinaria* males from sympatry or allopatry. Pairs of females were used in each trial to increase the frequency of male-female interactions. All females used in the study came from a sympatric population where *D. recens* and *D. subquinaria* overlap, but never from the same population as the male in that particular trial.

Females may respond differentially to different types of males, and males may adjust their courtship intensity in response to this (Dukas 2004). Therefore, to avoid conflating female mating preferences with male courtship intensity, females were carefully decapitated with a pair of micro-scissors under light CO_2 anesthetization 15 minutes prior to the start of a trial, following established protocols (Spieth 1966; Grossfield 1972). Only females that were able to stand unassisted 15 minutes after decapitation were subsequently used. Decapitation removes sensory organs associated with female mate choice in these species (Giglio & Dyer 2013) such that decapitated females almost uniformly reject courtship and mating attempts by all males (K. Kwok, unpublished observation).

All flies used in the experiment were collected as virgins within 24-hours of eclosion and separated by sex using light CO_2 anesthetization. These flies were held for five days at 10 individuals/vial to ensure sexual maturity. To reduce potential time-of-day effects, all trials were completed in 30-minute observation blocks within the first five hours of the lights turning on in the incubator. Trials were performed over five days with each of the 12 possible treatment × population combinations represented within each block on each day. Trials took place in ‘arenas’ consisting of a 35 × 10 mm plastic petri dish with 2 mL of a blended mushroom-agar medium evenly distributed across the bottom and which was partitioned in half by a sliding, translucent divider. Mushroom-based medium encourages mating but its dark color makes flies difficult to observe, so a semi-circular piece of white filter paper was placed on each side of the divider and on top of the medium to facilitate observation. A lightly anesthetized male and two decapitated females were added to opposite sides of the divider and were allowed to acclimatize for five minutes before a trial was initiated by sliding the divider out of the way.

During trials, males were observed for behaviors associated with courtship including wing vibrations, ‘circling’, and grooming a female with his forelimbs and/or ‘licking’ the female’s genitalia (Cobb et al. 1985; Dukas 2004, 2008). Each male (i.e. arena) was observed for one six-second period every minute over a total of 30 minutes. Each six-second observation period a separate binary score was given indicating whether each behavior was observed or not. Because wing vibrations and circling were not observed in any trial by males of either species, scores reflected the occurrence or not of grooming and licking. Observations were made by two assistants blind to male and female identity, with each observing six randomly assigned trials during a block.

Courtship intensity was calculated for a given trial as the total number of observation periods during which any courtship (i.e. grooming and/or licking) was observed by the male toward either female. Average courtship intensity was then calculated across all replicate trials using males from a given population with a given type of female (i.e. heterospecific or homospecific), thereby treating populations as replicates. Variation in average courtship intensity was analyzed, separately by male species, as a function of the fixed effects of female species (*D. recens* vs. *D. subquinaria*), male region (sympatry vs. allopatry), and their interaction. Both fixed effects were centered (i.e. coded symmetrically around zero) so that main effects would be interpretable in the presence of an interaction. The model was fit via least squares using the *lm* function in R v. 3.6.1 (R Core Team 2013) and employing sequential (i.e. type 3) tests using the *car* package (Fox and Weisberg 2019). Results were qualitatively unchanged if significance was instead determined via 10,000 permutations using the *lmPerm* package (unpublished results; Wheeler and Torchiano 2016).

Table 2.1 - *Details concerning experimental populations of D. subquinaria and D. recens*. Sympatry and allopatry refer to whether both or only one of the species occurs.

Region	Collection location	Species	Year collected	# Isofemale lines
Sympatry	Calgary, AB[†]	*D. recens*	2016	7
		D. subquinaria		11
	Peace River, AB[†]	*D. recens*	2016	7
		D. subquinaria		5
	Edmonton, AB[†]	*D. recens*	2016	8
		D. subquinaria		4
Allopatry	Smokey Mountains, TN[†]	*D. recens*	2012	3
	Fern Lake, NY[‡]	*D. recens*	2014	4
	Algonquin Park, ON[§]	*D. recens*	2017	5
	Sicamous, BC[†]	*D. subquinaria*	2012	4
	Portland, OR[†]	*D. subquinaria*	2010	5
	Revelstoke, BC[†]	*D. subquinaria*	2012	4

[†] Location details in Dyer et al. (2018).

[‡] Near Peru, NY.

[§] Collected at the Algonquin Park Wildlife Research Station on Sasajewun Lake.

Results

D. recens males discriminated against *D. subquinaria* females, courting them significantly less than their own females (main effect of female species, $F_{1,8} = 30.10$, $p = 0.0006$; Fig. 2.2A). This discrimination was also significantly stronger for sympatric as compared to allopatric males, generating a significant male region × female species interaction ($F_{1,8} = 6.52$, $p = 0.0340$). Finally, *D. recens* male courtship intensity did not differ significantly between males from sympatry vs. allopatry (main effect of region, $F_{1,8} = 3.74$, $p = 0.0892$), although there was a trend towards more courtship by sympatric males that approached significance.

In contrast to results for *D. recens* males, there was no evidence of overall discrimination by *D. subquinaria* males against heterospecific *D. recens* females (main effect of female species, $F_{1,8} = 1.44$, $p = 0.2642$, Fig. 2.2B). However, there was a male region × female species interaction that approached significance ($F_{1,8} = 3.84$, $p = 0.0857$) and that arose in large part from a weak preference of allopatric *D. subquinaria* males for heterospecific *D. recens* females that was not exhibited by sympatric males (which courted both types of females similarly). Allopatric males also courted more intensely than did sympatric males overall (main effect of region, $F_{1,8} = 17.53$, $p = 0.0031$).

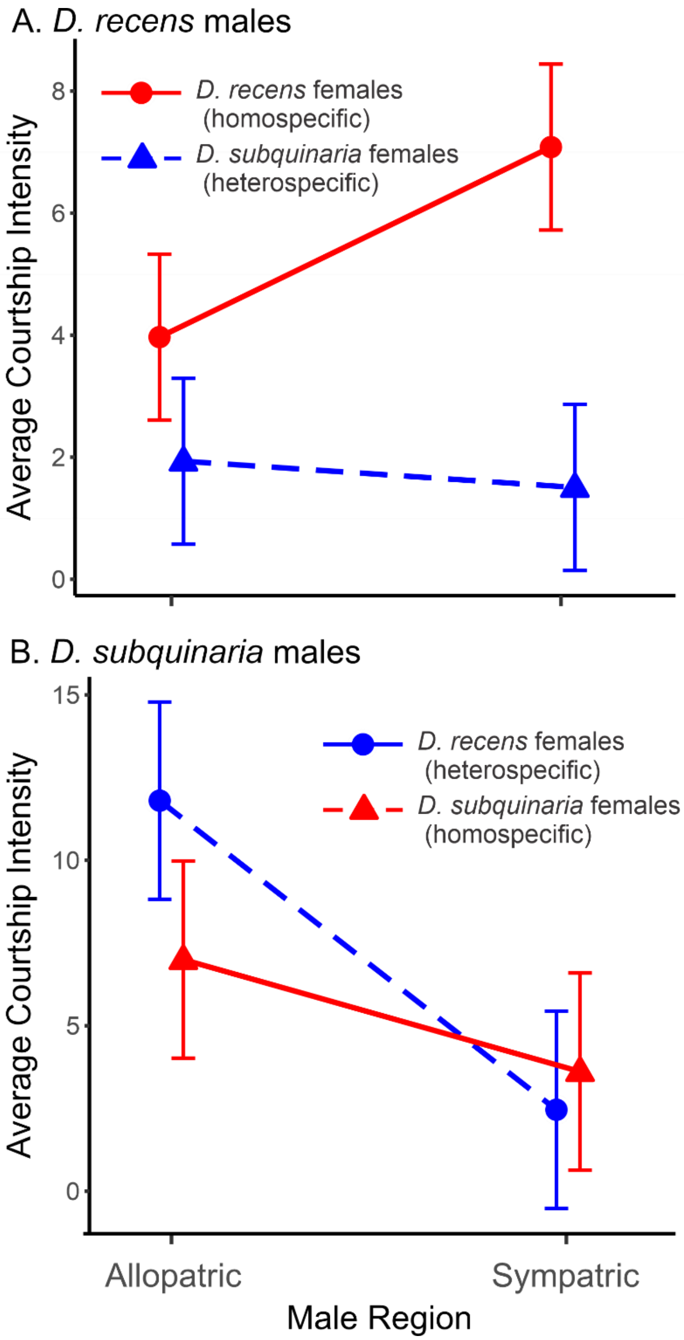

Fig. 2.2 – *Courtship intensity (least square means ± 95% confidence intervals) of allopatric vs. sympatric populations of (A) D. recens males or (B) D. subquinaria males*. Males were used in no-choice mating trials with either *D. subquinaria* females (triangles) or *D. recens* females (circles). Red solid/blue broken lines denote homospecific/heterospecific combinations respectively.

Discussion

The prevalence of reinforcement in the latter stages of speciation is unresolved, in particular with respect to sexual isolation arising from male behaviors. Here we quantified male mating preferences of replicate sympatric and allopatric populations of the *D. recens – D. subquinaria* species pair to test for a signature of reinforcement in both species. Sympatric *D. recens* males courted their own females more than they courted *D. subquinaria* females, demonstrating a contribution of historically overlooked male mating preferences to sexual isolation that mirrors recent observations in other *Drosophila* (Yukilevich and Peterson 2019). Allopatric *D. recens* males showed no such preference, however, and courted *D. recens* and *D. subquinaria* females similarly. In contrast, there was no evidence that sympatric or allopatric *D. subquinaria* males preferentially courted their own females over *D. recens* females. Thus, we do not find evidence of a contribution of male mating preferences to sexual isolation in *D. subquinaria*, nor do we find any indication that sexual isolation by these males has been enhanced in sympatry.

Considering both species, this asymmetrical pattern of enhancement of sexual isolation by males (i.e. in *D. recens* but not in *D. subquinaria*) is concordant with an existing asymmetry in postmating isolation in that there is a much greater cost of hybridization for *D. recens* compared to *D. subquinaria* males due to a *Wolbachia* infection that is only found in *D. recens* (Shoemaker et al. 1999; Jaenike et al. 2006). A note of caution is warranted in that, because the species were tested at different times, species effects are confounded with any temporal differences and were therefore analyzed separately. Ultimate confirmation of this asymmetry would require concordant testing. Nevertheless, concordant asymmetries in pre- and postmating isolation suggest reinforcement as the underlying cause because, while there are other processes that can result in stronger isolation in sympatry (Rundle & Schluter 1998; Coyne & Orr 2004;

Hopkins 2013), they do not predict concordance (Yukilevich 2012). Repeated, independent instances of concordant pre- and postmating isolation therefore provide particularly strong evidence of reinforcement, and such a pattern exists for the enhancement of sexual isolation by females across *Drosophila* (Yukilevich 2012) and other taxa (Bordenstein et al. 2000; Tiffin et al. 2001; Pfennig and Simovich 2002). In contrast, enhanced isolation by males appears rare in *Drosophila*; Yukilevich and Peterson (2019) detected no instances among 66 species pairs although it was detected in a separate study between *D. simulans* and *D. sechellia* (Shahandeh et al. 2018). It is possible that one or a small number of concordant asymmetries could have arisen simply by chance, although in the current case of *D. recens* other explanations for enhanced sexual isolation in sympatry appear unlikely. For instance, sexual isolation can be strengthened as a by-product of ecological character displacement in sympatry, but sympatric and allopatric *D. recens* are morphologically indistinguishable from one another (Wheeler 1960; Shoemaker et al. 1999). Furthermore, enhanced isolation was not detected in *D. recens* females (Jaenike et al. 2006; Dyer et al. 2018), as would be expected if it arose as a side-effect of resource competition or due to the biased extinction/fusion of weakly isolated populations. The absence of enhanced isolation in *D. recens* females also indicates that this pattern in males was not a correlated response to the evolution of females and further shows, consistent with results from other *Drosophila* species (Yukilevich and Peterson 2019), that male and female mating preferences can evolve independently.

Reinforcing selection arises from reduced hybrid fitness (Dobzhansky 1937; Coyne & Orr 2004), but there may also be direct selection on sympatric males not to waste their time and energy courting heterospecific females that are unlikely to accept them as mates. Indeed, sympatric *D. subquinaria* females currently always reject *D. recens* males (Jaenike et al. 2006;

Dyer et al. 2018), whereas allopatric *D. subquinaria* females will mate with *D. recens* males at a moderate rate. Evidence indicates that this discrimination by *D. subquinaria* females was reinforced in sympatry and sexual isolation was thus likely weaker upon secondary contact. As increased discrimination against *D. recens* males evolved in *D. subquinaria* females, the rate of hybridization would have declined and reinforcing selection on males would weaken (Jaenike et al. 2006; Dyer et al. 2018). At the same time, opportunity costs to *D. recens* males of courting *D. subquinaria* females would increase as these females reject them with higher probability. We do not know the relative contribution of these different sources of selection to the enhancement of isolation in males, and distinguishing them would be difficult.

