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Genetics of female interspecific mate rejection in species of Drosophila

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Abstract

Speciation can occur when accumulated differences in mating behaviour force diverging species to remain reproductively isolated from one another. A key determinant of behavioural isolation is the evolution of female mating preferences that prevent interspecific males from mating. However, no individual genes involved in species-specific preferences of females have yet been identified. Using various genetic mapping techniques available for studying strains and species of *Drosophila*, I identify candidate genes involved in *D. simulans* female discrimination against *D. melanogaster* males. One candidate gene in particular, *Katanin-60*, was selected for further characterization. *Katanin-60* is a gene encoding a microtubule severing protein that has been previously implicated in *Drosophila* behaviour. Transgenic rescue of *Katanin-60* expression using the GAL4/UAS system revealed the potential involvement of specific neural lobes of the Mushroom bodies in interspecific discrimination. Further characterization of the behaviour through modifying male mating signals showed that the type-aversive cue females are discriminating against is found in male wing song. However, this was not true of all strains and species tested, indicating that many means of mate assessment have diversified within the genus. One other species, *D. sechellia*, was additionally mapped for their females' discrimination against *D. simulans* males. Quantitative trait locus mapping identified two loci for interspecific preference that were compared to other maps of interspecific divergence between the two species. Together, these studies show how readily, and specifically, behaviour diverges between *Drosophila* groups. They also identify the first candidate genes for female interspecific preference, as well as validate a longstanding hypothesis that such genes should be found in regions of the genome where recombination is likely to be suppressed between diverging groups.

Keywords

Speciation, Prezygotic isolation, Mating preference, *Drosophila*

Co-Authorship Statement

I completed the work of this dissertation under the supervision of Dr. Amanda Moehring. I received help from an undergraduate honours thesis student (M. Wong) in the collection of genotyping data (Chapter 2). Several of the deficiency and P-element lines used for complementation mapping in Chapter 3 were completed by honours thesis students (Katrina Bruch, Ashley Renaud, Stephen Chan) and a graduate student (Meghan Laturney). Transgenic lines used in Chapter 3 were made by Aaron Allen. In addition, the experiment on *Drosophila* species females' use of antennae and aristae was conducted by laboratory technician (Tara Edwards).

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BC	Backcross
CHC	Cutitcular hydrocarbon
CM	Centimorgan
CNS	Central Nervous System
LOD	Logarithm Of the Odds
mB	megabase
MB	Mushroom Bodies
PCD	Programmed Cell Death
QTL	Quantitative trait locus
TE	Transposable element

Chapter 1

1 General Introduction

1.1 Speciation

Evolutionary processes that allow lineages to diverge are integral to the nature, diversity, and origins of species. The discovery of these evolutionary processes, and under what natural conditions they are enabled, has been a longstanding goal for the study of speciation. However, the lack of a clear-cut variation for taxonomic resolution among species has led to a debate among biologists known as the species problem (Queiroz and Donoghue 1988; Coyne and Orr 2004). Thus, before any examination of the processes of speciation, it is necessary to define first what constitutes a species, and to do so in such a way that is biologically relevant to species formation.

Many of the historical and contemporary definitions of a species use assorted forms of organic variation to separate one group of organisms from another similar group (i.e. Morphological Species Concept, Genetic Species Concept, Evolutionary Species Concept, etc). In contrast, Ernest Mayr's biological species concept organizes species as "groups of interbreeding natural populations that are reproductively isolated from other such groups" (Mayr 1942). In Mayr's definition, species can be recognized on the basis of incompatible characters that separate each population into distinct reproductive communities (Mayr 1942). Consequently, the evolutionary processes underlying the formation of incompatible characters are likely to be those processes that also underlie speciation (Mayr 1942; Coyne and Orr 2004).

The geographic distributions of diverging populations are an important determinant of potential sexual interactions between those populations. Speciation through allopatry involves populations evolving independently of one another in discrete geographic locations (Mayr 1963). If contact were ever re-established between these populations, successful reproductive interactions would not occur because of the divergence accumulated while geographically isolated. Speciation also occurs in contexts that rely less on extrinsic (geographic) factors and more on intrinsic (biological) factors for isolation. These contexts include allopatry (including peripatry), which

allows for gene flow after an initial period of isolation, parapatric isolation, whereby continuous ranges of populations are isolated except for small zones of overlap, and finally, sympatric isolation, which occurs without any geographic isolation (Coyne and Orr 2004). Mechanistically, intrinsic barriers manifest in three general categories: barriers that act in hybrid offspring, rendering them sterile or inviable (post-zygotic isolation), barriers that adapt species to non-overlapping ecological niches (ecological isolation), and barriers that preclude successful fertilization from occurring (pre-zygotic isolation) in the first place (Mayr 1963; Coyne and Orr 2004). If evolutionary divergence occurs in traits that would establish reproductive barriers, speciation is likely to proceed. Of special interest are candidate traits, called 'key features,' that are predicted to promote diversification and species richness among clades (Coyne and Orr 2004). These traits are theorized to include sexual dimorphism, size, host plant for phytophagy/pollination, dispersal, and traits associated with sexual selection (Coyne and Orr 2004).

1.2 Reproductive isolation barriers

Post-zygotic isolation refers to reproductive barriers that prevent fertile/viable offspring from developing when hybridization occurs (Mayr 1963). To date, several genes involved in post-zygotic isolation have been identified. Incompatible alleles of these genes underlie post-zygotic isolation through a variety of mechanisms including: epistatic interactions, selfish genetic conflicts, and genomic rearrangements (Orr and Presgraves 2000). For example, hybrid inviability occurs between the fruit fly species *Drosophila melanogaster* and *D. simulans* as a result of divergence in the gene hybrid male rescue (*Hmr*) (Barbash et al. 2003). Similarly, the gene *PRDM9* causes hybrid sterility between the mouse species *Mus musculus musculus* and *M. musculus dominicus* (Mihola et al. 2008). The alleles of both *Hmr* and *PRDM9* likely represent the mediation of a genomic conflict in one species of the pair, but not in the other, and now interact epistatically in hybrids as a result (Barbash et al. 2003; Mohole et al. 2008). In the sterility of *D. mauritiana/D. simulans* hybrids, the genomic region *too much yin* (*tmy)* suppresses a selfish genetic element of one species that is not present in the other (Tao et al. 2001). Finally, the transposition of *JYalpha* from the fourth chromosome to the third chromosome in *D. simulans* underlies hybrid dysfunction in later-generation hybrids of *D. simulans* and *D. melanogaster* (Masly et al. 2006). Given enough time for divergence, post-zygotic isolation is

expected among allopatric populations. However, if contact is resumed between incipient species, partial post-zygotic isolation may be sufficient to drive selection against hybridization. Such a process, referred to as reinforcement, ensures that females discriminate against interspecific males to prevent the formation of sterile or inviable hybrids (Dobzhansky 1940). Reinforcement may also occur if viable hybrids are produced, but maladapted to their environment (Schluter 1995). As a result, populations that adapt to niches that are discrete in some way are expected to remain as distinct species in ecological isolation from one another (Mayr 1947; Schluter 1995).

Ecological isolation is apparent among species that specialize in the use of unique habitats, hosts, and food sources (Schluter 1995; Lavistas-Llanos 2014). For example, *D. sechellia* has adapted to feeding on the toxic plant *Morinda citrifolia* (Legal et al. 1992; Farine et al. 1996), due to a mutation in the gene *catsup* (Lavistas-Llanos et al. 2014). *M. citrifolia* contains I-DOPA, which, in excess, complements the effects of the *catsup* mutation. The bearers of this allele used *M. citrifolia* as a host plant (which was avoided by non-bearers), creating a unique situation for the *D. simulans*-like ancestor of *D. sechellia* to exploit (Lavistas-Llanos et al. 2014). Another form of ecological isolation, allochronic isolation, occurs when asynchronicity in space utilization occurs between two different groups (Ording 2010). Two races of the pine processionary moth, *Thaumetopea pityocampa*, have alternative larval seasons (winter and summer). As a result, neither race is capable of reproductively interacting with one another (Satos 2007).

Pre-zygotic isolation refers to reproductive barriers that prevent successful mating from occurring. In the instance of mechanical isolation, anatomical divergence renders heterospecific mating impossible. For example, in millipedes of the *Parafontaria* complex, differences in body and genital size prevents mating from occurring (Soto and Tanabe 2010). Pre-zygotic post-mating (PZPM) isolation refers to incompatible interactions between the reproductive tissues, or gametes, of males and females. The result of PZPM is that fertilization cannot occur. Among *D. virilis, D. americana,* and *D. novamexicana*, the mortality of gametes from one species within the reproductive tract of another species is high (Patterson and Stone 1952). In another *Drosophila* species pair, *D. santomea* and *D. yakuba*, PZPM manifests through selective fertilization of eggs with only conspecific sperm (Matute 2010). Another form of prezygotic

isolation is behavioural isolation, which primarily relies on divergence in traits related to mating behaviours. Mating requires the co-ordination of sexual communication between sexes. If divergence occurs in a species' sexual signals, or in the perception of sexual signals, then rejection of heterospecific courtship occurs (West-Eberhard 1983). Examples of behavioural isolation will be covered in Chapter 1.3.

Comparisons of reproductive isolation mechanisms among dozens of species pairs indicate that pre-zygotic mechanisms generally evolve earlier than post-zygotic mechanisms (Coyne and Orr 1989). Behavioural isolation among sympatric species pairs accounts for much of this pattern (Coyne and Orr 1997). In general, mating behaviours are among the quickest traits to diversif, as they are often highly responsive to selection (Stelkens and Seehausen 2009; Gonzalez-Voyer 2011). Specifically, traits with sex-biased expression are correlated with faster evolution (Ellegren and Pasch 2009), and elevated species richness (see review by Danley et al. 2001; Proschel et al. 2006). The distinction of whether a reproductive barrier evolves early or not is important, as reproductive barriers that evolve later are redundant. For this reason, mechanisms of pre-zygotic isolation are thought to be especially important to the process of speciation.

1.3 Behavioural isolation

The expression, reception, and perception of sexual signals must be concordant between the sexes for successful mating to occur. Among most animal species, males bear signal traits on the basis of which females accept or reject copulation (see review by Ender 1992). For this reason, the divergence of female mating preferences between species is thought to be a key determinant in the development of assortative mating, and ultimately, species isolation (Mayr 1963). Numerous examples of species pairs across a diverse selection of taxa demonstrate behavioural incompatibilities that contribute to species isolation (Etges 2002).

Females of the butterfly *Pieris occidentalis* use visual cues (wing colouration) to reject mating attempts by males of *P. protodice* (Wiernasz and Kingsolver 1992). Darkening the wings of *P. protodice* mitigates *P. occidentalis* rejection, as the type-aversive cue against which females are visually discriminating against is masked (Wiernasz and Kingsolver 1992). The sympatric sea snakes, *Laticauda colubrina* and *L. frontalis*, maintain species boundaries on the basis of

different lipid-based contact pheromones (Shine et al. 2002). Conversely, sympatric races of the European corn borer, *Ostrinia nubialis*, express identical pheromones, but differ in the ratio of *cis*- to *trans*- isomers used (Kochansky et al. 1975). While the cases described above demonstrate that a single cue can be sufficient for species isolation, behavioural isolation is often mediated by multiple sensory modalities. For example, female *Passerina cyanea* discriminate against *P. amoena* males using both visual cues (plumage) and auditory cues (song) (Baker and Baker 1990).

The diversity of mating isolation mechanisms becomes especially apparent in clades for which many species pairs have been studied, such as *Drosophila*, birds, and cichlid fish. For example, among cichlids, recent rounds of adaptive radiation in the last 700,000 years have led to the proliferation of nearly 400 species (Danley and Kocher 2001). From the meta-analytics of mating behaviour, morphology, and genomic data, a working model for the succession of cladogenic events has been developed. The model includes an initial bifurcation of lineages along two major habitats, trophic specialization within each habitat, and then a round of divergent sexual selection for male nuptial colours (Danley and Kocher 2001).

The above example of cichlid speciation illustrates how both natural and sexual selection can contribute to the evolution of behavioural isolation. Natural selection operated directly on the visual acuity of cichlids living in different gradations of colour-filtration in Lake Victoria (Seehausen et al. 2008). Vision is also used by female cichlids during mating to assess male nuptial colours. Since female preference for vivid colouration benefits her in terms of increased survival and successful mate acquisition, selection maintains the preference. As new variants for visual acuity arise within a population, sub-populations form on the basis of each new variant for preference and signal. If each sub-population exhibits assortative mating and high mating success, sexual selection will cause both the preference and its corresponding signal to propagate within each sub-population. As sexual selection continues to act on the signals and on the preference for each signal, between-population mating will decrease, leading to behavioural isolation (West-Eberhard 1983). Several models have been proposed to describe how different dynamics of sexual selection may drive evolutionary diversification in mating (e.g. runaway, good genes, chase-away). It is important to note that diversity in mating preference often arises

due to the benefit provided to the offspring by the preference. In other words, the preference for a particular signal may result in the production of offspring that are more fit. Clearly, many evolutionary mechanisms cause mating behaviour to diversify, leading to speciation. The following section will cover some of the incompatible mating behaviours that have arisen among various species pairs.

1.4 Diversification of mating behaviour

A mating communication system consists of a signal, usually by a male, and the perception of that signal, usually by a female. If populations evolve variable communication systems for mating, and those systems diverge, heterospecific mating between populations decreases. However, for communication systems to diverge, the match between variant signals and variant preferences for those signals must be complementary. For this reason, sexually selected mating traits are expected to be under stabilizing selection (Coyne and Orr 2004). Evolutionary forces that would diversify signals and preferences must therefore overcome several challenges. These challenges include an initial need for trait variation that does not compromise fitness and can later be co-opted for mate selection. There must also be corresponding evolution for matching variants of the mating trait (signal/preference) in the opposite sex. Finally, the signal and preference are likely to be governed by different traits that are subject to different restrictions and pressures for diversification.

The evolution of assortative mating behaviour is expected to bypass the above challenges when certain factors are present. One factor is the genomic location of the genes involved in influencing behaviour. If genes controlling signal and preference are near one another, alleles of either gene will be inherited as a single linked unit. Since these units are rarely broken up by recombination, lineage-specific diversity can persist amidst gene flow (McGaugh and Noor 2012). In European crows, two species behaviourally assort on the basis of a single 2 Mb region. Notably, the region harbours a high density of fixed differences for a pigmentation gene and a gene involved with visual perception (Poelstra et al. 2014). Among species of the butterfly *Heliconius*, the loci encoding a specific wing colour, and the preference for that wing colour, map to a region that is only 5.5 cM (Kronforst et al. 2006). Hawaiian crickets are another such example where QTL (quantitative trait loci) influencing male courtship song and female

preference for that particular song co-localize. In *Laupala kohalensis* the region is 1cM, while in *L. paranigra* it is 4cM (Shaw and Lesnick 2009). The persistence of genomic regions that harbour both signal and preference loci may also occur if those regions are trapped near areas of low recombination such as centromeres, telomeres, within inversions, or outside the edges of inversions (Noor et al. 2007; Stevison et al. 2011). The extended linkage disequilibrium of these regions allows broader spans of genome to remain in association with one another. Consequently, novel behavioural variants may persist simply out of serendipitous placement within the genome (Laturney and Moehring 2012).

New signals and preferences can evolve when variation accumulates in mating traits. Novel expressions are expected to be initially irrelevant during mating, evolving neutrally until salient variation in the reciprocal signal/perception is acquired. In the parasitoid wasp *Nasonia vitripennis*, an ancestral gene duplication event created 4 tandem repeats of a gene involved in pheromone synthesis (Niehuis et al. 2013). One of these repeats became a neo-functional paralog that produces new variants of a male pheromone for which females now exhibit a preference (Niehuis et al. 2013). The new pheromone is imperceptible to females of the sister species *Nasonia giralti*, which suggests that the new pheromone would have been initially unutilized in the ancestral species to *N. vitripennis*. As a result, the new pheromone would have become an arousal cue in *N. vitripennis* females only after they later evolved the perceptual capacity to detect it (Niehuis et al. 2013).

Conversely, alternative perceptual capacities may evolve first, and subsequent evolution in the properties of signal traits will later fall within the new range of salience. In the process of sensory drive bias, signals between senders and receivers evolve to minimize signal degradation and maximize contrast with the environment (Vargas-Salinas and Amezquita 2013). One example is the evolution of visual preferences among female cichlids (Seehausen et al. 2008). Subpopulations of cichlids bear different alleles for the *LWS opsin* gene which each perform better at light absorption in different habitats. Male nuptial colours in each subpopulation then diversified to match the specific colour sensitivities of females (Seehausen et al. 2008). Relatedly, the perceptual range of a species may evolve alternative gradations for salience within an already existent signal range. One example of such evolution occurred in electric fish of the

family *Mormyridae,* which use species-specific electric signals to communicate. *Mormyridae* underwent a species radiation event concurrent with adaptations to brain areas involved in the refinement of communication signals (Carlson 2011).

Beyond adaptation, behavioural isolation may evolve as a result of genetic drift in founder populations (Templeton 1980). In this model, fluctuations in allele frequency for a small population are expected to result in rapid losses of genetic variation and fixation of alleles (Templeton 1980). Because inbreeding is prevalent in this scenario, alleles that promote overall genomic stability are favoured and behavioural traits may change without regard to their extrinisic value (Templeton 1980; Ahearn and Templeton 1989). However, experimental attempts to artificially induce evolution under founder conditions seldom yield incompatible phenotypes that result in behavioural isolation (Rundel et al. 1989).

The above examples illustrate some of the means by which type-specific mating preferences may evolve. In the context of speciation, it is also important to question how these type-specific preferences may play out in an interspecific mating context. Are females insufficiently aroused or are they excessively aggravated by interspecific males? In either case, rejection is expected, but the strength of rejection and the evolutionary pressures under which it arose are expected to differ (Boake 1997). For example, in the case of signal drive bias among Lake Victoria cichlids, the type-specific colouration of males is preferentially selected for by conspecfic females (Seehausen et al. 2008). In contrast, behavioural isolation between *D. pseudoobscura* and *D. persimilis* specific aversion behaviour has evolved to prevent maladaptive hybridizations from occurring (Koopman 1949). The former is likely to be a weaker form of mating isolation, as cases exist where disruptions to longstanding ecological boundaries lead to the collapse of former ‘species’ into intermediated lineages known as hybrid swarms (e.g. Hasselman et al. 2014). The latter is presumed to be a stronger barrier to mating as lineage distinctions are ‘reinforced’ by active discrimination against interspecific traits (Coyne and Orr 2004). Since interspecific rejection may harbour elements of both type-specific aversion and arousal, it is essential to understand the phenotypic underpinnings of female preferences.

1.5 *Drosophila* species as a speciation model

The species of the genus *Drosophila* are saprophagic feeders, from which they received their common and Latin names, fruit fly and "dew-loving", respectively (Meigen et al., 1869). Beginning with the work of T.H. Morgan, *D. melanogaster* has been an important cornerstone of genetic research for over a century. *D. melanogaster* is now also an important model organism for the study of development and behaviour. Several of the species in the melanogaster species subgroup have already been studied both independently and in relation to *D. melanogaster*. The extensive legacy of research and genetic tools already acquired in this subgroup allows opportunities for in-depth study of speciation that would be out of each reach among other subgroups of the *Sophorphora* subgenus.

In the *Drosophila* life cycle (Spindler-Barth 2012), females lay fertilized eggs in decaying organic matter, where embryos develop into larvae. Larvae feed and undergo three instar transitions before undergoing pupation. After several days, adults emerge, becoming sexually active within a few hours. Developmental time, from embryo to sexually mature adult, can take 10-16 days, depending on environmental conditions and species- or strain-specific traits. Females develop faster than males by approximately 8-12 hours and are usually larger in size (Miller and Demerec 1950). *Drosophila* husbandry is rapid and easy to perform within a laboratory for many species within the genus. Depending on the species of study, a number of sexually dimorphic characters differ between *Drosophila* males and females including the biochemical composition of cuticular hydrocarbons (CHC) that serve as pheromones during courtship (Pechine et al. 1985), the tibial bristles (aka sex combs) on male forelegs that grip females during mating (Carson and Bryant 1979), mating behaviour (Hall 1994), head morphology (Boake et al. 1997), and abdominal pigmentation (Kopp et al. 2000). Many of these dimorphisms have a role in courtship that will be described below in chapter 1.6.

A number of tools are available for genetic mapping and characterization in *D. melanogaster* (and increasingly in other *Drosophila* species). To study the effects of recessive alleles, deficiency mapping stocks have been developed with coverage for 98.4% of the euchromatic genome (Cook 2012). Deficiency stocks contain a single deletion at a known cytological location that partially overlaps in location with the deletions of other deficiency stocks. Crossing

deficiency stocks to stocks bearing the recessive element of interest will have the effects of the recessive allele unmasked in their progeny. By systematically crossing different deficiency stocks, various genomic regions can be ruled in or out for having an effect on the trait of interest (Cook 2012). If finer resolution of a locus of interest is required, other tools, such as point disruption lines generated by the *Drosophila* Gene Disruption Project, are available (Bellen et al. 2004).

Point disruptions are often created using transposable element (TE) insertions and, to date, over 9400 *Drosophila* genes are tagged with TEs such as *P, Minos,* and *Piggybac*. Additional tools have been engineered into TEs and incorporated into various *Drosophila* lines for increased utility (Bellen et al. 2004; Bellen et al. 2011). Nearly 70% of the annotated protein coding genes in *D. melanogaster* have been tagged with TEs. Furthermore, many of the genes tagged have multiple insertion sites within the *Drosophila* genome (Bellen et al. 2011). TEs inserted into different sites of a gene are useful, as sensitivity of gene function to TE placement varies (Spradling et al. 1999). For example, in the gene *smD3, P*-element insertion into the promoter region yields an aberrant neuronal differentiation phenotype, whereas insertion into the 5' UTR of the gene yields lethality (Schenkel 2002). Thus, the role of a putative candidate gene can be tested through the use of multiple *P*-element disruptions of the gene. Further confirmation can be procured with the use of transgenics for the gene. One of the most useful transgenic tools for the study of gene function in *Drosophila* has been the Gal4/UAS system. The two components of the system are the *Gal4* gene and a UAS (Upstream Activating Sequence) element linked to a gene of interest. The yeast transcription factor gene *Gal4* is inserted into the *D. melanogaster* genome. If the *Gal4* is inserted near a promoter or enhancer, it will produce the GAL4 protein in the tissue-specific pattern that would normally be produced by that enhancer or promoter. GAL4 binds the UAS, which subsequently activates transcription of the directly-adjoining candidate gene, enabling tissue-specific expression of the gene of interest (Brand and Perrimon 1993). Of note, many of the TE insertion lines are also tagged with UAS elements.

In the 1,579 or more *Drosophila* species known to exist (Brake 2008), many females display some form of discriminatory mating behaviour against interspecific males (Coyne 1989). If, as mentioned above, behavioural isolation is as widespread and readily evolved as predicted, then

there should be many instances of varying degrees of isolation among species of the genus. Examples of each are discussed below in section 1.6.

1.6 Courtship in *Drosophila* species

During courtship, *Drosophila* males perform a fixed sequence of moves, which includes tapping, circling, singing, licking and attempts at mounting the female (Hall 1994). Females appraise the visual, chemical, tactile and auditory signals received during courtship, and accept or reject mating on the basis of a species-specific composition of these cues (Billeter et al. 2009; Boake et al. 1997; Ewing and Bennet-Clark 1968; Hoikkala and Kaneshiro 1993).

1.6.1 Visual

Males rely on vision for at least two components of *Drosophila* mating rituals, namely orientation and chasing (Cook 1979). Visionless *Drosophila* males are capable of mating (Mei-ling and Griffith 1997), but take longer to court (Markow 1975), and are far less successful when compared to males with vision (Connolly 1969). The reduced mating occurs when males fail to follow a moving female (Tompkins 1982). Additionally, some sexually selected visual cues from males enhance female receptivity. Wing interference patterns from males affect the hue and saturation of colours and are a known mating signal in many transparently winged insects (Fuyama 1979). Females of *D. heteroneura* select for males on the basis of their 'hammer-head' width, which is a *D. heteronurea*-specific male elaboration (Boake et al. 1997). *D. heteronurea* females will still mate with males that do not possess the elaboration (i.e. interspecific males), but do so at much lower levels (Boake et al. 1997).

1.6.2 Auditory

The songs of *Drosophila* males during courtship are made with an outstretched wing. The degree to which wing song is important for mating varies on a species-by-species basis. Generally, wing song is an important component of courtship that enhances female mating when present and correctly executed during male *Drosophila* courtship rituals. *Drosophila* wing song often bears strain and species-specific information, which makes it a particularly important candidate for species recognition and discrimination (Blyth 2008; Gleason 2005;Tomaru 2004).

