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The evolution of pre-zygotic reproductive isolation

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ABSTRACT

Pre-zygotic barriers to interbreeding have received an increasing amount of attention during the past several decades. Emergent areas of interest include how novel sexual communication systems evolve, and intersexual conflict between sperm and the female reproductive tract. Here, I show for the first time that natural genetic variation between *Drosophila simulans* and *D. mauritiana* at a single genomic region can induce both species-specific female choosiness and the male trait they are discriminating against. Additionally, there were two separate regions of the genome that were individually capable of inducing this trait/preference combination, suggesting that trait/preference linkage may be widespread. In another study, I found that males of *Peromyscus* may be using sperm cooperation as an adaptation to obtain fertilizations. In addition, I observed that in *Peromyscus maniculatus*, where females mate multiply, the females have longer oviducts than in the monogamous *P. polionotus*. The longer oviducts may sexually select for more compatible (e.g. conspecific) sperm through cryptic female choice.

Key words: speciation, pre-zygotic reproductive isolation, genetic linkage, sexual selection, sperm cooperation, cryptic female choice

CO-AUTHORSHIP

Chapters 2 and 3 are co-authored by my supervisor, Dr. Amanda Moehring, who contributed to the creation of the *Drosophila* introgression lines, the experimental design, and the analysis and presentation of the results. Chapter 4 was completed under the supervision of Dr. Hopi Hoekstra, Dr. Heidi Fisher, and Emily Jacobs-Palmer (Harvard University).

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LIST OF ABBREVIATIONS

Aep2 - ATPase expression 2
ANOVA - A one-way analysis of variance
BC – Backcrossed
BSC – Biological Species Concept
GO - *Peromyscus gossypinus*
HCG - Human chorionic gonadotropin
Hmr - Hybrid male rescue
HP1 - Heterochromatin protein 1
IU – International units
LE - *Peromyscus leucopus*
M - *Drosophila mauritiana*
M_M – *Drosophila mauritiana* control introgression line
M_S - *Drosophila mauritiana* with a *D. simulans* introgression
MA - *Peromyscus maniculatus*
mau - *Drosophila mauritana*
Na+/K+ ATPase - Sodium-potassium adenosine triphosphatase
Nup96 - Nucleoporin 96
OdsH - Odysseus-site homeobox gene
Oli1 - Oligomycin resistance 1
Ovd – Overdrive
PO - *Peromyscus polionotus*
PMSG - Pregnant mare serum gonadotropin
Prdm9 - PR domain containing 9
PBS - Phosphate buffered saline
Prop copn court - Proportion of copulations of those pairings in which courtship occurred
Prop copn - Proportion of pairings where copulation occurs
Prop court - Proportion of males that court females
QTL – Quantitative trait locus
S - *Drosophila simulans*
S_M - *Drosophila simulans* with a *D. mauritiana* introgression
S_S – *Drosophila simulans* control introgression line
SE – Standard error
sim - *Drosophila simulans*
VERL - Vitelline envelope receptor for lysine
Zhr - Zygotic hybrid rescue

CHAPTER 1

General Introduction

Speciation, the process responsible for Earth's impressive biodiversity, has received an increasing amount of attention from evolutionary biologists over the past several decades (Coyne & Orr 2004). Awareness of how new species arise is not only significant in light of its critical role in our understanding of evolution, but also in light of its importance in conservation efforts and the maintenance of biodiversity.

The basic unit of biodiversity and speciation is the species. New species form when barriers to gene flow evolve between populations, permitting each population to follow a different evolutionary trajectory. Genetic differences accumulate over many generations, and eventually the diverged populations are no longer considered to be the same species. Even while we know the general logistics of how species are produced, actually defining these discrete units has been an area of much disagreement amongst biologists, and numerous species definitions have been introduced (at least 25; Coyne & Orr 2004). The focus of most of these species concepts includes morphology, phylogeny, or genetic differences. The lack of consensus in defining a species stems, in part, from the fact that none of the existing definitions are universal; each is subject to exceptions (Coyne & Orr 2004).

The leading concept used to define a species is the biological species concept (BSC). The BSC defines species as groups of individuals that can interbreed to produce fertile and viable offspring, and that are reproductively isolated from other such groups (Dobzhansky 1935; Mayr 1942). There are obvious limitations to this definition, the most

notable being the categorization of species that do not breed (i.e. asexual species). Even withstanding the non-universality of the BSC, this concept is useful in categorizing many sexually reproducing species.

GEOGRAPHY AND SPECIATION

According to the BSC, the essential condition for the evolution of new species is an impediment to successful sexual reproduction between populations. This reproductive isolation can arise in allopatry or sympatry.

Allopatric speciation

Allopatric speciation is well-supported, and is the most widely accepted model of speciation (Coyne & Orr 2004). In allopatric speciation, new species arise when a geographical barrier separates subpopulations over a long period of time. This period of separation facilitates genetic divergence between populations, whether by selection due to different environments, genetic drift, or the accumulation of different mutations. When these populations come back into contact, interbreeding is hindered by their genetic divergence.

Support for the evolution of reproductive isolation in allopatry exists both from laboratory experiments (e.g. Kilias *et al.* 1980) and from observations in nature (e.g. Knowlton *et al.* 1993). For example, Kilias *et al.* (1980) carried out a five-year selection experiment to determine whether they could induce sexual isolation between *Drosophila melanogaster* populations. The researchers varied the temperature and relative humidity of the environments of different subpopulations derived from a single ancestral

population. After five years, populations raised under different environmental conditions exhibited assortative mating: they preferred to mate with individuals from populations reared under the same conditions.

If two allopatric populations later come into contact, and reproductive isolation between them is not complete, then hybrids could be produced. If these hybrids are not fit (i.e. sterile, inviable), then selection will act to prevent the formation of these hybrid offspring through the development of pre-zygotic isolating mechanisms. This process of natural selection strengthening reproductive isolation is called reinforcement. Although the existence of reinforcement was controversial in the past (Coyne & Orr 2004), empirical evidence now supports its existence in nature (such as Ortiz-Barrientos *et al.* 2004; Matute 2010).

Sympatric speciation

The idea that reproductive isolation can occur in sympatry has historically been controversial. By definition, sympatric speciation occurs between populations living in the same geographical area. Given the absence of a geographical barrier, which is characteristic of allopatric speciation, there is often no way to prevent gene flow between populations. Therefore, in order for sympatric speciation to occur, divergent selection must be stronger than gene flow. As a result, with the exception of polyploid speciation in plants, which does not involve gene flow, sympatric speciation is rare. There are reported cases of sympatric speciation with gene flow (for examples see Barluenga *et al.* 2006; Savolainen *et al.* 2006), but the number of credible cases is small (Coyne & Orr 2004). A recent study by Papadopulos *et al.* (2011) suggests that sympatric speciation may not be

as rare as initially thought. The researchers identified 11 cases of species that likely diverged with gene flow on Lord Howe Island. However, even though sympatric speciation has been shown to occur, allopatric speciation remains considerably more prevalent.

MECHANISMS OF REPRODUCTIVE ISOLATION

According to the BSC, different species do not actually or potentially interbreed (Dobzhansky 1935). The use of the word 'potentially' implies that barriers to mating must exist upon secondary contact. Once subpopulations have diverged sufficiently to allow for reproductive isolation, a number of reproductive isolating mechanisms prevent these different species from merging upon later contact (Mayr 1942). There are two main classes of reproductive isolating barriers: post-zygotic and pre-zygotic.

Post-zygotic isolation

Post-zygotic barriers include the sterility and inviability of hybrids that result from the mating of different parent species (Dobzhansky 1935). Darwin found these unfit hybrids to be problematic (Darwin 1859): how could such hybrids, which do not produce offspring of their own, possibly be selected for in nature? The current view is that post-zygotic isolation evolves via genic speciation (Coyne & Orr 2004). Populations that are separated for long periods of time without gene flow accumulate genetic differences; these differences result from distinct random mutations that allow the populations to respond to selection in the two groups, or due to different selective pressures in the two groups. During the period of separation, there is no selection for one population's gene

pool to continue to be compatible with the other population's gene pool. As a result, when these two diverged genomes are merged in a hybrid, negative epistatic interactions between the two genomes can lead to hybrid sterility and/or inviability (Dobzhansky 1936; Muller 1942). These genic incompatibilities are known as Dobzhansky-Muller incompatibilities.

For example, Presgraves *et al.* (2003) identified a gene, Nucleoporin 96 (Nup96), that epistatically causes inviability in *D. melanogaster* / *D. simulans* hybrids. *Drosophila simulans* Nup96, which encodes a nuclear pore protein, interacts negatively with an unknown factor on the *D. melanogaster X* chromosome to cause lethality. In examining the evolutionary history of Nup96, Presgraves *et al.* observed a high ratio of non-synonymous to synonymous amino acid substitutions, a hallmark of positive natural selection, in both the *D. simulans* and *D. melanogaster* lineages. Thus, as a by-product of the adaptive evolution of Nup96 in the two species, an incompatibility arose between their genes which, when merged in the same hybrid, causes inviability.

Pre-zygotic isolation

Pre-zygotic barriers exert their influence prior to zygote formation. In nature, post-zygotic and pre-zygotic barriers often work concurrently to isolate a given pair of species. However, this is not always the case. Coyne and Orr (1989, 1998) found that, in many species of *Drosophila*, only one form of isolation is observed: either pre-zygotic or post-zygotic. For this reason, the two barriers are thought to evolve independently. Moreover, pre-zygotic isolation was found to occur alone much more often than post-zygotic isolation, thus, of the two types of barriers, pre-zygotic isolation is thought to be the

initial step in speciation. Pre-zygotic barriers include behavioural isolation (lack of attraction between species), habitat isolation (occupy different habitats even while living in the same geographic area), temporal isolation (breed at different times), and mechanical isolation (incompatibility of reproductive structures). Another type of pre-zygotic isolation, which has only recently begun to receive attention, is gametic (post-mating pre-zygotic) isolation (Coyne & Orr, 2004).

Gametic barriers act between copulation and fertilization, with the outcome being the prevention of fertilization. There are many types of gametic isolation, a few of which are poor storage of sperm in recipient females, inviability of sperm in foreign reproductive tracts, poor cross-attraction between the sperm and egg, and conspecific sperm precedence (reviewed in Birkhead *et al.* 2009). Price (1997) demonstrated conspecific sperm precedence in three *Drosophila* species. For example, when a *D. simulans* female mated with both a *D. simulans* male and a *D. mauritiana* male, the majority of the offspring were fathered by the conspecific *D. simulans* male.

The form of gametic isolation that has received the most attention is intrinsic gametic incompatibility, which involves a failure at the level of gametes in terms of their ability to fertilize heterospecifics. This form of isolation has been particularly well-studied in abalones (reviewed in Kresge *et al.* 2001) and sea urchins (reviewed in Birkhead *et al.* 2009). In abalones, species-specific gamete fertilization is facilitated by the sperm protein lysin and the egg protein VERL (vitelline envelope receptor for lysin). Lysin separates the VERL fibres of the egg, allowing access for sperm penetration. These lysin and VERL proteins allow for the recognition of conspecific gametes and the rejection of heterospecific gametes (Vacquier & Lee 1993; Swanson & Vacquier 1997).

Within the field of gametic isolation, the study of post-mating sexual conflict is of interest. Sexual conflict arises when the interests of males and females do not coincide with one another (Gavrilets & Hayashi 2005). For example, in polyandrous species there is often sperm competition, wherein the sperm of different males compete within the female's reproductive tract to obtain fertilizations, as well as sexual conflict arising from different fertilization optima in males and females. Males from populations that have sperm competition and sexual conflict undergo selection for increased fertilization efficiency (e.g., Price *et al.* 1999), while the females from these populations undergo selection to avoid loss of zygotes due to polyspermy, and to influence the paternity of their offspring (cryptic female choice; e.g., Clark *et al.* 1999). The result of this sexually antagonistic co-evolution is that the reproductive traits that are subjected to these selection pressures will diverge in different populations, facilitating speciation. For instance, Arnqvist *et al.* (2000) found that, in insects, speciation rates were significantly higher in promiscuous groups than in monogamous groups, as there are more opportunities for post-mating sexual selection in promiscuous mating systems.

Another area of pre-zygotic isolation that is of increasing interest to biologists is the study of the genetics of behavioural isolation (Coyne & Orr 2004). Behavioural isolation (also known as sexual isolation), involves differences between species that prevent them from initiating mating. A common area where this type of isolation comes into play is during courtship. Courtship involves the exchange of information between the sexes, and can involve one or more of the sensory modalities (i.e. touch, sound, taste, etc.). For example, there may be species-specific mating songs, dances, or pheromones (Coyne & Orr 2004). In some cases, isolation results from the male choosing not to court

a heterospecific female (e.g. Boake *et al.* 2000). However, more often, the male is non-discriminatory and will court females of another species. It is, therefore, up to the female to determine the compatibility of potential mates based on the signals/traits that he exhibits. The female can prefer (if the male is a conspecific) or discriminate against (if the male is a heterospecific) the courting male.

For example, there is species-specific assortative mating in the cichlids of Lake Malawi, which are closely related, but differ in coloration. Couldridge and Alexander (2002) found that, when presented with three heterospecific males, the females preferred the male with the colour pattern that most resembled their conspecific males. Another example of species-specific female preference is found in *Drosophila*. Females of *D. melanogaster*, *D. simulans*, and *D. sechellia* mated more quickly when a conspecific song accompanied the mute male they were exposed to (Ritchie *et al.* 1999). Moreover, in African weakly electric fish, females prefer to mate with males whose electric organ discharge matches the pattern of conspecific males (Feulner *et al.* 2009).

Until recently, understanding how behavioural isolation evolved was problematic (Coyne & Orr 2004). Since mating behaviour is dependent on the preference of one sex for a certain corresponding trait in the other sex, changes to either the trait or preference would be maladaptive. This maladaptive effect may be more profound for the trait as opposed to the preference: even while females may prefer a certain trait, they are likely to choose a trait from what is available even if it does not exactly match their preference. To avoid inabilities to attract a mate, mechanisms must exist for both the trait and preference to co-evolve. Several forces have been proposed that would allow this to happen (Coyne & Orr 2004). A non-genetic mechanism that can lead to behavioural isolation is

imprinting as observed in brood parasites. Brood parasites lay their eggs in the nests of other species of birds, and in some species the male parasitic offspring copy the song of their foster father (Payne *et al.* 1998). As for the female parasitic offspring, they will imprint on that same song, and learn to prefer it. Therefore, rapid behavioural isolation can result due to assortative mating based on the type of song produced and the female's song preference (Payne *et al.* 1998, 2000; Sorenson *et al.* 2003).

Another means by which behavioural isolation can evolve is via genetic drift. Models suggest that non-selective changes to preference can occur; however, this must be accompanied by selection on the trait to maintain the preference-trait combination (Nei *et al.* 1983; Wu 1985). The main force that has been proposed to account for the initial triggering of the evolution of behavioural isolation involves sexual selection acting on either the trait or preference.

SEXUAL SELECTION

The sight of the peacock's (genus: *Pavo*) tail made Charles Darwin sick with worry (Cronin 1991). Such an extravagant and cumbersome appendage would surely make peacocks easy prey. Moreover, the plumage must be costly to produce. Darwin eventually reconciled the ostensible defiance of the peacock's tail in the face of natural selection with his theory of sexual selection (Darwin 1871). This theory explains how ornamental traits in the males of many species come to be: the traits are selected for during the struggle for mates, rather than for survival. Female preference can select for elaborate traits in spite of associated costs, such as reduced lifespan.

Types of sexual selection

There are two main forms of sexual selection: intersexual selection (mate choice) and intrasexual selection (male-male or female-female competition; reviewed in Andersson 1994). In intersexual selection, there is an interaction between the sexes: one sex (usually, but not necessarily, the female) selects a mate from a group of the opposite sex, the members of which are all vying to be chosen. This type of sexual selection often leads to the formation of extravagant secondary sexual traits (reviewed in Andersson 1994). For example, it is the peahens who are selective in choosing a mate, and it is the peacocks who must display their wares – in this case, ornate tails – in the hopes of being chosen by a female. The more elaborate the tail, the more desirable the male (Petrie *et al.* 1991). As a result of this female preference, peacock tails have become increasingly ornate with time, in spite of the associated survival costs.

In intrasexual selection, individuals of a given sex (usually the males) compete amongst themselves to gain access to the other sex (usually females). This type of sexual selection often leads to the evolution of male armaments, such as antlers in deer. For example, in red deer (*Cervus elaphus*), the males often engage in aggressive encounters for access to females (reviewed in Appleby 1982). A male becomes dominant by intimidating and/or fighting off rival males, and gains exclusive mating privileges with a group of females (harem). The reward is enhanced reproductive success.

Why is there female preference?

Initially introduced in 1859 and expanded upon in 1871, Darwin's theory of sexual selection was neglected for nearly 100 years (Cronin 1991). This neglect stemmed, in

part, from the lack of satisfactory justifications presented to explain *why* females should prefer more exaggerated traits, and *how* these preferences could be maintained when male offspring suffered survival costs. The mid-1970s saw a rejuvenation of the field, however, and many instances of non-random mating have since been observed (reviewed in Andersson 1994).