As noted above, a recent study involving 66 species pairs of *Drosophila* found that while sexual isolation was stronger between sympatric than allopatric pairs, this was entirely due to stronger female mating preferences in sympatry, with no evidence of character displacement in male preferences (Yukilevich and Peterson 2019). Therefore, it is unclear why this pattern appears in *D. recens* when it is apparently uncommon in other *Drosophila* species. Servedio (2007) found that if female mating preferences diverge first, then selection for male preferences may be reduced or eliminated, so it is possible that the lack of divergence in female preferences in *D. recens* makes it more conducive to the evolution of male preferences. The cost of hybridization experienced by *D. recens* males is also strong: hybrid males is this direction of the cross are sterile and hybrid female survival is reduced by approximately 90% (Shoemaker et al. 1999). All else equal, such strong postmating isolation will generate strong selection for premating isolation. Additionally, *D. recens* males produce very long sperm (> 7 mm) (Pitnick et al. 1995) and thus may be sensitive to sperm limitation (see Jaenike 1996), although how this compares to other species-pairs has not been addressed. Whether variation in the strength of reproductive character displacement is associated with variation in opportunity costs of heterospecific courtship, sperm limitation, and other costs of hybridization is an interesting question for future study. Alternatively, assay design may matter (Dougherty and Shuker 2015). Our use of decapitated females reduces early rejection behaviors, meaning males may be less inclined to give up, potentially providing higher resolution data on male preferences compared to past studies that focus on presence/absence of courtship and thus lack data on intensity (e.g., Yukilevich and Peterson 2019).

The pattern of reproductive character displacement of male mating preferences indicates that this enhanced isolation has not spread to allopatry. Population structure in *D. recens* is weak, suggesting high levels of gene flow including between sympatric and allopatric populations (Dyer et al. 2018). The persistence of reproductive character displacement in males despite such gene flow suggests that selection may act against enhanced isolation in allopatry, as has been inferred in *D. serrata – D. birchii* species pair (Higgie et al. 2000; Higgie and Blows 2008). It is possible that gene flow could underlie the absence of reproductive character displacement in male *D. subquinaria*, although population structure is stronger in this species and females exhibit a pattern of enhanced isolation (Dyer et al. 2018), arguing against this.

Finally, our assay quantified the initial stages of male courtship using sexually inexperienced, virgin males and decapitated females. We used decapitated females to standardize their behavior across males, but males thus lacked feedback from females that may be used to decide whether to escalate or abort courtship (Grossfield 1972). It has also been shown in other *Drosophila* that males can learn from past experience (i.e. heterospecific rejections) to reduce subsequent heterospecific courtship (Dukas 2008). It is therefore possible that enhanced isolation exists in either species for male mate preferences expressed later in courtship, and/or learned from previous encounters. Reproductive character displacement of a male's ability to learn from past heterospecific encounters is an intriguing possibility for future studies as sympatric *D. subquinaria* females always reject *D. recens* males, whereas *D. recens* females will occasionally mate with *D. subquinaria* males (Jaenike et al. 2006; Dyer et al. 2018).

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Chapter 3

No Evidence that *Drosophila recens* males learn from courtship rejection by *D. subquinaria* females

Introduction

The study of speciation is inherently concerned with the evolution of barriers to gene exchange. Particular attention has been given to sexual isolation, a prezygotic barrier that arises from a behavioural preference for conspecific over heterospecific mates. Evidence suggests that sexual isolation is key to initiating speciation (Coyne & Orr 2004), and its reinforcement in response to reduced hybrid fitness may play an important role in the late stages of speciation as well (Paterson 1982; Harper & Lambert 1983; Servedio & Noor 2003; Bank et al. 2012). Sexual isolation is usually quantified via some sort of mate choice trial, or by measuring assortative mating among groups of individuals. To control for social environment and past experience, such studies typically use males and females that are collected as virgins and subsequently stored separately by sex prior to use. However, individuals in nature do not live in a social vacuum and are likely to interact with members of the opposite sex, and potentially members of other species as well. Such prior experiences may alter traits affecting sexual isolation (i.e. mate preferences and behavioural displays in future interactions), including the potential that individuals may learn from their past experiences (Dukas 2004; 2008; Knörnschild et al. 2010; Hunt et al. 2018). In the presence of such plasticity in sexual behaviour, the true extent of sexual isolation may differ from that under experimental conditions with inexperienced individuals.

Learning is a change in behaviour in response to prior experience (Johnston 1982). Learning in various contexts has been widely observed across both vertebrates and invertebrates (Dukas 2006; Hoedjes et al. 2010; Perry 2020). However, the ability to learn is associated with energetic and maintenance costs (Laughlin et al. 1998; Dukas 1999; Mery Kawecki 2003; 2004; 2005) and it is therefore only expected if benefits outweigh the costs (Dukas 2006). Consistent with this, the evolution of diminished cognitive performance has been associated with contexts in which learning does not appear to provide significant fitness benefits (Hollis & Kawecki 2014), and enhanced performance in contexts that favor it (Mery & Kawecki 2002).

Learned sexual behaviours are of particular interest because they may affect not just how sexual isolation is quantified, but by altering the strength of sexual isolation they may also impact population divergence and alter the likelihood of speciation. For example, if sexual displays and/or mate preferences are learned via imprinting, sexual isolation may arise or be maintained for long enough that genetic differences accumulate, thereby promoting speciation (Irwin & Price 1999; Servedio et al. 2009; Witte & Nöbel 2011). Consider mate preferences that are influenced by maternal identity in systems with parental care; Verzijden & ten Cate (2007) demonstrated that such imprinting helps maintain sexual isolation in Lake Victoria cichlids, with females developing a sexual preference for male partners of the same species as their mother. Imprinted mate preferences may also be particularly conducive to reinforcement, the process by which prezygotic barriers to gene exchange between incipient species, including sexual isolation, are strengthened as a consequence of reduced hybrid fitness (Dobzhansky 1937; Coyne & Orr 2004). This occurs because imprinting represents a 'one-allele' model *sensu* Felsenstein (1981) in which a single 'imprinting allele' can spread in both populations to create assortative mating (Servedio et al. 2009). This avoids the issue of recombination that hinders reinforcement in two-allele models that involve the spread of a preference and a population-specific trait. Imprinting is not limited to systems with direct parental care and can occur through sibling or early life peer identity, self-referent phenotype matching, eavesdropping, the audience effect, and mate-choice copying (Servedio et al. 2009; reviewed in Witte & Nöbel 2011).

Imprinting need not always promote speciation and could instead hamper it. Mis-imprinting is one example in which learning weakens sexual isolation, increasing the hybridization rate between two populations. For example, in sheep and goats Kendrick et al. (1998) found that offspring raised by heterospecific mothers strongly preferred to socialize and mate with heterospecific individuals. This preference for heterospecific mates did not differ after three subsequent years of association with homospecific individuals. Learning may also decrease the likelihood of reinforcement, hampering the latter stages of speciation. For instance, reinforcing selection arises from the production of low fitness hybrids, but if learned mate preferences cause a reduction in the formation of such hybrids, reinforcing selection is weakened (Servedio et al. 2009).

Sexual isolation can be affected by other forms of learning beyond imprinting, for example if individuals adjust their sexual behaviour in response to their success, or lack thereof, in previous mating encounters. Females have been shown to alter their mate preferences based on the males they have encountered (Dukas 2005b; Stoffer & Uetz 2015). My interest here, however, is with males. Though once thought to court indiscriminately, more recent work has demonstrated the presence of male mate preferences in multiple taxa including birds (Jones & Hunter 1993), fish (Amundsen and Forsgren 2001), and insects (Bonduriansky and Brooks 1998; Chapter 2). Furthermore, studies have shown that males can learn to avoid unreceptive females. Dukas (2004, 2008) found that both *D. melanogaster* and *D. persimilis* males that had experience being rejected by heterospecific females tended to reduce their courtship of such heterospecific females in subsequent interactions. Magurran and Ramnarine (2004) showed the same in male guppies. Verzijden et al. (2015) further demonstrated that *D. melanogaster* males could learn to associate arbitrary cues (i.e. eye colour) with female mating receptivity. Learning to avoid courting non-receptive individuals may be adaptive for males (Dukas 2006) by reducing energetic costs of wasted courtship (Wells & Taigen 1989; Mowles & Jepson 2015), by decreasing predation risk during courtship (Sih et al. 1990), and/or by minimizing opportunity costs in the form of missed chances to court potentially receptive females (Andrews et al. 1982).

Here, I investigate the potential contribution of learning to sexual isolation between *Drosophila recens* and its closely related sister species, *Drosophila subquinaria*. *D. recens* and *D. subquinaria* are both generalists on basidiomycete mushroom and are native to eastern and western North America, respectively. Though they were geographically isolated from one another during the last glacial maximum, they came into secondary contact ~12,000 years ago. Each species maintains regions of allopatry while also sharing a ~1,200 km wide region of sympatry east of the Rocky Mountains (Wheeler 1960; Jaenike et al. 2006; Dyer et al. 2018). It is likely that during this period of isolation, genetic incompatibilities accumulated such that F1 hybrid males are now sterile. Additionally, nearly all *D. recens* individuals are infected with the bacteria *Wolbachia* (Shoemaker et al. 1999). *Wolbachia* infection causes cytoplasmic incompatibility between infected males and uninfected females, the outcome being an approximately 90% reduction in offspring production in the *D. recens* male × *D. subquinaria* female cross. No similar effect is observed in the opposite cross (i.e. *D. subquinaria* male × *D. recens* females; Shoemaker 1999). This asymmetric cost of hybridization is reflected in enhanced sexual isolation, relative to allopatric populations, by both sympatric *D. subquinaria* females, which almost uniformly reject *D. recens* males (Jaenike et al. 2006; Dyer et al. 2018), and by sympatric *D. recens* males, which court their own females more intensely than they court heterospecific *D. subquinaria* females (Chapter 2).

Courting heterospecific *D. subquinaria* females is, at best, a waste of time and energy for *D. recens* males as they are rejected in the vast majority of cases, and it potentially exposes them to increased risk of predation and may result in lost mating opportunities with their own, potentially receptive, females. Selection may therefore favour *D. recens* males that learn from past rejections to avoid courting non-receptive heterospecific *D. subquinaria* females. To test for such learning, I use a slight modification of a method previously used to detect such learning in other *Drosophila* species (Dukas 2004, 2008). My goals are to: 1) determine whether *D. recens* males learn from past experiences to reduce their courtship of non-receptive heterospecific *D. subquinaria* females, and 2) test whether the magnitude of this effect is greater in *D. recens* males from sympatric as compared to allopatric regions. Such character displacement in learning has not, to my knowledge, been previously addressed. Its existence would provide additional evidence that the presence of *D. subquinaria* was the selective agent responsible for the evolution of this form of learning, and it would further suggest a potential role of learning in this speciation event.

Materials and Methods

Experimental Populations

Six experimental populations of *D. recens*, three from allopatry and three from sympatry, and three sympatric *D. subquinaria* populations, were created by pooling multiple isofemale lines collected from a given geographic location in the wild (Table 3.1). Isofemale lines from a given location were combined and allowed to mate *en masse* for two generations prior to conducting the experiment. All cultures were maintained with non-overlapping generations at 20°C and 75% relative humidity on a 12-hour light/dark cycle in glass vials (25 × 95 mm). Each vial contained 1.3 g of Instant *Drosophila* Medium (Formula 4-24; Carolina Biological Supply Company, Burlington NC), 6 mL of water, a piece of folded blotting paper (as a pupation substrate), and a piece of fresh commercial *Agaricus bisporus* mushroom (approximately 5 × 5 × 10 mm). These are a subset of the populations used in the experiments in Chapter 2 to study sexual isolation arising from male mating preferences and character displacement in this.

All flies used in the experiment were collected within 24-hours of eclosion and separated by sex using light CO_2 anesthetization to ensure all individuals were virgin and sexually inexperienced. Males were held for five days (10 individuals/vial) to ensure sexual maturity, whereas females of both species that were used in the experiment were held for three days (10 individuals/vial) so that they were not yet fully sexually mature. These immature females were used in both the ‘experience’ and ‘test’ phases of the experiment (see below) to control for female behaviour. A preliminary study demonstrated that *D. recens* males actively court such immature females (Fig. 3.1). If mature females were used instead, *D. recens* (but not *D. subquinaria*) females would be receptive to courtship by focal *D. recens* males and this could alter male courtship behaviour.