Wing songs have several characteristics organized among two domains of output: pulse song (rapid bursts) and sine song (extended bursts) (Kyriacou and Hall 1982). Auditory-ablated female mutants show reduced receptivity, as do females that are presented with no song or an aberrant song. In some instances (e.g. *D. sechellia* females x *D. simulans* males (Tomaru 2004) and *D. ananasae* females x *D. pallidosa* males (Doi 2001)) even greater rejection behaviour occurs for females presented with the wrong species song compared to no song. Interestingly, in the latter species pair, *D. pallidosa* females use their conspecific song to facilitate normal copulation, while *D. ananassae* females have no use for conspecific wing song in normal mating (Yamada et al. 2002). Consequently, wing song is likely utilized for a mixture of arousal and aversion preferences depending on the unique evolutionary history of each species. In some instances, specific components of song preference of the species are known (Tomaru 2004). In the melanogaster group, the interpulse interval (amount of time between pulses) appears to be an important conveyor of species-specific information (Ewing and Bennet-Clark 1968). In *D. virilis*, *D. montana*, and *D. lummei*, interspecific discrimination appears to be mediated by the properties of the pulse itself. (Hoikkala and Lumme 1987; Ritchie 1998). Wing song may also be a within-species signifier of male genetic quality as has been experimentally demonstrated in *D. montana*. Males with a specific song were preferred by females, and also produced progeny with a higher survival rate (Hoikkala et al. 1998).

1.6.3 Chemical

Drosophila cuticular hydrocarbons (CHC) are long chain fatty acids produced by specialized cells called oenocytes. CHCs are anti-desiccation compounds that also function as pheromonal compounds (Jallon and David 1987). CHC are used in both intra- and intersexual communication (Jallon and David 1987). *Drosophila* species use a species-specific blend of pheromones (sometimes strain-specific) that can be sexually monomorphic or dimorphic. For example, *D. melanogaster* are sexually dimorphic for CHCs with the females expressing 7,11-heptacosadiene (7,11-HD) as their most abundant CHC, while, male *D. melanogaster* (and both sexes of *D. simulans*) bear 7-tricosene (7-T) as their most abundant CHC (Veltsos et al. 2012). Genetically, the major determinant of a monomorphic or dimorphic pheromone profile depends, in part, on species-specific expression levels of the gene *desat-F* (Shirangi et al. 2009). Although they are

genetically determined, CHC blends are also known to vary based on factors related to climate, age and diet (Rouhalt et al. 2004).

In addition to interspecific variation, CHC blends vary on an intraspecific level as well. For example, *D. simulans* in West Africa utilize 7-pentacosene as their dominant CHC instead of 7-T (Bontonou et al. 2012). Desiccation resistance does not vary among *D. simulans* strains with different CHC blends, which suggests sexual selection may play a stronger role than natural selection in CHC diversification (Bontonou et al. 2012). CHC variation matters for both sex and species identification. In *D. melanogaster*, males lacking oenocytes (CHC producing cells) were courted by *D. melanogaster* males as if they were females (Billeter et al. 2009). It was only when coated with the primary male pheromone, 7-T, that normal perception of males as males was restored (Billeter et al. 2009). Similarly, oenocyte-less *D. melanogaster* females were hyper-attractive to *D. simulans* males until they were coated with the normal primary female *D. melanogaster* pheromone 7,11-HD (Billeter et al. 2009).

1.6.4 Ritual interactions

Complex actions and reactions occur between males and females during the events of *Drosophila* courtship. For example, *D. melanogaster* males are capable of issuing different songs and will dynamically switch between modes based on female reactions (Coen et al. 2014). Consequently, if the duration or protocol of an event is altered between species, then behavioural isolation may result. During *D. silvestris* courtship, females transition from one step to another much more quickly than do *D. plantibia* females. As a result, *D. silvestris* males fail to mate with *D. plantibia* females, having improperly navigated the species-specific lag in the female's co-ordination of mating (Hoikkala and Kaneshiro 1993).

1.7 Behavioural isolation of *Drosophila* species

1.7.1 Behavioural genes

Loci that influence within-species female receptivity to courtship, such as *spinster*, *dissatisfaction*, and *chaste*, have been found in mutation screens of *D. melanogaster* (Suzuki et al. 1997; Finley et al. 1997; Juni and Yamamoto 2009). It is unclear, however, whether these

genes possess naturally occurring behavioural variants necessary for evolutionary forces to act upon. The *spinster* mutants, as an example, also show neurodegenerative phenotypes, which may indicate the reduced mating receptivity is an epistatic by-product of *spinster*'s disrupted function (Suzuki et al. 1997). There are known naturally-occurring behavioural variants for male mating success. For example, the circadian rhythm gene *period* is involved in aspects of wing rhythm during courtship displays. Successful mating is enhanced when males present songs generated from the correct *period* allele to conspecific females. (Kyriacou 2002). Other alleles found to influence behaviour in natural populations are the rover/sitter variants of the *foraging* gene, which enable alternative locomotive behaviours (de Belle and Sokolowski 1987; Osborne 1997). Though not implicated in a speciation context, traits involved with dispersal are among those 'key factors' that correlate with species richness among clades (Coyne and Orr 2004).

Sexually dimorphic traits are often important modifiers of mating outcomes. As a result, when genes underlying sexual dimorphisms diversify, behavioural divergence may occur. The sex determination pathway of *Drosophila* includes a suite of genes that are differentially spliced during development to generate normal adult behaviour for males and females (Demir and Dickson 2005). Among these genes of the sex determination pathway are *doublesex* (*dsx*) and *fruitless* (*fru*), which are the major upstream determinants of male and female sex-specific behaviour. *dsx* is required for the genetic specification of neuronal cell lines involved in female receptivity (Zhou et al. 2014). Expression of *dsx* is necessary for normal female receptivity responses to male wing song and pheromones (Zhou et al. 2014). The gene *Abdominal-B* (*Abd-B*), is a downstream target of *dsx* regulation and is an important mediator of mating receptivity among virgin females (Bussell et al. 2014). The male-specific isoform of *fru* is the major determinant of male behaviour and anatomy (Demir and Dickson 2005). Males without *fru* expression display aberrant or missing male courtship behaviour and anatomy. Females that are transgenically modified to express FRU protein (the female version of the transcript is not translated) display male-like courtship and anatomy (Demir and Dickson 2005). Females with synaptically silenced *fru*-expressing neurons display reduced courtship and aberrant patterns of post-mating behaviour (Kvitsiani and Dickson 2006). Within the genus *Drosphila*, the utility of *fru* varies based on each species. For example, while female *fru* is not translated in *D. melanogaster* females, in *D. suzukii* females, *fru* transcripts are translated in the lamina and

ventral ganglia of the brain (Usui-Aoki et al. 2005). Thus species-specific differences in sexually dimorphic gene function may lead to behavioural divergence if populations differentially co-opt, suppress, or enhance the action of these traits in mating behaviour.

1.7.2 Speciation in *Drosophila* species

The genus *Drosophila* contains numerous instances of species pairs that are behaviourally isolated from one another. In Western North America, females of *Drosophila pseudoobscura* populations living sympatrically with *Drosophila persimilis* discriminate against males of *D. persimilis*. However, females of *D. pseudoobscua* living allopatrically with *D. persimilis* do not display similar levels of behavioural isolation (Noor 1995). Quantitative trait loci (QTL) mapping of introgressions from the sympatric *D. pseudoobscura* strain into the allopatric strain's background identified two loci associated with discrimination against *D. persimilis.* Interestingly, when one of the two loci identified, *Coy2*, was introgressed into a *D. persimilis* population, discrimination from *D. persimilis* females towards *D. pseudoobscura* males increased as well (Ortiz-Barrientos et al. 2004). The 'One-allele' hypothesis states that a single allele may enhance interspecific discrimination capabilities without affecting the normal within species mating dynamics. In addition to the example describing the 'One-allele' hypothesis, there are other examples, within *Drosophila* that may represent putative instances of incipient isolation. Within intraspecific populations of *D. melanogaster*, a Zimbabwe strain discriminates against a cosmopolitan strain of *D. melanogaster* on the basis of pheromone differences (Ting et al. 2001). Among allopatric populations of *D. montana*, mechanisms of assortative mating may be based on pheromonal and acoustic mating cues (Jenning et al. 2011).

Of the main groups of *Drosophila* used for behavioural isolation research, most work has been done with subsets of the melanogaster group, and in particular the simulans complex. Within the simulans complex, female interspecific discrimination developed in two closely related species against a common third species. *D. mauritiana* and *D. sechellia* females display a mating preference against *D. simulans* males, as well as the other males of the simulans complex. In contrast, *D. simulans* females accept mating from the males of *D. mauritiana* and *D. sechellia*. Of the three sibling species, it is unclear which two represent the sister species pair. All three species likely diverged in allopatry from a *D. simulans*-like ancestor nearly 242,000 years ago

(Garrigan 2012). The asymmetric network of mating preferences among these three species has led to preeminent usage of this complex as a behavioural isolation model in *Drosophila* (Coyne 1989; Coyne and Orr 2004). Since fertile female hybrids can be produced by crossing species in the non-choosy direction and the hybrid female's behaviour resembles that of their non-choosy mother, further backcrosses can be done to make regions of the genome homozygous for one species' alleles. In this way, contributions of different genomic regions to species-specific behaviour can be evaluated.

To date, multiple species pairings have been genetically mapped for genomic regions involved in female interspecific preference (Coyne 1992; Coyne et al. 1994; McNiven and Moehring 2013; Moehring et al. 2004; Chu et al. 2013; McNabney 2012). Most of the loci identified, however, do not map to identical locations. As a result, no single combination of traits and genes are likely to underlie any general mechanism of speciation in *Drosophila*. Instead, the genetic underpinnings of each form of isolation appear to map to a number of different loci. Therefore, multiple traits (or at least architectures involved in shaping their expression) may be poised to form and maintain discrete lineages according to their unique evolutionary circumstances.

Though the simulans complex of *Drosophila* is an interesting case of relatively recent speciation, the greater genus *Drosophila* contains not only more species with similar instances of asymmetrical behavioural isolation, but a broader range of genetic tools developed specifically within *D. melanogaster*, a species outside of the simulans complex. Additionally, over a dozen *Drosophila* species genomes have been sequenced, allowing for comparisons of interspecific gene divergence. *D. melanogaster* and *D. simulans* diverged from a common ancestor approximately 5.4 mya (Tamura 2004) in the tropical region of central Africa (Lachaise 1988). Since then, both species have spread with humans to a near global distribution (Lachaise 1988). Though both species are saprophagic feeders, possessing nearly identical morphologies and life histories, *D. simulans* differs from *D. melanogaster* in several ways, including lowered environmental temperature optima, behavioural avoidance of direct human commensalism, cuticular hydrocarbon profile (used by males as pheromones), male genital structure, male wing song properties (used in courtship) (reviewed in Capy and Gibert 2004), several fixed chromosome inversions, and substantially less transposable element invasion (Clark et al. 2007).

Crosses between *D. melanogaster* females and *D. simulans* males yield inviable hybrid males and viable, yet infertile, hybrid females (Robertson 1988). *D. simulans* females do not accept copulation from *D. melanogaster* males, whereas *D. melanogaster* females and F_1 interspecies female hybrids will accept mating from both *D. simulans* and *D. melanogaster* males. For reasons that are unclear, the dominance of non-choosiness over choosiness among hybrids appears to be a general pattern of continental *Drosophila* species where such mating asymmetries exist (Yohshimura 1997).

Despite the apparent importance that behavioural isolation plays in maintaining species as discrete units, no known genes account for how female interspecific preference arises. However, the genetics underlying isolation are not totally unknown. It is clear from previous studies using chromosomal substitutions in *D. melanogaster*, that genomic elements localizing to the third chromosome bear the largest effects on incipient discrimination (Ting et al. 2001). A broad-scale deficiency map of *D. simulans* discrimination against *D. melanogaster* found five regions on the right arm of the third chromosome (3R) that are likely to be involved in female interspecific discrimination (Laturney and Moehring 2012). Additionally, mapping data from the behavioural isolation of *D. mauritiana* to *D. simulans*, suggests that the right arm of the third chromosome (3R) may also harbour important loci (McNiven and Moehring 2013). The first QTL map for discrimination against *D. simulans* by *D. mauritiana* (Moehring et al. 2004) also yielded other loci (two on the X, two on the second, three on the third), which, in part, overlap with loci found from another QTL map identifying isolation between *D. santomea* and *D. yakuba* (Moehring et al. 2006). Alternatively, QTL maps have also implicated a strong role for the X chromosome with one and two loci identified in the isolation of *D. mauritiana* to *D. sechellia* and *D. simulans* to *D. sechellia*, respectively (McNabney 2012; Chu et al. 2013). In addition to interspecific mating preference, there are maps for divergence in behavioural traits that are known to be involved in species differences. The genetic analysis of pheromonal differences contributing to sexual isolation between *D. melanogaster* and *D. simulans* found that at least 4 loci seem to be involved, with the strongest effects localizing to 3R (Coyne 1996). 41% of the known gene effects contributing to variation in *Drosophila* wing song, an important component of *Drosophila* courtship, are found on 3R (Gleason 2005). The balance of evidence thus points to

3R as the most likely area to find a gene involved in female interspecific preference amongst melanogaster group *Drosophila* species.

1.8 Overview of dissertation

In this dissertation I map and characterize the genetics of behavioural isolation among *Drosophila* species pairs. My primary goals were 1) to identify regions/genes involved in behavioural isolation between various *Drosophila* species belonging to the melanogaster group, 2) to identify the neuro-anatomical and phenotypic composition of the traits involved in female discrimination processes, and 3) to compare sequence and behavioural divergence in these traits to establish putative models of behavioural diversification leading to species isolation. In Chapter 2 I use quantitative trait locus (QTL) mapping to identify a region of the *D. sechellia* genome that is significantly associated with female *D. sechellia* discrimination against *D. simulans* males. In Chapter 3 I use deficiency and TE disruption mapping to identify specific genes involved in the isolation of *D. simulans* females against *D. melanogaster* males. In Chapter 4 I check for the presence/absence of roles of these genes in mediating behavioural isolation among other strains and species of *Drosophila*. I also test the role of one of these genes, *Katanin-60,* in the sensory modalities typically associated with interspecific discrimination in *Drosophila*. In Chapter 4 I use GAL4/UAS lines with tissues-specific drivers for the mushroom bodies to ascertain which subsets of neuronal bodies are associated with mediating interspecific mating receptivity. In Chapter 5 I combine the results from the previous chapters into an integrated model that speculates on some of the genetic, developmental and phenotypic characterists of behavioural isolation in *Drosophila*. I additionally discuss the continued work required to complete these models, as well as some of the new hypotheses generated for speciation research.

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Chapter 2

2 Genetic mapping for behavioural isolation between *D. sechellia* and *D. simulans*

Species can be prevented from mating with one another when incompatibilities accumulate in behaviours necessary for successful mating. These incompatibilities are likely to occur when the mating preferences of females diverge. Behavioural isolation between *Drosophila sechellia* and *D. simulans* is maintained, in part, by female *D. sechellia* discrimination against *D. simulans* males during courtship. The genetic regions underlying differences between courtship traits in the two species (pheromone and wingsong) have been identified. Whether these regions control for traits involved with female discrimination against interspecific differences is unknown. Here, I used QTL mapping to identify two regions involved in female *D. sechellia* rejection of *D. simulans* males during courtship. The QTL centered on 46B co-localized with genes involved in auditory and mating receptivity behaviour. The QTL centered on 74B-C is in a region that has been previously implicated in pheromone divergence between the two species. Neither of these QTL matched regions previously identified in conspecific mate preference, suggesting that genes for aversion to heterospecific mating are not the same as those necessary for attraction in conspecific mating. Though there were differences in the duration of certain courtship features between the two species, none of these differences appear to be derived from, or of consequence to, the behavioural isolation of these two species.

2.1 Introduction

The females of many species discriminate against heterospecific males during mating, which can prevent divergent lineages from reproductively interacting with one another (Mayr 1942). For this reason, the evolutionary processes influencing female mating preferences are of special interest in our understanding of speciation (Coyne and Orr 1997; Coyne and Orr 2004). In particular, studying species-rich groups allows comparisons between mechanisms of behavioural isolation among multiple species pairs. For example, the cichlid fishes of Lake Malawi radiated

from a single species to nearly 400 species during the last 700,000 years (Danley and Kocher 2001). Additionally, female cichlids select conspecific mates on the basis of male nuptial colours (Dominey; 1984; Knight 2004). From the many studies of cichlid mating behaviour, morphology, and population genetics, we now possess an evolutionary model for the procession of selective pressures that likely gave rise to so many distinct lineages (Danley and Kocher 2001). A similar feat for understanding the genetics of interspecific preference can be achieved with the use of species from *Drosophila*, which bear many similar assets as a speciation model, as well as a robust variety of genetic tools.

Many genetic studies of female interspecific preference have been carried out in species of the genus *Drosophila*; in particular, the simulans species complex (reviewed in Laturney and Moehring 2012). The complex contains three sibling species, *D. simulans, D. sechellia*, and *D. mauritiana* that diverged from a *D. simulans*-like ancestor 900,000 years ago (Reis et al. 2011, Garrigan et al. 2012). These species have their genomes sequenced, distinct mating phenoypes, and are postzygotically (partial) and prezygotically isolated from one another (Lachaise et al. 1986). *D. simulans* originated from a tropical region of central Africa before spreading globally with humans (Lachaise et al. 1986). *D. sechellia*, on the Seychelles archipelago, and *D. mauritiana*, on the island of Mauritius, diverged allopatrically from one another (Lacaise et al. 1986). Females of *D. sechellia* and *D. mauritiana* discriminate against interspecific males in the complex, whereas *D. simulans* females do not discriminate against *D. mauritiana* or *D. sechellia* males (Lachaise et al. 1986; Coyne 1992).

Although hybrid males are sterile for all interspecies crosses of the simulans species complex, the hybrid females are fully fertile (Lachaise et al. 1986). Hybrid females can therefore be crossed to males of either parental species to produce backcross (BC) progeny. In this way, species-specific variations of preference behaviour can be associated with the inherited interspecific genetic regions through quantitative trait loci (QTL) mapping (Moehring et al. 2004). A QTL map for *D. mauritiana* mate discrimination against *D. simulans* identified seven loci: two on the X chromosome, two on the second chromosome, and three on the third chromosome, with the majority of the effect size coming from the third (Moehring et al. 2004; McNiven and Moehring 2013). However, a map of *D. mauritiana* discrimination against *D.*

sechellia identified a single loci of major effect in the middle of the X chromosome that did not match any interspecific preference locus previously identified (McNabney 2012). Together, these results indicate that alternative interspecific contexts can trigger different discriminatory behaviours, each controlled by different genetic loci. Another possibility may be that since different *D. mauritiana* strains were used in these studies, intraspecific behavioural variants may underlie different means of interspecific discrimination. More tests of different species pairs, in alternative pairings, can resolve which of the above scenarios is acting within this species group.

There are several major differences that differentiate *D. sechellia* from other species of Drosophila. *D. sechellia* use *Morinda citrifolia* as their primary breeding substrate and food source, which is toxic to other species of *Drosophila* (Louis and David 1986; Jones 2005). Female *D. sechellia* are more likely to mate with a male producing no courtship song than a male producing a heterospecific song (Tomaru 2004). In contrast, *D. melanogaster* and *D. simulans* females prefer to mate with males that generate any courtship song than with a male that produces none (Tomaru 2004). Thus *D. sechellia* females appear to be using type-specific auditory cues as an aversive signal for heterospecific mating, whereas the other species use these cues as an arousal signal for conspecific mating. *D. sechellia* has a sexually dimorphic pheromone profile, which is an ancestral feature, and not shared with the rest of the simulans complex. As a result, *D. sechellia* females are not as readily courted by simulans complex males since these males do not recognize *D. sechellia* female pheromones as appropriate female cues (Jallon and David 1987). A QTL map examining pheromone-based isolation of *D. simulans* males from *D. sechellia* females found a single region on the right arm of the third chromosome (3R) that controls interspecific aspects of courtship and copulation in *D. simulans* males (Civetta and Cantor 2003). Thus, the divergence of loci involved in audition, and the co-ordination of male courtship through olfaction are both likely components of female *D. sechellia* discriminatory processes.

An early chromosomal map of female *D. sechellia* discrimination against *D. simulans* identified the involvement of the second and third chromosomes (Coyne 1992). However, a subsequent QTL map, identifying genomic regions for female *D. simulans* conspecific preference for *D. simulans* males (instead of *D. sechellia* female interspecific rejection against *D. simulans* males)

found a role for each of the three major chromosomes, with the largest effects from two loci on either end of the X chromosome (Chu et al. 2013). The contrast of these two approaches ('conspecific arousal for' versus 'interspecific aversion against') allows questions about the genetic architecture of discriminatory behaviours to be addressed: How many loci are involved, what is their distribution in the genome, and how do their effects compare? Is the genetic basis of rejection of a heterospecific mate simply due to allelic variation of within-species mating preference? The loci underlying mating preference may be the same between both species if their preferences are sensitive to a specific range of stimuli within a larger range of variation, and this range differs between species (Ting et al. 2001). Alternatively, the range may remain the same, but the polarity of response to that range may differ between species. An example of such behaviour can be found in the European corn borer, where two races have reciprocal reactions to the pheromonal isomers produced by the opposite race (Kochansky et al. 1975). Therefore, a map looking for *D. sechellia* aversion could find the same locus encoding both *D. simulans* preference for *D simulans* and *D. sechellia* aversion against *D. simulans.* However, if intraspecific arousal and interspecific aversion are mediated by different mechanisms, it is likely that they are also under distinct genetic controls.

It is likely that the genetics of *D. sechellia*-specific aversion (interspecific mating isolation) differs from *D. simulans*-specific arousal behaviour (intraspecific sexual selection). Two different genetic maps of interspecific mating preference for *D. mauritiana* females did not even match each other (Moehring et al. 2004; McNabney 2012). However, those studies looked at *D. mauritiana* discrimination against different species (*D. simulans* or *D. sechellia*), and may only speak to the diversity of discriminatory behaviours available to *Drosophila* species in different mating contexts. Finally, the small and gene-poor 4th chromosome has yet to be tested for a role in behavioural isolation between *D. secehllia* and *D. simulans*. The 4th chromosome has been implicated in speciation of the melanogaster species subgroup at the level of postzygotic isolation (Masly et al. 2006). To address these questions, quantitative trait loci (QTL) mapping was conducted for female *D. sechellia* discrimination against *D. simulans* males. By comparing any identified loci to those already found in other mapping studies, it is possible to resolve which, if any, of the above hypotheses represent a general course of evolution for behavioural isolation in species of the simulans species complex.

2.2 Methods

Stocks and Crosses: *D. simulans* (from Winters, CA; stock #14021-0251.216) and *D. sechellia* (from Cousin Island, Seychelles; stock #14021-0248.25) were obtained from the Drosophila Species Stock Center (San Diego, CA). All flies were maintained in 8 dram (30 ml) plastic vials on standard Bloomington food recipe medium (Bloomington Drosophila Stock Center) under a 14:10 light:dark cycle at 25°C and 75% relative humidity.

F1 hybrids were created from crosses using 15 virgin *D. simulans* females, aged 5-14 days, paired with 15 *D. sechellia* males aged 1-14 days. Backcrosses (BC) were set up using 15 virgin F1 hybrid females, aged 5-14 days, paired with 15 *D. sechellia* males aged 1-14 days. *D. sechellia* males and BC females (used in mating assays) were collected as virgins within 8 hours of ecclosion using light CO_2 anaesthesia, and were separated by sex to maintain virginity.

No-Choice Mating assays: Within one hour of 'lights on', 5-7-day old virgin males were paired singly to 5-7-day old virgin females, via aspiration into a lightly misted 8 dram glass vial at 20-23°C. Pairings were observed for 45 minutes and scored for courtship and copulation. For pairings between *D. sechellia* males and BC females that copulated, mating pairs were stored at -20ºC for DNA extraction and genotyping. Pairings where males did not attempt to court the female were discarded. Pairings where males attempted to court but were rejected were set up for a 24-hour mating assay. Here, the mating pair was transferred by tipping into a food vial and stored at the original rearing conditions until dissection on the following day. Dissections checked for the presence or absence of sperm within the female reproductive tract and spermathecae (sperm storage organs). After dissection, the females were stored at -20°C for DNA extraction and genotyping. In total 575 assays were conducted, however 40 of the genotypes were dropped from the copulation analysis as these females either died or were lost between the time of courtship assay and the time of the sperm assay. As only a small portion of pairs (36 total) copulated within the 45 minute mating assay, which would not be sufficient for genetic mapping on its own, copulations are reported for pairings with BC females as the total sum of copulations that were recorded during the mating assays and the sperm assays.

DNA extraction and genotyping: DNA was extracted by homogenizing frozen flies in a buffer solution (1M Tris-HCl, 0.5M EDTA, and 5M NaCl) containing 200µg/mL Proteinase K. Samples were held at room temperature for 5-10 minutes before Proteinase K was inactivated at 95°C. Twenty-two markers (3 for Chromosome X, 8 for Chromosome 2, 10 for Chromosome 3, and 1 for Chromosome 4) corresponding to microsatellite repeat regions that vary in length between *D. sechellia* and *D. simulans* were amplified using PCR. The product length for the *D.simulans* and *D. sechellia* amplifications differ and were visualised on a 2% agarose gel for the presence of *D. sechellia* genome as one band or *D. sechellia*/*D. simulans* genome as two bands. The primer sequences and their approximate cytological locations are given in Table 2.2.