While Darwin observed that there was female preference for more elaborate traits, he was uncertain as to why females should prefer this showiness. Darwin posited that female preference was most likely the result of aesthetic inclinations and the novelty of more elaborate ornaments (Darwin 1871). There remained, however, the question of how female preference could possibly be maintained when the sons must pay the costs of having an elaborate trait that can reduce survival. Models that explain the evolution of female choice fall into two categories: direct and indirect benefits for females. Direct benefits are seen when females benefit directly by increasing their own fertility or chance of survival by mating with a more ornamented male. For example, peahens may gain direct benefits from mating with males that have brighter and therefore more easily detected eyespots, since the costs associated with searching for mates is reduced (Loyau *et al.* 2007).

Indirect benefits, on the other hand, are seen when the sexually selected trait is an indicator of the genetic quality of the male. These benefits are gained in the genetic contribution bestowed on offspring (Fisher 1930). For example, one model, the good genes theory, proposes that male ornaments are an indicator that the male has viability-enhancing genes. In satin bowerbirds (*Ptilonorhynchus violaceus*), the males offer

females multiple visual and acoustic signals, which may indicate heritable traits such as physiological condition (Doucet & Montgomerie 2003).

A similar indirect-benefits model proposed to account for sexual selection is Zahavi's Handicap principle (Fisher 1915; Zahavi 1975). Since sexual ornaments handicap male survival, the idea is that they must be honest signals for the underlying overall genetic quality of the male. Zahavi's model requires that the degree of exaggeration of the trait be dependent on the male's genetic quality: high quality males are able to produce more exaggerated displays. The handicaps therefore act as honest signals for underlying genetic quality; predation, disease and energetic constraints ensure the honesty of the signal.

A model that explains sexual selection in terms of a positive feedback runaway mechanism is Fisherian runaway selection. According to Fisher (1930), genes for preference and trait can spread throughout a population by positive feedback. The idea is that there is selection for the sexual traits that members of the opposite sex find desirable. Because of this preference, the trait becomes advantageous, since the male offspring that are produced will also have the desired trait and thus an increased chance of attracting a mate ('sexy sons' hypothesis; Weatherhead & Robertson 1979), which, in turn, makes having the preference for the trait advantageous. The process is referred to as 'runaway,' because with time, greater preference and more pronounced traits develop. So long as their associated partners are common, both the preference and trait confer a reproductive benefit (Fisher 1930). Runaway selection can lead to strong preferences for arbitrary traits that do not necessarily confer any benefits, and which may even decrease viability (Lande 1981; Kirkpatrick 1982).

For Fisher's runaway process to be set in motion, the ornament and preference genes must become genetically linked (Kirkpatrick 1982). Genetic linkage allows different loci to be inherited together without necessarily being physically linked in proximity on a chromosome. The runaway selection process continues until the costs associated with producing the trait balance the reproductive benefits associated with having it. A potential example of runaway sexual selection is seen in stalk-eyed flies (*Cyrtodiopsis dalmanni*), which carry their eyes on the ends of long, thin appendages (Wilkinson & Reillo 1994). Female stalk-eyed flies are choosy and prefer to mate with males that have longer eyestalk length, even beyond the length seen in nature. Eyestalk length is nonetheless limited in nature due to restrictions imposed by associated survival costs. The genetic correlation between male eyestalk length and female preference for longer stalks is consistent with Fisherian runaway selection.

Sexual selection as a force for behavioural isolation

Sexual selection can act in different directions and on different traits in separate populations. Moreover, the effects of sexual selection are dependent on the available underlying genetic variation in trait and preference alleles, which varies in different populations: genetic drift can randomly lead to the fixation of different alleles, different environmental pressures can lead to selection on different alleles, and the occurrence of different mutations can lead to novel allele variants (Coyne & Orr 2004). Since females will prefer to mate with males who possess the trait that their preference co-evolved with, behavioural isolation between populations can evolve (Coyne & Orr 2004).

GENETIC BASIS OF SPECIATION

Genetics of post-zygotic isolation

Hybrid Sterility

To date, studies delving into the genetic basis of speciation have focused primarily on post-zygotic isolation (Coyne & Orr 2004). The first hybrid sterility gene (i.e a gene that incidentally causes sterility in interspecies hybrids) to be identified, the *Odysseus-site homeobox* gene (*OdsH*), was discovered in *D. simulans* / *D. mauritiana* hybrids (Perez *et al.* 1993; Ting *et al.* 1998). Since the *OdsH* gene encodes a homeodomain, which is commonly found in transcription factors, the OdsH protein is thought to interact with DNA. Given that the DNA-binding domain of OdsH was shown to be rapidly evolving between *Drosophila* species (Ting *et al.* 1998), Bayes and Malik (2009) reasoned that the OdsH protein must, in turn, be interacting with DNA that is also rapidly evolving. The researchers discovered that OdsH binds to stretches of repetitive satellite DNA in *Y* chromosome heterochromatin. When *D. mauritiana* OdsH is found in a *D. mauritiana* / *D. simulans* hybrid background, it binds to *D. simulans Y* chromosome DNA in male reproductive tissues and interferes with the efficient packaging of its heterochromatin.

Another hybrid sterility gene, *Overdrive* (*Ovd*), was also found in *Drosophila*, but this time in the hybrids of two subspecies: *D. pseudoobscura* Bogota and *D. pseudoobscura* USA (Phadnis & Orr 2009). The male hybrids of these two species are mostly sterile, but with age, some fertility is rescued and they will produce a few female offspring. Both the segregation distortion and sterility of the hybrids of these species can be attributed to *Ovd*. While the function of the Ovd protein is unknown, it does carry a Myb (SANT-like) domain, which directs sequence-specific DNA binding. Just as rapid

evolution of *D. mauritiana* OdsH is associated with hybrid sterility in *D. simulans*, rapid evolution of Ovd in the Bogota lineage is also associated with hybrid sterility.

In yeast, a pair of hybrid sterility genes have been identified that result from an incompatibility between nuclear and mitochondrial genomes (Lee *et al.* 2008). Hybrids with the nuclear gene *Aep2* (*ATPase expression 2*) from *Saccharomyces cerevisiae* and the mitochondrial gene *Oli1* (*oligomycin resistance 1*) from *S. bayanus* are unable to sporulate or respire. When Aep2 binds to the 5'UTR of the *Oli1* transcript and facilitates its translation, *Oli1* encodes a subunit of ATP synthase allowing for ATP synthesis. *Saccharomyces cerevisiae* Aep2 fails to bind *S. bayanus* Oli1 mRNA due to sequence divergence in the two proteins, and thus ATP is not synthesized in these hybrids.

Another instance of genetic incompatibility is found in *D. melanogaster* / *D. simulans* hybrids and involves gene transposition (Masly *et al.* 2006). *JYAlpha*, which encodes the alpha subunit of a Na+/K+ ATPase and is essential for sperm motility, is found on the fourth chromosome of *D. melanogaster*, but on the third chromosome of *D. simulans*. As a result of this transposition in the *D. simulans* lineage, some F2 hybrids lack both copies of *JYAlpha*, and are sterile.

To date, the only hybrid sterility gene identified in vertebrates is *Prdm9* (*PR domain containing 9*), which contributes to sterility in male hybrids of *Mus musculus musculus* and *Mus musculus domesticus* (Mihola *et al.*, 2009). *Prdm9*'s protein product is a histone 3 lysine 4 trimethyltransferase that modifies chromatin. In *Mus m. musculus* / *Mus m. domesticus* hybrids, Prdm9, which is rapidly evolving, is no longer compatible with the chromatin regions it usually binds to, and therefore causes sterility by interfering with meiotic sex chromosome inactivation.

Hybrid Inviability

In addition to the previously discussed *Nup96* gene (Presgraves *et al.* 2003), there are a number of other genes known to cause interspecific hybrid lethality. In general, genes for lethality are thought to evolve less rapidly than genes for hybrid sterility (Wu 1992).

The hybrid females that result from matings between *D. simulans* females and *D. melanogaster* males are inviable. The non-coding *D. melanogaster* gene *Zygotic hybrid rescue* (*Zhr*), found in the centromeric heterochromatin of the *X* chromosome, is incompatible with an unknown autosomal *D. simulans* factor (Sawamura & Yamamoto 1997). *Zhr* contains *D. melanogaster*-specific 359 base pair repeats, and in hybrid females, there is improper condensation of this region, leading to mis-segregation (Ferree & Barbash 2009).

In the reciprocal parental cross, between *D. melanogaster* females and *D. simulans* males, it is the hybrid males that are inviable. *Hybrid male rescue* (*Hmr*), which has a DNA binding domain (myb (SANT-like) domain) and is found on the *X* chromosome of *D. melanogaster*, is incompatible with autosomal *Lethal hybrid rescue* (*Lhr*) of *D. simulans* (Barbash *et al.* 2003, Brideau *et al.* 2006). The Lhr protein interacts with heterochromatin protein 1 (HP1), which is involved in the regulation of heterochromatin. Overall, these hybrid incompatibilities - involving *Zhr* and *Hmr*/*Lhr* - show, along with the previously discussed *Odysseus* hybrid sterility gene, that divergence in heterochromatin and its regulation can lead to hybrid incompatibilities.

Another hybrid incompatibility that leads to lethality is found in *Xiphophorus* fish hybrids (Wittbrodt *et al.* 1989). *Xmrk-2* on the *X* chromosome of platyfish (*Xiphophorus maculatus*) encodes a receptor for tyrosine kinase, and is incompatible with an unknown

autosomal factor in swordtails (*X. helleri*; Wittbrodt *et al.* 1989; Schartl *et al.* 1999; Malitschek *et al.* 1995). When these two loci are both present in a hybrid, *Xmrk-2* is misexpressed, leading to the development of melanomas, and eventual early death (Malitschek *et al.* 1995; Schartl *et al.* 1994).

Genetics of pre-zygotic isolation

For its part, the genetic basis of pre-zygotic isolation has received comparatively less attention than that of post-zygotic isolation (Coyne & Orr 2004). While a number of studies have identified genomic regions that underlie mating discrimination (e.g. Moehring *et al.* 2004; Moehring *et al.* 2006; Shaw & Lesnick 2009), individual genes have not been identified. Given that behavioural isolation is arguably the primary cause of reproductive isolation (Coyne & Orr 1989, 1998), further elucidation of its genetic basis is of great interest. Coyne and Orr (2004) reviewed a number of studies addressing behavioural isolation between pairs of species and drew a number of conclusions about its genetic basis: 1) many genes as opposed to a single gene are required; 2) the genes tend to be found primarily on the *X* chromosome; 3) the same genes do not appear to underlie both the trait and preference; 4) preference genes appear to act recessively since hybrid females mate with both parental species and do not discriminate between them. While these are general trends, they are not necessarily universal.

The study of speciation is a burgeoning field. The role of the physical environment in species divergence is well understood (i.e. allopatric speciation). Moreover, we also know about some of the mechanisms that prevent gene flow between distinct species, such as the negative epistatic interactions between different genomes that

produce hybrid sterility or inviability (Dobzhansky-Muller incompatibilities). Another mechanism that assists with keeping species separate is sexual selection, which can be a force for behavioural isolation. To date, a number of genes associated with post-zygotic isolation have been identified. However, no genes have yet been identified that are associated with pre-zygotic isolation. Further studies in the field of reproductive isolation will help elucidate the events that lead to the formation of new species.

THESIS OUTLINE

The studies of this thesis were undertaken with the common goal of elucidating our understanding of the pre-zygotic barriers that reproductively isolate different species. I consider the genetic underpinnings of speciation, as well as the role of male-female sexual conflict, in isolating species.

In Chapters 2 and 3, I seek to contribute to the burgeoning field of the genetics of behavioural isolation. I do this by addressing whether single genomic regions introgressed into another species' genome are sufficient to induce behavioural isolation. Moreover, I seek to ascertain whether loci for female preference and male trait are physically linked within the *Drosophila* genome. Genetic linkage of these loci would provide an evolutionary means by which novel sexual communication systems could arise and be maintained, thereby facilitating species divergence. In Chapter 2, I look for male trait and female preference loci on the right-hand tip of the third chromosome. In Chapter 3, my goal was to determine whether preference/trait linkage is widespread in the genome, and I search for loci for male trait and female preference in the middle and left-hand tip of the third chromosome.

In Chapter 4, I consider the implications of male adaptations, such as sperm cooperation, on cryptic female choice. While cooperation with related sperm provides a competitive advantage to males of the sexually promiscuous *Peromyscus maniculatus* (Fisher & Hoekstra 2010), this behaviour has not been shown to occur *in vivo* in this species. In addition to verifying this behaviour, I will also address a potential female adaptation to biasing paternity – oviduct length. Given that polyandrous females often have the sperm from multiple males within their tracts, they could benefit from longer

oviducts, which provide a selection arena for the most compatible sperm. This form of cryptic female choice could act as a pre-zygotic barrier to reproduction by facilitating conspecific sperm precedence. To address this topic, I compare the oviduct lengths of a highly promiscuous species with less promiscuous ones.

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CHAPTER 2

Identification of genetically linked female preference and male trait

INTRODUCTION

New species form when barriers to gene exchange evolve between populations, most commonly through the reduced fitness of interspecies hybrid offspring or through the prevention of successful mating (e.g., behavioural isolation). Behavioural isolation is arguably the primary cause of reproductive isolation (Coyne & Orr 1989, 1998), and is generally thought to arise through selection acting on either a preference or a trait in one sex, which then leads to the modification of the corresponding trait or preference in the other sex (Engen & Saether 1985; Paterson 1985; Butlin & Ritchie 1989; Schluter & Price 1993; Doebeli 2005; McPeek & Gavrilets 2006). However, a novel preference, or a novel trait (or novel variant of an existing preference or trait), would be maladaptive if no matching trait or preference existed in the opposite sex of the population. The prevailing hypothesis proposed to account for how a novel preference and trait can arise is if they are maintained together through physical linkage on a chromosome (Alexander 1962; Hoy *et al.* 1977; Doherty & Hoy 1985; Butlin & Ritchie 1989; Boake 1991; Mead & Arnold 2004). This "genetic coupling" establishes a common genetic basis for the traits of the sender (e.g., the male and his trait) and the receiver (e.g., the female who demonstrates preference), thereby allowing for the evolution of trait and preference as a unit. The unit can be comprised of either a single gene controlling both trait and preference (pleiotropy), or by different genes that are physically linked in proximity on the chromosome.

This theory of genetic linkage can also apply to an enhancement of runaway sexual selection (Fisher 1930; Lande 1981; Kirkpatrick 1982). Most models suggest that runaway sexual selection occurs if linkage disequilibrium is present (Lande 1981; Kirkpatrick 1982; Barton & Turelli 1991; Otto 1991; Trickett & Butlin 1994; Takimoto *et al.* 2000). Reducing recombination that would separate trait and preference loci (e.g. through genetic coupling) further enhances the effectiveness of runaway sexual selection (Otto, 1991; Trickett & Butlin 1994; Takimoto *et al.* 2000).

To date, a few key studies have observed close genetic linkage for behavioural coupling. Three separate studies identified loci that show behavioural coupling when mutated (Marcillac *et al.* 2005; Fukamichi *et al.* 2009; Gumm *et al.* 2009), demonstrating that linkage is possible. However, it is not clear from these studies if coupling might occur with naturally occurring gene variants, and thus contribute to species isolation. Using natural variants, genomic regions were found to overlap for wing color and preference in *Heliconius* (Kronforst *et al.* 2006), acoustic signal and preference in Hawaiian crickets (Shaw & Lesnick 2009), and female preference and male copulation success in *Drosophila* (Moehring *et al.* 2004). These studies all found that some genomic regions for preference and trait overlapped, while others contributed to only the preference or the trait. What remains to be demonstrated is that the linked loci are sufficient to induce a preference/trait combination. In other words, for genetic coupling to be causal to behavioural isolation, a single, naturally-occurring genomic region would need to be sufficient to provide both the male trait and female preference necessary to induce the first stages of behavioural isolation.

In the previous study by Moehring *et al.* (2004), genomic regions responsible for behavioural isolation between two *Drosophila* species (*D. simulans* and *D. mauritiana*) were identified. These two species are asymmetrically sexually isolated: *D. mauritiana* females are choosy and nearly always reject *D. simulans* males, whereas *D. simulans* females are not choosy and will mate with *D. mauritiana* males. Thus, the two species are behaviourally isolated in response to female mating preference and the male traits that these females are discriminating against (Cobb *et al.* 1988; Coyne 1989; Carracedo *et al.* 2000; Moehring *et al.* 2004). At least seven genomic regions contributed to the preference of *D. mauritiana* females to selectively mate with conspecific males, and at least three genomic regions contributed to the male traits that those females select against (Moehring *et al.* 2004). One region for the male trait and female preference overlapped in genomic location on the right-hand tip of the third chromosome, suggesting that the genetic coupling hypothesis is possible. I focus in on this region in this study by characterizing the behaviour of introgression lines. The genomes of these introgression lines are almost entirely *D. simulans* (S) with a small piece of homozygous *D. mauritiana* (M) genome crossed in (hereafter referred to as S_M), or almost entirely *D. mauritiana* with a small piece of homozygous *D. simulans* genome crossed in (M_S). By pairing these introgression lines with pure-species individuals, the effect of the introgressed genomic region on mating behaviour can be determined relative to pure-species matings, and I can assess whether the genes for male trait and female preference are truly genetically coupled.

MATERIALS AND METHODS

Drosophila housing and strains

All fly lines were housed in 8-dram (~30ml) vials; with each vial containing approximately 7ml of standard Bloomington recipe fly food media. All flies were kept at room temperature (~23°C) on a 14:10 light:dark cycle.