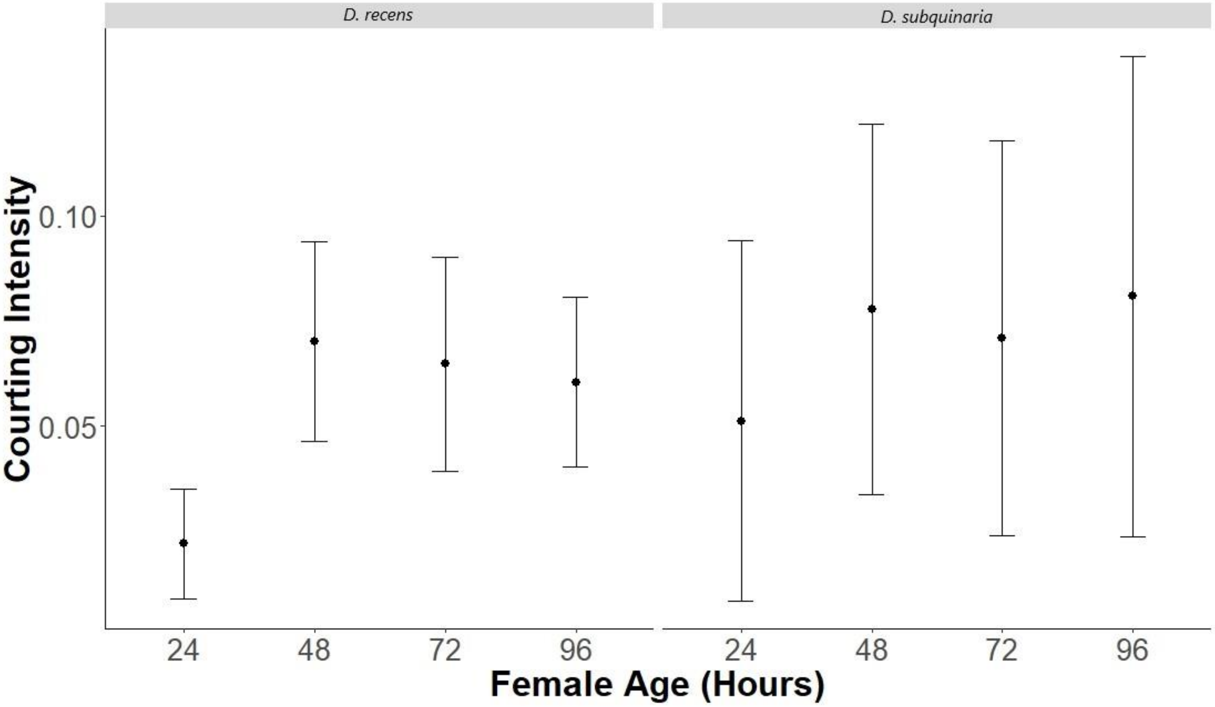

Fig. 3.1 – *Courtship intensity (mean ± 95% confidence intervals) of allopatric D. recens males when presented with allopatric D. recens or D. subquinaria females of varying ages (hours post-eclosion)*. A single virgin female was placed in a cylindrical glass vial (25 x 95 mm) with 7.5mL of mushroom-agar medium and capped with a foam plug. A five-day old virgin male was introduced to each female and were observed for courting behaviours (i.e. grooming, genital licking, and chasing) once every two minutes for 30 minutes. Courtship intensity is the log proportion of observation periods during which any courtship behaviour by the male was observed, over the total observation periods. Total observation periods were defined as the number of observations periods before mating occurred. The proportion of the observation period in which males courted females was used as courting was terminated when males successfully initiated copulation with 72- and 96-hour old females.

Male Learning Assay

To test whether *D. recens* males learned from previous courtship experience and whether there was character displacement in this, I used a 2 × 2 × 2 factorial design that measured the courtship by a *D. recens* male from either sympatry or allopatry, that either did or did not have previous experience being rejected by a *D. subquinaria* female, when subsequently presented with either a homospecific (i.e. *D. recens*) or heterospecific (i.e. *D. subquinaria*) female. Individuals were never used in more than one trial. In all cases, females came from a sympatric population so that differences in courtship intensity would reflect differences among males and not the females they interacted with. Males were never presented with females from their own population. Between 117 - 126 replicates of each of the eight treatment combinations were performed.

The design was based on Dukas (2004; 2008) with the addition of the two male types (i.e. sympatric vs. allopatric). Each trial consisted of two phases. In the first, 'experience' phase, *D. recens* focal males were either exposed to, and rejected by, heterospecific (i.e. immature *D. subquinaria*) females, yielding experienced males, or they were kept in isolation, yielding naïve (i.e. inexperienced) males. In the subsequent 'test' phase, courtship by the focal males was quantified when they were presented with either heterospecific (i.e. *D. subquinaria*) or homospecific (i.e. *D. recens*) females. By presenting focal *D. recens* males with their own vs. *D. subquinaria* females, I can differentiate any effect that past experience has on courtship in general (i.e. being previously rejected makes males less likely to court any female) vs. that specific to the rejecting female's phenotype (i.e. males previously rejected by *D. subquinaria* females court only these females less in future encounters).

In the experience phase, individual focal males were held for 30 minutes in a cylindrical glass vial (25 × 95 mm) containing 7.5 mL of mushroom-agar medium and capped with a foam plug. These males were either held individually (naïve males) or together with a single immature heterospecific (i.e. *D. subquinaria*) female, generating experienced males. Females were added to the vials before males and, after the addition of all flies, the foam plug was pushed down into the vial to restrict the area to approximately 25 x 30 mm (to increase the frequency of interactions). Vials were placed in an open-topped container with opaque sides to reduce visual disturbances from outside movement while allowing observation from above. After 30 minutes, the focal male was removed using light CO_2 anesthetization and transferred, via aspiration, directly to a test 'arena' for Phase 2, while females were discarded.

The second, 'test phase' took place five minutes later in 'arenas' consisting of a 35 × 10 mm plastic petri dish, partitioned in half by a sliding divider, that had 2 mL of the same mushroom-agar medium evenly distributed across the bottom. This medium encourages courtship but its dark color makes flies difficult to observe, so a semi-circular piece of white filter paper was placed on each side of the divider, and on top of the medium, to facilitate observation. A lightly anesthetized focal male (experienced or naïve) and a single immature female (*D. recens* or *D. subquinaria*) were added to opposite sides of the divider and were allowed to acclimatize for 5 minutes before sliding the divider out to initiate a trial.

Males were observed for behaviours associated with courtship including wing vibrations, 'circling', grooming a female with his forelimbs, 'licking' the female's genitalia, and 'chasing' (Cobb et al. 1985; Dukas 2004, 2008). Each male (i.e. arena) was observed for one six-second period every minute over a total of 30 minutes. Each six-second observation period was assigned a single binary score indicating whether or not any of these courtship behaviors was observed.

The experiment was divided into blocks consisting of 16 simultaneous trials. Each of the eight treatment combinations was tested twice in each block, one set of eight trials using one of the three sympatric populations and the other set of eight using one of the three allopatric populations. Each set of eight trials was monitored by a separate observer blind to the identity of the replicates. A total of nine blocks were performed consecutively on each day, testing each male population three times. This entire design was further replicated over seven days. This design fully balanced the experimental treatments by observer, block, and day.

Trials were performed within the first six hours of the lights turning on each morning. The order of which male populations were tested alternated throughout the day. The first population tested each day also alternated between days. Females used in the experiment were always drawn from a sympatric population that was different from that of the males used in the trial. All males and females used in each block originated from the same respective holding vials within each block × observer combination.

Statistical Analysis

Courtship intensity was calculated for a given trial as the total number of observation periods during which any courtship behaviour was observed. Average courtship intensity was then calculated across all replicate trials using males from a given population with a given level of experience (i.e. naïve vs. experienced) and particular type of female (i.e. heterospecific or homospecific), thereby conservatively treating male populations as the unit of replication. Variation in average courtship intensity was modeled as a function of the fixed effects of male type (sympatric vs. allopatric), female type (heterospecific vs. homospecific), male experience (naïve vs. experienced), and the two- and three-way interactions among these using a general linear model fit using R v. 3.6.1 (R Core Team 2013). P-values were obtained by 10,000 permutations via the lmPerm package (Wheeler and Torchiano 2016).

Table 3.1 – *Details concerning experimental populations of D. subquinaria and D. recens. Sympatry and allopatry refer to whether both or only one of the species occurs*.

Region	Collection location	Species	Year collected	# Isofemale Lines
Sympatry	Calgary, AB[†]	*D. recens*	2016	7
		D. subquinaria	2016	7
	Peace River, AB[†]	*D. recens*	2016	7
		D. subquinaria	2016	2
	Edmonton, AB[†]	*D. recens*	2016	7
		D. subquinaria	2016	2
Allopatry	Smokey Mountains, TN[†]	*D. recens*	2012	3
	Fernlake, NY[‡]	*D. recens*	2014	3
	Algonquin Park, ON[§]	*D. recens*	2017	5
	Sicamous, BC[†]	*D. subquinaria*	2012	4
	Portland, OR[†]	*D. subquinaria*	2010	4
	Revelstoke, BC[†]	*D. subquinaria*	2012	3

[†] Location details in Dyer et al. (2018).

[‡] Near Peru, NY.

[§] Collected at the Algonquin Park Wildlife Research Station on Sasajewun Lake.

Learning Avoidance of Interspecific Courtship in D. melanogaster and D. simulans

No evidence of learning by *D. recens* males was detected in the experiment above (see Results), so to gain additional insight into the experimental protocol I repeated the above procedure using the *D. melanogaster-D. simulans* species pair, focusing on learning by *D. melanogaster* males. Previous work has shown that male *D. melanogaster* can learn to reduce heterospecific courtship of unreceptive *D. simulans* females after experiencing past rejections by them (Dukas 2004). The current experimental design closely follows that of this earlier study and that of my *D. recens* experiment above. The assay used a single laboratory-adapted outbred population of *D. melanogaster* collected in 2005-2006 from around Dundas, ON (MacLellan et al. 2009), and a *D. simulans* population, recently collected by R. Dukas in Hamilton, ON, that had been held for 10 generations in the lab. A 2 × 2 factorial design was employed that manipulated the experience of

D. melanogaster males (i.e. naïve vs. experienced) and then the identity of the subsequent female with which they were tested (i.e. homospecific vs. heterospecific). During the experience phase, focal *D. melanogaster* males either interacted with, and were rejected by, *D. simulans* females, or were held in isolation, generating experienced and naïve males respectively. During the subsequent test phase I recorded courtship intensity by these focal males when presented with either an immature *D. melanogaster* or an immature *D. simulans* female.

Virgin *D. melanogaster* males were collected within 6-hours of eclosion and were held for five days to ensure sexual maturity. Immature virgin *D. melanogaster* and *D. simulans* females were collected within 2-hour of eclosion and were used in trials ~6 hours later. *D. melanogaster* males will court both *D. simulans* and *D. melanogaster* females that are 6-8 h post eclosion, but these females generally reject mating with these males (Dukas 2005a; 2010; Dukas and Scott 2015). The test phase was performed as above for the earlier experiment. All flies were raised and held on a standard cornmeal-based fly media of turbinado sugar, cornmeal, agar, 4-methylbenzoate diluted into methanol (1 g/10 mL), and propanoic acid; 7.5 mL of media was added to each of the holding vials and those used during the experience phase, while 2 mL was added to each of the arenas during the test phase. These arenas also contained two semicircular pieces of filter paper, as in the *D. recens* assay.

The assay was performed over six consecutive days with eight consecutive sets of trials each day. Each set included two replicates of each of the four possible treatment combinations and was observed by one of six assistants who were blind to the identity of each replicate. Observer identity was fully blocked with respect to the treatment combinations on any particular day. A total of 161-164 replicates of each treatment combination were performed (a few replicates were lost due flies escaping). Courtship intensity was calculated as above but, because only a single population of *D. melanogaster* was used, the effect of male experience, female type, and their interaction on courtship intensity was tested using a generalized linear model with a negative binomial distribution, thereby treating trials as replicates. A fixed effect of observer identity was also included in the model; interactions with observer identity were non-significant in all cases and were excluded included from the final model. The model was fit using the MASS package in R (Venables and Ripley 2002).

Results

D. recens males from populations allopatric to *D. subquinaria* courted females more intensely than did *D. recens* males from sympatric populations (main effect of 'Male region', Table 3.2; Fig. 3.2). No other effects were significant such that there was no evidence that *D. recens* males courted their own females more intensely than heterospecific females, nor that courtship intensity differed based on previous experience (i.e. between naïve and experienced males). Interactions among these were also non-significant, including between female species and male experience, providing no evidence that *D. recens* males learned to reduce courtship intensity in response to previous rejections. Furthermore, there was no evidence that learning differed between males from sympatric *vs*. allopatric populations (male region × female species × experience interaction; Table 3.2).

Table 3.2 – *Results of a general linear model analyzing sources of variation in D. recens male courtship intensity*.

Effect	***$F_{1,16\ df}$***	***p-value***
Male region	23.530	< 0.001
Female species	0.100	0.756
Experience	0.354	0.560
Male region × Female species	0.005	0.946
Male region *× Experience*	0.053	0.820
Female species *× Experience*	0.147	0.706
Male region *× Female* species *× Experience*	0.028	0.870

When the same protocol was applied to *D. melanogaster* males such that they either experienced previous rejection by *D. simulans* females or not, males courted their own (i.e. *D. melanogaster*) females significantly more that heterospecific (i.e. *D. simulans*) females (main effect of female species, $F_{1,640} = 76.834$, $p < 0.001$; Fig. 3.3). Additionally, previous rejection by *D. simulans* females increased male courtship intensity in the subsequent trials (main effect of male experience, $F_{1,640} = 4.435$, $p = 0.036$, Fig. 3.3, but there was no evidence of learning (i.e. female species × male experience interaction, $F_{1,640} = 0.007$, $p = 0.937$, Fig. 3.3). There was also an effect of observer identity ($F_{5,640} = 2.879$, $p = 0.014$).