QTL analysis: I correlated phenotype (copulation vs. non-copulation) to genotype (homozygous *D. sechellia* vs. heterozygous *D. sechellia/D. simulans*) through composite interval mapping (CIM) (Jansen 1994; Zeng 1994), using a forward selection model, 3 covariate markers, and a window size of 10. Analysis was conducted using the R/qtl package, which includes the CIM scheme from QTL cartographer (Broman et al. 2003). CIM calculates a likelihood ratio (LR) for each region bordered by two markers via the expectation-maximization (EM) algorithm. The LR, -2ln (H_0/H_a), represents the balance of whether a region between two given markers does not (H_0) or does (H_a) contain a QTL, with higher values describing the region as a probable QTL. Significance threshold is determined through 1000 permutations, which reduces the risk of committing Type I/Type II error by testing the correlated data against permuted variants of marker recombination rates and trait characteristics. The effect size of a QTL was characterized by the difference in phenotype averages among the QTL genotype groups. Here, I used the fitqtl function with a Haley-Knott regression in rQTL to calculate effect size (Broman and Sen, 2009). To determine epistasis, QTL pairs were isolated as an object and an additive QTL model, via the function fitqtl, was applied in rQTL. Single marker analysis for the 4th chromosome marker was conducted with a Chi-square test. The the null hypothesis for this test was that BC females do not differ in receptivity behaviour for being homozygous *D. sechellia* or heterozygous *D. sechellia/D. simulans*.

2.3 Results

During behavioural assays, the amount of time to initiate courtship (courtship latency, CUL), the amount of time to achieve copulation (copulation latency, CPL), and the duration of mating (copulation duration, CPD) were measured. Intraspecific mating assays were done as controls to establish that different averages exist between species for each of these measures. I compared the average *D. simulans* (n=10) and *D. sechellia* (n=14) CUL (717 s ± 137 s SE *vs.*690 s ± 123 s SE), CPL (373s ± 159 s SE *vs.* 761s ± 143 s SE) and CPD (1072s ± 218 s SE *vs.* 1661s ± 255 s SE). These differences were significant in a single-factor ANOVA at $p<0.05$ for CUL [$F(1,13) = 4.74$, $p = 0.048$], CPL [$F(1,16) = 4.49$, $p = 0.009$], and CPD [$F(1,15) = 4.54$, $p = 0.029$]. QTL analysis for CUL, CPL, CPD, and copulation success (as determined by behavioural and sperm assay results) was conducted on backcross (BC) females. Of the 575 mating assays observed, 460 courtships were measured (80% courtship), with 36 achieving copulation during the 50 minute assay (8% copulation). The total number of copulations in 24 hours, including those that occurred during the first 45 minutes, was 175 (38%). There was a significant difference in the average time of CUL between BC females that copulated and those that did not, indicating that the time of male courtship initiation affected female copulation occurrence. BC females that copulated did so after a CUL of 894 s, whereas BC females that did not had a CUL of 982 s ($F(1,459) = 13.47$, $p < 0.001$).

The logarithm of the odds (LOD) score estimates the likelihood of genetic linkage between a genetic marker and a trait of interest. For BC females, no QTL with a statistically significant influence on CUL (LOD = 2.23), CPL (LOD = 4.74), or CPD (LOD = 3.04) were identified ($\alpha = 0.05$). Two QTL influencing female preference as measured by copulation occurrence (LOD = 2.19; $\alpha = 0.05$) were found (Table 2.1; Figure 2.1). QTL #1 was located on the left arm of the second chromosome (2L) at 55cM (95% CI; 45-58cM), and QTL #2 was located on the left arm of the third chromosome (3L) at 75cM (95% CI; 65-78cM) (Table 2.1; Figure 2.1). QTL #1 and QTL #2 explain 2.13% and 9.03% of the phenotypic variance respectively (Table 2.1). All cytological locations were calculated by dividing the basepair distance between two markers by their recombination distance (bp/cM). Mapping unit quantities multiplied by this factor gave approximate physical locations which could be identified cytologically using a *D. simulans*

genome browser (UCSC Genome browser; last accessed December 14 2015). No epistatic interactions were detected between QTL 1 and QTL 2 (p=0.182). Because only one marker was located on the fourth chromosome, it could not be analyzed through confidence interval mapping. Instead it was tested through a single marker association test that did not find any association between the fourth chromosome marker and *D. sechellia* rejection behaviour. If anything, rejection behaviour was lower with BC females that were heterozygous for the 4th chromosome marker (X^2 = 6.362, p=0.012).

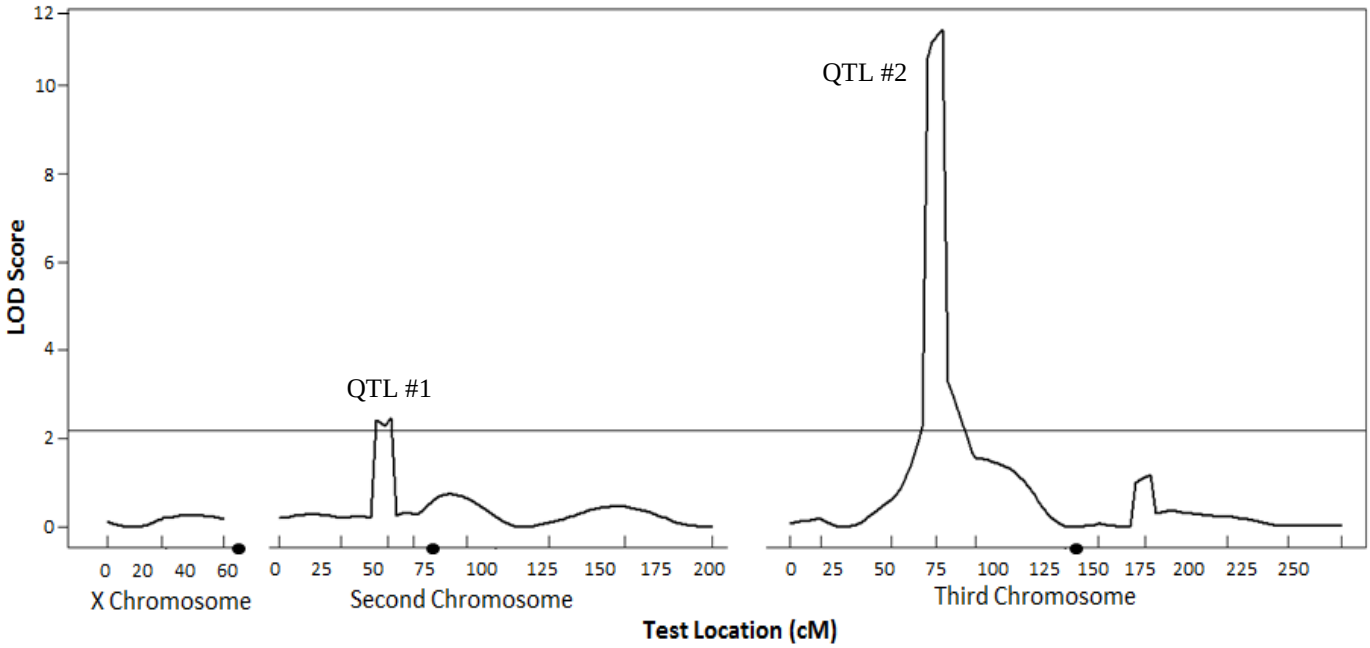

Figure 2.1: QTL for copulation occurrence among BC females paired with *D. simulans* males. The left arms of the second and third chromosome each contained a single statistically significant region. The LOD significance threshold value, 2.19 (α= 0.05), is represented as a horizontal line. Short vertical ticks along the x-axis represent the locations of molecular genotyping markers. Dots on the x-axis represent the locations of centromeres.

Table 2.1: Interspecific preference QTL locations and effects

Comparison	**QTL #[1]**	**Chrsm[2]**	**cM[3]**	**Range (cM)[4]**	**Max. LOD[5]**	**% V[6]**
Copulation vs. non-copulation	1	2L	55	45-58	2.47	2.12
	2	3L	75	65-78	11.27	9.03

[1]QTL peaks from left to right in **Figure 2.1**
[2]Chromosome (X, 2, 3, or 4) and arm (L or R)
[3]Position in centimorgans (cM) for the highest likelihood score as determined through CIM
[4]Span of the QTL as determined by a 95% confidence interval
[5]Maximum logarithm of the odds (LOD) score
[6]Percentage of phenotypic variance explained by the QTL

Table 2.2: Markers for differentiating *D. simulans* and *D. sechellia* microsatellite regions. Marker locations are based on Flybase (2003) *D. melanogaster* cytology. Markers were designed by R.M. Calhoun with the exception of those marked with the superscripts[1] (Dickman and Moerhing 2014) and [2](McNiven and Moehring 2013).

Marker name	Chromsome Arm	Cytological location	Forward primer	Reverse primer
5349	X	4A	CGGCACGCTACTTACCTTGT	CACCCGAAAAGTCGAAGTGT
9363	X	8E	TGGCCCTTTGTATGGATTTC	TTCCTTGCCGAATTAACACA
16836[1]	X	19F6	GGGCGGAAAGTAGAGAAGGT	GCCCACTGATTTGGCTATGT
517	2L	21D1	ATCGAACACCAACGAGGCTA	AGTAGTCCGGCATGAGCATT
9779	2L	30F5	GCACTTTGGGATCCTTTTGA	CACCAAGCTGTTCCACATTTT
14787	2L	35B	AACGGCTCACCCCTCGATCC	TACTTCTCCATGGCGTCCCG
16385[1]	2L	35F	CACACACCCATGCGAATAAG	TCCTTTGACCTCCACCTCTC
4976[2]	2R	47A	GAAATAGGATCATTTTGAATGGC	AATTAAAAACAAAAAACCTGAGCG
12933	2R	53F	GTCAGCGTGTTGCCACTTTA	CCTGCTGGCTTTTAGTTTGC
14938[1]	2R	56B	GTTTGGAGCAACAATTGCATCAGA	GTTTCTGGCCAAGTCGAGAAAAA
18646	2R	59A	TTTTACGGCCAGAAGATGCT	AAGACCTTGCCTTCCTGCTC
1457[1]	3L	62A	TGGGCCACCTGTGGGCGTCGT	TGGAGAGCGGCGTTCCCCTGTGT
3126	3L	63B	CTATGCCAACCACCATCTGC	TCTTTGTAGCCATTCACCGC
10062	3L	67D	TGGCAAACGAAACTGAAATC	GTTTACGATGGGAATGAAAATGGA
16008[1]	3L	73C	GTTTGGAGCAACAATTGCATCAGA	GTTTCTGGCCAAGTCGAGAAAAA
21864	3L	79A	TGGAGAAGTCGTAGGGATGG	GCAGCAGCAGAQACCAGTACA
668	3R	82D5	GTGATGACAGAGGCAGGGAG	GAAAAATGAATCCGTTGAAG
1309	3R	83A	TCTTTGCATGATAATGAAATCCAG	AAAGTTCTGTGGACTTGTGATG
17066[1]	3R	93D	GCGATTGTGTGCGAGTGTAT	GGGGGATTTTGTTTGTCATC
22471	3R	97C	CTATCAGCACCCAGCCAACT	CTGCTTTGGGGGTCTATGAA
27488[2]	3R	100E3	TGTCGGTGATGTTGAGTCTATC	GTTTGCGTCTGTTGAATTGTGTATC
121	4		CGGCACGCTACTTACCTTGT	CACCCGAAAAGTCGAAGTGT

2.4 Discussion

I mapped two loci in *D. sechellia* that are involved in female interspecific rejection against mating with *D. simulans* males. I sought to determine how many genetic elements are involved, how they are distributed throughout the genome, whether they interact epistatically, whether they represent notable candidate loci, and how they compare to the loci found in other maps of the simulans complex for mate preference. I identified two regions centered around the cytological locations 46B (located on 2L) and 74B-C (located on 3L) which had statistically significant associations with *D. sechellia* female rejection of interspecific males (Figure 2.1). Previous mapping for mating discrimination between these species found similar results; contributions from the second and third chromosomes, but not the X chromosome (Coyne 1992). In contrast, another QTL map of these species, examining intraspecific preference instead of interspecific rejection, found that each chromosome (X,2,3) had some contribution to mating preference (Chu et al. 2013). The largest effects found by Chu et al. were from two loci at either end of the X-chromosome (Chu et al. 2013). Thus, in this interspecific pairing, the loci influencing within-species attractiveness are different than those influencing between-species discrimination. This observation has been reached before when comparing other maps of mating receptivity in other *Drosophila* spp. (reviewed in Laturney and Moehring 2012).

The locations of the 2L and 3L QTL identified have noteworthy similarities and differences with other QTL map locations for traits associated with mating behaviour and ecology of *D. sechellia*. Uniquely among the simulans complex, *D. sechellia* has a sexually dimorphic pheromone profile (Gleason et al. 2009). Moreover, the genetic regions that produce the different pheromone profiles between *D. sechellia* and *D. simulans* have been identified for both males (Coyne 1996) and females (Gleason et al. 2009). The 3L QTL centers around cytological location 74B-C, which is approximately 0.8 Mb from the marker at 3R:1,600,800 (Figure 2.1). This region was found previously to influence interspecific differences in the production of 7-tricosene (7-T) between *D. sechellia* and *D. simulans* males (Civetta and Cantor 2003; Gleason 2009). This cuticular hydrocarbon has been shown to play an important role in mating behaviour across the melanogaster subgroup. 7-T influences mating behaviour in both sexes of *D. simulans* and *D. melanogaster* (Ferveur 1991; Grillet 2006). In *D. sechellia* mating, 6-tricosene (6-T) is used as the primary male pheromone instead of 7-T, which may partially influence *D. sechellia* female

rejection of *D. simulans* males (Coyne 1996). Similarly, the Sao Tome strain of *D. yakuba* (also an island endemic like *D. sechellia*) has decreased 7-T production (Denis et al. 2015). Instead, Sao Tome *D. yakuba* males produce more 7-heptacosene and 7-nonacosene, and their females discriminate against other strains of *D. yakuba* that produce predominantly 7-T (Denis et al. 2015). Thus, interspecific differences in 7-T utilization may be an important basis for behavioural isolation in some species of *Drosophila*. Genes involved in the synthesis and perception of 7-T may be important candidates for further analysis. Although there are no obvious candidate genes for pheromone detection or production within the significant region, the chemoreceptor gene *Odorant receptor 74a* (*Or74a*) is directly adjacent to the significant QTL peak (Robertson et al. 2003), and may warrant future examination in the interspecies mate rejection context.

Male *D. sechellia* courtship songs encode species-specific information towards which their females are responsive (Tamaru et al. 2004). Loci influencing interspecific features of *D. sechellia* song (interpulse interval) were found on the second and third chromosomes (Gleason and Ritchie 2004). One of these loci spans 42A-45E and contains the candidate song gene *croaker* (Gleason and Ritchie 2004). The second QTL, centered on 46B, overlaps with this region. While it is unlikely that a gene underlying male song production also controls female song preference, several examples exist of signal variants in tight linkage with the perceptual variant that acts upon them (Kronforst et al. 2006; Shaw and Lesnick 2009; McNiven and Moehring 2013). Several candidate genes can be found in just 46B alone, with functions in sensory perception of auditory cues (*trpl*), female receptivity (*lectin-46Ca, lectin-46Cb*), and peripheral nervous system development (*dila*) (Ram and Wolfner 2007; Ma and Jarman 2011; Senthilan et al. 2012). To confirm whether any of the above regions contain candidate genes that affect auditory or pheromone preferences, follow-up studies with additional backcrossing can be used to generate introgression lines which would further refine the current associations to smaller locations.

BC females that copulated had a significantly shorter courtship latency than BC females that did not. Since *D. sechellia* pheromones are sexually dimorphic, it remains possible that the non-copulating *D. simulans* males were inadequately stimulated to court, and less likely to achieve

copulation with these BC females that had a more *D. secehllia*-like blend of CHCs in their pheromone profile. If future studies are able to assess phenotypic variance of courtship traits known to be involved in isolation, in addition to the discrimination behaviour itself, it may be possible to determine which loci are involved and whether they target similar or different sets of male signals.

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Chapter 3

3 Mapping genes involved in species isolation between *D. melanogaster* and *D. simulans*

Diverging groups can remain reproductively isolated when barriers arise that prevent mating with one another. These barriers often occur when females evolve discriminatory mate preferences that let them reject heterospecific male courtship attempts. Though various species pairs of *Drosophila* have been genetically mapped for loci involved in this reproductive barrier, no individual genes have yet been identified for type-specific female preferences. Here I used fine-scale genome mapping, followed by tests of individual candidate genes, to identify *Katanin-60, fruitless,* and *Mekk-1* as genes underlying *Drosophila simulans* female rejection behaviour towards *D. melanogaster* males. All three genes were found in close proximity to the boundaries of two *D. simulans* inversions: 82F3;83B3 and 84F1;93F6-7. This appears to validate the long-standing hypothesis that alleles important for species mainteneance are likely to be found in genomic regions where recombination would have been low. *Katanin-60* is a phylogenetically-conserved microtubule severing enzyme with a well-established role in neuronal development. I attempted to validate the role of *Katanin-60* by rescuing the behavioural phenotype with transgenic constructs containing various *Katanin-60* alleles inserted into *D. melanogaster*. These manipulations yielded only *D. simulans*-like preference behaviour which suggests that the *D. melanogaster*-specific regulatory components of *Katanin-60* are outside of the focal genomic segment for this study.

3.1 Introduction

Behavioural isolation occurs when diverging populations acquire incompatibilities in traits necessary for courtship (Mayr 1942). Since mating preferences are important determinants of success and failure during courtship, divergence in these traits are important targets of inquiry for the study of speciation (Mayr 1942; Coyne and Orr 2004). To date, no individual genes involved in discrimination against interspecific mating have been identified. The genetic basis for interspecific mate preferences is beginning to be understood for several species pairs. In *Heliconius* (butterflies), wing colour patterns vary between females of different species. Males

prefer females that display conspecific wing colour patterns (Jiggins et al. 2001). Through quantitative trait locus mapping (QTL), a single locus, *wingless*, was identified that controls both wing colour pattern and preference for that particular pattern in multiple species pairs of *Heliconius* (Kronforst et al. 2006; Merrill et al. 2011). Similarly, in *Laupala* (Hawaiian crickets), QTL mapping identified small genomic regions underlying male courtship song and the female preference for that particular song (Shaw and Lesnick 2009). *Drosophila mauritiana* (fruit fly) females discriminate against *D. simulans* males on the basis of two genomic regions influencing male traits associated with female preference and the female preferences for those traits (McNiven and Moehring 2013). The above studies suggest that genes involved in preference-trait combinations associated with speciation are likely to be found in tight linkage with one another. Linkage ensures that lineage-specific combinations of alleles remain coupled with one another. The above studies are also in species pairs for which association mapping of genomic introgressions is used to link genotype to phenotype. One limitation of this method includes its dependence on fortuitous recombination events arising during continual backcrossing. Another limitation is that this approach cannot be used in species pairs that do not produce fertile hybrid offspring that can be used for successive backcrossing.

The species pair of *D. simulans* and *D. melanogaster* exhibit behavioural isolation dependent primarily upon female rejection behaviours. While the males of both species will court heterospecific females, *D. simulans* females strongly discriminate against *D. melanogaster* males (Carracedo et al. 1998). Since *D. melanogaster* females do not fully discriminate against *D. simulans* males, it is possible to produce viable female hybrids, although these females are sterile (Carracedo et al. 1998). Hybrids display the *D. melanogaster*-like lack of discrimination against *D. melanogaster* males, indicating that genes for *D. melanogaster*-like female receptivity are dominant (Carracedo et al. 1998). Previous studies have implicated each of the three main chromosomes in the behavioural isolation of these two species (Carracedo et al. 1998; Ting et al. 2001). The largest genetic contributions to female interspecific mate preference localize to the right arm of the third chromosome (*3R*) (Carracedo et al. 1998; Ting et al. 2001; Laturney and Moehring 2012).

One approach that can be applied to the first generation of offspring to genetically map loci for behaviour is the use of deficiency stocks, which are *D. melanogaster* lines with a known genomic deletion. Crosses using deficiency stock *D. melanogaster* females and *D. simulans* males produce hybrids that are hemizygous (possessing only the *D. simulans* locus) for the region of the deletion; the rest of the genome is heterozygous (heterospecific). If the region contains a gene affecting mate preference, the recessive *D. simulans* version of the trait (discrimination against *D. melanogaster* males) will be exhibited in the mating behaviour of hybrids. Regions underlying the *D. simulans*-like preference can be refined through the use of additional deficiencies that overlap within each region of interest. A study using deficiency mapping on 3R has already identified five regions of interest for *D. simulans*-like discrimination against *D. melanogaster* males (Laturney and Moehring 2012). Because deficiency lines do not exist for most single genes, the testing of candidate loci can be achieved using the same conceptual approach as with deficiency lines but through the use of *D. melanogaster* lines bearing transposable element (TE) insertions that disrupt gene function. (Spralding et al 1999; Bellen et al. 2004; Metaxakis et al. 2005).

Here I refine the mapping of two regions involved in behavioural isolation between *D. melanogaster* and *D. simulans* (Laturney and Moehring 2012). The candidate region (91B2-91F1), originally found using *Df(3R)Cha7*, was honed using the deletion *Df(3R)DG2* to refine its proximal border (Laturney and Moehring 2012). The breakpoints of this deletion have been updated recently, and likely includes 89E9;91A3-7, instead of 89E-89F;91B1-2 (Carpenter 2003). If this is the case, the candidate interval widens from 91B2-91F1 to 91A3-91F1 and includes 68 genes instead of 67. In addition to 91B2-91F1, I also map 82A-82F as a candidate region (Laturney and Moehring 2012).

I expected, as in the other mapping studies, to locate these genes in regions where recombination cannot easily disrupt new combinations of alleles as they emerge. If the genes identified have any previously described biological function, I hypothesized these functions to include key factors known to promote speciation. Key factors are traits associated with species richness in clades and are known to promote diversification (mating preferences, sexual dimorphisms, size variants, dispersal patterns, etc) (Coyne and Orr 2004). Through genetic crosses of *D.*

melanogaster lines, using either deficiencies or transposable elements, I identified three genes with an influence over *D. simulans*-like mating discrimination against *D. melanogaster* males. The three genes, *Katanin-60* (*Kat60*), *fruitless* (*fru*), and *Mekk-1*, map to two fixed inversions between *D. melanogaster* and *D. simulans*. Though *Mekk-1* has not yet been identified in behavioural context for *Drosophila* species, *Kat60* and *fru* have known roles in the developmental biology of *D. melanogaster* behaviour (Stewart et al. 2012; Ryner et al. 1996). Of note, the ortholog of *Kat60* in *Xenopus*, *katanin p60*, has been previously found to play a role in postzygotic species isolation (Loughlin et al. 2011). For these reasons, *Kat60* was selected for further experiments using transgenic insertions of *Kat60* alleles to rescue species-specific behaviours in hybrids. To provide further evidence that *Kat60* is a candidate gene for prezygotic isolation, I attempted to rescue species-specific behaviours in hybrids using transgenic insertions of various *Kat60* alleles.

3.2 Methods

***Drosophila* housing and strains:** One line of wild-type *D. simulans* (Stock #14021-0251.165, collected in Florida City) was obtained from Dr. Jerry Coyne; wild-type *D. melanogaster* (BJS1) were collected in 2009 in London, ON, Canada by Dr. Brent Sinclair. Most lines with deficiencies spanning the previously-identified significant regions (Laturney and Moehring 2012), as well as all transposable element (TE) disruption lines, were obtained from the Bloomington Drosophila Stock Center (Bloomington, IN, USA; Table 3.1). The deficiency stock bearing $Df(3R)^{fru4\text{-}40}$ was obtained from Dr. Barbara Taylor. All of the breakpoints were listed on the online database and initially provided by the donors. Nearly all TE insertions used are in the same orientation as the gene with which they are associated. The lines bearing TE insertions in genes for which they are oppositely oriented are: 15953, 15477, 24442, and 32790 (Table 3.1). The orientation of TE insertion is unknown in lines: 13748 and 13042. Chromosomes that contain a deletion (*Df)* or TE insertion (*In)* are collectively referred to as mutation chromosomes (*Mut)*. The homolog of these *Mut* lines contain balancer (*Bal*) chromosomes which feature a dominant visible marker and serial inversions to elminate viability of recombinant offspring. The *D. melanogaster* lines were maintained as *Mut/Bal*. Each line was kept in a standard 8-dram (30 ml) plastic vial and raised on ~7 ml of standard cornmeal and yeast medium (Bloomington Stock Center's standard medium recipe). All stocks were maintained in incubators with a LD 14:10

hour cycle, 25°C, and a relative humidity of 75%. A previous deficiency map found 82A-82F as a candidate region (Laturney and Moehring 2012). Further mapping reduced this region from 122 possible candidate genes to 9 genes located in 82F6 (Table 3.1; Figure 3.1; stocks 9224 and 24334 tested by M. Laturney). I used *P*-element insertions to disrupt eight of the nine functional genes within this area (Table 3.1; Figure 3.1; stocks 19220,15477,19914, 19578, and 1347 tested by S. Chan). Deficiency stocks 8683 and 6962 were used to refine 91B2-91F1 (Tested by M. Laturney and K. Bruch).