Dr. Amanda Moehring created the introgression lines used in this study (unpublished). Dr. Moehring crossed *D. mauritiana* "synthetic" (a mixture of 6 isofemale lines of *D. mauritiana* that were collected on Mauritius by O. Kitagawa in 1981 and combined in 1983; Coyne 1989) males to virgin *D. simulans* Florida City females. The resulting F_1 hybrid females (males are sterile) were backcrossed (BC) to either *D. mauritiana* (M) or *D. simulans* (S) males. The resulting virgin females were mated to M or S males, respectively, allowed to produce offspring, then genotyped for multiple species-specific markers evenly-spaced throughout the genome (see Appendix Table A.1 for primers used). Offspring of females that contained only the region of interest from the opposite species, but were homospecific at all other loci, were retained for further backcross generations. The region of interest for this study corresponded to the right hand tip of the third chromosome, where loci for male trait and female preference overlap (Moehring *et al.* 2004). Repeated backcrossing of BC females to males of the appropriate species was performed for 10 or more generations to reduce the size of the introgressed region through recombination and to ensure that the background genome was entirely of the BC species; additional molecular markers within the region were used to "track" the introgressed piece from one generation to the next and to define the boundaries of the

introgressed piece (Figure 2.1). At various intervals, BC females were crossed to BC males to attempt to make stable lines that were homozygous for the introgression. Due to recessive sterile loci and recessive lethal loci, these attempts were often unsuccessful, and additional backcrosses were employed to reduce the size of the region (and recombine out these sterile and lethal genes) before homozygous stable introgression lines were created. These introgression lines were either entirely *D. mauritiana* with a small piece of *D. simulans* genome (M_S) or entirely *D. simulans* with a small piece of *D. mauritiana* genome (S_M). Several lines with staggered introgression breakpoints were obtained for the same region at the right hand tip of the third chromosome (see Figure 2.1 for a schematic representation of introgression lines). Lines containing homospecific DNA in the introgression region (e.g. *D. simulans* genome for the introgression region, backcrossed to *D. simulans*; S_S) underwent the same crossing scheme as above in order to create introgression control lines with similar levels of inbreeding (hence called control introgression lines M_M and S_S). All introgression lines were created in parallel.

Behaviour assays

Assays were performed in 8-dram (~30ml) glass vials that had been heat-sterilized (90°C, 10 minutes). Vials were sprayed with a light mist of water to provide slight humidity, which increases the mating activity of *Drosophila*. All behavioural assays were carried out between zero and four hours after "lights on." This morning period is when *D. simulans* and *D. mauritiana* are most reproductively active (Sakai & Ishida 2001). *Drosophila* pairs were assayed at five to seven days old to ensure reproductive maturity and to limit the detrimental effects of enhanced age (Eastwood & Burnet 1977; Long *et*

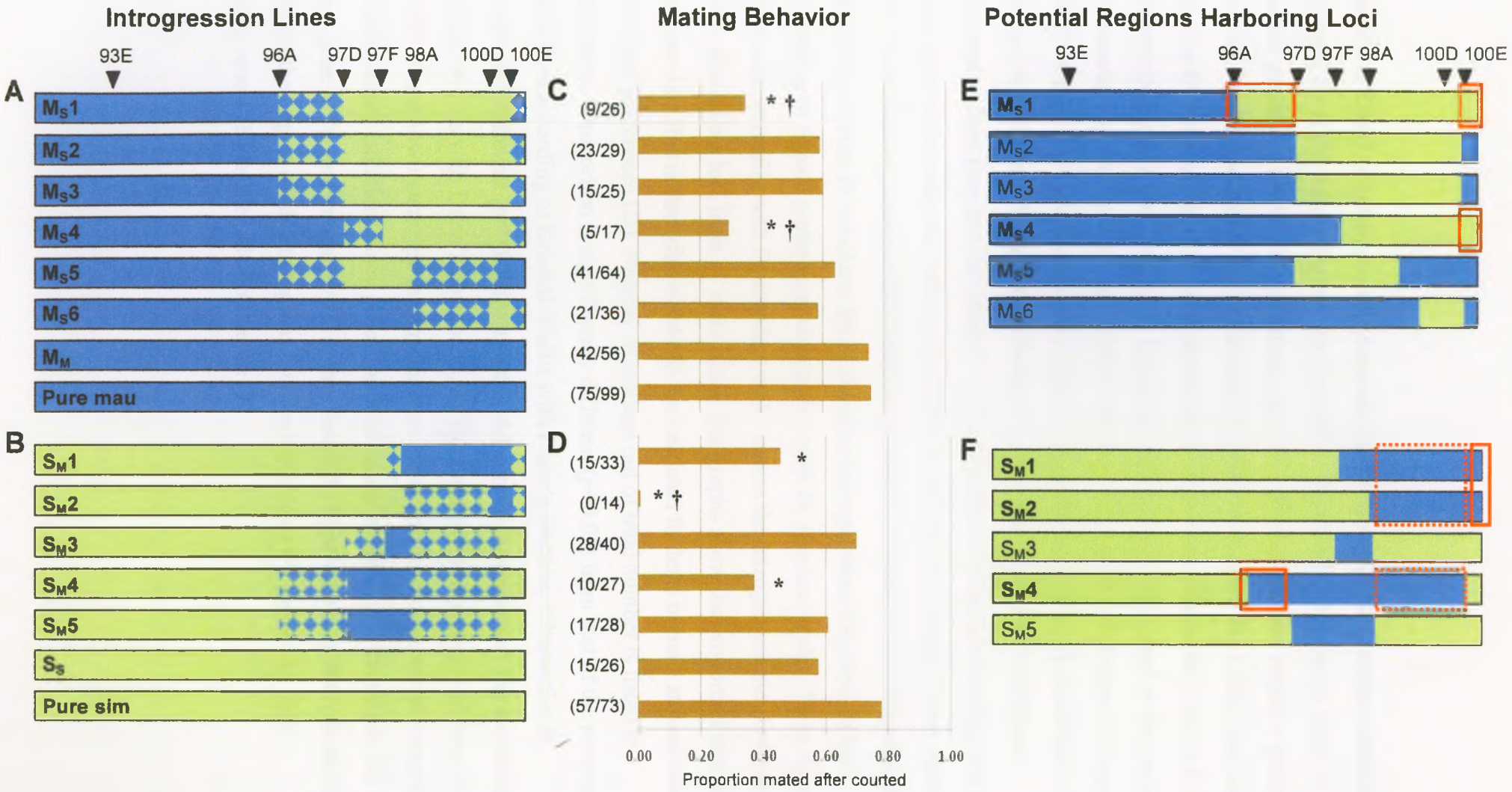

Figure 2.1. Effect of introgressed genes on species isolation. (Figure legend continued on next page)

Figure 2.1. Effect of introgressed genes on species isolation. Rectangles represent the right hand tip of the third chromosome for each of the tested introgression lines; *D. simulans* genome = green; *D. mauritiana* genome = blue; checkered region = genotype unknown. Molecular markers are represented by black triangles; the cytological location (based on *D. melanogaster*) is listed above the triangles (see Appendix Table A.1 for corresponding primers and base pair locations). Behaviour is measured as the proportion of successful copulations after courtship is initiated. M_M and S_S are control introgression lines, Pure mau and Pure sim are pure-species parental controls for *D. mauritiana* and *D. simulans*, respectively. M_S and S_M followed by a number represent the different introgression lines (see text for details). A) *D. mauritiana* introgressions (M_S) test for loci contributing to the male trait when paired with *D. mauritiana* females. These males should be attractive to conspecific females unless the introgressed piece contains key behaviour loci from *D. simulans*. B) *D. simulans* introgressions (S_M) test for loci contributing to female preference when paired with *D. simulans* males. These females should mate normally with *D. simulans* males unless the introgressed piece contains female choosiness loci from *D. mauritiana*. Bar graphs show the proportion that each corresponding line mated after courtship was initiated for tests of male attractiveness (C) and female preference (D). Number of matings out of total number is listed in parenthesis. *Proportion is significantly different ($p<0.05$) from that of the pure-species control line according to Kruskal-Wallis with Dunn's posthoc. †Proportion is significantly different ($p<0.05$) from that of the control introgression line according to Kruskall-Wallis with Dunn's posthoc. E, F) The most extreme example of how the regions of unknown genotype in significant *vs.* non-significant lines could contain loci for behavioural isolation. Since genes for behavioural isolation are thought to fall within the regions of "unknown genotype" in this study, the hypothetical genotypes that would produce the largest candidate regions (outlined in boxes) are shown for male attractiveness (E) and female preference (F).

al. 1980). A single virgin male and a single virgin female were placed together in a vial. The assays were "no choice" since only one mate of each sex was available.

The following variables were calculated: courtship latency, copulation latency, courtship duration, copulation duration, proportion courted (proportion of assays where courtship was initiated), proportion copulated (proportion of assays where copulation occurred), and proportion copulated after behaviour (proportion that copulated of those pairings in which courtship occurred).

During the behaviour assays, the commencement of courtship behaviour was determined if at least one of the following behaviours was produced: orientation of the male towards the female (if subsequently followed by another courtship behaviour), vibration of male wing in courtship song, male following of female, licking of female genitalia with male proboscis, or thrusting of male genitalia towards female. Copulation was determined when the male successfully linked genitalia with the female. Each assay was 45 minutes in length (i.e. each pairing was observed for 45 minutes). However, flies in the process of copulation at the time of the assay's end were monitored until they terminated copulation.

Four different types of pairings were carried out. 1) *D. mauritiana* males with introgressed *D. simulans* DNA (M_S) were paired with pure-species *D. mauritiana* females. Female rejection of the males would suggest that the introgressed *D. simulans* DNA contained genes that held some importance in turning away *D. mauritiana* females. As a control for general mating activity, M_S males were also paired with *D. simulans* females; *D. simulans* females do not normally discriminate against *D. mauritiana* males, and thus these pairings should display normal levels of mating. 2) *D. simulans* males with

introgressed *D. mauritiana* DNA (S_M) were paired with pure-species *D. mauritiana* females. Increased copulation would suggest that the presence of the *D. mauritiana* DNA rendered the males more attractive to *D. mauritiana* females. 3) *D. simulans* females with introgressed *D. mauritiana* DNA (S_M) were mated with *D. simulans* males. Decreased copulation would be expected if the *D. mauritiana* DNA made the *D. simulans* females more selective (paralleling the selectiveness of pure-species *D. mauritiana* females when mated with *D. simulans* males). As a control for general mating activity, S_M females were also paired with *D. mauritiana* males; *D. simulans* females mate readily with *D. mauritiana* males and any effect of the *D. mauritiana* introgression of the S_M females in repulsing *D. simulans* males should contribute to encouraging mating with *D. mauritiana* males if it relates to a species-specific mating preference. 4) *D. mauritiana* females with introgressed *D. simulans* DNA (M_S) were paired with pure-species *D. simulans* males. This pairing allowed the determination of whether the introgressed *D. simulans* DNA had a role in reducing the choosiness of *D. mauritiana* females, making them less likely to reject the *D. simulans* males.

In addition to the introgression lines that were tested, two different controls were also assayed. The first control was a pairing of the parental line that had been used for the backcross (for example, if testing S_M, then a pairing of pure *D. simulans* was used as a control). There is the potential for some lines to suffer from inbreeding depression, which can serve to reduce activity levels (Miller *et al.* 1993), thereby reducing the frequency at which the male and female encounter one another in the mating vial. To counteract this possible effect, the second control underwent the same selection for genotype as the introgression lines except that the conspecific alleles were chosen at the final stage when

the lines were made homozygous, making the entire genome that of a single species, but with approximately the same level of inbreeding as the introgression lines (called M_M and S_S). The second control therefore served to account for potential effects of inbreeding due to repeated selection and backcrossing.

Statistical Analysis

A Kruskal-Wallis test was used to compare the behaviours exhibited by the different lines in a given type of pairing; this was followed by Dunn's multiple comparison post-hoc test to compare the different lines to a control line. Statistical analyses were carried out using GraphPad Prism 5 software.

RESULTS AND DISCUSSION

One genomic region makes males less, but not more, likely to mate

D. mauritiana females readily mate with *D. mauritiana* (M) males, but strongly reject *D. simulans* (S) males. I tested whether *D. mauritiana* males whose genome contains a small introgressed piece of the *D. simulans* genome (M_S) achieve matings with *D. mauritiana* females. If the introgressed *D. simulans* alleles in M_S males contain loci for traits that females are discriminating against, then *D. mauritiana* females should allow fewer copulations with M_S males than with control males. To circumvent the confounding effects that the presence or absence of male courtship initiation may have on the interpretation of results, I focus on the trait most associated with female choice of these males: the proportion of copulations when only considering those pairings in which courtship occurred. This proportion directly relates to the female's rejection of the male.

When considering only those pairings in which courtship occurred, there was a significant effect of line on mating occurrence ($H=31.24$, $p<0.0001$). Dunn's post hoc test showed that there was a significantly lower proportion of males from two of the introgression lines (lines M_S1 and M_S4) that copulated with the *D. mauritiana* females relative to pure-species *D. mauritiana* control pairings ($p<0.05$; Figure 2.1A, 2.1C; Table 2.1) and relative to the M_M control introgression line ($p<0.05$). Males of these lines do not suffer from a general inability to obtain copulations, as they mate normally with *D. simulans* females (Table 2.1), demonstrating that this introgressed region reduces the mating success of these males in a species-specific manner. It should also be noted that this region reduces, but does not eliminate, male mating success, confirming previous results that loci for the male traits likely act additively (Moehring *et al.* 2004).

A comparison of the lines that exhibited a significant decrease in matings *vs.* those that did not can allow us to further reduce the size of the genomic region responsible for the species-specific reduction in male mating success. For example, lines M_S2 and M_S3 did not show a significant reduction in male mating success, and are known to contain *D. simulans* genome in the region between markers 97D and 100E (Figure 2.1A, 2.1C). We can, therefore, assume that this region does not contain loci contributing to this measure of behavioural isolation. Hence, when examining lines that *do* show a significant effect (lines M_S1 and M_S4), we presume that the effect is not caused by loci in the region 97D-100E, but rather is due to loci outside of this region. Since the exact breakpoints of the introgressions are unknown, it is likely that the differences between these lines are due to differences in the regions of unknown genotype. For example, it is possible that the non-significant lines have the least amount of introgressed genome in

Table 2.1. Results of pairings of *D. mauritiana* males containing a *D. simulans* introgression (M_S) with *D. mauritiana* or *D. simulans* females. (See Appendix Table A.2 for duration and latency times) The goal is to determine if an introgressed piece of *D. simulans* (sim) DNA can induce unattractiveness in *D. mauritana* (mau) males when paired with *D. mauritiana* females (top) and whether the introgressed region causes reduced mating in the control pairing with *D. simulans* females (bottom). M_M is a control introgression line and contains only *D. mauritiana* DNA. Pure mau and Pure sim are pure-species individuals that did not undergo any of the introgression crossing scheme. All proportions are listed as the number with trait/total number. Prop court = proportion of males that court females. Prop copn = proportion of pairings where copulation occurs. Prop copn court = proportion of copulations when considering only those pairings in which courtship occurred.

Female	Male	Prop court	Prop copn	Prop copn court	Percent copn court
Pure mau	**M_S1**	26/30	9/30*	9/26*†	34.6
Pure mau	**M_S2**	39/63*	23/63*	23/39	59.0
Pure mau	**M_S3**	25/34	15/34	15/25	60.0
Pure mau	**M_S4**	17/20	5/20*	5/17*†	29.4
Pure mau	**M_S5**	64/76	41/76	41/64	64.1
Pure mau	**M_S6**	36/63*	21/63*	21/36	58.3
Pure mau	**M_M**	56/77	42/77	42/56	75.0
Pure mau	**Pure mau**	99/117	75/117	75/99	75.8
Pure sim	**M_S1**	12/18	9/18	9/12	75.0
Pure sim	**M_S2**	16/19	12/19	12/16	75.0
Pure sim	**M_S3**	17/27	9/27†	9/17†	52.9
Pure sim	**M_S4**	15/17	9/17	9/15	60.0
Pure sim	**M_S5**	16/20	15/20	15/16	93.8
Pure sim	**M_S6**	13/17	9/17	9/13	69.2
Pure sim	**M_M**	17/20	17/20‡	17/17‡	100.0
Pure sim	**Pure mau**	18/19	13/19	13/18	72.2
Pure sim	**Pure sim**	17/23	10/23†	10/17†	58.8

*In a given column, value is significantly different ($p<0.05$) from the pure-species control line Pure mau according to Dunn's test. † In a given column, value is significantly different ($p<0.05$) from the control introgression line M_M according to Dunn's test. ‡ In a given column, value is significantly different ($p<0.05$) from the pure-species control line Pure sim according to Dunn's test.

these regions of genomic identity uncertainty, while the significant lines have the most introgressed genome, and this additional introgressed genome would contain the loci of interest (Figure 2.1E). For the male trait examined here, the area of overlap between the introgressed regions of the two significant lines that is not shared by the non-significant lines is restricted to the very right-hand tip of the third chromosome (Figure 2.1E, red squares), with a second possible locus in one of the lines between 96A and 97D. These regions likely contain loci for the *D. simulans* male trait that *D. mauritiana* females discriminate against. Thus, the tip of the third chromosome contains loci that are sufficient to induce the male trait that *D. mauritiana* females select against.

While this region alone can induce males to be less likely to mate with choosy females, it is not sufficient to make males successful at mating if they normally do not achieve matings. *D. simulans* males do not normally achieve any matings with *D. mauritiana* females (Coyne 1989). *D. simulans* males with a *D. mauritiana* introgression (S_M) were paired with pure-species *D. mauritiana* (choosy) females to test whether the presence of the *D. mauritiana* allele in these males allows them to achieve matings with *D. mauritiana* females. Although the tested males consistently courted the *D. mauritiana* females, the females did not allow them to copulate (Table 2.2).