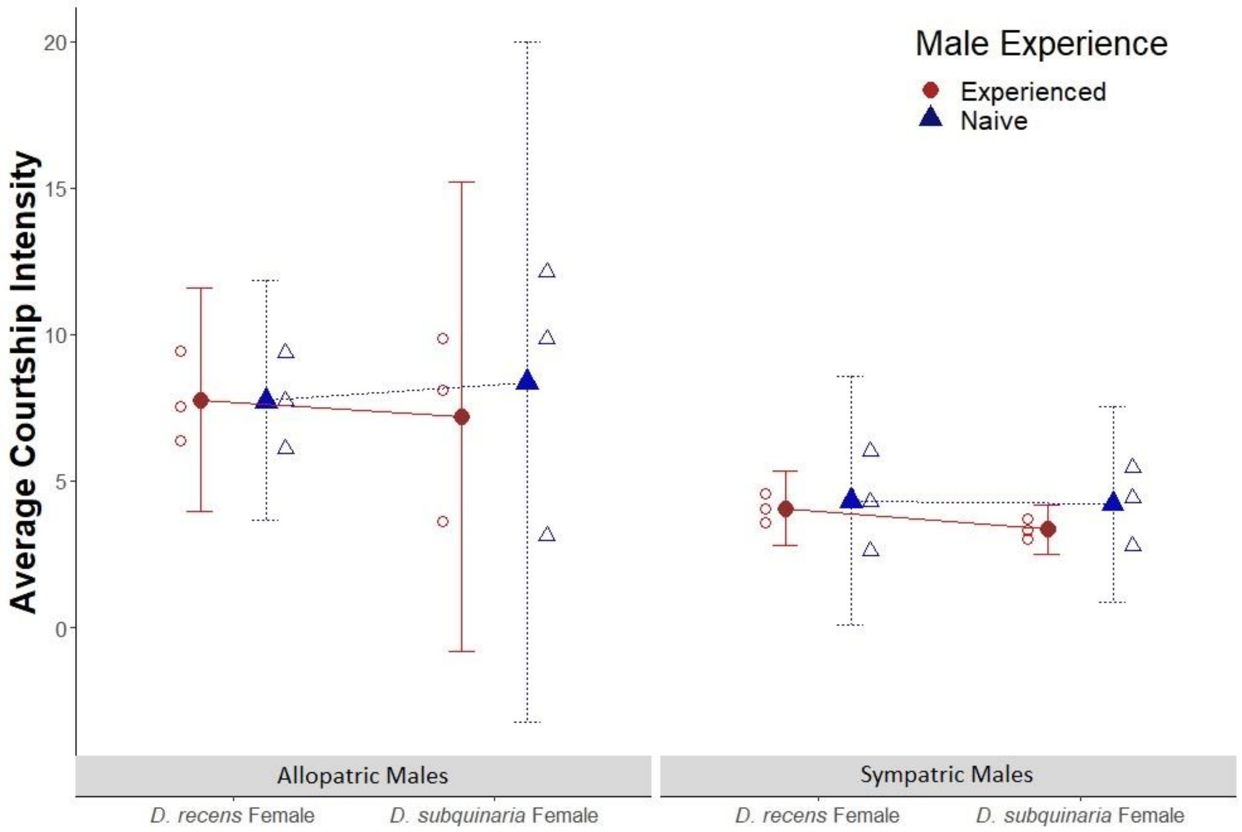

Fig. 3.2 – *Effects of prior experience on allopatric and sympatric D. recens male courtship of D. recens or D. subquinaria females*. Solid points represent mean courtship intensity (95% confidence interval) across the three replicate populations (open symbols) for each treatment combination.

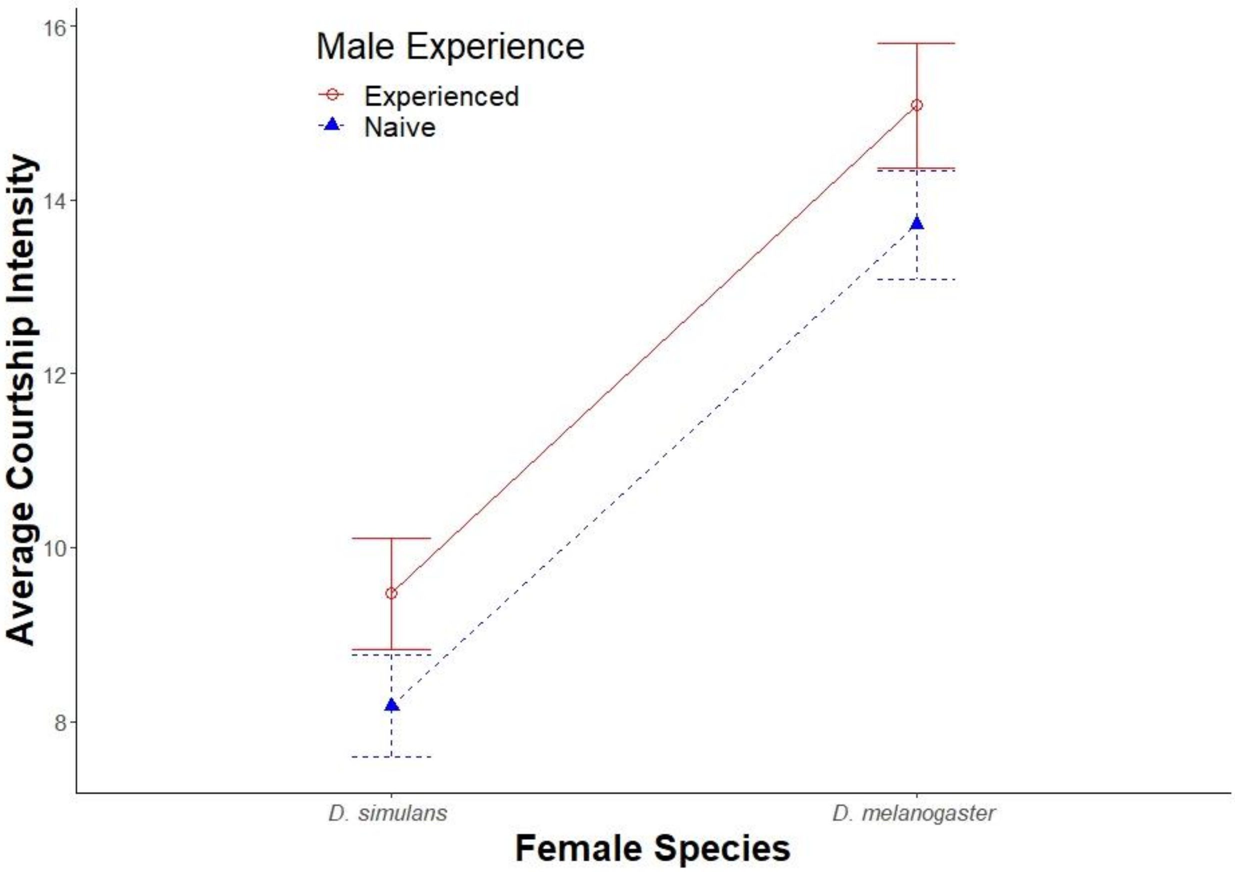

Fig. 3.3 – *Effects of prior experience on D. melanogaster average (± 95% confidence intervals) male courtship of D. simulans and D. melanogaster females*.

Discussion

Learning in D. recens males

Learning can alter sexual isolation in ways that may promote or inhibit population divergence and speciation, including via effects on the process of reinforcement (Irwin & Price 1999; Lachlan & Servedio 2004; Servedio et al. 2009). While attention has been given to the role of sexual imprinting, past experiences can also alter mate preferences such that previously indiscriminate individuals learn to become more selective, enhancing sexual isolation. *D. melanogaster*, *D. pseudoobscura*, and *D. persimilis* males can learn from past sexual encounters to avoid courting heterospecific females (Dukas 2004, 2008, 2009). Here, I investigated whether male *D. recens* were able to learn to reduce or avoid courting unreceptive heterospecific *D. subquinaria* females, and whether the ability to do so was enhanced in sympatric populations in which males are likely to encounter such females. My results provided no indication that allopatric or sympatric *D. recens* males learned from past heterospecific rejection, and consequently no evidence of reproductive character displacement in learning. Only the region from which males originated (i.e. allopatry vs sympatry) had a significant effect on courtship, with allopatric males courting more than sympatric males. Here I consider several possible explanations for this apparent lack of learning from prior sexual interactions in this species.

The first is simply that *D. recens* males do not learn to reduce courtship of *D. subquinaria* females following previous interactions with these females, or if they do, that the effect is relatively weak. Learning has been detected in at least three other *Drosophila* species (i.e. *D. melanogaster* (Dukas 2004, 2005a), *D. pseudoobscura* (Dukas 2009), *D. persimilis* (Dukas 2008)), and I am not aware of any species in which it has been tested but not found (although ‘file drawer’ effects may impact this). However, among-population heterogeneity in learning has also been detected (Kandul et al. 2006) and differential sensitivity to various experimental designs is known (e.g., Kandul et al. 2006, Dukas 2008). While learning itself can be beneficial (Andrews et al. 1982; Wells & Tiagen 1989; Sih et al. 1990; Mowles & Jepson 2015), it is also costly to maintain (Laughlin et al. 1998; Dukas 2006) and may thus trade-off with other fitness enhancing traits such that it is diminished in contexts that reduce its benefits (Mery & Kawecki 2003; 2004; 2005; Hollis & Kawecki 2014). The primary benefit of learning is the ability to respond and adjust to specific conditions. All else being equal, learning from prior heterospecific rejections would not be expected in allopatric populations of *D. recens*, unless it was a by-product of learning in other contexts, but in sympatric populations it could allow males to adopt a narrower criterion of females to court, thereby reducing costs associated with courting unreceptive females. A greater opportunity for learning is afforded under circumstances in which encounter rates with both sexually receptive and non-receptive females are high, and mating success with receptive females is also high (Dukas 2006). The sympatric *D. recens* populations used in this experiment come from regions in which *D. subquinaria* flies are present, but appear to be rare relative to *D. recens* flies (Dyer et al. 2018), although relative abundances are based on very limited data. If *D. subquinaria* are rare, it is possible that natural encounter rates are not sufficiently high for the advantage of learning to outweigh its maintenance costs in these populations. Furthermore, because the primary benefit of learning is an increased ability to adjust to specific conditions, and because *D. subquinaria* females nearly always reject courtship from *D. recens* males (Wheeler 1960; Jaenike et al 2006; Dyer et al. 2018), it is possible that *D. recens* males may benefit from a simpler and less costly strategy of fixed mating avoidance, as suggested in Chapter 2. However, I detected no evidence of that here.

It is possible that learning may be weaker than in other previously studied species pairs and therefore more difficult to detect. In the previous chapter, I found that there was an overall preference by naïve *D. recens* males for homospecific females relative to heterospecific ones, with a significantly stronger preference observed in sympatric *D. recens* males. This pre-existing mating discrimination against heterospecific females may reduce the negative experience of rejection during the training phase, as they would already spend less time courting and being rejected by these females, making a signal of learning weaker and thus harder to detect.

A second possibility is that *D. recens* males do learn, but that my experimental design was unable to detect it for some reason. Dukas (2006) commented that learning in *Drosophila* is not as ‘robust’ as in other model organisms (e.g., honey bees), and may thus be sensitive to small details of experimental design. For example, Kandul et al. (2006) failed to detect learning in the context of reduced courtship of heterospecific females by *D. pseudoobscura* males, whereas Dukas (2009) and Dukas et al. (2012) found evidence for this. Dukas (2008) suggested that the 10-minute training period used by Kandul et al. (2006) may have been too short; Siegel & Hall (1979) demonstrated that learning in *D. melanogaster* male courtship was most effective after training with mated females for 30 or more minutes, with training periods of less than 20 minutes being significantly less effective. In light of this, I used a 30-minute training period. However, I also previously showed that sympatric *D. recens* males court (headless) heterospecific females less than their own females (while still courting them; see Chapter 2). It is therefore possible that *D. recens* males in the current study experienced too few rejections by *D. subquinaria* females during the training phase to facilitate learning, although my preliminary assay provided no indication of reduced courtship of immature *D. subquinaria* over immature *D. recens* females (Fig. 3.1).

Dukas (2009) also demonstrated that the strength of the learning response can vary with the nature of the experience (i.e. “positive experience” with a receptive female vs. “negative experience” with a non-receptive female). He found that negative experiences alone were able to elicit a learned avoidance response, but positive experiences alone did not lead to a learned preference. Furthermore, positive experiences in conjunction with negative experiences elicited an even greater learned avoidance response than negative experience alone in *D. pseudoobscura*, but not in *D. persimilis*. It is therefore possible that *D. recens* males require positive experiences on their own, or in conjunction with negative experiences, to effectively learn.

Although my experimental design was closely based on that used by Dukas (2004, 2008), it did differ in a few ways. One difference of note is that Dukas (2004, 2008) performed their assay in the dark. The role of visual cues in *Drosophila* courtship varies among species (Shahandeh et al. 2020), and both *D. melanogaster* and *D. pseudoobscura*, the subjects of Dukas (2004, 2008), will mate in the dark (Markow & Grady 2005). However, in many species mating is partially or completely repressed in darkness (Markow & Grady 2005). Most importantly, visual cues have been shown to important to mating success in *D. recens* males and females (Giglio & Dyer 2013), with males typically using visual cues to align with females and initiate courtship. Giglio and Dyer (2013) found that mating rates significantly decreased after blinding individuals (i.e. painting their eyes), with a relatively greater reduction in males. While it is possible that eliminating visual feedback makes it easier to detect the use of other cues (e.g., pheromones) on which learning could be based, it is also possible that it alters courtship in biologically unrealistic ways such that learning detected under such conditions may not be relevant to that in nature. It is for these reasons that I decided to conduct my trials in the light.

Another difference from some earlier work (i.e. Dukas 2004, 2008) was my use of immature females, although they have been used before in other Drosophila species (i.e. Dukas 2005, Ejima et al. 2005). Immature *D. recens* females were used in the test phase to ensure that *D. recens* males did not mate with these females, which could alter their courtship behaviour. I chose to also use immature *D. subquinaria* females during the training and test phases to be consistent in this respect, and to further reduce the likelihood of even rare heterospecific matings. I was also originally planning on repeating the entire experiment using *D. subquinaria* males, and these females will mate with *D. recens* females at a reasonable rate. By ensuring all females were immature, between species comparisons would have been facilitated. In the end, given the apparent absence of learning in *D. recens* (the species in which it seems more likely to occur given stronger heterospecific rejection experienced by these males), I decided to invest subsequent effort instead in the follow-up study with *D. melanogaster* to gain additional insight into the experimental protocol.