Crosses: Female virgins of each *D. melanogaster (mel)* stock were collected 0-8 hours after eclosion and separated under CO_2 anaesthesia. Once separated, females were transferred to new vials at low densities (1-20) and housed for at least seven days to ensure virginity and reproductive maturity. Females from *D. melanogaster* stocks bearing either a deficiency or a TE insertion were maintained over a balancer (*Bal*) and crossed to wild-type *D. simulans* males. To create F_1 hybrid females, 10-15 female virgins (5-14 day-old) from each *D. melanogaster* stock and 20-25 *D. simulans* males (0-7 day-old) were placed in an 8-dram plastic vial with ~7 ml of food medium. Available space was reduced by pushing the cotton plug down to force increased interactions between the two species. Two types of heterospecific test hybrid females were produced from this cross: *sim*/*Bal* and *sim*/*Mut*. To control for effects of the balancer and *Mut* chromosome on general mating behaviour, ten *D. melanogaster* female virgins (5-14 day-old) from each deficiency, *P*-element, or *minos*-element stock and five *D. melanogaster* males (0-7 day-old) were placed in an 8-dram plastic vial with ~7 ml of food medium. In this way, BJS1 *D. melanogaster* were crossed with *Mut* stocks to produce *mel*/*Bal* and *mel*/*Mut*.

Mating assay: Test females were collected 0-8 hours after eclosion and separated on the presence/absence of the dominant marker (indicating the inheritance of the balancer chromosome) under light CO_2 anaesthesia. Virgin females were transferred to new vials of 1-10 flies, and housed for 5-7 days. Virgin wild-type *D. melanogaster* and *D. simulans* males were collected and housed the same way. For *D. melanogaster* assays intraspecies pairings, one test female was placed with one wild-type male for 45 minutes in an 8 dram glass vial (misted with water to increase humidity). Equal numbers of each type of test female (*mel/Bal* and *mel/Mut*) were observed simultaneously to control for environmental effects. Pairings where the females

were not courted by the males were discarded, as uncourted females cannot display acceptance or rejection behaviour. Copulation occurrence (proportion of the number of courted females that mated with *D. melanogaster*) was determined for each type of female in each line.

Hybrid female mating behaviour with *D. melanogaster* males is reduced in comparison to *D. melanogaster* females: only a very small number of both *sim*/*Mut, sim*/*Bal* mate with *D. melanogaster* males within the 45-minute mating assay. Therefore, to increase the number of matings for analysis, the length of the assay was increased and sperm presence was assessed. To perform the sperm assay, equal numbers of each type of hybrid test female (*sim*/*Bal* and *sim*/*Df* or *sim*/*Mut*) were paired with a *D. melanogaster* male partner in a plastic vial with food (as described above). After 24-50 hours, the female reproductive tract and spermathecae were dissected and scored for sperm presence under a light microscope. Preliminary observations indicated that all (or almost all) females were courted by males within the sperm assay time period, and thus sperm presence/absence was used to determine copulation occurrence. Hybrid females paired with *D. simulans* males were paired similarly to compensate for the long courtship latency of *D. simulans* males courting females with a primarily *D. melanogaster* pheromone profile (Billeter et al. 2009). The sperm assay was uninformative for determining whether there were differences between *Bal* and *Mut* pure intraspecific pairings, as all (or almost all) *D. melanogaster* females mate with *D. melanogaster* males during the longer time period.

Transgenic Constructs: Five different recombinant *Kat60* alleles (generated by Aaron Allen) were transgenically inserted in *D. melanogaster* to assess which species-specific DNA segments of *Kat60* could rescue female receptivity, and thus which components of the gene and its upstream promoter were responsible for the interspecific preferences of females. Two of the alleles were unmodified versions of *Kat60* (plus its upstream promoter region) from either species, and three of the alleles were chimeric DNA segments made up of different thirds of either species *Kat60* sequence. The regions where interspecific DNA segments were spliced to one another occurred at restriction sites in *Kat60* for XhoI and StruI, which are both present at the same site in both species' alleles. XhoI cuts once, just before the 2nd exon, and StuI cuts once, in the middle of the 5th exon. The five lines bearing these alleles had the transgene inserted into cytological region 51C5 (2R), so that transformants could be further crossed into lines bearing *P{EPgy2}EY09078* (3R).

All construction of recombinant *Kat60* alleles was performed by Aaron Allen at the University of Toronto (Toronto, ON). XhoI and StuI were the restriction enzymes used to cut the *D. melanogaster* and *D. simulans* alleles of *Kat*60 into thirds that could then be swapped and ligated to other interspecific sequences. The forward and reverse primers used for amplification and subcloning of *Kat60* were: F- ATAGGCGCGCCGTCATATGCCTTGGCGGTCAG, and R- ATAGCGGCCGCCCTCCAGCGGATTCTATCC. Recombinants were inserted into a pSinger-attB plasmid, which contains genetic elements necessary for transgenic insertion via the phiC31 integrase system. In total six different recombinants were made (Detailed methods in Appendix B). Injection of recombinant constructs was performed by Bestgene (Chino Hills, CA). Injections were made into strain 24482 (*y^1 M{vas-int.Dm}ZH-2A w^*; M{3xP3-RFP.attP'}ZH-51C*) which has its *attP* landing site in 51C1 (2R). The only modification made to the standard Bestgene protocol was lowering the injection and rearing temperatures to 18°C. This modification mitigated the toxic effects of *Kat60* overexpression during development, which had eliminated transformant viability in the first round of injections. Successful integration of the transgenes occurred for five out of the six lines which are termed: AA1, AA2, AA3, AA4, AA6 (Table 3.5).

Data analysis: For tests of mating propensity in behavioural assays, a four-way comparison was performed using a G-test of independence ($p<0.05$). Significance was determined after a False Discovery Rate (FDR) correction for multiple tests (Benjamini and Hochberg 1995). Data for all significant values were further assessed to ensure that the effect on the proportion mated was in the expected direction: a reduction in mating of *sim*/*Mut* compared to controls, assessed with the two criteria of (*sim/Bal* > *sim/Mut*) and [(*sim/Bal* - *sim/Mut*) > (*mel/Bal* - *mel/Mut*)]. For control assays using *D. simulans* males instead of *D. melanogaster* males the criterion was (*sim/Bal* $\leq$ *sim/Mut*). For tests of mating propensity among transgenic insertion lines, proportions mated were compared through a two-tailed Z test ($p<0.05$) with FDR correction for false positives due to multiple tests. For tests of the inserted *Kat60* transgenic rescue constructs, statistical comparisons were made for hybrids bearing: the transgenic allele (*In*), the TE insertion (*Tr*), both (*In/Tr*), or neither (WT).

3.3 Deficiency Mapping and *P*-element Results

3.3.1 Candidate Region 82F

In the 82F region, the only gene disruptions resulting in expression of a *D. simulans*-like preference against *D. melanogaster* males were for disruptions in the gene *Katanin-60* (*Kat60*) (Table 3.1). The insertion *P{Mae-UAS.6.11}Kat60*^{UY1645} has been previously shown to disrupt expression of *Kat60* (Nicolai et al. 2003). I tested an additional three lines bearing different insertions (two *P* and one *minos* element) in *Kat60*. The insertions were located within the upstream regulatory region, the 5'UTR, and the intronic region between exons 4 and 5 of *Kat60* (Table 3.1; Figure 3.1). Hybrids with each of these disruptions showed a similar *D. simulans*-like preference against mating with *D. melanogaster* males (Table 3.1). As expected, the preference is not identical to that of pure-species pairings; some females still accept copulations from heterospecific males, indicating that other genes contribute to the discrimination phenotype. When paired with *D. simulans* males, hybrids bearing a *Kat60* disruption from any of the four lines did not show a reduction in mating activity compared to hybrids not bearing a disruption. Therefore the behavioural discrimination observed is species-specific and not a general absence of female receptivity behaviour (Table 3.4).

3.3.2 Transgenic alleles of *Katanin-60*

The transgenic lines bearing both the disruption in *Kat60* and the transgenic rescue construct were crossed to *D. simulans*. The interspecies female hybrids produced from this cross were assessed for their mating receptivity. As hybrids bearing *P{EPgy2}EY09078* showed *D. simulans*-like mating preference, it was predicted that the insertion of a normal *D. melanogaster* copy (or portions of a normal copy) of *Kat60* would restore the normal *D. melanogaster*-like mating behaviour of hybrid females. In all five cases, no rescue of *D. melanogaster* behaviour was found (Table 3.5).

3.3.3 Candidate Region 91A-F

The revision of the breakpoints for *Df(3R)DG2* to include 91B2-91F1 agrees with our results (Carter 2003). All four deletions generate results congruent with the expectation for a candidate gene in this expanded interval (Table 3.2, Figure 3.2). This candidate gene is the sex-

determination gene *fruitless*. Further tests of this region with three TE insertion lines (two *minos*, one *P*) and a *fru*-specific deletion were conducted. All but one of the *minos* elements showed similar *D. simulans*-like mating results (Table 3.2, Figure 3.2). It is unclear why one of the *minos* elements had no effect when the deletion and other gene disruptions, all in different locations, did. However, given that *fru* is a complex gene with multiple splice variants, it is possible that the *minos* insertion in question did not disrupt the regular *D. melanogaster* function of the gene insofar as it would affect any interspecific preference functions of the gene. As was the case for the *Kat60* disruptions, intraspecific mating was not reduced for hybrids containing the *fru* gene disruptions (Table 3.2). Similarly, hybrid mating with *D. simulans* males was not affected (Table 3.4).

In addition to these findings on the proximal end of the interval, a further two deficiency lines were used to rule out the contributions of the distal end of the interval. The deficiency line *Df(3R)ED2* (tested by K. Bruch) had a significant effect on behaviour, but the overlappingdeficiency lines with deletions *Df(3R)ED5911* (tested by M. Laturney), and *Df(3R)Exel6180* (tested by K. Bruch), both yielded no effect on hybrid behaviour (Table 3.3; Figure 3.3). However, these overlapping deficiencies did not cover the entire span of the distal region under testing. Based on their predicted breakpoints, (18,740,468 and 18,742,927 respectively), an interval of ~2.5kb remained untested. The interval contained within it a single gene. The gene, *Mitogen-Activated Protein Kinase Kinase Kinase 1* (*Mekk-1*), encodes a protein kinase kinase involved in JNK signal pathways (Chen et al. 2002). I tested *Mekk-1* using three *P*-element gene disruption lines. Two of the three lines (20676 and 19991) yielded the *D. simulans*-like interspecific preference behaviour (Table 3.3; Figure 3.3), with no reductions in intraspecific mating behaviour (Table 3.4). These two TE insertions, and *Mekk1*, are minus strand orientated, whereas the orientation of the third (13748) TE insertion is unknown. For this reason, the *D. melanogaster* allele of *Mekk-1* may not have been disrupted if its function is insensitive to TE orientation in the plus strand.

Table 3.1. Effects of mutations in cytological region 82F on female hybrid mating with *D. melanogaster* males. Mutant *D. melanogaster stocks* (*Mut*) contain either a genomic deletion (*Df*), or a transposable element insertion (*In)* of a *P*-element (*P*) or *minos*-element (*Mi*). Mating behaviour of hybrid females bearing either a *Mut* or balancer chromosome (*Bal*) were generated from *D. melanogaster (mel)* males crossed to *D. simulans* (*sim)* females. Intraspecific crosses were made to control for the effects of possessing a *Bal* or *Mut.* Numbers are given for the number of courtships (Crt) and copulations (Cop) that occurred during behaviour assays. Hybrid females were additionally subjected to a sperm assay. Statistical significance was calculated through comparisons of the total number of hybrid females that copulated (behaviour + sperm) and the proportion of copulations for those intraspecific females that were courted during behaviour assays ((Cop/Crt)*N). Stocks are labelled 1-14 for ease of reference in **Figure 3.1**.

			Behaviour assay									+ Sperm assay			
			sim/Mut		*sim/Bal*		*mel/Mut*		*mel/Bal*			*sim/Mut*	*sim/Bal*		
Stock	**Mutant (*Mut*)**	**N**	**Crt**	**Cop**	**Crt**	**Cop**	**Crt**	**Cop**	**Crt**	**Cop**	**G test**		**Cop**	**Cop**	**G test**
(1) 9224	*Df(3R)ED5071*	13					12	9	9	8			11	9	$G=0.343, P=0.558$
(2) 8091	*Df(3R)ED5092*	23	14	7	14	2	19	11	15	13	$G=1.029, P=0.31$		21	8	$G=1.073, P=0.3$
(3) 24334	*Df(3R)BSC176*	72					41	32	41	33			5	17	$G=5.552, P=0.018$
(4) 19920	*P{EPgy2}CG1115^{EY06250}*	30	22	4	20	14	23	14	23	19	$G=2.356, P=0.125$		11	15	$G=0.0001, P=0.992$
(5) 25251	*Mi{ET1}Kat60^{MB06739}*	66	47	5	39	8	44	35	35	29	$G=0.519, P=0.471$		23	47	$G=4.557 P=0.036$
(6) 15953	*P{EPgy2}Kat60^{EY05598}*	60	39	1	34	8	46	27	46	27	$G=5.58, P=0.018$		7	26	$G=5.962, P=0.015$
(7) 17531	*P{EPgy2}EY09078*	64	39	2	30	7	51	38	44	28	$G=3.43, P=0.064$		7	42	$G=22.52, P<0.0001$
(8) 7345	*P{Mae-UAS.6.11}Kat60^{UT1645}*	70	51	4	47	10	52	34	44	27	$G=2.279, P=0.131$		21	49	$G=7.598, P=0.0059$
(9) 15477	*P{EPgy2}Mms19^{EY00797}*	30	20	1	20	1	23	15	23	13	$G=2.356, P=0.125$		7	10	$G=0.716, P=0.398$
(10) 13003	*P{SUPor-P}CG12163^{KG03153} CG33293^{KG03153}*	63	43	6	48	17	45	29	57	39	$G=3.844, P=0.05$		23	33	$G=0.75, P=0.386$
(11) 19914	*P{EPgy2}CG12163^{EY04405}*	30	23	12	20	13	24	20	23	17	$G=0.161, P=0.688$		13	14	$G=0.161, P=0.688$
(12) 19578	*P{EP}CG1113^{EP3693}*	30	14	5	19	8	22	16	22	17	$G=0.408, P=0.523$		10	14	$G=0.292, P=0.589$
(13) 13647	*P{SUPor-P}CG12173^{KG00232}*	30	20	4	18	7	24	19	26	21	$G=0.636, P=0.425$		12	11	$G=0.017, P=0.896$
(14) 18714	*PBac{WH}Hph^{f03923}*	30	19	0	15	1	21	15	18	13	$G=1.363, P=0.243$		5	11	$G=1.635, P=0.201$

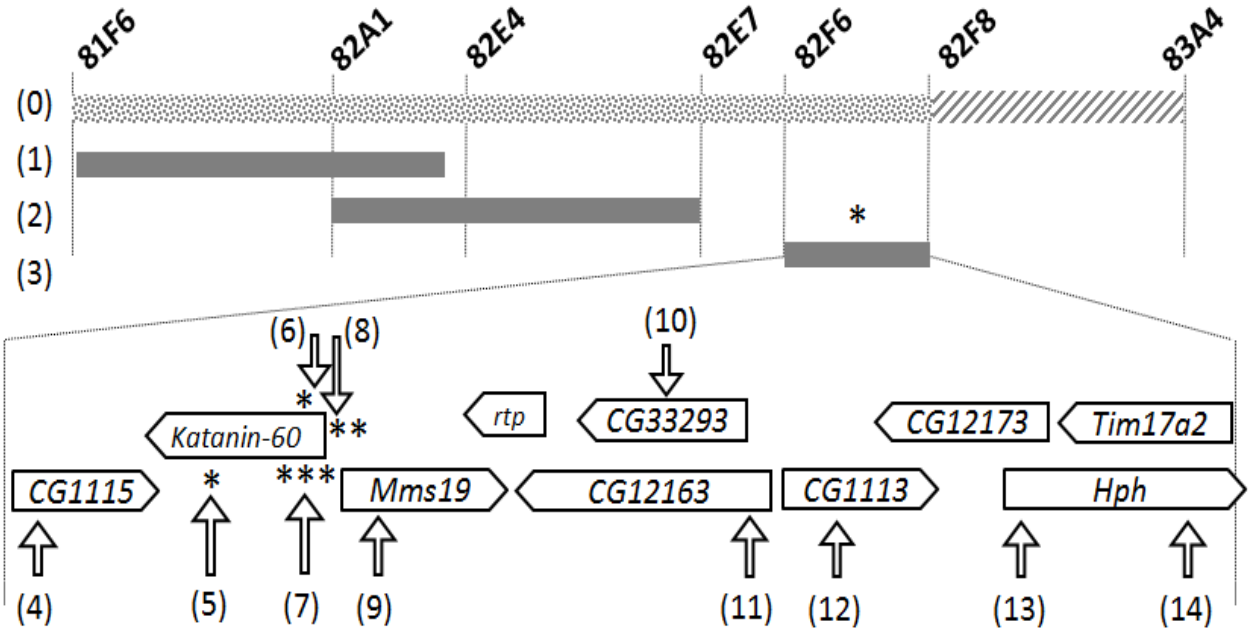

Figure 3.1. Approximate locations of deficiencies and TE insertions in cytological region 82F. Locations of deletions (grey bars) and transposable element insertions (arrows) relative to genes (white bars) in *D. melanogaster* genome. Gene orientation is in the direction of the point. (1-14) refers to data for corresponding element`s effects on mating discrimination of females (**Table 3.1**). Significant effects on behaviour when the corresponding regions were removed/disrupted are marked with * for $P<0.05$, ** for $P<0.005$, and *** for $P<0.0005$. Relevant cytological landmarks are shown with grey lines and listed in bold. The bar listed as region (0) represents findings from *Df(3R)ME15* (wavy bar) and *Df(3R)ED5156* (diagonal bar) which were reported Laturney and Moehring 2012 and so the raw data for those lines is not listed here. Lines with * were significant for an effect on interspecific behaviour at $P< 0.05$.

Table 3.2. Confirmation of species-specificity for candidate genes influencing female preference. Select *Mut* lines for candidate genes were tested with *D. simulans* (*sim*) males instead of *D. melanogaster* (*mel)* males. Mutant *D. melanogaster stocks* (*Mut*) contain either a genomic deletion (*Df*), or a transposable element insertion (*In)* of a *P*-element (*P*) or *minos*-element (*Mi*). Mating behaviour of hybrid females bearing either a *Mut* or balancer chromosome (*Bal*) were generated from *D. melanogaster* males crossed to *D. simulans* females. Numbers are given for the number of courtships (Crt) and copulations (Cop) that occurred during behaviour assays. Statistical comparisons using G tests were not possible as *D. simulans-165* males do not actively mate with hybrids. Instead effects of *Mut* were assessed on the hypothesis that *sim/Bal* mating would be approximately the same as (or less than) mating in *sim/Mut*.

			Behaviour assay				**+Sperm assay**	
	Transposable Element		***sim/Mut***		***sim/Bal***		***sim/Df***	***sim/Bal***
Stock	**Insertion**	**N**	**Crt**	**Cop**	**Crt**	**Cop**	**Cop**	**Cop**
15953	*P{EPgy2}EY09078*	21	2	1	3	0	17	8
17531	*P{EPgy2}Kat60*^{EY05593}	22	0	0	0	0	6	5
25251	*Mi{ET1}Kat60*^{MB06739}	24	1	0	1	0	16	12
13042	*P{SUPor-P}fru*^{KG00116}	28	0	0	0	0	6	8
19991	*P{EPgy2}Mekk1*^{EY02276}	20	0	0	0	0	16	1

Table 3.3. Effects of mutations in cytological region 91A-C on female hybrid mating with *D. melanogaster* males. Mutant *D. melanogaster stocks* (*Mut*) contain either a genomic deletion (*Df*), or a transposable element insertion (*In)* of a *P*-element (*P*) or *minos*-element (*Mi*). Mating behaviour of hybrid females bearing either a *Mut* or balancer chromosome (*Bal*) were generated from *D. melanogaster (mel)* males crossed to *D. simulans* (*sim)* females. Intraspecific crosses were made to control for the effects of possessing a *Bal* or *Mut.* Numbers are given for the number of courtships (Crt) and copulations (Cop) that occurred during behaviour assays. Hybrid females were additionally subjected to a sperm assay. Statistical significance was calculated through comparisons of the total number of hybrid females that copulated (behaviour + sperm) and the proportion of copulations for those intraspecific females that were courted during behaviour assays ((Cop/Crt)*N). Stocks are labelled 1-7 for ease of reference with **Figure 3.2**.

			Behaviour assay									+ Sperm assay			
			sim/Mut		*sim/Bal*		*mel/Mut*		*mel/Bal*			*sim/Mut*	*sim/Bal*		
Stock	**Mutant (*Mut*)**	**N**	**Crt**	**Cop**	**Crt**	**Cop**	**Crt**	**Cop**	**Crt**	**Cop**	**G test**	**Cop**	**Cop**	**G test**
(1) 13042	*P{SUPor-P}fru^{KG00116}*	68	41	5	31	3	41	36	45	36	$G=0.315, P=0.575$	17	45	$G=10.43, P=0.0012$
(2) 24442	*Mi{ET1}fru^{MB02472}*	12	6	0	5	0	11	10	11	8	$G=0.000, P=1.0$	8	8	$G=0.111, P=0.739$
(3) 32790	*Mi{MIC}fru^{MI01850}*	62	29	0	25	2	35	32	30	26	$G=2.845, P=0.092$	7	18	$G=2.356, P=0.032$
(4) fru$^{4-40}$	*Df(3R)$^{fru4-40}$*	51	27	0	17	0	28	19	19	14	$G=0.000, P=1.0$	0	17	$G=19.169, P=<0.0001$
(5) 6962	*Df(3R)ED2 , P{3'.RS5+3.3'}koko^{ED2}*	22	8	0	8	0	16	5	14	8	$G=0.000, P=1.0$	0	9	$G=7.818, P=0.0052$
(6) 2409	*Df(3R)07280*	48	21	2	25	7	25	7	38	33	$G=0.02, P=0.888$	7	18	$G=4.743, P=0.029$
(7) 25013	*Df(3R)BSC509*	20	15	0	18	0	18	15	14	10	$G=0.000, P=1.0$	1	20	$G=17.749, P<0.0001$

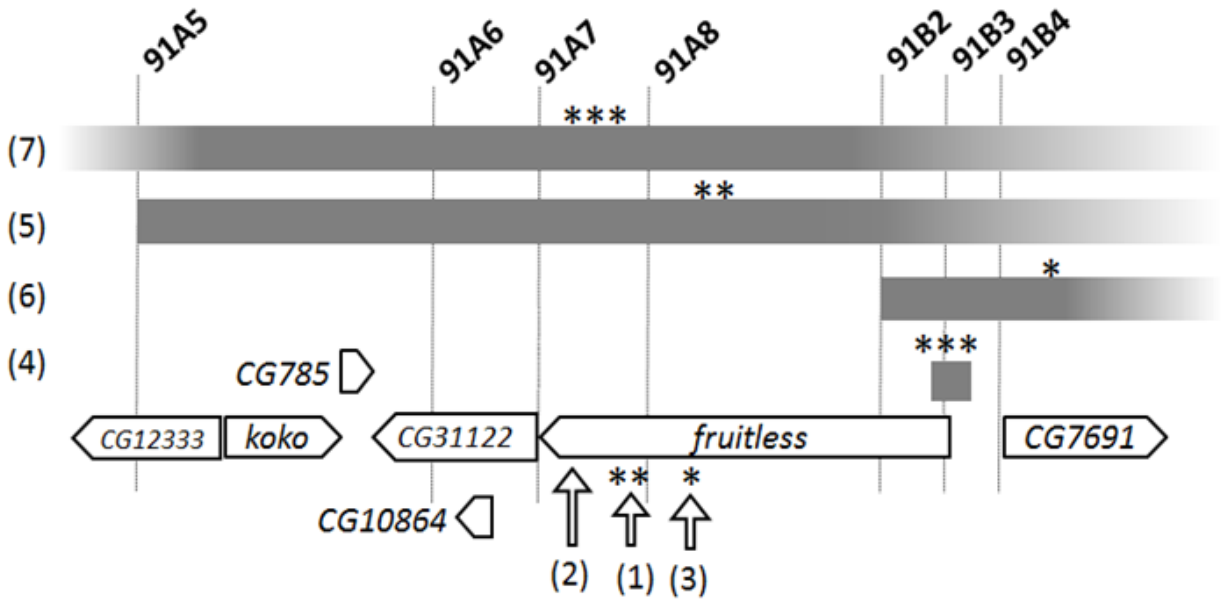

Figure 3.2. Approximate locations of deletions and TE insertions in cytological region 91A-C. Locations of deletions (grey bars) and transposable element insertions (arrows) relative to genes (white bars) in *D. melanogaster* genome. Gene orientation is in the direction of the point.(1-7) refers to data for corresponding element`s effects on mating discrimination of females (Table 3.2). Significant effects on behaviour when the corresponding regions were removed/disrupted are marked with * for $P<0.05$, ** for $P<0.005$, and *** for $P<0.0005$. Relevant cytological landmarks are shown with grey lines and listed in bold.