Hence, a single genomic region, of three possible main contributors (Moehring *et al.* 2004) is enough to reduce male mating success in a species-specific manner, but this same locus in an otherwise heterospecific genome is not sufficient to increase mating success. While it is possible that epistatic interactions among loci are confounding our results, no epistatic interactions were originally detected between the male trait or female preference loci (Moehring *et al.* 2004), making this unlikely.

Table 2.2. Results of pairings of introgression lines in order to determine if an introgressed region can alleviate behavioural isolation. (See Appendix Table A.3 for durations and latencies) M_M and S_S are control introgression lines and contain only *D. mauritiana* or *D. simulans* DNA, respectively. Pure mau and Pure sim are pure-species individuals that did not undergo any of the introgression crossing scheme. All proportions are listed as the number with trait/total number. Prop court = proportion of males that court females. Prop copn = proportion of pairings where copulation occurs. Prop copn court = proportion of copulations when considering only those pairings in which courtship occurred.

Female	Male	Prop court	Prop copn	Prop copn court	Percent copn court
M_S2	**Pure sim**	12/26	1/26	1/12	0.08
M_S5	**Pure sim**	11/23	0/23	0/11	0.0
M_S6	**Pure sim**	15/26	0/26	0/15	0.0
M_M	**Pure sim**	16/31	1/31	1/16	0.06
Pure mau	**Pure sim**	14/26	0/26	0/14	0.0
Pure mau	**S_M1**	28/38	0/38	0/28	0.0
Pure mau	**S_M3**	32/43	2/43	2/32	0.06
Pure mau	**S_M4**	28/39	0/39	0/28	0.0
Pure mau	**S_S**	25/34	1/34	1/25	0.04
Pure mau	**Pure sim**	18/35	0/35	0/18	0.0

None of the proportions in a given column is significantly different ($p<0.05$) from that of the control introgression line (M_M or S_S) or pure-species control, according to Dunn's test.

One genomic region makes females choosy, but not non-choosy

A single genomic region can induce males to be unsuccessful at mating in a species-specific manner, but can a single region induce a female to have a species-specific preference? Female choosiness may arise through a single locus, additively through multiple loci, or epistatically through genetic interactions among multiple loci. It has previously been shown that a single region alone can be sufficient to induce species-specific female preference (Doi *et al.* 2001). In this study, I expand upon these findings to ask whether a single region is sufficient to induce choosiness, whether that same region can alleviate choosiness, and whether that region is linked to loci for the male trait those females are discriminating against.

Females that were entirely *D. simulans* with a small piece of *D. mauritiana* crossed in (S_M) were tested with *D. simulans* males. If the introgressed *D. mauritiana* (choosy) alleles in S_M females are important for discrimination against *D. simulans* males, then those males should achieve fewer copulations with S_M females than with pure-species *D. simulans* females. Again, I focus primarily on the telling trait of the number of copulations that occur after a male has initiated courtship, and observed a significant effect of line on mating occurrence ($H=40.05$, $p<0.0001$). According to Dunn's posthoc test, there were significantly fewer copulations with females from three introgression lines compared to pure-species *D. simulans* pairings (lines S_M1, S_M2, and S_M4; $p<0.05$; Figure 2.1B, 2.1D; Table 2.3), and fewer matings of one line compared to the S_S introgression control (line S_M2; $p<0.05$); females from this line never allowed copulation with a *D. simulans* male. As demonstrated for the male trait, these females do

Table 2.3. Results of pairings of *D. simulans* females containing a *D. mauritiana* introgression (S_M) with *D. simulans* or *D. mauritiana* males. (See Appendix Table A.4 for durations and latencies) Goal is to determine if an introgressed piece of *D. mauritiana* (mau) DNA can induce choosiness in *D. simulans* (sim) females when paired with *D. simulans* males (top) and whether the introgressed region causes reduced mating in the control pairing with *D. mauritiana* males (bottom). S_S is a control introgression line and contains only *D. simulans* DNA. Pure mau and Pure sim are pure-species individuals that did not undergo any of the introgression crossing scheme. All proportions are listed as the number with trait/total number. Prop court = proportion of males that court females. Prop copn = proportion of pairings where copulation occurs. Prop copn court = proportion of copulations when considering only those pairings in which courtship occurred.

Female	Male	Prop court	Prop copn	Prop copn court	Percent copn court
S_M1	**Pure sim**	33/43	15/43 ‡	15/33 ‡	45.5
S_M2	**Pure sim**	14/41 ‡	0/41† ‡	0/14 †‡	0.0
S_M3	**Pure sim**	40/47†	28/47†	28/40	70.0
S_M4	**Pure sim**	27/44	10/44 ‡	10/27 ‡	37.0
S_M5	**Pure sim**	28/43	17/43	17/28	60.7
S_S	**Pure sim**	26/44	15/44 ‡	15/26	57.7
Pure sim	**Pure sim**	73/99	57/99†	57/73	78.1
S_M1	**Pure mau**	15/19	11/19	11/15	73.3
S_M2	**Pure mau**	19/20	13/20	13/19	68.4
S_M3	**Pure mau**	17/21	5/21 †‡	5/17 *†‡	29.4
S_M4	**Pure mau**	17/20	10/20	10/17	58.8
S_M5	**Pure mau**	14/17	11/17	11/14	78.6
S_S	**Pure mau**	26/27	17/27	17/26	65.4
Pure sim	**Pure mau**	16/19	13/19	13/16	81.3
Pure mau	**Pure mau**	18/23	13/23	13/18	72.2

*In a given column, value is significantly different ($p<0.05$) from the pure-species control line Pure mau according to Dunn's test. † In a given column, value is significantly different ($p<0.05$) from the control introgression line (S_S) according to Dunn's test. ‡ In a given column, value is significantly different ($p<0.05$) from the control line Pure sim according to Dunn's test.

not suffer from a general disinclination to mate, since they mate normally with *D. mauritiana* males (Table 2.3).

As before, I compared the area of overlap between the lines that exhibited a significant decrease in matings with those that did not. The area between cytological regions 98A and 100E likely contains loci for *D. mauritiana* female preference (Figure 2.1F, dashed red squares). An alternative possibility is that there is more than one locus on the tip of the third chromosome contributing to the trait; this scenario is suggested by the two closely-linked QTL peaks found in this region in the original study by Moehring *et al.* (2004). In this scenario, my results can instead be explained by a locus between 96A and 97D and a second locus between 100E and the telomere (Figure 2.1F, solid red squares). A third possibility is a more complex combination of loci in all three suggested regions. If we categorize significance solely on comparisons to the control introgression (rather than the pure-species line, which is less inbred), S_M2 is the only line that has a significant shift in behaviour. Line S_M1, which is not significant for this comparison, would then be presumed to not contain *D. mauritiana* at the telomere (contrary to what is drawn in Figure 2.1F), and subsequently the significant region would be between 100E and the telomere. In this scenario, loci for female preference and male trait both lie within a small region near the telomere, making it possible that the locus for female preference is the same as that for male trait.

Regardless of the exact combination of loci affecting behaviour, the tip of the third chromosome contains loci for *D. mauritiana* female discrimination against *D. simulans* males, and a single region alone is sufficient to induce species-specific female choosiness. The tip of the third chromosome also contains loci for the male traits in *D.*

simulans that *D. mauritiana* females are discriminating against, demonstrating that male trait and female preference loci are tightly linked on the chromosome.

While this region alone can induce females to show a species-specific preference, it cannot make normally choosy females "unchoosy." *D. mauritiana* females with a *D. simulans* introgression (M_S) were paired with *D. simulans* males. Pure-species *D. mauritiana* females would completely reject these males, and therefore the introgression females should only mate with those males if the presence of *D. simulans* ("unchoosy") alleles in that region removes the choosiness of *D. mauritiana* females towards these males. Although the males tested consistently courted M_S females, they were not allowed to copulate (Table 2.2). Therefore, replacing a single region (of seven possible regions; Moehring *et al.* 2004) in choosy females with the non-choosy allele did not remove the choosiness of those females, suggesting that the presence of choosy alleles elsewhere in the genome sufficed to induce choosiness even when this particular allele was removed. Hence, choosiness can be induced by a single locus, and it is possible that any single choosy locus in the genome may suffice to induce a level of female preference. Just as with the male trait, it appears that female choosiness is induced by many loci, and any one of those loci may be sufficient to induce choosiness. When a single locus is removed, other loci in the genome are sufficient to cause the choosiness phenotype to persist.

One genomic region induces both female preference and male trait

While it is notable that a single genomic region can induce females to become choosy and males to become unattractive to those females, it is of even greater consequence that these two regions correspond to the same genomic region, providing

evidence supporting the genetic coupling hypothesis (Butlin & Ritchie 1989; Boake 1991; Mead & Arnold 2004). This coupling can enhance Fisherian runaway sexual selection (Fisher 1930), in which the female's preference makes the corresponding male trait advantageous, thus leading to the coevolution of the preference and trait. If a novel variation arises in female choice and the male trait being selected upon, and those traits are physically in proximity on a chromosome, genetic linkage will allow those traits to remain together in subsequent generations since recombination will be less likely to separate them. Since a single locus can be sufficient to induce female choosiness, and a female may select for or against a single allele when choosing her mate, the implication is that a population containing these alleles may become genetically isolated from the parent population through selective mating; the genetic linkage of these alleles would allow this process to occur before recombination could separate the male trait and female preference alleles. Therefore, as a new preference and trait combination arises, they will be maintained and inherited as a unit, providing a mechanism for species isolation in the form of different preference/trait combinations. This single region reduced mating, but did not completely eliminate mating, suggesting that the level of behavioural isolation currently observed between these two species is likely due to the cumulative effect of multiple loci.

The mapped regions in this study, although refined, potentially include several hundred loci, depending on which portion of this region is truly causal. Due to the complex nature of *Drosophila* male courtship, and the relatively unknown basis of female preference, any of these loci could conceivably be involved in construction of the male traits or female preference (Hall 1994; Greenspan & Ferveur 2000; Ferveur 2010). Male

courtship behaviour in *Drosophila* involves the male orienting to the female, receiving and giving pheromonal cues, vibrating his wing in a courtship song, and subsequently attempting copulation. Females must sense these cues, process them neurally, and then respond. Thus, any gene that affects a morphological, sensory, chemical or neural pathway could potentially be involved in the formation of these traits and preferences. Conceivably, even non-coding sequence could contribute through its effect on the regulation of genes elsewhere in the genome. The localization of the polymorphisms contributing to interspecies differences in behaviour will, therefore, continue to be a long and arduous process. However, while the individual loci are still unknown, this information is not critical for the conclusions presented in this study. The tip of the third chromosome affects both male trait and female preference, and thus the genetic factors affecting these traits are physically linked on the chromosome.

The genetic coupling of attractiveness and choosiness, and a single locus inducing but not alleviating choosiness and unattractiveness, has significant implications for how these loci are able to arise and then persist. For example, a single locus can cause a female to be choosy and can cause males of the opposite species to appear unattractive. Physical linkage allows a novel variation in female choice and male trait to remain together in subsequent generations since recombination is unlikely to separate them. The ensuing behavioural isolation would reduce gene flow, making it possible for additional related loci that reinforce this phenotype to arise. If any of the loci are later mutated or lost, the phenotype would persist if a single locus is sufficient to maintain the isolation.

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CHAPTER 3

Is the genetic linkage of female preference and male trait common in the genome?

INTRODUCTION

Even though behavioural isolation is thought to play a larger role than post-zygotic isolation in the initial divergence of species (Coyne & Orr 1989, 1998), its genetic basis has received comparatively less attention (Coyne & Orr 2004). Behavioural isolation results from a lack of cross-attraction between species; it evolves when selection acts on a preference (usually of the female) or trait (usually of the male), causing the corresponding trait or preference in the opposite sex to evolve to match these changes (Engen & Saether 1985; Paterson 1985; Butlin & Ritchie 1989; Schluter & Price 1993; Doebeli 2005; McPeek & Gavrilets 2006).

The genetic coupling hypothesis offers an explanation of how preference and trait can co-evolve, thereby avoiding the maladaptive situation of having a new preference or trait variant with no matching partner. The hypothesis posits that a common genetic basis, such as control by a single gene, or physical linkage on a chromosome, would facilitate the joint inheritance of preference and trait (Alexander 1962; Hoy *et al.* 1977; Doherty & Hoy 1985; Butlin & Ritchie 1989; Boake 1991; Mead & Arnold 2004). Genetic linkage can enhance the effectiveness of runaway sexual selection, and is critical for the evolution of novel sexual communication systems (Fisher 1930; Lande 1981; Kirkpatrick 1982). As a result, genetic linkage can facilitate speciation.

A few studies have found that genetic linkage between trait and preference can occur (Moehring *et al.* 2004; Marcillac *et al.* 2005; Kronforst *et al.* 2006; Fukamichi *et*

al. 2009; Gumm *et al.* 2009; Shaw & Lesnick 2009). In Chapter 2 of this thesis, I complemented these previous studies by showing that a single genomic region is sufficient to provide both the male trait and female preference necessary to induce behavioural isolation. This previous study, as well as the current one, were carried out between two sister *Drosophila* species: *Drosophila simulans* and *D. mauritiana*.

Drosophila simulans and *D. mauritiana* are asymmetrically sexually isolated: *D. simulans* females are not choosy and will consent to mate with *D. mauritiana* males, whereas *D. mauritiana* females are very choosy (exhibit a strong preference) and will mate only rarely with *D. simulans* males (who carry (a) trait(s) discriminated against by *D. mauritiana* females). The males, for their part, are not choosy, and will attempt to mate with females of both species. Using quantitative trait locus (QTL) analysis, Moehring *et al.* (2004) identified seven genomic regions underlying *D. mauritiana* female preference, and three genomic regions underlying *D. simulans* male trait. One of these regions overlapped on the right-hand tip of the 3rd chromosome, suggesting the possibility of genetic linkage. In Chapter 2 of this thesis, I confirmed that this is indeed the case. Moreover, I showed that the trait and preference are sufficient to induce behavioural isolation (see Chapter 2).

While I have shown that genetic linkage occurs (Chapter 2), the question remains as to whether this is a widespread genomic phenomenon. Some studies have identified widespread linkage of QTL for preference and trait (Moehring *et al.* 2004; Wiley & Shaw 2010; Wiley *et al.* 2011), but it remains to be seen whether additional loci are not only genetically linked, but also sufficient to induce behavioural isolation. In this study, I look at two other regions within the *Drosophila* genome that potentially harbor loci for

behavioural isolation: the middle (cytological region 82) and left-hand tip (cytological region 62) of the 3rd chromosome (Moehring *et al.* 2004). Moehring *et al.*'s original QTL map localized male trait loci to both regions 62 and 82, and female preference loci to region 82.

MATERIALS AND METHODS

Materials and Methods for Chapter 3 are very similar to those of Chapter 2. The differences are highlighted below:

Drosophila housing and strains

The lines assayed in this study, and their corresponding genotypes, can be found in Figures 3.1-3.4.

Behaviour assays

Although measured in Chapter 2 of this thesis, the following variables were not computed: courtship latency, copulation latency, courtship duration, and copulation duration. The most relevant information required for the purposes of this study were the following: proportion courted, proportion copulated, and proportion copulated after the occurrence of courtship. Not having to record specific times, and focusing only on whether behaviour occurred (1 vs. 0), and whether copulation occurred (1 vs. 0), allowed for more mating pairs to be assayed at a given time.