It is also possible that immature females lack some species-specific phenotype that males use and on which learning is based. While mature *D. melanogaster* males discriminate against *D. simulans* females, Dukas and Scott (2015) show that this was not the case when these males were presented with sexually immature females of the two species. They speculated that species specific pheromones (i.e. cuticular hydrocarbons) may not have not fully developed, explaining the lack of mate discrimination. Previous work with the *D. recens – D. subquinaria* species pair used mature (five or more days post eclosion) individuals (Jaenike et al. 2006; Curtis et al. 2013; Dyer et al. 2018), including my experiment looking at male mate preferences in Chapter 2. Although I did confirm that males will court immature females, indicating that they view them as potential mates (Fig. 3.1), it is possible that one or more species-specific phenotypes were not yet fully developed or expressed, hampering the ability of males to discriminate.

Finally, I previously found mate discrimination by sympatric *D. recens* males against heterospecific *D. subquinaria* females (Chapter 2), but no such pattern was detected here. In the previous chapter, I controlled for female behaviour via decapitation, which greatly reduced female feedback to males, whereas here I used immature females to prevent homospecific mating and standardize female rejection behaviour. I avoided using decapitated females here because I was concerned that they may have lacked some key phenotype (e.g., behaviour) that males used for species recognition and may thus have been involved in learning. Decapitated females tended not to move during male courtship, but do attempt to flee if a male tries to mount, whereas immature females move much more, including to escape courting males. It is therefore possible that *D. recens* males are sensitive to this aspect of female rejection and thus terminated courtship more readily, making a preference for their own females harder to detect. This would help explain the difference in courtship intensities observed between Chapters 2 and 3.

Learning in D. melanogaster males

As noted above, it is difficult to determine whether this form of learning simply does not occur in *D. recens*, or whether the experimental design was unable to detect it. To provide some insight into the latter, I conducted a follow-up experiment with a species pair, *D. melanogaster – D. simulans*, in which learning had previously been detected using similar methods to those of my assay (Dukas 2004; 2005a; 2008). I detected no significant effect of prior rejection by *D. simulans* females on *D. melanogaster* male courtship intensity (i.e. male experience × female species interaction). *D. melanogaster* male courtship intensity was significantly greater with their own females than with the heterospecific *D. simulans* females, indicating an overall ‘species preference’ that would contribute to sexual isolation. There was also an effect of male experience such that males that had previously interacted with *D. simulans* females courted subsequent females of both types more intensely than did naïve males. These results indicate that *D. melanogaster* males were able to identify homospecific females, and that experience increased overall male courtship. This also demonstrates that the assay design was sensitive enough to detect male mate discrimination and differences in it, although no signal of learning was found. As with the previous assay, my protocol differed from Dukas (2004; 2008) in two main ways: the use of immature females and lightning conditions during the assay.

The experiments performed by Dukas (2004; 2008) were performed in the dark, thereby reducing or eliminating visual information, whereas my experiment was done in the light (on a lab bench with blinders to limit visual disturbance from observers). *D. melanogaster* will mate in the dark (Markow & Grady 2005) and courting and mating have been shown to be relatively light-independent compared to *D. simulans* (Shahandeh et al. 2020). It is therefore possible that light may contribute to an absence of learning in this species and, if so, this may be biologically relevant across many other *Drosophila* in which mating often occurs in the light (Markow & Grady 2005), including those within the *quinaria* group (i.e. *D. recens* and *D. subquinaria*). As previously noted, the relative importance of visual cues may decrease in the dark, and the importance of other female phenotypes, may be increased.

Conclusion

In summary, I found that *D. recens* males either did not learn from past courtship rejections or that modifications of my experimental design rendered it unable to detect learning in this species. The apparent absence of learning using the same protocol in *D. melanogaster*, in a context in which it has been previously demonstrated, seems to suggest the latter interpretation. If so, this implies that the detection of learning is extremely sensitive to the design of the experiment. This complicates inferences as to the importance of learning in natural populations and suggests caution when extrapolating lab result to the wild, especially if trials are performed under conditions under which mating does not normally occur in nature. However, further study will be required before definitive conclusions can be made.

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Chapter 4

Effects of Mating Environment on Sex Peptide Expression

Introduction

Mating induces a myriad of changes in the behaviour, physiology, and life history of female insects including altered lifespan (Wagner et al. 2001; Wigby and Chapman 2005; Papanastasiou et al. 2013), increased aggression and feeding (Chapman and Wolfner 2017), decreased likelihood of remating (Scott 1986; Chapman et al. 1996), and increased egg production and oocyte maturation rates (Soller et al. 1999; Sirot et al. 2011). These changes occur, at least in part, in response to seminal fluid proteins (SFP's) provided by males along with sperm (Chapman 2001; Avila et al. 2011; Laflamme and Wolfner 2013). In other words, via components of their ejaculate, males can alter female post-mating behaviour and physiology.

Reproduction has historically been considered a synergetic process in which males and females cooperate to produce offspring to their mutual fitness benefit. In the absence of mate competition, as can occur under monogamy, this is true as a male's fitness is entirely realized through his partner. In such a situation, selection will favour those traits in males that improve female fitness, and act against traits that harm her (Parker 1979; Holland and Rice 1999). Seminal fluid proteins are one potential means by which such co-operation may occur. In several taxa, for example, evidence suggests that females use the presence of seminal fluid proteins as a reliable cue by which to regulate their reproductive physiology to their benefit (Soller et al. 1999; reviewed in Chapman 2001; Wigby et al. 2009; Sirot et al. 2014).

In the presence of mate competition, however, the situation can be different because the reproductive interests of the sexes may not align. Traits can therefore evolve in males because they benefit them when competing with other males for mating and fertilization opportunities, even if these traits harm their mates (Parker 1979, 2006; Rice and Holland 1997; Arnqvist & Rowe 2005). This disassociation between male and female reproductive interests, and hence fitness, generates what is known as interlocus sexual conflict. While harm by males is never directly favoured by selection in such a situation, it can evolve as a by-product of traits that help males compete with one another for access to females and their gametes.

Evidence suggests that seminal fluid proteins may often be involved in such conflict, benefitting males in mate competition but harming females (Scott 1986; Chapman et al. 1996; Wigby and Chapman 2005). For example, seminal fluid protein SPF26a, known as ovulin, has been shown to increase short-term egg production in *Drosophila*, but possibly at the expense of future offspring production. This short-term increase in egg production would benefit a male's fitness by increasing the number of offspring produced using his sperm, whereas a later-life reduction in female fertility would not impact his fitness because the female would have long exhausted his sperm and it would be unlikely the he would encounter her again (Sirot et al. 2015). From the female perspective, however, her lifetime fitness may be reduced.

Seminal fluid proteins have also been shown to reduced female remating rates, thereby decreasing the potential for sperm competition but also reducing a female's ability to refresh sperm stores and/or to choose among competing sperm (Chapman et al. 2003; Smith et al. 2009; Lüpold et al. 2013). Consistent with sexual conflict over the impacts of seminal fluid proteins, Hollis et al. (2019) showed that expression of several seminal fluid protein genes evolved to be lower in *D. melanogaster* populations held in enforced monogamy, in which mate competition is absent, relative to populations held under polygamy, in which mate competition is present.

One seminal fluid protein, sex peptide, has received particular attention in *D. melanogaster*. Sex peptide helps activate juvenile hormone synthesis, which in turn increases oviposition (Moshitzky et al. 1996, Chapman et al. 2003) and oocyte maturation (Soller et al. 1999). It also decreases female remating rates (Chapman et al. 2003; Chapman and Wolfner

2017), thereby reducing the risk of sperm competition. Such effects are beneficial to males as they increase their reproductive success (Moshitzky et al. 1996; Wigby and Chapman 2005). However, despite these short-term benefits to female reproductive success, female longevity is also reduced such that the net effect is a decrease in female lifetime reproductive success when compared to females mated to sex peptide knockdown males (Wigby and Chapman 2005). These knockdown males lacked only sex peptide expression (i.e. other seminal fluid proteins were unaffected), providing direct evidence of sexual conflict over sex peptide expression.

Although much attention has been given to functional effects of sex peptide, the processes governing the evolution and divergence of this seminal fluid protein are not well understood, including the role of sexual conflict in this. For instance, it is well established that when populations evolve under enforced monogamy, male harm tends to decrease (Holland and Rice 1999; Martin and Hosken 2003; Crudgington et al. 2005, 2010). Do changes in sex peptide expression underlie at least some of this effect?

In this chapter I take advantage of a long-term evolution experiment, created by Li Yun and colleagues (Yun et al. 2018), that manipulated mating systems and the environment in which mating occurs to generate a series of populations under one of three mating treatments: i) enforced monogamy with single pairs of adults mating in a relatively simple environment (MC_{absent}); ii) polygamy with groups of adults mating in a relatively simple (i.e. a standard lab) environment (MC_{simple}); and iii) polygamy with groups of adults mating in a lower density environment that had added structural complexity ($MC_{complex}$) and multiple patches of food. The experiment was originally designed to investigate the effects of the mating treatments on adaptation and purging of deleterious mutations (Yun et al. 2018), but insight may also be gained into the evolution of seminal fluid protein expression.

Monogamy reduces or eliminates mate competition so expectations are clear in this case: if sex peptide expression is harmful to females, it should decrease in this treatment. The two environments in which polygamous mating occurs are of particular interest as space and structural complexity may mediate sexual interactions and hence the sexual selection and sexual conflict these generate. As Zuk et al. (2014) noted, in many non-monogamous species females have a large degree of control over mating interactions, for instance because they can avoid or escape males when they do not need to mate. Under such circumstances, mating rates, and hence the opportunity for postcopulatory mate competition, may be much lower. A female's ability to mediate sexual interactions is likely to depend on the environment in which mating occurs, as the available space and structural complexity of the environment may allow a female to hide and/or escape unwanted male attention. Indeed, the complex environment used in this experiment is known to reduce the frequency of sexual interactions, including mating rates, and hence to decrease the expression of male harm (Yun et al. 2017, 2019). In addition, Yun et al. (submitted) compared the evolution of male harm among these mating treatments. Consistent with expectation given the absence of mate competition under monogamy, males in the MC_{absent} treatment evolved to be less harmful, while males in the MC_{simple} mating treatment were harmful to females. Interestingly, males from the $MC_{complex}$ environment were not harmful, despite evidence that these males are superior mate competitors, performing similarly or better than all other males when tested in all three mating environments (Yun et al. 2019). This indicates that $MC_{complex}$ mates have evolved high mating success but do not harm their females in the process. Does sex peptide expression underlie these changes?

Here I quantify the effects of these mating treatments on the evolution of sex peptide expression in males from generation 74 of the evolution experiment. Using five-day old males from each treatment, I quantify both the amount of stored sex peptide and the amount of sex peptide encoding mRNA indicative of their current expression level. As noted above, if there is sexual conflict over sex peptide expression and this, at least in part, underlies the observed divergence in male harm, then I expect decreased sex peptide levels, in the form of stored quantitative and/or current expression levels, in the MC_{absent} populations, whereas these should remain relatively high in the MC_{simple} populations. Expectations for the $MC_{complex}$ populations are less clear; mating rates are reduced in these populations relative to the MC_{simple} populations (Yun et al. 2019), and males have evolved to be less harmful (Yun et al. *submitted*). Sex peptide expression could underlie these effects. However, mate competition still occurs in the $MC_{complex}$ populations and these males are excellent mate competitors compared to males from the other mating treatments, even when tested under conditions experienced by the MC_{simple} males (i.e. high mating rates and presumably strong sperm competition). It is therefore possible that changes in one or more of the other approximately 200 different seminal fluid proteins of *D. melanogaster* are responsible (Sirot et al. 2014).

Methods

Experimental Populations

My experiment used a subset of populations from a long-term evolution experiment created and maintained by Li Yun (Yun et al. 2018). 63 replicate populations were derived from a common lab-adapted ancestor in Sept. 2014 and split these equally across three different novel environments that varied the food and temperatures experienced during larval development (i.e. 21 populations/environment). These are referred to as ‘adaptation sets’ by Yun et al. (2018). I used the adaptation set (i.e. 21 populations) that had been evolving on a novel cornstarch-based food while experiencing a brief 37°C period of heat-shock every generation during larval development. Details concerning the maintenance of these populations are provided below; additional information about these populations and the ancestor can be found in Yun et al. (2018).

The 21 experimental populations were maintained on a three-week lifecycle and were split equally across three mating treatments that varied the opportunity for mate competition and the environment in which this occurred (i.e. seven replicate populations/mating treatment). Mate competition was eliminated in one treatment by enforcing monogamy via the random pairing of individuals ($\mathrm{MC}_{\mathrm{absent}}$). Mate competition was permitted in the other two treatments in either small and structurally simple fly vials ($\mathrm{MC}_{\mathrm{simple}}$) or in larger, structurally more complex 'containers' ($\mathrm{MC}_{\mathrm{complex}}$; Fig. 4.1).