Table 3.4. Effects of mutations in cytological region 91C5 on female hybrid mating with *D. melanogaster* males. Mutant *D. melanogaster stocks* (*Mut*) contain either a genomic deletion (*Df*), or a transposable element insertion of a *P*-element (*P*). Mating behaviour of hybrid females bearing either a *Mut* or balancer chromosome (*Bal*) were generated from *D. melanogaster (mel)* males crossed to *D. simulans* (*sim)* females. Intraspecific crosses were made to control for the effects of possessing a *Bal* or *Mut.* Numbers are given for the number of courtships (Crt) and copulations (Cop) that occurred during behaviour assays. Hybrid females were additionally subjected to a sperm assay. Statistical significance was calculated through comparisons of the total number of hybrid females that copulated (behaviour + sperm) and the proportion of copulations for those intraspecific females that were courted during behaviour assays ((Cop/Crt)*N). Stocks are labelled 1-5 for ease of reference with **Figure 3**. Note that the data from line 6962 is also presented in Table 2.

			Behaviour assay									+ Sperm assay			
			sim/Mut		sim/Bal		mel/Mut		mel/Bal			sim/Mut	sim/Bal		
Stock	**Mutant (*Mut*)**	**N**	**Crt**	**Cop**	**Crt**	**Cop**	**Crt**	**Cop**	**Crt**	**Cop**	**G test**	**Cop**	**Cop**	**G test**
(1) 20676	*P{EPgy2}Mekk1^{EY11461}*	66	37	0	33	4	45	41	42	32	$G = 6.122, P = 0.013$	7	37	$G = 6.122, P < 0.0001$
(2) 19991	*P{EPgy2}Mekk1^{EY02276}*	66	40	1	27	5	47	41	42	36	$G = 2.845, P = 0.092$	10	49	$G = 0.000, P < 0.0001$
(3) 13748	*P{SUPor-P}Mekk1^{KG02510}*	13	11	1	7	3	9	8	9	5	$G = 1.833, P = 0.176$	6	10	$G = 2.045, P = 0.153$
(4) 6962	*Df(3R)ED2 , P{3'.RS5+3.3'}koko^{ED2}*	22	8	0	8	0	16	5	14	8	$G = 0.000, P = 1.0$	0	9	$G = 6.207, P = 0.013$
(5) 8683	*Df(3R)ED5911*	21	20	2	17	0	19	11	17	17	$G = 3.819, P = 0.051$	5	9	$G = 0.004, P = 0.95$
(6) 7659	*Df(3R)Exel6180*	22	20	0	15	1	15	12	15	12	$G = 1.359, P = 0.244$	4	5	$G = 0.089, P = 0.766$

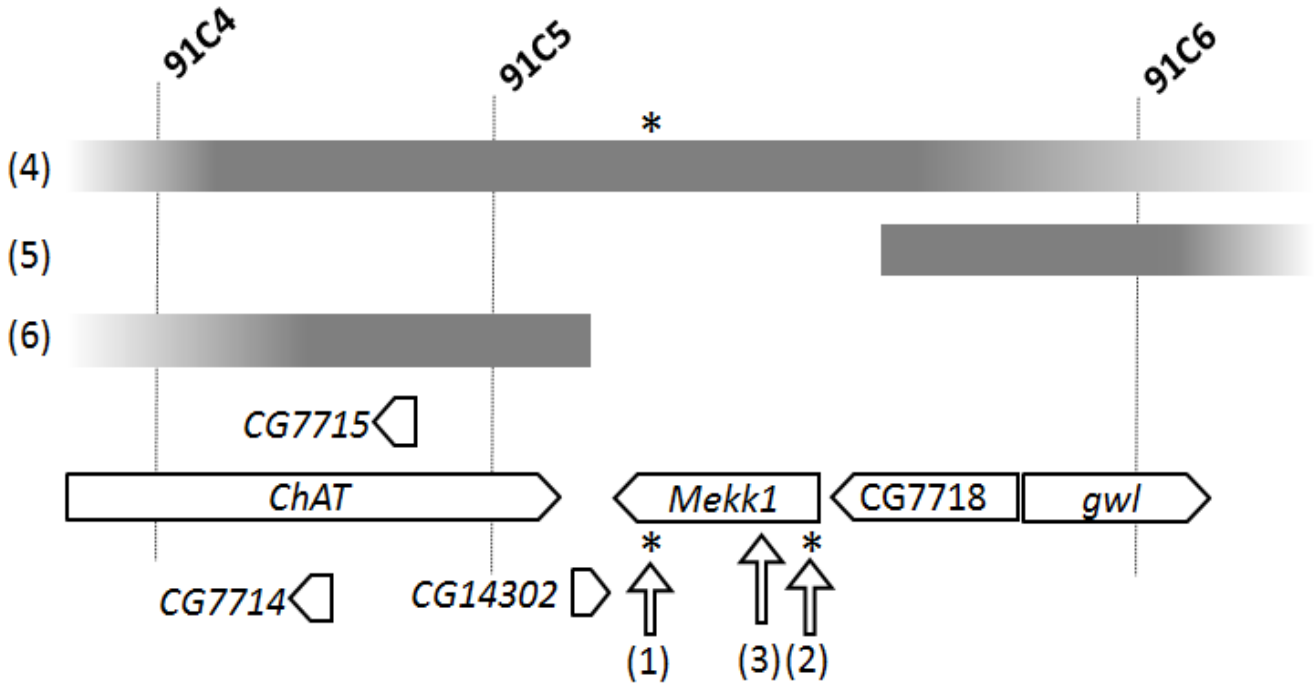

Figure 3.3. Approximate locations of deletions and TE insertions in cytological region 91A5-91B4. Locations of deletions (grey bars) and transposable element insertions (arrows) relative to genes (white bars) in *D. melanogaster* genome. Gene orientation is in the direction of the point. (1-6) refers to data for corresponding element`s effects on mating discrimination of females (**Table 3.3**). Significant effects on behaviour when the corresponding regions were removed/disrupted are marked with * for $p<0.05$. Relevant cytological landmarks are shown with grey lines and listed in bold.

Table 3.5. Hybrid female copulation using transgenic *Kat60* alleles. Hybrids bearing transposable element inserstion *P{EPgy2}EY09078* were previously shown to display *D. simulans*-like mating behaviour (**Table 3.1**). The mating of hybrid females bearing insertions (*In*) was compared to hybrid females bearing transnsgenic copies of *Kat60* (*Tr). Tr* were composed, to varying degrees, of *D. melanogaster* (dark grey bars) and *D. simulans* sequence (light grey bars). Wildtype (WT) hybrid females were used as a baseline for typical *D. melanogaster*-like mating behaviour among hybrids. Statistical significance was determined through a two-tailed Z-test ($p<0.05$).

Stock	Transgenic construct	Total Copulations (Behaviour + Sperm assays) N	*In/Tr*	*In*	*Tr*	WT	*p*-value (Z score)	*In*	*Tr*	WT
AA1		24	6	3	20	15	*In/Tr*	0.267 (1.11)	<0.01 (4.06)	0.01 (2.62)
							WT	<0.01 (-3.57)	0.11 (1.62)	
							Tr	<0.01 (4.91)		
AA2		26	10	12	22	19	*In/Tr*	0.57 (-0.56)	<0.01 (-3.42)	0.01 (-2.51)
							WT	0.05 (-1.98)	0.31 (-1.02)	
							Tr	<0.01 (-2.91)		
AA3		22	6	4	19	20	*In/Tr*	0.47 (0.72)	<0.01 (-3.96)	<0.01 (-4.29)
							WT	<0.01 (4.82)	0.63 (0.48)	
							Tr	<0.01 (-4.53)		
AA4		29	12	12	22	19	*In/Tr*	1 (0)	<0.01 (-2.67)	0.07 (-1.84)
							WT	0.02 (-2.32)	0.20 (-1.27)	
							Tr	<0.01 (-3.48)		
AA6		19	6	9	17	13	*In/Tr*	0.36 (-0.92)	<0.01 (-3.07)	0.04 (-2.02)
							WT	<0.01 (-2.79)	0.26 (-1.12)	
							Tr	0.03 (-2.22)		

3.4 Discussion

3.4.1 Chromosomal Analysis

Recombination frequency is reduced between heterokaryotypic regions of chromosomes. Without recombination in these regions, species-specific alleles are able to persist in association with one another (McGaugh and Noor 2012; Stevison et al. 2011). For this reason, chromosomal inversions are predicted to be important facilitators of speciation, allowing divergence to accumulate in those regions adjacent to the inversion breakpoints, and within the inversion itself (McGaugh and Noor 2012; Stevison et al. 2011). For example, *D. pseudoobscura* and *D. persimilis* differ by several fixed inversions (Stevison et al. 2011). Nucleotide divergence between these species is highest in regions that are close to telomeres, centromeres, inversion breakpoints, and the regions within inversions (Stevison et al. 2011). The maximum range outside an inversion for recombination suppression varies on an inversion-by-inversion basis. The most conservative estimates ranging from 2.18-2.44 Mb, 2.32-2.56 Mb, 2.75-2.84 Mb for three of the fixed inversions between *D. pseudoobscura* and *D. persimilis* (McGaugh and Noor 2012). Suppression has been found at the same inversions as far as 3.35 Mb, 4.55 Mb, and 3.0 Mb for the same inversions (Schaeffer et al. 2005). Therefore, a reasonable estimate of an inversion's average recombination suppression effect is in the range of 2.5-3.0 Mb. As a result, 5-6 Mb of total genome outside the inversion breakpoints can be added to the inversion's length of sheltered heterogeneity between species. It should be noted that two species groups as diverged as the pseudoobscura and melanogaster groups (estimated divergence 45.6 mya (Gao et al 2011)), may possess different factors that influence recombination in dissimilar ways. If the measures made of the pseudoobscura group recombination suppression boundaries are all similar on a genus-wide level, then my findings in *D. simulans/D. melanogaster* fall within the values predicted by the *D. pseudoobscura/D. persimilis* studies. The first candidate gene for *D. simulans*-like preference against *D. melanogaster* males, *Kat60*, was located within a *D. simulans*-specific inversion that spans cytological location 82F3;83B3 (Harrison 1939; Table 3.1; Figure 3.1). *Kat60* is approximately 50.4-58.7 kb from the closest inversion breakpoint at 82F3. In addition to being within an inversion, the proximal-to-distal reorientation of the 82F3;83B3 region places *Kat60* in closer proximity to yet another *D.simulans*-specific inversion that spans 84F1;93F6-7 (Ashburner and Lemeunier 1976). The location of a behavioural

isolation gene in inversion 82F3;83B3, and its proximity to the inversion at 84F1;93F6-7, supports the hypothesis that genes involved in species isolation are likely to be found in areas where recombination between divergent lineages is suppressed (McGaugh and Noor 2012; Stevison et al. 2011).

The second region I investigated contained two candidate genes, *Mekk1* and *fru*, and was also located within the *D. simulans* 84F1;93F6-7 inversion (Ashburner and Lemeunier 1976). *Kat60* is 2.90 Mb outside the 84F1 breakpoint (centromeric side of the inversion), *fru* is 3.1 Mb inside the 93F6-7 breakpoint and *Mekk1* is 2.98 Mb inside the 93F6-7 breakpoint. These distances are within the predicted boundaries of recombination suppression estimated from the above studies in *D. pseudoobscura*/*D. persimilis*. Additionally, the 82F3;83B3 and 84F1;93F6-7 inversions place *Kat60, Mekk1,* and *fru* in a genomic unit that is approximately 6.06 Mb long. The same interval in *D. melanogaster* is 13.51 Mb long (Figure 3.4). For these reasons, combinations of new alleles for the three candidate genes were more likely to be inherited as a unit in *D. simulans* than in *D. melanogaster*. Thus, the co-localization of *Kat60, Mekk1,* and *fru* to a similar genomic region support previous findings of genetic coupling among genes involved in species-specific mating preferences (Kronforst et al. 2006; Shaw and Lesnick 2009; McNiven and Moehring 2013). It is unknown whether a *D. simulans* male trait that *D. simulans* females prefer can also be found in the region. Currently, this hypothesis is impractical to test as generating hybrid males from the reciprocal cross is very difficult.

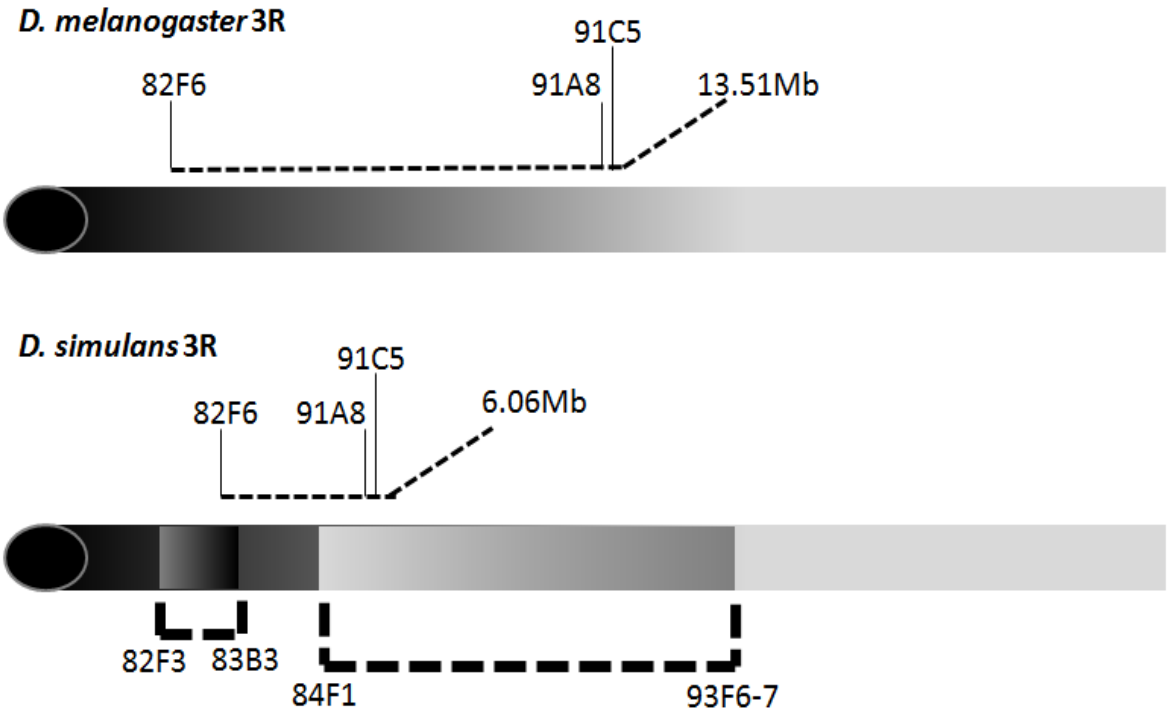

Figure 3.4. Relative locations of candidate genes in *D. melanogaster* and *D. simulans*. Relative locations of candidate gene cytological locations on the right arm of the third chromosome (3R). Physical distances (horizontal solid lines) of candidate genes (*Kat60*, *fru*, *Mekk1*) map much more closely in *D. simulans* than in *D. melanogaster* because of two fixed inversions (horizontal dashed lines) between the species.

3.4.2 *Katanin-60*

ATPases associated with various cellular activities (AAA) are a diverse family of proteins that can be found in all organisms (Frickey and Lupas 2004). Proteins of the AAA family feature a conserved C-terminal catalytic domain, and an N-terminal domain involved in protein-protein interactions (Lupas and Martin 2002). Katanins, a subclass of AAA, are involved in microtubule severing activities that underlie cell division, cell migration, cilia/flagella assembly, and neuronal structuring (Sharp and Ross 2011; Toyo-oka 2005). Katanin-60 binds and severs microtubules, either altering the length of microtubule spindles, or generating short fragments for the seeding of non-centrosomal microtubular arrays (Sharp and Ross 2001; Zhang et al. 2007, Zhang et al. 2011). Several Katanin proteins exist, and it is a well-known feature of these proteins to be involved in neuronal development, migration, and regeneration (Stone et al. 2012, Toyo-oka

2005). For example, Katanin p60-like1 shapes the dendritic arborizations (branch number and length) of class IV sensory neurons in *D. melanogaster*. If *Katanin p60-like1* is mis-expressed, these neurons produce poor arborisations with adverse behavioural consequences (decreased nocifensive response) (Stewart et al. 2012).

Our candidate gene, *Katanin-60* (*Kat60*), has activity during mitosis (Zhang et al. 2007), interphase microtubule dynamics, cell migration (Zhang et al. 2011), axonal/dendritic outgrowth (Yu et al 2008, Mao et al. 2014), and neuronal polarity (Yu et al. 2005). In *Drosophila* cell cultures, the ratio of *Kat60* to *Katanin-80* expression can have important consequences for neuronal morphology (Yu et al. 2005). To date, there is no known role for *Kat60* in mating behaviour, though it has been implicated as a potential source of species isolation (Loughlin et al. 2011). Interspecific differences in protein-level regulation have been demonstrated for the *Xenopus* ortholog of *Kat60,* - called *Katanin p60* (Loughlin et al. 2011). Serine 131 is a conserved phosphorylation site present in most identified Katanin proteins, however this site is polymorphic between *X. tropicalis* and *X. laevis* (Loughlin et al. 2011). As a result, postzygotic isolation exists between these species because of species-specific meiotic spindle lengths that are incompatible in hybrids (Loughlin et al. 2011). In addition to inviability between *Xenopus* species, Katanin mutants can cause sterility in mice. Katanin p80, the targeting subunit of the larger Katanin p60 complex, is essential for male fertility. Misexpression of *Katanin p80* leads to decreased sperm production (O'Donnell et al. 2012). The sperm that are produced are also morphologically aberrant, and completely immotile (O'Donnell et al. 2012). The influence of Katanins in postzygotic sterility, inviability, and now, mating behaviour, raises interesting questions of how divergence in a single class of genes can contribute to multiple types of incompatibilities involved in species isolation.

3.4.3 *fruitless*

The gene, *fruitless* (*fru,)* regulates sexually dimorphic development in *Drosophila* (Lee and Luo 2001; Kimura 2005). *fru* encodes several transcripts that are differentially spliced between sexes. The transcripts are translated into transcription factors which target different genes on the basis of their respective DNA binding affinities (Ryner et al. 1996). *fru* is the first gene in the sex determination pathway of *Drosophila* to function in the central nervous system (CNS), up-regulating the neurodevelopment genes *CadN*, *lola,* and *pdm2* (Nojima 2014). fru^{M}, the male-

specific transcript is essential to male specific behaviours (Lee et al. 2000). Misexpression of fru^M causes male courtship aberrations including courtship with other males (Lee et al. 2000). Futhermore, studies expressing fru^M transcripts in different mosaics of neural tissues have identified male-specific neuroanatomical structures (Lee et al. 2000). At a cellular level, the effects of fru^M`s are as a masculinization factor occur through the upregulation of cell surface markers that paint male neuronal identities on the *D. melanogaster* CNS. The male-specific cell surface markers prevent programmed cell death (PCD) for structures that would otherwise undergo PCD in the developing female (Kimura 2005). Differential PCD is a general mechanism of sexual dimorphism for many organisms. As an example, expression of *TRA-1A*, a gene in the sex-determination hierarchy in *C. elegans*, blocks PCD and preserves hermaphrodite-specific neurons. Currently there is no evidence of between species variation in *fru* that leads to variant male courtship behaviours (Cande et al. 2014). However, fru^M has divergent patterns of expression in different species of *Drosophila* that are responsible for anatomical differences between males of different species (Usui-Aoki 2005). Moreover, in *D. suzukii*, the canonically male-only transcript is translated in specific areas of the female brain (Usui-Aoki 2005). Thus, *fru*'s role as a source of interspecific variation in mating behaviour may be as a source of differential sexual dimorphism between species. If subsets of sexually dimorphic traits are preserved, altered, or removed between species, then the degree to which either species utilizes these traits for mating may vary as well.

3.4.4 *Mekk1*

Mitogen-Activated Protein Kinase (MAPK) cascades are signal pathways that connect a diverse set of circumstances (physiology, environmental stresses, pathology, etc) to the transcriptional machinery of the nucleus (Lopez-Ilasaca 1997). These cascades are typically composed of three kinases (MAPKKK->MAPKK->MAPK), of which *Mekk1* is a MAPKKK. *Mekk1* mediates stress responses in the p38 MAPK pathway under conditions of high osmolarity and high temperature, and in the JNK pathway in response to heavy metals (Inoue 2001; Ryabinina 2006). Additionally, in the JNK pathway, *Mekk1* demonstrates activity as an upstream mediator of apoptosis in response to reactive oxygen species (ROS) and microtubule destabilization (Kang 2012; Yujiri 1999). Interestingly *Mekk1* has a pro-apoptotic role in the ROS stress context, but is anti-apoptotic in response to microtubule destabilization (Kang 2012; Yujiri 1999).

Consequently, the polarity of *Mekk1*`s function may be conferred by its integration with downstream signalling products (Lin 2003). Though *Mekk1*'s involvement in behaviour may be less straightforward than that of *fru* and *Kat60*`s, *Mekk1*`s activity in PCD may be of consequence to neurodevelopmental processes. For example, PCD is an essential component of neuronal remodelling during pupation (Rusconi 2000). If divergent *Mekk1* alleles convey alternative patterns of PCD during development, variant systems of behaviour based on the inclusion/exclusion of specific cell clusters may emerge. Alternatively, if *Mekk1* is not involved in a developmental role, it may instead be involved directly in courtship. JNK signalling has been implicated in switching between genetic programs of neurotransmitter synthesis (Guemez-Gamboa 2014).

3.5 Conclusions

Previously, inversions were known to preserve heterospecificity between species because recombination was suppressed in those regions. For this reason, alleles yielding reproductive isolation between species are thought to be more likely to emerge in these regions. I support this hypothesis by identifying three candidate genes for *D. simulans* discrimination against *D. melanogaster* near the breakpoints of two inversions. As a result, I suggest future mapping efforts maximize the potential identification of other behavioural isolation genes by focusing first on heterokaryotypic regions that differentiate species from one another. I did not restore *D. melanogaster* behaviour with any of the transgenic insertions of *Kat60.* One reason may have been because modifications to the protocol for increasing viability of transformants (by lowering *Kat60* expression) were too severe. Another possibility is that the regulation of *Kat60* expression is complex, relying on cis-acting elements outside the boundaries of the gene itself. Species-specific differences in cis-regulatory regions have also been found between *Nasonia vitripennis* and *N. giraulti*. *N. vitripennis* possesses a duplication of the 5' UTR and its associated regulatory elements for the gene *doublesex*. This region is located in an intergenic segment between two transcription factors and is responsible for the species-specific differences in sexual dimorphism for wing size between these species (Loehlin 2010). If a similar dynamic is at work between *D. melanogaster* and *D. simulans*, then future studies may be able to identify the divergent intergenic sites involved in these species-specific mating behaviours.

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Chapter 4

4 Phenotypic components of the candidate gene *Katanin-60* in behavioural isolation of *D. simulans* and *D. melanogaster*

Species can remain reproductively isolated from one another when traits necessary for successful mating acquire type-specificity. An important mechanism in this process can be the evolution, in females, of alternative mating preferences for specific kinds of males. To understand how preferences diverge, it is necessary to identify the underlying components of these preferences, as well as the ways in which they vary between species. Female *D. simulans* discriminate against *D. melanogaster* males during courtship, in part, due to the influence of the candidate behavioural isolation gene *Katanin-60*. Here I examine the phenotypic basis of discrimination by examining female hybrids made to display either *D. melanogaster*-like or *D. simulans*-like mating preferences. I determined that *D. simulans* females potentially assess *D. melanogaster* male courtship songs unfavourably on the basis of neural processing that occurs in the α and β lobes of the mushroom bodies. I additionally tested these properties in other strains and other species to determine how general these mechanisms are within the genus *Drosophila* and found similar results in two other strains of *D. simulans* as well as in *D. mauritiana.*

4.1 Introduction

Reproductive isolation emerges between many animal species when barriers arise that prevent successful mating with one another (Mayr 1942). Mating incompatibilities often occur when female mating preferences evolve to target type-specific male courtship signals. As a result, females reject mating with heterospecific males for lack of necessary conspecific arousal cues, the presence of aversive cues, or both (Mayr 1963; Coyne and Orr 2004). To address questions of how female mating preferences diverge, it is necessary to understand which sensory modalities and neural structures are involved in the detection and processing of courtship signals. By studying these structures underlying behaviour I can understand how mating preferences

evolve type specificity, how that specificity can vary between closely-related strains and species, and how consequential these variations are to the process of speciation.