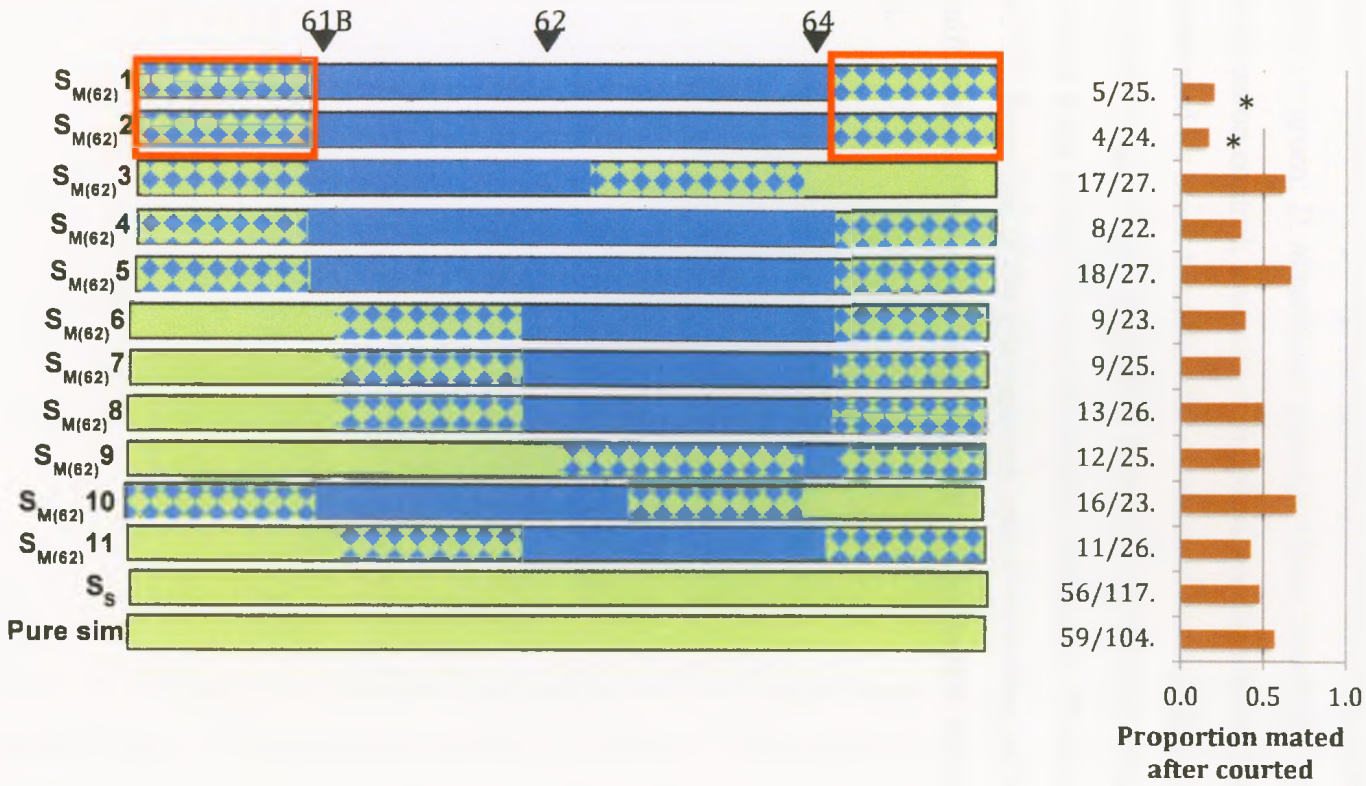

Figure 3.1. (See next page for figure legend)

Figure 3.1. Effect of introgressed *D. mauritiana* DNA in the left-hand tip of the 3rd chromosome on *D. simulans* female preference for *D. simulans* males. *D. mauritiana* introgressions (SM) test for loci contributing to female preference when paired with *D. simulans* males. These females should mate normally with their "own" males unless the introgressed piece contains female choosiness loci from *D. mauritiana*. Bar graphs show the proportion that each corresponding line mated after courtship was initiated for tests of female preference. Number of matings out of total number is listed in parentheses. Rectangles represent the left-hand tip of the third chromosome (cytological region 62) for each of the tested introgression lines; *D. simulans* genome = green; *D. mauritiana* genome = blue; checkered region = genotype unknown. S_S = introgression control; Pure sim = pure-species parental control. Molecular markers are represented by black triangles; the cytological location (based on *D. melanogaster*) is listed above the triangles (see Appendix Table A.1 for corresponding primers). Red boxes delineate potential areas of significance for female preference. *Proportion is significantly different ($p<0.05$) from that of the pure-species control according to Dunn's test. †Proportion is significantly different ($p<0.05$) from that of the control introgression line according to Dunn's test.

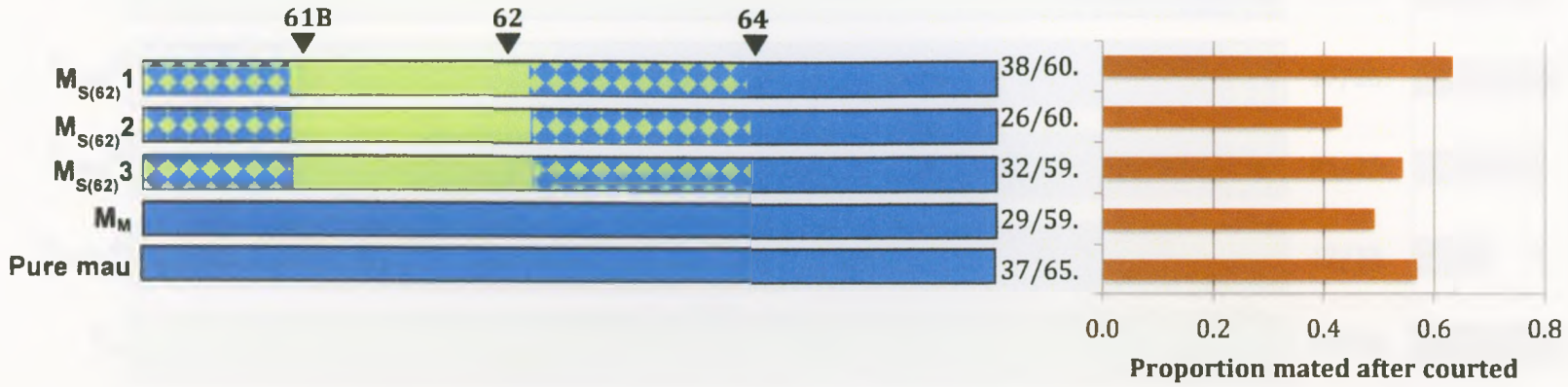

Figure 3.2. Effect of introgressed *D. simulans* DNA in the left-hand tip of the 3rd chromosome on *D. mauritiana* male attractiveness to *D. mauritiana* females. *D. simulans* introgressions (SM) test for loci contributing to male trait when paired with *D. mauritiana* females. *D. mauritiana* females should mate normally with their "own" males unless the introgressed piece contains male unattractiveness loci from *D. simulans*. Bar graphs show the proportion that each corresponding line mated after courtship was initiated for tests of female preference. Number of matings out of total number is listed in parentheses. Rectangles represent the left-hand tip of the third chromosome (cytological region 62) for each of the tested introgression lines; *D. simulans* genome = green; *D. mauritiana* genome = blue; checkered region = genotype unknown. M_M = introgression control; Pure mau = pure-species parental control. Molecular markers are represented by black triangles; the cytological location (based on *D. melanogaster*) is listed above the triangles (see Appendix Table A.1 for corresponding primers). None of the proportions are significantly different ($p<0.05$) from those of the pure-species or introgression control lines according to Dunn's test.

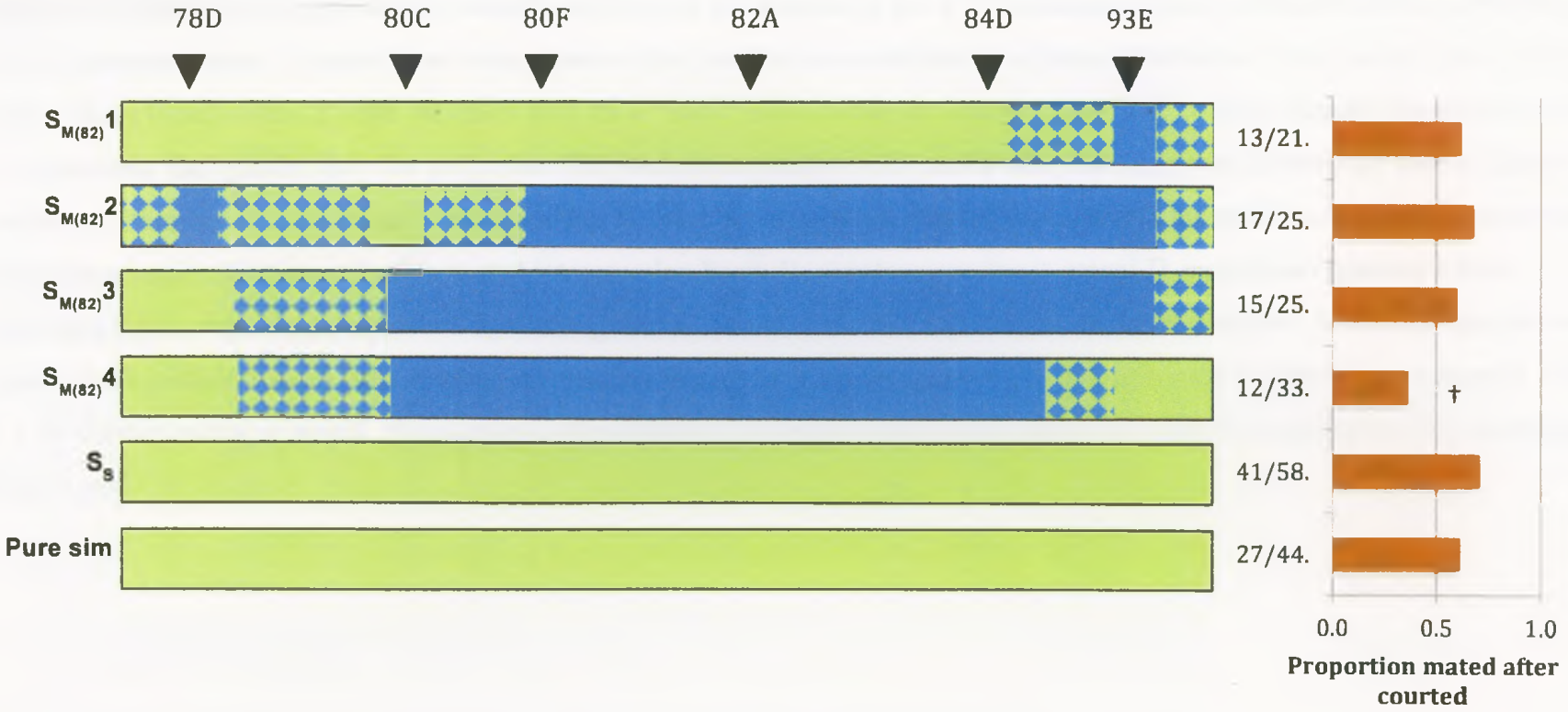

Figure 3.3. (See next page for figure legend)

Figure 3.3. Effect of introgressed *D. mauritiana* DNA in the middle of the 3rd chromosome on *D. simulans* female preference for *D. simulans* males. *D. mauritiana* introgressions (S_M) test for loci contributing to female preference when paired with *D. simulans* males. These females should mate normally with their "own" males unless the introgressed piece contains female choosiness loci from *D. mauritiana*. Bar graphs show the proportion that each corresponding line mated after courtship was initiated for tests of female preference. Number of matings out of total number is listed in parentheses. Rectangles represent the middle of the third chromosome (cytological region 82) for each of the tested introgression lines; *D. simulans* genome = green; *D. mauritiana* genome = blue; checkered region = genotype unknown. S_S = introgression control; Pure sim = pure-species parental control. Molecular markers are represented by black triangles; the cytological location (based on *D. melanogaster*) is listed above the triangles (see Appendix Table A.1 for corresponding primers). †Proportion is significantly different ($p<0.05$) from that of the control introgression line according to Dunn's test.

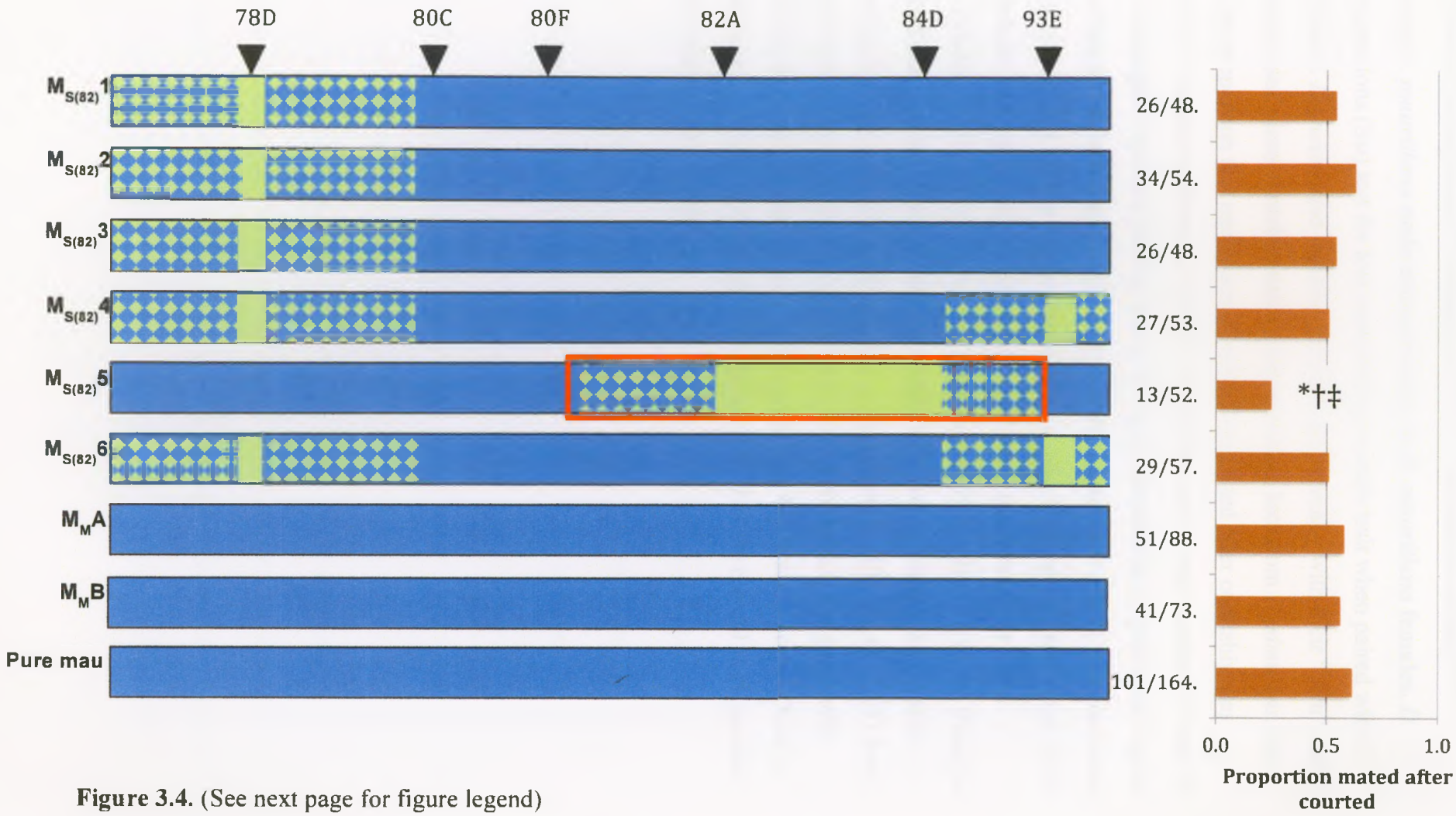

Figure 3.4. (See next page for figure legend)

Figure 3.4. Effect of introgressed *D. simulans* DNA in the middle of the 3rd chromosome on *D. mauritiana* male attractiveness to *D. mauritiana* females. *D. simulans* introgressions (S_M) test for loci contributing to male trait when paired with *D. mauritiana* females. *D. mauritiana* females should mate normally with their "own" males unless the introgressed piece contains male unattractiveness loci from *D. simulans*. Bar graphs show the proportion that each corresponding line mated after courtship was initiated for tests of female preference. Number of matings out of total number is listed in parentheses. Rectangles represent the middle of the third chromosome (cytological region 82) for each of the tested introgression lines; *D. simulans* genome = green; *D. mauritiana* genome = blue; checkered region = genotype unknown. M_M = introgression control; Pure mau = pure-species parental control. Molecular markers are represented by black triangles; the cytological location (based on *D. melanogaster*) is listed above the triangles (see Appendix Table A.1 for corresponding primers). Red boxes delineates potential region harbouring loci for male trait. *Proportion is significantly different ($p<0.05$) from that of the pure-species control according to Dunn's test. †Proportion is significantly different ($p<0.05$) from that of the control introgression line M_MA according to Dunn's test. ‡Proportion is significantly different ($p<0.05$) from that of the control introgression M_MB line according to Dunn's test.

Only the two types of pairings that produced significant alterations to female preference or male trait were repeated: *D. mauritiana* males with introgressed *D. simulans* DNA (M_S) were paired with pure-species *D. mauritiana* females, and *D. simulans* females with introgressed *D. mauritiana* DNA (S_M) were mated with *D. simulans* males. With respect to the other two pairings, chapter two of this thesis found that: 1) *D. simulans* males with introgressed *D. mauritiana* DNA (S_M) paired with *D. mauritiana* females were rejected by these females as if the males were purely *D. simulans*, and 2) *D. mauritiana* females with introgressed *D. simulans* DNA (M_S) rejected *D. simulans* males as if the females were purely *D. mauritiana.*

The regions of interest for this study corresponded to the middle and left-hand tip of the 3rd chromosome, where loci for male trait and female preference potentially overlap (Moehring *et al.* 2004). Several lines with staggered introgression breakpoints were obtained for the same region at the left-hand tip and middle of the 3rd chromosome (see Figures 3.1-3.4 for schematic representations of the introgression lines).

Some of the pairings tested had more than one control introgression line – to differentiate between them, an A or B designation was added (e.g. M_MA and M_MB).

RESULTS AND DISCUSSION

Single region sufficient to induce male unattractiveness

Drosophila mauritiana (M) females are normally choosy and mate only with males of their own species. In line with this, *D. mauritiana* females reject *D. simulans* (S) males. In Chapter 2 of this thesis, I found that *D. mauritiana* females discriminated against *D. mauritiana* males whose genome contained a small piece of introgressed *D. simulans*

genome (M_S). The introgressed *D. simulans* DNA in the M_S males harbored loci for traits that *D. mauritiana* females discriminated against. The introgression in this previous study was located on the right-hand tip of the 3rd chromosome. In the present study, I found that another region within the *Drosophila* genome is sufficient to induce behavioural isolation. A single *D. simulans* introgression at cytological region 82 (middle of 3rd chromosome), but not at cytological region 62 (left-hand tip of 3rd chromosome), is sufficient to cause males to be discriminated against by *D. mauritiana* females.