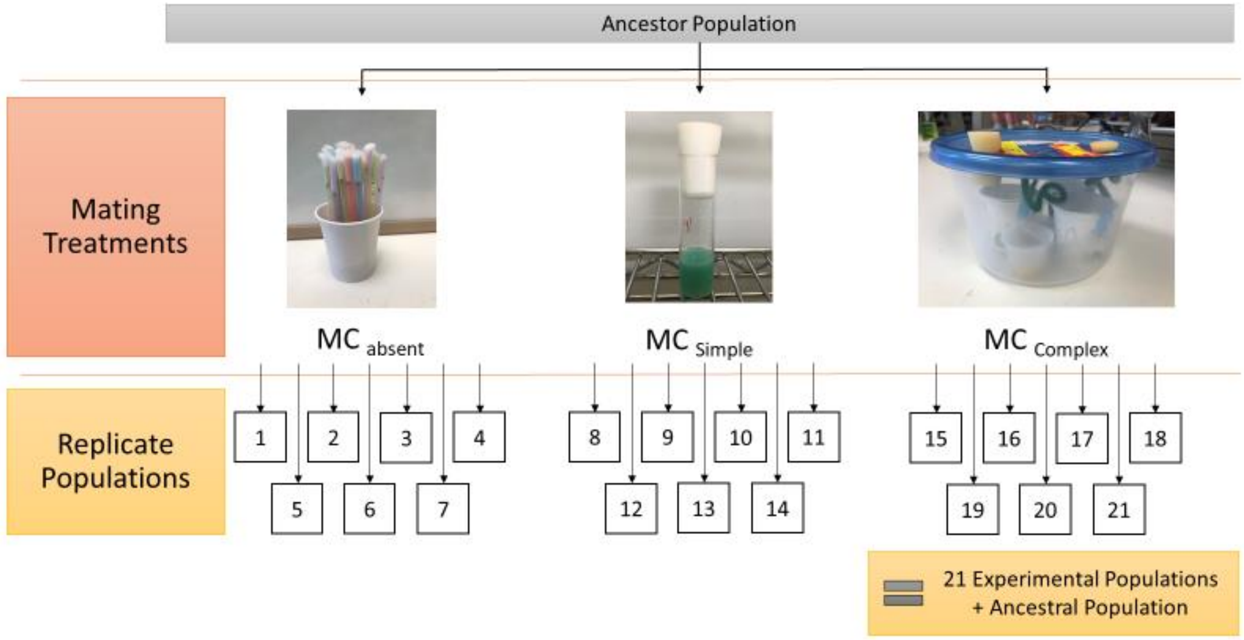

Fig. 4.1 – *Overview of the 21 experimental populations showing the different mating environments used in each.*

Adult flies were held in their respective mating treatments for six days every generation, transferring them to fresh media using light CO_2 anaesthetization on day 3 to minimize problems caused by larval-induced liquefaction of the food. MC_{simple} populations were held as 140 separate male/female pairs in individual cylindrical plastic straws (radius = 6 mm; height = 98.5 mm) with one end loosely plugged with cotton. 28 straws were inserted, via their open ends, into each of five 3 oz Dixie cups© with standard cornmeal-yeast media. MC_{simple} populations were held in standard *Drosophila* (i.e. cylindrical glass) vials (25 mm × 95 mm) with 7.5 mL of standard cornmeal-yeast media, at a density of 35 males and 35 females per vial. $MC_{complex}$ populations were held in cylindrical 1.65 L plastic Ziploc© containers at a density off 35 males and 35 females per container. Each container housed three 3 oz Dixie cups© and two 1 oz plastic cups containing 25 mL and 7.5 mL of standard cornmeal-yeast media respectively. Each container also had four curled pipe cleaners protruding from the inside of the lid into the interior of the container. The surface of the food in the three larger Dixie cups was further divided into two by inserting a plastic barrier into the medium. Containers are pictured in Fig. 4.1; more detailed images can be found in Yun et al. (2017).

Following six days in their mating treatments, 105 females were randomly collected from among the survivors from each population via light CO_2 anaesthetization and were distributed evenly across seven vials (i.e. 15 females/vial) to lay eggs for 24 hours, after which the females were discarded. Each vial contained the novel cornstarch-based food. Larval developed in these vials and on day 3 of development they were exposed to a two-hour (four hour from generation 6 onward) heat shock at 37°C. Offspring eclosed in these vials and on day 11, 140 adult males and 140 adult females were collected using light CO_2 anaesthetization from each population. To maintain a three-week generation time (to facilitate scheduling), adults were held in single-sex vials on standard cornmeal-agar media, at densities of 35 individuals per vial, for three days after which they were introduced to their respective mating environments.

The ancestor was maintained alongside the experimental populations via discrete, non-overlapping two week generations in glass bottles with approximately 100 mL of standard cornmeal-yeast media (25°C, 50% relative humidity, and 12:12 Light:Dark cycle).

Preparation and Collection of Experimental Flies

Sex peptide was quantified in four-day post-eclosion virgin adult males, raised and held under low, density-controlled conditions to reduce the influence of environmental and maternal effect. Males were frozen in liquid nitrogen and stored until either the quantity of sex peptide stored in these males, or sex peptide gene expression, was measured. Details are as follows.

Adult males were collected from the experimental populations, and the ancestor, at generation 74 of the evolution experiment. For each population, I collected adult flies emerging from their larval-cornstarch environments and released them into large Plexiglas© mating cages that contained 18 ‘egg-collection pads’. Egg-collection pads were polystyrene petri dishes (140 mm × 20 mm) that contained a hard agar-grape medium into which young larvae cannot burrow. A thin layer of yeast paste was spread over the surface of the medium to promote egg-laying. These dishes were left inside the mating cages for four hours before being discarded. Fresh egg-collection pads were then added to the mating cages for three additional hours. Following this, this second set of egg-collection pads was stored in a sealed Ziploc© plastic container in an incubator (25°C; 50% RH) for 24-hours. After 24 hours, larvae that hatched on these egg-collection pads were carefully collected with a blunted metal probe. Thirty larvae were transferred to each of six glass vials (25 mm × 95 mm) containing 7.5 mL of a cornmeal-yeast medium with a few live yeast pellets. Offspring developed in these vials and then virgin adult males were collected using light CO_2 anesthetization. These males were held in glass vials with 7.5 mL of a cornmeal-yeast medium and a few live yeast pellets, at a density of 12 males per vial for four days.

Once males had reached four days of age, ten males were randomly selected from each vial and lightly anesthetized with CO_2. These males were transferred to a plastic 1.5 mL Eppendorf tube. The tube was capped and immediately submerged in liquid nitrogen, then subsequently stored at -80°C prior to quantifying the amount of sex peptide present (via Immuno-Q-PCR) or current sex peptide expression levels (via qPCR). Different males were used for each of these assays.

Quantifying Sex Peptide Amounts in Males

Sex peptide was quantified using Immuno-Q-PCR following Rouhana (2018). This technique consisted of five primary stages:

1. Peptide extraction from males
2. Blocking ELISA plate wells
3. Addition of antibody-capture oligonucleotide-signal oligonucleotide complex
4. Cleaving signal oligonucleotide from antibody-capture oligonucleotide complex
5. RT-qPCR

Details concerning each stage are given below, followed by information concerning the construction of a standard curve and various controls that I employed during the assay.

1. Peptide extraction from males. The goal of this step is to extract peptides from frozen flies and coat the wells of an ELISA plate with it. In subsequent stages, sex peptide antibodies will be added to specifically bind to sex peptide coating the wells. For each population (experimental populations and the ancestor), a single sample was created by pooling five randomly chosen males from the ten that were collected. These males were transferred to a fresh 1.5 mL Eppendorf tube with 200 µL of protein extraction buffer (5% DPBS with a protease inhibitor cocktail tablet (cOmplete Tablets EASYpack; Roche, Switzerland)). Flies were finely ground using a plastic pestle for at least 30 seconds before being centrifuged at 12,000 rpm at -20°C for 5 minutes. The supernatant was transferred to another 1.5 mL Eppendorf tube and centrifuged at 12,000 rpm at -20°C for an additional 10 minutes. The resulting supernatant was then transferred to a final 1.5 mL Eppendorf tube. All stages outside of the centrifuge were performed on ice.

50 µL of each sample of five-pooled males of each population was then added to each of three wells on an ELISA plate (Immulon 4 HBX; Thermo Scientific, Massachusetts, USA), where the peptides would bind to the bottom of the wells. A series of synthetic sex peptide samples (Eurogentec, Seraing, Belgium) of known concentrations were also added (to allow construction of a standard concentration curve) along with experimental controls. These controls included a negative sex peptide control to detect non-specific binding of the antibody, a negative antibody-capture oligonucleotide-signal oligonucleotide control to determine whether RT-qPCR primers were annealing to non-target oligonucleotides in the sample, and blank control to detect cross-well contamination (see "*Standard Curve Construction and Controls*" below for further details). The plate was then covered with parafilm and incubated overnight (~16 hours) on a plate shaker at 4°C.

2. Blocking ELISA plate wells. Following incubation, the parafilm was discarded and all supernatant was removed by careful pipetting. Each well was then washed with 100 µL of wash solution (100mL of DPBS; 50 µL of TWEEN® 20) to remove any peptides that had not bound to the wells. The wash solution was immediately removed by careful pipetting and tapping on DNAse/RNAse paper towel roll. To block any remaining binding surfaces, 100 µL of a filtered blocking solution (50 mL of DPBS; 25 µL of TWEEN® 20 0.005%; 5% BSA (lysophilized powder, ≥96%; Sigma-Aldrich, Missouri, USA)) was then added to each well. This prevented the antibody-capture oligonucleotide-signal oligonucleotide complex from binding directly to the ELISA wells. The ELISA plate was then covered with parafilm and incubated overnight (~22 hours) on a plate shaker at 4°C.

3. Addition of antibody-capture oligonucleotide-signal oligonucleotide complex. Following incubation, the parafilm was removed and each well was washed with 100 µL of wash solution. To quantify the amount of sex peptide, 50 µL of blocking solution with an antibody-capture oligonucleotide-signal oligonucleotide complex (Appendix 1) was then added to each well and the plate was incubated at room temperature for one hour. The antibody specifically binds the complex to any sex peptide in the sample, producing a theoretical 1:1 ratio of sex peptide molecules in the sample and signal oligonucleotide. At the end of the incubation period, the blocking solution and any unbound antibody was removed by careful pipetting. Each well was then washed a total of five times with 150 µL of wash solution. Wash solution was removed during each wash by careful pipetting followed by firmly tapping the plate on DNAse/RNAse paper towel.

4. Cleaving signal oligonucleotide from antibody-capture oligonucleotide complex. After washing, 20 µL of a solution containing HindIII restriction enzyme (17.7 µL of sterile DNA/RNA free water; 2 µL of Buffer 10X (Buffer E 10X Buffer, Promega, Wisconsin, USA); 0.2 µL of BSA (Bovine Serum Albumin, acetylated; Promega, Wisconsin, USA); 0.1 µL of

HindIII (Ref: R604A; Promega, Wisconsin, USA) was added to each well to cleave a restriction site between the capture oligonucleotide and the signal oligonucleotide, thereby releasing the signal oligonucleotide into the solution. This was left to incubate at room temperature (~24°C) for two hours. Following the incubation period, the solution was transferred to a PCR plate (96-Well PCR Plate, Non-Skirted; Star Lab, Hamburg, Germany) and incubated at 65°C for 15 minutes to deactivate the HindIII restriction enzyme. Finally, the plate and solutions were stored at -80°C until analysis by RT-qPCR.

5. RT-qPCR. The quantity of signal oligonucleotide (and by proxy, sex peptide) was measured on a StepOnePlus Real-Time PCR System (Applied Biosystems, California, USA) and analyzed using StepOne (ver. 2.3) software. To do this, I transferred 5 µL of each final mixture to a PCR plate (MicroAmp® Fast Optical 96-Well Reaction Plate with Barcode (0.1 mL), Applied Biosystems, California, USA), along with 15 µL of a qPCR master mix (60 µL of 2 µM Forward Primer; 60 µL of 2 µM Reverse Primer; 1200 µL of iTaq SYBR Green (BioRad, California, USA); 480 µL of DNA/RNA free pure water; see Table 4.1 for Primer sequences). The qPCR cycle began with an initial activation stage of 95°C for five minutes, followed by 35 cycles of denaturation (95°C for 10 seconds), hybridization (50°C for 30 seconds), and elongation (72°C for 30 seconds). Fluorescence data were collected at the end of each of the 35 cycles. To test for contamination by impurities, a melt curve was constructed for each sample following the end of each cycle, after fluorescence data were collected (95°C for 10 seconds; 60°C for 1 minute; and 95°C for 15 seconds).

Table 4.1 – *Sequences of peptides, oligonucleotides, and primers used in Immuno-Q-PCR.*

Compound	**Length**	**Sequence**
Synthetic sex peptide	36 aa	H - WEW PWN RKP TKF PIP SPN PRD KWC RLN LGP AWG GRC - OH
Capture oligonucleotide	22 bp	5' - TGG ATC CTA AGC TTG AGC ATT T-3' * Biotin
Signal oligonucleotide	74 bp	5' - TGC TCA AGC TTA GGA TCC ATA GCG TGT ACC ATG TAA ACC TTA TAA CTT ACC TCA GAC TAG TTG GAA GTG TGG C - 3'
Forward primer	23 bp	5' - CCA TAG CGT GTA CCA TGT AAA CC - 3'
Reverse primer	21 bp	5' - GCC ACA CTT CCA ACT AGT CTG - 3'

* Red text is the complementary sequences between the capture oligonucleotide and signal oligonucleotide

Standard Curve Construction and Controls. A series of five-fold dilutions from a 10mM sample of artificial sex peptide (Eurogentec, Seraing, Belgium; Table 4.1) was added in triplicate to the ELISA plate (i.e. 10mM, 2mM, 0.4mM, 0.08mM, 0.016mM, and 0.0032mM) to construct a standard curve. This curve can be used to convert Ct-values from the RT-qPCR to absolute concentrations of sex peptide. However, unidentified problems with these sex peptide standards caused the triplicate technical replicates to be inconsistent with one another. Statistical analyses therefore used the Ct values themselves, as detailed below, and standard curves are not presented.