Much work in the study of behavioural isolation has been conducted with species of the genus *Drosophila* (reviewed in Nanda and Singh 2012). Though *Drosophila* species females assess visual, chemical, tactile, and auditory signals during courtship, it is most commonly the auditory and chemical features of mating that are assessed for variation between different strains and species (Nanda and Singh 2012). The auditory component of courtship is the pulse and frequency of a wing song performed by males through vibrations of their outstretched wings (Ewing and Bennet-Clark 1967). Females detect song vibrations through feather-like projections, the arista, located on the second segment of the antenna (Boekhoff-Falk and Eberl 2014). Primary mechano-sensory neurons within the Johnston's organ (also on the second antennal segment) acquire signals and then transmit them to the antennal mechano-sensory and motor center of the brain (Kamikouchi et al. 2006; Boekhoff-Falk and Eberl 2014). The neurons found in the antennal mechano-sensory and motor center of the brain are responsible for sex-specific song response (Zhou et al. 2015). The most noteworthy extensions created by these neurons are those made to the pC1 neurons, which surround the peduncles of the mushroom bodies (MB; Zhou et al. 2014). The MB are an important neuroanatomical component of many *Drosophila* behaviours and their role will be discussed in depth below.

The key components of song that females assess are the modes of output (pulse song or sine song), the interval between song bursts (interpulse interval), and other properties of the song itself (*e.g.*, carrier frequency) (Bennet-Clark and Ewing 1969; Kyriacou and Hall 1982; Ritchie et al. 1999). How these properties are evaluated by females varies both intraspecifically and interspecfically. For example, both *D. sechellia* and *D. ananasae* females discriminate against interspecific males on the basis of wing song (Doi et al. 2001; Tomaru 2004). However, interspecific males muted through surgical removal of their wings are able to achieve some level of copulation with normally discriminatory interspecific females. This dynamic indicates that females are able to recognize and discriminate against type-specific cues present in the song of interspecific males. Interestingly, in *D. ananassae*, the only role of wing song appears to be for rejecting heterospecific males, whereas in *D. sechellia*, conspecific wing song also stimulates

female receptivity (Tomaru 2004; Yamada et al. 2002). Intraspecifically, divergent wing songs are a partial source of behavioural isolation between allopatric populations of *D. montana* (Jenning et al. 2011). Therefore at the level of both strain and species, song can signal a mixture of arousal and aversion cues, with females of various lineages attuned to different song properties for different responses.

The olfactory component of *Drosophila* species courtship is mediated, in part, through cuticular hydrocarbons (CHC), which are surface compounds used dually as anti-desiccants and pheromones (Jallon and David 1987). Ligand detection of CHCs is primarily through olfactory sensory neurons (OSN) located in the antennae and the maxillary palps (See review by Keene and Waddell 2007). OSN make connections with the antennal lobe glomeruli where signals are sorted before being relayed to the mushroom bodies (MB) and the lateral horn. Depending on the species, the primary CHC utilized in the pheromone blend can either be sexually monomorphic or dimorphic for the two most abundant CHCs (Jallon and David 1987). Among species of the melanogaster group, *D. melanogaster* and *D. sechellia* are sexually dimorphic for pheromone blend, whereas *D. mauritiana* and *D. simulans* are sexually monomorphic (Jallon and David 1987). Males of monomorphic species have difficulty recognizing females from dimorphic species as viable courtship partners, and this may contribute to their behavioural isolation (Coyne et al. 1994). For example, perfuming experiments conducted with *D. sechellia* and *D. simulans* show that *D. simulans* males are less likely to mate with *D. simulans* females that have been perfumed with *D. sechellia* pheromones. Similarly, CHC-less *D. melanogaster* females were more attractive to *D. simulans* males (normally reluctant to mate with *D. melanogaster* females) until perfumed with the primary *D. melanogaster* pheromone 7,11-heptacosadiene (7,11-HD*;* Billeter et al. 2009). In this last study, it was also determined that CHC are important markers for intrasexual signals, as CHC-less *D. melanogaster* males are courted by other *D. melanogaster* males until perfumed with their primary male pheromone 7-tricosene (7-T). CHC have also been found to be important in marking species identity for females in the behavioural isolation of *D. serrata* against *D. birchii*, and *D. subquinaria* against *D. recens* (Blows and Alan 1998; Dyer et al. 2014). In these experiments, perfuming males with the pheromones of the reciprocal species increases their copulation success with interspecific females that normally reject their mating attempts. In addition to species-specific differences that contribute to behavioural isolation, some

strain-specific pheromone differences contribute to incipient cases of species isolation. For example, *D. melanogaster* females from a Zimbabwe strain prefer mating with males that have less 7-T. In contrast, a cosmopolitan strain displays a positively correlated preference for 7-T (Grillet et al. 2012).

In addition to understanding which cues are being discriminated against, I wanted to identify which neural structures are responsible for executing the evaluations. Several studies implicate the MB as a likely candidate structure for housing circuitry involved in mating receptivity decisions. The MB are a conserved neuroanatomical structure that have been implicated in complex insect behaviours since 1850, when Felix Dujardin demonstrated that the size of the MB positively correlated with behavioural complexity (Dujardin, 1850). Specifically, the MB are now known as a center for learning and memory, as well as a site where direct sensory inputs are integrated with contextual information before being relayed to higher order connections in the brain (Stausfeld et al. 1998; Keene and Waddell 2007). Specific behaviours mediated by the MB in *D. melanogaster* include saliency-based decision making (Zhang et al. 2007), temperature preference (Bang et al. 2011), sexual behaviour (O'Dell et al. 1995; Fleischmann et al. 2001), and associative odour learning (de Belle and Heisenberg 1994). To date the MB have not been associated with pre-mating receptivity, however, there is evidence that the MB are associated with the reductions in female receptivity that occur post-mating (Fleischmann et al. 2001). In these experiments, the detection of a male sex peptide also triggers the MB to de-repress oviposition and egg-laying behaviours. Additionally, chemical ablation of the MB in females also causes elevated rates of oviposition among virgin flies (Fleishmann et al. 2001). The case for MB involvement in female sexual behaviour can also be made from studies on female evaluations of male wing song and pheromones, which demonstrate a necessary role for *dsx*-expressing pC1 and pCd neurons, which surround the peduncles of the MB (Zhou et al. 2014)..

Compositionally, the MB are made up of Kenyon cells, which themselves can be subdivided into three classes of cells that cluster together in lobes (γ ,α, and β) (Lee et al. 1999). One of our candidate genes for female interspecific preference, *Katanin-60* (*Kat60*; see Chapter 3), has been implicated previously in mushroom body (MB) development in *D. melanogaster* (Nicolai et al. 2003). Removal of expression of *Kat60* with homozygous *P*-element insertion ($Kat60^{UY1645}$; also used to generate hemizygotes in the experiments conducted in Chapter 3) was observed to cause

neuron number defects and abnormal α lobe morphology in the MB (Nicolai et al. 2003). For these reasons, I test the hypothesis that the MB might have an inhibitory effect on mating behaviour that can be intensified or relieved on the basis of different mating signals.

Previously, I identified three candidate genes (*fruitless, Katanin-60, Mekk-1*) involved in the behavioural isolation of *D. simulans* females against *D. melanogaster* males (Chapter 3). By using *D. melanogaster* lines bearing gene disruptions for the *D. melanogaster* allele of *Katanin-60 (Kat60)* I was able to produce *D. melanogaster/D. simulans* female hybrids that displayed *D. simulans*-like mating preferences instead of the typical *D. melanogaster*-like receptive behaviour of hybrid females. By these means, it is possible to compare *D. melanogaster* and *D. simulans* behaviour among hybrid females of similar genetic backgrounds, with the key difference being whether the hybrid females inherit the transposable element insertion disrupting *D. melanogaster Kat60* function or not. This model allows us to test whether *Kat60* is involved in female discrimination against auditory or olfactory cues because any other features of *D. simulans* discrimination are still masked by the dominant *D. melanogaster* traits. Only those sensory modalities influenced by the *D. simulans* allele of *Kat60* are unmasked.

Usage of mating cues for aversion or arousal provides clues to the selective processes that have shaped female receptivity, especially when comparisons are made of cue use between intraspecific and interspecific mating contexts. To this end, I determined if wing song was a general discriminatory cue used by the females of other *D. simulans* strains, and of other simulans complex species. I also test whether *Kat60* plays a similar role in the interspecific preferences of other strains of *D. simulans,* as well as their sibling species *D. sechellia* and *D. mauritiana*. If *Kat60* is similarly involved in their behavioural isolation from *D. melanogaster*, then *Kat60* may represent an important gene for the evolution of mating preference within the genus. Fortuitously, the *P*-element insertion line $Kat60^{UY1645}$, used in my previous experiments (Chapter 3), bore a UAS element that can be utilized to drive tissue specific expression of the *D. melanogaster Kat60* allele in hybrid females (which display *D. simulans*-like behaviour) using the GAL4-UAS system. This allowed testing of hypotheses about the MB and whether they play a role in mating receptivity behaviour by using different GAL4 drivers to rescue *D. melanogaster* behaviour. The tissue-specificity of GAL4 expression allowed us to test which

subsets of the MB tissues may be involved. In testing the above hypotheses I found several interesting features of the influence of *Kat60* on behavioural isolation.

4.2 Methods

Genetic Crosses: Six tissue-specific second chromosome GAL4 driver lineswere used to drive expression in overlapping subsets of neurons within the MB region of the brain: *P{GawB}c747*, *P{GawB}Tab2*^{201Y}, *P{GawB}103Y*, *P{GawB}17D*, *P{w[+mW.hs]=GawB}1471*and *P{GawB}Hr39c739*. To make informative interspecies crosses, the GAL4 and UAS components had to be combined over balancer chromosomes within a single line so that I could generate and identify hybrid offspring containing the necessary components to induce expression of *D. melanogaster Kat60*. The final genotype of the *D. melanogaster* lines was: *w***; P{GawB}[Gal4 line]/CyO; P{Mae-UAS.6.11}Kat60*^{UY1645}*/MKRS,Sb*. Females from these *D. melanogaster* GAL4-UAS lines were crossed to *D. simulans* males to generate interspecies hybrids. Female receptivity towards *D. melanogaster* males was assessed with only the *P{Mae-UAS.6.11}Kat60*^{UY1645} insertion (the *D. melanogaster* allele of *Kat60* disrupted; only the *D. simulans* allele of *Kat60* is expressed) and compared to females that have both the insertion and a GAL4 driver that turns on the *D. melanogaster* allele of *Kat60* within the MB (both the *D. melanogaster* and *D. simulans* alleles of *Kat60* are expressed). Hybrid females containing only the GAL4 driver (but not the UAS) were assayed as a control for background genetic effects. Hybrid assays with *Kat60* disrupted using the *P*-element insertion, *P{EPgy2}*^{EY05593} (described in Chapter 3), were crossed according to the schemes laid out in Chapter 3.

Stocks and Rearing Conditions: All *Drosophila* spp.lines were reared in conditions similar to those described in Chapter 3. The only exceptions were the rearing conditions for hybrid crosses involving *D. sechellia*, and two out of the four tests of *D. mauritiana*. In these cases, hybrid crosses were reared at 18^0C instead of 25^0C. All *D. melanogaster* stocks (30813, 51631, 9465, 7362, 4440, 6494, 7345, 15953) were obtained from the Bloomington Drosophila Stock Center with the exception of the wildtype *D. melanogaster* strain (BJS1), which were provided by Dr. B. J. Sinclair. Wildtype *D. simulans* (14021-0251.165) were obtained from Dr. J. A. Coyne. Additional *D. simulans* strains (14021-0251.004, 14021-0251.166, 14021-0251.199, 14021-

0251.216, 14021-0251.288, 14021-0251.310), and *D. sechellia* (14021-0248.25) were obtained from the *Drosophila* species stock center at the University of California. *D. mauritiana* were collected from Rodrigues, Mauritiana by Christopher Austin.

RT-PCR: To assess general differences in *Kat60* gene expression levels between *D. simulans* and *D. melanogaster* I performed RT-PCR (Fig. S2). RNA was extracted from whole 5 day old virgin females using Purelink® RNA mini-kit (Life technologies). cDNA was synthesized from 100ng RNA using One step RT-PCR (Qiagen). Amplification of *Kat60* was performed using an exon-exon spanning primer 5'-CCATAACCTTACTGCGAGGTG-3' and 5'-CCGTGCTAATTTGGCATTCT-3'. The *spt6* housekeeping gene was amplified in multiplex with *Kat60* using primers 5'-GGAGAATCTGGGCGTCAAAGT-3' and 5'-CGCTTTCGTTGTCGTGGAT-3', which are in adjacent exons.

Statistics: Most assays of mating tested the proportion mated using a two-tailed Z-test, followed by FDR ($P \leq 0.05$) correction for multiple tests (Benjamini and Hochberg 1995). The only assays that used a different statistical test were those assessing the contributions of *Kat60* in other stains and species. These tests were performed using a G-test of independence ($p<0.05$).

Modality modifications and mating assays: Virgin hybrid *sim*/*mel*$^{Kat60-}$females (*P*-element insertion *P{EPgy2}*^{EY055938}) aged 3-5 days were anesthetised with CO_2 and had the last two antennal segments and aristae surgically removed (herein referred to as, ant-) using microdissection needles. Control females remained unaltered, with intact antennae and aristae (herein referred to as, ant+). Mating assays were performed approximately 48 hours after surgical removal of the anntenna/aristae. In order to assess the effects of wing song within species, wingless intraspecific males were compared to winged intraspecific males. An identical approach was taken to determine the effects of interspecific wing song between species. Wing removal (referred to as, wing-) were performed similarly to the described protocol above for aristae, with the exception that males wings were removed 24 hours prior to the mating assay. Wings were removed near the base and did not including any musculature. During the perfuming assays, I used a masking paradigm to confer species pheromonal identity on test males. If females use interspecific CHC as a basis for discrimination, then a drop in mating activity for

conspecific males perfumed with interspecific CHC would be measured. If however, females were using conspecific CHC as an arousal signal, I would instead measure an increase in mating activity among interspecific males that are normally discriminated against. Thus males were perfumed as such: *D. melanogaster* with *D. melanogaster* CHCs; *D. melanogaster* with *D. simulans* CHCs; *D. simulans* with *D. simulans* CHCs; *D. simulans* with *D. melanogaster* CHCs. CHC perfuming was conducted by crowding a single male in an 8mL vial with 15 other 'perfuming' males. Perfuming males were differentiated from the single test male by surgically removing their wings as described above. All test males were perfumed for 48 hours prior to placement within the mating assay. All modality assays involving *sim/mel* hybrids used *D. simulans*-165 (Strain #14021-0251.165)

4.3 Results

4.3.1 The role of *Katanin-60* in behavioural isolation of *Drosophila* spp.

D. melanogaster/D. simulans hybrid females accept mating from *D. melanogaster* males (Robertson 1988). Any *D. simulans* rejection behaviour in these hybrids is masked by the dominant *D. melanogaster* mating preferences. However, *D. melanogaster/D. simulans* hybrids bearing a *P*-element insertion (*P{Mae-UAS.6.11}Kat60*^{UY1645}) in the *D. melanogaster* allele of *Kat60* display *D. simulans*-like rejection behaviour (Chapter 3; Table 1). The strain of *D. simulans* used in these experiments (14021-0251.165) was originally collected from Florida City, FL, USA. I tested other strains of *D. simulans* from the Southeastern USA, and found similar results (Table 4.1). Female hybrids produced from *D. melanogaster* crossed with either *D. simulans* 288 (Athens, GA, USA) or *D. simulans* 166 (Islamorada, FL, USA) showed the same *D. simulans*-like mating rejection behaviour when the *D. melanogaster Kat60* allele was disrupted (N=55; G = 9.004, p=0.003 and N=29; p=0.013, G=6.23, Table 4.1). The other *D. simulans* strains, 004 (Australia) 199 (Nanyuki, Kenya), 216 (Winters, CA, USA), 310 (Nairobi, Kenya) continued to show the *D. melanogaster*-like acceptance of mating, despite the presence of a disruption to the *D. melanogaster* allele of *Kat60* (Table 4.1).

Beyond testing the above *D. simulans* strains, I tested other simulans complex species with the same methodology. Since the degree of postzygotic isolation between *D. melanogaster* and other

species differs, it was neccessary to rear crosses between *D. sechellia* and *D. melanogaster* at a lower temperature (18^0C) to obtain any viable offspring. In these assays, no discriminatory behaviour was displayed by hybrids, indicating that *D. sechellia* interspecific preference is not influenced by *Kat60* (Table 4.1). Given the difficulty of crossing *D. melanogaster* and *D. sechellia* at the normal rearing temperature (25^0C), it was assumed that similar precautions would be needed for crossing *D. mauritiana* and *D. melanogaster*. However after the first replicate of tests it was determined that such measures were unnecessary. Upon moving crosses to standard temperature incubation (25^0C), a different set of behavioural results were obtained that did not reflect those observed with hybrid females reared in the lower incubation condition (18^0C). To confirm the effects of rearing temperature on behavioural development, two more rounds of crosses were set up concurrently, with each reared at either the lower (L) or higer (H) temperatures. In both replicates using lower temperature conditions, hybrid females showed *D. mauritiana*-like rejection behaviour (L_1: N=36; G = 5.533, P = 0.02; L_2: N=35; G = 5.182, P = 0.02). Hybrid females in both of the higher temperature replicates showed only the usual *D. melanogaster*-like acceptance behaviour (Table 4.1).

4.3.2 Interspecific preference traits and *Katanin-60*'s influence

To understand how behavioural divergence has led to interspecific mating discrimination, it is necessary to determine how, and which sensory modalities evolved incompatible differences. *D. simulans* females discriminate against the multimodal courtship of *D. melanogaster* males, and removal of any one component of this male courtship does not rescue interspecies female rejection. *D. melanogaster* mating preferences are dominant in *D. melanogaster*/*D. simulans* hybrids. However, I was able to produce *D. melanogaster*/*D. simulans* hybrids bearing a disrupted *D. melanogaster* allele of *Kat60*, $P\{EPgy2\}^{EY05593}$ (*Kat60*-), which unmasks *D. simulans*-like interspecific mating preference (Table 4.1). This allows tests for *D. simulans* sensory modalities and evaluations that are influenced by *Kat60*, as any other redundant discriminatory processes are still masked dominantly by the *D. melanogaster* genetic background. To test which organs might receive sensory inputs important for interspecific discrimination decisions, the last two antennal segments and the arista were removed from *D. simulans*-165/*D. melanogaster* hybrid females (*sim/mel*). Removal did not reduce *sim/mel* receptivity towards males, nor did it relieve discrimination against *D. melanogaster* males from

sim/mel^{K60-} females (Table 4.2). Next I tested wing song generally by removing male wings, and thus their ability to produce courtship song (Tomaru et al. 2000). If song was being used to stimulate arousal, a drop in female mating receptivity towards w- males would be measured. If females were using wing song to discriminate against males, then w- males would bypass female discrimination and achieve mating. In both cases, if there is an element of type-specificity for these female preferences, the results for female mating receptivity with intraspecific males and interspecific males would differ. Removing male wings did not reduce *D. simulans*-165 or *sim/mel*$^{K60-}$ female receptivity towards *D. simulans*-165 males (Figure 4.1; Table 4.2). However, the absence of *D. melanogaster* song increased the frequency of interspecific copulation for both pure species *D. simulans* females (N=30; p=0.0048, Z= -2.8) and *sim/mel*$^{K60-}$ females (N=29; p=0.0015, Z=-3.17) (Figure 4.1; Table 4.2). Conversely, the absence of *D. melanogaster* song decreased the receptivity of *sim/mel* (N=29; p=0.0039, Z=-2.89), as has been previously reported in *D. melanogaster* females (Figure 4.1; Table 4.2; Tomaru and Oguma 2004). Thus, females bearing only the *D. simulans* allele of *Kat60* have decreased mating due to the presence of *D. melanogaster* male song, while females bearing at least one dominant allele of *D. melanogaster Kat60* have increased mating due to the presence of *D. melanogaster* song.

I also studied female use of wing song in intraspecific and interspecfic contexts among different strains and species of *Drosophila*. Females from D. *sechellia*, *D. mauritiana,* and several strains of *D. simulans* were presented with either a male of their own species, or another (*D. melanogaster*) , both with (wing+) and without (wing-) wings. These results show that male wing song is used as a conspecific arousal cue for *D. sechellia*, *D. mauritiana*, and the 288 strain of *D. simulans* (Table 4.2; Figure 4.2). There were no differences in female mating activity for the interspecific context (mating with wing+ or wing- *D. melanogaster*) that were significant for these species and strains (Table 4.2). Male wing song was used as a discriminatory cue by females from *D. simulans*-165 and *D. simulans*-166 (Figure 4.2; Table 4.2). The *D. simulans* strains 216, 310, and 004 had no differences in mating with wing+ or wing- males in either context (Figure 4.2; Table 4.2).

To test olfactory cues, I perfumed males with either their own species cuticular hydrocarbons (CHC) or CHCs of a different species (Coyne et al. 1994). Females using interspecific CHC as a

basis for discrimination, then a drop in mating activity for conspecific males perfumed with interspecific CHC would be measured. If however, females were using conspecific CHC as an arousal signal, I would instead measure an increase in mating activity among interspecific males that are normally discriminated against. In my experiments, only one interspecific arousal reaction was induced in females (Table 4.3). *D. simulans* males achieved more copulations with *sim/mel*$^{K60-}$ females when they were perfumed with the *D. melanogaster* CHC males instead of *D. simulans* CHC (N=20; p=0.01).

I hypothesized that the MBs are *Drosophila* neural structures that are likely involved in mediating mating decisions. I used *D. melanogaster* that have an UAS-bearing (*P{Mae-UAS.6.11}Kat60*^{UY1645}) transposable element insertion upstream of the promoter of *Kat60,* the presence of which disrupts *D. melanogaster* preference behaviour (Chapter 3; Table 4.1). When a GAL4-producing construct is crossed into the same genetic background as the UAS element, GAL4 binds to the UAS and activates the expression of the downstream gene; in this case, *Kat60*. The timing and location of the expression of GAL4 determines the timing and location of expression of the gene with the inserted UAS region (Brand and Perrimon 1993). Since many different tissue-specific GAL4 drivers exist for different tissues of the *Drosophila* brain, I was able to compare hybrids bearing these different drivers to narrow down which specific regions of the MB may be involved in female mating receptivity towards interspecific males.

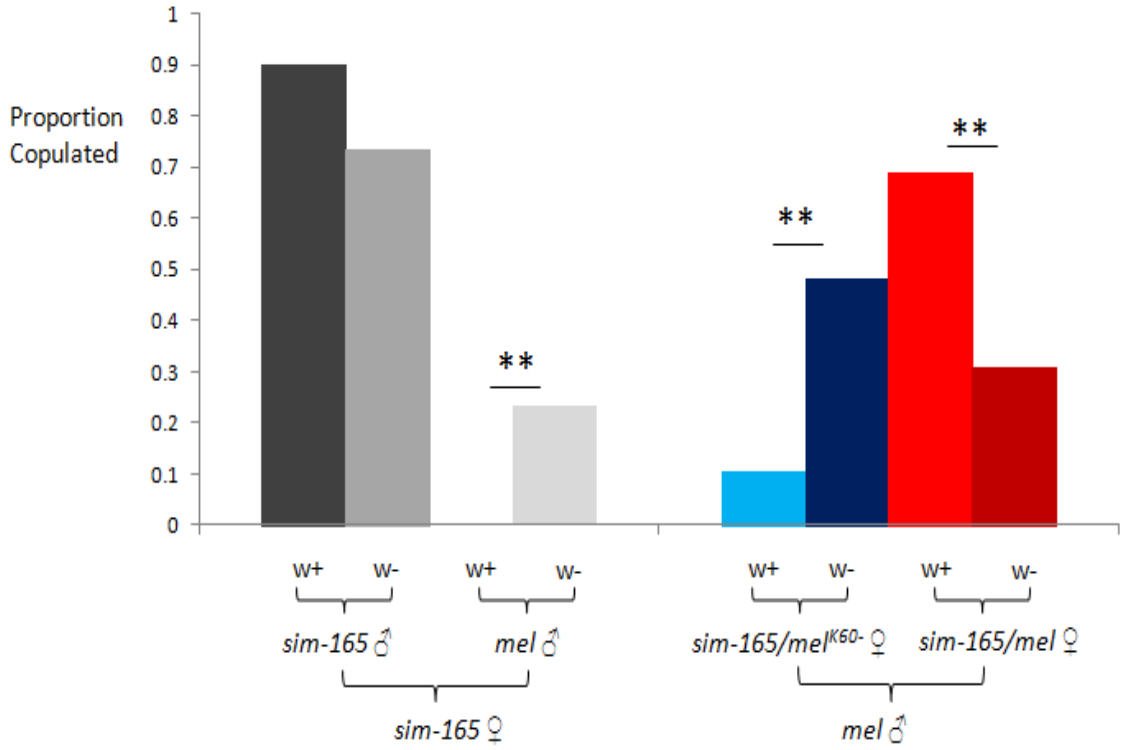
I tested six GAL4 drivers to induce expression at varying strengths for different subsets of neurons within the MB (α, α', β, β', γ). For three out of six of these different GAL4 lines (4440, 7362, 51631) *D. melanogaster-like* behaviour was restored in UAS-bearing hybrids (Figure 4.3). The Gal4/UAS hybrids of these lines achieved more copulations than hybrids bearing only the UAS element, and did not have statistically lower copulation levels than control hybrids that only bore the GAL4 driver or no Gal4/UAS elements at all (Table 4.4). In one line (9465) the difference between Gal4/UAS and UAS copulations was almost of statistical significance (N=24; p=0.08, Z=-1.76). This line may be another rescue as there is no statistical difference in copulations between the Gal4/UAS hybrids and the two control group females (Gal4-only hybrids and normal hybrids), which indicates that they are mating at the same level that *D. melanogaster* females would mate at with *D. melanogaster* males (Table 4.4). Comparison of the expression locations among these lines (both within and outside of the MB) reveals that there is

not a single neural substrate for *Kat60*'s effect on female rejection behaviour. By subtracting the GAL4 driven regions of the three lines where *D. melanogaster*-like mating receptivity was restored from the regions of expression in the three lines where it was not restored, I was able to narrow the circuitry and developmental timing involved (Table S1). From this analysis, I conclude that the development of *D. melanogaster*-like mating receptivity occurs in the α and β lobes of the MB during larval development, with an emphasis for the third instar stage (Table S1).