In assessing whether the 62 and 82 introgression lines (Figures 3.1-3.4) exhibit significant behavioural differences compared to controls, I focus on copulation occurrence of those pairings in which courtship occurred. This proportion relates directly to the female's rejection of the male, and is not confounded by whether or not the male initiates courtship.

Six lines with slightly different introgressions at cytological region 82 were tested to see if the introgressed *D. simulans* DNA in *D. mauritiana* males would cause *D. mauritiana* females to reject these otherwise conspecific males (Table 3.1; Figure 3.4). When considering the proportion of copulations of those pairings in which courtship occurred, a significant effect of line on mating occurrence was observed ($H=24.64$, $p=0.0018$). According to Dunn's posthoc test, males of line $M_{S(82)}5$ obtained significantly fewer copulations than the control pure-species ($p<0.05$) and introgression lines (M_MA, $p<0.05$). This observed difference in copulation occurrence cannot be attributed to a general disinclination on the part of the males to court the females, since there were no significant differences among any of the lines for courtship occurrence ($H=13.93$, $p=0.0837$).

Table 3.1. Proportions of courtship and mating behaviours of pairings of pure-species *D. mauritiana* females and *D. mauritiana* males containing a *D. simulans* introgression (M_S) at cytological regions 62 ($M_{S(62)}$) or 82 ($M_{S(82)}$). The goal is to determine if an introgressed region at 62 or 82 can induce male unattractiveness as it relates to behavioural isolation. M_M, M_MA and M_MB are control introgression lines and contain only *D. mauritiana* DNA. Pure mau are pure-species individuals that did not undergo any of the introgression crossing scheme. All proportions are listed as: number showing the trait/total number measured. Prop court = proportion of males that court females. Prop copn = proportion of pairings where copulation occurs. Prop copn court = proportion of copulations when considering only those pairings in which courtship occurred.

Female	Male	Prop court	Prop copn	Prop copn court	Percent copn court
Pure mau	**$M_{S(62)}1$**	60/74	38/74	38/60	20.0
Pure mau	**$M_{S(62)}2$**	60/72	26/72	26/60	43.3
Pure mau	**$M_{M(62)}3$**	59/81	32/81	32/59	54.2
Pure mau	**M_M**	59/84	29/84	29/59	49.2
Pure mau	**Pure mau**	65/84	37/84	37/65	56.9
Pure mau	**$M_{S(82)}1$**	48/50	26/50	26/48	54.2
Pure mau	**$M_{S(82)}2$**	54/64	34/64	34/54	63.0
Pure mau	**$M_{S(82)}3$**	48/56	26/56	26/48	54.2
Pure mau	**$M_{S(82)}4$**	53/63	27/63	27/53	50.9
Pure mau	**$M_{S(82)}5$**	52/58	13/58*†‡	13/52*†‡	25.0
Pure mau	**$M_{S(82)}6$**	57/60	29/60	29/57	50.9
Pure mau	**M_MA**	88/104	51/104	51/88	57.9
Pure mau	**M_MB**	73/80	41/80	41/73	56.2
Pure mau	**Pure mau**	164/201	101/201	101/164	61.6

*In a given column, for a given cytological region (62 vs. 82), value is significantly different ($p<0.05$) from the pure-species control line Pure mau according to Dunn's test. † In a given column, for a given cytological region (62 vs. 82), value is significantly different ($p<0.05$) from the control introgression line M_MA according to Dunn's test. ‡ In a given column, for a given cytological region (62 vs. 82), value is significantly different ($p<0.05$) from the control introgression line M_MB according to Dunn's test.

By comparing the genotypes (at different molecular markers) of the significant and non-significant lines, the size of the genomic region responsible for reducing male mating success can be reduced to the region spanning roughly cytological region 80F to 93E (red box in Figure 3.4). Additional molecular markers will need to be tested to further refine the boundaries of the introgressions.

Thus, this introgression study supports Moehring *et al.*'s (2004) QTL study that found that loci for male trait (i.e. what makes *D. simulans* males unattractive to *D. mauritiana* females) are located at cytological region 82. Moreover, the introgressed region of $M_{S(82)}5$ reduced, but did not eliminate, male mating success, thereby also confirming that male traits act additively. Of note is that since there were limitations as to the sizes and locations of the introgressions that were created for this study, the full genomic region identified in Moehring *et al.*'s (2004) QTL study was not tested here. Thus, it is possible that there are more loci acting in this centromeric region of the 3rd chromosome.

While cytological region 82 harbors loci for male unattractiveness, cytological region 62 does not. No significant differences were observed among the lines for courtship occurrence ($H=2.482$, $p=0.6479$), copulation occurrence ($H=5.762$, $p=0.2177$), or copulation occurrence of those pairings where courtship occurred ($H=5.573$, $p=0.2334$; Figure 3.2, Table 3.1). This failure to find loci for male trait in this region is in contrast to the findings of Moehring *et al.* (2004), who identified QTL for male trait in this region. However, only three lines with introgressions in the 62 region were tested in the present study, and they do not span the full QTL region. During the process of making the introgression lines, the uncovering of recessive sterile loci and recessive lethal loci

precluded many introgression lines from being created in this region (Amanda Moehring, personal communication). Thus, it is possible that loci for the male trait do reside in this region, and that the introgression lines tested in this study simply do not uncover them.

Thus, in addition to the right arm of the 3rd chromosome, near the telomere (Chapter 2), another region in the middle of the 3rd chromosome, near the centromere, is sufficient to reduce male mating success in a species-specific manner. The centromere is heterochromatic, and in *D. simulans* (but not in *D. mauritiana*) is subject to repressed recombination (True *et al.* 1996). Several studies have found that genes for traits relevant in speciation are often located in areas of reduced recombination in the genome (Williams *et al.* 2001; Feder *et al.* 2003). For instance, inversions are known to be areas of low recombination, and a number of QTL studies have found that traits involved in pre- and post-zygotic isolation map to inversions (e.g. Noor *et al.* 2001; Williams *et al.* 2001). Moreover, the quest for speciation genes has led to the identification of QTLs of interest in heterochromatic regions (e.g. Moehring *et al.* 2004). Novel variants of alleles (such as the male trait in this study) that arise in areas of low recombination will be more likely to be maintained in the presence of gene flow.

Single region sufficient to induce female preference

Drosophila simulans females, unlike their *D. mauritiana* counterparts, are normally not choosy, and will mate with *D. mauritiana* males. In Chapter 2 of this thesis, I found that *D. simulans* females with a piece of *D. mauritiana* DNA crossed in (S_M) discriminated against *D. simulans* males. Thus, this single *D. mauritiana* genomic region was sufficient to induce choosiness, and therefore harbors loci important for discriminating against *D.*

simulans males. In addition to this region located on the right-hand tip of the 3rd chromosome, the present study shows that two additional regions (at cytological regions 62, left hand tip of 3rd chromosome, and 82, middle of 3rd chromosome) are also sufficient to induce female preference.

For region 62, two lines showed an induction of female preference compared to the control lines (Figure 3.1, Table 3.2). When considering the proportion of copulations of those pairings in which courtship occurred, a significant effect of line on mating occurrence was observed ($H=34.62$, $p=0.0005$). Lines $S_{M(62)}1$ and $S_{M(62)}2$ exhibited significantly fewer copulations than the pure-species control ($p<0.05$) and, while not statistically significant, fewer copulations compared to the introgression control. No significant differences were observed for any of the introgression lines for courtship occurrence compared to either the pure-species or introgression control lines ($H=19.75$, $p=0.0720$). This observation that loci for female preference are found at region 62 is not supported by Moehring *et al.* (2004), who did not find a significant QTL at this region. This discrepancy is likely due to the original Moehring *et al.* study only being able to detect loci of major effect.

To further hone in on the specific region of introgression responsible for inducing female preference at cytological region 62, the lines that exhibited a significant reduction in matings were compared to those that did not. The region harboring loci for female preference is likely found to the left of 61B and/or to the right of 64 (red boxes in Figure 3.1). To further refine the boundaries of the introgressions, additional molecular markers will need to be tested.

Table 3.2. Proportions of courtship and mating behaviours of pairings of *D. simulans* males and *D. simulans* females containing a *D. mauritiana* introgression (S_M) at cytological regions 62 ($S_{M(62)}$) or 82 ($S_{M(82)}$). The goal is to determine if an introgressed region at 62 or 82 can induce female preference as it relates to behavioural isolation. S_S is a control introgression line and contains only *D. simulans* DNA. Pure sim are pure-species individuals that did not undergo any of the introgression crossing scheme. All proportions are listed as: number showing the trait/total number measured. Prop court = proportion of males that court females. Prop copn = proportion of pairings where copulation occurs. Prop copn court = proportion of copulations when considering only those pairings in which courtship occurred.

Male	Female	Prop court	Prop copn	Prop copn court
Pure sim	**$S_{M(62)}1$**	25/57	5/57*†	5/25*
Pure sim	**$S_{M(62)}2$**	24/45	4/45*†	4/24*
Pure sim	**$S_{M(62)}3$**	27/38	17/38	17/27
Pure sim	**$S_{M(62)}4$**	22/48	8/48	8/22
Pure sim	**$S_{M(62)}5$**	27/54	18/54	18/27
Pure sim	**$S_{M(62)}6$**	23/56	9/56	9/23
Pure sim	**$S_{M(62)}7$**	25/50	9/50	9/25
Pure sim	**$S_{M(62)}8$**	26/42	13/42	13/26
Pure sim	**$S_{M(62)}9$**	25/41	12/41	12/25
Pure sim	**$S_{M(62)}10$**	23/38	16/38	16/23
Pure sim	**$S_{M(62)}11$**	26/47	11/47	11/26
Pure sim	**S_S**	117/188	56/188	56/117
Pure sim	**Pure sim**	104/188	59/188	59/104
Pure sim	**$S_{M(82)}1$**	21/32	13/32	13/21
Pure sim	**$S_{M(82)}2$**	25/34	17/34	17/25
Pure sim	**$S_{M(82)}3$**	25/40	15/40	15/25
Pure sim	**$S_{M(82)}4$**	33/54	12/54†	12/33†
Pure sim	**S_S**	58/73	41/73	41/58
Pure sim	**Pure sim**	44/73	27/73	27/44

*In a given column, for a given cytological region (62 vs. 82), value is significantly different ($p<0.05$) from the pure-species control line Pure sim according to Dunn's test.
† In a given column, for a given cytological region (62 vs. 82), value is significantly different ($p<0.05$) from the control introgression line S_S according to Dunn's test.

An induction of female preference was also observed for region 82 (Figure 3.3, Table 3.2). When considering the proportion of copulations of those pairings in which courtship occurred, there was a significant effect of line on mating occurrence ($H=11.14$, $p=0.0250$). Line $S_{M(82)}4$ exhibited significantly fewer copulations than the introgression control ($p<0.05$), and, while not significant, fewer copulations than the pure-species control. This finding supports Moehring *et al.* (2004), who found a QTL of significant effect for female preference in this region. No significant differences were observed for any of the introgression lines for courtship occurrence compared to either the pure-species or introgression (S_S) control lines ($H=8.510$, $p=0.1303$).

The lines that had a significant reduction in matings were compared to those that did not, and the region likely containing loci for female preference is found between the markers at cytological regions 78D and 80C (red box in Figure 3.3). Additional molecular markers will need to be tested to further refine the boundaries of the introgressions.

Thus, the present study identifies loci for inter-specific female preference in both the centromeric and left-arm telomeric regions of the 3rd chromosome. Similar to as previously discussed for male trait, these heterochromatic regions are subject to reduced recombination (except for the centromere of *D. mauritiana*), which would prevent the loss of novel allelic variants due to gene flow.

Linkage of male trait and female preference

I have previously shown (Chapter 2) that a single region on the right-hand tip of the 3rd chromosome can cause species-specific female preference and male trait. Importantly,

this region showed linkage of the loci for female preference and for the male trait that the females discriminated against. The question remained, however, as to whether this linkage was a more widespread phenomenon throughout the genome. The present study shows that the middle of the 3rd chromosome (at cytological region 82) also shows linkage for male trait and female preference. Not only do these results support the genetic coupling hypothesis (Butlin & Ritchie 1989; Boake 1991; Mead & Arnold 2004), but they also show that genetic coupling may be more widespread in the genome, and is not restricted to a single region.

Two of the regions where I looked for significant effects of female preference and male trait showed linkage for female preference and male trait; these regions were both found in areas of low recombination: cytological region 82 (present study) is near the centromere, and cytological region 98 (Chapter 2) is near the telomere. Since both the centromere and telomere are heterochromatic, they experience reduced recombination. This low recombination means that the preference and trait loci will be less likely to be separated, and will therefore be more likely to be inherited as a unit (Alexander 1962; Hoy *et al.* 1977; Doherty & Hoy 1985; Butlin & Ritchie 1989; Boake 1991; Mead & Arnold 2004).

Different preference/trait combinations can be a powerful means of preventing gene flow between species. As such, sexual communication systems, and the evolution of novel variants of these systems, can play an important role in speciation. Genetic linkage of the traits and preferences that make up these communication systems can facilitate their rapid and concerted co-evolution via Fisherian runaway selection (Fisher 1930, Lande 1981). In runaway sexual selection, the female preference makes the

corresponding male trait advantageous, and vice versa, which leads to their co-evolution. Novel variants to the preference and trait would be maladaptive if they were not maintained together; genetic linkage facilitates this co-inheritance, since recombination between the preference and trait is suppressed.

Even though genetic linkage of trait and preference potentially plays an important role in speciation and sexual selection, it is not known how widespread it is in nature. This study, combined with my Chapter 2 study, serve to show that, at least within the *Drosophila* genome, genetic linkage as it relates to sexual communication and speciation is not an isolated occurrence.

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CHAPTER 4

Sexual selection, sperm cooperation, and cryptic female choice

INTRODUCTION

Although sexual selection is most widely known for its effects on behaviour and external morphology, it was not until the 1970s that it was discovered that sexual selection could also continue to act after copulation within the female reproductive tract (Parker 1970). Previously, it was assumed that the females of most species were sexually monogamous, and that the contest for fathering offspring was won upon successful copulation (Birkhead 2000). However, females of many species mate promiscuously, and this can lead to sperm competition. Sperm competition occurs when the sperm from the ejaculates of different males unite in a female's reproductive tract, and compete to fertilize the limited number of available ova (Parker 1970). As a result of this competition, there is selection for the male to have greater fertilization efficiency, which leads to the evolution of many male reproductive traits (Parker 1970; Birkhead & Møller 1998).

An adaptation that males of some species have evolved to contend with sperm competition is sperm cooperation (Moore *et al.* 2002). Sperm cooperate when they link to other sperm, forming aggregates, or 'trains,' and then swim as a collective to the ova. In their *in vitro* study of *Apodemus sylvaticus* (wood mouse) sperm, Moore *et al.* (2002) found that sperm trains have greater swimming velocity compared to individual sperm. Since the speed at which sperm swim correlates positively with fertilization success, sperm trains provide an advantage in inter-male sperm competition (e.g. Birkhead *et al.* 1999; Gage *et al.* 2004).

A more recent study by Fisher and Hoekstra (2010) showed that, in *Peromyscus* (deer mice), sperm preferentially aggregate with more closely-related sperm *in vitro*. This preference for more related sperm was seen for same-male sperm compared to conspecific brother sperm, as well as for conspecific compared to heterospecific sperm. This discrimination based on genetic relatedness is found in the highly promiscuous *P. maniculatus*, but not in its monogamous sister species, *P. polionotus*. Thus, sperm cooperation is likely an adaptation to sperm competition, and therefore mating system (polyandry vs. monogamy). While Moore *et al.* (2002) supported their findings by showing that clumping behaviour also occurs *in vivo* in *A. sylvaticus*, sperm clumping behaviour has not been verified *in vivo* in *Peromyscus* species. Showing that sperm aggregation behaviour is not the product of *in vitro* conditions is important for showing that this behaviour can indeed contribute to sexual selection and speciation. Here, I use *P. maniculatus* to determine whether sperm clumping occurs *in vivo*.

Sexual conflict occurs when male adaptations to obtain fertilizations, such as sperm cooperation, surpass the optimum number of fertilizations for females. The reproductive traits of the female will respond by co-evolving to counteract the male traits that impact her fitness (Eberhard 1996; Chapman *et al.* 2003). This sexually antagonistic co-evolution is a potential driver of speciation: females co-evolve with males from their own population, and thus are adapted to resisting potentially harmful traits in these males, but not in foreign males (Holland & Rice 1998; Parker & Partridge 1998; Chapman *et al.* 2003; Pizzari & Snook 2003).

As a result of sexual conflict, successful copulation and ejaculation does not guarantee fertilization. Sperm must pass through an 'obstacle course' on their way to the

female's ova, the site of fertilization (Birkhead 2000). Male sperm that are more adapted to a female's tract will be more successful in obtaining fertilizations - this phenomenon is known as cryptic female choice. In mammals, the oviduct represents the final region in the female reproductive tract where sperm competition can occur, and is therefore of interest when considering cryptic female choice and sexual selection.

There is a high degree of heritable variation in the morphology of mammalian oviducts, suggesting that selection may indeed be at work. Inter-specific differences are seen in oviduct length, in the morphology of the ciliated fimbria that help direct ova into the oviduct, and in the structure of the uterotubal junction (junction between the uterus and oviduct; Hunter 1988; Figure 4.1). Gomendio and Roldan (1993) found that, in 11 mammalian species, the greater the amount of sperm in male ejaculate, the longer the length of the oviducts of the females. Consistent with this, polyandrous species tend to have larger testes (reviewed in Gomendio *et al.* 1998), which have been shown to produce and release more sperm per ejaculate (Møller 1989). Moreover, Anderson *et al.*'s (2006) study suggests that oviduct length is positively correlated with promiscuity. Promiscuous females could benefit from having longer oviducts since the increased length would enable a more efficient selection area for females to exert cryptic female choice.