Three different controls were added to the ELISA plate to test for potential issues during the assay. The first was a negative sex peptide control. This involved the replacement of sex peptide for DNAse/RNAse water when sex peptide samples were initially added to the ELISA plate. All other stages of the protocol were completed as normal. Results were negative, indicating that the blocking solution was effective at limiting the antibody-capture oligonucleotide-signal oligonucleotide from binding directly to the ELISA plate and that washing was effective at removing antibody-capture oligonucleotide-signal oligonucleotide from the wells. The second of these controls added DNAse/RNAse water to the control wells instead of the antibody-capture oligonucleotide-signal oligonucleotide. No fluorescence was detected during the following RT-qPCR, the presence of which would indicate that primers were annealing to non-target oligonucleotides in the sample. Lastly, DNAse/RNAse water was added in place of the sex peptide samples, the blocking solution, and the antibody-capture oligonucleotide-signal oligonucleotide complex. Wells were washed and the restriction enzyme mix was added as per the normal protocol. Results were again negative, providing no indication of cross-well contamination. Finally, as an additional control 5 µL of DNAse/RNAse water was added to the PCR plate in place of the final sample during RT-qPCR. 15 µL of master mix was added as per usual. A positive result in this control would indicate significant primer-dimer interactions which could reduce primer efficiencies. No primer-dimer interactions were observed that would potentially reduce confidence of recorded Ct-values.

Quantifying Sex Peptide Gene Expression in Males

Five additional males from the original sample of 10, separate from those previously used for sex peptide quantification, were pooled from each population to create one sample per population. Sex peptide-encoding mRNA was isolated from each of these samples using the established protocols provided by the ThermoFisher Scientific mirVana^{TM} miRNA Isolation Kit (Thermo Fisher Scientific, Massachusetts, USA). RNA concentrations were determined with a Nanodrop 8000 Spectrophotometer (Thermo Fisher Scientific, Massachusetts, USA) and each sample was diluted to 200 ng/µL. The samples were then purified using the established protocols for the Ambion DNA-free^{TM} kit (Thermo Fisher Scientific, Massachusetts, USA) to remove any contaminating DNA. Sex peptide coding mRNA and the mRNA of two reference genes (i.e. CG13220 (Chintapalli et al. 2007; Van Hiel et al. 2009; Ling & Salvaterra 2011) and eIF1A (Yu & Marintchev 2018) were then reverse transcribed to cDNA using a Qiagen QuantiTect kit (Qiagen, Germany), using primers acquired from Eurofins Genomics (Luxembourg; Table 4.2). The final cDNA samples were stored at -20°C until quantification via qPCR.

cDNA samples were diluted to 2 ng/µL using a nanodrop. 5 µL of each sample, along with 15 µL of a master mix (1 µL of 2µM Forward Primer; 1 µL of 2 µM Reverse Primer; 10 µL of iTaq SYBR Green; 3 µL of DNA/RNA free pure water; Table 4.2), were then added to a PCR plate (MicroAmp® Fast Optical 96-Well Reaction Plate with Barcode (0.1 mL); Applied Biosystems, California, USA). Sex peptide and each reference gene were run on their own PCR plates due to the large number of samples. A DNAse/RNAse water control was included on each plate to test for significant primer-dimer interactions.

The qPCR cycle began with an initial activation stage of 95°C for 30 seconds, followed by 40 cycles of denaturation (95°C for 10 seconds), hybridization (50°C for 30 seconds), and elongation (72°C for 30 seconds). Fluorescence data were collected at the end of each of the 40 cycles. A melt curves was constructed from data collected at the end of each cycle (95°C for 10 seconds; 60°C for 1 minute; and 95°C for 15 seconds).

Table 4.2 – *Sequences of primers used in Immuno-Q-PCR.*

Primer	Length	Sequence
SP Forward	20 bp	5' - TGG GAA TGG CCG TGG AAT AG - 3'
SP Reverse	20 bp	5' - CCC CAA ATT AAG ACG GCA CC - 3'
CG13220 Forward	21 bp	5' - TGG TGA GCT ACG GAG CCC TTG - 3'
CG13220 Reverse	21 bp	5' - GGG GCC TGC CGT AAA TGT AGA -3'
eIF1A Forward	20 bp	5' - ATC AGC TCC GAG GAT GAC GC - 3'
eIF1A Reverse	20 bp	5' - GCC GAG ACA GAC GTT CCA GA - 3'

Statistical Analyses

Variation in sex peptide quantities among males from the different populations was analyzed using a one-way ANOVA, treating populations as replicates and mating treatment as a fixed effect. Due to problems with the standard curves, analysis was performed on Ct values directly, representing the number of replication cycles during qPCR that were required to produce a fluorescence signal that exceeded the threshold. A sample with greater quantities of sex peptide, and consequently greater quantities of signal oligonucleotide, would require fewer replication cycles and hence would yield a lower Ct value. While Ct values do not provide information on absolute quantities, they can be used to compare relative quantities among mating treatments.

To compare sex peptide gene expression among mating treatments, relative gene expression levels were calculated for each sample as primer efficiency to the negative power of the difference of sex peptide gene expression and the average gene expressions of the two reference genes (i.e. CG13220 and eIF1A) using the “Delta-delta method” (Pfaffl 2001). Variation in relative expression among the fixed effect of mating treatments was once again analyzed using a one-way ANOVA, treating populations as replicates.

Analyses were conducted via the lm function in R v. 3.6.1 (R Core Team 2013) with significance determined by via parametric p-values, but results were qualitatively unchanged if determined instead via 10,000 permutation using the lmp function in the lmperm package (Wheeler & Torchiano 2016). Post-hoc comparisons, when appropriate, were performed via a Tukey HSD test.

Results

Sex peptide quantities differed significantly among mating treatments ($F_{2,18} = 7.556$, $p = 0.0042$; Fig. 4.2). Sex peptide amounts were significantly lower, as indicated by the higher Ct values, in males from $MC_{complex}$ populations compared to those from both MC_{simple} ($p = 0.0037$) and MC_{absent} ($p = 0.0407$) populations, while MC_{simple} and MC_{absent} did not differ significantly from each other ($p = 0.508$).

Relative expression of sex peptide mRNA was highest males from the MC_{simple} mating treatment and lowest in males from the $MC_{complex}$ treatment, but there was substantial variation within mating treatments, in particular among the MC_{simple} populations, and differences among treatments were not significant ($F_{2,18} = 0.489$, $p = 0.621$, Fig. 4.3). This result was confirmed by permutation ($p = 0.7792$).

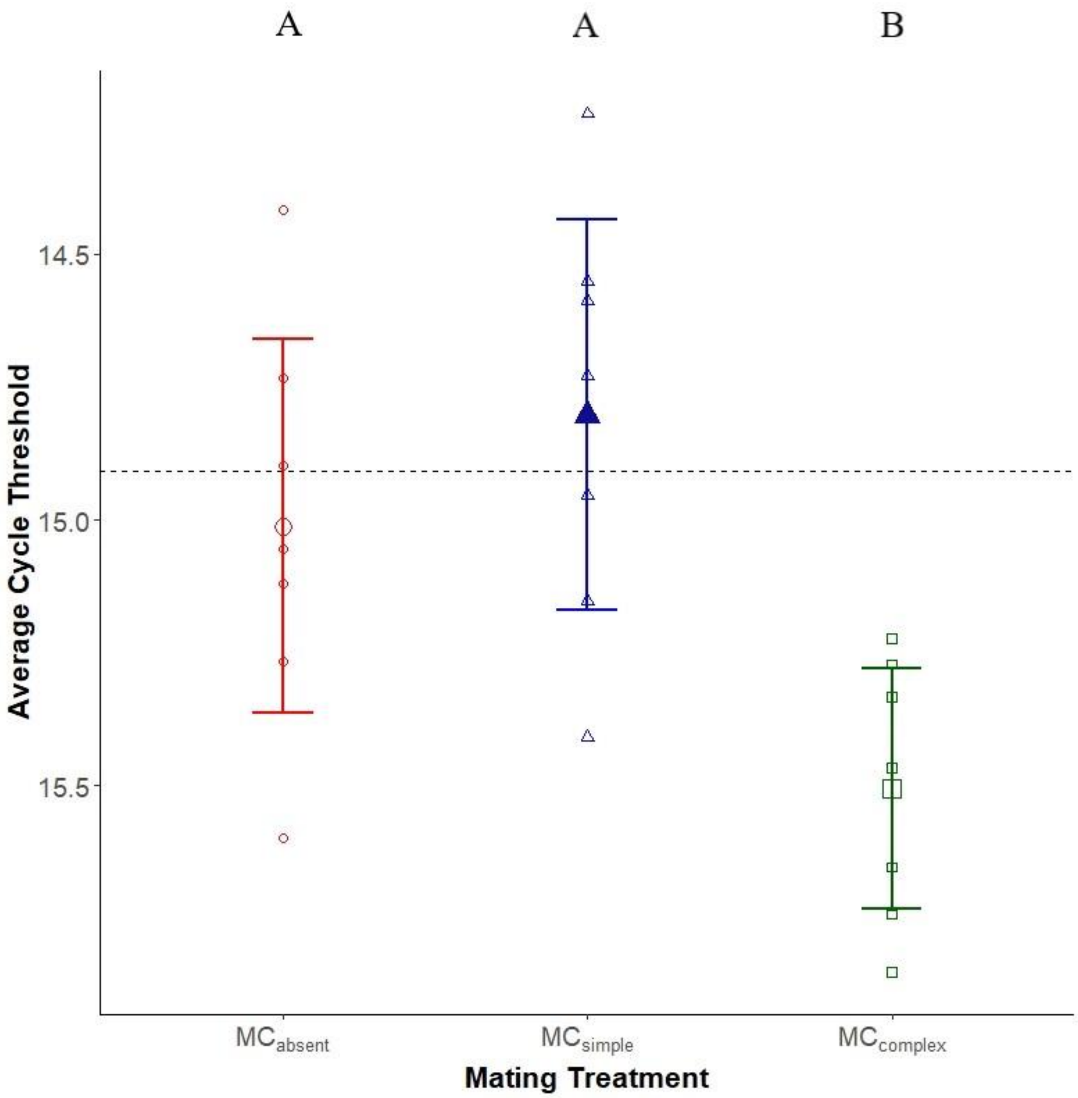

Fig. 4.2 – *Variation in sex peptide quantities among D. melanogaster males evolved under different mating treatments that manipulated the opportunity for mate competition and the environment*. Points represent the average cycle number at which amplification crossed the qPCR cycle threshold value such that a higher greater Average Cycle Threshold corresponds to lower average sex peptide expression (y-axis is reversed to aid interpretation). Solid points represent mating treatment means ± 95% confidence interval; open points represent individual populations within their respective mating treatment and the dashed line represents the ancestor population. Results of post-hoc comparisons are denoted by letter groups across the top.