Table 4.1: Role of *Kat60* in interspecific preference of different *D. simulans* strains and species of *Drosophila*. *D. melanogaster* (*Mut*) contain the *P*-element insertion *P{EPgy2}*^{EY05593}. Mating behaviour of hybrid females bearing either a *Mut* or balancer chromosome (Bal) were generated from *D. melanogaster (mel)* males crossed to the females from the listed strains and species[1]. Intraspecific crosses were made to control for the effects of possessing a *Bal* or *Mut.* Numbers are given for the number of courtships (Crt) and copulations (Cop) that occurred during behaviour assays. Hybrid females were additionally subjected to a sperm assay. Statistical significance was calculated for the comparisons of total copulations during the behaviour assay[2], and then again for the sum of the behaviour and sperm assays[3]. The number of intraspecific copulations that occurred were calculated by taking the proportion of copulations of intraspecific females that were courted during behaviour assays ((Cop/Crt)*N). *D. simulans-165* was tested previously and has been reprinted here for ease of reference (Chapter 3; Table 1)[4].

			Behaviour assay[2]									+ Sperm assay[3]			
			sim/*Mut*		sim/*Bal*		mel/Mut		mel/Bal			sim/Mut		sim/Bal	
Strains/Species[1]	**Discovery Location**	**N**	**Crt**	**Cop**	**Crt**	**Cop**	**Crt**	**Cop**	**Crt**	**Cop**	**G test**	**Cop**		**Cop**	**G test**
D. sechellia	Cousin, Seychelles	14	6	4	4	4	10	9	9	7	$G = 0.031, P = 0.86$	7		9	$G = 0.371, P = 0.54$
D. simulans-165[4]	Florida City, FL, USA	60	39	1	34	8	46	27	46	27	$G = 5.58, P = 0.018$	7		26	$G = 5.962, P = 0.015$
D. simulans-166	Islamorada, FL, USA	55	21	1	27	8	38	35	35	28	$G = 6.671, P = 0.01$	10		29	$G = 9.004, P = 0.003$
D. simulans-199	Nanyuki, Kenya	20	8	0	7	0	12	12	15	15	$G = 0.000, P = 1.00$	6		7	$G = 0.058, P = 0.81$
D. simulans-216	Winters, CA, USA	22	7	0	9	0	15	22	11	22	$G = 0.000, P = 1.00$	6		8	$G = 0.804, P = 0.37$
D. simulans-288	Athens, GA, USA	29	12	0	10	0	20	13	18	10	$G = 0.000, P = 1.00$	0		5	$G = 6.23, P = 0.013$
D. simulans-004	Australia	30	8	5	11	8	13	10	16	11	$G = 0.824, P = 0.36$	13		20	$G = 1.352, P = 0.25$
D. simulans-310	Nairobi, Kenya	33	20	12	16	10	22	17	17	8	$G = 0.286, P = 0.593$	20		19	$G = 0.745, P = 0.39$
D. mauritiana (H1)	Rodrigues, Mauritius	30	13	4	15	5	18	15	17	13	$G = 0.182, P = 0.67$	12		18	$G = 1.105, P = 0.29$
D. mauritiana (H2)	Rodrigues, Mauritius	30	10	2	13	4	19	15	25	22	$G = 0.772, P = 0.38$	13		17	$G = 0.581, P = 0.45$
D. mauritiana (L1)	Rodrigues, Mauritius	36	10	0	15	5	21	17	24	22	$G = 5.993, P = 0.014$	6		22	$G = 5.533, P = 0.02$
D. mauritiana (L2)	Rodrigues, Mauritius	35	13	3	11	6	34	28	28	25	$G = 3.471, P = 0.062$	8		25	$G = 5.182, P = 0.02$

Figure 4.1: Comparison of female *D. simulans*-165 mating to hybrid females displaying *D.*



***simulans*-like or *D. melanogaster*-like response to song.** *D. simulans-165* (*sim-165*) and *D. melanogaster* (*mel*) males with wings (w+) or no wings (w-) were paired with females. *D. melanogaster* mating preferences are dominant in hybrids (*sim/mel*) unless the *D. melanogaster* allele for *Kat60* is disrupted (*sim/mel*$^{K60-}$). *D. simulans-165* and *sim/mel*$^{K60-}$ females reject mating with w+ *mel* males, but accept mating from w- *mel* males. *sim/mel* display the typical *D. melanogaster*-like reduction in mating behaviour when no song is presented from w- *mel* males (Tamura and Oguma 2004). Statistical significance from Z-test comparions shown: ** = $P \leq 0.005$ (Table 4.2).

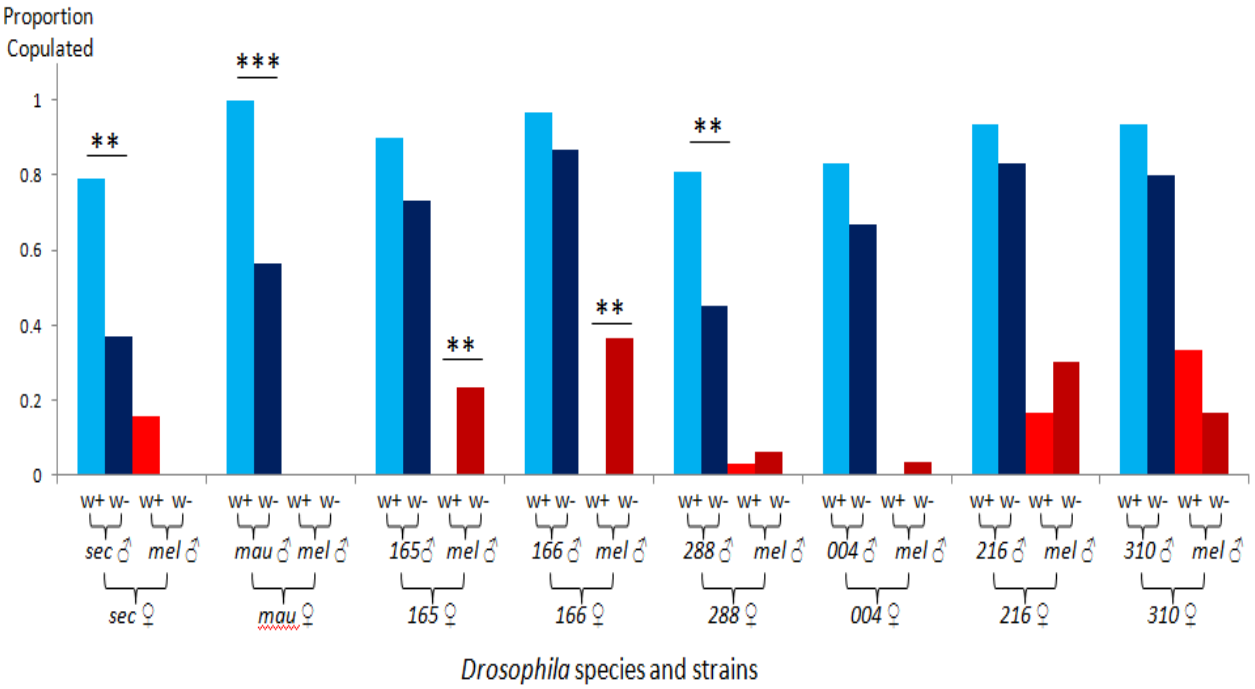


Figure 4.2: Response of simulans species complex females to male courtship song in intraspecific and interspecific contexts. To determine the relevance of song males with wings (w+) or no wings (w-) were paired with females. Females were tested in both an intraspecific context (blues) and an interspecific context (reds). Significance was determined through a two-tailed Z test (P<0.05) with FDR for false positives (Table 4.2). Significant differences in mating are marked as *: P<0.05; **: P<0.005, ***: P<0.0005. Simulans complex species *D. sechellia* (*sec*), *D. mauritiana* (*mau)*, and *D. simulans* strains 165, 166, *288*, *004*, *216*, and *310* were used.

Table 4.2: Effects of sensory modalities in the mating preferences of different strains and species of *Drosophila*. Mating proportions of females that had their antennae intact (ant+) or removed (ant-), or when males had their wings intact (wing+) or removed (wing-). Hybrid females that are *sim/melKat60-* have the *D. melanogaster* allele of *Kat60* disrupted with *P{EPgy2}Kat60*^{EY05593}. Significance was determined through a two-tailed Z test (p<0.05).

Female	Male	Treatment	N	# Copulations	*P*-value (Z-statistic)
sim/mel^{Kat60-}	*sim(165)*	ant +	37	36	0.69 (0.4)
sim/mel^{Kat60-}	*sim(165)*	ant -	46	44	
sim/mel^{Kat60-}	*mel*	ant +	42	7	0.64 (0.5)
sim/mel^{Kat60-}	*mel*	ant -	50	6	
sim-165	*sim(165)*	wing +	30	27	0.094 (-1.67)
sim-165	*sim(165)*	wing -	30	22	
sim-165	*mel*	wing +	30	0	0.0048 (-2.8)
sim-165	*mel*	wing -	30	7	
sim/mel^{Kat60-}	*mel*	wing +	29	3	0.0015 (-3.17)
sim/mel^{Kat60-}	*mel*	wing -	29	14	
sim/mel	*mel*	wing +	29	20	0.0039 (-2.89)
sim/mel	*mel*	wing -	29	9	
sec	*sec*	wing +	19	15	0.0085 (2.63)
sec	*sec*	wing -	19	7	
sec	*mel*	wing +	19	3	0.072 (1.80)
sec	*mel*	wing -	19	0	
mau	*mau*	wing +	23	23	0.0004 (3.57)
mau	*mau*	wing -	23	13	
mau	*mel*	wing +	23	0	-
mau	*mel*	wing -	23	0	
sim-004	*sim(004)*	wing +	30	25	0.14 (-1.49)
sim-004	*sim(004)*	wing -	30	20	
sim-004	*mel*	wing +	30	0	0.31 (-1.01)
sim-004	*mel*	wing -	30	1	
sim-216	*sim(216)*	wing +	30	28	0.23 (1.21)
sim-216	*sim(216)*	wing -	30	25	
sim-216	*mel*	wing +	30	5	0.22 (-1.22)
sim-216	*mel*	wing -	30	9	
sim-310	*sim(310)*	wing +	30	28	0.13 (1.52)
sim-310	*sim(310)*	wing -	30	24	
sim-310	*mel*	wing +	30	10	0.14 (1.49)
sim-310	*mel*	wing -	30	5	
sim-288	*sim(288)*	wing +	31	25	0.0039 (2.89)
sim-288	*sim(288)*	wing -	31	14	
sim-288	*mel*	wing +	31	1	0.56 (-0.59)
sim-288	*mel*	wing -	31	2	
sim-166	*sim(166)*	wing +	30	29	0.16 (1.40)
sim-166	*sim(166)*	wing -	30	26	
sim-166	*mel*	wing +	30	0	0.0002 (-3.67)
sim-166	*mel*	wing -	30	11	

Table 4.3: Response of females to interspecific CHC. *D. simulans-165/D. melanogaster h*ybrid (*sim*/mel) females were paired with either *D. simulans-165* (*sim-165)* or *D. melanogaster* (*mel*) males. Males were perfumed with either (*mel*) or (*sim-165)* CHC. Hybrids did not copulate differently with *D. melanogaster* males perfumed in either conspecific or interspecific pheromones. **S**ignificance was determined through a two-tailed Z test ($p<0.05$).

Female	Male	Treatment	N	# Copulations	*P*-value (Z-statistic)
sim/mel^{K60-}	*mel*	*(mel)*	20	1	
sim/mel^{K60-}	*mel*	*(sim-165)*	20	4	0.15 (1.43)
sim/mel	*mel*	*(mel)*	20	14	
sim/mel	*mel*	*(sim-165)*	20	17	0.25 (1.14)
sim/mel^{K60-}	*sim-165*	*(mel)*	20	12	
sim/mel^{K60-}	*sim-165*	*(sim-165)*	20	4	0.01 (2.58)
sim/mel	*sim-165*	*(mel)*	20	9	
sim/mel	*sim-165*	*(sim-165)*	20	6	0.33 (0.98)
sim-165	*mel*	*(mel)*	20	0	
sim-165	*mel*	*(sim-165)*	20	0	-
sim-165	*sim-165*	*(mel)*	21	16	
sim-165	*sim-165*	*(sim-165)*	21	16	-

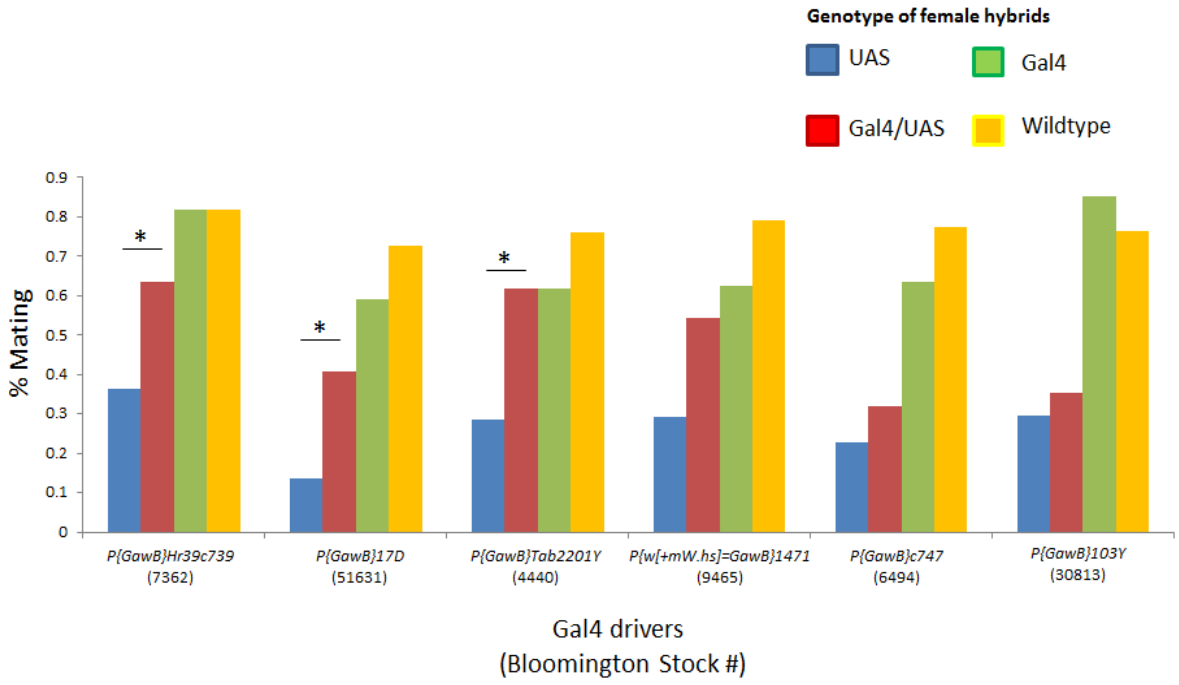


Figure 4.3: Effects on mating behaviour of tissue-specific MB expression for the *D. melanogaster* allele of *Kat60* in hybrid females. Mating rate of hybrid females bearing different GAL4 drivers for expression of *D. melanogasterKat60* in various neural tissues. Three different GAL4/UAS restored *D. melanogaster*–like mating levels ($p<0.05$). FDR correction for multiple tests ruled all three significant lines in as true positives.

Table 4.4. Hybrid female copulation with tissue-specific expression of the *D. melanogaster* allele of *Kat60*. Hybrids bearing transposable element (*P{Mae-UAS.6.11}Kat60*^{UY1645}) were previously shown to display *D. simulans*-like mating behaviour (Chapter 3; Table 3.1). GAL4 drivers that selectively drive expression of UAS bearing genes in specific neural tissues were used to test whether *D. melanogaster*-like mating receptivity was restored. Behaviour of GAL4/UAS hybrid females was compared to UAS-only hybrid females which display the *D.* simulans-like rejection behaviour. Hybrids bearing only GAL4 or neither GAL4 or UAS (WT) were used to control for the presence of the GAL4 element. Significance was determined through a two-tailed Z-test ($p<0.05$).

Bloomington	TE insertion	Copulations					Z-test			
Stock #		n	UAS	GAL4/UAS	GAL4	WT		UAS	GAL4	WT
7362	P{GawB}Hr39c739	54	18	31	42	39	GAL4/UAS	0.01 (-2.53)	0.02 (-2.31)	0.10 (-1.64)
							WT	<0.01 (-4.09)	0.50 (-0.69)	
							GAL4	<0.01 (-4.70)		
30813	P{GawB}103Y	34	10	12	29	26	GAL4/UAS	0.60 (-0.52)	<0.01 (-4.21)	<0.01 (3.42)
							WT	<0.01 (-3.89)	0.35 (-0.93)	
							GAL4	<0.01 (-4.66)		
51631	P{GawB}17D	22	3	9	13	16	GAL4/UAS	0.04 (-2.031)	0.23 (-1.21)	0.03 (-2.13)
							WT	<0.01 (-4.00)	0.34 (-0.95)	
							GAL4	<0.01 (-3.13)		
9465	P{w[+mW.hs]=GawB}1471	24	7	13	15	19	GAL4/UAS	0.08 (-1.76)	0.56 (-0.59)	0.07 (-1.84)
							WT	0.02 (-2.32)	0.20 (-1.27)	
							GAL4	<0.01 (-3.48)		
4440	P{GawB}Tab2201Y	21	6	13	13	16	GAL4/UAS	0.03 (-2.17)	1 (0)	0.32 (-1.00)
							WT	<0.01 (-3.09)	0.32 (-1.00)	
							GAL4	0.03 (-2.17)		
6494	P{GawB}c747	22	5	7	14	17	GAL4/UAS	0.50 (-0.68)	<0.01 (3.03)	0.03 (2.11)
							WT	<0.01 (-2.74)	0.32 (0.99)	
							GAL4	0.01 (-3.62)		

4.4 Discussion

4.4.1 *Kat60* in behavioural isolation of *Drosophila* species

I tested the phenotypic and evolutionary significance of the candidate behavioural isolation gene *Kat60* in various strains and species of *Drosophila*. Though *D. melanogaster* mating preferences are dominant over *D. simulans* mating preferences in female hybrids, I was able to induce *D. simulans*-like discriminatory behaviour in hybrids using lines bearing various *P*-element insertions in the *D. melanogaster* allele of *Kat60* (Chapter 3; Table 3.1). I was able to replicate this effect in two other *D. simulans* strains (166, 288) collected from a similar geographic region (Table 4.1). However, four other strains of *D. simulans* (004 199, 216, 310) did not show discrimination against *D. melanogaster* when the *D. melanogaster Kat60* allele was similarly disrupted. Finding only three out of seven strains with the same genetic basis for interspecific preference is not surprising given similar findings from mapping studies of other *Drosophila* species pairs. For example, two loci influencing *D. pseudobscura* discrimination against *D. persimilis* were found in a QTL mapping study. The same experimental design applied to six other strains of *D. pseudoobscura* did not find any genetic correlation between the previously identified regions and interspecific preference of *D. pseduoobscura* females (Barnwell and Noor 2008). Thus, I replicate here the finding that any pattern of isolation identified in a specific strain may not represent a general pattern of isolation among other strains for that species. Moreover, when loci underlying behavioural isolation identified against one species are identified, it is unlikely that the same genetic basis will be found to underlie behavioural isolation against another species. For example, *D. mauritiana* loci involved in discrimination against *D. simulans* (Moehring et al. 2004) did not match loci involved their discrimination against *D.sechellia* (McNabney 2012) or *D. melanogaster* (Chapter 3; Table 4.1). This makes sense as the males of different species likely possess different signal cues, and thus trigger rejection behaviours on the basis of different transgressions.

Did *Kat60*-mediated discrimination evolve during an era that could have contributed to species isolation? Among the *D. simulans* strains tested, I only found *Kat60*-mediated isolation in the behavioural isolation of strains from the South-Eastern USA. The simplest explanation is that the

preference evolved after these strains were relocated from their ancestral location in central Africa. However, there is evidence that this form of discrimination is ancestral (and therefore lost by the other global strains of *D. simulans* tested here) because of the results obtained from *D. mauritiana*. *D. mauritiana*-like discrimination was unmasked in hybrids where the *D. melanogaster* allele of *Kat60* was disrupted. However this was only the case when hybrids were reared at a lower temperature. If the former hypothesis is true (*Kat60* discrimination against *D. melanogaster* is recent), then it is possible that *Kat60* is generally important to the evolution of mating behaviour in *Drosophila* species. If the latter hypothesis is true (*Kat60* discrimination evolved pre-speciation of *D.mauritiana/D. simulans*, post-speciation of *D. simulans*/*D. melanogaster*) then *Kat60* may represent the kind of gene that would have aided early reproductive isolation between the two species. Regardless of which hypothesis is correct, *Kat60* appears to be an important gene for explaining interspecific differences in behaviour between *Drosophila* species.

4.4.2 Sensory modalities usage and mediation by *Kat60*

In addition to establishing whether *Kat60* was involved in the mating preferences of these various strains and species, I also sought to determine which sensory modality conveys the type-specific information upon which these decisions are made. The ability to produce hybrids that display either *D. melanogaster*-like or *D. simulans*-like mating preferences (because of *P{EPgy2}Kat60*^{EY05593}), allowed us to assess only those component preferences influenced by *Kat60*. Any additional modalities *D. simulans* may use to discriminate against *D. melanogaster* are still masked by the dominant *D. melanogaster* background. Specifically, I tested whether *Kat60* encodes for discrimination against either auditory or chemical cues from *D. melanogaster* males, since both of these modalities have been shown to influence mating receptivity in *D. simulans* females (Coyne et al. 1994; Ritchie et al. 1999). The first surgical manipulations tested (Performed by Tara Edwards) removed the antennae and aristae, which are the primary systems for detecting auditory and volatile chemical signals (Burnet et al 1971; Cline et al. 1997). Removal of these organs did not cause a reduction in female receptivity towards conspecific males (Table 4.1). It also did not increase receptivity in females paired with heterospecific males, demonstrating that *D. simulans* discrimination is not based on the detection of an aberrant heterospecific signal via these organs (Table 4.1). The removal of aristae has been found to not

have an effect on mating receptivity before in both *D. subquinaria* and *D. recens* (Dyer et al. 2014). Thus, *Kat60* does not appear to influence discrimination through these particular sensory organs, which means future searches must be expanded to other chordotonal organs located on the legs, thorax, and abdomen in future experiments.

To specifically test the role of song I compared copulation success among normal males, and wingless males. I hypothesized that if females recognized aversive cues in interspecific males then w- males would achieve more copulations than w+ males, and conspecific males would similarly achieve copulation regardless of being w+ or w-. I also hypothesized that if conspecific males possessed necessary arousal cues, then w+ conspecific males would achieve more copulations than w- conspecific males, and interspecific males would be similarly discriminated against regardless of being w+ or w-. Song was important for discrimination against interspecific males in *D. simulans*-165 and *D. simulans*-166. Interestingly, *D. simulans*-165 and *D. simulans*-166 are from similar geographic regions (Florida City, FL and Islamorada, FL), so it is likely that each is derived from a similar ancestral strain (Figure 4.1). When I tested *Kat60*$^{-}$ hybrids (display *D. simulans*-like preference), I found the same result: hybrids did not discriminate against w- *D. melanogaster* males (Figure 4.1). Conversely, among normal hybrids (display *D. melanogaster*-like preference), copulation decreased with w- males, which is the expected result for *D. melanogaster* females (Tomaru and Oguma 2004). Thus the behavioural effects of *Kat60* alleles on hybrid behaviour is not likely due to mis-expression of the gene, as I was able to generate behaviours in hybrids that matched either parent's preference behaviours. Conspecific song was also important for stimulating copulation with *D. mauritiana, D. sechellia,* and *D. simulans*-288 females (Table 4.1; Figure 4.2). The *D. sechellia* results contradict previous studies (Tamura and Oguma. 2004) which showed that *D. sechellia* use song for both intraspecific arousal and interspecific aversion. However, Tomaru and Oguma (2004) used different strains from the ones tested here, which may account for the differences in interspecific responses, a phenomenon seen here with these *D. simulans* strains as well (Table 4.1). For the species that did not discriminate against *D. melanogaster* on the basis of song it should be noted that they may still discriminate against other strains and species on the basis of auditory cues, just not *D. melanogaster* auditory cues.