It remains to be seen, however, whether sister species with divergent mating systems (promiscuity vs. monogamy) exhibit differences in oviduct length. To test whether there is an association between oviduct length and mating system, I compared

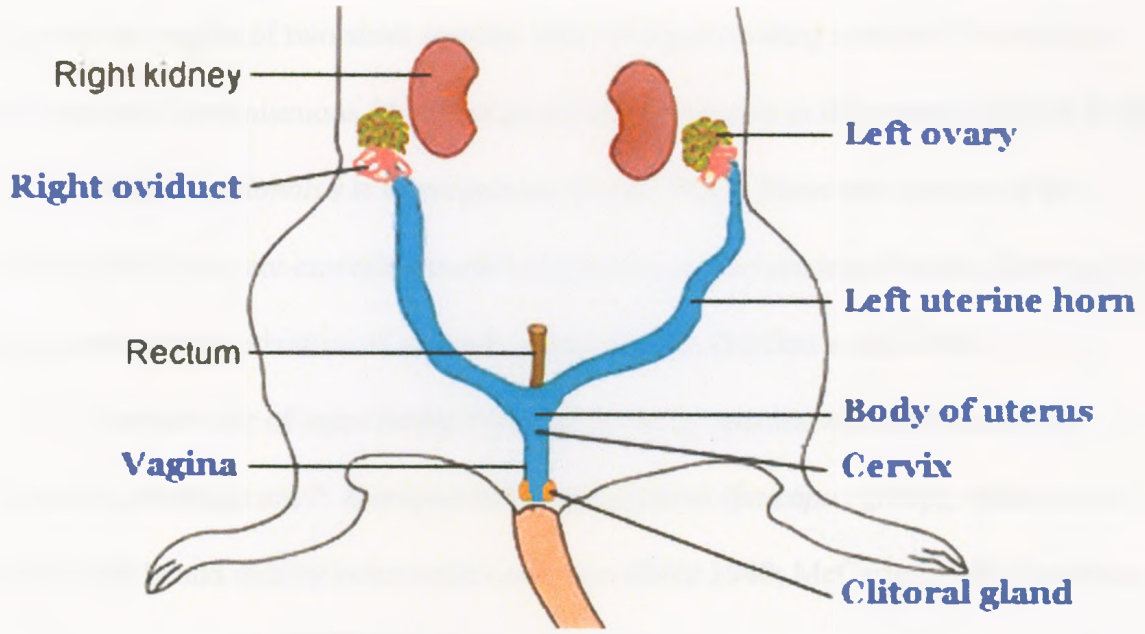

Figure 4.1. Schematic representation of murine female reproductive tract (as found in *Peromyscus*). Sperm travels from vagina through uterus, uterine horns, and oviducts to reach ovaries. Image adapted from Conti *et al*. (2004).

the oviduct lengths of two sister species with divergent mating systems: *Peromyscus maniculatus* is promiscuous (the most promiscuous species in the genus; Birdsall & Nash 1973), while *P. polionotus* is monogamous (Foltz 1981). These two species of the *maniculatus* group are emerging model organisms as they are inter-fertile, allowing for the genetic characterization of sexually selected traits (Mullen *et al.* 2006).

Another pair of inter-fertile *Peromyscus* sister species which are receiving increasing attention are *P. leucopus* and *P. gossypinus* (*leucopus* group), which occur sympatrically and exhibit behavioural isolation (Dice 1940; McCarley 1964; Bradshaw 1968; Lovecky *et al.* 1979). *Peromyscus leucopus* has been observed to have both monogamous and promiscuous mating systems (Mineau & Madison 1977; Kirkland & Layne 1989), while the mating type of *P. gossypinus* is believed to be promiscuous (Heidi Fisher, personal communication), but has not, as of yet, received attention in the literature. In this study, I will determine whether oviduct length is correlated with mating system.

MATERIALS AND METHODS

The Hoekstra laboratory currently maintains colonies of *P. maniculatus* (MA), *P. polionotus* (PO), *P. leucopus* (LE), and *P. gossypinus* (GO) (originally obtained from the Peromyscus Genetic Stock Center; University of South Carolina). Laboratory-reared females and males were housed at 22°C with a 16:8 hour light:dark cycle in single-sex groups of two to four until they were used.

Natural mating and *in vivo* clumping

Proven *P. maniculatus* male breeders (had previously fathered litters) were paired with *P. maniculatus* virgin females; both the males and females were over the age of 60 days and thus reproductively mature. To ensure that females were in oestrus, and therefore sexually receptive at the time of pairing, they were superovulated via intraperitoneal injections of PMSG (pregnant mare serum gonadotropin) and HCG (human chorionic gonadotropin). A 10 IU PMSG injection was given to the females, followed by a 10 IU HCG injection 48 hours later. Following injections, one-on-one pairings were immediately set up of one superovulated female with one male; courtship and mating behaviours were observed. The females were sacrificed at two, three, and six hours after copulation, and their reproductive tracts were removed immediately. Cuts were made at different regions in the tract, such as the uterine horn and oviducts (Figure 4.1), and the internal liquid was pipetted out and placed on a slide for imaging. The presence of sperm clumps was determined visually. Sperm clumps were scored as present when two or more sperm heads were linked together and the grouping was swimming forward.

Female tract isolation and imaging

Following sacrifice, females were weighed and the following lengths measured: body (base of tail to snout tip), tail (base of tail to tip of tail), hind foot (base of foot to longest toe), and ear (base of ear to tip of ear). The sample size varies across traits for a given species due to missing values for some individuals (Table 4.1). The reproductive tracts were then excised: the lower bound included the uterus and cervix, and the upper part included the ovaries. The tract was placed in PBS (phosphate buffered saline) and imaged

Table 4.1. Descriptive statistics for average values (±SE) of different traits in four different *Peromyscus* species.

Species	Average Oviduct Length (mm)	Hindfoot Length (mm)	Weight (g)	Body Length (mm)	Tail Length (mm)	Ear Length (mm)
P. maniculatus (MA)	22.31±0.49 (N=15)	19.73±0.25 (N=11)	17.18±0.69 (N=8)	87.75±0.75 (N=8)	59.75±1.77 (N=8)	14.50±0.20 (N=8)
P. polionotus (PO)	15.39±0.64 (N=8)	17.63±0.28 (N=7)	13.77±1.09 (N=3)	81.33±2.48 (N=3)	44.67±2.16 (N=3)	15.00±0 (N=3)
P. gossypinus (GO)	18.49±0.74 (N=21)	23.24±0.22 (N=21)	24.29±1.26 (N=21)	90.67±1.25 (N=21)	66.43±1.17 (N=21)	17.40±0.26 (N=21)
P. leucopus (LE)	16.76±0.52 (N=21)	20.76±0.20 (N=21)	18.63±0.47 (N=21)	85.86±0.74 (N=21)	75.70±1.13 (N=20)	16.25±0.13 (N=20)

The N value below each average trait value is the sample size; the values are not consistent across all traits for a given species since, for some individuals, certain trait values were missing.

within two hours of dissection. Surgical blades were used to cut the connective tissue and straighten the oviducts; care was taken not to stretch the oviducts during this process of straightening. Measurements of oviduct length were obtained using AxioVision software (Carl Zeiss).

Statistics for oviduct length

To correct for body weight, a regression of oviduct length and body weight was carried out and residuals were calculated. A univariate analysis of variance followed by Tukey's post-hoc multiple comparison test was used to determine differences between the residual means of the four species: *P. maniculatus*, *P. polionotus*, *P. leucopus*, and *P. gossypinus*.

RESULTS

***In vivo* sperm clumping**

Three separate *P. maniculatus* females were observed to possess sperm clumps at two (N=1) and three (N=1) hours post-copulation (see Figure 4.2 for image of sperm clumps), but not at six (N=1) hours post-copulation. Thus, sperm clumping occurs *in vivo*, and appears to be a time-dependent phenomenon. Sperm clumps were observed in both the body of the uterus and in the base and upper regions of the uterine horns. Although not formally scored, I observed a severe attrition in the number of sperm from the body of the uterus to the oviducts. The observed sperm clumping behaviour is not due to any external buffers since none were used: samples of sperm were taken directly from the female reproductive tracts, and not diluted in any external media.

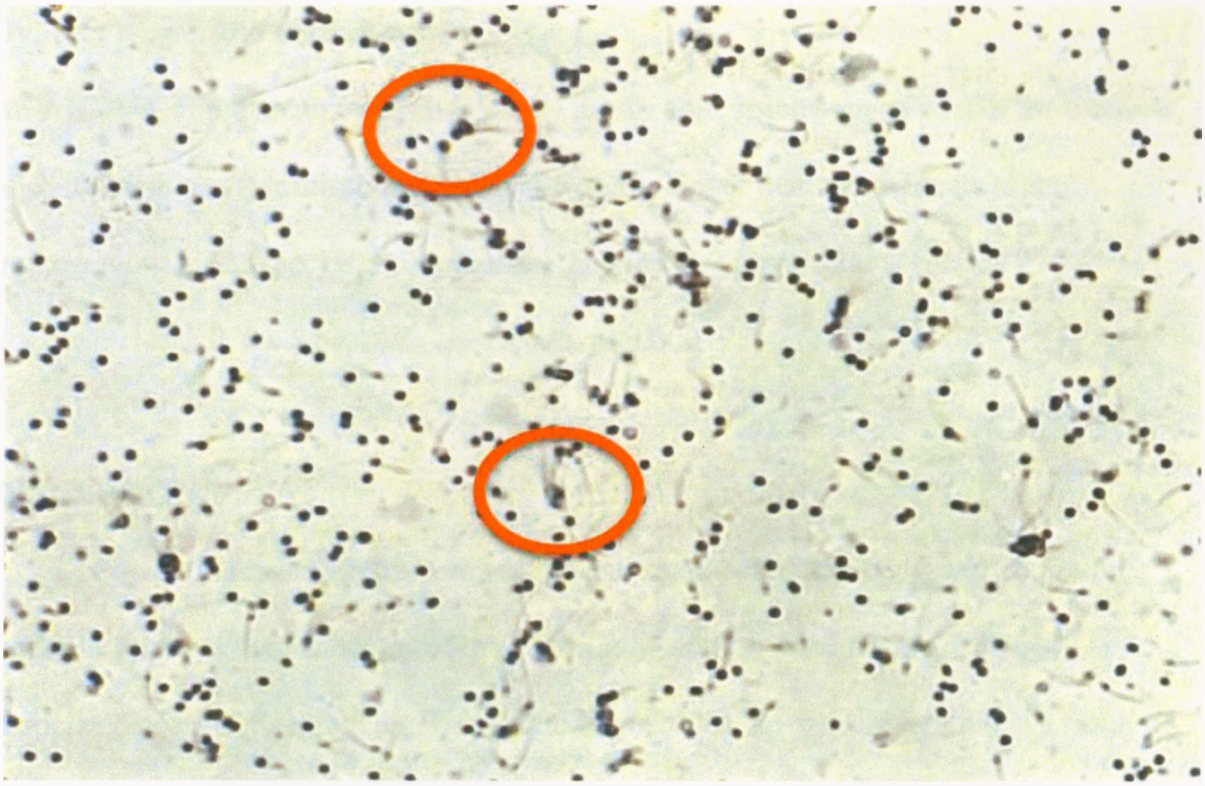

Figure 4.2. Evidence of sperm clumping *in vivo*. The image shows a still-image capture of fluid extracted from the uterine horn of a female *P. maniculatus* two hours post-copulation with a *P. maniculatus* male. The red circles highlight two sperm clumps: the bundle of heads are at one end (near the bottom portion of the circle in each case) and the tails extend to the opposite end of the red circles (towards the top right in the upper circle and towards the top in the lower circle).

Oviduct length and mating system

An ANOVA of the mean oviduct residuals for the four species revealed that the oviducts of *P. maniculatus* (promiscuous) are significantly longer than the oviducts of its monogamous sister species, *P. polionotus*, and the less promiscuous species *P. gossypinus* and *P. leucopus*. ($F_{(3,49)}$=10.096, p<0.0001; Figure 4.3).

DISCUSSION

Populations with sperm competition and sexual conflict undergo selection for males to improve the efficiency of their fertilizing ability; this occurs in spite of associated costs to females (e.g. Gomendio *et al.* 2006). Sperm cooperation is a result of selection upon males to deal with sperm competition. The present study found that sperm clumping occurs *in vivo* in *P. maniculatus*. Previously, this behaviour had only been seen *in vitro* for this species (Fisher & Hoekstra 2010), leading to the caveat that *in vitro* conditions may have produced the aggregations. With the newfound knowledge that this is not the case, and that sperm cooperation is a naturally occurring phenomenon, its role in sexual selection and speciation is supported. Sperm cooperation is of interest to the study of pre-zygotic reproductive isolation since, while it represents an adaptation to intraspecific competition, it may also be involved in interspecific competition. Specifically, the preferential cooperation of related sperm provides a means by which conspecific sperm can outcompete heterospecific sperm, enabling conspecific sperm precedence (Howard 1999). This increased fertilization success of conspecific sperm compared to heterospecific sperm represents a form of gametic isolation, and facilitates the segregation of different species.

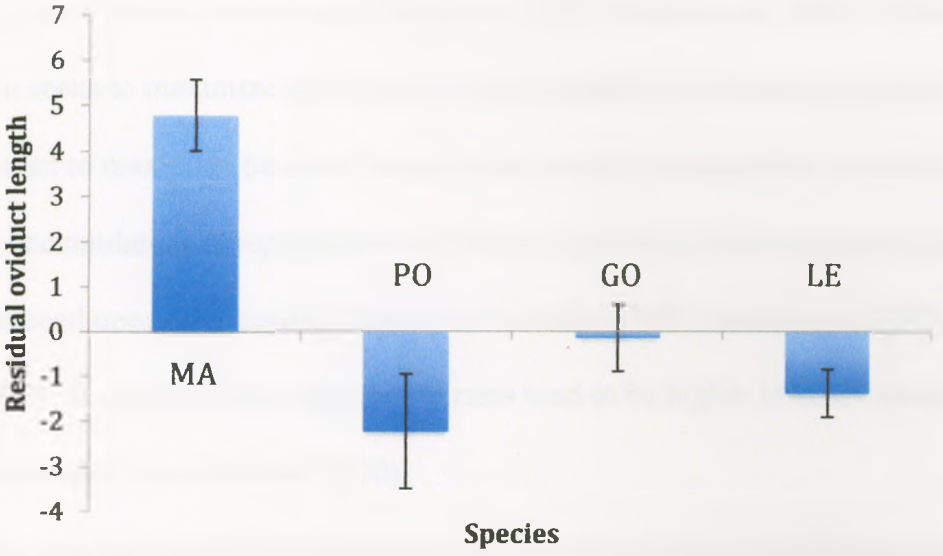

Figure 4.3. Average residual oviduct length (± SE) observed in different *Peromyscus* species. MA = *P. maniculatus* (highly promiscuous; N=8); PO = *P. polionotus* (monogamous; N=3); GO = *P. gossypinus* (promiscuous; N=21); LE = *P. leucopus* (promiscuous; N=21). MA and PO are sister species within the *maniculatus* group, and GO and LE are sister species within the *leucopus* group. Histogram bars associated with PO, GO, and LE, are not significantly different from one another, but are significantly different from MA, according to Tukey's test ($p<0.0001$).

For their part, females also undergo selection to influence the paternity of their offspring (cryptic female choice; e.g. Clark *et al.* 1999; Yeates *et al.* 2009). While sperm cooperation seeks to maximize the fitness of a given male, it is curbed by traits in the female that act to maximize her own fitness. Such sexually antagonistic arms races can lead to the accumulation of reproductive differences such that different populations may fail to interbreed upon later contact (Parker & Partridge 1998; Panhuis *et al.* 2001; Ritchie 2007). In support of this, speciation rates tend to be higher in taxa where sexual conflict is present (Arnqvist *et al.* 2000).

Sexual selection predicts that males of polyandrous species will have greater post-copulatory competition and therefore experience greater selection than males of monogamous species (Andersson 1994; Birkhead & Møller 1988). In addition to the selection that results from competition with other males, there is also selection conferred by cryptic female choice. Thus, mating system (polyandry vs. monogamy) is crucial to the evolution of reproductive traits (Shuster & Wade 2003). An arena where females can exert cryptic choice is within their oviducts, the last region of the female reproductive tract prior to the site of fertilization. Longer and more coiled oviducts present a more challenging obstacle course for sperm, allow a greater time period for sperm to be in competition with other sperm, and allow a greater time for sperm to be impacted by the conditions present in the female reproductive tract. For example, the female tract can retain chemical barriers such as low pH and viscous mucus, which select for sperm that are able to survive in these adverse conditions (reviewed in Suarez & Pacey 2006).

Thus, the female's anatomy in the form of oviduct length may represent a form of sexual selection. In challenging the sperm of rival males, gametes with the greatest

reproductive potential may be selected for. With this reasoning, the shorter oviduct lengths of *P. polionotus* observed in this study suggest that there has not been a selective force on genital specialization in this species. This also accords with the smaller testes size observed in this species (Heidi Fisher, personal communication). On the other hand, *P. maniculatus* was observed to have very long oviducts (and males also have larger testes; Heidi Fisher, personal communication). Given that the females of this species mate multiply, this increased length provides a means by which only the most efficient, and therefore compatible, sperm will reach the ova.