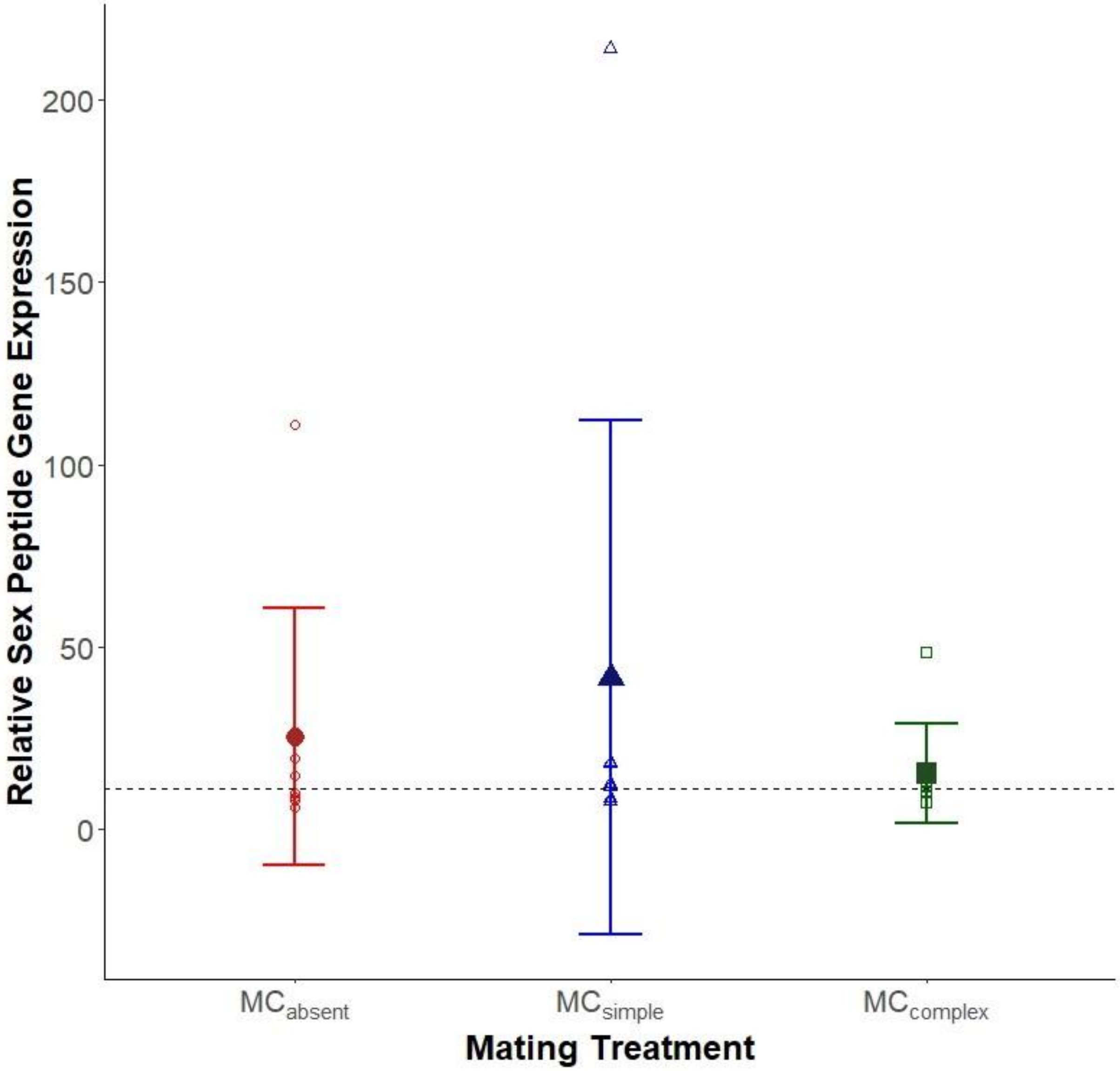

Fig. 4.3 – *Relative gene expression of sex peptide of five-pooled Drosophila melanogaster males evolved in the different mating environments*. Points represent the expression (±95% confidence intervals) of sex peptide relative to two reference genes, correcting for primer efficiency using the "delta-delta method. Solid points represent mating treatment means, whereas open points represent individual populations within their respective mating treatment. The dashed line represents the ancestor population.

Discussion

Several seminal fluid proteins have been implicated as a source of sexual conflict (Sirot et al. 2015; Wigby & Chapman 2005), increasing male fitness at the expense of female fitness. Though male harm is unlikely to evolve on its own, harmful behaviours, biochemical traits and morphologies can evolve as a by-product of mate competition. One such biochemical trait is the seminal fluid protein sex peptide, which has been shown to decrease female lifetime reproductive output while increasing short-term reproductive benefits for males (Wigby & Chapman 2005). The factors governing the evolution and divergence of sex peptide expression, and its contribution to varying male harmfulness, are not well understood. To gain insight into these, I took advantage of a long-term evolution experiment in which replicate *D. melanogaster* populations were adapting to one of three different mating treatments that varied the opportunity for mate competition and the environment in which it occurred. Using four-day old virgin males from these populations that were raised in a common environment, I measured both the quantity of sex peptide they were carrying and current sex peptide gene expression levels. The quantity of stored sex peptide was greatest in males that evolved in the presence of mate competition in a structurally simpler lab environment, which is characteristic of most *Drosophila* lab populations (MC_{simple} treatment). Quantities were somewhat lower, but not significantly so, in males that evolved in an environment absent of mate competition (i.e. under monogamy; MC_{absent} treatment), while quantities were lowest, and significantly so compared to the other mating treatments, in males that evolved with mate competition in a structurally somewhat more complex mating environment ($MC_{complex}$ treatment). In contrast, there was no significant effect of these mating treatments on sex peptide gene expression levels.

Variation in Sex Peptide Quantities

The quantity of stored sex peptide in males from the MC_{simple} treatment were similar to that of the ancestor, which is perhaps unsurprising given that this mating treatment most closely resembles the maintenance routine of the ancestor. Quantities in $MC_{complex}$ males were significantly reduced compared to this, consistent with expectation given that mating rate is reduced in the complex relative to the simple mating environment, presumably because females can more readily avoid or escape from males (Yun et al. 2019). A lower mating rate should reduce sperm competition and also cause the reproductive interests of the sexes to more closely align, weakening interlocus sexual conflict and potentially generating selection against male harm. In line with this, $MC_{complex}$ males are less harmful to females than are MC_{simple} males (Yun et al. 2019, Yun et al. submitted). This current result suggests that decreased sex peptide quantities in $MC_{complex}$ males may, in part, be responsible for their decreased harmfulness, which could arise if males carrying less tend also to transfer less when mating. How these complex males achieve high reproductive success relative to MC_{simple} males, even when tested in the simple mating environment in which sperm competition is presumably strong (Yun et al. 2019), is unclear. Results from the MC_{absent} mating treatment are harder to explain as, unexpectedly, these males carried relatively high quantities of sex peptide, significantly more than $MC_{complex}$ males and similar (although non-significantly less) amounts to that of MC_{simple} males. There are several possible explanations for this.

First, I measured the quantity of sex peptide stored in virgin males, not the amount transferred to females during mating. Beneficial effects of sex peptide for males, and harmful effects on females, result from that transferred to females during copulation. It is therefore possible that reduced harmfulness of MC_{absent} males was achieved by a reduction in the quantity of seminal fluid protein transferred per mating, instead of by decrease sex peptide stores. Why reduced harmfulness would also not be achieved in the same way in $\mathrm{MC}_{\mathrm{complex}}$ males is unclear, but perhaps selection in monogamy favours a general reduction in many or all seminal fluid components that is most readily achieved by reducing the total amount transferred, whereas in the $\mathrm{MC}_{\mathrm{complex}}$ mating treatment selection may have favoured a specific reduction in sex peptide so other seminal fluid proteins could be maintained. *D. melanogaster* males have been shown to manipulate ejaculate composition, including the relative amounts of different seminal fluid proteins transferred to females during mating (Wigby et al. 2009), and this can occur in response to varying social environment (Garbaczewska et al. 2013; Hopkins et al. 2019), female mating status (Sirot et al. 2011) and perceived female quality (Lüpold et al. 2010). Investigating differential effects of mating treatments on sex peptide transfer would require q, but could be tested by quantifying amounts in females immediately following a single mating.

A second possibility is that effects of sex peptide, male benefit and/or female harm, may arise from its interaction with other seminal fluid proteins. If this was the case, then selection may have acted on the suite of seminal fluid proteins as a whole, rather than individually on its isolated components (i.e. sex peptide), and patterns in any one may be misleading when considered in isolation. For instance, the harmfulness of $\mathrm{MC}_{\mathrm{absent}}$ males may have decreased due to altered expression of one or more other seminal fluid proteins with which sex peptide interacts. With over 200 seminal fluid proteins identified (Swanson et al. 2001; Ravi Ram and Wolfner 2007; Findlay et al. 2008), and evidence that several are involved in the same post-mating effects (Avila et al. 2009), it is feasible that phenotypic effects may result from underlying changes in different seminal fluid proteins in different mating treatments. Consistent with this, Hollis et al. (2019) found that the expression of numerous seminal fluid proteins genes differed between 48-hour old males from monogamous vs. polygamous populations, with the expression of 27 decreasing in the monogamous populations relative to the polygamous populations, but several remaining unchanged and two even increasing. The differential decrease in some seminal fluid proteins, and increases in others, suggests potentially complex interactions between them, implying caution in interpreting changes in individual components in isolation.

With respect to sex peptide in particular, at least eight other seminal fluid proteins are required to bind it to sperm tails (Ravi Ram & Wolfner 2009; Findlay et al. 2014). Binding to sperm tails allow sex peptide to persist longer within the female reproductive tract, and to be slowly be released over time (Chapman et al. 2003; Peng et al. 2005). In the absence of such binding, it quickly degrades (Ravi Ram & Wolfner, 2009). It is therefore possible that high levels of sex peptide in MC_{absent} males improves female reproductive output over 1-2 days (Chapman et al. 2003), while longer term costs are ameliorated by the absence of other necessary components to allow it to bind to sperm tails and persist. Some seminal fluid proteins also undergo post-transfer changes within females and females can impact this. For example, the seminal fluid protein ovulin is cleaved within the female reproductive tract by female-produced proteases (Heifetz et al. 2005), and Findlay et al. (2014) demonstrated that at least three female reproductive tract proteins were required for the long-term persistence of sex peptide responses. It is therefore possible that male harm is reduced in MC_{absent} populations via differential female processing of sex peptide. Further insight into interactions could be gained by examining additional seminal fluid proteins and female components among these experimental populations, or via proteomic studies of the male accessory gland.

A third possibility is that sex peptide may not be harmful to females in the context of these mating treatments and the life cycle they impose, and it is therefore under selection for other reasons. Wigby and Chapman (2005) demonstrated that females continuously mated to sex peptide-knockdown males enjoyed significantly greater lifetime offspring production than when continuously mated to males that produced sex peptide normally, in part due to increased survivorship of these females. Differences in survivorship became apparent later in life (i.e. approximately 22 days after introduction of males). Females in my populations are discarded at 11-days of age, so they may not live long enough for costs of sex peptide to be manifested. If so, selection on sex peptide may arise for other reasons. For example, sex peptide (and other seminal fluid proteins) may be energetically expensive to synthesize and maintain, and males from populations of the $MC_{complex}$ treatment may have relatively more constrained energetic budgets due to the added demands of searching for, courting, and chasing females in a their more spacious and structurally complex mating environment. Pre-mating male-male competition in the form of territorial defence may also occur. Selection may favour a decrease in seminal fluid protein production and/or storage in such an environment, but not in the simple MC_{absent} environment in which such energy demanding activities are absent. To the best of my knowledge, costs of sex peptide synthesis have not been directly studied. Although it is a relatively small molecule, such a process could operate on seminal fluid proteins more generally.

Lastly, it is worth quickly addressing the possibility that sex peptide itself has evolved in response to different selective pressures characteristic of each mating treatment. This is not unheard of as variation in other seminal fluid proteins has been shown to have fitness consequences in females (e.g., CG17331, a gene in proteasome subunit beta; Fiumera et al. 2006). With respect to sex peptide, it is possible that a less harmful allelic variant is a high frequency, or has fixed, in the populations from MC_{absent} mating treatment, explaining why these males are less harmful to females despite carrying relatively high quantities of sex peptide.

However, sex peptide has not been found to be highly variable, even between closely related *Drosophila* species (Cierera & Aguadé 1997). Therefore, it seems unlikely that allele frequency differences in sex peptide variants are responsible, though sequence data would be required to address this.

Variation in Sex Peptide Gene Expression

In contrast to the quantity results, there was no evidence that expression of sex peptide RNA differed significantly among the mating treatments, indicating that in four-day old virgin males were synthesizing sex peptide at similar rates in all populations. This suggests the treatment effects on the amount of sex peptide carried by males must have arisen from differences in sex peptide production rates at a younger age, and/or differences in its degradation. It is possible that, once storage capacity is reached (and matings do not occur), expression levels represent maintenance only and treatment effects on this are small or non-existent. Consistent with my results, Hollis et al. (2019) did not find a difference in sex peptide encoding mRNA expression between four-day old males from monogamous (similar to my MC_{absent}) and polygamous (similar to my MC_{simple}) populations. However, expression levels did differ in one- and three-day old males, with lower expression in males from the monogamous populations. Insight into this could be gained by quantifying sex peptide expression at earlier ages in my populations, as well as following mating.

Conclusions

Here, I provide evidence that mate competition, and the environment in which it occurs, can have evolutionary consequences on the quantity of sex peptide stored in *D. melanogaster* males, though the specific selective pressures that gave rise to the observed variation is less clear. Further work investigating the quantity of sex peptide transferred to females during copulation and the consequence of the mating treatments on other seminal fluid proteins, as well as female components, would shed further light on the consequences of mate competition for sex peptide expression and how this impacts male harmfulness.

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Appendix 1 – Preparation of the antibody-capture oligonucleotide-signal oligonucleotide complex

The antibody consisted of a polyclonal antibody generated against a sex peptide-specific amino acid region (i.e. H-CKPTKFPIPSPNPRD-NH_2) through the injection of purified sex peptide into two rabbits (Eurogentec, Belgium). The extracted antibodies were purified and stored as a dehydrated powder until reconstitution by the addition of 1mL of DNA/RNA/Protein free water. This was then further diluted to a concentration of 1g/mL and stored at -20°C until use. The antibody was then conjugated to streptavidin following established protocols using a Lightning Link Streptavidin Conjugation Kit (Innova BioSciences Ltd., Cambridge, UK). 10 µL of LL-Modifier reagent and 100 µL of sex peptide (2.15 µg/µL) was gently mixed. The mix was then pipetted directly into Lightning mix streptavidin tubes and incubated for three hours at room temperature. 10 µL of quencher reagent was added and incubated again at room temperature for 30 minutes. The antibody-streptavidin complex was stored at -18°C until use.

The 10 µL of biotin-capture oligonucleotide (5.6×10^{15} copies/µL) and 100 µL signal oligonucleotides (5.75×10^{15} copies/µL) were bound together by incubating them together for 15 minutes at 48°C and then stored at -80°C until use. The antibody-capture oligonucleotide-signal oligonucleotide complex was generated by biotinylation of the streptavidin-antibody complex, and the bound oligonucleotides described above. 1 µL of antibody and 1 µL of bound oligonucleotides were incubated in 800 µL of filtered DPBS with 5% BSA for one hour at room temperature. This mixture was used immediately after incubation.