To assess the role of *Kat60* in pheromone discrimination, I perfumed test males with CHC from either their own species or a different one. Female hybrids with the TE insertion (displaying *D. simulans*-like behaviour) did not accept more *D. melanogaster* males perfumed with *D. simulans* CHC. This result indicates that *Kat60* is not involved in discrimination against *D. melanogaster* CHC. The only result with a statistically significant difference between pheromone treatments was for increased copulation between *Kat60*$^{-}$ hybrid females and *D. simulans* males perfumed with *D. melanogaster* male CHC (N=20; p=0.01; Table 4.3). I interpret this result to mean that even though *D. simulans* discrimination is unmasked in these hybrids, there are other arousal systems of *D. melanogaster* that are still being expressed, and one of these systems is governed by *D. melanogaster* male CHC. I believe this to be concordant with other results, because *Kat60* hybrids still mate with *D. melanogaster* at a low level (as opposed to *D. simulans* which do not mate at all; Chapter 3). This trend was also present in normal (control) hybrids for the same treatment, but I did not achieve statistical significance for their difference in copulation success (Table 4.4). Even though there was no statistically significant difference between treatments (*D. melanogaster* perfume vs. *D. simulans* perfume) for control hybrids, there was also no statistically significant difference between hybrid classes (hybrid vs. *Kat60*$^{-}$ hybrid) for either perfuming treatment of *D. melanogaster* perfume (N= 20; p=0.34, Z=-0.95) or *D. simulans* perfume (N=20; p=0.47, Z=-0.73). Future experiments with larger samples may be able clarify the ambiguity in these results.

4.4.3 Expression of *Kat60* in the MB

Alternative mating behaviours can exist as a result of variation in gene expression. Various alleles of the circadian rhythm gene *period* influences female sexual behaviour (Sehgal et al. 1994). Different expression patterns of *fruitless* influences male sexual behaviour in *Drosophila* (Goodwin et al. 2000). Because the severing activity of *Kat60* is concentration-dependent, it is likely that differential expression of the gene during development explains species differences as opposed to protein differences (Zhang et al. 2007B). However, since the effect on behaviour is potentially due to a very specific core set of neurons, it is not surprising that neither I nor others could report any species-specific differences in expression of *Kat60* (Figure 4.3; Graze et al. 2009). Expression differences in these neurons, if present, would only contribute a small amount of transcripts to the overall *Kat60* transcript pool and may only be present transiently during

specific stages of development. A significant difference in expression levels of *Kat60* was not detected between the two species (Figure S1; Wang et al. 2007), and there is no mis-expression or dominance of expression of *Kat60* in hybrid females (Wang et al. 2007).

To confirm the role of *Kat60* in behavioural isolation, I induced expression of the *D. melanogaster* allele of *Kat60* using the GAL4-UAS system. This was possible because the *P{Mae-UAS.6.11}Kat60*^{UY1645} insertion used to disrupt *D. melanogaster Kat60* also contained a UAS element which allowed us to drive tissue specific expression of *Kat60*. Three different GAL4 lines restored *D. melanogaster* like mating behaviour in *Kat60*$^{-}$ hybrids. Comparisons of their expression patterns revealed that they were all expressed in the α and β lobes of the MB during larval development, with an emphasis for the third instar stage (Supplementary Table 4.1). At this stage, *D. melanogaster* larva are ~120-124 hours old, and begin undergoing developmental changes leading into pupation (Doane 1967). During pupation, the MBs undergo extensive remodelling, including: pruning and regrowth of neuronal processes, programmed cell death (PCD), and trans-differentiation (Veverysta and Allan 2013). Given the reliance of these cellular processes on microtubule dynamics, *Kat60* could play a role in this stage of development of the MB. *Kat60*`s action could occur through several different activities including: disassembly of MT during the pruning of larval neurite networks, generation of short microtubule fragments to seed the rearborization of Kenyon cells, facilitating PCD, or some mixture of each (Moore 2008; Roll-Mecak and McNally 2010). Further study of these regions at various stages of development could be done using the temperature sensitive *shibire* system to negatively select these regions of interest. Such an approach could shed further insight into the different circuitry used by these two species in preference behaviour.

4.5 Conclusion

Females of *D. simulans* strains of the Southeastern USA bear a *Kat60* allele that influences their mating preferences against *D. melanogaster* males. The discrimination is against interspecific signals present in *D. melanogaster* wing song, and requires neural processing in the α and β lobes of the MB. This behaviour is recessive in hybrids of the two species, which instead display *D. melanogaster* reliance on wing song for arousal cues. The differential usage of wing song between the two species may explain the mating asymmetry between the two species (*D.*

simulans females reject *D. melanogaster* males but not the other way around). The finding that *D. mauritania* had a temperature-dependent requirement for *Kat60*-mediated discrimination indicates that variant behavioural phenotypes may only be realized under certain environmental influences during development. Though it is unlikely that this discrimination behaviour was present in *D. simulans* ancestors, the fact that it is a type-specific aversion suggests that there may have been an episode of sexual selection, in recent evolutionary history, against unfavourable male mating partners.

4.6 Literature cited

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Chapter 5

5 General Discussion

5.1 Thesis Summary

The loci underpinning behavioural isolation between species do not appear to be those underpinning genetic variation within species. In song QTL among *Drosophila* spp., the within species variation in wing song for *D. melanogaster* matches none of the between species genetic differences found between *D. simulans* and *D. sechellia* (Gleason 2004). There are no known alleles of *fruitless* that produce variant *D. melanogaster* male morphology, but other melanogaster group species males have morphological differences that are attributable to their version of *fruitless.* The QTL for conspecific prefence of *D. simulans* females for *D. simulans* males did not share any loci with my QTL map for *D. sechellia* female rejection of *D. simulans* males (Chapter 3). On the basis of these results, it seems reasonable to conclude that many unique outlets for diversification appear to exist, each yielding alternative mating preferences between species, and each underwritten from contributions of different genetic loci. Though *Kat60* was identified as a candidate behavioural isolation gene in both *D. simulans* and *D. mauritiana*, the action and developmental conditions of their discrimination against *D. melanogaster* males varied from one another (Chapter 3; Chapter 4). Thus, even when a single gene underlying behavioural isolation is identified, there are potential intra- and interspecific variants of genes in the same pathway from which species-specific charactersitics could be derived. One such candidate gene for female *D. mauritiana* discrimination against *D. simulans* males has already been mapped to a small region containing only three genes, one of which is the microtubule binding protein *Map205* (McNiven and Moehring 2013). Lastly, it is also possible that the same pathway may give rise to multiple forms of reproductive isolation, such as hybrid sterility. For example, the cellular activity of Katanin-60 is sometimes modulated by Katanin-80, a protein that can result in male sterility when mutated (O'Donnell et al. 2012). If prezygotic and postzygotic mechanisms of isolation co-occur in the same pathway, then the reinforcement of reproductive barriers between species could occur readily.

Genomic recombination is suppressed between heterokaryotypic regions of homologous chromosomes. As a result these regions have increased levels of nucleotide divergence between species, and are predicted to have a higher likelihood of harbouring alleles important for the maintenance of species as distinct groups (Noor et al. 2007; Stevison et al. 2011). The identification of the candidate behavioural isolation genes *Kat60*, *Mekk-1*, and *fruitless* near the borders of two *D. simulans* chromosomal inversions appears to validate this hypothesis. The finding that genes involved in behavioural isolation are coincident with regions of elevated interspecific divergence is important as this prediction is at the intersection of two important species concepts. The genotypic species cluster concept is our most powerful means of defining distinct lineages from one another but does not speak to whether reproductive isolation exists between any given species pair (Mallet 1995). The biological species concept is the most reflective of how speciation dynamics emerge in nature, but is experimentally unfeasible to test on the myriad combinations of closely related species that might reproductively interact with one another (Mayr 1976). Thus it is possible to make predictive claims about the behavioural isolation that might exist between a species pair given the divergent features present within the genome, or vise versa. This may be especially true of inversions which may not only shelter divergent regions, but possess adaptive value that distinguish and diversify the group bearing them. For example, inversions between various strains of *D. pseudoobscura* are selectively maintained between heterogeneous environments because of their ability to hold different combinations of adaptive loci in specific combinations (Schaeffer 2008). Another putative mechanism might be that once a region is inverted, either end of the inversion might be introduced to new gene regulatory environments, inducing its divergence (Heard and Bickmore 2007). The *D. simulans* breakpoint at 84F1 (near *Kat60*) is directly in front of a Hox gene cluster (84F1-84B2), which are classical examples of the influence of chromosomal territory on gene function (Graham et al. 1989). The breakpoint at 93F6-7 (near *Mekk-1* and *fruitless*) is directly after the neuronal response gene *Insulin-like receptor* (*InR*). *InR* is noteworthy in the context of female *D. melanogaster* mating behaviour as it has been shown to regulate sex-specific female courtship responses to pheromones (Lebreton et al. 2015). Thus inversions may be an important substrate for evolutionary dynamics that are likely to lead to speciation.

5.2 Genetics of behavioural isolation in *Drosophila*

The loci underpinning behavioural isolation between species do not appear to be those underpinning variation within species. In song QTL among *Drosophila* spp., the within species variation in wing song for *D. melanogaster* matches none of the between species genetic differences found between *D. simulans* and *D. sechellia* (Gleason 2004). There are no known alleles of *fruitless* that produce variant *D. melanogaster* male morphology, but other melanogaster group species males have morphology differences that are attributable to their version of *fruitless.* The QTL for conspecific prefence of *D. simulans* females for *D. simulans* males did not share any loci with my QTL map for *D. sechellia* female rejection of *D. simulans* males (Chapter 3). On the basis of these results, it seems reasonable to conclude that many unique outlets for diversification appear to exist, each yielding alternative mating preferences between species, and each underwritten from contributions of different genetic loci. Though *Kat60* was identified as a candidate behavioural isolation gene in both *D. simulans* and *D. mauritiana*, the action and developmental conditions of their discrimination against *D. melanogaster* males varied from one another (Chapter 3; Chapter 4). Thus, even when a single gene underlying behavioural isolation is identified, there are potential intra- and interspecific variants of genes in the same pathway from which species-specific charactersitics could be derived. One such candidate gene for female *D. mauritiana* discrimination against *D. simulans* males has already been mapped to a small region containing only three genes, one of which is the microtubule binding protein *Map205* (McNiven and Moehring 2013). Lastly, it is also possible that the same pathway may give rise to multiple forms of reproductive isolation, such as hybrid sterility. For example, the cellular activity of Katanin-60 is sometimes modulated by Katanin-80, a protein that can result in male sterility when mutated (O'Donnell et al. 2012). If prezygotic and postzygotic mechanisms of isolation co-occur in the same pathway, then the reinforcement of reproductive barriers between species could occur readily.

Genomic recombination is suppressed between heterokaryotypic regions of homologous chromosomes. As a result these regions have increased levels of nucleotide divergence between species, and are predicted to have a higher likelihood of harbouring alleles important for the mainteneance of species as distinct groups (Noor et al. 2007; Stevison et al. 2011). The

identification of the candidate behavioural isolation genes *Kat60*, *Mekk-1*, and *fruitless* near the borders of two *D. simulans* chromosomal inversions appears to validate this hypothesis. The finding that genes involved in behavioural isolation are coincident with regions of elevated interspecific divergence is important as this prediction at the intersection of two important species concepts. The genotypic species cluster concept is our most powerful means of defining distinct lineages from one another but does not speak to whether reproductive isolation exists between any given species pair (Mallet 1995). The biological species concept is the most reflective of how speciation dynamics emerge in nature, but is experimentally unfeasible to test on the myriad combinations of closely related species that might reproductively interact with one another (Mayr 1976). Thus it is possible to make predictive claims about the behavioural isolation that might exist between a species pair given the divergent features present within the genome, or vise versa. This may be especially true of inversions which may not only shelter divergent regions, but possess adaptive value that distinguish and diversify the group bearing them. For example, inversions between various strains of *D. pseudoobscura* are selectively maintained between heterogeneous environments because of their ability to hold different combinations of adaptive loci in specific combinations (Schaeffer 2008). Another putative mechanism might be that once a region is inverted, either end of the inversion might be introduced to new gene regulatory environments, inducing its divergence (Heard and Bickmore 2007). The *D. simulans* breakpoint at 84F1 (near *Kat60*) is directly in front of a Hox gene cluster (84F1-84B2), which are classical examples of the influence of chromosomal territory on gene function (Graham et al. 1989). The breakpoint at 93F6-7 (near *Mekk-1* and *fruitless*) is directly after the neuronal response gene *Insulin-like receptor* (*InR*). *InR* is noteworthy in the context of female *D. melanogaster* mating behaviour as it has been shown to regulate sex-specific female courtship responses to pheromones (Lebreton et al. 2015). Thus inversions may be an important substrate for evolutionary dynamics that are likely to lead to speciation.

5.2.1 Divergent sexual dimorphism

Evolutionary diversification of traits associated with sexual dimorphism are predicted to be key factors of speciation (Coyne and Orr 2004). *fruitless*, a necessary determinant of sexually dimorphic anatomy and behaviours in *Drosophila* species, was identified as a candidate gene (Chapter 3). In Chapter 3 I covered briefly how *fruitless* may contribute to divergent behaviours

between species, and will expand on this discussion here with a more speciation-centric perspective. The first notable behaviour attached to the gene *fruitless* was its role in inhibiting courtship behaviour of males towards other males (Ryner et al. 1996). *fruitless* has, since then, been found to be an important determinant of proper behavioural and anatomical development of male *D. melanogaster* (Lee et al. 2000; Usui-Aoki et al. 2005). However, the degree to which *fruitless* establishes sexually dimorphic systems varies on a species-by-species basis. For example in the lamina of the *Drospohila* CNS, *fru* is expressed by *D. virilis* males, by *D. suzukii* males and females, and not at all among seven other *Drosophila* species (Usui-Aoki et al. 2005). Together, these two facts suggest that females, when evolving aversive mating preferences, may sometimes borrow those same systems that males use to identify and inhibit their own mating behaviour towards other males. One advantage to shifting intersexual boundaries in this way is that the system already exists and avoids the evolutionary hurdle of having to develop a new one from scratch. A potential consequence may be that, for the male signifier to be sexually selected against, males bearing feminized/neutral traits are favoured during mating. This may partially explain the mating asymmetry between *D. melanogaster* (sexually dimorphic for CHC) and *D. simulans* (sexually monomorphic for CHC). If such a dynamic is prevalent, it would be straightforward to measure whether females are generally more likely to discriminate against interspecific males possessing traits that were ancestrally present in conspecific males, but have since been lost.

5.3 Evolution of traits involved in behavioural isolation

D. melanogaster and *D. simulans* have species-specific differences in auditory cues and olfactory cues that play a role their behavioural isolation from one another (reviewed in: Capy and Gibert 2004). I determined that one of the means female *D. simulans* used to discriminate against *D. melanogaster* males was through a system that involved: aversion towards cues present in male wing song, neural structures found within the MB, and the expression of *Kat60* - a gene located in a region of the *D. simulans* genome which would have been sheltered from recombination as it diverged (Chapter 3, Chapter 4). This system did not rely on the antennae or aristae to detect these signals, and male CHC were not discriminated against (Chapter 4). These patterns were not uniform across the other strains and species of melanogaster group *Drosophila* that I tested. In total, three different usages of song by various strains and species of the melanogaster group

were found: as a bearer of conspecific arousal cues, as a bearer of interspecific aversion cues, and with no apparent use for species recognition at all. Taken together, these results indicate that *Drosophila* species can readily diversify by using different subsets of mating traits, or similar subsets with different trait salience. The evolution of conspecific male arousal signals likely represents a process of sexual selection for male traits that signify adaptive qualities (Seehausen et al. 2008). Thus in *D. mauritiana, D. simulans-288*, and *D. mauritiana* I expect some episode of ecological adaptation to explain their interspecific preference that targets conspecific males and bears no regard towards interspecific males (Chapter 4). *D. simulans-165* and *D. simulans-166* likely acquired their interspecific aversion to *D. melanogaster* males under different evolutionary circumstances. During reinforcement, maladaptive or inviable offspring produced from interspecific mating are prevented through the evolution of female discriminatory processes that recognize the interspecific males that would produce such offspring (Koopman 1949). As a result, females are able to detect and reject a signifier present in the interspecific males but not conspecific males. Alternatively, if the females recognize the signifier as a means of avoiding sexually anagonistic conspecific males, then interspecific males, which presumably still bear this ancestral trait, could also be selected against. This latter interpretation has some support in the context of *Kat60*, which is highly upregulated in female-specific versus male-specific tissues (Innocenti and Morrow 2010). Genes with sexually antagonistic functions are more likely to arise from genes with sex-biased expression (Reviewed in Ellegen and Parsch 2007). Alternatively, no selective mechanisms are required if alleles for new behaviours were acquired through genetic drift (Niehuis 2013), and were subsequently sheltered by inhabiting a region with suppressed recombination, such as an inversion (Stevison et al. 2011). Given that the candidate genes were located in such an interval, this may also be a plausible evolutionary explanation.

5.4 Concluding remarks

In conclusion, female mechanisms of discrimination against interspecific males can arise through many different pathways. One pathway I have identified here in *D. simulans* appears to rely on genes that are involved in the selective cultivation of specific neuronal populations or connections during development. More work is required to identify the genetic components of these alleles that vary between species and are responsible for behavioural isolation. This

includes using inducible enhancer-mediated expression to determine developmental timing of the behavioural effect, and immunohistochemistry to determine the location of expression within various structures of the CNS, such as the MB, in the two species.

Based on analyses of the discriminatory system found in *D. simulans-165*, and its comparison to other strains and species of *Drosophila*, I suggest that the discrimination shown by *D. simulans-165* females against *D. melanogaster* males evolved relatively recently, evolved in response to past sexual antagonism, and evolved through redundant or secondary means of detection of auditory signals. To determine whether any these dynamics represent general patterns of species formation in *Drosophila*, we will need to expand our catalogue of species pairs, their reproductive interactions with one another, and upon what phenotypic bases these interactions occur. Such studies of courtship are becoming easier and more nuanced with the introduction of software-based analytics that are better able to capture details that are not apparent to a human observer. For this reason, we are increasingly becoming aware that the flow of information and assessment during courtship may be more bidirectional and more influenced by context than has been previously noted. I look forward to further addressing the ideas explored in this chapter, and I hope they find wider usage in the speciation research community.

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Appendices

Appendix A: Chapter 3 supplementary methods

Cloning of the *Kat60* gene from *D. melanogaster* and *D. simulans*
Aaron M. Allen

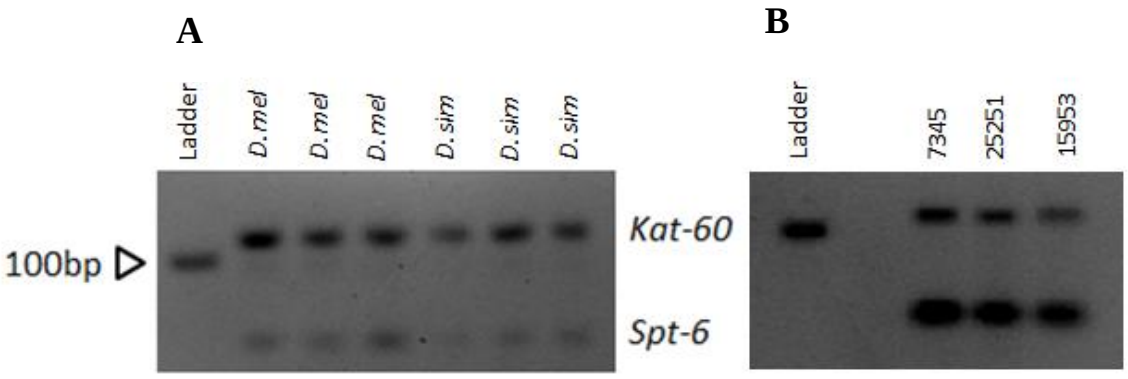
PCR amplification of the *Kat60* alleles:

Kat60 alleles were amplified through PCR. Restriciton sites (AscI, NotI) as well as an "ata" spacer were subcloned into the alleles with the primers ATA GGC GCG CCG TCA TAT GCC TTG GCG GTC AG and ATA GCG GCC GCC CTC CAG CGG ATT CTA TCC. NEB Phusion High-Fidelity polymerase was used (cat # M0530) following the manufacturers instructions.

Digestion, ligation and transformation of the PCR amplified *Kat60* alleles:

All enzymes used were purchased from NEB and used following the manufacturers instructions. The PCR amplicons were directly digested with AscI and NotI. The pStinger-attB plasmid was also digested with AscI and NotI. The resulting digestions were gel electrophoresed and the corresponding bands were purified using Bio Basic gel extraction kit (cat# BS654) following the manufacturers instructions. The insert and vector were ligated using NEB's T4 DNA ligase (cat# M0202). The resulting ligation was used to transform NEB 10 beta cells (cat# C3020K) following the manufacturers instructions. Isolated colonies were used to generate overnight liquid cultures. Plasmid DNA mini-prepartions (Bio Basic cat# BS614) were performed to isolate the resulting recombinant DNA.

Appendix B: Chapter 4 supplementary material



Supplementary Figure 1: RT-PCR of *Kat60* from *Drosophila* species females (A) RT-PCR of *Katanin-60* (*Kat60*) in *D. melanogaster* (*D. mel*) and *D. simulans*(*D. sim*); the housekeeping gene *Spt-6* was used as an internal control.**(B)** Expression of *Katanin-60* in three *D. melanogaster* lines bearing different *P*-elements associated with *Katanin-60*. *D. mel* (7345) is the line with *P{Mae-UAS.6.11}Kat60*^{UY1645}.

Supplementary Table 1. Comparison of regions with tissue-specific expression from different GAL4 drivers. Hybrids bearing transposable element (*P{Mae-UAS.6.11}Kat60*^{UY1645}) were previously shown to display *D. simulans*-like mating behaviour (Chapter 3; Table 3.1). GAL4 drivers that selectively drive expression of UAS bearing genes in specific neural tissues were used to test whether *D. melanogaster*-like mating receptivity was restored. Hybrids bearing only GAL4 or neither GAL4 or UAS (WT) were used to control for the presence of the GAL4 element. Significance was determined through a two-tailed Z-test ($P<0.05$). All expression data was obtained from Flybase (Date accessed: March 31 2016).

Neuroanatomical region	Developmental stage	Stock
adult mushroom body	third instar larval stage	51631
adult pars intercerebralis	third instar larval stage	51631
adult pars intercerebralis	adult stage	4440
adult protocerebral SMP Tachykinin neuron	adult stage	7362
adult tritocerebrum	third instar larval stage	51631
alpha lobe core layer	third instar larval stage	51631
alpha lobe surface layer	third instar larval stage	51631
alpha/beta Kenyon cell	third instar larval stage	51631
alpha-lobe	larval stage - adult stage	7362
alpha-lobe	pupal stage P5 - P6 restricted	7362
alpha-lobe	pupal stage P5	4440
alpha-lobe	third instar larval stage	51631
alpha'-lobe	larval stage	4440
alpha'-lobe	second instar larval stage -- third instar larval stage	7362
alpha-lobe	larval stage	4440
alpha-lobe	second instar larval stage -- third instar larval stage	7362
beta lobe core layer	third instar larval stage	51631
beta lobe surface layer	third instar larval stage	51631
beta-lobe	pupal stage P5 – P6 & restricted	7362
beta-lobe	pupal stage P5	4440
beta-lobe	third instar larval stage	51631
beta-lobe	larval stage	4440
beta-lobe	second instar larval stage -- third instar larval stage	7362
body calyx	larval stage	4440
body calyx	larval stage	7362
calyx of adult mushroom body	adult stage	4440
dorsal anterior lateral neuron of the protocerebrum	adult stage	7362
embryonic/larval mushroom body	first instar larval stage -- second instar larval stage	4440
embryonic/larval mushroom body	third instar larval stage	7362
embryonic/larval mushroom body	larval stage	4440
eye	adult stage	7362
GABAergic local interneuron of the adult antennal lobe	adult stage	7362
gamma-lobe	pupal stage P6 – P7	4440
gamma-lobe	pupal stage P6	7362
gamma-lobe	third instar larval stage -- pupal stage P6	4440
gamma-lobe	larval stage	4440
gamma-lobe	second instar larval stage -- third instar larval stage	7362
haltere disc	third instar larval stage L2	7362
intrinsic neuron of mushroom body alpha-lobe	third instar larval stage	51631
intrinsic neuron of mushroom body beta-lobe	third instar larval stage	51631
Kenyon cell	first instar larval stage -- second instar larval stage	4440

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