Peromyscus gossypinus and *P. leucopus* were also observed to have shorter oviduct lengths than the highly promiscuous *P. maniculatus*. This suggests that these two species of the *leucopus* group have also not experienced pronounced selection on their oviduct length to deal with sperm competition. Given that *Peromyscus leucopus* has the potential to adopt both monogamous and promiscuous mating systems (Mineau & Madison 1977; Kirkland & Layne 1989), it is possible that there has not been a long enough history of polyandry within this species to encourage selection to act on oviduct length. Moreover, while the mating type of *P. gossypinus* is believed to be promiscuous, given observations in the laboratory (Heidi Fisher, personal communication), this does not reflect how this species behaves in the wild. Further studies are required to ascertain the mating systems of both *P. leucopus* and *P. gossypinus*.

The different selective pressures found in monogamous and promiscuous species can potentially lead to interspecific gamete incompatibility, and hence a pre-zygotic barrier to reproduction. The relationship observed between oviduct length and mating system in *Peromyscus* suggests that oviduct length is undergoing selection to deal with

sperm competition in *P. maniculatus*. The sexual conflict that has lead to the co-evolution between male and female traits (such as sperm cooperation and oviduct length) could potentially have occurred via Fisherian runaway processes (Fisher 1930). In Fisherian runaway sexual selection, there is a spread of preference and trait via positive feedback. For example, cryptic female choice (such as oviduct length) is a way for females to exert their preference for superior sperm competitors (sexually selected sperm hypothesis; reviewed in Pizzari & Birkhead 2002). As a consequence, females will produce sons who are also good sperm competitors ('sexy sons' hypothesis).

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CHAPTER 5

General Discussion

Pre-zygotic barriers to reproduction are a powerful means of preventing gene flow between species. Moreover, barriers that act prior to the formation of the zygote avoid fitness costs associated with producing sterile or inviable hybrids. In this thesis, I considered two types of pre-zygotic isolating barriers: behavioural isolation and gametic isolation.

Genetic linkage and the induction of behavioural isolation

New species often evolve when new sexual communication systems evolve in separate populations (Ritchie 2007). Since different species are characterized by unique sexual signals and preferences, gene flow can easily be prevented between even recently diverged groups. In spite of the importance of the evolution of new sexual communication systems for speciation, only a few studies have empirically looked at the underlying genetics of these systems (reviewed in Shaw *et al.* 2011). One of the aims of this thesis is to contribute to this area of study.

In Chapters 2 and 3, I used *Drosophila simulans* and *D. mauritiana* to locate regions of the genome that can induce inter-specific behavioural isolation. In Chapter 2, I found that a single genomic region was sufficient to induce species-specific female choosiness. Similarly, a single genomic region was sufficient to induce species-specific male unattractiveness. This induction of behavioural isolation via changes to a single genomic region is a significant finding.

Also noteworthy is the fact that the loci that induced female preference and male trait were physically linked in proximity on the 3rd chromosome. While linkage for preference and trait has previously been observed (Moehring *et al.* 2004; Kronforst *et al.* 2006; Shaw & Lesnick 2009), I have expanded upon these previous studies by showing that a single, naturally occurring genomic region is sufficient to provide both the male trait and female preference necessary to induce behavioural isolation. Not only does genetic linkage facilitate the co-inheritance of trait and preference alleles by reducing recombination between them, it also facilitates runaway sexual selection (Fisher 1930; Lande 1981; Kirkpatrick 1982). Thus, rapid and coordinated evolution of sexual communication systems, and species, can occur.

In Chapter 3, I lend further support to the role of genetic linkage in behavioural isolation by showing that a second region within the *Drosophila* genome also shows linkage for trait and preference; this region is also sufficient to induce behavioural isolation. These findings suggest that the genetic linkage of preference and trait may be widespread within the genome.

Of note is that the two regions identified in this thesis that show genetic linkage for preference and trait are found in regions of low recombination (heterochromatic regions near the centromere and telomere of the 3rd chromosome). These areas of reduced recombination may facilitate the evolution of novel variants of trait and preference, since the genetic linkage of these traits would be more likely to be maintained in the face of gene flow.

The behavioural isolation that I observed in Chapters 2 and 3 was characterized by a significant decrease in, but not elimination of, matings. Thus, of the preference and trait

loci identified in this thesis, a single locus was not sufficient to completely behaviourally isolate *D. simulans* and *D. mauritiana*. It would be of interest to see the effect of combining the loci that I identified in a single line – will behavioural isolation be magnified? Another area of future research relates to the large size (up to several megabases) of the genomic regions that I have uncovered. Decreasing the size of these introgressions and pinpointing the specific genes that underlie female preference and male trait will be an important future step.

In general, the study of the genetic basis of behavioural isolation is an emerging field, and many questions still remain to be addressed, such as: How many genes contribute to pre-zygotic reproductive isolation? What do these genes encode? Where are these genes located in the genome? Is the genetic linkage of trait and preference loci evident throughout the genome, and in other species as well?

Gametic isolation: Sperm cooperation and cryptic female choice

According to sexual selection theory, males of species where females mate multiply will experience greater post-copulatory selection (Andersson 1994). In addition to the selective forces caused by male-male competition, there is also selection caused by cryptic female choice. Given that males and females have different fitness optima, sexual conflict can result (Ritchie 2007).

In Chapter 4 of this thesis, using *Peromyscus*, I considered sexual conflict and its potential role in sexual selection and speciation. First, I addressed the male adaptation of sperm cooperation, and found that this behaviour occurs *in vivo* in the sexually promiscuous *P. maniculatus*. Importantly, I discovered that this previously studied

behaviour is not the product of *in vitro* conditions, therefore supporting its role in sexual selection.

In response to male adaptations to maximize fertilization success, females can have adaptations to bias which male's sperm will be successful in fertilization (Eberhard 1996; Chapman *et al.* 2003). Such cryptic female choice could occur in the oviducts (Anderson *et al.* 2006). Longer oviducts increase the sperm's challenges during the journey to the site of fertilization, with only the most compatible sperm reaching the ova (Suarez & Pacey 2006). In Chapter 4, I found that oviduct length correlated positively with promiscuity: the highly promiscuous *P. maniculatus* had longer oviducts than the other, less promiscuous, species observed. This suggests that oviduct length may be a sexually selected trait that depends on mating type. Females that mate multiply require a more selective environment (such as longer oviducts) to weed out incompatible sperm.

An important future step for a better understanding of male sperm competition and cooperation would be to set up contests between conspecific and heterospecific sperm and score fertilization success. Moreover, if such competition assays could be observed within the female reproductive tract (such as via fluorescent labeling), this would provide information about cryptic female choice. Another future direction for elucidating gametic isolation involves identifying genomic regions that underlie fertilization success. A comparison of the genetic variation between species in their ability to fertilize a given female's ova would provide important information about the genes that underlie such adaptations as conspecific sperm precedence.

In summary, this thesis provides an important addition to the growing field of the evolution of pre-zygotic reproductive isolation.

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APPENDIX

Table A.1. Molecular markers used to differentiate *D. simulans* and *D. mauritiana*.

Chr.	Cyto. region	Marker Name	Forward Primer	Reverse Primer	Notes
X	4F	Dmu566661	TATTTCGCTAACAAACCGGC	AACGCGATCACAAACATCAA	
	8B	Dso9021	GATCTTTTCATGTGTTATTT	CCGTTTTGTTTGGCAACTTT	
	13F	Droexo2	TGCAGGGCACCTTCTCTCCA	GAACGCTTGATTTAGATTTGGG	
	18C	simmau_18C3	TCTTTGCATGATAATGAAATCCAG	AAAGTTCTGTGGACTTGTGGATG	
2	21C	Droexpand	GTGATCGATCCCGCTGTC	TCCGGYTTCCAATFAGCTTG	
	30A	AC005889	GCGTGGCTGGCATATAG	TAAGCCCCCTCGTGTAATTG	
	38E	AC004759	ACAGACGGAAAGCCAAAATG	CACTCCGCCTCGTTTCTTAC	
	47A	Drogpad	GAAATAGGAATCATTTTGAATGGC	AATTAAAAACAAAAAACCTGAGCG	
	54B	Ds003617	CAACCACCCACAAGCACAC	CCTCTCCGGTTGGGCTAC	
	59C	twi	TCCCTGCAGCAGATCATCCC	ATCACTCGAGCTGAGCATGC	digest with enzyme HinfI for 1hr at 37°C
3	61B	3L_173	GTGAATCGGAGGGACAAAGA	GACGGATTTGCCAAACAAAC	
	62A	ve	GAGAACCCAACGCAGAATGT	ATATCCTCCGACTCCGGAAG	digest with enzyme PstI for 1hr at 37°C
	63D	AC004658	ATTTGGTCCACGAGAGATTT	TGGGAAAACTGTGCCACATA	
	73A/B	Dm22f11t	GGATGCTCGGATACCAAAAA	TCGCCTGTGACTTAGATTGC	
	78D	simmau_78D8	TTTGAGTATCGCTTGGATGC	GCGGACCATTTAAATTCGAG	
	81F6	CG12582	CCCAAGTGCTGGACTCCTAC	CGTGAAACGTCAGGTTCATG	
	84D5	Mel84Db	AAAAAACTGCATTTGGCAGCCG	GAGAGCAGAAATCGAGAATCAGGC	
	91F	Dronanos	CGCAAGTATTCATTTCAACACA	TGCTGGCGGTTGTTTCAT	
	93E*	3R_4051	TTCTGTTATTGCCGCTGACA	ACTGCTTGCTCACCCAATCT	base position 4051000
	96A*	3R_20104	CCCGAGATAATTGCGTCTTT	CGGCTCGTGTTGTTTCCTAT	base position 20104000
	97D*	3R_22436	ACAAACAGAGGAGCGCAGAT	CAGCGACTTGTCATCGCTAA	base position 22436000
	97F*	3R_23001	TAGCTGCCATCGAGTGTGTC	GTTTTGCGGCTAATGAGAGG	base position 23001000
	98A	simmau_98A	CAGTAATGTGATTACCGAAGGAGAT	CCCTTCATTGGCTAAATATTTCATA	base position 23018000
	100D*	3R_27081	GTGCGCGTCAACAGAAATTA	CAGACACCTTGCTACGTGGA	base position 27081000
	100E	3R_27488	CATCGGATTCCACGATGTTT	TGGCGTCTGTTGAATTGTGT	base position 27488000

"Cytogenetic region" is based on the *D. melanogaster* cytology. * Markers were only used to refine the introgression breakpoints. Base position is the approximate base location on the right arm of the third chromosome in *D. simulans* (1 is at the centromere; 27,517,382 is at the telomere). Note that there is an inversion in *D. simulans* in relation to *D. melanogaster* from region 84F1 to 93F6; therefore the marker at 93E is much closer to the centromere than it would be in *D. melanogaster*.

Table A.2. Results of pairings of *D. mauritiana* males containing a *D. simulans* introgression (M_S) with *D. mauritiana* or *D. simulans* females. Courtship and copulation durations and latencies in minutes (±SE). M_M is a control introgression line and contains only *D. mauritiana* DNA. Pure mau and Pure sim are pure-species individuals that did not undergo any of the introgression crossing scheme.

Female	Male	Courtship latency	Copulation latency	Courtship duration	Copulation duration
Pure mau	**M_S1**	10.85±2.30	19.80±2.98	12.79±3.39	12.15±0.64
Pure mau	**M_S2**	10.73±1.79	19.21±2.58*	10.31±2.24	12.41±1.09
Pure mau	**M_S3**	10.02±1.99	20.99±3.13*	9.14±1.98	12.53±1.20
Pure mau	**M_S4**	14.32±3.48*	9.92±1.69	5.87±1.14	12.87±2.67
Pure mau	**M_S5**	9.13±1.21	13.63±1.25	4.69±0.63	13.23±0.72
Pure mau	**M_S6**	11.38±1.89	19.01±2.36	9.48±1.58	12.55±0.98
Pure mau	**M_M**	8.67±1.18	14.43±1.42	6.92±1.42	11.23±0.61
Pure mau	**Pure mau**	6.88±0.83	12.80±1.02	7.13±0.85	11.39±0.46
Pure sim	**M_S1**	14.64±3.22	14.47±1.76	4.45±1.56	5.29±0.87‡
Pure sim	**M_S2**	11.48±2.48	15.05±2.68	4.07±1.80	6.80±1.13‡
Pure sim	**M_S3**	11.75±2.08	20.78±3.67	12.49±3.69†*	7.14±1.68‡
Pure sim	**M_S4**	14.48±3.51	16.74±4.96	3.56±0.92	9.53±1.50‡
Pure sim	**M_S5**	7.62±2.35	12.45±2.95	5.30±2.58	6.62±0.73‡
Pure sim	**M_S6**	9.55±2.74	16.83±4.79	5.01±2.70	8.17±1.11‡
Pure sim	**M_M**	5.45±0.68	8.06±0.72	2.60±0.69	8.04±0.65‡
Pure sim	**Pure mau**	11.56±2.08	15.95±3.28	4.34±1.87	6.78±0.86‡
Pure sim	**Pure sim**	8.61±1.90	16.23±2.84	8.08±1.43	24.15±0.99†*

*In a given column, value is significantly different ($p<0.05$) from the pure-species control line Pure mau according to Dunn's test. † In a given column, value is significantly different ($p<0.05$) from the control introgression line M_M according to Dunn's test. ‡ In a given column, value is significantly different ($p<0.05$) from the pure-species control line Pure sim according to Dunn's test.

Table A.3. Results of pairings of introgression lines in order to determine if an introgressed region can alleviate behavioural isolation. Courtship and copulation durations and latencies in minutes (±SE). M_M and S_S are control introgression lines and contain only *D. mauritiana* or *D. simulans* DNA, respectively. Pure mau and Pure sim are pure-species individuals that did not undergo any of the introgression crossing scheme. N/A = not applicable.

Female	Male	Courtship latency	Copulation latency	Courtship duration	Copulation duration
M_S2	**Pure sim**	14.49±3.41	29.72*	0.55*	22.53*
M_S5	**Pure sim**	11.21±3.19	N/A	N/A	N/A
M_S6	**Pure sim**	10.39±2.49	N/A	N/A	N/A
M_M	**Pure sim**	14.13±2.70	8.00*	2.43*	11.93*
Pure mau	**Pure sim**	6.82±1.44	N/A	N/A	N/A
Pure mau	**S_M1**	4.92±1.29†	N/A	N/A	N/A
Pure mau	**S_M3**	7.00±1.40†	6.43±3.12	5.27±4.17	20.96±4.91
Pure mau	**S_M4**	9.59±1.91	N/A	N/A	N/A
Pure mau	**S_S**	13.80±2.29	7.50	6.03	28.88
Pure mau	**Pure sim**	9.64±2.00	N/A	N/A	N/A

*These values are not averages, and represent a single pairing. † In a given column, value is significantly different ($p<0.05$) from the control introgression line (M_M or S_S) pairing according to Dunn's test.

Table A.4. Results of pairings of *D. simulans* females containing a *D. mauritiana* introgression (S_M) with *D. simulans* or *D. mauritiana* males. Courtship and copulation durations and latencies in minutes (±SE). M_M and S_S are control introgression lines and contain only *D. mauritiana* or *D. simulans* DNA, respectively. Pure mau and Pure sim are pure-species individuals that did not undergo any of the introgression crossing scheme.

Female	Male	Courtship latency	Copulation latency	Courtship duration	Copulation duration
S_M1	**Pure sim**	11.74±2.00	21.77±2.53	13.69±0.84 ‡	22.61±1.44
S_M2	**Pure sim**	11.42±3.13	N/A	N/A	N/A
S_M3	**Pure sim**	14.16±1.99	22.47±2.42 ‡	9.73±2.30	20.07±1.28
S_M4	**Pure sim**	15.89±2.41	16.71±3.18	3.11±0.61	25.67±1.20†
S_M5	**Pure sim**	13.98±1.87	16.39±2.59	2.93±0.70	24.06±0.88†
S_S	**Pure sim**	14.96±2.25	15.88±2.43	6.15±2.34	19.92±1.01
Pure sim	**Pure sim**	11.49±1.11	14.86±1.32	5.39±0.98	22.84±0.55
S_M1	**Pure mau**	13.01±3.75	19.03±4.35	8.67±2.87	7.37±1.27*
S_M2	**Pure mau**	8.39±1.83	14.42±3.47	6.47±2.71	8.86±0.75
S_M3	**Pure mau**	10.06±2.86	13.47±4.50	7.98±2.72	9.08±1.43
S_M4	**Pure mau**	8.80±1.83	14.23±2.49	7.67±2.32	6.86±0.99*
S_M5	**Pure mau**	6.58±1.94	8.68±2.10	3.23±0.80	7.95±1.03
S_S	**Pure mau**	7.71±1.40	10.50±1.56	4.27±1.35	8.19±0.76
Pure sim	**Pure mau**	9.42±2.48	10.40±1.65	3.54±0.98	8.93±0.80
Pure mau	**Pure mau**	6.93±1.90	15.96±2.19	9.85±2.16	10.99±0.69

*In a given column, value is significantly different ($p<0.05$) from the pure-species control line Pure mau according to Dunn's test. † In a given column, value is significantly different ($p<0.05$) from the control introgression line (S_S) according to Dunn's test. ‡ In a given column, value is significantly different ($p<0.05$) from the control line Pure sim according to Dunn's test.